Maternal Blood Glucose Levels Determine the Severity of Diabetic Embryopathy in Mice with Different Expression of Copper-Zinc Superoxide Dismutase (CuZnSOD)

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Excess oxygen radical formation is suggested to be involved in the etiology of diabetic embryopathy. We aimed to investigate the effects of altered maternal antioxidative status in conjunction with a varied severity of the maternal diabetic state on embryonic development by using mice with different gene expression of CuZn superoxide dismutase (CuZnSOD). The mice were wild-type (WT), transgenic (TG), or knockout (KO) with regard to CuZnSOD. Alloxan was used to induce diabetes (DWT, DTG, DKO) in female mice before pregnancy and, noninjected mice served as controls (NWT, NTG, NKO). The minimum alloxan dose required to induce diabetes was 80 mg/kg for WT, 100 mg/kg for TG, and 65 mg/kg for KO mice. When KO mice were made diabetic with 80 mg/kg alloxan, they produced no living offspring. The pregnancies were interrupted on gestational day 18, when maternal diabetic state, that is, blood glucose concentration, as well as fetal outcome, genotype and hepatic isoprostane levels were assessed. The mean maternal blood glucose levels were positively associated with the alloxan dose, that is, the DWT and DTG groups had higher blood glucose concentration than the DKO group, and the DWT and DTG fetuses increased their hepatic isoprostane levels, whereas the DKO fetuses did not. However, in all diabetic groups, increased maternal blood glucose concentration was associated with higher resorption and malformation rates as well as lowered fetal and placental weight. Furthermore, diabetes increased the fraction of WT offspring in the TG and KO groups. We conclude that both fetal antioxidative capacity and maternal diabetic state affect the development of the offspring. However, the maternal diabetic state is the major teratogenic factor and overrides the influence of fetal antioxidative capacity.

Key Words: CuZnSOD; transgenic mice; knockout mice; oxidative stress; alloxan; diabetes in pregnancy.

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Mills et al., 1979). The maternal diabetic state leads to metabolic disturbances such as hyperglycemia (Sadler, 1980), zinc deficiency (Styrud et al., 1986), copper deficiency (Didion et al., 2002; Hawk et al., 1998; Yang et al., 2006), and the generation of free oxygen radicals (Eriksson and Borg, 1991, 1993; Hagay et al., 1995; Sivan et al., 1997; Weksler-Zangen et al., 2003). These factors may either singly or in combination lead to embryonic maldevelopment and depend on the concentrations of the metabolites, disturbances in the maternal-fetal transport (Brent and Fawcett, 1998; Pinter et al., 1986), teratological period (Eriksson et al., 1989a, b; Mills et al., 1979), and the genetic constitution of the embryo (Cederberg et al., 2000; Eriksson, 1988; Weksler-Zangen et al., 2003).

It has been demonstrated that excess production of reactive oxygen species (ROS) yields oxidative stress (Oberley, 1988; West, 2000). This state can be determined by measuring levels of the isoprostane 8-epi-PGF2α (Cederberg and Eriksson, 1997; Gerber et al., 2000; Wentzel et al., 1997) which is the result of a direct peroxidation of arachidonic acid by ROS (Morrow et al., 1990). Furthermore, it has been observed that both maternal diabetic rats and their fetuses display high levels of isoprostane 8-epi-PGF2α (Cederberg and Eriksson, 1997; Wentzel et al., 1997). CuZn superoxide dismutase (CuZnSOD) is a ROS scavenger and its activity and transcriptional level have been widely studied in various species including human, rat and mouse (Benedetto et al., 1991; Hsu et al., 1992; Levanon et al., 1985). A correlation study on the antioxidant enzymes CuZnSOD, MnSOD, Catalase, and GPX has shown that gene expression increases linearly with increased protein expression and activity (Tiedge et al., 1997). The CuZnSOD exon-intron organization of human, rat and mouse are identical, encoded by five exons and four introns (Benedetto et al., 1991; Hsu et al., 1992; Levanon et al., 1985). Mutation in the CuZnSOD gene is known to cause familiar amyotrophic lateral sclerosis (Rosen, 1993) and premature aging (Yarom et al., 1988). In contrast, mice overexpressing the enzyme CuZnSOD decrease pulmonary oxygen toxicity and pulmonary cellular damage (White et al., 1991). Another recent report concluded that maternal mice overexpressing CuZnSOD display...
diminished ethanol-induced fetal dysmorphogenesis (Wentzel and Eriksson, 2006) and display a protective effect against diabetes-associated embryopathy (Hagay et al., 1995). However, there are no previous reports of diabetes-associated embryopathy in mice lacking CuZnSOD. Our working hypothesis was that low levels of fetal antioxidative defense and the severity of the maternal diabetic state would affect the fetal development. We therefore investigated fetal outcome in mice with different CuZnSOD expression levels and different degrees of maternal diabetes.

