QSOX sulfhydryl oxidase in rat adenohypophysis: localization and regulation by estrogens

A Tury*, G Mairet-Coello*, F Poncet, C Jacquemard, P Y Risold, D Fellmann and **B Griffond**

Laboratoire d'Histologie, E.A. Estrogènes, Expression Génique et Pathologies du Système Nerveux Central, IFR INSERM 133, Université de Franche-Comté, Faculté de Médecine et de Pharmacie, 19 rue Ambroise Paré, 25041 Besançon Cedex, France

(Requests for offprints should be addressed to B Griffond; Email: bernadette.griffond@univ-fcomte.fr)

*(A Tury and G Mairet-Coello contributed equally to this work)

Abstract

The expression of the rat quiescin sulfhydryl oxidase (rQSOX) and its putative regulation by estrogens were investigated in the adenohypophysis. Immunohistochemical observations revealed that rQSOX protein is abundantly expressed throughout the anterior lobe of the pituitary, and can be found in almost all the different cell populations. However, as shown by double immunohistochemistry, the cells displaying the strongest rQSOX labeling belong to a subset of gonadotrophs. Immunoelectron microscopy showed that, in adenohypophyseal cells, the protein is linked to the membranes of the rough endoplasmic reticulum, the Golgi apparatus and to dense-core secretory granules. These results are consistent with the secretion of the protein and its presumed role in the extracellular matrix. According to its sulfhydryl oxidase function, rQSOX could also participate in the intracellular folding of secreted proteins or hormones like LH and FSH and act as an endogenous redox modulator of hormonal secretion. A semiquantitative RT-PCR analysis of *rQSOX* level across the estrous cycle and the fact that chronic administration of 17 β-estradiol to ovariectomized rats led to a sustained up-regulation of *rQSOX* in the pituitary suggest that *rQSOX* expression is controlled by sex hormone levels. Further investigations are needed in order to elucidate its precise roles in that gland and the mechanisms of its regulation.

Journal of Endocrinology (2004) **183,** 353–363

Introduction

The rat quiescin sulfhydryl oxidase (rQSOX), a 64 kDa glycoprotein, is a member of the quiescin Q6/FAD (flavin adenine dinucleotide)-dependent sulfhydryl oxidase (QSOX) family (Hoober *et al.* 1999*a*, Benayoun *et al.* 2001, Thorpe *et al.* 2002). QSOX proteins display a highly conserved structure with an N-terminal PDI (protein disulfide isomerase)-like thioredoxin domain and a C-terminal ERV1 (essential for respiration and vegetative growth)-like domain which contains the FAD-binding site and the redox-active CXXC motif (Coppock *et al.* 1998). Among them, the most investigated are the human quiescin Q6, whose mRNA level is increased when lung fibroblasts enter reversible quiescence (Coppock *et al.* 1993, 1998), sulfhydryl oxidases from chicken egg white (Hoober *et al.* 1996, 1999*a*, Hoober & Thorpe 1999), guinea-pig endometrial cells (SOx-3, Musard *et al.* 2001), mouse epidermis (Matsuba *et al.* 2002) and human neuroblastoma cells (SOXN, Wittke *et al.* 2003). QSOX proteins catalyze the formation of disulfide bonds in peptides and proteins with reduction of molecular oxygen to hydrogen peroxide (H_2O_2). They are secreted and are thought to be involved in a broad range of essential cell functions such as elaboration of the extracellular matrix, protein folding, control of the cell cycle and regulation of the redox state through their oxidative effects (Coppock *et al.* 1998, Hoober & Thorpe 1999, Hoober *et al.* 1999*b*, Thorpe *et al.* 2002).

rQSOX was first identified in the reproductive tract (Chang & Morton 1975) and its mRNA was recently sequenced from seminal vesicles where the protein is highly expressed (Benayoun *et al.* 2001). It was also detected in a wide range of rat tissues (Benayoun *et al.* 2001, Mairet-Coello *et al.* 2002). In a previous paper, we demonstrated that two *rQSOX* transcripts are expressed throughout the rat brain, the predominant one probably corresponding to the unique transcript detected in peripheral organs (Mairet-Coello *et al.* 2002). We also mapped the rQSOX protein in the adult rat brain (Mairet-Coello *et al.* 2004) showing that it is differentially distributed in the central nervous system, notably with a strong expression in neuron populations displaying a high amount of disulfide bond-containing peptides. In the supraoptic

DOI: 10.1677/joe.1.05842
0022–0795/04/0183–353
Online version via http://www.endocrinology-journals.org

nucleus, where its ultrastructural localization was investigated, it is essentially present in the Golgi apparatus and dense-core granules.

An interesting field of research is the study of the estrogen responsiveness of *QSOX* genes. Effects of the estrogen status on these enzymes are not well documented yet. The *SOx-3* and *Q6* genes have been shown to be down-regulated by estrogens respectively in endometrial cells (Musard *et al.* 2001) and MCF-7 breast cancer cells (Inoue *et al.* 2002); but no investigations have been undertaken concerning estrogen effects on *rQSOX* expression. Estrogens are important physiological regulators of the secretory activity of the anterior pituitary lobe. In that gland, they exert gene- and cell-specific regulations with positive or negative actions. For example, estrogens stimulate prolactin (PRL) synthesis and secretion in the lactotrophs (Perez *et al.* 1986, Shull *et al.* 1987, Maeda *et al.* 1996) and regulate luteinizing hormone (LH) and follicle-stimulating hormone (FSH) synthesis and secretion in the gonadotrophs (Shupnik 1996, McNeilly *et al.* 2003). While mapping rQSOX distribution in rat brain, our attention was attracted by an intense labeling of many adenohypophyseal cells. In the present work, we thus investigated *rQSOX* expression in the adult rat pituitary using RT-PCR and immunohistochemistry; we determined its subcellular localization and characterized pituitary cell populations expressing it by double-immunohistochemical procedures. Finally, a semiquantitative RT-PCR protocol was used to analyze the putative changes of *rQSOX* mRNA levels in pituitaries of adult female rats throughout the estrous cycle as well as in ovariectomized rats treated or not with 17 β-estradiol.

Materials and Methods

Animals and experimental protocols

Adult male and female 250–300 g Sprague–Dawley rats (IFFA Credo, L'Arbresle, France) were housed in a temperature-controlled environment under natural light conditions with food and water available *ad libitum*. All animal manipulations and experimental protocols were performed according to the recommendations of our institution and under the supervision of authorized investigators.

Estrous cycle experiments

The estrous cyclicity was monitored by daily cytological examination of vaginal smears between 1000 h and 1200 h. Only rats exhibiting at least two consecutive 4-day estrous cycles were selected for the study. Animals were killed by decapitation at 1400 h on each day of the estrous cycle: proestrus (P), estrus (E), metestrus (M) and diestrus (D) (five animals were used for each data point of the cycle). Pituitary glands were rapidly removed, frozen over liquid nitrogen and stored at −80 °C until RNA isolation.

