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Formation of Protein–Glutathione Mixed Disulfides in the Developing Rat Conceptus Following Diamide Treatment In Vitro

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ABSTRACT Protein–glutathione mixed disulfide (protein–S-SG) formation was investigated in developing rat conceptuses during early organogenesis (gestational day 10, GD 10) using the whole embryo culture system. Low levels of protein–S-SG (25.0 ± 6.6 pmoles resolved GSH/conceptus) were found in conceptuses under normal culture conditions. Incubation of the conceptuses with 75–500 μM diamide (a thiol oxidant) resulted in rapid increases in protein–S-SG (to 2- to 16-fold that of control values) in a dose-dependent manner during 30 min of the culture period. Approximately 20% of the observed cytosolic glutathione (GSH) depletion following diamide (500 μM) could be accounted for as mixed disulfides of protein sulfhydryls, when determined in whole conceptual tissues after 15 min. The most extensive S-thiolation of protein sulfhydryls by GSH was observed in visceral yolk sac (VYS) when compared to embryo proper and ectoplacental cone. This result indicates that the most abundant, sensitive, or accessible protein sulfhydryls were found in the VYS. Inhibition of glutathione disulfide reductase activity by pretreatment of the conceptuses with 25 μM BCNU for 2 hr potentiated protein–S-SG formation elicited by 75 μM diamide. Reincubation of the conceptuses in fresh media, following the 15-min treatment with 500 μM diamide, reversed both the GSH depletion and the protein–S-SG formation in conceptal tissues. The reduction of the protein–S-SG was dependent on adequate intracellular GSH levels and was inhibited when GSH was rapidly depleted by subsequent addition of *N*-ethylmaleimide (NEM, 100 μM). Under the same experimental conditions, addition of 1 mM dithiothreitol (DTT) did not significantly enhance the GSH restoration rate nor the protein–S-SG reduction rate. The results also indicated that low levels of intracellular cysteine do not play an important role in the reduction of protein–S-SG. Protein–S-SG formation may be important for cellular regulation and in mediating the embryotoxicity elicited by diamide or other oxidative stresses.

Reduced glutathione (GSH), the most abundant intracellular thiol, contributes the greatest source of cellular reducing equivalents and is known to be important in the control of cellular redox status. Glutathione participates, either directly or indirectly, in various critical cellular processes including detoxication (Ketterer et al., '83; Jones et al., '86) and maintenance of membrane integrity (Kosower et al., '69a). The regulation of cellular proliferation, differentiation, and development have also been shown to be associated with GSH status (reviewed in Allen and Balin, '89). Glutathione levels may be depleted considerably as a result of detoxication activities and may result in cellular toxicity and altered cell functions as GSH-dependent protective mechanisms are oversaturated.

Glutathione is important for the protection of developing conceptuses against embryotoxicity produced by several xenobiotics both in vitro and in vivo (Faustman-Watts et al., '86; Harris et al., '87; Wong et al., '89). Although underlying mechanisms could involve the chemical's ability to elicit toxicity directly, it is also probable that altered GSH status produced as a consequence of detoxication processes may contribute to embryotoxicity. Our observations that incubation of developing rat conceptuses (gestational day 10, GD 10) with diamide, a thiol oxidant, causes dysmorphogene-

Abbreviations: BCNU, 1,3-bis (2-chloroethyl)-1-nitrosourea; EPC, ectoplacental cone; DTT, dithiothreitol; DMSO, dimethylsulfoxide; DTPA, diethylenetriaminepentaacetic acid; GD, gestational day; GSH, reduced glutathione; GSSG, glutathione disulfide; GSSG-reductase, glutathione disulfide reductase; HBSS, Hanks' balanced salt solution; HEPPS, (*N*-[2-hydroxyethyl]-piperazine-*N*'-[3-propane-sulfonic acid); MSA, methanesulfonic acid; NEM, *N*-ethylmaleimide; MBBr, monobromobimane; PPB, pentose phosphate shunt pathway; protein–S-SG, protein–glutathione mixed disulfide; VYS, visceral yolk sac.

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sis provide additional evidence that glutathione redox status may have an important role in developmental processes and embryotoxicity (Hiranruengchok and Harris, '93). Moreover, redox cycling compounds, which are capable of causing oxidative stress and altered GSH status, have been reported to be embryotoxic and dysmorphogenic in vivo and in vitro (Juchau et al., '86). It has been suggested that the consequences of altered redox status that occur during cellular oxidation may influence gene expression and the regulatory control of development (Allen and Balin, '89). In spite of considerable evidence showing an association between redox status and cellular function, the regulation of these processes has not been thoroughly investigated. It is assumed that structural and functional modifications of proteins or critical enzymes may be responsible. Various studies suggest that protein mixed disulfide formation (or protein S-thiolation) represents one possible cellular mechanism for regulation of enzymatic activity and cellular function in vivo (Ziegler, '85).

