

# Minimal ovarian upregulation of glutamate cysteine ligase expression in response to suppression of glutathione by buthionine sulfoximine

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## Abstract

The antioxidant tripeptide glutathione (GSH) protects ovarian follicles against oxidative damage that may lead to apoptotic death. The rate-limiting step in synthesis of GSH is catalyzed by glutamate cysteine ligase (GCL), a heterodimer composed of a catalytic subunit (GCLC), and a modifier subunit (GCLM). We hypothesized that GSH depletion in vivo or in vitro with buthionine sulfoximine (BSO), a specific inhibitor of GCL activity, would increase ovarian and granulosa cell GCL subunit expression. Ovarian glutathione levels are lowest on proestrous morning and increase to their highest levels on estrus and metestrus. Therefore, we treated rats on proestrous morning or on proestrous morning and again 12 h later to prevent the normal increase in ovarian glutathione between proestrus and estrus. Ovarian *Gclc* and *Gclm* mRNA levels and GCLC protein levels increased transiently by 1.4–1.5-fold at 8 h, but not at 12 or 24 h, after a single dose of BSO administered to adult rats on the morning of proestrus. GCLC protein levels were also modestly increased 1.4-fold at 12 h after a second dose of BSO. GCLM protein levels increased 1.4-fold at 24 h after a single dose of BSO, but not at other time points. BSO treatment did not significantly alter ovarian GCL enzymatic activity or the intraovarian localization of either GCL subunit mRNA. Treatment of a human granulosa cell line or primary rat granulosa cells with BSO suppressed intracellular GSH; however, there was no compensatory upregulation of GCL subunit protein or mRNA levels. These results demonstrate that ovarian follicles and granulosa cells are minimally able to respond to acute GSH depletion by upregulating expression of GCL.

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## 1. Introduction

The tripeptide glutathione (GSH) is the most abundant intracellular, non-protein thiol. GSH constitutes a critical component of the antioxidant defense system, functions in cysteine transport and storage, maintains cellular redox status, and serves other important functions [1]. GSH is present at moderately high concentrations in the ovary [2–4]. As it does in other tissues, ovarian GSH likely plays important roles in detoxifying reactive oxygen species and in conjugating electrophilic toxicants via glutathione-S-transferase

catalyzed reactions. Indeed, GSH depletion increases atresia, the apoptotic process of follicular degeneration [5,6], whereas enhancement of GSH levels rescues cultured ovarian follicles from apoptosis [7].

GSH synthesis occurs via two ATP-dependent enzymatic reactions [8–10]. The first and rate-limiting step forms  $\gamma$ -glutamylcysteine and is catalyzed by the enzyme glutamate cysteine ligase (GCL, also known as  $\gamma$ -glutamylcysteine synthetase). The second step is catalyzed by glutathione synthetase. GCL is a heterodimer composed of a catalytic (GCLC) and a modifier (GCLM) subunit that are joined by disulfide bonds [10,11]. GCLC possesses all the catalytic activity and is catalytically active alone in vitro [12]. However, under normal physiological conditions, GCLC is

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thought to be catalytically inactive unless it forms a heterodimer with GCLM [12]. Transcriptional regulation of both GCL subunit genes is an important mechanism for modulating intracellular GSH synthesis [10,11]. Interestingly, transcriptional regulation of GCL subunits varies considerably among cell types, and within the same cell type the two subunits are independently regulated [13]. GCL enzymatic activity is under negative feedback regulation by GSH [10,11]. Formation of the heterodimer with GCLM decreases the  $K_m$  for glutamate and increases the  $K_i$  for GSH [10,11]. GSH synthesis is also dependent on the availability of cysteine [9].

In the ovary, granulosa cells and oocytes of healthy, growing follicles display high levels of *Gclm* mRNA expression, whereas follicles undergoing the apoptotic process of atresia lack *Gclm* expression [14]. Other ovarian cell types display minimal *Gclm* expression, except for a transient, but large, increase in theca cells after an ovulatory gonadotropin stimulus [15]. In contrast, GCLC protein and mRNA are more ubiquitously expressed throughout the ovary [14,15]. Ovarian GSH synthesis increases in response to gonadotropin hormone stimulation of follicular development, mediated by increased GCLC and GCLM protein expression and increased GCL enzymatic activity [4,15].

Buthionine sulfoximine (BSO) inhibits GSH synthesis by blocking the active site of GCL [16]. Suppression of GSH synthesis using BSO in vitro has been shown to upregulate GCL subunit mRNA levels in some cell types [17–19], but not in others [20]. In vivo treatment of mice with BSO did not alter hepatic GCL mRNA or protein expression [21]. No previous studies have tested the ability of the ovary or of different cell types within the ovary to respond to GSH depletion by up-regulating GCL subunit expression.

The present study was, therefore, designed to investigate the hypothesis that suppression of ovarian GSH synthesis using BSO would cause compensatory increases in ovarian GCL subunit protein and mRNA expression and, further, that this increase would be localized to growing follicles. We tested this hypothesis in cycling rats in vivo and in cultured granulosa cells. Our results demonstrate that, unlike other cell types, ovarian cells are unable to respond to GSH depletion by up-regulating expression of GCL, the rate-limiting enzyme in its synthesis.

## 2. Materials and methods

### 2.1. Materials

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO) unless otherwise noted. All tissue culture reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA).

### 2.2. Experimental animals

Adult (9–10 weeks old) or pre-pubertal (22 days old), female Sprague–Dawley (CrI:CD(SD)IGS BR) rats were purchased from Charles River Laboratories (Wilmington, MA). Upon arrival, animals were housed 3 to a cage in an AAALAC-accredited facility on a 14 h light/10 h dark cycle. Adult rats were allowed to acclimate for 7 days. Deionized water and standard laboratory rodent chow were provided ad libitum. Vaginal cytology was performed for at least two 4-day estrous cycles before the in vivo experiments began. All experimental protocols were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* [22] and were approved by the Institutional Animal Care and Use Committee at the University of California at Irvine.

