The aim of the present study was to investigate whether diabetic embryopathy may be associated with the inhibition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) resulting from an excess of reactive oxygen species (ROS) in the embryo. Recent demonstrations of enhanced ROS production in mitochondria of bovine aortic endothelial cells exposed to high glucose have supported the idea that the pathogenesis of diabetic complications may involve ROS-induced GAPDH inhibition. We investigated whether a teratogenic diabetic environment also inhibits embryonic GAPDH activity and alters GAPDH gene expression and whether antioxidants diminish such GAPDH inhibition. In addition, we determined whether the inhibition of GAPDH with iodoacetate induces dysmorphogenesis, analogous to that caused by high glucose concentration, and whether antioxidants modulated the putative teratogenic effect of such direct GAPDH inhibition. We found that embryos from diabetic rats and embryos cultured in high glucose concentrations showed decreased activity of GAPDH (by 40–60%) and severe dysmorphogenesis on gestational days 10.5 and 11.5. GAPDH mRNA was decreased in embryos of diabetic rats compared to control embryos. Supplementing the high-glucose culture with the antioxidant N-acetylcysteine (NAC) increased GAPDH activity and diminished embryonic dysmorphogenesis. Embryos cultured with iodoacetate showed both decreased GAPDH activity and dysmorphogenesis; supplementing the culture with NAC increased both parameters toward normal values. In conclusion, dysmorphogenesis caused by maternal diabetes is correlated with ROS-induced inhibition of GAPDH in embryos, which could indicate that inhibition of GAPDH plays a causal role in diabetic embryopathy. *Diabetes* 52:1222–1228, 2003

Earlier studies have shown that infants of diabetic mothers have an increased risk for congenital malformations and growth disturbances compared with infants of nondiabetic mothers (1). The cellular processes causing the maldevelopment in diabetic pregnancy are not fully understood. It is likely that the developmental disturbances are induced by several different mechanisms and that the cause is multifactorial (2–5). In embryos exposed to a diabetes-like environment, one teratological pathway involves enhanced activity of reactive oxygen species (ROS) (6), decreased antioxidative protection (7,8), or both. Exogenous supplementation with antioxidative agents diminishes diabetic embryopathy in vivo (9–13) and glucose-induced embryonic dysmorphogenesis in vitro (3,6). The mechanisms by which ROS excess may influence intracellular processes of developmental importance (e.g., glucose metabolism) have not been explained.

In line with the concept of ROS-mediated embryopathy, an overproduction of superoxide in mitochondria of tissues exposed to a high glucose concentration (14) has recently been suggested as a common mechanism for all diabetic complications (15). A possible consequence of excess ROS generation in the mitochondria would be increased ROS leakage and inhibition of the cytosolic glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). This enzyme has displayed sensitivity to ROS in several different conditions of oxidative stress (16). This sensitivity resides in the thiol group of cysteine residue 149 in the active site of the enzyme (17,18). Oxidation of the thiol group by NO or ROS leads to decreased enzyme activity (19), and blocking of this process by antioxidants protects the activity of the enzyme (20).

Inhibition of GAPDH has pronounced metabolic consequences, such as accumulation of glycolytic substrates before the GAPDH enzyme (16,21) and enhanced activity in the polyol and hexosamine pathways (22). In addition, alterations in phosphoinositide metabolism and enhanced formation of glycosylation products are possible effects of inhibited glycolysis at the GAPDH step (15). All of these pathways may be involved in the induction of embryonic dysmorphogenesis.

Many studies have shown that GAPDH has an essential role in apoptosis and age-related neuronal disorders, and that several antioxidative agents (i.e., glutathione) affect GAPDH structure and alter its function (17,23). It is therefore conceivable that oxidatively altered GAPDH may have a role in the disrupted intracellular signal transduction of diabetic embryopathy. For example, it has been shown that oxidative stress may enhance GAPDH gene.
expression (24) without increasing its activity, thereby causing increased migration of GAPDH to the cell nucleus (25).

