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Opposing Effects of Glutathione Depletion and Follicle-Stimulating Hormone on Reactive Oxygen Species and Apoptosis in Cultured Preovulatory Rat Follicles

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Oxidative stress and depletion of the antioxidant glutathione (GSH) trigger apoptosis in many systems. Previous work showed that antioxidants prevented apoptosis as effectively as FSH in preovulatory follicles. We aimed to test the hypotheses that follicular reactive oxygen species (ROS) initiate apoptosis and that follicular GSH protects against apoptosis. Preovulatory follicles were isolated from ovaries of immature rats primed with pregnant mare serum gonadotropin. Negative control (0-h) follicles were processed immediately. Others were cultured for 2 to 48 h with 1) medium alone, 2) 75 ng/ml ovine FSH, or 3) FSH plus 100 μM buthionine sulfoximine (BSO), a specific inhibitor of GSH synthesis. Total GSH concentrations declined in follicles cultured without FSH for 48 h, whereas FSH increased GSH levels above those observed at 0 h. BSO suppressed GSH to undetectable levels. Treatment with FSH prevented apoptosis in granulosa cells, measured by terminal dUTP transferase-mediated nick-end-labeling and activated caspase 3 immunohistochemistry. Addition of BSO partially and significantly reversed the antiapoptotic effect of FSH on granulosa cells; supplementation of GSH completely prevented BSO-induced granulosa cell apoptosis. Whole-follicle ROS production, measured as dichlorofluorescein and rhodamine fluorescence using confocal microscopy, was significantly increased by 4 h of culture and increased further thereafter. FSH significantly suppressed ROS production, and the addition of BSO partially overcame this effect of FSH. These findings provide evidence that oxidative stress induces apoptosis in preovulatory follicles and that the antiapoptotic effect of FSH is mediated in part by stimulation of follicular GSH synthesis and suppression of ROS production.

The VAST MAJORITY of ovarian follicles does not mature to ovulation, but rather undergoes degeneration by an apoptotic process called atresia. Most atresia in the adult ovary occurs in antral follicles (1, 2). In these follicles, atresia is driven by apoptosis of the granulosa cells, which secondarily leads to oocyte death (2). The pituitary gonadotropin hormones, LH and follicle-stimulating hormone (FSH), are the primary endocrine factors regulating apoptosis in ovarian follicles both in vivo and in vitro (3–6). The antiapoptotic effects of gonadotropins appear to be mediated in part by inhibition of the mitochondrial or damage-induced apoptotic pathway. Gonadotropins induce expression of prosurvival molecules such as X-linked inhibitor of apoptosis (7) and decrease expression of proapoptotic molecules such as BAX (6), apoptotic protease activating factor-1 (8), and caspase 3 (9).

Reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide, and hydroxyl radical, are produced as byproducts of normal cellular metabolism (10). ROS can react spontaneously with DNA, RNA, protein, and lipids. Oxidative stress occurs when levels of ROS overwhelm the cell’s antioxidant defenses. Electron transport associated with steroidogenesis is an important site of oxygen radical generation in the adrenal gland (11) and testis (12) and likely is also in the ovary. Inadequate protection from ROS that are formed in steroidogenically active granulosa cells could be a potential trigger for follicular atresia. ROS promote apoptosis in various model systems (13), but the role of ROS in apoptosis in ovarian follicles has received limited attention. Although apoptosis induction by treatment with ROS has not been investigated in ovarian follicles, treatment with hydrogen peroxide is toxic to granulosa cells in that it inhibits FSH-stimulated cAMP accumulation and progesterone production (14). Indirect evidence for a proapoptotic role of ROS in ovarian follicles comes from the observation that treatment with several antioxidants prevents apoptosis in cultured follicles (15).

The tripeptide glutathione (GSH, γ-glutamylcysteinylglycine) detoxifies ROS and electrophilic toxicants by spontaneously reacting with them or by providing reducing equivalents for enzymes like glutathione peroxidase and glutathione-S-transferases (16). The reversible glutathionylation of cellular proteins is also an important regulatory mechanism of protein function (17). Under normal conditions, the reduced form of GSH is present far in excess of the oxidized form (GSSG), because of the action of GSH reductase, as well as de novo synthesis of GSH (18). In situations of...
oxidative stress, the ratio of GSH to GSSG decreases. GSH is synthesized by the successive action of glutamate cysteine ligase (GCL) and GSH synthetase. GCL, the rate-limiting enzyme, is composed of a catalytic (GCLC) and a modifier (GCLM) subunit (18, 19). Gonadotropin hormones regulate ovarian expression of GCL subunit protein and mRNA (20, 21). A gonadotropin stimulus that decreases ovarian apoptosis in vivo (5) also significantly increases ovarian GCLC and GCLM protein levels (20, 21). Depleting GSH by blocking its synthesis induces apoptosis directly (22–25) in some cell types and sensitizes other cell types to apoptotic stimuli (26, 27). We recently demonstrated that suppression of GSH synthesis in vivo increases atresia of antral follicles in rats (28). Other studies have shown that treatments that enhance cellular GSH concentrations protect against apoptotic stimuli in various cell types (29, 30).

Taken together, these previous studies led us to hypothesize that GSH, under regulation by gonadotropins, serves to protect ovarian follicles against ROS that could cause apoptosis if left unchecked. The present studies used a well-characterized model of follicular apoptosis in which cultured antral follicles undergo apoptosis in the absence of gonadotropin support, and follicles treated with gonadotropin are rescued from apoptosis (3–5, 31, 32). This model was used to test the specific hypotheses that 1) FSH enhances GSH synthesis in cultured preovulatory follicles, 2) GSH protects follicles against the proapoptotic effects of ROS and, therefore, GSH depletion promotes apoptosis, and 3) intracellular ROS rise in the absence of FSH, leading to the initiation of apoptosis.

