Genetic and environmental influence on diabetic rat embryopathy

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Ejdesjö A, Wentzel P, Eriksson UJ. Genetic and environmental influence on diabetic rat embryopathy. Am J Physiol Endocrinol Metab 300: E454–E467, 2011. First published November 30, 2010; doi:10.1152/ajpendo.00543.2010.—We assessed genetic and environmental influence on fetal outcome in diabetic rat pregnancy. Crossing normal (N) and manifestly diabetic (MD) Wistar Furth (W) and Sprague-Dawley (L) females with W or L males yielded four different fetal genotypes (WW, LL, WL, and LW) in N or MD rat pregnancies for studies. We also evaluated fetal outcome in litters with enhanced or diminished severity of maternal MD state, denoted MD^W^L and MD^L^W rats. The MDWW litters had less malformations and resorptions (0% and 19%) than the MDLW litters (17% and 30%). The MDWL litters (0% and 8%) were less maldeveloped than the MDLW litters (9% and 22%), whereas the MD^W^L (3% and 23%) and MD^L^W (1% and 17%) litters showed increased and decreased dysmorphogenesis (compared with MDWL and MDDL litters). The pregnant MDW rats had lower serum levels of glucose, fructosamine, and branched-chain amino acids than the pregnant MDL rats, whereas the pregnant MD^W^L and MD^L^W rats had levels comparable with those of the MDL and MDW rats, respectively. The 8-iso-PGF2α levels of the malformed MDDL offspring were increased compared with the nonmalformed MDDL offspring. Diabetes decreased fetal heart Ret and increased Bmp-4 gene expression in the MDDL offspring and caused decreased GDNF and Shh expression in the malformed fetal mandible of the MDDL offspring. We conclude that the fetal genome controls the embryonic dysmorphogenesis in diabetic pregnancy by instigating a threshold level for the teratological insult and that the maternal genome controls the teratogenic insult by (dis)regulating the maternal metabolism.

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The incidence of cardiovascular, skeletal, urinary, gastrointestinal, and caudal dysgenesis-related defects is higher in offspring of women with preexisting diabetes compared with infants of nondiabetic women (4, 18, 27, 38). Despite extensive research and optimized clinical care, diabetic embryopathy has remained etiologically enigmatic and difficult to prevent (40). The diabetes-induced alterations in embryonic development are similar to changes caused by several other teratogens, suggesting that the mechanism of diabetic dysmorphogenesis is multifactorial.

The maternal environment and the fetal-maternal genotypes have been implicated in the teratogenicity of diabetic pregnancy. Thus, in diabetic women, the metabolic control (Hb A1c levels) during the proximal part of the first trimester (29) is a strong predictor of the risk for congenital malformation (28). In experimental work, maternal metabolic deregulation has been confirmed as an important teratogenic factor (14, 17, 25), and a teratogenic interval has been identified in rat diabetic pregnancy gestational days 6–10 (10). In addition, several teratological consequences of a diabetic environment have been identified by animal studies. Diabetic teratogenesis has been associated with oxidative stress (5, 7, 12, 13, 42, 56), enhanced lipid peroxidation (5, 52), decreased antioxidative defense capacity (21, 33, 44, 47, 48), sorbitol accumulation (15, 23), disturbed arachidonic acid metabolism (52), decreased GAPDH activity (49), alteration in PKC activity (20, 22, 53), decreased Pax-3 expression (37, 39), increased apoptosis (17, 19, 35, 39, 46), and enhanced JNK1 and JNK2 activity (41) in the embryos. Indeed, a multitude of environmental changes with teratological importance for diabetic embryopathy have been identified, several of which are likely to be causally interrelated.

The teratological importance of the fetal and maternal genome(s), on the other hand, has not been characterized in detail so far. There is anecdotal clinical evidence, especially from the preinsulin era, of diabetic women with several pregnancies ending in stillbirth or malformation, which would indicate a familial “clustering” of fetal-embryonic maldevelopment (54). Furthermore, in experimental diabetic pregnancy, there are several observations of varied teratological susceptibility in different strains of rodents (11, 34, 36) in support of the presence of a genetic predisposition for congenital malformations and/or compromised fetal development. However, the exact nature of the genetic predisposition is not clarified.

The aim of the present work was to assess the importance of the fetal/maternal genotype and environmental factors for the induction of diabetic embryopathy. We used two inbred rat strains, denoted W (Wistar-Furth) and L (Sprague-Dawley). The offspring of diabetic W rats do not show skeletal malformations and display a minimal rate of cardiac malformations compared with the offspring of diabetic L rats, whereas the L rats are an inbred line of the outbred Sprague-Dawley substrain of rats (U; malformation prone), which display skeletal (14) and cardiac (30, 43) malformations in response to maternal diabetes.

In the present study, we proceeded in three steps. First, we mated diabetic and nondiabetic W and L females with nondiabetic males to obtain baseline maternal metabolites and fetal outcomes, thereby yielding the experimental groups NWL [offspring of normal W (NW) female and W male], NLL [offspring of normal L (NL) female and L male], MDDL [offspring of manifestly diabetic W (MDW) female and W male], and MDLL [offspring of manifestly diabetic L (MDL) female and L male]. We then proceeded to mate diabetic and nondiabetic W females with nondiabetic L males and diabetic and nondiabetic L females with nondiabetic W males, thereby obtaining the groups NWL (offspring of MW female and L male), NLW (offspring of NL female and W male), MDWL (offspring of MDW female and L male), and MDDL (offspring of MDL female and W male) (Fig. 1). We further attempted to obtain pregnancies in diabetic W and L rats.
of the latter for the induction of diabetic embryopathy, and, consequently, by keeping the fetal genotype constant and varying the maternal environment we attempted to assess the influence of the fetal environment for the induction of diabetic embryopathy.

