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Cellular Stress, Excessive Apoptosis, and the Effect of Metformin in a Mouse Model of Type 2 Diabetic Embryopathy



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Increasing prevalence of type 2 diabetes in women of childbearing age has led to a higher incidence of diabetes-associated birth defects. We established a model of type 2 diabetic embryopathy by feeding 4-week-old female mice a high-fat diet (HFD) (60% fat). After 15 weeks on HFD, the mice showed characteristics of type 2 diabetes mellitus (DM) and were mated with lean male mice. During pregnancy, control dams fed a normal diet (10% fat) were maintained on either normal diet or HFD, serving as a control group with elevated circulating free fatty acids. DM dams produced offspring at a rate of 11.3% for neural tube defect (NTD) formation, whereas no embryos in the control groups developed NTDs. Elevated markers of oxidative stress, endoplasmic reticulum stress, caspase activation, and neuroepithelial cell apoptosis (causal events in type 1 diabetic embryopathy) were observed in embryos of DM dams. DM dams treated with 200 mg/kg metformin in drinking water ameliorated fasting hyperglycemia, glucose intolerance, and insulin resistance with consequent reduction of cellular stress, apoptosis, and NTDs in their embryos. We conclude that cellular stress and apoptosis occur and that metformin effectively reduces type 2 diabetic embryopathy in a useful rodent model.

Globally, nearly 60 million women of reproductive age (18–44 years old) have diabetes, and this number is expected to double by 2030 (1,2). Pregestational maternal type 1 and 2 diabetes is strongly associated with high rates of severe

structural birth defects, including neural tube defects (NTDs) and congenital heart defects (3–6). The use of insulin during pregnancy in both animal models and humans greatly reduces the incidence of diabetes-induced embryonic malformations (7,8). However, euglycemia is difficult to achieve and maintain in women with pregestational type 1 or type 2 diabetes. Thus, offspring of women with diabetes still have significantly higher rates of birth defects than those of mothers without diabetes (5). Diabetic embryopathy remains a significant health problem for both women with diabetes and their children, and additional effective therapeutic options are needed.

Previous studies in a type 1/insulin-deficient diabetic embryopathy model demonstrated that oxidative stress, endoplasmic reticulum (ER) stress, and cellular stress-induced cell apoptosis (8–16) are causative events in NTD formation. We and others have observed that maternal diabetes induces oxidative stress by suppressing endogenous expression of antioxidant enzymes while simultaneously increasing production of cellular reactive oxygen species (17–20). Maternal diabetes triggers a spectrum of ER stress markers (12). Treatment of embryos *in vitro* with 4-phenylbutyric acid, an ER stress inhibitor, ameliorates NTD formation induced by hyperglycemia (12). Excess apoptosis is observed in the neuroepithelium of rodent embryos exposed to maternal diabetes, and deletion of proapoptotic kinase genes reduces NTD incidence in these embryos (9,11,12,21). In addition, maternal diabetes-induced apoptosis is caspase-8 dependent (9,11,12,21).

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Prior research to delineate mechanisms underlying diabetic embryopathy has almost exclusively been performed in mouse models of type 1 diabetic/insulin-deficient animals (9,15,16,22,23). Thus, the question remains whether similar mechanisms underlie type 2 diabetic embryopathy, a health problem of increasing importance. Hyperglycemia, a prominent feature of both type 1 and type 2 diabetes is a major contributing factor to the teratogenicity of maternal diabetes (19,24,25). Nevertheless, many aspects of the pathophysiology of type 1 and type 2 diabetes are quite different. In particular, insulin resistance plays a prominent role in the pathophysiology of type 2 diabetes, whereas insulin deficiency is the primary defect in type 1 diabetes. Thus, effects of type 2 diabetes on developing embryos may be distinct from effects of type 1 diabetes. As the number of women with type 2 diabetes continues to increase (1), a type 2 diabetic embryopathy model is urgently needed to develop safe and effective therapeutic interventions to complement current therapies inadequate to oppose the rising incidence and prevalence of diabetes-induced embryopathy.

To date, animal models of type 2 diabetic embryopathy have not been well described or studied. In C57BL/6J mice, use of a high-fat diet (HFD) to cause diet-induced obesity (DIO) recapitulates many features of the natural history of human obesity, metabolic syndrome, and frank type 2 diabetes (26,27). This obese type 2 diabetic model manifests the characteristics of human type 2 diabetes (26,27). After 15 weeks on HFD, female mice exhibit high fasting glucose levels, hyperinsulinemia, glucose intolerance, and insulin resistance (26,27). In the current study, we use the DIO mouse model to characterize type 2 diabetic embryopathy and evaluate the effects of metformin therapy.

RESEARCH DESIGN AND METHODS

Mice and HFD Treatment

The procedures for animal use were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee. Four-week-old female C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were maintained in a temperature-controlled room on a 12-h light-dark cycle. After arrival, mice were divided into two groups and fed either an HFD (Research Diets, New Brunswick, NJ) or a normal diet (Teklad; Harlan) for 15 weeks. The HFD contained 20% protein, 20% carbohydrates, and 60% fat. The normal diet contained 20% protein, 70% carbohydrates, and 10% fat. Body weight was recorded weekly. After 15 weeks, BMI was determined using the Lee index. Both HFD- and normal chow-fed mice were mated with lean male mice. During pregnancy, mice in the normal diet group were either maintained on the normal diet (control group 1 [Ctrl 1]) or subsequently fed the HFD to serve as the high fatty acid control group (Ctrl 2). Embryonic day 0.5 (E0.5) of pregnancy was established at noon on the day when a vaginal plug was observed. Fasting and random blood glucose levels were determined

after 15 weeks on either HFD or normal diet and at E8.5. Blood was collected from orbital veins, and sera were extracted by centrifugation and reserved to determine circulating free fatty acid (FFA) and insulin concentrations. For long-term storage, sera were kept at -80°C .

Blood Glucose Measurement, Glucose Tolerance Test, and Insulin Tolerance Test

Blood glucose was measured using a handheld glucometer with appropriate test strips (FreeStyle Lite). For the glucose tolerance test (GTT), mice were fasted overnight and then injected intraperitoneally with glucose 2 g/kg body weight. Blood glucose levels were measured before injection and at 15, 30, 60, 90, and 120 min after injection. The trapezoidal rule was used to determine the area under the curve (AUC) for GTT. For the insulin tolerance test (ITT), mice were fasted for 5 h and then injected intraperitoneally with insulin 0.75 units/kg body weight. Blood glucose levels were measured before injection and at 15, 30, 60, 90, and 120 min after injection.