MATERIAL AND METHODS

Ethical Consideration

All animal experimental procedures had full approval from the Animal Ethics Committee of Uppsala University.

Animals

Wild-type (WT), transgenic (TG), and knockout (KO) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). The mice were housed at the Laboratory Animal Resources of the Biomedical Center in Uppsala and were maintained at an ambient temperature of 22°C with 12-h light/dark cycle. All mice had free access to pelleted food R36 (Lactamin AB, Stockholmi, Sweden) and tap water.

Wild-type. Mating pairs from C57/Bl/6J inbred mice (genetic background (B6129SF2/J)) expressing normal CuZnSOD gene and protein activity served as a control strain.

Transgenic. Mating pairs of identity (B6; 129S7-CuZnSODtm1Leb/J) served as a KO substrain. These mice have a targeted mutation in the CuZnSOD gene, in which exon 5 of the CuZnSOD gene has been replaced with a neomycin resistant cassette, which results in decreased CuZnSOD gene expression and protein activity (Matzuk et al., 1998). The htz KO mice display 50% of the normal CuZnSOD gene expression since only one of the CuZnSOD alleles is functional. Moreover, the hoz KO mice have completely abolished gene expression and protein activity. The htz KO mice are phenotypically normal, whereas hoz KO female mice are infertile and males reproduce normally (Matzuk et al., 1998). Only htz KO mice were used in the experiments.

Induction of Diabetes

The minimum dose of alloxan monohydrate (Sigma-Aldrich Sweden AB, Sweden) required to obtain manifestly diabetic mice was determined. WT mice required a single injection of 80 mg/kg alloxan in the tail vein (denoted DWT80). A group of hzt TG female mice were injected with 100 mg/kg alloxan (denoted DTG100). Two groups of hzt KO female mice were injected with 65 mg/kg (denoted DKO65) and 80 mg/kg (denoted DKO80) alloxan, respectively (Table 1). Five days after alloxan injection, the maternal blood glucose levels of the different groups of mice were determined with a glucose analyzer (Abbott Scandinavia AB, Freestyle, Abbott Diabetes Care, Solna, Sweden). Mice with a blood glucose level above 15 mmol/l were considered manifestly diabetic and denoted DWT80, DTG100, DKO65, or DKO80. Noninjected (N) female mice were denoted NWT, NTG, or NKO. These groups were mated during the night with N males originating from similar genetic substrains.

Pregnancy

The day of a positive vaginal plug was denoted gestational day 0. Irrespective of genetic background the NWT, NTG, and NKO mice received a vaginal plug sooner than their respective diabetic groups, in which the DWT80 and DKO65 and DKO80 mice became pregnant about 1 week after induction of diabetes and the DTG100 mice became pregnant about 2 weeks after induction of diabetes. Pregnancy was interrupted on gestational day 18 by cervical dislocation of the pregnant mouse and the uterus was dissected out. The total number of pregnant mice in the different experimental groups varied from 7 to 15 (Table 1). The intrauterine position of each fetus from right and left uterine horn was recorded, in which first implantation site next to the ovary was denoted position 1 following in order to the last position in the uterine horn. Fetuses were examined for external anomalies with the severity of the maternal diabetic state would affect the fetal outcome. We therefore investigated fetal outcome in mice with different CuZnSOD expression levels and different degrees of maternal diabetes.

