Teratogenicity of 3-Deoxyglucosone and Diabetic Embryopathy

Ulf J. Eriksson, Parri Wentzel, Harjit S. Minhas, and Paul J. Thornalley

The increased rate of embryonic dysmorphogenesis in diabetic pregnancy is correlated with the severity and duration of the concurrent hyperglycemia during early gestation. Whole embryo culture was used to investigate a possible association of hyperglycemia-induced disturbances of embryo development with tissue levels of the three α-oxoaldehydes: glyoxal, methylglyoxal, and 3-deoxyglucosone (3-DG). Rat embryos exposed to high glucose levels in vitro showed severe dysmorphogenesis and a 17-fold increased concentration of 3-DG compared with control embryos cultured in a low glucose concentration. Exogenous 3-DG (100 μmol/l) added to the medium of control cultures yielded an increased embryonic malformation rate and a 3-DG concentration similar to that of embryos cultured in high glucose. Addition of superoxide dismutase (SOD) to the culture medium decreased the malformation rates of embryos exposed to either high glucose or high 3-DG levels, but it did not decrease the high embryonic 3-DG concentrations caused by either agent. Our results implicate the potent glycating agent 3-DG as a teratogenic factor in diabetic embryopathy. In addition, the anti-teratogenic effect of SOD administration appears to occur downstream of 3-DG formation, suggesting that 3-DG accumulation leads to superoxide-mediated embryopathy. *Diabetes* 47:1960–1966, 1998

There is a threefold increased risk of embryo malformations in the early stages of pregnancy in diabetes (1,2). The etiology of this embryopathy is currently unknown. Improved glycemic control from intensive metabolic care of the pregnant diabetic mother decreases the risk of embryo malformation (3–5), but perinatal mortality in diabetic pregnancy is still twice that of the normal population (6), making further search for etiologic mechanisms imperative.

Increased rates of congenital malformations have been found in the children of diabetic women (1,6), in offspring of animals with experimentally induced diabetes (7–13), and in embryos kept under diabetes-like conditions in vitro (7,8,14–27). Hyperglycemia has been identified as a distinct teratogenic agent, since high HbA_{1c} values in early diabetic pregnancy predict increased risk for congenital malformation (5,28–30). Experimental results support this notion, since high glucose levels in vivo (31) and in vitro (7,8,14,15,17–27) cause embryonic maldevelopment. On the other hand, moderately increased serum glucose levels in pregnant diabetic women are not statistically associated with an increased risk for congenital malformation (32). Furthermore, in experimentally diabetic rats, factors other than maternal glucose levels also correlate with embryonic dysmorphogenesis (31), and serum from diabetic rats retains teratogenic activity in vitro despite normalization of the glucose levels (26,33). In vitro studies show that high concentrations of not only glucose (14,15) but also β-hydroxybutyrate (16,23) and branched-chain amino acids (31) (α-ketoisocaproate [23]) induce embryo malformation, thereby providing support for the notion of a multifactorial mechanism behind the embryonic dysmorphogenesis in diabetic pregnancy (33–36).

Accepting the view of hyperglycemia as the principal teratogen with several supporting and predisposing factors contributing to the diabetes-induced disruption of embryogenesis, a number of studies have attempted to block the teratogenic activity of maternal diabetes in vivo (7,10,12,37–47) and of high glucose concentration in vitro (7,18–21,23–27,48–50). Excluding insulin therapy (37,38), most pronounced antiteratogenic effects have been reported by authors using agents with antioxidative action in vivo (12,39–44,46,47) and in vitro (21,23–27,46). Thus, dietary supplements of butylated hydroxytoluene (39,46), vitamin E (40–42,46,47), and vitamin C (43) and overexpression of superoxide dismutase (SOD) (12) decrease the incidence of teratogenesis in experimental diabetes in vivo. Furthermore, addition of *N*-acetylcysteine (26,27), glutathione monoester (25), SOD (21,23,26,27), catalase (21), and glutathione peroxidase (21) and use of embryos transgenic for SOD (24) diminishes embryonic malformation induced by a high glucose concentration in vitro.

