Spatial Activities and Induction of Glutamate-Cysteine Ligase (GCL) in the Postimplantation Rat Embryo and Visceral Yolk Sac

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Glutathione (GSH) synthesis is differentially regulated in the embryo and visceral yolk sac (VYS) of the developing rat conceptus. The innate capacity to respond to environmental insult and chemical exposure by inducing de novo GSH synthesis may help to determine overall cell sensitivity and/or resistance to chemically induced malformation. Specific activities of glutamate-cysteine ligase (GCL), the rate limiting enzyme in GSH synthesis, were determined by measuring the formation of γ-glutamylcysteine (GC) in homogenates prepared from rat embryos and VYSs. GC formation increased linearly with time and with relative protein concentration. Specific activities were found to be 60.5 ± 3.2 and 118.9 ± 4.2 pmol GC/mg protein/min in the gestational day (GD) 10 embryo and VYS, respectively, and 22.7 ± 0.4 and 71.3 ± 0.6 pmol GC/mg protein/min in the respective GD 11 embryo and VYS. Apparent kinetic constants determined from embryo and VYS homogenates gave respective apparent K_m values for glutamate of 0.75 and 1.38 mM and for cysteine 0.03 mM in both tissues. Apparent V_max values were higher in the VYS in each case, corresponding with a lower apparent K_m and higher GCL activity. GCL specific activities increased significantly following a 24 h in vitro exposure to diethyl maleate (DEM) and diamide, but remained unchanged following exposure to prostaglandin A_2 (PGA_2) and t-butylated hydroxytoluene (BHT). Basal expression of GCL catalytic subunit (GCLR) and regulatory subunit (GCLR) was 59- and 25-fold higher in VYS, respectively, compared to the embryo. Quantitative real-time fluorescence reverse transcriptase polymerase chain reaction (RT-PCR) showed that following DEM and diamide treatment, GCLR expression increased up to 19-fold in embryonic tissues but was not induced in the VYS. Only DEM increased the expression of the light/regulatory subunit GCLR in the embryo (8-fold). Densitometry of immunoblots revealed approximately 75% more GCLR in the VYS than in the embryo. Following treatments, a marked increase was induced in embryonic GCLR content with both DEM (85%) and diamide (19%), but in the VYS, only DEM caused an increase in GCLR protein (38%).

Key Words: glutamate-cysteine ligase; glutathione; visceral yolk sac; rat conceptus; γ-glutamylcysteine; rat embryo.

Cellular glutathione (GSH) protects embryos from chemical toxicants (Berberian et al., 1996; Harris et al., 1988; Hiranruengchok and Harris, 1993, 1995a,b; Slott and Hales, 1987; Stark et al., 1987). Glutathione is found in most cells at relatively high concentrations (>2 mM) and interacts directly with several types of reactive chemical intermediates including electrophiles, epoxides, and free radicals to protect cells from damage (Jones et al., 1986; Meister, 1984; Meister and Anderson, 1983). In addition, GSH status is now recognized as an important regulator of the intracellular redox environment that is necessary to control homeostasis and numerous metabolic and dynamic functions (Schafer and Buettner, 2001). As the most abundant non-protein thiol, GSH accounts for nearly 90% of the total cellular reducing equivalents (Cogreave and Gerdes, 1998). The GSH/glutathione disulfide (GSSG) redox couple is the primary determinant of intracellular redox environment and appears to aid in the normal function of enzymes, to maintain structure, to regulate macromolecules involved in biosynthesis, to facilitate transport of amino acids and ions, to preserve membrane integrity, and to control pathways such as those involved in receptor function, signal transduction, and transcription factor activation and DNA binding (Kosower and Kosower, 1978; Meister and Anderson, 1983; Schafer and Buettner, 2001). The latter functions are also important in the control and regulation of many processes that are vital for normal development.

