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ORIGINAL ARTICLE

FSH and bFGF stimulate the production of glutathione in cultured rat Sertoli cells

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Summary

Migration of developing germ cells from the basal to the adluminal compartment of the seminiferous epithelium requires extensive tissue restructuring, resulting in the production of reactive oxygen species. Sertoli cells are involved in this process. Glutathione (GSH), produced by Sertoli cells, has an essential role in cell protection against oxidative stress. Intracellular GSH content is maintained by *de novo* synthesis, involving glutamate-cysteine ligase catalytic (GCLC) and modulatory (GCLM) subunits, and by recycling from oxidized GSH, catalysed by glutathione reductase (GR). To assess whether follicle-stimulating hormone (FSH) and basic fibroblast growth factor (bFGF) modulate GSH production in Sertoli cells by regulating the expression of GCLC, GCLM and/or GR, we performed in vitro studies using rat Sertoli cells in primary culture. FSH and bFGF stimulation increased Sertoli cell GSH levels after 24 h incubation. The simultaneous addition of FSH and bFGF did not produce any further effect. GCLM expression was upregulated by FSH and bFGF 6 h. At 24 h, only the FSH-mediated effect was still observed. FSH and bFGF also upregulated GR expression. In conclusion, our results show that FSH and bFGF increase GSH levels in Sertoli cells through stimulation of the *de novo* synthesis and recycling by upregulating GCLM and GR expression respectively. Therefore, protection of germ cells against oxidative stress seems to be regulated by hormones and germ cell-released growth factors capable of influencing the production of Sertoli cell GSH.

Introduction

Developing germ cells in the testis, in addition to undergoing complex cellular changes, must migrate progressively from the basal to the adluminal compartment of the seminiferous epithelium. Sertoli cells are structurally and nutritionally involved in this process. This migration requires extensive tissue restructuring in the seminiferous tubules, resulting in the production of reactive oxygen species (ROS) (Mruk *et al.*, 2002). Glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) is the most abundant non-protein thiol in cells and has an essential role in cell protection against oxidative stress and xenobiotic detoxification (Meister & Anderson, 1983; Griffith, 1999; Sies, 1999; Dickinson & Forman, 2002). High levels of GSH have been localized in testes and specifically in Sertoli cells (Den Boer *et al.*, 1989; Bauché *et al.*, 1994; Castellón, 1994; Dickinson & Forman, 2002). Significant functions of GSH in reproductive processes and spermatogenesis have also been reported (Li *et al.*, 1989; Castellón, 1994; Knapen *et al.*, 1999; Fujii *et al.*, 2003).

There are several mechanisms by which cells maintain their intracellular GSH content: *de novo* synthesis from its constituent amino acids, GSH redox recycling from oxidized glutathione – catalysed by glutathione reductase (GR) and direct uptake.

Glutamate-cysteine ligase (GCL) is the initial and rate-limiting enzyme in the *de novo* synthesis of GSH. This enzyme catalyses the binding of L-glutamate and L-cysteine to form L-gamma-glutamyl-cysteine in an ATP-dependent manner. Its activity is feedback-inhibited by GSH. GCL is a heterodimer composed of a heavy catalytic subunit (GCLC,

72 kDa) and a light modulatory subunit (GCLM, 30 kDa) (Reid *et al.*, 1997a,b). The presence of GCL enzymatic activity has been demonstrated in rat Sertoli cells (Castellón, 1994).

Glutathione reductase catalyses the reduction of oxidized GSH (GSSG) to reduced GSH using NADPH as the electron donor. GR is a dimer formed by two identical 50 kDa subunits. High levels of GR have been described in rat Sertoli and germ cells (Bauché *et al.*, 1994; Kaneko *et al.*, 2002).

Sertoli cells are regulated by follicle-stimulating hormone (FSH), androgens and locally produced growth factors. There is extended evidence that FSH plays a major role in both the development and maturity of Sertoli cells (Allan & Handelsman, 2005). In addition, several members of the fibroblast growth factor (FGF) family have been shown to influence testis function (Gnessi *et al.*, 1997). Basic FGF (bFGF) is one of the most studied FGF family members and it has been shown to stimulate differentiation and Sertoli cell growth prepubertally (Jaillard *et al.*, 1987; Schteingart *et al.*, 1999; Skinner, 2005). In testes, little information is available concerning the regulation of GSH and related enzymes by hormones and growth factors (Castellón, 1999; Benbrahim-Tallaa *et al.*, 2002a,b).