The purpose of our study was to investigate whether diabetes in vivo or hyperglycemia in vitro, both of which cause embryonic dysmorphogenesis, also decrease GAPDH activity, and whether supplementing culture with antioxidants at antiteratogenic concentrations would be able to block such enzyme inhibition. We also wanted to measure mRNA levels of GAPDH in embryos subjected to diabetes in vivo or hyperglycemia in vitro. We chose to perform these investigations in an experimental system using rat embryos that have previously displayed skeletal, central nervous system, and cardiac malformations in vivo (26,27), and neural tube closure defects in vitro (28), all of which closely resemble malformations found in human diabetic pregnancy. In addition, we aimed to investigate if the inhibition of GAPDH with iodoacetate (IA) induces a dysmorphogenesis in rat embryos analogous to the maldevelopment caused by high glucose concentration, and whether antioxidants could modulate the putative teratogenic effect of direct GAPDH inhibition as they do with glucose-induced embryonic dysmorphogenesis.

RESEARCH DESIGN AND METHODS

Animals. Embryos were obtained from a local outbred SD rat strain with an increased incidence of congenital malformations in diabetic pregnancy (26,29). All rats were fed a commercial pelleted diet (AB Analyenc, LdiKoping, Sweden) and had free access to food and tap water. They were maintained at an ambient temperature of 22°C with 12-h light/dark cycles. Female and male rats were caged together during the night. The morning that conception was verified by the presence of sperm in a vaginal smear was designated gestational day 0.

Induction of diabetes. Diabetes was induced in a subset of female rats by injection of 40 mg/kg streptozotocin (Pharmacia AB, Kalamazoo, MI) into the tail vein 1 week before mating commenced (1–3 weeks before conception). The diabetic state was confirmed 1 week after the injection by the presence of a serum glucose level >20 mmol/l (Beckman Glucose Analyzer II; Beckman Instruments, Fullerton, CA).

Whole embryo culture. Pregnant nondiabetic rats were killed by cervical dislocation on gestational day 9 between 1200 and 1400 h. The embryos in their intact yolk sacs were cultured (four to five embryos per 50-ml culture tube; Falcon) in 5 ml of 80% immediately centrifuged rat serum (30) and 20% saline. The saline fraction was used to add various compounds from stock solutions, yielding an end concentration of 10 or 30 mmol/l glucose (Apoteksbolaget AB, Stockholm, Sweden), 2–4 μmol/l IA (BDH Chemicals, Poole, U.K.), or 0.5 mmol/l N-acetylcyesteine (NAC; Sigma). These concentrations were obtained from previous pilot experiments where different dosages of the compounds were tested, such as 1–5 μmol/l IA and 0.1–2.0 mmol/l NAC. All compounds were readily dissolved in saline.

Measurement of DNA and protein. Each embryo was homogenized by ultrasonic disruption (20 kHz, 60 W for 5 s; Vibra Cell; Sonic and Materials, Danbury, CT) in 1 ml 0.5 mol/l NaOH and kept at 4°C until protein and DNA were measured. The protein content of the homogenates was determined by the method of Lowry et al. (32) using BSA as standard, and DNA was measured as described by Kissane and Robins (33) and Hinegardner (34).

Measurement of GAPDH activity. Material used to measure GAPDH activity was obtained from Sigma-Aldrich Sweden AB: triethanolamine hydrochloride, (−)3-phosphoglyceric acid, magnesium sulphate, nicotinamide adenine dinucleotide (reduced form), and adenosine 5-triphosphate (3-phosphoglyceric phosphokinetase.

Embryos cultured (24–48 h) in vitro and day 10 and 11 embryos from normal and diabetic rats were used to determine the activity of GAPDH by the method described by Bergmeyer (35) and modified by Mohamed et al. (36).

The embryos were dissected free of surrounding membranes and homogenized in an ethanolic buffer (pH 7.6). The GAPDH activity was estimated by observing (in a spectrophotometer) the conversion of NADH to NAD+ at 340 nm in the backward reaction starting with glyceraldehyde-3-phosphate using glyceraldehyde-3-phosphate dehydrogenase (PGK) + ATP as an auxiliary enzyme (see below). The absorbance difference per minute was estimated from the linear initial part of the recording.

