Oxidative Stress–Induced JNK1/2 Activation Triggers Proapoptotic Signaling and Apoptosis That Leads to Diabetic Embryopathy

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Oxidative stress and apoptosis are implicated in the pathogenesis of diabetic embryopathy. The proapoptotic c-Jun NH_2-terminal kinases (JNK)1/2 activation is associated with diabetic embryopathy. We sought to determine whether *1*) hyperglycemia-induced oxidative stress is responsible for the activation of JNK1/2 signaling, *2*) JNK1 contributes to the teratogenicity of hyperglycemia, and *3*) both JNK1 and JNK2 activation cause activation of downstream transcription factors, caspase activation, and apoptosis, resulting in neural tube defects (NTDs). Wild-type (WT) embryos from nondiabetic WT dams and WT, superoxide dismutase (SOD) 1–overexpressing, *jnk1*^{+/−}, *jnk1*^{−/−}, and *jnk2*^{−/−} embryos exposed to maternal hyperglycemia were used to assess JNK1/2 activation, NTDs, activation of transcription factors downstream of JNK1/2, caspase cascade, and apoptosis. SOD1 overexpression abolished diabetes-induced activation of JNK1/2 and their downstream effectors: phosphorylation of c-Jun, activating transcription factor 2, and E twenty-six–like transcription factor 1 and dephosphorylation of forkhead box class O3a. *jnk1*^{−/−} embryos had significantly lower incidences of NTDs than those of WT or *jnk1*^{+/−} embryos. Either *jnk1* or *jnk2* gene deletion blocked diabetes-induced activation of JNK1/2 signaling, caspases 3 and 8, and apoptosis in Sox1^{+} neural progenitors of the developing neural tube. Our results show that JNK1 and JNK2 are equally involved in diabetic embryopathy and that the oxidative stress–JNK1/2–caspase pathway mediates the proapoptotic signals and the teratogenicity of maternal diabetes. *Diabetes* 61:2084–2092, 2012

T he high rate of birth defects associated with diabetic pregnancy is a significant public health problem that results in major congenital malformations in up to 10% of newborn babies (1). Major malformations in children of pregestational diabetic women are neural tube defects (NTDs) and cardiovascular defects. The recent rise in the number of diabetic women (2) makes this pregnancy complication a continuing issue (3). Because glycemic control in diabetic women is difficult to achieve and maintain (4), malformation rates of offspring in diabetic women, even under modern preconception care, are approximately six times higher than those in nondiabetic women (3). Therefore, therapeutic interventions other than the achievement of euglycemia are needed to avert diabetes-associated adverse pregnancy outcomes. Mechanistic studies provide important insights in the development of accessible, convenient, and effective prevention strategies for diabetic embryopathy.

Experimental data support the conclusion that congenital malformations during maternal hyperglycemia are the result of a disruption in the balance between intracellular reactive oxygen species and endogenous antioxidant capacities (5–10). Thus, embryonic malformations under hyperglycemic conditions are the result of oxidative stress. Oxidative stress induced by hyperglycemia stimulates apoptosis in a variety of cell types (11). Maternal hyperglycemia increases cell apoptosis in the embryo (12–14). Apoptosis is specifically seen in neuroepithelial cells, which are particularly susceptible to hyperglycemic damage (14). Hyperglycemia-induced apoptosis involves caspase cascade activation (15), and caspase inhibitors abolish hyperglycemia-induced apoptosis and prevent hyperglycemia-induced malformations (16). Caspases are classified as initiator caspases that trigger apoptosis and effector caspases that execute apoptosis and serve as apoptosis indices. We have identified caspase 8 as the initiator caspase in diabetic embryopathy (17) and have consistently used cleaved caspase 3 as an indicator for apoptosis (15). Although multiple studies suggest that excess cell death, at least in the central nervous system, may contribute to the abnormal development of structures in the embryos of diabetic animals (18,19), it is elusive how oxidative stress induces apoptosis in diabetic embryopathy.

c-Jun NH_2-terminal kinases (JNK)1/2 mediate oxidative stress–induced apoptosis in a variety of cellular systems. In maternal diabetes, JNK1/2 activation in the embryos and yolk sacs correlates with excessive apoptosis and NTDs (15,20,21). Antioxidant supplementation blocks hyperglycemia-induced JNK1/2 activation, resulting in prevention of diabetic embryopathy (22). This finding suggests that maternal hyperglycemia-induced oxidative stress is responsible for JNK1/2 activation and the subsequent activation of apoptotic pathways. We have recently demonstrated that a specific pharmacological JNK1/2 inhibitor decreases hyperglycemia-induced malformations, and a JNK1/2 activation inducer mimics the teratogenic effect of hyperglycemia (20). Most striking, *jnk2* gene deletion significantly reduces maternal diabetes-induced embryonic malformations (20). These findings suggest that JNK1/2 activation plays a causative role in the induction of diabetic embryopathy. However, the precise mechanism underlying JNK1/2 activation–mediated diabetic embryopathy needs to be further investigated.

The JNK1/2 pathway specifically responds to stress-induced signals that drive apoptosis. The specific molecular targets of JNK1/2 include transcription factor AP-1 (mainly c-Jun, JunB, and activating transcription factor 2 [ATF-2]),

forkhead box class o (Foxo) factors (23), and many other nontranscription factors, such as Bcl-2 proteins, which are closely related to apoptotic cell death factors (24). Substantial genetic and pharmacological evidence suggests that JNK1/2 serve as key proapoptotic mediators during oxidative stress (25). Mice having null mutations in any single JNK1/2 gene develop normally. Double knockout mutants ($jnk1^{-/-}$ $jnk2^{-/-}$) die in utero as a result of abnormal apoptosis in the brain (26). In addition, JNK1 and JNK2 share 83% homology in their protein sequences (27). These observations suggest that JNK1 and JNK2 are functionally redundant in development. JNK1 and JNK2 are also functionally distinct because they have differential substrate preferences and localization selectivity (28). The JNK1/2 single gene null mice, thus, are useful models for delineating the redundant or distinct roles of JNK1 and JNK2 in diabetic embryopathy.

We previously reported that superoxide dismutase (SOD) 1 overexpression significantly reduces diabetes-induced malformations (29). Therefore, we used SOD1 transgenic (Tg) mice in the current study to determine whether oxidative stress is responsible for diabetes-induced JNK1/2 activation. Because JNK1 and JNK2 mediate similar biological effects, we sought to determine whether JNK1, like JNK2 (20), was critically involved in the induction of diabetic embryopathy. We found that the oxidative stress–induced JNK1/2 activation mediated the teratogenicity of maternal diabetes through the induction of apoptosis.

