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Tilapia Follicle-Stimulating Hormone (FSH): Immunochemistry, Stimulation by Gonadotropin-Releasing Hormone, and Effect of Biologically Active Recombinant FSH on Steroid Secretion[1]

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ABSTRACT

In fish, FSH is generally important for early gonadal development and vitellogenesis. As in mammals, FSH is a heterodimer composed of an alpha subunit that is noncovalently associated with the hormone-specific beta subunit. The objective of the present study was to express glycosylated, properly folded, and biologically active tilapia FSH (tFSH) using the *Pichia pastoris* expression system. Using this material, we aimed to develop a specific ELISA and to enable the study of FSH response to GnRH. The methylotrophic yeast *P. pastoris* was used to coexpress recombinant genes formed by fusion of mating factor alpha leader and tilapia *fshb* and *cga* coding sequences. Western blot analysis of tilapia pituitary FSH, resolved by SDS-PAGE, yielded a band of 15 kDa, while recombinant tFSH beta (rtFSH beta) and rtFSH beta alpha had molecular masses of 17–18 kDa and 26–30 kDa, respectively. Recombinant tFSH beta alpha was found to bear only *N*-linked carbohydrates. Recombinant tFSH beta alpha significantly enhanced 11-ketotestosterone (11-KT) and estradiol secretion from tilapia testes and ovaries, respectively, in a dose-dependent manner (similar to tilapia pituitary extract, affinity-purified pituitary FSH, and porcine FSH). Using antibodies raised against rtFSH beta, FSH-containing cells were localized adjacent to hypothalamic nerve fibers ramifying in the proximal pars distalis (PPD), while LH cells were localized in a more peripheral region of the PPD. Moreover, FSH is under the control of hypothalamic decapeptide GnRH, an effect that was abolished through the use of specific bioneutralizing antisera, anti-rtFSH beta. It also reduced basal secretion of 11-KT.

anterior pituitary, estradiol, follicle-stimulating hormone, gonadotropin-releasing hormone, luteinizing hormone

INTRODUCTION

FSH is a heterodimeric glycoprotein synthesized and secreted by the anterior pituitary gland. This hormone is involved in the regulation of essential vertebrate reproductive processes such as gametogenesis and follicular growth. Like other members of the pituitary glycoprotein hormone family (e.g., LH and thyroid-stimulating hormone [TSH]), FSH is composed of a common α subunit and a unique β subunit that confer biological specificity to each hormone [1]. These glycoprotein hormones are structurally and functionally conserved in various vertebrates and have been identified in most lineages of actinopterygian (bony) fish [2].

Thus far, cDNA sequences encoding gonadotropin subunits have been isolated and characterized from more than 19 fish species, representing seven teleostean orders [3], and this number is increasing. The two gonadotropins, LH and FSH, have distinct temporal expression and release profiles and are synthesized by two different cell types in the pituitary of several teleosts [4], including tilapia [5]. In most fish, FSH is generally prevalent during early gonadal development and the vitellogenic phase, while LH secretion remains at low levels during these phases and exhibits a sharp peak around final oocyte maturation and ovulation or during spermiation [3]. In mammals, it has been shown that, while measurements of FSH and LH in the peripheral circulation indicate their basal release, only LH appears to be released in a clear pulsatile manner, with no close relationship to the release patterns of FSH [6]. Taken together, these results suggest that the control of FSH release in fish and mammals may be different from that of LH. We are still far from understanding the regulatory mechanisms involved in the control of piscine FSH synthesis, secretion, and function. Part of the problem relates to its molecular heterogeneity and the unavailability of FSH standard and assay methods capable of distinguishing between FSH and LH in fish. The paucity of information on the regulation of piscine FSH prompted this study.

The objective of this study was to express glycosylated, properly folded, and biologically active tilapia FSH (tFSH) using an expression system capable of producing large quantities of the bioactive recombinant protein. In addition, we expected to provide sufficient quantities of tFSH for the development of specific antibodies that could be used in assays to quantify FSH and to determine its response to GnRH.

MATERIALS AND METHODS

Recombinant DNA Technology and Construction of the Expression Plasmid

Amplification of the α and β subunit sequences of tFSH, subcloning of fusion genes into *Pichia pastoris* expression vector, and expression of recombinant tFSH (rtFSH) were performed using a procedure that has been used to express tilapia LHβα (tLHβα) [7]. Briefly, primers were designed for amplification of the tilapia α-glycoprotein subunit gene (*cga*) and *fshb* sequences, forming fusion genes to be subcloned into the *P. pastoris* expression vector. The oligonucleotide primers used to clone the subunit DNAs from the pituitary of *Oreochromis niloticus* were designed according to the nucleotide sequences of *Oreochromis mossambicus cga* [8] and *fshb* [9]. *O. niloticus* and *O. mossambicus* belong to the genera *Oreochromis* and *Tilapia*, which share more than 95% homology between their DNA sequences. Mature protein-coding sequences were joined to form a fusion gene that encodes a "tethered"

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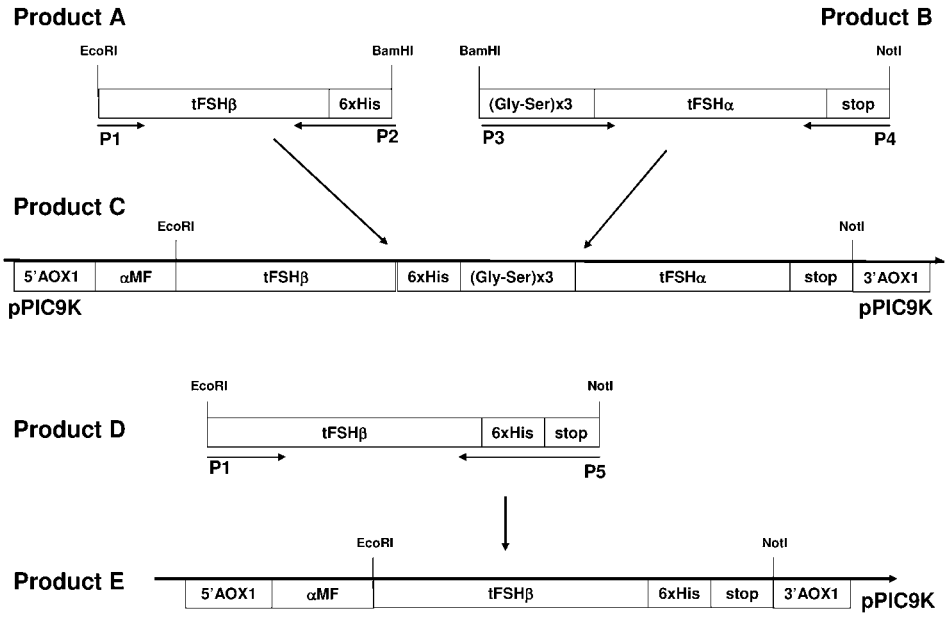

