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Glutathione Oxidation and Embryotoxicity Elicited by Diamide in the Developing Rat Conceptus *in Vitro*

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This study was performed in the rat whole-embryo culture system to investigate the effects of glutathione oxidation by diamide, a thiol oxidant, in developing rat conceptuses during early organogenesis. The effects of diamide on reduced glutathione (GSH), glutathione disulfide (GSSG), and embryotoxicity were found to be concentration and time dependent. Diamide at concentrations of 75 and 100 μM produced abnormal axial rotation (62–89%), decreased viability (to 69% by 100 μM diamide), and reduced protein and DNA content in the embryo and visceral yolk sac (VYS) when evaluated on Day 11. High concentrations of diamide (250–500 μM) resulted in 100% mortality. GSH and GSSG levels in the conceptuses were not significantly affected during 2 hr following diamide addition at concentrations of 50 to 100 μM. At concentrations of 250 and 500 μM, rapid GSH depletion (50% of control) was seen within 5 min of exposure and was followed at 5–30 min by a significant increase in GSSG relative to control values. Diamide (500 μM) exposure for only 15 min on Gestational Day 10 was sufficient to elicit malformations (53% of exposed conceptuses with abnormal axial rotation) without significant loss of viability. After 30 min of exposure to the high concentration (500 μM), viability was decreased to 71% and defects of axial rotation increased to 87% in surviving conceptuses. This indicates that events associated with initial exposure are critical for expression of toxicity. Inhibition of glutathione disulfide reductase (GSSG reductase) activities in embryo and VYS with 1,3-bis(2-chloroethyl)-1-nitrosourea prior to diamide addition potentiated the embryotoxicity of diamide (75 μM) and resulted in corresponding reductions in GSH/GSSG ratios as determined during the first 2 hr of exposure. Inhibition of new GSH synthesis with L-buthionine-[*S,R*]-sulfoximine during diamide (75 μM) exposure also exacerbated toxicity compared to diamide treatment alone. These results implicate the involvement of GSH synthesis and GSSG reductase activity in mediating the embryotoxicity of diamide. © 1993 Academic Press, Inc.

Intracellular reduced glutathione (GSH)[1] status has been shown to modulate the embryotoxicity elicited by various chemicals *in vitro* and *in vivo* (Faustman-Watts *et al.*, 1986; Harris *et al.*, 1987, 1988; Little and Mirkes, 1990; Slott and Hales, 1987; Wong and Wells, 1989; Wong *et al.*, 1989). Alterations of thiol levels are important in mechanisms of toxicity since GSH and related low-molecular-weight thiols (LMWT) are involved in several essential cellular functions, as well as being necessary for chemical detoxification. GSH, the major LMWT in cells, is known to exert significant protection against chemical toxicity both via nonenzymatic and enzymatic reactions (Ketterer *et al.*, 1983; Jones *et al.*, 1986). GSH is also known to regulate several critical cellular functions such as macromolecule synthesis (protein and DNA synthesis), microtubule assembly, maintenance of cellular structure and integrity, and modulation of protein conformation and enzyme activities (Kosower and Kosower, 1978). Evidence that alterations of intracellular GSH levels influence incidence and severity of dysmorphogenesis suggests that interactions of GSH with embryotoxins or their metabolites occur during biotransformation and detoxification processes. The chemical interactions with GSH may result in different outcomes with respect to intracellular thiol status, such as GSH depletion (via adduct formation or oxidation), inhibition of GSH synthesis, or changes in redox ratios. Understanding the consequences of chemical interaction with GSH will lead to insights into the mechanism of action and cellular adaptations that occur following exposure to embryotoxins.

Recently, it has been reported that several compounds that undergo redox cycling in biological systems are embryotoxic both *in vivo* and *in vitro* (Juchau *et al.*, 1986). These compounds include quinones (adriamycin, mitomycin, paraquat), aromatic nitro compounds (nitrofurazone, nitrofurantoin, niridazole, 2-acetylaminofluorene, nitrosofluorene), and trypan blue. Redox cycling agents are capable of undergoing single-electron reduction by cellular re-

[1] 1,3-bis(2-chloroethyl)-1-nitrosourea, BCNU; L-buthionine-(*S,R*)-sulfoximine, BSO; dimethyl sulfoxide, DMSO; reduced glutathione, GSH; glutathione disulfide, GSSG; glutathione disulfide reductase, GSSG reductase; Hanks' balanced salt solution, HBSS; low-molecular weight thiol, LMWT; visceral yolk sac, VYS.

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ducing agents to yield radical intermediates (Kappus, 1986). Depending on their one electron redox potentials, the reactive intermediates are reoxidized to the original parent compounds by transferring one electron to O_2 with the resulting formation of superoxide anion radical ($O_2^{\cdot-}$). This process is termed redox cycling. The superoxide radical formed readily dismutates to hydrogen peroxide (H_2O_2) either spontaneously or via superoxide dismutase. H_2O_2, if not metabolized, can be converted to highly reactive and toxic hydroxy radical ($^{\cdot}OH$) in the presence of transition metals such as Fe^{2+} and Cu^{2+}. Metabolism of the redox cycling compounds can result in the oxidation of GSH to form GSSG (glutathione disulfide) through glutathione peroxidase activity as cells attempt to remove H_2O_2 and protect themselves against toxicity of the subsequent reactive oxygen intermediates. Additional protective effects of GSH by direct interaction of sulfhydryl groups with free radical intermediates can also lead to GSH depletion and GSSG formation (Reed, 1985). In addition to redox cycling, redox active quinone compounds such as menadione react directly with GSH to form GSH conjugates which can still redox cycle by forming the semiquinone radical and eventually lead to the formation of active oxygen species (DiMonte *et al.*, 1984; Ross *et al.*, 1985; Wefers and Sies, 1983). Exposure of cells to redox cycling agents would be expected to deplete intracellular GSH and/or increase GSSG levels, particularly when cellular counteracting mechanisms are overwhelmed or disrupted. During GSH oxidation, not only GSH synthesis but also the enzyme glutathione disulfide reductase (GSSG reductase) become important in maintaining GSH/GSSG redox status. GSSG reductase is the enzyme responsible for reduction of GSSG to GSH, at the expense of NADPH (Jones *et al.*, 1986; Kosower and Kosower, 1978). The involvement of GSH and GSSG reductase in protecting cells against chemically induced oxidative challenges, including redox cycling, has been relatively well established in isolated cell culture systems (Babson *et al.*, 1981; Nathan *et al.*, 1980; Ross *et al.*, 1986; Stark and Farber, 1985).

