Analysis of serum FSH bioactivity in a patient with an FSH-secreting pituitary microadenoma and multicystic ovaries: A case report

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FSH-secreting pituitary adenoma (FSHoma) is often associated with increased levels of serum FSH and ovarian hyperstimulation syndrome (OHSS). The OHSS has historically been attributed to elevated FSH production by the FSHoma; however, some FSHoma patients with OHSS have normal serum FSH levels. OHSS may result not from increased FSH levels, but also from increased bioactivity of the FSH derived from the adenoma. To address this, we measured the FSH bioactivity in the serum of a 40-year-old woman with an FSHoma and OHSS, whose FSH levels were normal. Chinese hamster ovary cells stably expressing FSH receptors were prepared and transfected with a cAMP-responsive element-driven luciferase reporter plasmid. Cells were then treated with recombinant human FSH (rhFSH), the patient’s sera, or sera from controls, collected at different time points, and subjected to a luciferase assay. Luciferase activity was increased in response to rhFSH in a dose-dependent manner. The responsiveness was further augmented by co-addition of a 3-methyl isobutylxanthine, which improved the sensitivity of our assay. Unexpectedly, the serum FSH bioactivity/immunoactivity ratio of the patient was mostly equal to that of normal subjects. This was confirmed with a granulosa cell aromatase assay. This case report suggests that alternate explanations may exist for the OHSS phenotype seen in some FSHoma patients.

Keywords: FSH; pituitary adenoma; bioactivity; ovarian hyperstimulation

Introduction

Follicle stimulating hormone (FSH)-producing pituitary adenomas (FSHomas) are difficult to diagnose because they produce few, clinically recognizable symptoms. In postmenopausal women, the physiological increase in FSH levels further complicates the diagnosis. In general, FSHomas only give rise to a clinical syndrome once the mass has reached a critical threshold. Enlarged multicystic ovaries with an elevated serum estradiol (E2) concentration in a reproductive-aged women, however, are suggestive of an FSHoma (Djerassi et al., 1995; Christin-Maitre et al., 1998; Valimaki et al., 1999; Shimon et al., 2001; Maruyama et al., 2005; Sugita et al., 2005; Ghayuri and Liu, 2007). Thus, the mechanism by which an FSHoma induces OHSS remains elusive. A few studies, however, have noted that the FSH produced by the adenoma had enhanced bioactivity (Galway et al., 1990; Borgato et al., 1998; Shimon et al., 2001).

To further explore the hypothesis of increased FSH bioactivity, we tested the serum of a patient with an FSHoma and OHSS whose serum FSH levels were in the normal range. We evaluated the FSH bioactivity of the patient’s sera collected at different time points during her follow-up without treatment. Bioactivity was measured with a luciferase reporter assay and a rat granulosa cell aromatase bioassay.

Case report

A 40-year-old woman presented with multicystic ovaries and mild menstrual irregularity. The medical and gynecologic history of this patient has been reported previously (Maruyama et al., 2005). Briefly, on the first evaluation, her serum E2 and prolactin concentrations were mildly elevated, ranging as high as...
respectively. FSH and LH concentrations were 8.8 IU/l (range for the follicular phase is 2.9–12.3) and were stored frozen until the FSH bioassays.

Figure 1: Clinical and laboratory data of a patient with an FSH-secreting pituitary microadenoma and multicystic ovaries (A) Transvaginal ultrasonographs of the right ovary taken 3 months after the initial visit. (B) T2-weighted coronal magnetic resonance imaging performed 12 months after the initial visit revealing an intra-cellular tumor measuring 9 mm in diameter, with high signal intensity in the left side of the pituitary (arrow). (C) Concentrations of pituitary hormones and E2 in serum samples collected from the patient during an 18-month follow-up period. Sample names are designated as indicated (a–d). Bars, 1 cm

