Gonadotropin regulation of glutathione synthesis in the rat ovary☆

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Abstract

Glutathione (GSH), an antioxidant and conjugator of electrophilic toxicants, prevents toxicant-mediated destruction of ovarian follicles and oocytes. Ovarian GSH has previously been shown to change with estrous cycle stage in rats, suggesting that the gonadotropin hormones may regulate ovarian GSH synthesis. The present studies tested the hypotheses that [1] estrous cycle-related changes in ovarian GSH result from cyclic changes in protein and mRNA expression of the rate-limiting enzyme in GSH synthesis, glutamate cysteine ligase (GCL, also called γ-glutamylcysteine synthetase), and [2] that these changes result from gonadotropin-mediated regulation of GCL subunit expression. In the first experiment, ovaries were harvested from cycling adult female rats on each stage of the estrous cycle. In the second experiment immature female rats were injected with pregnant mare's serum gonadotropin (PMSG) to stimulate follicular development or with vehicle and killed 8, 24, or 48 h later. In both experiments the ovaries were harvested for [1] total GSH assay, [2] Western analysis for GCL catalytic (GCLc) and regulatory (GCLm) subunit protein levels, or [3] Northern analysis for *Gclc* and *Gclm* mRNA levels. Ovarian GSH concentrations and *Gclc* and *Gclm* mRNA levels, but not GCL subunit protein levels, varied significantly with estrous cycle stage. PMSG administration significantly increased ovarian GSH concentrations 24 and 48 h later. GCLm protein levels increased significantly at 24h and 48h following PMSG. GCLc protein levels did not increase significantly following PMSG. *Gcl* subunit mRNA levels were not significantly increased at any time point by the planned ANOVA; however, an increase in *Gclc* at 48 h was identified by *t*-testing. These results support the hypothesis that gonadotropins regulate ovarian GSH synthesis by modulating GCL subunit expression. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Glutathione; Estrous cycle; Glutamate cysteine ligase; γ-Glutamylcysteine synthetase; Luteinizing hormone; Follicle-stimulating hormone; Ovary

1. Introduction

Destruction of ovarian follicles by environmental toxicants can result in permanent loss of fertility because female mammals possess a finite complement of oocytes. Oxidative damage is a key putative mechanism by which toxicants can destroy ovarian follicles and oocytes [1]. The tripeptide glutathione (γ–glutamylcysteinylglycine, GSH) plays critical roles in protecting cells from oxidant injury by both endogenous and exogenous agents [2]. GSH also acts as a cofactor for enzymes, regulates protein and DNA synthesis by altering redox status, and participates in microtubule assembly [2].

Although GSH is ubiquitous in mammalian cells, there is considerable interorgan variability in the roles it plays and in the regulation of its synthesis. As in other tissues, ovarian GSH likely protects ovarian cells from electrophilic toxicants [3]. In addition to protection from exogenous toxicants, ovarian GSH may also protect maturing follicles from the reactive oxygen species (ROS) such as superoxide, which arise as byproducts of normal cellular processes, particularly steroid synthesis by the steroidogenic cytochrome P450 enzymes in follicle cells [4–6]. ROS produced by cellular processes or by toxicant exposure may promote follicular apoptosis. The GSH precursor, N-acetyl cysteine, inhibits apoptosis as effectively as the gonadotropin follicle-stimulating hormone in cultured antral follicles [7]. Following ovulation, the high concentrations of GSH in mature oocytes are critical for early preimplantation embryo development. Depletion of oocyte GSH before in vitro fertilization blocks the normal formation of both the female

and the male pronuclei and prevents normal pronuclear apposition [8–11]. Moreover, depleting GSH in two-cell embryos blocks their further normal development [12–14].

Despite the evidence that ovarian GSH is an important line of defense against oxidant damage and that oocyte GSH is important in early embryo development, relatively little is known about the regulation of GSH synthesis in the ovary. Total ovarian GSH content and release of GSH from in vitro perfused ovaries have been reported to vary significantly with estrous cycle stage in adult rats [15]. These changes may depend on stimulation of ovarian follicular development by pituitary gonadotropin hormones (luteinizing hormone, LH, and follicle-stimulating hormone, FSH). Evidence for this possibility comes from experiments in which immature rats were treated with exogenous gonadotropin. Ovarian GSH content increased during the period of follicular growth induced by exogenous gonadotropin stimulation and declined during luteal regression in immature rats [16]. Gonadotropin injection resulted in partial recovery of ovarian GSH concentrations, which had been suppressed by administration of an inhibitor of GSH synthesis, in immature rats [17].

GSH synthesis has been shown to be regulated via several mechanisms in other tissues, and these mechanisms likely are also relevant in the ovary [2,18,19]. The synthesis of GSH occurs via two steps: the first and rate-limiting step, involving formation of a gamma peptide bond between cysteine and glutamate forming γ–glutamylcysteine, is catalyzed by glutamate cysteine ligase (GCL, also called γ-glutamylcysteine synthetase); the second step, involving formation of a peptide bond between cysteine and glycine, is catalyzed by glutathione synthetase [2]. GCL consists of two subunits, GCLc, the catalytically active subunit, and GCLm, the regulatory or modulatory subunit [18,20]. GCLc may be catalytically active alone, but GCLm binding via a disulfide bridge decreases the K_m for glutamate and increases the K_i for GSH inhibition of GCL catalytic activity [18,20]. In addition to regulation of GCL catalytic activity, GSH synthesis is also modulated by transcriptional and translational regulation of *Gcl* subunit gene expression [18, 20].

The purpose of the studies described herein was to test the hypotheses that [1] estrous cycle-related changes in ovarian GSH concentrations are the result of cyclic changes in GCL subunit protein and mRNA expression, and [2] that these changes result from gonadotropin-mediated regulation of GCL subunit expression.