### 2.3. Experimental protocols

In the first experiment, adult female rats weighing 205–260 g were injected intraperitoneally (i.p.) with 5 mmol/kg (1110 mg/kg) buthionine sulfoximine in 0.9% saline or saline alone on the morning (07:00–08:00 h) of proestrus and killed by decapitation 8, 12, or 24 h later or were injected at 07:00 and 19:00 h on proestrus and were killed the following morning of estrus (07:00 h). The dose of BSO was chosen to maximally suppress GSH synthesis. Given the solubility of BSO and the desire to limit the injection volume to 5 mL or less [23], 5 mmol/kg was the maximum achievable dose. The day of proestrus was chosen because we have previously observed that ovarian GSH concentrations increase significantly between the morning of proestrus and the morning of estrus [4]. Dosing twice with BSO on proestrus was designed to prevent this normal rise in ovarian GSH levels. Trunk blood was collected for estradiol and progesterone assays. Examination of the uterus for ballooning (accumulation of 100 mg or more of uterine fluid) on proestrus allowed for further verification of cycle stage [24]. Ovaries were dissected, trimmed of fat, weighed, and processed for GSH assay, Western analysis, or Northern analysis. Ovaries from animals killed 24 h after a single dose of BSO or saline were also processed for GCL enzymatic activity assay. This assay was not performed at earlier time points because the mechanism of action of BSO is to suppress GCL enzymatic activity [16].

In the second experiment, proestrous or estrous female rats weighing 209–295 g were injected i.p. with 5 mmol/kg BSO in 0.9% saline or saline alone at 07:00 and 19:00 h. Animals were killed by decapitation at 0700 h the following day (estrus or metestrus), and ovaries were dissected and processed for GSH assay or for in situ hybridization as described below. Because we observed no effects of suppressing GSH synthesis during the proestrus to estrus transition when ovarian GSH levels are normally rising in the first experiment, we added a second time point, designed to suppress ovarian GSH synthesis when levels are normally high [4].

#### 2.4. COV434 human granulosa cell culture

COV434 cells (gift of Dr. Peter Schrier, University Hospital of Leiden, Netherlands) are derived from a human granulosa cell tumor [25]. They possess many characteristics of normal granulosa cells [26]. A preliminary dose–response experiment showed that culture for 24 h with concentrations of BSO from 100 to 1000  $\mu$ M suppressed intracellular GSH to undetectable levels. Therefore, 100  $\mu$ M was chosen for the subsequent experiments.

COV434 cells were plated  $5 \times 10^6$  cells per 75 cm<sup>2</sup> flask in DMEM F12 with GlutaMAX, 9% fetal bovine serum, 100 mg/mL streptomycin, and 100 IU/mL penicillin. After 24 h the cells were washed twice with PBS, and the medium was replaced with (1) serum-free control medium (DMEM-F12), (2) medium plus 100  $\mu$ M BSO, (3) medium plus 200 ng/mL recombinant human follicle stimulating hormone (rhFSH; Purchased from Dr. A.F. Parlow, National Hormone and Peptide Program, NIDDK), or (4) rhFSH plus 100  $\mu$ M BSO. Treatment media were removed after 20 h and replaced with control, serum-free medium for 6 h. Cells were harvested by trypsinization for GSH assay or by scraping with a cell scraper for protein or RNA extraction. Cells were routinely assessed for mycoplasma contamination using the Mycotect Kit (Invitrogen) and were found to be free of contamination.

Cell viability was assessed after trypsinization by Trypan Blue exclusion. After incubation for 20 h with treatment media followed by 6 h in control media, the percentage of dead cells did not exceed 7.5% in any treatment group. Cells were then centrifuged at  $300 \times g$  at room temperature for 5 min and were processed for GSH assay, protein extraction, or RNA extraction.

#### 2.5. Primary rat granulosa cell culture

Granulosa cells from small antral follicles are not fully differentiated and differentiate in response to FSH and androgen treatment [27,28]. Granulosa cells from small antral follicles were obtained from prepubertal rats primed once daily for 3 days beginning at 24 days of age with estradiol injections (1.5 mg 17 $\beta$ -estradiol/0.2 mL propylene glycol administered subcutaneously). Twenty-four hours after the last injection, the animals were killed by carbon dioxide asphyxiation and ovaries were dissected out under aseptic technique. Granulosa cells were isolated directly into DMEM-F12 medium with GlutaMAX, penicillin, and streptomycin in a Petri dish from follicles 300 to 400  $\mu$ m in diameter by puncture with 26 gauge needles under a stereomicroscope. Cells were cultured in (1) serum-free medium alone, (2) medium plus 200 ng/mL recombinant human FSH (National Hormone and Peptide Program, NIDDK), or (3) FSH plus 100  $\mu$ M BSO, at a density of  $1 \times 10^6$  cells per 60 mm fibronectin-coated Petri dish (Becton Dickinson). After 18 h, FSH plus BSO medium was replaced with DMEM F12 and culture was continued for another 6 h. Cells were harvested by trypsinization for GSH

assay or by scraping with a cell scraper for Western analysis at 24 h after the initiation of culture.

#### 2.6. GSH assay

Fresh ovaries were immediately homogenized in 1:4 (w/v) 5% sulfosalicylic acid on ice. Cell pellets were pipetted up and down in buffer containing 20 mM Tris base, 1 mM EDTA, 250 mM sucrose, 2 mM L-serine, and 20 mM borate (TES-SB buffer). After removing an aliquot of suspension for protein assay (BCA Assay Kit, Pierce, Rockford, IL), 1/4 volume 5% sulfosalicylic acid was added. After 30 min incubation on ice, cells or tissue suspensions were centrifuged at  $15,800 \times g$  for 10 min at 4 °C. The supernatant was removed and stored at –70 °C until assay as described [4], except that the assay was scaled down to a 96-well microplate format and absorbances were read using a VersaMax tunable microplate reader (Molecular Devices, Sunnyvale, CA). The interassay coefficient of variation was 13.6% for a rat liver pool.

#### 2.7. Northern blot analysis

Total ovarian or granulosa cell RNA was prepared using the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Samples of RNA (20  $\mu$ g) were analyzed by separation in 1% agarose/formaldehyde-containing gels, followed by capillary transfer to nylon membranes and hybridization with 32P-labeled nucleic acid probes. 32P-labeled random-primed probes were prepared using template full-length cDNAs of the mouse *Gclc*, *Gclm* [29,30] and *Gapdh* (Ambion, Austin, TX) genes with the DECAprime II DNA Labeling Kit (Ambion, Austin, TX). Visualization was by autoradiography. The rat has one *Gclc* transcript (size 4.1 kb) and two *Gclm* transcripts (size 1.8 and 5.2 kb) [11]. Semiquantitative analysis of autoradiographs was performed using a Strata-gene molecular documentation and image analysis system with EagleSight software. The absorbance readings of the *Gclc* band and the sum of the absorbance readings for the two *Gclm* bands were normalized to control mRNA (*Gapdh*) and relative differences among treatment groups were calculated.