The proper redox environment is dependent on GSH status and is regulated through total concentrations, ratios of reduced and oxidized forms, and the capacity for GSH restoration via enzymatic reduction and de novo synthesis (Schafer and Buettner, 2001). Some tissues, such as intestinal epithelium and kidney proximal tubules, possess specific GSH transporters, which facilitate transport of intact GSH into cells (Hagen and Jones, 1987; Lash and Jones, 1983), but most cells require de novo synthesis to replenish GSH lost through oxidation, adduct formation, and routine catabolism. (Hiranruengchok and Harris, 1993; Jones et al., 1986).

Glutathione biosynthesis and turnover requires a series of six enzyme-catalyzed reactions, identified collectively as the γ-glutamyl cycle (Meister, 1974, Meister and Anderson, 1983).
The glutathione component of the γ-glutamyl cycle may also be important as a means to facilitate transport of GSH precursors and other amino acids needed for new protein synthesis through the activity of γ-glutamyl transeptidase, which is found in plasma membranes (Meister, 1984). Glutathione synthesis occurs via two consecutive enzymatic reactions (Meister, 1974; Snoke and Bloch, 1954). In the first step, glutamate is coupled with cysteine to form γ-glutamylcysteine (GC) by a cystolic enzyme, glutamate-cysteine ligase (EC 6.3.2.2, GCL), a process requiring ATP and Mg2+ as cofactors. Glycine is subsequently added to the GC cysteine by a second reaction that is catalyzed by glutathione synthetase (EC 6.3.2.3, GS), which also requires ATP and Mg2+. The first reaction is the rate-limiting step in GSH synthesis, and is primarily regulated by the availability of cysteine and through feedback inhibition by GSH (Kaplowitz et al., 1985; Richman and Meister, 1975). Although GSH de novo synthesis is well documented in rat liver, kidney, erythrocytes, and other tissues (Orlowski and Meister, 1971; Seelig and Meister, 1985a,b), less is known about GSH synthesis in developing embryos in spite of the well-documented protective functions of GSH against embryotoxicity and teratogenicity produced by xenobiotics (Harris et al., 1995).

The mouse GCL holoenzyme is a heterodimer consisting of a 72 kD, heavy subunit (GCLC) that contains the catalytic active site with corresponding glutamate and cysteine binding domains. The 27 kD, light subunit (GCLR) provides regulatory control for GSH synthesis through a thiol-sensitive sulfhydryl bridge that interacts with the intracellular GSH/redox environment to facilitate the feedback inhibition that is activated when GSH levels rise. GCLC and GCLR originate from separate genes and appear to be independently regulated (Diaz et al., 2002; Tsuchiya et al., 1995). GCL is identified as the rate-limiting enzyme of GSH synthesis in biological systems and the rate limiting substrate is usually cysteine. Deficits in cysteine transport or availability have been shown to significantly slow the ability to restore GSH through de novo biosynthesis (Meister, 1974).

Study of GCLC expression in developing mouse embryos has shown that expression is observed as early as gestational day 3 (blastocyst stage; Gardiner and Reed, 1995). By gestation day 10, expression is found in the neuroepithelium, spinal cord, branchial arches, and liver, and by gestational day (GD) 16, expression is detected in virtually every tissue (Diaz et al., 2002). Mouse fetuses (GD 12/16) treated with methyl mercury showed oxidation of GSH in both fetuses and VYS, but only the VYS showed an increase in GCL activity (Thompson et al., 2000). These experiments demonstrate the regulation and inducibility during development at later stages of development but do not address these responses in early organogenesis stage embryos (mouse: gestational day 8 and rat: gestational day 10).

In the present study, intrinsic GSH synthesis has been characterized in the rat embryo and VYS during early organogenesis using an in vitro GCL assay. Presence of active GCL has been demonstrated in the embryo and VYS by measuring the formation of γ-glutamylcysteine (GC) in tissue extracts. Relative activities of GCL and apparent, tissue-specific kinetic properties of GCL for the utilization of precursor amino acids have been determined in extracts of embryos and VYSs. Furthermore, GCL mRNA inducibility and protein expression will also be addressed to assess conceptual responses following exposure to substances that cause oxidative stress.