Changes in glutathione levels due to detoxification activity could elicit deleterious changes in GSH-mediated defense mechanisms against reactive radical intermediates or oxygen metabolites and also alter other important cellular functions known to be affected by GSH/GSSG status. Currently, mechanisms underlying the embryotoxicity elicited by redox cycling agents are not well understood and have not been thoroughly investigated. Consequences of reactive radical intermediate formation expressed as altered intracellular glutathione status and changes in status of other cellular reducing equivalents may be involved in the mechanism of embryotoxicity. While several studies of the modulation and maintenance of intracellular GSH levels have been performed in adult mammals and also in cell culture systems, very little is known concerning the regulation of intracellular GSH in the developing conceptus and the consequences of altered thiol status on normal growth and development.

The present study evaluates the effects of altered intracellular thiol status via GSH oxidation using diamide [$(CH_3)_2N$-CO-N = N-CO-$N(CH_3)_2$], a thiol oxidant, in the developing rat conceptuses during the period of early organogenesis using the rat whole-embryo culture system. Diamide exposure results in direct and rapid oxidation of GSH to GSSG without concomitant formation of radicals (Kosower *et al.*, 1969). GSH, under these conditions, is depleted and GSSG accumulates. Diamide has been used to alter GSH status in the study of thiol-related cellular processes such as protein, DNA and RNA synthesis, radiosensitization, microtubule assembly, enzyme functions, amino acid, sugar and ion transport, actions of hormones, neurotransmitter release, and mitochondrial functions (Harris, 1979). The effects of diamide, however, on the developing conceptuses have not yet been reported. Our study explores the embryotoxicity of diamide, alterations of GSH/GSSG levels, and the roles of the enzyme GSSG reductase (via inhibition with BCNU) and new GSH synthesis (via inhibition with L-buthionine-[*S,R*]-sulfoximine, BSO) in restoring GSH levels and mediating the effects of diamide.

METHODS

Chemicals. Diamide and BSO were purchased from the Sigma Chemical Co. (St. Louis, MO). BCNU was obtained from the Bristol-Myers Company. All other chemicals and reagents were of the highest purity available.

Embryo culture and assessment. Time-mated Sprague–Dawley rats were obtained on Days 7–9 of gestation from the University of Michigan Reproductive Science Program; P-30 Small Animal Core Facility. All animals were allowed free access to food and water. The morning following copulation, indicated by a positive vaginal smear, is designated as Day 0 of gestation. Pregnant dams were anesthetized with ether on Day 10 of gestation and blood collected from the abdominal aorta was prepared for serum, heat-inactivated, and used in the embryo culture media. Conceptuses were removed from the decidua and the remnants of the parietal yolk sac and the Riechert's membrane were removed, allowing the embryo to develop properly in culture. Conceptuses were grown in 125-ml roller bottles in medium consisting of 33% heat-inactivated rat serum and sterile Hanks' balanced salt solution (HBSS, pH 7.4) with potassium penicillin G (100 IU/ml) and streptomycin (50 μg/ml) in a total volume of 15 ml. Embryos having 8–10 somites were chosen for culture. The culture medium was saturated with 20% O_2:5% CO_2:75% N_2. Diamide and BSO were dissolved in distilled water and were added directly to the culture medium. BCNU was dissolved in redistilled dimethyl sulfoxide (DMSO) and also added directly to the culture medium.

Malformations and growth parameters were assessed under a dissecting microscope after 26 hr in culture on the afternoon of Gestational Day 11. Bottles containing conceptuses were saturated with a mixture of 95% O_2:5% CO_2 on the morning of Day 11. Conceptuses regarded as viable were those having a positive heartbeat and active vitelline circulation in the visceral yolk sac (VYS). Only viable conceptuses were used for statistical analysis of malformation incidence and biochemical data. Embryonic

growth parameters such as crown–rump length, somite number, abnormal axial rotation, closure of the anterior neural tube, and other morphological defects were evaluated. Embryo and VYS were placed individually in 0.5 ml of HBSS at the completion of the assessment and frozen at −74°C for protein and DNA analysis. Protein content was determined using the method of Bradford (1976) as modified for use with a 96-well plate and analyzed in a microtiter plate spectrophotometer. Bovine plasma γ-globulin was used for preparation of the standard curve. DNA was determined by the method of Labarca and Paigen (1980).

Intracellular glutathione assay. Analysis of reduced and oxidized glutathione was carried out by high-performance liquid chromatography (HPLC) using the method of Fariss and Reed (1987). The conceptuses were harvested from culture at the appropriate times and rinsed twice with warm HBSS (pH 7.4, 37°C) to remove chemicals. Each conceptus was placed in a microcentrifuge tube containing 250 μl of HBSS, 5 μl of 10 mM γ-glutamyl-glutamate (an internal standard), 25 μl of 15 mM bathophenanthrolinedisulfonic acid, and 50 μl of 70% perchloric acid and quickly frozen in liquid nitrogen. The samples were stored at −74°C until analyzed by HPLC. When evaluation of individual embryos and VYS was required, the conceptuses were dissected, pooled as needed, and frozen immediately. Thawed samples were homogenized by ultrasonic cell disruption, precipitated protein was removed by centrifugation, and the supernatant was used for GSH/GSSG analysis. The remaining residue was solubilized with NaOH and assayed for protein content using the method of Bradford (1976) as described above except NaOH was used as a diluent and a standard was prepared using bovine serum albumin.

