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Altered Levels of Scavenging Enzymes in Embryos Subjected to a Diabetic Environment

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Maternal diabetes during pregnancy is associated with an increased rate of congenital malformations in the offspring. The exact molecular etiology of the disturbed embryogenesis is unknown, but an involvement of radical oxygen species in the teratological process has been suggested. Oxidative damage presupposes an imbalance between the activity of the free oxygen radicals and the antioxidant defence mechanisms on the cellular level. The aim of the present study was to investigate if maternal diabetes *in vivo*, or high glucose *in vitro* alters the expression of the free oxygen radical scavenging enzymes superoxide dismutase (CuZnSOD and MnSOD), catalase and glutathione peroxidase in rat embryos during late organogenesis. We studied offspring of normal and diabetic rats on gestational days 11 and 12, and also evaluated day-11 embryos after a 48 hour culture period in 10 mM or 50 mM glucose concentration. Both maternal diabetes and high glucose culture caused growth retardation and increased rate of congenital malformations in the embryos. The CuZnSOD and MnSOD enzymes were expressed on gestational day 11 and both CuZnSOD, MnSOD and catalase were expressed on day 12 with increased concentrations of MnSOD transcripts when challenged by a diabetic milieu. There was a good correlation between mRNA, protein, and activity levels, suggesting that the regulation of these enzymes occurs primarily at the pretranslational level. Maternal diabetes *in vivo* and high glucose concentration *in vitro* induced increased MnSOD expression, concomitant with increased total SOD activity, and a tentative decrease in catalase expression and activity in the embryos. These findings support the notion of enhanced oxidative stress in the embryo as an etiologic agent in diabetic teratogenesis.

Key words: scavenging enzyme, free oxygen radical, rat embryo, diabetes, malformation, gene expression

Abbreviations: MnSOD, superoxide dismutase localized to the mitochondria containing manganese; CuZnSOD, superoxide dismutase localized to the cytoplasm containing copper and zinc; N, and MD, normal, and (manifestly) diabetic rats; N11, N12, offspring of N rats on gestational day 11, and 12; MD11, MD12, offspring of MD rats on gestational day 11, and 12.

INTRODUCTION

Diabetic pregnancy is despite major improvements in medical and obstetrical care, still associated with a 2–3 fold increased incidence of major malformations in the offspring.[1,2] The exact cellular and molecular mechanisms are largely unknown. A diabetes-like environment *in vitro* or

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maternal diabetes *in vivo* have been shown to produce both growth retardation and congenital malformations in experimental animals.[3] The search for teratological agents in various experimental models of diabetic pregnancy, both *in vivo* and *in vitro*, has identified a handful of possible compounds and mechanisms. Thus, metabolites which are elevated in a diabetic milieu, such as glucose, β-hydroxybutyrate, triglycerides, and branched chain amino acids have shown an association with compromised embryonic development *in vivo*.[4] Furthermore, addition of excess glucose,[5] β-hydroxybutyrate,[6] and a metabolite of leucine, α-ketoisocaproate,[7] to rodent embryo culture *in vitro* have demonstrated that these agents have strong teratogenic activity. Notably, these compounds are all oxidizable substrates and are likely to be metabolized by the embryo.

Recently it has been suggested that the embryonic oxidative metabolism is directly coupled to the teratogenic process via excess of free oxygen radicals in the embryo.[7,8] Free oxygen radicals are known to generate disturbed *in vitro* development *per se*,[9] and exogenous supplementation of the free oxygen radical scavenging enzyme superoxide dismutase to embryo culture with high concentration of glucose, β-hydroxybutyrate and α-ketoisocaproate protects against malformations.[7,8] Therefore, it seems plausible that oxidative stress constitutes a link between the diabetes-like environment *in vitro* and embryonic dysmorphogenesis.