**MATERIALS AND METHODS**

**Chemicals.** L-Glutamic acid monopotassium salt, L-cysteine, ATP disodium salt, γ-glutamylcysteine trifluoroacetate salt, GSH, and L-buthionine-S,R-sulfoximine (BSO) were obtained from Sigma Chemical Company (St. Louis, MO). L-[35S]-Cysteine was purchased from DuPont Company (NEN Research Products, Wilmington, DE). Tris-HCl and KCl were obtained from Schwarz/Mann Biotech (Cleveland, OH) and J. T. Baker Inc. (Phillipsburg, NJ), respectively. MgCl2·6H2O and (Ethylene dinitrilo)-tetraacetic acid disodium salt (EDTA) were purchased from Mallinkrodt Inc. (Paris, Kentucky). Monobromobimane (Thiolate) was purchased from Calbiochem (La Jolla, CA). All other chemicals and reagents were purchased from commercial sources and were of the highest available purity.

**Animals.** Primagravida Sprague-Dawley rats were obtained from the Reproductive Sciences Program Small Animal Core, University of Michigan or Charles River (Portage, MI) on GD 6–9. Day 0 was determined by a sperm-positive vaginal smear on the morning following copulation. Pregnant rats were maintained on a 12-h light/12-h dark cycle until explantation on GD 10. Food and water were given ad libitum. Conceptuses were explanted on day 10 of gestation and cultured for 2 h in a medium consisting of 33% (v/v) serum in Hanks’ balanced salt solution (HBSS, pH 7.4) and saturated with a gas mixture containing 20% O2/5% CO2/5% N2 (v/v/v). The conceptuses were withdrawn from the culture and rinsed (3X) with homogenization buffer (100 mM Tris·Cl, 50 mM KCl, 2 mM MgCl2 and 2 mM EDTA, pH 7.4 at 37°C). Embryos were dissected free of the VYSs and placed in separate microcentrifuge tubes containing buffer. Embryos and VYSs were homogenized by ultrasonic disruption and centrifuged at 14,900 × g for 10 min. The resulting supernatants were used for the determination of GCL activity.

**Assay of GCL activity.** GCL activity was determined in a reaction mixture containing 100 mM Tris·Cl, 50 mM KCl, 20 mM MgCl2, 2 mM EDTA, 6 mM ATP, 8 mM glutamic acid, 0.1 mM cysteine, and tissue extracts (equivalent to 200 μg of protein) in a 150 μl final volume (pH 8.2 at 37°C). The reaction was incubated at 37°C for 10 to 40 min, and terminated by adding 50 μl of 800 mM methanesulfonic acid on ice. The amount of GC produced in the reaction was determined by HPLC analysis following derivatization with monobromobimane as described in a previous study (Lee and Harris, 1995). GCL specific activity was expressed as pmol GC/mg protein/min.

**Incorporation of [35S]-cysteine into GC and GSH.** [35S]-Cysteine (1200 Ci/mmol) was diluted with deionized water to obtain specific activities of 240,000 dpm/pmol. Tissue extracts were prepared from GD 10 embryos and VYSs. The enzyme reaction was initiated by adding [35S]-cysteine into the reaction mixtures which does not contain unlabeled cysteine. After 20 min of incubation at 37°C, the reaction was terminated, and the products were resolved by HPLC. Fractions eluted from HPLC were further analyzed by scintillation counting to determine the amount of labeled GC and GSH produced by incorporation of [35S]-cysteine.

**HPLC analysis.** Glutathione and GC levels were determined by reverse phase HPLC analysis according to the method previously described (Fenton and Fahey, 1986). Samples were resolved on a NovaPak C-18 4 μm column (Waters, Millipore Corporation, Milford, MA) with an isocratic mobile phase of 14.2% (v/v) acetonitrile and 0.25% (v/v) acetic acid in water at a flow rate of 1.0 ml/min. Products were detected by a Waters Model 470 scanning fluorescence detector (l excitation 360 nm; l emission 455 nm). The column was washed with 90% (v/v)
methyl alcohol and 0.25% (v/v) acetic acid in water to re-equilibrate before each run. Authentic standards (GC and GSH) were prepared for quantification of each sample.