#### 2.8. Western blot analysis

Ovaries were homogenized and cells were lysed in RIPA lysis buffer (PBS, 1% Nonidet-P-40, 0.5% sodium deoxycholate, 0.1% SDS) with protease inhibitors on ice. Lysates were incubated on ice for 30 min and were centrifuged at  $15,800 \times g$  for 10 min at 4 °C. Supernatants were stored at –70 °C. Gel electrophoresis, Western blotting for GCLC, GCLM, and  $\beta$ -actin and semiquantitative analysis of images were performed as previously described [4]. Briefly, 40  $\mu$ g of protein extract from each ovary or 25  $\mu$ g of protein from cell extracts were loaded onto 12% Tris HCl polyacrylamide gels (BioRad, Hercules, CA), separated by electrophoresis,

transferred to polyvinylidene difluoride membranes, blocked, and incubated with GCLM and GCLC antisera [31]. For the in vivo experiments, samples for each time point were run on separate gels (three to six samples per treatment group per gel). Blots were subsequently reprobed with  $\beta$ -actin antiserum (Sigma–Aldrich, St. Louis, MO) as a loading control. The second antibody was HRP-conjugated goat antirabbit (for GCLC and GCLM) or antimouse (for  $\beta$ -actin) immunoglobulin G (Amersham Pharmacia Biotech, Piscataway, NJ). Visualization was accomplished using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Piscataway, NJ) followed by exposure to Hyperfilm ECL (Amersham). Semiquantitative analysis of films was performed using a Stratagene molecular documentation and image analysis system with EagleSight software. For statistical analyses, the mean of the absorbance readings for the control (saline-treated) samples on a given blot was calculated and the absorbance reading for each individual band was then divided by the mean of the control values for that blot and multiplied by 100 to express the absorbance as “percent control”.

## 2.9. GCL enzymatic activity assay

GCL activity was measured essentially as described by White et al. [32]. Ovaries homogenized in TES-SB buffer were centrifuged and the supernatant was diluted again in TES-SB for a final dilution of 1:24 w/v. Fifty microlitres of sample (in duplicate) or GSH standards in TES-SB were added to prewarmed 1.5 ml microcentrifuge tubes containing GCL reaction cocktail (400 mM Tris, 40 mM ATP, 20 mM L-glutamic acid, 2 mM EDTA, 20 mM boric acid, 2 mM L-serine, 40 mM  $\text{MgCl}_2$ ) in a 37 °C water bath. After 11 min preincubation, the reaction was initiated by the addition of 50  $\mu\text{l}$  of 2 mM L-cysteine to one of each of the two sample tubes. After exactly 20 min incubation, the reaction was stopped by the addition of 50  $\mu\text{l}$  of 200 mM sulfosalicylic acid to all tubes. Then, 50  $\mu\text{l}$  of cysteine was added to the second tube for each sample (the baseline GSH tube) and to the standard tubes. Samples were vortexed, incubated on ice for 20 min, and centrifuged at  $1500 \times g$  at 4 °C for 5 min. Twenty microlitres of supernatant (in triplicate) were pipetted into the wells of a 96 well microplate. One hundred and eighty microlitres of naphthalenedicarboxaldehyde solution (1.4 parts 50 mM Tris base, pH 10; 0.2 parts 0.5N NaOH; 0.2 parts 10 mM 2,3-naphthalenedicarboxaldehyde in DMSO) was then added to each well. The plate was incubated at room temperature for 30 min and read at an excitation wavelength of 485 and emission wavelength of 530 in a Biotek FL600 spectrofluorometer microplate reader (Biotek Instruments, Winooski, VT). Results were expressed as the nanomoles of GSH synthesized above the baseline GSH level per minute per milligram ovary or per milligram protein (determined using the Pierce BCA Protein Assay Kit, Pierce, Rockford, IL).

## 2.10. Fluorogenic 5'-nuclease mRNA quantification (real time PCR)

RNA was extracted using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Two micrograms of RNA from each sample were incubated for 30 min at 37 °C with DNase I (0.25 U), dithiothreitol (10 mM), and RNase inhibitor (10 U), then inactivated at 70 °C. Oligo d(T)15 primer (0.13  $\mu\text{g}$ ; Invitrogen) was added and the mixture was incubated at 70 °C for 5 min. Finally, reverse-transcription was carried out by adding RT Master Mix containing Superscript II RNase H-Reverse Transcriptase (100 U), dNTPs (1.43 mM), first strand buffer, and dithiothreitol (14.3 mM) (Invitrogen) at 45 °C for 1 h. RT reactions were shipped on dry ice to the University of Washington where fluorogenic 5'-nuclease assays (TaqMan<sup>®</sup>) were carried out using an ABI Prism 7700 Sequence detection system (Perkin-Elmer Applied Biosystems). The thermal cycling conditions comprised an initial denaturation step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 20 s and 62 °C for 60 s. The human gene-specific sequences of primer pairs and probes used in the TaqMan assays are listed in Table 1. A standard curve derived from serial dilutions of rat kidney RNA (crossing point as a function of log dilution) was used to convert the crossing points for *Gclc*, *Gclm*, and *Gapdh* mRNAs in samples to log dilutions of the standard for data analysis. The rat gene-specific primer pairs and probes used for the standard curves were previously reported [15].

## 2.11. Estradiol and progesterone assays

A standard curve was prepared in charcoal-stripped, ovariectomized rat serum using a stock solution of 17 $\beta$ -estradiol (Sigma, St. Louis, MO) dissolved in 100% ethanol at a concentration of 100 ng/mL that was serially diluted to concentrations of 7.8, 15.6, 62.5, 125, 250, 500, and 1000 pg/mL. The other reagents were from the Estradiol Double Antibody Radioimmunoassay Kit (Diagnostic Products Corporation, Los Angeles, CA), and the assay was performed

Table 1  
Primer pairs and probes used in real time PCR assays

cDNA GenBank Accession #	Type of oligo	Sequence
<i>Gclc</i> ; BC039894	Primer (forward)	tggatgtggaccagatgtagtattc
	Primer (reverse)	tgcttgctgtagtcaggatggttt
	Probe (anti-sense)	ttctccagatgctctctctt
<i>Gclm</i> ; L35546	Primer (forward)	tgactgcatttgctaacaatttga
	Primer (reverse)	cgtgcgcttgaatgacagg
	Probe (anti-sense)	caatgatccaaaagaactgct
<i>Gapdh</i> ; BT006893	Primer (forward)	tcttcaccaccaactgctt
	Primer (reverse)	gaggggccatccacagtctt
	Probe (anti-sense)	ctcatgaccacagtcctcatccac



as previously described [5]. The progesterone assays utilized the Progesterone CL Radioimmunoassay Kit (Diagnostic Systems Corporation, Webster, TX), as previously described [5].

