Increased mRNA Levels of Mn-SOD and Catalase in Embryos of Diabetic Rats From a Malformation-Resistant Strain

Jonas Cederberg, Joakim Galli, Holger Luthman, and Ulf J. Eriksson

Previous studies have suggested that reactive oxygen species (ROS) are mediators in the teratogenic process of diabetic pregnancy. In an animal model for diabetic pregnancy, offspring of the H rat strain show minor dysmorphogenesis when the mother is diabetic, whereas the offspring of diabetic rats of a sister strain, U, display major morphologic malformations. Earlier studies have shown that embryonic catalase activity is higher in the H than in the U strain, and maternal diabetes increases this difference in activity. The aim of this study was to characterize the influence of genetic predisposition on diabetic embryopathy by comparing the mRNA levels of ROS-metabolizing enzymes in the two strains. We determined the mRNA levels of catalase, glutathione peroxidase, γ-glutamylcysteine-synthetase, glutathione reductase, and superoxide dismutase (CuZn-SOD and Mn-SOD) in day 11 embryos of normal and diabetic H and U rats using semiquantitative reverse transcription-polymerase chain reaction. The mRNA levels of catalase and Mn-SOD were increased in H embryos as a response to maternal diabetes, and no differences were found for the other genes. Sequence analysis of the catalase promoter indicated that the difference in mRNA levels may result from different regulation of transcription. Sequence analysis of the catalase cDNA revealed no differences between the two strains in the translated region, suggesting that the previously observed difference in the electrophoretic mobility in zymograms is due to posttranslational modifications. An impaired expression of scavenging enzymes in response to ROS excess can thus be an integral part of a genetic predisposition to embryonic dysmorphogenesis. Diabetes 49:101-107, 2000

Material type 1 diabetes during pregnancy has been known for many years to be associated with an increased risk for congenital malformations in the offspring (1-4). Clinical studies have estimated the risk for a malformed fetus in a type 1 diabetic pregnancy to be in the range of 5-10% (5-9). The malformations are induced before the 7th postconceptional week in human diabetic pregnancy (10). In rats, the teratogenic process is believed to occur during organogenesis (11). Recent reports, however, indicate that a diabetic environment may decrease the inner cell mass (12) and that a high glucose concentration can lead to increased apoptosis already in the preimplantation embryo (13). The teratogenic process and its predisposing factors are not known in detail. In pregnancies with poorly controlled diabetes, however, there is a correlation between the level of HbA1c in maternal blood and the risk for having a malformed child (5,14,15).

The hypothesis has been put forward that an excess of reactive oxygen species (ROS) mediates the teratogenicity of diabetic pregnancy (16-19). The oxygen radicals may be harmful to the cell and its functions by reacting with unsaturated fatty acids in membranes, yielding lipid peroxides and causing decreased membrane fluidity (20) and the formation of reactive aldehydes, which may in turn diffuse to other parts of the cell and react with macromolecules (21,22). The oxygen radicals can also react directly with proteins, resulting in, for instance, cross-linking of collagen with DNA, causing damage to both bases and sugars (23,24).

There are three major types of findings indicating a role for an excess of free oxygen radicals in diabetic embryopathy. First, diabetes in vivo (25) and hyperglycemia in vitro (26,27) cause an increase in lipid peroxides and free radicals in the offspring (28). Second, developmental defects similar to those seen in diabetic rat pregnancies in vivo and in high-glucose embryo cultures in vitro can be induced by enzymatic production of superoxide ions in embryo culture systems (29). Third, several different scavengers of free oxygen radicals added to the diet (18,30-32) decrease the malformation rate in diabetic rat pregnancy. In vitro, the increased malformation rates caused by both high glucose (16,17) and enzymatic radical production (33) in the culture medium can be diminished by radical scavengers. In addition, radical scavengers normalize glucose-induced overproduction of the isoprostane 8-iso-prostaglandin F2α in cultured rat embryos (28). The ROS theory is also strengthened by the suggestion that embryonic catalase activity is higher in the H than in the U strain, and maternal diabetes increases this difference in activity. The aim of this study was to characterize the influence of genetic predisposition on diabetic embryopathy by comparing the mRNA levels of ROS-metabolizing enzymes in the two strains. We determined the mRNA levels of catalase, glutathione peroxidase, γ-glutamylcysteine-synthetase, glutathione reductase, and superoxide dismutase (CuZn-SOD and Mn-SOD) in day 11 embryos of normal and diabetic H and U rats using semiquantitative reverse transcription-polymerase chain reaction. The mRNA levels of catalase and Mn-SOD were increased in H embryos as a response to maternal diabetes, and no differences were found for the other genes. Sequence analysis of the catalase promoter indicated that the difference in mRNA levels may result from different regulation of transcription. Sequence analysis of the catalase cDNA revealed no differences between the two strains in the translated region, suggesting that the previously observed difference in the electrophoretic mobility in zymograms is due to posttranslational modifications. An impaired expression of scavenging enzymes in response to ROS excess can thus be an integral part of a genetic predisposition to embryonic dysmorphogenesis. Diabetes 49:101-107, 2000

