Trehalose prevents neural tube defects by correcting maternal diabetes-suppressed autophagy and neurogenesis

Cheng Xu,1 Xuezheng Li,1 Fang Wang,1 Hongbo Weng,1 and Peixin Yang1,2

1Department of Obstetrics, Gynecology, and Reproductive Sciences and 2Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore, Maryland

Submitted 2 April 2013; accepted in final form 19 July 2013

Xu C, Li X, Wang F, Weng H, Yang P. Trehalose prevents neural tube defects by correcting maternal diabetes-suppressed autophagy and neurogenesis. Am J Physiol Endocrinol Metab 305: E667–E678, 2013. First published July 23, 2013; doi:10.1152/ajpendo.00185.2013.—Preexisting maternal diabetes increases the risk of neural tube defects (NTDs). The mechanism underlying maternal diabetes-induced NTDs is not totally defined, and its prevention remains a challenge. Autophagy, an intracellular process to degrade dysfunction protein and damaged cellular organelles, regulates cell proliferation, differentiation, and apoptosis. Because autophagy impairment causes NTDs reminiscent of those observed in diabetic pregnancies, we hypothesize that maternal diabetes-induced autophagy impairment causes NTD formation by disrupting cellular homeostasis, leading to endoplasmic reticulum (ER) stress and apoptosis, and that restoration of autophagy by trehalose, a natural disaccharide, prevents diabetes-induced NTDs. Embryos from nondiabetic and type 1 diabetic mice fed with or without 2 or 5% trehalose water were used to assess markers of autophagy, ER stress, and neurogenesis, numbers of autophagosomes, gene expression that regulates autophagy, NTD rates, indices of mitochondrial dysfunction, and neuroepithelial cell apoptosis. Maternal diabetes suppressed autophagy by significantly reducing LC3-II expression, autophagosome numbers, and GFP-LC3 punctate foci in neuroepithelial cells and by altering autophagy-related gene expression. Maternal diabetes delayed neurogenesis by blocking Sox1 neural progenitor differentiation. Trehalose treatment reversed autophagy impairment and prevented NTDs in diabetic pregnancies. Trehalose resolved homeostatic imbalance by correcting mitochondrial defects, dysfunctional proteins, ER stress, apoptosis, and delayed neurogenesis in the neural tubes exposed to hyperglycemia. Our study demonstrates for the first time that maternal diabetes suppresses autophagy in neuroepithelial cells of the developing neural tube, leading to NTD formation, and provides evidence for the potential efficacy of trehalose as an intervention against hyperglycemia-induced NTDs.

diabetic embryopathy; autophagy; trehalose; neurogenesis; neural tube defects

PREGESTATIONAL DIABETES significantly increases the risk of neural tube defects (NTDs), also known as diabetic embryopathy. There are three to 10 times more NTDs in the offspring of diabetic mothers than in those of nondiabetic mothers (3, 9, 36). Because optimal glycemic control is difficult to achieve and maintain, and even transient exposure to diabetes causes NTDs, maternal diabetes-induced NTDs are significant health problems for both the mother and child. The seriousness of these relationships is emphasized by the upsurge in diabetic pregnancies; nearly 3 million American women and 70 million women worldwide of reproductive age (18–44 yr) have diabetes today, and this number is expected double by 2030. Although diabetic mellitus is a complex metabolic disease, hyperglycemia is the sole mediator of diabetes teratogenicity. Indeed, clinical studies have revealed a strong correlation between the degree of maternal hyperglycemia and the rate and severity of birth defects (15, 29). When whole rodent embryos are cultured in high concentrations of glucose, high glucose induces NTDs similar to those observed in human fetuses exposed to maternal diabetes (55). Therefore, we and others have used an internationally accepted rodent model of type 1 diabetes that mimics the human condition seen in type 1 and type 2 maternal diabetes to study the pathogenesis of maternal diabetes-induced NTDs.

Studies from our group (23, 24) and others (1, 14, 22, 34, 37) reveal that cellular stress, including oxidative stress and endoplasmic reticulum (ER) stress, and enhanced neural progenitor apoptosis play central roles in the induction of NTDs by maternal diabetes. However, the molecular intermediates downstream of hyperglycemia remain elusive. Autophagy, also referred to as macroautophagy, is an intracellular process that degrades dysfunctional proteins and damaged cellular organelles, including ER and mitochondria. Autophagy is initiated by the formation of a double-membrane structure, the autophagosome (13). At least 14 genes are essential for autophagosome formation (30). Multiple lines of indirect evidence suggest that autophagy plays a role in diabetic embryopathy. First, diminished autophagy resulting from deletion of the autophagy/Beclin-1 regulator 1 (Ambra1) gene induces neural progenitor apoptosis and NTDs (12) reminiscent of those observed in diabetic embryopathy. Second, both oxidative stress and ER stress, two central players in diabetic embryopathy, regulate autophagy (6). Third, autophagy can act as either a prosurvival or proapoptotic mediator under physiological and pathological conditions (21). Finally, dysfunctional proteins (48) and damaged cellular organelles such as mitochondria (56) and ER (24) accumulate in cells of neurulation-stage embryos exposed to maternal diabetes.