FIG. 1. Construction of the expression vector for tilapia FSHβ^{His}, *cga*, and FSHβα^{His}. The expression vector pPIC9K is an *Escherichia coli-P. pastoris* shuttle vector with sequences required for selection in each host. It has the 5′ promoter and 3′ transcription termination sequences of the alcohol (methanol) oxidase gene (*AOX1*) flanking the cloning site into which the tilapia FSHβ^{His}, *cga*, or FSHβα^{His} was introduced. The vector has an α yeast mating factor signal peptide downstream of the *AOX1* promoter to which the recombinant proteins were fused.

polypeptide in which one of the β-chains forms the *N*-terminal domain and the α-chain forms the C-terminal domain. A "linker" sequence of six amino acids (three Gly-Ser pairs) was placed between the β- and α-chains to assist in chimerization of the subunits, and a six-His (His^6) tail was placed at the end of the β subunit to enable purification of the recombinant protein (Fig. 1). The gene encoding tFSHβ (containing a His^6-tag at the C-terminus; product D in Fig. 1) was cloned into the pPIC9K vector (product E in Fig. 1), while tFSHβα (containing a His^6-tag at the C-terminus and a linker between the β [product A in Fig. 1] and α [product B in Fig. 1] subunits) was cloned into the *Eco*RI-*Not*I sites of the pPIC9K vector (Invitrogen, Carlsbad, CA), giving rise to product C in Figure 1. The pPIC9K plasmid contained the α yeast mating factor secretion signal that directs the recombinant protein into the secretory pathway. The constructs were digested with *Bgl*II and were used to transform *P. pastoris* strain GS115 by electroporation (products C and E in Fig. 1, for tFSHβα and tFSHβ, respectively). This resulted in insertion of the construct at the *AOX1* locus of *P. pastoris*, generating a His$^+$ Mut^s phenotype. Transformants were selected for the His$^+$ phenotype on 2% agar containing regeneration dextrose-biotin medium (1 M sorbitol, 2% dextrose, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, and 0.005% of L-glutamic acid, L-methionine, L-lysine, L-leucine, and L-isoleucine) and were then further selected for high copy number by their ability to grow on 2% agar containing 1% yeast extract, 2% peptone, 2% dextrose medium, and the antibiotic G418 at various concentrations (0.5–2 mg/ml; Invitrogen). The protein was expressed in a shaker flask and was harvested at 72 h after induction by methanol. Recombinant tFSHβ and rtFSHβα were purified using nickel nitrilotriacetic acid-agarose (Ni-NTA; Qiagen, Alameda, CA) according to the method by Kasuto and Levavi-Sivan [7]. As a negative control, *P. pastoris* was transformed using an expression vector that did not contain the tFSH cDNA, and fractions were prepared in the same manner.

Recombinant tFSHβ Antibody Production

Antibodies against rtFSHβ were raised in two rabbits. The purified rtFSHβ protein (50 μg) was dissolved in 1 ml of 0.9% NaCl and was emulsified with complete Freund adjuvant (1 ml; Sigma, Ness Ziona, Israel). Each rabbit received four subcutaneous injections at 3-wk intervals. Test bleedings, to determine antiserum titers, were carried out at 2 wk after the third injection. The rabbits were bled at 2 wk after the final injection, and the serum was aliquoted and lyophilized.

Biotinylation of Antibodies

The antibodies (anti-rtFSHβ) were purified using a protein A column and an ImmunoPure immunoglobulin G kit (Pierce Chemical Company, Rockford, IL) according to the manufacturer's instructions. The antibodies were rinsed three times with 4 ml of ice-cold PBS (pH 8) dialyzed against 0.1 M $NaHCO_3$ (pH 8.5) and were then incubated for 10 min at room temperature in 0.5 ml of PBS containing sulfo-NHS-LC-biotin (0.5 mg/ml; Pierce Chemical Company). The biotinylation reagent was removed with four washes of 4 ml of ice-cold PBS (pH 7.2). The antibodies were then dissolved in lysis buffer (150 mM NaCl, 20 mM Tris/HCl [pH 7.6], and 1% Triton X-100) for 30 min on ice.

Tilapia Pituitary FSH Isolation Using Anti-rtFSHβ

Immobilized streptavidin was used to affinity purify FSH from tilapia pituitaries, using the biotinylated specific antibodies. Tilapia pituitaries (n = 104) were homogenized in 0.2 M ammonium acetate (pH 6.1) containing 0.02 M phenylmethylsulforyl fluoride and 0.05 M EDTA on ice using a Polytron homogenizer. The homogenate was stirred for 2 h at 4°C and was centrifuged at 10 000 × *g* for 30 min. The pellet was reextracted to maximize recovery. The resulting supernatant was immediately subjected to the affinity column.

The biotinylated antibodies (2 mg/ml) were loaded onto an ImmunoPure streptavidin column (ImmunoPure AffinityPak Immobilized Streptavidin Prepacked column; Pierce Chemical Company) for 1 h at 4°C. The column was washed four times with three volumes of PBS (0.1 M phosphate and 0.15 M NaCl [pH 7.2]). The biotinylated antibodies were added to the column, allowed to enter the gel bed, and incubated at room temperature for 30 min. The column was washed with PBS, and the bound FSH was eluted with 50 mM sodium acetate and 0.5 M NaCl (pH 4.0). The fractions containing the affinity-purified pituitary tFSH (taFSH) were immediately neutralized with 1 M Tris (pH 9.5).

Glycosylation Analysis of rtFSHβα or rtFSHβ

Based on supplier recommendations (Roche Applied Science, Mannheim, Germany), 100 ng of reduced and denatured rtFSHβα or rtFSHβ was incubated for 2 h at 37°C in the presence or absence of *N*-glycosidase F (PNGase F), which hydrolyzes all types of *N*-glycan chains. Deglycosylated proteins were analyzed by Western blot analysis.

Gel Electrophoresis and Western Blot Analysis

Reduced samples that were from culture supernatants or that had been nickel purified were electrophoresed on 15% SDS-polyacrylamide running gels with a 5% stacking gel. Gels were blotted onto nitrocellulose membranes (Schleicher and Schuell, Dassell, Germany) and were blocked with 5% low-fat milk. Recombinant tFSHβα^{His}, tFSHβ^{His}, and tilapia pituitary extract (TPE) were visualized with the antibody against rtFSHβ (see the "Recombinant tFSHβ Antibody Production" subsection) or with antibodies against the His-tail (QIAexpress anti-His antibodies; Qiagen).When using anti-His, the membranes were treated according to the manufacturer's recommendations (dilution, 1:6000); when using anti-rtFSHβ, the membranes were incubated in PBS plus 1% nonfat milk with the antibodies (dilution, 1:10 000) for 1 h at room temperature and then with goat antirabbit horseradish peroxidase conjugate (dilution, 1:5000; Jackson ImmunoResearch Laboratories, Inc., West Grove,

PA) for 1 h at room temperature. After washing, all membranes were treated with enhanced chemiluminescence reagent (Biological Industries, Beit Haemek, Israel) to reveal immunoreactive bands.