2. Materials And Methods

2.1. Animals

For experiment 1, adult, female Sprague-Dawley rats 9 to 10 weeks old weighing 195 to 229 g were purchased from Charles River Laboratories. For experiment 2, 20-day-old immature, female Sprague Dawley rats were purchased from Charles River Laboratories. Upon arrival, the animals were housed in an AAALAC-accredited facility, 3 or 4 to a cage, with free access to deionized water and standard laboratory chow, on a 12-h/12-h (experiment 1) or a 14-h/10-h (experiment 2) light/dark cycle. The experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Washington (experiment 1) or at the University of California at Irvine (experiment 2), where the experiments were performed.

2.2. Experimental protocol, experiment 1

After a 7-day acclimatization period, daily vaginal cytology was performed for at least two four-day estrous cycles prior to initiation of the experiment. Rats were rapidly killed by decapitation between 0900 h and 1100 h on each day of the estrous cycle as determined by vaginal cytology: $n = 14$ on metestrus, 15 on diestrus, 15 on proestrus, and 15 on estrus. Trunk blood was collected for estradiol and progesterone assays to verify estrous cycle stage (data not shown). Estrous cycle stage was further verified by examination of the uterus for ballooning on proestrus [21]. Ovaries were rapidly dissected out, trimmed of fat, and subjected to one of the following: 1) freezing on dry ice for storage at –70 C for subsequent protein or RNA extraction or 2) homogenization immediately on ice in 1:4 (w/v) 5% sulfosalicylic acid for total GSH and oxidized glutathione (GSSG) assay. The two ovaries from an individual animal were used for two different assays.

2.3. Experimental protocol, experiment 2

After a 5-day acclimatization period, 25-day-old female rats were injected subcutaneously (s.c.) with 10 IU pregnant mare's serum gonadotropin (PMSG, gift of Dr. A.F. Parlow, Director, NIDDK National Hormone and Pituitary Program) to stimulate ovarian follicular development. PMSG possesses both LH and FSH activity. Eight, 24, or 48 h later, the animals were rapidly killed by decapitation. Ovaries were dissected out, trimmed of fat and [1] immediately homogenized on ice in 1:6 (w/v) 5% sulfosalicylic acid, followed by centrifugation (15,800 g) and removal of the supernatant for storage at –70 C prior to total GSH assay, or [2] rapidly frozen on dry ice and stored at –70 C for subsequent protein or RNA extraction. The data from one control animal were not included in the analyses because the animal was found at autopsy to have vaginal opening, indicating that it was postpubertal.

2.4. Total glutathione (GSSG plus reduced GSH) and oxidized glutathione (GSSG) assay

For experiment 1, supernatants from 5% sulfosalicylic acid homogenates 1:4 W/V were immediately processed either for reduced GSH or for GSSG. For GSSG 100 μl of

sample, 400 μl of TES buffer, 4 μl of 2-vinylpyridine, and 50 μl of 10% triethanolamine (pH 5.5) were vortexed for 1 h at room temperature. This process binds all reduced GSH to 2-vinylpyridine. Excess 2-vinylpyridine was then extracted from samples by addition of 500 μl of chloroform followed by vortexing for 15 min. Following chloroform extraction, samples were centrifuged at 15,800 g for 5 min and 100 μl aliquots in triplicate of the aqueous phase were added to 200 μL of 100 mM sodium phosphate/1 mM EDTA (pH 7.5). Fifty microliters of 1 mM NADPH/20 units/mL GSH reductase solution were then added and samples were incubated for 1 h at room temperature. Samples were then derivatized with 20 μl of 12.5 mM monobromo-bimane, followed by incubation in the dark for 30 min. After derivatization, 150 μl of 5% sulfosalicylic acid was added, and the samples were centrifuged at 15,800 g for two minutes. A 50-μl aliquot of sample was then injected on the HPLC. For reduced GSH the 1:4 W/V sample supernatant was further diluted 1:10 with 5% sulfosalicylic acid. Next, 100 μl of the diluted sample, 400 μl TES, and 50 μl of 10% triethanolamine were mixed. A 100-μl aliquot of the mixture was added to 200 μl buffer (100 mM NaH_2PO_4, 1 mM EDTA), and 50 μl water. Derivatization and HPLC were as for GSSG. All samples were assayed in triplicate.

The samples were analyzed using a Shimadzu LC-6A tertiary gradient HPLC equipped with an Alltech 15 $\times$ 0.5-cm C_{18} reverse-phase column maintained at ambient temperature. Peaks were eluted using a binary gradient program with solvent A = 1.0 mM tetrabutylammonium phosphate adjusted to pH 3.0 with 10% phosphoric acid and solvent B = methanol. The flow-rate was 1.5 ml/min, and starting conditions were 95% A/5% B, maintained for 0.5 min. Linear gradients were generated as follows: 80% A/20% B at 1 min, 70% A/30% B at 10 min, and 40% A/60% B at 12 to 14 min. The gradient program then re-equilibrated the column at the starting conditions over the next 5 min. Fluorescence was monitored at λ_{ex} = 375, λ_{em} = 475 and determined with a Shimadzu model RF-10Axl fluorescence detector. The GSH-bimane peak was identified by coelution with a prepared GSH-bimane standard. Peak areas were integrated using a Shimadzu C-R5A Chromatopac integrator and converted to GSH equivalents using a standard curve.