RESEARCH DESIGN AND METHODS

Reagents. C57BL/6J mice (median body weight 22 g) were purchased from The Jackson Laboratory (Bar Harbor, ME). Sustained-release insulin pellets (Linplant) were purchased from LinShin Canada. Streptozotocin (STZ) from Sigma-Aldrich was dissolved in sterile 0.1 mol/L citrate buffer (pH 4.5).
In vivo mouse model of diabetic embryopathy and morphologic assessment of NTDs. The procedures for animal use were approved by the institutional animal care and use committee.

For decades, we (20,29,30) and others (31–36) have used an internationally accepted rodent model of STZ-induced diabetes in research of diabetic embryopathy. Ten-week-old (median body weight 22 g) $jnk1$ heterozygous ($jnk1^{+/-}$), $jnk2$ homozygous ($jnk2^{-/-}$), and wild-type (WT) female mice in C57BL/6J background (The Jackson Laboratory) were intravenously injected daily with 75 mg/kg STZ during a 2-day period to induce diabetes. Hemizygous SOD1-Tg mice in C57BL/6J background were revived from frozen embryos by The Jackson Laboratory, and a subsequent colony of SOD1 mice was established in the laboratory. The transgene in the SOD1-Tg mice is the full-length human SOD1 gene (hSOD1) (37). To generate SOD1 embryos, we crossed SOD1-Tg male mice with diabetic WT female mice. Nondiabetic WT and $jnk1^{+/-}$ female mice with vehicle injection served as nondiabetic controls and were mated with respective genotypes of male mice. Blood glucose levels were monitored daily by tail vein puncture and using the FreeStyle Blood Glucose Monitoring System (TheraSense; Abbott Diabetes Care, Alameda, CA). Diabetes was defined as 12-h fasting blood glucose levels ≥250 mg/dL, which normally occurred at 3–5 days after STZ injections. To successfully establish pregnancies, insulin pellets were subcutaneously implanted in diabetic mice to restore euglycemia (80–150 mg/dL) prior to mating. WT, $jnk1^{+/-}$, or $jnk2^{-/-}$ female mice were mated with male mice of the same respective genotype. Embryos from nondiabetic WT mice and from diabetic WT mice crossed with SOD1-Tg male mice and $jnk2^{-/-}$ embryos from $jnk2^{-/-}$ mice were harvested at embryonic day (E)8.75 (2:00 P.M. at E8.5) for biochemical and molecular analysis. $jnk1^{+/-}$ female diabetic mice mated with $jnk1^{+/-}$ male mice produced WT ($jnk1^{+/+}$), $jnk1^{+/-}$, and $jnk1^{-/-}$ embryos under the same maternal diabetic conditions for malformation assessment. STZ has a very short (30 min) half-life, and pregnancies in our study were established after 1–2 weeks of STZ injections. Our previous study using the STZ/insulin-administered and the non–STZ-administered animals in the mouse model (15) and in the rat model (7,38) did not detect any difference in embryonic development. Therefore, there is no residual toxic effect of STZ in our animal model.

On the basis of a published report (39), male and female mice were paired at 3:00 P.M., and day 0.5 (E0.5) of pregnancy was established by the presence of the vaginal plug the next morning (8:00 A.M.). On day 5.5 of pregnancy (E5.5),

insulin pellets were removed to permit frank hyperglycemia (>250 mg/dL glucose level) so the developing embryos would be exposed to a hyperglycemic environment during the critical period of closure of the neural tube (neurulation) (E8–E10.5). Therefore, our mouse model of diabetic embryopathy specifically impacts neurulation and induces NTDs. On the basis of our extensive studies (15,20), insulin treatment from E0.5 to E5.5 is essential for successful implantation establishment and, thus, prevents early embryonic lethality (resorption) caused by hyperglycemic exposure at early embryonic stages (≤E5.5). Control nondiabetic pregnant mice were sham operated on for insulin implantation and removal. E10.5 embryos were examined for malformations. Normal embryos were classified as possessing a completely closed neural tube and no evidence of other malformations. Malformed embryos were classified as showing evidence of failed closure of the anterior or posterior neural tubes resulting in anencephaly, a major type of NTD. Even at E15.5, we still observed NTD embryos with no sign of delayed embryonic development (Fig. 2D and E). At late embryonic stages, anencephaly leads to the absence of skull and, consequently, an uncovered brain (Fig. 2E). Embryos were first examined for the open neural tube structure under a Leica MZ16F stereomicroscope (Bannockburn, IL) to identify NTDs. Images of embryos were captured by a DFC420 5 MPix digital camera with software (Leica). In Fig. 2A–C, the open neural tube structure in embryos with NTDs was verified by series sections through the anterior or posterior neural tubes, and the open neural tube structure at the ventral side was shown. Because our model specifically induces NTDs at E10.5, other major structural malformations, such as cardiovascular defects, which are not formed until late embryonic stages (E15.5), were not examined.
Genotyping of embryos. We genotyped embryos from $jnk1^{+/-}$ female mated with $jnk1^{+/-}$ male mice, and embryos from diabetic WT mice crossed with SOD1-Tg male mice, by using the yolk sac DNA according to The Jackson Laboratory's protocol.
Western blotting. Western blotting was performed as previously described (20). The following antibodies were used: phospho (p)-JNK1/2, SOD1, JNK2, JNK1, p-E twenty-six–like transcription factor 1 (p-Elk1) (Ser383), p-c-Jun (Ser63), p-ATF-2 (Thr71), Sox1, and p-Foxo3a (Thr32) (Cell Signaling, Beverly, MA), anti-caspase 3 (Chemicon, Billerica, MA), anti-caspase 8 (Alexis Biochemicals, San Diego, CA), and anti–β-actin (Abcam, Cambridge, MA).
Determining numbers of apoptotic cells using transferase-mediated dUTP nick-end labeling assay. Frozen 10 μm-thick embryonic sections through the anterior neural tube were fixed with 4% paraformaldehyde in PBS and incubated with transferase-mediated dUTP nick-end labeling (TUNEL) reaction agents (In Situ Cell Death Detection kit; Roche, Indianapolis, IN) with or without Sox1 immunostaining. TUNEL$^+$ cells in the neural tube area of each section were counted. On the basis of total cell nuclei counts, the sizes of the neural tube areas of each section were relatively constant. Thus, apoptotic cell numbers were expressed as total TUNEL$^+$ cells per neural tube area per section. TUNEL assays were performed without the observer knowing the experimental group of the embryo examined.
Statistical analysis. Densitometric data were presented as means ± SE. One-way ANOVA was performed using SigmaStat 3.5 software. In ANOVA analysis, Tukey test was used to estimate the significance of the results. Significant difference between groups in malformation incidences was analyzed by χ^2 test. Statistical significance was accepted at $P < 0.05$.