A few other agents are able to decrease hyperglycemia/diabetes-induced teratogenesis. Thus, arachidonic acid supplementation diminishes the teratogenicity of diabetes in vivo (7,44) and a high glucose concentration in vitro (7,18,19,27), and the reaction product, prostaglandin E_2, exerts similar effects on embryos exposed to high glucose (19,27) and/or diabetic serum (49) in vitro. Likewise, inositol supplementation in vitro (17,19,20,22) and in vivo (10,45) diminishes the teratogenic effects of a high glucose concentration or maternal diabetes.

There is evidence to suggest that these metabolic pathways in the pathogenesis of embryonic dysmorphogenesis are interrelated and affect each other (27,51). In addition, metabolic factors related to the early steps of hyperglycemia

From the Department of Medical Cell Biology (U.J.E., P.W.), University of Uppsala, Uppsala, Sweden; and the Department of Biological Sciences (H.S.M., P.J.T.), University of Essex, Colchester, Essex, U.K.

Address correspondence and reprint requests to Dr. Ulf J. Eriksson, MD, PhD, Department of Medical Cell Biology, University of Uppsala, Biomedicum, P.O. Box 571, SE-751 23 Uppsala, Sweden. E-mail: ulf.eriksson@medcellbiol.uu.se.

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AGE, advanced glycation end product; 3-DG, 3-deoxyglucosone; RAGE, receptor for advanced glycation end product; SOD, superoxide dismutase.

may predispose diabetic subjects to the other events caused by the disease (27). One particular process, the formation of the end-stage adducts of the reactions of glucose and glucose-derived metabolites with proteins and nucleotides, advanced glycation end products (AGEs), may have a role in diabetic teratogenesis. Of special interest are the observations that oxidative stress may potentiate AGE formation (11,52–54), and also that the reverse process is of importance, i.e., AGEs stimulate reactive oxygen species formation (55,56). Furthermore, physiological α-oxoaldehyde precursors of AGEs—glyoxal, methylglyoxal, and 3-deoxyglucosone (3-DG)—are increased in several tissues in the diabetic state (57–59).

In the present work, we aimed to elucidate whether the teratogenicity of a diabetic environment may be channeled via an accumulation of α-oxoaldehydes, which could lead to the formation of advanced glycated proteins, which subsequently would disturb embryonic development. The putative involvement of α-oxoaldehydes in glucose-induced embryo malformation was investigated by measuring the concentrations of 3-DG, glyoxal, and methylglyoxal in rat embryos exposed to different glucose concentrations and by determining the teratogenicity of an increased α-oxoaldehyde concentration in vitro.

RESEARCH DESIGN AND METHODS

Reagents. Glyoxal (40% aqueous solution) and SOD (E.C. 1.15.1.1) were purchased from Sigma (St. Louis, MO). Methylglyoxal, 3-DG, and 1,2-diamino-4,5-dimethoxybenzene dihydrochloride were prepared, purified, and characterized as described previously (59).

Embryo culture and assessment of embryopathy. Embryos were obtained from outbred female Sprague-Dawley rats, maintained at the Biomedical Center in Uppsala (U strain [8]). Rats had free access to a commercial pelleted diet (R 36; Analycen, Lidköping, Sweden) and tap water. They were maintained at an ambient temperature of 22°C with a 12-h light/dark cycle. Female and male rats were caged together during the night. Conception was verified by the presence of sperms in a vaginal smear, and the morning of the day of conception was designated gestational day 0. Between 1100 and 1300 on gestational day 9, the pregnant rats were killed by cervical dislocation, and the embryos were recovered. Conceptuses were explanted, and a culture of whole embryos was performed as previously outlined (21,60). Embryos, within their intact yolk sacs, were maintained in polypropylene tubes (Falcon 2070) in a roller incubator at 38°C and 60 rpm. Each tube contained four conceptuses in 4 ml of culture medium (80% vol/vol rat serum and 20% vol/vol isotonic saline). Rat serum was obtained from freshly drawn arterial blood after immediate centrifugation (60) and subsequently supplemented with sodium benzylpenicillinate and streptomycin to give a final concentration of 60 and 100 mg/l, respectively. Serum was stored frozen and was heat-inactivated at 56°C for 1 h immediately before use. The final glucose concentration (10, 30, or 50 mmol/l), 3-DG concentration (0.1, 0.5, or 1.0 mmol/l), and SOD activity (2,500 U/ml) of the culture media were established with aliquots of stock, i.e., concentrated sterile solutions of the additives. Tubes were initially gassed with 5% oxygen, 5% carbon dioxide, and 90% nitrogen (vol/vol/vol) and capped tightly. After 24 h, the conceptuses were transferred to new culture tubes with fresh medium gassed with 20% oxygen, 5% carbon dioxide, and 75% nitrogen. After a further 20 h of culture, tubes were gassed with 40% oxygen, 5% carbon dioxide, and 55% nitrogen for 10 min. Tubes were harvested 4–6 h later. The total duration of the culture was 48 h, and the time of harvesting corresponds to gestational day 11.7. Conceptuses were transferred to Petri dishes containing isotonic saline. The embryo proper and its yolk sac and amniotic membranes were separated by gentle dissection, viewed in a stereo microscope at a magnification of 10–20×, subsequently snap-frozen in individual tubes, and stored at –80°C until biochemical analysis.