NADPH-dependent glutathione disulfide reductase (GSSG reductase) assay. For Day 10 analysis, conceptuses were removed from culture media after 2 hr of equilibration (1:00 PM) and washed twice with HBSS to remove chemicals. Embryo and VYS were separated, pooled as needed (four to six embryos or VYS on Gestational Day 10 and two embryos or VYS on Gestational Day 11) and placed in microcentrifuge tubes containing 600 μl of 0.2 M potassium phosphate buffer (pH 7) and stored at −74°C until analyzed for GSSG reductase activity. The thawed tissue was homogenized by ultrasonication. Homogenate fractions containing the enzyme were separated from cell debris by centrifugation for 10 min at 4000*g*. GSSG reductase activity was measured at room temperature in a CARY219 spectrophotometer using the spectrophotometric method described by Carlberg and Mannervik (1985). The reaction mixture consisted of 500 μl of 0.2 M potassium phosphate buffer (pH 7) containing 2 mM EDTA, 50 μl of 2 mM NADPH in 10 mM Tris–HCl (pH 7) and 50 μl of 20 mM GSSG in deionized water. Final reaction volume was adjusted to 1.0 ml by adding deionized water. In a reference cuvette, water, instead of GSSG, was used. The reaction was initiated by addition of the supernatant to the cuvette. The oxidation of NADPH was followed by the decrease in absorbance at 340 nm compared to the reference cell. Enzyme activity is defined as nanomoles of NADPH oxidized per minute per milligram protein in the supernatant. Day 11 conceptuses were analyzed using the same procedure by removing two embryos or VYS at 2:00 PM. Recovery of GSSG reductase activity following BCNU inhibition on Day 10 was determined by removing conceptuses into fresh media after 2 hr and conducting assays at 3-hr intervals.

Statistical analyses. Statistical differences of GSH and GSSG levels between treatments were determined using analysis of variance (ANOVA), general linear models procedure. Data on embryonic growth parameters were assessed using one-way ANOVA. Unless otherwise indicated in the figure, multiple pairwise comparisons of the treatments were made by Tukey's test. Differences were considered to be statistically significant when $p < 0.05$. For abnormal axial rotation studies, the Fisher's exact test (two tail) was carried out to determine statistical significance where the corrected level of significance for multiple comparisons was $p < 0.05/n$; n, number of multiple comparisons (Wang *et al.*, 1990).

RESULTS

Initial experiments characterized the concentration effects of diamide on viability, common growth parameters (crown–rump length, somite number, protein, and DNA content), and morphology in the conceptuses exposed to diamide on Gestational Day 10 and evaluated after 26 hr in culture (Table 1). Addition of diamide (50 μM) produced no detectable adverse effects on viability or growth parameters and no abnormal embryos were observed. Increased concentrations of diamide (75 and 100 μM) resulted in the significant reduction of protein and DNA contents, and the embryos exhibited increased incidence of abnormal trunk morphology characterized by a disruption in the normal dorsal-to-ventral axial rotation usually seen during this stage of development (Gestational Days 10–11). The displacement of the trunk was normally observed at the level of the upper limb bud. Diamide (75 μM) produced abnormal rotation in 62% of exposed conceptuses while maintaining 100% viability. Concentrations of 100 μM increased the incidence of rotation defects to 89% and decreased viability to 69%. In addition, VYS vasculature was reduced and prosencephalic hypoplasia was observed with 100 μM diamide (data not shown). Under the same conditions, diamide concentrations of 250 and 500 μM (which are the concentrations commonly used in other cell culture systems) resulted in 100% mortality and, thus, malformations were not assessed. Conceptuses exposed to high concentrations of diamide (500 μM) on Day 10 for short periods of time (15–30 min) remained viable but had significant morphological defects similar to those seen at lower concentrations and where diamide remained in the media during the entire culture period (Table 2). Exposure to diamide for 15 min produced abnormal axial rotation in 53% of exposed conceptuses as well as significant reductions in VYS protein and DNA of 20 and 28% of control, respectively. After 30 min of exposure, viability was decreased to 71% of control and axial rotation defects increased to 87% in surviving conceptuses. Crown–rump determinations could not be accurately made in these embryos. In addition to the obvious lack of axial rotation, embryos exposed to 500 μM diamide for 30 min also exhibited apparent necrosis in the region of the neural tube characterized by the presence of opaque debris in the anterior tube lumen. Swollen areas in the prosencephalon and further significant reductions in both embryonic and VYS protein and DNA contents were also seen (Table 2). Exposure times in excess of 30 min resulted in rapid loss of viability as determined on Day 11. Diamide at any concentration did not adversely affect anterior neural tube closure in the embryo.

Changes in the GSH and GSSG status as a result of diamide exposure were determined after allowing the conceptuses to equilibrate in control media for 2 hr prior to di-

TABLE 1
Concentration Effects of Diamide[a] on Axial Rotation and Embryonic Growth Parameters from Rat Conceptuses Exposed for 26 Hr in Whole-Embryo Culture

Treatment	Viability (%)	Abnormal axial rotation (%)	Crown–rump length (mm)	Somite number	Protein (μg)		DNA (μg)	
					Embryo	Visceral yolk sac	Embryo	Visceral yolk sac
Control	100 (12/12[b])	0 (12)	3.21 ± 0.17[c] (12)	26.17 ± 1.03 (12)	252.75 ± 37.97 (12)	153.97 ± 18.25 (12)	34.06 ± 6.42 (12)	11.19 ± 1.67 (12)
Diamide								
50 μM	100 (13/13)	0 (13)	3.12 ± 0.10 (13)	26.08 ± 0.90 (13)	225.49 ± 54.73 (13)	135.76 ± 26.23 (13)	30.66 ± 5.53 (13)	10.37 ± 1.28 (13)
75 μM	100 (13/13)	62 (13)[d]	3.10 ± 0.10 (5)	26.20 ± 0.45 (5)	185.04 ± 47.51[d] (13)	116.68 ± 27.72[d] (13)	26.00 ± 6.70[d] (13)	9.28 ± 1.45[d] (13)
100 μM	69 (9/13)	89 (9)[d]	—	—	102.00 ± 49.23[d] (9)	85.89 ± 23.81[d] (9)	13.61 ± 6.86[d] (9)	7.13 ± 1.14[d] (9)

[a] Diamide was added at the beginning of the culture period (Gestational Day 10).
[b] Number of viable conceptuses/total number of conceptuses.
[c] Values represent the mean ± SD (*n*) of viable conceptuses.
[d] Significantly different from control.