For experiment 2 a modification of the enzymatic assay developed by Griffith [22] was used. Twenty-μL aliquots of the supernatants from 5% sulfosalicylic acid homogenized ovaries or of standards containing known concentrations of GSH were incubated at 30 C for 10 min with 700 μl of 0.3 mM NADPH, 100 μl of 6 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and 180 μl of deionized water. The mixture was then transferred to a cuvette containing 10 μl of 50 U/mL glutathione reductase, and absorbance was monitored continuously for 60 s at 412 nm using a Beckman DU 640 spectrophotometer. The slope of the absorbance versus time plot is proportional to the concentration of GSH in the cuvette. Concentrations of GSH in the sample were calcu-lated from the standard curve using Systat software (SPSS, Evanston, IL). All samples were run in triplicate. The limit of quantification of this assay was 1 nmol total GSH.

2.5. Western blot analysis

Protein extracts were prepared from whole ovaries by homogenization in RIPA buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) with protease inhibitors (100 μg/mL Pefabloc, 10 μg/mL TLCK, 1 μg/mL Pepstatin A, 1 μg/mL Leupeptin). Total protein concentration in the extracts was determined using the Pierce BCA Protein Assay Kit (Pierce, Rockford, IL). Forty micrograms of each protein extract were loaded onto polyacrylamide gels, separated by electrophoresis, transferred to PVDF membranes, and stained with antisera raised against ovalbumin conjugates of the peptide ETLQEKGERTNPNHPT for GCLm and the peptides EKINPDEREEMKVSAK and ESNS-SSSTRSAVD for GCLc [23]. The peptide sequences were chosen to have 100% homology between human, mouse, and rat GCLc and GCLm amino acid sequences. The second antibody was HRP-conjugated goat antirabbit immunoglob-ulin G (Amersham Pharmacia Biotech, Piscataway, NJ). Visualization was accomplished using enhanced chemilu-minescence (ECL, Amersham Pharmacia Biotech, Piscat-away, NJ) followed by exposure to Hyperfilm ECL (Amer-sham). Semiquantitative analysis of films was performed using a BioRad GelDoc and Molecular Analyst software (experiment 1) or Stratagene molecular documentation and image analysis system with EagleSight software (experi-ment 2).

2.6. Northern blot analysis

Total ovarian RNA was prepared using the TRIzol re-agent kit (Life Technologies, Gaithersburg, MD). Samples of RNA were analyzed by separation in 1% agarose/form-aldehyde-containing gels, followed by capillary transfer to nylon membranes and hybridization with ^{32}P-labeled nu-cleic acid probes. ^{32}P-labeled random-primed probes were prepared using template full-length cDNAs of the mouse *Gclc, Gclm* [24,25], *GAPDH* (Ambion, Austin, TX), and cyclophilin genes with the DECAprime II DNA Labeling Kit (Ambion, Austin, TX). Visualization was by phosphor-imaging (experiment 1) or by autoradiography (experiment 2). Semiquantitative analysis of phosphorimages or autora-diographs was performed using a BioRad or a Stratagene, respectively, molecular documentation and image analysis system. *Gclc* and *Gclm* mRNA were normalized to control mRNA (cyclophilin or GAPDH) and relative differences among treatment groups were calculated.

2.7. Statistical analysis

Analysis of covariance (ANCOVA) was used to deter-mine significant differences among estrous cycle stages for
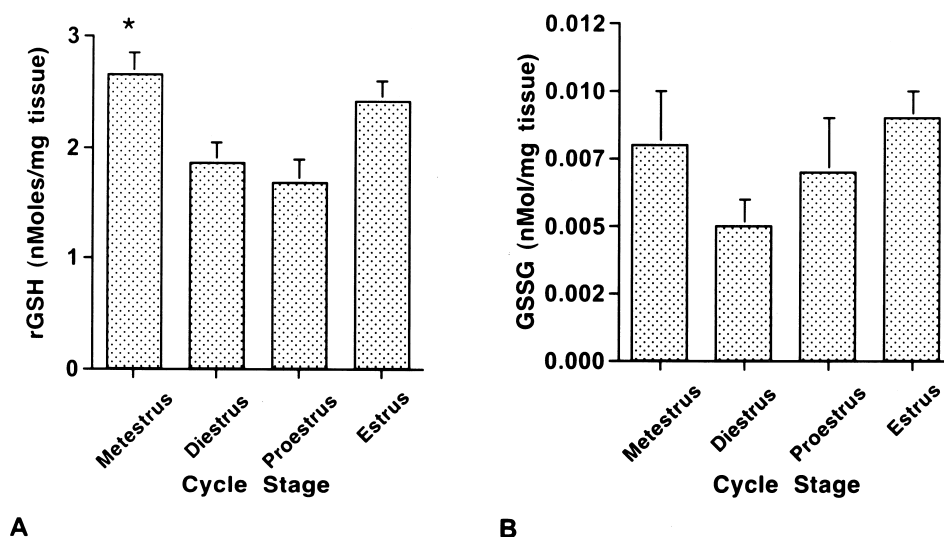
Fig. 1. Reduced (rGSH) glutathione, but not oxidized glutathione (GSSG), in whole ovary homogenates varies during the estrous cycle. The graphs depict the mean ± SEM from 6 to 9 ovaries for each cycle stage. a) Levels of rGSH varied significantly by cycle stage ($P < 0.01$ by ANOVA). *rGSH levels on metestrus were significantly higher than on diestrus and proestrus by the Bonferroni test. b) GSSG content did not vary significantly by cycle stage. GSSG represented 0.5% to 0.9% of the total GSH during the cycle. Note the different y-axis magnitudes for rGSH and GSSG.

experiment 1. For Northern and Western data, blot was found to be a significant covariate. For the GSH and GSSG assays, assay number was not found to be a significant covariate. Therefore, the results presented are from ANOVAs for the latter and ANCOVAs with blot as covariate for the Northern and Western data. Comparisons among cycle stages were made using the Bonferroni test. ANOVA was used to test for overall effects of treatment or time on ovarian weight and total GSH content for experiment 2. Comparisons between PMSG and saline treated groups were made using Fisher's Least Significant Difference test. ANOVA with treatment (PMSG versus saline) and blot number as independent variables was used to test for differences in GCL subunit protein and mRNA levels between the control and PMSG-treated groups at each time point. All analyses were performed using Systat software (SPSS, Chicago, IL).