Glutathione, as the major thiol in cells, is also quantitatively the most important thiol involved in forming mixed disulfides with proteins. The protein–glutathione mixed disulfides (protein–S-SG) are formed when glutathione disulfide (GSSG) reacts with protein sulfhydryls of cysteine residues in proteins to form mixed disulfides. Cellular oxidations, which cause depletion of GSH and increased levels of GSSG, can therefore result in increased formation of protein–S-SG. The formation of protein–S-SG by oxyradical-initiated mechanisms other than through direct interaction with GSSG via thiol–disulfide exchange has also been proposed (Miller et al., '90). Various cellular functions may be affected by protein–S-SG formation, especially when the protein thiol being oxidized represents a critical structural element or is involved in active site function of an enzyme, receptor, or transporter. Recently, specific S-thiolated proteins have been described in isolated cells under conditions of oxidative stress induced by diamide (Park and Thomas, '88), *t*-butylhydroperoxide (Guarnieri et al., '87), and menadione (Tsukahara et al., '87). Some of these S-thiolated proteins have been identified as creatine kinase, phosphorylase b (Collison et al., '86; Collison and Thomas, '87), carbonic anhydrase III (Chai et al., '91), glyceraldehyde 3-phosphate dehydrogenase (Schuppe-Koistinen et al., '94), and a proteinase inhibitor (Tsukahara et al., '87).

The biochemical consequences of altered GSH status on protein mixed disulfide formation in tissues of the developing conceptuses have not yet been directly investigated. Some of the cellular processes shown to be modulated by formation of the protein mixed disulfides and known to be important in maintaining the growth and development of mammalian embryos include proteolysis and glucose metabolism (glycolysis and the pentose phosphate shunt pathway) (Brigelius, '85). It is therefore probable that embryotoxicity induced by altered glutathione redox status during oxidative stress may be mediated through biological consequences of protein mixed disulfide formation. Quantitation of the protein mixed disulfides is important in understanding this possible mechanism of thiol oxidation-induced embryotoxicity. As a continuation of our previous studies, the effects of GSH oxidation by diamide on protein mixed disulfide formation have been investigated in organogenesis-stage rat conceptuses (GD 10) using the rat whole embryo culture system (New, '73). The formation and subsequent reduction of total protein mixed disulfides and accompanying alterations in thiol status have been evaluated. In addition, the role of glutathione disulfide reductase (GSSG-reductase) in formation of the protein mixed disulfide was also examined. It was shown in this study that oxidation of GSH by diamide can lead to increased formation of protein–S-SG in tissues of the conceptuses and this effect was enhanced by inhibition of GSSG-reductase activity. The protein–S-SG formation by diamide was a reversible process and excess intracellular GSH was found to be important for reduction of the mixed disulfides. Correlations of increased protein–S-SG formation and the embryotoxicity caused by diamide (Hiranruengchok and Harris, '93) implicate a possible role of the protein S-thiolation in mechanisms of embryotoxicity elicited by chemically induced oxidative stress.

MATERIALS AND METHODS

Chemicals

Diamide, dithiothreitol (DTT), *N*-ethylmaleimide (NEM), and GSH were obtained from Sigma Chemical Co. (St. Louis, MO) as were glutathione disulfide reductase and glucose 6-phosphate dehydrogenase. 1,3-Bis-(2-chloroethyl)-1-nitrosourea (BCNU) was provided by the Bristol Myers Company (Syracuse, NY). Other chemicals and reagents were of the highest grade commercially available.

Animals

Time-mated primagravida Sprague-Dawley rats were used in all experiments. Animals were obtained on days 6–9 of gestation from the Reproductive Science Program, Small Animal Core, University of Michigan, and maintained on a 14-hr light/10-hr dark cycle until explantation on GD 10. All animals had free access to food and water. The morning following copulation, indicated by a sperm-positive vaginal smear, was designated as day 0 of gestation (GD 0).

Rat whole embryo culture and tissue preparation

Pregnant dams were anesthetized with ether on GD 10. Preparation of conceptuses and serum for culture have been described in detail elsewhere (Hiranruengchok and Harris, '93). The conceptuses used in culture experiments consisted of intact visceral yolk sac (VYS),

ectoplacental cone (EPC), amnion, and embryo. Embryos having 8–10 somites were carefully chosen for experiments and generally cultured 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 (41 IU/ml) and streptomycin (41 μg/ml) in a total volume of 10 or 15 ml (one conceptus per 1–2 ml of medium). The culture medium was previously warmed and saturated with 20% O_2/5% CO_2/75% N_2. The culture bottles were placed in a roller-incubator and maintained at 37°C throughout the culture period. Conceptuses were normally allowed to equilibrate in culture media for approximately 1 hr prior to addition of chemical agents. Diamide, DTT, and NEM were dissolved in distilled water; BCNU was dissolved in redistilled dimethylsulfoxide (DMSO). Exposure to chemical agents was accomplished by direct addition to the culture medium.

At specific time points, conceptuses were taken from the culture media, rinsed to remove chemicals and media, placed in 200 μl of methanesulfonic acid (MSA, 200 mM), quickly frozen in liquid nitrogen and kept at 74°C until analyses of GSH, cysteine, and protein–glutathione mixed disulfide (protein–S-SG), which were performed as subsequently described. When determinations of protein–S-SG in individual embryos, VYS, or EPC were to be carried out, the conceptuses removed from the media were rinsed with HBSS (pH 7.4) containing 10 mM NEM and dissected in the same solution under a dissecting microscope. Pooled embryos, VYSs, or EPCs were also placed in 200 μl of MSA (200 mM) and processed as were whole conceptuses.