Measurement of GAPDH expression

Preparation of total RNA. Total RNA from embryos was isolated with an Rneasy mini kit (Qiagen, Merck, Eurosalab) according to the manufacturer’s protocol. Briefly, embryos were lysated in 350 μl buffer, after which 350 μl of 70% ethanol was added to the homogenates and the samples were mixed and applied to Rneasy mini-spin columns sitting in 2 ml collection tubes. The columns and tubes were centrifuged for 1 min at 13,000 rpm, the flow-through was discarded, and the columns were washed with 350 μl buffer and spun at 13,000 rpm for 1 min. The samples were DNase treated with 10 μl DNase I and 70 μl buffer and incubated at room temperature for 15 min. Thereafter, the columns were washed with 350 μl buffer and spun at 13,000 rpm for 1 min. The columns were subsequently washed with 500 μl buffer twice, with the flow-through being discarded after each centrifugation. The columns were transferred to new collection tubes, and 50 μl RNase-free water was applied to each column twice; the accumulated flow-through was collected after the centrifugations (total RNA sample). A volume of 1 μl of RNA guard (RNase inhibitor) was added to each sample.

Preparation of cDNA. Reverse transcription was performed with 1 μg of total RNA. First-strand cDNA synthesis was performed using first-strand

FIG. 1. GAPDH activity in embryos of normal (N) and diabetic (D) rats on gestational days 10 and 11, expressed as arbitrary enzyme units per μg protein. Number of embryos for each experiment indicated on bar. Data are means ± SE. *P < 0.05 vs. normal embryos of same age (ANOVA).
beads (Ready To Go; Pharmacia Biotech, Uppsala, Sweden), according to the manufacturer’s description. Then 30 μl RNA-free water containing 1 μg RNA was heated at 65°C for 10 min and chilled on ice for 2 min. The RNA solution was transferred to the first-strand reaction mix beads. The chosen primer was oligo(dT). The mixture was incubated at 37°C for 60 min, and the resulting cDNA was diluted threefold with water treated with diethyl pyrocarbonate. Analysis of GAPDH mRNA levels. For the analysis, 1 μl of the cDNA purified from embryos (containing 10 ng of converted total RNA) was amplified and measured with real-time PCR using the LightCycler (Roche Diagnostics, Mannheim, Germany). Specific primers were made for GAPDH by Amersham Pharmacia Biotech (Uppsala, Sweden) and were 5'-TGGCCGC (sense) and 5'-ATTGGCCGT ATTGCCGC (antisense). After the LightCycler protocol, 1 μl of the cDNA was amplified in a final volume of 10 μl containing 6.2 μl RNA-free water, 1 μl FastStart DNA Master SYBR Green I (Roche Molecular Biochemicals, Mannheim, Germany), 2 mmol/l MgCl₂, and 0.5 μmol/l of the sense and antisense primers. For relative quantification, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as control. GAPDH sense and antisense primers, 5'-ATTGACCACTACCTGGGCAA and 5'-GAG ATACAATCCAAACACTTGACC (TIB Molbiol, Berlin, Germany), respectively, were used in the same manner as for GAPDH.

The LightCycler Run (Version 5.32) program was used with the following parameters: denaturation at 95°C for 10 min and amplification with a total of 45 cycles, with each cycle having a denaturation temperature at 95°C for 15 s, annealing temperature at 60°C for 15 s, and elongation temperature at 72°C for 15 s.

Controls were included in each run of the real-time PCR assay; for each primer pair, one sample with no cDNA (containing only RNA free water) was included. Furthermore, to exclude the possibility of remaining DNA fragments in the samples, 10 ng of total RNA of each sample were amplified in the LightCycler. We found no PCR product in the water or in the total RNA samples. Results were analyzed for each sample with relative quantification comparing the difference between sample and control crossing point (Cp) values. The differences were subsequently transformed according to the formula ΔCp = Cp(sample) - Cp(control) to yield GAPDH expression relative to GAPDH expression.