We hypothesized that FSH and bFGF modulate GSH production in Sertoli cells by regulating the expression of GR and GCL. To test these hypotheses, we performed in vitro studies using rat Sertoli cells in primary culture.

Materials and methods

Materials

Ovine FSH (NIH-oFSH-S-16) was obtained from the National Hormone and Pituitary Program, National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK, Bethesda, MD, USA). Tissue culture media was purchased from Grand Island Biological Co. (Grand Island, NY, USA). Basic fibroblast growth factor (bFGF; human recombinant) was purchased from Invitrogen Tech-Line (Carlsbad, CA, USA). Other drugs and reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Sertoli cell isolation and culture

Sertoli cells from 18-day-old Sprague–Dawley rats were isolated as previously described (Schteingart *et al.*, 1989). Briefly, decapsulated testes were digested with 0.1% collagenase and 0.006% soybean trypsin inhibitor in Hanks' balanced salt solution (HBSS) for 5 min at room temperature. Seminiferous tubules were saved, cut and submitted to 1 M glycine–2 mM EDTA (pH 7.4) treatment to remove peritubular cells. The washed tubular pellet was then digested again with collagenase for 10 min at room temperature to remove germ cells. The Sertoli cell suspension, collected by sedimentation, was resuspended in culture medium consisting of a 1 : 1 mixture of Ham's F-12 and Dulbecco's modified Eagle medium, supplemented with 20 mM Hepes, 1.8 mg/mL sodium bicarbonate, 100 IU/mL penicillin, 2.5 μg/mL amphotericin B, 10 μg/mL transferrin, 5 μg/mL insulin, 5 μg/mL vitamin E and 4 ng/mL hydrocortisone. Sertoli cells were cultured at 34 °C in a mixture of 5% CO_2 : 95% air.

Purity of Sertoli cells reached 95% after 5 days in culture as seen by phase contrast microscopy. No myoid cell contamination was revealed when a immunoperoxidase technique was applied to Sertoli cell cultures using a specific antiserum to α-smooth muscle actin. Remaining cell contaminants were of germ cell origin.

Culture conditions

Sertoli cells were allowed to attach for 48 h in the presence of insulin (5 μg/mL); the medium was then replaced with insulin-free fresh medium; bFGF (30 ng/mL) and/or FSH (100 ng/mL) were added 48, 24 or 6 h before the end of the culture period. When indicated, H_2O_2 (50 or 500 μM) was added 3 h before the end of the culture period. Cells were harvested on day 5 and disrupted by sonication. Adequate aliquots for DNA determinations were saved and analysed by the method of Labarca & Paigen (1980).

GSH assay

A modified (Baker *et al.*, 1990) method was used to determine intracellular concentration of total GSH. Briefly, Sertoli cell monolayers in 24-multiwell plates (10 μg DNA/well, corresponding to approximately 1.4×10^6 cells/well, at the beginning of the culture,) were homogenized in 0.15 M Tris-HCl buffer (pH 7.4) and disrupted by sonication. Adequate aliquots for DNA determinations were saved. In microtitulation plates, two different aliquots of the homogenate were added to the reaction buffer (0.1 mL) consisting of a mix of 100 mM sodium phosphate, pH 7.5 – 1 mM EDTA (5.75 mL), 1 mM NADPH (5 mL) and 1 mM 5.5′-Dithiobis (2-nitrobenzoic acid) (DTNB, 5 mL). GR from baker's yeast (20 U) was added to the mix to initiate the assay. The rate of 5-thio-2-nitrobenzoic acid formation (TNB) was followed at 405 nm for every 2-min intervals for 10 min using a spectrophotometer. Absorbance was proportional to the sum of GSH and GSSG present. It was compared to a standard curve generated with GSH. Results were expressed as pmol GSH/μg DNA.