In Vitro Bioassay

The bioassay procedure was performed according to that described by Bogomolnaya and Yaron [10] for tilapia ovaries and by Kasuto and Levavi-Sivan [7] for tilapia testes. Briefly, ovaries from early vitellogenic tilapia (mean ± SEM, 26.1 ± 1.27 g of body weight [BW]; gonadosomatic index [GSI] [i.e., gonadal weight percentage of BW], 0.98% ± 0.07%) or testes from maturing males (mean ± SEM, 19.75 ± 0.62 g of BW; GSI, 1.32% ± 0.25%) were divided into uniformly sized fragments (of about 20 mg each). The fragments were preincubated for 3 h in a 96-well culture plate at 26°C in the presence of 0.2 ml/well of basal medium eagle containing $NaHCO_3$ (4 mM), penicillin (100 IU/ml), streptomycin (0.1 mg/ml), and nystatin (1.25 IU/ml) (all from Biological Industries) and 0.05% BSA (Sigma) buffered to pH 7.4 with 2.1 mM Hepes. The gonadal fragments were rinsed three times every hour, and then the medium was replaced with the same medium containing 0.2 mM isobutylmethylxanthine (Sigma) with or without the gonadotropin being tested. Stimulation with tFSHβα, TPE, or porcine FSH (pFSH) (Sigma), at graded doses was continued for another 18 h. These incubations were performed in triplicate wells per treatment. The incubation medium was collected and stored at −20°C until assayed for 11-ketotestosterone (11-KT) or estradiol (E_2) concentration by ELISA.

ELISAs for 11-KT and E_2

The presence of 11-KT and E_2 was determined by specific ELISAs according to established protocols [11, 12] using acetylcholinesterase as a label. The anti-11-KT was donated by Dr. David E. Kime (University of Sheffield, Sheffield, England) and is described in Cuisset et al. [13]. All samples were analyzed in duplicate, and a separate standard curve was run for each ELISA plate. The lower limit of detection was 1.56 pg/ml for E_2 and 11-KT. The intra-assay and inter-assay coefficients of variation were less than 7% and less than 11%, respectively. Steroid levels in the medium determined by ELISA were validated by verifying that serial dilutions were parallel to the relevant standard curve.

ELISAs for tFSH and tLH

Specific and homologous ELISAs for the determination of tFSH and tLH were performed according to the protocol by Levavi-Sivan et al. [14]. Competitive ELISAs were performed as described by Mananos et al. [15] using primary antibodies against tLHβ [16] or rtFSHβ and recombinant tLHβα (rtLHβα) [7] or rtFSHβα for the standard curves. The wells were coated with rtLHβ or rtFSHβ (0.5 ng/well), and the antibodies were diluted 1:5000 (for LH) or 1:50 000 (for FSH). The intra-assay and inter-assay coefficients of variation were 7.2% and 14.8%, respectively, for LH and 8.0% and 12.5%, respectively, for FSH. The sensitivities of the assays were 0.65 ng/ml and 0.55 ng/ml for LH and FSH, respectively. When ELISA plates were coated with one of the gonadotropins and the antibodies were against the second gonadotropin, no standard curve was obtained. More details on the ELISAs for tLH and tFSH will be published elsewhere.

Immunohistochemical Staining

Immunohistochemical staining for tFSH and tLH was performed using the high polymer method (Histofine simple stain MAX PO; Nichirei Co., Tokyo, Japan). Tissue samples were fixed in 10% formalin and were embedded in paraffin, and 6-μm sections were mounted on slides. After dewaxing and rehydration, the sections were incubated in 3% hydrogen peroxide for 10 min at room temperature to quench endogenous peroxidase activity. To allow unmasking of the antigen, microwaving (at 350 W for 5 min) in 0.01 M citrate buffer (pH 6) was performed. After 30 min, the specimens were preincubated with 0.1% BSA for 10 min to reduce nonspecific reactions. They were then incubated with rabbit anti-rtFSHβ (dilution, 1:1000) or with rabbit anti-rtLHβ (dilution, 1:250) for 1 h. The specimens were rinsed three times with PBS for 5 min and were incubated with Histofine simple stain for 30 min. Peroxidase activity was visualized by treatment with 3-amino-9-ethylcarbazole (Lab Vision Corporation, Fremont, CA) for 5 min. After rinsing in water, nuclei were counterstained with hematoxylin. The sections were then dehydrated, cleared, and mounted. All steps of the procedure were carried out at room temperature. The sections were observed under a light microscope (Olympus, Tokyo, Japan) and were digitally photographed. The specificity of the immunoreaction was confirmed by incubating the sections with normal rabbit serum instead of the specific anti-rtFSHβ or anti-LHβ.

In Vivo Experiments

Tilapia (*O. niloticus*) were kept and bred in the fish facility unit at Hebrew University in 500-L tanks at 26°C under 14L:10D photoperiod. Fish were fed every morning ad libitum with commercial pellets and flakes containing 50% protein, 6% fat, 5.6% ash, and 2.6% cellulose (Zemach Feed Mills, Zemach, Israel). All experimental procedures were in compliance with the Animal Care and Use Guidelines of Hebrew University and were approved by the local Administrative Panel on Laboratory Animal Care Committee.

Effect of Salmon GnRH Analog on LH and FSH Release. Fish were anesthetized with 2-phenoxyethanol (Sigma) at a concentration of 1 ml/L before being weighed and injected i.p. with 15 μg/kg of BW of salmon GnRH analog ([D-Arg^6,Pro^9-NEt]-GnRH) (sGnRHa) (Bachem Inc., Torrance, CA) dissolved in 0.85% saline. Tilapia LH and FSH were measured after 6 h. Controls were injected with saline. Fish received sGnRHa at an injection volume of 1 μl/g of BW. Blood was collected from the caudal vasculature into heparinized syringes from anesthetized fish. After centrifugation, the plasma was stored at −20°C until analysis.

Effect of Immunoneutralization with Anti-rtFSHβ on 11-KT Levels. Fish were allocated to four groups with 15 fish each. The control group was injected i.p. with normal goat serum. The second, third, and fourth groups were injected with 15 μg/kg of BW of sGnRHa. The time of sGnRHa injection was defined as time 0 h. The second group was injected with sGnRHa only. The third group was injected with sGnRHa and was immunized with 0.2 ml of rtFSHβ antiserum together with the sGnRHa injection. The fourth group was immunized i.p. with 0.2 ml of rtFSHβ antiserum at 12 h before and together with the sGnRHa injection. A fifth group was injected only with anti-rtFSHβ at time −12 h. Blood samples were collected, as already described, at 4-h intervals until 24 h after treatment and were stored at −20°C until assay for 11-KT.

