Dominant negative FADD dissipates the proapoptotic signalosome of the unfolded protein response in diabetic embryopathy

Fang Wang,1,∗ Hongbo Weng,1,∗ Michael J. Quon,2 Jingwen Yu,1,∗ Jian-Ying Wang,3 Anne-Odile Hueber,4 and Peixin Yang1,5

1Department of Obstetrics, Gynecology, and Reproductive Sciences, 2Department of Medicine, 3Cell Biology Group, Department of Surgery, and 4Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore, Maryland; and 5Institut de Biologie de Valrose, UMR CNRS 7277, UMR Institut National de la Sante et de la Recherche Medicale 1091, Université de Nice-Sophia-Antipolis, Nice, France

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Wang F, Weng H, Quon MJ, Yu J, Wang J, Hueber A, Yang P. Dominant negative FADD dissipates the proapoptotic signalosome of the unfolded protein response in diabetic embryopathy. Am J Physiol Endocrinol Metab 309:E861–E873, 2015.—Endoplasmic reticulum (ER) stress and caspase 8-dependent apoptosis are two interlinked causal events in maternal diabetes-induced neural tube defects (NTDs). The inositol-requiring enzyme 1α (IRE1α) signalosome mediates the proapoptotic effect of ER stress. Diabetes increases tumor necrosis factor receptor type 1 (TNF-α)–associated death domain (TRADD) expression. Here, we revealed two new unfolded protein response (UPR) regulators, TRADD and Fas-associated protein with death domain (FADD). TRADD interacted with both the IRE1α-TRAF2-ASK1 complex and FADD. In vivo overexpression of a FADD dominant negative (FADD-DN) mutant lacking the death effector domain disrupted diabetes-induced IRE1α signalosome and suppressed ER stress and caspase 8-dependent apoptosis, leading to NTD prevention. FADD-DN abrogated ER stress markers and blocked the JNK1/2-ASK1 pathway. Diabetes-induced mitochondrial translocation of proapoptotic Bcl-2 members mitochondrial dysfunction and caspase cleavage were also alleviated by FADD-DN. In vitro TRADD overexpression triggered UPR and ER stress before manifestation of caspase 3 and caspase 8 cleavage and apoptosis. FADD-DN overexpression repressed high glucose- or TRADD overexpression-induced IRE1α phosphorylation, its downstream proapoptotic kinase activation and endonuclease activities, and apoptosis. FADD-DN also attenuated tunicamycin-induced UPR and ER stress. These findings suggest that TRADD participates in the IRE1α signalosome and induces UPR and ER stress and that the association between TRADD and FADD is essential for diabetes- or high glucose-induced UPR and ER stress.

maternal diabetes; dominant negative Fas-associated protein with death domain; neural tube defects; unfolded protein response; endoplasmic reticulum stress; apoptosis

However, recent studies have demonstrated that multivitamins containing folic acid failed to prevent maternal diabetes-induced NTDs (7, 19). Therefore, there is an urgent need in developing effective interventions against diabetic embryopathy.

Mechanistic studies are first steps toward the development of effective interventions for diabetes-associated birth defects. Oxidative stress-induced apoptosis and gene dysregulation in the developing neuroepithelium play causal roles in NTD formation under maternal diabetic conditions (8, 9, 16, 17, 20, 21, 23, 30, 32, 33). Recently, we have revealed that maternal diabetes increases the activity of forkhead transcription factor 3a (FoxO3a), which in turn induces the expression of proapoptotic factor tumor necrosis factor (TNF) receptor type 1-associated death domain (TRADD) (35).

TRADD is a key adaptor protein in TNF signaling cascade that mediates apoptosis (12, 13). TRADD interacts with Fas-associated protein with death domain (FADD) through their mutual death domains. The TRADD-FADD complex triggers caspase 8 cleavage, by the FADD death effector, leading to apoptosis (5, 12). Whereas our previous study demonstrated that increased FoxO3a activity is involved in NTD induction (35), the functional role of increased TRADD expression and the consequent formation of the TRADD-FADD complex in diabetic embryopathy are unclear.

In addition to its interaction with FADD, TRADD binds to another TNF receptor adaptor protein, TNF receptor-associated factor 2 (TRAF2) (12). The TRADD-TNF-FADD complex induces kinase signaling (13). TRAF2 is a critical signal transducer of the unfolded protein response (UPR) because TRAF2 directly interacts with phosphorylated IRE1α (18), a major UPR sensor that transduces stress signals from the endoplasmic reticulum (ER) (27). The IRE1α-TRAF2 interaction recruits and activates apoptosis signal-regulating kinase 1 (ASK1), leading to JNK1/2 activation and apoptosis (18). Our previous study demonstrated that maternal diabetes-induced ER stress in the developing embryo causes proapoptotic JNK1/2 activation, neuroepithelial cell apoptosis, and NTD formation (30). Because the bona fide TRADD-binding partner TRAF2 is critically involved in ER stress (18), we hypothesize that the two proapoptotic pathways, the TRADD-FADD-caspase 8 pathway and the ER-UPR pathway, are interlinked in maternal diabetes-induced ER stress, apoptosis, and NTD formation.

