Significance of Glutathione-Dependent Antioxidant System in Diabetes-Induced Embryonic Malformations

Hiroyuki Sakamaki, Shoichi Akazawa, Miwa Ishibashi, Kiyohiro Izumino, Hirofumi Takino, Hironori Yamasaki, Yoshihiko Yamaguchi, Shinji Goto, Yoshishige Urata, Takahito Kondo, and Shigenobu Nagataki

Hyperglycemia-induced embryonic malformations may be due to an increase in radical formation and depletion of intracellular glutathione (GSH) in embryonic tissues. In the past, we have investigated the role of the glutathione-dependent antioxidant system and GSH on diabetes-related embryonic malformations. Embryos from streptozotocin-induced diabetic rats on gestational day 11 showed a significantly higher frequency of embryonic malformations (neural lesions 21.5 vs. 2.8%, *P* < 0.001; nonneural lesions 47.4 vs. 6.4%, *P* < 0.001) and growth retardation than those of normal mothers. The formation of intracellular reactive oxygen species (ROS), estimated by flow cytometry, was increased in isolated embryonic cells of diabetic rats on gestational day 11. The concentration of intracellular GSH in embryonic tissues of diabetic pregnant rats on day 11 was significantly lower than that of normal rats. The activity of γ-glutamylcysteine synthetase (γ-GCS), the rate-limiting GSH synthesizing enzyme, in embryos of diabetic rats was significantly low, associated with reduced expression of γ-GCS mRNA. Administration of buthionine sulfoxamine (BSO), a specific inhibitor of γ-GCS, to diabetic rats during the period of maximal teratogenic susceptibility (days 6–11 of gestation) reduced GSH by 46.7% and increased the frequency of neural lesions (62.1 vs. 21.5%, *P* < 0.01) and nonneural lesions (79.3 vs. 47.4%, *P* < 0.01). Administration of GSH ester to diabetic rats restored GSH concentration in the embryos and reduced the formation of ROS, leading to normalization of neural lesions (1.9 vs. 21.5%) and improvement in nonneural lesions (26.7 vs. 47.4%) and growth retardation. Administration of insulin in another group of pregnant rats during the same period resulted in complete normalization of neural lesions (4.3 vs. 21.5%), nonneural lesions (4.3 vs. 47.4%), and growth retardation with the restoration of GSH contents. Our results indicate that GSH depletion and impaired responsiveness of GSH-synthesizing enzyme to oxidative stress during organogenesis may have important roles in the development of embryonic malformations in diabetes. *Diabetes* 48:1138–1144, 1999

From the First Department of Internal Medicine (H.S., M.I., K.I, H.T., H.Y., Y.Y., S.N.), the Unit of Metabolism/Diabetes and Clinical Nutrition (S.A.), and the Department of Pathological Biochemistry (S.G., Y.U., T.K.), Atomic Disease Institute, Nagasaki University School of Medicine, Nagasaki, Japan.

Address correspondence and reprint requests to Shoichi Akazawa, MD, Unit of Metabolism/Diabetes and Clinical Nutrition, Nagasaki University School of Medicine, Nagasaki 852-8501, Japan. E-mail: akazawa@net.nagasaki-u.ac.jp.

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BSO, buthionine sulfoxamine; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; γ-GCS, γ-glutamylcysteine synthetase; GPX, glutathione peroxidase; GSH, glutathione; GSSG, oxidized glutathione; PBS, phosphate-buffered saline; PSL, photostimulated luminescence; ROS, reactive oxygen species; SOD, superoxide dismutase; STZ, streptozotocin.

The incidence of congenital malformations in neonates of diabetic mothers is higher than that in the general neonatal population (1–3). These congenital malformations, which may occur before the 7th week of gestation, include the caudal regression syndrome; a variety of central nervous system deformities such as anencephaly, spina bifida, and hydrocephalus; and cardiac anomalies (4). Results of several animal experiments suggest that these congenital malformations result from diabetes-associated alterations in serum factors in the intrauterine environment (5–9). Several serum factors are suspected to adversely affect the normal development of cultured embryos. These include hyperglycemia (10–12), hypoglycemia (13–15), hyperketonemia (16–20), hypoinsulinemia (21), and excess branched chain amino acid (22) and somatomedine inhibitor (19,20,23).

Although the exact biological mechanisms of teratogenicity are not known, several teratogenic factors have been identified in the hyperglycemia-induced malformations, including sorbitol accumulation (24), myo-inositol deficiency (25), arachidonic acid deficiency (26,27), and altered prostaglandin metabolism (28). The role of reactive oxygen species (ROS) in embryonic dysmorphogenesis in diabetic pregnancy has been suggested based on the protective role of the radical scavenger enzymes against excess glucose and other oxidative substrates (ketones, intermediary metabolites, branched chain amino acids) (29–31). In the rat embryo during the major period of organogenesis (days 9 to 10 of gestation), functional analysis of the scavenger enzymes, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX), indicates their immaturity at that stage, in agreement with the low activity of mitochondrial oxidative pathways (32,33). We have reported that oxygen-induced embryonic malformations were caused by the production of ROS in the presence of an immature free radical scavenger system (34).