Statistical Analysis

Data are presented as mean ± SEM. The significance of differences between group means of hormone levels was determined by one-way ANOVA, followed by Newman-Keuls test using Graph-Pad Prism software (GraphPad, San Diego, CA). To test for parallelism between different regression lines, we used analysis of covariance (http://home.ubalt.edu/ntsbarsh/Business-stat/otherapplets/ANOCOV.htm).

RESULTS

Chemical Properties of Tilapia Recombinant and Pituitary FSH

Among the 300 His$^+$/Mut^s clones that contained rtFSHβ or rtFSHβα sequences in their genome at the *AOX1* locus, 10 clones were selected according to their resistance to a high dose of G418. The highest-secreting clone of each construct was chosen for further purification. The rtFSHβ and rtFSHβα were purified to apparent homogeneity using one-step nickel affinity chromatography (data not shown). In a typical preparation, 1 ml of the induced culture supernatant was obtained after concentrating 1000 ml of culture medium through a 1-ml bed volume of Ni-NTA beads. The eluted fraction contained 6.5 mg of rtFSHβ^{His} and 100 μg of rtFSHβα^{His}. The eluted fraction was dialyzed against PBS and was resolved by SDS-PAGE for Western blot analysis. Figure 2, A and B, shows the results of a Western blot analysis of supernatant from nontransfected GS115 cells transformed with pPIC9KtFSHβ^{His} or pPIC9KtFSHβα^{His} together with TPE. These proteins were immunoreacted with antibodies against the His-tag (Fig. 2A) or against rtFSHβ (Fig. 2B). Under reducing conditions, the immunoreactive rtFSHβα and rtFSHβ were revealed with both antibodies as bands of 26–30 kDa (Fig. 2, A and B, lane 1) and 17–18 kDa (Fig. 2, A and B, lane 3), respectively. When the homogenate of vitellogenic female tilapia pituitaries was subjected to SDS-PAGE under reducing conditions, a protein of approximately 15 kDa was specifically reacted to anti-rtFSHβ (Fig. 2B, lane 5), and (not surprisingly) no reaction was revealed with the anti-His (Fig. 2A, lane 5). Transformation with the vector alone (i.e., GS115/pPIC9 [Mut^s)]) (Fig. 2, A and B, lane 7) served as a negative control.

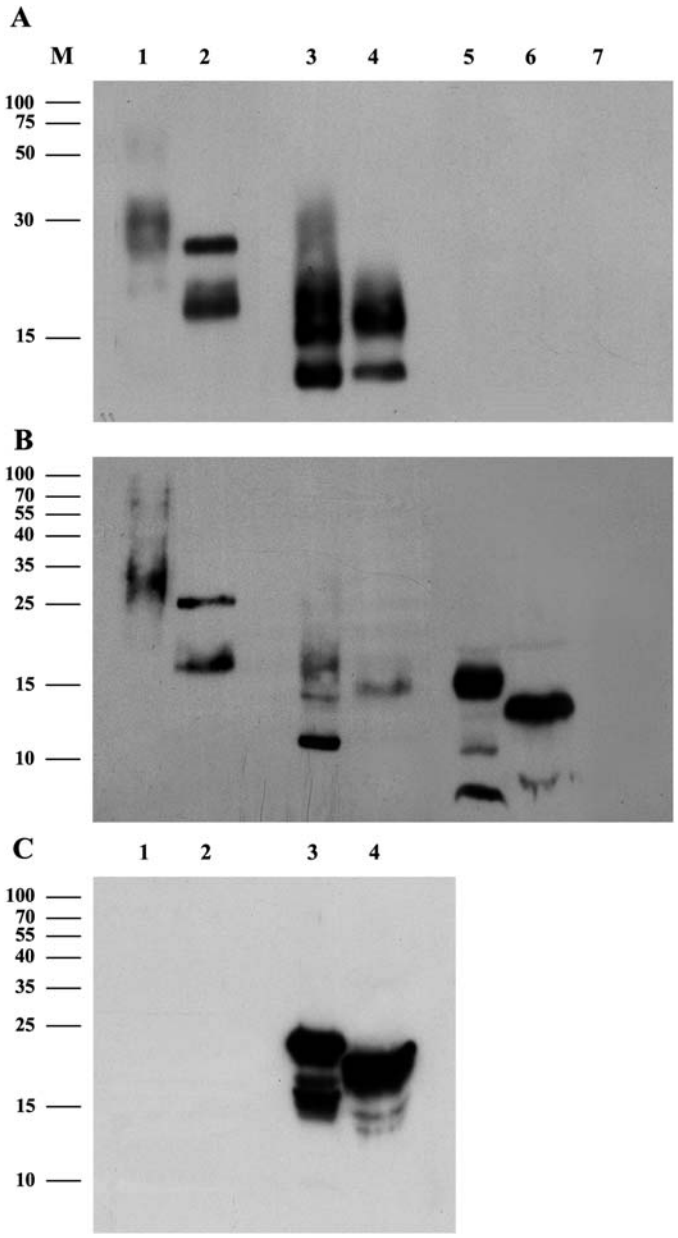

FIG. 2. Characterization of *P. pastoris*-expressed rtFSHβ and rtFSHβα by Western blot analysis. Total proteins from tilapia pituitary or supernatants of methanol-induced transformed *P. pastoris* cultures were separated by 15% SDS-PAGE and were immunoreacted with antibodies against His (**A**), against rtFSHβ (**B**), or against rtLHβ (**C**). **A** and **B**) Lanes 1 and 2 represent rtFSHβα^{His} (2 μl from a culture of 1 L that was nickel batch purified); lanes 3 and 4, rtFSHβ^{His} (0.2 μl from a culture of 1 L that was nickel batch purified); lanes 5 and 6, TPE (1:10 of a pituitary); lane 7, culture supernatant of yeast transformed with an expression vector not containing the tFSH subunit cDNA (10 μl from a culture of 50 ml);and lanes 2, 4, and 6, deglycosylated samples of those shown in lanes 1, 3, and 5, respectively. **C**) Lanes 1 and 2 represent rtFSHβα^{His} (2 μl from a culture of 1 L that was nickel batch purified); lanes 5 and 6, TPE (1:10 of a pituitary); and lanes 2 and 4, deglycosylated samples of those shown in lanes 1 and 3, respectively. M indicates molecular marker.