### 2.12. In situ hybridization

Upon dissection, ovaries were immediately fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in phosphate-buffered saline (PBS) at 4 °C for 1 h, dehydrated in 15% sucrose in PBS at 4 °C for 3 to 4 h, embedded in Tissue-Tek O.C.T. (Sakura Finetek, Torrance, CA), and stored at –70 °C until serial sectioning at 10 µm onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) using a cryostat. Slides were stored at –70 °C with dessicant until in situ hybridization. The hybridization procedure was adapted from Wilcox [33] as previously described [14], utilizing 35S-labeled antisense and sense riboprobes transcribed from full-length 0.82 kb mouse GCLM cDNA in pCR II [29] or a 0.6 kb mouse GCLC cDNA fragment in pBluescript II [34]. Sections of kidney were used as “positive controls” because of the known high levels of GCL subunit mRNA in this organ [35]. Negative control slides were incubated with sense *Gclc* or *Gclm* riboprobes.

Serial sections of ovaries were examined under light microscopy and follicles were classified as primary (Pederson stages 1–3), secondary (Pederson stages 4–5), or antral (Pederson stages 6–8) [36,37]. The follicle granulosa cells and oocytes were scored for *Gclm* hybridization and for *Gclc* hybridization (absent, weak, or strong signal) using dark field microscopy.

### 2.13. Statistical analysis

The effects of BSO treatment on GSH concentrations in ovaries and on serum estradiol and progesterone concentrations were analyzed by two-way analysis of variance (ANOVA) with treatment (saline versus BSO) and time as independent variables. The effect of BSO on GCL subunit protein and mRNA levels at each time point was analyzed by independent samples *t*-test. The effect of BSO treatment on the intraovarian localization and intensity of *Gclc* and *Gclm* riboprobe hybridization was analyzed by ANOVA. Treatment and estrous cycle stage were the independent variables and the arcsine transformed percentages [38] of follicles with strong hybridization signal were the dependent variables. Separate analyses were also performed to assess the effects of BSO treatment on GCL subunit hybridization in antral follicles alone, secondary follicles alone, and primary follicles alone. The effects of BSO and FSH treatments on GCL subunit protein and mRNA levels in cultured granulosa cells were analyzed by two-way ANOVA. Analyses were performed using SPSS 11 for the MacIntosh (SPSS, Chicago, IL).

## 3. Results

### 3.1. Ovarian GSH concentrations after BSO treatment

Mean ovarian GSH concentrations in animals treated with BSO were significantly decreased to 58% of control levels at 8 h after a single BSO injection and gradually increased towards control levels at 12 and 24 h after a single BSO injection; ovarian GSH levels were suppressed to 53% of control levels at 12 h after a second BSO injection ( $p < 0.001$ , effect of treatment by two-way ANOVA; Fig. 1A). Ovarian GSH lev-

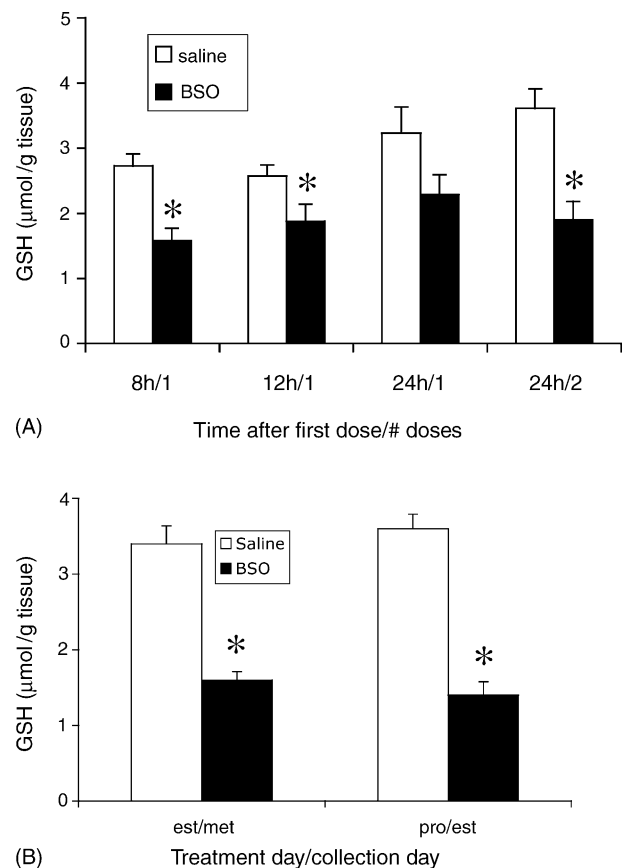


Fig. 1. BSO treatment suppresses ovarian GSH levels. (A) Proestrous adult female rats were injected i.p. with 5 mmol/kg BSO in 0.9% saline or saline alone at 07:00 h and sacrificed 8 h (8 h/1), 12 h (12 h/1), or 24 h (24 h/1) later or were injected at 07:00 and 19:00 h and sacrificed the following morning at 07:00 h (24 h/2). Ovaries were subjected to total GSH assay by the enzymatic recycling method. Ovarian GSH levels, expressed as mean  $\pm$  S.E.M., were significantly suppressed after BSO treatment ( $p < 0.001$ , effect of treatment by two-way ANOVA). (\*) BSO-treated significantly different from respective saline control. Ovarian GSH concentrations in estrous saline control animals (24 h/1, 24 h/2) were significantly higher than in proestrous control animals (8 h/1, 12 h/1) by *t*-test,  $p = 0.003$ ;  $N = 5$ –10 per group. (B) Proestrous (pro/est) or estrous (est/met) adult female rats were injected i.p. with 5 mmol/kg BSO in 0.9% saline or saline alone at 07:00 and 19:00 h and sacrificed the following morning of estrus or metestrus, respectively, at 0700 h. Ovarian GSH levels, expressed as mean  $\pm$  S.E.M., were suppressed by about half in both BSO-treated groups compared to the respective saline control ( $p < 0.001$ , effect of treatment by two-way ANOVA; (\*) indicates significantly lower than respective saline control by *t*-test). There was no effect of estrous cycle stage.

