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Normal testicular function without detectable follicle-stimulating hormone. A novel mutation in the follicle-stimulating hormone receptor gene leading to apparent constitutive activity and impaired agonist-induced desensitization and internalization

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ABSTRACT

Activating mutations in the follicle-stimulating hormone (FSH) receptor (FSHR) gene are rarely detected due to the absence of a clearly defined phenotype, particularly in men. We here report the biochemical features of a novel mutation in the first extracellular loop of the FSHR. The mutation (N431I) was detected in an asymptomatic man exhibiting normal spermatogenesis, suppressed serum FSH, and normal or elevated levels of biochemical markers of FSH action. Employing different experimental strategies on HEK-293 cells transiently expressing the N431I FSHR mutant, we found that the mutation led to decreased cell surface plasma membrane expression of the receptor protein, but conferred a low level of constitutive activity associated with markedly altered agonist-stimulated desensitization and internalization. These latter features may contribute and/or amplify the persistent activation of the receptor in both absence and presence of agonist and provide new insights into opportunities for adjuvant therapies based on disruption of these processes.

1. Introduction

The follicle-stimulating hormone (FSH) receptor (FSHR) belongs to a highly conserved subfamily of the G protein-coupled receptor (GPCR) superfamily and is mainly expressed in specific cells in the gonads (Dias et al., 2002; Simoni et al., 1997; Ulloa-Aguirre and Timossi, 1998). In the female, the FSHR is expressed in the granulosa cells where it regulates growth and maturation of ovarian follicles as well as estrogen production, whereas in the testis it supports the metabolism of Sertoli cells, thereby indirectly maintaining spermatogenesis (Ulloa-Aguirre et al., 2007). Upon agonist binding, the activated FSHR stimulates a number of intracellular signaling pathways. Although the canonical Gαs/cAMP/PKA signaling pathway has been recognized as a key effector mechanism of action of gonadotropins, the FSHR has also been reported to couple to other G protein subtypes and activate a number of distinct transduction mechanisms (Ulloa-Aguirre et al., 2011) depending on the particular developmental stage of the host cells (Musnier et al., 2009).

Mutations in GPCRs may lead to either loss-of-function or gain-of-function of the receptor molecule (Ulloa-Aguirre and Conn, 1998). Several loss-of-function mutations in the FSHR gene have been described in women with hypergonadotropic hypogonadism (Meduri et al., 2008); depending on their location in the receptor protein, inactivating mutations may provoke defective traffic of the receptor to the cell surface plasma membrane or impaired signal transduction (Huhtaniemi and Themmen, 2005). Although loss-

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of-function mutations in the FSHR gene do not apparently affect fertility in men, they may alter sperm quality to several extents (Tapanainen et al., 1997). Activating or gain-of-function mutations in the FSHR are extremely rare. Naturally occurring mutations in several transmembrane domains (TMD) (which participates in receptor activation and signal transduction) of the FSHR have been reported in women presenting ovarian hyperstimulation syndrome during pregnancy (Costagliola et al., 2005; De Leener et al., 2006; Montanelli et al., 2004; Smits et al., 2003; Vasseur et al., 2003). Although the pathogenesis of this particular syndrome has been explained by promiscuous activation of the receptor by the high levels of choriogonadotropin, some of these mutations (D567N, T449A, and I545T) also provoke low levels of constitutive receptor activity (De Leener et al., 2006; Montanelli et al., 2004; Smits et al., 2003). In men, the only apparently activating mutation in the FSHR gene has been described by Gromoll and colleagues (1996) in a hypophysectomized, testosterone-treated man with preserved normal spermatogenesis in spite of undetectable gonadotropins. The rarity of activating FSHR mutations is not surprising considering the absence of a clearly defined phenotype, particularly in men.

We here report a novel mutation in the FSHR located in the first extracellular loop (EL1) of the receptor protein. The mutation (N431I) was detected in a man with normal spermatogenesis, suppressed serum levels of FSH and normal or elevated circulating levels of biochemical markers of FSH action, initially suggesting that the mutation conferred constitutive activity to the receptor. We show here that this particular mutation additionally provokes impaired agonist-induced desensitization and internalization of the modified receptor. These abnormalities, concurrently with the low levels of constitutive activity, may explain the biochemical phenotype detected in the patient.

2. Materials and methods

Informed written consent for genomic testing and *in vitro* and *in vivo* studies was obtained from the index subject and the normal donors. All procedures were approved by the ethics committees of the institutions involved in the study.

2.1. DNA sequence analysis and identification of the mutation at the FSHR gene

Genomic DNA from the patient and relatives present in peripheral blood applied on Whatman FTA classic cards was used as template for amplification by PCR. Exon 10 of the FSHR gene was divided into 3 overlapping fragments (Fig. S1) that were individually amplified by PCR. A segment of 690 bp corresponding to segment 151–840 bp of exon 10 of the human FSHR was amplified using the oligonucleotide primers shown in Fig. S1. PCR amplification was exposed to 30 cycles of denaturation at 98 °C for 10 s, annealing at 58 °C for 1 min, and polymerization at 72 °C for 2 min. The reaction was then loaded on an agarose gel stained with 1.2% ethidium bromide and the band corresponding to 690 bp (segment 10A) was isolated from the gel and used either for direct sequencing or for subsequent cloning into the pGEM-T vector (Promega, Madison, WI, USA) to obtain the construct pGEM-T/segment A. The presence and position of the identified substitution in the cloned segment were verified by sequence alignment of the exon 10 sequence from the wild-type (WT) FSHR cDNA cloned in pcDNA3.1(+) (Invitrogen, Carlsbad, CA, USA) with that of segment A cloned in pGEM-T.

Blood samples from 60 healthy male donors born in the same town as the index subject were analyzed by allele-specific PCR (Akada et al., 2001) for the presence of the *FSHR* mutation identified in the index subject. Genomic DNA samples from 3 mm-diameter FTA classic card punches were subjected to PCR to specifically amplify a fragment of 634 bp from exon 10 corresponding to the WT sequence or to amplify a fragment of the same length corresponding to the mutant sequence. The PCR products were electrophoresed in 10% polyacrylamide gels and visualized by staining with ethidium bromide. Samples of genomic DNA from the index case as well as WT and N431I mutant FSHR cDNA constructs (in pcDNA3.1) were used as controls for the procedure.

2.2. Construction of the mutant FSHR

Construction of the mutant FSHR was performed employing the full-length WT human FSHR cDNA [GenBank Accession Number S59900] cloned in the expression vector pcDNA3.1. Asn431 (Fig. S2 E) was replaced with Ile (FSHR N431I) using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), as previously described (Uribe et al., 2008).

2.3. Cell culture and transfection

Human embryonic kidney (HEK)-293 cells were maintained in an humidified atmosphere of 5% CO_2 at 37 °C in high-glucose DMEM supplemented with 5% fetal calf serum (FCS) and antibiotics. Cells were grown to 70–80% confluency in 10 cm diammeter Petri dishes, replated on 60 mm diameter dishes and cultured for 24 h at 37 °C. Subconfluent cells were then transfected with 2–4 μg WT or mutant FSHR cDNAs by liposome-mediated endocytosis in OPTIMEM (Life Technologies, Grand Island, NY) or by Fugene 6 (Roche Diagnostics, Basel, Switzerland). Transfected cells were incubated for an additional 24 h before replating in 24-well plates for subsequent experiments or processed for immunoblotting. Co-transfections of WT or mutant FSHR with empty vector or cDNA constructs of β-arrestins [β-arrestin 1 and 2, or a dominant-negative form of β-arrestins (β-arrestin 319–418) (Krupnick et al., 1997)] were performed employing 3 μg of WT or mutant FSHR cDNA plus either 1 μg β-arrestin 1, β-arrestin 2, β-arrestin 319–418, or empty vector.

2.4. Measurement of cAMP production

Wild-type or mutant FSHR cDNA-transfected cells were incubated for 2 h at 37 °C in a humidified 5% CO_2 incubator in supplemented DMEM containing 0.125 mM 3-isobutil-methyl-xanthine (IBMX) in the absence or presence of increasing doses of recombinant FSH (recFSH) (Merk Serono, Geneva, Switzerland). At the end of the incubation, the medium and cells were collected and measured for total cAMP content by RIA (Zambrano et al., 1996).