RESULTS

SOD1 overexpression blocks hyperglycemia-induced JNK1/2 activation and its downstream signaling. We hypothesized that oxidative stress is responsible for diabetes-induced JNK1/2 activation and its downstream signaling in embryonic tissues. To test this hypothesis, E8.75 embryos were harvested from nondiabetic WT and diabetic WT mice that were mated with SOD1-Tg male mice to avoid any potential maternal influence by the SOD1 transgene. 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. p-JNK1/2 levels in WT embryos exposed to maternal hyperglycemia were significantly higher than those in WT embryos from nondiabetic WT mice (Fig. 1A and B). SOD1-overexpressing embryos expressed high levels of hSOD1 protein (Fig. 1A and C). SOD1-overexpressing embryos had significantly lower levels of p-JNK1/2 and their downstream effectors, p-c-Jun,

p-ATF-2, and p-Elk1, compared with those in WT embryos from the same group of diabetic WT mice mated with SOD1-Tg male mice (Fig. 1A–D). Levels of p-JNK1/2, p-c-Jun, p-ATF-2, and p-Elk1 in SOD1-overexpressing embryos exposed to maternal hyperglycemia did not differ from those in WT embryos from nondiabetic WT mice (Fig. 1A–D). Maternal hyperglycemia-suppressed p-Foxo3a levels, indices of Foxo3a inactivation, were restored in SOD1-overexpressing embryos (Fig. 1C and D). Under nondiabetic conditions, SOD1 overexpression did not affect the basal levels of p-JNK1/2 (Fig. 1E).

jnk1 gene deletion ameliorates diabetes-induced NTDs. Western blotting detects two bands of p-JNK1/2: p54JNK and p46JNK. Both p54JNK and p46JNK are highly phosphorylated in embryos exposed to maternal diabetes. Since the *jnk1* gene contributes to both p54JNK and p46JNK, we proposed that JNK1 activation plays a role in the induction of diabetic embryopathy. $jnk1^{+/-}$ mice were used to test this hypothesis because $jnk1^{-/-}$ mice are poor breeders. To generate $jnk1^{-/-}$ embryos, diabetic $jnk1^{+/-}$ female mice were mated with $jnk1^{+/-}$ male mice. The mating of $jnk1^{+/-}$ male and female mice produced WT, $jnk1^{+/-}$, and $jnk1^{-/-}$ embryos under the same maternal diabetic

conditions (Fig. 2). This mating scheme resulted in effective comparison in malformation rates of these three genotype embryos while it minimized the maternal influence. Average blood glucose levels in $jnk1^{+/-}$ mothers at E8.5–E10.5, a critical period of organogenesis, were 371.8 ± 27.2 (SE) mg/dL. E10.5 embryos from 11 $jnk1^{+/-}$ mothers were examined for malformations (Table 1). As described above, malformed embryos were classified as showing evidence of failed closure of the anterior or posterior neural tubes, namely, NTDs. Other defects, such as heart defects, were not examined because they develop at late embryonic stages (E15.5). In total, 80 embryos were harvested, and the ratios of WT, $jnk1^{+/-}$, and $jnk1^{-/-}$ embryos were close to the expected ratio, 1:2:1 (Table 1). There was a total of 22 $jnk1^{-/-}$ embryos, with 4 of them having NTDs. The malformation rate of $jnk1^{-/-}$ embryos was 18.2% (Table 1). Of 40 $jnk1^{+/-}$ embryos harvested, 22 of them had NTDs (Table 1). The malformation rate of $jnk1^{+/-}$ embryos was 55.0% (Table 1). A total of 10 out of 18 WT embryos exhibited NTDs, and the malformation rate of WT embryos was 55.6%, which was comparable with that in $jnk1^{+/-}$ embryos (Table 1). A representative litter from a diabetic $jnk1^{+/-}$ female mated with a $jnk1^{+/-}$ male
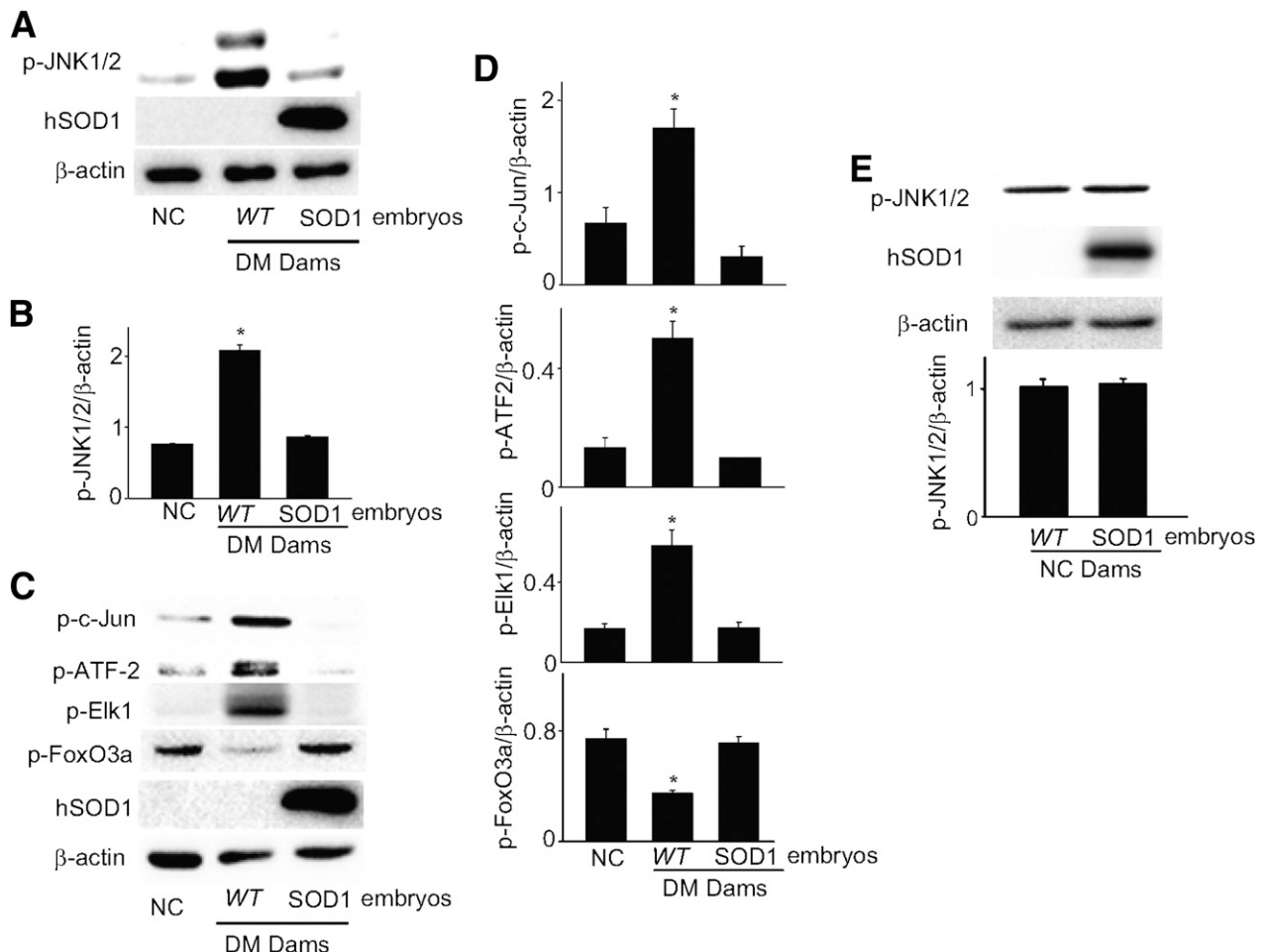