**Quantitative fluorescence PCR.** Embryos and VYS were collected, washed with HBSS and placed in Trizol (Invitrogen, Carlsbad, CA) for RNA extraction by the manufacturer’s suggested protocols and followed by a clean up with the RNeasy kit (Qiagen, Valencia, CA). Reverse transcription (RT) of RNA and synthesis of cDNA were performed with M-MLV RT (Promega, Madison, WI) using 1 μg of isolated RNA. RT mix containing RT buffer (Promega), 40 mM dithiothreitol, 0.5 mM dNTPs, 10 U RNAase inhibitor (Promega), and 200 ng random primers (Promega). The reaction was incubated for 1 h at 37°C. GCLC and GCLR primers were obtained from Integrated DNA Technologies (Coralville, IA) (GCLC forward primer: 5'-TGTTAATCCGGCACGCTCA-3'; GCLC reverse primer: 5'-ATCCCTAGTTCGATCACAT-3'; GCLR forward primer: 5'-CTCTGAGTCTAGACAAAAC-ACAGT-3'; GCLR reverse primer: ACATGCAAAACCACACATTCAAC-3'); β-actin polymerase chain reaction (PCR) was used as an internal control (forward primer: 5'-TACCCCA CACTGTGCCCATGTACGA-3'; reverse primer 5'-CTCTGAGTCTAGACAAAAC-ACAGT-3').

Real-time PCR was performed using a LightCycler (Roche, Indianapolis, IN) and the LightCycler FastStart DNA Master SYBR Green 1 kit (Roche Applied Science, Indianapolis, IN). Reactions were carried out as described by the manufacturer’s suggestions and protocols. Briefly, real-time fluorescence PCR analysis consisted of reactions using 50 ng of cDNA that included a preincubation period (95°C for 5 min), an amplification cycle repeated up to 45 times (cycle: 95°C for 1 sec, 60°C for 21 s, 72°C for 21 s) and melting curve analysis for verification of specific, desired product. Products were also run on a 2% agarose gel to verify product size. The β-actin amplification cycle consisted of annealing at 60°C for 7 s and elongation at 72°C for 21 s.

GCLC protein detection and quantification by immunoblot. Following treatment and incubation in whole embryo culture, embryos and VYSs were collected in lysis buffer and snap frozen in liquid nitrogen. Protein amounts were determined by the Lowry method with the Dc protein assay kit (BioRad, Hercules, CA). Loading buffer was added to 25 μg of each sample and boiled for 5 min prior to loading on a 10–20% polyacrylamide ReadyGel (SDS-PAGE) (BioRad). Following separation samples were transferred to a nitrocellulose membrane and probed for GCLC with a primary anti-goat antibody (Santa Cruz Biotechnology, Santa Cruz, CA). An Alexafluor 680 nm donkey anti-goat secondary antibody (Molecular Probes, Eugene, OR) was used to reveal GCLC. Membranes were visualized on an Odyssey fluorescence detection system as described by the manufacturer (Li-Cor, Lincoln, NE). Densitometry was determined with the Odyssey software.

**RESULTS**

The specific activity of GCL was determined in the early organogenesis-stage rat embryo and VYS in vitro by measuring the formation of GC, the product of the enzyme reaction, as determined by HPLC analysis using a concentration curve constructed with authentic standards. Product formation was linear with respect to time and protein concentration during a 40 min reaction period. The basal levels of GC measured 0.55 and 0.67 nmol/mg protein in GD 10 embryo and VYS, respectively. The specific activities were 60.5 ± 3.2 and 118.9 ± 4.2 pmol GC/mg/min in the embryo and VYS, respectively, showing two-fold greater activity in the VYS than in the embryo proper (Table 1). GC generation in GD 11 embryos and VYS also increased linearly with time, and specific activities of GCL were found to be 22.7 ± 0.4 and 71.3 ± 0.6 pmol/mg/min for GD 11 embryos and VYSs, respectively. The specific activity of GCL in the embryo versus VYS was greater at GD 11 than GD 10, showing a three-fold higher GCL activity in the VYS than in the embryo.