Glutathione (GSH) is present in most mammalian cells and plays an important role in cellular defense against oxidative stress by reducing protein disulfides and other cellular molecules. It also acts as a scavenger of free radicals of ROS (35). GSH is synthesized intracellularly by two GSH-synthesizing enzymes, γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase. γ-GCS catalyzes the rate-limiting step of GSH synthesis (36). In many cells, the GSH redox cycle is catalyzed by both glutathione peroxidase and glutathione reductase. During oxidative stress, the reduced form of GSH is converted by glutathione peroxidase to oxidized glutathione

(GSSG). We have reported the presence of low GSH and high GSSG concentrations in erythrocytes of diabetic patients (37) and in endothelial cells of diabetic rabbits (38). Furthermore, we have shown that γ-GCS is reduced specifically in human erythrocytes of diabetic patients (37) and in endothelial cells cultured under hyperglycemic conditions (39).

Using the whole embryo culture system, we have shown that embryos cultured under hyperglycemic conditions showed increased formation of ROS and depletion of GSH contents, as well as reduced synthesis of GSH (40). To further extend these studies, we investigated in the present study the pathologic significance of GSH metabolism in embryos of diabetic mothers. To this effect, we examined whether maternal diabetes is associated with diminished GSH contents, and if so, whether correction of such deficiency by GSH during the critical period of organogenesis could prevent growth retardation and malformation. In addition, we also examined the effects of further reductions in GSH by administering buthionine sulfoxamine (BSO), a specific inhibitor of γ-GCS, to pregnant diabetic rats during the same period, inducing increased frequency of embryonic malformations.

RESEARCH DESIGN AND METHODS

Animals and induction of diabetes. Virgin female Wistar rats obtained from Shizuoka Laboratory Animal Center (Shizuoka, Japan) were housed at 22°C in a 12 h/12 h light-dark environment (light on at 7:00 A.M. and off at 7:00 P.M.) and provided with standard laboratory diet (Oriental Yeast, Tokyo) and tap water ad libitum. The experimental protocol was approved by the Animal Care and Use Committee at our institution. Experimental diabetes was induced by intraperitoneal injection of 65 mg/kg body wt streptozotocin (STZ) (Sigma, St. Louis, MO), dissolved in 0.1 mol/l sodium citrate buffer (pH 4.7), into healthy female rats. Induction of diabetes was confirmed by the presence of plasma glucose concentration >20 mmol/l at 24 h and 48 h after STZ injection. Diabetic female rats injected with STZ at least 3 days earlier and normal female rats were mated overnight with nondiabetic males of the same strain, and pregnancy was timed at midnight preceding the morning when sperm was present in vaginal smears. Blood samples were taken from tail veins of rats at 10:00 A.M. daily from day 0 to day 11 of gestation, and glucose levels were measured by the glucose oxidase method.

Treatment with GSH or insulin. Diabetic pregnant rats were treated with an intraperitoneal injection of GSH ester (Sigma) dissolved in Hanks' buffer solution at a dose of ~0.5 mmol/kg body wt or with a similar dose of BSO (Sigma), a specific inhibitor of γ-GCS, once daily from day 6 to 11 of gestation. Another group of diabetic pregnant rats was treated with insulin (from bovine pancreas; Wako Pure Chemical Industries, Tokyo) from day 6 to day 11 of gestation, by inserting an Alzet osmotic minipump (no. 2ML2; Alza, Palo Alto, CA) in the dorsal neck region. For this purpose, the rat was anesthetized with ether and a skin incision was made in the neck region. After establishing a pocket in the subcutaneous tissue, the pump was inserted into the pocket and the incision was closed with three or four sutures. The osmotic pump had a total volume of 2 ml and released 5 μl of solution per hour. The pump was filled with approximately 54 U/ml of insulin, and the rats received 0.27 U insulin/h at a constant rate. The rats that showed glucose levels >10 mmol/l after day 7 of gestation were eliminated from the insulin-treated diabetic group. On day 11 of gestation (at noon), pregnant female rats were killed by cervical dislocation. The uterus was excised rapidly and examined for the number of resorptions and fetuses, and individual embryo units consisting of the embryo and extraembryonic membranes (yolk sac and amnion) were freed from the surrounding decidua under a dissecting microscope. The embryos were frozen at –80°C until examined. The overall growth and differentiation of the embryo were carefully assessed by direct measurement of crown–rump length and somite number, respectively. Dysmorphogenesis was evaluated by visual inspection using a detailed checklist (34,40). Such assessment was performed by an individual who was unaware of the maternal condition. Malformations were divided into two categories. Major lesions, representing specific lesions of neural plate development, included open neural tube, fusion of anterior and posterior neural folds, and brain anomalies. Minor lesions included all extraneural lesions, such as abnormalities of axial rotation, lesions involving the optic and otic vesicles, heart size, pericardial cavity, and somite.