<table>
<thead>
<tr>
<th>Group</th>
<th>Alloxan (mg/kg)</th>
<th>No. of dam</th>
<th>Maternal body weight (g)</th>
<th>Maternal blood glucose (mmol/l)</th>
<th>No of impl.</th>
<th>Mean maldeveloped (%)</th>
<th>Mean resorbed (%)</th>
<th>Mean malformed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NWT</td>
<td>Not inj.</td>
<td>15</td>
<td>38.8 ± 0.6</td>
<td>5.6 ± 0.2</td>
<td>9.7 ± 0.3</td>
<td>13 ± 4</td>
<td>12.6 ± 3.9</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>DWT80</td>
<td>80</td>
<td>7</td>
<td>32.5 ± 1.6</td>
<td>23.5 ± 1.2*</td>
<td>9.3 ± 0.7</td>
<td>42 ± 12*</td>
<td>40.6 ± 11.1*</td>
<td>1.4 ± 1.4*</td>
</tr>
<tr>
<td>NTG</td>
<td>Not inj.</td>
<td>11</td>
<td>35.3 ± 1.5</td>
<td>6.6 ± 0.3</td>
<td>7.5 ± 0.6</td>
<td>17 ± 6</td>
<td>22.3 ± 8.8</td>
<td>0.9 ± 0.9</td>
</tr>
<tr>
<td>DTG100</td>
<td>100</td>
<td>7</td>
<td>30.6 ± 1.5</td>
<td>25.0 ± 2.0*</td>
<td>7.7 ± 0.6</td>
<td>41 ± 9*</td>
<td>33.7 ± 8.1</td>
<td>7.0 ± 3.6</td>
</tr>
<tr>
<td>NKO</td>
<td>Not inj.</td>
<td>12</td>
<td>36.4 ± 0.7</td>
<td>5.1 ± 0.2</td>
<td>10.0 ± 1.0</td>
<td>21 ± 8</td>
<td>20.9 ± 8.2</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>DKO65</td>
<td>65</td>
<td>7</td>
<td>31.7 ± 2.0</td>
<td>17.4 ± 1.3*</td>
<td>8.6 ± 0.4</td>
<td>38 ± 13</td>
<td>34.8 ± 11.7</td>
<td>3.2 ± 3.2*</td>
</tr>
<tr>
<td>DKO80</td>
<td>80</td>
<td>12</td>
<td>22.1 ± 0.5*#</td>
<td>24.0 ± 1.3*#</td>
<td>0.0 ± 0.0*#</td>
<td>100 ± 0*#</td>
<td>100 ± 0.0*#</td>
<td>Not detected</td>
</tr>
</tbody>
</table>

Note. Mean maternal body weight, maternal blood glucose status, and fetal outcome of WT, TG, and KO mice of N and D groups. $7 < n < 15$, mean ± SEM. Significances by Student’s t-test: *$p < 0.05$ versus the N group within the same subgroups. #$p < 0.05$ versus D groups within each of the TG and KO subgroups.
Genotyping

From all TG and KO offspring either a piece of the tail or a piece of resorbed tissue was collected for DNA preparation. Each sample tube contained Tris buffer (50mM TrisBase, 100mM ethylenediaminetetraacetic acid [EDTA], and 100mM NaCl, 20% sodium dodecyl sulfate, pH 7.5) with 10 mg/ml Proteinase K (Sigma-Aldrich Sweden AB) and was incubated at 55°C overnight. Samples were then vortexed and centrifuged at 13,000 x g for 10 min. To precipitate DNA, 300 µl of each supernatant was transferred to a new tube and mixed with 200 µl of 2-propanol (Kebo lab AB, Spånga, Sweden) and gently shaken. Next, the samples were centrifuged at 13,000 x g for 10 min. The supernatant was discarded and 1 ml of 70% ethanol was added to the pellets. Samples were then centrifuged at 13,000 x g for 5 min. Subsequently, the supernatant was removed and the pellets were dried and resuspended in RNase-free water. Finally the DNA concentration was estimated by measuring the absorbance of the samples.