The main goal of our mechanistic studies is to develop accessible, convenient, and effective prevention strategies against maternal diabetes-induced NTDs. Folate supplements are able to prevent about 70% of NTDs in human (47) and reduce maternal diabetes-induced NTDs in animal models (50). However, recent studies suggest that high maternal folate supplementation during pregnancy may increase the risk of breast cancer (27) and inflammatory bowel diseases (39) in offspring. Studies have shown that inositol can prevent some of the folate-resistant NTDs (16). Like folate, inositol is an intracellular signaling molecule that regulates various cellular processes (51) and thus may adversely affect the offspring of diabetic mothers in the long term. Furthermore,
Trehalose is a naturally occurring disaccharide present in a wide variety of organisms, including plants, bacteria, yeast, and invertebrates. The main function of trehalose in these organisms is to protect cells against various environmental stresses (5). Recently, trehalose was characterized as an autophagy inducer in vitro (38). Here, we show that maternal diabetes diminishes autophagy in the developing neuroepithelium. Trehalose reactivates autophagy and restores cellular homeostasis by preventing cellular stress, mitochondrial defects, and delayed neurogenesis. We propose that trehalose may be another intervention for preventing maternal diabetes-induced NTDs.

MATERIALS AND METHODS

Mice. All animal procedures 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. The GFP-LC3 strain on a C57BL/6J background was created originally by Dr. Noboru Mizushima (31).

In vivo mouse model of diabetic embryopathy and morphologic assessment of NTDs. Our mouse model of diabetic embryopathy was described previously (23, 24). Briefly, female mice were intravenously (iv) injected daily with 75 mg/kg streptozotocin (STZ) over 2 days to induce diabetes. Nondiabetic WT with vehicle injection served as controls. Diabetes was defined as 12-h fasting blood glucose levels of ≥250 mg/dl, which normally occurred at 3–5 days after STZ injections. There is no residual toxic effect caused by STZ in this animal model. Embryos were harvested at embryonic day (E)8.75 (2 PM at E8.5) for analysis and at E11.5 for NTD examination.

Trehalose treatment and trehalose measurement. Concentrations of either 2 or 5% [g/vol (ml)] trehalose (cat. no. T9531; Sigma) were given to WT nondiabetic and diabetic pregnant mice at E5.5 in drinking water. Trehalose levels in maternal serum and embryos were determined by the Trehalose Kit from Megazyme (Wicklow, Ireland).

Electron microscopy. The structures of autophagosome and mitochondria were examined by transmission electron microscopy (EM) in our University EM core facility. Thick sections (1 μm) were cut and visualized at ×100 magnifications to identify the neuroepithelia of E8.75 embryos. Thin sections (80 nm) of identified neuroepithelia were cut and viewed with an electron microscope (Joel JEM-1200EX) under high-power resolution (10, 12, and 25 K) for identification of cellular organelle structures.

Green fluorescent protein (GFP)-LC3 puncta analysis. GFP-LC3 Tg mice were used to quantify autophagy in vivo. E8.75 embryos were harvested, fixed in 4% paraformaldehyde (PFA) in PBS for 30 min at 4°C, quickly frozen, and cryosectioned at 5 μm at 4°C, quickly frozen, and cryosectioned at 5 μm at 4°C. Green fluorescent protein (GFP) fluorescent images in embryonic neuroepithelial cells were recorded by confocal microscopy using a laser-scanning microscope (LSM 510 META; Zeiss). GFP-LC3 punctate foci with a diameter ≥20 pixels in each cell were calculated by Image J software according to the manufacturer’s manual.

Western blotting. Western blotting was performed as described previously (24, 54, 55). Briefly, E8.75 embryos from different experimental groups were sonicated in 80 mM Na-orthovanadate, 1 mM MOPS, and 1% Triton 100 containing a protease inhibitor cocktail (Sigma, St. Louis, MO). Equal amounts of protein and the Precision Plus Protein Standards (Bio-Rad) were resolved by SDS-PAGE gels, 12% gel for caspase 3 and 8 and 10% gel for all other proteins, and transferred onto Immobilon-P or Immobilon-PD™ (for cleaved caspase) membranes (Millipore). To prevent nonspecific bindings, all membranes were incubated with 5% nonfat milk in PBS, pH 7.0, for 45 min and then for 18 h at 4°C with the following primary antibodies at 1:1,000 to 1:2,000 dilutions in 5% nonfat milk: LC3, binding immunoglobulin protein (BiP), CEBP homologous protein (CHOP), phosphor- (p–) eukaryotic initiation factor-2α (eIF2α), p-JNK1/2, p-C-Jun (JunE), and p-Bad (Cell Signaling Technology, Beverly, MA); anti-malondialdehyde (MDA) (no. 442730; EMD); anti-nitrotyrosine, anti-4-hydroxynonenal (4-HNE), and anti-ubiquitin (Millipore); anti-BID (no. PC645; Calbiochem); anti-caspase 3 (Chemicon International, Billerica, MA); and rat caspase 8 (mouse specific) (Alexis Biochemicals, San Diego, CA), which detects both procaspase 8 and cleaved caspase 8. Membranes were exposed to goat anti-rabbit or anti-mouse (Jackson Immunoresearch Laboratories, West Grove, PA) 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 an Amersham ECL Advance Detection Kit (GE Healthcare, Piscataway, NJ) or a Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific), and chemiluminescence emitted from the bands was captured directly using a UVP Bioimage EC3 system (UVP, Upland, CA). Densitometric analysis of chemiluminescence signals was performed by VisionWorks software (UVP). All experiments were repeated three times with the use of independently prepared tissue lysates.