Embryo maldevelopment was assessed by measuring the crown–rump length and counting the number of somites. Embryos were categorized as morphologically normal or showing malformations of varied severity. An average morphological score was calculated for each experimental condition. Normal embryos and embryos with minor, less severe, or severe malformations were assigned individual scores of 0, 1, 5, and 10, respectively, as outlined previously (26). Briefly, a malformation score of 0 indicated a completely normal embryo, fully rotated with a closed neural tube (Fig. 1*A*). Embryos given a score of 1 showed one minor, and only one, deviation from this pattern, mainly an open posterior end of the neural tube. A score of 5 signified one major, and only one, malformation, mainly an open neural tube in the rhombencephalon area or a slight tail twist, whereas a score of 10 indicated an embryo with multiple major malformations, such as open neural tube, rotational defects, and/or heart enlargement (Fig. 1*B*).

Before the study, the complete research protocol, including all experimental procedures involving animals, was approved by the animal ethical committee of the medical faculty of the University of Uppsala.

Assay of α-oxoaldehydes. The concentrations of 3-DG, glyoxal, and methylglyoxal were determined in embryos, yolk sac membranes, and culture medium by derivatization of the α-oxoaldehydes with 1,2-diamino-4,5-dimethoxybenzene by the method described previously (59). Samples were initially acidified with 100 mmol/l acetic acid for storage at –80°C and transit (on cardice) between collaborating groups before derivatization.

Statistics. Differences between means were evaluated by one-way analysis of variance, where the applied test was Fisher's protected least significant difference at the 95% significance level (61) or χ^2 statistics, whichever method was applicable (62). Comparisons between different experimental groups were based on individual embryos, except for evaluation of the malformation score, where Fisher's exact χ^2 test for 2 × 2 tables (with Yates' correction) was used, and we therefore combined the scores (one group with scores 0 and 1, another group with scores 5 and 10).

RESULTS

Culture in 10 mmol/l glucose produced normal embryos with a crown–rump length of 4 mm, with 30 somites, and a malformation score close to 0 (Table 1). Increasing the glucose concentration in the culture medium to 30 and 50 mmol/l decreased crown–rump length and somite number, and increased the malformation score, in a dose-dependent manner. Thus, culture in 30 mmol/l glucose resulted in decreased crown–rump length and somite number by 15 and 22%, respectively, compared with the 10 mmol/l glucose culture, and also increased the malformation score to 6.4. Culture in 50 mmol/l glucose yielded a massive teratological effect with a 45 and 65% decrease in crown–rump length and somite number, respectively, as well as the highest possible malformation score of 10 (Table 1).

Addition of SOD to the 10 mmol/l glucose culture exerted no effect, and addition of SOD to the 30 mmol/l glucose culture diminished the changes in embryonic parameters; in fact, the SOD addition normalized the somite number, despite the high glucose concentration (Table 1).