For desensitization experiments, cells transfected with 4 μg WT or mutant FSHR cDNAs, were replated on poly-D-lysine-coated plates and incubated in the absence or presence (12–1200 ng/ml) of recFSH as described above. Cells were washed twice with warm PBS, replaced with fresh culture medium (with IBMX) containing 1200 ng/ml recFSH, and returned to the incubator for 30 min. Thereafter, the plates were put on ice and the medium and cells were collected for measurement of total cAMP.

2.5. SDS- PAGE and Western blotting

SDS–PAGE (7.5%) and Western blotting of protein extracts from cells transfected with the WT or mutant N431I cDNAs were performed as described previously (Uribe et al., 2008) employing the primary antibody mAb 106.105 (Nechamen and Dias, 2003). Equal protein loading was confirmed in a stripped, washed and reprobed membrane for glyceraldehyde-3-phosphate dehydrogenase detection.

2.6. Radioreceptor assay

Highly purified pituitary FSH was radiolabeled as described previously (Lindau-Shepard et al., 1994) to an average specific activity of 28 μCi/μg protein. Cells transfected with 2 μg of WT or mutant N431I FSHR cDNA plasmids, were replated in 24-well culture plates (150,000 cells/well) and assayed for specific ^{125}I-FSH binding (Uribe et al., 2008). Cell surface bound ^{125}I-FSH was eluted with elution buffer (50 mM glycine/100 mM NaCl, pH 3.0) for 20 min on ice and the eluate was removed to a glass tube and counted.

For displacement-binding assays, cells were transfected as described above, transferred to 12 × 75 mm culture tubes, and incubated in the presence of increasing concentrations of unlabelled pituitary FSH and 20 ng ^{125}I-FSH for 18 h at room temperature (Uribe et al., 2008). At the end of the incubation, 3 ml cold assay buffer were added and the cells were pelleted at 3000 rpm for 30 min and counted.

2.7. Internalization of FSHR under equilibrium and non-equilibrium conditions

Equilibrium-binding internalization assays were performed as described previously (Uribe et al., 2008). Transfected cells replated in 24-well plates were exposed to 20 ng/ml ^{125}I-FSH in serum-free DMEM in the presence or absence of 1 μg/ml unlabeled recFSH for 1 h at 37 °C. After the preincubation period and at each time point, cells were removed from the incubator, placed on ice, washed with PBS, and incubated on ice in elution buffer for 20 min. After the elute was removed for counting, the cells were washed with PBS and solubilized in 2 M NaOH for 1 h at room temperature to allow measurement of cell-associated counts per minute.

Internalization under non-equilibrium binding conditions was measured by binding assays performed as described above, but ommiting the 1 h preincubation step and with ^{125}I-FSH removed at different times [0 (immediately after addition of labeled agonist), 5, 10, 15, 30, 45, 60, and 90 min]. Cells were processed as described above for measuring cell surface and cell-associated ^{125}I-FSH.

2.8. Recycling and degradation of the internalized FSHR

Recycling and degradation of the FSHR were assessed following the procedure described previously (Kluetzman et al., 2011). This assay is a pulse-chase paradigm in which the relative levels of total recycled ^{125}I-FSH [the sum of trichloroacetic acid (TCA)-insoluble radioactivity in the medium and surface bound radioactivity) and degraded ^{125}I-FSH (TCA-soluble radioactivity in the medium) are determined during a period of 4 h after allowing internalization of the receptor-ligand complex. At each time point, the recycled ^{125}I-FSH is an indirect measurement of the amount of internalized receptor that was recycled back to the plasma membrane, whereas the amount of degraded ^{125}I-FSH represents the fraction of the internalized receptor that was targeted to lysosomes and/or proteasomes for degradation (Kluetzman et al., 2011; Krishnamurthy et al., 2003b).

2.9. FRET assay

HEK-293 cells transiently expressing the WT or N431I FSHR were transiently transfected with the cAMP (ICUE) plasmid-encoded FRET sensor (DiPilato et al., 2004; Zhang et al., 2001) and processed for the FRET assay as described previously (Tranchant et al., 2011). Baseline signals at the wavelength corresponding to YFP and CFP were recorded for 5 min and then the first recFSH dose (1.5 ng/ml) was added to the dish. After signal recording for 10 min, cells received a second recFSH challenge (100 ng/ml) to assess for desensitization and the signal was recorded for an additional 10 min period before adding forskolin (20 μM) as a positive control for the integrity of the $G_{\alpha s}$–adenylyl cyclase–cAMP pathway. Fluorescent intensity of non-transfected cells was substracted from the intensity of fluorescent cells expressing the sensor in order to quantify the specific signal. The mean (±SEM) FRET CFP/YFP *ratio* was calculated from at least 15 individual cell responses.

2.10. Reporter gene assay

Cells were co-transfected with the WT or N431I FSHR cDNA and the pSOMLuc reporter gene as previously described (Lazari et al., 1999; Troispoux et al., 1999). Forty-eight hours after transfection, cells were serum-starved for 24 h, then stimulated for 6 h with forskolin. Cells were then washed twice with cold PBS and lysed with passive lysis buffer. The luciferase activity was measured using a luciferase assay system (Promega). Each sample (20 μl) was mixed with 50 μl of luciferase assay reagent, containing the substrate. The light produced was measured in a luminescence counter and expressed as a percentage of maximum response by the WT FSHR.

2.11. Hormone assays

Serum LH and FSH levels were measured by immunoradiometric assay employing commercial kits (Diagnostic Products Corporation, Los Angeles, CA, USA); the sensitivity of these assays were 0.3 IU/L (FSH) and 0.15 IU/L (LH), and their intra- and inter-assay coefficients of variation were ⩽3.0% and ⩽5.0%, respectively. Serum T levels were measured by a previously reported in-house RIA (Scaglia et al., 1991). Serum anti-Müllerian hormone (AMH) and Inhibin B (Inh-B) levels were measured employing enzyme-linked immunoassays from Diagnostic System Laboratories Inc. (Webster, TX, USA); the sensitivity of these assays were 0.06 ng/ml (AMH) and 7.0 pg/ml (Inh-B), and their intra- and inter-assay coefficients of variation were ⩽5.0% and ⩽8%, respectively.

2.12. Statistical analysis

Statistical analysis was performed employing one-way-analysis of variance (ANOVA) (for comparing the means from 3 independent groups) followed by the Tukey's multiple comparison test or by the unpaired Student's *T* test (to compare the means in 2 groups). For the dose–response curves, the curve parameters were determined using the software GraphPad PRISM 4.0 (GraphPad Software, Inc., La Jolla, CA). Values are expressed as means ± SEM. from three or more independent experiments. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Patient

The patient was a 21 year old man from the southwest of Buenos Aires Province in Argentina, who was originally referred for testicular pain during sexual intercourse. His previous history was uneventful, with a normal pubertal development beginning at the age of 11 years. No sexual dysfunction or impaired libido were reported. At physical examination, normal secondary sexual characteristics, including testicular volume and penis size, were found. A left varicocele was detected by Doppler color echography. Two spermograms performed at 2 different laboratories showed normal results in all parameters examined, including sperm count (36 and 42 × 10^6/ml), motility (70% and 75%), and morphology. Basal serum thyroid-stimulating hormone, free T_4, prolactin, testosterone, luteinizing hormone, and inhibin-B (Inh-B) levels were within

Table 1
Basal, GnRH-, recFSH- and hCG-stimulated serum concentrations of FSH, LH, AMH, Inh-B and T in the index case.