Whole embryo culture. The procedure of whole embryo culture has been described elsewhere previously (53, 55). C57BL/6J mice were paired overnight. The next morning was designated E0.5 if a vaginal plug was present. Mouse embryos at E8.5 (8 AM) were dissected out of the uteri in PBS (Invirogen, La Jolla, CA). The parietal yolk sac was removed using a pair of fine forceps, and the visceral yolk sac was left intact. Embryos (4/bottle) were cultured in 4 ml of rat serum at 38°C at a 30-rpm rotation in the roller bottle system. The culture bottles were gassed with 5% O2-5% CO2-90% N2. Embryos were cultured for 12 h under control (100 mg/dl glucose) and high-glucose (250 mg/dl glucose) conditions. The lysosomal inhibitor chloroquine (Chlo) was added to the cultures in a final concentration of 25 μM.

Real-time PCR. Total RNA was isolated from E8.75 embryos using an RNeasy Mini Kit (Qiagen, Valencia, CA). RT-PCR for ULKI, Beclin1, Atg3, Atg5, Ambra1, p62, Dap, CHOP, BiP, calnexin, eukaryotic translation initiation factor-2α kinase 3, eukaryotic translation initiation factor-2A, and p53, protein disulfide isomerase family A, member 3, IRE-1α and β-actin were performed using ABI TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA). RNA was reverse transcribed by using the high-capacity cDNA archive kit (Applied Biosystems). RT-PCR and subsequent calculations were performed by the Step One Plus Real-time PCR system (Applied Biosystems), which detected the signal emitted from fluorogenic probes during PCR.

Immunofluorescent staining. E8.75 embryos were fixed in the methanol-chloroform-glacial acetic acid solution (6:3:1), embedded in paraffin, and sectioned at 5 μm through the anterior neural tube. DEparaffinized sections were incubated with TuJ1 antibody (no. 21C10; EMD; anti-nitrotyrosine, anti-4-hydroxynonenal (4-HNE), and anti-ubiquitin (Millipore); anti-BID (no. PC645; Calbiochem); anti-caspase 3 (Chemicon International, Billerica, MA); and rat caspase 8 (mouse specific) (Alexis Biochemicals, San Diego, CA), which detects both procaspase 8 and cleaved caspase 8. Membranes were exposed to goat anti-rabbit or anti-mouse (Jackson Immunoresearch Laboratories, West Grove, PA) 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 an Amersham ECL Advance Detection Kit (GE Healthcare, Piscataway, NJ).
Closure at the forebrain-midbrain boundary is an essential step for closure of the anterior neural tube, and failed closure in this area results in exencephaly (2, 8). TUNEL-positive cells were counted in the neural tube area of each section. Based on the counting of total cell nuclei, the sizes of the neural tube areas of each section were relatively constant. Thus, apoptotic cell numbers were expressed as total TUNEL-positive cells per neural tube area. TUNEL assays were performed without the observer knowing the experimental group of the embryo examined.

**Statistical analyses.** Statistical differences were determined by Student’s t-test for two group comparisons and one-way ANOVA for more than two group comparisons using SigmaStat 3.5 software. In ANOVA analysis, a Tukey test was used to estimate the significance of the results. Significant difference between groups in NTD incidence was analyzed by Chi-square test.

**RESULTS**

Maternal diabetes suppresses autophagy in neuroepithelial cells during embryonic neurulation. To determine whether maternal diabetes alters autophagy, we assessed levels of LC3-II (microtubule-associated protein 1 light chain 3), a reliable autophagy marker, in E8.75 embryos. Maternal diabetes decreased LC3-II levels significantly (Fig. 1A). However, LC3-II reduction by maternal diabetes in vivo or high glucose in vitro was not due to increased lysosomal activity, because lysosomal inhibition by Chlo did not block high glucose-induced LC3-II reduction (Fig. 1B).

We then examined whether autophagy impairment occurs specifically in neuroepithelial cells by subjecting sections from the E8.75 developing neural tube (neuroepithelium) to EM. Consistent with the prior finding that autophagy is essential for neurulation (12), our EM study revealed that abundant autophagosomes were present in embryonic neuroepithelial cells under nondiabetic conditions (Fig. 1C). In contrast, the number of autophagosomes in embryonic neuroepithelial cells exposed to diabetes was significantly lower than that in neuroepithelial cells under nondiabetic conditions (Fig. 1C).