Ovariectomy and estrogen administration

Twelve adult female rats were bilaterally ovariectomized (OVX) under ketamine/xylazine anesthesia. After a 3-week recovery period, OVX rats were divided into two groups of six rats. The first group (OVX+E_2, $n=6$) received a daily estrogen injection (β-estradiol 3-benzoate, Sigma; 25 μg/kg body weight, diluted in 5% ethanol, s.c.) for 9 days. The second group (OVX, $n=6$) received the ethanol vehicle injection (equivalent volume, s.c.) for the same period. The rats were killed by decapitation 4 h after the last injection and the pituitary glands were collected, frozen over liquid nitrogen and stored at −80 °C until RNA preparation. The experiment was repeated twice. To assess the effects of estradiol chronic administration, all rats were weighed before the first and after the last injection; then, body weights of the treated group were compared with those of the control group. In addition, at the end of the experiments, uteruses were isolated and macroscopically examined to compare their size in treated animals and controls.

Light microscope immunohistochemistry

Four male and four female rats were deeply anesthetized with 7% chloral hydrate solution (5 ml/kg body weight). After transcardial perfusion of the animals with 300 ml 0·9% NaCl followed by 300 ml of fixative (ice-cold 4% paraformaldehyde solution in 0·1 M phosphate buffer (PB), pH 7·2), pituitaries were removed, post-fixed in the same fixative for 2 h at 4 °C, and immersed overnight in a 15% cryoprotective sucrose solution in 0·1 M PB at 4 °C. They were embedded in a commercial medium (Cryomatrix, Shandon, Pittsburgh, PA, USA), quickly frozen over liquid nitrogen, then serially cut at a thickness of 10 μm with a cryostat-microtome; the sections were mounted on gelatinized glass-slides and stored at −45 °C until treatment.

For rQSOX detection, after rinsing in 0·1 M PBS (pH 7·2), containing 0·3% triton X-100 (PBS-T), sections were submitted to a classical indirect immunofluorescence protocol. As previously described (Mairet-Coello *et al.* 2004), sections were incubated overnight with the rabbit rQSOX polyclonal antiserum prepared by Benayoun *et al.* (2001) and diluted 1/500 in a PBS-T solution containing 10% lactoproteins, 1% BSA and 0·01% sodium azide. After washing in PBS-T, sections were then exposed for 1 h to secondary goat anti-rabbit Alexa Fluor 488-conjugated immunoglobulin G (Molecular Probes, Interchim, Montluçon, France; diluted 1/400 in the same solution as the primary). The rQSOX antiserum we used revealed only one protein as attested by Western blots on

seminal vesicle fluid and whole brain homogenates (Benayoun *et al.* 2001, Mairet-Coello *et al.* 2004). In the present study, specificity of the stainings was also checked by incubating the rQSOX antiserum (1/500) for 4 h at room temperature with the antigen solution (0·03 nmol of rQSOX protein purified from seminal vesicle fluid per microliter of non-diluted antiserum) prior to using it for immunostaining. Sections were observed under a fluorescence microscope (Olympus BX51).

In order to characterize which anterior pituitary cell populations express rQSOX, double- immunohistochemical procedures were performed either on cryostat or on semi-thin sections by combining peroxidase anti-peroxidase (PAP) and indirect immunofluorescence methods. Cryostat sections were first incubated overnight with the rQSOX antiserum and revealed through the PAP (DAKO, Trappes, France) procedure using 3,3′-diaminobenzidine (DAB), then treated with the second primary (anti-LH (1/200), anti-FSH (1/200), anti-growth hormone (GH, 1/200), anti-PRL (1/200), anti-β-endorphin (1/200) from Biogenex Laboratories, San Ramon, CA, USA; and anti-thyroid stimulating hormone (TSH, 1/50) from DAKO) which was revealed using a secondary anti-rabbit antibody conjugated to Alexa Fluor 488 (1/400) or a fluorescein isothiocyanate (FITC)-labeled anti-mouse antibody (1/100, Jackson Immunoresearch Laboratories Inc., Interchim, Montluçon, France) for the monoclonal anti-TSH antibody. Semi-thin sections were obtained from thick sections treated with the rQSOX antiserum and revealed through the PAP procedure before embedding (see below). They were incubated overnight with the second primary and revealed as described above.

Electron microscope immunohistochemistry

The technique was adapted from Li *et al.* (2000). Briefly, male rats anesthetized with 7% chloral hydrate solution were perfused at room temperature with 0·1 M PB containing 4% paraformaldehyde, 15% saturated picric acid (v/v) and 0·05% glutaraldehyde. Pituitary glands were quickly removed. The anterior and the posterior lobes of the pituitaries were separated, placed in 0·1 M PB and immersed for 30 min in 0·05 M PB containing 25% sucrose and 10% glycerol (v/v) for cryoprotection. Anterior pituitaries were then quickly frozen over liquid nitrogen and cut into serial sections (40 μm thick) on a slicer microtome. Slices were immersed for 1 h in 0·05 M Tris-buffered saline (pH 7·4) containing 20% normal goat serum. After a 60 h incubation at 4 °C with the rQSOX antiserum (1/500 in 0·05 M Tris-buffered saline, pH 7·4, containing 2% goat serum), they were exposed for 24 h to goat anti-rabbit immunoglobulin G (1/100, P.A.R.I.S., Compiègne, France) followed by a 24 h incubation with PAP (1/200). Peroxidase was revealed with 0·02% DAB in 0·05 M Tris–HCl containing 0·003% H_2O_2. Slices were then embedded in araldite and cut into 1 μm sections for double immunohistochemistry. For the electron microscope study, slices were incubated for 1 h in 1% osmium tetroxide in 0·1 M PBS, then embedded and sectioned in ultrathin sections before being examined on a Jeol 1230 electron microscope.

Photomicrograph production

For light microscopy, digital image acquisitions were carried out using a DP50 Olympus camera and the AnalySIS 3·1 software (Soft Imaging System). Electron micrographs were also numerized through the camera associated with the Jeol microscope using AnalySIS software. Images were imported in Adobe Photoshop 7·0 software for treatment. Transformation of color images into black and white images was performed using gray-scale functions minimally altering the captured images.

Total RNA extraction

Total RNAs were extracted from individual pituitaries using an RNA extraction kit (RNA NOW, Ozyme, Saint Quentin en Yvelines, France) according to the manufacturer's instructions and as previously described (Mairet-Coello *et al.* 2002). RNA concentrations were determined by 260 nm absorbance using a spectrophotometer (Eppendorf, Le Pecq, France).

RT-PCR and semiquantitative RT-PCR

Denatured total RNAs (1 μg) were reverse transcribed into cDNAs in a 20 μl reaction mixture consisting of 4 μl of 5 × reverse transcriptase reaction buffer (Promega), 5 μM random hexamer primers (Amersham), 1·25 mM mixture of the four deoxyribonucleotides (Roche), 20 U RNasin® (Promega) and 100 U Moloney murine leukemia virus reverse transcriptase (Promega). RT reactions were incubated for 10 min at 23 °C, 1 h at 42 °C, 5 min at 95 °C and cooled to 4 °C in a PTC-200 thermocycler (MJ-Research, Fontenay-sous-Bois, France).