Hormone	Basal	Minutes post iv GnRH		Hours post im recFSH or hCG		
		30'	60'	24 h	48 h	72 h
FSH (IU/L)	<0.3 (1.0–8.0)*	1.3 (4.0–22.0)	0.7 (5.0–30.0)	–	–	–
LH (IU/L)	4.0 (0.7–9.7)	42 (20.0–60.0)	30 (15.0–55.0)	–	–	–
AMH (ng/ml)	10.5 (2.0–5.9)	–	–	8.4	9.1	7.8
Inh-B (pg/ml)	128 (109–305)	–	–	131	570	130
T (ng/ml)	6.5 (3.0–9.0)	–	–	9.0	10.4	–

* Normal range in 40 healthy adult men.

normal limits, while serum levels of anti-Müllerian hormone (AMH) were elevated (Table 1). Since undetectable concentrations (<0.3 IU/L) of serum FSH were found in two independent samples obtained in separate visits, a GnRH *i.v.* stimulation test was performed. After the exogenous GnRH challenge (100 μg), serum LH levels rose normally and FSH levels increased 4.3-fold above the minimal detectable dose of the immunoassay employed, albeit still well below the values found in young healthy men (Table 1). Given the normal results from the spermograms as well as the normal and increased levels of serum Inh- B and AMH, respectively, in the patient, 300 IU of recombinant FSH (Serono Argentina S.A., Buenos Aires, Argentina) were administered *i.m.* and blood samples were collected 24, 48, and 72 h after the injection. Serum inhibin B rose normally whereas AMH remained virtually unmodified (Table 1). Normal spermatogenesis in the presence of undetectable serum FSH levels strongly suggested that the patient may bear a gain-of-function mutation in the FSHR. To determine if a mutation in the FSHR gene existed, a study was designed with informed consent, and further blood samples were obtained from the patient and relatives as well as from 60 normal male individuals living in the same town as the patient.

3.2. Detection of the FSHR mutation

Comparative analysis of the sequences obtained from the three FSHR exon 10 overlapping segments from the patient and the

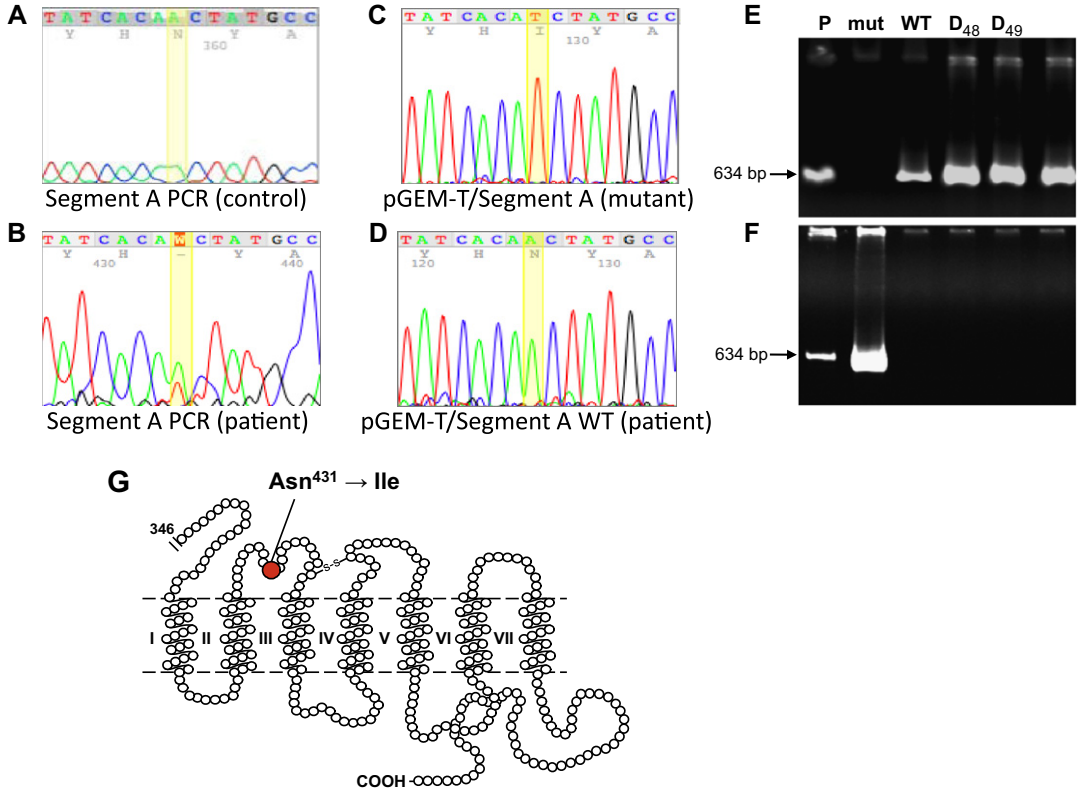

Fig. 1. Identification of the heterozygous mutation in the FSHR gene of the patient. AAC in codon 431 of the *FSHR* was mutated to ATC, generating the amino acid substitution of aspargine with isoleucine at the EL1 of the receptor protein. (A) Representative electropherogram of a PCR product from a control sequence (the father of the index subject), showing codon 431 (AAC, with the WT A shaded in yellow) of the WT FSHR cDNA. (B) Segment A (see text and Fig. S1) of exon 10 of the *FSHR* showing the allelic forms (AAC and ATC) of codon 431 ("W" at the top) detected in the PCR product from the patient's sample. (C and D) Sequence of two different clones of segment A cloned into pGEM-T showing the mutant (C) and WT (D) DNA strands of the patient, individually. The mutant T or WT A in codon 431 are shaded in yellow. The "W" at the top of (B) indicates the uncertainty between adenine and thymine at this position. (A–D) are representative from at least 3 independent PCR amplifications of 3 individual samples. (E) Polyacrylamyde gel (10%) electrophoresis from a representative PCR assay run with the WT oligonucleotide, showing the amplification products of genomic DNA from the patient (P) and 3 healthy male donors born in the same town as the index subject (D_{48}-D_{50}), as well as from the WT and N431I FSHR cDNAs cloned in pDNA3.1 (WT and mut, respectively). Note the absence of a 634 bp band in the lane corresponding to the mutant plasmid. (F) Same as E but from a PCR assay run with the mutant oligonucleotide. Note the absence of the 634 bp band in the lanes corresponding to the WT FSHR cDNA plasmid and the genomic DNA from the control subjects. Similar results were found in PCR assay runs of genomic DNA from the remaining 57 controls. (G) Schematic representation of the FSHR structure showing the location of the N431I mutation (red circle). The structure does not show amino acid residues corresponding to the large extracellular ectodomain of the receptor.

reported sequence of the WT FSHR, revealed a discrepancy at nucleotide 437 of segment A (Fig. 1B). The mutation was located at codon 431 of the *FSHR* where AAC was mutated to ATC, generating the amino acid substitution of aspargine with isoleucine at the EL1 of the receptor protein (Fig. 1A, C and E). The change could be detected as heterozygous in the PCR sequence as a trace of adenine and thymine overlapped at position 437 of segment A (Fig. 1B). Subsequently, the presence of the mutant sequence (ATC) was detected only in one individual strand whereas the WT sequence (AAC) was found in the other as disclosed by sequence analysis of segment A cloned into pGEM-T (Fig. 1C and D). Therefore, the patient was heterozygous for the missense mutation 1292A > T in exon 10 of the *FSHR* that led to the expression of a N431I mutant receptor. No further mutations were detected in exon 10 of the *FSHR* in this patient.

Screening of the N431I mutation in samples from 8 family members (including the parents) of the index subject and from 60 normal men did not identify the mutation in any of the individuals (Fig. 1E and F).

3.3. ^{125}I-FSH binding to the N431I FSHR and Western blot analysis

The mutant receptor cDNA was transfected in HEK-293 cells to assess plasma membrane expression of the N431I FSHR mutant by radioreceptor binding assay and Western blotting. Compared to cells transfected with the WT receptor, the capability of cells transfected with 2 μg cDNA to maximally bind ^{125}I-FSH was considerably reduced at levels <50% from those exhibited by the WT FSHR counterpart (Fig. 2A, inset); meanwhile, co-transfection of WT and N431I FSHR cDNAs at a 1:1 WT to mutant cDNA ratio, yielded specific ^{125}I-FSH binding levels that where intermediate between those shown by the WT receptor and the N431I mutant. To compare the receptor affinity of the WT and N431I mutant FSHRs, a competition RRA was performed on transiently transfected HEK-293 in the presence of 20 ng/ml ^{125}I-FSH and increasing concentrations of unlabeled FSH (Fig. 2A). The K_i value calculated for the N431I mutant (0.804 ± 0.102 nM) was modestly higher ($p < 0.05$) than that showed by the WT FSHR (0.341 ± 0.174 nM), indicating a reduced ligand-binding affinity of the mutant receptor.

Western blot analysis of protein extracts from cells transfected with the mutant N431I cDNA further confirmed the reduced plasma membrane expression levels of the mutant receptor as disclosed by the reduced intensity of the ~80 kDa band representing the mature, fully glycosylated FSHR (Fig. 2B).