After deglycosylation with PNGase F, anti-His and anti-rtFSHβ were reacted with 23-kDa and 16-kDa proteins for rtFSHβα and rtFSHβ, respectively (Fig. 2, A and B, lanes 2 and 4). These results clearly show that rtFSHβα and rtFSHβ decreased their molecular mass by deglycosylation of the *N*-glycosylation type. Deglycosylation of the pituitary proteins

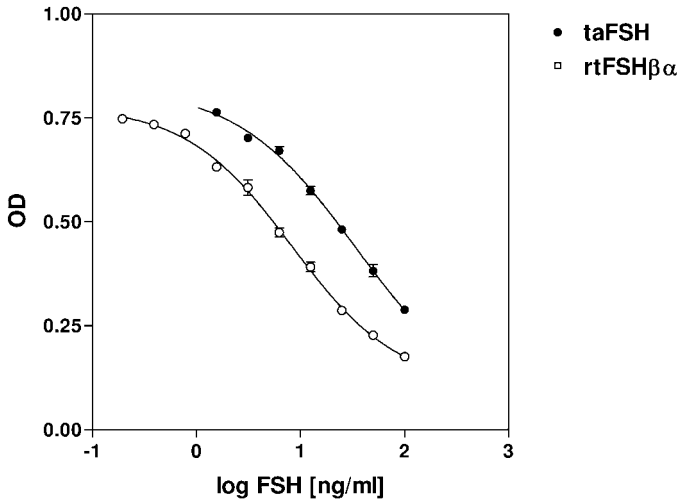

FIG. 3. Parallelism of taFSH and serially diluted concentrations of purified rtFSHβα^{His} using one-step nickel batch purification (n = 3 for each concentration).

yielded a band at around 13 kDa of the heterodimer (Fig. 2B, lane 6). In addition to the main bands, the rtFSHβ supernatant and the glycosylated rtFSHβα supernatant contained labeled bands of 13 kDa and 17 kDa, respectively (Fig. 2, A and B). It is important to note that no bands were detected when anti-rtLHβ was reacted with rtFSHβα and deglycosylated rtFSHβα (Fig. 2C, lanes 1 and 2, respectively), while 23-kDa and 15-kDa proteins were detected in tilapia pituitary (Fig. 2C, lane 3), which were reduced to 20-kDa and 13-kDa proteins after deglycosylation; these bands probably represent the heterodimer and the β subunit of tLH, respectively (Fig. 2C, lanes 3 and 4).

Serial dilutions of taFSH were found to parallel the standard curve (Fig. 3). The slope of the displacement curve obtained with rtFSHβα^{His} (slope, -0.282 ± 0.012) was not significantly different from that obtained with native pituitary FSH (slope, -0.287 ± 0.012), suggesting that the recombinant gonadotropin is immunologically similar to the authentic glycoprotein. These results confirmed the specificity of the antibodies raised against rtFSHβ to the native pituitary heterodimer.

In Vitro Biological Activity of rtFSHβα

To examine the biological activity of rtFSHβα, we tested its ability to bind to a cognate receptor and to elicit steroid secretion, compared with TPE, taFSH, and pFSH. Tilapia FSHβα significantly enhanced 11-KT (Fig. 4C) and E_2 (Fig. 5C) secretion in a dose-dependent manner at concentrations between 120 and 1200 ng/ml. Similarly, serially diluted TPE, taFSH, and pFSH (Figs. 4 and 5, A, B, and D, respectively) stimulated 11-KT and E_2 secretion from tilapia testes and ovaries, respectively. While the maximal stimulation obtained with rtFSHβα was comparable to that obtained with taFSH with respect to 11-KT and E_2 secretion, the potency of pFSH (stimulated in the milligram range [1 IU = 1 mg of pFSH] was higher. The culture supernatants from yeast that were transformed with "empty" vector (control) or from yeast that were transformed with rtFSHβ did not stimulate 11-KT or E_2 production (data not shown).

Immunohistochemistry

Using antibodies raised against rtFSHβ or rtLHβ, differential staining of pituitary cells was obtained. FSH-containing

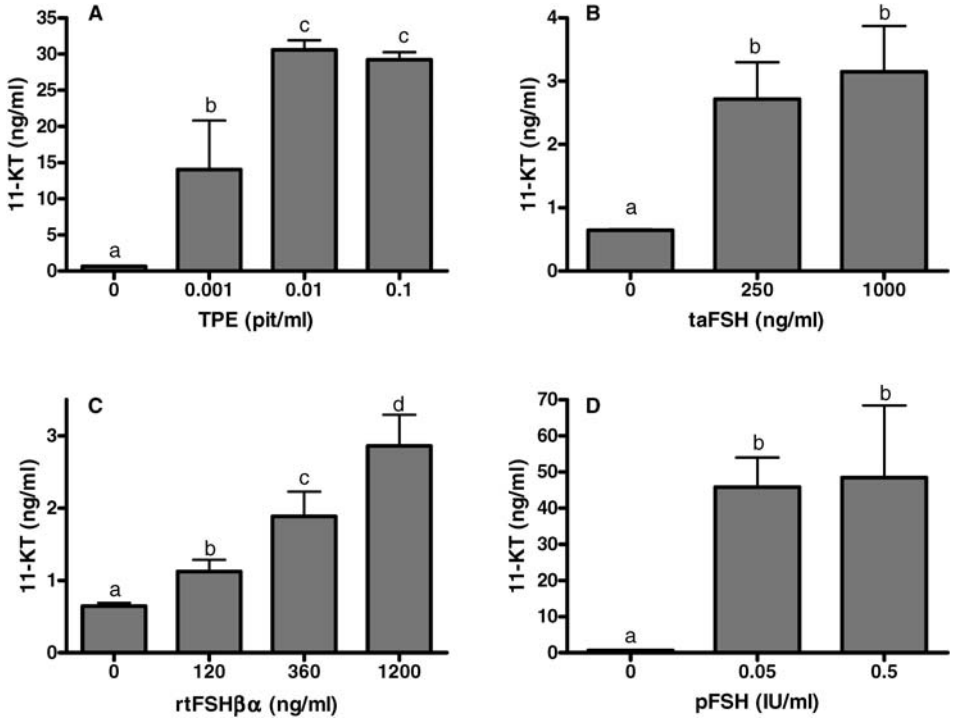

FIG. 4. Secretion of 11-KT from maturing tilapia testes (mean ± SEM, 19.75 ± 0.62 g of BW; GSI, 1.32% ± 0.25%) in response to various sources of FSH. **A**) TPE. **B**) Affinity-purified tilapia pituitary FSH. **C**) Culture supernatant of *P. pastoris* transformed with rtFSHβα cDNA that was purified using one-step nickel batch purification. **D**) Porcine FSH. Means marked by different letters differ significantly ($P < 0.05$).

cells were localized adjacent to the palisade of the somatotrophs that overlie the hypothalamic nerve fibers ramifying in the proximal pars distalis (PPD) (Fig. 6, A and B), while LH-containing cells were localized in a more peripheral region of the PPD (Fig. 6, C and D). The amount of FSH cells was much lower than that of LH.

