Superoxide Dismutase 1 In Vivo Ameliorates Maternal Diabetes Mellitus–Induced Apoptosis and Heart Defects Through Restoration of Impaired Wnt Signaling

Fang Wang, PhD; Steven A. Fisher, MD; Jianxiang Zhong, PhD; Yanqing Wu, BS; Peixin Yang, PhD

Background—Oxidative stress is manifested in embryos exposed to maternal diabetes mellitus, yet specific mechanisms for diabetes mellitus–induced heart defects are not defined. Gene deletion of intermediates of Wingless-related integration (Wnt) signaling causes heart defects similar to those observed in embryos from diabetic pregnancies. We tested the hypothesis that diabetes mellitus–induced oxidative stress impairs Wnt signaling, thereby causing heart defects, and that these defects can be rescued by transgenic overexpression of the reactive oxygen species scavenger superoxide dismutase 1 (SOD1).

Methods and Results—Wild-type (WT) and SOD1-overexpressing embryos from nondiabetic WT control dams and nondiabetic/diabetic WT female mice mated with SOD1 transgenic male mice were analyzed. No heart defects were observed in WT and SOD1 embryos under nondiabetic conditions. WT embryos of diabetic dams had a 26% incidence of cardiac outlet defects that were suppressed by SOD1 overexpression. Insulin treatment reduced blood glucose levels and heart defects. Diabetes mellitus increased superoxide production, canonical Wnt antagonist expression, caspase activation, and apoptosis and suppressed cell proliferation. Diabetes mellitus suppressed Wnt signaling intermediates and Wnt target gene expression in the embryonic heart, each of which were reversed by SOD1 overexpression. Hydrogen peroxide and peroxynitrite mimicked the inhibitory effect of high glucose on Wnt signaling, which was abolished by the SOD1 mimetic, tempol.

Conclusions—The oxidative stress of diabetes mellitus impairs Wnt signaling and causes cardiac outlet defects that are rescued by SOD1 overexpression. This suggests that targeting of components of the Wnt5a signaling pathway may be a viable strategy for suppression of congenital heart defects in fetuses of diabetic pregnancies. (Circ Cardiovasc Genet. 2015;8:665-676. DOI: 10.1161/CIRCGENETICS.115.001138.)

Key Words: apoptosis ■ congenital ■ diabetes mellitus ■ heart defects ■ pregnancy ■ oxidative stress ■ Wnt signaling pathway

Congenital heart defects (CHD) are the most prevalent birth defects occurring in ≈4 to 10 per 1000 live births.¹ To date, there have been multiple breakthroughs in understanding the genetic basis of CHD. However, many epidemiological evidences suggest a significant contribution of environmental factors to the induction of CHD.²⁻⁴ Unfortunately, there is a lack of mechanistic information about the non-genetic factors that adversely affect heart development. The panel of maternal diabetes mellitus is a non-genetic factor that is associated with a 5-fold increase in CHD risk.³⁻⁴ The CHD commonly seen in diabetic pregnancies are ventricular septal defects (VSD)³⁻⁵ and outflow tract defects.³⁻⁴ The same types of CHD are also observed in diabetic mouse models.⁶⁻⁷ Studies of animal models have suggested that apoptosis⁸⁻¹⁰ and gene dysregulation⁸⁻¹¹ are involved in diabetes mellitus–induced CHD. However, the mechanism underlying defective heart formation under diabetic conditions is not clearly defined.

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Wnt signaling is essential for normal heart development.¹² Targeted gene deletion of key Wnt intermediates results in heart defects similar to those observed in human diabetic pregnancies.¹³⁻¹⁴ Altered Wnt signaling has been implicated in diabetes mellitus–induced birth defects.¹⁵ However, the role of the Wnt pathway in diabetes mellitus–induced heart maldevelopment has not been clarified. Wnt signaling has traditionally been classified into the canonical and the non-canonical pathways.¹² In the canonical pathway, Wnt ligands bind to the membrane-spanning receptor proteins, Frizzleds, and subsequently trigger the phosphorylation of Dishevelled protein (Dvl). Dvl activates a signaling cascade that results in the stabilization and nuclear localization of β-catenin, which interacts with the T-cell–specific factor/lymphoid enhancer-binding factor transcription factor to induce the transcription of genes involved in heart development.

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From the Departments of Obstetrics, Gynecology, and Reproductive Sciences (F.W., J.Z., Y.W., P.Y.), Medicine (S.A.F.), and Biochemistry and Molecular Biology (P.Y.), School of Medicine, University of Maryland, Baltimore.


Correspondence to Peixin Yang, PhD, Department of Obstetrics, Gynecology, and Reproductive Sciences, University of Maryland School of Medicine, BB11-039, 655 W. Baltimore St, Baltimore, MD 21201. E-mail pyang@fpi.umaryland.edu

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of canonical Wnt signaling target genes. In the absence of Wnt proteins, β-catenin is phosphorylated by glycogen synthase kinase-3β (GSK3β) and degraded through the ubiquitin-proteosome pathway. The noncanonical Wnt ligand, Wnt5a, activates the Ca²⁺/calcineurin signaling pathway, which is critically involved in cardiogenesis. Wnt5a activates calcineurin and its downstream transcriptional effector, the nuclear factor of activated T cells (NFAT). In the nucleus, NFAT is associated with transcription factors such as activator protein 1 and myocyte enhancer factor 2 to induce gene transcription. The Ca²⁺/calcineurin/NFAT signaling pathway is essential for stress. Using the SOD1-Tg mouse model, we aimed to determine whether maternal diabetes mellitus abrogates oxidative stress. Our previous studies showed that superoxide dismutase (SOD1) overexpression in SOD1 transgenic (SOD1-Tg) mice abrogates maternal diabetes mellitus–induced oxidative stress. Using the SOD1-Tg mouse model, we aimed to determine whether maternal diabetes mellitus alters Wnt signaling in the developing heart.