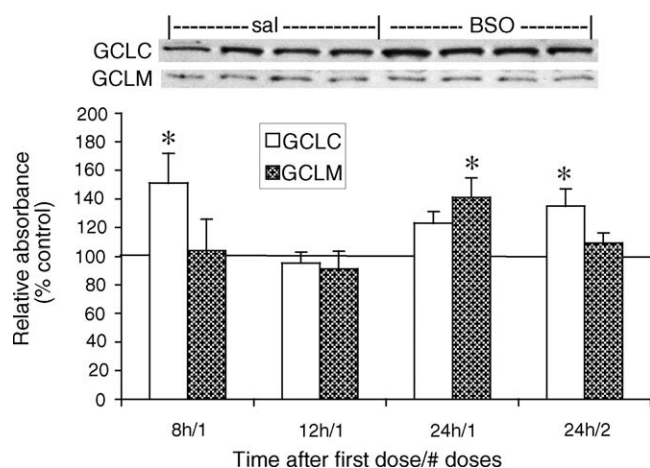


Fig. 2. BSO treatment modestly increases ovarian GCLC protein levels. The experimental protocol was as described under Fig. 1, except that ovaries were subjected to protein extraction, polyacrylamide gel electrophoresis, and Western blotting with anti-GCLC and anti-GCLM antibodies. Densitometry was performed on each band and all values were divided by the mean of the saline values for the same blot. (A) Representative Western blot showing GCLC and GCLM protein expression in extracts from ovaries 8 h after injection with saline or BSO. The graph summarizes the mean  $\pm$  S.E.M. of densitometry results for several blots for each time point expressed as percent of saline control. The horizontal line indicates 100% of control. (\*) BSO-treated differed significantly from saline-treated for same time point by independent samples *t*-test,  $p < 0.05$ ;  $N = 4$ –15/group.

els were significantly higher in control estrous ovaries than in control proestrous ovaries ( $p = 0.014$ , by *t*-test), as previously reported [3,4]. In the second experiment, mean ovarian GSH concentrations were suppressed by more than half at 12 h after the second BSO injection compared to saline treated controls ( $p < 0.001$ , effect of treatment by ANOVA; Fig. 1B). Ovarian GSH levels did not differ between estrous and metestrous stages of the estrous cycle, consistent with previous observations [4]. Collectively, these results show that repeated injections of BSO administered at 12 h intervals can maintain ovarian GSH concentrations at about 50% or less of control levels.

### 3.2. Ovarian GCL subunit protein and mRNA expression after BSO treatment

Ovarian GCLC protein levels were statistically significantly increased by 1.5-fold at 8 h after a single BSO injection and by 1.4-fold at 12 h after a second BSO injection compared to saline-treated controls (Fig. 2). GCLM protein levels were significantly increased by 1.4-fold compared to saline controls at 24 h after a single BSO injection, but not at other time points (Fig. 2). *Gclc* and *Gclm* mRNA levels were increased by 1.4-fold at 8 h after a single BSO injection (Fig. 3). *Gclm* mRNA levels were significantly decreased at 12 h after a single BSO injection (Fig. 3).

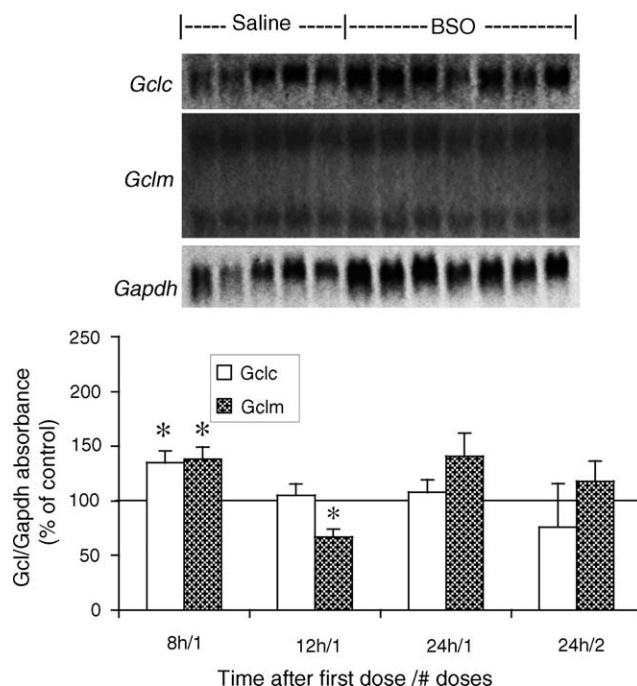


Fig. 3. Effect of BSO treatment on ovarian GCL subunit mRNA levels. The experimental protocol was as described under Fig. 1, except that ovaries were subjected to RNA extraction, agarose-formaldehyde gel electrophoresis, and Northern blotting with  $^{32}$ P-labeled *Gclc*, *Gclm*, and *Gapdh* cDNA probes. Densitometry was performed on each band and all *Gcl* values were normalized to *Gapdh*. Each normalized value was then divided by the mean of the saline values for the same blot. At top is a representative Northern blot showing *Gclc* and *Gclm* mRNA expression in extracts of ovaries 12 h after injection of saline or BSO. The graph summarizes mean  $\pm$  S.E.M. of normalized densitometry results expressed as percent of saline control. The horizontal line indicates 100% of control. (\*) BSO-treated differed significantly from saline-treated for same time point by independent samples *t*-test,  $p < 0.05$ ;  $N = 4$ –11/group.

### 3.3. Ovarian GCL enzymatic activity after BSO treatment

Ovarian GCL enzymatic activity was  $66.8 \pm 6.4$  pmol GSH/(mg ovary/min) ( $1.06 \pm 0.06$  nmol/(mg protein/min)) at 24 h after a single dose of BSO compared to  $54.9 \pm 4.3$  pmol GSH/(mg ovary/min) ( $0.89 \pm 0.07$  nmol/(mg protein/min)) after saline. These differences were not statistically significant ( $p = 0.17$ ,  $p = 0.08$ , respectively;  $N = 10$ /group).

### 3.4. Serum sex steroid concentrations and organ weights after BSO

BSO treatment did not alter serum estradiol or progesterone concentrations (Table 2). Estradiol concentrations were significantly higher at 16:00 h on proestrus than at the other time points ( $p < 0.001$ ). Progesterone concentrations were significantly higher at both 16:00 and 20:00 h on proestrus than on estrous morning ( $p < 0.001$ ). There was no effect of BSO on body weight, ovarian weight, uterine

Table 2  
Serum estradiol and progesterone concentrations after BSO treatment

Injection time/sac time	Treatment	Mean $\pm$ S.D.	
		Estradiol (pg/mL)	Progesterone (ng/mL)
08:00 h pro/16:00 h pro	Saline	70.7 $\pm$ 49.1	67.9 $\pm$ 33.8
	BSO	52.6 $\pm$ 25.7	69.7 $\pm$ 23.2
08:00 h pro/20:00 h pro	Saline	22.4 $\pm$ 18.4	64.5 $\pm$ 10.6
	BSO	30.7 $\pm$ 34.4	67.5 $\pm$ 13.4
08:00 h pro/08:00 h est	Saline	13.8 $\pm$ 11.7	27.4 $\pm$ 15.9
	BSO	22.0 $\pm$ 24.1	29.6 $\pm$ 11.9
07:00 h, 19:00 h pro/07:00 h est	Saline	16.7 $\pm$ 2.8	39.7 $\pm$ 13.1
	BSO	25.6 $\pm$ 23.9	42.5 $\pm$ 17.6

Estradiol concentrations were significantly higher at 16:00 h on proestrus (pro) than at the other time points ( $p < 0.001$ , effect of time by two-way ANOVA and post hoc comparison with 16:00 h time point by Fisher's LSD test). Progesterone concentrations were significantly higher at both time points on proestrus evening than on estrous (est) morning ( $p < 0.001$ ). There was no effect of BSO treatment on either hormone.  $N = 7$ –18/group for estradiol;  $N = 12$ –17/group for progesterone.

weight, or on vaginal cytology on estrous morning (data not shown).