FIG. 1. Oxidative stress drives maternal hyperglycemia-induced JNK1/2 activation and its downstream signaling. Levels of p-JNK1/2 (*A* and *B*) and p-c-Jun, p-ATF-2, p-Elk1, and p-Foxo3a (*C* and *D*) were determined in E8.75 WT embryos from nondiabetic control (NC) and WT embryos and SOD1-overexpressing embryos from diabetic (DM) WT mice mated with SOD1-Tg male mice. *E*: p-JNK1/2 in WT and SOD1-overexpressing embryos in the NC group. SOD1-overexpressing embryos harbor the hSOD1 transgene, and SOD1 is detected by a human-specific antibody. Experiments were repeated three times using samples from different dams in each group. In *B* and *D*, densitometric analysis showed that SOD1 overexpression blocked maternal hyperglycemia-induced JNK1/2 activation and its downstream signaling. *$P < 0.05$ compared with the other two groups.
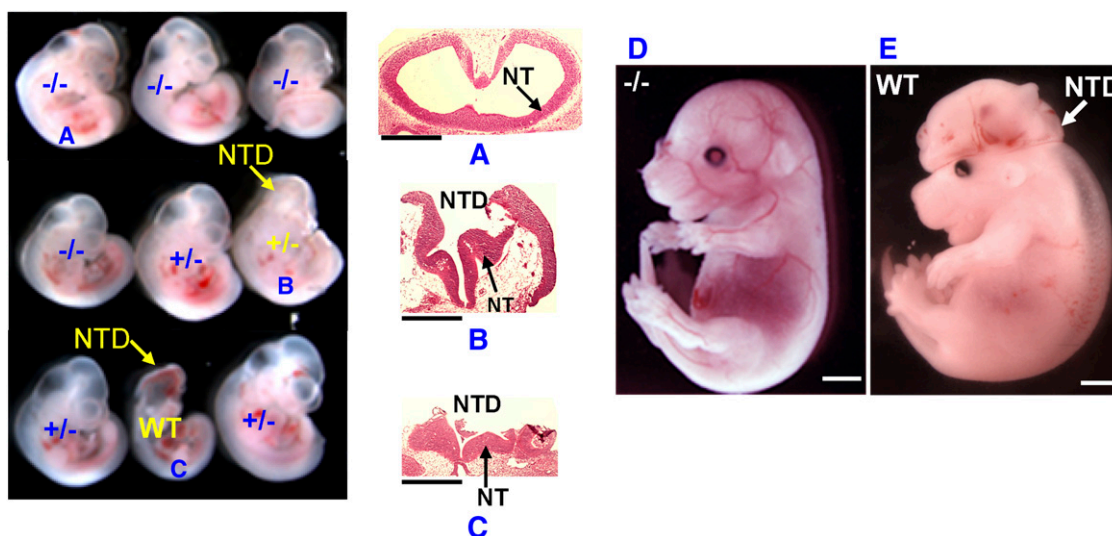
FIG. 2. One representative litter from one of the diabetic *jnk1*^{+/−} dams in Table 1. *jnk1*^{−/−} (−/−) embryos have lower incidences of NTDs than those in WT (*jnk1*^{+/+}) and *jnk1*^{+/−} (+/−) E10.5 embryos under the same maternal diabetic conditions. *A–C*: Representative images of whole embryos (*left*) from one litter of *jnk1*^{+/−} diabetic mice mated with *jnk1*^{+/−} male mice. Arrows point to NTD embryos with open neural tubes (NTs). Pictures of frontal sections (*middle panel*) show open NT structures in a *jnk1*^{+/−} embryo (*B*) and a WT embryo (*C*) that exhibit NTDs, whereas a normally developed *jnk1*^{−/−} embryo has a closed NT structure (*A*). *D* and *E*: Under diabetic conditions, a normally developed E15.5 *jnk1*^{−/−} embryo (*D*) and a WT embryo at E15.5 with anencephaly (NTD) (*E*) in which the brain was uncovered. Scale bars = 1 mm. (A high-quality color representation of this figure is available in the online issue.)

showed NTDs in WT (Fig. 2*C*) and *jnk1*^{+/−} (Fig. 2*B*) embryos, whereas none of the four *jnk1*^{−/−} embryos in the same litter had NTDs (Fig. 2*A*). The NTDs observed were typically anencephaly (Fig. 2). In agreement with our previous study (15,20), we still observed NTD embryos at E15.5 (Fig. 2*D* and *E*) with no sign of delayed development. Consistent with the previous observation that targeted deletion of *jnk1* gene does not affect embryonic development, no NTDs were observed in WT, *jnk1*^{+/−}, and *jnk1*^{−/−} embryos from nondiabetic *jnk1*^{+/−} mice injected with saline (Table 2).

JNK1/2 deficiency in either *jnk2*^{−/−} or *jnk1*^{−/−} embryos blocks hyperglycemia-induced downstream signals of JNK activation. Because both JNK1 (Table 1) and JNK2 deficiency (20) reduce maternal diabetes-induced NTDs, we propose that JNK1 and JNK2 equally contribute to the activation of JNK1/2 downstream effectors in diabetic embryopathy. To this end, E8.75 *jnk2*^{−/−} embryos from JNK2KO mice and *jnk1*^{−/−} embryos from

jnk1^{+/−} females mated with *jnk1*^{+/−} males were used to detect the phosphorylated levels of critical JNK1/2 downstream effectors c-Jun, ATF-2, Elk1, and Foxo3a. The activation of these transcription factors is critical for the induction of apoptotic genes. Levels of p-c-Jun, p-ATF-2, and p-Elk1 in WT embryos exposed to maternal hyperglycemia were significantly higher than those in embryos from nondiabetic WT mice (Fig. 3*A* and 4*A*). This finding suggests that hyperglycemia activates c-Jun, ATF-2, and Elk1. Hyperglycemia significantly reduced p-Foxo3a levels in WT embryos exposed to maternal hyperglycemia (Fig. 3*B* and 4*B*), demonstrating that Foxo3a activities are induced by hyperglycemia because dephosphorylation of Foxo3a correlates with its transcriptional activities. Levels of p-c-Jun, p-ATF-2, and p-Elk1 in *jnk2*^{−/−} embryos exposed to maternal hyperglycemia were significantly lower than those in WT embryos from diabetic mice and were comparable with those in embryos from nondiabetic mice (Fig. 3*A* and *C–E*). In a similar manner, *jnk1*^{−/−} embryos exposed to hyperglycemia had significantly lower levels of p-c-Jun, p-ATF-2, and p-Elk1 than those in WT embryos from the same group of diabetic *jnk1*^{+/−} mothers (Fig. 4*A*