To confirm these EM findings, we employed the GFP-LC3 transgenic mouse (GFP-LC3 Tg) to quantify GFP-LC3 punctate foci, which is the most reliable in vivo method for determining autophagy activity (31). Robust autophagic GFP-LC3 puncta were observed in embryonic neuroepithelial cells under nondiabetic conditions (Fig. 1D). In contrast, GFP-LC3 displayed a diffused pattern of expression in neuroepithelial cells exposed to diabetes (Fig. 1D). Maternal diabetes reduced autophagic GFP-LC3 puncta in neuroepithelial cells to approximately one-tenth the level observed in the nondiabetic group.
Fig. 2. Trehalose prevents maternal diabetes-induced neural tube defects (NTDs) and autophagy impairment. A: the trehalose treatment schematic. Trehalose was administrated in drinking water from E5.5. B: NTD rates in ND and DM dams with or without 5% trehalose. NTD rates were calculated by litters; n = 12 dams. C: nos. of normally developed embryos and NTD embryos. Chi² test was used in statistical analysis. D: morphology of E11.5 embryos and histological view of the neural tubes (NT). The blue lines indicate the levels of sections shown below. Bars, 1 mm. E: total resorption rates calculated by group. F: blood glucose levels. G: serum and embryonic levels of trehalose in treated DM dams; n = 12 dams. H: LC3-II levels in E8.75 embryos. I: quantitative GFP-LC3 punctate foci in E8.75 neuroepithelial cells. J: no. of autophagosomes. K: gene expression levels in E8.75 embryos; n = 5 (embryos from 5 dams/group). *Significant difference compared with other groups. In H–J, n = 3 (embryos from 3 dams/group).
(Fig. 1, D and E). We also found that autophagy induction only occurs in neuroepithelial cells during the period of neurulation. Indeed, compared with the neuroepithelial cells, nonneuroepithelial cells under nondiabetic conditions had negligible numbers of autophagic GFP-LC3 puncta, which were not altered by diabetes (Fig. 1F). Collectively, these findings support the hypothesis that maternal diabetes specifically induces autophagy impairment in the neuroepithelial cells.

To determine whether altered gene expression leads to autophagy impairment in diabetes, we surveyed all 17 known genes that regulate autophagy. Maternal diabetes suppressed the expression of ULK1 (Atg1 in yeast), Atg3, Atg5, Beclin1 (Atg6), and Ambra1 (Fig. 2K), all of which are essential for autophagosome formation. Maternal diabetes also simultaneously increased the expression of p62 (Fig. 2K), whose accumulation serves as an index of impaired autophagy (17), and Dap (Fig. 2K), which negatively regulates the autophagy processes. Thus, maternal diabetes induces autophagy impairment by altering gene expression that regulates autophagy.

Trehalose aneolorates maternal diabetes-induced NTDs by reactivating autophagy in neuroepithelial cells. We then sought nontoxic reagents to reverse autophagy impairment in diabetic embryopathy. Trehalose, a naturally occurring disaccharide, has been shown to possess autophagy-stimulating activities in vitro (38). We hypothesized that trehalose treatment would prevent maternal diabetes-induced NTDs by restoring autophagy activity. The NTD rate (4.0 ± 2.2%) in embryos from diabetic dams treated with 5% trehalose water (wt/vol) was comparable with those in embryos from nondiabetic dams with or without trehalose treatment (0.0 ± 0.0%; Fig. 2, A–D) and was significantly lower than that in the diabetic group without trehalose treatment (28.3 ± 4.7%; Fig. 2, A–D).

Treatment with 2% trehalose also significantly reduced maternal diabetes-induced NTDs (Table 1), but to a lesser extent than 5% trehalose treatment, demonstrating that trehalose reduces maternal diabetes-induced NTDs in a dose-dependent manner. Resorption rates did not differ between nondiabetic and diabetic dams with or without trehalose treatments (Fig. 2E), demonstrating that resorption is not a contributing factor in trehalose’s ability to reduce NTDs. In agreement with one published report (42), trehalose treatment did not affect blood glucose levels in either nondiabetic or diabetic mice (Fig. 2F).

Both 2 and 5% trehalose treatment resulted in 162.0 ± 16.9 and 503.9 ± 19.3 μg/ml trehalose, respectively, in maternal blood of diabetic dams (Fig. 2G), and embryonic trehalose levels were 15.8 ± 1.0 and 53.9 ± 3.7 μg/g tissue in the 2 and the 5% groups, respectively (Fig. 2G). Thus, embryonic levels were proportionally related to maternal trehalose levels, demonstrating that maternal exposure to trehalose can effectively reach the developing embryo.

Trehalose treatment also restored maternal diabetes-diminished LC3-II expression (Fig. 2H). The number of autophagic GFP-LC3 puncta in neuroepithelial cells of the diabetic group treated with trehalose was restored to the level of that observed in the nondiabetic groups with or without trehalose treatment (Fig. 2I) and was significantly higher than that of the diabetic group without trehalose treatment (Fig. 2I). Because trehalose did not affect basal autophagy activities in neuroepithelial cells of the nondiabetic group (Fig. 2, H and I), subsequent analyses excluded the nondiabetic group treated with trehalose. Trehalose treatment blocked maternal diabetes-repressed autophago-

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Total Embryos</th>
<th>Total NTD Embryos</th>
<th>NTD Rate</th>
<th>No. of Dams</th>
<th>Resorption Rates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nondiabetic</td>
<td>70</td>
<td>0</td>
<td>0</td>
<td>11</td>
<td>4.2</td>
</tr>
<tr>
<td>Nondiabetic + 2% trehalose</td>
<td>29</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>4.8</td>
</tr>
<tr>
<td>Diabetic</td>
<td>83</td>
<td>23*</td>
<td>27.7</td>
<td>12</td>
<td>4.6</td>
</tr>
<tr>
<td>Diabetic + 2% trehalose</td>
<td>41</td>
<td>4</td>
<td>9.8</td>
<td>5</td>
<td>4.7</td>
</tr>
</tbody>
</table>

NTD, neural tube defect; E10.5, embryonic day 10.5. *Significant difference (P < 0.05) when compared with other groups using Chi2 test. NTD rate = total NTD embryos/total embryos.