The scavenging enzymes – catalase, glutathione peroxidase and superoxide dismutase – are prominent members of the cellular antioxidant defence which metabolizes the reactive oxygen derivatives such as hydrogen peroxide (catalase and glutathione peroxidase) and the superoxide ion (superoxide dismutase). In addition, there are two intracellular isoenzymes of superoxide dismutase – CuZnSOD and MnSOD. The former enzyme is localized in the cytoplasm and the latter in the mitochondria of the cell.[10]

The possibility that a diabetic environment induces congenital malformations in the offspring via increased oxygen radical activity prompted us to investigate whether maternal diabetes and high glucose *in vitro* may alter the expression and/or activity of CuZnSOD, MnSOD, catalase and glutathione peroxidase in rat embryos during the critical period of organogenesis. In a previous study we found marked changes in gene expression of the two extracellular matrix proteins laminin and fibronectin in embryos subjected to a diabetic or hyperglycemic environment.[11] The aim of this study was to identify effects on the cellular antioxidant defence mechanisms exerted by a diabetes-like milieu.

MATERIALS AND METHODS

Animals and Embryo Culture

Female Sprague-Dawley rats from a malformation prone U substrain were made diabetic by a single *i.v.* injection of streptozotocin at a dose of 40 mg/kg body weight. Two to three weeks after the injection diabetic females (>20 mM serum glucose) were caged overnight with non-diabetic male Sprague-Dawley rats. The following morning was denoted gestational day 0 if a positive vaginal smear was found. Rat embryos from normal and diabetic mothers were excised on day 11 and 12, examined under a stereo microscope and stored at −80°C for subsequent RNA and protein studies. Day-9 embryos from normal mothers were explanted and cultured for 48 hours in 10 or 50 mM glucose as outlined in earlier reports.[7,8] After culture, morphological analysis was performed as previously described.[7,8]

RNA Studies

Total RNA was extracted with the guanidine isothiocyanate method[12] and poly $(A)^+$ RNA isolated by oligo-dT affinity chromatography (Fast Track mRNA Isolation Kit; Invitrogen, San Diego, CA, USA). The RNA samples, either total or poly-A^+ RNA fraction, were subjected to electrophoresis on 1% agarose gel containing 2.2 M formaldehyde, transferred to a nylon membrane and

hybridized to ^{32}P-labelled cDNA probes coding for human CuZnSOD,[13] human MnSOD,[14] rat catalase,[15] mouse glutathione peroxidase,[16] and human γ-actin.[17] Hybridization was performed at 42°C in a solution containing 50% formamide, 5X SSPE, 2.5X Denhart's solution, 0.1% SDS and 100 mg/ml denatured salmon sperm DNA. The membranes were washed in 0.1X SSPE, 0.1% SDS at 55°C and incubated with Kodak X-Omat AR film at −80°C. Densitometric analysis of the autoradiograms was performed after non-saturating exposures with a Quick Scan Jr densitometer (Helena Laboratories, Beaumont, TX, USA). Hybridization to γ-actin, a transcript not altered by maternal diabetes, was used as an internal control to correct for loading inequalities.

Immunoblot Analysis

Embryos were sonicated and about 30 μg protein per well were resolved by 14% SDS-polyacrylamide gel electrophoresis. The polypeptide bands were transferred to a nitrocellulose membrane using electroblotting (Bio-Rad Laboratories, Richmond, CA, USA) and incubated with polyclonal rabbit antibody to rat CuZnSOD and rat MnSOD.[18] Secondary horseradish peroxidase-labelled sheep anti-rabbit antibodies were subsequently applied and the antigen-antibody complex was visualized by chemiluminescence (ECL western blotting analysis system, Amersham International, Little Chalfont, Buckinghamshire, UK), detected by a Kodak X-Omat AR film. The bands were quantitated densitometrically and data were expressed as arbitrary densitometric units per μg protein loaded in each well. The protein content of the sonicated embryos was determined by the method of Lowry *et al.*[19] using bovine serum albumin as a standard.

