Influence of maternal metabolism and parental genetics on fetal maldevelopment in diabetic rat pregnancy

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Despite optimal clinical care, diabetic pregnancies show two to five times higher frequency of congenital malformations compared with nondiabetic pregnancies (1, 8, 18, 29). The most important predictor for malformations in human diabetic pregnancy is maternal HbA1c concentration in early gestation (47, 79), whereas optimal preconceptional care appears to be the most effective clinical prevention for malformations (29, 58, 65).

Studies of biological pathways underlying diabetes-associated malformations have suggested that the teratological impact of a diabetic environment partly depends on excess of reactive oxygen species (ROS) in the embryo (23) as a consequence of either increased free oxygen radical formation (22, 60, 78), decreased capacity of ROS-scavenging enzymes (9, 31, 63, 70, 72, 80), or both. Previous studies have also suggested an association between increased sorbitol accumulation and dysmorphogenesis in the embryo (26, 32, 34, 67). However, attempting to diminish such accumulation by using inhibitors of aldose reductase (AR) did not prevent the maldevelopment in the offspring (26, 34). Another key enzyme in glycolysis, glyceraldehyde-3-phosphate dehydrogenase (Gapdh), is inhibited in rat embryos subjected to diabetes in vivo and high glucose in vitro (73). This Gapdh inhibition is suggested to result from poly(ADP-ribose)ylation of Gapdh by activated poly(ADP-ribose) polymerase (PARP) (16), attempting to repair diabetes-induced DNA damage (43, 44). The decreased glycolytic flux proximal to Gapdh (16) and the presence of increased ambient glucose levels will yield enhanced flux in the sorbitol (26, 34) and hexosamine pathways (36). An increased availability of proximal glycolytic intermediaries would also increase diacylglycerol (DAG) production and cause activation of several protein kinase C (PKC) isoforms (30, 33), as well as enhancing the flux in the advanced glycation end product (AGE) pathway (14, 27). Several of the consequences of inhibited Gapdh activity may thus contribute to different facets of the teratogenic outcome in diabetic pregnancy.

There are several reports of racial differences in rates of specific malformations, such as an association between Native American origin and increased risk for cleft palate (71). Other studies have addressed the question of predisposing genomic factors for skeletal (12) and cardiac (51) malformations (76). With regard to diabetes in pregnancy, there are several anecdotal tales of familiar clustering of malformations and dysmorphogenesis in women with diabetes. Regarding genetic susceptibility for malformations, it is established that maternal polymorphism in the methylene-tetrahydrofolate-reductase (Mthfr) gene increases the risk for neural tube defects (NTD) in the offspring (55).

NTD are overrepresented in diabetic pregnancies compared with the nondiabetic population (59). It is also evident that the 677C→T polymorphism in the Mthfr gene is more common in women with type 1 diabetes than in the background population (42). It is tempting to conclude that such genetic predisposition as in the case of reduced Mthfr activity in pregestational diabetic pregnancy would increase the risk for NTDs even further, but up to date there are no studies of Mthfr polymorphism and malformation outcome in diabetic pregnancy available. In experimental work, different susceptibilities to diabetes-induced congenital malformations have been demonstrated in different rodent strains (21, 54, 56). We have previously reported that both the maternal and fetal genome are likely to
metabolic and genetic factors in diabetic embryopathy

Affect the rate of diabetes-induced malformations in studies comparing the fetal outcome of diabetic rats of different strains (21). There has also been demonstrated that a specific chromatographic variant of the catalase enzyme (Cs-1a) is present in malformation-prone U rats, whereas another variant of the catalase protein (Cs-1b) is present in rats of an outbred Sprague-Dawley strain, malformation resistant (H) rats that do not develop malformations in response to maternal diabetes (25). Furthermore, it has been shown that embryonic catalase activity was lower in embryos from normal U rats than in embryos from normal H rats on gestational day 11 (corresponding to gestational week 5 in human pregnancy) and that maternal diabetes augments this difference (9). We sequenced catalase cDNA and the promoter region of the catalase gene in U and H rats (10). Sequence analysis showed one nucleotide mutation in the 5′-untranslated region of the U rat cDNA and a heterozygocity in the U rat gene promoter, indicating different regulation of transcription (promoter) and posttranscriptional modifications (cDNA mutation) causing the noted catalase differences (10).