The amounts of glyoxal and methylglyoxal in embryos and yolk sac membranes, and concentrations in the culture medium (mean ± SE; n = 3), were determined. In the 10 mmol/l glucose cultures, we found 0.08 ± 0.01 and 0.19 ± 0.03 nmol in embryos, 0.80 ± 0.09 and 0.24 ± 0.06 nmol in membranes, and 0.17 ± 0.02 and 0.21 ± 0.02 μmol/l in the medium, respectively. These values did not increase significantly in incubations with 30 and 50 mmol/l glucose (Fig. 2*B–D*) or by SOD treatment (results not shown). The corresponding levels of 3-DG were 0.31 ± 0.13 nmol in embryos, 0.80 ± 0.09 nmol in membranes, and 0.84 ± 0.24 μmol/l in the medium after culture in 10 mmol/l glucose. In contrast to glyoxal and methylglyoxal, these levels increased markedly with increasing glucose concentration. In incubations with 50 mmol/l glucose, the concentration of 3-DG increased 17-fold in embryos, 7-fold in the membrane fraction, and 9-fold in the culture medium compared with 10 mmol/l glucose culture (Fig. 2*B–D*).

Culture of embryos in 10 mmol/l glucose with 0.1–1.0 mmol/l 3-DG also induced malformations, the severity of which became increasingly pronounced with increasing 3-DG concentrations (Table 2). Thus, embryo culture with 0.1 mmol/l 3-DG resulted in moderate changes in embryo devel-

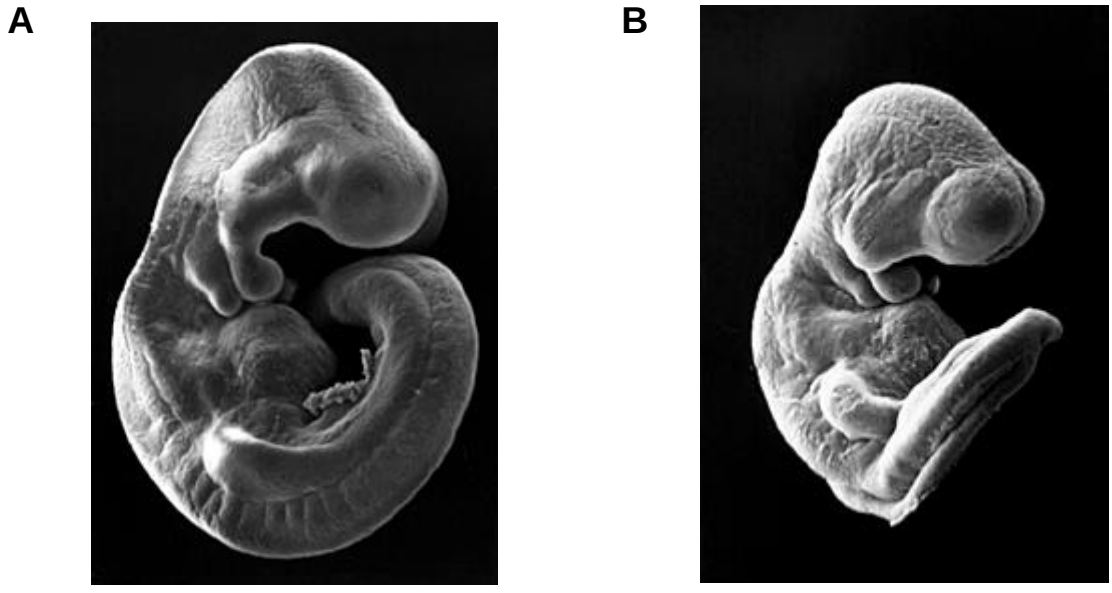

FIG. 1. *A*: Rat embryo cultured in 10 mmol/l glucose showing a completely normal appearance and having a malformation score of 0. *B*: Rat embryo cultured in 50 mmol/l glucose showing major malformation (rotational defect) and having a malformation score of 10. Original magnification ×18.

opment, such as decreased crown–rump length by 6%, diminished somite number by 19%, and a malformation score of 4.0. Increasing the concentration of 3-DG to 0.5 mmol/l yielded greater disturbances, with decreases of crown–rump length and somite number by 15 and 23%, respectively, and a malformation score of 6.2. Culture in 1.0 mmol/l 3-DG induced major disturbances in embryo development, i.e., decreased crown–rump length by 23%, decreased somite number by 37%, and a malformation score of 7.7 (Table 2). In addition, supplementation of the culture medium with SOD diminished the developmental damage exerted by 3-DG. At 0.1 mmol/l 3-DG, the SOD effect was evident with respect to somite number, and it tended to show in the malformation score. At 0.5 mmol/l 3-DG, the SOD addition tended to increase the crown–rump length, but it had a clear-cut effect on somite number and malformation score. At 1 mmol/l 3-DG, addition of SOD diminished the teratogenic effect of the α-oxoaldehyde in all three embryonic parameters (Table 2).