Some formation in neuroepithelial cells (Fig. 2J). Next, we determined whether trehalose treatment corrects altered autophagy-related gene expression. Trehalose treatment blocked the downregulation of ULK1, Beclin1, Atg3, Atg5, and Ambra1 and the upregulation of p62 and Dap (Fig. 2K).

Trehalose blocks maternal diabetes-induced ER stress and its associated JNK1/2 activation. Previously, we have demonstrated that ER stress and JNK1/2 activation are two interdependent causal events in maternal diabetes-induced NTD formation (24). We proposed that the autophagy activator trehalose could block ER stress and JNK1/2 activation in diabetic embryopathy. Consistent with our previous finding (24), maternal diabetes triggered ER stress (Fig. 3, A–E), and trehalose treatment abrogated maternal diabetes-increased expression or phosphorylation of the ER chaperone proteins BiP (Binding immunoglobulin protein), CHOP (C/EBP-homologous protein), and eukaryotic initiation factor 2α (eIF2α) (Fig. 3, A–D). These ER chaperone proteins all serve as ER stress markers.

Prolonged ER stress leads to X-box binding protein-1 (XBP-1) mRNA splicing (24), another reliable ER stress marker. Embryos under maternal diabetic conditions exhibited robust XBP-1 splicing, which was manifested by a 179-bp band of the PCR products (Fig. 3E). On the other hand, trehalose treatment diminished XBP1 mRNA splicing (Fig. 3E). ER stress also is known to activate the proapoptotic c-Jun NH2-terminal kinase 1/2 (JNK1/2) (45), which mediates the teratogenicity of diabetes (55). Trehalose completely abolished maternal diabetes-induced JNK1/2 activation (Fig. 3F) and its major downstream effector c-Jun (Fig. 3F).

In addition, maternal diabetes increased the expression of seven ER chaperone genes, and this increase was abolished by trehalose treatment (Fig. 4).

Trehalose removes defective mitochondria and oxidized proteins, thereby protecting mitochondrial function and preventing neuroepithelial cell apoptosis.

Mitochondrial dysfunction is associated with diabetic embryopathy (36). We found that maternal diabetes increased the number of defective mitochondria significantly (Fig. 5, A and B), whereas it did not affect the total number of mitochondria (Fig. 5B). Under diabetic conditions, trehalose treatment significantly reduced the number of defective mitochondria to a level comparable with that in the nondiabetic control group (Fig. 5B). The activation of proapoptotic members of the Bcl-2 family is linked to mitochondrial dysfunction. We found that the level of phosphorylated Bad (inactive form), a proapoptotic Bcl-2 member, was significantly reduced by maternal diabetes but was restored by trehalose treatment (Fig. 5C). Another proapoptotic Bcl-2 member, BID, was activated by cleavage...
under diabetic conditions, whereas its cleavage was prevented by trehalose (Fig. 5D). Cleaved BID translocates to mitochondria and induces mitochondrial damage (25). Thus, trehalose treatment prevents maternal diabetes-induced mitochondrial dysfunction presumably by removing defective mitochondria through autophagy.

Previously, we have identified caspase 8 and caspase 3 as initiator and effector caspases, respectively, in maternal diabetes-induced apoptosis (23, 24). In this study, we demonstrated that trehalose treatment abolished maternal diabetes-induced caspase 3 and 8 cleavage (Fig. 5E) and consequently blocked neuroepithelial cell apoptosis (Fig. 5, F and G).

Fig. 3. Trehalose restored cellular homeostasis by blocking maternal diabetes-induced endoplasmic reticulum (ER) stress and JNK1/2 activation. The protein expression of ER markers, binding immunoglobulin protein (BiP; A and B), C/EBP homologous protein (CHOP; A and C), and phosphorylated eukaryotic initiation factor-2α (p-eIF2α; A and D) in E8.75 embryos of the ND, DM, and DM with 5% trehalose group (DM-Tre). E: X-box binding protein 1 (XBP-1) splicing. F: Levels of p-JNK1/2 and p-c-Jun. *Significant difference compared with other groups; n = 3 (embryos from 3 dams/group).

Fig. 4. Trehalose suppresses maternal diabetes-induced ER chaperone gene expression. mRNA levels of eIF2as1, calnexin, eIF2ak3, BiP, PDIA3, IRE-1α, and CHOP in E8.75 embryos of the ND, DM, and DM-Tre groups. Four embryonic samples (n = 4) from 4 different dams/group were used for RT-PCR. *Significant difference (P < 0.05) compared with the other 2 groups in the same gene.
removes dysfunctional protein caused by oxidative stress. To
determine whether trehalose-induced autophagic activities ef-
fectively clear oxidized proteins under diabetic conditions, we
assessed the levels of two lipidperoxidation markers, 4-HNE
and MDA, as well as nitrotyrosine or ubiquitin-modified pro-
teins. Trehalose blocked maternal diabetes-increased levels of
all of these modified proteins (Fig. 6, A–D).

Trehalose functionally restores maternal diabetes-impaired
neurogenesis during embryonic neurulation. Our findings and
those of others (12) support the conclusion that autophagy is
essential for neural tube closure; however, the functional im-
 pact of autophagy on neurogenesis is unclear. Either delayed
(26) or advanced (18) neurogenesis causes NTDs. To investigate
whether maternal diabetes alters neurogenesis and whether tre-
alose could correct aberrant neurogenesis, we compared patterns
of neural stem cell and neuron marker expression in neural tubes
of embryos under nondiabetic and diabetic conditions with or
without trehalose treatment.