MATERIALS AND METHODS

Animals. The Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985; http://grants1.nih.gov/grants/olaw/ references/phspol.htm) guide was followed. The Uppsala Regional Ethical Committee on Animal Experiments approved the research protocol, including all experimental procedures involving animals beforehand. All animals were maintained at an ambient room temperature of 22°C with a 12:12-h light-dark cycle. They were fed a commercial pelleted diet (R36; Analycon, Linköping, Sweden) and had free access to food and tap water.

Animals were from either a W strain (purchased from B & K, Sollentuna, Sweden) or locally housed Sprague-Dawley-derived inbred strain (L). The L strain has increased incidence of mandibular and cardiac malformations in diabetic pregnancy (14). Manifestly diabetic (MD) and control (N) W and L female rats were mated with males from the same strain in the first set of experiments. In a subsequent set, MD and N females from the W and L strains were cross-bred with males from the opposite strain to achieve two different F1 hybrid fetuses regarding maternal heritage (Fig. 1). Female rats were caged with male rats overnight. Conception was verified by the presence of sperm in vaginal smear and designated gestational day 0.

MD was induced in W and L rats (denoted MDW and MDL) by injection of 40 mg/kg streptozotocin (Sigma-Aldrich Stockholm, Stockholm, Sweden) and in a subset of L rats with 35 mg/kg streptozotocin (denoted MDL¹), into the tail vein (Fig. 1). MD was confirmed within 1 wk after the injection (Freestyle Mini; Abbot Laboratories, Chicago, IL). A blood glucose value >20 mmol/l denoted MD. N animals were not injected at all. In a subset of MDW rats, denoted MDW, 2% glucose was added to the drinking water from gestational day 0 and throughout pregnancy (Fig. 1).

At gestational day 10, 400 μl of blood was drawn from the tail vein of all pregnant rats. Blood was centrifuged, and the serum was used for analysis of glucose/lipid compounds, free amino acids, and isoprostanes. Animals were euthanized on gestational day 20 by cervical dislocation after mild ether anesthesia, and the uterine horns were quickly dissected out. From each horn, fetuses and resorptions were removed, morphologically assessed, and subsequently decapitated. All placentae were inspected and weighed. External malformations of the fetuses were recorded; these were mainly alterations of facial skeleton, i.e., micrognathia and agnathia (cf. Fig. 2). Fetal liver was collected for isoprostane evaluation. Heart and mandible bone with cartilage were dissected free from surrounding soft tissue and divided into two portions. One part from heart and mandible was submerged in lysis buffer (Buffer RLT; Qiagen, Hilden, Germany), and the other part was snap-frozen in liquid nitrogen for later determination of enzyme activities.

Laboratory procedures. Serum concentrations of D-glucose, fructoseamine, triglycerides, and cholesterol were measured with a Konelab 30 (KONE Instruments, Espoo, Finland). All analyses were performed with standard reagent kits.

After deproteinization of 100 μl of serum with 200 μl of sulfosalicylic acid, serum concentrations of free amino acids were determined chromatographically utilizing a Biochrom 20 and 30 (Biochrom, Cambridge, UK). Norleucine was used as internal standard.

Total RNA from each fetal heart and mandible was isolated with an RNeasy minikit (Qiagen) according to the manufacturer’s instructions for fibrous tissues. To each sample, 1 μl of RNase inhibitor (RNA-guard; Amersham Biosciences, Piscataway, NJ) was added. Reverse transcription of mRNA was performed with 1 μg of total RNA utilizing a first-strand cDNA synthesis kit (Ready-To-Go You-Prime

**Fig. 1. Schematic outline of the various cross-matings of the normal (N), manifestly diabetic (MD), less diabetic (MD¹), and severely diabetic (MD²) animals from the inbred Wistar-Furth malformation-resistant (W) and Sprague-Dawley malformation-prone (L) rat substrains. Note that all male rats are normal (nondiabetic) and that the offspring is denoted as a combination of the parental abbreviations. WL, offspring of W female and L male; LW, offspring of L female and W male; NW, normal W rat; NL, normal L rat; MDW, manifestly diabetic W rat; MDL, manifestly diabetic L rat injected with 40 mg/kg streptozotocin; MDW², manifestly diabetic W rat given 2% glucose in drinking water; MDL¹, offspring of MDW female and L male; MDL², manifestly diabetic L rat injected with 35 mg/kg streptozotocin; MDL‘W, offspring of MDL female and W male.**

(denoted MDW and MDL) with a diabetic state similar to that of MDL and MDW rats, respectively. This was done by supplementing a group of MDW rats 2% glucose in the drinking water throughout pregnancy (these rats are denoted MDW²) and by treating one group of MDL rats with a lower dose of streptozotocin (35 instead of 40 mg/kg) prior to gestation (subsequently denoted MDL¹) (Fig. 1). Blood sampling on gestational day 10 enabled assessment of the metabolic status of the pregnant rats at the end of the estimated teratogenic period of gestational days 6–10 (10). The pregnancies were interrupted by caesarean delivery on gestational day 20, when morphological assessment of the fetuses (e.g., sex determination and identifying external malformations) and placenta was performed.

In previous work, we and others have found evidence in favor of a reactive oxygen species (ROS)-associated etiology of the diabetic embryopathy (5, 7, 12, 13, 42, 56); therefore, we measured the maternal serum levels and fetal hepatic concentrations of 8-iso-PGF₂α isoprostane to estimate the degree of oxidative stress in the mother and offspring (6, 52). We have established teratological linkage to several regions in the L/W genome (Nordquist N, Luthman H, Petterson U, and Eriksson JU, unpublished observations) and thereby designated the aldose reductase (AR) (15, 23) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (49) genes as candidate genes. Therefore, the fetal heart and mandible were assessed for expression of genes involved in ROS scavenging and development as well as enzyme activity of AR and GAPDH.