FADD is an adapter protein that was originally isolated as a transducer of apoptotic signals for death domain-containing receptors (3). Dominant negative FADD (FADD-DN), which lacks its death effector domain, has been shown to inhibit TRADD-induced apoptosis in vivo and in vitro (12). Here, we

* F. Wang, H. Weng, and J. Yu contributed equally to this article.

Address for reprint requests and other correspondence: P. Yang, Univ. of Maryland School of Medicine, Dept. of Obstetrics, Gynecology, and Reproductive Sciences, BRB11-039, 655 W. Baltimore St., Baltimore, MD 21201 (e-mail: pyang@fpi.umd.edu).
used a mouse transgenic model of FADD-DN lacking the death effector domain (26) to unravel the functionality of the TRADD-FADD complex in diabetic embryopathy. Our study revealed that TRADD participates in the IRE1α signalsome through its interaction with TRAF2. We also found that FADD-DN overexpression blocks caspase 8-dependent neuroepithelial cell apoptosis, disrupts the IRE1α signalsome, diminishes ER stress, and ameliorates NTD formation. Overexpressing TRADD in vitro has a similar effect to high glucose in vivo on the induction of IRE1α signalsome and ER stress. FADD-DN overexpression alleviated high glucose-, TRADD overexpression-, and tunicamycin-induced IRE1α signalsome and ER stress, supporting our hypothesis that the TRADD-FADD pathway interacts with IRE1α signalsome and activates the ER stress pathway.

MATERIALS AND METHODS

Animals and reagents. The procedures for animal use were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee. Wild-type (WT) C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). FADD-DN overexpression in C57BL/6J background mice were described previously (26), in which the human FADD-DN transgene was driven by the β-actin promoter.

Mouse models of diabetic embryopathy. Our mouse model of diabetic embryopathy has been described previously (16, 29–31, 37, 38). Briefly, 8-wk-old WT female mice were injected intravenously daily with 75 mg/kg streptozotocin (STZ; Sigma, St. Louis, MO), dissolved in sterile 0.1 M citrate buffer (pH 4.5), over 2 days to induce diabetes. Using STZ to induce diabetes is not a complicating factor because STZ is cleared from the bloodstream rapidly (STZ serum half-life is 15 min), and pregnancy is not established until 1–2 wk after STZ injection. Diabetes was defined as a 12-h fasting blood glucose level of ≥14 mM. After diabetes in the mice was established, males and females were paired at 3 PM, and day 0.5 of pregnancy (E0.5) was established by the presence of the vaginal plug the next morning (8 AM). WT female mice were treated with vehicle injections to serve as nondiabetic controls. At 6 PM on E8.75, mice were euthanized and conceptuses dissected out of the uteri, and the embryos, taken from the decidua and without the yolk sacs, were used for analyses.

Overall, the use of spontaneous diabetic mouse models in research of diabetic complications is premature (14). There are two spontaneous diabetic mouse models, the AKITA mice and the NOD mice. AKITA mice possess gene mutations, which may affect pregnancy outcomes and add further genetic complexities in our genetically modified mice (14). The NOD mice are in a rare type of genetic background that is not comparable with the C57BL/6J background used in our study. The onset of diabetes in AKITA mice is very early at 3–4 wk of age and is associated with ER stress (14). For these reasons, the STZ-induced diabetic model used in a relatively short window (~5 wk) is ideal for research of diabetic embryopathy.

Cell culture, transplantation, glucose, and tunicamycin treatment. C17.2 mouse neural stem cells, originally obtained from the European Collection of Cell Culture (Salisbury, UK), were maintained in DMEM (5 mM glucose) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO2. TRADD plasmid (Myc-DDK-tagged, MR223646) was obtained from Santa Cruz Biotechnology (Dallas, TX). The pcDNA3.1-GFP-FADD-DN construct was made by cloning the cDNA coding for human FADD-DN (residues 80–208) into the pcDNA3.1/GFP vector (Invitrogen, Grand Island, NY). Lipofectamine 2000 (Invitrogen) was used according to the manufacturer’s protocol for transfection of plasmid into the cells. After the cells were seeded overnight, C17.2 mouse neural stem cells were transfected with TRADD and FADD-DN plasmid and then cultured for 24 h. Cells transfected with TRADD plasmid were harvested directly, and cells transfected with FADD-DN plasmid were treated with high glucose for another 48 h before being harvested and used in assays. To assess the effect of FADD-DN on TRADD-induced ER stress, cells were cotransfected with TRADD and FADD-DN plasmid and cultured for 24 h before being assayed. To determine the effect of 4-phenylbutyric acid (4-PBA) on TRADD-induced apoptosis, cells were changed to DMEM medium with 2 mM 4-PBA at 6 h after transfection with TRADD plasmid. After 48 h of treatment with 4-PBA, cells were assayed and analyzed. Tunicamycin (77765, 4 μg/ml; Sigma) or DMSO (final concentration is 0.04% DMSO volume/total medium volume) as control was added to growth medium for another 6 h, and then cells were harvested.