HPLC analysis of GSH, cysteine, and protein–glutathione mixed disulfides (protein–S-SG)

Concentrations of intracellular thiols (cysteine and GSH) were determined as their monobromobimane (MBBr, Thiolyte, Calbiochem, La Jolla, CA) adducts using the high-performance liquid chromatography (HPLC) method modified from Fahey and Newton ('87) and Harris ('93). Thawed whole conceptal cells or pooled embryos, VYSs, or EPCs (in 200 μl of 200 mM MSA) were homogenized by ultrasonic tissue disruption. An equal volume of sodium methanesulfonate (4 M) was then added and precipitated protein was separated by centrifugation (14,000*g*, 10 min). Supernatant was removed from the protein pellets, and the pH of the supernatant was increased to 8.0 by the addition of HEPPS (1 M) containing 5 mM diethylenetriaminepentaacetic acid (DTPA) (pH 8.5). MBBr was then added at a final concentration of 0.2 mM, and the reaction was allowed to proceed for 20 min in the dark at room temperature. Next, MSA was added to 200 mM, and the samples were stored at −74°C until analyzed by HPLC. HPLC analysis procedures have been described in detail elsewhere (Harris, '93).

For protein–S-SG assays (Lou et al., '87), the protein pellets obtained from the preceding procedure were washed three times with ice-cold 80% ethanol to eliminate acid soluble thiols and to completely denature proteins. The pellets were then suspended in sodium pyrophosphate (pH 8.4); the enzymatic reduction system consisting of 5 mM EDTA, 1 mM DTT, 0.2 mM $NADP^+$, 2.5 mM glucose 6-phosphate (G6P), 2 μg/ml glucose 6-phosphate dehydrogenase (G6PD) (Lenconostoc mesenteroides; Sigma Type XXIII) and 4 μg/ml glutathione disulfide reductase (yeast; Sigma Type III) was then added to reduce the disulfide bonds and release free GSH. The sample was incubated at 37°C for 30 min, cooled and MSA was added (to a final concentration of 200 mM) to stop the reaction. Next, an equal volume of 4 M of sodium methansulfonate was added to precipitate the protein pellets. The sample was centrifuged and the pH of the supernatant was carefully raised to 8.0 by adding an appropriate amount of the HEPPS–DTPA buffer as described above and MBBr was added to a final concentration of 0.2 mM. After a 20-min incubation in the dark at room temperature, the reaction was terminated by addition of MSA to a final concentration of 200 mM, and the samples were stored at −74°C until analysis of released GSH by HPLC. The amount of the protein–S-SG was determined from this procedure and expressed as pmoles of resolved GSH.

Determination of protein

Tissues were placed in HBSS, frozen, and kept at 74°C for analysis of protein, using the method of Bradford ('76), as modified for use with a 96-well plate and analyzed in a microtiter plate spectrophotometer. Bio-Rad reagent (200 μl) was added to the tissue homogenate (15 μl) in each well. The mixture was incubated for 30 min at room temperature prior to measurement of the absorbance (at 595 nm). Bovine plasma γ-globulin was used for preparation of the standard curve.

When protein content was determined from the acid-precipitated protein pellets (after removal of the supernatant used for GSH and cysteine analysis) (see Fig. 5), the remaining residue was solubilized with NaOH (0.25 N) and assayed for protein content using the method of Bradford ('76), as described previously. In this case, NaOH was used as a diluent, and a protein standard curve was prepared using bovine serum albumin.

Inhibition of glutathione disulfide reductase (GSSG–reductase)

Glutathione disulfide–reductase activity was inhibited by incubation of the conceptuses in culture medium containing BCNU (25 μM added at the start of the culture period) for 2 hr. At the end of the incubation period, GSSG–reductase activities were inhibited to 20% and 40% of control levels in the VYS and embryo, respectively (Hiranruengchok and Harris, '93). The

conceptuses were rinsed to free BCNU and recultured in new medium for subsequent treatments.

Statistical analysis

For all experiments, statistical analysis was performed by ANOVA, general linear models procedure (SAS), followed by Tukey's Studentized Range (HSD) test. Significance was accepted when $P<0.05$.

RESULTS

Diamide, a thiol oxidant (Kosower et al., '69b), which has been shown in our previous studies (Hiranruengchok and Harris, '93) to oxidize GSH to GSSG and cause embryotoxicity, is now also shown to elevate protein–glutathione mixed disulfide (protein–S-SG) formation concomitantly in the cultured rat conceptus. Incubation of conceptuses (GD 10) with increasing concentrations of diamide (75–500 μM) resulted in dose-dependent increases in protein-S-SG formation (Fig. 1). Low levels of protein–S-SG (25.0 ± 6.6 pmoles of GSH per conceptus, mean ±SE) were measured in the conceptuses under normal culture conditions. The increase in mixed disulfides formed by diamide was a rapid process. Peak levels of the mixed disulfide formed by 75–250 μM diamide were reached within 5–15 min. Those formed by 500 μM diamide continued to increase and reached the maximum level at 30 min. During this incubation period, protein–S-SG levels were increased by approximately twofold in conceptuses treated with low concentrations of diamide (75 and 100 μM), when compared to control values. A more extensive protein–S-SG formation (approximately 6- to 16-fold increase over controls) was produced by diamide at concentrations of 250 and 500 μM, respectively. A transient increase in protein–S-SG seen at time 0 min suggested that increased GSSG, formed by the initial GSH oxidation with diamide, rapidly oxidizes the accessible sulfhydryl groups of proteins and forms the mixed disulfide bonds. Diamide has been reported to easily diffuse through cell membranes and oxidize GSH within seconds (Kosower et al., '69b).