The 3-DG content of embryos was markedly increased by the addition of 0.1–1.0 mmol/l 3-DG to the culture medium: with 0.1 mmol/l 3-DG, the embryonic 3-DG content was 7.6–30.8 nmol. The 3-DG content of embryos incubated with 50 mmol/l glucose was similar to that in embryos incubated with 0.1 mmol/l 3-DG (5.5 ± 0.07 vs. 7.6 ± 1.3 nmol; $P > 0.05$). Similar increases in 3-DG concentration were found in the membrane fraction and the culture medium. Addition of SOD did not significantly decrease the 3-DG levels of embryos, membrane fraction, or culture medium (Fig. 3).

DISCUSSION

In the present study, we were able to show that the embryonic concentration of the reactive glycosylating agent 3-DG is glucose-dependent, that increased 3-DG concentration is teratogenic, and that this effect can be diminished by SOD supplementation.

3-DG is formed by the degradation of fructosamines in glucose-modified proteins and by the elimination of phosphate from fructose-3-phosphate in the metabolism of fructose. Glyoxal is formed by glucose autoxidation, glycation, and lipid peroxidation. Methylglyoxal is formed mainly by phosphate elimination from triosephosphates and ketone bodies, with small amounts also from protein glycation (63). Glycation reactions lead to the formation of these α-oxoaldehydes in all compartments of the culture. The formation of fructose-3-phosphate in the embryo may be increased during

TABLE 1
Effects of glucose and SOD addition on embryogenesis in vitro

Culture condition	*n*	Crown–rump length (mm)	Somite number	Malformation score
10 mmol/l glucose	20	3.98 ± 0.08	29.9 ± 0.3	0.1
10 mmol/l glucose + SOD	8	3.88 ± 0.08	29.9 ± 0.4	0.0
30 mmol/l glucose	21	3.39 ± 0.09*	23.2 ± 1.5*	6.4*
30 mmol/l glucose + SOD	12	3.68 ± 0.08*†	27.7 ± 1.0†	2.5*†
50 mmol/l glucose	13	2.20 ± 0.22*†	10.4 ± 1.6*†	10.0*†

Data are means ± SE or *n*. For estimation of malformation score, see METHODS. *$P < 0.05$ vs. 10 mmol/l glucose; †$P < 0.05$ vs. 30 mmol/l glucose.