TABLE 1

Malformation rates and genotypes of E10.5 embryos from JNK1^{+/−} diabetic female mice mated with JNK1^{+/−} male mice

Embryos of JNK1^{+/−} diabetic mice	Total number of embryos	Total number of malformed embryos	NTD rate (%)
WT (*JNK1*^{+/+})	18	10[a]	55.6
Heterozygous (*JNK1*^{+/−})	40	22[a]	55.0
Homozygous (*JNK1*^{−/−})	22	4[b]	18.2

Embryos are from 11 JNK1^{+/−} diabetic mice. Average glucose level was 371.8 ± 27.2 mg/dL (mean ± SE) during E8.5–E10.5. Malformation rates = malformed embryos/total number of embryos. The resorption rate of embryos from JNK1^{+/−} diabetic mice is 1.25%, which is the same as that of embryos from JNK1^{+/−} nondiabetic mice. Values denoted with [a] are not significantly different, and the value denoted with [b] indicates significant difference when compared to the other two groups by χ² test (P < 0.05).

TABLE 2

Malformation rates and genotypes of E10.5 embryos from JNK1^{+/−} nondiabetic female mice mated with JNK1^{+/−} male mice

Embryos of JNK1^{+/−} nondiabetic mice	Total number of embryos	Total number of malformed embryos	NTD rate (%)
WT (*JNK1*^{+/+})	19	0	0
Heterozygous (*JNK1*^{+/−})	37	0	0
Homozygous (*JNK1*^{−/−})	19	0	0

Embryos are from 11 JNK1^{+/−} nondiabetic mice. Average glucose level was 173.8 ± 11.9 mg/dL (mean ± SE) during E8.5–E10.5. Malformation rates = malformed embryos/total number of embryos. The resorption rate is 1.25%.
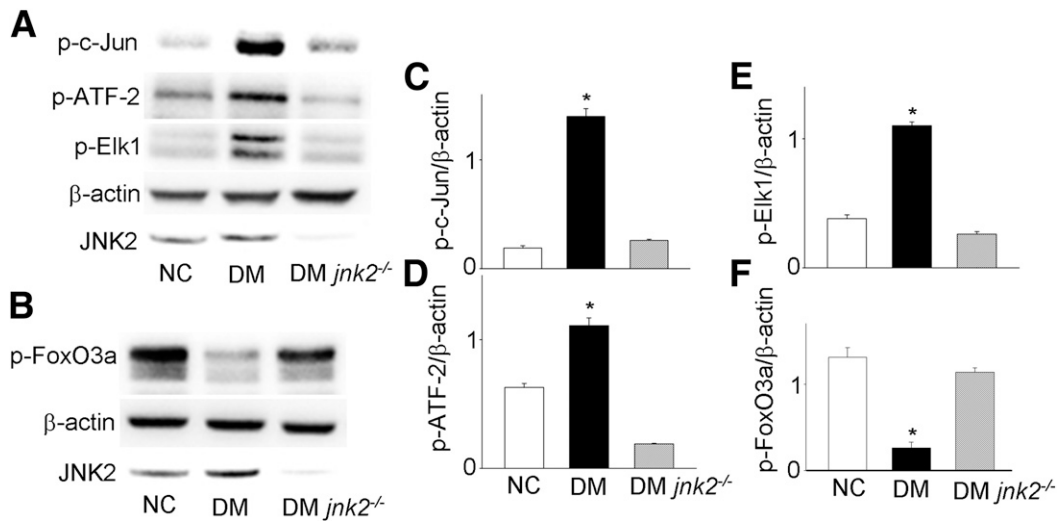
FIG. 3. JNK2 deficiency in *jnk2*$^{-/-}$ embryos blocks the activation of transcriptional factors downstream of JNK1/2. p-c-Jun, p-ATF-2, p-Elk1 (*A*), and p-Foxo3a (*B*) were detected in E8.75 embryos from nondiabetic control (NC), diabetic (DM), and diabetic JNK2KO (DM *jnk2*$^{-/-}$) mice. Experiments were repeated three times using samples from different dams in each group. Data of densitometric analysis are shown in *C* for p-c-Jun, *D* for p-ATF-2, *E* for p-Elk1, and *F* for p-Foxo3a. *$P < 0.05$.

and *C–E*). Levels of p-FoxO3a in *jnk2*$^{-/-}$ embryos exposed to hyperglycemia were significantly higher than those in embryos from diabetic WT mice and were comparable with those in embryos from nondiabetic mice (Fig. 3*B* and *F*). *jnk1* gene deletion in *jnk1*$^{-/-}$ embryos prevented maternal hyperglycemia-induced p-Foxo3a reduction (activation) (Fig. 4*B* and *F*).

JNK1/2 deficiency in either *jnk2*$^{-/-}$ or *jnk1*$^{-/-}$ embryos blocks hyperglycemia-induced caspase cascade activation. Caspase activation is manifested in diabetic embryopathy. However, the mechanism whereby hyperglycemia