Maternal diabetes mellitus increases the production of reactive oxygen species and impairs endogenous antioxidant activities leading to oxidative stress during embryonic organogenesis. Antioxidant treatments elicit some beneficial effects against hyperglycemia-induced CHD. In addition, inducing oxidative stress by an inhibitor of the mitochondrial respiratory chain replicates the effect of maternal diabetes mellitus on CHD formation. These studies implicate a critical role of oxidative stress in maternal diabetes mellitus–induced CHD. To unravel the molecular pathway downstream of oxidative stress leading to CHD in diabetic pregnancies is an essential step toward the development of effective interventions, which directly target identified molecular intermediates downstream of oxidative stress. Our previous studies showed that superoxide dismutase 1 (SOD1) overexpression in SOD1 transgenic (SOD1-Tg) mice abrogates maternal diabetes mellitus–induced oxidative stress.

Methods

Mouse Models of Diabetic Embryopathy

Mouse models of maternal diabetes mellitus–induced embryonic malformations were described previously. The procedures for animal use were approved by the Institutional Animal Care and Use Committee of University of Maryland School of Medicine.

Statistical Analyses

Data on heart defect rates were analyzed by Fisher exact test or χ² test. Data on protein and mRNA expression are presented as mean±SE. The nonparametric Mann–Whitney U test was performed using the Statistical Package for the Social Sciences (SPSS) Statistics software to estimate significance. Statistical significance was accepted when P<0.05 in multiple comparisons.

Results

SOD1 Overexpression Suppresses Maternal Diabetes Mellitus–Induced Oxidative Stress in the Developing Heart

Staining with DHE of sections in a frontal plane with respect to the heart as shown in Figure 1A was used to detect superoxide in the embryonic heart. E12.5 hearts from nondiabetic dams showed minimal fluorescent signal, whereas embryonic hearts from diabetic mice exhibited robust Dihydroethidium (DHE) staining (Figure 1B). Maternal diabetes mellitus also significantly increased the abundance of lipid hydroperoxide, an index of lipid peroxidation, in the developing heart (Figure 1C). SOD1 overexpression suppressed maternal diabetes mellitus–increased DHE staining (Figure 1B) and lipid peroxidation (Figure 1C).

Suppression of Oxidative Stress Reduces the Frequency of Maternal Diabetes Mellitus–Induced Heart Defects

Maternal diabetes mellitus significantly induced heart defects. Embryos from diabetic dams did not appear to be growth retarded as determined by somite numbers. Serial cross sections of E17.5 hearts in embryos exposed to diabetes mellitus revealed large VSDs in 18% of hearts (Table; Figure 2A). The VSDs in anterior positions (Figure 2A) were in the cardiac outlet and associated with an over-riding aorta (Figure 2A). More posterior VSDs were large and in the muscular septum. Indian ink injections indentified persistent truncus arteriosus (PTA) in 4 of 50 embryos exposed to diabetes mellitus (Table; Figure 2B). We next examined the incidence of heart defects in wild-type and SOD1-overexpressing embryos from wild-type dams mated with wild-type or SOD1 transgenic males. Under nondiabetic conditions, SOD1 overexpression did not affect heart development (Table). The incidence of heart defects in wild-type embryos (n=50) from diabetic wild-type dams mated with wild-type males was 26% (Table). The types of heart defects were VSDs with or without aorta over-riding (Figure 2A) and PTAs (Figure 2B). Likewise, a similar high incidence of heart defects was also present in wild-type embryos from diabetic wild-type dams mated with SOD1-Tg male mice (Table; Figure 2B). In contrast, there were no heart defects in SOD1-overexpressing embryos from diabetic wild-type dams mated with SOD1-Tg male mice (Table). To reveal whether reduced hyperglycemia by insulin treatment ameliorates diabetes mellitus–induced heart defects, insulin pellets remained in diabetic dams throughout the course of the experiments. Insulin treatment effectively reduced blood glucose levels in diabetic dams and significantly reduced the incidence of heart defects (Table). These results support our hypothesis that hyperglycemia-induced oxidative stress mediates the teratogenic effect of maternal diabetes mellitus on the developing heart.

SOD1 Overexpression Inhibits Caspase Activation and Apoptosis and Restores Cell Proliferation in the Developing Heart Exposed to Diabetes Mellitus

Previous studies have reported that maternal diabetes mellitus induces apoptosis in cells of the developing heart. We sought to determine whether oxidative stress is responsible for diabetes mellitus–induced apoptosis. By TUNEL assay, apoptotic cells were present with increased frequency in the endocardial cushions of the outflow tract (OFT) and AV canal and the epicardial lining of the ventricles of E12.5 wild-type embryos from diabetic dams (Figure 3A and 3B). Under diabetic conditions, SOD1-overexpressing hearts had significantly fewer apoptotic cells than wild-type hearts (Figure 3A and 3B). Maternal diabetes mellitus induces apoptosis in a caspase-dependent manner in neurulation.
stage embryos. To determine whether maternal diabetes mellitus induces caspase activation in the heart, cleaved caspase 3 and caspase 8 were assessed. Maternal diabetes mellitus induced caspase 3 and caspase 8 cleavage in wild-type embryos, but cleaved caspase products were significantly reduced in SOD1-overexpressing embryos (Figure 3C).

We used p-Histone H3 staining to evaluate cell proliferation. Maternal diabetes mellitus significantly reduced the number of cells with p-Histone H3 staining, and SOD1 overexpression blunted the reduction in the number of p-Histone H3–positive cells (Figure 3D).