Northern blots

Northern blot analyses for GR and GCLC were performed. Total RNA was isolated from Sertoli cells cultured in 25 cm^2 tissue culture flasks with Tri Reagent (Sigma Chemical Co.). The amount of RNA was estimated by spectrophotometry at 260 nm. RNA (30 μg/lane) was electrophoresed on 1% agarose–17% formaldehyde gel. After migration, RNAs were transferred to Hybond-N nylon membrane (Amersham Pharmacia Biotech, Buenos Aires, Argentina) by capillary transfer with 10× SCC (10× stock solution: 1.5 M NaCl and 0.15 M sodium citrate, pH 7.4) and fixed with UV Stratalinker (Stratagene Cloning Systems, La Jolla, CA, USA). Blots were pre-hybridized for 4 h in 50% formamide, 0.75 M NaCl, 20 mM sodium phosphate (pH 7.5), 1 mM EDTA, 5× Denhardt solution, 10% dextran sulphate, 0.5% SDS and 100 μg/mL Herring sperm DNA. Hybridization was then performed overnight in the same hybridization solution containing a ^{32}P-labelled probe. Rat GR cDNA probe was generously provided by Dr J. Fujii (Yamagata, Japan) (Fujii *et al.*, 2000) and mouse GCLC cDNA was generously provided by Dr T. Kavanagh (Seattle, USA) (Reid *et al.*, 1997a). Probes were labelled with ^{32}P-dCTP using the Random Prime Labeling kit (Promega Corporation, Madison, WI, USA). Membranes hybridized with rat GR cDNA were washed twice in 2× SSC–0.1% SDS (55 °C) and twice in 0.2× SSC–0.1% SDS. Membranes hybridized with mouse GCLC cDNA were washed twice in 2× SSC–0.1% SDS (room temperature) and twice in 0.2× SSC–0.1% SDS (55 °C). Membranes were exposed to Kodak X-Omat S film (Eastman Kodak, Rochester, NY, USA) for appropriate periods at −70 °C. Finally, blots were stripped and re-probed with a ^{32}P-labelled rat 18 S cDNA probe for normalization of data with regard to sample loading and transfer of RNA. The intensities of the autoradiographic bands were estimated by densitometric scanning using NIH Image software (Scion Corporation, Frederick, MD, USA).

Western blots

Western blot analyses for GR, GCLC and GCLM were performed. Sertoli cell monolayers in 6-multiwell plates were disrupted by sonication on PBS (NaCl 137 mM, ClK 2.7 mM, Na_2HPO_4 8 mM, KH_2PO_4 1.5 mM, pH 7.4) containing 1% protease inhibitor cocktail from Sigma (P-8340) and phenylmethylsulphonyl fluoride 2 mM. Proteins (20–40 μg/lane) were resolved in 12.5% SDS-PAGE and then transferred onto polyvinylidene fluoride membrane using a MiniTrans-blot Cell (Bio-Rad, Hercules, CA, USA). Protein concentration was determined according to Bradford (1976). Membranes were then blocked by incubation with 5% skim milk in TBS (Tris 20 mM, ClNa 137 mM, pH 7.6) containing 0.1% Tween-20 (TBS–Tween) for 3 h at room temperature. After washing them with TBS-Tween, membranes were incubated overnight with a rabbit anti-GR antibody (1 : 6000 dilution) generously provided by Dr J. Fujii (Fujii *et al.*, 2000); a rabbit anti-GCLC antibody (1 : 10 000 dilution) or a rabbit anti-GCLM antibody (1 : 12 000 dilution), generously provided by Dr T. Kavanagh (Thompson *et al.*, 1999). Membranes were then washed with TBS-Tween and incubated with peroxidase-conjugated goat anti-rabbit immunoglobulin G (Cell Signal Technology Inc., Beverly, MA, USA) for 1–2 h. The antibody–antigen complexes were detected by chemiluminescence (Phototope®-HRP Western Blot Detection System, Anti-rabbit IgG HRP-linked; Cell Signal Technology Inc.).

Blots were subsequently re-probed with β-actin antiserum (monoclonal anti-β-actin antibody produced in mouse, A-5441; Sigma Chemical Co.) as a loading control. The intensities of the autoradiographic bands were estimated by densitometric scanning using NIH Image software (Scion Corporation).