The working hypothesis was that specific maternal and fetal genotypes are both necessary, but not individually sufficient, for induction of embryonic/fetal maldevelopment in diabetic pregnancy. By keeping the maternal genotype constant and varying the fetal genotype we attempted to assess the influence...
First-Strand Beads; GE Healthcare, Uppsala, Sweden) according to the manufacturer’s description. The cDNA was diluted to a final volume of 100 μl with diethyl pyrocarbonate (DEPC)-treated water. For analysis of mRNA expression, cDNA was amplified using the MJMini Personal Thermal Cycler (Bio-Rad Laboratories, Sundbyberg, Sweden) and measured using the MiniOpticon Real-Time PCR Detection System (Bio-Rad Laboratories). All primers were from TIB Molbiol (Berlin, Germany) (primer sequences, cf. Supplemental Table S1; Supplemental Material for this article is available online at the AJP-Endocrinology and Metabolism web site). For each sample, 1 μl of the final cDNA was amplified in a total volume of 10 μl containing 5 μl of iQ SYBR Green Supermix (Bio-Rad Laboratories), 3 μl of DEPC-treated water, and 0.5 μl of sense and antisense primers (10 μM each). PCR procedure was denaturation and activation step at 95°C for 180 s followed by 36 cycles with denaturation at 95°C for 10 s, annealing at 51–52°C for 10 s, and elongation at 72°C for 15 s. Each PCR was ended with a melting point analysis. A blank control for each primer pair was added in every run. Glucose-6-phosphate dehydrogenase was used as housekeeping gene. Each fetal sample (heart or mandible) was assessed individually for mRNA levels, and each sample (10–18 per group) was run in duplicate. Relative quantification was calculated as the difference between sample and housekeeping gene crossing point (Cp) values yielding ΔCp values yielding ΔCp, which was transformed using the formula 2−ΔCp; however, all statistical calculations, including assessment of mean and standard deviations, were performed on the nontransformed values.

Heart and mandible tissue were homogenized on ice by ultrasound disruption (20 kHz, 60 W for 5 s) (Vibra Cell; Sonics & Materials, Danbury, CT) in 140 μl of 100 mM triethanolamine buffer (pH 7.6) and centrifuged at 4°C for 40 min at 13,000 rpm. AR activity was measured with modifications of the method described by Wu et al. (55). All chemicals were purchased from Sigma-Aldrich. For heart and mandible, 80 and 50 μl, respectively, of the supernatant was added to a reaction mixture, giving a final volume of 500 μl containing 0.4 M ammonium sulphate and 0.2 mM NADPH in a 5-mM sodium phosphate buffer, pH 6.3, and preincubated at 37°C for 20 min. The reaction was started by adding of 50 μl 100 mM DL-glyceraldehyde, followed by 5 min in a spectrophotometer (UVMini 1240; Shimadzu, Kyoto, Japan) at 340 nm. The difference in absorbance over time (ΔA) in the linear part of the reaction was used for determination of enzyme activity. Enzyme activity was calculated as ΔA·C/Protein in sample.

GAPDH activity was measured with modifications of the method by Bergmeyer (1), as described before (49). All chemicals were purchased from Sigma-Aldrich Sweden (Stockholm, Sweden). For the assay, 20 and 50 μl of the supernatant were used from heart and mandible, respectively. Enzyme activity was calculated as ΔA·C/Protein in sample.

Protein concentration in 10 μl from sample homogenates was measured according to the method described by Bradford (2), using bovine serum albumin as standard.
Table 1. Serum concentration of glucose and lipid compounds on gestational day 10 in N and MD female rats of W or L strain mated with males of same strain

<table>
<thead>
<tr>
<th>Mother</th>
<th>NW</th>
<th>NL</th>
<th>MDW</th>
<th>MDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-Glucose, mmol/l</td>
<td>6.9 ± 0.2</td>
<td>6.3 ± 0.3</td>
<td>33 ± 2*</td>
<td>41 ± 1b,c</td>
</tr>
<tr>
<td>S-Fructosamine, μmol/l</td>
<td>197 ± 6</td>
<td>207 ± 5</td>
<td>326 ± 6*</td>
<td>336 ± 8*</td>
</tr>
<tr>
<td>S-Cholesterol, mmol/l</td>
<td>1.9 ± 0.1</td>
<td>2.0 ± 0.04</td>
<td>1.9 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>S-Triglycerides, mmol/l</td>
<td>1.0 ± 0.1</td>
<td>1.2 ± 0.2</td>
<td>1.7 ± 0.2a</td>
<td>1.3 ± 0.1</td>
</tr>
</tbody>
</table>

Values are means ± SE (ANOVA). N, nondiabetic; MD, manifestly diabetic; W, Wistar-Furth inbred strain, malformation resistant; L, inbred Sprague-Dawley strain, malformation prone; NW, normal W rat; NL, normal L rat; MDW, manifestly diabetic W rat; MDL, manifestly diabetic L rat, injected with 40 mg/kg streptozotocin. Significance: *P < 0.05 vs. NW; bP < 0.05 vs. NL; cP < 0.05 vs. MDW.

Estimations of 8-iso-PGF2α in fetal liver and maternal serum were performed largely in accordance with the instructions from the manufacturer (Cayman Chemical, Ann Arbor, MI), as described in previous work (31, 50). The protein content of the liver samples was estimated by the method of Lowry et al. (26), using bovine serum albumin as standard. For sequencing of AR and GAPDH transcripts, liver tissue from MD pregnant W and L rats was used for cDNA synthesis, as described above. All primers were from TIB Molbiol (primer sequences, cf. Supplemental Table S2). PCR was performed with a total volume of 20 μl containing 1× GeneAmp PCR Gold Buffer, 3.5 mM MgCl2, 200 μM of each GeneAmp dNTP, 1.0 μM of sense and antisense primer, 0.5 U AmpliTaq Gold, and 2 μl of sample cDNA. All reactions were from Applied Biosystems (Foster City, CA). PCR was performed on an MJMini Thermal Cycler (Bio-Rad Laboratories). Initial denaturation and activation step was performed at 95°C for 10 min. Thereafter, individual numbers of cycles and annealing temperatures were performed for each primer pair. For each cycle, there was denaturation at 95°C for 30s, annealing at 60°C for 60 s, and elongation at 72°C for 60s. PCR was ended at 72°C for 180 s.