In order to confirm the presence of active GCL in the conceptual tissues, assays were conducted with a specific GCL inhibitor, buthionine sulfoximine (BSO), or without the addition of the conceptal extracts (Fig. 1). When conceptual extracts were preincubated with BSO for 5 min before initiation of the assay, GC formation decreased by 85% compared to control. Reactions incubated without the addition of conceptual extracts yielded no GC. These findings demonstrate that GD 10 rat embryo and VYS possess active GCL, and GC in the reaction mixture was generated by GCL activity. In addition, assays conducted without the addition of glutamate decreased the formation of GC by 73% compared to control and demonstrated the precursor substrate role of glutamate for the formation of GC. Trace amounts of GC were detected in the assay that most likely originated from endogenous glutamate pools contained in conceptual extracts.

**TABLE 1**

<table>
<thead>
<tr>
<th>Specific activity (pmol GC/mg/min)</th>
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<tr>
<td>GD Embryo VYS</td>
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<tr>
<td>10  60.5 ± 3.2  118.9 ± 4.2</td>
</tr>
<tr>
<td>11  22.7 ± 0.4  71.3 ± 0.6</td>
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**FIG. 1.** GC formation in conceptual homogenates. Reaction mixtures were incubated for 20 min with the conceptual homogenates pretreated with BSO for 5 min, or without the addition of conceptual homogenates. The requirement for glutamate was examined by incubating reaction mixtures without adding glutamate. Each value represents the mean ± SE obtained from at least three determinations. Asterisks indicate significant differences (p < 0.05) compared to control in Student’s t-test.
Embryonic and VYS GCL activity was further verified by the incorporation of [35S]-cysteine into GC and GSH (Fig. 2). The reaction mixture was incubated with [35S]-cysteine (0.015 μCi/ml) for 20 min, separated using high performance liquid chromatography (HPLC). The radioactivity for each product of the HPLC effluent was determined by fraction collection and liquid scintillation counting. The pattern of [35S]-radioactivity in HPLC fractions collected from the 20 min incubation mixtures, showed a peak at 16 min, indicating the formation of [35S]-labeled GC (Figs. 2B and 2D). The peak area of the radioactivity was 272,000 and 384,000 DPM/mg of protein in the embryo and VYS, respectively, in a pattern consistent with direct GC measurements. Radiolabeled GSH was produced, but in much smaller quantities as indicated by the peak of radioactivity at a retention time of 19 min.

The apparent GCL enzyme kinetics were studied in embryo and VYS homogenates by varying the concentrations of glutamate and cysteine. As shown in Figure 3, the rate of the reaction increased proportionally with cysteine at low concentrations and approached a maximum as the concentration was increased, exhibiting the typical saturation effect. The apparent Km of cysteine and Vmax for GCL were determined from the double reciprocal plot of the data. The Km for cysteine was similar in embryos and VYSs throughout repeated experiments, and the average value was 0.03 mM in both the embryonic and VYS extracts (Table 2).

![Graphs showing enzyme kinetics](https://example.com/fig3.png)
The GC formation versus glutamate concentrations was also hyperbolic, and apparent Km of glutamate and Vmax were determined from the linear plot (Fig. 3). The apparent Km of glutamate was nearly 2-fold greater in embryos than in VYSs, and the average values were 1.38 mM and 0.75 mM in embryos and VYSs, respectively. The apparent Vmax for both cysteine and glutamate was higher in the VYS compared to the embryo, which is consistent with the finding that GCL activity is greater in the VYS, as shown in Table 1. Concentrations of ATP that produced the maximum enzyme activity were found to be 6–12 mM under the conditions described (Fig. 3). At ATP concentrations greater than 12 mM the activity of GCL was decreased.

Addition of the GSH depleting agents diethyl maleate (DEM, depletion through GSH conjugation) and diamide (depletion through chemical oxidation of GSH to GSSG) directly to the culture medium containing GD 10 conceptuses resulted in increased GCL activities on the order of 25–125% in embryos and 65–165% in VYS after 24 h (Fig. 4). Increased GCL specific activities were statistically significant for DEM and diamide in the VYS. Other known inducers of GSH synthesis such as PGA2 and BHT, exposed using the same protocol, had no effect on cultured rat embryo or VYS GCL activities (Fig. 4).