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CuZn-SOD, cytoplasmic form of SOD; γ-GCS, γ-glutamylcysteine-synthetase; GR, glutathione reductase; GSH, glutathione; GSH-Px, glutathione peroxidase; GSSG, oxidized glutathione; HD, embryos of diabetic H rats; RN, embryos of normal H rats; Mn-SOD, mitochondrial form of SOD; PCR, polymerase chain reaction; ROS, reactive oxygen species; SOD, superoxide dismutase; UD, embryos of diabetic U rats; UN, embryos of normal U rats.
that ROS excess is a teratologic mediator of ethanol (34), phentyoин (35), and thalidomide (36).

There are three main intracellular ROS scavenging enzymes: superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSHpx). The three differ in subcellular distribution and type of catalyzed reaction. SOD catalyzes the conversion of superoxide ions into oxygen and hydrogen peroxide and exists in a cytoplasmatic form (CuZn-SOD) and a mitochondrial form (Mn-SOD). Catalase, on the other hand, is distributed mainly to the peroxisomes and catalyzes the decomposition of hydrogen peroxide into water and oxygen. GSHpx reduces hydrogen peroxide to water using glutathione (GSH), which in turn is oxidized to oxidized glutathione (GSSG). The embryonic expression of these three enzymes seemed relevant to investigate against the background of a possible role for oxygen radicals in diabetic teratogenicity. Hyperglycemia in vivo has been found to decrease embryonic GSH content (27), as has also been demonstrated in vivo in embryos of diabetic rats (37). The enzyme γ-glutamylcystein synthetase (γ-GCS) catalyzes the rate-limiting step in synthesis of GSH, while glutathione reductase (GR) reduces GSSG back to GSH in a NADPH-dependent reaction. To get a more comprehensive view of glutathione metabolism in the embryo, therefore, we investigated the mRNA levels of the enzymes γ-GCS and GR.

We have used two substrains of the Sprague-Dawley rat, denoted U and H. The U rat developed spontaneously out of the H strain and has been shown in several studies to have a high frequency of congenital malformations in maternal diabetes (38,39). Both strains are now kept under outbreeding conditions. We also studied whether the mRNA levels would differ between the H and U substrains. We have used two substrains of the Sprague-Dawley rat, denoted H and U. The U rat developed spontaneously out of the H strain and has been shown in several studies to have a high frequency of congenital malformations in maternal diabetes (38,39). Both strains are now kept under outbreeding conditions.

The aim of this study, therefore, was to investigate if the embryonic mRNA levels of ROS-scavenging and GSH-metabolizing enzymes were different between the H and U substrains. We also studied whether the mRNA levels would change in a state of maternal diabetes. Furthermore, we determined and compared the H and U nucleotide sequences of catalase cDNA and catalase promoter region with the aim of finding discrepancies that may explain the strain-related differences in catalase activity and protein structure.
The cDNA sequence of the catalase was determined using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit and specific primers in the ABI PRISM 377 DNA semiautomatic sequencer (PE Biosystems, Foster City, CA). The sequence of the PCR products were determined using the ABI PRISM 377 DNA sequencer (PE Biosystems). In one of the H alleles, a deletion of two nucleotides (AA) was found in the U strain (Fig. 4). The DNA sequence upstream of the catalase transcription start site revealed no differences between H and U, except for a heterozygosity in the H strain. In all other groups, the catalase gene was denatured at 94°C for 10 min and finished with 5 extra min at 72°C.