3.4. Basal, FSH- and forskolin-stimulated cAMP production by HEK-293 expressing the N431I FSHR

Signaling activity of the N431I mutant transiently expressed by HEK-293 cells was measured by its basal cAMP production and the response to increasing doses of recombinant FSH (Fig. 3A and B, and Table 2). Binding of ^{125}I-FSH was measured on cells in parallel so that cAMP induction could be normalized to cell surface expression of FSHR (Fig. 3C and D). In basal (unstimulated) conditions, cAMP production was decreased by ~25% in cells transfected with 2 μg of the N431I mutant cDNA or co-transfected with the WT and N431I mutant FSHR cDNAs at a 1:1 WT to mutant cDNA ratio (Fig. 3A). Follicle-stimulated hormone-stimulated cAMP production was also significantly decreased in cells transiently expressing the N431I mutant or both the WT receptor and the mutant (Fig. 3B). Nevertheless, when cAMP values were normalized to ^{125}I-FSH binding, the mutation enhanced basal cAMP and its response to agonist stimulation by ~2.3- and ~1.5-fold, respectively, over those shown by the WT counterpart (Fig. 3C and D, and Table 2). These results suggested that the Asn to Ile substitution at amino acid residue 431 conferred a low level of constitutive activity to the FSHR.

Since enhancement of forskolin-induced cAMP production due to spontaneous coupling to signal transduction pathways seems to be a general characteristic of constitutively active G_s-coupled receptors (Alewijnse et al., 1997; Parma et al., 1993), HEK-293 cells

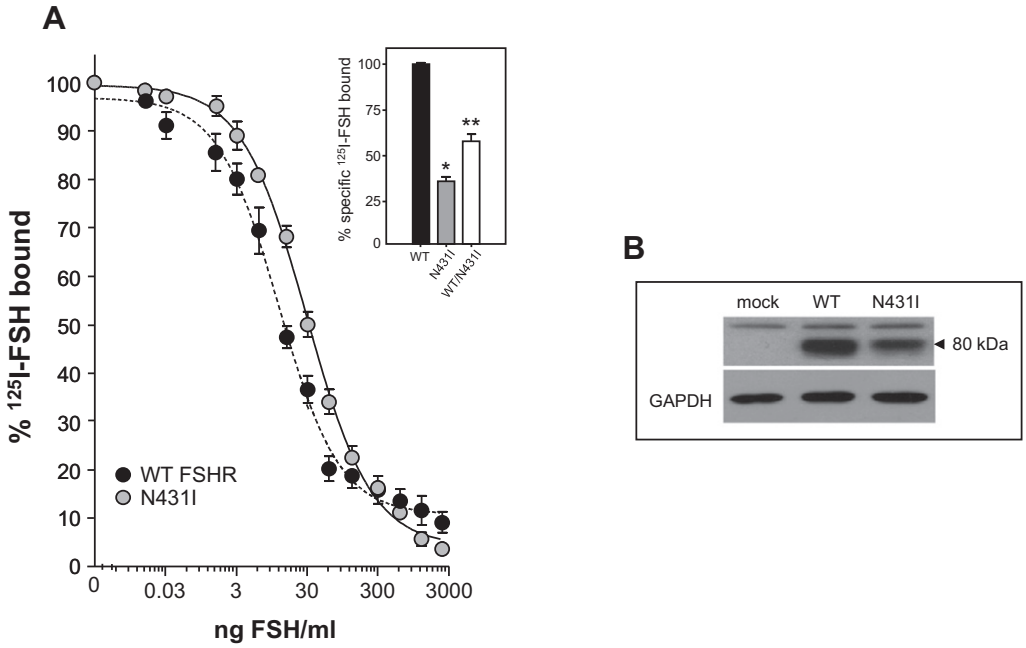

Fig. 2. Plasma membrane expression of the WT and N431I FSHR. (A) Displacement of ^{125}I-FSH by increasing doses of unlabeled recombinant human FSH in HEK-293 cells transfected with the WT FSHR and the mutant N431I FSHR. *Inset*: specific labeled agonist binding to HEK-293 cells transiently expressing the WT, N431I or both WT and N431I FSHRs. $^{*}p < 0.01$ vs. WT and WT/N431; $^{**}p < 0.01$ vs. WT. (B) Relevant portion of an autoradiogram from an immunoblot of the WT FSHR and N431I mutant FSHR. HEK-293 lysates transiently expressing the WT or N431I FSHRs were resolved by SDS–PAGE, transferred to Immobilon-P membranes and probed with anti-FSHR mAb 106.105. The arrowhead indicates the position of the mature, cell surface membrane-expressed 80 kDa form of the FSHR. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

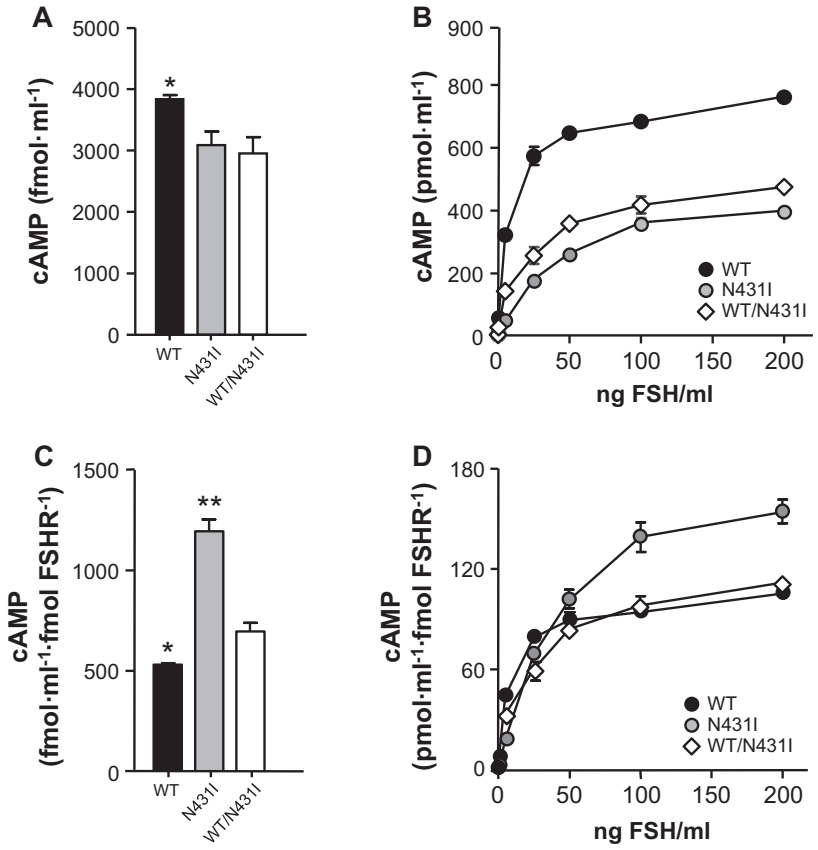

Fig. 3. Basal (A and C) and FSH-stimulated (B and D) cAMP production in HEK-293 cells transiently expressing the WT, N431I, or both WT and N431I FSHRs. Basal cAMP levels in cells transfected with empty vector were 721 ± 30 fmol ml^{-1}. The data in (C and D) were normalized to fmoles of FSHR protein according to parallel ^{125}I-FSH binding assays. $^{*}p < 0.01$ vs. N431I and WT/N431I; $^{**}p < 0.01$ vs. WT/N431I.

Table 2
Basal and maximal FSH-stimulated cAMP production (R_{max}), and effective FSH concentration (ED_{50}) (means ± S.D.; $n = 3$ independent experiments) in HEK-293 cells transiently expressing the WT FSHR, the N431I mutant or both WT and mutant FSHRs.

