Objective—To characterize normal and malformed embryos within the same litters from control and diabetic rats for expression of genes related to metabolism of reactive oxygen species (ROS) or glucose as well as developmental genes.

Research design and methods—Embryos from nondiabetic and streptozotocin-induced diabetic rats were collected on gestational day 11 and evaluated for gene expression (PCR) and distribution of activated caspase-3 and glutathione peroxidase (Gpx)-1 by immunohistochemistry.

Results—Maternal diabetes (MD group) caused growth retardation and an increased malformation rate in the embryos of MD group rats compared with those of controls (N group). We found decreased gene expression of Gpx-1 and increased expression of vascular endothelial growth factor-A (Vegf-A) in malformed embryos of diabetic rats (MDm group) compared with nonmalformed littersmates (MDn group). Alterations of messenger RNA levels of other genes were similar in MDm and MDn embryos. Thus, expression of copper zinc superoxide dismutase (CuZnSOD), manganese superoxide dismutase (MnSOD), and sonic hedgehog homolog (Shh) were decreased, and bone morphogenetic protein-4 (Bmp-4) was increased, in the MD embryos compared with the N embryos. In MDm embryos, we detected increased activated caspase-3 immunostaining in the first visceral arch and cardiac area and decreased Gpx-1 immunostaining in the cardiac tissue; both findings differed from the caspase/Gpx-1 immunostaining of normal embryos.

Conclusions—Maternal diabetes causes growth retardation, congenital malformations, and decreased general antioxidative gene expression in the embryo. In particular, enhanced apoptosis of the first visceral arch and heart, together with decreased cardiac Gpx-1 levels, may compromise the mandible and heart and thus cause an increased risk of developing congenital malformation. Diabetes 57:3344–3352, 2008
ROS-induced DNA damage (29) may directly disrupt development via altered expression of key genes. In addition, cellular DNA repair processes may activate poly(ADP-ribose) polymerase (PARP), which may cause glyceraldehyde-3-phosphate dehydrogenase (GAPDH) inhibition by poly(ADP-ribosylation) (32). The net result would be diminished embryonic GAPDH activity, which has been demonstrated in rat embryos subjected to diabetes in vivo and high glucose in vitro (33). Furthermore, decreased glycolytic flux proximal to GAPDH (32) and the presence of increased ambient glucose levels will yield enhanced flux in the polyol (34,35) and hexosamine (36) pathways. An increased availability of proximal glycolytic intermediaries would increase diacylglycerol production and cause activation of several protein kinase C isozymes (37,38), as well as enhance the flux in the advanced glycosylation end product pathway (39). All of the consequences of inhibited GAPDH activity may thus contribute to the teratogenic outcome of diabetic pregnancy. Evidently, there are multiple ways for a diabetes-induced state of oxidative stress in the embryo to disturb embryonic/fetal development, several of which enjoy considerable experimental support.

Another consequence of a state of oxidative stress would be enhanced apoptosis in embryonic/fetal tissue (40), which has been described (26,41–43) and has been suggested to be mediated by enhanced Jun-amino-terminal (40), which has been described (26,41–43) and has been enhanced apoptosis in embryonic/fetal tissue (40), which has been described (26,41–43) and has been suggested to be mediated by enhanced Jun-amino-terminal kinase-1 and -2 activity (19,44). It has also been suggested that maternal diabetes induces an inflammatory state in the embryo, where proinflammatory cytokines (i.e., tumor necrosis factor-α [TNF-α] [45,46]) act to downregulate the principal ROS-scavenging enzymes via increased activity of mitogen-activated protein kinases (47). The exact relation between enhanced apoptosis and induction of malformations is still unclear, mainly since we do not fully comprehend the specific transmission of a general increase in programmed cell death into precisely restricted developmental damage to embryonic organs or organ systems (42).

Based on earlier studies (7,48,49) and on a question that has been raised several times—which genes are involved in diabetes-induced embryonic dysmorphogenesis? (5,8,26,42,50)—we wanted to add to the teratological knowledge by identifying differences in gene expression between the malformed and nonmalformed offspring within the same litters of diabetic animals.