Sample preparation. Embryos were homogenized in lysis buffer containing 10 mmol/l NaH_2PO_4, 1.0 mmol/l EDTA, 250 mmol/l sucrose, 150 mmol/l KCl, 1.0 mmol/l phenylmethylsulfonyl fluoride and centrifuged at 15,000 rpm for 15 min. The supernatants were used for assays as described below.

Measurement of GSH. Samples were treated with the same volume of ice-cold 10% trichloroacetic acid immediately after collection. The intracellular concentration of GSH was measured photometrically using the neutralized supernatant of trichloroacetic acid extract, as described by Beutler (41).

Enzyme assay. The activity of γ-GCS was estimated using L-α-amino-*N*-butyric acid and [^{32}P]ATP (New England Nuclear, Boston, MA) as substrates, as described previously (42). The activities of glutathione peroxidase and glutathione S-transferase were estimated photometrically as described by Beutler (41). Protein concentration was estimated according to the method of Lowry et al. (43) using bovine serum albumin as a standard. One unit of enzyme activity was expressed as 1 mol substrate change per minute.

Northern blot analysis. The γ-GCS probe (267 bp, corresponding to 54–320 of rat kidney γ-GCS) was [^{32}P]-radiolabeled using the random primer technique. The methods used for the isolation of cytoplasmic RNA and Northern blot analysis were essentially based on those described by Sambrook et al. (44). Cytoplasmic RNA isolated from embryos was subjected to electrophoresis in 1% agarose gel containing 0.6 mol/l formaldehyde, transferred to nylon membrane filters, and later hybridized with [^{32}P]-labeled nick-translated probe for γ-GCS. After stripping these probes, the papers were rehybridized with [^{32}P]-labeled glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe. The relative radioactivity of the autographed filters was analyzed using a Fuji Bio-Analyzer BAS-2000 (Fuji Photo Film, Tokyo) (45). The relative radioactivity was expressed as photostimulated luminescence (PSL) corrected for the intensity of β-actin.

Cell isolation. We measured intracellular levels of ROS in embryonic cells on day 11 of gestation. For this purpose, embryonic cells were isolated by a modification of the method described by Guguen-Guillozo et al. (46). Briefly, the embryo was placed in 0.5% collagenase in RPMI and incubated at 37°C for 45 min with gentle shaking. The embryonic cells were dispersed using a transfer pipette. RPMI 1640 (12 ml) was then added, and the cells were centrifuged at 1,000 rpm for 5 min. The isolated cells were filtered through a nylon mesh and resuspended in phosphate-buffered saline (PBS) (9 parts of 0.154 mol/l NaCl and 1 part of 0.1 mol/l NaH_2PO_4/Na_2HPO_4, pH 7.4) containing 5 mmol/l glucose and 1 mmol/l $MgCl_2$. The cells were then counted in a hemocytometer, and viability was assessed by trypan blue dye exclusion.

Intracellular ROS. Chemiluminescence was measured using a FACScan (Becton Dickinson, Oxford, U.K.) based on a modified method of Bass et al. (47). For this purpose, isolated embryonic cells were resuspended in PBS buffer and incubated with 5 μmol/l 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Eastman Kodak, Rochester, NY), a substrate that reacts with hydrogen peroxide, dissolved in DMSO at 37°C for 30 min with continuous shaking. The cells were transferred

TABLE 1
Maternal body weight on days 0 and 11 of gestation, weight gain during gestation during the same period, and resorption rates on day 11 of gestation

	Mothers (*n*)	Body weight (g)		Weight gain (%)	Resorption (%)
		Day 0	Day 11		
Control	26	248.5 ± 4.4	296.7 ± 5.3	19.5 ± 1.0	2.9
Diabetes	27	248.3 ± 3.3	262.0 ± 4.0*	5.6 ± 1.1*	17.6*
Diabetes + insulin	23	242.0 ± 3.9	296.3 ± 4.0	22.7 ± 1.4	11.9*
Diabetes + GSH	9	245.0 ± 6.9	260.6 ± 5.9*	6.6 ± 1.9*	11.0*
Diabetes + BSO	9	240.7 ± 10.7	244.3 ± 8.4*	1.8 ± 1.2*	63.6*†
Control + BSO	5	243.0 ± 10.1	276.0 ± 13.5	13.5 ± 1.7	4.7

Data are means ± SE. **P* < 0.01 vs. control; †*P* < 0.01 vs. diabetes.