E14 mouse embryonic stem cells were obtained from the American Type Culture Collection (Manassas, VA) and then seeded onto a culture plate with a layer of STO feeder cells maintained in embryonic stem cell culture medium [DMEM (25 mM glucose) supplemented with 10% FBS, 1% NEAA, 1% β-mercaptoethanol, 2 mM l-glutamine, 1,000 U/ml LIF, 100 U/ml penicillin, and 100 μg/ml streptomycin] at 37°C in a humidified atmosphere of 5% CO2. At second passage, E14 cells were transferred to feeder cell-free plates and maintained in embryonic stem cell culture medium supplemented with 15% FBS. After two passages, feeder cell-free E14 cells were changed to and maintained in low glucose embryonic stem cell culture medium (5 mM glucose DMEM) for at least eight passages. Plasmid transfection and high glucose treatment were the same as described previously (31).

Immunoprecipitation and immunoblotting. Immunoprecipitation (IP) and immunoblotting were performed as described previously (16, 30, 31). Protease inhibitor cocktail (Sigma), lysis buffer (Cell Signaling Technology), protein A magnetic bead slurry (New England BioLabs, Ipswich, MA), and rabbit anti-ASK1 antibody (Santa Cruz Biotechnology) were used for IP. For immunoblotting, equal amounts of protein and the Precision Plus protein standards (Bio-Rad, Hercules, CA) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto Immunobilon-P membranes (Millipore, Billerica, MA). Membranes were incubated in 5% nonfat milk for 45 min and then incubated for 18 h at 4°C with the following primary antibodies at 1:1,000 to 1:2,000 dilution: c-Jun, p-NF-kB, Bax, Bak, Bim, caspase 3, caspase 8, caspase 9, caspase 12, caspase 3 (Millipore), phosphorylated (p)-FADD, TRADD, p-ASK1, p-IRE1α, p-JNK1/2, p-NF-κB, p-ATF2 (activating transcription factor 2), p-Erk1/2, p-PERK (protein kinase R-like endoplasmic reticulum kinase), p-eIF2α (eukaryotic initiation factor 2), BiP, CCAAT enhancer-binding protein (C/EBP) homologous protein (CHOP), p-JNK1/2, p-c-Jun, p-ATF2 (activating transcription factor 2), p-Erk1, p-FoxO3a, tbid, p-Bad, Bax, Puma, Bak, Bim, and prohibitin (Millipore). Signals were detected using the SuperSignal West Femto Maximum Sensitivity Substrate kit (Thermo Scientific, Waltham, MA) and a Chemidoc XRS Bio Imaging System (Biorad, Hercules, CA). Membranes were exposed to goat anti-rabbit or anti-mouse secondary antibodies. To ensure that equivalent amounts of protein were loaded among samples, membranes were stripped and probed with a mouse antibody against β-actin (Abcam, Cambridge, MA) and prohibitin (Millipore). Signals were detected using the SuperSignal West Femto Maximum Sensitivity Substrate kit (Thermo Scientific, Waltham, MA). All experiments were repeated three times with the use of independently prepared tissue lysates.

RNA extraction and real-time PCR. Total RNA was isolated from embryos using an RNeasy Mini Kit (Qiagen, Frederick, MD) and reverse transcribed using the high-capacity cDNA archive kit (Applied Biosystems, Life Technologies, Grand Island, NY). MicroRNA was extracted by using the mirVana miRNA isolation kit (Ambion, Life Technologies) and reverse transcribed using NCode VILO miRNA cDNA synthesis kit (Invitrogen, Life Technologies). RT-PCR for ER stress chaperone genes (calnexin, BiP, CHOP, IRE1α, PDI, and GRP94), β-actin, mmu-mir-34a, mmu-mir-96, and mmu-mir-U6 was performed using Maxima SYBR Green/ROX qPCR Master Mix assay (Thermo Scientific). RT-PCR

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and subsequent calculations were performed by a 7700 ABI PRISM sequence detector system (Applied Biosystems).