Our working hypothesis was that genes of the malformed offspring with an expression pattern different from that of genes in the nonmalformed offspring within the same litter may be associated, either directly or indirectly, with the teratogenic process. We thus decided to compare gene expression of embryos that were morphologically normal and those that were malformed in litters of normal and diabetic rats. By comparing gene expression and protein distribution in embryos of the same age and with exposure to an identical intrauterine milieu, we aimed to control for possible confounding factors of the teratogenic process. From earlier work, we knew that the two tissues of the rat fetuses that are particularly vulnerable to the diabetic state are the mandible and the heart (17) (Fig. 1). We also knew that a susceptible period in diabetic rat pregnancy occurs during gestational days 6–10 (51), which corresponds to weeks 2–4 in human pregnancy. Bearing in mind that the size and relative immaturity of day-10 embryos makes them difficult to evaluate for developmental defects, we chose to interrupt the rat pregnancies on gestational day 11 in order to be able to clearly distinguish between malformed and non malformed embryos. We decided to relate embryonic (mal)development to expression of the major oxidative defense genes (SODs, glutathione peroxidase [Gpx]-1, Gpx-2, and catalase), to key genes of glucose metabolism (aldose reductase [AR] and GAPDH), to developmental/teratological genes [poly(ADP-ribose) polymerase (PARP)], to tumor protein 53 (p53), to bone morphogenetic protein-4 (Bmp-4), to Ret proto-oncogene (Ret), to sonic hedgehog homolog (Shh), to vascular endothelial growth factor-A (VEGF-A), to TNF-α, to interleukin-6 (IL-6), and to the tissue distribution of activated caspase-3 (denoting apoptotic rate) and Gpx-1.

**FIG. 1.** A: Fetuses displaying micrognathia (*left fetus*) and normal morphology (*right fetus*). B: Outcome of pregnancy in the control (N) and manifestly diabetic (MD) groups, distributed as normal ( ), malformed ( ), and resorbed ( ) embryos on gestational day 11. *P < 0.05 vs. N (χ² statistics). (Please see http://dx.doi.org/10.2337/db08-0630 for a high-quality digital representation of this figure.)

**RESEARCH DESIGN AND METHODS**

Nondiabetic and diabetic rats from a local Sprague-Dawley strain (bred in the animal facility of the Biomedical Center of Uppsala University) with an increased incidence of congenital malformations in diabetic pregnancy were used as embryo donors (12). The rats had free access to food (AB Analyce, Lidköping, Sweden) and tap water and were maintained at an ambient temperature of 22°C with a 12-h light/dark cycle.

Diabetes was induced with a single injection of 40 mg/kg streptozotocin (STZ) (Sigma-Aldrich Chemie, Steinheim, Germany) into a tail vein (12). Within 1 week after STZ injection, blood glucose concentration was measured with a Free Style Mini Glucose Meter (Thera Sense, Alameda, CA). Rats with a glucose concentration >20 mmol/L were considered to be manifestly diabetic (MD group), and female rats without STZ-induced diabetes were denoted as...
TABLE 1