Serum FFA and Insulin Measurements

FFA levels were determined using the Free Fatty Acid Quantitation Kit (Sigma-Aldrich) according to the manufacturer's instructions. Palmitic acid standards and 6 μL serum per sample were incubated with reaction agents. Absorbance was measured at 570 nm in each sample to determine concentrations of FFA. The Rat/Mouse Insulin ELISA EZRMI-13K Kit (Millipore, Bedford, MA) was used to assess the level of plasma insulin. Insulin standards and serum samples were reacted with assay buffer. Absorbance was read at 450 and 590 nm for each sample using a 96-well plate reader within 5 min. QUICKI was calculated using the following equation: $1/[\log(\text{fasting insulin, } \mu\text{U/mL}) + \log(\text{fasting glucose, mg/dL})]$.

Dihydroethidium Staining

Dihydroethidium (DHE) staining was used to detect superoxide. DHE reacts with superoxide bound to cellular components, including protein and DNA, and exhibits bright red fluorescence. E8.5 embryos were fixed in 4% paraformaldehyde for 30 min, washed three times with PBS (5 min per wash), and embedded in optimum cutting temperature medium. Ten-micrometer frozen embryonic sections were incubated with 1.5 $\mu\text{mol/L}$ DHE for 5 min at room temperature and then washed three times with PBS for 5 min per wash. Sections were counterstained with DAPI and mounted with aqueous mounting medium (Sigma, St. Louis, MO).

Lipid Hydroperoxide Quantification

The degree of lipid peroxidation, an index of oxidative stress, was quantitatively assessed with a lipid hydroperoxide (LPO) assay as described by others (28) using the Calbiochem Lipid Hydroperoxide Assay Kit (Millipore). Briefly, E8.5 embryos were homogenized in high-performance liquid chromatography-grade water. LPOs were extracted from embryos by deoxygenated chloroform, and measurements of absorbance at 500 nm were taken after reaction with chromogen. Results were expressed as micromole LPO per

microgram protein. Protein concentrations were determined with the DC protein assay (Bio-Rad, Hercules, CA).

Real-Time PCR

Using the RNeasy Mini Kit (QIAGEN), mRNA was isolated from E8.75 embryos and reversed transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Grand Island, NY). Real-time PCR for calnexin, GRP94, PDIA, BiP, IRE1 α , CHOP, and β -actin were performed using the Maxima SYBR Green/ROX qPCR Master Mix Assay (Thermo Scientific, Rockford, IL) in the StepOnePlus system (Applied Biosystems). Primer sequences used are listed in Supplementary Table 1.

Immunoblotting

Immunoblotting was performed as previously described (29,30). Briefly, embryos from the different experimental groups were sonicated in lysis buffer containing a protease inhibitor cocktail (Sigma). Equal amounts of protein and the Precision Plus Protein standards (Bio-Rad) were resolved by SDS-PAGE and transferred onto Immobilon-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 primary antibodies. To determine whether equivalent amounts of protein were loaded among all samples, membranes were stripped and incubated with a mouse antibody against β -actin (Abcam) to generate a signal used as a loading control. Signals were detected using the SuperSignal West Femto Maximum Sensitivity Substrate kit (Thermo Scientific). The sources and dilutions of antibodies used in each experiment are listed in Supplementary Table 2.

TUNEL Assay

The TUNEL assay was performed using the ApopTag Fluorescein Direct In Situ Apoptosis Detection Kit (Chemicon) as previously described (9,11,12,21). Briefly, 10- μ m frozen embryonic sections were fixed with 4% paraformaldehyde in PBS and incubated with TUNEL reaction agents. Three embryos from three different dams per group were used, and two sections per embryo were examined. TUNEL-positive cells in an area (~200 cells) of neuroepithelium were counted. The percentage of TUNEL-positive cells was calculated as a fraction of the total cell number multiplied by 100, and values were averaged within the sections of one embryo.

Metformin Treatment

Dams were given metformin (Sigma, St. Louis, MO) in their drinking water from E0.5 to E10.5. The water consumption per mouse per day (24 h) was predetermined and monitored daily during the course of metformin treatment. Because daily water consumption differed between lean control dams and obese diabetic dams, metformin concentrations in drinking water were adjusted accordingly to achieve comparable dosing. The purpose of giving metformin in drinking water was to maintain a constant level of metformin. Metformin is typically given to patients in an extended release form so that the effects of metformin can be sustained over long durations (31). The final metformin intake per mouse per day was ~200 mg/kg body weight, a dose previously described as safe and effective in mice (31–33). At

E8.5, fasting glucose levels were determined, and GTT and ITT were performed as previously described. At E10.5, embryonic NTD formation was examined in metformin-treated mice.

Statistical Analyses

Data are presented as mean \pm SE. Each set of experiments were repeated independently at least three times with comparable results, and embryonic samples from each replicate were taken from different dams. Statistical differences were evaluated using one-way ANOVA with SigmaStat 3.5 software. For one-way ANOVA analyses, a Tukey test was used to estimate the significance of the results, with $P < 0.05$ indicative of statistical significance. The χ^2 test was used to estimate the significance of differences in NTD rates among experimental and control groups.

RESULTS

Induction of Obese Type 2 Diabetes in Female Mice

Four-week-old female mice were fed an HFD or a normal diet (control) for 15 weeks. Mice in the HFD group continuously gained weight from 2 weeks onward and after 15 weeks. Mean body weight in the HFD group was significantly greater than in the control group (35.02 ± 0.81 vs. 22.96 ± 0.49 g, $P < 0.05$) (Fig. 1A). BMI in the HFD group was also significantly higher (Fig. 1B).

Fasting glucose levels and random glucose levels in the HFD group were significantly elevated compared with the control group (Fig. 1C and D). Plasma insulin levels in the HFD group were approximately twofold higher (Fig. 1E), indicating hyperinsulinemia. QUICKI was used to assess insulin resistance. Mean QUICKI values in the HFD group were significantly less than in the control group (Fig. 1F). Additionally, high FFA levels were observed in the HFD group (Fig. 1G).