Using L, an inbred U rat strain (20% fetal skeletal malformations in diabetic pregnancy), and inbred Wistar Furth rats (no diabetes-inducible skeletal malformations), we performed a global gene linkage analysis of the skeletal malformations. There was a strong association to seven regions on chromosomes 4, 10, 14, 18, and 19 and a weaker association to 14 other loci on several other chromosomes (53). From these regions, 20 candidate genes, and 2 candidate proteins, AR (chromosome 4) and Gapdh (chromosome 10), were chosen for further studies. In earlier experimental work, we have shown that the severity of the diabetic state is important but not completely decisive for the development of malformations in rat diabetic pregnancy (19). The fetal genotype as well as derangements in the uterine environment, i.e., overload of glucose and lipid compounds, disturbed amino acid levels, and enhanced oxidative stress, play different roles in the induction and direction of dysmorphic/genesis pathways. To investigate metabolic and genetic hereditary patterns that may influence fetal development, we evaluated fetal outcome in two different F1 hybrids. These F1 hybrids were created by a crossbreed of two strains, W and L, with low (0%) and high (20%) malformation rate, respectively. The F1 offspring was designated WL (W female mated with L male) and LW (L female mated with W male). The WL and LW F1 females share the same chromosomal setup, whereas the WL and LW F1 males differ in XY chromosomal origin. The working hypothesis was that dissimilarities in maternal metabolic state and genetic features from W and L parental strains would contribute to differences in outcome between the two F1 hybrids reflected in different malformation and resorption frequency, fetal ROS defense, and disturbances in the gene expression of developmental genes in fetal heart and mandible.

Materials and Methods

Animals. The “Principles of Laboratory Animal Care” (National Institutes of Health publication no. 85–23, revised 1985; http://grants1.nih.gov/grants/olaw/references/phspol.htm) were 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; Analycen, Linköping, Sweden) and had free access to food and tap water.

Animals were either from a Wistar Furth strain (denoted W; purchased from B&K, Sollentuna, Sweden) or from a locally housed Sprague-Dawley-derived inbred strain, denoted L. The L strain has increased incidence of mandibular and cardiac malformations in diabetic pregnancy (24). W and L female rats were crossbred with males from the opposite strain to produce two different F1 hybrids regarding maternal heredity designated WL and LW, respectively. Nondiabetic control and manifestly diabetic female WL rats and female LW rats were caged with nondiabetic males from the same F1 generation overnight. Conception was verified by the presence of sperms in vaginal smear the next morning, which was designated gestational day (GD) 0.

Manifest diabetes (MD) was induced in 3-mo-old female WL and LW rats (denoted MD-WL and MD-LW) by injection of 40 mg/kg streptozotocin (Sigma-Aldrich Stockholm, Stockholm, Sweden) into the tail vein. MD was confirmed within a week after the injection (Freestyle Mini; Abbot Laboratories). A blood glucose value >20 mmol/l was considered to denote MD. Control (N) animals were not injected at all.

At GD 10, venous blood was drawn from the tail vein of pregnant diabetic and nondiabetic rats. Blood was centrifuged, and the serum was used for analysis of glucose/lipid compounds, free amino acids, and isoprostanes.