| Gene       | Forward primer                                   | Reverse primer                                   |
|------------|--------------------------------------------------|--------------------------------------------------|
| G6PDH      | gTCATgCAggACCCAgCCCTCT                          | ACATACgTCgCCAggACCCAgCCCTCT                    |
| CuZnSOD    | AAgCggTgACCCAgTTgTg                            | CAgAggTgCCAggACCCAgCCCTCT                       |
| MnSOD      | gTgATgAggACCCAgCCCTCT                          | CAggATgAggACCCAgCCCTCT                          |
| Gpx-1      | gTgCggTgACCCAgCCCTCT                          | gTgCggTgACCCAgCCCTCT                          |
| Gpx-2      | gTgAGAAttgAggAGCAAggACCCAggACCCAgCCCTCT         | gTgAGAAttgAggAGCAAggACCCAggACCCAgCCCTCT         |
| Catalase   | gTCCTACAgCTTAggACCAACgTCCTTAggACCA              | gTCCTACAgCTTAggACCAACgTCCTTAggACCA              |
| Aldose reductase | gTCCTACAgCTTAggACCAACgTCCTTAggACCA   | gTCCTACAgCTTAggACCAACgTCCTTAggACCA   |
| GAPDH      | gTgAGAAttgAggAGCAAggACCCAggACCCAgCCCTCT         | gTgAGAAttgAggAGCAAggACCCAggACCCAgCCCTCT         |
| PARP       | gTCCTACAgCTTAggACCAACgTCCTTAggACCA              | gTCCTACAgCTTAggACCAACgTCCTTAggACCA              |
| p53        | gTCCTACAgCTTAggACCAACgTCCTTAggACCA              | gTCCTACAgCTTAggACCAACgTCCTTAggACCA              |
| Shh        | gTCCTACAgCTTAggACCAACgTCCTTAggACCA              | gTCCTACAgCTTAggACCAACgTCCTTAggACCA              |
| Ret        | gTCCTACAgCTTAggACCAACgTCCTTAggACCA              | gTCCTACAgCTTAggACCAACgTCCTTAggACCA              |
| Bmp-4      | gTCCTACAgCTTAggACCAACgTCCTTAggACCA              | gTCCTACAgCTTAggACCAACgTCCTTAggACCA              |
| VEGF-A     | gTCCTACAgCTTAggACCAACgTCCTTAggACCA              | gTCCTACAgCTTAggACCAACgTCCTTAggACCA              |
| TNF-α      | gTCCTACAgCTTAggACCAACgTCCTTAggACCA              | gTCCTACAgCTTAggACCAACgTCCTTAggACCA              |
| IL-6       | gTCCTACAgCTTAggACCAACgTCCTTAggACCA              | gTCCTACAgCTTAggACCAACgTCCTTAggACCA              |

nondiabetic controls (N group). After establishing diabetes status, the MD and N rats were mated overnight with nondiabetic male rats. A positive vaginal smear (containing sperm) the following morning designated gestational day 0. On gestational day 11, the MD and N rats were killed by cervical dislocation after light ether anesthesia. This gestational day was chosen because it corresponds with an early stage of embryogenesis during which malformations of the heart, neural tube, and general rotation pattern can be identified and, consequently, their severity can be assessed. The embryos were disected and evaluated with regard to presence of malformations (cardiac hypertrophy, neural tube defects, and somatic malrotation), crown rump length, and somite number. Viability of the embryos was confirmed by the presence of a heartbeat. Embryos were prepared for immunostaining (four N and four MD litters) and gene expression determination (four N and five MD litters).

Preparation of total RNA. Total RNA from each embryo was isolated with an RNAeasy mini kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. Briefly, each embryo was lysed in 350 μl buffer. The samples were DNase treated (RNase-free DNase; Qiagen) and incubated at room temperature for 25 min. The isolated total RNA was washed twice with 50 μl RNase-free water, and the accumulated flow-through was collected, yielding the total RNA sample. Lastly, 1 μl of an RNase inhibitor (RNA Guard; Amersham Pharmacia, Uppsala, Sweden) was added to each sample.

Preparation of complementary DNA. One microgram of total RNA was amplified for complementary DNA (cDNA) preparation. For cDNA synthesis, we used first-strand beads (Ready To Go; Amersham Pharmacia Biotech), according to the manufacturer’s instructions. The resulting cDNA was diluted threefold with RNAse-free water.

Analysis of messenger RNA levels. Four litters with 32 embryos yielded the messenger RNA (mRNA) samples of the N group, whereas five litters with 35 embryos produced the mRNA samples of the MD group. One microliter of cDNA purified from each embryo with 10 ng of converted total RNA was amplified and measured in duplicate with real-time PCR using the MiniOpticon system (Bio-Rad Laboratories, Hercules, CA), with SYBR green used to detect the PCR product. Specific primers for CuZnSOD, MnSOD, Gpx-1, Gpx-2, catalase, AR, G6PDH, p53, PARP, Shh, Ret, Bmp-4, VEGF-A, TNF-α, and IL-6 were constructed with the aid of the Primer3 free software (available at http://primer3.sourceforge.net/) and subsequently purchased from TIB Molbiol (Berlin, Germany) (Table 1). We have previously assessed the stability of expression of various housekeeping genes and found the glucose-6-phosphate dehydrogenase (G6PDH) gene to be constant in day-10 and day-11 embryos compared with the difference between sample and reference crossing point (Cp) values. The relative abundance of mRNA/G6PDH in each sample was determined using the following equation to yield the ratio sample to G6PDH:

\[ \frac{2^{(-\Delta \Delta Ct)}}}{C_{p}} \]