Materials and Methods

Materials

All chemicals were purchased from Sigma Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA), unless otherwise noted. Tissue culture reagents were from Invitrogen (Carlsbad, CA). Pregnant mare serum gonadotropin (PMSG) and ovine FSH (NIDDK-oFSH-20; 4453 IU/mg) were obtained from Dr. A. F. Parlow (National Hormone and Peptide Program, National Institute of Diabetes and Digestive and Kidney Diseases, Torrance, CA). WST-1 reagent, diaminobenzidine substrate and terminal transferase-mediated nick-end labeling (TUNEL). The cell viability WST-1 assay was performed using cultured granulosa cells collected from preovulatory follicles of PMGS-primed immature rats. Treatments with 100, 250, and 500 μM BSO for 24 h all suppressed GSH below the limit of detection in our assay (0.04 nmol, data not shown). Therefore, the 100-μM dose of BSO was used in the present studies. An additional experiment tested whether the effect of BSO treatment on follicular apoptosis was specifically caused by GSH depletion. There were four experimental groups: 1) 75 ng/ml FSH, 2) FSH plus 100 μM BSO, 3) FSH plus BSO plus 1 mm GSH ethyl ester (GEE, a cell-permeable analog of GSH) (37), or 4) FSH plus BSO plus 5 mm GEE. After 48 h, follicles were processed for GSH assay or terminal dUTP transferase-mediated nick-end labeling (TUNEL) assay.

At the time of collection, follicles were processed for several assays as follows. For GSH assay, five or six follicles were immediately homogenized on ice in 65 μl 5% sulfosalicylic acid, incubated on ice for 15 min, and centrifuged at 14,000 × g for 2 min at 4 C. DNA was extracted from pools of six follicles immediately after termination of culture. For protein extraction, follicles were immediately incubated in 15% sucrose in PBS for 90 min at 4 C, embedded in Tissue Tek OCT (Sakura Finetek, Torrance, CA), and stored at −70 C until sectioning at 10 μm thickness using a cryostat. For DNA extraction, DNA was extracted from pools of six follicles immediately after termination of culture. For protein extraction, follicles were immediately homogenized in RIPA lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS in PBS) with protease inhibitors on ice. After incubation on ice for 30 min, lysates were centrifuged at 15,000 × g, and the supernatants were stored at −70 C. The cell viability WST-1 assay and the ROS assays were performed immediately after termination of culture.

Preovulatory follicle isolation and culture

Isolation and culture of preovulatory follicles were adapted from previously described methods (5, 31). This whole-follicle culture system preserves the interactions among the oocyte, granulosa cells, and theca cells that are present in vivo. The 25- to 26-d-old female rats were injected sc with 10 IU PMSG in 0.1 ml sterile 0.9% NaCl to promote growth of a cohort of preovulatory follicles (34). PMSG has both FSH and LH activity in the rat (35). Forty-eight hours after the PMSG treatment, the animals were euthanized for collection of ovaries using aseptic technique. Ovaries were placed on ice in serum-free medium pregassed with 95% O2 and 5% CO2. Healthy preovulatory follicles (eight to 12 per ovary) 600–800 μm in diameter were isolated using iris forceps and 270-gauge needles under a dissecting microscope using aseptic technique. To minimize spontaneous apoptosis during follicle preparation, only one ovary was processed at one time, and isolated follicles were kept at 4 C until ready to culture.

Isolated preovulatory follicles were pooled, and three to six follicles per treatment group per experiment were cultured in 2 ml of 1) MEM (Eagle’s MEM, supplemented with 2 mm t-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 0.1% fatty acid-free BSA) alone, 2) MEM plus 75 ng/ml ovine FSH (0.33 IU/ml), or 3) 75 ng/ml FSH and 100 μM buthionine sulfoximine (BSO), a specific inhibitor of GCL, the rate-limiting enzyme in GSH synthesis (36), in sterile 20-ml glass scintillation vials. Each vial was gassed with 95% O2 and 5% CO2 every 12 h, sealed with petroleum jelly, covered with Parafilm (Pechiney Plastic Packaging, Menasha, WI), and incubated in a shaking water bath at 37 C for varying durations of time ranging from 2–48 h. Two or three replicate experiments were performed per time point for each end point. Each replicate experiment had its own 0-h control group of follicles that were processed immediately after dissection without culturing and that served as negative controls for apoptosis. The MEM groups served as positive control groups for apoptosis. The dose of ovine FSH was chosen based on a dose-response experiment that compared the amount of oligonucleosomal DNA fragmentation in 0-h control follicles (no apoptosis) with follicles cultured for 24 h with one of five different doses of ovine FSH (5, 25, 75, 200, and 500 ng/ml). DNA fragmentation was assessed by ethidium bromide gel electrophoresis. The results indicated that the antiapoptotic effect of FSH was maximal at 75 ng/ml (data not shown). The dose of BSO was chosen based on preliminary dose-response experiments using cultured granulosa cells collected from preovulatory follicles of PMGS-primed immature rats. Treatments with 100, 250, and 500 μM BSO for 24 h all suppressed GSH below the limit of detection in our assay (0.04 nmol, data not shown). Therefore, the 100-μM dose of BSO was used in the present studies. An additional experiment tested whether the effect of BSO treatment on follicular apoptosis was specifically caused by GSH depletion. There were four experimental groups: 1) 75 ng/ml FSH, 2) FSH plus 100 μM BSO, 3) FSH plus BSO plus 1 mm GSH ethyl ester (GEE, a cell-permeable analog of GSH) (37), or 4) FSH plus BSO plus 5 mm GEE. After 48 h, follicles were processed for GSH assay or terminal dUTP transferase-mediated nick-end labeling (TUNEL) assay.