PCRs were performed to amplify a 797 bp fragment of *rQSOX* cDNA (base 17 to base 813, GenBank accession number NM_053431) using the fluorescent *rQSOX* sense primer 5′-6-FAM-ACTTGAGCGAGGTGGACAGTC AAG-3′ and the *rQSOX* antisense primer 5′-AGCACA GGCACTCGGGAA-3′ (Eurogentec, Seraing, Belgique; Benayoun *et al.* 2001, Mairet-Coello *et al.* 2002). For quantitative experiments, additional primers amplifying a 509 bp fragment of cyclophilin cDNA (*CYC*, GenBank accession number M19533), a housekeeping gene, were included in the PCR mixture as co-amplified internal control for normalization (*CYC* sense primer 5′-CGCC GCTTGCTGCAGACATGG-3′ and fluorescent *CYC* antisense primer 5′-6-FAM-GAGTTGTCCACAGTCG GAGATGG-3′). All PCR amplifications were carried out

in the PTC-200 thermocycler using the FastStart Taq DNA polymerase kit (Roche) in a 20 µl final reaction mixture. Each PCR reaction contained 0·8 µl of the RT product, 2 µl of 10 × PCR buffer, 3·75 mM $MgCl_2$, 0·5 mM mixture of the four deoxyribonucleotides, 1 U Taq DNA polymerase, and *rQSOX* and *CYC* primers at optimal concentrations (see Results). Conditions of amplification were established simultaneously with the two sets of primers, by varying the primer dilutions, the cDNA matrix concentration and the number of cycles. Optimal cycle number was determined over a range of 21–35 cycles and was chosen at the beginning of the exponential phase of amplification. Thermocycling parameters were as follows: one step of PCR enzyme activation at 95 °C for 5 min followed by 27–30 cycles (see Results) of denaturation at 95 °C for 30 s, annealing at 63 °C for 30 s and extension at 72 °C for 1 min 30 s. The first investigations to know whether *rQSOX* is expressed in the rat pituitary involved a standard 30 cycle PCR using a single set of non-fluorescent *rQSOX* primers (1·5 µM for each primer). For the qualitative detection of *rQSOX* in the pituitary, 18 µl of *rQSOX*-PCR product were separated by electrophoresis on a 1·5% agarose gel containing ethidium bromide and visualized under u.v. illumination using Gel Doc 2000 (BioRad) and the BioRad Quantity One 4·2·3 software. For the quantitative part of the protocol, each sample was denatured by heating for 2 min at 95 °C and snap-cooled before loading. Fluorescent PCR products (1 µl) were separated on a 24 cm 6% polyacrylamide denaturing gel for 6 h at 1200 V on a 373 A DNA sequencer (Applied Biosystems, Courtaboeuf, France). In each electrophoresis lane, intensities of cDNA fluorescent fragments, corresponding to *rQSOX* (797 bp) and *CYC* (509 bp) were detected at an absorbance of 500 nm. Electrophoretic data were automatically analysed and sized in base pairs by GENESCAN Analysis software and reconstructed as a gel image.

Statistical analyses

Although internal control compensates for the inherent inter-assay variability of RT-PCR reactions, two RTs were performed for each sample followed by two PCRs for the estrous cycle study and one for the estrogen replacement experiment. Results used for statistics correspond to the average obtained for each animal. *rQSOX* fluorescence intensity was normalized to *CYC* fluorescence. All data are presented as percentages of the controls. The controls (100%) correspond to the value at metestrus for estrous cycle experiments and to the value of vehicle-treated animals for estrogen injection experiments. Statistical analyses were performed using the GraphPad InStat 3·05 software. Statistical significance was evaluated by unpaired one-way ANOVA test for multiple groups and by unpaired parametric Student's *t*-test for comparisons between two groups. Differences were considered statistically significant when $P<0·05$.

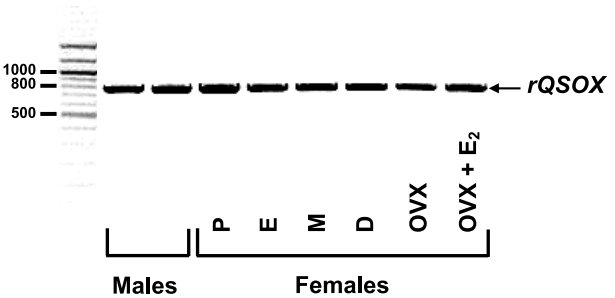

Figure 1 RT-PCR detection of *rQSOX*. RT-PCR were performed on 1 µg of total RNAs extracted from each sample, using specific *rQSOX* primers. After agarose gel electrophoresis and ethidium bromide staining, a band of 797 bp was detected in pituitaries of male (two separate individuals), cycling female (P, proestrus; E, estrus; M, metestrus; D, diestrus) and untreated (OVX) or 17 β-estradiol ($OVX+E_2$)-treated ovariectomized rats.

Results

Detection of QSOX expression in rat pituitary

Using RT-PCR analysis, an *rQSOX* fragment of the 797 bp expected size was reproducibly observed in pituitary homogenates from male rats, gonadally intact female rats and ovariectomized vehicle-(OVX) or E_2 ($OVX+E_2$)-treated rats (Fig. 1). The amplified fragment corresponds to a part of the 5′ region common to the two transcripts previously identified in the brain (Mairet-Coello *et al.* 2002).

Light and electron immunocytochemical observations

On cryostat sections, numerous cells were more or less intensely labeled by the rQSOX antiserum throughout the anterior pituitary. The labeling was located in the cytoplasm, being either concentrated at one pole of the cell or staining the whole cytoplasm and exhibiting a more or less granular aspect (Fig. 2A). Preincubating the antiserum with the corresponding antigen completely abolished the immunolabeling (Fig. 2B and C). At the ultrastructural level, rQSOX immunoreactivity was associated with the rough endoplasmic reticulum and golgian compartments; more precisely, a dark labeling underlined the membranes of the rough endoplasmic reticulum cisternae (Fig. 2D) and saccules of the Golgi apparatus (Fig. 2E). A deposit was also noticed either in the dense core (Fig. 2F) or sometimes along the membrane (Fig. 2D) of secretory granules dispersed among large amounts of unlabeled ones. Double immunostainings with antisera to the main pituitary hormones did not allow clear definition of the nature of all rQSOX-positive cells. Only some cells detected by

