OBSTETRICS

# Superoxide dismutase 1 overexpression in mice abolishes maternal diabetes—induced endoplasmic reticulum stress in diabetic embryopathy

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**OBJECTIVE:** Both oxidative stress and endoplasmic reticulum stress (ER stress) are causal events in diabetic embryopathy. We tested whether oxidative stress causes ER stress.

**STUDY DESIGN:** Wild-type (WT) and superoxide dismutase 1 (SOD1)—overexpressing day 8.75 embryos from nondiabetic WT control with SOD1 transgenic male and diabetic WT female with SOD1 transgenic male were analyzed for ER stress markers: C/EBP-homologous protein (CHOP), calnexin, eukaryotic initiation factor 2$\alpha$ (eIF2$\alpha$), protein kinase ribonucleic acid (RNA)-like ER kinase (PERK), binding immunoglobulin protein, protein disulfide isomerase family A member 3, kinases inositol-requiring protein-1$\alpha$ (IRE1$\alpha$), and the X-box binding protein (XBP1) messenger RNA (mRNA) splicing.

**RESULTS:** Maternal diabetes significantly increased the levels of CHOP, calnexin, phosphorylated (p)-eIF2$\alpha$, p-PERK, and p-IRE1$\alpha$; triggered XBP1 mRNA splicing; and enhanced ER chaperone gene expression in WT embryos. SOD1 overexpression blocked these diabetes-induced ER stress markers.

**CONCLUSION:** Mitigating oxidative stress via SOD1 overexpression blocks maternal diabetes—induced ER stress in vivo.

**Key words:** diabetic embryopathy, endoplasmic reticulum stress, oxidative stress, superoxide dismutase 1 transgenic mice

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Preexisting maternal diabetes increases the risk of neural tube defects (NTDs).[1] In mouse models of diabetic embryopathy, embryos exposed to maternal diabetes during neurulation develop a high rate of NTDs.[2-5] Although the mechanism for this is still being elucidated, we do know that maternal diabetes increases the production of several reactive oxygen species[5, 6] and simultaneously impairs the activities of endogenous antioxidant enzymes,[7] resulting in oxidative stress.

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On the other hand, we also know that both dietary antioxidant supplements in vivo[8] and antioxidant treatment to cultured embryos in vitro[3] can ameliorate the incidence of NTDs in diabetic embryopathy. Indeed, transgenic (Tg) mice overexpressing an endogenous antioxidant, superoxide dismutase 1 (SOD1), have been shown to have a significantly reduced NTD rate, even under maternal diabetic conditions.[9]

These multiple lines of experimental evidence support the hypothesis that oxidative stress plays a causal role in the induction of NTDs in diabetic embryopathy. The endoplasmic reticulum (ER) is a likely candidate to play a role in this pathological pathway because it is a critical organelle for the posttranslation modification and correct folding of newly synthesized proteins. A group of ER-resident chaperone proteins, including binding immunoglobulin protein (BiP) and calnexin, is essential for maintaining normal ER function. An overload of misfolded and/or aggregated proteins in the ER lumen induces ER dysfunction, which results in ER stress and the induction of cell apoptosis.[10]

ER stress triggers the unfolded protein response (UPR), which enhances the expression of ER chaperone proteins.[11] Elevated ER chaperone proteins thus are indicators of ER stress. In mammalian cells, 2 kinases, the inositol-requiring enzyme 1$\alpha$ (IRE1$\alpha$) and the protein kinase RNA-like ER kinase (PERK), are responsible for the activation of UPR.[12,13] IRE1$\alpha$ activation induces messenger RNA (mRNA) splicing of the transcription factor, X-box-binding protein1 (XBP1), and converts this XBP1 into a potent activator that induces the expression of many UPR-responsive genes,[14] whereas PERK activation inhibits protein translation and increases the expression of the proapoptotic C/EBP-homologous protein (CHOP) through the phosphorylation of eukaryotic initiation factor 2$\alpha$ (eIF2$\alpha$).[15]

We have recently demonstrated that maternal diabetes induces ER stress, and an ER stress inhibitor, 4-phenylbutyrate, blocks ER stress and thus suppresses the induction of diabetic embryopathy.[16] We also have demonstrated that the activation of c-Jun-N-terminal kinase 1 and 2 (JNK1/2) causes diabetic embryopathy.[17,18]

Indeed, in our laboratory the targeted deletion of either *jnk1* or *jnk2* gene diminished maternal diabetes–induced ER stress, supporting the causal role of JNK1/2 activation in maternal diabetes–induced ER stress.[16] Because our experimental evidence has demonstrated that oxidative stress triggers JNK1/2 activation, we hypothesize that oxidative stress is responsible for ER stress induced by maternal diabetes in diabetic embryopathy.