Animals were killed on GD 20 by cervical dislocation after mild ether anesthesia, and the uterine horns were quickly dissected out. From each horn, fetuses were dissected free from their surrounding membranes and umbilical cord. Fetuses where further weighed, morphologically evaluated with gender determination, and subsequently decapitated. Placentas were dissected free from adherent membranes and wiped on a filter paper to exclude excess fluid and further weighed. Resorptions were counted, weighed, and morphologically evaluated. External malformations of the fetuses were recorded; these were mainly alterations of facial skeleton, i.e., micrognathia, agnathia, and cleft lip and palate (cf. Fig. 1). 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 the 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, fructosamine, triglycerides, and cholesterol were measured with a Konelab 30 analyzer (ThermoFisher Scientific, Vantaa, Finland). All analyses were performed with standard reagent kits for d-glucose, cholesterol, fructose, and isoprostanes (Hirubax ABX, Montpellier, France). Serum concentrations of β-hydroxybutyrate were performed on a Cobas MIRA Multichannel analyzer (Roche Diagnostica, Basel, Switzerland) with reagent kit LiquiColor Procedure No. 2440 (Stanbio Laboratories, Boerne, TX).

After deproteinization of 100 μl serum with 200 μl sulfosalicylic acid, serum concentrations of free amino acids were determined chromatographically using a Biochrom 20 (Biochrom, Cambridge, UK) and a 4.6 × 200 mm high-resolution PEEK column with Ultracap 8 resin (Biochrom). Norleucine was used as internal standard.

Estimations of 8-iso-PGF2α, in fetal liver and maternal serum were largely performed in accordance with the instructions from the manufacturer (Cayman Chemical, Ann Arbor, MI) as described in previous work (50, 74). The protein content of the liver samples was estimated by the method of Lowry (45) using BSA as a standard.

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

For analysis of mRNA expression, cDNA was amplified and measured using the MyIQ Optical Thermal Cycler (Bio-Rad Laboratories, Sundbyberg, Sweden). All primers were from TIB Molbiol (Berlin, Germany), and their corresponding sequences are listed in Table 1. 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 RNase-free water, and 0.5 μl of sense and antisense primers (10 μM each). The PCR procedure was as follows: 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. We have previously assessed the stability of primer sequences for semiquantitative real-time PCR

<table>
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<th>Primer</th>
<th>Sequence 5’ to 3’</th>
<th>Tm, °C</th>
<th>Product Size, bp</th>
<th>Annealing, Temperature, °C</th>
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</thead>
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<td></td>
<td>Rev</td>
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</tr>
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<td>Fw</td>
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<tr>
<td></td>
<td>Rev</td>
<td>AGT CCA AGA GGA AAT GG</td>
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Tm, melting temperature; G6PDH, glucose-6-phosphate dehydrogenase; AR, aldose reductase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CuZnSOD, copper zinc superoxide dismutase; MnSOD, manganese superoxide dismutase; ECSOD, extracellular superoxide dismutase; GPx-1 and 2, glutathione peroxidase-1 and -2, respectively; GDNF, glial cell line-derived neurotrophic factor; SHH, sonic hedgehog homolog; Ret, Ret proto-oncogene; BMP-4, bone morphogenetic protein-4; Fw, forward; Rev, reverse.

Table 1. Primer sequences for semiquantitative real-time PCR
Table 2. Fetal outcome at gestational day 20 in N and MD WL and LW rats

<table>
<thead>
<tr>
<th>Offspring</th>
<th>N-WLWL</th>
<th>N-LWLW</th>
<th>MD-WLWL</th>
<th>MD-LWLW</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of litters</td>
<td>6</td>
<td>6</td>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td>No. of implantations</td>
<td>73</td>
<td>60</td>
<td>204</td>
<td>196</td>
</tr>
<tr>
<td>Fetal wt, g</td>
<td>3.63 ± 0.06</td>
<td>3.74 ± 0.13</td>
<td>2.95 ± 0.06a</td>
<td>2.88 ± 0.06b</td>
</tr>
<tr>
<td>Male wt, g</td>
<td>3.56 ± 0.06</td>
<td>3.70 ± 0.12</td>
<td>2.96 ± 0.05a</td>
<td>2.89 ± 0.08b</td>
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<tr>
<td>Placental wt, g</td>
<td>0.52 ± 0.02</td>
<td>0.55 ± 0.02</td>
<td>0.58 ± 0.02</td>
<td>0.57 ± 0.01</td>
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<tr>
<td>No. of normal, %</td>
<td>71 (97)</td>
<td>58 (97)</td>
<td>175 (86)</td>
<td>177 (90)</td>
</tr>
<tr>
<td>No. of malformed, %</td>
<td>2 (3)</td>
<td>2 (3)</td>
<td>20 (10)a</td>
<td>9 (5)b</td>
</tr>
<tr>
<td>No. of female, %</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>9 (4)c</td>
<td>8 (8)</td>
</tr>
<tr>
<td>No. of male, %</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>2 (2)</td>
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<tr>
<td>No. of micro/agnathia, %</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>9 (100)</td>
<td>6 (60)</td>
</tr>
<tr>
<td>No. of cleft lip and palate, %</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>4 (40)</td>
</tr>
</tbody>
</table>