Under nondiabetic conditions, postmitotic neurons express-
ing TuJ1 (class III β-tubulin), a marker of terminal neuronal
differentiation, first appeared in the floor plate at E8.75 (Fig. 7 A),
expanding along the two ventral sides of the developing neural
tube at E9.0 (Fig. 7 B). They then gradually spread to the entire
neural tube from E9.5 to E10.5 (Fig. 7, C and D). However,
under maternal diabetic conditions, TuJ1-expressing neurons
were absent in E8.75 and E9.0 neural tubes (Fig. 7, A and B)
and appeared in the two ventral sides of closed neural tubes at
E9.5 to much less of a degree than their counterparts in the
nondiabetic group (Fig. 7C). At E9.5, TuJ1-expressing neurons were still absent in the open neural tubes of NTD embryos in the diabetic group (Fig. 7C).

At E10.5 the extent of TuJ1-expressing neurons was comparable in closed neural tubes in both the nondiabetic and the diabetic groups (Fig. 7D), whereas the open neural tubes in NTD embryos of the diabetic group displayed relatively lower amounts of TuJ1-expressing neurons (Fig. 7D). As expected, trehalose reversed delayed neurogenesis due to maternal diabetes (Fig. 7, B–D). With trehalose treatment, TuJ1-expressing neurons were observed in E9.0 neural tubes exposed to diabetes with expression patterns that were identical to those in the nondiabetic group (Fig. 7B). They also expanded in a pattern similar to the pattern observed in the nondiabetic group from E9.5 to E10.5 (Fig. 7, C and D).

Furthermore, Sox1, a reliable marker of neural precursors, was expressed in most parts of the E8.75 neural tubes (Fig. 8A), but there was reduced expression in the floor plate (where the first neuron appears) in both the nondiabetic and the diabetic with trehalose treatment groups (Fig. 8B). On the other hand, there was strong Sox1 expression in the entire neural tube of the nontreated diabetic group (Fig. 8B), which had no sign of neurogenesis (Fig. 7B). At E9.5 and E10.5, Sox1 expression rapidly faded in the closed neural tubes of the three groups, whereas unclosed neural tubes in the diabetic group retained robust Sox1 expression (Fig. 8, C and D). These data suggest that neural precursors are unable to differentiate into neurons under diabetic conditions and thus lead to failed neural tube closure and NTD formation.

On the other hand, we found that trehalose treatment corrected maternal diabetes-blocked differentiation of Sox1-positive neural progenitors and thus prevented NTD formation. The critical timing of neural tube closure is between E9.0 and E9.5. Under nondiabetic conditions this is when neurons gradually appeared in the dorsal side, where apoptotic cells were present mainly in the diabetic group (Fig. 5F). Thus, our finding also suggests that Sox1 neural progenitors undergoing neuronal differentiation might undergo apoptosis due to autophagy impairment caused by maternal diabetes.

**DISCUSSION**

Autophagy is highly active and essential for early embryonic development (44). The precise role of autophagy in early embryogenesis may be explained by its clearance of unnecessary cellular components, which thus facilitates remodeling during differentiation (30). Neurulation is a complex process involving multiple and coordinated steps of growth, apoptosis, and differentiation. Imbalanced growth, apoptosis, or differentiation in the developing neural tube results in NTDs. Basal autophagy is essential for normal neurulation, and impaired autophagy caused by deletion of the autophagy regulator Ambral leads to NTDs (12). Using multiple approaches, we have determined that autophagy is highly active in neuroepithelial cells of the developing neural tube, and maternal diabetes suppresses this neuroepithelial cell-specific autophagic activity. Studies in other disease models have suggested that diabetes may induce autophagy (1, 46). Our observation that diabetes suppresses autophagy is consistent with the finding that activities of the autophagy marker GAPDH (7) are decreased by hyperglycemia in embryos at neurulation stages (49). Consistent with the fact that NTDs induced by autophagy impairment (12) resemble those observed in diabetic embryopathy, our data support the hypothesis that impaired autophagy contributes to the pathogenesis of maternal diabetes-induced NTDs.
Maternal diabetes induces 28% NTDs in the C57BL/6J background in our study. This is consistent with the finding from two other groups that maternal hyperglycemia induces more than 22% NTDs (exencephaly) in the C57BL/6J background (19, 41). Our current observation also is in line with our previous finding (23, 24). One report (33) shows no significant increase in NTDs in the C57BL6J strain; however, this study is inconclusive because embryos from nondiabetic C57BL/6J dams have more than 12% NTDs (normal incidence 0–1% NTDs), and the sample size is very small (n = 4 dams). In 2001, there was a report demonstrating that intravenous alloxan-induced diabetes in C57BL/6J induces 14.6 ± 5.0% exencephaly, and the nondiabetic control has 0% NTDs (28). Although STZ-induced diabetes and alloxan-induced diabetes may be different, the findings in these two models come to the same conclusion, i.e., that C57BL/6J is not resistant to diabetic embryopathy. Beside NTDs, maternal diabetes induces more than 20% heart defects in C57BL/6J background (28). By realizing this conflict, we have extensively characterized and validated our models in this particular background by including surgical (anesthesia) controls and insulin-treated diabetic controls (54).