TABLE 2

| n | Crown rump length (mm) | Somites |
|---|-----------------------|---------|
| N group (normal) | 66 | 3.85 ± 0.03 | 29.8 ± 0.2 |
| N group (malformed) | 1 | 1.50 | 17.0 |
| MD group (normal) | 36 | 2.95 ± 0.07* | 25.7 ± 0.3* |
| MD group (malformed) | 27 | 2.20 ± 0.11** | 15.8 ± 1.2** |

RESULTS

Maternal diabetes increased malformation and resorption rates in embryos of diabetic pregnancy compared with embryos of normal pregnancy (Fig. 1). We also found decreased crown rump length as well as diminished somite number in embryos of control (N group) and manifestly diabetic (MD group) rats.
somite number in embryos from diabetic rats compared with embryos from nondiabetic rats (Table 2).

The expression patterns of the antioxidative enzymes (AOEs) are shown in Fig. 2. We found decreased gene expression of CuZnSOD, MnSOD, and Gpx-1 in MD embryos (both nonmalformed embryos of STZ-induced diabetic rats [MDn group] and malformed littermates [MDm group]) compared with N embryos. In addition, Gpx-1 mRNA levels were more decreased in MDm than in MDn embryos, and Gpx-2 and catalase expression did not differ between the groups (Fig. 2). No significant differences were found with regard to gene expression of AR, GAPDH, p53, and PARP (Fig. 3).

Shh was decreased, Ret was unaltered, and Bmp-4 mRNA levels were increased in MD embryos compared with N embryos (Fig. 4). VEGF-A mRNA levels were increased in both the MDn and MDm embryos compared with N embryos, and, in addition, the MDm embryos had higher mRNA levels than the MDn embryos (Fig. 4). The TNF-α mRNA levels were decreased in the MD embryos (Fig. 4), whereas we could not demonstrate IL-6 mRNA in the embryos (data not shown).

We detected increased activated caspase-3 immunostaining in the first visceral arch and cardiac area in the MDm embryos compared with the MDn and N embryos (Fig. 5). The immunostaining of Gpx-1 displayed a general accumulation of positive cells in the cardiac tissue of all embryos; however, the MDn embryos had less staining than the N embryos, and the MDm embryos had less staining than the MDn embryos (Fig. 6).

DISCUSSION

One important finding in the present study was that maternal diabetes diminishes the antioxidative defense gene expression in the embryos, as evidenced by decreased gene expression of CuZnSOD, MnSOD, and Gpx-1 in MDn and MDm offspring, which may be responsible for a decrease in AOE activity (52). This observation is in concert with previous reports (7–9,48) of diminished AOE activity in embryonic tissues exposed to diabetes; however, it is not in concert with all previous reports (2,7,9), which may be related to differences in mode and time of exposure to the diabetes milieu. In addition, in the present study we detected decreased Gpx-1 mRNA levels in MDm embryos and a corresponding reduction of Gpx-1 protein in the cardiac tissues of the MDm embryos, both findings differing from MDn embryos of the same litters, as well as from Gpx-1 estimations in N embryos. In a similar rat model, Gpx activity was found decreased in embryos with
neural tube defects compared with nonmalformed embryos from the same litters of diabetic rats (7).

The specific decrease in Gpx-1 expression/occurrence in MDm embryos may suggest an association between the abundance of this isoform of glutathione peroxidase and the teratogenicity of diabetes, tentatively, with the processes behind cardiac malformations. Such an association has not been suggested before, to our knowledge, and the localization of Gpx-1 protein in the cardiac wall on day 11 may suggest the occurrence of a protective antioxidative mechanism. The net effect of the Gpx-1 deficiency (further enhanced by the decreased levels of the other AOE)s would be enhanced cellular damage, as suggested by mice with a targeted Gpx-1 mutation; they display mitochondrial dysfunction (53) and apoptosis (54)—changes that are in line with a teratological disturbance of normal development. Evidently, however, the association between malformation and reduced Gpx-1 expression may also be the opposite; the embryonic maldevelopment may actually induce the alteration in Gpx-1 mRNA levels and, putatively, decreased activity of the enzyme (7).