Data are means ± SE. N, nondiabetic; MD, manifestly diabetic; WL, offspring of W female and L male; LW, offspring of L female and W male. ANOVA, Fisher’s exact test, and χ²-statistics: aP < 0.05 vs. N-WLWL; bP < 0.05 vs. N-LWLW; cP < 0.05 vs. MD-WLWL; and dP < 0.05 vs. females of same experimental group.

RESULTS

Fetal outcome. There was no difference in the number of implantations between any of the groups (Table 2). Fetal weight was decreased by maternal diabetes in both MD-WLWL and MD-LWLW litters compared with their respective nondiabetic control group. Female MD-WLWL offspring weighed less than corresponding male offspring, whereas female MD-LWLW offspring only tended to be smaller (0.05 < P < 0.10) than corresponding male offspring. No effect of strain, maternal diabetes, or fetal gender was found regarding placental weight (Table 2).

MD-WLWL offspring had a higher resorption rate (10%) compared with N-WLWL offspring (3%). MD-LWLW offspring showed no differences in resorption rate compared with N-LWLW offspring, 5 and 3% respectively. There was also no difference in resorptions rate between N-WLWL and N-LWLW. MD-WLWL offspring had thus higher resorption rate compared with MD-LWLW offspring. Of the MD litters examined, 6 of 18 MD-WLWL litters and 5 of 16 MD-LWLW litters presented with malformed fetuses.

MD-WLWL had increased malformation frequency (4%) compared with N-WLWL (0%). MD-LWLW had increased malformation rate (5%) compared with N-LWLW (0%). Furthermore, malformed MD-WLWL offspring presented with 100% agnathia/micrognathia, whereas malformed MD-LWLW offspring had agnathia/micrognathia and cleft lip and palate with a frequency of 60 and 40%, respectively (P = 0.05). Malformation types are displayed in Fig. 1B. Maternal metabolic state. The maternal serum concentration at GD 10 of glucose, fructosamine, cholesterol, triglycerides, and β-hydroxybutyrate in nondiabetic and manifestly diabetic WL and LW rats is depicted in Table 3. MD increased maternal serum glucose and fructosamine levels in both MD-WL and MD-LW rats. N- and MD-WL rats had lower serum cholesterol than N- and MD-LW rats, respectively. No differences were
found regarding serum levels of triglycerides. Furthermore MD-WL and MD-LW rats had higher serum β-hydroxybutyrate levels than their respective nondiabetic control, whereas MD-WL rats had higher serum β-hydroxybutyrate compared with MD-LW rats.

Serum concentrations of amino acids are depicted in Fig. 2. MD-WL compared with N-WL rats (Fig. 2A) had increased levels of threonine, asparagine, proline, alanine, citrulline, valine, isoleucine, leucine, and tyrosine and a decrease in the levels of glutamic acid and lysine. MD-LW compared with N-LW rats (Fig. 2B) had increased levels of asparagine, glutamic acid, glutamine, proline, alanine, valine, isoleucine, leucine, tyrosine, phenylalanine and arginine and decreased levels of ornithine and lysine. In addition, MD-WL dams (Fig. 2C) had increased levels of citrulline, cystine, leucine and tryptophan as well as decreased levels of taurine, asparagine, glutamic acid and tyrosine compared with MD-LW dams.