TUNEL assay. The TUNEL assay was performed as described previously by using the In Situ Cell Death Detection Kit (Millipore) (30). Cells were seeded on the eight-well Nunc Lab-Tek Chamber Slide system (Sigma). After transfection and 4-PBA or high glucose treatment, cells were fixed with 4% paraformaldehyde in PBS and incubated with TUNEL reagent, counterstained with DAPI, and mounted with aqueous mounting medium (Sigma). TUNEL-positive cells in each well were counted. The percentage of apoptotic cells was calculated as the number of TUNEL-positive (apoptotic) cells divided by the total number of cells multiplied by 100.

Statistical analysis. Data are presented as means ± SE. Three embryos from three different dams were used for immunoblotting, and five embryos for five different dams were studied for RT-PCR. One-way ANOVA was performed using SigmaStat 3.5 software, and a Tukey test was used to estimate the significance. Chi-square test was used to estimate the significance of NTD formation among different groups. Statistical significance was accepted when $P < 0.05$.

RESULTS

FADD-DN ameliorates maternal diabetes-induced caspase activation, apoptosis, and NTD formation. Maternal diabetes induces caspase 8-dependent apoptosis in the neuroepithelium of the developing embryo (30, 33, 35, 39). Caspase 8 is an initiator caspase whose cleavage or activation leads to the activation of the executive caspase 3. Indeed, the cleavage of caspase 3 and caspase 8 was observed in embryos exposed to maternal diabetes (Fig. 1A). FADD-DN overexpression abolished maternal diabetes-induced caspase 3 and caspase 8 cleavage and TRADD upregulation (Fig. 1A). Consistent with caspase activation, the number of apoptotic cells was significantly increased in the neuroepithelium of embryos exposed to maternal diabetes compared with that in the neuroepithelium of embryos from nondiabetic dams with or without FADD-DN overexpression (Fig. 1B). FADD-DN overexpression suppressed maternal diabetes-induced neuroepithelial cell apoptosis (Fig. 1B) and NTD formation (Table 1 and Fig. 1C). These findings support the hypothesis that FADD-DN blocks maternal diabetes-induced neuroepithelial cell apoptosis, leading to reduction of diabetic embryopathy.

FADD-DN suppresses ER stress by disrupting the interaction of TRADD-FADD with the IRE1α/ASK1 complex. Maternal diabetes activates IRE1α (17, 30), which forms a complex with TRAF2 and ASK1 (18, 31). Because TRADD interacts with TRAF2 (12), we sought to determine whether the maternal diabetes-induced IRE1α signalosome, the IRE1α-TRAF2-ASK1 complex, interacts with TRADD and FADD and whether FADD-DN disrupts the interaction of IRE1α signalosome with TRADD. ASK1 immunoprecipitation using a rabbit anti-ASK1 antibody pulled down p-IRE1α, p-ASK1, TRAF2, TRADD, and FADD in embryonic lysates from nondiabetic or diabetic dams (Fig. 2A), whereas control (normal rabbit IgG) did not pull down any of these proteins (Fig. 2A), indicating that p-IRE1α forms a complex with TRAF2, p-ASK1, TRADD, and FADD. The abundance of p-IRE1α, TRAF2, p-ASK1, TRADD, and FADD proteins in the ASK1 immunoprecipitates was significantly higher in embryos from diabetic dams than in embryos of nondiabetic dams (Fig. 2A). FADD-DN overexpression diminished the increased presence of p-IRE1α, TRAF2, p-ASK1, TRADD, and FADD in ASK1 immunoprecipitates under diabetic conditions (Fig. 2A).

To test whether the disruption of the IRE1α signalosome by FADD-DN overexpression has any impact on the UPR pathway, whose prolonged activation leads to ER stress, levels of p-IRE1α, p-PERK, and eIF2α were measured. FADD-DN overexpression suppressed maternal diabetes-induced phosphorylation of IRE1α, PERK, and eIF2α (Fig. 2, B–D). Because the two UPR sensors IRE1α and PERK were deactivated by FADD-DN, we predicted that ER stress was relieved. The levels of ER stress markers CHOP and BiP and ER chaperone gene expression in embryos of diabetic dams that overexpressed FADD-DN were reduced to levels comparable with those in embryos of nondiabetic dams (Fig. 3, A–C). Another index of ER stress, X-box-binding protein-1 mRNA splicing, was also diminished by FADD-DN overexpression (Fig. 3D). These results demonstrate that FADD-DN disrupts the assembly of the IRE1α signalosome, leading to blockage of ER stress.

FADD-DN represses the proapoptotic ASK1-JNK1/2 pathway in the developing embryo. One of the functional readouts of the IRE1α signalosome is the activation of the proapoptotic ASK1-JNK1/2 pathway. Diabetes-induced phosphorylation of ASK1 and JNK1/2 was blocked by FADD-DN overexpression (Fig. 4, A and B). Furthermore, the phosphorylation of three transcription factors, c-Jun (Fig. 4C), Elk1 (Fig. 4D), and ATF-2 (Fig. 4E), that are downstream effectors of JNK1/2 activation was blunted by FADD-DN overexpression. FoxO3a is also downstream of JNK1/2, and its activation relies on dephosphorylation. Maternal diabetes induced FoxO3a dephosphorylation, and FADD-DN overexpression restored FoxO3a phosphorylation (or inactivation; Fig. 4F). These findings reveal the inhibitory role of FADD-DN in the ASK1-JNK1/2 pathway in diabetic embryopathy.