Ethical and statistical evaluation. The Animal Ethical Committee of the Medical Faculty of Uppsala University approved the research protocol, including all experimental procedures involving animals. The comparisons between different experimental groups were based on individual embryos. Differences between means were evaluated by ANOVA, where the applied post hoc test was Fisher’s protected least significant difference at the 95% significance level. The malformation score was evaluated with the aid of χ² statistics by comparing frequencies of malformation scores (0, 1, 5, or 10) in the different groups. Student’s two-tailed t test was used to compare the differences of crossing point values of embryos. P < 0.05 was considered to denote a statistically significant difference between groups. The calculations were performed with the Macintosh version of the statistical program Statview.

Results
Both day-10 and day-11 embryos from diabetic rats had lower GAPDH activity compared to embryos from nondiabetic mothers (Fig. 1). These embryos also showed increased malformation scores (Fig. 2, Table 1) and decreased crown-rump length and somite numbers (Table 1). Similarly, the embryos cultured in high glucose concentration (30 mmol/l) showed lower GAPDH activity than embryos cultured in 10 mmol/l glucose, after both 24 and 48 h of culture (Fig. 3). The high glucose—cultured embryos displayed severe dysmorphogenesis with increased malformation scores (Table 2, Fig. 4) and decreased crown-rump length, somite number, and DNA and protein content (Table 2). The addition of 0.5 mmol/l NAC to the culture medium attenuated all metabolic and morphological changes caused by the high glucose concentration. Thus the GAPDH activity was increased (Fig. 3), the morphological score was decreased (Fig. 4), and the crown-rump length, somite number, and DNA and protein content were increased to normal values in the 30G + NAC group (Table 2).

Supplementing the culture medium (10 mmol/l glucose)
with 2 μmol/l IA decreased GAPDH activity (Fig. 3), and the addition of 2–4 μmol/l IA increased morphological score (Fig. 4) and decreased other morphological parameters compared with embryos cultured in 10 mmol/l glucose (Table 2). The addition of 0.5 mmol/l NAC to the culture medium at each concentration of IA increased GAPDH activity (Fig. 3) and improved the malformation score (Fig. 4) as well as other morphological parameters (Table 2).

Estimation of GAPDH mRNA levels in the embryos showed that maternal diabetes decreased GAPDH gene expression at gestational days 10.5 and 11.5, and that there were no differences between the glucose-exposed embryos and the controls at any time point (Fig. 5).

**DISCUSSION**

The main result of this study was that GAPDH activity was decreased in embryos subjected to a teratogenic diabetic environment in vivo (maternal diabetes) or diabetes-like environment in vitro (increased glucose concentration). When the disturbed embryonic development in vitro was corrected by the addition of the antioxidant NAC to the culture medium with high glucose concentration, GAPDH activity increased toward normal values. This strongly suggests the existence of common ROS-associated elements in the pathogenesis of glucose-induced congenital malformations and glucose-induced inhibition of embryonic GAPDH activity.

To probe the relation among glucose-induced embryonic maldevelopment, GAPDH inhibition, and ROS excess, we removed the glucose challenge by directly inhibiting GAPDH with IA. We found that IA supplementation to the culture medium yielded embryonic maldevelopment similar to the glucose-induced dysmorphogenesis and, furthermore, that the addition of NAC diminished both the GAPDH inhibition and the disturbance in embryonic development. This finding suggests that GAPDH inhibition may be causally related to the induction of congenital malformations via enhanced ROS metabolism.

There is ample clinical and experimental evidence showing diabetes per se as a state of oxidative stress (14,37–40). Likewise, diabetic pregnancy is associated with oxidative stress (41–43), and the assumption that diabetic embryopathy may be induced by enhanced ROS metabolism is supported by several studies. Markers of lipid peroxidation (28), protein carbonylation (44), and DNA damage (45,46) are increased in offspring of diabetic animals. Increased thiol oxidation from diamine exposure yields embryonic dysmorphogenesis and GAPDH inhibition (47). Indeed, antioxidative treatment in vivo (9–12) and in vitro (3,6,48,49) has been successful in preventing diabetes- or hyperglycemia-induced embryonic dysmorphogenesis. It has recently been demonstrated that bovine endothelial cells produce excess superoxide when exposed to high glucose levels (14), and that this state of ROS excess may inhibit GAPDH. It is notable that inhibited GAPDH activity, as a consequence of oxidation by endogenous aldehydes, has been suggested as an important etiologic element of diabetic neuropathy (50). In addition, as our present work indicates, GAPDH inhibition caused by ROS excess may be involved in the etiology of diabetic embryopathy.