Conceptuses removed from the culture media at time 0 min (as indicated in Figs. 1 and 3) are normally exposed to diamide for approximately 10–20 sec, the time needed for mixing and removal of conceptuses from the media. Under these experimental and assay conditions, the value of protein–S-SG formed by 15 min of exposure to diamide (500 μM) was 263.0 ± 18.7 pmoles of GSH per conceptus (Fig. 1), which corresponds to approximately 2.58 nmoles of GSH per mg conceptal protein. The total protein content of conceptuses in this case was found to be equal to that of conceptuses incubated with 500 μM diamide for 15 min, as shown in the subsequent experiments (see Fig. 5; 0.1 ± 0.02 mg/conceptus). During the same 15-min period, GSH was depleted from initial concentrations of 25.3 ± 5.8 nmoles per mg protein (data not shown) to 12.6 ± 1.1

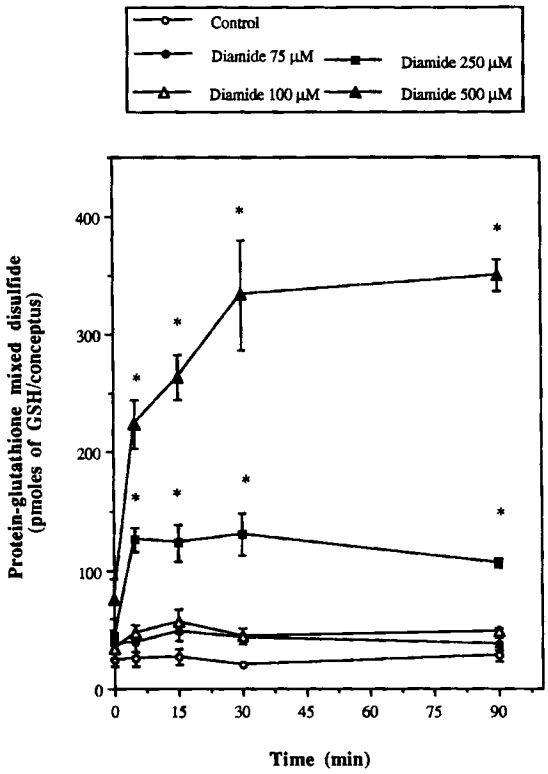

Fig. 1. Dose response of diamide on protein–glutathione mixed disulfide (protein–S-SG) formation in rat conceptuses (gestational day 10). Conceptuses were cultured in media containing various concentrations of diamide. At times indicated, pooled conceptuses (4) were taken from the culture media and processed for quantitation of protein–S-SG as described under Materials and Methods. Immediately after addition of diamide to the media conceptuses were removed, this was designated as 0 min. Protein–S-SG were quantitated as amounts of GSH (released from the mixed disulfide bonds) per one conceptus. Data are represented as mean ±SE (n = 3–4). Asterisk (*) indicates a statistically significant difference from control values ($P<0.05$).

nmoles per mg protein in the conceptus (see Fig. 5A, time 0 min). Assuming that GSH depletion by diamide was primarily due to the total oxidation of thiols, it appears that 20% of depleted cytosolic GSH was due to the formation of mixed disulfides with protein thiols. Depletion of GSH by pretreatment with the γ-glutamylcysteine synthetase inhibitor, BSO (1mM), did not result in any significant increases in protein–S-SG formation and the data is, therefore, not shown.

Determinations of the distribution of protein–S-SG formation showed that diamide caused increases in the mixed disulfides in all three compartments measured: embryo proper, VYS, and EPC (Fig. 2). It is not unexpected that higher levels of mixed disulfides were found in VYS and EPC, since these tissues were directly exposed to the diamide added to the culture media (Fig. 2A). When these values were normalized to the protein content in each tissue, it was found that most extensive S-thiolation of protein thiols by GSH

