

Brief Genetics Report

Polymorphic Susceptibility to the Molecular Causes of Neural Tube Defects During Diabetic Embryopathy

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Previously, we demonstrated that neural tube defects (NTDs) are significantly increased in a mouse model of diabetic pregnancy. In addition, expression of *Pax-3*, a gene encoding a transcription factor required for neural tube development, is significantly decreased. This suggests that diabetic embryopathy results from impaired expression of genes regulating essential morphogenetic processes. Here, we report that in one mouse strain, C57Bl/6J, embryos are resistant to the effects of maternal diabetes on NTDs and *Pax-3* expression, in contrast to a susceptible strain, FVB, in which maternal diabetes significantly increases NTDs ($P = 0.02$) and inhibits *Pax-3* expression ($P = 0.01$). Resistance to NTDs caused by diabetic pregnancy is a dominant trait, as demonstrated by heterozygous embryos of diabetic or nondiabetic mothers of either strain. There was no significant difference between strains in expression of genes that regulate free radical scavenging pathways, suggesting that susceptibility to oxidative stress does not account for the genetic differences. Understanding the genetic bases for differential susceptibility to altered gene expression and NTDs in diabetic mice may be important in delineating the mechanisms by which maternal hyperglycemia interferes with embryo gene expression. Moreover, if susceptibility to diabetic embryopathy is variable in humans as well as in mice, it may be possible to screen individuals at increased risk for this complication of diabetes. *Diabetes* 51: 2871–2874, 2002

Maternal diabetes significantly increases the risk of congenital malformations in humans and in animal models (1,2). These malformations arise at the beginning of organogenesis, during the first 8 weeks of gestation in human embryos, and during the first 7–10 days in mouse and rat embryos (1). Diabetic embryopathy can affect any developing organ system, although defects of the neural tube and heart are among the most common (3). In studying neural tube defects (NTDs) in a mouse model of diabetic embryopa-

thy, we showed that expression of *Pax-3*, a gene that regulates neural tube closure (4,5), is significantly reduced in embryos of diabetic mice before the manifestation of morphological defects (6). Because loss-of-function *Pax-3* mutation causes the same kind of NTDs as those caused by maternal diabetes (open neural tube defects, particularly exencephaly and spina bifida) in 100% of mutant embryos (5), this suggests that impaired expression of *Pax-3* is sufficient to prevent normal formation of the neural tube. We also showed that the excess glucose surrounding the embryo, which is a consequence of maternal diabetes, is responsible for the adverse effects of diabetic pregnancy (7). Therefore, a unifying mechanism possibly explaining the spectrum of malformations associated with diabetic embryopathy is that exposing the embryo to excess glucose at critical times during induction of developmental control genes can compromise subsequent morphogenetic processes.

Many studies have demonstrated that antioxidants can prevent congenital malformations associated with maternal diabetes in rodent embryos (8–15). We recently demonstrated that antioxidants prevent the increased lipid peroxidation, decreased *Pax-3* expression, and NTDs in embryos exposed to excess glucose (Chang et al., submitted manuscript). This suggests that oxidative stress in the embryo caused by maternal hyperglycemia contributes to the altered gene expression and NTDs associated with diabetic pregnancy.

In humans, susceptibility to diabetic complications, such as nephropathy, is influenced by genetic background (16), indicating that there are polymorphic genes that modify the cellular responses to hyperglycemia. In animal models, penetrance or expressivity of a phenotype associated with a genetic mutation is well known to be influenced by strain background (17), although the specific modifier gene(s) responsible are seldom characterized. Interestingly, effects of maternal diabetes on congenital malformations in rat embryos is strain dependent (18). Recently, Cederberg et al. (19) found that embryos from a malformation-resistant rat strain express increased levels of Mn⁺⁺-superoxide dismutase (SOD) and catalase mRNA, suggesting that higher levels of free radical scavenging enzymes protect these embryos from oxidative stress caused by maternal diabetes.

Most of our studies on the molecular regulation of neural tube defects in the embryos of diabetic mice have been performed using the outbred ICR mouse strain (6,7,20,21). Upon testing other mouse strains for effects of

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GSH, glutathione; NTD, neural tube defect; SOD, superoxide dismutase.