Glucose intolerance is the defining characteristic of type 2 diabetes. GTT was performed after 15 weeks of feeding with different diets. After injecting mice in both the HFD and the control groups with glucose, blood glucose levels in all mice were increased during early time points of the GTT (Fig. 1H). At 30 min, peak mean glucose levels in the HFD group were much higher, and glucose disappearance thereafter was much slower compared with the control group (Fig. 1H). Indeed, the mean AUC for GTT in mice fed an HFD was significantly higher than in mice fed a normal diet (Fig. 1H). Thus, HFD induced significant and substantial glucose intolerance in mice after 15 weeks.

ITT was used to evaluate insulin resistance in the mice. After insulin administration, blood glucose levels declined as expected in both groups; however, this decrease was significantly less in the HFD group (Fig. 1I). During ITT, glucose levels in the HFD group were higher at every time point than in the control group, clearly demonstrating insulin resistance in the HFD group. Taken together, these findings are consistent with DIO from HFD causing insulin resistance and key characteristics of type 2 diabetes. Therefore, the HFD group is referred to as the diabetes mellitus (DM) group hereafter.

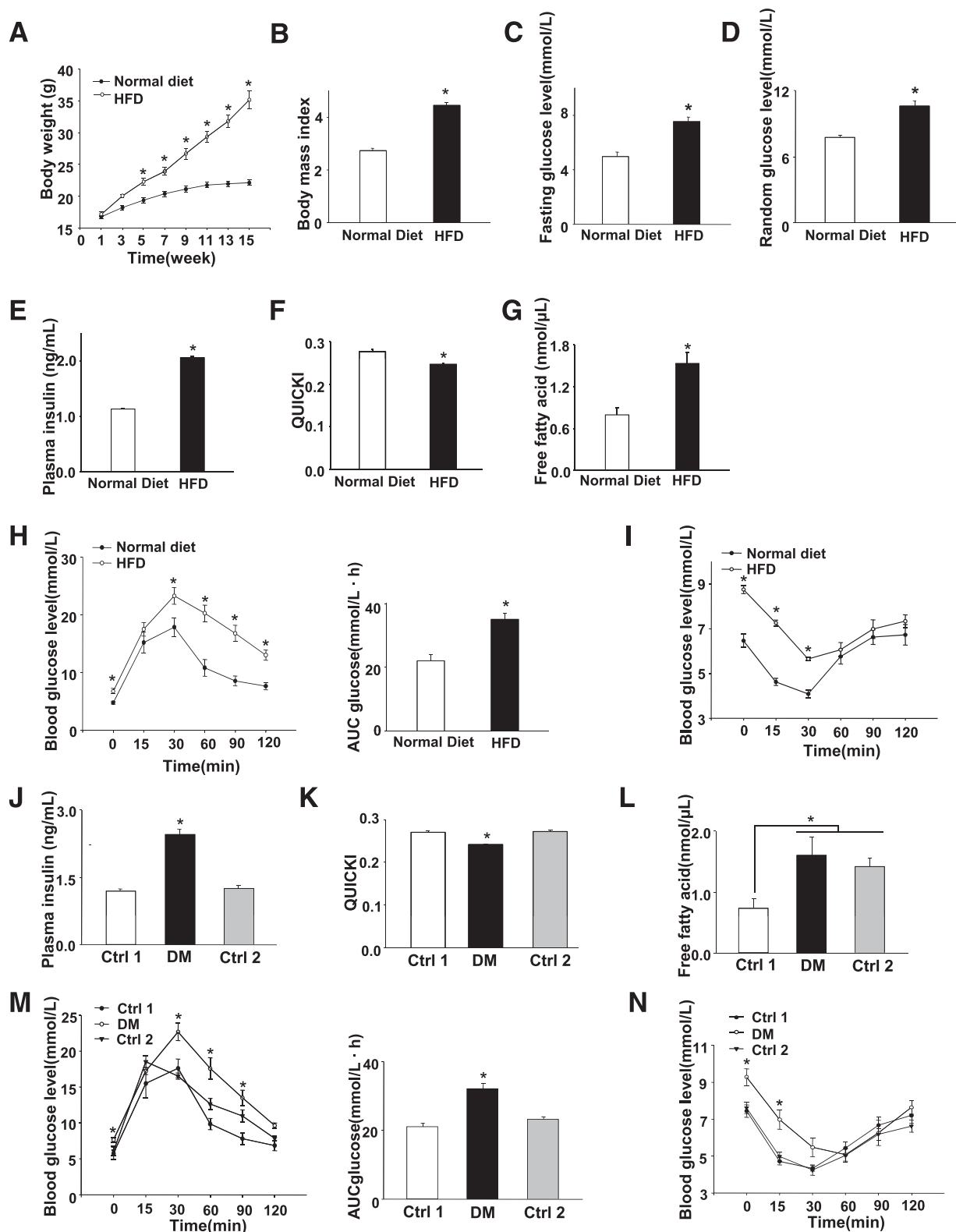


Figure 1—Induction of obese type 2 diabetes in female mice and metabolic indices of obese type 2 diabetic dams. (A) Weekly body weight measurements during feeding with normal control diet (10% fat) ($n = 15$) or HFD (60% fat) ($n = 15$). BMI in normal diet and HFD groups ($n = 8$) (B), fasting glucose level ($n = 12$) (C), random glucose level ($n = 16$) (D), plasma insulin level ($n = 5$) (E), QUICKI ($n = 5$) (F), and FFA level ($n = 7$) (G) after 15 weeks of feeding. (H) Blood glucose levels and AUC_{glucose} level during GTT ($n = 13$) after 15 weeks of feeding with different diets. (I) Blood glucose levels during ITT ($n = 6$) after 15 weeks of feeding with different diets. *Significant differences compared with the other group. Plasma insulin levels (J), QUICKI (K), and FFA levels (L) in Ctrl 1 and Ctrl 2 and the obese DM group at E8.5 ($n = 10$). (M) Blood glucose levels and AUC_{glucose} levels during GTT at E8.5 ($n = 6$). (N) Blood glucose levels during ITT at E8.5 ($n = 7$). *Significant differences compared with the other two groups.

Table 1—Maternal type 2 diabetes induces NTD formation

Group	Fasting glucose (mmol/L)	Random glucose (mmol/L)	Number of embryos	Resorption rate (%)	NTD rate (%)
Ctrl 1 (n = 15)	5.08 ± 0.54	7.06 ± 0.35	119	3 (2.5)	0 (0.0)
DM (n = 16)	7.92 ± 0.51*	9.56 ± 0.23*	106	26 (19.7)*	12 (11.3)*
Ctrl 2 (n = 15)	5.76 ± 0.54	7.38 ± 0.26	107	4 (3.6)	0 (0.0)

Data are mean ± SD unless otherwise indicated. Ctrl 1 group fed normal diet, DM group fed HFD, and Ctrl 2 group fed 60% HFD during pregnancy. *Significant differences compared with the other two groups as analyzed by the Tukey or χ^2 test.