**RESULTS**

The mean crown-to-rump lengths were equal in HN and UN groups, whereas embryos with diabetic mothers of both substrains were smaller than embryos from controls (Table 2). The decrease in size induced by maternal diabetes was 14% in the H strain and 18% in the U strain. The mean somite number was ~25 and similar in all four groups (Table 2).

The catalase-to-actin ratio, reflecting the catalase mRNA levels, was two times as high in the HD group as in the HN group (Fig. 1). There was no difference in mRNA levels of catalase between the UN and UD groups (Fig. 1). The catalase mRNA level in the HD group was higher than in all other groups.

No differences between any of the groups were detected in the mRNA levels of GSHpx, γ-GCS, or GR (Fig. 2). The GSHpx-to-actin ratio in the HD group appeared to be numerically higher than in any of the other embryo groups, but the variations in all groups were considerable, which precluded any statistically significant difference. The pattern (Fig. 2), however, was remarkably similar to that seen for catalase expression (Fig. 1).

The CuZn-SOD mRNA levels did not display any variation in response to diabetes or between the subgroups (Fig. 3). In the H strain, embryos from diabetic mothers displayed increased Mn-SOD mRNA levels compared with embryos from normal mothers; the ratios were 3.4 and 0.9, respectively. Mn-SOD expression was unchanged by maternal diabetes in embryos of the U strain (Fig. 3).

To further investigate a possible role of the catalase gene and Mn-SOD in the process of malformation in the U strain, we determined the DNA sequence of the catalase cDNA as well as 1.3 kb of the promoter region in both H and U rats. No differences were found between H and U in the coding region. In the 3' untranslated region 178 bp downstream of the translation stop codon, however, an A-to-G substitution was found in the U strain (Fig. 4). The DNA sequence upstream of the catalase transcription start site revealed no differences between H and U, except for a heterozygosity in the H strain.

**DISCUSSION**

The main finding of this study was that maternal diabetes causes an increase in the mRNA levels of the radical scavenging enzymes catalase and Mn-SOD in embryos from the malformation-resistant rat strain (H), while there is no such increased expression in embryos of a malformation-prone strain (U). The catalase result for the U embryos is in line with earlier studies showing no differences in mRNA levels between embryos of normal and diabetic U rats (43). Interestingly, in the previous study, Mn-SOD tended to be increased in embryos of diabetic rats (43), similar to the result in the present investigation. It may be speculated, therefore, that maternal diabetes could induce a small increase in U-strain mRNA levels of Mn-SOD. Such an increase would escape detection due to small magnitude and methodologic imperfection, and, at any rate, be of less functional importance than the clear-cut response to maternal diabetes in the mRNA levels of Mn-SOD in H embryos. In addition, we did not detect any differences between the H and U strains in the mRNA levels of either CuZn-SOD or the three GSH-metabolizing enzymes.

**TABLE 1**

<table>
<thead>
<tr>
<th>cDNA</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>PCR program</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>5'-CTGACCGAGCTGGTAC</td>
<td>5'-CCTGCTTGCTGAT</td>
<td>94/55/30/72/45/22</td>
</tr>
<tr>
<td>Catalase</td>
<td>5'-GGCAGCTGGGACTGGC</td>
<td>5'-CCGACCTCCTCCTGCTGAC</td>
<td>94/55/30/72/15/30</td>
</tr>
<tr>
<td>GSHpx</td>
<td>5'-CTCCTCCCGTTGCACAGT</td>
<td>5'-CCACCGCGGGTCCGACAT</td>
<td>94/60/70/32/30/20/28</td>
</tr>
<tr>
<td>γ-GCS</td>
<td>5'-ACACGGAGATTACAGGAGCC</td>
<td>5'-CCACAAATACACATAGGACAG</td>
<td>94/57/30/72/20/32</td>
</tr>
<tr>
<td>GR</td>
<td>5'-CTCAGACCGGCGCTGCT</td>
<td>5'-TCACTGCTCCGGACAT</td>
<td>94/57/30/72/20/32</td>
</tr>
<tr>
<td>CuZn-SOD</td>
<td>5'-GGTTCGGAGGGCGGGC</td>
<td>5'-GTCGCCCATATTGGGAC</td>
<td>94/55/30/72/20/28</td>
</tr>
<tr>
<td>Mn-SOD</td>
<td>5'-CTGAGGAGAGAGCCGCGT</td>
<td>5'-CTTTGCGACGCGCTCGT</td>
<td>94/55/30/72/30/22</td>
</tr>
</tbody>
</table>