FADD-DN rescues mitochondrial function altered by maternal diabetes. TRADD-FADD interacts with the IRE1α signalosome, which activates the proapoptotic ASK1-JNK1/2 pathway. The ASK1-JNK1/2 pathway induces apoptosis through the intrinsic mitochondrial pathway. Mitochondrial dysfunction was manifested in diabetic embryopathy (34). To reveal whether FADD-DN overexpression affects mitochondrial dysfunction induced by maternal diabetes, a set of mitochondrial dysfunction markers was assessed. The activation and mitochondrial translocation of the proapoptotic members of the Bcl-2 family reflect mitochondrial dysfunction. Maternal diabetes triggered Bad dephosphorylation (activation) and Bid cleavage, whereas FADD-DN overexpression prevented these changes (Fig. 5A). Maternal diabetes-induced Bax, Bak, and Puma, and Bim mitochondrial translocation was also blocked by FADD-DN overexpression (Fig. 5B), indicating that FADD-DN restores mitochondrial function that is suppressed by maternal diabetes.

TRADD mimics high glucose to induce UPR and ER stress. Because TRADD participates in the IRE1α signalosome, we tested whether TRADD overexpression is sufficient to induce the UPR and ER stress. Ectopic TRADD overexpression in neural stem cells activated the two UPR arms by inducing phosphorylation of IRE1α, PERK, and eIF2α (Fig. 6, A and B). TRADD overexpression also upregulated the ER stress markers CHOP and BiP (Fig. 6B). The activation of key components of the IRE1α signalosome, p-ASK1 and p-JNK1/2, was induced by TRADD overexpression (Fig. 6C). Levels of miR17, miR34a, and miR96, indices of the IRE1α endonuclease ac-
activities (31), were significantly downregulated by TRADD overexpression (Fig. 6D). UPR activation and ER stress induction by TRADD were not secondary effects of apoptosis because they occurred before TRADD-induced apoptosis. TRADD-induced UPR and ER stress took place as early as 24 h after TRADD transfections (Fig. 6, B–D), whereas the appearance of apoptotic cells, cleaved caspase 3, and cleaved caspase 8 were observed after 36 h onward (Fig. 6, E–G). The ER stress inhibitor 4-PBA (17, 31) significantly reduced TRADD-induced apoptosis (Fig. 6, E–G), suggesting that ER stress is essential for TRADD-induced apoptosis. The above results suggest that TRADD stimulates UPR and ER stress, leading to apoptosis.

**FADD-DN blocks high glucose- or TRADD-induced UPR and ER stress in neural stem cells.** The neuroepithelium during embryonic neurulation contains essentially neural stem cells, and maternal diabetes imposes an adverse effect on the developing neuroepithelium by inducing neural stem cell apoptosis.
The C17.2 neural stem cell line was derived from neonatal cerebellum and immortalized by retroviral transduction with v-myct (25). We have demonstrated that the C17.2 cell line mimics the in vivo diabetic embryopathy model in ER stress and apoptosis induction when exposed to high glucose (REF). Therefore, the C17.2 cell line was chosen for our in vitro experimentations. We used FADD-DN to determine whether the TRADD functional output through FADD mediates the stimulatory effect of high glucose on the UPR and ER stress.

Table 1. *FADD-DN overexpression ameliorates diabetes-induced NTDs*

<table>
<thead>
<tr>
<th>Experimental Group: FADD-DN (M) × WT (F)</th>
<th>Glucose Level, mM</th>
<th>Embryo Genotypes</th>
<th>Total No. of Embryos</th>
<th>No. of NTD Embryos</th>
<th>NTD Rate, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND dams (16 litters)</td>
<td>7.83 ± 0.37</td>
<td>WT</td>
<td>69</td>
<td>1</td>
<td>1.4</td>
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<tr>
<td></td>
<td></td>
<td>FADD-DN</td>
<td>51</td>
<td>0</td>
<td>0</td>
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<tr>
<td>DM dams (15 litters)</td>
<td>24.0 ± 0.86</td>
<td>WT</td>
<td>51</td>
<td>10*</td>
<td>19.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FADD-DN</td>
<td>47</td>
<td>1</td>
<td>2.1</td>
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Glucose values are means ± SE. FADD-DN, dominant negative Fas-associated protein with death domain transgenic mice or overexpressing embryos; NTD, neural tube defect; M, male; F, female; ND, nondiabetic; DM, diabetic; WT, wild-type mice or embryos. Males are all ND. *Significant difference compared with WT and FADD embryos from the nondiabetic group and FADD-DN embryos from the diabetic group using Chi² tests or Fisher exact tests.