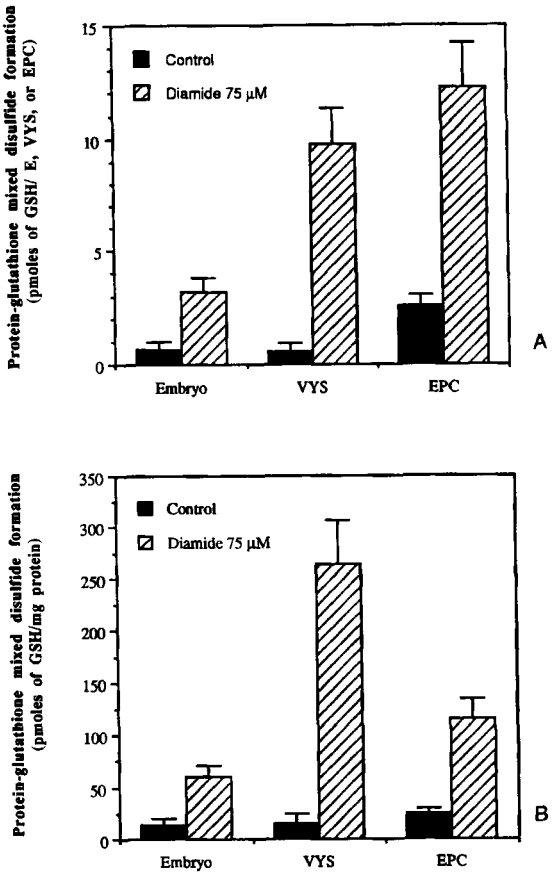

Fig. 2. Distribution of protein–glutathione mixed disulfides (protein–S-SG) in embryos, visceral yolk sacs (VYS), and ectoplacental cones (EPC). Conceptuses (gestational day 10) were incubated for 15 min in the presence of diamide (75 μM), then rinsed and dissected in HBSS (pH 7.4) containing (10 mM) *N*-ethylmaleimide (NEM) in order to prevent removal of glutathione bound to proteins and to inhibit thiol–disulfide exchange. Ten embryos or VYS or EPC were pooled for determination of the protein-S-SG [expressed per conceptus (**A**) or per mg protein (**B**)]. Data are represented as mean ±SE (n = 3–4) of three separate, identical experiments.

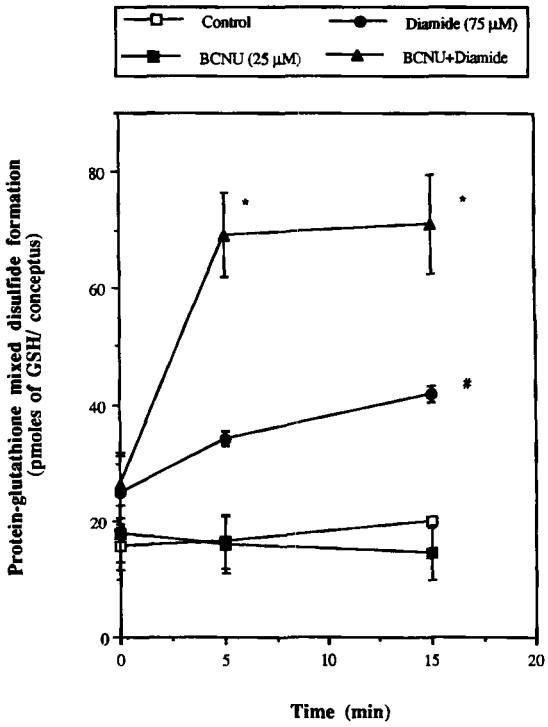

Fig. 3. Formation of protein–glutathione mixed disulfides (protein–S-SG) by diamide (75 μM) in control and BCNU-pretreated conceptuses (gestational day 10). Conceptuses were previously incubated with BCNU (25 μM, dissolved in DMSO) for 2 hr to inhibit glutathione disulfide reductase, then washed and recultured in fresh media with and without diamide (75 μM). In control groups, dimethylsulfoxide (DMSO, 5 μl), instead of BCNU, was added. At the indicated intervals, four conceptuses were removed and pooled for quantitation of protein–S-SG. Results are expressed as mean ±SE (n = 3–4) of three separate experiments. Asterisk (*), statistically significant difference ($P<0.05$) from diamide, BCNU and control (DMSO) groups; #, statistically significant difference from BCNU and control (DMSO) groups.

was present in the VYS (Fig. 2B). Because we were unable to determine the accurate protein contents when the protein pellets had been used for assay of protein–S-SG, another identical experiment was conducted in parallel, with each embryo, VYS, and EPC prepared solely for determination of protein content (described in Materials and Methods) and used for comparison. The protein contents of each embryo, VYS, and EPC was 47.7 ± 7.1, 38.7 ± 6.1, and 103.2 ± 29.5 μg, respectively.

Similar to GSSG formation reported previously (Hiranruengchok and Harris, '93), the protein–S-SG formation elicited by 75 μM diamide was potentiated by pretreatment of the conceptuses with BCNU (Fig. 3). Pretreatment with BCNU (25 μM) for 2 hr resulted in approximately 80% and 60% inhibition of GSSG-reductase activities in VYS and embryo, respectively (Hiranruengchok and Harris, '93). However, BCNU alone did not significantly elevate the level of mixed disulfide formation. Inhibition of GSSG-reductase by BCNU resulted in an increase of GSSG supply during diamide (Hiranruengchok and Harris, '93), which enhanced formation of mixed disulfides with protein sulfhydryls. As a result of observations that higher activity of GSSG-reductase (Hiranruengchok and Harris, '93) and a greater degree of protein–S-SG formed by diamide are found in VYS, it is likely that increased protein–S-SG in VYS is responsible for the increase in total protein–S-SG in the whole conceptus pretreated with BCNU (Fig. 3).