**TABLE 2**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Crown-to-rump length (mm)</th>
<th>Somite number</th>
</tr>
</thead>
<tbody>
<tr>
<td>HN</td>
<td>10</td>
<td>3.94 ± 0.21</td>
<td>25.4 ± 0.58</td>
</tr>
<tr>
<td>HD</td>
<td>18</td>
<td>3.38 ± 0.14*</td>
<td>26.2 ± 0.79</td>
</tr>
<tr>
<td>UN</td>
<td>16</td>
<td>3.83 ± 0.10†</td>
<td>24.9 ± 0.49</td>
</tr>
<tr>
<td>UD</td>
<td>12</td>
<td>3.14 ± 0.11‡</td>
<td>24.3 ± 0.73</td>
</tr>
</tbody>
</table>

Data are means ± SE. Significant difference versus *HN, †HD, and ‡UN.
Another finding was that a diabetic environment hampers embryonic growth, measured as crown-to-rump length. This observation is in line with earlier studies showing decreased inner cell mass in blastocysts from diabetic rats (44). The crown-to-rump length is likely a reflection of a general environmental stress factor during embryonic growth, whereas the fetal malformation difference between H and U strains relates to specific genetic susceptibilities expressed under diabetic conditions. The somite number is approximately the same in all the groups. Because this parameter is a measure of the developmental stage of the embryo, we can conclude that our observations were performed at the same developmental stage in all four groups—an important consideration, since the pattern of gene expression varies between different developmental stages. The findings suggest that embryonic growth is hampered in maternal diabetes by a process different from that causing malformations, and that the developmental stage of the embryos in this study is largely unaffected by maternal diabetes.

It has been shown that catalase activities from H and U rats behave differently in zymograms (40). We wanted to find out if this difference was due to a structural difference in the catalase gene. We discovered that there is a base pair exchange in the 3' untranslated region of the catalase cDNA in the malformation-prone U strain compared with the H strain and the sequence shown by Furuta et al. (45). The relevance of this finding is not clear, but the change could affect mRNA stability or processing. In addition, embryos of the H and U strains also respond differently to maternal diabetes with respect to catalase activity (39). We therefore sequenced the catalase promoter region, since the differences in embryonic catalase activity and effect of maternal diabetes could be due to promoter sequence differences. The rat catalase promoter is a TATA-less promoter containing three CCAAT boxes and contains at least eight transcription starting points (46). The 5'-flanking region of catalase has different regulatory effects on reporter genes in dif-

**FIG. 1.** The catalase expression in all groups expressed as mean ratios of catalase/β-actin mRNA (± SE); n = 9-10. a, significant difference versus HN; b, significant difference versus HD; c, significant difference versus UN.

**FIG. 2.** The mean ratios (± SE) of GSHPx mRNA (A), γ-GCS (B), and GR (C) to β-actin; n = 8-10. No significant differences were found.
ferent cell types in cell-line experiments (47). We did not find any difference in DNA sequence of the promoter region between H and U strains, apart from a heterozygosity in the H strain that is not likely to affect expression. This finding may indicate that the differences in catalase activity and mRNA levels between H and U embryos are due to differences in the transcriptional regulation rather than the genes and promoters per se. This conjecture supports the notion that the difference in catalase activity is not the primary lesion leading to malformations; rather, it is a reflection of differences between the H and U strains in regulation of gene expression.