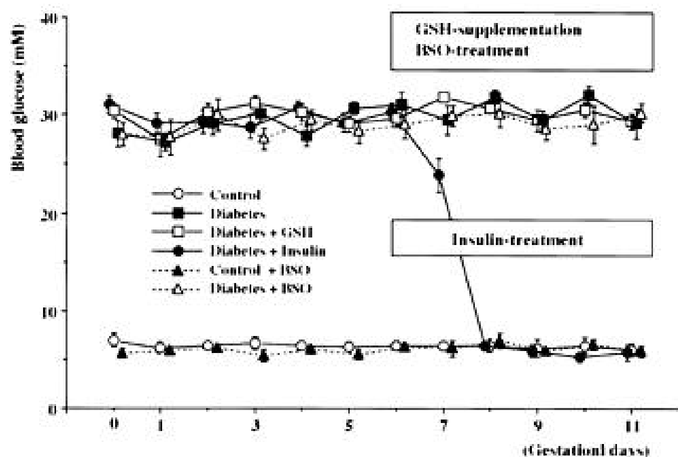

FIG. 1. Serial changes in blood glucose levels during the first 11 days of gestation in normal (control), diabetic, GSH ester-treated diabetic, insulin-treated diabetic, BSO-treated normal, and BSO-treated diabetic pregnant rats. An insulin-releasing minipump was implanted subcutaneously in each insulin-treated diabetic rat on day 6 of gestation, and GSH- or BSO-treated rats were injected intraperitoneally with GSH ester or BSO once a day from day 6 to day 11 of gestation.

to an ice bath and the formation of 2′,7′-dichlorofluorescein was analyzed by flow cytometry. Estimation of fluorescence with an excitation wavelength at 488 nm and emission at 530 nm was determined with a photomultiplier gain setting such that all intensities of cellular fluorescence were recorded between 10 and 90% of the full scale of 1,000 channel resolution of the instrument. Cell gating was used because of the mixed-cell population, and 10,000 cells were examined in each sample. The formation of ROS was expressed as relative fluorescence intensity (fluorescence index).

Statistical analysis. Values are expressed as mean ± SE. Statistical analyses were performed by two-tailed Student's t test and Wilcoxon's rank-sum test for nonparametric analysis of unpaired data. Intergroup differences in the frequency of embryonic morphologic lesions were assessed by χ^2 analysis. A P value <0.05 was considered significant.

RESULTS

Changes in body weight. The mean body weight of each group of pregnant rats at 0 and 11 days of gestation is shown in Table 1. The baseline body weight was not different among the groups. However, the mean weight gain (%) in diabetic rats during the first 11 days of pregnancy was significantly lower than that in normal rats, except for insulin-treated diabetic rats, which had a normal rate of weight gain.

Changes in blood glucose concentrations. Figure 1 shows the serial changes in blood glucose concentrations for each group. The concentration of blood glucose in STZ-induced diabetic rats measured on each day (range 25–33 mmol/l) was not different from that of GSH ester–supplemented diabetic rats and BSO-administered diabetic rats. Blood glucose concentrations in insulin-treated diabetic rats decreased dramatically to normal glycemic levels following the use of the subcutaneously implanted minipump on day 6 of gestation, maintaining normal levels from day 8 to 11 of gestation.

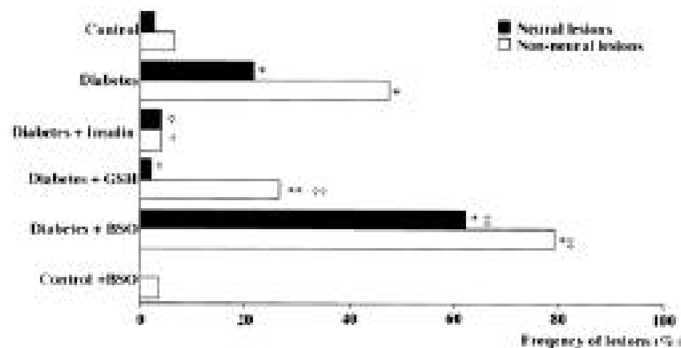

FIG. 2. The frequency of embryonic abnormalities in control, STZ-induced diabetic, insulin-treated diabetic, GSH ester–treated diabetic, BSO-treated diabetic, and BSO-treated normal pregnant rats on embryonic development. Neural lesions included neural plate anomalies, e.g., open neural tube, fusion of anterior and posterior neural folds, and brain anomalies. Nonneural lesions included extraneural lesions. *P < 0.001, **P < 0.01 vs. control; †P < 0.001, ‡P < 0.01, ††P < 0.05 vs. diabetes.

Frequency of congenital malformations. Compared with embryos of normal mothers, those from diabetic rats showed a significantly higher frequency of neural (21.5 vs. 2.8%, P < 0.001) and nonneural (47.4 vs. 6.4%, P < 0.001) malformations (Fig. 2) and growth retardation according to crown–lump length and somite number (Table 2). The resorption rate of embryos of diabetic rats was significantly higher than in of normal pregnant rats (Table 1).

