Maternal Diabetes In Vivo and High Glucose In Vitro Diminish GAPDH Activity in Rat Embryos

Parri Wentzel, Andreas Ejdesjö, and Ulf J. Eriksson

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

From the Department of Medical Cell Biology, Uppsala University, Uppsala, Sweden.

Address correspondence and reprint requests to Dr. Parri Wentzel, Department of Medical Cell Biology, Uppsala University, Biomedical Center, P.O. Box 571, SE-751 23 Uppsala, Sweden. E-mail: parri.wentzel@medcellbiol.uu.se.

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DAG, diacylglycerol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPDH, glycerol-6-phosphate dehydrogenase; IA, iodoacetate; NAC, *N*-acetylcysteine; PGK, glycerate-3-phosphate kinase; PKC, protein kinase C; ROS, reactive oxygen species.

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

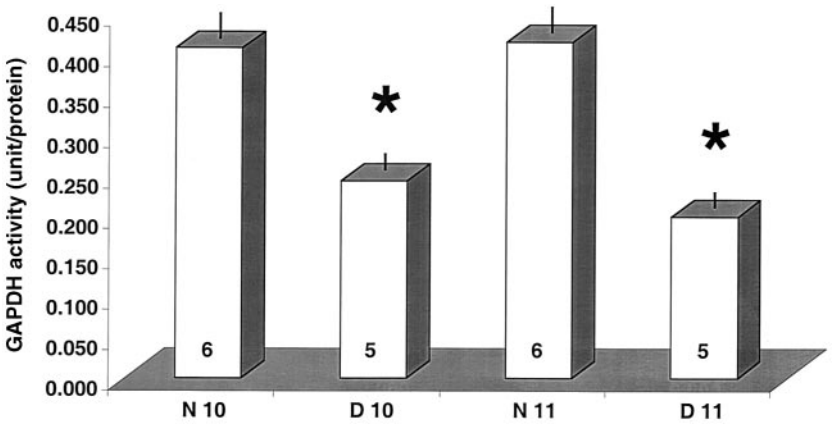

FIG. 1. GAPDH activity in embryos of normal (N) and diabetic (D) rats on gestational days 10 and 11, expressed as arbitrary enzyme units per μg protein. Number of embryos for each experiment indicated on bar. Data are means ± SE. *$P < 0.05$ vs. normal embryos of same age (ANOVA).

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 Analycen, Lidköping, 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 in a roller incubator at 60 rpm for 48 h at 38°C. Afterward, the embryos were dissected out of their yolk sacs and examined under a stereo microscope. The malformation score of the embryos was determined by assigning 0, 1, 5, or 10 points, respectively, to no, minor, less severe, or severe malformation (31). A malformation score of 0 indicated a completely normal embryo, fully rotated with a closed neural tube. Embryos given a score of 1 showed a single minor deviation from this pattern, mainly an open posterior end of the neural tube. A score of 5 signified one major malformation, most often an open neural tube in the rhombencephalon area or a slight tail twist, and a score of 10 indicated an embryo with multiple major malformations such as open neural tube, rotational defects, and/or heart enlargement. In addition, we determined the crown-rump length and somite number of each embryo.

Culture conditions. The embryos were cultured (four to five embryos per 50-ml culture tube; Falcon) in 5 ml of 80% immediately centrifuged rat serum 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*-acetylcysteine (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 ultrasound disruption (20 kHz, 60 W for 5 s; Vibra Cell; Sonics & 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, D(-)3-phosphoglyceric acid, magnesium sulphate, nicotinamide adenine dinucleotide (reduced form), and adenosine 5-triphosphate 3-phosphoglyceric phosphokinase.

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 ethanolamine 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 glycerate-3-phosphate using glycerate-3-phosphate kinase (PGK) + ATP as an auxiliary enzyme (see below). The absorbance difference per minute was estimated from the linear initial part of the recording.

$$\text{Glycerate-3-phosphate} + \text{ATP} \xrightarrow{\text{PGK}} \text{Glycerate-1,3-diphospate} + \text{ADP}$$

$$\text{Glycerate-1,3-diphosphate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{GAPDH}} \text{GAP} + \text{NAD}^+ + \text{P}_i$$

Measurement of GAPDH expression

Preparation of total RNA. Total RNA from embryos was isolated with an RNeasy minikit (QiaGEN, Merck, Eurolab), according to the manufacturer's description. 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