The 22- to 23-d-old female Sprague Dawley rats weighing 40–51 g were obtained from Charles River Laboratories (Wilmington, MA). Upon arrival, two to three animals per cage were housed in an Association for Assessment and Accreditation of Laboratory Animal Care accredited facility, with free access to deionized water and standard laboratory chow, on a 14-h light, 10-h dark cycle. The experimental protocols were carried out according to the Guide for the Care and Use of Laboratory Animals (33) and were approved by the Institutional Animal Care and Use Committee at the University of California, Irvine.
μl MEM and 20 μl WST-1 reagent. Blank wells contained culture medium and WST-1 reagent with no follicle. The plate was incubated for 4 h at 37 C in a humidified atmosphere of 95% air, 5% CO2. The absorbance at 440 nm was then read using a VersaMax tunable microplate reader (Molecular Devices, Sunnyvale, CA). The average of the blank absorbance values was subtracted from the absorbance value for each well.

**Total GSH assay**

Supernatants from homogenized follicles were analyzed for total GSH content using a microplate enzymatic assay, modified from Griffith (20, 21, 38). Briefly, 5 μl of sample or GSH standards were pipetted in triplicate with 33 μl deionized water. The microplate was incubated for 10 min at 30 C. Reaction mixture (162 μl of 0.62 U/ml GSH reductase, 0.26 mM NADPH, and 0.74 mM 5,5’-dithiobis(2-nitrobenzoic acid) in a buffer of 143 mM Na2HPO4 and 6.3 mM EDTA) was added, and color development was monitored every 10 sec for 6.5 min at 412 nm using a Versamax tunable microplate reader (Molecular Devices). The sensitivity of this assay in our hands is 0.04 nmol. The samples from the 24- and 48-h experiments were assayed in one assay, and the samples from the 4- and 12-h experiments were assayed in a second assay. The intra-assay coefficients of variation were 10.9 and 6%, respectively. The average GSH content per follicle was calculated as follows. The total number of nanomoles of GSH in the 65-μl sample homogenate was calculated from the concentration determined from the assay (concentrations that were undetectable were arbitrarily set equal to half the limit of detection of 0.008 nmol). The nanomoles of GSH were then divided by the number of follicles in the homogenate (five or six follicles per sample that had been cultured in the same vial).

**Western blotting**

Gel electrophoresis and Western blotting were carried out as previously described (20, 21). Briefly, 20 μg of protein extracts were separated by electrophoresis in 12% Tris-HCl polyacrylamide gels (Bio-Rad, Hercules, CA) and transferred to polyvinylidene difluoride membranes. After transfer, blots were routinely stained with Ponceau Red to verify equal protein loading. Immunostaining was performed using GCLC and GCLM antibodies (39). Each blot was subsequently reprobed using β-actin antibody (Sigma-Aldrich) as another loading control. After incubation with the primary horseradish peroxidase-conjugated secondary antibody, visualization was accomplished by ECL with ECL reagents and exposure to Hyperfilm ECL. Semiquantitative analysis of images was accomplished using a Stratagene molecular documentation and image analysis system with EagleSight software. Densitometry values for GCLC and GCLM bands were normalized to β-actin values. The average normalized value for the 0-h controls on a given blot was then calculated from the number of normalized absorbance for each band that was expressed as the fold of the average 0-h control value for that blot.

**Oligonucleosomal DNA fragmentation detection**

DNA was extracted from follicles using the DNeasy tissue kit according to the manufacturer’s instructions. After DNA extraction, 100 ng DNA per sample was 3’-end labeled with [α-32P]deoxyxymoecdite-ATP (3000 Ci/mmol; Amersham) using terminal transferase enzyme, followed by ethanol precipitation and resuspension in Tris-EDTA buffer according to the method described by Tilley and Hsueh (40).

One hundred nanograms of labeled DNA per lane were loaded onto a 2% agarose gel made with modified Tris-acetate-EDTA buffer. DNA samples were separated by electrophoresis at 50 V for 90 min. After electrophoresis, the gel was placed on Whatman filter paper and dried in a gel dryer at 50 C for 45 min. Dried gels were sealed in plastic bags and placed on Biomax MS film (Eastman Kodak, Rochester, NY) with an intensifying screen for 5 min or longer, depending on the signal intensity. After gel electrophoresis and autoradiographic analysis, the low-molecular-weight portion of each lane of the gel was cut out with a razor blade. The total amount of radiolabel incorporated into the low-molecular-weight DNA portions of the gel was quantified by scintillation counting to estimate the degree of apoptotic DNA fragmentation in any given sample as described (40). The counts per minute for each of the MEM, FSH, and FSH- plus BSO-treated samples were divided by the average of the MEM-positive controls for the same experiment. These fold control values were used for statistical analysis and for presentation of the data.

**TUNEL assay for in situ detection of apoptosis**

TUNEL was performed using the in situ cell death detection kit as described (41). Briefly, slides were blocked with 3% H2O2 in methanol, permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate, blocked with 5% BSA, and treated with terminal deoxynucleotidyl transferase solution with fluorescein-labeled dUTP. After washing, sections were again blocked with 3% BSA, washed, and incubated with peroxidase converter solution, followed by color development with diaminobenzidine. Last, slides were counterstained with hematoxylin, dehydrated in graded ethanol and xylene washes, and mounted with Permount. Negative control slides incubated with label solution without terminal transferase and positive control slides pretreated with DNase 1 were included with each experiment.