Metabolic Indices of Obese Type 2 Diabetic Dams

The mice in the normal diet group were randomized into two control groups: Ctrl 1 (normal diet the whole time, including pregnancy) and Ctrl 2 (mice switched to HFD after becoming pregnant to serve as the high-FFA control group). The metabolic indices of the DM group and the two control groups were determined at E8.5, an important time point for embryonic neural tube formation. The fasting and random glucose levels in the DM group were significantly increased compared with the two control groups (Table 1). Fasting insulin level in the DM group was more than two-fold greater than either of the two control groups (Fig. 1J). Furthermore, QUICKI in the DM group was significantly less than in either of the control groups (Fig. 1K). The FFA level in the DM group was significantly higher than in Ctrl

1 but comparable with Ctrl 2 (Fig. 1L). DM dams retained the glucose intolerance and insulin resistance observed before pregnancy (Fig. 1M and N).

Maternal Type 2 Diabetes Induces NTD Formation

To assess whether embryos from DM dams exhibit increased NTD formation, we examined NTD formation at E10.5. As shown in Table 1, 12 of 106 embryos (11.3%) from DM dams had NTDs, whereas no NTD was detected in embryos from either Ctrl 1 or Ctrl 2 dams. Histological examination of embryo sections of NTD embryos confirmed the presence of open neural tube structures (Fig. 2A). In addition, 26 of 132 embryos (19.7%) from DM dams were resorbed, whereas Ctrl 1 and Ctrl 2 had lower resorption rates (Table 1). Blood glucose levels in the NTD embryos were significantly higher

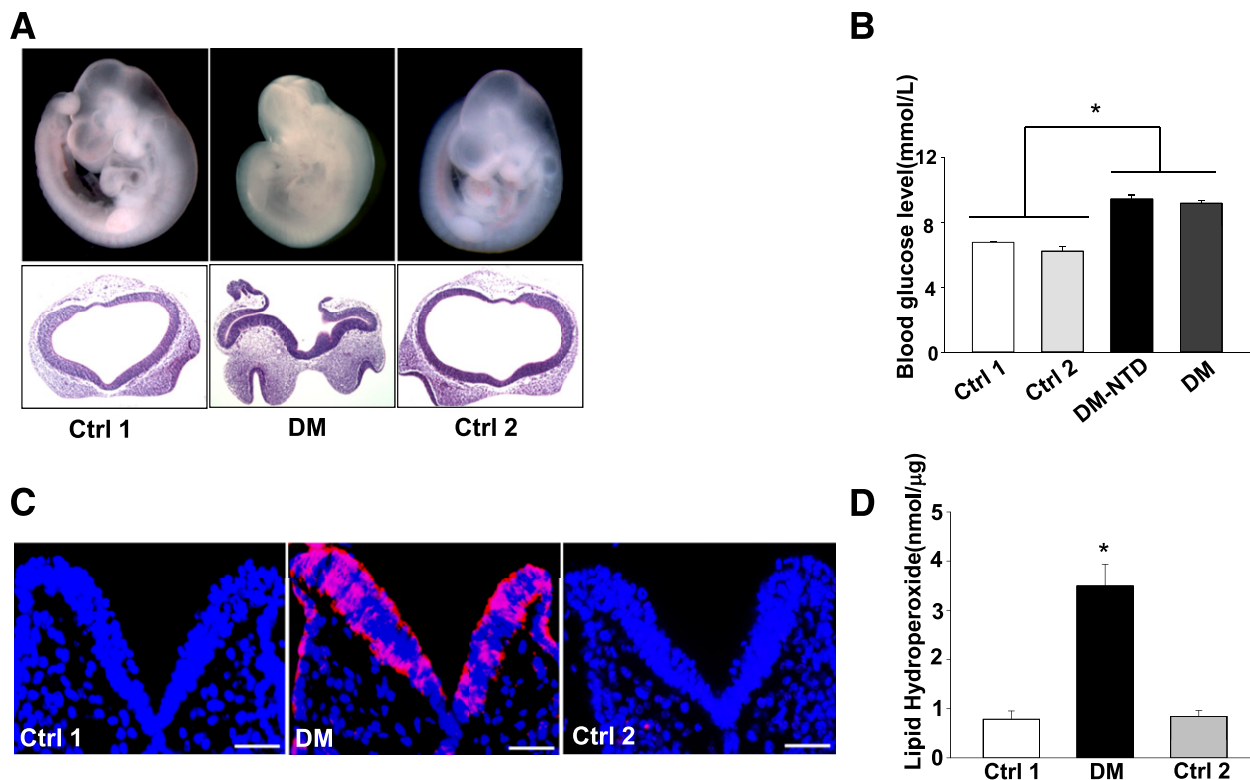


Figure 2—Maternal type 2 diabetes induces NTD formation and oxidative stress in the developing embryo. (A) Closed and open neural tube structures of E10.5 embryos from control and DM dams. **(B)** Random blood glucose levels in the DM-NTD group (n = 10). Sample sizes for the other three groups are shown in Table 1. **(C)** Representative images of DHE staining. Red signals were observed in the V-shaped neuroepithelium at E8.5. All cell nuclei were stained with DAPI (blue). Scale bar = 30 μm. **(D)** LPO levels in E8.5 embryos. Experiments were performed using three embryos from three different dams per group. *Significant differences compared with the other groups.

than those in the two control groups but were only slightly higher than those in the overall DM group (Fig. 2B).

Maternal Type 2 Diabetes Triggers Oxidative Stress, ER Stress, and Caspase Activation and Apoptosis in the Developing Embryo

Previous studies showed that oxidative stress and ER stress are involved in the induction of maternal type 1 diabetic embryopathy. In the current study, we examined whether maternal type 2 diabetes also induces oxidative stress and ER stress. The abundance of superoxide was determined by DHE staining. DHE-positive signals in the neuroepithelia of embryos from DM dams were robust (Fig. 2C). By contrast, minimal to no DHE signals were

observed in either of the two control groups (Fig. 2C). In addition, lipid peroxidation levels in embryos of DM dams were significantly higher than those in the two control groups (Fig. 2D).