397 ng/ml (normal level for the follicular phase is <97) and 24.7 ng/ml (the range for the follicular phase is 2.4–18.7), respectively. FSH and LH concentrations were 8.8 IU/l (the range for the follicular phase is 2.9–12.3) and <0.2 IU/l (the range for the follicular phase is 1.4–8.6), respectively. She had enlarged ovaries with multiple cysts. These were still present on transvaginal ultrasound three months after the initial visit (Fig. 1A). Gadolinium-enhanced dynamic T2-weighted magnetic resonance imaging revealed a 9 mm intrasellar tumor with high signal intensity within the left side of the pituitary. This remained unchanged 12 month after the initial visit (Fig. 1B). The patient exhibited paradoxical secretory responses of FSH and LH to TRH, which were characteristic of an FSH-producing pituitary adenoma (Daneshdoost et al., 1991). The patient was followed without any medical treatment for 18 months. During this period, serum samples were collected at different time points (Fig. 1C). As controls, serum samples were collected during the follicular phase from two healthy, consenting women who had regular menstrual cycles, no endocrinological disorders, and took no medications. Sera collected from the patient and the two volunteers were identically handled and were stored frozen until the FSH bioassays.

Materials and Methods

Plasmids
An expression plasmid encoding the human FSH receptor (hFSHR), pcDNA3-hFSHR, was a generous gift from Dr Aaron J.W. Hsueh (Division of Reproductive Biology, Department of Gynecology and Obstetrics, Stanford University Medical Center). A firefly luciferase reporter vector, pE1b-Luc, was constructed as described previously (Yamada et al., 2000). Oligonucleotides 5'-ctagcctctctctgacgtcagccaaaggagg-3' and 5'-ctagcctcctttggctgacgtcaga-3' were annealed, phosphorylated by T4 polynucleotide kinase (New England Biolabs, Inc., Beverly, MA), and subcloned into the Nhel site of pE1b-Luc to generate the p3×CRE-Luc vector. This vector contained three copies of the canonical cAMP responsive element (CRE) derived from the rat somatostatin gene promoter region (Montminy et al., 1986). A sea pansy luciferase expression vector pRL-CMV was purchased from Promega (Madison, WI). All plasmids for transfection experiments were prepared using a Genopure Plasmid Maxi Kit (Roche Molecular Biochemicals, Indianapolis, IN).

Establishment of an hFSHR-expressing stable line
CHO-K1 cells (8×10⁶ cells) purchased from the American Type Culture Collection (Manassas, VA) were inoculated in a 60-mm culture dish on the day prior to transfection. The cells were transfected with the FuGene6 reagent (Roche Molecular Biochemicals) using 2 μg of pcDNA3-hFSHR. Two days after transfection, the cells were cultured in the presence of the neomycin analogue G418, and 10 resistant colonies were isolated by limiting dilution. Colonies were individually analyzed for expression of hFSHR by using RT–PCR and western blotting techniques, and the clone with the highest expression was selected, designated as CHO-hFSHR, and used for further analysis.

Transient transfection and luciferase assay
On the day prior to transfection, 5×10⁶ per well of CHO-hFSHR cells were inoculated in a 96-well plate. The cells in each well were then transfected with 40 ng of pE1b-Luc or p3×CRE-Luc firefly luciferase reporter vector, and 1 ng of pRL-CMV sea pansy luciferase expression vector using the FuGene6 reagent. Twenty-four hours after transfection, various doses of recombinant human FSH (rhFSH; Gonal-F®, Serono, Aubonne, Switzerland) or serum samples were added, and if necessary, 200 mM of 3-methylisobutylxanthine (IBMX) was added at the same time. Then after 6 h, the firefly and sea pansy luciferase assays were performed with the dual luciferase assay system (Promega) according to the manufacturer’s protocol. Luciferase activities were determined using a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA). Firefly luciferase activities were normalized with the sea pansy luciferase activities, and described as relative light units (RLU). The serum FSH bioactivity/immunoactivity ratio (B:I ratio) was determined by dividing the RLU by the FSH concentration.

Isolation of rat granulosa cells
Rat granulosa cells were collected as described previously (Kajitani et al., 2004). Briefly, immature, 21-day-old Kwl:Wistar female rats were treated with 2 mg of diethylstilbestrol (Sigma Chemical Co., St. Louis, MO) in 0.1 ml of sesame oil once daily, for 4 days, to stimulate the proliferation of ovarian granulosa cells. The ovaries were then excised, and the follicles punctured with a 26-gauge needle to release the granulosa cells which were collected. The cells were washed and collected by a brief centrifugation at 500g for 5 min at room temperature, and cell viability was determined by trypan blue staining. Cell viability was in excess of 90%. All animal studies were approved by our Institutional Review Boards.