Transgenic. Extracted DNA was amplified in a final volume of 10 µl reaction mix. Each reaction mix contained 5.80 µl of RNase-free water, 25mM MgCl2, 10× PCR gold buffer, 2.5mM deoxy nucleotidytriphosphate (dNTP) (Ampli Taq Gold with Gene Amp, Applied Biosystems, Branchburg, NJ), 20µM of each mouse CuZnSOD and human CuZnSOD sense and antisense primers (TIB, Molbiol, Berlin, Germany; Table 2) were used in the PCR reaction. WT genotype is denoted if only the mouse endogenous CuZnSOD PCR product is amplified (product size of 324 bp). Htz genotype is denoted if similar amounts of both the hCuZnSOD and mouse endogenous CuZnSOD PCR product are amplified (product size of 324 and 236 bp, respectively). Hoz genotype is denoted if a higher amount of the inserted hCuZnSOD PCR product is amplified.

Knockout. Extracted DNA was amplified in a final volume of 10 µl reaction mix. Each reaction mix contained 15.3 µl of RNase-free water, 25mM MgCl2, 10× PCR gold buffer, 5mM dNTP (Ampli Taq Gold with Gene Amp), 20µM of each HPRT (hypoxanthine guanine phosphoribosyl transferase), and CuZnSOD sense and antisense primers (TIB, Molbiol; Table 2). The HPRT primers amplify a DNA fragment, which includes the CuZnSOD promoter region and exon 2, whereas the primers for mouse endogenous CuZnSOD amplify a DNA fragment from the wt allele on exon 2. Wt genotype is denoted if only the mouse endogenous CuZnSOD PCR product is amplified (product size of 123 bp). Htz genotype is denoted if PCR products positive for both HPRT and mouse CuZnSOD are amplified. Hoz genotype is denoted if PCR products positive for only HPRT are amplified (product size of 240 bp). Separate PCR cycling programs were used for TG and KO samples. The following parameters were used: TG: (1) denaturation at 95°C for 3 min, (2) amplification with a total of 35 cycles, each cycle with denaturation temperature at 95°C for 30 s, annealing temperature at 60°C for 30 s, elongation temperature at 72°C for 2 min. KO: (1) denaturation at 94°C for 3 min, (2) amplification with a total of 35 cycles, each cycle with denaturation temperature at 94°C for 30 s, annealing temperature at 61°C for 1 min, elongation temperature at 72°C for 2 min.

Isoprostane 8-epi-PGF2α

Measurement of Isoprostane 8-epi-PGF2α was performed according to the manufacturer’s instructions (Cayman Chemical Co, Ann Arbor, MI). Liver from WT, TG, and KO offspring of N and D mice was homogenized on ice in 1 ml of homogenizing buffer (0.1M phosphate buffer [pH 7.4], containing 1mM EDTA and 10µM indomethacin). Equal volume of 15% KOH was added to each liver homogenate, and the samples were incubated at 40°C for 60 min. Next, two to four volumes of ethanol were added to each sample, which was vortexed, incubated at 4°C for 5 min, and centrifuged at 1500 x g for 10 min. The supernatant of each sample was transferred to a new tube, whereas the precipitates were used for protein determination. The protein contents were determined according to Lowry et al. (1951), in which bovine serum albumin was used as standard. The ethanol content in each of the supernatants was evaporated and 30% acetic acid was added to each sample to reach acidification (pH 4.0). Each sample was passed through a C-18 Reverse Phase Cartridge Tube separately (Supelco DSC-18, Bellefonte, PA), in which the cartridge was previously activated by rinsing with 5 ml of methanol followed by 5 ml of Ultra Pure water. The sample was passed through the cartridge and the cartridge was rinsed with 5 ml of Ultra Pure water followed by 5 ml of hexane (HPLC-graded; Sigma-Aldrich Sweden AB). Subsequently 5 ml of ethyl acetate containing 1% methanol (Sigma-Aldrich Sweden AB) was added to the column to elute the absorbed 8-epi-PGF2α. The eluates were placed in a vacuum centrifuge (SpeedVac SVC 100, Savant Instruments, Inc., Farmingdale, NY) to evaporate the ethyl acetate in the samples. Then the samples were analyzed in a spectrophotometric plate reader (Integrated EIA Management System, Labsystems, Helsinki, Finland) at a wavelength between 405 and 420 nm according to the manufacturer’s instructions (Cayman Chemical Company).