To determine the level of ER stress in embryos, we examined a number of ER stress markers. Protein levels of p-PERK, p-eIF2 α , p-IRE1 α , and CHOP were significantly upregulated in the embryos from DM dams compared with those from control dams (Fig. 3A–D). Furthermore, maternal diabetes significantly increased mRNA levels of calnexin, GRP94, PDIA, BiP, and CHOP (Fig. 3E). Only the mRNA level of IRE1 α did not differ among the three groups (Fig. 3E).

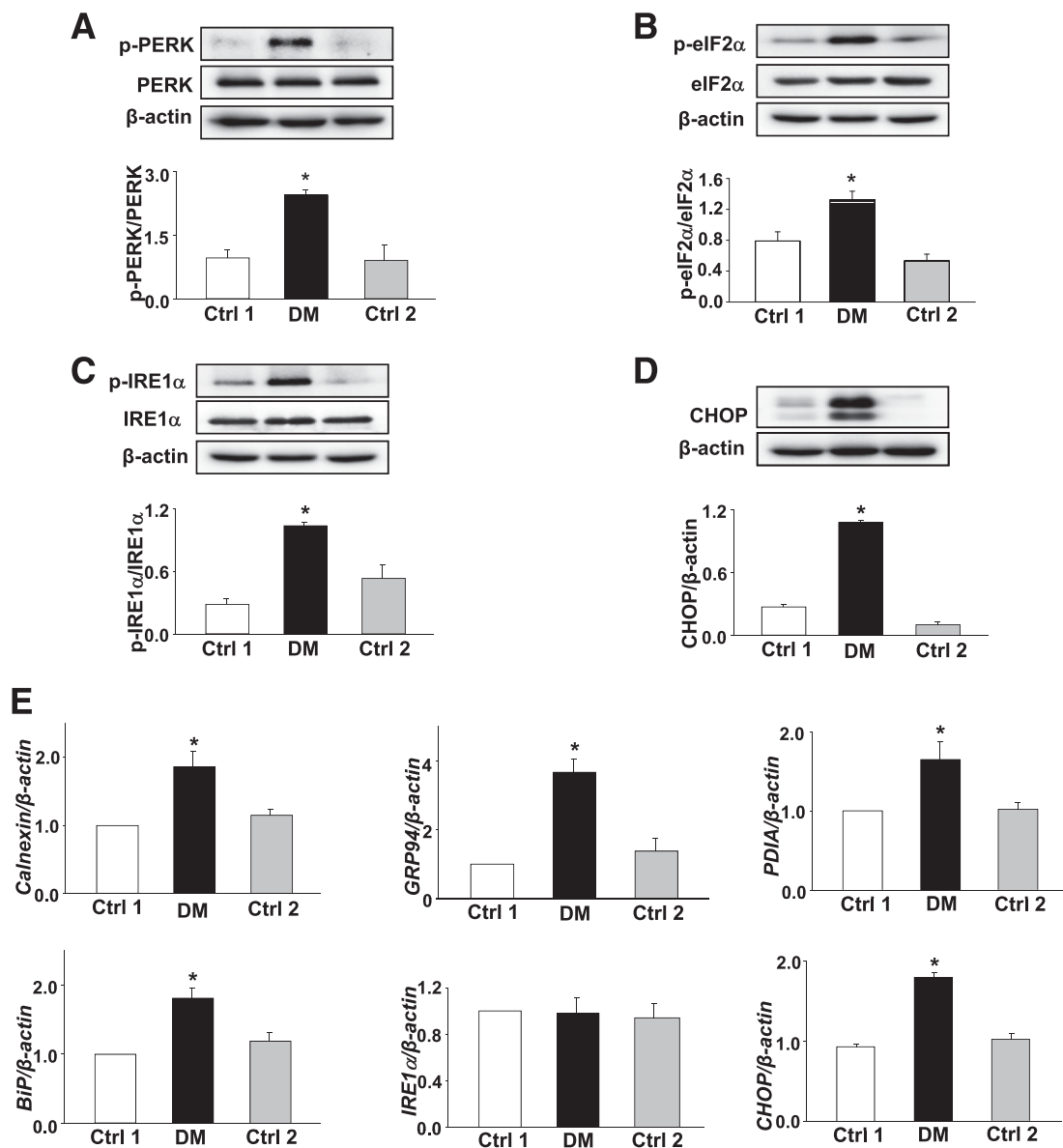


Figure 3—Maternal type 2 diabetes triggers ER stress in the developing embryo. Levels of p-PERK (A), p-eIF2 α (B), p-IRE1 α (C), and CHOP (D) in E8.5 embryos. (E) mRNA levels of calnexin, GRP94, PDIA, BiP, IRE1 α , and CHOP. Experiments were performed using three embryos from three different dams per group. *Significant differences compared with the other two groups.

To test whether excessive cell apoptosis is involved in type 2 diabetic embryopathy, we used a TUNEL assay. The number of apoptotic cells in the neuroepithelia of embryos from DM dams was much greater than in the two control groups (Fig. 4A). Cleaved caspase-8 (an initiator caspase) and cleaved caspase-3 (an executive apoptosis molecule) levels in embryos of DM dams were significantly increased compared with the two control groups (Fig. 4B).

Metformin Treatment Partially Normalizes the Adverse Metabolic Phenotypes of Type 2 Diabetic Dams

Metformin is an effective antidiabetic drug that increases insulin sensitivity and peripheral glucose uptake while inhibiting hepatic glucose production in type 2 diabetes (34). We assessed the effect of metformin treatment on adverse metabolic phenotypes of DM dams. Fasting and random glucose levels (Table 2), plasma insulin levels, and QUICKI (Fig. 5A and B) were comparable in the two control groups treated with metformin as well as in the DM group treated with metformin. DM dams treated with metformin demonstrated an amelioration of diabetic phenotype compared with those with no metformin treatment (Table 2 and Fig. 5A and B). GTT and ITT were performed to evaluate the effects of metformin therapy in ameliorating glucose intolerance and insulin resistance. Metformin treatment partially improved glucose

intolerance and completely reversed insulin resistance in DM dams (Fig. 5C and D).