amide (50–500 μM) addition (Fig. 1). The time course of effects of diamide on GSH and GSSG levels was determined for the 2-hr period after diamide addition to culture media. The effects of diamide at 500 μM were only followed through 60 min of exposure due to the rapid loss of viability mentioned above. Concentrations of 50 to 100 μM diamide failed to deplete GSH levels significantly from the control values in the tissues of the whole conceptus. Significant decreases in GSH were seen, however, in the conceptuses exposed to diamide at concentrations of 250 and 500 μM. The GSH depletion was rapid, indicated by a 50% reduction of the control GSH within 5 min of exposure. Similar determinations of GSH in individual embryos and VYS from conceptuses exposed to diamide (500 μM) show that the GSH loss is due almost exclusively to depletion of the VYS (Fig. 2). GSH pools in the VYS were reduced by 70% within 15 min of diamide exposure while embryonic levels decreased by only 25%. Significant elevations in GSSG were seen in the diamide (500 μM) treatment group (Fig. 1). When only the values obtained from the first 30 min following exposure were evaluated statistically, diamide at concentrations of 250 and 500 μM each produced a significant increase in GSSG levels. With 500 μM diamide, GSSG levels were increased threefold over controls at 30 min.

The role of GSSG reductase in maintaining GSH levels by reduction of GSSG was also evaluated with respect to diamide toxicity. Specific enzyme activities for GSSG reductase in both embryo and VYS were comparable on Gestational Days 10 and 11 (Table 3). On both days, however, the VYS had a six- to eightfold higher GSSG reductase activity than did the embryo proper. BCNU, an inhibitor of GSSG reductase, was used to determine the importance of

TABLE 2
Short-Term Exposure Effects of Diamide[a] (500 μM) on Axial Rotation and Embryonic Growth Parameters in Gestational Day 10 Rat Conceptuses

Treatment	Viability (%)	Abnormal axial rotation (%)	Crown–rump length (mm)	Somite number	Protein (μg)		DNA (μg)	
					Embryo	Visceral yolk sac	Embryo	Visceral yolk sac
Control	100 (19/19[b])	0 (19)	2.95 ± 0.17[c] (18)	25.94 ± 1.86 (18)	278.43 ± 48.34 (19)	177.45 ± 34.27 (19)	29.00 ± 7.03 (19)	11.12 ± 2.36 (19)
Diamide								
0 min	100 (20/20)	20 (20)	3.10 ± 0.35 (16)	25.81 ± 0.54 (16)	279.32 ± 54.93 (20)	178.52 ± 39.60 (20)	30.76 ± 7.22 (20)	11.48 ± 2.89 (20)
5 min	96 (22/23)	18 (22)	2.98 ± 0.20 (18)	25.27 ± 0.80 (15)	269.87 ± 46.18 (22)	165.89 ± 29.36 (22)	28.52 ± 7.28 (22)	11.16 ± 3.07 (22)
15 min	95 (19/20)	53 (19)[d]	2.90 ± 0.24 (10)	25.67 ± 0.71 (9)	252.74 ± 60.15 (19)	142.21 ± 40.05[e] (19)	25.93 ± 6.21 (19)	8.17 ± 1.95[e] (19)
30 min	71 (15/21)	87 (15)[d]	—	—	199.74 ± 72.66[e] (15)	125.14 ± 25.36[e] (15)	19.94 ± 9.84[e] (15)	7.07 ± 2.58[e] (15)

[a] Conceptuses were equilibrated in the media 2 hr before addition of diamide. After exposure conceptuses were transferred to diamide-free media and cultured normally for 24 hr.
[b] Number of viable conceptuses/total number of conceptuses.
[c] Values represent the means ± SD (*n*) of viable conceptuses.
[d] Signficantly different from control.
[e] Signficantly different from control and diamide, 0 min.