8-Isoprostane. Serum levels of 8-isoprostane (Fig. 3A) were increased by maternal diabetes in both MD-WL and MD-LW rats compared with their respective nondiabetic control group. No differences in serum 8-isoprostane levels were found between the strains or by comparing manifestly diabetic rats that gave birth to nonmalformed or malformed litters (Fig. 3C).

Hepatic concentrations of 8-isoprostane are shown in Fig. 3, B and D. MD-WLWL and MD-LWLW offspring had higher levels of 8-isoprostane compared with N-WLWL and N-LWLW offspring, respectively (Fig. 3B). No difference in hepatic 8-isoprostane levels was found between N-WLWL and N-LWLW offspring or between MD-WLWL and MD-LWLW. Furthermore, no differences were found between nonmalformed and malformed offspring of manifestly diabetic rats, although a numerical difference (P = 0.11) was observed between nonmalformed and malformed MD-LWLW offspring (Fig. 3D).

AR activity and gene expression. There was no effect of maternal diabetes or parental strain on cardiac or mandibular AR activity in WLWL and LWLW offspring (Fig. 4A and B). However, cardiac AR gene expression tended to be decreased by maternal diabetes in MD-WLWL and MD-LWLW offspring (0.05 < P < 0.10) compared with nondiabetic offspring (Fig. 4C). Furthermore, malformed MD-WLWL offspring had a decreased mandibular gene expression of AR compared with nonmalformed MD-WLWL offspring (Fig. 4D).

Gapdh activity and gene expression. Maternal diabetes had no effect on cardiac Gapdh activity in WLWL and LWLW offspring (Fig. 5A). However, cardiac Gapdh gene expression was decreased in both MD-WLWL and MD-LWLW offspring compared with N-WLWL and N-LWLW, respectively (Fig. 5C). There were no differences in cardiac Gapdh activity or gene expression between nonmalformed and malformed offspring of MD-WLWL and MD-LWLW, respectively (Fig. 5, B and D).

Mandibular Gapdh activity was higher in N-WLWL compared with MD-LWLW offspring (Fig. 5A). Furthermore, mandibular Gapdh activity was increased in MD-WLWL offspring in relation to MD-LWLW fetuses (Fig. 5A). Malformed MD-WLWL offspring had decreased mandibular activity and gene expression of Gapdh compared with nonmalformed MD-WLWL offspring (Fig. 5B and D).

ROS scavengers. Maternal diabetes decreased cardiac gene expression of copper zinc superoxide dismutase (CuZn-SOD), manganese superoxide dismutase (MnSOD), extracellular superoxide dismutase (EC-SOD), and catalase in both MD-WLWL and MD-LWLW fetuses (Fig. 6, A, B, C, and D). Furthermore, cardiac gene expression of glutathione peroxidase-1 (Gpx1) was decreased in MD-WLWL fetuses compared with N-WLWL (Fig. 6E). Cardiac Gpx2 gene expression was increased in MD-LWLW fetuses compared with N-WLWL and MD-WLWL (Fig. 6F). Maternal diabetes decreased mandibular gene expression of EC-SOD and Gpx1 in MD-WLWL offspring but not in MD-LWLW offspring (Fig. 6, C and E). Mandibular Gpx2 gene expression was decreased in both MD-WLWL and MD-LWLW offspring (Fig. 6F). Furthermore, maternal gene expression of CuZnSOD, MnSOD, EC-SOD, and Gpx1 was lower in MD-WLWL compared with MD-LWLW fetuses (Fig. 6, A, B, C, and E). When comparing gene expression of scavengers between nonmalformed and malformed fetuses of the same MD group, we found that the MD-LWLW fetuses displayed a decreased cardiac and mandibular Gpx2 gene expression compared with nonmalformed MD-LWLW fetuses (data not shown).

Developmental genes. Maternal diabetes decreased cardiac gene expression of Ret proto-oncogene (Ret) in both MD-WLWL and MD-LWLW offspring (Fig. 6G). Cardiac bone morphogenetic protein 4 (Bmp4) expression was unchanged by diabetes in MD-WLWL offspring, whereas it was increased in MD-LWLW (Fig. 6H). Cardiac Bmp4 expression was lower in MD-LWLW compared with MD-LWLW fetuses with nonmalformed MD-LWLW (Fig. 6J).