PCR products were purified with Exonuclease I and Shrimp Alkaline Phosphatase (ExoSAP-IT PCR Clean-up Kit; GE Healthcare) according to the manufacturer’s description and quantified on 1.5% agarose gel. Sequencing cycling of 5 ng of PCR product was performed with BigDye Terminator version 3.1 (Applied Biosystems) on an ABI 3730 (Applied Biosystems) with initial denaturation at 94°C for 30 s followed by 35 cycles at 94°C for 25 s, 50°C for 15 s, and 60°C for 120 s. For sequence separation and detection, an ABI 3730 (Applied Biosystems) was used. Sequencing data were analyzed with 4 Peaks version 1.7.2 for MacOS X (Mekentosj.com, Amsterdam, The Netherlands), and sequence comparisons were made with ApE version 1.11 for MacOS X (http://wwwbiology.utah.edu/jorgensen/wayned/ape/M. Wayne Davis, Salt Lake City, UT).

Statistical evaluation was made with SigmaStat for Windows version 3.5. Applied tests were one-way χ2-statistics (numerical estimations of dysmorphogenesis), Student’s t-test (means of malformed and nonmalformed fetuses of the same experimental group), or ANOVA with Fisher’s protected least significant difference test applied as post hoc test (means of normal and experimental groups). P < 0.05 was considered significant.

RESULTS

Maternal metabolic state in WW and LL pregnancies. In W and L females, which were mated with males of the same strain, the diabetic rats (MDW and MDL) had markedly increased serum glucose levels compared with the nondiabetic rats (NW and NL); in addition, the MDW rats had slightly lower serum glucose concentration compared with the MDL rats (Table 1). The serum fructosamine levels increased to the same extent in the MDW and MDL groups. The serum cholesterol levels were similar in all groups, and the MDW rats displayed increased serum triglyceride levels (Table 1).

Fetal outcome in WW and LL pregnancies. In the offspring of W and L females, which had been mated with males of the same strain, maternal diabetes decreased fetal body weights and increased placental weights in both strains (MDWW vs. NW, MDLL vs. NLL); these changes were more pronounced in the MDLL pregnancies (Table 2). Distribution of female and male normal fetuses was similar in three groups (NWW, NLL, and MDWW), whereas the MDLL group showed a tendency toward male preponderance (Table 2). Maternal diabetes increased resorption rates in the MDWW and MDLL pregnancies. In addition, the MDLL litters contained 17% malformed fetuses, two-thirds of which were female, a significant difference in sex distribution compared with that of the nonmalformed offspring (Table 2).

Maternal metabolic state in WL and LW pregnancies. In the W and L females, which were mated with males of the opposite strain, the two parameters representing maternal glucose metabolism, serum glucose and fructosamine, were markedly increased by maternal diabetes, and both glucose and fructosamine concentrations were higher in MDL serum compared with MDW serum (Table 3). The maternal serum cholesterol levels did not differ between the groups, whereas the MDL rats

Table 2. Fetal outcome on gestational day 20 in N and MD female rats of W or L strain mated with males of same strain

<table>
<thead>
<tr>
<th>Offspring</th>
<th>NW</th>
<th>NLL</th>
<th>MDWW</th>
<th>MDLL</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of litters</td>
<td>8</td>
<td>10</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>No. of implantations</td>
<td>71</td>
<td>110</td>
<td>120</td>
<td>130</td>
</tr>
<tr>
<td>Implantations/litter</td>
<td>8.9 ± 0.9</td>
<td>11.0 ± 1.0</td>
<td>8.6 ± 0.8</td>
<td>9.3 ± 0.6</td>
</tr>
<tr>
<td>Fetal weight, g</td>
<td>3.09 ± 0.07</td>
<td>3.13 ± 0.10</td>
<td>2.78 ± 0.08a</td>
<td>2.40 ± 0.08b,c</td>
</tr>
<tr>
<td>Placental weight, g</td>
<td>0.52 ± 0.02</td>
<td>0.46 ± 0.01a</td>
<td>0.71 ± 0.04a</td>
<td>0.54 ± 0.03b,c</td>
</tr>
<tr>
<td>No. of normal fetuses (%)</td>
<td>62 (87)</td>
<td>99 (90)</td>
<td>97 (81)</td>
<td>69 (53)</td>
</tr>
<tr>
<td>Females (%)</td>
<td>34 (55)</td>
<td>53 (56)</td>
<td>54 (56)</td>
<td>28 (41)</td>
</tr>
<tr>
<td>Males (%)</td>
<td>28 (45)</td>
<td>43 (44)</td>
<td>43 (44)</td>
<td>41 (59)</td>
</tr>
<tr>
<td>No. of resorptions (%)</td>
<td>9 (13)</td>
<td>11 (10)</td>
<td>23 (19)a</td>
<td>39 (30)b</td>
</tr>
<tr>
<td>No. of malformed fetuses (%)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>22 (17)b,c</td>
</tr>
<tr>
<td>Females (%)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>16 (73)</td>
</tr>
<tr>
<td>Males (%)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>6 (27)</td>
</tr>
</tbody>
</table>

Values are means ± SE (ANOVA and χ2-statistics). Offspring are from mothers listed in Table 1. NWW, offspring of NW female and W male; NLL, offspring of NL female and L male; MDWW, offspring of MDW female and W male; MDLL, offspring of MDL female and L male. *P < 0.05 vs. NWW; bP < 0.05 vs. NLL; cP < 0.05 vs. MDWW.
had higher serum triglyceride concentrations than the NW and NL groups (Table 3).

Fetal outcome in WL and LW pregnancies. The offspring of diabetic W and L females, which were mated with males of the opposite strain (i.e., the MDWL and MDLW fetuses), had lower body weight and higher placental weight compared with NWL and NLW offspring (Table 4). The MDLW group had higher resorption rate than the NWL, NLW, and MDWL groups and, in addition, was the only group with malformations among these four groups (Fig. 2). The fetal sex distribution did not vary in the different groups. In contrast, there was a clear female dominance among the malformed offspring (90% female fetuses) (Table 4).