For quantification of apoptosis, the numbers of TUNEL-positive granulosa cells or theca cells were counted in a blinded manner in five ×400 fields per follicle using an Olympus BX60 microscope. The averages of the five granulosa cell counts and the five theca cell counts were calculated for each follicle, and these values were used for statistical analyses.

**Immunohistochemistry**

Immunohistochemistry was performed essentially as described (21). For antigen retrieval, slides were heated at 80°C in a conventional microwave in 1 mM sodium citrate, 1 mM citric acid buffer for 10 min. The slides were blocked with 3% hydrogen peroxide in methanol for 10 min, 1.5% goat serum in PBS for 20 min, and avidin D and biotin blocking solutions for 15 min each. The slides were incubated with primary anti-cleaved caspase 3 antibody (1:5) in PBS overnight at 4 C, washed, incubated with biotinylated goat antirabbit IgG in PBS for 30 min, washed, incubated with ABC reagents for 30 min, washed, and incubated with diaminobenzidine substrate for 10 min. Finally, the slides were counterstained with hematoxylin, dehydrated in graded ethanol washes, washed in xylene, and mounted with Permount. Negative controls included slides incubated with nonimmune rabbit serum without primary antibody and slides incubated in primary antibody that had been preincubated with activated caspase 3 peptide.

For quantification of caspase 3 activation, the numbers of cleaved caspase 3-positive granulosa cells or theca cells were counted in a blinded manner in five ×400 fields per follicle using an Olympus BX60 microscope. The averages of the five granulosa cell counts and the five theca cell counts were calculated for each follicle, and these values were used for statistical analyses.

**In situ ROS detection**

Preovulatory follicles were subjected to the same treatments as for the previous experiments, except that a hydrogen peroxide-treated group (5% H2O2 for 0.5 h) was added as a positive control for oxidative stress. After 0, 2, 4, 8, 12, 24, or 48 h of culture, treatment media were removed, and the follicles were washed with PBS. The follicles were then incubated with 100 μM 2’,7’-dichlorofluorescin diacetate (H2DCFDA) (Molecular Probes, Eugene OR) in MEM for 30 min or with 100 μM dihydrodorhamine 123 (DHR) (Molecular Probes) for 30 min, and were washed again with PBS. H2DCFDA is taken up by cells, where it is converted by esterases to the nonfluorescent compound dichlorodihydrofluorescein, which in the presence of ROS is oxidized to the fluorescent dichlorofluorescein (DCF). DHR is a nonfluorescent compound that diffuses into cells, where it is oxidized in the presence of ROS to the cationic and fluorescent compound rhodamine 123 that localizes to mitochondria (42, 43). DCF or DHR fluorescence was viewed and quantified using a Zeiss LSM 510 META laser scanning confocal microscope using a Plan-Neofluor 10×/0.3 objective with Cy2/alexa/fluorescein isothiocyanate excitation and a BP-500–530 filter for emission. The follicle was scanned and the fluorescence intensity was measured for 15 equally spaced layers from the top to the bottom of the follicle (10–25 μm thickness). The area under the curve calculated from the histogram...
of the 15 DCF or DHR fluorescence intensity measurements per follicle was used for statistical analyses and presentation of data.

**Statistical analyses**

Data from replicate experiments were pooled for statistical analyses. Differences in GSH concentrations, low-molecular-weight DNA laddering, TUNEL cell counts, activated caspase 3 cell counts, and cell viability among treatment groups (0 h, MEM, FSH, and FSH plus BSO) were evaluated by one-way ANOVA for each time point (4, 12, 24, and 48 h). Differences in ROS measurements after various times in culture (0, 2, 4, 8, and 12 h) were assessed separately for each treatment group (MEM, FSH, and FSH plus BSO) and for each time point by one-way ANOVA. If the overall \( P \) value indicated statistical significance (\( P < 0.05 \)), post hoc least significant difference (LSD) tests were applied to assess differences among groups based on a priori hypotheses. Levene’s test was used to assess homogeneity of variances. If variances were not homogeneous, a square root transformation was applied to the data before ANOVA. If transformation did not result in homogeneous variances, a nonparametric test, the Kruskal-Wallis test, was used to assess the overall effect of treatment. If the latter was statistically significant at \( P < 0.05 \), the Mann-Whitney test was then used for intergroup comparisons. SPSS version 11.0 for Macintosh was used for all statistical analyses.

**Results**

**FSH enhances and BSO depletes GSH concentrations in cultured preovulatory follicles**

Culture in serum-free medium resulted in a time-dependent decline in follicular GSH concentrations from 1.0 ± 0.2 nmol/follicle at 0 h to 0.4 ± 0.1 nmol/follicle in the MEM group at 48 h (\( P = 0.02 \), MEM at 48 h vs. 0 h; Fig. 1A). Treatment with 75 ng/ml FSH not only prevented the decline in follicular GSH, but also increased GSH concentrations to greater than 0-h control levels at 12, 24, and 48 h of culture (\( P < 0.05 \), FSH at 12, 24, and 48 h vs. respective 0-h control; Fig. 1A). By 48 h of FSH treatment, GSH concentrations reached 2.4 ± 0.5 nmol/follicle. Treatment with 100 \( \mu \)M BSO, a specific inhibitor of GCL enzymatic activity, suppressed GSH concentrations to less than 15% of 0-h control levels after 12 h and to less than 10% of 0-h control levels after 24 and 48 h (\( P < 0.01 \), BSO at 12, 24, and 48 h vs. respective 0-h control).
The FSH-induced increase in follicular GSH was associated with a statistically significant increase in protein levels of GCLM, the rate-limiting enzyme in GSH synthesis (P = 0.03, FSH vs. MEM and FSH plus BSO vs. MEM; Fig. 1B). Cotreatment with BSO did not modify the enhancement of GCLM protein levels by FSH. Levels of GCLC did not differ among treatment groups. GCL subunit protein levels were assessed after 24 h of treatment only.