**SOD1 Overexpression Blocks the Inhibitory Effect of Maternal Diabetes Mellitus on Canonical Wnt Signaling in the Developing Heart**

We analyzed the mRNA expression of 5 canonical Wnt ligands, Wnt1, Wnt2a, Wnt3a, Wnt7b, and Wnt8a, and observed no significant changes in hearts from wild-type embryos or SOD1-overexpressing embryos under both non-diabetic and diabetic conditions (data not shown). Because SOD1 overexpression under non-diabetic conditions does not affect heart development, cell apoptosis, and proliferation, subsequent analyses will not include this group.

### Table. Heart Defect Rates in E17.5 Embryos of Nondiabetic and Diabetic Dams

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Glucose Level, mg/dL</th>
<th>Embryo Genotype</th>
<th>Total Embryos</th>
<th>Total Heart Defect Embryos</th>
<th>VSD</th>
<th>VSD With AO</th>
<th>PTA</th>
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<td>ND</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>WT ♂×WT ♀ (8 L)</td>
<td>140.8±9.3</td>
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<td>48</td>
<td>0</td>
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<tr>
<td>SOD1-Tg ♂×WT ♀ (14 L)</td>
<td>124.8±12.6</td>
<td>WT</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>WT ♂×WT ♀ (9 L)</td>
<td>433.8±20.6</td>
<td>WT</td>
<td>50</td>
<td>13 (26%)*</td>
<td>6</td>
<td>3</td>
<td>4</td>
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<tr>
<td>SOD1-Tg ♂×WT ♀ (14 L)</td>
<td>443.6±14.7</td>
<td>WT</td>
<td>40</td>
<td>10 (25%)*</td>
<td>5</td>
<td>2</td>
<td>3</td>
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<td>SOD1</td>
<td>45</td>
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<td>0</td>
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<tr>
<td>SOD1-Tg ♂×WT ♀ (9 L)</td>
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<td>2 (6.0%)†</td>
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* indicates male; ♀, female (dams); AO, aorta over-riding; DM, diabetes mellitus; DM+Insulin: insulin pellets were maintained throughout the course of experiments; ND, nondiabetic; SOD1-Tg, superoxide dismutase 1-transgene; PTA, persistent truncus arteriosus; VSD, ventricular septal defects; and WT, wild-type.

*Significant difference compared with SOD1-overexpressing embryos and embryos in the ND group.
†Significant difference compared with WT embryos from WT males and WT DM dams.
WT embryo/ND Dam  WT embryo/DM Dam

Figure 2. Maternal diabetes mellitus (DM) induces outflow tract and ventricular septal defects (VSDs). A, Hematoxylin and eosin (H&E)-stained E17.5 hearts from anterior to posterior: normal heart (left) from wild-type (WT) embryos of nondiabetic (ND) dam, heart with an isolated VSD (middle) representative images of H&E-stained sections, whereas B is representative images of India ink dye injections in E17.5 embryonic hearts. A, Morphologically normal heart (left) from WT embryos of ND dam, a typical VSD heart (middle), and aorta-overriding VSD heart (right) from WT embryos of DM dam. B, E17.5 heart and blood vessels imaged in whole mount after injection of India Ink: a normal heart from WT embryos of nondiabetic dam and a heart with persistent truncus arteriosus (PTA) from WT embryos of diabetic dam. Scale bars, 300 μm. ao indicates aorta; la, left atrium; lv, left ventricle; pa, pulmonary artery; ra, right atrium; and rv, right ventricle.

Because Wnt expression at the mRNA level is not changed, we turned our attention to factors that may block Wnt protein binding. The availability of Wnt ligands for their receptors is regulated by secreted Wnt antagonists. The mRNA expression of 3 major Wnt antagonists was assessed. The mRNA expression of the Wnt antagonist, WIF1 (Wnt inhibitory factor 1), was not affected by maternal diabetes mellitus (Figure 4A). However, the mRNA expression of the other 2 Wnt antagonists, secreted frizzled-related protein 1 (sFRP) and Dickkopf WNT Signaling Pathway Inhibitor 1 (DKK1), was significantly increased in hearts of wild-type embryos from diabetic dams when compared with that in hearts of wild-type embryos of nondiabetic dams and SOD1-overexpressing embryos of diabetic dams (Figure 4A).

To determine whether diabetes mellitus–induced increases in sFRP1 and DKK1 expression impact canonical Wnt signaling, we analyzed the phosphorylation status of key canonical Wnt signaling intermediates. Consistent with the increase of Wnt antagonists, maternal diabetes mellitus significantly decreased Dvl2 phosphorylation, a positive canonical Wnt signaling transducer and increased GSK3β activity by reducing the levels of phosphorylated GSK3β (an inactive form of GSK3β), a negative canonical Wnt signaling regulator (Figure 4B). SOD1 overexpression blunted maternal diabetes mellitus–induced Dvl2 dephosphorylation and GSK3β phosphorylation (Figure 4B). All the above upstream Wnt signaling components eventually converge on β-catenin, the central mediator of the canonical Wnt pathway. Total β-catenin protein abundance was decreased by diabetes mellitus, and SOD1 overexpression blocked this effect (Figure 4B). Under nondiabetic conditions, β-catenin was robustly present in the ventricular myocardium, the endocardial cushion, and outflow tract (Figure 4C). Maternal diabetes mellitus suppressed β-catenin protein staining in the ventricular myocardium, the endocardial cushion, and the outflow tract area (Figure 4C). These results suggest that maternal diabetes mellitus blocked the canonical Wnt signaling in the developing heart. Under diabetic conditions, SOD1 overexpression restored β-catenin expression in the heart (Figure 4C).