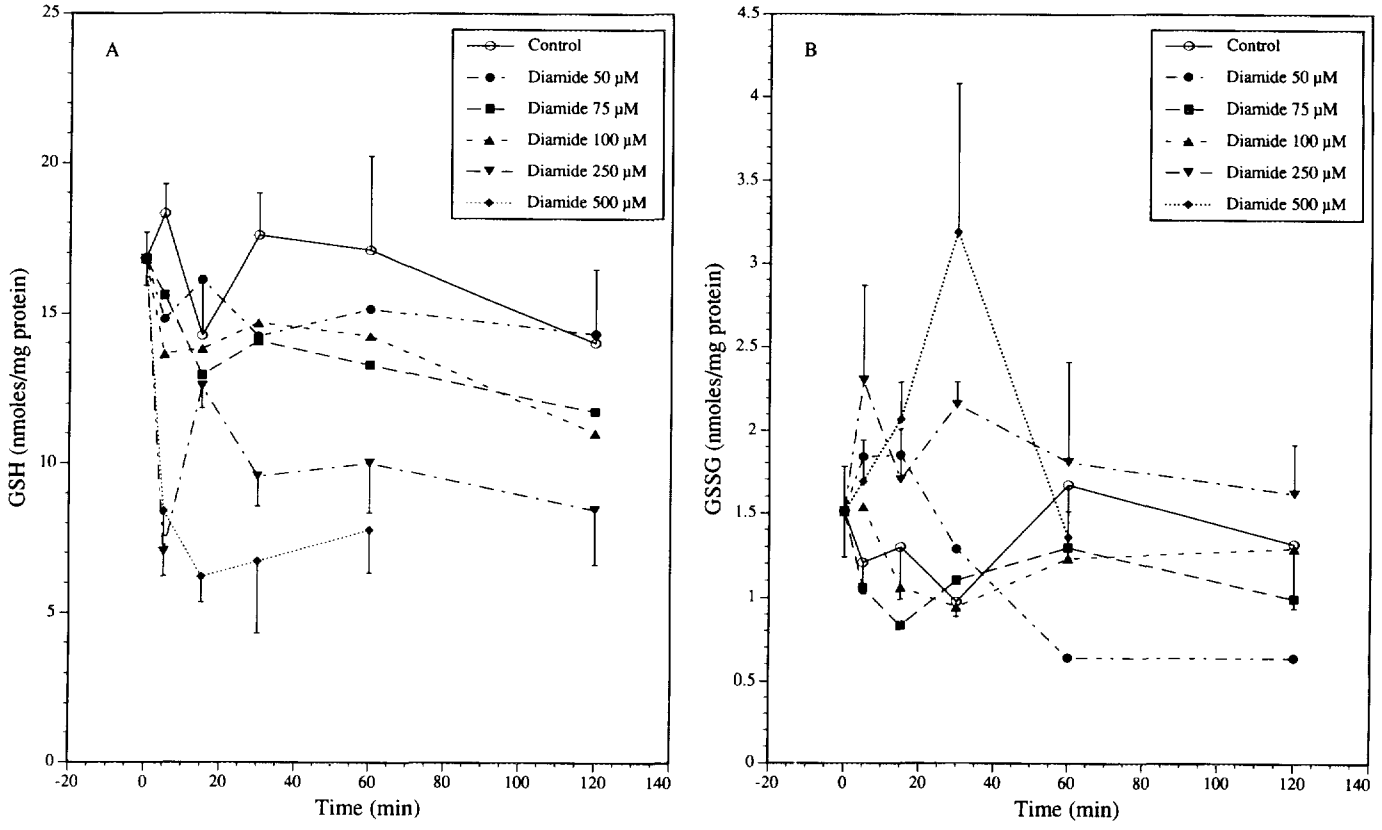

FIG. 1. Effects of diamide on GSH (A) and GSSG (B) levels in the Day 10 rat conceptus. Each value is expressed per milligram of whole conceptal protein. The conceptuses were allowed to equilibrate in control media for 2 hr before addition of diamide. Each conceptus, removed at times indicated after diamide addition, was assayed for GSH and GSSG as described under Methods. Each point represents the mean of at least three determinations and error bars represent the SE (omitted for diamide at 50, 75, and 100 μM for clarity). The 5 min values at 75 μM diamide (A and B) are the average of two determinations. Statistical analyses were performed between control and each treatment groups (five comparisons) with the corrected level of significance for multiple comparisons as 0.05/5 = 0.01. Overall effects on GSH following 250 and 500 μM diamide were significantly different compared to controls. GSSG values of the 500 μM diamide concentration were significantly different from control.

GSSG reductase in maintaining GSH status following diamide exposure. Following 2 hr of incubation with BCNU (25 μM), the activities of GSSG reductase in the embryo and VYS were inhibited to approximately 50 and 20% of control, respectively (Fig. 3). After removal of conceptuses to media not containing BCNU, the VYS gradually regained GSSG reductase activity to about 40% of control within 6 hr. The activity in the embryo remained inhibited during this same period. The 2-hr inhibition of GSSG reductase with BCNU was found to be slightly embryotoxic because conceptuses removed from culture and grown in BCNU-free media for 24 hr showed reduced embryonic (not VYS) DNA content (Table 4). Some embryos also exhibited slight hypoplasia of the prosencephalon, which has been reported previously (Kirby *et al.*, 1987). Under the same conditions, neither significant increases in the incidence of abnormal axial rotation nor altered growth parameters were seen in the conceptuses exposed to diamide (75 μM). BCNU pretreatment (to inhibit GSSG reductase), followed by diamide (75 μM) did, however, result in increased toxicity (Table 4). Protein and DNA contents, both in embryo and VYS, were greatly and significantly decreased compared to those of control, BCNU, or diamide alone. Incidence of abnormal axial rotation significantly increased to 79%, but 100% viability was maintained. When GSH and GSSG levels were determined, neither BCNU nor diamide alone significantly altered GSH and GSSG levels (Fig. 4). BCNU plus diamide appeared to reduce GSH levels over the first 15 min but these changes were not statistically significant. In contrast, BCNU plus diamide did cause a significant increase in GSSG compared to control and BCNU alone. Pairwise comparisons also showed that GSSG concentrations at 5 min for BCNU plus diamide were significantly different from those of diamide alone ($p < 0.05$). The elevation of GSSG levels occurred rapidly and were approximately twofold higher than control levels when observed at 5 min following diamide addition. GSSG levels following BCNU plus diamide returned to control levels in 120 min.

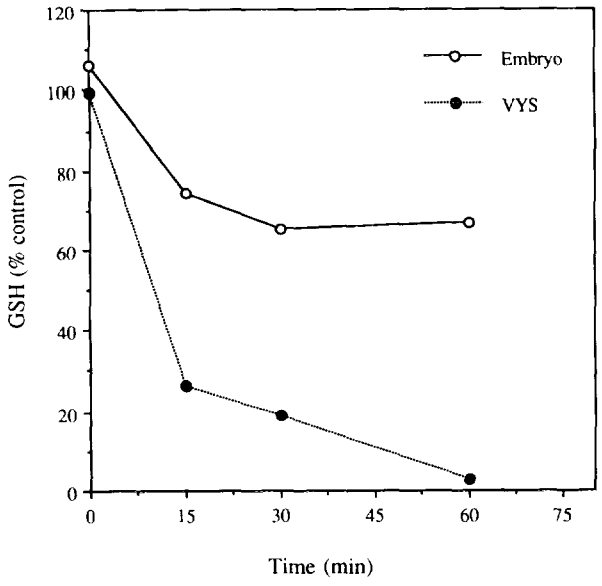

FIG. 2. Differential depletion of embryonic and VYS glutathione in Day 10 rat conceptuses following exposure to diamide (500 μM). The data presented represent the mean of two separate determinations obtained as described under Methods. Additional determinations were also made using an independent HPLC method (Fenton and Fahey, 1986) and results were found to be indistinguishable. Starting at Time 0 min GSH concentrations of 18.9 ± 0.9 and 26.4 ± 2.3 nmol/mg protein were reduced to 13.6 ± 1.4 and 9.4 ± 2.0 nmol/mg protein after 15 min of diamide exposure for embryo and VYS, respectively.

Inhibition of GSH synthesis by BSO 15 min prior to diamide addition also potentiated embryotoxicity of diamide (75 μM) (Table 4). BSO treatment alone did not interfere with viability, produce abnormal axial rotation, or alter any other embryonic growth parameters. Diamide alone and BSO plus diamide caused significant increases in axial rotation defects to 67 and 80% of exposed conceptuses, respectively. BSO plus diamide also decreased viability to 53% and significantly reduced protein and DNA contents in both the embryo and VYS to about 65% of control. Crown–rump length and somite number were not affected under this condition.

