OBSTETRICS

Type 2 diabetes mellitus induces congenital heart defects in murine embryos by increasing oxidative stress, endoplasmic reticulum stress, and apoptosis

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BACKGROUND: Maternal type 1 and 2 diabetes mellitus are strongly associated with high rates of severe structural birth defects, including congenital heart defects. Studies in type 1 diabetic embryopathy animal models have demonstrated that cellular stress-induced apoptosis mediates the teratogenicity of maternal diabetes leading to congenital heart defect formation. However, the mechanisms underlying maternal type 2 diabetes mellitus–induced congenital heart defects remain largely unknown.

OBJECTIVE: We aim to determine whether oxidative stress, endoplasmic reticulum stress, and excessive apoptosis are the intracellular molecular mechanisms underlying maternal type 2 diabetes mellitus–induced congenital heart defects.

STUDY DESIGN: A mouse model of maternal type 2 diabetes mellitus was established by feeding female mice a high-fat diet (60% fat). After 15 weeks on the high-fat diet, the mice showed characteristics of maternal type 2 diabetes mellitus. Control dams were either fed a normal diet (10% fat) or the high-fat diet during pregnancy only. Female mice from the high-fat diet group and the 2 control groups were mated with male mice that were fed a normal diet. At E12.5, embryonic hearts were harvested to determine the levels of lipid peroxides and superoxide, endoplasmic reticulum stress markers, cleaved caspase 3 and 8, and apoptosis. E17.5 embryonic hearts were harvested for the detection of congenital heart defect formation using India ink vessel patterning and histological examination.

RESULTS: Maternal type 2 diabetes mellitus significantly induced ventricular septal defects and persistent truncus arteriosus in the developing heart, along with increasing oxidative stress markers, including superoxide and lipid peroxidation; endoplasmic reticulum stress markers, including protein levels of phosphorylated-protein kinase RNA-like endoplasmic reticulum kinase, phosphorylated-IRE1α, phosphorylated-eIF2α, C/EBP homologous protein, and binding immunoglobulin protein; endoplasmic reticulum chaperone gene expression; and XBP1 messenger RNA splicing, as well as increased cleaved caspase 3 and 8 in embryonic hearts. Furthermore, maternal type 2 diabetes mellitus triggered excessive apoptosis in ventricular myocardium, endocardial cushion, and outflow tract of the embryonic heart.

CONCLUSION: Similar to those observations in type 1 diabetic embryopathy, maternal type 2 diabetes mellitus causes heart defects in the developing embryo manifested with oxidative stress, endoplasmic reticulum stress, and excessive apoptosis in heart cells.

Key words: apoptosis, endoplasmic reticulum stress, heart defects, oxidative stress, type 2 diabetes mellitus

Introduction

Both maternal type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM) increase the risk that offspring will have cardiovascular malformations 5-fold compared with the general population, even with modern preconception care.[1-4] The most common maternal diabetes-associated congenital heart defects (CHDs) include cardiac septation defects such as ventricular septal defects (VSDs) and conotruncal cardiac anomalies such as persistent truncus arteriosus (PTA).[4-6] However, the mechanisms underlying these anomalies remain largely unknown. Because the number of women of reproductive age with T2DM is increasing rapidly, understanding the molecular pathways involved in maternal T2DM-induced CHDs is essential for developing new strategies to prevent these types of birth defects.

Oxidative stress is triggered by an imbalance in intracellular reduction-oxidation (redox) homeostasis, and is sustained by the generation of reactive oxygen species.[7] Our previous studies have revealed that both T1DM and T2DM significantly induce oxidative stress by increasing the levels of superoxide and lipid peroxidation in the developing neuroepithelium (T1DM and T2DM) and the embryonic heart (T1DM).[8-26] Overexpression of the antioxidant enzyme, superoxide dismutase 1 (SOD1), diminishes maternal T1DM-induced oxidative stress in developing neuroepithelium and the embryonic heart, and thus ameliorates neural tube defects (NTDs) and CHDs in offspring of diabetic dams.[20-22,24,26]

Animal studies have shown that both maternal T1DM and T2DM trigger endoplasmic reticulum (ER) stress in neurulation-stage embryos (T1DM and T2DM) and in embryonic hearts (T1DM).[13,18,20,24,26] ER stress occurs when misfolded proteins accumulate in the ER lumen and cause ER dysfunction.[27] Both maternal T1DM and T2DM disrupt ER luminal homeostasis by enhancing transcription of the proapoptotic C/EBP homologous protein (CHOP), and increasing other ER stress markers, such as binding immunoglobulin protein (BiP) and calnexin, in the developing neuroepithelium.[28,29] In

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TABLE 1
Sequences of primers used in real-time quantitative polymerase chain reaction