Enzymatic Activity Studies

Activity measurements was performed as previously described.[8] Briefly, superoxide dismutase activity was measured as inhibition of xanthine oxidase-generated luminol chemiluminescence.[20] Peroxidative activity of catalase was utilized for spectrophotometric determination by the production of formaldehyde from methanol.[21] Glutathione peroxidase activity was established by estimating absorbance changes due to the hydroperoxide-specific oxidation of NADPH in an enzymatic cycling assay.[22] The DNA content of the embryos was measured fluorometrically as described by Kissane and Robins[23] and Hinegardner.[24]

Statistical Analysis

Differences between means were evaluated with the aid of Student's two-tailed unpaired *t*-test, or with X^2-statistics with Yates' correction, whichever method was applicable.[25]

TABLE 1 Embryo morphology

	number of observations	crown-rump length (mm)	number of somites	malformation score (0–10)
10 G	20	4.1 ± 0.3	29.3 ± 0.5	0.2
50 G	31	2.7 ± 0.2*	22.3 ± 0.4*	8.9*
N 11	42	4.5 ± 0.1	29.1 ± 0.3	0.2
MD 11	55	2.9 ± 0.1*	25.1 ± 0.2*	4.5*
N 12	16	6.6 ± 0.1	35.2 ± 0.3	0.2
MD 12	30	4.7 ± 0.2*	29.7 ± 0.3*	2.1

Crown rump length, somite number and malformation score in embryos cultured in 10 mM or 50 mM glucose concentration (10 G, 50 G), and in day-11 and day-12 embryos of normal (N) and diabetic rats (MD).
Significance: * = $p < 0.05$ versus either 10 G embryos *in vitro*, or N 11 or N 12 embryos *in vivo*. Student's two-tailed unpaired *t*-test, or X^2-statistics (with Yates' correction) (25).

TABLE 2 Enzymatic activity

	superoxide dismutase	catalase	glutathione peroxidase
10 G	12.0 ± 0.9 (12)	3.2 ± 0.4 (8)	12.2 ± 1.8 (9)
50 G	16.5 ± 1.5* (12)	3.6 ± 0.3 (8)	10.9 ± 1.3 (9)
N 11	14.4 ± 1.1 (4)	1.1 ± 0.1 (6)	7.9 ± 2.7 (3)
MD 11	18.5 ± 1.1* (3)	0.8 ± 0.1 (6)	7.2 ± 1.4 (5)
N 12	10.7 ± 0.6 (4)	0.9 ± 0.1 (3)	5.7 ± 0.4 (4)
MD 12	15.3 ± 1.6* (3)	1.0 ± 0.2 (2)	6.0 ± 0.5 (2)

Activities of superoxide dismutase, catalase, and glutathione peroxidase in embryos cultured in 10 mM or 50 mM glucose concentration (10 G, 50 G), and in day-11 and day-12 embryos of normal (N) and diabetic rats (MD). Superoxide dismutase activity is expressed as mU/μg DNA, catalase and glutathione peroxidase are expressed as pKat/μg DNA. Number of observations in parentheses.
Significance: * = $p < 0.05$ versus either 10 G embryos *in vitro*, or N 11 or N 12 embryos *in vivo*. Student's two-tailed unpaired *t*-test (25).

TABLE 3 Superoxide dismutase expression

	mRNA		Protein	
	CuZnSOD	MnSOD	CuZnSOD	MnSOD
50 G	94 ± 20 (6)	134 (2)	109 ± 67 (13)	124 ± 64 (13)
MD 11	95 ± 39 (7)	164 (2)	ND	ND
MD 12	112 ± 37 (6)	121 (3)	93 ± 51 (12)	138 ± 82* (20)

Superoxide dismutase mRNA and protein levels in embryos cultured in 50 mM glucose concentration (50 G), and in day-11 and day-12 embryos of diabetic rats (MD 11 and MD 12). Data are expressed as percent of either embryos cultured in 10 mM glucose *in vitro*, or day-11 or day-12 embryos from normal rats *in vivo*. (mean ± SD), "ND" denotes not determined value.
Significance: * = $p < 0.05$ versus either 10 G embryos *in vitro*, or N 11 or N 12 embryos *in vivo*. Student's two-tailed unpaired *t*-test (25).

RESULTS

The morphological analysis of the embryos were in agreement with previously published data[4,7,8,26]: Embryos from diabetic rats were smaller than offspring from normal rats, culture in 10 mM glucose produced embryos with a morphology closely corresponding to that occurring *in vivo*, while culture in 50 mM glucose had a profound growth retarding and teratogenic effect, yielding decreased crown rump length, decreased somite number and increased malformation score (Table 1).