DISCUSSION

The present study investigates the effects of intracellular GSH oxidation by diamide and the role of GSSG reductase in restoring GSH homeostasis in the developing rat conceptus during organogenesis. Diamide has been shown to cause direct oxidation of GSH in biological systems resulting in decreased GSH through conversion to GSSG (Kosower *et al.*, 1969). Because a number of known embryotoxins such as nitrosofluorene, niridazole, adriamycin, paraquat, and diethylstilbestrol have been shown to undergo redox cycling (Juchau *et al.*, 1986) and could possibly result in reduced GSH/GSSG ratios, it was proposed that diamide may be useful in determining how the conceptus responds to toxic agents which cause these types of changes in thiol homeostasis.

Our initial study evaluated the concentration response of diamide (50–500 μM) on embryotoxicity and alterations in GSH and GSSG levels. Only when concentrations of 250 and 500 μM diamide were used were significant changes in GSH and GSSG observed (Fig. 1). These concentrations, added to the culture media containing conceptuses on Day 10 and evaluated on Day 11, resulted in a complete loss of viability, perhaps suggesting that conceptuses had lost the ability to maintain normal GSH/GSSG ratios necessary for survival. These manifestations of embryotoxicity seen on Day 11 with high concentrations of diamide could be elicited with a relatively short exposure. Diamide (500 μM) produced significant reductions in viability and growth parameters, as well as increased dysmorphogenesis following exposures of only 15 to 30 min on Day 10 (Table 2). These short-term effects suggest that the acute initial consequences of diamide exposure may be most important for elicitation of defects and embryotoxicity terminating as a loss of viability.

GSH, oxidized to GSSG as a result of chemical action, can be restored by enzymatic reduction requiring NADPH and the enzyme GSSG reductase. GSSG reductase activity appears to be important for the conceptus in maintaining GSH/GSSG ratios and providing protection against the embryotoxicity elicited by diamide. We have demonstrated significant GSSG reductase activity in both embryo and VYS at this stage of development which is capable of converting GSSG to the reduced form (GSH). GSSG reductase activities in the VYS (Gestational Day 11, 27.76 nmol/min/mg protein) are similar to those measured in adult rat lung and liver. Nakagawa (1987) has shown that GSSG reductase activities in supernatant fractions of tissue homogenates from adult rat lung and liver were 24.2 ± 2.5 and 37.9 ± 1.4 nmol/mg protein/min, respectively. The need for GSSG reductase activity for maintaining GSH was shown to be important by inhibiting GSSG reductase in both embryo and VYS with BCNU and seeing significantly in-

TABLE 3

Glutathione Disulfide Reductase Activity (GSSG Reductase) in the Day 10 and 11 Rat Embryo and Visceral Yolk Sac (VYS)

	Embryo	VYS
Day 10	3.37 ± 0.41 (4)[a]	20.73 ± 1.32 (4)
Day 11	3.30 ± 0.26 (4)	27.76 ± 1.21 (4)

[a] Values are the means ± SE (*n*). GSSG reductase activity is expressed as nanomoles of NADPH oxidized/min/mg protein.

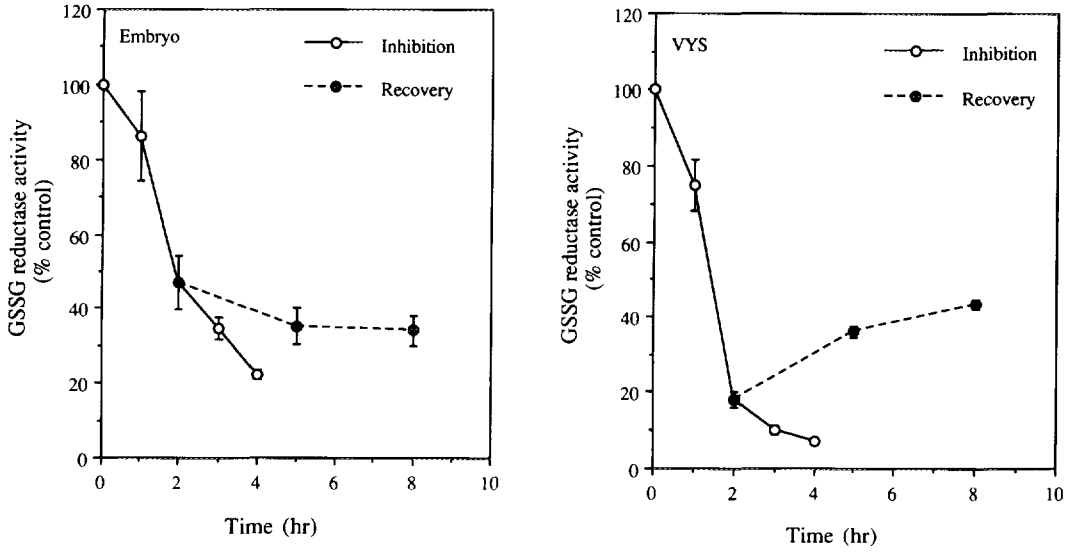

FIG. 3. GSSG reductase inhibition by BCNU (25 μM) in the Day 10 rat embryo and VYS. BCNU 25 μM dissolved in DMSO was added at the onset of the culture period. In the control, the same volume of DMSO was added. At times indicated the conceptuses were harvested and dissected to separate embryo and VYS for analysis of GSSG reductase activity. For determination of recovery, conceptuses were removed at 2 hr, rinsed, and cultured in media not containing BCNU. GSSG reductase activity was defined as nanomoles of NADPH oxidized per minute per milligram protein of embryo or VYS. The values of GSSG reductase activity remaining (in the figure) are presented as percentages of control. Data represent the means ± SE obtained from at least three determinations.