Treatment with BSO to deplete GSH partially reverses the antiapoptotic effect of FSH on granulosa cells of cultured preovulatory follicles, and supplementation of GSH completely prevents the BSO-induced apoptosis

Preovulatory follicles cultured without gonadotropin support begin to undergo apoptosis within 24 h, whether cultured in the absence (3) or presence (32) of serum. FSH treatment prevents apoptosis in these follicles (3). To investigate whether the enhancement of follicular GSH levels by FSH plays a role in the suppression of apoptosis by FSH, we depleted GSH with BSO in the presence of FSH.

Using TUNEL to localize apoptotic cells within cultured follicles, we observed differential effects on granulosa cells and theca cells of FSH treatment alone compared with combined FSH and BSO treatment (Figs. 2 and 3). Culture in serum-free medium caused a marked, time-dependent increase in both granulosa cell and theca cell apoptosis as judged by TUNEL, with no increase at 4 h, but statistically significant increases at 12, 24, and 48 h (P < 0.001, MEM at 12, 24, and 48 h vs. respective 0 h; Figs. 2 and 3, A and B). FSH treatment brought about a statistically significant decrease in the number of TUNEL-positive granulosa cells at all three later time points (P < 0.001, effect of treatment by ANOVA for 12-, 24-, and 48-h experiments, with FSH different from MEM at P < 0.01 by LSD test). In contrast, FSH treatment enhanced theca cell apoptosis at 24 h (P < 0.001, FSH vs. MEM) but not at 4, 12, or 48 h (Fig. 3B).

Cotreatment with FSH and BSO partially overcame the antiapoptotic effect of FSH on granulosa cells. BSO treatment led to a statistically significant increase in granulosa cell apoptosis compared with follicles treated with FSH alone (Figs. 2 and 3A). The number of TUNEL-positive granulosa cells was significantly increased in FSH plus BSO-treated groups compared with FSH treatment alone at 24 and 48 h (P = 0.03 and P < 0.001, BSO vs. FSH at 24 and 48 h, respectively; Fig. 3A). The magnitude of granulosa cell apoptosis in the FSH plus BSO group at 48 h was similar to that in the MEM group at 24 h. BSO enhanced the proapoptotic effect of FSH on theca cell apoptosis at 24 h (P = 0.04, BSO vs. FSH at 24 h), but did not enhance apoptosis at 4, 12, or 48 h (Figs. 2 and 3B). To test whether the BSO-induced granulosa cell apoptosis was a specific effect of GSH depletion, we cultured follicles for 48 h with FSH, FSH plus BSO, or FSH plus BSO plus GEE, a cell-permeable analog of GSH. Treatment with 5 mM GEE completely prevented the suppression of follicular GSH concentrations by BSO. GSH concentrations were 2.3 ± 0.3, 0.04 ± 0.0, and 2.4 ± 0.3 nmol/follicle in the FSH, FSH plus BSO, and FSH plus BSO plus 5 mM GEE groups, respectively. The 5 mM GEE dose also completely prevented the BSO-induced increase in granulosa cell apoptosis (Fig. 3C). Both the 1 and 5 mM doses of GEE suppressed theca cell apoptosis in the presence of BSO (Fig. 3C).

We next used an antibody directed against the activated (cleaved) form of caspase 3 as an additional marker of apoptosis. Caspase 3 is activated by both the mitochondrial and the receptor-mediated apoptotic pathways (44). The numbers of activated caspase 3-positive granulosa cells and theca cells increased in a time-dependent manner upon culture in the absence of gonadotropin support. The increase in caspase 3-positive granulosa cells was statistically significant at 4, 12, 24, and 48 h of culture (P < 0.008, MEM vs. respective 0-h controls; Fig. 4B). The increase in caspase 3-positive theca cells was statistically significant at 24 and 48 h (P < 0.001, MEM vs. respective 0-h controls; Fig. 4C). Treatment with FSH caused a significant decline in caspase 3-positive granulosa cells at 12, 24, and 48 h (P < 0.03, FSH vs. MEM). In contrast, FSH increased theca cell caspase 3 activation at 24 and 48 h (P < 0.001, FSH vs. MEM). Combined FSH plus BSO treatment partially overcame the suppressive effect of FSH on activated caspase 3 immunostaining in granulosa cells, and the difference between FSH plus BSO and FSH-treated groups was statistically significant at both 24 and 48 h (P < 0.007; Fig. 4). Thus, activated caspase 3 immunostaining closely paral-