Effect of GnRH on Gonadotropin Release

The effect of GnRH on LH secretion is well documented in tilapia and in other fish species (reviewed by Yaron et al. [3]). Hence, it was not surprising that at 6 h after injection of 15 μg/kg of BW of sGnRHa the plasma LH levels increased from 5.12 ± 1.21 ng/ml to 13.61 ± 1.52 ng/ml (n = 15, $P < 0.001$) (Fig. 7). However, plasma FSH levels also increased from 6.11 ± 0.91 ng/ml to 10.91 ± 1.06 ng/ml (n = 15, $P < 0.01$).

Effect of Immunoneutralization with Anti-rtFSHβ on 11-KT Levels

To find the correlation between FSH and steroid secretion in tilapia, we tested the effects of immunoneutralization of endogenous FSH on the ability of GnRH peptide to stimulate 11-KT. The 11-KT levels increased from 0.65 ± 0.16 ng/ml to 10.48 ± 2.18 ng/ml at 4 h following sGnRHa injection.

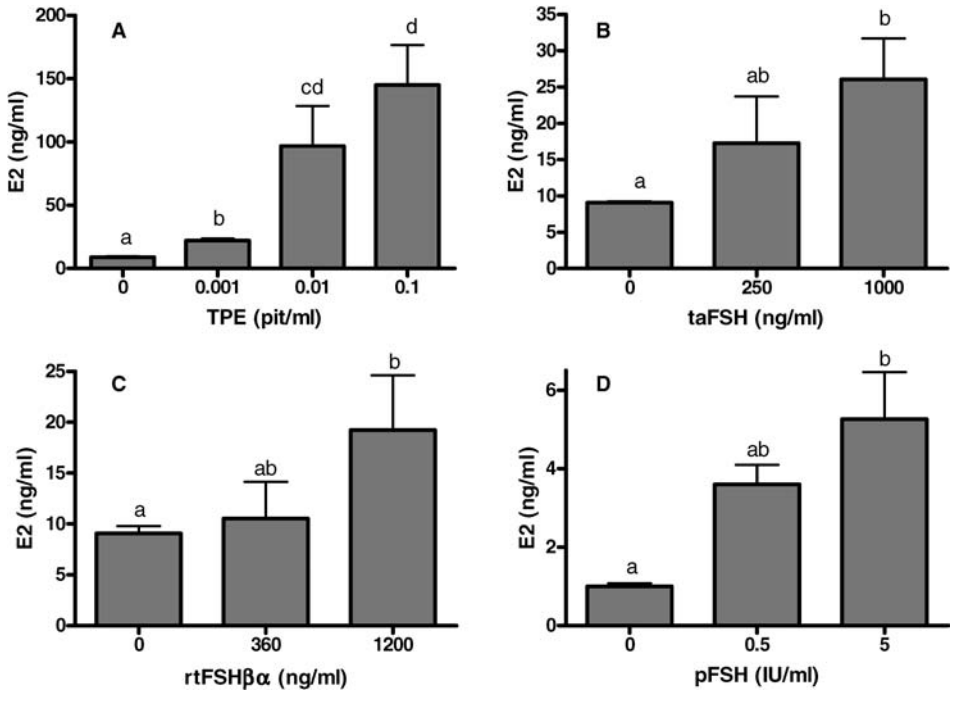

FIG. 5. Secretion of E_2 from vitellogenic tilapia ovaries (mean ± SEM, 26.1 ± 1.27 g of BW; GSI, 0.98% ± 0.07%) in response to various sources of FSH. **A**) TPE. **B**) Affinity-purified tilapia pituitary FSH. **C**) Culture supernatant of *P. pastoris* transformed with rtFSHβα cDNA that was purified using one-step nickel batch purification. **D**) Porcine FSH. Means marked by different letters differ significantly ($P < 0.05$).

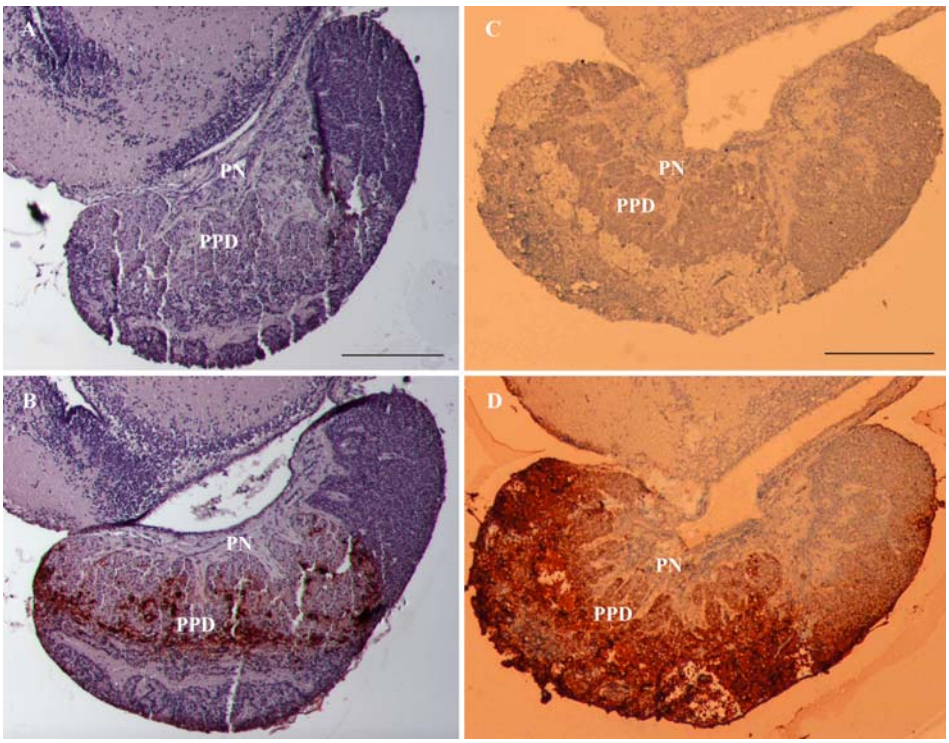

FIG. 6. Immunohistochemistry of tilapia pituitary. Sagittal section of an adult pituitary immunostained with normal rabbit serum (**A** and **C**), anti-rtFSHβ (**B**), or anti-rtLHβ (**D**). PN indicates pars nervosa. Bar = 200 μm.

However, a concomitant injection of sGnRHa and anti-rtFSHβ postponed the 11-KT peak by 4 h (Fig. 8). Fish that received two injections of anti-tFSHβ at 12 h before and together with sGnRHa also delayed their 11-KT peak by 4 h; however, the level of 11-KT was suppressed, reaching only 4.39 ± 1.27 ng/ml (Fig. 8). Injection of anti-rtFSHβ alone at 12 h before sGnRHa injection resulted in a dramatic decrease in 11-KT levels (from 1.02 ± 0.21 ng/ml to 0.14 ± 0.02 ng/ml) (Fig. 8, lower panel). Injection of the antibodies alone resulted in a sharp decrease in 11-KT levels that lasted for 24 h. These results may indicate that FSH is responsible not only for the GnRH-stimulated 11-KT secretion but also for the basal steroid secretion.