Diabetes is known to induce the production of free radicals in rats (48). It is also known that the addition of free radical scavengers in vitro (16) and in vivo (25,31) can reduce maldevelopment in rat embryos cultured in high glucose concentrations and in embryos from diabetic mothers. The mechanism and location of radical production are still disputed; however, a probable site of production is the mitochondrion (49). It can be speculated that radicals are formed within the embryonic mitochondria, since they are subjected to substrate overload in the diabetic environment. In favor of this hypothesis are the facts that diabetes induces increased uterine blood flow in early rat pregnancy (50), GLUT-1 is not

**FIG. 3.** The ratio (± SE) of CuZn (A) and Mn-SOD (B) to β-actin mRNA; n = 8-10. a, significant difference versus HN; b, significant difference versus HD; c, significant difference versus UN.

**FIG. 4.** A schematic figure of the rat catalase promoter region (A) and cDNA (B), outlining the differences in DNA sequence between the H and U strains.
downregulated in embryonic tissues despite exposure to a diabetic environment (51), and pyruvate uptake inhibitors decrease glucose-induced malformations in vitro (17). Embryonic rat mitochondria also show high-amplitude swelling in diabetic pregnancy, a phenomenon prevented by maternal antioxidant treatment (52). Also, more superoxide is produced from the neuroepithelium of rat embryos when cultured in high glucose than in normal glucose concentrations, a finding paralleled by inhibition of oxygen uptake and increased glucose utilization (26). Earlier studies have shown both increased malformation rate and low catalase activity in our malformation-prone U strain (39). The catalase mRNA results in the present study display the same pattern as the previous activity data; that is, the U embryos tend to have less protection against hydroxyl radical production when the mother is diabetic, whereas maternal diabetes seems to have the opposite effect on the H embryos. Apart from catalase, Mn-SOD expression is also increased in HD embryos. Hence, these embryos most likely have an increased protection against both superoxide ions and hydrogen peroxide and subsequently lower hydroxyl radical formation. One recent study has shown a correlation between low activities of SOD and catalase and neural tube defects in rat embryos; however, the authors did not find any correlation with diabetes (19). Mn-SOD is the mitochondrial form of SOD, and the mRNA increase is in line with the notion that the mitochondria are the main sites of free radical production in embryos exposed to maternal diabetes. It is known, however, that overexpression of CuZn-SOD in mice protects embryos from malformation in a diabetic environment (53), which may be due to a large surplus of cytosolic SOD. None of the investigated enzymes involved in GSH metabolism were affected by maternal diabetes, which may indicate that the GSH system is mainly reducing basal levels of hydrogen peroxide, whereas catalase would be more inducible by high amounts of oxygen radicals. This idea is in line with indications from RNA and enzyme activity studies that GSHpX is more important than catalase under normal conditions in mouse embryos (54). In embryos exposed to high glucose in vitro, Trocino et al. (27) found decreased expression of $\gamma$-GCS, a finding we were unable to repeat in embryos of diabetic rats. The reasons for this discrepancy are not completely clear but may be related to the use of different techniques for measuring mRNA and differences in the experimental conditions, such as the use of different rat strains.

One possible way to interpret the role of free oxygen radicals in diabetic embryopathy is that the radicals could induce apoptosis in the embryos of diabetic mothers, resulting in too few cells to produce a well-formed embryo (13). Apoptosis has recently been shown to be associated with the development of neural tube defects in embryos of diabetic mice (55). It has also been shown that oxidative stress induces apoptosis in cultured neurons from rat fetuses (56). The neural crest cells have been proposed to be a prime target of oxygen radicals in diabetic pregnancy (57), a hypothesis supported by the facts that tissues displaying malformations are those that are neural crest–derived (58) and that the migratory capacity of neural crest cells is reduced by high glucose in vitro (59).

Mn-SOD and catalase enzymes are thus likely to be involved in the protection of embryos against diabetes-induced malformations. An impaired response to high levels of free radicals in the expression of these enzymes may therefore cause increased embryonic vulnerability to maternal diabetes.

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