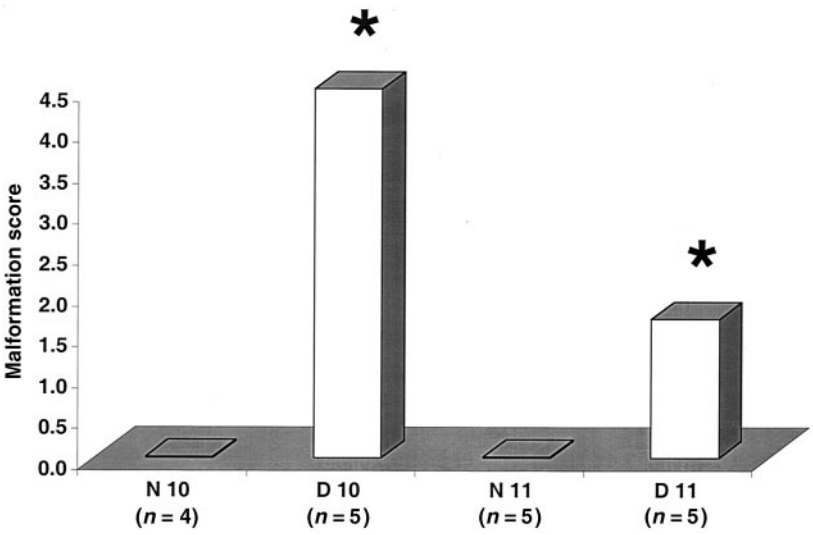

FIG. 2. Malformation score in embryos of normal (N) and diabetic (D) rats on gestational days 10 and 11. *$P < 0.05$ vs. normal embryos of same age (χ^2).

beads (Ready To Go; Pharmacia Biotech, Uppsala, Sweden), according to the manufacturer's description. Then 30 μl RNase-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′-TTGGCCGT ATTGGCCGC (sense) and 5′-GTGCCATTGAACTTGCCGTG (antisense). After the LightCycler protocol, 1 μl of the cDNA was amplified in a final volume of 10 μl containing 6.2 μl RNase-free water, 1 μl FastStart DNA Master SYBR Green I (Roche Molecular Biochemicals, Mannheim, Germany), 2 mmol/l $MgCl_2$, and 0.5 μmol/l of the sense and antisense primers. For relative quantification, glycerol-6-phosphate dehydrogenase (GPDH) was used as control. GPDH sense and antisense primers, 5′-ATTGACCACTACCTGGGCAA and 5′-GAG ATACACTTCAACACTTTGACCT (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 10 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 RNase 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 $2^{-(\text{Cp GAPDH} - \text{Cp GPDH})}$ to yield GAPDH expression relative to GPDH 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 χ^2 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)

TABLE 1
Crown-rump length and number of somites in embryos of normal and diabetic rats on gestational days 10 and 11 and morphologic distribution of the embryos

Type	n	Crown-rump length (mm)	Number of somites	No malformations (%)	Minor malformations (%)	Major malformations (%)
N 10	6	2.08 ± 0.05	19.7 ± 0.4	100	0	0
D 10	20	1.33 ± 0.06*	12.5 ± 0.7*	55	0	45*
N 11	11	4.03 ± 0.04	30.5 ± 0.2	100	0	0
D 11	24	3.41 ± 0.10*	27.0 ± 0.6*	79	8	13*

Data are means ± SE unless otherwise indicated. N, normal; D, diabetic. *$P < 0.05$ vs. normal embryos of same age (ANOVA or χ^2).