Statistical analysis

Data presented as mean ± SD were analysed by Student's *t*-test or one-way ANOVA followed by Tukey's multiple comparison test, as adequate, using GraphPad InStat version 3.00 (GraphPad Software, San Diego, CA, USA). Results were considered significantly different if $p < 0.05$.

Results

FSH and bFGF stimulation increases Sertoli cell GSH levels

The effect of FSH and bFGF treatments on total GSH levels in primary Sertoli cell cultures of 18-day-old Sprague–Dawley rats is shown in Fig. 1. Although no effect was observed at 6 h, both FSH and bFGF increased GSH content at 24 and 48 h. The simultaneous addition of FSH and bFGF did not produce any further effect.

FSH and bFGF increase GCL levels

To assess whether FSH and bFGF stimulation of GSH production was mediated by an upregulation of GCL, the rate-limiting enzyme involved in the *de novo* synthesis of GSH, we studied the effect of FSH and bFGF on the expression of GCLC and GCLM subunits.

Glutamate-cysteine ligase catalytic mRNA expression was upregulated by FSH and bFGF as early as 6 h (FSH: 218 ± 45% and bFGF: 178 ± 44%, considering basal as 100%). No additive effect was observed when both factors

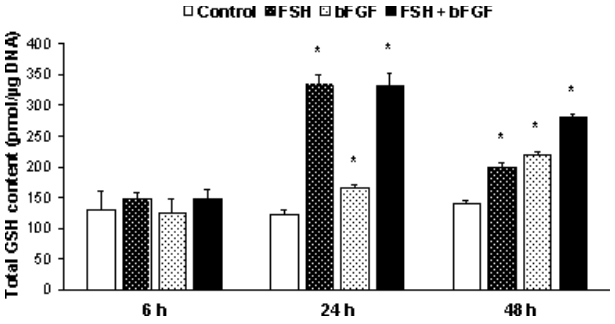

Figure 1 Total GSH levels in Sertoli cell cultures treated for 6, 24 and 48 h with FSH and bFGF. Results are expressed as mean ± SD from four different experiments (*$p < 0.05$ statistically different from control. anova and Tukey's multiple comparison test).

were added simultaneously (FSH + bFGF: 225 ± 40% compared with basal level). At 24 h, only the FSH-mediated effect was still observed (FSH: 153 ± 30%; bFGF: 107 ± 20%; FSH + bFGF: 149 ± 21%, compared with basal level). Unexpectedly, neither FSH nor bFGF had a significant effect on GCLC protein levels (Fig. 2a).

Conversely, an increase of more than 1.8-fold in modulatory subunit (GCLM) protein levels was observed in Sertoli cells treated with FSH and bFGF at 6 h (Fig. 2b). At 24 h, only the FSH effect persisted.

FSH and bFGF upregulate GR expression

We also investigated whether the increase in GSH levels in Sertoli cells could be mediated by an upregulation of GR as well. Both mRNA and protein levels were significantly increased by FSH and bFGF as early as 6 h (Fig. 3). The effect persisted on mRNA levels at 24 h, but unexpectedly no persistent effect was observed at the protein level.

FSH protects against oxidative stress condition generated by hydrogen peroxide

To evaluate the protective effect of FSH under oxidative stress, Sertoli cells were cultured for 24 h with FSH and then exposed to different doses of H_2O_2 during the last 3 h (Table 1). Treatment with 500 μM H_2O_2 decreased intracellular GSH content; however, pre-treatment with FSH hampered H_2O_2 effect.

Discussion

Recent evidence indicates that sperm quality is declining, resulting in decreasing trends in fertility rates in humans (Skakkebaek *et al.*, 2006). Changing lifestyle and environmental exposures may underlie male reproductive health