Mandibular Ret gene expression was lower in both N-WLWL and MD-WLWL offspring compared with N-LWLW and MD-LWLW offspring, respectively (Fig. 6G). Maternal diabetes decreased mandibular gene expression of glial cell line-derived neurotrophic factor in both MD-WLWL and MD-LWLW fetuses but increased mandibular Sonic hedgehog homolog (Shh) expression only in MD-LWLW (Fig. 6, J and K). Mandibular Shh expression was lower in both MD-WLWL compared with MD-LWLW fetuses.

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Table 3. Serum concentration of glucose and lipid compounds at gestational day 10 in N and MD WL and LW rats

<table>
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<th>N-LW</th>
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<th>MD-LW</th>
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<tr>
<td>Glucose (mmol/l)</td>
<td>7.8 ± 0.2</td>
<td>7.2 ± 0.3</td>
<td>36 ± 1a</td>
<td>34 ± 1b</td>
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<tr>
<td>Fructosamine (μmol/l)</td>
<td>209 ± 1</td>
<td>214 ± 3</td>
<td>301 ± 3a</td>
<td>294 ± 3b</td>
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<tr>
<td>Cholesterol (mmol/l)</td>
<td>1.7 ± 0.03</td>
<td>2.0 ± 0.1a</td>
<td>1.8 ± 0.02</td>
<td>1.9 ± 0.04c</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.1 ± 0.1</td>
<td>1.2 ± 0.1</td>
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</tr>
<tr>
<td>β-Hydroxybutyrate (mmol/l)</td>
<td>0.07 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.20 ± 0.01a</td>
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</tbody>
</table>

Data are means ± SE. ANOVA: *P < 0.05 vs. N-WL; **P < 0.05 vs N-LW; and ***P < 0.05 vs. MD-WL.
Mandibular gene expression of Bmp4 and Shh was decreased in malformed MD-WLWL fetuses, although there was a tendency (0.05 < P < 0.10) of decreased mandibular Shh expression in MD-LWLW (Fig. 6, I and L). Finally, mandibular Ret expression was higher in malformed MD-LWLW fetuses compared with nonmalformed MD-LWLW (data not shown).

**DISCUSSION**

The most intriguing result in the present study was the marked variations in fetal dysmorphology between the WLWL and LWLW offspring in diabetic pregnancy. The question why two similar (almost identical) fetal genotypes exposed to two similar (almost identical) maternal environments gives rise to
nonidentical fetal phenotypes is fundamental in the following discussion.

Maternal factors. One important aspect of the teratogenic mechanism would be the influence of maternal factors, i.e., the maternal genome and metabolic state, on the dysmorphogenic process. In the present study, the WL and LW dams differ only in the origin of mitochondria and possible autosomal imprinting. The mitochondria are of the W and L types in WLWL and LWLW offspring, respectively. In previous studies, the W and L mitochondrial genomes have been found to contain several sites of polymorphism. However, no clustering of fetal dysmorphogenesis was found to be associated with W or L mitochondrial identity (53). In the present study, W and L specific genomic imprinting may also be important, although we have no clear appreciation of the methylation pattern in the WLWL and LWLW offspring.