creased GSSG and exacerbated embryotoxicity subsequent to addition of diamide (75 μM) (Fig. 4 and Table 4). Slight reductions of embryonic growth and DNA contents by BCNU may be mediated through its alkylating effects (Weinkam and Lin, 1982). We observed that embryotoxicity of diamide may depend on the time of exposure, where younger conceptuses having complete dorsal flexure and open (45°) anterior neural tubes were more sensitive to diamide than those exposed at later developmental stages. These differences become apparent when we compare malformation incidence for 75 μM diamide in the dose–response analysis in Table 1 to the data in Table 4 where diamide (75 μM) was routinely added 2 hr later in development (due to 2-hr BCNU pretreatment). The conceptuses used for diamide exposure in the BCNU pretreatment group (Table 4) were larger on Day 11 than all others used in concurrent experiments based on crown–rump length, somites, protein, and DNA. Thus, we assume that they were developmentally advanced at the time of exposure and that this may account for the decreased sensitivity toward diamide toxicity. Despite temporal differences with diamide alone, BCNU inhibition of the GSSG reductase resulted in significant additional effects on axial rotation and growth parameters, such as reduced DNA and protein contents. Increased GSSG levels were not accompanied by significant GSH depletion following BCNU plus diamide treatment. This suggests that GSH may be replenished by other pathways such as new GSH synthesis or that the extent of total GSH oxidation may be small relative to the total GSH pool and, therefore, undetectable. The potentiation of embryotoxicity under these conditions suggests that maintenance of GSH/GSSG ratios may be a critical factor in diamide-induced toxicity. Additional experiments with simultaneous inhibition of GSH synthesis with BSO show significantly decreased viability, protein, and DNA contents in the embryo and VYS, indicating that new GSH synthesis may also be important in modulating the embryotoxicity of diamide (75 μM). The potentiation of diamide toxicity with BSO is not believed to be due to GSH depletion per se because previous experiments have shown that 15 min is not a sufficient amount of time to deplete GSH using this method (Harris *et al.*, 1986). We have, however, not yet investigated changes in GSH and GSSG contents in the conceptuses exposed to diamide when GSH synthesis is inhibited.

Although altered GSH/GSSG homeostasis may be involved in embryotoxicity of diamide, other consequences of diamide exposure unrelated to changes in GSH/GSSG levels may be possible. Direct interaction of diamide with critical cellular components could lead to dysmorphogenesis. Diamide has been shown to directly interact with cellular oxidizable components other than GSH such as pyridine nucleotides, cysteine, coenzyme A, lipoic acid, and protein thiols, although at a much slower rate than reactions occur with GSH (Harris and Biaglow, 1972; Kosower *et al.*, 1972). Freeman *et al.* (1987) have demonstrated that

TABLE 4

Effects of Glutathione Modulation by 1,3-bis (2-chloroethyl)-1-nitrosourea [BCNU (25 μM)][a] and L-Buthionine-[*S*,*R*]-Sulfoximine [BSO (1 mM)][b] on Embryotoxicity of Diamide (75 μM)

Treatment group	Treatment	Viability (%)	Abnormal axial rotation (%)	Crown–rump length (mm)	Somite number	Protein (μg)		DNA (μg)	
						Embryo	Visceral yolk sac	Embryo	Visceral yolk sac
BCNU	Control	100 (12/12)[c]	8 (12)	3.24 ± 0.19[d] (11)	23.33 ± 1.67 (12)	278.08 ± 42.13 (12)	175.41 ± 30.50 (12)	23.91 ± 5.08 (12)	7.71 ± 1.28 (12)
	BCNU	100 (14/14)	7 (14)	3.16 ± 0.20 (13)	23.77 ± 1.67 (13)	243.78 ± 30.22 (14)	173.67 ± 21.14 (14)	18.09 ± 3.13[e] (14)	6.50 ± 1.05 (14)
	Diamide	100 (11/11)	9 (11)	3.37 ± 0.13 (10)	24.00 ± 1.05 (10)	297.11 ± 39.82 (11)	183.21 ± 18.90 (11)	26.30 ± 4.06 (11)	7.80 ± 1.10 (11)
	BCNU + Diamide	100 (14/14)	79[e] (14)	3.17 ± 0.15 (4)	23.20 ± 0.84 (5)	191.42 ± 49.94[f] (14)	140.83 ± 25.80[f] (14)	12.27 ± 5.10[f] (14)	4.40 ± 1.53[f] (14)
BSO	Control	100 (18/18)	17 (18)	3.1 ± 0.23 (15)	25.81 ± 1.76 (16)	249.88 ± 40.08 (18)	171.51 ± 20.46 (18)	22.19 ± 5.53 (18)	9.17 ± 1.49 (18)
	BSO	100 (18/18)	6 (18)	2.99 ± 0.21 (17)	25.72 ± 1.18 (18)	233.82 ± 39.30 (18)	181.24 ± 25.02 (17)	21.72 ± 4.97 (18)	9.22 ± 1.80 (17)
	Diamide	100 (18/18)	67[e] (18)	3.05 ± 0.24 (6)	26.29 ± 1.25 (7)	217.63 ± 47.49 (18)	160.31 ± 22.30 (18)	19.41 ± 4.78 (18)	7.96 ± 1.19 (18)
	BSO + Diamide	53 (10/19)	80[e] (10)	3.00 (2)	25.00 ± 1.00 (3)	164.31 ± 48.11[g] (10)	114.32 ± 34.04[g] (10)	14.28 ± 4.60[g] (10)	6.22 ± 1.73[g] (10)

[a] Conceptuses were preincubated with BCNU (dissolved in DMSO) or DMSO alone (for control culture) for 2 hr following which the conceptuses were rinsed to free BCNU/DMSO and recultured in new media in the presence and absence of diamide for the remaining 24 hr.

[b] BSO was added at the beginning of culture period 15 min before addition of diamide.

[c] Number of viable conceptuses/total number of conceptuses.

[d] Means ± SD; (*n*), number of viable conceptuses assessed for malformations and embryonic growth parameters.

[e] Significantly different from control.

[f] Significantly different from control, BCNU, and diamide.

[g] Significantly different from control, BSO, and diamide.