We found a 30–40% increase in the activity of superoxide dismutase in embryos cultured in 50 mM glucose compared with 10 mM glucose culture, as well as in embryos from diabetic rats compared with embryos of normal rats (Table 2). Exposure to a diabetic environment did not appear to affect the activity of glutathione peroxidase, whereas catalase tended to be decreased by about 30% in the MD 11 embryos compared to the N 11 embryos ($0.05 < p < 0.1$, Table 2).

To determine the relative contribution of CuZnSOD and MnSOD to the increase in total SOD activity, and to dissect the mechanisms underlying this effect of the diabetic milieu we measured both mRNA and protein levels of the CuZnSOD and MnSOD. Both CuZnSOD mRNA and protein levels were unchanged in the diabetic compared to the control conditions, both *in vivo* and *in vitro* (Table 3). In order to detect the rare message of MnSOD we had to use polyA selected mRNA from pooled RNA of 10–20 embryos. This diminished the number of determinations, thereby precluding statistical comparison. However, since the results were reproducible in 2–3 Northern blots, each representing 10–20 embryos, the reported mean values should be regarded as highly representative of the expression of this gene (Table 3, Figures 1 and 2). Both MnSOD mRNA and protein levels were thus increased in embryos from diabetic rats and in high glucose culture. The increase in MnSOD mRNA and protein levels were compatible with the increased superoxide dismutase activity in the embryos exposed to a diabetic environment. Thus, the MnSOD protein was significantly increased in day-12 embryos of diabetic rats, although in the high-glucose cultured embryos the numerical difference failed to reach statistical significance ($0.05 < p < 0.1$, Table 3).

In accordance with the activity data, maternal diabetes or high glucose culture had no significant effect on the expression of catalase and glutathione

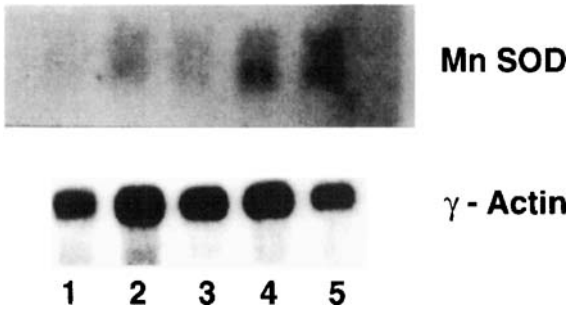

FIGURE 1 Northern blot analysis of poly-A^+ RNA from day-11 rat embryos cultured in vitro for 48 h in 10 and 50 mM glucose. Hybridization was to cDNAs coding for MnSOD and γ-actin. Lane 1: 50 mM glucose – 0.5 mg RNA, Lane 2: 10 mM glucose – 1 mg RNA, Lane 3: 50 mM glucose – 1 mg RNA, Lane 4: 10 mM glucose – 2 mg RNA, Lane 5: 50 mM glucose – 2 mg RNA. The steady state levels of MnSOD transcripts is elevated in high glucose-cultured embryos when normalized with the mRNA levels of γ-actin, the latter indicating the total amount of RNA.

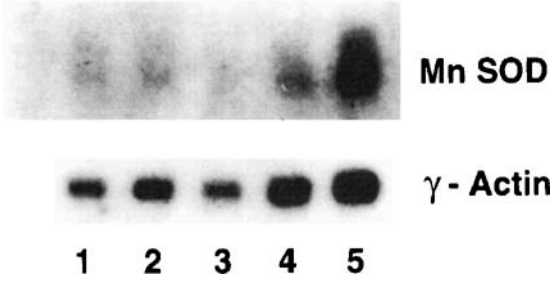

FIGURE 2 Northern blot analysis of poly-A^+ RNA from day-12 rat embryos from normal (N) and diabetic (D) rats. Hybridization was to cDNAs coding for MnSOD and γ-actin. Lane 1: N – 1.25 mg RNA, Lane 2: D – 1.25 mg RNA, Lane 3: D – 1.25 mg RNA, Lane 4: N – 2.5 mg RNA, Lane 5: D – 2.5 mg RNA. Increased MnSOD mRNA concentrations are evident in embryos from diabetic rats.

peroxidase in the embryos (Table 4). The catalase activity level of 70% seen in MD 11 embryos corresponded to a catalase mRNA level of 76% in these embryos, however, without statistically significance versus the N 11 embryos (Table 4).