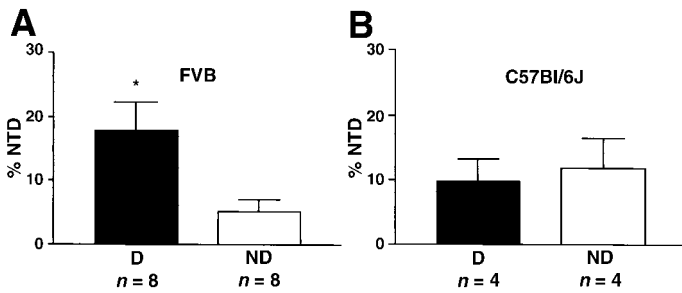


FIG. 1. Percent NTDs in day 10.5 embryos of diabetic (D, ■) or nondiabetic (ND, □) FVB (A) or C57Bl/6J mice (B). Blood glucose levels in FVB mice were 146 ± 6 and 538 ± 27 mg/dl in nondiabetic and diabetic mice, respectively, on day 7.5, the day that is sensitive to hyperglycemia-induced NTDs. Blood glucose levels in C57Bl/6J mice were 144 ± 3 and 354 ± 64 mg/dl in nondiabetic and diabetic mice, respectively. Values represent the mean \pm SEs, and *n* refers to numbers of pregnancies analyzed. **P* = 0.02 vs. embryos of nondiabetic mice.

maternal diabetes to increase NTDs, several strains displayed responses similar to the ICR strain, but one, C57Bl/6J, did not. Therefore, we compared the effects of maternal diabetes on embryos of a susceptible inbred strain, FVB, with embryos of the resistant C57Bl/6J strain. As shown in Fig. 1, maternal diabetes increased NTDs 3.4-fold (*P* = 0.02) in day 10.5 embryos of the inbred FVB strain, as was seen previously with ICR mice (6). In contrast, maternal diabetes did not increase NTDs in C57Bl/6J embryos. Embryos did not differ with regard to developmental stage, as determined by somite number, or crown-rump length, regardless of strain or diabetes status of mothers (data not shown). To test whether normal neural tube formation was correlated with levels of Pax-3 mRNA, a sensitive quantitative RT-PCR assay was performed using RNA obtained from day 8.5 embryos (the day when Pax-3 expression in neuroepithelium and neural tube closure began). As shown in Fig. 2, Pax-3 mRNA was reduced almost twofold (*P* = 0.01) in FVB embryos of diabetic mice, but there was no effect of maternal diabetes on Pax-3 mRNA in C57Bl/6J embryos. This demonstrates that there are strain differences in susceptibility to NTDs induced by maternal diabetes that correlate with effects of maternal diabetes on expression of a gene that is essential for neural tube closure.

From the preceding experiments, it could not be determined whether the failure of maternal diabetes to inhibit Pax-3 expression and induce NTDs in C57Bl/6J embryos was due to the genetic background of the mother or the embryo. For example, hyperglycemia in diabetic C57Bl/6J

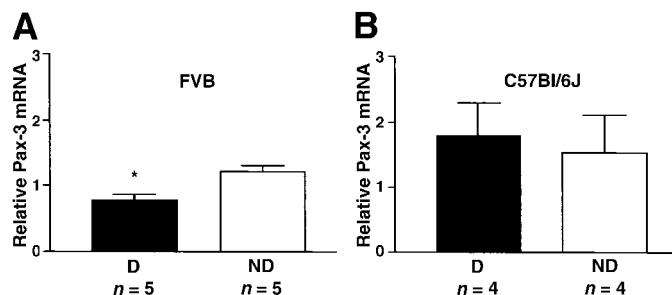


FIG. 2. Pax-3 mRNA normalized to GAPDH mRNA from day 8.5 embryos of diabetic (D, ■) or nondiabetic (ND, □) FVB (A) or C57Bl/6J mice (B) assayed by quantitative RT-PCR. **P* = 0.01 vs. embryos of nondiabetic mice.

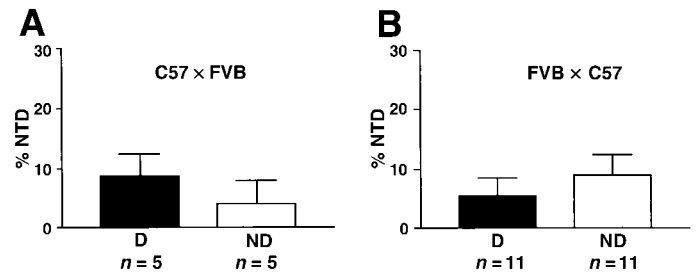


FIG. 3. Percent NTDs in day 10.5 embryos of diabetic (D, ■) or nondiabetic (ND, □) C57Bl/6J females mated with FVB males (A) or diabetic or nondiabetic FVB females mated with C57Bl/6J males (B). Blood glucose levels in diabetic C57Bl/6J and FVB mice were 384 ± 48 and 449 ± 28 mg/dl, respectively.