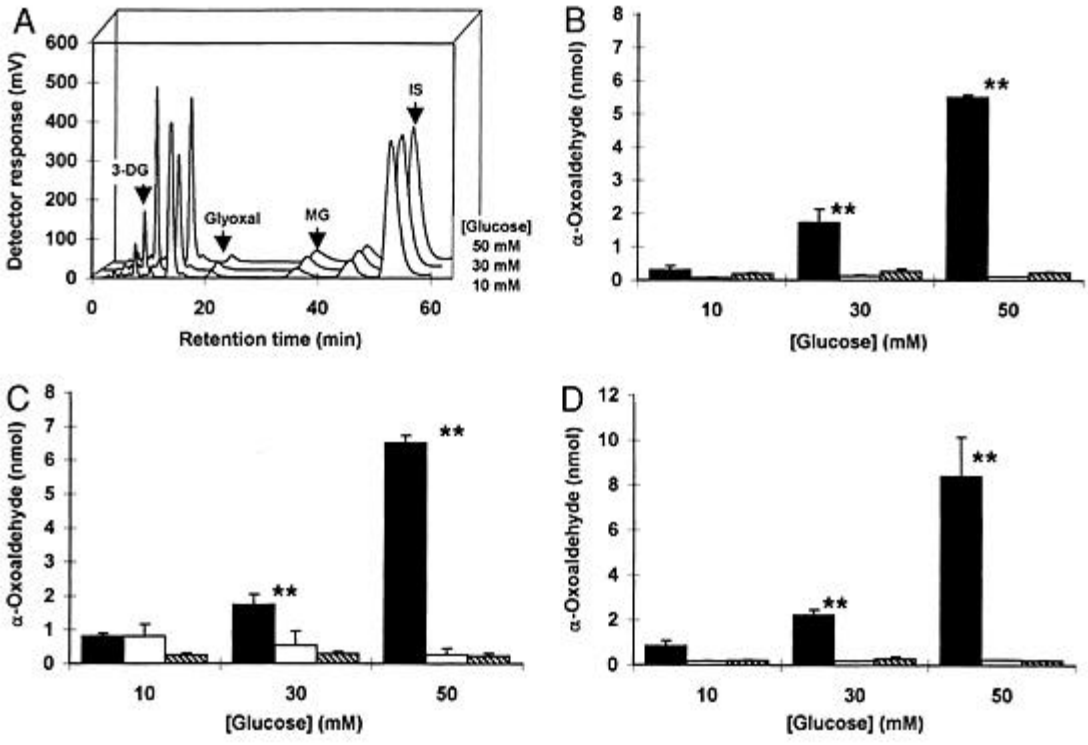

FIG. 2. Effect of hyperglycemia on the concentrations of 3-DG, glyoxal, and methylglyoxal in rat embryo cultures. *A*: High-performance liquid chromatography chromatograms of α-oxoaldehyde analysis of embryos incubated with 10, 30, and 50 mmol/l glucose. α-Oxoaldehyde concentrations of embryos (*B*), membranes (*C*), and culture medium (*D*). ■, 3-DG; □, glyoxal; ▨, methylglyoxal. Data are means ± SE (n = 3 cultures). Significance (Student's t test, with respect to 10 mmol/l glucose control): **P < 0.01.

hyperglycemia as a consequence of increased embryonic fructose concentration (64) from increased flux through the polyol pathway in the embryo (17,20,65). Inhibition of fructose formation via the polyol pathway with an aldose reductase inhibitor, however, did not prevent glucose-induced embryo malformations (17,20,65), and fructose addition (3–13 mmol/l) did not induce embryo malformations in vitro (66). These results suggest that fructose and polyol-derived fructose phosphates are not the principal substrates for the enhanced, and teratogenic, accumulation of 3-DG in rat embryos exposed to a high glucose concentration.

In recent experimentation, however, the size of the congenital cataract in offspring of diabetic rat pregnancy can be diminished by aldose reductase treatment of the pregnant rat (K. Berg, G. Dorner, personal communication). This may indicate a varied susceptibility among the embryonic cell populations to the metabolic and developmental consequences of an increased flux in the polyol pathway, a teratological susceptibility that also may vary with the time point of gestation.

3-DG reacts with proteins to form a reversible hemithioacetal adduct of cysteine residues and irreversible adducts with

TABLE 2
Effects of 3-DG and SOD addition on embryogenesis in vitro

Culture condition	n	Crown–rump length (mm)	Somite number	Malformation score
10 mmol/l glucose	20	3.98 ± 0.08	29.9 ± 0.3	0.1
10 mmol/l glucose + 0.1 mmol/l 3-DG	18	3.75 ± 0.08	24.3 ± 1.3*	4.0*
10 mmol/l glucose + 0.1 mmol/l 3-DG + SOD	12	3.74 ± 0.09	28.3 ± 1.0†	1.3
10 mmol/l glucose + 0.5 mmol/l 3-DG	18	3.38 ± 0.11*	23.1 ± 1.2*	6.2*
10 mmol/l glucose + 0.5 mmol/l 3-DG + SOD	12	3.55 ± 0.09*	27.3 ± 0.9†	1.7†
10 mmol/l glucose + 1 mmol/l 3-DG	22	3.08 ± 0.10*	18.8 ± 1.2*	7.7*
10 mmol/l glucose + 1 mmol/l 3-DG + SOD	16	3.53 ± 0.10*†	26.3 ± 1.0*†	2.3*†

Data are means ± SE or n. For estimation of malformation score, see METHODS. *P < 0.05 vs. 10 mmol/l glucose; †P < 0.05 vs. corresponding 3-DG concentration without SOD addition.