Basal expression of the GCLC and GCLR subunits and changes as a result of DEM or diamide exposure were evaluated in embryos and VYS using real-time fluorescence quantitative RT-PCR. Constitutive expression of GCLC was observed to be 59-fold higher and GCLR 25-fold higher in the VYS when compared to the embryo proper (Table 3). Significant increases in embryonic GCLC expression occurred as a result of DEM exposure (18.6-fold) in the direction of constitutive levels of expression already seen in the VYS. Diamide also increased GCLC expression in the embryo proper, but to a lesser degree than DEM (4.2-fold). No significant changes were induced in the VYS in either case (Table 3). GCLR expression was significantly induced only in the embryo (8.2-fold) following exposure to DEM. There were no significant changes in the expression of

### TABLE 2

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<th>Tissue-Specific Apparent Km and Apparent Vmax Values for γ-Glutamate Cysteine Ligase (GCL), Determined from Homogenates of Gestational Day 10 Rat Embryo and Visceral Yolk Sac (VYS)</th>
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<tbody>
<tr>
<td>Km (mM)</td>
</tr>
<tr>
<td>Cysteine</td>
</tr>
<tr>
<td>Embryo</td>
</tr>
<tr>
<td>VYS</td>
</tr>
<tr>
<td>Glutamate</td>
</tr>
<tr>
<td>Embryo</td>
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<tr>
<td>VYS</td>
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*Note. Mean ± SE obtained from three independent determinations are shown. Embryos and VYSs were homogenized and centrifuged at 14,900 × g. The resulting supernatant was used to determine kinetic parameters.*

![FIG. 4. GCL specific activities expressed as nmol GC formed/mg protein/min from GD 11 embryo and VYS homogenates following exposure to DEM (500 μM), diamide (100 μM), PGA2 (60 μM), and BHT (200 μM) in whole embryo culture for 24 h. Test chemicals were added directly to the culture medium at the onset of culture. Each value represents 3–5 separate determinations and are expressed as mean ± SE. Values significantly different from control are denoted by an * and are significant at p < 0.05 as determined by the Student’s t-test.](image-url)
ALTERATIONS IN GCLC mRNA EXPRESSION WERE FOUND TO BE PROPORTIONAL TO HIGHER GCLC PROTEIN CONCENTRATIONS AS DETERMINED BY IMMUNOBLOTTING OF CONCEPTAL HOMOGENATES. DIAMIDE AND DEM TREATMENTS INCREASED PROTEIN LEVELS, RANGING FROM 120% FOR DIAMIDE TO OVER 180% FOR DEM IN THE EMBRYO. SMALLER INCREASES OF 113% TO 138% WERE SEEN FOR THE SAME TWO RESPECTIVE COMPOUNDS IN THE VYS (FIG. 5).

DISCUSSION

The ability to maintain adequate concentrations of GSH is a nearly universal requirement for restoration of normal function in cells altered by a variety of physical and chemical insults. Different cell types, distinguished only by their intrinsic capacities to restore GSH following its oxidation or adduct formation as a result of extreme environmental exposures, may suffer a spectrum of outcomes ranging from normal recovery to death and necrosis. Glutathione that has been oxidized to GSSG or protein-S mixed disulfides and/or GSH that has formed covalent adducts with chemical electrophiles may remain extant within cells, to be recovered via various biochemical pathways involving glutathione reductases, thioredoxins, peroxiredoxins, glutaredoxins, thiol-disulfide reductases, γ-glutamyltranspeptidases, and other enzyme systems. GSSG that is refractory to reduction back to GSH due to the lack of appropriate enzymes, GSH that is irreversibly covalently bound, or GSH that is lost due to cellular export would require cells to revert to a strategy of de novo GSH synthesis in order to replenish lost stores. Due to the complexity of cell-cell interactions, timing of developmental events and differentiation, the mechanism and regulation of GSH synthesis becomes crucial in the overall determination of cell fate following chemical and environmental insult.