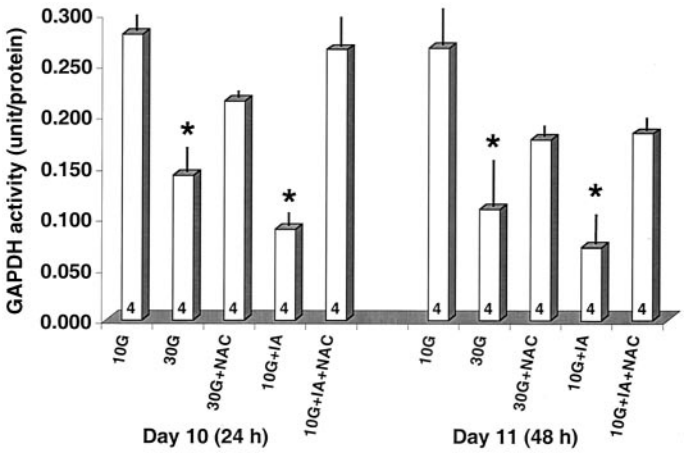

FIG. 3. GAPDH activity in embryos cultured in 10 or 30 mmol/l glucose (10G or 30G), with or without addition of NAC or IA after 24 or 48 h of culture (corresponding to gestational days 10 and 11), expressed as arbitrary enzyme units per μg protein. Data are means ± SE. *$P < 0.05$ vs. 10G embryos with same culture time (ANOVA). Number of embryos for each experiment indicated on bar.

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

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

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 χ^2).

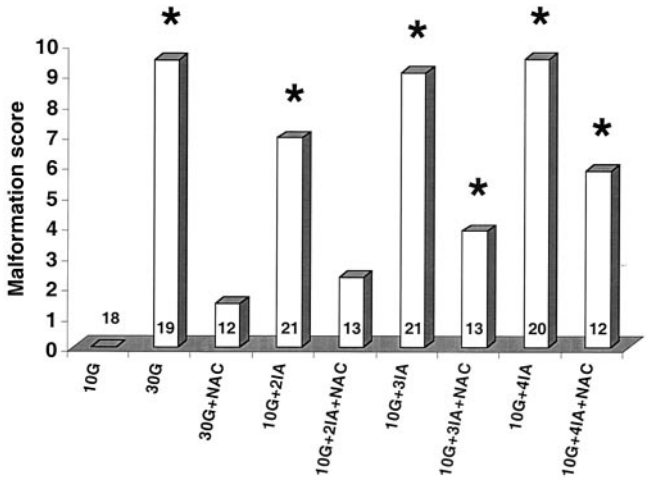

FIG. 4. Malformation score in embryos cultured in 10 or 30 mmol/l glucose (10G or 30G), with or without addition of NAC or IA after 48 h of culture (corresponding to gestational day 11). Number of embryos for each experiment indicated on bar. *P < 0.05 vs. 10G embryos (χ^2).

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

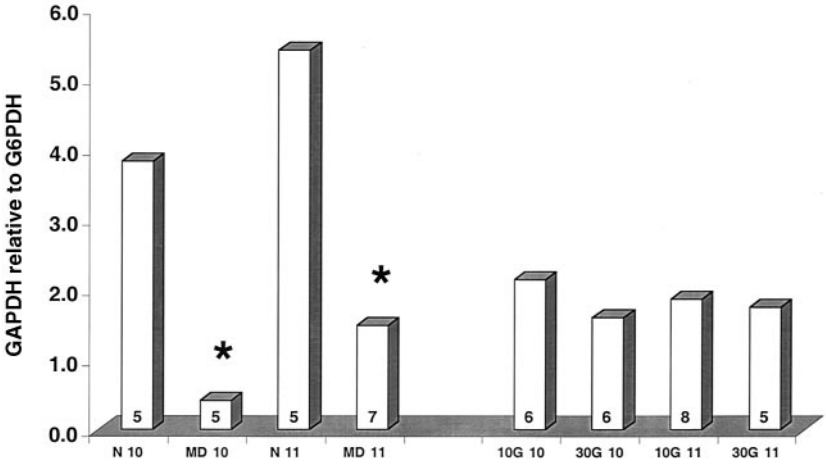

FIG. 5. Ratio between mRNA levels of GAPDH and GPDH in embryos from normal (N) and diabetic (MD) rats on gestational days 10 and 11 and in embryos cultured in 10 or 30 mmol/l glucose (10G or 30G) after 24 or 48 h of culture (corresponding to gestational days 10 or 11). Number of embryos for each experiment indicated on bar. *P < 0.05 vs. normal embryos of same age (Student's t test).