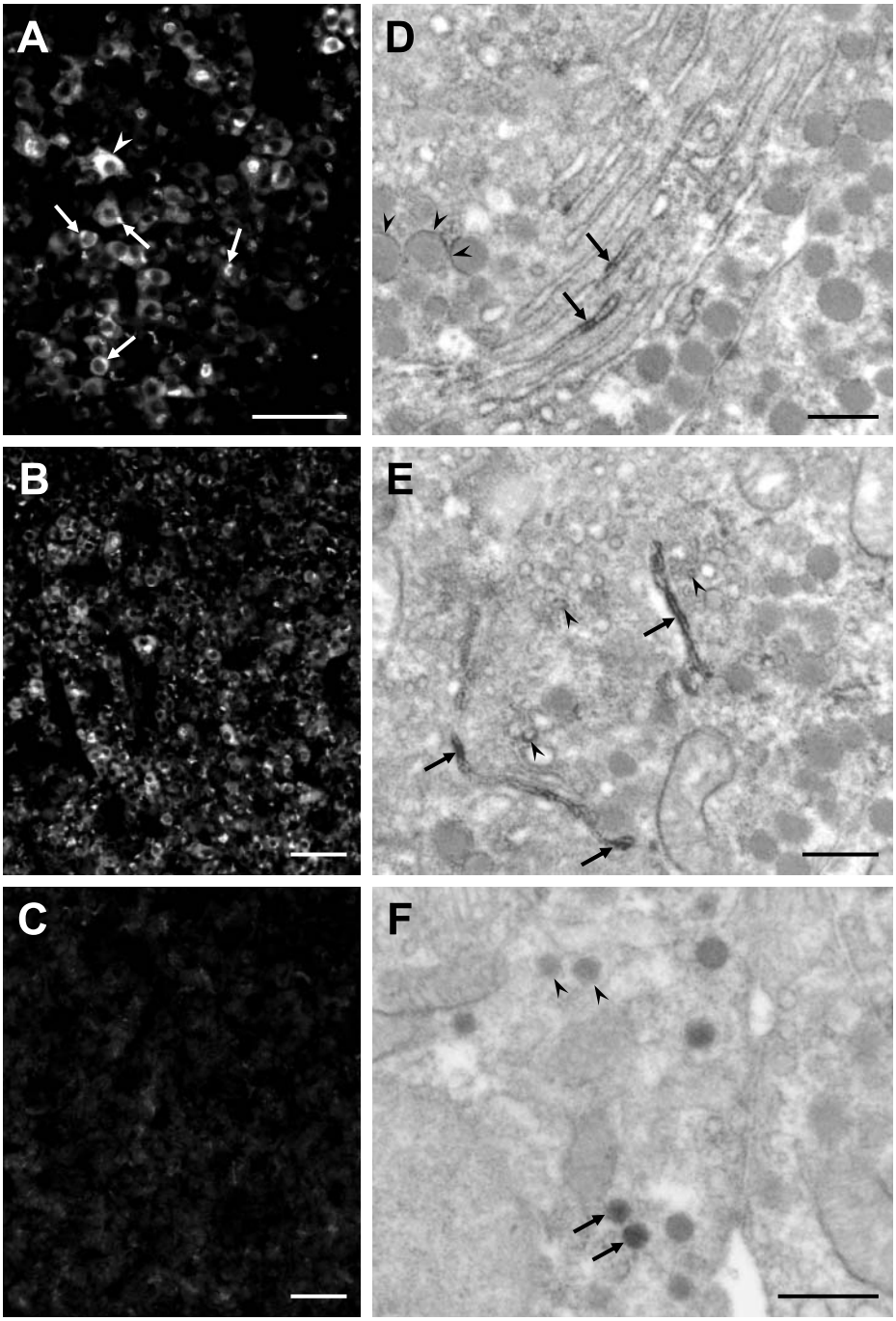

Figure 2 Immunolocalization of rQSOX. (A, B and C) Light microscope photographs: (A) in adenohypophyseal cells, the rQSOX immunolabeling is located in the whole cytoplasm (arrowhead) or concentrated at one pole of the cell (arrows); (B and C) preincubation of the rQSOX antiserum with the antigen completely abolished the labeling (C) observed in the precedent section (B). (D, E and F) Electron microscope photographs: (D) the labeling is associated with the membranes of ergastoplasmic cisternae (arrows) and the membranes of some secretory granules (arrowheads); (E) labeled membranes of saccules of the Golgi apparatus cut into longitudinal (arrows) or transversal (arrowheads) planes; (F) immunoreactive dense-core granules (arrows) among unlabeled ones (arrowheads). Scale bars: 50 µm (A, B and C); 500 nm (D, E and F).

LH and FSH antiserums undoubtedly showed a double labeling. In order to clarify these observations, antisera to the different pituitary hormones were applied to semi-thin sections obtained after an rQSOX-PAP detection performed at a pre-embedding step (see Materials and Methods). Results confirmed that cells showing the strongest rQSOX labeling were those recognized by LH (Fig. 3A and B) and FSH antisera (Fig. 3C and D). Few GH (Fig. 3E and F) and TSH (Fig. 3G and H) cells were lightly stained by the rQSOX antiserum. Very few lactotrophs and corticotrophs were slightly labeled.

Study of a putative effect of estrogens on QSOX expression in rat adenohypophysis

During the estrous cycle It is well established that estrogen plasma concentrations undergo large variations during the estrous cycle. Thus, we examined the effect of these physiological variations on *rQSOX* mRNA expression in intact cycling rats. The optimization of semiquantitative RT-PCR conditions (see Materials and Methods) was carried out on pituitaries from rats killed at the metestrus stage. Primers were used at 187·5 nM for each rQSOX primer and 37·5 nM for each CYC primer and PCR amplifications were performed for 27 cycles. The amounts of *rQSOX* mRNA in the pituitary showed moderate changes during the course of the estrous cycle (Fig. 4). Relative *rQSOX* mRNA levels (normalized to the housekeeping gene cyclophilin) were highest during estrus and then declined to reach the lowest levels at the proestrus stage (35% decrease compared with the estrus stage).

After ovariectomy and chronic 17 β-estradiol administration The responsiveness of the animals to the treatment was verified by comparison of body weights between the control (OVX) and the estradiol-treated (OVX+E_2) groups before the first and after the last vehicle or estradiol injections. Before treatment, the average weights of the two groups of ovariectomized rats were not significantly different (control group, 344·6 ± 11·19 g; estradiol-treated group, 327·2 ± 6·91 g; $P>0·1$). Conversely, after the ninth injection, the vehicle-treated ovariectomized rats (349·6 ± 11·61 g) were significantly heavier than those that had received estradiol (306·5 ± 6·39 g, $P<0·005$). Between the beginning and the end of the treatment, the estradiol-treated group lost weight (− 20·7 ± 1·71 g) whereas the control group gained weight (+5 ± 2·1 g, $P<0·0001$). Moreover, macroscopic examination showed that uteruses of the estradiol-treated rats were larger than those of the controls. As for the amounts of pituitary *rQSOX* mRNA, optimal conditions of PCR amplifications were established for vehicle-treated rat pituitaries. Each *rQSOX* primer was used at 1·5 μM, each *CYC* primer was diluted at 8·3 nM and PCRs were performed for 30 cycles. The treatment of OVX rats with E_2 caused a significant average increase of 56% in the ratio of *rQSOX* mRNA/*CYC* mRNA compared with the OVX control group ($P=0·0069$) (Fig. 5A and B).

Discussion

Expression of rQSOX in the anterior pituitary

Previous studies showed that rQSOX is widely distributed throughout the rat brain (Mairet-Coello *et al.* 2002, 2004) and is present in most of the peripheral organs with a high amount in the male reproductive tract (Benayoun *et al.* 2001, Mairet-Coello *et al.* 2002). Despite this ubiquitous expression, few studies have been devoted to sulfhydryl oxidases. The present data demonstrate the occurrence of rQSOX in rat pituitary. It is expressed both in male and female animals and the protein is abundant throughout the anterior lobe. The subcellular localization of rQSOX in the adenohypophysis is in agreement with our previous data showing the association of the protein with the Golgi apparatus and the dense-core granules in neurons of the supraoptic nucleus (Mairet-Coello *et al.* 2004). In anterior pituitary cells, the protein was also found to be linked to the membranes of the ergastoplasmic compartment, as was shown for human QSOX (Thorpe *et al.* 2002). These results suggest that QSOX proteins, which possess a putative signal peptide, can follow the secretory pathway and probably play a role in the extracellular space, as previously proposed (Coppock *et al.* 1993, Hoober & Thorpe 1999, Hoober *et al.* 1999*b*, Benayoun *et al.* 2001, Thorpe *et al.* 2002, Mairet-Coello *et al.* 2004).