3. Results

3.1. Experiment 1

Ovarian total and reduced GSH concentrations, but not GSSG concentrations, varied significantly ($P < 0.01$) with estrous cycle stage (Fig. 1). Significantly higher ($P < 0.05$) levels of total and reduced GSH were observed on metestrus than on diestrus or proestrus. The percentage of total GSH that was oxidized (GSSG) was 0.6% on metestrus, 0.5% on diestrus, 0.9% on proestrus, and 0.9% on estrus. This variation during the cycle was not statistically significant ($P = 0.49$).

Neither GCLc nor GCLm protein levels showed signif-

icant estrous cycle stage-related variation (Fig. 2). In contrast to GCL subunit protein levels, *Gclc* and *Gclm* mRNA both varied significantly ($P < 0.05$) with estrous cycle stage (Fig. 3). For *Gclc* the highest levels were observed on metestrus with the lowest levels on estrus. For *Gclm* the highest levels were observed on proestrus. The results of the analyses were essentially the same whether *Gclc* and *Gclm* were normalized to cyclophilin (Fig. 3) or to GAPDH (not shown).

3.2. Experiment 2

The injection of 25-day-old rats with 10 IU PMSG, but not saline vehicle, significantly increased ovarian GSH concentrations at 24 h and 48 h (Fig. 4; $P = 0.02$, overall effect of treatment by ANOVA). The experiment was performed twice with essentially the same results each time. As expected, PMSG treatment also significantly increased ovarian weight by 1.5-fold at 24 h and 1.8-fold at 48 h ($P < 0.003$). Fig. 5 shows the combined GCL protein data from five Western blots. GCLm protein levels were increased 1.5, 2.0, and 1.7-fold at 8, 24, and 48 h following PMSG administration, respectively. This effect was statistically significant at 24h ($P = 0.007$, compared to controls) and 48 h ($P = 0.03$), but not 8 h ($P = 0.06$) after PMSG injection. Although GCLc protein levels appeared to increase after PMSG, there were no significant differences after PMSG injection: apparent increases were 2.6-fold ($P = 0.098$) and 1.6-fold ($P = 0.20$) at 24 and 48 h after PMSG treatment, respectively. *Gclc* mRNA levels were not significantly increased by ANOVA at 48 h following PMSG treatment ($P = 0.12$; Fig. 6), although the difference was significant by the less stringent t test ($P = 0.01$). There was no signif-
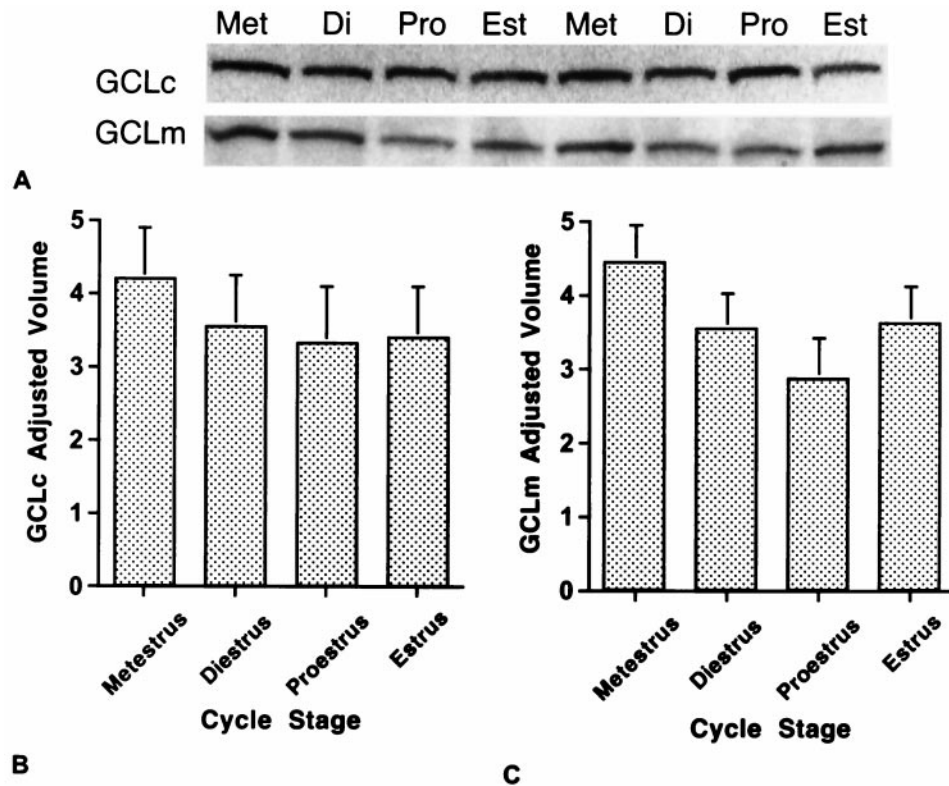
Fig. 2. GCLc and GCLm protein levels in whole ovary homogenates do not vary significantly with estrous cycle stage. a) Representative Western blot, run as described in Methods. Met: metestrus; Di: diestrus; Pro: Proestrus; Est: Estrus. b) Adjusted means ± SEM of GCLc densitometric readings determined from film images for 5 or 6 ovaries per cycle stage. c) Adjusted means ± SEM of GCLm densitometric readings determined from film images for 5 or 6 ovaries per cycle stage. There was no significant effect of estrous cycle stage on either GCLc or GCLm protein levels.

icant effect of PMSG administration on *Gclm* mRNA levels at any of the time points (Fig. 6).