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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REFERENCES

1. Pedersen J: *The Pregnant Diabetic and Her Newborn: Problems and Management*. 2nd ed. Copenhagen, Munksgaard, 1977
2. Sadler TW, Hunter ES, Wynn RE, Phillips LS: Evidence for multifactorial origin of diabetes-induced embryopathies. *Diabetes* 38:70–74, 1989
3. Eriksson UJ, Borg LAH: Diabetes and embryonic malformations: role of substrate-induced free-oxygen radical production for dysmorphogenesis in cultured rat embryos. *Diabetes* 42:411–419, 1993
4. Buchanan TA, Denno KM, Sipos GF, Sadler TW: Diabetic teratogenesis: in vitro evidence for a multifactorial etiology with little contribution from glucose per se. *Diabetes* 43:656–660, 1994
5. Wentzel P, Eriksson UJ: Insulin treatment fails to abolish the teratogenic potential of serum from diabetic rats. *Eur J Endocrinol* 134:459–446, 1996
6. Eriksson UJ, Borg LAH: Protection by free oxygen radical scavenging enzymes against glucose-induced embryonic malformations in vitro. *Diabetologia* 34:325–331, 1991
7. Trocino RA, Akazawa S, Ishibashi M, Matsumoto K, Matsuo H, Yamamoto H, Goto S, Urata Y, Kondo T, Nagataki S: Significance of glutathione depletion and oxidative stress in early embryogenesis in glucose-induced rat embryo culture. *Diabetes* 44:992–998, 1995
8. Cederberg J, Eriksson UJ: Decreased catalase activity in malformation-prone embryos of diabetic rats. *Teratology* 56:350–357, 1997
9. Eriksson UJ, Simán CM: Pregnant diabetic rats fed the antioxidant butylated hydroxytoluene show decreased occurrence of malformations in the offspring. *Diabetes* 45:1497–1502, 1996
10. Viana M, Herrera E, Bonet B: Teratogenic effects of diabetes mellitus in the rat: prevention with vitamin E. *Diabetologia* 39:1041–1046, 1996
11. Simán CM, Eriksson UJ: Vitamin C supplementation of the maternal diet reduces the rate of malformation in the offspring of diabetic rats. *Diabetologia* 40:1416–1424, 1997
12. Wiznitzer A, Ayalon N, Hershkovitz R, Khamaisi M, Reece EA, Trischler H, Bashan N: Lipoic acid prevention of neural tube defects in offspring of rats with streptozocin-induced diabetes. *Am J Obstet Gynecol* 180:188–193, 1999
13. Sakamaki H, Akazawa S, Ishibashi M, Izumino K, Takino H, Yamasaki H, Yamaguchi Y, Goto S, Urata Y, Kondo T, Nagataki S: Significance of glutathione-dependent antioxidant system in diabetes-induced embryonic malformations. *Diabetes* 48:1138–1144, 1999
14. Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardino I, Brownlee M: Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* 404:787–790, 2000
15. Brownlee M: Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813–820, 2001
16. Janero DR, Hreniuk D, Sharif HM: Hydroperoxide-induced oxidative stress impairs heart muscle cell carbohydrate metabolism. *Am J Physiol* 266: C179–C188, 1994
17. Rivera-Nieves J, Thompson WC, Levine RL, Moss J: Thiols mediate superoxide-dependent NADH modification of glyceraldehyde-3-phosphate dehydrogenase. *J Biol Chem* 274:19525–19531, 1999
18. Ishii T, Sunami O, Nakajima H, Nishio H, Takeuchi T, Hata F: Critical role of sulfenic acid formation of thiols in the inactivation of glyceraldehyde-3-phosphate dehydrogenase by nitric oxide. *Biochem Pharmacol* 58:133–143, 1999