FSHR	BASAL		R_{max}		ED_{50}
	fmol ml^{-1}	fmol ml^{-1} fmol FSHR^{-1}	pmol ml^{-1}	pmol ml^{-1} fmol FSHR^{-1}	ng/ml
WT	3836 ± 85*	531 ± 9*	762 ± 49*	105 ± 2***	6 ± 0.1*
N431I	3097 ± 209	1195 ± 59**	399 ± 58**	154 ± 7**	34 ± 0.9
WT/N431I	2951 ± 256	697 ± 43	474 ± 52	112 ± 4	25 ± 4.0

* $p < 0.01$ WT vs. N431I and WT/N431I.
** $p < 0.05$ N431I vs. WT/N431I.
*** $p < 0.05$ WT vs. N431I.

co-transfected with the cAMP-sensitive reporter plasmid pSOMLuc and the N431I mutant or WT receptor cDNAs were stimulated with increasing doses of forskolin. As shown in Fig. 4, forskolin increased luciferase activity in cells transfected with the WT and the mutant FSHRs in a dose-dependent manner. Nevertheless and despite the reduced expression levels of the mutant receptor (Fig. 4 inset), forskolin-induced luciferase activity in cells expressing the N431I mutant was higher than that exhibited by cells expressing the WT FSHR. These results confirmed that the N431I mutant FSHR is a constitutively active receptor.

3.5. Desensitization and internalization of the N431I mutant FSHR

Since constitutively active mutant GPCRs may or may not exhibit constitutive desensitization and down-regulation, depending on the particular receptor and the location of the activating mutation (Frenzel et al., 2006; Pei et al., 1994; Quellari et al., 2003), we studied the kinetics of desensitization and internalization of the N431I mutant FSHR. For this purpose, HEK-293 transiently transfected with the WT and mutant FSHR cDNAs were incubated with increasing concentrations of FSH for 2 h and then exposed to high concentrations of agonist for 30 min. During the first 2 h of incubation, FSH-stimulated cAMP production was significantly reduced in cells transiently expressing the N431I mutant or both the WT receptor and the N431I mutant (Fig. 5A). Upon re-exposure to high doses of agonist, the WT FSHR displayed desensitization regardless of the FSH dose used during pretreatment, whereas the N431I mutant showed a clear reduction in desensitization (Fig. 5B). In cells co-expressing both receptors (at a 1:1 WT to mutant ratio) the decrease in cAMP response provoked by re-exposure to FSH was intermediate between those shown by cells expressing only the WT receptor or the N431I mutant.

To corroborate the above described results, a FRET assay in living cells was designed in which HEK-293 cells transiently expressing the WT or the N431I FSHR and the cAMP (ICUE) FRET sensor were exposed to 2 consecutive doses (1.5 and 100 ng/ml) of FSH. As shown in Fig. 5C, FRET experiments revealed that the robust and rapid cAMP response to 1.5 ng/ml FSH exhibited by cells expressing the WT receptor was decreased in cells expressing the N431I mutant. Conversely, addition of the second (100 ng/ml) FSH dose led to reduced responsiveness of the WT receptor and to a robust response of the N431I mutant. In both conditions adenylyl-cyclase activity remained inducible as revealed by the addition of forskolin. Interestingly, the cAMP response to forskolin as detected by FRET was higher in cells expressing the N431I than

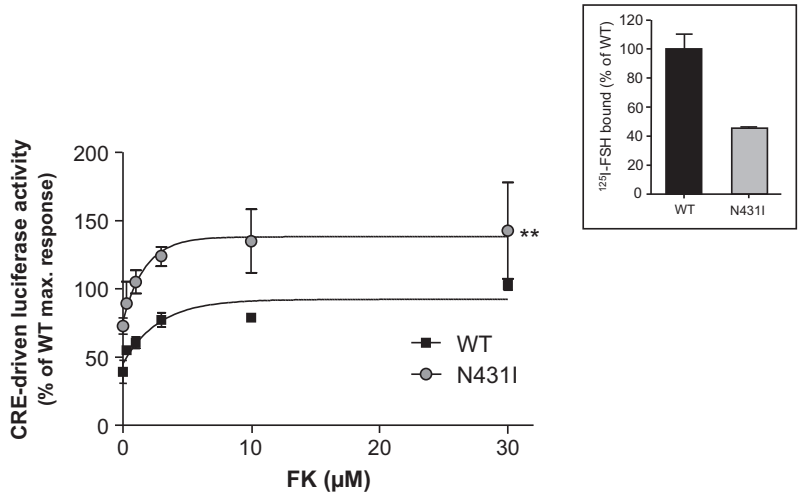

Fig. 4. Increased $G_{\alpha s}$–adenylyl cyclase constitutive coupling in HEK-293 cells transiently expressing the WT or N431I FSHRs. HEK-293 cells were transiently co-transfected with the cAMP-sensitive reporter plasmid pSOMLuc and either WT or N431I FSHR. Forty-eight hours after transfection, cells were serum-starved for 24 h and then stimulated for 6 h with increasing amounts (0.33–30 μM) of forskolin (FK). Luciferase activity was then measured in total cell extracts. Results are expressed as mean ± S.E.M. of 4 independent measurements. $^{**}p < 0.01$, when compared to WT FSHR. *Inset*: relative expression levels of the WT and N431I FSHR as assessed binding assay with ^{125}I-FSH. Data corresponding to WT FSHR were arbitrarily chosen as 100%.