mice was not as severe during pregnancy as in diabetic FVB mice (354 ± 64 vs. 538 ± 27 mg/dl, *P* = 0.01), although it was well above the threshold of 250 mg/dl, the level at which NTDs in embryos of diabetic ICR mice are significantly increased (7). There might also be anatomical or physiological differences between pregnant C57Bl/6J mice and susceptible strains serving to preserve metabolic homeostasis in embryo. Alternatively, genes expressed by the embryo may confer differential sensitivity to hyperglycemia or to pathways activated by glucose metabolism. To determine whether the genetic background of the mother, the embryo, or both was responsible for differential susceptibility to diabetes-induced NTDs, diabetic or nondiabetic FVB or C57Bl/6J females were mated with males of the opposite strain, and NTDs were scored in heterozygous embryos. Notably, heterozygous embryos were resistant to NTDs caused by maternal diabetes whether the C57Bl/6J genome was of maternal or paternal origin (Fig. 3). Because maternal diabetes failed to increase NTDs in heterozygous embryos, even when mothers were of the susceptible FVB strain, this demonstrates that 1) the genotype of the embryo, and not the mother, is responsible for resistance to NTDs associated with diabetic pregnancy, 2) epigenetic or mitochondrial mechanisms are not responsible for differential susceptibility, and 3) resistance to NTDs caused by diabetic pregnancy is dominant over susceptibility.

To test whether the genetic variation in embryo susceptibility to diabetes-induced malformations in these two mouse strains is associated with altered expression of genes that regulate oxidative stress, as suggested by studies with the rat (19), we compared the mRNA levels of several enzymes that either scavenge free radicals or regulate the synthesis of the cellular antioxidant, reduced glutathione (GSH). As shown in Fig. 4, there was no significant difference in the level of any mRNA examined between embryos of different strains, and there was no significant effect of maternal diabetes to affect any mRNA level. Thus, differential expression of several genes regulating the accumulation of reactive oxygen intermediates in response to maternal hyperglycemia does not appear to explain the differential effects of maternal diabetes on inhibition of Pax-3 expression and NTDs between FVB and C57Bl/6J embryos. However, genetic polymorphism affecting either enzyme activity or stability, or different levels of other stressors such as iron, cannot be ruled out at this time.