The major metabolic effect of inhibited GAPDH would be accumulation of glycolytic intermediates upstream of this enzyme, which could lead to enhanced activity in the polyol and hexosamine pathways (22,51) and to increased glycation. There is also evidence to support the idea that inhibited GAPDH leads to diabetic embryopathy. For example, sorbitol formation is enhanced in embryos sub-

**TABLE 2**

Crown-rump length, number of somites, and protein and DNA content in embryos and morphological distribution of embryos

<table>
<thead>
<tr>
<th>Type</th>
<th>n</th>
<th>Crown-rump length (mm)</th>
<th>Number of somites</th>
<th>Protein content</th>
<th>DNA content</th>
<th>No malformations (%)</th>
<th>Minor malformations (%)</th>
<th>Major malformations (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10G</td>
<td>18</td>
<td>3.8 ± 0.05</td>
<td>29.9 ± 0.2</td>
<td>419 ± 9</td>
<td>35.7 ± 0.6</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30G</td>
<td>19</td>
<td>2.6 ± 0.1*</td>
<td>16.2 ± 0.9*</td>
<td>176 ± 23*</td>
<td>11.4 ± 1.3*</td>
<td>5</td>
<td>0</td>
<td>95*</td>
</tr>
<tr>
<td>30G + NAC</td>
<td>12</td>
<td>3.8 ± 0.1†</td>
<td>28.2 ± 1.3†</td>
<td>377 ± 18†</td>
<td>31.2 ± 2.0†</td>
<td>75</td>
<td>25</td>
<td>0†</td>
</tr>
<tr>
<td>2 IA</td>
<td>21</td>
<td>3.4 ± 0.1*</td>
<td>22.0 ± 1.1*</td>
<td>312 ± 20*</td>
<td>21.4 ± 1.9*</td>
<td>14</td>
<td>33</td>
<td>52*</td>
</tr>
<tr>
<td>2 IA + NAC</td>
<td>13</td>
<td>3.8 ± 0.1†</td>
<td>28.0 ± 1.1†</td>
<td>392 ± 22†</td>
<td>32.2 ± 2.2†</td>
<td>69</td>
<td>16</td>
<td>15†</td>
</tr>
<tr>
<td>3 IA</td>
<td>21</td>
<td>3.1 ± 0.1*</td>
<td>19.3 ± 0.8*</td>
<td>257 ± 14*</td>
<td>15.0 ± 1.4*</td>
<td>5</td>
<td>9</td>
<td>86*</td>
</tr>
<tr>
<td>3 IA + NAC</td>
<td>13</td>
<td>3.6 ± 0.1†</td>
<td>25.8 ± 1.5†</td>
<td>378 ± 27†</td>
<td>28.5 ± 2.6†</td>
<td>54</td>
<td>15</td>
<td>31†</td>
</tr>
<tr>
<td>4 IA</td>
<td>20</td>
<td>3.0 ± 0.1*</td>
<td>17.0 ± 1.1*</td>
<td>230 ± 15*</td>
<td>13.4 ± 1.3*</td>
<td>5</td>
<td>0</td>
<td>95*</td>
</tr>
<tr>
<td>4 IA + NAC</td>
<td>12</td>
<td>3.5 ± 0.2†</td>
<td>24.0 ± 1.4†</td>
<td>305 ± 20†</td>
<td>23.6 ± 2.3†</td>
<td>17</td>
<td>50</td>
<td>33†</td>
</tr>
</tbody>
</table>

Data are means ± SE unless otherwise indicated. Embryos were cultured in 10 or 30 mmol/l glucose (10G or 30G), with or without addition of NAC or IA after 48 h of culture (corresponding to gestational day 11). *P < 0.05 vs. 10G embryos; †P < 0.05 vs. analogous embryo without NAC addition (ANOVA or χ²).
jected to a diabetic environment in vivo (52) and in vitro (53). However, attempts to block dysmorphogenesis by inhibiting aldose reductase have not been particularly successful (52–54). Evidence in favor of enhanced hexosamine pathway activity has been found in embryos of diabetic mice (55) as well as in the enhanced formation of the reactive glycosylating agent deoxyglucosone (56) in embryos subjected to high glucose concentration. The teratological potential of these pathways is currently under investigation.