induces caspase activation is not clear. The JNK1/2 pathway is a proapoptotic signaling pathway that mediates caspase activation in other systems. We, therefore, reasoned that JNK1/2 activation leads to caspase activation in diabetic embryopathy. Since our previous studies (15,17) define the initiator caspase, caspase 8, and the effector caspase, caspase 3, in diabetic embryopathy, we determined levels of cleaved (active) caspase 3 and 8 to test this hypothesis. Cleaved caspase 3 and 8 were robustly present in WT embryos exposed to hyperglycemia of diabetes but were not present at all in WT embryos from nondiabetic mice
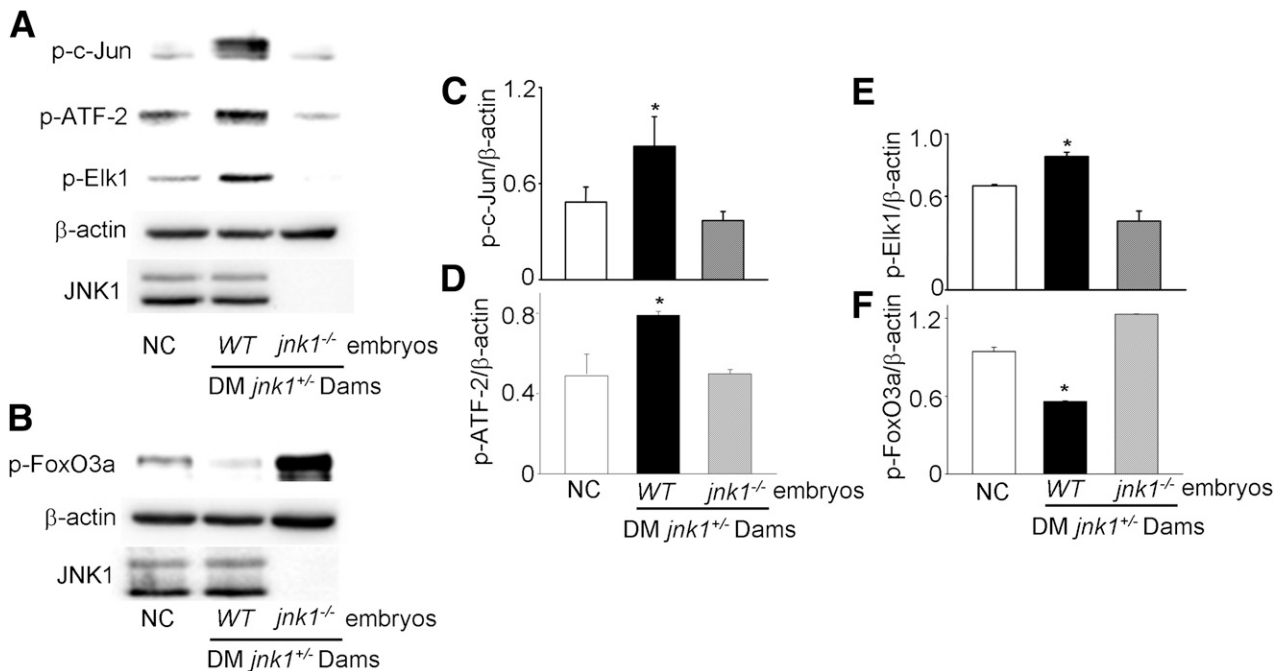

FIG. 4. JNK1 deficiency in *jnk1*$^{-/-}$ embryos blocks the activation of transcriptional factors downstream of JNK1/2. p-c-Jun, p-ATF-2, p-Elk1 (*A*), and p-Foxo3a (*B*) were detected in E8.75 WT embryos from nondiabetic control (NC) and WT embryos and *jnk1*$^{-/-}$ embryos from diabetic *jnk1*$^{+/-}$ mice (DM *jnk1*$^{+/-}$) mated with male *jnk1*$^{+/-}$ mice. The JNK1 antibody detected the two JNK1 protein products, 54 kDa and 46 kDa. Experiments were repeated three times using samples from different dams in each group. Data of densitometric analysis are shown in *C* for p-c-Jun, *D* for p-ATF-2, *E* for p-Elk1, and *F* for p-Foxo3a. *$P < 0.05$.

A

caspase 8

Cleaved
caspase 8

caspase 3

Cleaved
caspase 3

β-actin

JNK2

NC   DM   DM $jnk2^{-/-}$

B

caspase 8

18 kDa

Cleaved
caspase 8

Casp 3

Cleaved
Casp 3

β-actin

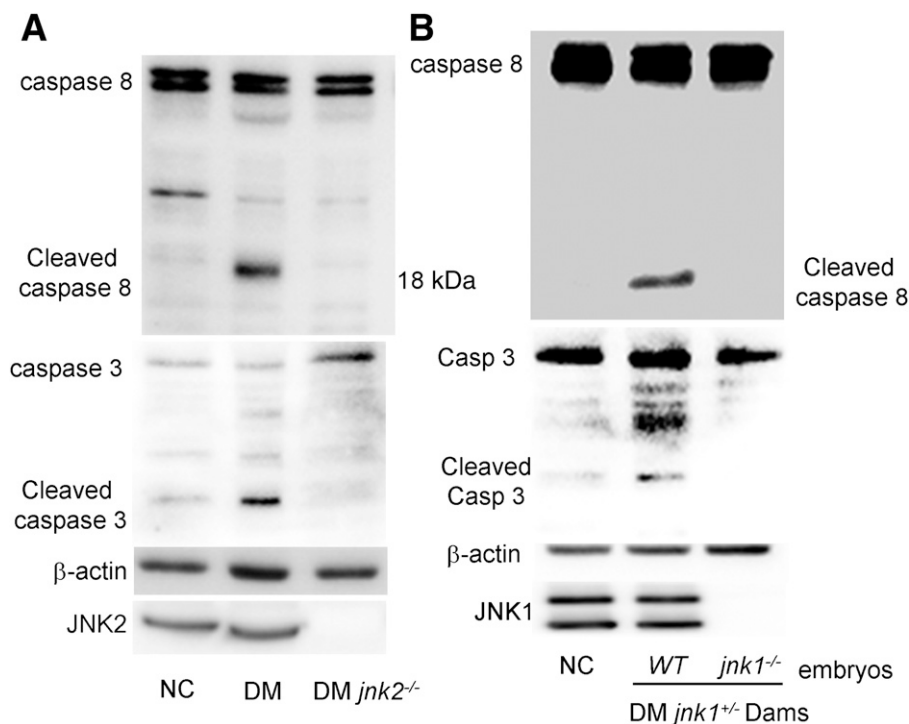JNK1

NC   *WT* $jnk1^{-/-}$ embryos
DM $jnk1^{+/-}$ Dams

FIG. 5. Targeted deletions of either *jnk1* or *jnk2* gene abolish maternal hyperglycemia-induced caspase 3 and 8 cleavage. In *A*, cleaved caspase 3 and 8 were analyzed in E8.75 embryos from nondiabetic control (NC), diabetic (DM), and diabetic JNK2KO (DM $jnk2^{-/-}$) mice. In *B*, cleaved caspase 3 and 8 were assessed in E8.75 WT embryos from NC and WT embryos and $jnk1^{-/-}$ embryos from diabetic $jnk1^{+/-}$ mice (DM $jnk1^{+/-}$) mated with male $jnk1^{+/-}$ mice. Experiments were repeated three times using samples from different dams in each group and similar results were obtained. Representative images are shown.

(Fig. 5*A* and *B*). Similar to observations in WT embryos from nondiabetic mice, we observed no cleaved product of caspase 3 and 8 in $jnk2^{-/-}$ embryos exposed to hyperglycemia (Fig. 5*A*). Neither were cleaved caspase 3 and 8 detected in $jnk1^{-/-}$ embryos exposed to hyperglycemia (Fig. 5*B*). In contrast, WT embryos, which were from the same group of diabetic $jnk1^{+/-}$ mothers as the $jnk1^{-/-}$ embryos, had robust cleaved caspase 3 and 8 (Fig. 5*B*). These results support that both JNK1 and JNK2 activation lead to caspase cascade activation.