The rate-limiting step in GSH synthesis involves the enzyme glutamate cysteine ligase (GCL), which is comprised of two subunits; a heavy, catalytic subunit (GCLC) and a light, regulatory subunit (GCLR), which facilitates the ligation of glutamate and cysteine. The two subunits are products of different genes, independently regulated, and may be distributed unequally between cells due to their selective expression. Our data show that, within the organogenesis-stage rat conceptus, constitutive specific activity for GSH synthesis differs significantly between the embryo proper and the associated VYS. Specific activities decrease as gestational age progresses, but the relative differences between embryo and higher activity VYS persist through mid-gestation.

Tissue-specific apparent kinetic values derived from cysteine and glutamate saturation curves in conceptal homogenates show high and equal affinities for cysteine in embryo and VYS and lower and tissue-specific affinities for glutamate, where the embryonic apparent Km value is twice that of the VYS Km.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fold expression change from control</th>
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<tbody>
<tr>
<td>GCLC</td>
<td></td>
</tr>
<tr>
<td>Diamide</td>
<td>+4.2 ± 1.9*</td>
</tr>
<tr>
<td>DEM</td>
<td>+18.6 ± 2.2*</td>
</tr>
<tr>
<td>GCLR</td>
<td></td>
</tr>
<tr>
<td>Diamide</td>
<td>+1.9 ± 0.6</td>
</tr>
<tr>
<td>DEM</td>
<td>+8.2 ± 1.3*</td>
</tr>
</tbody>
</table>

Note. Constitutive GCLC expression in VYS was 59-fold higher than in the corresponding embryo proper. Constitutive GCLR expression in VYS was 25-fold higher than in the corresponding embryo proper. Data for DEM and diamide treatments are represented as fold changes from control tissue values after normalization to β-actin expression. Treated samples are compared to similar tissue controls. Asterisks (*) denote significant (p < 0.05) alterations from control. Means (±SEM) represent three separate experiments.

FIG. 5. GCLC immunoblot analysis. GCLC protein levels in both embryos and VYSs (A) treated with DEM and diamide were determined by immunoblotting. (B) Densitometry measurements of immunoblots. DEM and diamide treatments results in an increase of GCLC protein in the embryo, but only DEM caused an increase of GCLC protein in the VYS.
value on gestational day 10. The observed high affinity of con-
etional GCL for cysteine may be an embryonic adaptation to
compensate for reported low levels of cysteine in the conceptus
(Beckman et al., 1990; Hansen et al., 1999). Differences for
the apparent Km values for glutamate between embryo and VYS do
not necessarily imply that distinct isoforms of the proteins exist.
To our knowledge, there are no structural variations between
embryonic and VYS GCLC; that could account for these differ-
ences, suggesting that inherent differences in the tissue homo-
genates, possibly the presence of endogenous inhibitors or
regulators, may be contributing to the differences. For both
glutamate and cysteine, the VYS is capable of producing
more GC than the embryo. Although the specifics for the
observed disparities in the apparent catalytic binding affinity
have not yet been characterized, it does suggest some significant
implications for embryonic susceptibility and VYS resistance to
chemical-induced oxidative stress and GSH depletion.

Previous studies with the electrophile, DEM, showed that
considerable differences exist within the conceptus with regard
to rates of GSH synthesis following DEM exposure and GSH
depletion (Harris, 1993). Exposure of conceptuses to DEM in
rats grown in whole embryo culture resulted in rapid and equiva-
 lent depletion of GSH in both embryo and VYS, but immediate
rehemishment occurred only in the VYS. Embryo GSH repletion
lagged for several hours (Harris, 1993). This observation agrees
well with our determination that 2–3-fold higher basal GCL
specific activities occur in the VYS when compared to the
embryo (Table 1). The initial inability of embryos to rapidly
respond to GSH depletion and resynthesize GSH was interpreted
to be due to the lack of amino acid precursors in the embryo and a
renewable supply of precursor in the VYS through constant
degradation of maternal proteins. The current data show that
basal activities and enzyme inducibility may also contribute
to the differential behavior.