(Fig. 2) FADD-DN overexpression blocks maternal diabetes-enhanced formation of the inositol-requiring enzyme 1α (IRE1α)-apoptosis signal-regulating kinase 1 (ASK1)-TNF receptor-associated factor 2 (TRAF2)-TRADD-FADD complex, ER stress, and the ASK1-JNK1/2 signaling pathway. A: representative images of phospho (p)-IRE1α, TRAF2, TRADD, and FADD in ASK1 immunoprecipitates. Relative protein abundance was normalized by their respective levels in 10% input and is shown in the bar graphs. Normal rabbit IgG was used as a negative control. Protein levels of p-IRE1α (B), p-PERK (protein kinase R-like endoplasmic reticulum kinase; C), p-eIF2α (eukaryotic initiation factor; D), and activating transcription factor 6 (ATF6; E). Experiments were repeated 3 times using embryos from 3 different dams (n = 3/group). *Significant differences compared with the other groups. IP, immunoprecipitation; WB, Western blot.
eIF2α phosphorylation (Fig. 7A), diminished the increase of ER stress markers CHOP and BiP (Fig. 7A), terminated the activation of ASK1 and JNK1/2 by high glucose (Fig. 7B), blunted the decrease of miRNAs downstream of IRE1α (Fig. 7C), and blocked caspase activation and apoptosis (Fig. 7, A and D). Likewise, FADD-DN abolished TRADD-induced IRE1α, PERK, and eIF2α phosphorylation (Fig. 7E) and upregulation of CHOP and BiP (Fig. 7E). These results indicate that FADD-DN is able to suppress TRADD-induced ER stress.

We further examined the effect of FADD-DN on tunicamycin-induced UPR and ER stress in C17.2 cells. FADD-DN blocked tunicamycin-induced IRE1α phosphorylation and CHOP upregulation and attenuated PERK phosphorylation and BiP upregulation (Fig. 7F). These findings demonstrate that FADD-DN blocks TRADD-, high glucose-, or tunicamycin-induced ER stress.

Both TRADD and high glucose induce the UPR and ER stress in embryonic stem cells. Although the findings in the C17.2 cell line were supportive of in vivo results, the C17.2...
cell line may not truly reflect the cell biology of neural stem cells in the developing neuroepithelium. The C17.2 cell line is developmentally more differentiated than neural stem cells in the neuroepithelium of the neurulating embryo (25). Additionally, maternal diabetes adversely impacts the entire period of embryogenesis, including the stages of embryonic stem cells. Thus we chose E14 embryonic stem cells to recapitulate the finding that TRADD triggers UPR and ER stress in neural stem cells. We examined the effect of TRADD overexpression in an embryonic stem cell line. TRADD overexpression activated the two UPR sensors IRE1 and PERK and increased the ER stress markers CHOP and BiP (Fig. 8A).

Maternal diabetes adversely impacts the entire period of embryonic development and thus may also affect the function of embryonic stem cells. High glucose induced the phosphorylation of IRE1α and PERK and upregulated ER stress markers in embryonic stem cells. FADD-DN abrogated high glucose-induced UPR and ER stress (Fig. 8B).

FIG. 4. FADD-DN overexpression inhibits maternal diabetes-induced activation of the ASK1-JNK1/2 pathway. p-ASK1 (A), p-JNK1/2 (B), p-c-Jun (C), p-Elk1 (D), p-ATF2 (E), and p-FoxO3a (F) in wild-type (WT) and FADD-DN overexpressing embryos from ND and DM dams mated with FADD-DN males. Experiments were repeated 3 times using embryos of 3 different dams (n = 3/group). *Significant differences compared with other groups.

DISCUSSION

Programmed cell death caused by the oxidative stress-ASK1-FoxO3a pathway culminates in the upregulation of TRADD and consequent caspase 8 activation, leading to diabetic embryopathy (35). The proapoptotic function of TRADD is carried out through its interaction with FADD (5). By suppressing TRADD upregulation and blocking the functional output of the TRADD-FADD complex, FADD-DN lacking the FADD death effective domain diminishes maternal diabetes-induced caspase 8 activation and apoptosis in the developing neuroepithelium and significantly curtails NTD formation in diabetic pregnancies.