Maternal metabolic state in MD+W and MD−L pregnancies. The pregnant MDW females with 2% glucose in the drinking water (MD+W) had serum glucose and serum fructosamine

Fig. 3. Serum concentration of free amino acids in maternal N or MD serum at gestational day 10 in W and L rats. Bars showing relation in percent between MDW and NW (A), MDL and NL (B), and MDL and MDW (C) (Student’s t-test). Significance: *P < 0.05 vs. N (A and B) and MDW (C).
values that were similar to those of the MDL pregnant females (Table 3). The pregnant MD⁻L rats (given 35 instead of 40 mg/kg streptozotocin iv) lowered their serum glucose and serum fructosamine levels down to the range of the MDW females (Table 3). The maternal serum cholesterol levels did not differ between the MDW, MD⁺W, MDL, and MD⁻L rats. The serum triglyceride levels of the MD⁺W rats were similar to those of the MDL rats, whereas the MD⁻L rats had markedly decreased serum triglyceride levels compared with MDL and MDW females (Table 3).

**Fetal outcome in MD⁺WL and MD⁻LW pregnancies.** When 2% glucose in the drinking water was given to pregnant MDW female rats mated with male L rats, the resulting MD⁺WL fetuses were numerically smaller and their placentae numerically larger than the MDWL and MDLW fetuses and placentae, respectively (0.05 < P < 0.10; Table 4). The MD⁺WL

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Fig. 4. Serum concentration of free amino acids in maternal serum at gestational day 10 in MDW and MDL rats, in MDW rats with enhanced diabetes due to 2% glucose added to the drinking water (MD⁺W rats), and in MDL rats with diminished diabetes by injection of less streptozotocin (MD⁻L rats). Bars showing relation in percent between MD⁺W and MDW (A) and MD⁻L and MDL (B) (Student's t-test). Significance: *P < 0.05 vs. MDW (A) and MDL (B).

Fig. 5. A: serum concentration of 8-Iso-PGF2α in N (open bars) and MD (gray bars) W and L rats at gestational day 10. B: hepatic concentration of 8-Iso-PGF2α at gestational day 20 in WL and LW fetuses from N (open bars) and MD (gray bars) W and L rats. Means ± SE (ANOVA and Student’s t-test). Significance: *P < 0.05 vs. N; † P < 0.05 vs. MDL with no malformations.
group had higher resorption rate than the MDWL group (23 vs. 8%) and was similar to the rate of the MDLW group (22%). In contrast, the malformation rate of the MD-WL fetuses (3%) was intermediary to that of the MDWL (0%) and MDLW groups (9%) (Table 4).

When 35 instead of 40 mg/kg streptozotocin was given to MDL females before they were mated with W males, the resulting MD-LW fetuses were numerically larger and their placentae numerically smaller than the MDLW fetuses and placentae (0.05 < P < 0.10; Table 4). The resorption rate in the MD-LW group was numerically smaller (15 vs. 22%, 0.05 < P < 0.10), but the malformation rate was decreased (1 vs. 9%) compared with the MDLW group (Table 4).

Maternal serum amino acid concentrations in WL and LW pregnancies. Diabetes in the mother caused increased levels of asparagine, glutamine, proline, alanine, valine, isoleucine, and leucine and decreased lysine in both strains, whereas the MDW rats showed increased levels of taurine and glutamic acid and decreased cysteine and tryptophan, and the MDL rats showed increased citrulline and ornithine (Fig. 3, A and B). Comparison between the MDL and MDW rats showed that the MDL rats had lower taurine and...
glutamic acid as well as higher glutamine, citrulline, valine, cysteine, methionine, leucine, ornithine, and lysine compared with the MDW rats (Fig. 3C).

Maternal serum amino acid concentrations in MD^+^WL and MD^-^LW pregnancies. The differences in maternal serum amino acid levels between MD^+^W and MDW as well as MD^-^L and MDL are shown in Fig. 4. MD^+^W compared with MDW rats (Fig. 4A) show decreased levels of taurine, aspartic acid, asparagine, glutamic acid, and arginine as well as increased levels of threonine, glutamine, proline, valine, cysteine, methionine, isoleucine, leucine, histidine, and tryptophan. MD^-^L compared with MDL rats (Fig. 4B) had decreased levels of threonine, serine, asparagine, proline, glycine, alanine, citrulline, valine, isoleucine, leucine, and ornithine and an increase in tryptophan.

Maternal serum and fetal hepatic isoprostane concentrations. The 8-iso-PGF2α levels in maternal serum and fetal liver are shown in Fig. 5. In the maternal diabetic serum, the isoprostane was increased compared with control levels, with no difference between MDW and MDL (Fig. 5A). Likewise, the fetal hepatic isoprostane levels were increased but did not differ between the MDWL and MDLW fetuses. However, the isoprostane levels in liver of malformed MDLW fetuses were increased compared with the liver values of the nonmalformed MDLW fetuses (Fig. 5B).