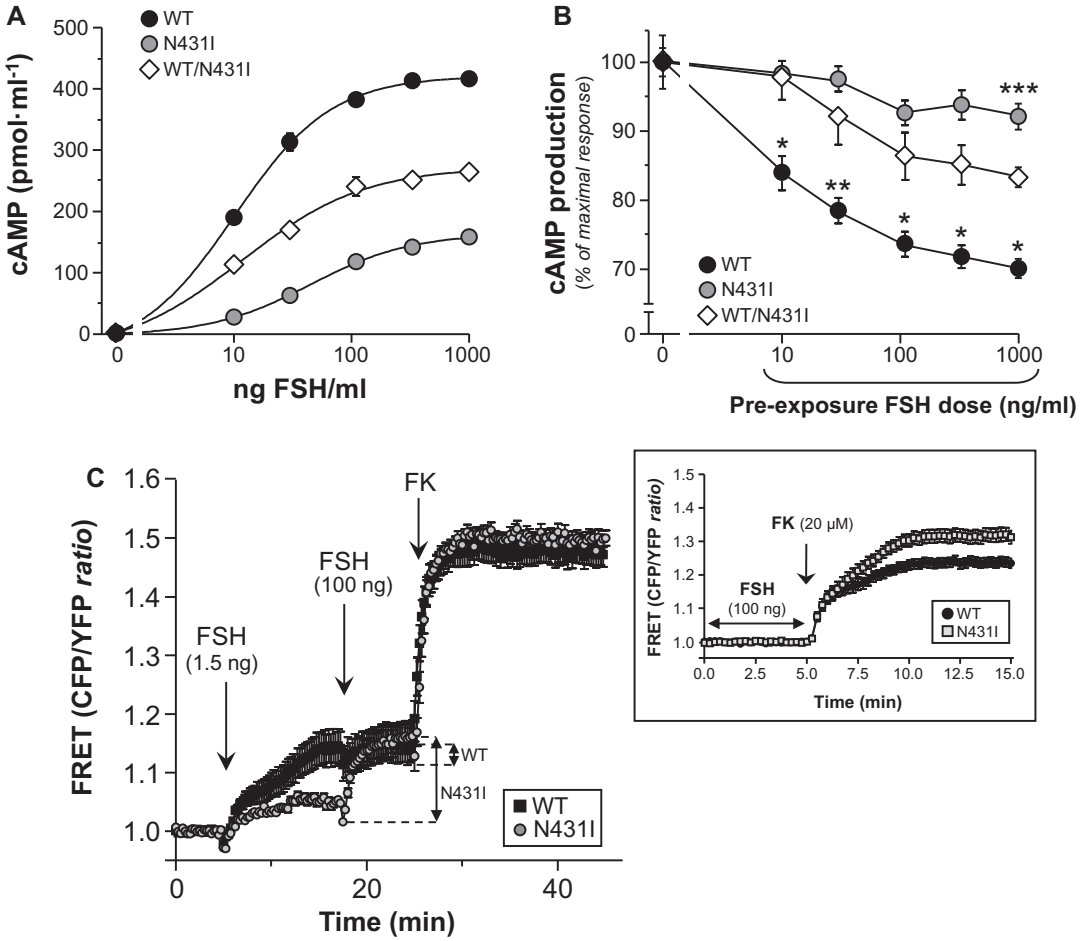

Fig. 5. Altered desensitization of the N431I FSHR. (A) Serum-starved HEK-293 cells transiently expressing either the WT, the N431I or both WT and N431I FSHRs were stimulated for 2 h with increasing amounts of recombinant FSH in the presence of IBMX, and cAMP in the incubation media was determined by RIA. (B) After stimulation with FSH, cells were washed twice and then re-challenged with a saturating (1200 ng/ml) dose of FSH in the presence of IBMX; total (intra- and extracellular) cAMP was then determined. The results are the means ± S.E.M. from three independent experiments. $^{*}p < 0.02$ vs. N431 and WT/N431I; $^{**}p < 0.01$ vs. N431I and $p = 0.05$ vs. WT/N431I; $^{***}p < 0.02$ vs. WT/N431I. (C) HEK-293 cells transiently expressing either the WT or N431I FSHR and the cAMP FRET sensor ICUE were exposed to a low (1.5 ng/ml) dose of recombinant FSH and after 10 min re-challenged with a high (100 ng/ml) dose of the hormone. At the end of the experiment, forskolin (FK) (20 μM) was added as a positive control for the integrity of the $G_{\alpha s}$–adenylyl cyclase–cAMP pathway. Signals at the wavelength corresponding to YFP and CFP were recorded during the entire experiment. The areas that did not respond to forskolin were removed from the analysis. Double-head arrows limit the magnitude of the response to the high-dose FSH challenge. *Inset:* Net responses to forskolin after the high-dose FSH challenge in the experiment shown in (C); YFP/CFP ratios corresponding to the WT and N431I responses were set at 1.0. The results shown are representative of a single FRET experiment; similar results were found in a second, independent experiment.

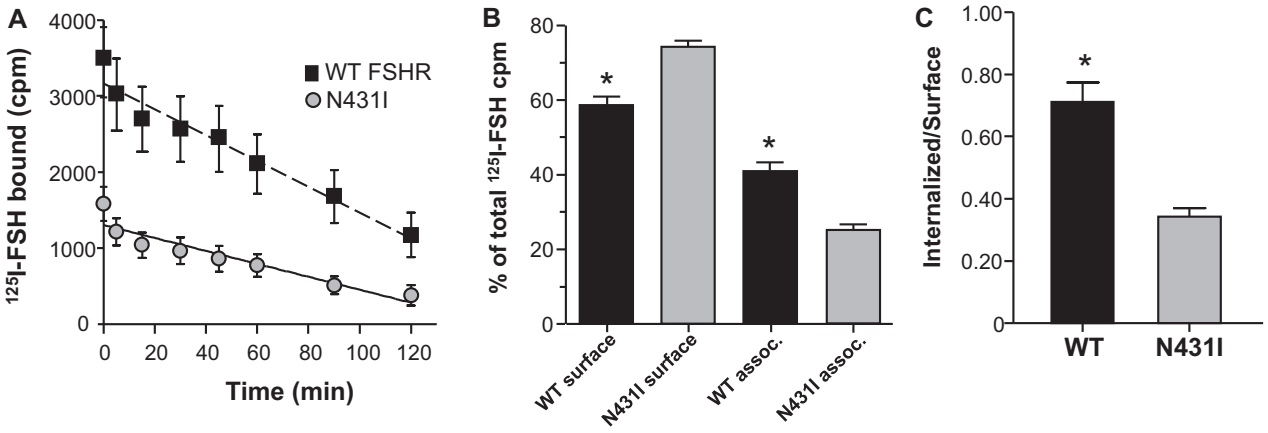

Fig. 6. Internalization dynamics of the WT and N431I FSHRs in equilibrium binding conditions. (A) Disappearance of cell surface ^{125}I-FSH bound to the WT and N431I mutant FSHRs in transiently transfected HEK-293 cells as a function of time. (B) Cell surface and cell associated ^{125}I-FSH at the end of the internalization experiment (120 min). More radiactivity was detected in the surface of cells expressing the N431I mutant receptor. (C) Internalized (cell associated)/surface ^{125}I-FSH ratio in HEK-293 cells transiently expressing either the WT or the N431I mutant receptor at the end of the internalization experiment. $^{*}p < 0.01$ vs. N431I. Data are the means ± S.E.M. of three independent experiments.

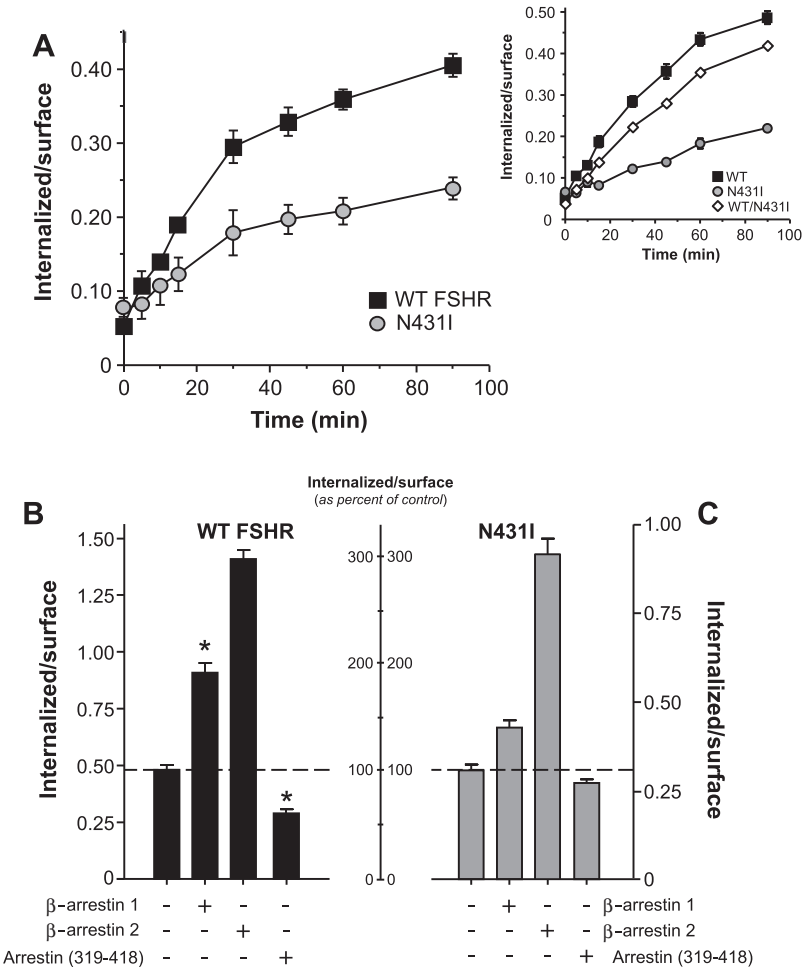

Fig. 7. Internalization of ^{125}I-FSH measured under non-equilibrium binding conditions is impaired in cells expressing the N431I mutant. (A) Internalized (cell associated)/surface ^{125}I-FSH ratio in HEK-293 cells transiently expressing either the WT or the N431I mutant receptor as a function of time. Specific FSH binding was assessed by removing free hormone, and surface-bound radiolabeled FSH was eluted and quantitated after adjusting for nonspecific binding. Cells were then lysed, and cell-associated radiolabeled hormone was determined. *Inset:* Internalized/surface ^{125}I-FSH ratio in HEK-293 cells transiently expressing either the WT, the N431I, or both the WT and N431I FSHRs. Agonist-provoked internalization of the FSHR in cells expressing the N431I mutant was consistently reduced. Both graphs are the means ± S.E.M. from at least 3 independent experiments. (B and C) Internalization of ^{125}I-FSH at 90 min measured under non-equilibrium binding conditions in cells transiently co-transfected with either the WT or the N431I FSHR and β-arrestin 1, β-arrestin 2 or dominant-negative arrestin (319–418). $^{*}p < 0.01$ WT vs. N431I for the same co-transfection conditions.