Trehalose reactivates autophagy by restoring LC3-II levels and autophagosome formation in neuroepithelial cells of the developing neural tube and thus prevents maternal diabetes-induced NTDs. Although trehalose has been shown to be an mTOR-independent autophagy inducer (38), the mechanism underlying trehalose’s autophagy stimulatory effect is unknown. Because trehalose prevents alteration of gene expression that regulates autophagy, trehalose may activate autophagy by modulating gene expression. Previous studies have shown that JNK1/2 activation (23, 24), TGFβ pathway disturbance (40), and diminished Wnt signaling (35) are critically involved in the induction of diabetic embryopathy. These three signaling pathways regulate the autophagy process (20, 32, 52). Because trehalose serves as a signaling molecule to control certain pathways in yeast and plants (11), it may activate autophagy in mouse embryonic neuroepithelial cells by modulating the activities of the JNK1/2, TGFβ, and/or Wnt signaling pathways. LC3-II lipidation and its specific recruitment to autophagosomes are crucial steps in autophagy, and trehalose is an integral component of various glycolipids in nonmammalian cells (11). Therefore, trehalose may trigger autophagy by directly participating in the lipidation step of autophagy. Because of the scope of the current study, the mechanism by which trehalose induces autophagy may be explored in our future studies.

We use 2 and 5% trehalose doses in our study. These doses are similar to those reported previously. In a mouse model of Huntington’s disease, 0.2, 2, and 5% trehalose in drinking water are used in a dose-dependent study (42), and 2% trehalose is most effective in the prevention of Huntington’s disease. Two percent trehalose in drinking water prevents muscular dystrophy in a mouse model (10). The optimal dose, 2% trehalose, in the above two studies is lower than our most effective dose, 5% trehalose. However, low doses of trehalose,
such as 2%, require prolonged treatment, from several weeks to several months, to obtain preventive effect (10, 42). We treat pregnant mice with 5% trehalose only for 3 or 5 days, which is remarkably effective in the prevention of diabetes-induced NTDs.

Maternal diabetes induces ER stress, which is responsible for embryonic neuroepithelial cell apoptosis, a causal event leading to NTD formation (24). Autophagy is required for cells under ER stress to survive (4). Indeed, trehalose effectively suppresses ER stress markers and subsequently blocks neuroepithelial cell apoptosis. These functions of trehalose are attributed presumably to its autophagy stimulatory property.

Autophagy is the only cellular process to remove damaged cellular organelles such as the ER and the mitochondria. Mitochondrial dysfunction is a key mechanism underlying diabetic embryopathy (56). A significant number of defective mitochondria are observed in neuroepithelial cells exposed to maternal diabetes, and trehalose is able to completely remove these damaged mitochondria. Our previous studies have demonstrated that oxidized proteins are constant features of diabetic embryopathy (48). We observed dysfunctional proteins, including lipid peroxidation markers (4-HNE and MDA), nitrotyrosine, and ubiquitin-modified proteins, in neurulation stage embryos under maternal diabetic conditions. Trehalose effectively removes these dysfunctional proteins. Thus, trehalose restores cellular homeostasis by cleaning dysfunctional cellular components and consequently prevents cells from undergoing apoptosis and NTD formation. We also assessed whether maternal diabetes impacts cell proliferation, using the bromodeoxyuridine (BrdU) incorporation assay. We injected BrdU 1, 2, and 4 h before embryo harvest and did not observe any difference in neuroepithelial cell proliferation assessed by BrdU immunostaining between the nondiabetic group and the diabetic group. One reason for this observation is that virtually all cells are proliferating in the neuroepithelium, and the impact of diabetes on cell proliferation is masked by the high basal proliferative activity. The other reason is that the BrdU incorporation method may not be suitable in assessing cell proliferation under this circumstance. Whether maternal diabetes influences cell proliferation warrants further investigation.

We have demonstrated that excessive apoptosis is present in Sox1-positive neural progenitors of the developing neural tube under maternal diabetic conditions (23). Neural progenitor elimination by apoptosis affects cell differentiation in the neural tube and thus may adversely impact the ontogeny of neurons, a process called neurogenesis. In fact, altered neurogenesis, either delayed or advanced neurogenesis, causes NTD formation (18, 26). Maternal diabetes delays the appearance of TuJ1-positive neurons, and embryos with delayed neurogenesis exhibit unclosed neural tubes in which neural progenitors remain undifferentiated. Because autophagy impacts cell proliferation, differentiation, and apoptosis (12), it may positively regulate neurogenesis by facilitating neural tube closure. Indeed, trehalose acts as an autophagy inducer by facilitating neural progenitor differentiation and correcting delayed neu-