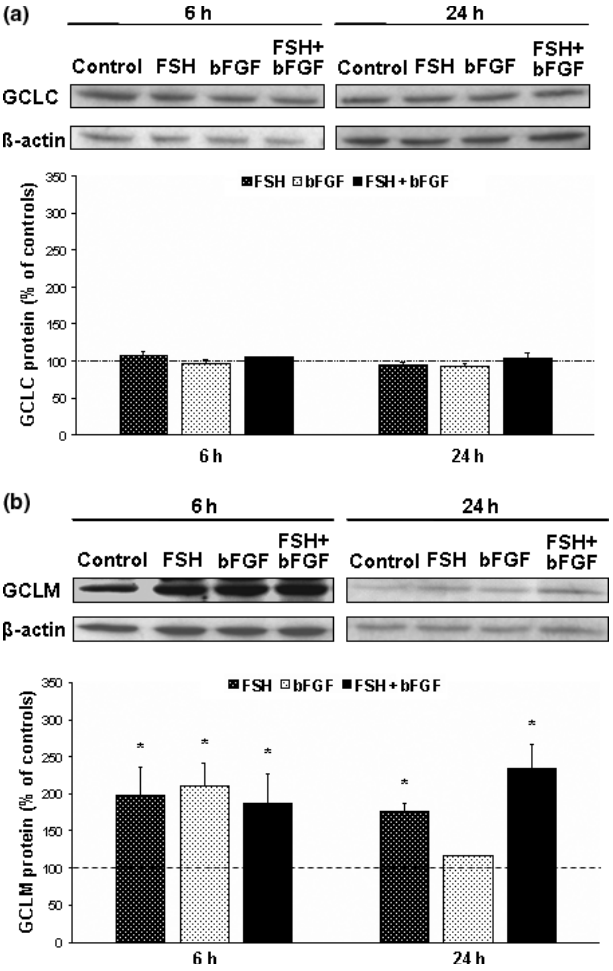

Figure 2 Analysis of GCLC and GCLM expression in Sertoli cells under FSH and/or bFGF treatment for 6 and 24 h. (a) Top: representative Western blot for GCLC. Bottom: quantification of data (mean ± SD) from four different experiments, expressed as percentage of the untreated controls (*$p < 0.05$ statistically different from control. anova and Tukey's *t*-test). (b) Top: representative Western blot for GCLM. Bottom: quantification of data (mean ± SD) from four different experiments, expressed as percentage of the untreated controls (*$p < 0.05$ statistically different from control. anova and Tukey's multiple comparison test).

problems. The pathophysiology of defective sperm production may be explained in part by an excessive generation of ROS (Agarwal *et al.*, 2003). In normal conditions, testes appear to be continuously exposed to ROS produced by their active metabolism (Fujii *et al.*, 2003), mainly after pubertal onset when the spermatogenic process becomes particularly active. Consequently, it is logical to presume that GSH, a central molecule in the detoxification of ROS, is physiologically regulated. GSH maintenance in germ cells is dependent upon interactions with somatic cells, notably Sertoli cells, as already shown in *Xenopus laevis* (Li *et al.*, 1989). In this study, we