4. Discussion

GSH conjugation is an important Phase 2 biotransformation mechanism for many exogenous toxicants. GSH also detoxifies reactive oxygen species, maintains cellular redox status, and participates in microtubule assembly [2]. Although the roles of ovarian GSH have not been as extensively studied as in other tissues, GSH appears to perform similar functions in the ovary [3,9,10,26]. Ovarian GSH concentrations are very low (<0.65 μmol/g) in one week old Sprague-Dawley rats, increase 6 to 8-fold between 1 and 2 weeks of age, and increase another 1.5-fold by about 8 weeks of age [3]. This pattern is reminiscent of the recruitment of primordial follicles into the actively growing pool that increases dramatically during the second week of life, concomitantly with an increase in serum FSH levels, and then declines [27]. This similarity suggested that ovarian GSH synthesis might also be under gonadotropin control. This hypothesis is further supported by the observation that ovarian GSH increases significantly following gonadotropin injection to stimulate follicular development in immature rats. Behrman and coworkers reported that ovarian GSH

concentrations increased almost 3-fold at 60 h after gonadotropin injection in 26-day-old rats [16]. Therefore, in the present study we sought to discover whether gonadotropins modulate the transcription and/or the translation of the catalytic and regulatory subunit genes for GCL, the rate-limiting enzyme in GSH synthesis.

Significant estrous cycle related-changes in ovarian GSH concentrations observed in the present study and by others [15] and significant cyclic changes in *Gclc* and *Gclm* mRNA levels in the present study support the hypothesis that GSH synthesis is regulated by LH and FSH. Ovarian GSH concentrations were increased on estrous and metestrous mornings compared to proestrous morning. These observations suggest that the preovulatory gonadotropin surge, which occurs on the evening of proestrus, may stimulate GSH synthesis in ovarian follicles. However, the cyclic changes in *Gcl* subunit mRNA did not parallel the changes in GSH concentrations, suggesting that the relationship between GCL transcription and GSH synthesis is not a linear one at the level of the whole ovary and/or that there are cyclic variations in *Gcl* subunit mRNA stability. Both *Gcl* subunit genes have been shown to be transcriptionally and translationally regulated, and posttranscriptional stabilization of *Gcl* subunit mRNA has also been reported [18,20]. Therefore, the cyclic changes in *Gcl* sub-
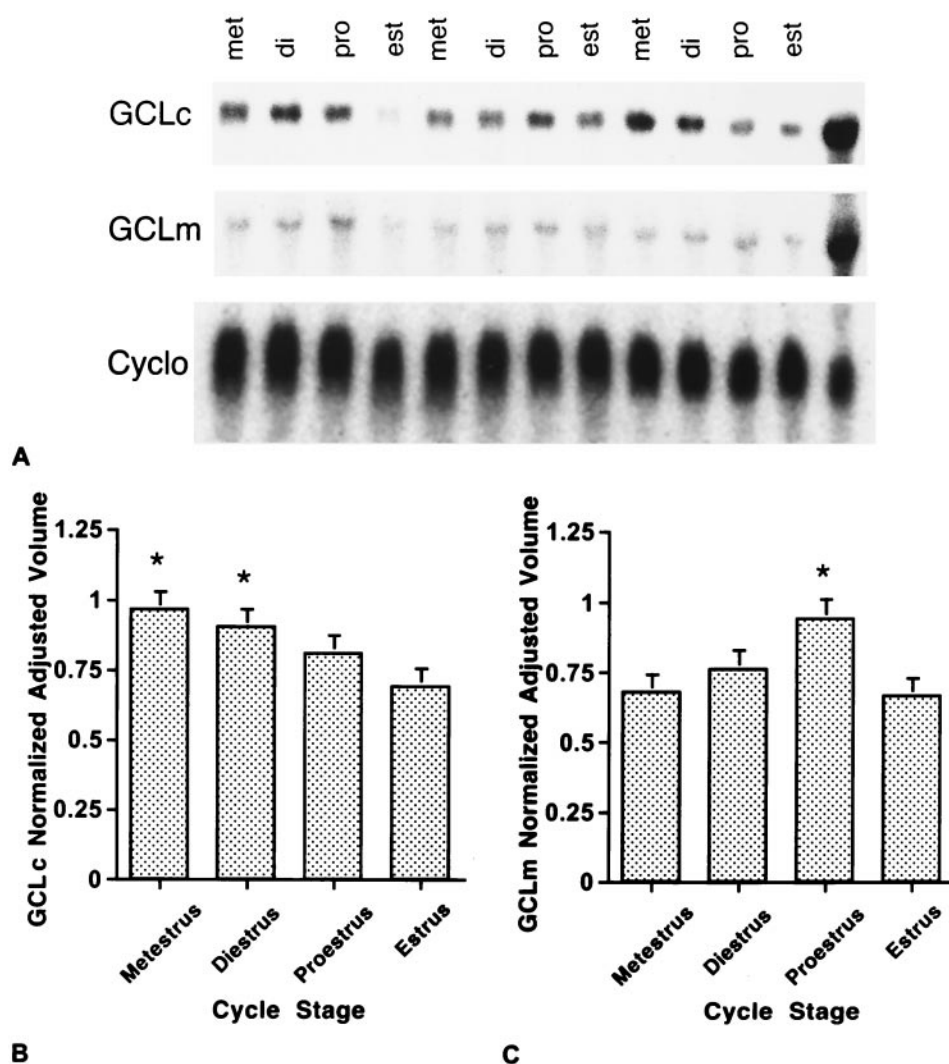
Fig. 3. Gclc and *Gclm* mRNA levels in whole ovary homogenates vary significantly with the estrous cycle. a) Representative Northern blot, performed as described in Methods, and probed with ^{32}P-labeled cDNA probes for mouse Gclc, Gclm, or cyclophilin (cyclo). Met: metestrus; Di: diestrus; Pro: Proestrus; Est: Estrus. RNA extracted from an adult female mouse kidney was run as a positive control for *Gcl* expression because *Gcl* is known to be strongly expressed in this organ (for right lane). b) Mean ± SEM of the adjusted volume for *Gclc* normalized to cyclo for 7 ovaries per stage determined from phosphorimages using Molecular Analyst software. c) Mean ± SEM of the normalized adjusted volume for *Gclm* for 7 ovaries per stage. ANCOVA with blot as covariate revealed significant differences among cycle stages for both *Gclc* and *Gclm*. *Gclc expression was significantly higher on mestestrus and diestrus than on estrus, and *Gclm* expression was significantly higher on proestrus than on metestrus or estrus by Bonferroni test, $P < 0.05$.