The strong expression of rQSOX in a secretory gland such as the hypophysis is not surprising. Indeed, sulfhydryl oxidases are enzymes that catalyze the formation of disulfide bonds (Hoober *et al.* 1996, Benayoun *et al.* 2001) and most eukaryote secreted proteins contain disulfide bridges (Wittrup 1995). Disulfide bonds and cysteine thiols could play a decisive role in protein folding and secretion; their manipulation affects intracellular transport and release (Wittrup 1995, Van Horssen *et al.* 1998, Gorr *et al.* 1999). QSOX proteins have been shown to be abundantly expressed in secretory tissues producing disulfide-containing peptides and proteins such as skin apocrine glands, parotid glands, pancreas and small intestine (Thorpe *et al.* 2002); in addition, we recently reported an intense rQSOX immunoreactivity in neurons synthesizing neuropeptides or neurohormones which possess disulfide bridges such as melanin-concentrating hormone, hypocretin/orexin, vasopressin and ocytocin (Mairet-Coello *et al.* 2004). Although rQSOX immunoreactivity was more or less detected in most of the hormone-secreting cell types of the adenohypophysis (gonadotrophs, somatotrophs, lactotrophs and thyrotrophs), double-immunolabeling experiments provided evidence that the

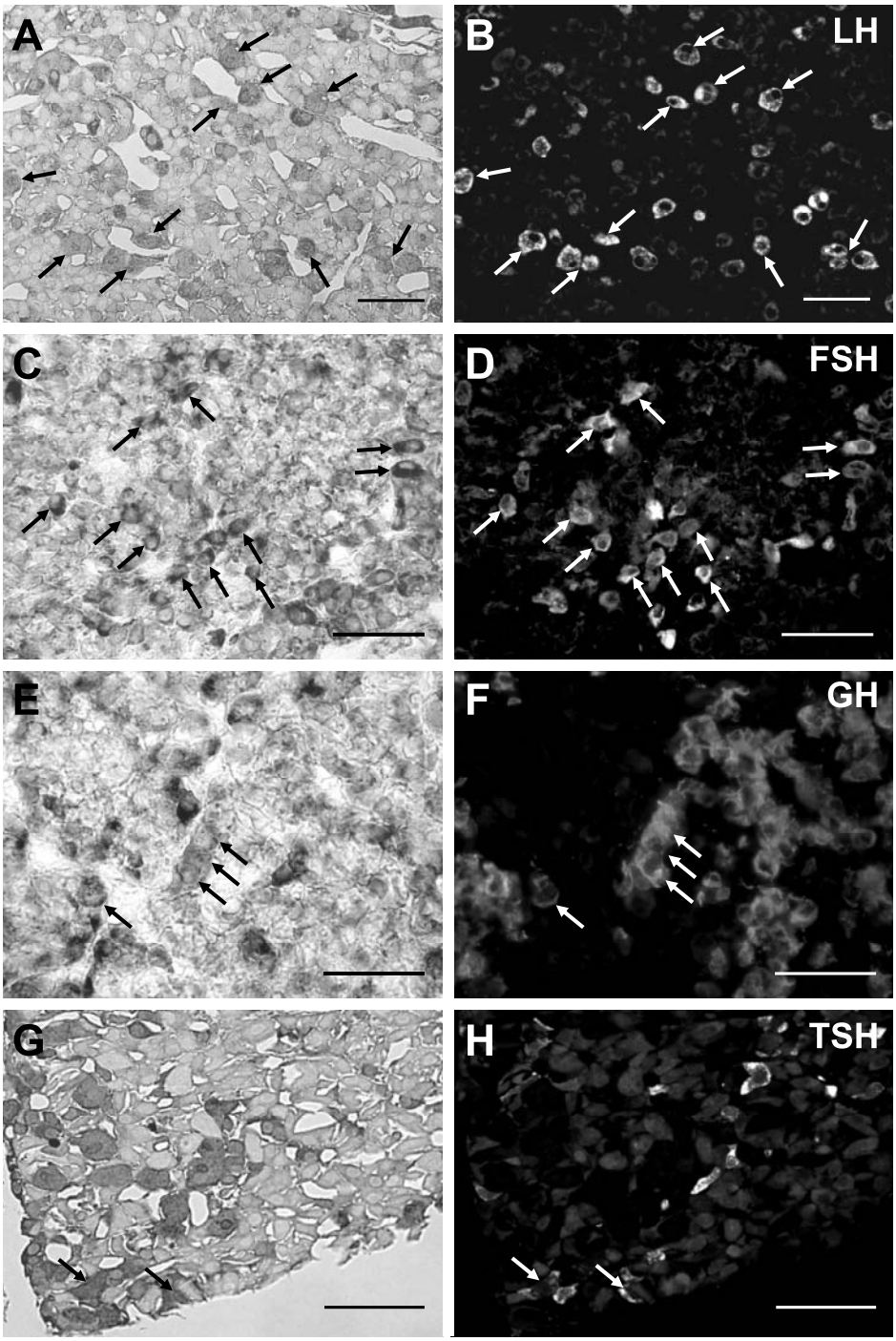

Figure 3 Double immunohistochemistry for rQSOX (PAP) and the main anterior pituitary hormones (immunofluorescence). (A and B) Numerous LH-positive cells (B) are also positive for rQSOX (A). (C and D) Most of the FSH gonadotrophs (D) are also labeled for rQSOX (C). (E and F) Some somatotrophs (F) are slighly stained by the rQSOX antiserum (E). (G and H) Few cells are double labeled for TSH (H) and rQSOX (G). Scale bars: 50 µm.

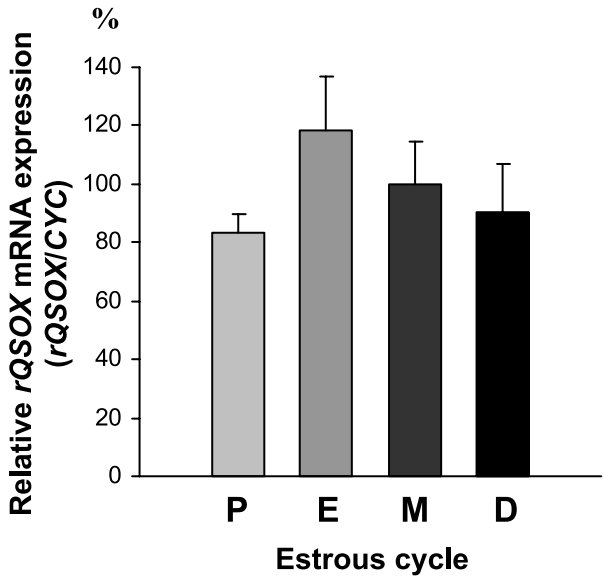

Figure 4 Variations of *QSOX* mRNA levels in pituitary glands of female rats across the estrous cycle as determined by semiquantitative RT-PCR. Pituitaries were collected at 1400 h on proestrus (P), estrus (E), metestrus (M) and diestrus (D) stages. Total RNAs (1 µg) from individual pituitaries were reverse transcribed and relative quantitative PCR analyses were performed using fluorescent primers. The intensity of the fluorescent products was quantified on a 373 A DNA sequencer and analysed with the GENESCAN Analysis software. *rQSOX* cDNA level was normalized to that of the coamplified internal control cDNA signal (cylophilin, *CYC*). For each stage, data from five rats are shown as means ± S.E.M. of two independent RTs followed by two PCRs per RT. Results are expressed as a percentage after setting the level in the metestrus stage at 100%.