19. Morgan PE, Dean RT, Davies MJ: Inhibition of glyceraldehyde-3-phosphate dehydrogenase by peptide and protein peroxides generated by singlet oxygen attack. *Eur J Biochem* 269:1916–1925, 2002
20. McKenzie SM, Doe WF, Buffinton GD: 5-aminosalicylic acid prevents oxidant mediated damage of glyceraldehyde-3-phosphate dehydrogenase in colon epithelial cells. *Gut* 44:180–185, 1999
21. Danshina PV, Schmalhausen EV, Avetisyan AV, Muronetz VI: Mildly oxidized glyceraldehyde-3-phosphate dehydrogenase as a possible regulator of glycolysis. *IUBMB Life* 51:309–314, 2001
22. Du XL, Edelstein D, Rossetti L, Fantus IG, Goldberg H, Ziyadeh F, Wu J, Brownlee M: Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation. *Proc Natl Acad Sci U S A* 97:12222–12226, 2000
23. Sirover MA: New insights into an old protein: the functional diversity of mammalian glyceraldehyde-3-phosphate dehydrogenase. *Biochim Biophys Acta* 1432:159–184, 1999
24. Ito Y, Pagano PJ, Tornheim K, Brecher P, Cohen RA: Oxidative stress increases glyceraldehyde-3-phosphate dehydrogenase mRNA levels in isolated rabbit aorta. *Am J Physiol* 270:H81–H87, 1996
25. Berry MD, Boulton AA: Glyceraldehyde-3-phosphate dehydrogenase and apoptosis. *J Neurosci Res* 60:150–154, 2000
26. Eriksson UJ, Dahlstrom E, Larsson KS, Hellerstrom C: Increased incidence of congenital malformations in the offspring of diabetic rats and their prevention by maternal insulin therapy. *Diabetes* 31:1–6, 1982
27. Siman CM, Gittenberger-De Groot AC, Wisse B, Eriksson UJ: Malformations in offspring of diabetic rats: morphometric analysis of neural crest-derived organs and effects of maternal vitamin E treatment. *Teratology* 61:355–367, 2000
28. Wentzel P, Welsh N, Eriksson UJ: Developmental damage, increased lipid peroxidation, diminished cyclooxygenase-2 gene expression, and lowered PGE2 levels in rat embryos exposed to a diabetic environment. *Diabetes* 48:813–820, 1999
29. Eriksson UJ: Importance of genetic predisposition and maternal environment for the occurrence of congenital malformations in offspring of diabetic rats. *Teratology* 37:365–374, 1988
30. New DA: New DA. Whole-embryo culture and the study of mammalian embryos during organogenesis (Review). *Biol Rev Camb Philos Soc* 53:81–122, 1978
31. Wentzel P, Thunberg L, Eriksson UJ: Teratogenic effect of diabetic serum is prevented by supplementation of superoxide dismutase and N-acetylcysteine in rat embryo culture. *Diabetologia* 40:7–14, 1997
32. Lowry OH, Rosebrough. NJ, Farr AL, Randall RJ: Protein measurement with the folin phenol reagent. *J Biol Chem* 193:265–275, 1951
33. Kissane JM, Robins E: The fluorometric measurement of deoxyribonucleic acid in animal tissues with special reference to the central nervous system. *J Biol Chem* 233:184–188, 1958
34. Hinegardner RT: An improved fluorometric assay for DNA. *Anal Biochem* 39:197–201, 1971
35. Bergmeyer HU: *Methods of Enzymatic Analysis*. Weinheim, Germany, Verlag Chemie, 1983, p. 211–213
36. Mohamed AO, Ronquist G, al Bayoumi R: Increased membrane activity of glyceraldehyde 3-phosphate dehydrogenase in erythrocytes of patients with homozygous sickle cell anaemia. *Clin Chim Acta* 209:189–195, 1992