unit mRNA levels observed in this study could reflect both changes in transcription and changes in mRNA stability.

One difficulty with studying normally cycling rats is that the ovaries contain follicles at many different stages of development and degeneration. Therefore, the second experiment was conducted using an immature rat model in which the development of a synchronized cohort of follicles was stimulated by injection of gonadotropin [28]. GSH concentrations were significantly increased by 24 h and 48 h after PMSG injection. This finding was consistent with the increased ovarian GSH content observed in experiment 1 on estrus and metestrus, 15 and 27 h after the preovulatory gonadotropin surge. Concomitant with the increase in GSH levels, GCLm protein levels approximately doubled at 24

and 48 h after PMSG. If GCLc protein is more abundant than GCLm protein in the ovary, one would expect an isolated increase in GCLm protein to result in increased catalytically active GCL enzyme (GCLc/GCLm heterodimer). There were no discernible increases in *Gcl* subunit mRNA levels until possibly 48 h after PMSG injection, when *Gclc* mRNA was significantly increased, but only by *t*-testing rather than the planned ANOVA. These results are consistent with an early effect of gonadotropins on GSH synthesis mediated by upregulation of GCLm subunit translation, followed by a later upregulation of *Gclc* transcription. Little is known concerning the factors that affect GCLc and GCLm translation. However, as with other proteins, phosphorylation and dephosphorylation of protein synthesis
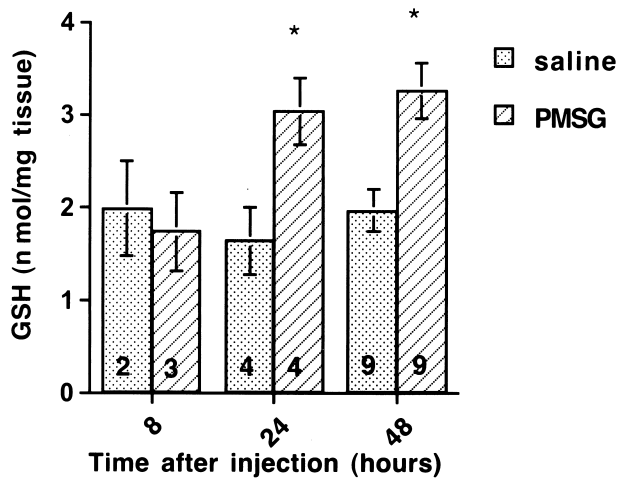
Fig. 4. Gonadotropin treatment increases ovarian GSH concentration. 25-day-old female rats were injected sc with 10 IU pregnant mare's serum gonadotropin or saline vehicle and killed 8, 24, or 48h later. Ovaries were homogenized in 5% sulfosalicylic acid and GSH was measured using the enzymatic recycling method as described under Materials and Methods. The graph depicts mean ± SEM concentrations for the number of animals per group indicated at the bottom of each bar. Compared to saline controls, PMSG treatment increased the ovarian GSH concentration at 24 h and 48 h, but not at 8 h. *$P = 0.02$, overall effect of treatment by ANOVA, $P = 0.016$ and $P = 0.001$ PMSG- versus saline-treated at 24 and 48 h, respectively, by Fisher's Least Significant Difference test.

initiation factors [29] and subcellular localization of mRNA transcripts [30] may be important. Binding of gonadotropins to their receptors activates adenylate cyclase, resulting in increased intracellular cyclic adenosine monophosphate (cAMP) levels [31]. It is therefore possible that gonadotropins may regulate GCL subunit translation via cAMP-dependent phosphorylation of initiation factors or other proteins involved in translation of the mRNA transcripts. Gonadotropins may also affect GSH synthesis more directly by cAMP-dependent phosphorylation of GCL subunits. In contrast to the increase in GSH synthesis observed in the present study following gonadotropin administration, phosphorylation of GCLc by protein kinase A has been shown to decrease GCL enzymatic activity [18,20]. Future studies should therefore investigate the effects of gonadotropins on GCL enzymatic activity and on GCL subunit phosphorylation in the ovary. Cyclic AMP also modulates gene expression by phosphorylating the transcription factor cAMP response element binding protein [32]. *Gclc* and *Gclm* are not known to have cAMP response elements (CRE) in their promoter regions [18,20], but gonadotropins acting via cAMP could affect *Gcl* mRNA expression indirectly by cross-talk with other transcription factors, such as other members of the bZip family or members of the small Maf family [33], which appear to modulate *Gclc* and *Gclm* transcription by binding to the electrophile response elements found in the promoter regions of both genes [34].