### 3.5. Localization of *Gclc* and *Gclm* mRNA within the ovary after BSO treatment on proestrus or estrus

*Gclm* antisense riboprobes hybridized strongly to granulosa cells and oocytes of healthy, growing follicles, but not to atretic follicles, or to the most immature (primordial) follicles in saline controls (Fig. 4A–D), as we have previously reported [14]. *Gclc* riboprobe hybridized more ubiquitously throughout the ovary in the saline controls (Fig. 4E–H), also as previously reported [14]. Neither *Gclm* nor *Gclc* hybridization appeared altered by BSO treatment (Fig. 4B, C, G, H). Statistical analyses confirmed that the distribution and intensity of hybridization of *Gclm* and *Gclc* antisense riboprobes within the ovary did not vary with BSO treatment or estrous cycle stage (data not shown).

### 3.6. Effect of GSH depletion with BSO on GSH concentrations and GCL subunit protein and mRNA levels in a human granulosa cell line

Treatment of COV434 cells with 100  $\mu$ M BSO for 20 h, followed by 6 h in control media, suppressed intracellular GSH to 14% of control levels ( $106.8 \pm 12.4$  nmol/mg protein in controls versus  $14.8 \pm 3.7$  nmol/mg protein in FSH + BSO,  $p = 0.012$ ). Treatment with BSO in the presence or absence of FSH for 20 h, followed by 6 h in control media without BSO or FSH, did not significantly alter GCLC or GCLM protein or mRNA levels compared to cells cultured in control media (Fig. 5A and B).

### 3.7. Effect of GSH depletion with BSO on GSH concentrations and GCL subunit protein levels in cultured primary rat granulosa cells

Treatment of granulosa cells with 100  $\mu$ M BSO plus 200 ng/mL FSH for 18 h, followed by 6 h in control medium without BSO or FSH did not alter GCLC or GCLM protein levels compared to cells cultured in control medium or medium with FSH alone (Fig. 6). There was an apparent stimulatory effect of FSH treatment on GCL protein levels that was not statistically significant. However, we have observed statistically significant stimulation of GCL protein levels in granulosa cells after longer durations of culture with FSH (data not shown).

## 4. Discussion

We observed small increases in ovarian *Gclc* and *Gclm* mRNA levels and in GCLC protein levels at 8 h after administration of BSO on proestrus to inhibit GSH synthesis. GCLC protein and mRNA levels returned to baseline by 12 and 24 h after BSO. Ovarian GCLM protein levels were slightly increased at 24 h after a single dose of BSO, but the concomitant increase in *Gclm* mRNA levels was not statistically significant. BSO treatment did not alter the distribution of GCL subunit mRNA expression within the ovary or ovarian GCL enzymatic activity. In experiments using BSO to deplete GSH in a human granulosa cell line and in primary rat granulosa cells, we again observed no upregulation of GCLC or GCLM protein or mRNA levels after BSO treatment. These data show that acute depletion of ovarian GSH in vivo or of granulosa cell GSH in vitro using BSO does not lead to a robust upregulation of *Gclc* and *Gclm* transcription, in contrast to some other cell culture models. To our knowledge this is the first study to investigate the effects of BSO treatment on ovarian GCL expression.

Our observations that suppression of ovarian GSH levels with BSO did not result in dramatic upregulation of ovarian GCL subunit expression are similar to results in the liver [21]. Kitteringham and coworkers observed no effects on hepatic *Gclc* mRNA levels at various time points (1, 2, 3, 10, or 24 h) after a single BSO injection in mice. GCLC and GCLM protein levels were assessed only at 24 h after BSO treatment and were not changed at that time [21]. Hepatic GCL enzymatic activity was increased at 24 h after BSO injection [21]. In contrast, we observed no significant difference in ovarian GCL enzymatic activity at 24 h after a single BSO injection compared to saline. Treatment of primary hepatocyte cultures [18,19] and the L2 rat lung epithelial cell line [17] with BSO has been reported to increase both *Gclm* and *Gclc* mRNA steady state levels and transcription. However, BSO treatment did not increase *Gclm* or *Gclc* mRNA levels in mouse hepatoma cells [20].

The lack of an effect of in vivo treatment with BSO on liver GCL expression versus the pronounced upregulation of