In this study, increases in GCLC specific activity were observed
after 24 h in both embryos and VYSs following DEM exposure,
and to a lesser degree following oxidation of GSH by diamide.
While these values correlate with changes in GCLC and GCLR
mRNA and GCLC and GCLR protein levels in the embryo, no
substantial induction was observed in the VYS. Quantitative
PCR of embryos and VYS exposed to DEM and diamide showed
significant increases in GCLC expression only in the embryo
proper. Twenty-four hour GCLC protein concentrations were
also proportional to the changes in gene expression in the
embryo, suggesting that the induced expression of GCLC in
the embryo was highest for DEM but that it did not exceed
the basal expression levels seen constitutively in the VYS.
This result implies that the increase in specific activity observed
in the VYS is not due to induction (increased message and
protein) per se, but may involve other regulatory events, possibly
involving increased precursor availability, or feedback regula-
tion and activation related to intracellular GSH status and redox
environment. Cysteine, the rate limiting precursor to glutathione
synthesis, is nearly 3-fold greater in the VYS (Hansen et al.,
1999), again suggesting that the VYS may respond more com-
pletely and quickly than the embryo during periods of GSH
depletion and resynthesis.

GCLC and GCLR gene expression and protein synthesis were
more responsive and robust in the embryo following DEM treat-
ment than observed with diamide treatments. Although both
chemicals effectively deplete GSH, depletion mechanisms are
very different; DEM depletion occurs through covalent adduct
formation and diamide occurs through chemical oxidation to
GSSG. While oxidation of GSH to GSSG by diamide shifts the
GSH:GSSG ratio and intracellular environment, the total
amount of thiol does not necessarily change. Glutathione
disulfide reductase (GSSG-Rd) can convert GSSG back to
GSH following oxidant exposures, such as with diamide, but
not in cases of GSH adduct formation, such as with DEM. VYS
GSSG-Rd activity is 2–3 fold greater than that measured in the
embryo and may account for the VYS’s superior ability to re-
pond to chemically induced oxidation (Choe et al., 2001,
Hiranruengchok and Harris, 1993). Glutathione recovery
from GSSG via GSSG-Rd is faster than de novo synthesis,
the means by which GSH is restored in DEM treatment, yielding
a shorter period of redox imbalance.

In response to conditions of GSH depletion and/or oxidative
stress where a significant percentage of GSH becomes oxidized
or lost, induction of GCLC and GCLR are possible by virtue of
selective binding of redox-sensitive transcription factors such as
AP-1 and NF-kB. AP-1 has been shown to be responsible for
most changes in GCL expression. Ozolins et al. (2002) have
determined that the changes in conditions that accompany the
introduction of conceptuses into culture is sufficient to induce a
shift in the GSSG/GSH ratio, inducing oxidative stress and acti-
vating AP-1 expression and DNA binding. Post-translational
regulation of AP-1 activation and DNA binding differ in embryo
and VYS and may contribute to observed organ specific differ-
ences in inducibility and activity related to the apparent constit-
tutive production of GCLC in the VYS (Ozolins and Hales, 1997,
1999a,b). Diamide may not be as effective an inducer of GCL
due to the inherent cellular capacity to rapidly reduce GSSG and
restore intracellular GSH without the need for de novo GSH
biosynthesis.

The idea of redox regulation of transcription factors such as
AP-1, Ref-1, NF-kB, and nr2 has been studied (Abate et al.,
1990; Erickson et al., 2002; Ozolins and Hales, 1999a,b; Solis
et al., 2002). Our data in conceptuses suggest that redox-sensitive
transcription factors may be more sensitive to overall thiol deple-
tion (DEM) than conversion to an oxidized disulfide. Thus, the
nature of GSH depletion, conditions of oxidative stress and the
intrinsic abilities of conceptual tissues to restore GSH through
new GSH synthesis may determine the overall susceptibility to
chemical embryotoxicants and teratogens. Induction of GCLC
and GCLR as well as differential modes of amino acid precursor
supply are likely to contribute heavily to selective cell and tissue
selectivity to toxicants an may act to regulate other redox-
sensitive aspects of normal development.
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REFERENCES