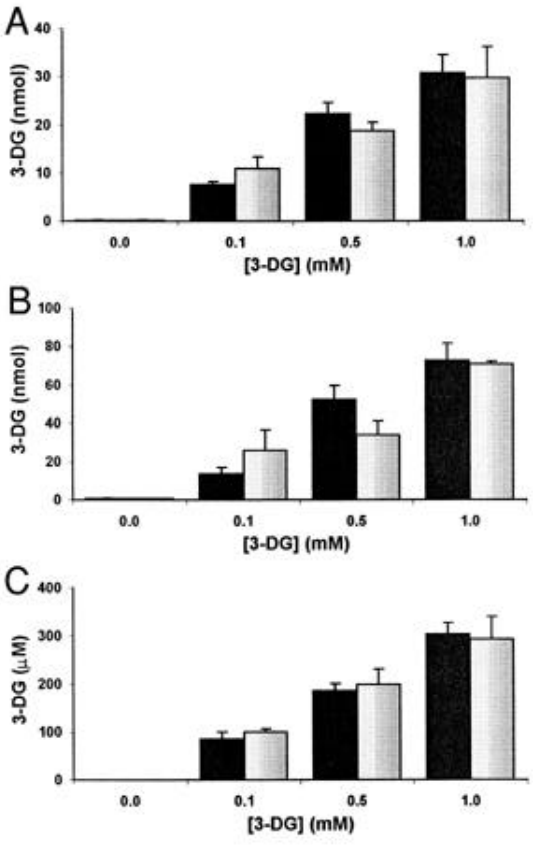

FIG. 3. Effect of exogenous 3-DG on the concentration of 3-DG in rat embryo cultures. α-Oxoaldehyde concentrations of embryos (*A*), membranes (*B*), and culture medium (*C*). Data are means ± SE (n = 3). ■, + 3-DG; ■ ,+ 3-DG + SOD. All 3-DG estimates were higher than control cultures with 10 mmol/l glucose without added 3-DG ($P < 0.001$), and there was no significant effect of SOD on 3-DG concentration (P > 0.05).

lysine and arginine residues (pyrraline, hydroimidazolone, imidazolone, and bis[lysyl]imidazolium cross-link) (54) (Fig. 4). The formation of hydroimidazolone derivatives has recently been implicated as an AGE receptor (RAGE) recognition factor (67). Binding of AGE-modified proteins to RAGE induced oxidative stress that was prevented by SOD, catalase, glutathione peroxidase, and *N*-acetylcysteine (68). The inhibition of hyperglycemia- and 3-DG–induced embryo malformation by SOD herein and the inhibition of hyperglycemia-induced embryo malformation by antioxidants reported previously (21,23–27,39–43,47) are consistent with 3-DG forming AGE-modified proteins and increased superoxide formation by AGE-protein/RAGE interaction. This advance provides a novel explanation for hyperglycemia-induced embryopathy. 3-DG, like other α-oxoaldehydes, may also modify guanylyl nucleotides (69). These modifications in DNA are associated with mutagenesis (70) and apoptosis (71). In recent studies, a diabetic or hyperglycemic environment was found to be associated with embryonic DNA damage (11,72) and with diminished expression of the developmentally important gene *Pax-3* in the neural tube (73), thereby supporting the notion of a glucose-induced effect on gene expression in embryos, possibly exerted by 3-DG accumulation.

The embryonic levels of 3-DG obtained after direct addition of the compound to the culture medium were slightly higher than those reached when the embryos were subjected to a high glucose concentration, for each level of embryonic dysmorphogenesis. This is compatible with the notion that hyperglycemia causes a number of metabolic effects in the embryonic tissue, changes that have different teratological capacity but seem to converge into a state of embryonic ROS excess (74). The substantial glucose-induced increase in 3-DG seems to be of major importance for the diabetes-induced embryopathy, possibly via the glycating capacity of the