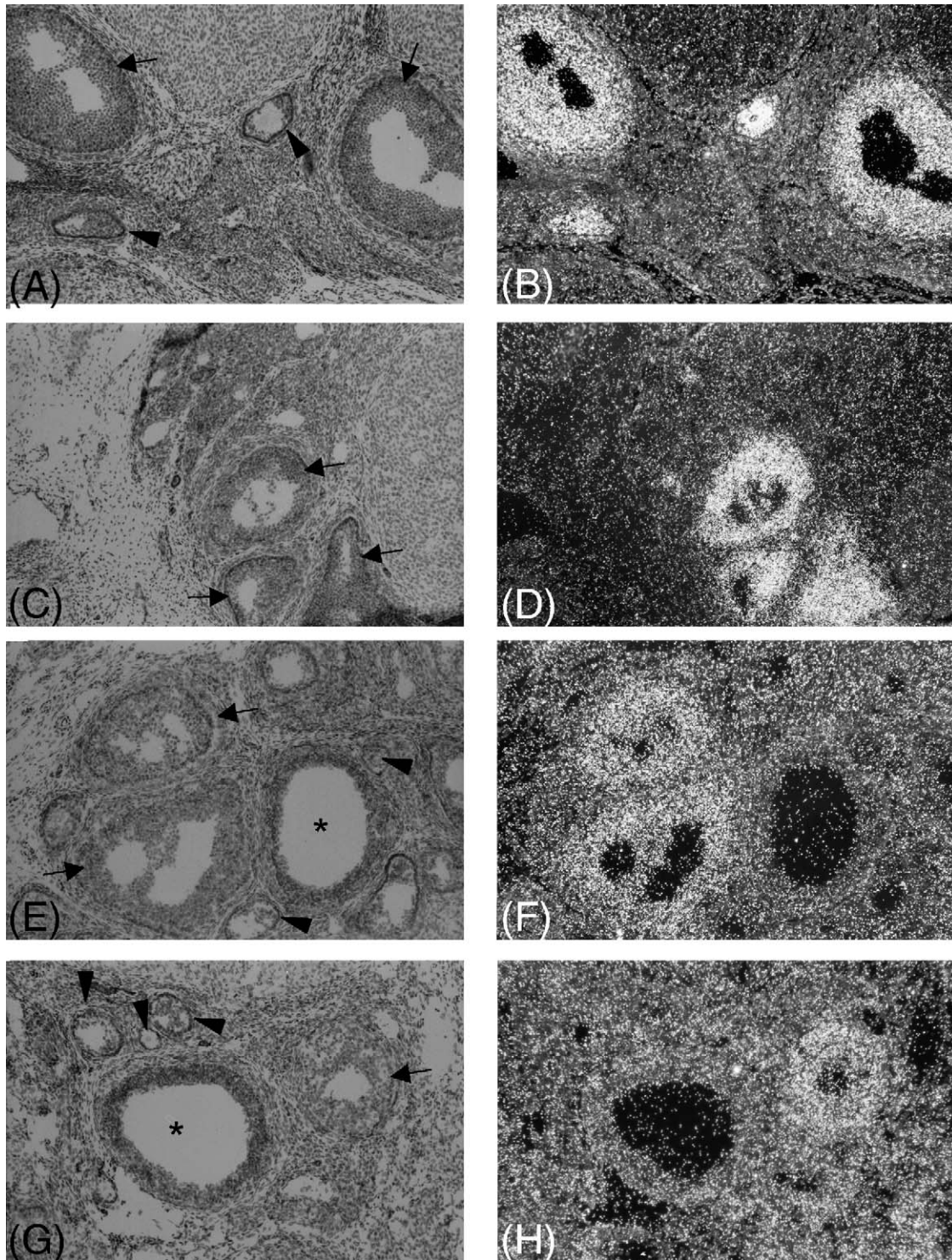


Fig. 4. Effect of BSO treatment on in situ *Gclc* and *Gclm* mRNA expression. (A) Bright field view of metestrous ovary from animal treated with saline on estrus. (B) Dark field view of same section showing *Gclm* riboprobe hybridizing to granulosa cells and oocytes of antral (arrows) and secondary (arrowheads) follicles. (C) Bright field view of metestrous ovary from animal treated with BSO on estrus. (D) Dark field view of same field as in C shows *Gclm* riboprobe hybridizing predominantly to granulosa cells of several antral follicles (arrowheads). (E) Bright field view of estrous ovary from animal treated with saline on proestrus. (F) Dark field view of same field as in E shows strongest *Gclc* hybridization to granulosa cells of healthy antral follicles (arrows), with less hybridization to atretic antral follicle (\*) and secondary follicles (arrowheads). (G) Bright field view of estrous ovary from animal treated with BSO on proestrus. (H) Dark field view of same field as in G shows strongest *Gclc* hybridization to healthy antral follicle (arrow), with less hybridization to atretic antral follicle (\*) and smaller growing follicles (arrowheads). Original magnification of all images  $\times 33$ .



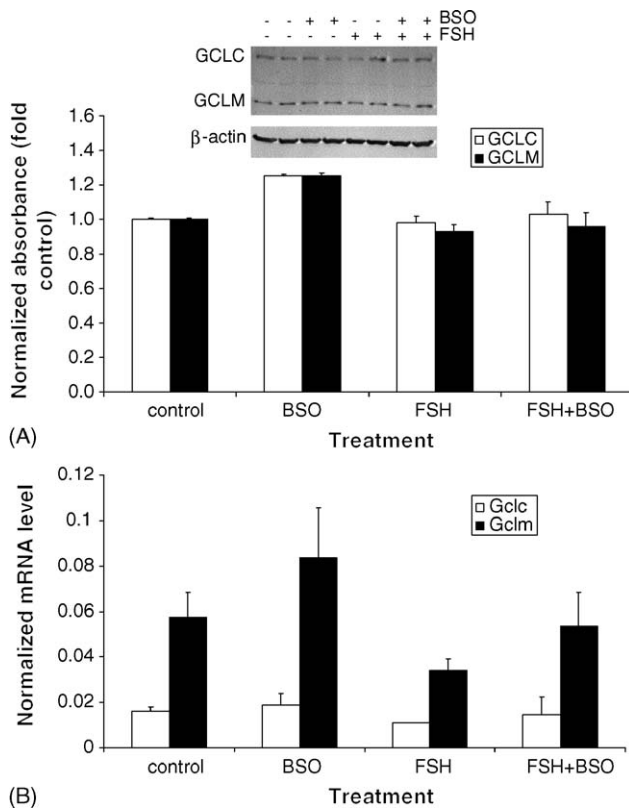


Fig. 5. Effect of BSO treatment on GCL subunit protein and mRNA expression in human granulosa (COV434) cells. COV434 cells were cultured as detailed in Section 2. (A) Cells were treated with control medium (DMEM F12), 100  $\mu$ M BSO (BSO), 200 ng/mL FSH (FSH), or 200 ng/mL FSH and 100  $\mu$ M BSO (FSH + BSO) for 20 h. Medium was then replaced with control medium and the cells were incubated for an additional 6 h. (A) Cells were collected for protein extraction and quantification, followed by gel electrophoresis and Western blotting, as detailed in Section 2. A representative Western blot is shown. The graph shows the mean  $\pm$  S.E. of the normalized GCLC and GCLM subunit absorbances expressed as fold of the mean of the control absorbances for the same blot. There was no significant effect of BSO treatment on GCL subunit protein levels.  $N=4$ –5/group from three separate experiments. (B) Cells were collected for RNA extraction and quantification, followed by reverse transcription and real time PCR, as detailed in Section 2. The graph shows the mean  $\pm$  S.E. of the *Gclc* and *Gclm* subunit concentrations normalized to *Gapdh*. There was no significant effect of BSO treatment on GCL subunit mRNA levels.  $N=3$ –4/group from two separate experiments.