The GSH involved in forming mixed disulfide bonds with protein sulfhydryls was rapidly released when conceptuses were recultured in diamide free media (Fig. 4). At 5 min after removal of diamide, more than 50% of the protein–S-SG formed during 15 min of in-

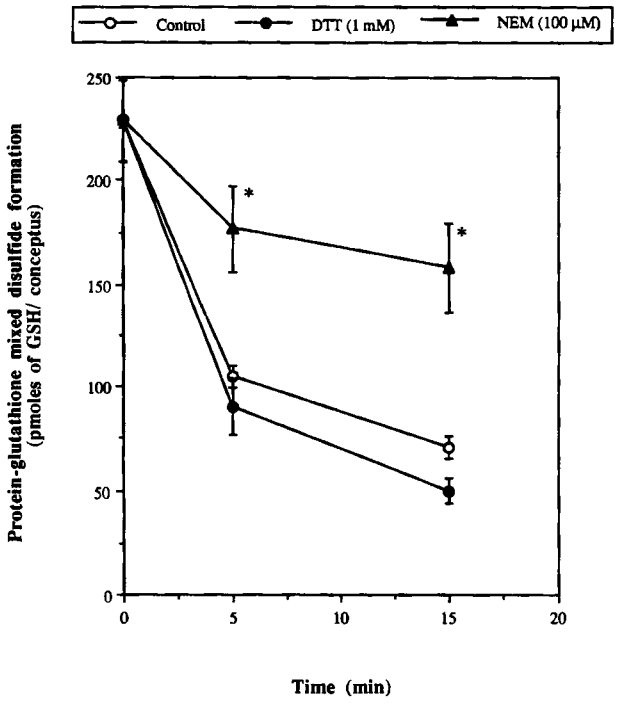

Fig. 4. Effects of dithiothreitol (DTT) and *N*-ethylmaleimide (NEM) on reduction of protein–glutathione mixed disulfides (protein–S-SG) formed by diamide (500 μM) in conceptuses (gestational day 10). Conceptuses were incubated for 15 min in the presence of diamide (500 μM), washed and recultured (designated as 0 min) in fresh media alone (control), or supplemented with DTT (1 mM) or NEM (100 μM). Four conceptuses from each group were taken at the indicated times and pooled for quantitation of protein–S-SG. Results (expressed per one conceptus) are represented as mean ±SE (n = 3) of three identical experiments. Asterisk (*), statistically significant difference ($P<0.05$) from control and DTT treatment.

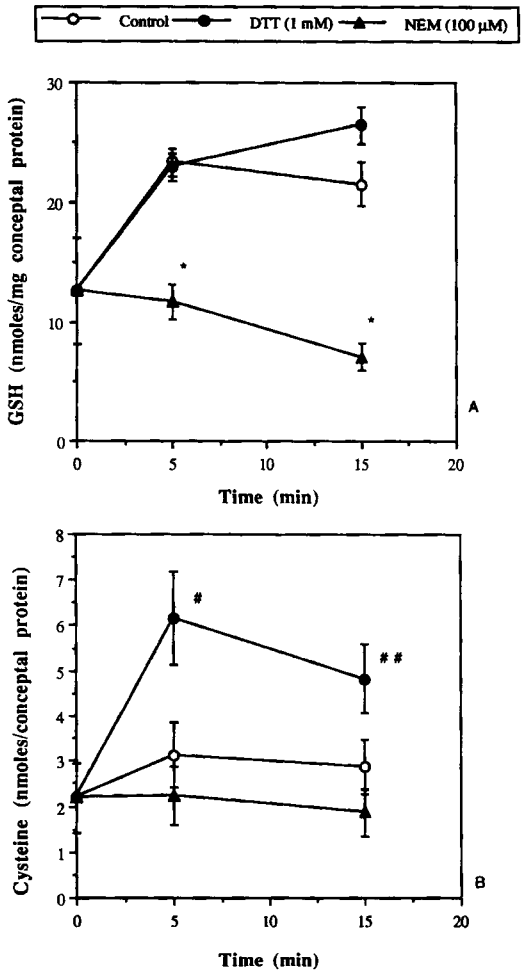

Fig. 5. Recovery of glutathione (**A**) and cysteine (**B**) levels in whole conceptuses in response to dithiothreitol (DTT, 1 mM) and *N*-ethylmaleimide (NEM, 100 μM). Experiments were conducted as described in Figure 4. One conceptus was taken and processed for determination of intracellular GSH and cysteine. Results are means ±SE (n = 8) of three separate experiments. A statistically significant difference was determined when a $P<0.05$ was found. **A:** NEM was significantly different from control and DTT (*). **B:** DTT was significantly different from control and NEM at 5 min (#) and NEM only at 15 min (##).