Metformin Treatment Alleviates Maternal Type 2 Diabetes-Induced NTD Formation

To determine whether metformin treatment reduced type 2 diabetes-induced NTD formation, embryonic NTDs were examined in the absence and presence of metformin therapy. Similar to a previous study reporting no adverse effects of metformin treatment on neurulation stage (35), metformin treatment in the current study did not induce NTD in embryos of the control dams (Table 2). The NTD rate in embryos from DM dams treated with metformin was only 1.5%, which was significantly lower than that in embryos from DM dams without metformin treatment (11.9%) (Table 2). Metformin treatment of DM dams reduced the NTD rate to that observed in both control groups (Table 2). Metformin treatment reduced the resorption rate in DM dams but did not completely prevent maternal diabetes-induced resorption (Table 2). Moreover, metformin treatment alleviated oxidative stress, ER stress, and apoptosis by reducing levels of LPO, p-PERK, eIF2 α , and IRE1 α ; upregulation of CHOP; and cleavage of caspase-3 and -8 (Fig. 6A–G). These data support the hypothesis that metformin is effective in treating type 2 diabetes and associated diabetic embryopathy.

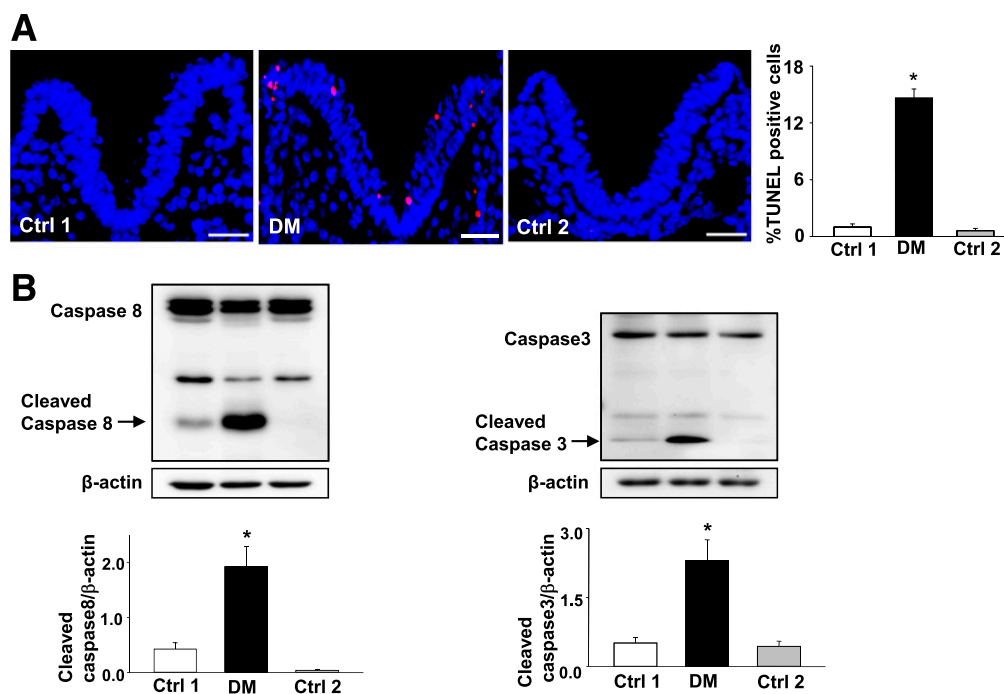


Figure 4—Maternal type 2 diabetes activates caspase and induces neuroepithelial cell apoptosis in the developing embryo. (A) Representative images of the TUNEL assay showing apoptotic cells (red signal). Cell nuclei were stained with DAPI (blue). The bar graph shows the quantification of TUNEL-positive cells. Three embryos from three different dams per group and two serial sections per embryo were analyzed. Scale bar = 30 μ m. (B) Protein levels of cleaved caspase-3 and caspase-8 in E8.5 embryos. Experiments were performed using three embryos from three different dams per group. *Significant differences compared with the other two groups.

Table 2—Metformin treatment alleviates maternal type 2 diabetes-induced NTD formation

Group	Fasting glucose (mmol/L)	Random glucose (mmol/L)	Number of embryos	Resorption rate (%)	NTD rate (%)
Ctrl 1 + Met (<i>n</i> = 6)	5.20 ± 0.25	7.09 ± 0.31	50	2 (3.8)	0 (0.0)
Ctrl 2 + Met (<i>n</i> = 8)	5.38 ± 0.22	7.67 ± 0.34	62	3 (4.6)	0 (0.0)
DM (<i>n</i> = 10)	8.34 ± 0.55*	9.46 ± 0.27*	67	15 (18.3)**	8 (11.9)*
DM + Met (<i>n</i> = 10)	6.05 ± 0.33	8.03 ± 0.25	64	9 (12.3)	1 (1.5)

Data are mean ± SD unless otherwise indicated. Ctrl 1 + Met group fed normal diet and treated with metformin, Ctrl 2 + Met group fed 60% fat diet and treated with metformin, DM group fed HFD, and DM + Met group fed HFD and treated with metformin during pregnancy. *DM group significantly different compared with the DM + Met, Ctrl 1 + Met, and Ctrl 2 + Met groups. **DM group significantly different compared with the Ctrl 1 + Met and Ctrl 2 + Met groups but not significantly different compared with the DM + Met group. Statistical differences were analyzed by the Tukey test or χ^2 test.

DISCUSSION

Prior animal studies elucidating the mechanisms underlying diabetic embryopathy have been almost exclusively performed using a type 1 diabetes/insulin-deficient animal model (9,15,16,22). With the increasing epidemic

of type 2 diabetes in women of reproductive age, a useful animal model of type 2 diabetic embryopathy will be essential for understanding the mechanisms of and potential therapies for diabetic embryopathy in women with type 2 diabetes. In the current study, we developed and