Statistical Evaluation

Comparisons between different experimental groups were based on litter means. For the group of fetal dysmorphogenesis, both malformations and resorptions were pooled as one group and the differences between groups were evaluated by Chi-squared test. Differences between means were evaluated by Student’s two-tailed t-test at the 95% significance level. A value of p < 0.05 was considered to denote a significant difference between groups. A value of 0.05 < p < 0.1 denoted a “trend.” The calculations were performed with the aid of the Windows version of the statistical program SigmStat (SigmStat 2.0 for Windows, SPSS Inc., Chicago, IL, USA).

RESULTS

Induction of Diabetes

We found that the minimum dose of alloxan required to obtain manifestly diabetic mice was substrain dependent and associated with the expression level of CuZnSOD. In

<table>
<thead>
<tr>
<th>Oligo name</th>
<th>Sequences (5’–3’)</th>
<th>Annealing temp. (°C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG. Internal standard F</td>
<td>CTAGGCCACAGAATTGAAAGATCT</td>
<td>60</td>
<td>324</td>
</tr>
<tr>
<td>TG. Internal standard R</td>
<td>GTAGGTTGGAAATTTCTGACATCC</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>TG. hCuZnSOD F</td>
<td>CATCAGCCTAATTCATCTGA</td>
<td>60</td>
<td>236</td>
</tr>
<tr>
<td>TG. hCuZnSOD R</td>
<td>CGCGACTAACAATCAAGTG</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>KO. Internal standard F</td>
<td>TGAACACAGTTGTGTTGTCAGG</td>
<td>61</td>
<td>123</td>
</tr>
<tr>
<td>KO. Internal standard R</td>
<td>TCCATCAGTGGTCACTAGCC</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>KO. HPRT F</td>
<td>TTGTTCCTCTCCCTCCTATCTC</td>
<td>61</td>
<td>240</td>
</tr>
<tr>
<td>KO. HPRT R</td>
<td>ACCCCTTCCAATCTCAGC</td>
<td>61</td>
<td></td>
</tr>
</tbody>
</table>
particular, the required alloxan dose for WT mice was 80 mg/kg, for TG mice 100 mg/kg, and for KO mice 65 mg/kg (Table 1). KO mice that were injected with the similar dose of alloxan as the WT group did not achieve any successful implantations (Table 1; Fig. 1).

**External Anomalies**

We found similar malformations which are typical for fetuses from diabetic mice in the WT, TG, and KO groups irrespective of CuZnSOD expression. The malformations were spina bifida hematoma, exencephaly, anophthalmia, and malrotation of the tail.

**Maldevelopment Rate**

Increased maldevelopment rate (res + malf) was found in the DWT80 group compared with the NWT offspring. Furthermore, the maldevelopment rate was increased in the DTG100 group compared with the NTG group (Fig. 1) and in the DKO65 group compared with the NKO group (Fig. 1). In the DKO80 group, consisting of 12 female mice injected with 80 mg/kg alloxan and subsequently displaying a vaginal plug, 100% resorption rate was observed (Table 1; Fig. 1).

Despite the diabetes-induced differences found in all groups, when the resorptions and malformations were pooled together, we found that large standard deviations (interlitter variations) in the groups precluded the demonstration of significant differences between all diabetic and normal offspring with respect to resorption and malformation percentages (Table 1). Thus, the WT fetuses showed clearcut increases in both resorption and malformation percentages (DWT80 vs. NWT, Table 1). However, the TG animals only showed numerical differences (DTG100 vs. NTG, Table 1), suggestive of a difference but not statistically significant, whereas the KO fetuses displayed one increased malformation percentage (DKO65 vs. NKO) and one increased resorption percentage (DKO80 vs. NKO).