cubation with diamide (500 μM) were reduced. This was followed by a slower reduction process throughout the subsequent 15-min period, at which time the protein–S-SG still remained at levels approximately two-fold those found in untreated conceptuses. Addition of dithiothreitol (DTT), a thiol reducing agent, at a concentration of 1 mM to the media did not significantly enhance the reduction rate of the protein–S-SG. By contrast, addition of a thiol alkylating agent, *N*-ethylmaleimide (NEM, 100 μM), inhibited the protein–S-SG reduction (Fig. 4). Reduction of protein–S-SG via thiol–disulfide exchange requires thiols such as GSH and cysteine as cofactors (Bellomo et al., '87). Therefore, under the same experimental condition, as described in Figure 4, intracellular GSH and cysteine status in whole conceptal tissues was determined. It was shown that exposure of the conceptuses to 500 μM diamide for 15 min (designated by 0 min in Fig. 5) resulted in GSH depletion to approximately 50% of initial values (25.3 ± 5.8 nmoles/mg protein). Following removal of diamide, GSH levels in the exposed conceptuses recultured in fresh media were recovered within 5 min (Fig. 5) concomitant with reduction of protein–S-SG (Fig. 4). The GSH levels may be recovered from GSSG or other mixed disulfides of GSH (e.g., protein–S-SG). Dithiothreitol did not produce significant additional effects on the regeneration of GSH in the 15 min following diamide removal, suggesting that effective cellular reducing systems are present in the developing conceptus. The recovery of GSH was inhibited by NEM. NEM quickly alkylates free GSH and therefore, reduced GSH cofactor supply for further reduction of protein–S-SG. Like other cellular systems, free cysteine in developing rat conceptuses was present at much lower concentrations compared to GSH. In vivo GSH is more important in this role because it is the most abundant

intracellular thiol. The removal of diamide appeared to restore cysteine levels; however, these values were not statistically significant. No recovery of cysteine was seen by the subsequent treatment of the conceptuses with NEM as expected. In contrast to GSH, intracellular cysteine was increased following the addition of DTT to levels higher than those seen in the control group.

DISCUSSION

This study presents data indicating that mixed disulfides are formed between GSH and protein sulfhydryls during diamide exposure in organogenesis-stage rat conceptuses (GD 10). Because of its known ability to oxidize GSH directly, the mechanism of diamide-induced formation of protein–glutathione mixed disulfides (protein–S-SG) may involve oxidation of GSH to GSSG, followed by interaction with sulfhydryl groups of proteins to form mixed disulfides according to the reaction (Thomas and Sies, '91):

$$\text{GSSG} + \text{protein-SH} \rightleftharpoons \text{protein–S-SG} + \text{GSH}.$$

This reversible protein–S-SG formation may occur either spontaneously or enzymatically through the activity of thioltransferases (Mannervik and Axelsson, '75, '80). The reduction reactions catalyzed by thioltransferase are most specific for GS-containing mixed disulfides (Gravina and Mieyal, '93) and require GSH as a cosubstrate. The role of GSH in conceptal mixed disulfide formation was confirmed by showing that a rapid depletion of GSH by NEM also inhibited the overall reduction of protein–S-SG. Reduction of the mixed disulfide bonds via thiol–disulfide exchange may occur spontaneously, requiring only a certain threshold level of GSH. It has previously been reported that diamide-induced protein–S-SG in human erythrocytes are readily reduced by GSH via thiol–disulfide exchange (Heast et al., '79). Dithiothreitol (DTT) is a reducing agent that has been widely used to study protein sulfhydryls and thiol–disulfide exchange reactions. Dithiothreitol was shown to remove protein mixed disulfides efficiently (Bellomo et al., '87) and reverse the inhibitory effects of diamide on the activity and mixed disulfide formation of the microsomal glutathione S-transferases (Aniya and Naito, '93). In our study, however, addition of DTT (1 mM) did not enhance the rate of protein–S-SG reduction. This result indicates that the high levels of intracellular GSH restored after removal of diamide and supported by a high rate of GSH synthesis are sufficient for the rapid reduction of protein sulfhydryls, without requiring an additional reducing agent.

The addition of DTT did not alter GSH levels but did increase cysteine, probably via reduction of the disulfide bonds of cystine, the oxidized form of cysteine. In addition to GSH, cysteine is also able to reduce a substantial portion of the protein–S-SG, as demonstrated by Bellomo et al. ('87). However, cysteine in intact conceptal tissues did not appear to play this role, since the increased cysteine levels produced by DTT did not result in an increase in the rate of reduction of protein sulfhydryls. This is probably due to the relatively low concentrations of cysteine compared to GSH under these experimental conditions.

The high levels of protein–S-SG (expressed per mg protein) found in VYS, when compared to EPC (a maternally derived tissue) and the embryo proper, indicate that GSH and/or the thioltransferases responsible for their formation are more abundant in the VYS. An analysis of distribution and characterization of thioltransferases in the conceptus has not been made but GSH is abundant. A more likely explanation for observed differences is that sulfhydryl groups of VYS proteins may simply be more sensitive and/or accessible to GSSG to form the mixed disulfides. The lower levels of protein–S-SG seen in the embryo proper suggest that there may also be a reduced accessibility to diamide, determined as a function of the VYS barrier. This was evident in previously reported observations that showed a lower degree of GSH oxidation in the embryo when compared to the VYS (Hiranruengchok and Harris, '93). Absolute concentrations of accumulated diamide have not yet been determined in the embryo and VYS but can be indirectly inferred from previous studies by the magnitude of GSH oxidation (Hiranruengchok and Harris, '93).