37. Ceriello A, Bortolotti N, Falleti E, Taboga C, Tonutti L, Crescentini A, Motz E, Lizzio S, Russo A, Bartoli E: Total radical-trapping antioxidant parameter in NIDDM patients. *Diabetes Care* 20:194–197, 1997
38. West IC: Radicals and oxidative stress in diabetes. *Diabet Med* 17:171–180, 2000
39. Shin CS, Moon BS, Park KS, Kim SY, Park SJ, Chung MH, Lee HK: Serum 8-hydroxy-guanine levels are increased in diabetic patients. *Diabetes Care* 24:733–737, 2001
40. Ceriello A: Nitrotyrosine: new findings as a marker of postprandial oxidative stress. *Int J Clin Pract Suppl* Jul:51–58, 2002
41. Myatt L, Kossenjans W, Sahay R, Eis A, Brockman D: Oxidative stress causes vascular dysfunction in the placenta. *J Matern Fetal Med* 9:79–82, 2000
42. Cederberg J, Siman CM, Eriksson UJ: Combined treatment with vitamin E and vitamin C decreases oxidative stress and improves fetal outcome in experimental diabetic pregnancy. *Pediatr Res* 49:755–762, 2001
43. Persson B: Prevention of fetal malformation with antioxidants in diabetic pregnancy. *Pediatr Res* 49:742–743, 2001
44. Cederberg J, Basu S, Eriksson UJ: Increased rate of lipid peroxidation and protein carbonylation in experimental diabetic pregnancy. *Diabetologia* 44:766–774, 2001
45. Lee AT, Plump A, DeSimone C, Cerami A, Bucala R: A role for DNA mutations in diabetes-associated teratogenesis in transgenic embryos. *Diabetes* 44:20–24, 1995
46. Lee AT, Reis D, Eriksson UJ: Hyperglycemia induced embryonic dysmorphogenesis correlates with genomic DNA mutation frequency in vitro and in vivo. *Diabetes* 48:371–376, 1999
47. Hiranruengchok R, Harris C: Diamide-induced alterations of intracellular thiol status and the regulation of glucose metabolism in the developing rat conceptus in vitro. *Teratology* 52:205–214, 1995
48. Ornoy A, Zaken V, Kohen R: Role of reactive oxygen species (ROS) in the diabetes-induced anomalies in rat embryos in vitro: reduction in antioxidant enzymes and low-molecular-weight antioxidants (LMWA) may be the causative factor for increased anomalies. *Teratology* 60:376–386, 1999
49. Zaken V, Kohen R, Ornoy A: Vitamins C and E improve rat embryonic antioxidant defense mechanism in diabetic culture medium. *Teratology* 64:33–44, 2001
50. Novotny MV, Yancey MF, Stuart R, Wiesler D, Peterson RG: Inhibition of glycolytic enzymes by endogenous aldehydes: a possible relation to diabetic neuropathies. *Biochim Biophys Acta* 1226:145–150, 1994
51. Du XL, Edelstein D, Dimmeler S, Ju Q, Sui C, Brownlee M: Hyperglycemia inhibits endothelial nitric oxide synthase activity by posttranslational modification at the Akt site. *J Clin Invest* 108:1341–1348, 2001
52. Eriksson UJ, Naeser P, Brolin SE: Increased accumulation of sorbitol in offspring of manifest diabetic rats. *Diabetes* 35:1356–1363, 1986
53. Hod M, Star S, Passonneau JV, Unterman TG, Freinkel N: Effect of hyperglycemia on sorbitol and myo-inositol content of cultured rat conceptus: failure of aldose reductase inhibitors to modify myo-inositol depletion and dysmorphogenesis. *Biochem Biophys Res Commun* 140: 974–980, 1986
54. Hashimoto M, Akazawa S, Akazawa M, Akashi M, Yamamoto H, Maeda Y, Yamaguchi Y, Yamasaki H, Tahara D, Nakanishi T, Nagataki S: Effects of hyperglycaemia on sorbitol and myo-inositol contents of cultured embryos: treatment with aldose reductase inhibitor and myo-inositol supplementation. *Diabetologia* 33:597–602, 1990
55. Loeken MR, Horal M: Regulation of transcription and morphogenesis by glucosamine: does hexosamine flux mediate the molecular effects of high glucose metabolism on embryogenesis? (Abstract). *Diabetes* 49 (Suppl. 1):A274, 2000
56. Eriksson UJ, Wentzel P, Minhas HS, Thornalley PJ: Teratogenicity of 3-deoxyglucosone and diabetic embryopathy. *Diabetes* 47:1960–1966, 1998
57. Hiramatsu Y, Sekiguchi N, Hayashi M, Isshiki K, Yokota T, King GL, Loeken MR: Diacylglycerol production and protein kinase C activity are increased in a mouse model of diabetic embryopathy. *Diabetes* 51:2804–2810, 2002
58. Wentzel P, Wentzel CR, Gareskog MB, Eriksson UJ: Induction of embryonic dysmorphogenesis by high glucose concentration, disturbed inositol metabolism, and inhibited protein kinase C activity. *Teratology* 63:193–201, 2001
59. Alcazar O, Qiu-yue Z, Gine E, Tamarit-Rodriguez J: Stimulation of islet protein kinase C translocation by palmitate requires metabolism of the fatty acid. *Diabetes* 46:1153–1158, 1997
60. Kim JR, Kwon KS, Yoon HW, Lee SR, Rhee SG: Oxidation of proteinaceous cysteine residues by dopamine-derived H2O2 in PC12 cells. *Arch Biochem Biophys* 397:414–423, 2002
61. Matthews RT, Ferrante RJ, Jenkins BG, Browne SE, Goetz K, Berger S, Chen IY, Beal MF: Iodoacetate produces striatal excitotoxic lesions. *J Neurochem* 69:285–289, 1997