Measurement of E2 concentrations in the culture media of rat granulosa cells
Isolated rat granulosa cells (2×10⁶ cells) were inoculated on collagen type-I coated 35-mm dishes and cultured in Ham F-12: Dulbecco’s modified Eagle’s medium (1:1, v:v) supplemented with antibiotics and 0.1% BSA. After 24 h, 200 mM IBMX was added, and the cells cultured for 48 h in the presence or absence of 10 μM of rhFSH or 100 μl of a serum sample. After 48 h, the medium was replaced with fresh medium containing 10⁻⁷ M androstenedione. The medium was collected after 3 h of culture, and E2 concentrations were measured using an RIA (DPC Estradiol Kit, Mitsubishi
Kagaku Iatron, Inc., Tokyo, Japan). The serum FSH B:I ratio was determined by dividing the concentration of E2 by the concentration of FSH.

Results
Establishment of the luminescence-based in vitro FSH bioassay
FSH exerts most of its intracellular actions via a cAMP-mediated signaling pathway that activates a number of CRE-driven promoters. Several in vitro FSH bioassays have been developed that take advantage of this mechanism (Rose et al., 2000). The most sensitive assay for FSH bioactivity employs the luciferase activity of reporter plasmids bearing CRE-driven promoters in primary cells or cell lines stably overexpressing the hFSHR (Rose et al., 2000).

We established a CHO-hFSHR line that was then transfected for 24 h with pE1b-Luc or p3×CRE-Luc. The cells were then treated with various doses of rhFSH for 6 h and subjected to luciferase assay. As shown in Fig. 2A, rhFSH treatment of CHO-hFSHR cells harboring p3×CRE-Luc, but not pE1b-Luc (no CREs), enhanced the luciferase activity. The increase was

![Figure 2: Quantitative assessment of the FSH bioactivity of the sera collected from the patient and normal subjects using a luciferase assay and a granulosa cell aromatase assay](image)

(A) Dose-dependent increases in luciferase activity by rhFSH in the established FSH bioassay. Each value represents the mean ± SEM of four independent transfection experiments. (B) Enhancement of the sensitivity of the established FSH bioassay by co-addition of IBMX. Each value represents the mean ± SEM of four independent transfection experiments. (C) None of the patient’s samples demonstrated an increased FSH B:I ratio (a–d) as determined by the luminescence-based bioassay. Each value represents the mean ± SEM of four independent transfection experiments. (D) None of the patient’s samples demonstrated an increased FSH B:I ratio (a–d) as determined by the granulosa cell aromatase bioassay. Each value represents the mean ± SEM of three independent cultures. (E) There was a significant correlation between the B:I ratios determined by the luciferase assay and the aromatase assay.
dose-dependent and ranged from 10 to $1 \times 10^4$ mIU/l. This confirmed that our assay system could specifically and quantitatively assess the activation of the FSH/FSHR/cAMP-mediated signaling pathway. However, when the rhFSH concentration was within the physiological range, i.e. <10 mIU/l, the FSH bioactivity could not be quantitatively evaluated (Fig. 1A). To improve the sensitivity of the assay we protected the intracellular cAMP from degradation (Jia and Hsueh, 1986), by adding IBMX, a phosphodiesterase inhibitor. As illustrated in Fig. 2B, in the presence of IBMX, treatment with 10 mIU/l of FSH significantly increased the luciferase activity compared with the control. This enabled us to quantitatively assess the FSH activity of sera derived from the two normal subjects (control #1 and #2). Both sera enhanced the p3CRE-Luc reporter activity more effectively than the untreated control, whereas pE1b-luc reporter activities were constant across treatments (Fig. 2B). This confirmed that the assay was suitable for measuring the FSH bioactivity in the serum samples.

**Evaluation of serum FSH bioactivity by the luciferase reporter assay**

To evaluate the FSH bioactivity in the patient’s sera, serum samples collected at four different time points (Fig. 1) were subjected to the luciferase reporter assay. Unexpectedly, none of the samples exhibited high FSH B:I ratios, compared with those from two healthy volunteers (Fig. 2C). Thus, it is unlikely that the OHSS phenotype results from increased FSH bioactivity and stimulation of the FSH/FSHR/cAMP-mediated signaling pathway.