Fig. 2. TUNEL localization of apoptotic cells in cultured preovulatory follicles: effects of FSH treatment and GSH depletion with BSO. Isolated preovulatory follicles were cultured for 4, 12, 24, or 48 h as described for Fig. 1 and were processed for TUNEL, as detailed in Materials and Methods. A, Preovulatory follicle from 0-h control shows healthy granulosa cells and theca cells. B and C, Preovulatory follicles cultured in serum-free medium (MEM) for 24 h (B) or 48 h (C) show time-dependent increase in TUNEL-positive, brown stained granulosa cells and TUNEL-positive theca cells. D and E, Preovulatory follicles cultured with FSH for 24 h (D) or 48 h (E) show fewer TUNEL-positive granulosa cells but more TUNEL-positive theca cells when compared with the follicles in B, F and G. Preovulatory follicles treated with FSH and BSO for 24 h (F) or 48 h (G). Original magnification, ×132.
leled the results with TUNEL (Fig. 3), providing additional evidence that GSH depletion induces apoptosis in granulosa cells of cultured preovulatory follicles. We also sought to demonstrate a reversal of the protective effect of FSH on preovulatory follicle apoptosis using DNA gel electrophoresis to detect oligonucleosomal DNA fragmentation in DNA from whole follicles. Culture of preovulatory follicles in serum-free medium induced apoptosis, as evidenced by oligonucleosomal DNA fragmentation at 24 and 48 h of culture (Fig. 5A). No oligonucleosomal DNA fragmentation was observed in 0-h control follicles (Fig. 5A). Treatment with FSH significantly suppressed oligonucleosomal DNA fragmentation by 67 and 60% at 24 and 48 h, respectively (Fig. 5B; \( P < 0.001 \), effect of treatment by ANOVA at 24 and 48 h). Depletion of GSH with BSO partially reversed the antiapoptotic effect of FSH, but this was not statistically significant (\( P < 0.07 \) and \( P < 0.16 \), BSO vs. FSH at 24 and 48 h, respectively). The absence of a statistically
significant effect of GSH depletion on whole-follicle DNA laddering compared with the significant effects observed using two in situ assays for apoptosis, TUNEL and immunostaining for activated caspase 3, highlights the greater sensitivity of the in situ assays.

Effects of FSH and GSH depletion on the viability of preovulatory follicles

To show that follicles cultured in the presence of FSH remain fully viable during the 48-h culture period, we used WST-1, a tetrazolium salt that is cleaved to formazan by mitochondrial dehydrogenases of viable cells. Follicle viability decreased with increasing duration of culture in serum-free medium, falling to 49% of control levels at 48 h (Fig. 6; $P < 0.001$, MEM at 24 and 48 h vs. 0 h). After 24 h of culture, WST-1 cleavage in FSH- and FSH- plus BSO-treated follicles was also decreased compared with 0-h control follicles but did not differ from MEM-treated follicles. Interestingly, at 48 h, FSH-treated follicles had significantly greater WST-1 cleavage than 0- or 48-h MEM controls ($P < 0.001$, FSH vs. MEM at 0 h; Fig. 5). BSO treatment partially reversed the stimulatory effect of FSH on WST-1 cleavage at 48 h, with WST-1 absorbance in FSH- plus BSO-treated follicles not statistically different from the absorbance in the 0-h controls (Fig. 6). These results again paralleled the effects of BSO and FSH observed on apoptosis, further demonstrating the ability of GSH depletion to reverse the protective effect of FSH on preovulatory follicles.
Oxidative stress has been linked to apoptosis in various systems (13). However, the evidence that oxidative stress induces apoptosis in ovarian follicles has heretofore been indirect (15). We have shown for the first time that the onset of apoptosis in preovulatory follicles cultured in the absence significantly suppressed compared with MEM treatment at the same time points (P < 0.05, FSH vs. MEM at 4 and 24 h; Fig. 7, A and B). Cotreatment with FSH and BSO appeared to slightly reverse the suppressive effect of FSH on ROS production (Fig. 7). However, the difference between FSH- and FSH- plus BSO-treated groups was not statistically significant at any time point.

Similar to the results with DCF, culture of preovulatory follicles in the absence of FSH led to a statistically significant increase in DHR fluorescence at 4 h and all subsequent time points (P = 0.002, MEM at 4, 8, and 12 h vs. 0 h). Treatment with FSH significantly suppressed DHR fluorescence at 4 and 12 h (P = 0.002, FSH vs. MEM). Cotreatment with BSO reversed the suppression of DHR fluorescence by FSH; this effect was statistically significant by 12 h (P = 0.009, FSH plus BSO vs. FSH). Treatment with 5% hydrogen peroxide for 30 min caused an increase in DHR fluorescence similar in magnitude to that observed in MEM groups (2.0 ± 0.3-fold increase over 0-h negative control).

Taken together, the DCF and DHR data show that follicular ROS are significantly increased after 4 h of culture without gonadotropin support and that FSH suppresses ROS production. Both methods showed a partial reversal by BSO of the suppressive effect of FSH on ROS, but this was statistically significant only when DHR was used as a probe for ROS.

**Discussion**

Oxidative stress has been linked to apoptosis in various systems (13). However, the evidence that oxidative stress induces apoptosis in ovarian follicles has heretofore been indirect (15). We have shown for the first time that the onset of apoptosis in preovulatory follicles cultured in the absence...
of gonadotropin is preceded by a rise in follicular ROS. Furthermore, we have shown that FSH treatment enhances follicular GSH content and suppresses follicular ROS. Using a specific inhibitor of GSH synthesis we have also demonstrated that GSH depletion partially reverses both the suppression of ROS and the inhibition of granulosa cell apoptosis by FSH.

There is evidence that ROS play roles in the induction of both the death receptor and mitochondrial apoptotic pathways (13). Oxidative stress caused by the addition of exogenous oxidants induces apoptosis in many types of mammalian cells, including hepatocytes (46), epithelial cells (47), and fibroblasts (48). Endogenous production of ROS has also been associated with the induction of apoptosis (24). However, there is controversy as to whether ROS signaling is critical for the induction of apoptosis or is merely a by-product of apoptosis (13, 49). We assessed follicular ROS production using the probes H₂DCFDA and DHR (42, 43, 45).