The lack of correlation between *Gcl* mRNA levels with GSH concentrations during the estrous cycle together with the correlation between GCL subunit expression and GSH

concentrations in synchronized ovaries suggests that gonadotropin effects on GSH synthesis in ovarian follicles may depend on the stage of follicular development. This idea is supported by a preliminary study using in situ hybridization to localize *Gcl* subunit mRNA within the rat ovary, which shows that follicles in various stages of development express *Gclc* and *Gclm* differentially [35]. For example, granulosa cells of primary follicles do not express *Gclm* whereas granulosa cells of larger preantral and antral follicles do (unpublished data). Alternatively, GSH in adult, cycling ovaries may be synthesized in structures other than follicles. In support of this alternative, in situ hybridization data demonstrate that *Gclc* mRNA is expressed in corpora lutea and interstitial cells as well as in follicles, whereas *Gclm* is expressed almost exclusively in follicles and oocytes [35].

Although regulation of GCL subunit expression by gonadotropin hormones has not previously been investigated, effects of steroid hormones and of insulin on GCL have been noted. Treatment of rat hepatocyte cultures with insulin or corticosterone increased steady state GCLc mRNA and protein levels [36]. To our knowledge, no one has investigated the effect of estrous cycle stage on levels of GSH in organs other than the ovary. A number of studies have investigated the effects of sex steroids on GSH concentrations in various non-ovarian tissues. Neither castration nor diethylstilbestrol treatment had any effect on brain or liver GSH concentrations in male rats [37]. Both estradiol and testosterone treatment in immature female rats increased uterine GSH, but not GSSG, levels [38]. In male mice, castration or estradiol treatment lengthened the half life of renal and hepatic GSH, and the effect of castration was reversed by administration of testosterone [39]. In randomly cycling female mice, the renal and hepatic half-lives of GSH were more than twice as long as in males, and neither ovariectomy nor estradiol treatment affected the half-life [39]. Treatment of females with testosterone shortened the renal and hepatic half lives of GSH, though not to the male levels [39]. These results suggest that GSH metabolism or transport may be modulated by sex steroids. This raises the possibility that the effects of gonadotropins on GCL subunit expression observed in the present study may be mediated by gonadotropin-induced increases in follicular estradiol synthesis.

Mean ovarian concentrations of total GSH observed in the present study are quite similar to concentrations observed in two previous studies of Sprague-Dawley rats [3,15]. However, in contrast to one previous report that GSH concentrations on estrus were 1.3 to 1.6-fold higher than on the other three cycle stages in Sprague-Dawley rats [15], we observed 1.4- to 1.5-fold higher concentrations on both metestrus and estrus than on diestrus or proestrus. One difference between the two studies is the age of the rats used. Clague and coworkers studied 7 to 8-week-old rats, whereas all the animals in the present estrous cycle experiment were older than 10 weeks at the time of sacrifice. The time of day could be another important difference. In the
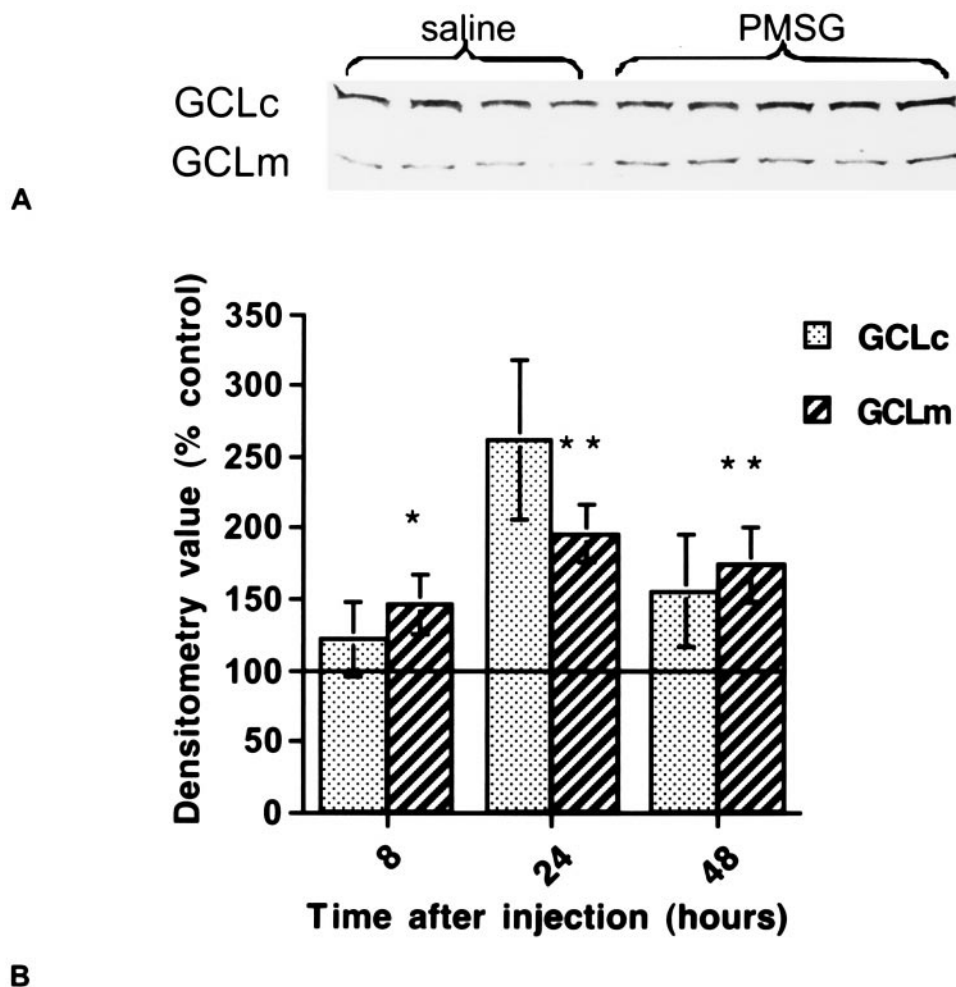
Fig. 5. Gonadotropin treatment increases ovarian GCLm protein expression. Immature female rats were treated with PMSG or saline vehicle as for Fig. 4. Ovaries were collected ($n = 8$ for 8 h and 24 h groups, $n = 4$ for 48 h control, $n = 5$ for 48 h PMSG), and Western analysis was performed as described in Methods. Band density was determined using a Stratagene molecular documentation and image analysis system with EagleSight software. The graph depicts the combined data from 5 different Western blots. a) representative Western blot with ovaries collected 48 h after saline (lanes 1–4) or PMSG (lanes 5–9) injection. b) Mean ± SEM GCL subunit protein levels expressed as percent of control value (densitometric units for GCLc or GCLm in PMSG-treated ovaries were divided by the mean densitometric units for GCLc or GCLm in saline controls at the same time point on the same blot). The horizontal line indicates 100% of the control value. **The difference in GCLm protein levels between PMSG-treated animals and controls was significant by ANOVA with treatment and blot number as independent variables at 24 h ($P = 0.007$) and 48 h ($P = 0.03$), and *approached significance at 8 h ($P = 0.06$).