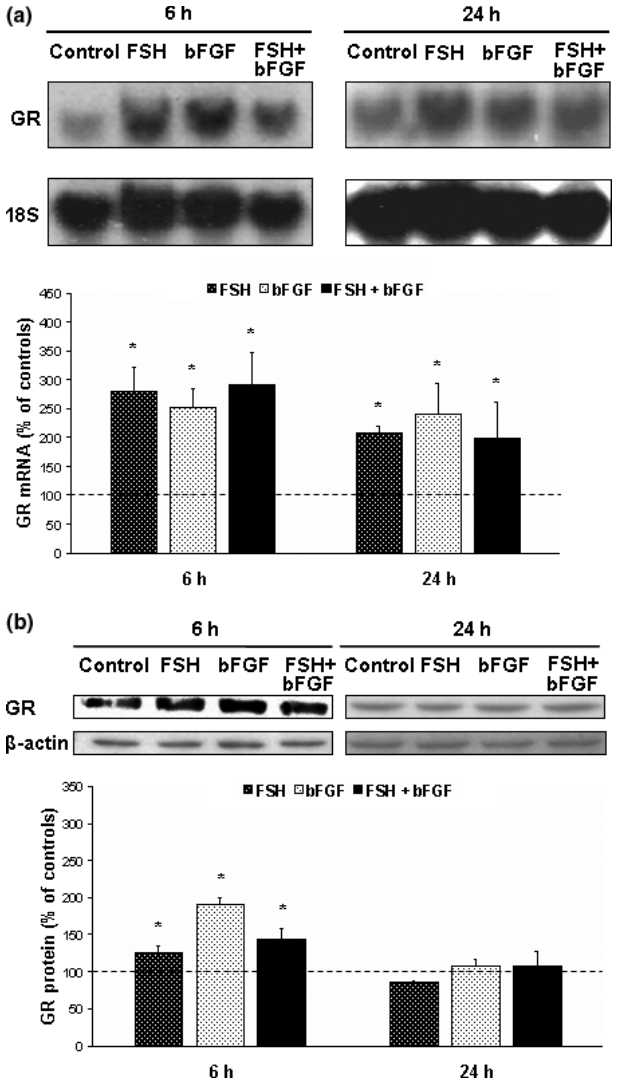

Figure 3 Analysis of GR expression in Sertoli cells under FSH and/or bFGF treatment for 6 and 24 h. (a) Top: representative Northern blot for GR. Bottom: quantification of data (mean ± SD) from four different experiments, expressed as percentage of the untreated controls (*$p < 0.05$ statistically different from control. anova and Tukey's *t*-test). (b) Top: representative Western blot for GR. Bottom: quantification of data (mean ± SD) from four different experiments, expressed as percentage of the untreated controls (*$p < 0.05$ statistically different from control. anova and Tukey's multiple comparison test).

demonstrate that FSH and bFGF increase GSH levels, by stimulating GCLM and GR enzymes, in Sertoli cells from 18-day-old rats, an age when pubertal spermatogenesis is in active expansion. During pubertal development, there is an increase in both FSH, as a consequence of the reactivation of the hypothalamic-gonadotrophic axis, and bFGF, resulting from the appearance and rapid increase in the number of primary spermatocytes (Gnessi *et al.*, 1997).

Table 1 Effect of exposure to H_2O_2 on total GSH in Sertoli cells cultured under basal conditions or treated with FSH for 24 h

	GSH (pmol/μg DNA)	
H_2O_2 (μM)	Basal	FSH (100 ng/mL)
0	176.1 ± 25.6	267.2 ± 23.5**
50	171.4 ± 34.9	238.2 ± 50.7**
500	116.4 ± 32.9*	224.5 ± 44.1**

Different concentrations of H_2O_2 were added in the last 3 h of culture. Results are expressed as mean ± SD of six observations. The effect of different H_2O_2 treatments within basal or FSH-treated conditions was evaluated by anova and Tukey's *t*-test (*$p < 0.05$ vs. 0 μM). The effect of FSH-treated conditions against basal conditions for each H_2O_2 concentration was analyzed by Student's *t*-test (**$p < 0.05$ vs. basal condition).

The mechanism by which Sertoli cells facilitate GSH maintenance in spermatogenic cells is unknown, but it appears to involve GSH replacement rather than degradation or export (Li *et al.*, 1989; Fujii *et al.*, 2003). It has been suggested that Sertoli cells may secrete GSH or GSH precursors for uptake by germ cells (Li *et al.*, 1989). A similar mechanism has been described in rat brains: GSH, released from astrocytes, is a substrate for the astroglial ectoenzyme γ-glutamyl transpeptidase (γ-GT). The resulting product, the dipeptide CysGly, serves as a precursor of neuronal GSH synthesis (Dringen *et al.*, 2000). Our laboratory has previously demonstrated that FSH and bFGF stimulate γ-GT activity in rat Sertoli cells (Schteingart *et al.*, 1989, 1999), thus accounting for an eventual release of the dipeptide CysGly towards germ cells. Alternatively, mammal Sertoli cells might provide GSH to the spermatogenic cells as a source of cysteine and a reducing power (Kaneko *et al.*, 2002; Fujii *et al.*, 2003).