GCL expression observed after in vitro treatment of primary hepatocytes with BSO may be due to the lesser extent of GSH depletion that was achieved using BSO in vivo compared to in vitro. In the latter situation, GSH concentrations were suppressed to less than 10% of control levels within 12–24 h [18]. In vivo, hepatic GSH levels were maximally suppressed to 25–40% of control levels at 2–5 h after injection, and returned to initial levels by about 18–24 h after injection [16,21]. Similarly, in the current in vivo study, ovarian GSH levels were 58% of control levels at 8 h after a single dose of BSO and were not statistically different from control levels by 24 h after BSO (Fig. 1). However, in our granulosa cell models, even depletion of GSH below 15% of control levels did

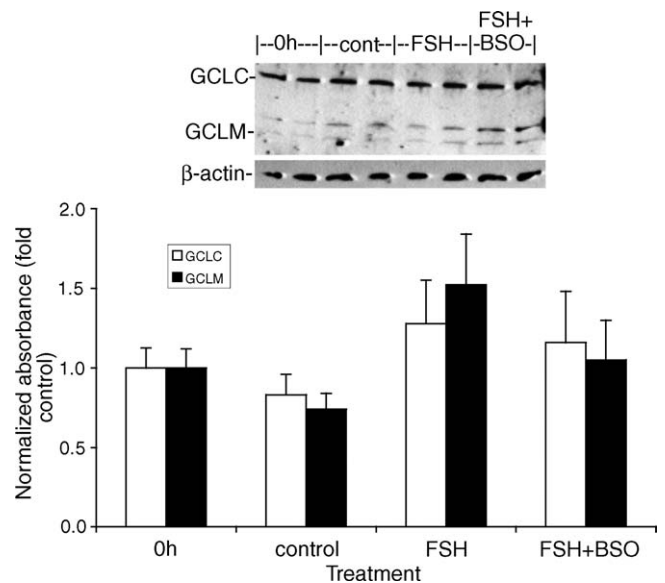


Fig. 6. Effect of BSO treatment on GCL subunit protein expression in primary rat granulosa cells. Granulosa cells were collected and cultured as detailed in Section 2. (A) Cells were treated with control medium (DMEM F12) or 200 ng/mL FSH (FSH) for 24 h. A third experimental group was treated with 200 ng/mL FSH and 100  $\mu$ M BSO (FSH + BSO) for 18 h, at which time the medium was replaced with control medium and the cells were incubated for an additional 6 h. Additional cells were processed immediately after harvesting without culturing (0 h). Cells were collected for protein extraction and quantification, gel electrophoresis, and Western blotting as detailed in Section 2. The representative Western blot shows the GCLC band at 73 kDa and the GCLM band at 30 kDa. The third band between the two may be a degradation product of GCLC. The graph shows the mean  $\pm$  S.E. of the normalized GCLC and GCLM subunit absorbance values expressed as fold of the mean of the 0 h absorbances for the same blot. There was no significant effect of BSO or FSH treatment on GCL subunit protein levels.  $N=4$ /group from two separate experiments.

not result in upregulation of GCLC or GCLM expression, suggesting that the negative findings in our in vivo study were not due to inadequate suppression of GSH synthesis and further suggesting that granulosa cells lack the ability to respond to GSH depletion by upregulating GCL subunit expression.

Agents that deplete GSH by mechanisms other than inhibition of GCL have varied effects on GCL subunit mRNA and protein expression. Diethylmaleate (DEM), which depletes GSH via glutathione-S-transferase-mediated conjugation, increases both *Gclc* and *Gclm* mRNA levels in cultured hepatocytes [19]. Hepatic *Gclc* mRNA levels were significantly increased at 30 min and 3 h, but not 1, 2, 10, or 24 h, after in vivo administration of DEM [21]. This dose of DEM also upregulated GCLC, but not GCLM, protein levels and GCL enzymatic activity at 24 h [21]. Culture of day 1 (cleavage stage) or day 3 (blastocyst stage) mouse embryos for 3 h with DEM, followed by a 3 h recovery for RT-PCR or a 5 h recovery for Western blotting, did not result in increased GCLC protein or mRNA levels in embryos of either developmental stage [39]. In contrast, treatment of cultured day 10 rat embryos with DEM for 24 h significantly increased *Gclc*

and *Gclm* mRNA levels and GCLC protein levels (GCLM was not measured), but did not significantly increase GCL enzymatic activity [40]. Acetaminophen, which also depletes GSH by glutathione-S-transferase mediated conjugation, had a different effect upon hepatic GCL expression than DEM. *Gclc* mRNA levels were increased at 30 min and 24 h after acetaminophen, but not at 1 or 2 h [21]. GCLC protein levels, but not GCLM protein levels, were also increased at 24 h, but GCL enzymatic activity was suppressed at 24 h after acetaminophen treatment [21]. *Tert*-butyl hydroquinone, an agent that depletes GSH by inducing oxidative stress, upregulated *Gclc* and *Gclm* mRNA levels in cultured hepatocytes [19] and in Hepa-1c1c7 hepatoma cells [20]. Similarly, treatment with *tert*-butyl hydroperoxide, another agent that induces oxidative stress, increased GCLC protein and mRNA levels of cleavage stage embryos, but not of blastocyst stage embryos [39]. Taken together the results of these studies suggest that the GCL response to GSH depletion depends on both the mechanism of GSH depletion as well as the tissue or cell type in which depletion occurs.

Only one other study of which we are aware has investigated the effects of agents that deplete GSH on ovarian GCL expression. The anti-cancer drug and ovarian toxicant cyclophosphamide suppressed ovarian GSH levels at 24 h, but not 8 h, after an in vivo dose in adult rats. This suppression of GSH levels was associated with an increase in ovarian *Gclc* and *Gclm* mRNA levels at 8 h, but not at 24 h, and no effect on GCL subunit protein levels [5].

GSH appears to play an anti-apoptotic role in ovarian follicles. Depletion of GSH with BSO increases granulosa cell apoptosis in cultured preovulatory follicles [6] and increases atresia of antral follicles in vivo [5]. Treatment with the GSH precursor *N*-acetylcysteine prevents apoptosis induced by serum-withdrawal in cultured preovulatory follicles [7]. Reactive metabolites of the pro-apoptotic follicular toxicants cyclophosphamide, polycyclic aromatic hydrocarbons, and 2-vinylcyclohexene are detoxified by GSH conjugation [41–44]. The inability to respond to GSH depletion by upregulating GCL subunit expression may contribute to the sensitivity of ovarian follicles to the induction of apoptosis by stimuli that suppress GSH.

This time-course study of the effects of inhibition of GSH synthesis with BSO on ovarian expression of GCL, the rate-limiting enzyme in GSH synthesis, showed no more than 1.5-fold increases in GCL subunit protein or mRNA levels at any of the time points tested. GCL subunit mRNA localization within the ovary was also not affected by BSO treatment. GCL enzymatic activity was not increased at 24 h after BSO injection. Suppression of GSH synthesis with BSO in cultured granulosa cells also did not result in increased GCL subunit protein or mRNA expression. Taken together these results demonstrate that ovarian follicles are minimally able to respond to acute GSH depletion by upregulating GCL subunit expression. This may be one reason for ovarian sensitivity to toxicants that are detoxified by GSH conjugation.

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