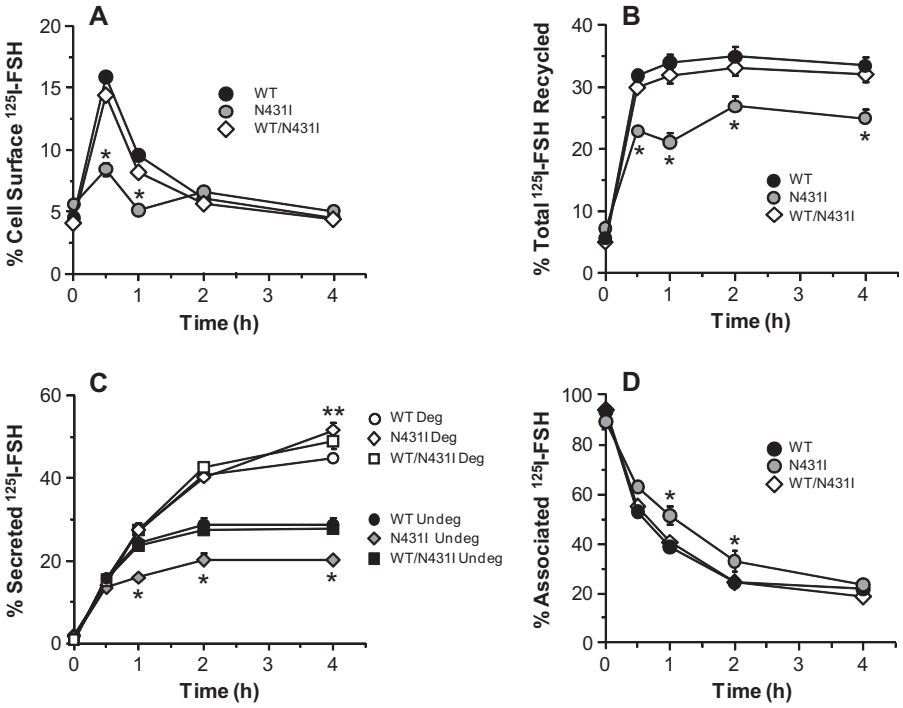

Fig. 8. Recycling back to the plasma membrane of the N431I FSHR after agonist-induced internalization is reduced. (A) Cell surface ^{125}I-FSH (presumably bound to the FSHR) recycled back to the plasma membrane throughout the second, 4 h incubation (see Section 2.8); $^{*}p < 0.01$ vs. WT and WT/N431I. (B) Total ^{125}I-FSH/FSHR recycled back to the plasma membrane [cell surface ^{125}I-FSH (Fig. 8A) *plus* TCA-precipitable ^{125}I-FSH (Fig. 8C)]; $^{*}p < 0.03$ vs. WT and WT/N431I. C: TCA-soluble (degraded) and –precipitable (undegraded) radioactivity recovered from the culture medium throughout the second, 4 h incubation. Less undegraded tracer is secreted to the medium in cells transiently expressing the N431I mutant FSHR; $^{*}p < 0.05$ vs. WT and WT/N431I (undegraded); $^{**}p < 0.05$ N431I vs. WT (degraded). (D) Cell associated ^{125}I-FSH recovered throughout the second incubation; $^{*}p < 0.05$ vs. WT and WT/N431I.

in those expressing the WT receptor (Fig. 5C, inset). These results confirmed the impaired agonist-stimulated desensitization as well as the constitutively active nature of the N431I mutant FSHR.

Given that agonist-induced early desensitization is followed by internalization of the agonist-bound receptor and that in both processes, β-arrestins play a critical role (Freedman and Lefkowitz, 1996), we next examined the internalization kinetics of the N431I mutant and its regulation by β-arrestin 1 and 2. In equilibrium binding conditions, the internalization of the N431I was significantly delayed as disclosed by the lower disappearance rate of ^{125}I-FSH [and presumably receptor as well (Kluetzman et al., 2011; Krishnamurthy et al., 2003b)] from the cell surface during a pulse-chase paradigm (slope values of the regression line generated by graphing the surface-associated hormone as a function of time: N431I mutant, −7.76 ± 1.47; WT FSHR, −16.90 ± 3.61; $p = 0.02$) (Fig. 6A). Further, after 2 h of the chase phase the total amount of acid dissociable ^{125}I-FSH that remained at the cell surface was significantly higher and the level of internalized, cell associated ^{125}I-FSH significantly was lower in FSHR-N431I transfected HEK-293 cells than in WT-FSHR transfected cells ($p < 0.01$) (Fig. 6B). Accordingly, the internalization index (defined as the relationship between the amount (cpms) of internalized and surface-bound ^{125}I-FSH at time 120 min) was significantly lower for the N431I receptor (Fig. 6C). Under non-equilibrium binding conditions (which does not allow for saturation of the recycling/degradative pathways), the internalization kinetics of the N431I mutant was also significantly ($p < 0.05$) delayed [area under the curve (AUC) of the internalized/surface ratio/90 min in cells expressing the N431I receptor: 14.4 ± 3.9; WT FSHR: 27.7 ± 3.9] (Fig. 7A). Similar results were found when internalization of the liganded receptor was tested in cells co-transfected with the WT and N431I mutant receptor cDNAs (at a 1:1 WT:mutant ratio), albeit in this case the delay in internalization dynamics was less marked than in cells transfected with the N431I mutant (AUC of the internalized/surface ratio/90 min in cells expressing the WT and N431I FSHRs: 24.2 ± 1.2; $p < 0.05$ vs. WT and N431I FSHRs) (Fig. 7A, inset).

Fig. 7B and C show the internalization index of ^{125}I-FSH in cells transfected with the WT and N431I mutant cDNAs, after 90 min of exposure to the hormone under over-expression conditions of β-arrestins [β-arrestin 1 and 2, or a dominant-negative form of β-arrestins (β-arrestin 319–418)]. Over-expression of WT β-arrestin 1 increased by nearly 2-fold and 1.5-fold the internalization of the WT and N431I mutant FSHRs, respectively ($p < 0.05$ N431I vs. WT) (Fig. 7B and C), whereas the internalization provoked by over-expression of WT β-arrestin 2 was similar in both receptors. Interestingly, over-expression of the dominant-negative form of β-arrestin reduced internalization of the WT receptor by 40 ± 5% whereas in the case of the N431I mutant internalization was only marginally affected (12 ± 4% reduction). These data suggested that the delayed desensitization and internalization of the N431I mutant receptor was probably due to failure to recruit endogenous β-arrestin 1 properly.

3.6. Degradation and recycling of the N431I mutant FSHR

In order to test if the N431I mutation altered the fate of the receptor following agonist-provoked internalization, a pulse-chase experiment was performed in which the relative levels of total recycled ^{125}I-FSH (the sum of TCA-insoluble radioactivity in the medium and surface bound radioactivity) and degraded (TCA-soluble radioactivity in the medium) were determined (see Section 2.8). As shown in Fig. 8, cells transfected with the N431I mutant FSHR appear to recycle internalized ^{125}I-FSH back to the plasma membrane less efficiently than those transfected with the WT receptor or co-transfected with the WT and mutant receptor (Fig. 8A and B)

(AUC of total ^{125}I-FSH recycled/240 min in cells transfected with the N431I FSHR: 94.4 ± 5.4; WT FSHR: 128.6 ± 13.2; WT/N431I: 121.8 ± 13.8; $p < 0.05$ N431I vs. WT and WT/N431I). Accordingly, less TCA precipitable ^{125}I-FSH (which represents the main fraction of FSHR recycled back to the plasma membrane) was recovered within the medium of cells transfected with the N431I mutant (AUC of secreted TCA-precipitable ^{125}I-FSH/240 min in cells transfected with the N431I mutant: 69.7 ± 7.2; WT FSHR: 98.7 ± 13.0; WT/N431I: 94.4 ± 12.8; $p < 0.05$ N431I vs. WT and WT/N431I). Thus, FSHR-N431I transfected cells degraded more (Fig. 8C) and exported less (Fig. 8B) internalized hormone back to the plasma membrane than those expressing the WT receptor or both the WT and N431I FSHRs.