**Distribution of Fetal Genotype**

The fetuses of NWT and DWT80 mice displayed only wt genotype as expected. Fetal genotype fractions did not differ between either of the N and D groups of each subgroup (Fig. 2). In particular, the fraction of wt genotype was less than that of the hoz and htz genotypes in both the NTG and DTG100 groups (Fig. 2). Furthermore, the fraction of the different genotypes in the NKO fetuses displayed a Mendalian distribution, in which a tendency toward 1:2:1 was evident (Fig. 2). In the DKO65 group the fraction of wt fetuses was larger than the fraction of hoz fetuses. Among the maldeveloped offspring the genotype fractions did not differ (Fig. 3). However, maternal diabetes caused increased fraction of wt genotype among the maldeveloped offspring of DTG100 and DKO65 groups (Fig. 3).

**Body and Placental Weight**

Decreased maternal weight was found in the DWT80 and DTG100 mice compared with their respective NWT and NTG
groups (Table 1). The maternal weight of the DKO65 tended to decrease compared to the NKO group. Furthermore, maternal weight was decreased in the DKO80 group compared with both its DKO65 and NKO groups (Table 1).

Fetal and placental weight was decreased in the DWT80 group compared with its NWT group. Furthermore, decreased fetal weight was observed in the DTG100 and DKO65 groups compared with their respective N groups (Fig. 4). However, no difference in placental weight was found in the DTG100 and DKO65 groups compared with their respective control groups. No fetal and placental weights for the DKO80 group were displayed since there was no alive offspring in this group.

When we examined the influence of maternal diabetic state on fetal and placental weight in wt fetuses of the WT, TG, and KO mice, a pattern similar to that in Figure 4 was found.

**Fetal Hepatic Isoprostane Concentration**

Maternal diabetes caused increased fetal hepatic isoprostane levels to the same degree in the DWT80 and DTG100 group, but not in the DKO65 group (Fig. 5).

**DISCUSSION**

One important finding in the present study was that both fetal antioxidative capacity and severity of the maternal diabetic state affect the development of the offspring. However, the maternal diabetic state is the major teratogenic factor and overrides the influence of fetal antioxidative capacity. Moreover, we found that maternal diabetes affects intrauterine growth and development by decreasing fetal weight and by increasing maldevelopmental rate in all experimental groups, regardless of fetal genotype. Furthermore, maternal diabetes increased fraction of wt genotype among the malformed offspring of TG and KO mice.

We found that the minimum dose of alloxan required to obtain a manifestly diabetic state in the recipient was strain dependent and/or proportional to the expression level of CuZnSOD. Thus, the required alloxan dose was 80 mg/kg for WT, 100 mg/kg for TG, and 65 mg/kg for KO mice. However, it is known that a single intravenous injection of alloxan in laboratory animals yields excess superoxide production which destroys the pancreatic β-cells and consequently induces diabetes (Dunn *et al.*, 1943). Since we found that TG mice expressing enhanced levels of CuZnSOD also require a higher dose of alloxan to become manifestly diabetic compared with mice expressing normal or lower levels of CuZnSOD, our result indicates that increased expression levels of CuZnSOD protect against alloxan-induced diabetes. In addition, our result is in concert with previous studies showing that CuZnSOD display protection against alloxan-induced oxidative stress in β-cells (Kubisch *et al.*, 1997) as well as protection against alloxan-induced islet DNA strand breaks (Uchigata *et al.*, 1982).

Increased levels of serum isoprostane 8-epi-PGF$_{2α}$ levels in rat diabetes pregnancy (*Palmer et al.*, 1998; Wentzel and Eriksson, 2002) have been associated with elevated production of ROS (Oberley, 1988; West, 2000), suggested to be causally involved in diabetic embryopathy (Eriksson and Borg, 1991, 1993; Hagay *et al.*, 1995). In line with these results, increased fetal hepatic 8-epi-PGF$_{2α}$ isoprostane levels were found in the present study in the diabetic WT and TG mice compared with their N groups, but not in the KO mice, indicating a more severe diabetic state in WT and TG mice. It was expected that markers of oxidative stress would appear in the D groups since it is known that alloxan-induced diabetes leads to oxidative stress (Oberley, 1988; West, 2000). The fact that the fetuses of the DKO mice did not increase their hepatic isoprostane levels despite maternal alloxan administration is likely to be related to the relatively mild diabetic state induced in this group.