In vivo SOD1 overexpression in SOD1-Tg mice effectively suppresses maternal diabetes–induced oxidative stress[19,20] and significantly ameliorates maternal diabetes–induced NTDs.[9] Thus, in the present study, we used SOD1-Tg mice to determine whether reducing oxidative stress by SOD1 overexpression blocks maternal diabetes-induced ER stress and found that SOD1 overexpression diminished ER stress markers, suggesting that oxidative stress causes ER stress in diabetic embryopathy.

## Materials and Methods

### Animals and reagents

C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). SOD1-Tg mice in a C57BL/6J background were revived from frozen embryos by the Jackson Laboratory (stock no. 002298). Streptozotocin (STZ) from Sigma (St. Louis, MO) was dissolved in sterile 0.1 M citrate buffer (pH 4.5). Sustained-release insulin pellets were purchased from Linplant (Linshin, CA).

### Mouse models of diabetic embryopathy

The procedures for animal use were approved by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine. Our mouse model of diabetic embryopathy has been described previously.[16,17] Briefly, 8 week old wild-type (WT) mice were intravenously injected daily with 75 mg/kg STZ over 2 days to induce diabetes. Once a level of hyperglycemia indicative of diabetes (≥250 mg/dL) was achieved, insulin pellets were subcutaneously implanted in these diabetic mice to restore euglycemia prior to mating. The mice were then mated with SOD1-Tg male mice at 3:00 PM to generate WT (SOD1 negative) and SOD1-overexpressing embryos.

We designated the morning when a vaginal plug was present as embryonic day (E) 0.5. On E5.5 (day 5.5), insulin pellets were removed to permit frank hyperglycemia (>250 mg/dL glucose level), so the developing conceptuses would be exposed to a hyperglycemic conditions from E7 onward. WT, nondiabetic female mice with vehicle injections and sham operation of insulin pellet implants served as nondiabetic controls. On E8.75, mice were euthanized, and conceptuses were dissected out of the uteri for analysis. E8.75 embryos are suitable models to study the impact of diabetes on embryonic neural tube closure. This is based on the fact that the E8.75 is a key stage of neurulation, and the major part of E8.75 embryos is the developing neural tube. The average litter sizes of nondiabetic and diabetic dams do not differ and are about 5-6 embryos per dam. To avoid any redundancy, data of NTD incidences were not collected because these have been published elsewhere.[9]

### Genotyping of embryos

Embryos from WT diabetic dams mated with SOD1-Tg male mice were genotyped according to the Jackson Laboratory's protocol using the yolk sac deoxyribonucleic acid (DNA).

### Western blotting

Western blotting was performed as previously described.[16,17] Briefly, embryos from different experimental groups were sonicated in 80 μL ice-cold lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 10 mM NaF, 2 mM Na-orthovanadate, 1 mM pheylmethylsulfonyl fluoride, and 1% Triton X-100) containing a protease inhibitor cocktail (Sigma). Equal amounts of protein and the Precision Plus protein standards (Bio-Rad Laboratories, Hercules, CA) were resolved by sodium dodecyl sulfate-polyacryamide gel electrophoresis and transferred onto Immunobilon-P membranes (Millipore, Bedford, MA).

Membranes were incubated in 5% nonfat milk for 45 minutes and then were incubated for 18 hours at 4°C with the following 5 primary antibodies at 1:1000 to 1:2000 dilutions in 5% nonfat milk: (1) CHOP, (2) calnexin, (3) phosphorylated (p)-eIF2α, (4) p-PERK, and (5) SOD1 (Cell Signaling Technology, Beverly, MA). After that, the membranes were exposed to goat antirabbit or antimouse 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). Signals were detected using SuperSignal West Femto maximum sensitivity substrate kit (Thermo Scientific, Rockford, IL). All experiments were repeated 3 times with the use of independently prepared tissue lysates.

### Detection of XBP1 mRNA splicing

The mRNA was reverse transcribed to complementary DNA (cDNA) using QuantiTect reverse transcription kit (QIAGEN, Hilden, Germany). The PCR primers for XBP1 were: forward, 5′-GAA CCAGGAGTTAAGAACACG-3′ and reverse, 5′-AGGCAACAGTGTCAGAGT CC-3′. If no XBP1 mRNA splicing occurred, a 205 bp band was produced. A 205 bp and a 179 bp (main band) bands were produced when XBP1 splicing exists.

### Real-time polymerase chain reaction

Total RNA was isolated from embryos using an RNeasy minikit (QIAGEN, Valencia, CA) and reverse transcribed by using the high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA). Real-time polymerase chain reaction (RT-PCR) for BiP, calnexin, IRE1α, eIF2αk3, eIF2αs1, protein disulfide isomerase family A, member 3 (PDIA3), and β-actin were performed using Maxima SYBR Green/ROX quantitative PCR master mix assay (Thermo Scientific). Primers are listed in the Table. RT-PCR and subsequent calculations were performed using a 7700 ABI PRISM sequence detector system (Applied Biosystems).

### Statistical analysis

Densitometric data were presented as means ± SE. Three embryos from 3