Fetal gene expression. The gene expression of the principal ROS-scavenging enzymes in the fetal heart and mandible is depicted in Fig. 6, A–F. In the fetal heart, maternal diabetes decreased mRNA levels of CuZnSOD (only...
Fig. 7. Gene expression of aldose reductase (AR; A and E) and GAPDH (B and F) in heart and mandible at gestational day 20 in WL and LW fetuses from N (open bars) and MD (gray bars) W and L rats. Transformed means ± SE. Enzyme activity of AR (C and G) and GAPDH (D and H) in heart and mandible at gestational day 20 in WL and LW fetuses from N (open bars) and MD (gray bars) W and L rats. Means ± SE (ANOVA and Student’s t-test). Significance: *P < 0.05 vs. N; #P < 0.05 vs. WL; †P < 0.05 vs. MDLW fetuses with no malformations.
MDW), MnSOD, EC-SOD, catalase, and Gpx-1 but increased Gpx-2 mRNA (Fig. 6, A–F). CuZnSOD levels in the NLW fetal heart were decreased compared with NWL hearts, whereas, in contrast, the mRNA levels of CuZnSOD, MnSOD, and Gpx-1 were increased in MDLW fetal heart compared with MDWL hearts (Fig. 6, A, B, and E). In the fetal mandible, maternal diabetes decreased CuZnSOD (only MDWL), EC-SOD, and catalase but increased CuZnSOD (only MLDW). Furthermore, the CuZnSOD levels of the NLW mandibles were lower, and those of the MLDW mandibles were higher, than the corresponding CuZnSOD mRNA levels of the NWL and MDWL mandibles (Fig. 6, A, C, and D).

The gene expression of several developmental genes is depicted in Fig. 6, G–I. In the fetal heart, maternal diabetes decreased Ret mRNA levels but increased Bmp-4 mRNA levels (only MDLW). Both Ret and Bmp-4 mRNA were increased in MLDW hearts compared with MDWL hearts (Fig. 6, I and J). In the fetal mandibles, maternal diabetes decreased GDNF, Ret, and Shh (only MDLW). Furthermore, the malformed MLDW mandibles had lower levels of GDNF and Shh than the nonmalformed MLDW mandibles (Fig. 6, G, H, I, K, and L).

**Fetal gene expression and activity of AR and GAPDH.** The AR mRNA levels were increased in the mandibles of MDW and MDL fetuses, whereas maternal diabetes decreased the mRNA levels of GAPDH (only MDLW) in the fetal heart. No differences in mRNA levels of AR and GAPDH in heart and mandible were seen between nonmalformed and malformed fetuses in MLDW group (Fig. 7, A, B, E, and F). The AR activity baseline was decreased in the hearts from NLW fetuses compared with hearts from NWL fetuses. The AR activity of the fetal hearts was decreased by maternal diabetes in the MDWL group but not in the MLDW group. Furthermore, hearts from malformed MLDW fetuses displayed decreased AR and GAPDH activity compared with hearts from nonmalformed MLDW fetuses (Fig. 7, C, G, and H). The AR activity in the fetal mandible was markedly decreased by maternal diabetes, with no difference between nonmalformed and malformed mandibles. The NLW mandibles had higher GAPDH activity than the NWL mandibles, and maternal diabetes distinctly depressed the MDWL and MLDW GAPDH enzyme activity in fetal mandibles (Fig. 7, C, D, G, and H).

**Fetal AR and GAPDH mRNA sequences.** The primers used for sequencing the AR and GAPDH mRNA are shown in Fig. 8. There were no detectable differences between the W and L AR and GAPDH mRNA sequences, which also corresponded to the published rat AR and GAPDH mRNA sequences.

**DISCUSSION**

The most important finding in the present work was the strong teratogenic impact of the maternal L genome/intrauterine environment compared with the maternal W genome/environment in diabetic pregnancy, increased fetal maldevelopment in MDD/W vs. MDWW and in MDDW vs. MDWL, with a clear teratological threshold, since fetal maldevelopment was drastically reduced in the MD- L vs. MDDL, when the severity of the maternal diabetic state was diminished. However, teratological resistance of the maternal W genome/environment was also found, with less fetal maldevelopment in MDW- W vs. MDDL, despite similar severity of maternal diabetes.

The MDDL offspring display increased rates of malformations and resorptions compared with the MDWL offspring. This result may be viewed in two ways. If the LW and WL fetuses are regarded as genetically equal in a teratological sense, the difference in fetal development should be related solely to differences in environment, i.e., the diabetic intrauterine milieu. If, on the other hand, the LW and WL fetuses are not genetically equal in a teratological sense; i.e., their different mitochondria and differences in genomic imprinting are important for the embryonic (mal)development, then we need to postulate a teratological “cross-talk” between mother and fetus as a component in the pathogenesis of diabetic embryopathy.

In support of the first alternative, that the maternal environment completely determines the fetal dysmorphogenesis, we observe that the pregnant MDDL rats when mated with W males displayed 9% malformations and higher serum glucose and fructosamine levels than the pregnant MDWW rats mated with L males, which produced no malformed offspring. Moreover, the triglycerides and branched-chain amino acids (valine, leucine, isoleucine) were more increased in the MDL rats than in the MDWW rats. The malformation-prone LW offspring were obviously exposed to a more severe intrauterine “diabetic milieu” than the malformation-resistant WL offspring. The teratological importance of the maternal metabolic deregulation was further illustrated by the effects of enhanced severity of the diabetic state in the MDW- W rats and diminished diabetic severity in the MD- L rats; in these groups, the maternal serum parameters were enhanced and diminished, respectively. Also the malformation rates were enhanced and diminished com-

**Fig. 8.** Primer setup for sequencing cDNA from AR (A) and GAPDH (B) transcripts in MD pregnant W and L rats. Arrowheads show direction and insertion point for each primer. Yellow boxes represent PCR products for each primer pair. 3'-UTR, 3'-untranslated region.
pared with the rates in the MDW and MDL groups, respectively. Obviously, the severity of the maternal diabetic state exerts a major teratological influence in this model of diabetic pregnancy.

The differences in fetal outcomes in the four diabetic groups, however, may indicate a teratological role also for the fetal genome. Thus, by enhancing the maternal diabetic state from MDW to MD+W, the malformation rate increased from 0 to 3%. Likewise, diminishing the maternal diabetic state from MDL to MD-L yielded a decrease in malformation rate from 9 to 1%. Since the diabetic states of the MD+W and MDL rats are of comparable severity with the MDW and MD-L states, the results suggest that the WL offspring are less prone to develop malformation than the LW offspring also in a similar diabetic environment.