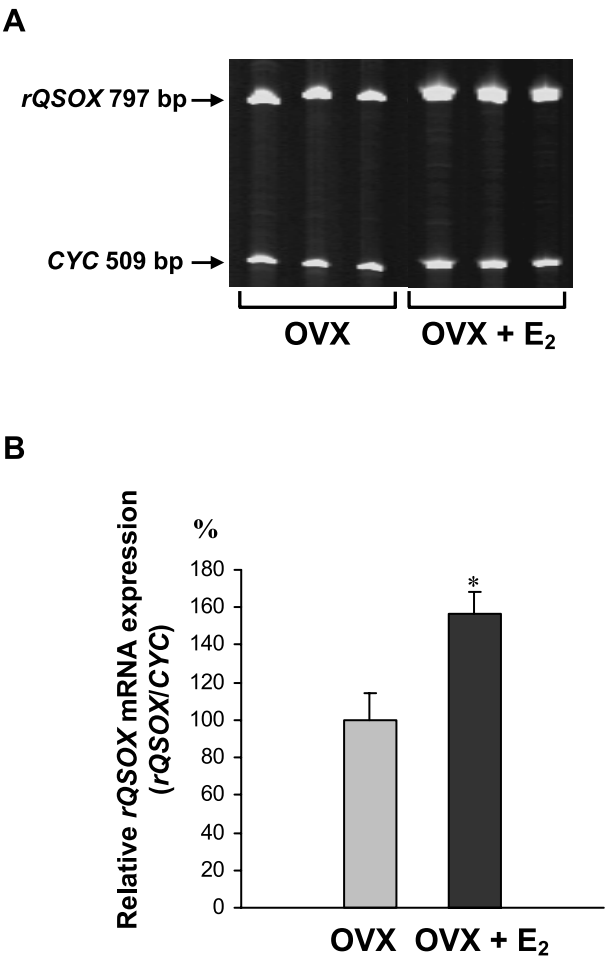

Figure 5 Effects of 17 β-estradiol replacement on *QSOX* mRNA content in pituitaries of ovariectomized rats. After ovariectomy, animals received, for 9 days, a daily s.c. injection of vehicle (OVX) or estradiol benzoate (25 µg/kg per day, OVX+E_2). Total RNAs were prepared from each pituitary and analysed by semiquantitative RT-PCR as indicated in Fig. 4. (A) Representative fluorescent PCR signals from OVX and OVX+E_2 rats on polyacrylamide gel reconstructed by GENESCAN software. (B) Bars represent the ratio of *rQSOX* cDNA levels normalized to the cyclophilin (*CYC*) signal. Data are shown as means ± S.E.M. of two independent series, each containing six control rats (OVX) and six rats treated by estradiol (OVX+E_2); two independent RT-PCR were assayed for each sample. Results are expressed as a percentage after setting the level in the vehicle control at 100%. The asterisk indicates that the values are significantly different between the two groups, using Student's *t*-test (*P=0·0069).

anterior pituitary cells strongly expressing rQSOX correspond to a subset of gonadotrophs. However, more detailed analyses are needed to clarify whether rQSOX-positive cells belong to the subpopulation of gonadotrophs which expresses both LH and FSH and/or to monohormonal subsets (Kovacs & Horvath 1985). LH and FSH are members of the glycoprotein hormone family which regroups non-covalently bounded heterodimers made up of the assembly of a common α-subunit and a distinct β-subunit that confers biological specificity. Interestingly, each subunit contains several cysteine amino acid residues that are involved in multiple intramolecular disulfide linkages which are required to allow subunit interactions to form the biologically active dimers (Reeve & Pierce 1981, Chin 1985, Muyan *et al.* 1998). Thus, in addition to its action on extracellular components, rQSOX could also participate intracellularly in different steps of oxidizing folding of secreted proteins, from the initial formation of disulfide linkages that occurs in the rough endoplasmic reticulum until the maturation and the maintenance of the disulfide-bonded protein structure in the golgian compartment and the secretory vesicles. It still remains to be verified whether rQSOX and disulfide-containing hormones like LH and FSH are colocalized in the same compartments and whether they are co-secreted. Although TSH, GH and PRL contain intra-chain disulfide bridges (Reeve & Pierce 1981, Chin 1985, Seo 1985), rQSOX was slightly expressed by thyrotrophs, somatotrophs and lactotrophs. This means that rQSOX preferentially exerts its oxidizing activities on LH and FSH

while the other hormones probably use other sulfhydryl oxidases or mechanisms of disulfide bond production.

Interestingly, an intense rQSOX labeling was observed in neurosecretory neurons of the hypothalamus that control the adenohypophyseal secretions – such as the parvicellular cells of the anterior periventricular, median preoptic, paraventricular and arcuate nuclei (Mairet-Coello *et al.* 2004). The cellular redox state might regulate secretion and action of hormones; for example, nitric oxide and thioredoxin, two redox-modulatory substances, could be implicated in the hypothalamic–pituitary functions, notably in the modulation of GH (Kato 1992, Hata *et al.* 2001) and LH secretions (McCann *et al.* 1996, 2003); cellular glucocorticoid hormone responsiveness is negatively modulated by antisense thioredoxin expression and treatment with H_2O_2 (Makino *et al.* 1996). A rapidly increasing body of evidence indicates that reactive oxygen species, in particular H_2O_2, could play an important role as signaling molecules involved, for example, in cellular proliferation or activation, growth inhibition or cell death (Nakamura *et al.* 1997, Gabbita *et al.* 2000, Hensley *et al.* 2000). Through the production of H_2O_2, rQSOX could thus act as an endogenous cellular redox modulator in the hypothalamic–pituitary axis.

Effect of estrogens on rQSOX expression in the pituitary

Our experimental data, together with the variations of *rQSOX* expression during the estrous cycle, suggest that *rQSOX* expression is related to the estrogen level. The quantitative experiments were conducted on homogenates from whole pituitaries and so, they show global variations of *rQSOX* expression. However, it cannot be excluded that the variations could be restricted to specific cell populations of the pituitary. rQSOX immunoreactivity (see above) was present in almost all of the adenohypophysis cell types but was predominant in the gonadotrophs which constitute 5–10% of the total anterior pituitary cells (Dada *et al.* 1983). If the changes of its expression are restricted to this small population (or another), it could partially explain the moderate variations detected across the estrous cycle and the lack of statistical significance. Moreover, two *rQSOX* transcripts, probably arising from an alternative splicing, have been identified in rat brain by Northern blot, but only the short transcript was revealed in several peripheral organs (Mairet-Coello *et al.* 2002). A sulfhydryl oxidase activity has been shown for the protein encoded by the short mRNA (Benayoun *et al.* 2001), but no investigations have been undertaken yet for the putative protein encoded by the long transcript. The primers we used for the semiquantitative PCR analysis are located in the 5′ part of the cDNA sequence common to the two presumed transcripts and this did not enable us to distinguish them by PCR. We do not know yet whether the second transcript is also expressed in the pituitary; if this is the case, the quantifications realized could correspond to the expression of both mRNAs; however, it is also possible that they are differentially regulated by estrogens. The existence of such a differential regulation has been previously reported in the literature; for example, several estrogen receptor mRNA isoforms were specifically modulated in rat pituitaries throughout the estrous cycle as well as in response to steroid hormones (Friend *et al.* 1995, 1997). Further experiments are thus necessary to determine whether both *rQSOX* splice variants are present in the pituitary and, if that is the case, to analyze each of them separately.