In diabetic embryopathy, we found that TRADD interacts with the IRE1α signalosome, a major UPR sensor, through its binding to TRAF2. The critical involvement of TRADD in the UPR pathway is not a previously recognized function of TRADD. Our study showed that FADD-DN overexpression not only blocks TRADD-FADD-induced caspase 8 activation...
but also disrupts the IRE1α signalosome, thereby inhibiting ER stress. Although the exact mechanism leading to the disassembly of the IRE1α signalosome by FADD-DN is not clear, our study does provide a potential explanation for the action of FADD-DN overexpression in suppressing ER stress. Our results indicate that the IRE1α/TRAF2-ASK1-TRADD complex recruits FADD to trigger caspase 8-mediated apoptosis. In the presence of FADD-DN, the proapoptotic function of the IRE1α/TRAF2-ASK1-TRADD complex was blocked, leading to the dissociation of the IRE1α signalosome and the formation of inactive TRADD-FADD-DN complexes, which did not trigger caspase 8 activation and apoptosis. Our data also support the hypothesis that FADD-DN reduces tunicamycin-induced ER stress.

Although maternal diabetes-induced ER stress is different than tunicamycin-induced ER stress, these findings suggest that FADD’s inhibition on ER stress may be applicable to conditions under chemical ER stressors.

Our study revealed that TRADD is a critical component of the IRE1α signalosome, the IRE1α/TRAF2-ASK1 complex. TRADD binds to the TRAF domain of TRAF2 protein (12), and IRE1α and ASK1 also bind to TRAF2 through its TRAF domain (18), implicating that multiple TRAF2 protein molecules are incorporated in the IRE1α complex.

Although there are three UPR pathways, the UPR pathway initiated by IRE1α appears to be most important in the induction of ER stress because IRE1α activation is responsible for the entire UPR in the yeast (4). Additionally, IRE1α possesses
Fig. 6. TRADD in vitro overexpression induces unfolded protein response (UPR) and ER stress. A: TRADD mRNA and protein levels after TRADD plasmid transfections. Control (Ctrl) vector: the backbone vector of the TRADD construct. B: protein levels of p-IRE1α, p-PERK, p-eIF2α, BiP, and CHOP after TRADD plasmid transfections; Ctrl vector transfection. C: protein levels of p-ASK1 and p-JNK1/2 after TRADD plasmid transfections. D: miRNA levels. E: representative images of the TUNEL assay. Apoptotic cells were labeled as red, and cell nuclei were stained by DAPI (blue). F: levels of cleaved caspase 3; experiments were repeated 3 times (n = 3). G: levels of cleaved caspase 8; experiments were repeated 3 times (n = 3). *Significant differences compared with other groups (in A, B, C, D, F, and G); * and #significant differences (P < 0.05) compared with each other or between the other two groups (in E).
Fig. 7. FADD-DN suppresses high glucose- (HG; 25 mM) or TRADD overexpression-induced UPR and ER stress. A: protein levels of p-IRE1α, p-PERK, p-eIF2α, CHOP, and BiP and cleaved caspase 3 under HG conditions with or without FADD-DN. B: protein levels of p-ASK1 and p-JNK1/2. C: miRNA levels. D: representative images of the TUNEL assay; apoptotic cells were labeled as red, and cell nuclei were stained as blue. E: protein levels of p-IRE1α, p-PERK, p-eIF2α, BiP, and CHOP under TRADD overexpression with or without FADD-DN cotransfections. F: protein levels of p-IRE1α, p-PERK, p-eIF2α, BiP, and CHOP by tunicamycin treatment (4 μg/ml for 6 h) with or without FADD-DN overexpression. Experiments were repeated 3 times (n = 3). *Significant differences compared with other groups.
both proapoptotic kinase and endoribonuclease activities (10). Transient IRE1α activation resolves the homeostatic imbalance in the ER lumen by the accumulation of unfolded/misfolded proteins (22), whereas prolonged IRE1α activation leads to ER stress (24). Prolonged IRE1α activation requires autophosphorylation and subsequently aggregation of IRE1α (2). Because FADD-DN overexpression disassembles the IRE1α-TRAF2-ASK1-TRADD-FADD complex. The IRE1α signalosome induces prolong UPR and ER stress and triggers apoptosis by activating the ASK1-JNK1/2 pathway and concomitantly inducing mitochondrial dysfunction, all of which leads to caspase 8 activation. FADD-DN blocks the UPR and ER stress by disassembling the IRE1α signalosome.