**SOD1 Overexpression Restores Maternal Diabetes Mellitus–Impaired Noncanonical Wnt Signaling in the Developing Heart**

We next investigated whether the noncanonical Wnt signaling is also affected by maternal diabetes mellitus. Abundance of the major noncanonical Wnt ligand, Wnt5a mRNA and protein, was significantly decreased in hearts of wild-type embryos of diabetic dams when compared with those in hearts of wild-type embryos from nondiabetic dams and SOD1-overexpressing embryos from diabetic dams (Figure 5A). Immunofluorescent staining revealed that Wnt5a was predominately localized in the cardiac outflow tract (Figure 5C). Maternal diabetes mellitus suppressed and SOD1 overexpression restored Wnt5a expression in the outflow tract area (Figure 5C).

Wnt5a is essential for normal cardiac outflow tract development and deletion of Wnt5a gene induces cardiac outflow tract defects similar to those observed in diabetic pregnancies. Wnt5a binds to the receptor tyrosine kinase Ror1/2 leading to activation of the noncanonical Ca2+/NFAT pathway. NFAT2 and NFAT4 are 2 members of the NFAT family that are critical for cardiogenesis. The level of phosphorylated NFAT4 (inactive form) was significantly upregulated by maternal diabetes mellitus (Figure 5D). Maternal diabetes mellitus–induced NFAT4 phosphorylation was diminished by SOD1 overexpression. NFAT2 protein abundance in nuclear fractions was significantly decreased by maternal diabetes mellitus, and SOD1 overexpression restored NFAT2 nuclear accumulation (Figure 5E). On the contrary, levels of the phosphorylated (active) CaMKII, a negative regulator of...
the noncanonical Ca\textsuperscript{2+}/NFAT pathway\textsuperscript{28} were significantly increased by maternal diabetes mellitus (Figure 5F) and this increase was blunted by SOD1 overexpression (Figure 5F).

These results support that oxidative stress is responsible for maternal diabetes mellitus–induced impairment of the noncanonical Wnt5a-Ca\textsuperscript{2+}/NFAT pathway.
SOD1 Overexpression Restores Maternal Diabetes Mellitus–Suppressed Wnt Target Gene Expression in the Developing Heart

Because maternal diabetes mellitus suppresses the canonical Wnt signaling, we tested 3 target genes of the canonical Wnt signaling: Islet1, GJA1, and Versican. The mRNA levels of these 3 genes were significantly decreased by maternal diabetes mellitus (Figure 6A) and restored by SOD1 overexpression (Figure 6A). Similarly, the mRNA levels of 3 NFAT target genes (Mrtf-b, Tpm1, and Rcan1) were significantly lower in hearts of wild-type embryos exposed to diabetes mellitus than those in hearts of wild-type embryos from nondiabetic dams (Figure 6B). SOD1 overexpression restored the expression of Mrtf-b, Tpm1, and Rcan1 that was suppressed by maternal diabetes mellitus (Figure 6B).

**Figure 4.** Superoxide dismutase 1 (SOD1) overexpression rescues diabetes mellitus (DM)–increased canonical Wnt antagonists and restores the expression of key canonical Wnt intermediates. A, mRNA abundance of the 3 canonical Wnt antagonists. B, Representative Western blots of phosphorylated and nonphosphorylated dishevelled protein (Dvl)-2, phosphorylated by glycogen synthase kinase-3β (p-GSK3β), and β-catenin. The dot graphs showed the quantification of the immunoblotting. A and B, Three hearts from embryos of 3 dams (n=3) per group were analyzed. C, Images of β-catenin immunostaining. Rabbit normal IgG served as controls. Red signal was β-catenin, and cell nuclei were stained by DAPI (4',6-diamidino-2-phenylindole) (blue). Three E12.5 hearts from embryos of 3 dams (n=3) per group, and 3 serial sections per heart were analyzed, and similar results were obtained. Bars, 150 μm. *Significant difference compared with other groups. DKK1 indicates Dickkopf WNT signaling pathway inhibitor 1; ND, nondiabetic; sFRP, secreted frizzled-related protein 1; Tg, transgene; WIF1, Wnt inhibitory factor 1; and WT, wild-type hearts.
Both Hydrogen Peroxide and Peroxynitrite Mimic the Inhibitory Effect of High Glucose on Wnt Signaling

Next, we sought to determine whether ROS or RNS suppression of Wnt signaling directly mediates the teratogenic effect of maternal diabetes mellitus on the developing heart by exposing cultured embryos to these agents in vitro. Treatment with either 100 μmol/L H₂O₂ or 1 μmol/L peroxynitrite mimicked high glucose suppression of β-catenin and Wnt5a expression, increased DKK1 expression, and inactivated the key noncanonical intermediate, NFAT4, through phosphorylation (Figure 7B). The ROS scavenger (SOD-mimetic) Tempol (5 mmol/L) restored high glucose–inhibited canonical and noncanonical Wnt signaling (Figure 7A). Thus SOD1 overexpression in vivo and tempol treatment in vitro abrogated Wnt signaling inhibition by diabetes mellitus or high glucose.

Figure 5. Maternal diabetes mellitus (DM) impairs the noncanonical Wnt pathway, and this impairment is abolished by superoxide dismutase 1 (SOD1) overexpression. A, mRNA levels of Wnt5a in E12.5 hearts. B, Wnt5a protein levels in E12.5 hearts. C, Images of Wnt5a immunostaining. Rabbit normal IgG served as controls. Red signal was Wnt5a, and cell nuclei were stained by DAPI (4',6-diamidino-2-phenylindole) (blue). Three hearts from embryos of 3 dams (n=3) per group, and 3 serial sections per heart were analyzed, and similar results were obtained. Bars, 150 μm. D, Levels of phosphorylated nuclear factor of activated T cells (NFAT)-4. E, Levels of nuclear NFAT2. F, Levels of p-CaMKII (phosphorylated Ca²⁺/calmodulin-dependent protein kinase II). B–F, Quantification of immunoblotting is shown in the dot graph. A, B, D, E, and F, Experiments were repeated 3× using 3 E12.5 hearts from embryos of 3 dams (n=3) per group. *Significant difference compared with other groups. ND indicates nondiabetic; Tg, transgene; and WT, wild-type hearts.
supporting ROS inhibition of Wnt signaling playing a causal role in diabetes mellitus–induced heart defects.