Changes in GSH. The concentration of intracellular GSH in embryos of diabetic rats was 23.4% less than in those of normal rats (26.6 ± 2.4 vs. 34.7 ± 2.1 nmol/mg protein, P < 0.05; Fig. 3) and the activity of γ-GCS, the rate-limiting GSH synthesizing enzyme, in those embryos was also low (P < 0.05; Table 3). γ-GCS mRNA expression was significantly lower in embryos of diabetic rats than in those of normal rats (Fig. 4). On the other hand, GPX and GST activities in embryos of diabetic rats were significantly higher than in those of normal rats (Table 3).

Changes in intracellular ROS. Estimates of intracellular ROS from collagenase-treated isolated embryonic cells in each group are shown in Fig. 5. The production of intracellular ROS in embryos from diabetic rats was markedly increased, with a shift in the peak intensity to the right on the fluorescence curve (mean fluorescence index, diabetic 610 ±

TABLE 2

Effects of STZ-induced diabetes, insulin treatment, GSH ester treatment and administration of BSO on growth in embryos on day 11 of gestation compared with normal pregnant rats

	Embryos (n)	Crown-rump length (mm)	Somite (n)
Control	116	3.78 ± 0.04	29.4 ± 0.1
Diabetes	114	3.03 ± 0.06*	25.7 ± 0.4*
Diabetes + insulin	113	3.73 ± 0.06	29.2 ± 0.2
Diabetes + GSH	104	3.28 ± 0.04†‡	27.9 ± 0.2†‡
Diabetes + BSO	58	2.68 ± 0.06*§	23.8 ± 0.5*§
Control + BSO	69	3.78 ± 0.03	28.8 ± 0.2

Data are means ± SE. *P < 0.0001 vs. control; †P < 0.01 vs. control; ‡P < 0.01 vs. diabetes; §P < 0.05 vs. diabetes.

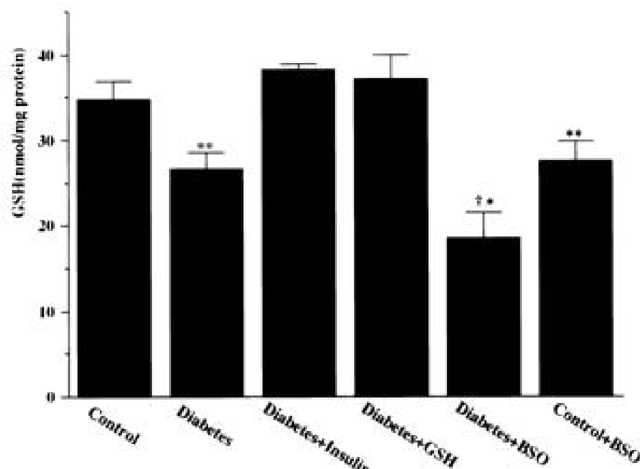

FIG. 3. Intracellular GSH concentration in embryos from normal, untreated diabetic, insulin-treated diabetic, GSH ester–treated diabetic, BSO-treated diabetic, and BSO-treated normal pregnant rats. $*P < 0.01$, $**P < 0.05$ vs. control; $†P < 0.05$ vs. diabetes.

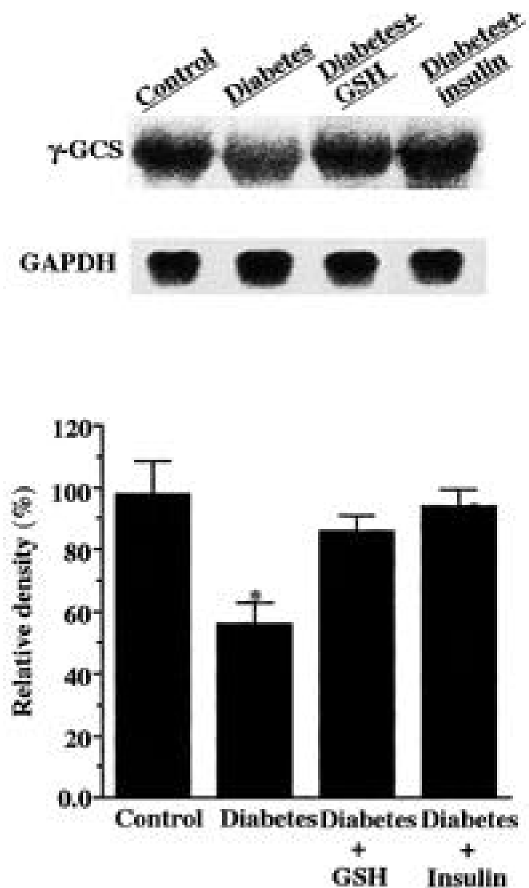

FIG. 4. Northern blot analysis of γ-GCS mRNA in embryos of normal, untreated diabetic, GSH ester–treated diabetic, and insulin-treated diabetic pregnant rats. Total cytoplasmic RNA (40 μg) isolated from embryos was electrophoresed in 1% agarose gel, transferred to nylon membrane filters, and hybridized with [^{32}P]-labeled nick-translated probe for γ-GCS. Densitometric data of γ-GCS mRNA ($n = 3$) are normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RNA. The level of mRNA in embryos from normal pregnant rats was expressed as 100%. $*P < 0.05$ vs. control.