Primers name	Primer sequences
BiP	Forward primer 5'-ACTTGGGGACCACCTATTCCT-3'
	Reverse primer 5'-ATCGCCAATCAGACGCTCC-3'
CHOP	Forward primer 5'-CGGAACCTGAGGAGAGAGTG-3'
	Reverse primer 5'-CTGTCAGCCAAGCTAGGGAC-3'
Calnexin	Forward primer 5'-ATGGAAGGGAAGTGGTTACTGT-3'
	Reverse primer 5'-GCTTTGTAGGTGACCTTTGGAG-3'
IRE1α	Forward primer 5'-ACACCGACCACCGTATCTCA-3'
	Reverse primer 5'-CTCAGGATAATGGTAGCCATGTC-3'
PDIA	Forward primer 5'-CGCCTCCGATGTGTTGGA-3'
	Reverse primer 5'-CAGTGCAATCCACCTTTGCTAA-3'
GRP94	Forward primer 5'-TCGTCAGAGCTGATGATGAAGT-3'
	Reverse primer 5'-GCGTTTAACCCATCCAACTGAAT-3'
β-Actin	Forward primer 5'-GAACCAGGAGTTAAGAACACG-3'
	Reverse primer 5'-AGGCAACAGTGTCAGAGTCC-3'

BiP, binding immunoglobulin protein; *CHOP*, C/EBP homologous protein.
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addition, we have shown in vitro that the ER stress inhibitor, 4-phenylbutyric acid, inhibits NTD formation in cultured embryos exposed to high glucose.[30]

Oxidative stress— and ER stress-induced cell apoptosis are causative events in maternal T1DM- and T2DM-induced NTDs.[8,13,18,30-33] Apoptosis is a precisely controlled cellular event that is essential to many biological processes.[34] However, our previous studies have demonstrated that maternal T1DM and T2DM induces excessive apoptosis, leading to defective neurulation and failed neural tube closure.[13,18,32] Furthermore, we have found that deletion of the gene for apoptosis signal-regulating kinase 1 significantly ameliorates diabetes-induced NTDs.[24] Therefore, we hypothesize that oxidative stress, ER stress, and subsequent excessive apoptosis are contributing factors for maternal T2DM-induced CHDs.

Here, we use a high-fat diet (HFD)-induced T2DM mouse model to explore whether oxidative stress and ER stress-induced excessive apoptosis are present in T2DM-induced CHDs. A previous study demonstrated that this murine T2DM model exhibits modest hyperglycemia, glucose intolerance, insulin resistance, and hyperinsulinemia,[18] all of which are characteristics of human T2DM. We showed that the levels of oxidative stress, ER stress, and apoptosis markers were increased in hearts of embryos from T2DM dams. We also found that maternal T2DM specifically induced VSDs and PTA. By investigating the role of abnormal cellular processes in embryonic heart development, we elucidated possible mechanisms in T2DM-induced CHDs.

TABLE 2
Maternal type 2 diabetes mellitus induces heart defects in developing embryos

Group	Dams, n	Glucose level, mmol/L	Date	Hearts, n	VSD rate (%)	PTA rate (%)
Ctrl 1	5	5.8 $\pm$ 0.46	E17.5	47	0 (0.0)	0 (0.0)
DM	7	9.3 $\pm$ 0.31[a]	E17.5	46	6 (13.0)[a]	2 (3.7)[a]
Ctrl 2	5	6.9 $\pm$ 0.35	E17.5	44	0 (0.0)	0 (0.0)

Ctrl 1, control group fed normal diet; *Ctrl 2*, control group fed 60% high-fat diet during pregnancy; *DM*, group fed high-fat diet; *PTA*, persistent truncus arteriosus; *VSD*, ventricular septal defect.
[a] Significant differences compared with other 2 groups as analyzed by 1-way analysis of variance followed by Tukey (blood glucose levels) or χ^2 (VSD and PTA rates) test.
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Materials and Methods

Animal model of embryopathy

The procedures for animal use were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee. The mouse model of T2DM was established as previously described.[18] 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-hour light-dark cycle. After arrival, mice were divided into 2 groups and fed either a HFD (Research Diets Inc, New Brunswick, NJ) or a normal diet (Harlan Laboratories, Indianapolis, IN) for 15 weeks. The HFD contained 20% protein, 20% carbohydrate, and 60% fat. The normal diet contained 20% protein, 70% carbohydrate, and 10% fat. HFD mice and mice fed the normal diet were mated with lean male mice. During pregnancy, mice in the normal diet group were either maintained on the normal diet (control group 1) or subsequently fed HFD to serve as the high circulating free fatty acid control group (control group 2). Male and female mice were paired at 3:00 PM, and day 0.5 (E0.5) of pregnancy was established at noon of the day when a vaginal plug was present. Only CHDs were examined in these animals. NTDs were not the subject of the present study.

India ink injection and hematoxylin-eosin staining

After euthanizing the pregnant dams at E17.5, fetuses were excised from uteri