In our conditions, higher *rQSOX* expression was seen at estrus and the minimum expression at proestrus, suggesting that it may be linked to the levels of physiological sex steroids. In the rat, the estrogen peak is reached in the late afternoon of the proestrus stage, approximately 8 h before the beginning of estrus (El Meskini *et al.* 1997). This implies either that *rQSOX* responds to the elevation of the estrogen level with a delay, or that the peak of *rQSOX* expression happens during a period of the estrous cycle that we did not examine, i.e. between the afternoon of proestrus and the early afternoon of estrus. Then, *rQSOX* expression declines slowly after the estrogen level falls to reach a minimum at the proestrus stage just before the following peak of estrogens. The administration of 17 β-estradiol to ovariectomized rats showed that *rQSOX* expression is up-regulated by estrogens. But we cannot exclude an influence of progesterone impregnation as well. The guinea-pig sulfhydryl oxidase *SOx-3* was discovered by looking for estrogen-regulated genes and its levels of expression in the endometrium changed across the estrous cycle. Conversely to *rQSOX* in rat pituitary, *SOx-3* expression in guinea-pig endometrium was lowered during the estrus phase and was supposed to be down-regulated by estrogens (Musard *et al.* 2001). Moreover, a screening of estrogen genomic effects has recently demonstrated that the human quiescin *Q6* gene is repressed by estrogens in MCF-7 breast cancer cells (Inoue *et al.* 2002). These data suggest species- and/or tissue-specific modulations of *QSOX* gene expression. Tissue-dependent regulation by estrogens has been previously described for several other genes such as vasoactive intestinal peptide (Kasper *et al.* 1992), angiotensinogen (Gordon *et al.* 1992) and gastrin-releasing peptide (Whitley *et al.* 2000).

The apparent delayed response during the estrous cycle and the sustained increase of *rQSOX* level in pituitary after chronic administration of estrogens raise the possibility of indirect estrogenic effects. Indeed, estrogens are known to affect pituitary function either directly or indirectly via the hypothalamus. The majority of gonadotrophs do not express estrogen receptors (Mitchner *et al.* 1998) and there is a general consensus that estrogens affect the gonadotrophs mainly via the hypothalamus (McCann *et al.* 1998). For example, it is well established that estrogens regulate the reproductive hormones LH and FSH by primarily altering the release of gonadotropin-releasing hormone

(GnRH) (Shupnik 1996). In the same way, estrogens could affect *rQSOX* expression in pituitary indirectly through the hypothalamic gonadoliberins.

In conclusion, we report the occurrence of QSOX throughout the anterior lobe of rat pituitary, mainly in gonadotrophs. Associated with intracellular compartments belonging to the secretory pathway, this enzyme may modulate secretory activity by participating in the folding of hormones containing disulfide bonds and by regulating the redox state. We also show that *rQSOX* expression is correlated to estrogen levels. However, further investigations are needed to elucidate the mechanisms of its regulation and its precise role in the pituitary.

Acknowledgements

The authors thank Dr F Esnard and Dr A Esnard-Fève (INSERM EMI-U-0010, Université François Rabelais, Tours, France) for providing the rQSOX antiserum and Dr A Gouget (Service de Cytogénétique, Centre Hospitalier Universitaire, Besançon, France) for her expert technical advice.

Funding

This work was supported by the Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche. There are no conflicts of interest in this study.

References

Benayoun B, Esnard-Feve A, Castella S, Courty Y & Esnard F 2001 Rat seminal vesicle FAD-dependent sulfhydryl oxidase. Biochemical characterization and molecular cloning of a member of the new sulfhydryl oxidase/quiescin Q6 gene family. *Journal of Biological Chemistry* **276** 13830–13837.

Chang TS & Morton B 1975 Epididymal sulfhydryl oxidase: a sperm-protective enzyme from the male reproductive tract. *Biochemical and Biophysical Research Communications* **66** 309–315.

Chin WW 1985 Organization and expression of glycoprotein hormone genes. In *The Pituitary Gland, Comprehensive Endocrinology*, pp 103–125. Ed H Imura. New York: Raven Press.

Coppock DL, Kopman C, Scandalis S & Gilleran S 1993 Preferential gene expression in quiescent human lung fibroblasts. *Cell Growth and Differentiation* **4** 483–493.

Coppock DL, Cina-Poppe D & Gilleran S 1998 The quiescin Q6 gene (QSCN6) is a fusion of two ancient gene families: thioredoxin and ERV1. *Genomics* **54** 460–468.

Dada MO, Campbell GT & Blake CA 1983 A quantitative immunocytochemical study of the luteinizing hormone and follicle-stimulating hormone cells in the adenohypophysis of adult male rats and adult female rats throughout the estrous cycle. *Endocrinology* **113** 970–984.

El Meskini R, Delfino C, Boudouresque F, Hery M, Oliver C & Ouafik L 1997 Estrogen regulation of peptidylglycine alpha-amidating monooxygenase expression in anterior pituitary gland. *Endocrinology* **138** 379–388.

Friend KE, Ang LW & Shupnik MA 1995 Estrogen regulates the expression of several different estrogen receptor mRNA isoforms in rat pituitary. *PNAS* **92** 4367–4371.

Friend KE, Resnick EM, Ang LW & Shupnik MA 1997 Specific modulation of estrogen receptor mRNA isoforms in rat pituitary throughout the estrous cycle and in response to steroid hormones. *Molecular and Cellular Endocrinology* **131** 147–155.

Gabbita SP, Robinson KA, Stewart CA, Floyd RA & Hensley K 2000 Redox regulatory mechanisms of cellular signal transduction. *Archives of Biochemistry and Biophysics* **376** 1–13.

Gordon MS, Chin WW & Shupnik MA 1992 Regulation of angiotensinogen gene expression by estrogen. *Journal of Hypertension* **10** 361–366.

Gorr SU, Huang XF, Cowley DJ, Kuliawat R & Arvan P 1999 Disruption of disulfide bonds exhibits differential effects on trafficking of regulated secretory proteins. *American Journal of Physiology, Cell Physiology* **277** C121–C131.

Hata I, Shigematsu Y, Ohshima Y, Tsukahara H, Fujisawa K, Hiraoka M, Nakamura H, Masutani H, Yodoi J, Kotsuji F, Sudo M & Magumi M 2001 Involvement of thioredoxin in the regulation of growth hormone secretion in rat pituitary cell cultures. *American Journal of Physiology – Endocrinology and Metabolism* **281** E269–E274.

Hensley K, Robinson KA , Gabbita SP, Salsman S & Floyd RA 2000 Reactive oxygen species, cell signaling, and cell injury. *Free Radical Biology and Medicine* **28** 1456–1462.

Hoober KL & Thorpe C 1999 Egg white sulfhydryl oxidase: kinetic mechanism of the catalysis of disulfide bond formation. *Biochemistry* **38** 3211–3217.

Hoober KL, Joneja B, White HB 3rd & Thorpe C 1996 A sulfhydryl oxidase from chicken egg white. *Journal of Biological Chemistry* **271** 30510–30516.

Hoober KL, Glynn NM, Burnside J, Coppock DL & Thorpe C 1999*a* Homology between egg white sulfhydryl oxidase and quiescin Q6 defines a new class of flavin-linked sulfhydryl oxidases. *Journal of Biological Chemistry* **274** 31759–31762.