Notably, the recorded differences in fetal outcome in the present study were related to proposed differences between W and L genome, although complete genomic identity between individuals in the two inbred lines is not likely to be present. To the best of our knowledge, however, we have no indication that marked genomic variation among LW or WL offspring would explain our findings, although we have not formally evaluated such a possibility of structural variation.

In previous cross-breeding experiments, it was concluded that both maternal and fetal genomes affect the outcome (11, 34). Roughly, the more malformation prone the genome present on both sides of the placenta, the higher the risk for embryofetal maldevelopment, but this relationship does not appear to be linear and does not seem to work via the same genes/gene products in the mother and offspring. A comparison of the development of nonobese diabetic and Institute for Cancer Research mouse embryos after blastocyst transferral to a normal or diabetic recipient of the other strain led the authors of that study to conclude that both the maternal environment and the fetal genome are of importance for the diabetes-induced maldevelopment (34). Resistance to diabetes-induced neural tube defects, on the other hand, appears to be a dominant trait of the fetal genome in mice (36), indicating a distinct regulating role for the genetic makeup of the offspring. Taken together, these findings and the present study would suggest that the risk of developing diabetic embryopathy is determined by the concomitant presence of a malformation-enabling fetal genome together with a maternal diabetic state, whose severity modulates the degree of fetal damage.

Pondering the nature of the fetal genetic contribution to the teratogenesis of the present model, it appears evident that the genotypes of the WL and LW fetuses are essentially identical with regard to autosomes (all offspring have one L and one W autosomal chromosome) and sex chromosomes in females (one X chromosome from each parent) but differ regarding sex chromosomes in males (X_W Y_L vs. X_L Y_W). Furthermore, the WL and LW offspring differ with regard to mitochondrial type (WL fetuses have W mitochondriae, LW fetuses have L mitochondriae) and with regard to imprinting patterns of gene expression. We aimed to differentiate between these possible mechanisms.

When we inspect the outcome in the WW and LL pregnancies, a clear sex-specific pattern was evident. The viable offspring showed a female preponderance (about 55% females and 45% males) in the NW, NL, and MDWW groups, whereas this proportion was largely reversed in the MDLL group (41% females and 59% males), where we also found a

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**Table 3. Serum concentration of glucose and lipid compounds on gestational day 10 in N and MD female rats of W or L strain mated with males of opposite strain**

<table>
<thead>
<tr>
<th>Mother</th>
<th>NW</th>
<th>NL</th>
<th>MDW</th>
<th>MDL</th>
<th>MD-W</th>
<th>MD-L</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-Glucose, mmol/l</td>
<td>7.4 ± 0.2</td>
<td>6.9 ± 0.2</td>
<td>29 ± 1*</td>
<td>38 ± 1*</td>
<td>40 ± 1*</td>
<td>28 ± 1*</td>
</tr>
<tr>
<td>S-Fructosamine, μmol/l</td>
<td>211 ± 12</td>
<td>201 ± 10</td>
<td>278 ± 3*</td>
<td>310 ± 3*</td>
<td>315 ± 6*</td>
<td>276 ± 3*</td>
</tr>
<tr>
<td>S-Cholesterol, mmol/l</td>
<td>1.9 ± 0.1</td>
<td>1.9 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>2.0 ± 0.04</td>
<td>1.8 ± 0.1</td>
<td>1.8 ± 0.05</td>
</tr>
<tr>
<td>S-Triglycerides, mmol/l</td>
<td>1.0 ± 0.1</td>
<td>1.1 ± 0.2</td>
<td>1.4 ± 0.1</td>
<td>1.8 ± 0.2b</td>
<td>1.7 ± 0.2a</td>
<td>0.8 ± 0.04c,d</td>
</tr>
</tbody>
</table>

Values are means ± SE (ANOVA). MD+* W and MD+* L, manifestly diabetic W and L rats, respectively, with extra glucose in drinking water and given less streptozotocin. Significance: *P < 0.05 vs. NW; **P < 0.05 vs. NL; ***P < 0.05 vs. MDW; ****P < 0.05 vs. MDL.

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**Table 4. Fetal outcome on gestational day 20 in N and MD female rats of W or L strain mated with males of opposite strain**

<table>
<thead>
<tr>
<th>Offspring</th>
<th>NWL</th>
<th>NLW</th>
<th>MDWL</th>
<th>MDLW</th>
<th>MD-WL</th>
<th>MD-LW</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of litters</td>
<td>6</td>
<td>4</td>
<td>10</td>
<td>12</td>
<td>13</td>
<td>9</td>
</tr>
<tr>
<td>No. of implantations</td>
<td>45</td>
<td>45</td>
<td>110</td>
<td>106</td>
<td>116</td>
<td>89</td>
</tr>
<tr>
<td>Implantations/litter</td>
<td>7.5 ± 0.6</td>
<td>11 ± 0.8</td>
<td>8.8 ± 0.5</td>
<td>8.8 ± 1.0</td>
<td>9.1 ± 0.5</td>
<td>9.9 ± 0.8</td>
</tr>
<tr>
<td>Fetal weight, g</td>
<td>3.89 ± 0.21</td>
<td>3.55 ± 0.06</td>
<td>3.09 ± 0.05a</td>
<td>3.05 ± 0.09b</td>
<td>2.87 ± 0.10a</td>
<td>3.29 ± 0.10</td>
</tr>
<tr>
<td>Placental weight, g</td>
<td>0.59 ± 0.02</td>
<td>0.51 ± 0.01</td>
<td>0.72 ± 0.02a</td>
<td>0.77 ± 0.06b</td>
<td>0.78 ± 0.04a</td>
<td>0.71 ± 0.02b</td>
</tr>
<tr>
<td>No. of normal fetuses (%)</td>
<td>40 (89)</td>
<td>42 (95)</td>
<td>81 (92)</td>
<td>73 (69)</td>
<td>88 (75)</td>
<td>73 (82)</td>
</tr>
<tr>
<td>Females (%)</td>
<td>22 (55)</td>
<td>24 (57)</td>
<td>44 (54)</td>
<td>31 (42)</td>
<td>38 (43)</td>
<td>40 (55)</td>
</tr>
<tr>
<td>Males (%)</td>
<td>18 (45)</td>
<td>18 (43)</td>
<td>37 (46)</td>
<td>42 (58)</td>
<td>50 (57)</td>
<td>33 (45)</td>
</tr>
<tr>
<td>No. of resorptions (%)</td>
<td>5 (11)</td>
<td>9 (23)</td>
<td>1.7 (8.5)</td>
<td>23 (22)bc</td>
<td>27 (23)bc</td>
<td>15 (17)bc</td>
</tr>
<tr>
<td>Females (%)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>10 (9)b</td>
<td>3 (3)</td>
<td>1 (1)d</td>
</tr>
<tr>
<td>Males (%)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (10)</td>
<td>1 (33)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