100 μM diamide did not affect GSH concentrations during 60 min of incubation, but produced moderate degree of resistance to hyperthermal toxicity in the cultured Chinese hamster ovary (CHO) cells. Only 40–50% reductions in GSH were observed in the CHO cells upon exposure to 400 μM diamide. In agreement with our data, lower concentrations of diamide (75–100 μM) failed to cause significant GSH depletion or GSSG accumulation but the embryotoxicity and malformations were seen. It is possible, however, that localized alterations in GSH/GSSG may be masked because the whole conceptus was evaluated in this study. GSH depletion in individual tissues of the embryo and the VYS differ following 500 μM diamide exposure. We found that approximately 70% of the GSH in the VYS was depleted (within 15 min) while only 25 and 50% of control GSH was depleted in the embryo proper and the whole conceptus, respectively (Figs. 1 and 2). Thus, determinations of GSH depletion in the whole conceptus using the lower concentrations of diamide may not be sufficiently sensitive to detect the small changes in GSH or GSSG levels if they occur locally or only in the VYS. A greater degree of GSH depletion in the VYS may also indicate that a smaller proportion of diamide is reaching the embryo proper. Diamide, 75 μM, seemed to produce general toxicity in the form of abnormal rotation, similar to that seen with 2-nitrosofluorene (NF) (Harris *et al.*, 1987). Recently, it has been shown that NF-induced abnormal rotation may be mediated primarily through its effects on the VYS (Stark and Juchau, 1989). Evidence from our study, such as the susceptibility of VYS to GSH depletion, high GSSG reductase activity found in the VYS, and the absence of any specific defects in addition to abnormal axial rotation may indicate that VYS is a primary target for the toxic effects of diamide. This hypothesis requires further investigation. In addition, other cellular consequences underlying embryotoxicity and mediated through GSH oxidation by diamide such as reduction of NADPH supply, GSSG efflux, and protein–GSH mixed disulfide formation also need to be evaluated. Thiol–disulfide exchange reactions can form protein–GSH mixed disulfides and several enzyme activities have been shown to be regulated by these thiol and disulfide exchange mechanisms (Ziegler, 1985). The effects of increased GSSG on specific enzymes important to development are yet to be determined.

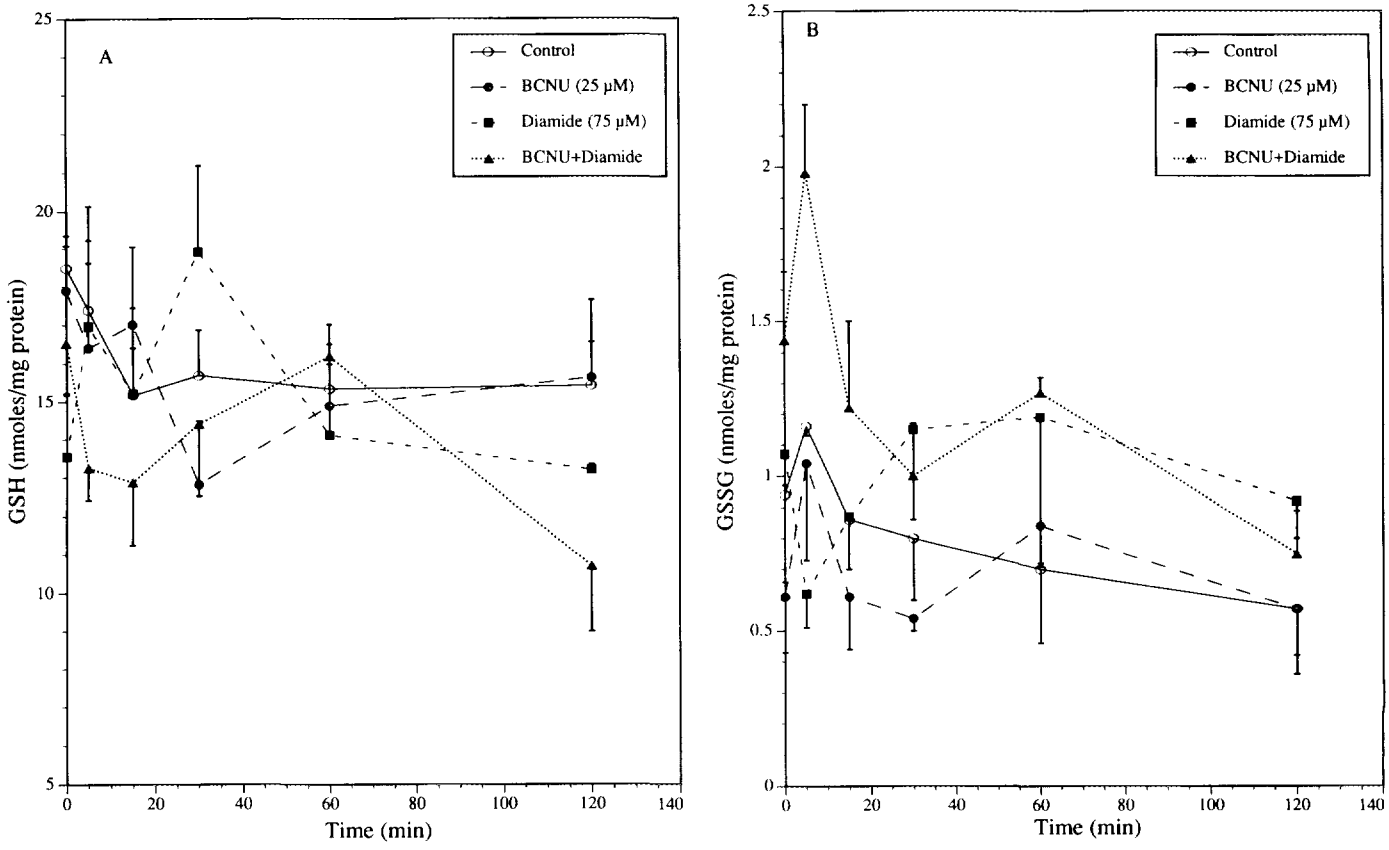

FIG. 4. Effects of diamide (75 μM) on GSH (A) and GSSG (B) following GSSG reductase inhibition by BCNU (25 μM). Conceptuses were preincubated with BCNU for 2 hr, rinsed to free BCNU, and returned to new culture media. Diamide was then added (Time 0 min) and the conceptuses were removed at time indicated for GSH and GSSG analysis. Each value represents the mean ± SE (n = 3–4). In control cultures the same volume of DMSO was added instead of BCNU. No significant differences in GSH levels were seen in any experimental group. Changes in GSSG levels in the BCNU plus diamide group were significantly different from those of control and BCNU-treated groups ($p < 0.05$). Pairwise comparisons using the Tukey test show that GSSG concentrations at 5 min for BCNU plus diamide are significantly different from those for diamide alone ($p < 0.05$).

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