Hoober KL, Sheasley SL, Gilbert HF & Thorpe C 1999*b* Sulfhydryl oxidase from egg white. A facile catalyst for disulfide bond formation in proteins and peptides. *Journal of Biological Chemistry* **274** 22147–22150.

Inoue A, Yoshida N, Omoto Y, Oguchi S, Yamori T, Kiyama R & Hayashi S 2002 Development of cDNA microarray for expression profiling of estrogen-responsive genes. *Journal of Molecular Endocrinology* **29** 175–192.

Kasper S, Popescu RA, Torsello A, Vrontakis ME, Ikejiani C & Friesen HG 1992 Tissue-specific regulation of vasoactive intestinal peptide messenger ribonucleic acid levels by estrogen in the rat. *Endocrinology* **130** 1796–1801.

Kato M 1992 Involvement of nitric oxide in growth hormone (GH)-releasing hormone-induced GH secretion in rat pituitary cells. *Endocrinology* **131** 2133–2138.

Kovacs K & Horvath E 1985 Morphology of adenohypophyseal cells and pituitary adenomas. In *The Pituitary Gland, Comprehensive Endocrinology*, pp 25–55. Ed H Imura. New York: Raven Press.

Li JL, Wang D, Kaneko T, Shigemoto R, Nomura S & Mizuno N 2000 Relationship between neurokinin-1 receptor and substance P in the striatum: light and electron microscopic immunohistochemical study in the rat. *Journal of Comparative Neurology* **418** 156–163.

McCann SM, Karanth S, Kimura M, Yu WH & Rettori V 1996 The role of nitric oxide (NO) in control of hypothalamic-pituitary function. *Revista Brasileira de Biologia* **56** 105–112.

McCann SM, Kimura M, Walczewska A, Karanth S, Rettori V & Yu WH 1998 Hypothalamic control of gonadotropin secretion by LHRH, FSHRF, NO, cytokines, and leptin. *Domestic Animal Endocrinology* **15** 333–344.

McCann SM, Haens G, Mastronardi C, Walczewska A, Karanth S, Rettori V & Yu WH 2003 The role of nitric oxide (NO) in control of LHRH release that mediates gonadotropin release and sexual behavior. *Current Pharmaceutical Design* **9** 381–390.

McNeilly AS, Crawford JL, Taragnat C, Nicol L & McNeilly JR 2003 The differential secretion of FSH and LH: regulation through genes, feedback and packaging. *Reproduction Supplement* **61** 463–476.

Maeda T, Ikegami H, Sakata M, Yamaguchi M, Wada K, Koike K, Adachi K, Kurachi H, Hirota K & Miyake A 1996 Intraventricular administration of estradiol modulates rat prolactin secretion and synthesis. *Journal of Endocrinological Investigation* **19** 586–592.

Mairet-Coello G, Tury A, Fellmann D, Jouvenot M & Griffond B 2002 Expression of SOx-2, a member of the FAD-dependent sulfhydryl oxidase/quiescin Q6 gene family, in rat brain. *Neuroreport* **13** 2049–2051.

Mairet-Coello G, Tury A, Esnard-Feve A, Fellmann D, Risold PY & Griffond B 2004 The FAD-linked sulfhydryl oxidase QSOX: topographic, cellular and subcellular immunolocalization in adult rat central nervous system. *Journal of Comparative Neurology* **473** 334–363.

Makino Y, Okamoto K, Yoshikawa N, Aoshima M, Hirota K, Yodoi J, Umesono K, Makino I & Tanaka H 1996 Thioredoxin: a redox-regulating cellular cofactor for glucocorticoid hormone action. Cross talk between endocrine control of stress response and cellular antioxidant defense system. *Journal of Clinical Investigation* **98** 2469–2477.

Matsuba S, Suga Y, Ishidoh K, Hashimoto Y, Takamori K, Kominami E, Wilhelm B, Seitz J & Ogawa H 2002 Sulfhydryl oxidase (SOx) from mouse epidermis: molecular cloning, nucleotide sequence, and expression of recombinant protein in the cultured cells. *Journal of Dermatological Science* **30** 50–62.

Mitchner NA, Garlick C & Ben-Jonathan N 1998 Cellular distribution and gene regulation of estrogen receptors alpha and beta in the rat pituitary gland. *Endocrinology* **139** 3976–3983.

Musard JF, Sallot M, Dulieu P, Fraichard A, Ordener C, Remy-Martin JP, Jouvenot M & Adami P 2001 Identification and expression of a new sulfhydryl oxidase SOx-3 during the cell cycle and the estrous cycle in uterine cells. *Biochemical and Biophysical Research Communications* **287** 83–91.

Muyan M, Ruddon RW, Norton SE, Boime I & Bedows E 1998 Dissociation of early folding events from assembly of the human lutropin beta-subunit. *Molecular Endocrinology* **12** 1640–1649.

Nakamura H, Nakamura K & Yodoi J 1997 Redox regulation of cellular activation. *Annual Review of Immunology* **15** 351–369.

Perez RL, Machiavelli GA, Romano MI & Burdman JA 1986 Prolactin release, oestrogens and proliferation of prolactin-secreting cells in the anterior pituitary gland of adult male rats. *Journal of Endocrinology* **108** 399–403.

Reeve JR Jr & Pierce JG 1981 Disulfide bonds of glycoprotein hormones. Their selective reduction in the beta subunits of bovine lutropin and thyrotropin. *International Journal of Peptide and Protein Research* **18** 79–87.

Seo H 1985 Growth hormone and prolactin: chemistry, gene organization, biosynthesis, and regulation of gene expression. In *The Pituitary Gland, Comprehensive Endocrinology*, pp 57–82. Ed H Imura. New York: Raven Press.

Shull JD, Walent JH & Gorski J 1987 Estradiol stimulates prolactin gene transcription in primary cultures of rat anterior pituitary cells. *Journal of Steroid Biochemistry and Molecular Biology* **26** 451–456.

Shupnik MA 1996 Gonadotropin gene modulation by steroids and gonadotropin-releasing hormone. *Biology of Reproduction* **54** 279–286.

Thorpe C, Hoober KL, Raje S, Glynn NM, Burnside J, Turi GK & Coppock DL 2002 Sulfhydryl oxidases: emerging catalysts of protein disulfide bond formation in eukaryotes. *Archives of Biochemistry and Biophysics* **405** 1–12.

Van Horssen AM, Van Kuppeveld FJ & Martens GJ 1998 Manipulation of disulfide bonds differentially affects the intracellular transport, sorting, and processing of neuroendocrine secretory proteins. *Journal of Neurochemistry* **71** 402–409.

Whitley JC, Giraud AS, Mahoney AO, Clarke IJ & Shulkes A 2000 Tissue-specific regulation of gastrin-releasing peptide synthesis, storage and secretion by oestrogen and progesterone. *Journal of Endocrinology* **166** 649–658.

Wittke I, Wiedemeyer R, Pillmann A, Savelyeva L, Westermann F & Schwab M 2003 Neuroblastoma-derived sulfhydryl oxidase, a new member of the sulfhydryl oxidase/Quiescin6 family, regulates sensitization to interferon gamma-induced cell death in human neuroblastoma cells. *Cancer Research* **63** 7742–7752.

Wittrup KD 1995 Disulfide bond formation and eukaryotic secretory productivity. *Current Opinion in Biotechnology* **6** 203–208.

Received 3 June 2004
Accepted 27 July 2004
Made available online as an Accepted Preprint
12 August 2004