Several lines of evidence show that DCF fluorescence indicates production of hydroxyl radical, peroxynitrite, and, in the presence of peroxidases, hydrogen peroxide (50). We observed significant increases in follicle DCF and DHR fluorescence by 4 h of culture in the absence of gonadotropin (Figs. 7 and 8), which preceded the increase in granulosa cell apoptosis, consistent with the hypothesis that the increase in ROS initiates the apoptotic cascade in granulosa cells. The suppression of follicular ROS by FSH treatment (Figs. 7 and 8), which also inhibited granulosa cell apoptosis, further supports this hypothesis. Depletion of GSH with BSO in the presence of FSH partially reversed the suppression of ROS by FSH (Figs. 7 and 8). Finally, the observations that FSH treatment enhanced follicular levels of the antioxidant GSH (Fig. 1) and that GSH depletion partially reversed the anti-apoptotic effects of FSH (Figs. 2–4) provide additional evidence that the rise in ROS initiates the apoptotic cascade in these follicles. There has been discussion in the literature that...
increases in DCF and DHR fluorescence may not indicate an increase in ROS but rather may be indicative of cytochrome c release, an early event of apoptosis (43, 51). These authors reported that cytochrome c is capable of catalyzing the oxidation of dichlorofluorescin by hydrogen peroxide (51) and of catalyzing the oxidation of DHR by hydrogen peroxide (43). It appears unlikely that the observed increases in DCF and DHR fluorescence in the present studies were a result of cytochrome c release in the presence of no change in cellular hydrogen peroxide levels. We observed no increase in cytochrome c in cytosolic preparations of follicles cultured for 4 h in MEM, compared with 0-h control follicles (data not shown). Activation of caspase 3 occurs almost concomitantly with cytochrome c release (52, 53), and we observed only a very small number of granulosa cells with activated caspase 3 immunostaining at 4 h (Fig. 4). In contrast, we observed large increases in DCF and DHR fluorescence throughout the follicles at 4 h of culture (Figs. 7 and 8).

Thus, our results provide evidence that GSH exerts an antiapoptotic effect in cultured preovulatory follicles. Our results further suggest that the antiapoptotic effect of GSH is a result of its ability to detoxify ROS that are generated in the absence of gonadotropin support. Additional evidence for this hypothesis comes from previous observations that treatment of preovulatory follicles with N-acetylcysteine, a precursor of GSH, as well as several other antioxidants, prevents apoptosis as effectively as FSH (15). However, GSH may also be acting at other points in the apoptotic cascade. GSH extrusion from cells has been shown to be necessary for cytochrome c release and apoptosis induction in some cells, occurring as early as 6 h after the application of an apoptotic stimulus (44, 54). This raises the question of whether the decline in intrafollicular GSH levels in follicles cultured in serum-free medium that we observed in the present study was a result of GSH extrusion. This appears unlikely for two reasons. First, we were unable to detect GSH in the culture media (data not shown); however, it is possible that GSH was present in the media at levels below the detection limit of our assay. Second, intrafollicular GSH did not begin to decline until 24 h of culture in serum-free medium, and GSH was not significantly decreased until 48 h (Fig. 1). In contrast, clear increases in granulosa cell apoptosis were evident at 12 h of
culture in serum-free medium (Fig. 4), before the decline in follicular GSH levels.

Most previous studies that demonstrated antiapoptotic effects of FSH on follicular apoptosis measured oligonucleosomal DNA fragmentation in DNA from whole-follicle or whole-ovary homogenates (3–5, 55). These studies and the present study showed that FSH decreased apoptosis using this method. Using TUNEL to localize DNA fragmentation and immunostaining to localize activated caspase 3 within cultured preovulatory follicles, we have confirmed a previous report (32) that FSH exerts antiapoptotic effects on granulosa cells and paracrine effects on theca cells of cultured preovulatory follicles. In the present study, FSH suppressed granulosa cell apoptosis in preovulatory follicles at 12, 24, and 48 h of culture (Figs. 3A and 4B). In contrast, FSH enhanced theca cell apoptosis in preovulatory follicles at 24 and 48 h but not at 4 or 12 h (Figs. 3B and 4C). Coincident with its suppression of granulosa cell apoptosis, FSH treatment also enhanced follicular GSH concentrations, supporting a role for GSH in mediating the antiapoptotic effect of FSH on granulosa cells. The partial reversal of the antiapoptotic effect of FSH by the GSH synthesis inhibitor BSO (Figs. 3, A and C, and 4B) further supports this hypothesis. The complete prevention of the BSO-induced increase in granulosa cell apoptosis by GSH supplementation (Fig. 3C) demonstrates that this proapoptotic effect of BSO is specifically a result of GSH depletion. The increase in follicular GSH concentrations in response to FSH in the present study was associated with increased protein levels of GCLM, the rate-limiting enzyme in GSH synthesis (Fig. 1). Although protein levels of the catalytic subunit were not increased by FSH treatment, an isolated increase in the modifier subunit likely increased levels of the holoenzyme, which is a heterodimer of the catalytic and modifier subunits (56, 57). We have previously reported that ovarian protein and mRNA levels of GCLM are dramatically up-regulated by 4 h after an ovulatory dose of human chorionic gonadotropin in PMSG-primed rats (21).

The present data provide evidence that this isolated increase in GCLM levels in vivo may lead to localized increases in GSH synthesis in preovulatory follicles.

In addition to up-regulating ovarian GSH synthesis, gonadotropins also enhance other antioxidant responses in the ovary. Ovarian mRNA levels of secreted and manganese-containing superoxide dismutase increase after PMSG treatment in rats (15, 58). PMSG treatment also increases ovarian concentrations of vitamin A (59). FSH enhances uptake of ascorbic acid by cultured granulosa cells (60). Up-regulation of other antioxidant responses in ovarian follicles by FSH may explain why GSH depletion in the present studies did not fully overcome the suppressive effect of FSH on follicular ROS generation or granulosa cell apoptosis in the present study.