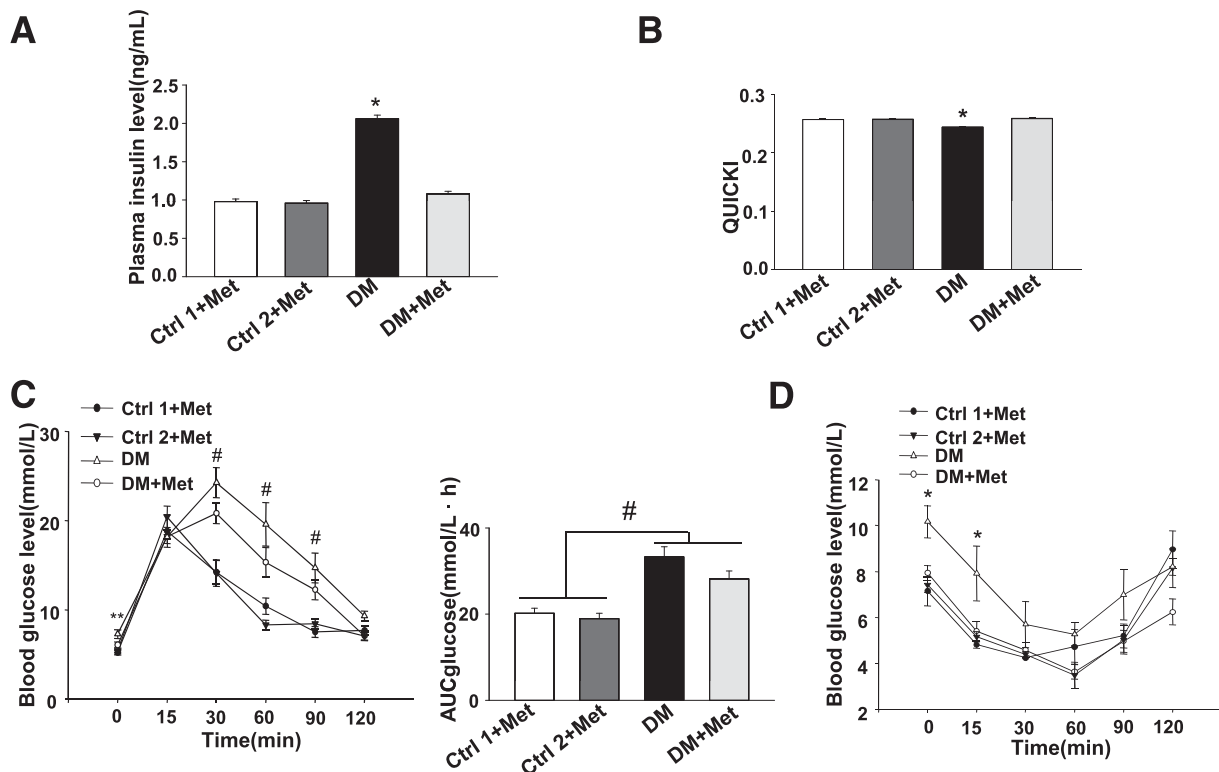


Figure 5—Metformin treatment normalizes some of the adverse metabolic phenotypes in type 2 diabetic dams. Plasma insulin levels (A) and QUICKI (B) after metformin treatments from E0.5 to the timing of assessment (E8.5). To reach a sustained effect, metformin was given through drinking water. Daily water consumption and body weight were monitored, and metformin concentrations in the drinking water were adjusted accordingly. Dams were housed individually. The final amount of metformin given to one dam was 200 mg/kg body weight per 24 h. In A and B, there were four experimental groups: Ctrl 1 + Met, Ctrl 2 + Met, DM + Met, and DM without metformin treatment. (C) Blood glucose levels and AUC_{glucose} levels during GTT at E8.5 after treating with metformin. (D) Blood glucose levels during ITT at E8.5 after treating with metformin. During GTT, the number of dams per group were eight in the Ctrl 1 + Met group, six in the DM + Met group, eight in the Ctrl 2 + Met group, and four in the DM group. During ITT, the number dams per group were three in the Ctrl 1 + Met group, nine in the DM + Met group, five in the Ctrl 2 + Met group, and six in the DM group. *DM group was significantly different compared with the DM + Met, Ctrl 1 + Met, and Ctrl 2 + Met groups. **DM group and DM + Met group were not significantly different, but the DM group was significantly different compared with the Ctrl 1 + Met and Ctrl 2 + Met groups. #DM and DM + Met groups were not significantly different, but they were significantly different compared with the Ctrl 1 + Met and Ctrl 2 + Met groups. Met, metformin.