110 vs. control 105 ± 21/10^5 cells, $P < 0.05$). Intracellular ROS formation was also measured by a method using dihydrorhodamine, a converted green fluorescence compound after reaction with intracellular ROS (48). The results using this method were similar to those by DCFH-DA assay (fluorescence index, diabetic 34.9 ± 5.0 vs. control 26.5 ± 2.9/10^4 cells, $P < 0.05$).

Effects of GSH. To examine the effect of GSH on embryonic growth, development, and organogenesis, GSH ester was administered daily by intraperitoneal injection in diabetic pregnant rats. This resulted in improvement in the frequency of neural lesions (1.9 vs. 21.5%, $P < 0.001$), nonneural lesions (26.7 vs. 47.4%, $P < 0.05$), and growth retardation (Table 2) in their embryos (Fig. 2). In addition, such treatment resulted in a complete restoration of intracellular GSH concentration in embryos from GSH-treated diabetic rats to normal concentrations (37.0 ± 3.0 vs. 26.6 ± 2.0 nmol/mg protein; Fig. 3). A similar pattern of improvement in γ-GCS activity and the level of its mRNA was observed (Table 3, Fig. 4). As shown in Fig. 5, GSH treatment reduced the formation of intracellular ROS in the isolated embryonic cells to levels comparable to normal (160 ± 32/10^5 cells, $P < 0.05$, vs. diabetic).

To further examine the effect of reductions in intracellular GSH concentrations in the diabetic state on embryonic growth and development, we administered BSO to diabetic pregnant rats during the same period. BSO administration resulted in a marked increase in the frequency of neural (62.1 vs. 21.5%, $P < 0.01$) and nonneural (79.3 vs. 47.4%, $P < 0.01$) lesions (Fig. 2), growth retardation (Table 2), and resorption (Table 1) compared with those observed in embryos of diabetic rats. The activity of γ-GCS and concentration of GSH in embryos of BSO-treated diabetic rats were further diminished compared with untreated diabetic rats (4.31 ± 2.1 vs. 11.4 ± 1.7 μU/mg protein, $P < 0.05$; 18.52 ± 3.0 vs. 26.6 ± 2.0 nmol/mg protein, $P < 0.05$, respectively) (Table 3, Fig. 3).

TABLE 3
Intracellular activities of GPX, GST, and γ-GCS

	Experiment (n)	GPX	GST	γ-GCS
Control	7	503.7 ± 57.9	12.5 ± 0.6	22.9 ± 3.5
Diabetes	7	850.9 ± 129.8†	16.8 ± 1.1*	11.4 ± 1.7†
Diabetes + insulin	6	601.1 ± 82.3	12.5 ± 0.9	17.1 ± 5.1
Diabetes + GSH	6	812.8 ± 46.3*	16.6 ± 1.0*	18.5 ± 5.0
Diabetes + BSO	5	1,223.0 ± 170.7*	24.3 ± 2.6*‡	4.32 ± 2.1‡
Control + BSO	4	480.0 ± 44.6	16.2 ± 0.6†	8.61 ± 1.8†

Data are means ± SE. GPX, GST, and γ-GCS activities are expressed in microunits per milligram protein. $*P < 0.01$, $†P < 0.05$ vs. control, $‡P < 0.05$ vs. diabetes.