DISCUSSION

In this study we observed that a diabetic environment *in vivo* and *in vitro* increases the total superoxide dismutase activity by increasing the expression of the MnSOD gene. Since generation of reactive oxygen species has been found to enhance the expression of MnSOD,[27] this finding supports the notion of enhanced oxidative stress in diabetic pregnancy. We found also, however, a trend towards decreased catalase mRNA concentration and decreased catalase activity in MD 11 embryos. This tentative change may surpass the increase in SOD activity, and makes it difficult to predict the net effect of a diabetic environment on the total activity of radical scavenging enzymes in the embryo. Since the antioxidative status in the cell is dependent on the equilibrium of different antioxidant compounds, a rise in SOD activity in combination with impaired catalase activity may cause an increased steady state concentration of hydrogen peroxide in the embryo, leading to increased production of hydroxyl radicals via the Fenton reaction. In high-glucose cultured embryos there was no decrease in catalase activity despite a tentative diminution of catalase mRNA. The superoxide dismutase activity was, however, increased in analogy to the MD 11 embryos.

In previous studies, exogenous supplementation of the free oxygen radical scavenging enzymes superoxide dismutase, catalase and glutathione peroxidase protected against malformations in 50 mM glucose culture.[8] Superoxide dismutase also repressed the teratogenic effects of

TABLE 4 Catalase and glutathione peroxidase mRNA

	catalase	glutathione peroxidase
50 G	84 ± 39 (6)	ND
MD 11	76 ± 44 (7)	ND
MD 12	119 ± 42 (6)	134 ± 63 (15)

mRNA levels of catalase and glutathione peroxidase in embryos cultured in 50 mM glucose concentration (50 G), and in day-11 and day-12 embryos of diabetic rats (MD). Data is either expressed as percent of embryos cultured in 10 mM glucose *in vitro*, or day-11 or day-12 embryos from normal rats *in vivo* (mean ± SD), "ND" denotes not determined value. Number of observations in parentheses.

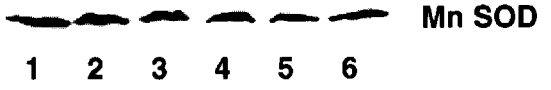

FIGURE 3 Western blot of protein from day-12 rat embryos from normal (N) and diabetic (D) rats. The antibody used was a polyclonal rabbit-anti mouse MnSOD and 30 mg protein from each homogenized embryo was applied to the wells. Lane 1–3: D embryos, lane 4–6: N embryos. Note the slight upregulation of MnSOD protein in the D embryos.

excessive concentrations *in vitro* of the intermediate metabolites, pyruvate, β-hydroxybutyrate, and α-ketoisocaproate, all of which are elevated in diabetes.[7] It was thus hypothesized that embryonic excess of oxidizable metabolites followed by intramitochondrial overload might result in increased free oxygen radical leakage from the electron transport chain.[3]

In a recent series of morphological studies, high amplitude swelling, reduced number of cristae and ruptured membranes of mitochondria in neural epithelial cells in embryos from diabetic rats or exposed to high glucose during culture *in vitro* have been demonstrated.[28]

A transgenic mouse strain containing an increased gene dosage and activity of CuZnSOD[29] has recently been shown to be more resistant to the teratogenic effects of high glucose concentrations *in vitro*,[30] as well as to the negative influence of a diabetic intrauterine milieu *in vivo*.[31] These findings suggest that the embryonic antioxidative capacity is of prime importance for modulation of the teratogenic effect of a diabetic environment.

In view of these studies suggesting an involvement of embryonic free oxygen radicals in the teratogenic process, we decided to investigate if the embryonic enzymatic antioxidant defence would be affected by maternal diabetes at the mRNA and/or protein levels.