JNK1/2 deficiency in either $jnk2^{-/-}$ or $jnk1^{-/-}$ embryos prevents hyperglycemia-induced apoptosis in the developing neural tube. Hyperglycemia-induced apoptosis in the developing neural tube causes NTDs. Since JNK1/2 activation mediates hyperglycemia-induced caspase cascade activation, which is a biochemical hallmark of apoptosis, it is reasoned that JNK1/2 activation is a proapoptotic signal that is responsible for diabetes-induced apoptosis in the developing neural tube. To determine whether JNK1/2 deficiency blocks maternal diabetes-induced apoptosis in the developing neural tube, we used the TUNEL assay to assess apoptosis. We detected fewer apoptotic cells in WT embryos from nondiabetic mice and $jnk2^{-/-}$ and $jnk1^{-/-}$ embryos from diabetic mice (Fig. 6*A* and *B*). WT embryos from diabetic mice exhibited significantly higher numbers of apoptotic cells in the developing neural tube compared with those observed in WT embryos from nondiabetic mice and $jnk2^{-/-}$ and $jnk1^{-/-}$ embryos from diabetic mice (Fig. 6*A* and *B*). Although double TUNEL/Sox1 staining was not as sensitive as the TUNEL assay alone, it detected the apoptotic cells at the dorsal side of the neural tube as Sox1$^+$ neural progenitors (Fig. 6*C* and *D*).

DISCUSSION

Using genetically modified mice, our studies provide strong evidence that hyperglycemia-induced oxidative stress activates the proapoptotic JNK1/2 signaling, which triggers apoptosis in the developing neural tube resulting in NTDs. In a separate study (submitted for publication), we demonstrated that SOD1 overexpression inhibits maternal diabetes-induced caspase activation and apoptosis. SOD1-overexpressing (29), $jnk2^{-/-}$ (20), and $jnk1^{-/-}$ embryos (Table 1) are all resistant to diabetes-induced embryonic malformations, demonstrating that oxidative stress and JNK1/2 activation are key steps in the induction of diabetic embryopathy. JNK1/2 activation mediates the downstream events of oxidative stress and plays a causative role in the induction of diabetic embryopathy. In this study, we used a mouse diabetic model in C57BL/6J background to induce sustained maternal hyperglycemia (≥250 mg/dL glucose) that mimics the human condition seen in type 1 and type 2 maternal diabetes. In our studies (15,20) and those of others (32,33), this mouse model in C57BL/6J background consistently produces >22% NTDs in embryos exposed to hyperglycemia. In contrast, embryos from nondiabetic control mice display ~1% NTDs. This rate of spontaneous NTD formation is lower than previously reported (35). The high NTD rate in our study is probably due to the calculation based on the gross NTD rate per genotype. Under the same maternal diabetic environment, it is also possible that reduced susceptibility of $jnk1^{-/-}$ embryos increases NTD incidences of WT and $jnk1^{+/-}$ embryos. Nevertheless, another group has reported comparable malformation rates in diabetic mice (32).
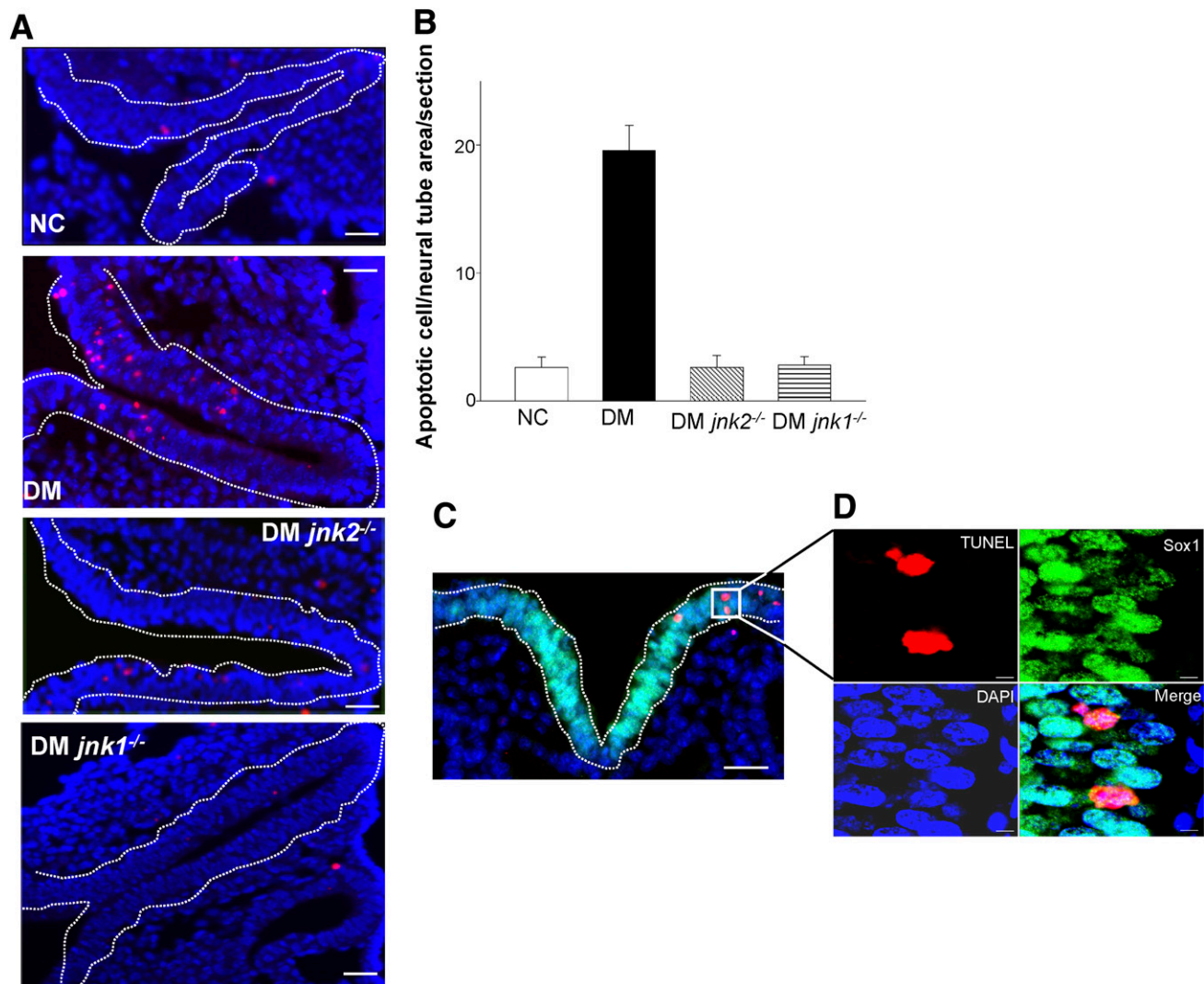
FIG. 6. Targeted deletions of either *jnk1* or *jnk2* gene suppress maternal hyperglycemia-induced apoptosis in cells of the developing neuro-epithelium. Representative images of the TUNEL assays are shown in *A*. Apoptotic cells are labeled in red, and all cells are labeled in blue. The dense blue areas, which are marked by white dash lines, are neuroepitheliums of E8.75 WT embryos from nondiabetic control (NC) and diabetic (DM) mice, *jnk2*$^{-/-}$ embryos from diabetic JNK2KO (DM *jnk2*$^{-/-}$) mice, and *jnk1*$^{-/-}$ embryos from diabetic *jnk1*$^{+/-}$ (DM *jnk1*$^{+/-}$) mice mated with male *jnk1*$^{+/-}$ mice. Experiments were repeated three times using samples from different dams in each group. Quantifications of apoptotic cell number are shown in *B*. Apoptotic cells of the whole neuroepithelium, including the dorsal and ventral domains. Five serial coronal sections through the anterior neural tubes of each embryo were analyzed. Six embryos per group were used. The numbers of apoptotic cells were counted in the neural tube area of each section and averaged for each embryo. In *C* and *D*, apoptotic cells labeled by TUNEL (red) staining are Sox1$^+$ (green) neural progenitors in the DM group. Cell nuclei are stained by DAPI (blue). Bars are 30 μmol/L in *A* and *C* and 3.5 μmol/L in *D*. (A high-quality digital representation of this figure is available in the online issue.)