present experiment the rats were killed in the morning. The time of sacrifice was not reported by Clague et al. It may be that ovarian GSH levels remain elevated from estrous morning through metestrous morning, falling to diestrous levels later on metestrus. Two- to ten-fold higher total GSH concentrations than observed in ovaries in the present study have been reported for kidney, liver, lung, and red blood cells, with comparable levels in heart and diaphragm in male Fisher 344 rats [40,41]. GSSG concentrations have not previously been reported for the ovary. Reduced GSH to GSSG ratios of greater than 100, as were observed in the present study, are comparable to ratios observed in other tissues at normal levels of oxidative stress [2,18].

In summary, the present experiments show that GSH concentrations and *Gcl* subunit mRNA levels change significantly with estrous cycle stage in the rat ovary. In addi-

tion, induction of ovarian follicular development by exogenous gonadotropin treatment in immature rats increases ovarian GSH concentrations as well as GCL subunit protein levels. Taken together, these results support the hypothesis that gonadotropin hormones regulate ovarian GSH synthesis in part by modulating GCL subunit expression. This gonadotropin-mediated increase in GSH synthesis may serve to protect maturing follicles against both endogenously produced reactive oxygen species and exogenous toxicants.
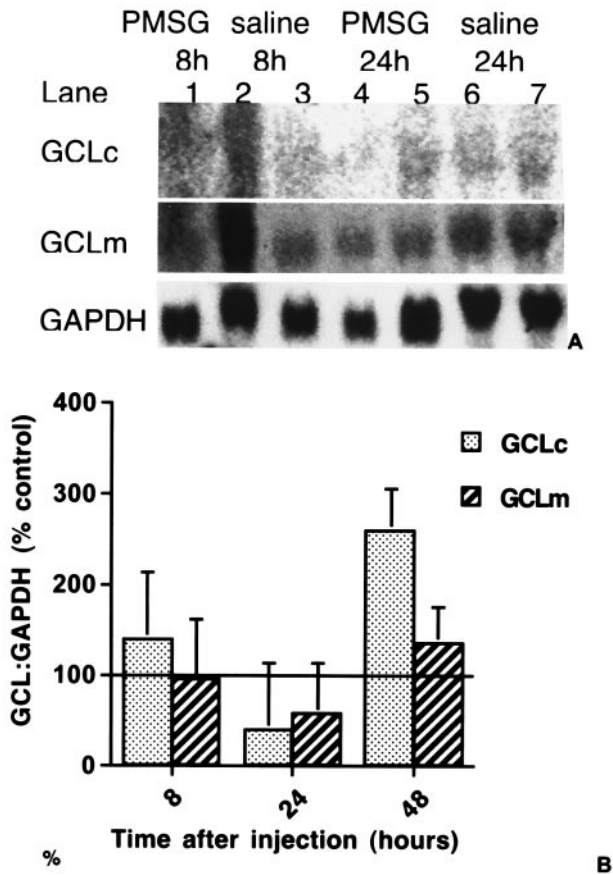
Acknowledgments

Fig. 6. Gonadotropin treatment does not significantly affect ovarian Gcl mRNA expression. Immature female rats were treated with PMSG or saline vehicle as for Fig. 4. Ovaries were removed and Northern analysis was performed as described in Methods using ^{32}P-labeled cDNA probes for mouse *Gclc, Gclm,* or *GAPDH* ($n = 3$–4 per group, except $n = 8$ for PMSG-treated 48 h group). a) representative Northern blot with ovaries collected 8 h after PMSG (lane 1) or saline (lanes 2 and 3) injection or 24 h after PMSG (lanes 4 and 5) or saline (lanes 6 and 7) injection. b) Mean ± SEM *Gcl* subunit to *GAPDH* ratio expressed as percent of control value (densitometric units for *Gclc* or *Gclm* normalized to *GAPDH* in PMSG-treated ovaries were divided by the mean densitometric units for *Gclc* or *Gclm* normalized to *GAPDH* in saline controls at the same time point and on the same blot). The horizontal line indicates 100% of the control value. *Gclc* mRNA levels in PMSG-treated animals were not significantly higher than in saline controls at 48 h by ($P = 0.12$ by ANOVA with treatment and blot number as independent variables).

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