The maternal metabolic state is different between WL and LW dams. Interestingly, the major markers of severity of diabetic state, serum glucose, fructosamine, cholesterol, triglycerides, and two of the branched-chain amino acids (valine and isoleucine), are remarkably similar in the pregnant MD-WL and MD-LW rats. The identifiable differences between the pregnant MD-WL and MD-LW rats are the serum concentrations of β-hydroxybutyrate (both increased compared with N, but MD-WL > MD-LW) and increased serum leucine, the third branched-chain amino acid, as well as different levels of taurine in serum. In previous experimental work, β-hydroxybutyrate has been identified as a potent teratogenic agent in diabetic pregnancy (6, 37, 62). Further differences were observed with regard to amino acid levels, where the MD-WL dams had lower serum taurine, asparagine, glutamic acid, and tyrosine as well as higher citrulline, cystine, leucine, and tryptophan compared with MD-LW dams. The teratogenic association with increased branched-chain amino acids has been shown earlier (66, 75). Altered levels of amino acids have been associated with teratogenic effects in chicken such as tryptophan (49) and leucine (4). Ketoisocaproate, an intermediate metabolite of leucine, was teratogenic in rat embryo culture (22), inducing increased mitochondrial ROS production. Taurine, which was decreased in MD-WL dams compared with MD-LW pregnant rats, is an aminosulfonic acid not incorporated into proteins. In diabetic pregnant rats, taurine is decreased in both maternal and fetal serum (2). Some known beneficial effects of taurine in diabetes are decreased oxidative stress (38) and decreased vascular dysfunction (38, 48). Furthermore, taurine supplementation to low-protein-fed rats restored insulin secretion in fetal endocrine pancreas (13). Taurine has also been shown to facilitate in vitro preimplantation development in mice (17). However, there is little known of the effects of taurine supplementation in diabetic embryopathy. In one study of hyperglycemic mice, taurine supplementation decreased the NTD rate (46). The metabolic differences found in the present study are thus likely to be involved in the different teratogenic processes in MD-WLWL and MD-LWLW offspring.

Embryonic factors. Another important aspect of diabetic teratogenicity would be the effect of embryonic factors, mainly the embryonic genetic setup. The embryonic genotype differs interindividually in the WLWL and LWLW offspring because of stochastic division and distribution of W and L autosomes in F1 germ line meiosis. This genomic diversity in the offspring renders embryos with a range of 0–100% W and L genome,
whereas the mean distribution over several litters reaches a
distribution of 50% W and 50% L genome. This hereditary
equilibrium would in theory lead to similar fetal outcome in the
two F2 generations; however; the higher resorption rate in
MD-WLWL and the different craniofacial malformations in
the LWLW offspring need further consideration. As with WL
and LW dams, the WLWL and LWLW offspring differ with
regard to their mitochondrial heredity and genomic imprinting,
which are W and L strain specific in WLWL and LWLW
fetuses, respectively. Furthermore, the W and L origin of X and
Y sex chromosomes is different between WLWL and LWLW
offspring, which may be of importance since there is a pre-
ponderance of female gender among the malformed offspring
in both MD-WLWL and MD-LWLW. This may implicate the
X chromosome(s) in the teratological, and also developmental,
processes, and a search for genes enabling (diabetic) embry-
opathy should be considered. In one study, pregnant women
with preexisting diabetes were reported to be more likely to
give birth to a malformed male infant than a female infant with
an odds ratio of 3.5 (28), although other recent studies showed
no gender differences (18). As discussed above, different
diabetes in diabetic mice have shown a decrease in gene expression of several embryonic chromatin-
modifying factors involved in transcriptional regulation as well
as increased variability in gene expression in diabetes-exposed
embryos, suggesting a disturbed epigenetic signal for gene
expression in diabetic embryopathy (61). In the present study,
the weight of evidence supports an influence of the embryonic
genetic setup on the teratogenic outcome in diabetic preg-
nancy.

Maternal and embryonic interactions. The malformation
rate was 4 and 5% in MD-WLWL and MD-LWLW offspring.
In earlier work, MD-WL and MD-LW offspring had 0 and 9%
malformations, whereas parental MD-WW and MD-LL dia-
betic pregnancies presented with 0 and 17% malformation rates
(19). The protection from diabetes-induced malformations in
MD-WL offspring (compared with MD-LW offspring) was
hypothesized to be caused mainly by maternal protective fac-
tors, i.e., the W uterine milieu. In contrast, the increase of
malformation rate from 0% in MD-WL offspring to 4% in
MD-WLWL offspring indicates a loss of protective W mater-
nal factors, since the maternal genome goes from 100% W to
50% W. On the other hand, the reduction of malformation rate
from MD-LL fetuses (17%) to MD-LW fetuses (9%) may be
viewed as the result of a gene dilution effect in the offspring.
i.e., less L genome in the embryo, exposed to the same intrauterine milieu (100% L). The noted decrease of malformation rate from 9% in MD-LW fetuses to 5% in MD-LWLW fetuses further elucidates the importance of protective W genome in the mother, in this case L vs. LW maternal genome. A similar effect of inductive and protective genomes on the outcome of diabetic pregnancy was previously noted in a study of outbred diabetic rats (21).