In previous work, we have only been able to secure suggestive evidence of enhanced regional apoptotic activity in embryos subjected to a diabetic environment. We thus regard the results of the immunohistochemistry efforts of the present study with great interest. We thus found increased caspase-3 immunostaining, in the first visceral arch and in cardiac tissue of the MDm embryos compared with that of MDn and N embryos, indicative of enhanced apoptosis in the two regions corresponding to the two major types of diabetes-induced malformations in this rat model, micrognathia and cardiac outflow defects (Figure 1).

We found only one malformed N embryo, which displayed gene expression patterns similar to the MDm embryos, further supporting the view that generalized changes of the gene clusters pave the way for specific morphogenetic alterations. In particular, the SODs, Gpx-1, and Shh genes decreased and VEGF increased in the Nm embryo, thus indicating a coupling to oxidative stress and neural crest cell development. However, the small sample size (n = 1) precludes any far-reaching conclusions.

The effect on AOE gene expression was strikingly similar in CuZnSOD, MnSOD, and Gpx-1, which were all decreased. The finding of these diabetes-related genes suggests, in concert with previous studies (4,5,7,9), that maternal diabetes causes a general downregulation of the principal AOE)s in rat embryos prone to develop congenital malformations and allows the speculation that this putative weakening of the antioxidative defense may be involved in the teratogenic process. The physiological reasons for diminished ROS-scavenging gene expression in the diabetes-exposed embryos are not clear and may, therefore, currently be subject to speculation only. It is noteworthy that in the present study, we found decreased TNF-α mRNA levels and could not detect IL-6 in the embryos; therefore, a “generalized inflammation” causing the diminished AOE gene expression does not seem to be likely, at least not at this embryonic stage (45,46).

The metabolic state of the severely STZ-induced diabetic non–insulin-treated rat has been characterized previously by us (10,25). In general, the diabetic state yields blood glucose levels in the range of 20–25 mmol/l, as well as increased concentrations of ketone bodies (10) and increased levels of branched chain amino acids (25). The particular characteristics of the diabetic state, which may be primarily affecting gene expression, are not clear, but increased glucose levels should be of importance because in vitro exposure of embryos to high glucose levels promotes gene expression changes analogous to those found in vivo. Furthermore, existence of a functioning glucose transporter gene has been shown to be of fundamental importance for diabetes-induced embryonic (mal)development (27), illustrating the importance of high intracellular glucose levels for normal embryonic development.
levels of glucose in diabetic embryopathy. Regarding the genes controlling glucose metabolism, we and others have previously identified an association between increased sorbitol accumulation and dysmorphogenesis in the embryo (34,35). However, attempting to diminish such accumulation by using aldose reductase inhibitors did not prevent the maldevelopment in the offspring (34,35).

Another reason for the interest in the AR and GAPDH

![Graphs showing gene expression]

**FIG. 4.** Gene expression of Shh, Ret, Bmp-4, VEGF-A, and TNF-α on gestational day 11 in the control (N) and manifestly diabetic (MD) groups containing 29 and 32 embryos, respectively. Nonmalformed and malformed N and MD offspring are denoted Nn (28 embryos), MDn (20 embryos), Nm (1 embryo), and MDm (12 embryos), respectively. Data are means ± SE. *P < 0.05 vs. N and #P < 0.05 vs. MDn (ANOVA).

![Images showing activated caspase-3 protein]