Another possible effect of inhibited GAPDH activity is the accumulation of diacylglycerol (DAG) and, consequently, increased protein kinase C (PKC) activity. Recently, researchers reported increased DAG concentrations and increased PKC activity in mouse embryos exposed to high glucose concentration in vitro and diabetes in vivo (57). In a previous study, however, we found no direct evidence for PKC activation in rat embryonic tissues exposed to high glucose concentration in vitro (58). When we added a PKC inhibitor, GF-109203X, to high glucose cultures of rat embryos, we worsened the dysmorphogenesis, and when the inhibitor was added to a low glucose culture, it was found to be teratogenic (58). The difference in results might be attributable to a species difference with respect to embryonic tissue between rat and mice. In an ongoing experimental series, we are measuring PKC activity (by estimating the degree of enzyme translocation) (59) of several PKC isoenzymes in embryos of normal/diabetic rats and in rat embryos subjected to various glucose concentrations; thus far we have likewise failed to demonstrate an increased PKC activation.

The GAPDH protein has functions other than participating in the glycolysis; in particular, GAPDH has a role in the initiation of apoptosis (23), a process that may well be induced by oxidative stress (25). Altered GAPDH function has also been suggested as an integral element in the etiology of several neurodegenerative disorders, such as Parkinson (60) and Huntington (61) diseases. The notion that nonglycolytic properties of the GAPDH protein are important for diabetic embryopathy is an exciting possibility; however, these nonglycolytic effects are often associated with a simultaneous overexpression of GAPDH, which was not the case in the embryos of the present investigation.

We found decreased GAPDH activity and decreased GAPDH gene expression in embryos of diabetic rats on gestational days 10.5 and 11.5. In contrast, GAPDH gene expression was not affected in embryos subjected to high glucose concentration in vitro, either on day 10.5 (corresponding to 24-h embryo culture) or day 11.5 (48-h embryo culture). Therefore, the data suggest that effects of a diabetic/hyperglycemic environment on GAPDH gene expression take days to develop, and that the differences in GAPDH enzyme activity induced by maternal diabetes in vivo and, in particular, by high glucose in vitro, are likely to be the result of posttranslational changes in GAPDH protein structure and function. Because we did not measure GAPDH protein levels in the present study, we are unable to state to what extent GAPDH activity per GAPDH protein was altered by the different experimental conditions. Our main conclusion remains, however, that short-term exposure of embryos to high glucose and long-term exposure of embryos to a diabetic environment do indeed decrease the embryonic GAPDH activity.

The possible teratological events consistent with the results of the present study are that maternal diabetes in vivo or hyperglycemia in vitro causes embryonic hyperglycemia, leading to embryonic mitochondrial overproduction of superoxide (14). The resulting ROS excess inhibits
GAPDH (16), which leads to an accumulation of glycolytic intermediates upstream of GAPDH (16,21), and the ensuing metabolic changes (15) trigger embryonic maldevelopment. However, this may not be the only, or the most important, mechanism for induction of diabetic embryopathy. For example, both IA and NAC may affect cellular constituents with higher teratogenic capacity than GAPDH, thereby reducing the importance of the observed covariation between low GAPDH activity and a high rate of embryo maldevelopment. On the other hand, because IA is a widely used GAPDH inhibitor and the major function of NAC is to serve as an antioxidant, the interpretation of the findings would seem to favor a teratological role for inhibited GAPDH activity in embryos subjected to a diabetes-like environment. Evidently, the exact role of GAPDH in diabetic embryopathy has to be resolved by future studies.

The present work shows that a diabetic/hyperglycemic environment diminishes embryonic GAPDH activity. This decrease in enzyme activity is likely to be an effect of oxidative stress in the embryo. We therefore conclude that ROS-induced inhibition of embryonic GAPDH activity may be partly responsible for embryonic dysmorphogenesis in diabetic pregnancy.

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