In conclusion, there are mechanisms in C57Bl/6J em-

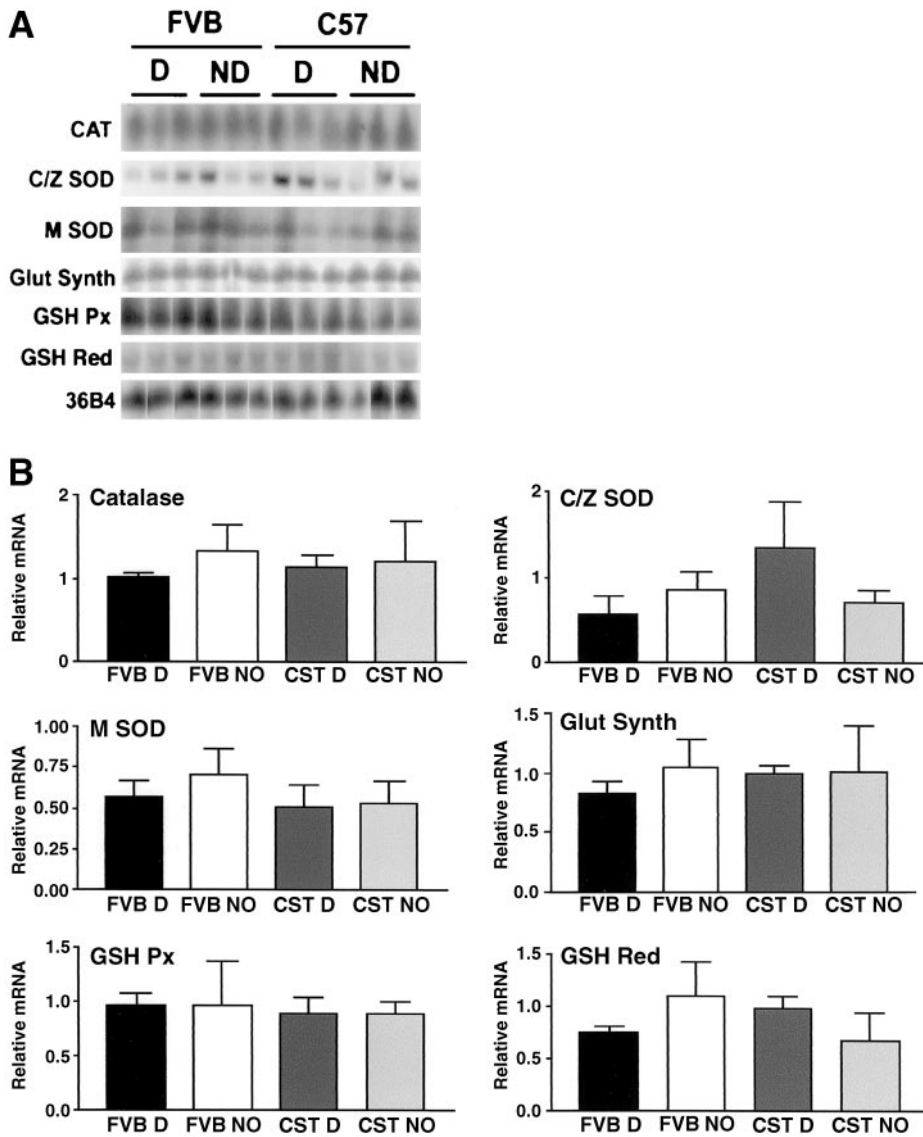


FIG. 4. A: RT-PCR of RNA from embryos of three diabetic (D) or nondiabetic (ND) FVB or C57Bl/6J mice. CAT, catalase; C/Z SOD, Cu⁺⁺/Zn⁺⁺ SOD; M SOD, Mn⁺⁺-SOD; Glut Synth, γ -glutamylcystein-synthetase; GSH Px, GSH peroxidase; GSH Red, GSH reductase. **B:** Quantitation of PCR products from **A** relative to 36B4 (means \pm SE). Each data set was analyzed by ANOVA and Newman-Keuls test, and no differences were found to be statistically significant.

bryos that prevent glucose inhibition of *Pax-3* expression and that are not operational in FVB embryos. Resistance to the molecular causes of NTDs associated with diabetic embryopathy is a dominant trait but does not appear to be due to increased expression of the same genes involved in free radical inactivation that are associated with resistance to diabetic embryopathy in rat embryos. Thus, the existence of multiple modifier genes affecting susceptibility to diabetic embryopathy in rodents may indicate that there are multiple signaling mechanisms by which hyperglycemia can interfere with developmental programs. Use of congenic or recombinant inbred strains derived from susceptible and resistant strains may be useful in identifying the gene(s) responsible for resistance to diabetic embryopathy. This knowledge will be important for understanding the biochemical mechanisms by which maternal diabetes disturbs expression of genes regulating essential developmental processes. In addition, it may be possible to screen for genetic polymorphism in humans to identify those at increased risk for diabetic embryopathy or other diabetic complications.

RESEARCH DESIGN AND METHODS

Experimental animals. Diabetes was induced in FVB (Taconic, Germantown, NY) and C57Bl/6J mice (The Jackson Laboratories, Bar Harbor, ME) with streptozotocin and treated with subcutaneously implanted insulin pellets as described (6). Mice were mated with nondiabetic age-matched mice, and day 0.5 of pregnancy was determined to be noon on the day that copulation plugs were found. Embryos were recovered from uteri on day 8.5 or 10.5 to assay mRNA (day 8.5) or to score NTDs (day 10.5) as described (6).

RT-PCR. For quantitative RT-PCR of *Pax-3* mRNA, RNA from pooled day 8.5 embryos from individual pregnancies was prepared and reverse transcribed, as previously described (6), in quadruplicate. Real-time quantitative PCR was performed using a Perkin Elmer 7700 Sequence Detection System (Foster City, CA) as described by Chang et al. (submitted manuscript) in an online appendix at <http://diabetes.diabetesjournals.org>. Amplified *Pax-3* cDNA was normalized to amplified GAPDH cDNA, which was assayed using Pre-Developed TaqMan Assay Reagents (PE Biosystems, Foster City, CA) according to the manufacturer's recommendations. *Pax-3* mRNA from each pregnancy was expressed relative to one of the nondiabetic pregnancies. For semiquantitative RT-PCR of RNA encoding free radical-scavenging enzymes or GSH-metabolizing enzymes, RNA was reverse transcribed, three- to four-fold serial dilutions were amplified by PCR using sequence-specific primers, and PCRs were electrophoresed as described (6). Bands on autoradiographs from PCR products that amplified within a linear range were quantitated by scanning densitometry and expressed relative to the PCR product for 36B4, a gene not regulated by diabetes (20). Primer sequences for GSH reductase, Cu⁺⁺/Zn⁺⁺-SOD, and Mn⁺⁺-SOD were as previously described (22); for GSH

reductase corresponding to nucleotides 55-72 and 197-177 were as previously described (23); for catalase were GCAGCCATGGAGAGCC (forward) and CTGACGTCCACCCTGACT (reverse); and for γ -glutamylcystein-synthetase were ATTAGGAGATCTACGAGCAGC (forward) and CCACAGATACCACAT AGGCAG (reverse). Primers for catalase and γ -glutamylcystein synthetase were obtained by comparison of rat sequence primers (19), with corresponding mouse sequences in GenBank.

Statistical analyses. Statistical analyses were performed using an unpaired Student's *t* test or ANOVA and post hoc Newman-Keuls test using Prism 3 software (GraphPad Software, San Diego, CA). Statistical significance was set at $P < 0.05$.

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