Fig. 8. FADD-DN blocks TRADD overexpression or HG-induced UPR and ER stress in embryonic stem cells. A: protein levels of p-IRE1α, p-PERK, p-eIF2α, BiP, and CHOP after TRADD overexpression in E14 embryonic stem cells. B: protein levels of p-IRE1α, p-PERK, p-eIF2α, BiP, and CHOP under HG conditions with or without FADD-DN overexpression. Experiments were repeated 3 times (n = 3). *Significant differences compared with other groups. C: a schematic diagram depicts the role of TRADD and its partner FADD in UPR and ER stress in diabetic embryopathy. Maternal diabetes or HG in vitro upregulates TRADD, which facilitates the assembly of the IRE1α signalosome, the IRE1α-TRAF2-ASK1-TRADD-FADD complex. The IRE1α signalosome induces prolong UPR and ER stress and triggers apoptosis by activating the ASK1-JNK1/2 pathway and concomitantly inducing mitochondrial dysfunction, all of which leads to caspase 8 activation. FADD-DN blocks the UPR and ER stress by disassembling the IRE1α signalosome.
with other components, TRAF2 and ASK1, in the IRE1α signalosome, act as scaffold proteins to hold the whole complex together and achieve IRE1α aggregation. Additionally, TRADD may be essential for the relay of the proapoptotic signals resulting from prolonged IRE1α activation though the ASK1-JNK1/2 pathway. The latter is supported by our observation that displacement of TRADD from the IRE1α signalosome by FADD-DN represses the activation of the ASK1-JNK1/2 pathway.

The total shutdown of the three UPR arms would be detrimental to the developing embryo. However, FADD-DN blocks prolonged activation of the UPR. Basal activities of the UPR still exist under nondiabetic or diabetic conditions with or without FADD-DN. For an example, eIF2α phosphorylation, which suppresses new protein synthesis, was attenuated by FADD-DN but was still expressed. Similarly, ATF6 cleavage was reduced but not totally abolished by FADD-DN. These basal UPR activities may be sufficient to restore ER homeostasis and sustain cell viability. Suppression of prolonged UPR by FADD-DN inhibited maternal diabetes-induced apoptosis.

Maternal diabetes increases TRADD expression through the ASK1-JNK1/2-FoxO3a pathway (35). In addition, TRADD promotes ASK1 and JNK1/2 activation through the active IRE1α signalosome. Therefore, a positive feedback loop between TRADD and the ASK1-JNK1/2-FoxO3a pathway appears to exist in diabetic embryopathy. TRADD overexpression in vitro induces cell apoptosis (13); however, the mechanism underlying TRADD-induced apoptosis is not entirely clear. Because TRADD is a key adaptor protein of the TNFα cell death receptor pathway (12), whose activation results in caspase 8-dependent apoptosis (13), it is thought that TRADD overexpression induces apoptosis through its interaction with FADD and consequent caspase 8 activation. This is also true in diabetic embryopathy because the abundance of TRADD-FADD complexes is increased along with cleaved caspase 8. It appears that additional mechanisms are involved in TRADD-induced apoptosis. Our in vivo observation revealed that TRADD may be a key component of the IRE1α signalosome. TRADD overexpression in vitro activated the IRE1α UPR pathway and induced ER stress before cell death was manifested. These results support our hypothesis that TRADD overexpression induces apoptosis through the induction of ER stress. Our findings suggest that the IRE1α signalosome is disrupted by FADD-DN, leading to ER homeostasis, which in turn attenuates the other two UPR arms, the PERK-eIF2α pathway and the ATF6 pathway. Resolving ER imbalance by FADD-DN may enable the ER chaperone BiP to interact with PERK and ATF6 and subsequently block their activation. FADD-DN may also restore the expression of endoplasmic reticulum-associated protein degradation (ERAD) genes. Future studies may investigate the effect of FADD-DN on the functions of ER chaperones and the expression of ERAD genes.

We showed that FADD-DN abolishes IRE1α activation and ER stress induced by TRADD overexpression in vitro. These findings suggest that the TRADD-FADD interaction plays an important role in high glucose-induced UPR activation and ER stress. Maternal diabetes induces ER stress in the neuroepithelium of the neurulation stage embryo, leading to NTD formation (17, 31). Neural stem cells of the embryonic neuroepithelium are the primary targets of maternal diabetes and ER stress (17, 34). Although the C17.2 neural stem cell line we studied comes from the external germinai layer of neonatal mouse cerebellum (25), it is vulnerable to insults from high glucose and TRADD overexpression undergoing the UPR and ER stress seen in embryonic neuroepithelia. In addition, both TRADD overexpression and high glucose induce UPR activation and ER stress in embryonic stem cells.

In summary, we have found that FADD-DN overexpression blocks the activation of the IRE1α signalosome, ER stress, ASK1-JNK1/2 activation, mitochondrial dysfunction, and neuronal epithelial cell apoptosis (Fig. 8C). FADD-DN effectively blocks apoptosis caused by increased TRADD expression under maternal diabetic conditions. TRADD participates in the IRE1α signalosome, whereas FADD-DN overexpression in vivo and in vitro disrupts the IRE1α signalosome. Our study is among the first to demonstrate a critical role of TRADD and FADD in ER stress induction and suggest a protective role of FADD-DN in diabetic embryopathy.

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DISCLOSURES

No potential conflicts of interest relevant to this article, financial or otherwise, are reported by the authors.

AUTHOR CONTRIBUTIONS


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