The initial formation of protein–S-SG proceeds through GSSG formation and the subsequent reduction of protein–S-SG by thioltransferase and GSH results in the regeneration of GSSG. The inability to remove GSSG via the activity GSSG-reductase may increase the rate of protein–S-SG formation and reduce the available pools of GSH. In this context, it is of interest to note that significant increases in protein–S-SG were seen at doses of diamide that did not produce significant elevations of GSSG (Hiranreungchok and Harris, '93). This apparent discrepancy can be resolved when we consider that GSSG is being rapidly used in the formation of protein–S-SG and is also being reduced to GSH by the NADPH-dependent enzyme glutathione disulfide reductase (GSSG-reductase). This would be especially true in the VYS, where GSSG–reductase activities were found to be sixfold higher than in the corresponding embryo (Hiranruengchok and Harris, '93). As expected, inhibition of GSSG-reductase with BCNU results in the significant increase in protein–S-SG formation (Fig. 3) and a concomitant increase in GSSG (Hiranruengchok and Harris, '93) at doses at which no measurable increases were otherwise seen. Protein–S-SG formation may therefore be a more reliable indicator of acute oxidative stress in conceptuses than the measurement of GSSG or the GSH/GSSG ratio.

Incubation of the conceptuses with L-buthionine-

(S,R)-sulfoximine (BSO, 1 mM), a selective inhibitor of GSH synthesis, did not result in embryotoxicity (Hiranruengchok and Harris, '93) and did not result in increased protein–S-SG formation. However, intracellular GSH levels of the BSO exposed conceptuses were shown to be depleted (Harris et al., '87) to a comparable degree to those exposed to diamide (500 μM) (Hiranruengchok and Harris, '93). These observations suggest that embryotoxicity or protein–S-SG formation elicited by diamide was not due to GSH depletion per se and that consequences of GSSG and protein mixed disulfide formation may be involved. Increased GSSG, even in the presence of excess GSH, has been shown to have a significant effect on enzymes and cellular functions (Kosower and Kosower, '74). The mechanisms by which embryotoxicity and dysmorphogenesis are elicited during the short exposures to diamide are not yet understood. The deleterious effects in conceptuses that occur as consequences of increased GSSG and mixed disulfide formation must be initiated during the short period of acute exposure. Although intracellular thiol oxidation induced by diamide was reversed when the chemical was removed, a portion of the protein remained oxidized and dysmorphogenesis was not eliminated following this short-term exposure to diamide (Hiranruengchok and Harris, '93). A wide variety of enzymes and transport activities have been shown to be affected by the formation of mixed disulfides with functional protein sulfhydryls (Brigelius, '85). These include enzymes in glucose metabolism pathways such as glycolysis and the pentose phosphate shunt pathway (PPP, Mannervik and Axelsson, '80; Mieyal et al., '91; Shen et al., '91). Embryos during this organogenesis period of development (GD 10 to 11) are heavily dependent on these pathways for energy production and nucleic acid synthesis (Shepard et al., '70; Freinkel et al., '84; Shum and Sadler, '90; Hunter and Tugman, '92). According to these reports, oxidative stress and protein mixed disulfide formation result in regulation of these enzymes by thiol–disulfide exchange and may result in the shift of glucose metabolism from glycolysis to the PPP. In a similar manner, cellular exposure to thiol oxidants such as diamide (Tuttle et al., '92), paraquat (Rose et al., '76), and *t*-butylhydroperoxide (Brigelius, '83) have been shown to increase the PPP activity, in concert with increases in protein–S-SG formation (Brigelius and Schult, '84). This adaptive response, especially during prolonged exposure to oxidative stress, may not be compatible with cell survival. A blockage of the glycolytic pathway during oxidative insults could be a crucial event leading to embryotoxicity during a critical period of organogenesis, where glycolysis is considered a major pathway of energy production (Freinkel et al., '84).

This study suggests that VYS functions may be selectively vulnerable to consequences of oxidative stress-induced GSH oxidation and protein–S-SG formation. The VYS is of considerable importance during postimplantation development of the rodent embryo, especially before the time when the chorioallantoic placenta becomes functional (Jollie, '90). The VYS digests proteins captured by pinocytosis to produce free amino acids, which are incorporated into embryonic proteins. Thiol protease activity, which is responsible for the degradation of protein following pinocytosis, has also been shown to be inhibited by NEM or iodoacetic acid (thiol alkylating agents), indicating that sulfhydryl groups of this enzyme are important for its activity (Grubb et al., '91). Alterations of proteolytic activity via formation of mixed disulfides with the active sulfhydryl groups of the enzyme could be possible.

In summary, our studies have demonstrated the conceptal formation and removal of protein mixed disulfides under conditions of chemically induced GSH oxidation. Glutathione disulfide-reductase and GSH are important for maintenance of cellular redox status and control of formation and reduction of protein–S-SG. It will be important to further investigate whether protein–S-SG may provide a mechanism for regulation of cellular functions or whether they are simply a consequence that contributes to toxic effects of oxidative stress in developing conceptuses. Findings will provide additional information to aid our understanding of the importance of GSH and cellular redox status in embryonic development.

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