We found increased maldevelopmental rate in all D groups regardless of CuZnSOD expression and alloxan dose. The most vulnerable group was the KO group since they displayed similar maldevelopmental rate as the WT and TG mice, despite the mildest diabetic state of all D mice (mean blood glucose concentration 17 mmol/l in the DKO65 group) a consequence
of receiving the lowest alloxan dose. In contrast, the TG group showed comparable degree of fetal maldevelopment, despite a severe maternal diabetes with a blood glucose level of 25 mmol/l, achieved after injection of the highest alloxan dose, indicating that the TG mice are the most protected group against alloxan-induced diabetic embryopathy. These results indicate that CuZnSOD expression, and possibly the expression of other ROS scavenging enzymes, is a protective/modulating factor affecting free radical induced oxidative stress in diabetic embryopathy.

We found decreased number of implantations in both normal and diabetic TG mice compared to the normal and diabetic WT mice. This finding is not easily explained since SOD-TG animals are not reported to display decreased reproductive function, as for example, the SOD-KO animals frequently do (Matzuk et al., 1998). More malformations and fewer resorptions were found in the TG group compared to the WT and the KO groups. Furthermore, fewer malformations and more resorptions were found in the KO group compared with the WT and TG groups. Previous studies have demonstrated that maternal diabetes may lead to early resorptions (Moley et al., 1998; Pampfer et al., 1990, 1997). Usually unsuccessful implantation or embryonic development leads to the embryo becoming resorbed. Our findings suggest that since the TG substrain displayed enhanced protection against embryopathy, maldeveloped embryos that should have become resorbed continued developing, explaining why we observed most malformations and fewest resorptions among this group. Along with this line of thinking, the KO substrain with decreased protection against embryopathy also displayed fewest malformations and most resorptions. Our result is in concert with previous studies, reporting an increased resorption and malformation rate among embryo and fetuses of diabetic rodents in vitro and in vivo (Eriksson and Borg, 1991, 1993; Hagay et al., 1995).

Comparing the three substrains, the TG mice were most protected against the alloxan-induced diabetes, and the KO mice appeared to be the most sensitive to alloxan-induced ROS formation, as well as to the resulting diabetic condition. Indeed, the group of KO mice (DKO80) that were injected with similar dose of alloxan as the WT mice (DWT80) only displayed swollen corpora lutea and no implantations. In support of our explanation, it has been demonstrated that mice that are homozygous for the targeted mutation of CuZnSOD display reduced fertility and altered ovarian morphology; hence the CuZnSOD is needed for ovarian function (Matzuk et al., 1998). Furthermore, the resorption and malformation rate in the present study was less in the NKO group compared with the DKO groups. The maternal blood glucose level of the DKO80 group was on average 24 mmol/l. In contrast, the DKO65 group achieved successful implantations and thus increased resorption and malformation rate were observed. The successful implantations in the DKO65 group may be explained by the milder diabetic state in these mice with an average maternal blood glucose level of only 17 mmol/l.

In the present study the maternal genotype chosen in the experimental design was htz irrespective of different CuZnSOD expression. The fetuses of these htz maternal TG or KO mice may theoretically obtain a 1:2:1 fraction of wt, htz, or hoz fetal genotype. We found no difference in fetal genotype fraction between either of the N and D groups irrespective to CuZnSOD expression. Among the normal fetuses of the diabetic mice with increased CuZnSOD expression (DTG), we found a lower fraction of normal fetuses with wt genotype and an increased fraction of maldevelopment fetuses with wt genotype compared to hoz and htz genotype. However, this was not observed for the fetuses of KO mice. Furthermore, maternal diabetes per se in WT, TG and KO mice decreased the body and placental weight of the wt fetuses. These results suggest that fetuses with increased CuZnSOD expression are more likely to survive in uterine milieu of a mother with increased CuZnSOD expression.

In summary, we found that both fetal antioxidative capacity and maternal diabetic state affect the development of the offspring. However, the maternal diabetic state is the major teratogenic factor and overrides the influence of fetal antioxidative capacity.

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