The occurrence of cleft-lip palate (CLP) in MD-LWLW offspring, a malformation not seen before in W- or L-derived diabetic pregnancies, needs further consideration. In humans, the incidence of CLP is diverse among different ethnic groups, and sex differences are also evident regarding the different forms of CLP (15). Beside embryonic genetic predisposition, maternal (39) and environmental (7, 35) factors have been implicated as a causative mechanism for CLP. Pregestational diabetes or obesity increases the risk for development of CLP in the human fetus (1, 11, 64). Embryonic genetic disturbances also imply several mechanisms for CLP. Indeed, Bmp4 mutations as well as disturbed expression patterns of Bmp4 and Shh (68, 69) have been shown to be involved in the pathogenesis of CLP. Tp63 knockout embryos with CLP (69) present with increased Bmp4 and decreased Shh expression in the facial processes concordant with the findings of increased Bmp4 and decreased Shh gene expression in MD-LWLW offspring in this present study. Experiments with different lines of inbred F1 hybrids from C57Bl/6J and A/J mice show that susceptibility genes for teratogen-induced orofacial clefts is resident on both C57Bl/6J and A/J genome, although only the A/J strain exhibits the induced malformations (20). The same association for susceptibility regions on both resistant (W) and susceptible (L) rat genome was noted in earlier work (53). Genotypic alterations of the two genes clf1 and clf2 in the A/WySn mice are both required for spontaneous CLP development, but maternal factors are also strongly influencing the penetrance (40). Wnt9b gene modification has been shown to be the cause to the clf1 mutation (41, 57).

A previous embryo transfer study (52) of CLP-susceptible CL/Fr strain embryos transferred to CL/Fr or C57BL dams showed that the embryonic genotype was important for CLP malformations to occur but also that the uterine environment in CL/Fr dams had an important role for the frequency of CLP and fetal outcome. In our previous work with WL and LW F1 offspring, a combination of embryonic susceptibility and maternal milieu appeared as the most plausible explanation for differences in fetal outcome (19), a notion applicable also on the results in the present study.

In conclusion, as a general assessment, the differences in maternal milieu are not solely responsible for the differences in fetal outcome between the WL and LW pregnancies. Differences in fetal genetic setup are likely to be of importance, although the precise influence cannot be ascertained at present. The possibility, therefore, that the fetal genetic setup enables the malformations to occur, given a maternal diabetic state of a certain severity, appears reasonable and should be further scrutinized in the future.
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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: A.E., P.W., and U.E. conception and design of research; A.E. performed experiments; A.E. and U.E. analyzed data; A.E., P.W., and U.E. interpreted results of experiments; A.E. prepared figures; A.E.

Fig. 6. Gene expression of reactive oxygen species (ROS) scavenging enzymes (A–F) and developmental genes (G–L) in heart and mandible at gestational day 20 in WLWL and LWLW fetuses from N (open bars) and MD (filled bars) WL and LW rats. CuZnSOD, copper zinc superoxide dismutase; MnSOD, manganese superoxide dismutase; ECSOD, extracellular superoxide dismutase; Gpx-1 and 2, glutathione peroxidase-1 and -2, respectively; Ret, Ret proto-oncogene; Bmp4, bone morphogenetic protein 4; Gdnf, glial cell line-derived neurotrophic factor; Shh, sonic hedgehog homolog. Transformed means ± SE (ANOVA and Student’s t-test). Significance: *P < 0.05 vs. N; #P < 0.05 vs. WLWL (A–C, E–H, and K) and ‡P < 0.05, MD fetuses with no malformations vs. MD fetuses with malformations of the same strain (I and L).
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