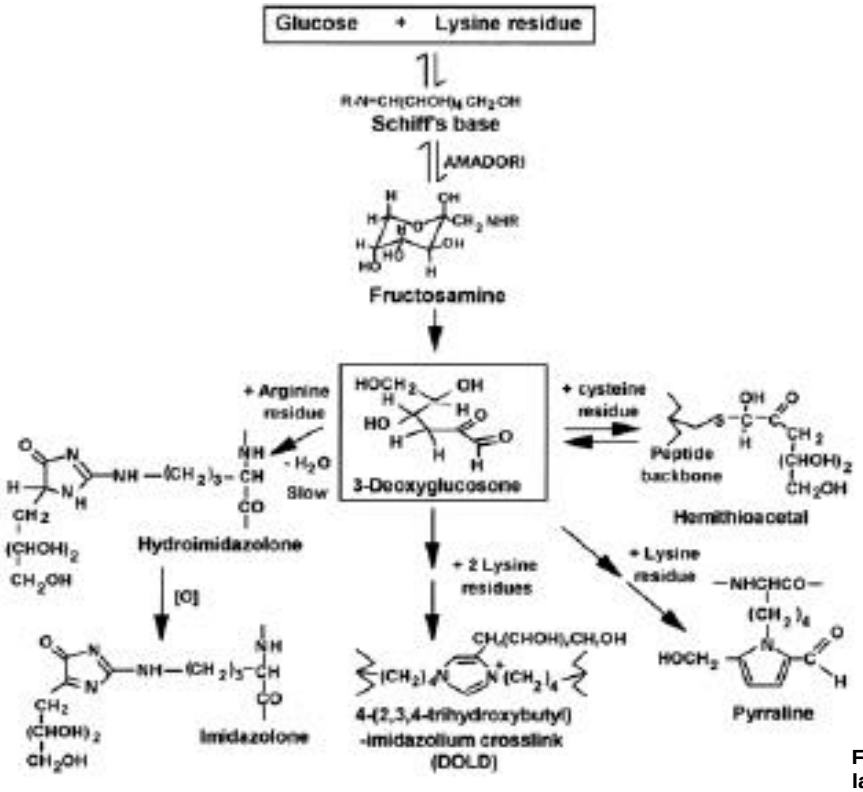

FIG. 4. Interactions of 3-DG with other cellular compounds.

oxoaldehyde (54), whereas the disturbed prostaglandin metabolism (7,18,19,27,51) and diminished inositol levels (10,19,45,75,76) in the embryos are also likely to contribute to the disturbance of development in a diabetic environment.

The embryo appears to be particularly susceptible to the accretion of 3-DG and not to the accumulation of glyoxal and methylglyoxal. We investigated the teratogenicity of methylglyoxal and found that it was only weakly teratogenic even when embryonic methylglyoxal concentration was increased fivefold above that found in embryos incubated with 50 mmol/l glucose (data not shown). However, both the rate of formation and the rate of enzymatic detoxification of α-oxoaldehydes determine the steady state of 3-DG, methylglyoxal, and glyoxal in the embryonic tissue. 3-DG is detoxified by 3-DG reductase, a 3-DG dehydrogenase, and methylglyoxal and glyoxal are detoxified mainly by glyoxalase I (77). The high activity of glyoxalase I, characteristic of embryonic tissue (77), may prevent teratogenicity of methylglyoxal and glyoxal in hyperglycemia. This raises the prospect for novel therapeutic intervention in diabetic embryopathy to prevent the accumulation of 3-DG and/or the effects of 3-DG accumulation. This may be achieved directly by scavengers of 3-DG, such as aminoguanidine (78), or indirectly by antioxidants that may prevent depletion of NADPH and tissue thiols and thereby decrease the irreversible modification of proteins and nucleotides by 3-DG (54), a concept presently under experimental scrutiny.

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Author Queries (please see Q in margin and underlined text)

Q1: Fisher's PLSD spelled out correctly?

Q2: Spell out ROS.

Q2a: The following *P* values are not shown in Figure 2: "*$P < 0.05$ and ***$P < 0.001$. Should they be deleted from the legend? Or should Figure 2 be revised to show the values? Please advise.

Q3: Please supply first initials of Berg and Dorner.

Q4: "and" or "a" 3-DG dehydrogenase meant?

Q5: Has ref. 47 been published yet? If so, please provide journal, vol, pages, and year.

Q6: Has ref. 51 been published yet?