**SOD1 Overexpression Is Unable to Prevent Heart Defects in Wnt5a Null Embryos**

Because SOD1 overexpression restored the noncanonical Wnt signaling by both preventing Wnt5 downregulation and inactivating the negative regulator, CaMKII, it is possible that SOD1 may act downstream of Wnt5a. To test whether SOD1 rescues the noncanonical Wnt signaling by directly inhibiting negative regulators downstream of Wnt5a, Wnt5a−/− females were crossed with Wnt5a−/−;SOD1 males to generate Wnt5a null embryos with or without SOD1 overexpression (Table I and Figure I in the Data Supplement). As previously reported,16 Wnt5a null embryos exhibited heart defects (VSD and PTA) with 100% penetrance similar to those in embryos of diabetic dams (Table I in the Data Supplement). SOD1 overexpression did not reduce the incidence of heart defects in Wnt5a null embryos (Table I in the Data Supplement), suggesting that the heart defects in the Wnt5a knockout model are not ROS mediated. These findings support the hypothesis that the beneficial effect of SOD1 is because of its direct effect on Wnt5a by preventing Wnt5a downregulation.

**Discussion**

In the current study, we demonstrate that a mouse model of maternal diabetes mellitus induces a spectrum of heart defects similar to that of human diabetic pregnancies.3,5 In humans, maternal diabetes mellitus induces ≈5% VSDs and 12% outflow tract defects.3 These incidences are comparable with the incidences of our STZ-induced diabetic mouse model. The heart defect incidences also are in agreement with those of other animal studies.6,7 The cause of these heart defects under maternal diabetic conditions is not clear. Although it has been shown that oxidative stress mimics the teratogenicity of maternal diabetes mellitus leading to heart defects,9 no direct evidence supports the hypothesis that oxidative stress causes heart defects in diabetic pregnancies. Our study demonstrates the presence of superoxide and lipidperoxidation in the developing heart exposed to diabetes mellitus. Furthermore, we provide direct evidence supporting the oxidative stress hypothesis by showing that SOD1 overexpression, which effectively mitigates maternal diabetes mellitus–induced oxidative stress in the developing heart, prevents diabetes mellitus–induced heart defects. In addition, our ex vivo studies demonstrated that superoxide, hydrogen peroxide, and peroxynitrite form a hierarchy of oxidants and are all necessary for the suppression of the Wnt signaling. The SOD1 mimetic, tempol, effectively prevents high glucose–induced impairment of Wnt signaling. In agreement with our findings, a recent study demonstrated that the antioxidant, N-acetylcysteine, suppresses diabetes mellitus–induced oxidative stress–heart defects.29 Our findings advance the oxidative stress hypothesis by revealing perturbed Wnt signaling as a mediator of oxidative stress—heart defects. Additional studies are...
needed to identify specific cellular superoxide sources mediating impairment of Wnt signaling in the developing heart. NADPH oxidases and mitochondrial dysfunction are potential sources of cellular ROS and subsequent formation of RNS. 

Embryonic heart development is an intricate process that requires fine-tuned cell proliferation, apoptosis, and differentiation. Excess apoptosis and reduced cell proliferation have been implicated in CHD formation and myocardial hypoplasia. Enhanced apoptosis and reduced cell proliferation are observed in embryonic hearts exposed to diabetes mellitus. The apoptotic cells are present in the endocardial cushion and the myocardium, which may contribute to VSD formation. 

Figure 7. Superoxide dismutase 1 (SOD1) mimetic, tempol, abolishes high glucose–suppressed Wnt signaling, and both ROS and RNS inhibit Wnt signaling in cultured hearts. A and B, DKK1, β-catenin, Wnt5a and phosphorylated nuclear factor of activated T cells (NFAT)-4 levels in cultured hearts. Experiments were repeated 3×. C, Schematic of oxidative stress-mediated Wnt signaling impairment in the developing heart leading to heart defects under maternal diabetic conditions. Oxidative stress induced by maternal diabetes inhibits both the canonical and the noncanonical Wnt signaling pathways through 2 distinct mechanisms. Maternal diabetes mellitus suppresses the canonical Wnt signaling by increasing its antagonist expression and the activity of its negative regulator, whereas it inhibits the noncanonical Wnt signaling by downregulating Wnt5a. Major types of heart defects associated with gene deletion of key Wnt intermediates are indicated. *Significant difference compared with other groups. DKK1 indicates Dickkopf WNT signaling pathway inhibitor 1; DVL, disheveled protein; GSK3, glycogen synthase kinase-3β; p-NFAT, phosphorylated nuclear factor of activated T cells; p-CaMKII, phosphorylated Ca2+/calmodulin-dependent protein kinase II; RNS, reactive nitrogen species; ROS, reactive oxygen species; sFRP, secreted frizzled-related protein 1; VSD, ventricular septal defect; and WIF1, Wnt inhibitory factor 1.
induction, and in the outflow tract, which may be responsible for PTA formation. The mechanism underlying maternal diabetes mellitus–induced embryonic heart cell apoptosis is elusive. In neurulation stage embryos, caspase 3 and 8 activation are critically involved. Caspase 8 is an initiator caspase, whereas caspase 3 is an executive caspase. Both caspase 3 and 8 are activated in the embryonic heart by maternal diabetes mellitus, and this activation is blocked by SOD1 overexpression. Oxidative stress can induce caspase activation and subsequent apoptosis by suppressing prosurvival signaling, activating proapoptotic signaling, or both. The Wnt pathway triggers prosurvival signaling and promotes cell proliferation by stimulating cell cycle gene expression. Maternal diabetes mellitus inhibits heart cell proliferation, and SOD1 abrogates this inhibition. Although other signaling pathways may also be involved, based on our results, it seems that impaired Wnt signaling mainly mediates the promoting effect of apoptosis and the inhibitory effect of cell proliferation downstream of oxidative stress in diabetes mellitus–induced CHD.