Using *jnk1*$^{+/-}$ male and female mice, we produced *jnk1*$^{-/-}$ embryos, which, when exposed to diabetes, had lower malformation rates than those in *jnk1*$^{+/-}$ or *jnk1*$^{+/+}$ (WT) embryos. These findings clearly show that JNK1 mediates the teratogenicity of maternal diabetes. Because double deletion of *jnk1* and *jnk2* genes is lethal, our initial plan was to generate *jnk2*$^{-/-}$*jnk1*$^{-/+}$ or *jnk2*$^{+/-}$*jnk1*$^{-/-}$ mice and, thus, produce *jnk2*$^{-/-}$*jnk1*$^{-/+}$ or *jnk2*$^{+/-}$*jnk1*$^{-/-}$ embryos to study the synergistic effects of JNK1 and JNK2 on diabetic embryopathy. Unfortunately, *jnk2*$^{-/-}$*jnk1*$^{-/+}$ or *jnk2*$^{+/-}$*jnk1*$^{-/-}$ mice are poor breeders, and no successful pregnancies were established from these matings. Nevertheless, we found that both *jnk1*$^{-/-}$ and *jnk2*$^{-/-}$ embryos (20) from diabetic dams have significantly lower malformation rates than those in WT embryos exposed to hyperglycemia. Targeted deletions of either *jnk1* or *jnk2* gene are able to completely block downstream events of

JNK1/2 activation, including activation of transcription factors, caspase cascade activation, and apoptosis in the developing neural tube induced by maternal diabetes. The current study provides compelling evidence that hyperglycemia-induced apoptosis in the developing neural tube accounts for NTD formation.

The expression of JNK1 and JNK2 in the neural tube starts at E7, whereas JNK3 is expressed at E11 (26). Therefore, JNK3 appears not to be involved in hyperglycemia-induced NTDs. Under normal conditions, virtually all neural tube cells are Sox1$^+$ neural progenitors at E9.5 (40). In addition, we have shown that the apoptotic cells are Sox1$^+$ neural progenitors, and no glia cells exist until E12.5 (41). Therefore, it is clear that JNK1 and JNK2 are expressed in Sox1$^+$ neural progenitors along with apoptosis in some of these cells in the dorsal side of the neural tube. Double deletion of *jnk1* and *jnk2* increases

apoptosis in the forebrain and decreases apoptosis in the lateral edges of the hindbrain. In contrast, single deletion of either *jnk1* or *jnk2* does not affect apoptosis in the neural tube. Therefore, JNK1KO and JNK2KO mice are suitable models in studying JNK1/2 activation–induced apoptosis in diabetic embryopathy.

A robust increase in JNK1/2 activities, which is associated with NTDs, is observed in both in vivo maternal diabetes models and in vitro embryo cultures exposed to high glucose (15). Subsequent pharmacological and genetic data (20), including these in the current study, confirm the causative role of JNK1/2 activation in the induction of diabetic embryopathy. Thus, JNK1/2 are the key intracellular kinases mediating the proapoptotic signals in diabetic embryopathy. JNK1 and JNK2 are cytoplasmic kinases and relay their proapoptotic signals to the nucleus through activation of an array of transcriptional factors. The current study demonstrates that the transcription factors c-Jun, ATF-2, Elk1, and Foxo3a, which are known downstream effectors of JNK1/2 activation, are activated in diabetic embryopathy. The activation of these transcription factors are presumably responsible for altered gene expression in diabetic embryopathy (36,42,43). Our study uncovers a proapoptotic signaling cascade emanating from JNK1/2 activation in diabetic embryopathy.

Studies suggest that Pax3 deficiency (13), transforming growth factor (TGF)-β pathway disturbance (44), and diminished Wnt signaling (45) are involved in diabetic teratogenesis. Because homeobox (*Hox*) genes are essential for Pax3 gene expression (46) and *Hox* genes suppress JNK1/2 activation (47), *Hox* genes may mediate the interaction between Pax3 and the JNK1/2 pathway. JNK1/2 inhibit the Wnt/β-catenin pathway (48), suggesting that JNK1/2 may contribute to the altered Wnt signaling in diabetic embryopathy (45). JNK1/2 inhibition enhances TGF-β1/Smad2 signaling in embryonic tissues (49), which suggests that JNK1/2 may be responsible for the TGF-β signaling disturbance (44). Therefore, there is potential crosstalk between the JNK1/2 pathway and other identified factors/pathways in mediating the teratogenicity of maternal diabetes.

In summary, the activation of the JNK1/2 pathway in diabetes-induced NTDs is the result of oxidative stress. Targeted deletion of *jnk1* gene ameliorates diabetes-induced NTDs, and both JNK1 and JNK2 contribute to the induction of diabetic embryopathy. JNK1/2 activation induces activation of its downstream transcription factors and is responsible for diabetes-induced caspase activation and apoptosis. Thus, our study shows the causative role of the oxidative stress cascade (JNK1/2-caspase-apoptosis) in the induction of diabetic embryopathy.

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