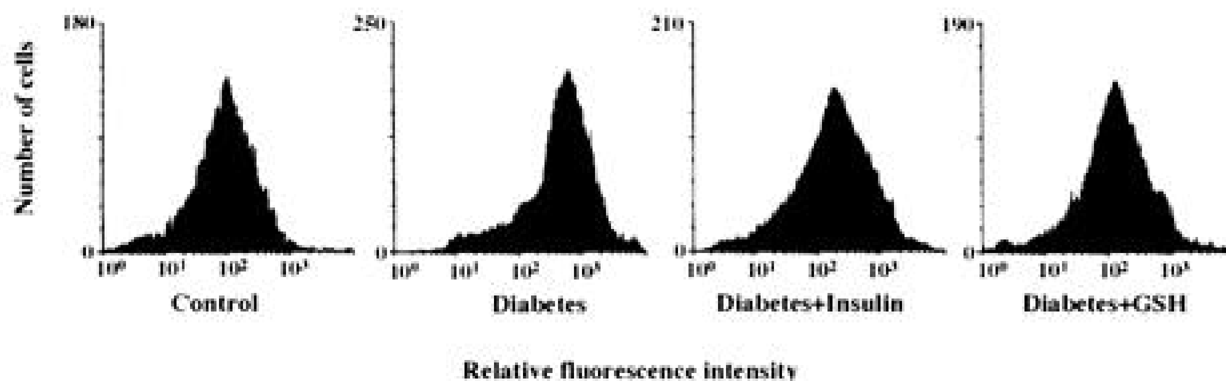

FIG. 5. Intracellular formation of ROS. Fluorescence distribution of DCFH-DA oxidation in embryos of normal, untreated diabetic, insulin-treated diabetic, and GSH ester–treated diabetic pregnant rats. Embryos were incubated for 30 min with 5 µmol/l DCFH-DA followed by measurement of the fluorescence index. Histograms represent the number of cells as a function of relative fluorescence intensity. The figure is a representative example of three experiments with similar results.

However, when BSO was administered to normal pregnant rats, no adverse effects on the development and growth of embryos were observed, although it resulted in a significant reduction in the activity of γ-GCS and concentration of GSH in their embryos (8.61 ± 1.8 vs. 22.9 ± 3.5 µU/mg protein, $P < 0.05$; 27.5 ± 2.4 vs. 34.73 ± 2.1 nmol/mg protein, $P < 0.05$, respectively) compared with normal control rats (Tables 2 and 3, Figs. 2 and 3).

Effect of insulin. Insulin treatment resulted in a complete normalization of dysmorphogenesis (neural lesions 4.3%, nonneural lesions 4.3%) and crown–lump length and somite number (Fig. 2, Table 2). The concentration of intracellular GSH in embryos from insulin-treated rats was restored to normal (38.1 ± 0.8 nmol/mg protein). There were no significant differences in the activity of γ-GCS, GPX, or GST in embryos of insulin-treated and normal pregnant rats (Table 3). The expression of γ-GCS mRNA in embryos of insulin-treated diabetic rats was not different from that of normal rats (Fig. 4). These embryos showed a decrease in the production of intracellular ROS to values comparable to normal levels (220 ± $24/10^5$ cells, $P < 0.05$, vs. diabetic) (Fig. 5). The mean activity of γ-GCS in these embryos significantly decreased compared with BSO-untreated diabetic and BSO-untreated normal pregnant rats (4.31 ± 2.1 vs. 11.4 ± 1.7 µU/mg protein, $P < 0.05$; 8.61 ± 1.8 vs. 22.9 ± 3.5 µU/mg protein, $P < 0.05$, respectively). These results confirmed that the activity of γ-GCS was inhibited by administration of BSO and that it was similar to the level of intracellular GSH concentration.

DISCUSSION

We investigated in the present study the importance of the glutathione-dependent antioxidant system and the effect of GSH on diabetic embryonic malformations. The major findings of our study were that reduced concentrations of embryonic intracellular GSH and increased formation of ROS were associated with increased frequency of malformations in embryos of diabetic mothers. Furthermore, the activity of γ-GCS, a rate-limiting GSH synthesizing enzyme, and its mRNA in these embryos was significantly reduced. A direct evidence for the involvement of GSH in the development of neural tube malformations was provided by administration of BSO, a specific inhibitor of γ-GCS, or GSH ester to pregnant diabetic rats. BSO caused a further reduction in intracellular GSH and consequently increased the number of embryos with neural tube defects. Restoration of GSH contents in embryos of diabetic mothers normalized embryonic neural tube development. Our results indicate that GSH depletion during high levels of activity of oxidative stress is instrumental in diabetes-induced embryonic malformations.

Previous studies have shown that embryonic malformations in rat embryos exposed to hyperglycemia in vitro were reduced following the addition of free radical scavenging enzymes (e.g., SOD, catalase, GPX, and *N*-acetylcysteine) to culture media (29,30). Studies from our laboratories have also shown that in rat embryos cultured under hyperglycemic conditions (72 mmol/l glucose) during the first 9–11 days of gestation showed increased generation of ROS and depletion of GSH (40). Restoration of GSH levels in embryonic tissue by exogenously administered GSH ester reduced ROS formation and prevented growth retardation and embryonic dysmorphogenesis (40).