Maternal diabetes mellitus alters signaling pathways such as the BMP4 pathway that is essential for normal cardiogenesis. Beyond Wnt inhibition, sFRP1 antagonizes the BMP4 signaling. It is possible that elevated sFRP1 expression is responsible for maternal diabetes mellitus–impaired BMP4 signaling. Both the canonical and the noncanonical Wnt signaling pathways are crucial for heart development. In the canonical Wnt pathway, Wnts bind to the Frizzled receptors and trigger Dvl2 activation, which results in the inhibition of GSK3β activity and the subsequent stabilization of its target β-catenin, which leads to gene transcription. Gene deletion of key intermediates in the canonical pathways including Dvl2 and β-catenin causes outflow tract defects including pulmonary atresia, PTA and VSD, which are observed in diabetic pregnancies, suggesting a link between impaired canonical Wnt signaling and maternal diabetes mellitus–induced heart defects. Maternal diabetes mellitus does not affect the expression of traditionally canonical Wnt members but increases the expression of secreted Wnt antagonists DKK1 and sFRP1. The impact of increased Wnt antagonists is translated into decreased Dvl2 phosphorylation, increased GSK3β activation and destabilizing β-catenin. Deletion of β-catenin results in the loss of cardiac neural crest cells. Because it has been demonstrated that maternal diabetes mellitus induces the loss of cardiac neural crest cells, we reason that decreased β-catenin may contribute to the loss of these cells leading to outflow tract defects, β-catenin null mutations reduce cell proliferation in the myocardium. Correlated with outflow tract defects and VSDs in diabetic pregnancies, β-catenin is expressed in the outflow tract area and in the myocardium. Therefore, impaired canonical Wnt signaling may contribute to outflow tract defects and VSD formation.

DKK1 and sFRP1 expressions is known to be responsive to oxidative stress. Our data support that maternal diabetes mellitus increases the expression of DKK1 and sFRP1 through oxidative stress. Because the stress kinases, c-Jun-N-terminal kinases 1/2, is responsible for the induction of DKK1 by oxidative stress in vitro, maternal diabetes mellitus–induced oxidative stress may stimulate Wnt antagonist expression through c-Jun-N-terminal kinases 1/2, which is activated in embryos exposed to maternal diabetes mellitus. Other stress kinase such as the protein kinase C pathway is also activated by maternal diabetes mellitus and is downstream of oxidative stress and could also play a role in impaired Wnt activation in our mouse model. Epigenetic modifications may be also involved in diabetes mellitus–induced sFRP1 and DKK1 expression because studies have suggested that epigenetic mechanisms such as DNA methylation and histone modifications mediate the dysregulated expression of these genes. The mechanism underlying maternal diabetes mellitus–induced Wnt antagonist expression warrants further investigation. The major types of VSDs in our diabetic pregnancy models are perimembranous VSDs, which likely reflect defects in endocardial cushion development. The defective endocardial cushion development is manifested by enhanced ROS production and apoptosis and diminished β-catenin expression on exposure to diabetes mellitus. Deletion of the central canonical Wnt mediators, Frizzled 2 and β-catenin, results in VSDs along with outflow tract defects, implicating that impaired canonical Wnt signaling caused by diabetes mellitus exposure may be responsible for VSD formation. In these Wnt mutants, both increased apoptosis and decreased cell proliferation likely contribute to the failed closure of the ventricular septum. The affected cell types include the neural crest cells and cardiomyocytes. In our model, impaired canonical Wnt signaling may enhance apoptosis and reduce proliferation in cells of endocardial cushion leading to VSD formation. The impaired Wnt signaling may also translate to decreased gene expression that is essential for ventricular septum closure.

Maternal diabetes mellitus–induced oxidative stress suppresses Wnt target gene expression, which is critical for normal cardiogenesis. Deletion of the canonical Wnt target gene, Versican, induces exclusively VSDs, whereas null mutations of the noncanonical Wnt target gene, Mrgf-l, mainly causes outflow tract defects. These findings support the hypothesis that the canonical Wnt pathway is essential for the development of both ventricular septum and the outflow tract, whereas the noncanonical Wnt pathway is essentially involved in the development of the outflow tract. Human mutations of the GJA1 gene, a canonical Wnt target gene, associating with hypoplastic left heart syndrome, provide additional evidence that the canonical Wnt pathway supports cardiac ventricular development. However, depending on cellular context, the canonical and noncanonical Wnt signaling pathways can interact or counteract with each other. Because deletion of negative regulators of the Wnt pathway such as DKK1 and GSK3β also produces heart defects similar to those in null mutations of Wnt-positive regulators, a fine-tuned Wnt signal, not less or not too much, is required for the formation of a structurally normal heart.

SOD1 overexpression did not rescue Wnt5a null-induced heart defects, indicating that heart defects that arise with knockout of Wnt5a are not because of oxidative stress. In contrast, under conditions of high oxidative stress as in diabetes mellitus, Wnt5a signaling is suppressed and both its signaling and CHDs are rescued by SOD1 overexpression.

In summary, we provide direct in vivo evidence that oxidative stress mediates the teratogenicity of maternal diabetes mellitus leading to heart defects modeling those of diabetic
human pregnancies. Maternal diabetes mellitus–induced oxidative stress suppresses both the canonical and the noncanonical Wnt pathways through different mechanisms (Figure 7C). Whereas the canonical Wnt pathway is impeded because of the upregulation of its antagonists, the noncanonical pathway is blocked through the suppression of noncanonical Wnt5a expression. SODI restores the noncanonical Wnt signaling by preventing Wnt5a downregulation. In the absence of Wnt5a, SODI is unable to restore the noncanonical Wnt signaling. Our results support the hypothesis that oxidative stress is the cause of impaired Wnt signaling in the developing heart and subsequent CHD formation in diabetic pregnancies.