The CuZnSOD in the cytoplasm and the mitochondrial MnSOD[32] catalyze the same reaction converting the superoxide ion (O^{2-}) to hydrogen peroxide (H_2O_2). Glutathione peroxidase and catalase metabolize the breakdown of hydrogen peroxide to water. MnSOD is the main inducible scavenging enzyme, since MnSOD expression in adult tissues has been shown to be augmented by oxidative stress by hydrogen peroxide and superoxide ion,[33] X-ray irradiation,[27] cytokines,[34,35] and phorbol esters.[36]

In experimental animals the CuZnSOD mRNA levels correlated well with the enzymatic activity, indicating that regulation of the enzyme was exerted mainly at the transcriptional level.[37] CuZnSOD mRNA and glutathione peroxidase mRNA levels were not linked to oxygen consumption or oxygen tension in fetal or neonatal rats.[38] In neonatal lungs, though, the activities of total SOD, catalase and glutathione peroxidase were increased after exposure to normobaric hyperoxia.[39]

The diabetic state has been shown to affect the antioxidant status in several tissues. Diabetic children exhibited lower serum antioxidant activity, expressed as lowered capacity to trap peroxyl radicals.[40] In streptozotocin-diabetic rats, the urinary excretion of thiobarbituric acid positive material was increased, indicating a high level of oxidative stress in these animals.[41] Activities of catalase, glutathione reductase and CuZnSOD were elevated in the pancreas of streptozotocin-induced diabetic rats while hepatic enzymatic activities were downregulated in the same animals. Interestingly, 4 weeks of insulin treatment reversed all the alterations in enzymatic activities.[42] Dobashi and coworkers reported that in hypertrophied kidneys from diabetic rats the total CuZnSOD content was normal, but CuZnSOD was increased in degenerating tubules. MnSOD levels were elevated in thick ascending limbs of Henle's loops, indicating that the cells might adapt to oxidative stress.[43] Another possible mechanism of diabetes to increase the effect of the oxidative stress was suggested by Arai and collaborators who reported that glycosylated erythrocyte CuZnSOD has lower activity than non-glycosylated enzyme.[44] Of interest are also the findings that connective tissue stiffening in diabetic patients was shown to be related to serum lipid peroxide concentration,[45]

and that antioxidant treatment of diabetic animals can diminish the neurological complications of the disease.[46,47] If the embryonic dysmorphogenesis is an analogous process to the induction of complications, it follows that antioxidant treatment should block also the teratogenic effect of diabetic pregnancy, and, indeed, there are results indicating the validity of such an approach.[48]

Little is known about the antioxidant capacity of the developing embryo. El-Hage and coworkers first detected scavenging enzyme mRNA in gestational day-8 mouse embryos. The *in utero* mRNA concentrations were higher than in adult tissue while the specific enzymatic activities increased with age.[49] It was speculated that the mRNAs accumulated *in utero* and were not translated until after birth when the fetus adapts to aerobic respiration, at which time the activities of the antioxidant enzymes increase considerably.[49,50] CuZnSOD and MnSOD protein have previously been found in day-12 rats embryos, but only in cardiomyocytes and later in hepatocytes.[18] Recent attempts to determine antioxidant capacity in the offspring have indicated decreased embryonic α-/tocopherol/MDA ratio in day-11 embryos of manifestly diabetic rats, the effect of a combined decrease in vitamin E levels, and an increase of MDA concentration in these embryos.[51]

Our data in the present investigation show that the CuZnSOD and MnSOD enzymes are expressed on gestational day 11 and both CuZnSOD, MnSOD and catalase are expressed on day 12 with increased concentrations of MnSOD transcripts when challenged by a diabetic milieu. Moreover, there was a good correlation between mRNA, protein, and activity levels, suggesting that the regulation of these enzymes occurs primarily at the pretranslational level.

The embryos are apparently not able to protect themselves against the reactive oxygen species at the levels present in our systems, perhaps due to the fact that embryos normally are exposed to a anaerobic milieu, in line with the findings of El-Hage and coworkers.[49] The imbalance in activity levels of superoxide dismutase and catalase in the embryo observed in the present study could be interpreted as an indirect evidence of embryonic oxidant stress exerted by abnormal maternal metabolism leading to anomalous embryonic development.

In conclusion, this study has shown a diabetes-induced increase in MnSOD expression, concomitant with increased total SOD activity, and a tentative decrease in catalase expression and activity. These findings support the notion of enhanced oxidative stress in the embryo as an etiologic agent in diabetic teratogenesis.

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