**FIG. 5.** Distribution of activated caspase-3 protein in nonmalformed N embryo (A), nonmalformed MD embryo (B), and malformed MD embryo (C). The first visceral arch (square) and cardiac area (circle) are marked. Note differences in caspase-3 staining between the embryos. Space bar = 400 μm. (Please see http://dx.doi.org/10.2337/db08-0830 for a high-quality digital representation of this figure.)
genes is a recent linkage study of skeletal malformation of diabetic rats, in which we found linkage to the chromosomal regions harboring these two genes (U.J.E., N. Nordquist, H. Luthman, U. Pettersson, unpublished data). In the present study, however, we found no significant changes of mRNA levels of AR and GAPDH in the MD embryos, as noted previously with regard to the GAPDH expression (33), a difference we are unable to explain. The previously demonstrated diabetes-induced decrease in GAPDH enzyme activity (33) may thus be a ROS-mediated alteration of the GAPDH enzyme itself (either directly or via DNA damage-induced PARP activity) (32), thereby causing a block in the glycolytic pathway with a number of metabolic effects with teratological importance, such as diacylglycerol-mediated activation of (several isoforms of) protein kinase C (37,38), increased hexosamine flux (36), and enhanced production of glycosylation precursors/products (39).

Maternal diabetes did not alter embryonic p53 (as noted previously [43]) and PARP expression in the present study, despite a putative PARP activity increase due to the presence of DNA damage in the MD embryos (29). The increased Bmp-4 levels of the embryos of STZ-induced diabetic rats suggested specific affection of the precursors of the skeletal system, which backs earlier findings of delayed and defective chondrogenesis and osteogenesis in the offspring (55).

With regard to the diabetes-induced malformations of this rat model (Fig. 1), suggested to have a neural crest cell-derived origin (17), the two neural crest-associated genes, Shh and Ret, were expected to be affected. Whereas the Ret gene was unaltered by maternal diabetes, we found a diabetes-induced decrease in the Shh mRNA levels in the MD embryos. Despite the absence of a significant difference in Shh mRNA levels between the MDn and MDm embryos, the decreased Shh expression in the MD offspring is of particular interest in relation to the findings of embryonic craniofacial (56) and aortic (57) anomalies resulting from direct inhibition of Shh, as well as the findings of an early role for Shh in securing the neural crest cell survival in the early development of the lower jaw (58). Taken together, these results support a diabetes-induced disturbance in the neural crest cell development (17,59) of the embryos of the present model and, putatively, in human diabetic pregnancy as well. However, since isolated neural stem cells exposed to glucose in vitro show increased gene expression of both Shh and Bmp-4 (50), the exact relationship(s) between gene expression changes in the surrounding tissues and the neural crest cells themselves caused by exposure to a diabetic environment will have to be subject to further studies.

We found markedly increased VEGF-A expression in the MD embryos and further increased expression in the malformed embryos compared with the nonmalformed MD embryos. This is of particular interest with regard to the coupling between VEGF alterations and hampered embryonic development (60), although an increased VEGF expression has not been previously reported in association with embryonic dysmorphogenesis. We suggest that the enhanced embryonic VEGF-A levels in the present study may be interpreted as a compensatory change in response to a diabetes-induced general developmental delay. The demonstrated gene expression changes in malformed (and nonmalformed) embryos of diabetic rats will evidently demand future work efforts in order to clarify the association to embryonic maldevelopment (cause, effect, or no relation at all—efforts that, to some extent, are ongoing.

One major, and slightly surprising, observation was that there was no clear individual pattern of gene expression in the embryos for any of the genes studied (online appendix [available at http://dx.doi.org/10.2337/db08-0830]). Thus, when examining individual gene expression values, there was no evident relation to uterine position, growth, or morphological status (with the possible exceptions of Gpx-1 and Vegf-A). The Gpx-1 mRNA was markedly decreased and the VEGF-A mRNA was markedly increased in malformed compared with nonmalformed embryos from the same litter of diabetic rats. Further efforts
in the search for the etiology of diabetes-induced congenital malformations may therefore benefit from considering a putative impact of a decrease in embryonic Gpx-1 expression and, possibly, function as well as considering the importance of enhanced VEGF-A expression for embryonic development.

We conclude that maternal diabetes causes growth retardation, congenital malformations, and decreased general antioxidative gene expression in the embryo. In particular, enhanced apoptosis of the first visceral arch and heart, together with decreased cardiac Gpx-1 levels, may cause the mandible and heart to have an increased risk of developing congenital malformation.

ACKNOWLEDGMENTS

Financial support was obtained from the Ernfors Family Fund, the Swedish Diabetes Association, the Novo Nordisk Foundation, and the Swedish Research Council (grant no. 12X-7475).

The authors are indebted to Ing-Marie Mörssæ for valuable technical support.

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