**Evaluation of serum FSH bioactivity by the granulosa cell aromatase bioassay**

Since FSH stimulates aromatase activity, FSH bioactivity can also be determined by measuring estrogen production in granulosa cell aromatase assay (Jia and Hsueh, 1986). The results of the aromatase assay and the luciferase assay were consistent. None of the patient’s sera samples had significantly higher FSH B:I ratios compared with the samples from the healthy volunteers (Fig. 2D). Furthermore, there was a significant correlation ($r^2 = 0.89, P = 0.008$) between the B:I ratios determined by each of the assays (Fig. 2E). This substantiates the role of intracellular cAMP accumulation as the major determinant of aromatase activity (Fitzpatrick and Richards, 1991), and also verifies the validity of our experimental procedures. Taken together, it is unlikely that FSH bioactivity *per se* may contribute to the formation of the OHSS phenotype in FSHoma patients.

**Discussion**

FSH exhibits considerable variability in the structure of its polypeptide backbone and in its glycosyl residues. This gives rise to a large number of isoforms that differ not only in molecular mass and overall charge, but also in biological and immunological activity (Rose et al., 2000). Indeed, the fluctuation in FSH bioactivity over the menstrual cycle is attributable to the different FSH forms produced at different stages of the cycle (Rose et al., 2000). Aberrant FSH bioactivity is a feature of some FSHomas (Galway et al., 1990; Borgato et al., 1998; Shimon et al., 2001), and has been thought to contribute to the clinical symptoms such as OHSS and menstrual irregularities (Shimon et al., 2001).

As our FSHoma patient developed OHSS in the setting of normal levels of serum FSH, we postulated that the FSH produced by her adenoma was biologically hyperactive. However, we found somewhat unexpectedly that the patient’s serum FSH bioactivity, as determined by both a luciferase reporter assay and a granulosa cell aromatase assay, did not exceed that of control subjects. This is in agreement with a previous report of another patient with an FSHoma and OHSS who also had normal FSH levels with normal FSH B:I ratios (Christin-Maitre et al., 1998). Thus, an explanation other than FSH bioactivity likely exists to explain the OHSS phenotype in FSHoma patients.

Intracellular signaling by FSH is a complex process, which involves many potential pathway interactions including not only cAMP-mediated PKA and PKB activation, but also the MAPK pathway, the phosphatidylinositol 3-kinase pathway and intracellular Ca$^{2+}$ elevations (Conti, 2002). The discrepancies in the literature may be attributed to the differences between experimental cell models and species tested (Silva et al., 2006). The *in vitro* bioassays used in this study were limited to rat granulosa cells and FSHR-expressing CHO cells, and may not be generalizable to the whole human ovary. Indeed, the interaction between theca and granulosa cells, e.g. is central to ovarian physiology and pathology. It is possible that the *in vitro* single cell population-based assays may fail to replicate the behavior of the patient’s sera *in vivo*. Thus, our findings do not completely exclude the possibility that altered FSH bioactivity mediates OHSS in FSHoma patients. Another limitation of *in vitro* FSH bioassays is that no comparisons of biological half-life can be made. Therefore, if FSHoma-derived FSH was post-translationally modified resulting in a longer half-life, our assay system would not detect this change. With regard to our patient, her FSH levels were within the normal range, but were at the upper limit. This chronic low-grade elevation in FSH may have been sufficient to prevent atresia of the non-dominant follicles, thereby resulting in the development of polycystic ovaries. Since FSH secretion is intermittent, the patient may have had higher levels at times other than when we sampled. Additionally, even though FSH secretion may have been intermittent, it may have been sufficient to cause sustained E$_2$ release by the numerous antral follicles. Lastly, certain types of hFSHR mutations result in unusual ligand selectivity that has been linked to OHSS (Smits et al., 2003). Our patient may have had a mutation in her hFSHR gene or one of its regulatory promoter regions. This may have increased her sensitivity to FSH. Our study highlights the need for further research into the mechanisms underlying the development of OHSS in patients with FSHomas.

In summary, the FSH bioactivity of our patient as determined by measuring the activation of a cAMP/CRE-mediated pathway and aromatase activity was similar to that of the normal subjects. This suggests that a mechanism other than enhanced bioactivity may be responsible for the development of OHSS in FSHoma patients.
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Author’s contributions
T.K. and S.L. participated in all of the experiments and data analysis. T.K. and T.M. designed the study. H.U., R.S., H.M., T.N., M.O. and T.A. participated in data collection and supporting the experiments. T.K., T.M. and Y.Y. wrote the manuscript.

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