Our results are consistent with previous findings that activation of the executioner caspase, caspase 3, is involved in both granulosa and theca cell apoptosis in cultured preovulatory follicles. Our data for activated caspase 3 immunostaining in granulosa and theca cells of preovulatory follicles mirrored our TUNEL data. These data are consistent with previous in vivo observations that procaspase 3 is expressed in granulosa cells of atretic follicles, but not of healthy follicles, in the rat ovary (61) and that activated caspase 3 is expressed in granulosa cells of atretic, but not healthy, follicles in the mouse ovary (9). Moreover, a critical role for caspase 3 in granulosa cell apoptosis in the mouse ovary has been demonstrated, because caspase 3 knockout mice have delayed and abnormal granulosa cell apoptosis (9). Because granulosa cells possess FSH receptors, but theca cells do not (62), the effects of FSH on apoptosis in granulosa cells are likely to be direct, whereas the effects of FSH on theca cells are likely to be indirect. FSH treatment in vivo has been shown to suppress apoptotic protease activating factor-1 (APAF-1) protein expression and bax mRNA expression in granulosa cells (6, 8). Because both BAX and apoptotic protease activating factor-1 act upstream of caspase 3 activation in the mitochondrial apoptotic pathway, these data collectively show that FSH acts to inhibit several key points in this pathway in granulosa cells. The present study shows that the full antiapoptotic effect of FSH on granulosa cells cannot occur in the face of follicular GSH depletion. We are unaware of any studies that investigated paracrine signals by which FSH stimulates theca cell apoptosis in cultured follicles. However, FSH has been reported to enhance protein and mRNA expression of granzyme-like proteins in primary human and immortalized rat granulosa cells (63). Granzyme B released from granules within cells can act on adjacent cells to directly activate caspase 8 and other caspases (63), making granzyme-like proteins possible candidates as mediators of the proapoptotic effect of FSH on theca cells of cultured follicles.

In addition to measures of apoptosis, we also assessed follicle health using a cell viability assay. This assay measures metabolic activity, assessed by the cleavage of the tetrazolium salt WST-1 to formazan by mitochondrial dehydrogenases in viable cells. As expected, there was a decline in WST-1 cleavage in follicles cultured for 24 h in serum-free media compared with 0-h control follicles that were not cultured, indicating a decline in follicle viability. Surprisingly, we observed a statistically significant increase in WST-1 cleavage after 48 h of treatment with FSH, compared with 0-h control follicles. This implies that proliferation of follicular cells occurred upon FSH stimulation. However, granulosa cells from preantral, small antral, and preovulatory follicles do not proliferate in response to FSH stimulation alone in vitro (64–66), and cultured preantral follicles also show minimal growth in response to FSH alone (55). Although FSH does not promote the proliferation of isolated granulosa cells in vitro, it does enhance the growth-promoting effects of other factors, such as transforming growth factor β (64), activin (65), and growth differentiation factor 9 (66), on granulosa cells. This suggests that FSH may stimulate the release of such paracrine or autocrine factors, which have mitotic actions, from granulosa or theca cells of cultured preovulatory follicles. BSO treatment in the presence of FSH reversed the stimulatory effect of FSH on WST-1 cleavage, consistent with the increased apoptotic granulosa cell death observed in the presence of BSO (Figs. 3 and 4). Another explanation for the increased WST-1 cleavage in the FSH-treated follicles is that FSH stimulated the activity of mitochondrial dehydrogenases. Although it is well established that FSH enhances the expression and activity of the mito-
chondrial cytochrome P450 cholesterol side chain cleavage enzyme (67,68), there is little information available about the actions of FSH on other aspects of mitochondrial function. It was recently reported that whole-ovary mitochondrial content, mitochondrial oxygen uptake, mitochondrial electron transfer activities, and mitochondrial indicators of oxidative damage all increased in response to FSH in a hyperstimulated estrous cycle in adult rats (69). Thus, our observations that FSH increased WST-1 cleavage of preovulatory follicles may be related to up-regulation of mitochondrial activity by FSH. It has recently been shown that mitochondrial NADP⁺-dependent isocitrate dehydrogenase enzymatic activity is suppressed by glutathionylation (70). Glutathionylation of proteins is favored when the cellular redox balance of reduced to oxidized glutathione (GSH-GSSG) shifts toward GSSG (17). This occurs in situations of oxidative stress and GSH depletion (17). ROS have also been shown to induce glutathionylation of proteins even in the absence of a detectable change in GSH-GSSG (17). Thus, FSH could enhance the activity of mitochondrial dehydrogenases by shifting cellular redox balance toward reduced glutathione and decreased glutathionylation.

In summary, we have shown that culture of preovulatory follicles in the absence of gonadotropin support increased follicular ROS levels by 4 h and increased granulosa cell and theca cell apoptosis by 12 h. Treatment with FSH delayed and decreased the production of ROS, enhanced follicular GSH concentrations by 12 h, and suppressed granulosa cell apoptosis by 12 h. Cotreatment with FSH and BSO, a specific inhibitor of GSH synthesis, partially and significantly reversed the suppression of ROS production and granulosa cell apoptosis by FSH. Collectively, our results support the hypothesis that apoptosis in cultured preovulatory follicles is initiated by oxidative stress and that the antioxidant GSH plays a role in mediating the antiapoptotic effect of FSH on granulosa cells of preovulatory follicles.

Our results further suggest that abnormalities in follicular glutathione systems could decrease the ability of follicles to respond to oxidative stress or toxicant exposures, causing increased follicular apoptosis and even ovarian failure.

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