Fig. 8. Trehalose corrected maternal diabetes-delayed neurogenesis. A–D: Sox1 neural progenitors (red) in the developing neural tubes of ND, DM, and DM-Tre mice. Cell nuclei were stained by DAPI (blue). Embryos from 3 dams/group were examined, and representative images are shown. Bars: 30 (A), 60 (B and C), and 150 μm (D).
REFERENCES
C.X. and P.Y. approved the final version of the manuscript; P.Y. contributed
H.W., and P.Y. analyzed the data; C.X., X.L., F.W., and P.Y. interpreted the
GRANTS
of Maryland School of Medicine for editing the manuscript.
ACKNOWLEDGMENTS
We thank Dr. Noboru Mizushima at the Tokyo Medical and Dental
University for providing the GFP-LC3 mice and Dr. Graham Aberdeen
and Hua Li, both at the University of Maryland School of Medicine, for their
technical assistance. We are grateful to Dr. E. Albert Reece at the University
of Maryland School of Medicine for editing the manuscript.
GRANTS
This study is supported by National Institute of Diabetes and Digestive and
Kidney Diseases Grants R01-DK-083243, R01-DK-083770, and R56-DK-
095360.
DISCLOSURES
There are no potential conflicts of interest relevant to this article to report.
The authors declare no competing financial interests.
AUTHOR CONTRIBUTIONS
C.X., L.W., and H.W. performed the experiments; C.X., L.L., F.W.,
H.W., and P.Y. analyzed the data; C.X., L.L., F.W., and P.Y. interpreted the
results of the experiments; C.X., L.L., F.W., and P.Y. prepared the figures;
C.X. and P.Y. approved the final version of the manuscript; P.Y. contributed
to the conception and design of the research; P.Y. drafted the manuscript; P.Y.
edited and revised the manuscript.
REFERENCES
1. Adastra KL, Chi MM, Riley JK, Moley KH. A differential autophagic
response to hyperglycemia in the developing murine embryo. Reproduction
2. Barbera JP, Rodriguez TA, Greene ND, Weninger WJ, Simeone A,
Copp AJ, Beddington RS, Dunwoodie S. Folic acid prevents exencephal-
malformations, as the only known effective prevention against NTDs,
caused by other factors.
3. Correa A, Gilboa SM, Besser LM, Botto LD, Moore CA, Hobbs CA,
Cleves MA, Riehle-Colarusso TJ, Walker DK, Reece EA. Diabetes
mellitus and birth defects. Am J Obstet Gynecol 199: 237 e231–e239,
2008.
4. Davies JE, Sarkar S, Rubinsztein DC. Trehalose reduces aggregate
formation and delays pathology in a transgenic mouse model of oculo-
5. Elbein AD, Pan YT, Pustuszak I, Carroll D. New insights on trehalose:
6. Finia GM, Stoykova A, Romagnoli A, Giunta L, Di Bartolomeo S,
Nardacci R, Corazzari M, Fuoco C, Ucar A, Schwartz P, Gruss P,
Piacentini M, Chowdhury K, Cecconi F. Ambral regulates autophagy and
7. Fujita N, Itoh T, Omori H, Fukuda M, Noda T, Yoshimori T. The
Atg16L complex specifies the site of LC3 lipidation for membrane
in vivo and high glucose concentration in vitro increases apoptosis in rat
9. Greene MF, Hare JW, Cloherty JP, Benacerraf BR, Soeldner JS.
First-trimester hemoglobin A1c and risk for major malformation and
spontaneous abortion in diabetic pregnancy. Teratology 39: 225–231,
1989.
10. Greene ND, Copp AJ. Insolot prevents folate-resistant neural tube
11. Inami Y, Waguri S, Sakamoto A, Kouno T, Nakada K, Hino O,
Watanabe S, Ando J, Iwadate M, Yamamoto M, Lee MS, Tanaka K,
Komatsu M. Persistent activation of Nrf2 through p62 in hepatocellular
F. Targeted disruption of mammalian hairy and Enhancer of split ho-
omolog-1 (Hes-1) leads to up-regulation of neural helix-loop-helix factors,
premature neurogenesis, and severe neural tube defects. Genes Dev 9:
13. Kamimoto Y, Sugiyama T, Kihira T, Zhang L, Murabayashi N,
Umekawa T, Nagao K, Ma N, Toyoda Y, Yodol J, Sagawa N. Trans-
genric mice overproducing human thioredoxin-1, an antioxidative and
anti-apoptotic protein, prevents diabetic embryopathy. Diabetes 53:
MR, Sugimoto K, Miyazono K. Autophagy is activated by TGF-beta and
potentiates TGF-beta-mediated growth inhibition in human hepatocellular
16. Li R, Chase M, Jung SK, Smith PJ, Loeken MR. Hypoxic stress in
diabetic pregnancy contributes to impaired embryo gene expression and
defective development by inducing oxidative stress. Am J Physiol Endo-
JNK1/2 activation triggers proapooptotic signaling and apoptosis that leads
18. Li X, Xu C, Yang P. c-Jun NH2-terminal kinase 1/2 and endoplasmic
reticulum stress as interdependent and reciprocal causation in diabetic
interacting protein, mediates cytochrome c release from mitochondria in
response to activation of cell surface death receptors. Cell 94: 481–490,
1998.
20. Lupu F, Alves A, Anderson K, Doyle V, Lacy E. Nuclear pore compos-
sition regulates neural stem/progenitor cell differentiation in the mouse
Doppman JL. Effect of maternal and postwean-
Diabetic embryopathy in C57BL/6J mice. Altered fetal sex ratio and
altered development by inducing oxidative stress. Am J Physiol Endo-
23. Rees EA, Yang P. Oxidative stress-induced JNK1/2 activation triggers
24. Miller E, Hare JW, Cloherty JP, Dunn PJ, Gleason RE, Soeldner JS,
Kitzmiller JL. Elevated maternal hemoglobin A1c in early pregnancy and
TREHALOSE-INDUCED AUTOPHAGY IN DIABETIC EMBRYOPATHY