Values are means ± SE (ANOVA and χ²-statistics). Offspring are from mothers listed in Table 3. NWL, offspring of NW female and L male; NLW, offspring of NL female and W male; MDWL, offspring of MDW female and L male; MDLW, offspring of MDL female and W male; MD+W, offspring of MD+W female and L male; MD-LW, offspring of MD-L female and W male. Significance: *P < 0.05 vs. NWL; **P < 0.05 vs. NLW; ***P < 0.05 vs. MDWL; ****P < 0.05 vs. MDLW.
large proportion of resorptions (30%) and malformations (17%), the latter with a strong female preponderance (73% females and 27% males). This suggests that fetal maldevelopment in diabetic pregnancy (resorptions and malformations) of the present model preferentially occurs in female fetuses. We regard the possibility that the altered female/male proportion in the MDLL group is due to a high proportion of selective mortality only in malformed male fetuses (and not female malformed fetuses) to be less likely.

Furthermore, among the viable offspring in the cross-bred groups of this study, we also found an increased proportion of female fetuses compared with male fetuses in all groups (about 55% females vs. 45% males), with the exception of the MDLW and MD+WL groups, where a clear male preponderance was present (about 42% females vs. 58% males). Notably, in the two latter groups, the resorption rate was much increased (about 23% compared with 5–17% in the other groups), which suggests that a majority of the resorbed offspring in the MDLW and MD+WL pregnancies was of the female sex. In concert with the hypothesis of increased proportions of resorptions among the female offspring was the observation that the malformed fetuses in the MDLW and MD+WL groups also showed a clear female preponderance. Therefore, in the two groups of diabetic pregnancies with the highest teratological potential (highest serum levels of glucose, fructosamine, and triglycerides) in the present animal model, the teratological impact is more pronounced in the female offspring, suggesting enhanced female embryonic vulnerability in a severe diabetic environment. Since the female WL and LW offspring share the same chromosomal setup, the difference in teratological outcome between WL and LW fetuses may be related to nonchromosomal factors such as different mitochondrial types or different imprinting influences. In a previous study, we found no linkage between diabetic dysmorphogenesis and type of mitochondria (W or L) in the offspring (Nordquist N, Luthman H, Pettersson U, and Eriksson UJ, unpublished observations); therefore, the diabetic embryopathy of the present model may be related to epigenetic changes resulting from differences in gene imprinting (9). However, this notion remains speculative at present, since we lack experimental epigenetic support from the present study.

The increased rate of female dysmorphogenesis in the rat is in contrast to a recent study of maldevelopment in human type 1 diabetic pregnancy, where a higher proportion of male compared with female fetuses was found (16). The reasons for this discrepancy are not evident.

Here the threshold for fetal maldevelopment in the MDLW group appears to be related to the serum levels of glucose, triglycerides, and branched-chain amino acids as well as to the serum concentration of citrulline and ornithine. There is strong experimental support for excess glucose as an important teratogenic factor (8, 13, 17, 25), although other serum components may be of significance (51), e.g., B-hydroxybutyrate (3), triglycerides (10), and branched-chain amino acids (12). The increased citrulline and ornithine levels have been observed before (45) and may reflect an enhanced urea cycle activity associated with the teratogenic process. In addition, there was a selective decrease in serum levels of taurine in the MDL rats compared with the MDW rats, suggesting a possible role for this nonessential amino acid in the teratogenesis (24). However, determination of which of these serum parameters (or combinations) have direct teratological action will have to await further studies.

There was no difference in maternal 8-iso-PGF2α levels of the MDW and MDL rats, indicating that the oxidative stress was of similar degree in the W and L maternal compartments. However, the 8-iso-PGF2α levels of the malformed MDLW offspring were increased compared with the non malformed MDLW offspring, which supports a role for oxidative stress in the pathogenesis of diabetic embryopathy. Furthermore, maternal diabetes suppresses the expression of most ROS-scavenging enzymes in the fetal heart, whereas the expression changes are not as clear-cut in the fetal mandible.

The developmental gene expression in the fetal heart shows considerable diabetes-induced decrease of Ret and increased Bmp-4, changes most pronounced in the MDLW offspring. Furthermore, in the fetal mandible, maternal diabetes caused decreased GDNF and Shh expression, both of which were most pronounced in the malformed MDLW offspring. These changes indicate that the neural crest cells are involved in the teratogenic processes.

The effect of maternal diabetes on the expression of the two candidate genes, AR and GAPDH, and the activity of their gene products in the fetal tissues were somewhat divergent. The pertinent findings were decreased AR and GAPDH activity in MD mandibles and decreased AR and GAPDH activities in malformed MDLW fetal hearts, supporting a role for inhibited GAPDH activity in the teratogenic process of diabetic pregnancy (49).

In conclusion, the present study supports the notion that the fetal genome enables the occurrence of embryonic dysmorphogenesis by instigating a threshold level for the teratological insult, and the maternal genome controls the extent of embryonic/fetal maldevelopment by (de)regulating the maternal metabolism. These findings further emphasize the importance of normalized maternal metabolism for a normalized fetal outcome in diabetic pregnancy (38).

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

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