Studies have indicated a protective effect of orally administered antioxidants, such as vitamin E and butylated hydroxytoluene, in pregnant diabetic rats (49,50), further implicating the generation of ROS or decreased antioxidative status in the teratogenic process of diabetic pregnancy. The results of the present study also showed increased ROS formation in embryonic cells isolated from diabetic rats, as estimated by DCFH-DA, as substrates that reacted with hydrogen peroxide (Fig. 5), although it is possible that we may have underestimated the levels of ROS, since unstable ROS might decay during the manipulation. High serum levels of oxidative substrates such as glucose, ketone bodies, intermediary metabolites of glucose, and branched chain amino acids, could overload the mitochondria and contribute to the generation of free radicals (22). In this regard, morphologic changes suggestive of mitochondrial overload (e.g., high-amplitude swelling, loss of matrix density, and disturbed crystals) are usually detected at days 10 and 11 of gestation in embryos of diabetic rats and in embryos cultured with D-glucose, pyruvate, and metabolites of leucine and α-keto isocaproate (51). The oxidative stress may arise from oxidative degradation of glycated protein, monosaccharide auto-oxidation, and lipid peroxidation in the embryo. Che et al. (52) used the method of DCFH-DA assay and demonstrated that ROS were generated through glycation reaction. They revealed that 3-deoxyglucosone, which is a major and highly reactive intermediate in the glycation reaction, caused significant increase in intra-

cellular ROS. 3-Deoxyglucosone has been shown to be increased in the smooth muscle cells of blood vessels under diabetic conditions (53) and in embryos cultured in hyperglycemic conditions (54).

In the presence of a low activity of mitochondrial oxidative glucose metabolism during early organogenesis, the activity of the scavenging enzymes is also low during this period. Preliminary results from our laboratory using rat embryos at days 9–10 have shown an extremely low catalase activity and diminished GSH contents and activities of GSH-related enzymes (5–10% of those measured in the fetus; S.A., M.I., unpublished observations). In this study, we demonstrated that GSH content in embryos of diabetic mothers was more significantly reduced in streptozotocin-induced diabetic rats compared with control rats.

The level of intracellular GSH is maintained through reduction of GSSG by NADPH and de novo GSH synthesis (35). Supply of NADPH is very low in rat embryos from diabetic mothers because of the low activity of the hexose monophosphate shunt during early organogenesis (33) and the increased activity of aldose reductase, which requires NADPH as a cofactor (55). Based on these findings, GSH regeneration by GSSG conversion coupled with the reaction of NADPH to NADP is theoretically insufficient for the maintenance of intracellular GSH during organogenesis under diabetic conditions. De novo synthesis of GSH is accomplished through a two-step process catalyzed by γ-GCS and GSH synthetase. γ-GCS is the rate-limiting step in GSH synthesis, and previous studies have demonstrated that elevation of GSH concentration is dependent on increased γ-GCS activity and its mRNA level (56–58). Our results also showed that the concentration of GSH and activity of γ-GCS were further reduced in embryos of diabetic rats and did not increase in response to oxidative stress, while other GSH-related enzymes were significantly increased compared with controls (Table 3).

We also studied the effects of BSO-induced reductions in GSH contents on neural malformations. Administration of BSO to diabetic pregnant mothers further reduced intracellular GSH content by 46.7% (Fig. 3), resulting in more severe malformations. On the other hand, administration of BSO to normal pregnant rats did not have adverse effects on embryogenesis by visual inspection, despite the reduction (20.9%) of intracellular GSH content. Inhibition of cellular antioxidants in aerobic cells that generate highly ROS renders the organism more susceptible to oxygen toxicity, while in anaerobic cells, which generate little ROS, no toxic cell injury is produced even by further reductions of antioxidant components (59). However, it should be noted that toxic effects of BSO in normal pregnancy cannot be ruled out, because specific organs for dysmorphogenesis were evaluated visually.

We have reported similar phenomena in the mechanism of hyperoxygen-induced embryonic malformations (34). We found that embryos exposed to higher oxygen in culture media had increased ROS formation, with a significant decrease of GSH concentration, and addition of BSO to culture media further increased embryonic malformations, with further decrease of GSH. On the other hand, addition of BSO to culture media exposed to low oxygen did not cause malformation (34). That study, together with the present article, indicated that GSH depletion and impaired responsiveness of GSH-synthesizing enzyme to oxidative stress were important factors causing embryonic malformation.

The present results showed that intraperitoneal administration of GSH ester in diabetic pregnant rats reduced the formation of ROS, normalized GSH contents of embryonic cells, and diminished the rate of development of neural lesions. It should be noted, however, that the administration of GSH did not completely normalize nonneural lesions and growth retardation, indicating that other factors—e.g., somatomedin inhibitor (19,23), arachidonic acid deficiency (26), myoinositol deficiency (25)—might also be involved in diabetic embryopathy.

In conclusion, our results show that the concentration of GSH and the activity of its rate-limiting enzyme, γ-GCS, were significantly reduced in embryos of diabetic rats. Furthermore, the frequency of neural tube defects in embryos of diabetic rats correlated with intracellular GSH concentration. Our results indicate that in the presence of oxidative stress, GSH depletion may play an important role in the development of embryonic malformation in diabetic pregnancy.

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