DISCUSSION

Since the cloning of two gonadotropins from salmon [17] and with the advent of sensitive molecular techniques, there is a growing body of evidence on the regulation and patterns of gonadotropin gene expression (reviewed by Yaron et al. [5]).

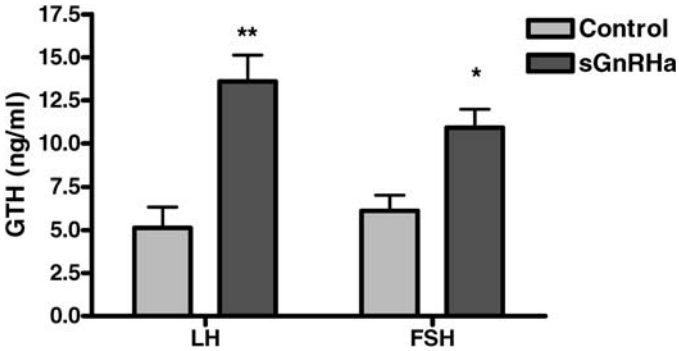

FIG. 7. Effect of sGnRHa on the levels of the gonadotropins (GTH) LH and FSH in mature female tilapia. Fish (mean ± SEM, 94.7 ± 7.45 g of BW; GSI, 2.85% ± 0.50%) were injected with sGnRHa (15 μg/kg of BW) and were examined at 6 h later. The results are expressed as mean ± SEM (n = 15). $^{**}P < 0.001$; $^{*}P < 0.01$.

Nevertheless, our understanding of the unique biological functions of the two gonadotropins in fish is still incomplete primarily because of a lack of purified hormones, particularly FSH. Herein, we demonstrate the production of a biologically active rFSH and its use as a tool for revealing the biological relevance of FSH in tilapia as a model fish.

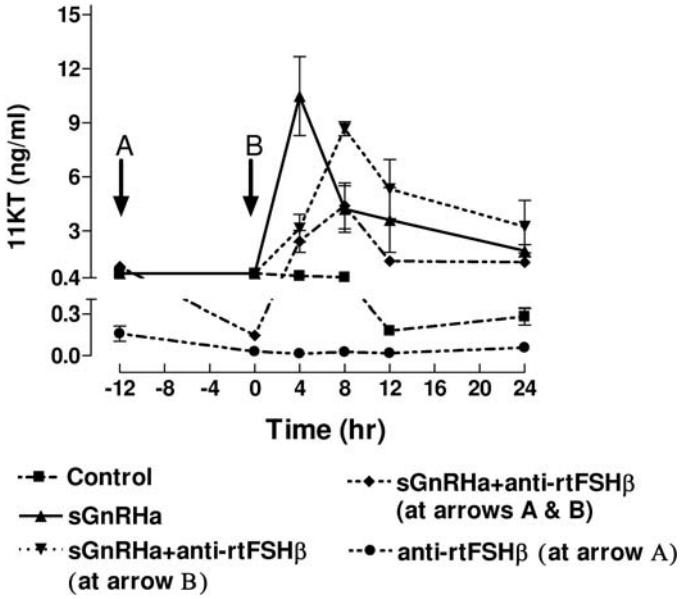

FIG. 8. Immunoneutralization study. Male fish (mean ± SEM, 189.7 ± 9.86 g of BW; GSI, 0.71% ± 0.16%) were injected i.p. with normal goat serum (control; squares); anti-rtFSHβ at the time indicated by arrow A (0.2 ml of anti-rtFSHβ; circles); sGnRHa at the time indicated by arrow B (15 μg/kg of BW of sGnRHa; triangles); sGnRHa (15 μg/kg of BW) and 0.2 ml of anti-rtFSHβ both at the time indicated by arrow B (sGnRHa together with anti-rtFSHβ; inverse triangles); or 0.2 ml of anti-rtFSHβ at the time indicated by arrows A and B and sGnRHa (15 μg/kg of BW) injection only at the time indicated by arrow B (sGnRHa together with anti-rtFSHβ; diamond).

Earlier work conducted in our laboratory has shown that the *P. pastoris* expression system can be used to achieve single-strand tethered tLH [7]. That study showed that *P. pastoris*-expressed hormone subunits yield a recombinant protein that is conformationally similar to the natural hormone and can elicit a biological response in vitro. In the present study, we used this expression system to produce rtFSH.

Overall, the purification procedure yielded 6.5 mg of pure rtFSHβ or 100 μg of pure rtFSHβα from 1 L of medium. The yield of the heterodimer was significantly lower than that of the monomer probably because of the complex structure of the former. This yield is lower than that reported for tethered ovine FSH (6.1 mg/ml [18]), pFSH (10 mg/L [19]), or tethered pFSH using a commercial fermenter (280 mg/L [20]). Very high expression levels were reported for human rFSH expressed in a Chinese hamster ovary cell line and grown in perfusion culture (8 mg/ml [21]) and for catfish FSH in an S2 *Drosophila* cell line (8 mg/L [22]). Kamei et al. [23] reported on 2 mg/L of Japanese eel (*Anguilla japonica*) FSH obtained using *P. pastoris*.

Using antibodies directed against the His-tag or against rtFSHβ, Western blot analysis labeled a product of approximately 26–30 kDa. Because the predicted size of the secreted 204-amino acid protein of the rtFSHβα fusion protein is 23.01 kDa, the additional size detected probably reflects glycosylation of the fusion protein, as the proteins were found to be of the expected size after deglycosylation. In addition to a 23-kDa product for the rtFSHβα protein, Western blot analysis showed that there was a product of approximately16 kDa. We suggest that this product is likely a degraded form of rtFSHβα. The molecular mass estimates of rtFSHβα and rtFSHβ were in the range of those reported for other teleost species (although estimates vary considerably), between 15 kDa and 28 kDa for the subunits and between 29 kDa and 50 kDa for the intact hormones (Mediterranean yellowtail [*Seriola dumerilii*] [24], *Cichlasoma dimerus* [25], bonito [*Katsuwonus plelamis*] [26], hybrid striped bass [27], tuna [*Thunnus obesus*] [28], chum salmon [17], coho salmon [29], seabream [*Pagrus major*] [30], killifish [*Fundulus heteroclitus*] [31], common carp [32], goldfish [33], Japanese eel [23], and Atlantic halibut [*Hippoglossus hippoglossus*] [34]. Because the amino acid composition is similar for each individual subunit of tilapia and other teleost species [5], the different molecular mass estimates may result from differences in the degree of glycosylation or in the method of molecular mass determination.