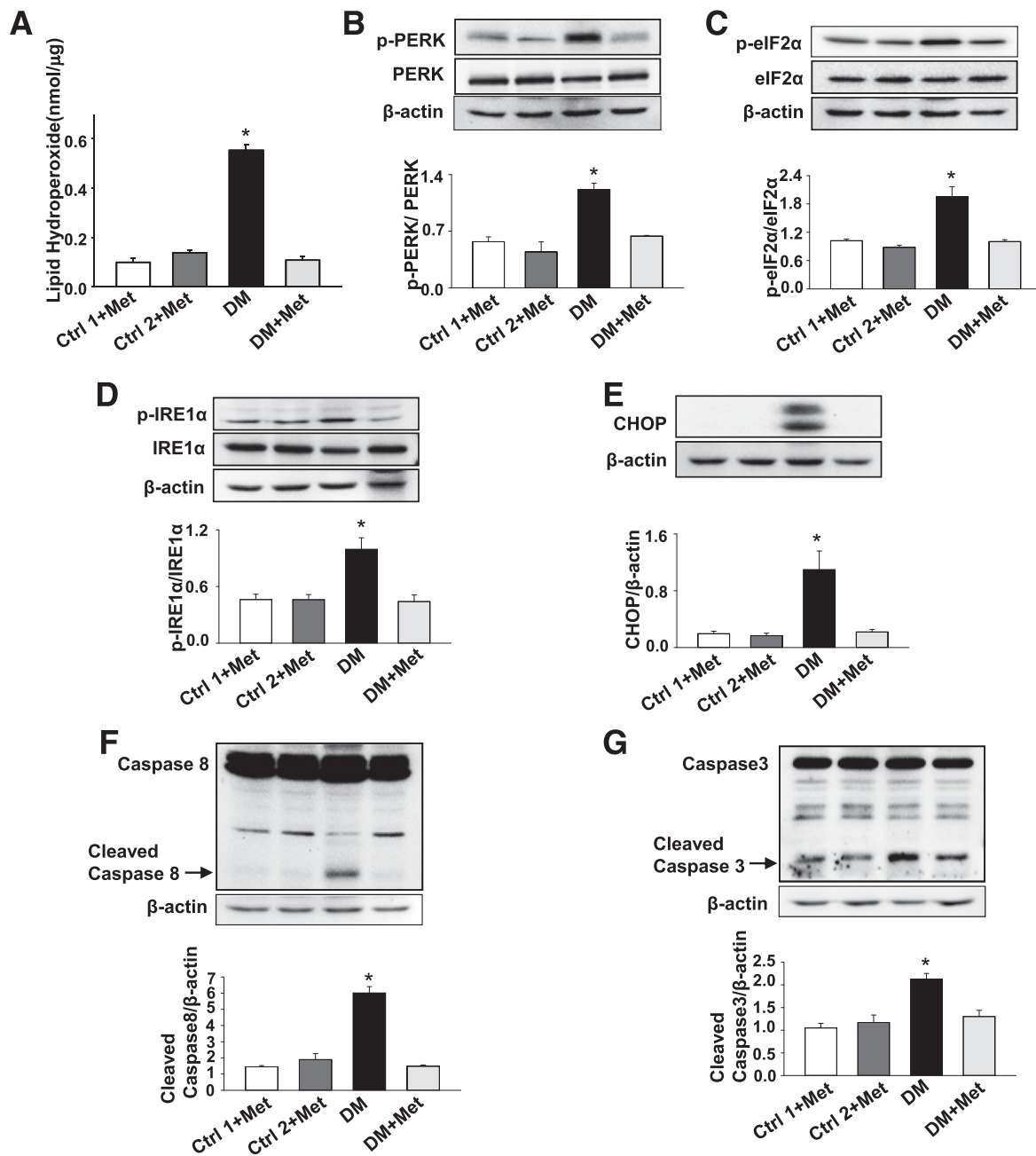


Figure 6—Metformin treatment alleviates maternal type 2 diabetes–induced cellular stress and excessive apoptosis in the developing embryo. (A) LPO levels in E8.5 embryos. Protein levels of p-PERK (B), p-eIF2α (C), p-IRE1α (D), CHOP (E), cleaved caspase-8 (F), and caspase-3 (G) in E8.5 embryos. Experiments were performed using three embryos from three different dams per group. *Significant differences compared with the other three groups. Ctrl 1 + Met, the control normal diet group in which mice were continuously fed chow for 15 weeks and during pregnancy with metformin treatment; Ctrl 2 + Met, the control group in which mice were fed chow for 15 weeks and then HFD during pregnancy with metformin treatment.

characterized a model of type 2 diabetes embryopathy using DIO in C57BL/6J mice fed an HFD. Although type 2 diabetes is a complex metabolic disorder, hyperglycemia with resulting glucotoxicity is a major mediator of diabetes teratogenicity. The modest hyperglycemia (an average blood glucose level of 9.56 ± 0.23 mmol/L) present in our type 2 diabetes animal model causes significant birth defects, specifically NTDs, when compared with

normal mice, even those on an HFD after pregnancy to achieve comparable FFA levels and lipotoxicity. Our model of type 2 diabetes pregnancy generated lower rates of NTD than models of type 1 diabetes, which have an average blood glucose level of 21.3 ± 1.2 mmol/L. These findings are consistent with observations in a human study that the increase of hyperglycemia linearly increases the incidence of birth defects (24).

In this study, treatment with metformin, a standard therapy for pregnant women with type 2 diabetes, inhibited gluconeogenesis in the liver (34), effectively normalizing hyperglycemia in our type 2 diabetes animal model. We found that metformin improves glucose metabolism, reduces hyperglycemia, and significantly ameliorates NTD formation. However, metformin treatment only partially prevented glucose intolerance. The incomplete correction of defective glucose metabolism by metformin may still allow transient hyperglycemia, which we may not have detected. Transient increases in maternal glucose may explain why we observed a higher NTD rate (1.5%) in diabetic dams treated with metformin than in nondiabetic dams. Indeed, *in vitro* embryo culture studies have demonstrated that transient exposure to high glucose induces NTD formation (36).

The type 2 diabetic embryopathy model we used displays hyperinsulinemia and high FFA levels. Hyperinsulinemia associated with type 2 diabetes is unlikely to cause NTD formation because maternal insulin does not cross the blood/placental barrier (37). In addition, NTDs are not caused by embryonic pancreas-derived insulin because pancreatic β -cells do not produce insulin until E11, which is after the neural tube closes (38). Although high fatty acid levels may still affect embryonic development, the current findings demonstrate that high fatty acid levels did not contribute to NTD formation in our type 2 diabetic embryopathy model; lean mice fed an HFD during pregnancy did not produce embryos with NTDs.

Mechanistic studies performed in type 1 diabetic embryopathy models revealed that oxidative stress, ER stress, and caspase-dependent neuroepithelial cell apoptosis are causal events leading to NTD formation (8–16,39–43). In the current study, we found that superoxide production and apoptosis were induced only in the developing neuroepithelia of embryos exposed to maternal type 2 diabetes. We also observed that maternal type 2 diabetes triggered the unfolded protein response and ER stress in the developing embryo. Additionally, we found that the cellular stress and apoptotic signaling pathways in embryos of type 2 diabetic dams mirrored that of type 1 diabetic embryopathy, suggesting that both type 1 and type 2 diabetic embryopathy share common mechanisms underlying NTD formation.

We used the C57BL/6J background in this study. A previous report showed no significant increase of NTDs in the C57BL/6J strain; however, it was inconclusive because embryos from nondiabetic C57BL/6J dams have >12% NTDs (normal incidence 0–1% NTDs), and the study sample size was very small ($n = 4$ dams) (44). That study contradicts a report in 2001 demonstrating that intravenous alloxan-induced diabetes in wild-type C57BL/6J significantly increases the occurrence of NTDs (45). More recent studies from us (9,12,21) and others (46,47) demonstrated that streptozotocin-induced diabetes produces >22% NTDs in the C57BL/6J background. Thus, the C57BL/6J strain responds well to

maternal diabetes. The 11.3% NTD rate in embryos from type 2 diabetic dams is significantly or many-fold higher than the 0% NTD rate from the nondiabetic group. In humans, maternal diabetes induces two- to six-fold higher NTD rates than those from the general population.

In summary, we used obese type 2 diabetic mice to examine embryopathy caused by type 2 diabetes. We found that type 2 diabetes induces oxidative stress and ER stress in the developing neuroepithelium, leading to NTD formation. Metformin treatment significantly reduced NTD formation through partial normalization of the metabolic defects in the type 2 diabetic embryopathy model. Hyperglycemia rather than lipotoxicity seems to be the predominant pathogenic feature inducing embryopathy. The type 2 diabetic embryopathy model is unique and useful, containing key aspects of the metabolic pathophysiology present in women with type 2 diabetes. Thus, this new model may be valuable in elucidating underlying causes of embryopathy in type 2 diabetes that may lead to novel therapeutic interventions for diabetes-induced birth defects.

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