Our results in Sertoli cells show that FSH and bFGF cause an upregulation in GCLM and GR expression as early as 6 h. GCLM and GR expression remained increased even 24 h after FSH treatment, whereas only GR mRNA persisted at high levels 24 h after bFGF treatment. These observations seem to indicate that FSH is a more potent regulator of the enzymes involved in GSH synthesis in Sertoli cells than bFGF. GSH levels increased after 24 h treatment and remained elevated at 48 h. These observations suggest that an early increase in the expression of GCLM and GR with FSH and bFGF treatments could be reflected in a later increment in GSH levels. The delay in GSH content increase after GCLM and GR enzyme activation needs to be elucidated. GCLC does not seem to be involved in GSH increment under FSH or bFGF treatments. Our observation of increment in GCLC mRNA levels without a concomitant increment in GCLC protein is an intriguing finding. Other laboratories have found an increment in mRNA GCLC levels which was

not accompanied by a parallel increment in protein levels (Diaz *et al.*, 2004; Krzywanski *et al.*, 2004). It has been suggested that translational regulation is involved in the production of GCL subunit protein from the GCL subunit messages that has not been elucidated yet (Krzywanski *et al.*, 2004).

To test our hypothesis that maintenance or increase in GSH levels would be protective against ROS in the testis, we incubated Sertoli cells with H_2O_2 in the presence of FSH, which increases GSH content. Our results show that treatment of H_2O_2, which induces oxidative stress, decreased intracellular GSH content; this was not observed in cells pre-treated with FSH. This seems to indicate that FSH can maintain a high GSH level in Sertoli cells in spite of oxidative stress. Recently, it has been demonstrated in preovulatory follicles that FSH prevents the decline in follicular GSH levels after 48 h in culture. This FSH-induced increase in GSH was associated with an increase in GCLM protein levels and a suppression of ROS production (Tsai-Turton & Luderer, 2006).

The intracellular pathways and molecular mechanisms involved in the increase in the expression of both GCL enzymes and GR in response to FSH and bFGF were not explored in this work. Potential mediators of FSH stimulation on GCLC gene expression could be nuclear factor kappa B (NFκB), AP-1 and/or AP-2, for which putative binding sites have been identified on the promoter region (reviewed by Wild & Mulcahy, 2000). AP-1 and AP-2 binding sites also exist on the GCLM gene promoter (Wild & Mulcahy, 2000). In fact, NFκB and AP-2 have been shown to mediate FSH upregulation of the AMH gene promoter in Sertoli cells (Lukas-Croisier *et al.*, 2003). On the other hand, AP-1 is induced by cyclic AMP (Muller *et al.*, 1989), a well-known second messenger of FSH. Alternatively, regulation could involve transcription factor Nrf2, a critical regulator of both GCL subunits and GR. Interestingly, Nrf2 translocation to the nucleus is activated by phosphatidylinositol 3 kinase (PI3K) (Nakaso *et al.*, 2003) and mitogen-activated protein kinases (MAPKs) (Kong *et al.*, 2001). PI3K mediates FSH signalling (Meroni *et al.*, 2002) whereas PI3K and MAPKs mediate bFGF signalling (Riera *et al.*, 2003) in Sertoli cells. Nrf2-null mice do not seem to have major fertility impairment until 2 months of age, but they develop a large range of signs of illness thereafter (Ma *et al.*, 2006), which unfortunately precludes detailed studies of adult testicular function.

Loss of intracellular GSH can be due to its conjugation to various electrophiles catalysed by glutathione-*S*-transferases (GST) (Griffith, 1999). In rat and pig Sertoli cells, FSH increases GST activity (Castellón, 1999; Benbrahim-Tallaa *et al.*, 2002b). Also testosterone and tumor necrosis factor-α modify GST-α expression in pig Sertoli cells (Benbrahim-Tallaa *et al.*, 2002a). These enzymes, which were not studied in this work, coexist with the previously presented mechanisms of the *de novo* synthesis and recycling on the regulation of the levels of intracellular GSH in Sertoli cells. Another pathway involved in the control of GSH levels is GPX4, an enzyme regulated by gonadotropins (Roveri *et al.*, 1992) and linked to male fertility (Foresta *et al.*, 2002). However, GPX4 is present in germ cells but not in Sertoli cells (Roveri *et al.*, 1992), and was therefore not studied in this work.

In conclusion, our results show that FSH and bFGF increase GSH levels in Sertoli cells through stimulation of the *de novo* synthesis and recycling by upregulating GCLM and GR expression respectively. Therefore, it is possible that the protection of germ cells against oxidative stress and xenobiotic detoxification is regulated by hormones and germ cell-released growth factors capable of influencing the production of Sertoli cell products, like GSH. Further research is necessary to elucidate the molecular mechanisms involved in this process.

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