The tilapia *fshb* subunit carries one *N*-linked oligosaccharide that consists of a heterogeneous array of neutral, sulfated, and sialylated structures. The sugars, amounting to more than 30% of the FSH mass, form the major chemical basis of the charge heterogeneity of FSH isoforms in the pituitary and bloodstream of mammals and are essential for FSH intracellular folding, secretion, clearance from the blood, and binding and signaling at the target-cell level [35]. Deglycosylation of rtFSHβ, rtFSHβα, and pituitary gonadotropins with PNGase F, which hydrolyzes all types of *N*-glycan chains, reduced the glycosylated forms to the nascent translated protein. This implies that the carbohydrate modifications on the mature protein occur exclusively through *N*-linked (and not at all through *O*-linked) glycosylation, similar to findings for Japanese eel FSH [23] and tLH [7]. In contrast, mammalian glycoprotein hormones carry *O*-linked and *N*-linked oligosaccharides. Recently, *N*-linked oligosaccharide chains were found to have a minor role in receptor binding of glycoprotein hormones yet are critical for bioactivity, while *O*-linked oligosaccharide chains were found to have a minor role in receptor binding and signal transduction yet are critical for in vivo half-life and bioactivity [36]. The role of glycosylation in the piscine gonadotropins is still unclear and should be elucidated. Despite the fact that glycosylation moieties synthesized by *P. pastoris* are of the high-mannose type (characteristic of yeast [37]) and thus differ from those of vertebrate cells, it is important to note that the biological potency of the recombinant gonadotropin was in the same range as that of mammalian FSH and pituitary-derived gonadotropin.

The specific functions of FSH in fish are not yet clearly understood. In this study, we show that FSH (synthetic and native) increases the secretion rates of E_2 and 11-KT in females and males, respectively. This corroborates similar findings in other species; FSH and LH of salmon have been found to be equally potent in stimulating E_2 secretion from the vitellogenic ovary of amago and coho salmon, but FSH was less potent in stimulating secretion of 17α,20β,dihydroxy-4-pregnen-3–1 from postvitellogenic oocytes [29, 38]. FSH also increased E_2 levels in common carp [32], tuna [28], Atlantic halibut [34], red seabream [30], and Japanese eel [39]. In line with its activity in the secretion of E_2, FSH was found to increase aromatase activity and the expression of the ovarian form of the P450arom gene (*cyp19a1a*) in brown trout [40] and in gilthead seabream [41]. FSH has been reported to stimulate the incorporation of vitellogenin into the ovaries of rainbow trout [42], which is corroborated by the surge of FSH concomitant with the new generation of vitellogenic oocytes in trout [43] and in tilapia [14].

Using antibodies generated against rtFSHβ, a method for affinity purification was established that produces biologically active protein. Despite the harsh conditions required to break the streptavidin-biotin bond, taFSH was not affected, as reflected by the biological activity of the hormone eluted from the column. The high degree of parallelism found between serial dilutions of the recombinant and native pituitary-derived FSH, together with the biological activity of the pituitary FSH, may be a sign of similar structures of the recombinant and native pituitary-derived FSH.

In mammals, it is well documented that LH stimulates androgen production through activation of LH receptors on Leydig cells, while FSH directly regulates Sertoli cell functions through FSH receptors on Sertoli cells [44]. However, the situation in fish seems to be more complex because both gonadotropins showed prominent steroidogenic potency [45]. We show herein that rtFSH, as well as pituitary-derived and mammalian FSH, stimulate a dose-dependent increase in 11-KT secretion from maturing tilapia testes. Eel rFSH also stimulated 11-KT secretions from immature eel testes [46]. This is in accord with FSH being the most important hormone regulating spermatogenesis in fish via the stimulation of 11-KT secretion [47].

The effect of GnRH on LH secretion is well documented in tilapia and in other fish species (reviewed by Yaron et al. [3]). We show herein that a single injection of sGnRHa is sufficient to increase FSH release by 1.8-fold. These results show for the first time (to our knowledge) that FSH release in female tilapia is also under the control of the hypothalamic decapeptide GnRH. This contrast with the situation in mammals in which most of the FSH secretion occurs independent of signals arising from the GnRH receptor to the release mechanisms [6]. Although the pulsatile nature of GnRH secretion is believed to be evident in all vertebrates [48], there is no conclusive evidence for such pulsatility in fish. Taken together, the results shown herein with respect to the direct effect of GnRH on the release of FSH, as well as the fact that in teleosts FSH and LH

are secreted from different cells (reviewed by Yaron et al. [5]), render the fish pituitary an excellent model to study the differences between LH and FSH secretion patterns.

The fact that the release of LH in response to GnRH was more robust than that of FSH may be due to differences in the control of the two gonadotropins. Alternatively, it may relate to the fact that the quantity of LH in the pituitary is larger than that of FSH [49] (Fig. 6).

One of the most effective approaches to determine the physiological role of a biological factor is its elimination from the system in question. In recent years, transgenic technology and knockout mice have become the cutting-edge technology used in the biological sciences to answer questions related to physiological function. However, this method requires that the gene encoding the protein under study be fully known and characterized. An alternative means of eliminating a physiological factor from a model system is immunoneutralization (i.e., the administration of antisera produced against the factor). This method provides relative specificity in eliminating only the factor in question. In the present study, injecting antibodies against rtFSHβ into male tilapia reduced and delayed the 11-KT response to sGnRHa. Moreover, in the presence of anti-FSH, not only the GnRH-stimulated 11-KT level but also the basal steroid levels were reduced. This strongly implies involvement of FSH in 11-KT secretion in tilapia. In adult male rats, immunoneutralization of endogenous FSH also decreases serum hormone levels, testicular function, and fertility [50].

Using antibodies raised against rtFSHβ, FSH-containing cells were localized adjacent to the palisade of the somatotrophs that overlie the hypothalamic nerve fibers ramifying within the PPD. This is in agreement with the cells that were found to react with tilapia *fshb* mRNA in an in situ hybridization study [3] and in immunohistochemical investigations using heterologous antibodies [51]. However, anti-rtLHβ reacted with a different population of cells localized in a more peripheral region of the PPD, the same region that was detected in the previous study using in situ hybridization [3]. This distinguishable recognition of cells in the tilapia pituitary suggests antibody specificity.

The glycoprotein hormones are an interesting group of molecules not only because of their importance in reproduction and overall physiology but also because of their unique structural features. The mammalian gonadotropins have proven to be useful models for understanding protein folding and protein-protein interactions, as well as for studying the role of carbohydrates in protein function. The availability of rtFSH in sufficient quantity should enable us to better define its physiological role and pituitary control and to further explore the usefulness of fish as a model system for understanding gonadotropin function in vertebrates. Taken together, the parallelism between the native and rFSH and their similar potency in eliciting biological responses, as well as the generation of antibodies that label FSH cells in the pituitary and are able to neutralize GnRH-stimulated secretion of 11-KT, sheds new light on the role of FSH and on its regulation in tilapia.

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