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Disclosures
None.

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14. Bushdhi PB, Osimka H, Waclaw RR, Molkentin JD, Yutzey KE. NFATc3 and NFATc4 are required for cardiac development and microvascular function. Circ Res. 2003;92:1305–1313. doi: 10.1161/01.RES.0000077045.84689.9F.
Congenital heart defects (CHDs) are the most common birth defects, have a significant impact on morbidity and mortality of newborns, and cost billions of dollars in health care to treat children and adults. More than 32,000 infants are born with CHD each year in the United States. Understanding the underlying causes of abnormal heart formation is an essential step toward developing new therapeutic treatments or preventive measures for CHD. Environmental factors contribute to the majority of newborns, and cost billions of dollars in health care to treat children and adults. More than 32,000 infants are born with CHD each year in the United States. Understanding the underlying causes of abnormal heart formation is an essential step toward developing new therapeutic treatments or preventive measures for CHD.
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Fang Wang, Steven A. Fisher, Jianxiang Zhong, Yanqing Wu and Peixin Yang

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SUPPLEMENTAL MATERIAL

METHODS

Mice and reagents

C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). SOD1-Tg mice in a C57BL/6J background were revived from frozen embryos by the Jackson Laboratory (stock no. 002298). Wnt5a+/− mice were provided by Dr. Terry Yamaguchi at the Center for Cancer Research, NCI-Frederick, NIH, as previously described1. SOD1-Tg male mice were mated with nondiabetic or diabetic female WT mice to generate SOD1 overexpressing and WT embryos. SOD1-Tg male mice mated with Wnt5a+/− female mice to generate SOD1; Wnt5a+/− male mice. SOD1; Wnt5a+/− male mice mated with Wnt5a+/− female mice to Wnt5a null embryos with or without SOD1 overexpression. Streptozotocin (STZ) from sigma (St. Louis, MO) was dissolved in sterile 0.1M citrate buffer (pH 4.5). Sustained-release insulin pellets were purchased from Linplant (Linshin, CA).

Mouse models of diabetic embryopathy

Twelve-week old wild-type (WT) female mice were intravenously injected with 75 mg/kg streptozotocin (STZ) on consecutive days. Diabetes was defined as 12 hours fasting blood glucose levels ≥ 250 mg/dL. Insulin pellets were subcutaneously implanted in these diabetic mice to restore euglycemia prior to mating. Insulin implantation during early pregnancy stages is essential for successful embryo implantation2,3. Diabetic wild-type female (DM-WT) mice were then mated with SOD1 transgenic or wild-type male mice at 3:00 P.M to produce wild-type and SOD1 overexpressing embryos. Pregnancy was established by the presence of the vaginal plug the next morning and noon of that day was designated as Embryonic day 0.5 (E0.5). On E5.5,
insulin pellets were removed to ensure that the developing embryos would be exposed to hyperglycemia during the critical period of cardiogenesis (E8.5-E13.5). Nondiabetic WT (ND-WT) female mice with vehicle injection and sham operation of insulin pellet implantation/removal served as nondiabetic controls. On E11.5, E12.5 and 17.5, embryonic hearts were harvested for analyses. The findings in E11.5 hearts in molecular and biochemical analyses were identical or similar to those of E12.5 hearts. To avoid redundancy, data in E11.5 hearts was not included.

*India ink injection and Hematoxylin-eosin staining*

E17.5 hearts were collected for morphological examination. For India ink injections, mouse embryos were collected at E17.5 and diluted (1:100) India ink was injected into the left ventricle and perfused through the vascular system using μTIP (TIP10TW1-L, world precision instrument, Inc., Sarasota, FL). Hearts were then fixed in methacarn (methanol, 60%; chloroform, 30%; glacial acetic acid, 10%), embedded in paraffin, and cut into 8-μm sections. After deparaffinization and rehydration, all specimens then underwent hematoxylin and eosin (H&E) staining in a standard procedure. All heart sections were photographed and examined for heart defects.

*LPO (Lipid Hydroperoxide) Assay*

The degree of lipidperoxidation in the developing heart was assessed by the LPO assay using the Calbiochem Lipid Hydroperoxide Assay Kit (Millipore, Bedford, MA) as per the manufacturer’s instructions. Briefly, E12.5 hearts were homogenized in HPLC-grade water. The lipid hydroperoxides of each heart were extracted by deoxygenated chloroform, and reacted with chromogen. The optical density was then measured at the absorbance of 500 nm. The results
were expressed as μM lipid hydroperoxides per gram protein. Protein concentrations were determined by the BioRad DC protein assay kit (BioRad, Hercules, CA).

**Ex vivo embryonic heart culture**

*Ex vivo* embryonic heart culture was performed as described by Hisayuki Hashimoto *et al*[^4]. Briefly, E11.5 embryonic hearts were quickly explanted from nondiabetic WT (ND-WT) dams and placed in a 24-well plate casted with collagen gel (A10483-01, BD Gibco). The collagen gel was prepared in 5mM (low glucose, LG) in M199 culture media (M4530, Sigma) and then hydrated by warmed Opti-MEM media plus 1% fetal bovine serum (FBS, Gibco) and insulin-transferrin-selenium (ITS, Corning). After incubated overnight at 37°C in a humidified atmosphere of 5% CO₂, hearts were cultured under 5 mM, or 25 mM D-glucose (high glucose, HG) conditions with or without 5 mM Tempol, (Enzo Life Science) 1μM sodium peroxynitrite (CAS14042-01-4, Cayman Chemical Company Inc.), or 100μM H₂O₂ (Sigma) for 24 hours.

**Western blotting**

Western blotting was performed as previously described[^5-7]. Briefly, E12.5 embryonic hearts were sonicated in ice-cold RIPA lysis buffer (Millipore, Bedford, MA) containing a protease inhibitor cocktail (Sigma, St Louis, MO). Nuclear protein extraction was prepared by using the EpiQuik Nuclear Extraction Kit (Epeigentek Group Inc, Farmingdale, NY). Proteins from different experimental groups were separated by 6%-12% SDS-PAGE and immunoblotted using primary antibodies at 1:1000 to 1:2000 dilutions in 5% nonfat milk. Antibodies of Dvl2, β-catenin, phosphor-(p-)GSK3β, p-CaMKII, NFAT2, NFAT4 and SOD1 were from Cell Signaling Technology (Boston, MA). Anti-caspase3 and anti-caspase8 were from Millipore (Bedford, MA). The antibody of Wnt5a was from R&D system (Minneapolis, MN). The intensity of the protein
bands were determined by densitometry and normalized by the densities of β-actin (Abcam, Cambridge, MA), or Histone H3 (Cell Signaling Technology, Boston, MA) for nuclear proteins or corresponding total proteins for phosphorylated proteins in the same preparation. Signals were detected using SuperSignal West Femto Maximum Sensitivity Substrate kit (Thermo Scientific, Rockford, IL). All experiments were repeated three times with the use of independently prepared tissue lysates.

Real-time PCR

Total RNA was isolated from embryos using an RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse transcribed by using the high-capacity cDNA archive kit (Applied Biosystem, Grand Island, NY). RT-PCR for Wnt ligands (Wnt1, 2a, 3a, 5a, 7b, 8a), Wnt antagonists (WIF1 (wnt inhibitory factor 1), sFRP1 (secreted frizzled-related protein 1), DKK1 (dickkopf 1), Wnt target genes (Islet1, GJA1 (gap junction alpha 1), Versican, Mrtf-b (myocardin related transcription factor B), Tpm1 (alpha tropomyosin 1), Rcan1 (regulator of calcineurin)) and β-actin were performed using Maxima SYBR Green/ROX qPCR Master Mix assay (Thermo Scientific, Rockford, IL). RT-PCR and subsequent calculations were performed by using the StepOnePlus real-time PCR system (Applied Biosystem, Grand Island, NY).

Immunofluorescence and DHE staining

E12.5 hearts were fixed in 4% paraformaldehyde overnight followed by embedding in OCT (optimal cutting temperature, Sakura finetek, Torrance, CA) compound. 10-μm cryosections of heart tissues were antigen-unmasked using citrate buffer and blocked in 5% bovine serum albumin in PBST (0.1% Triton X-100 in PBS) for 1 hour. The following antibodies were used as primary antibodies: β-catenin (1:200) (Cell Signaling Technology,
Boston, MA), Wnt5a (1:50) (ThermoFisher Scientific, Rockville, MD) and p-Histone H3 (1:100) (Millipore, Bedford, MA). Normal rabbit or mouse IgG at the same dilutions as those for antibodies was used as controls. Sections were counterstained with DAPI and mounted with aqueous mounting medium (Sigma, St Louis, MO). Images were captured using an inverted microscope (Nikon Eclipse E1000M). For fluorescence detection of superoxide, the frozen heart sections were incubated with 1.5μM DHE (dihydroethidium) for 5 min at room temperature and then washed for 3 times with PBS, 5 min each time. Sections were counterstained with DAPI and mounted with aqueous mounting medium (Sigma, St Louis, MO). The DHE staining has been successfully used in assessing superoxide levels in tissue sections such as kidney sections. However, the DHE → E reaction may detect ROS broadly including hydrogen peroxide and hydroxyl, in addition to superoxide. For the evaluation of cell proliferation, p-Histone H3 positive cells were counted on three heart sections from three different dams per group.

**TUNEL Assay**

The TUNEL assay was performed as previously described by using the In Situ Cell Death Detection Kit (Millipore, Billerica, MA). 10-μm serial heart frozen sections were fixed with 4% paraformaldehyde in PBS and incubated with TUNEL reagent. Sections were counterstained with DAPI and mounted with aqueous mounting medium (Sigma, St Louis, MO). TUNEL-positive cells in the heart of each section were counted. Heart sections from three embryos of different dams per group and three sections per heart were analyzed. The percentage of apoptotic cells was calculated as number of apoptotic cells divided by 200 cells in a selected area.


**Supplementary Table 1.** Effect of SOD1 overexpression on heart defect incidence in Wnt5a null embryos

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Blood glucose levels (mg/dl)</th>
<th>Embryo Genotype</th>
<th>Number of embryos</th>
<th>Number with heart defects</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND</td>
<td>103.2 ± 12.6</td>
<td>SOD1; Wnt5a+/+</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wnt5a+/+</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wnt5a+/-</td>
<td>15</td>
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<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SOD1; Wnt5a+/+</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
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<td></td>
<td>SOD1; Wnt5a+/-</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>SOD1; Wnt5a-/-</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

ND: nodiabetic; +/+: wild-type; +/-: heterozygous; -/-: Wnt5a null.
Supplementary Figure 1. Heart defects in Wnt5a null embryos with or without SOD1 overexpression. A, Representative images of India ink injections revealed persistent truncus arteriosus (PTA) in Wnt5a⁻/⁻ (null) embryos with or without SOD1 overexpression. B, Representative images of serial heart sections showed ventricular septum defect (VSD) in Wnt5a null embryos. AO: Aorta; PA: Pulmonary Artery; LA: Left Atrium; RA: Right Atrium; LV: left ventricle; RV: right ventricle.