4. Discussion

Constitutive activation of GPCRs is characterized by signaling in the absence of agonist (Parnot et al., 2002); this phenomenon, resulting from point mutations is a well-recognized cause of an array of disorders (Tao, 2008). In the glycoprotein hormone receptors, constitutive activation of the LH and TSH receptors leads to male pseudohermaphroditism and hyperthyroidism, respectively (Tao, 2008). Nevertheless, in the case of the FSHR, there is not a clearly defined phenotype or disease associated with this particular molecular alteration in receptor function. In the present study, we identified both a novel mutation in the FSHR gene and a previously unrecognized modulation of FSHR activity in an asymptomatic man that exhibited completely normal spermatogenesis associated with undetectable serum FSH concentrations and normal (Inh-B) or high (AMH) levels of biochemical markers of FSH activity. The *de novo* heterozygous mutation detected was located in the region coding the EL1 of the FSHR gene and conferred the receptor protein markedly reduced plasma membrane expression and apparent constitutive activity associated with impaired desensitization and internalization as well as with reduced recycling to the plasma membrane after agonist-provoked internalization. Interestingly, co-expression of both WT and N431I FSHR in HEK-293 cells (emulating what is presumably expressed by the patient) also resulted in altered basal activity, and significantly reduced desensitization and internalization, which may concurrently explain the biochemical phenotype detected in the patient. Nevertheless, it should be emphasized in that, given that transfection of the WT receptor cDNA led to significantly higher FSHR expression levels, it might be that when equivalent amounts of both the WT and N431I plasmids were cotransfected, an overwhelming majority of WT receptor was eventually expressed, which could in turn lead to less marked differences with the WT receptor. In any case, the mutation teaches how extracellular loops of the FSHR can modulate receptor activation, and more important, intracellular trafficking, suggesting that small molecule modulators of FSHR extracellular loop conformation could lead to agonist independent fertility enhancement by decreasing desensitization following stimulation with endogenous hormone – a potential fertility booster.

Along these lines, N431 lies in the middle of the EL1, which is an unusual location for a mutation associated with constitutive activity of GPCRs. Nevertheless, constitutively active mutations located in the extracellular loops of other structurally related glycoprotein hormone receptor, the TSH receptor, have been previously detected in patients with functioning thyroid adenomas (Fuhrer et al., 1997; Parma et al., 1995; Tonacchera et al., 2000). As previously discussed for activating mutations in the EL1 of the TSHR (Parma et al., 1995), in the case of the N431I FSHR mutation the substitution of asparagine, a polar and hydrophilic amino acid residue, with the hydrophobic isoleucine might distort the EL1, alter its molecular conformation, and lead to modifications in the relative position of TMDs 2 and/or 3, mimicking a conformation associated with receptor activation and/or G protein interaction (Ulloa-Aguirre et al., 2007), and additionally preventing desensitization and arrestin binding (discussed below). The former effect could conceivably occur by a distortion in the EL1 provoked by the mutation secondarily altering the configuration of the NH_2-terminal end of the loop (whose first five amino acids form a turn of helix as an extension of the TMD2), which has been suggested to be involved in agonist binding and receptor activation (Ji and Ji, 1995). A second alternative explanation for the constitutive activity of this particular mutant is that the EL1 may be involved in the interaction between the ectodomain of the unliganded FSHR and its serpentine domain (via the extracellular loops as outward projections of the TMDs), interaction that presumably "locks" the latter domain in a predominantly inactive conformation (Vassart et al., 2004; Vlaeminck-Guillem et al., 2002). In this scenario, alterations in the configuration of the EL1 might partially interfere with the inhibitory ectodomain-TMD interactions, releasing the constraints imposed on receptor activation in the absence of ligand.

It was noteworthy to find that the cell surface plasma membrane expression of the mature form of the mutant N431I FSHR was consistently reduced when compared head-to-head with its WT counterpart. In fact, constitutive activation (as canonically defined) was detected only when basal cAMP production levels were normalized to the amount of plasma membrane-expressed FSHR protein, as revealed by ^{125}I-FSH binding studies, as well as by observing spontaneous mutant receptor coupling to the G_s/cAMP signaling pathway through exposing the cells to forskolin. Reduced plasma membrane expression of the mutant receptor might be due in part to impaired trafficking from the endoplasmic reticulum to the plasma membrane resulting from failure of the mutant receptor to couple with cytoplasmic interacting proteins, leading to its rapid degradation in the proteasomes. In fact, it has been recently shown that the EL1 of the FSHR is not masked by its large ectodomain and it is surface accessible (Angelotti et al., 2010; Dupakuntla and Mahale, 2010), an observation that suggests that its abnormal hydrophobic shape may be susceptible for recognition by the quality control system of the cell (Angelotti et al., 2010; Ulloa-Aguirre and Conn, 2009).

Propagation as well as termination of signaling by agonist-occupied GPCRs involves phosphorylation of the intracellular domains of the receptor by G protein-coupled receptor kinases (GRKs) (Reiter and Lefkowitz, 2006). The phosphorylated receptor then recruits β-arrestins, which are scaffold molecules that uncouple the receptor and G protein thereby leading to desensitization of the agonist-stimulated cAMP signaling while continuing signaling through cAMP independent pathways (Reiter et al., 2012; Ulloa-Aguirre et al., 2011). β-arrestins recruitment also leads to internalization of the receptor protein functioning as adaptor proteins that link the receptor to components of the endocytic machinery. The internalized receptor may be then targeted to lysosomes and/or proteasomes for degradation or recycled back to the cell surface, allowing preservation of potential responsiveness of the receptor to further hormonal stimulation (Koenig and Edwardson, 1997; Pitcher et al., 1998; Reiter and Lefkowitz, 2006). In the case of the FSHR it has been shown that both phosphorylation and β-arrestins recruitment map to the intracellular loops 1 and 3, and the carboxyl-terminal domain (Ascoli, 1996; Kara et al., 2006; Krishnamurthy et al., 2003a; Nakamura et al., 1998a). In studying the desensitization and internalization kinetics of the N431I mutant FSHR, it was surprising to find that in contrast to the WT receptor, which displayed desensitization to repetitive stimulation by agonist, the response of the mutant was considerably reduced as disclosed by two different experimental strategies. Further, agonist-

stimulated internalization of the mutant receptor in both equilibrium and non-equilibrium conditions was also substantially impaired. These findings indicate that failure to desensitize and internalize properly might amplify the apparent constitutive activity exhibited by the mutant receptor, particularly considering that increased desensitization and internalization is what should be expected to occur for a receptor bearing an active conformation (Pei et al., 1994). Changes in the configuration of the TMD2 and the intracellular loop 1 resulting from the conformational alteration in the EL1 may interfere with the accessibility to proper receptor phosphorylation by GRKs, leading to impaired β-arrestin recruitment. In fact, Nakamura and colleagues (1998a) showed that agonist-induced phosphorylation of the rat FSHR maps on serine and threonine residues located in the first intracellular loop (which in the human FSHR correspond to T388, T389, S390 and T396) and that mutations in these phosphorylation sites, severely impaired agonist-stimulated phosphorylation, uncoupling, and internalization of the receptor, thus emphasizing on the critical role of this particular loop in GRK interactions and arrestin-promoted desensitization. Our results showing that co-expression of β-arrestin 1 led to impaired internalization of the mutant N431I receptor and that over-expression of β-arrestin 319–418 failed to reduce its internalization as it did with the WT receptor, support the possibility that the mutation led to impaired β-arrestin recruitment and failure to desensitize and internalize properly. At this time, we do not have a satisfactory explanation for the normal internalization response observed when the mutant receptor was co-transfected with β-arrestin 2. In this regard, it is interesting in the previous observation that over-expression of this particular form of non-visual arrestin completely rescued internalization of mutant rat FSHRs with impaired ability to promote agonist-stimulated signal transduction, phosphorylation and internalization (Nakamura et al., 1998b).

As discussed above, it was noteworthy that plasma membrane expression of the mutant FSHR was persistently reduced compared with that presented by the WT receptor. Besides the probably impaired trafficking of the N431I from the endoplasmic reticulum to the cell surface, reduced recycling to the plasma membrane after agonist-stimulated internalization might additionally contribute to the reduced levels of net receptor protein detected at the cell surface. Although the subserving mechanism(s) for this downward trafficking defect are unknown at this time, it is tempting to speculate that both increased proteasome-mediated degradation at the endoplasmic reticulum and reduced recycling to the plasma membrane after internalization might represent undermining mechanisms by which the intracellular trafficking system of the cell normally engenders surface membrane expression of receptors.

In summary, we have described herein the physiopathogenic mechanisms of a new, naturally occurring, gain-of-function mutant of the FSHR. Although detected in an asymptomatic man with a biochemical phenotype compatible with receptor coupling to effector in the absence of ligand, at this time it cannot be substantiated that persistent spermatogenesis is solely due to the apparent constitutive activity conferred by the mutation, given the low levels of both plasma membrane expression and constitutive activity showed by the mutant receptor. However, the mutant receptor exhibited markedly impaired agonist-stimulated desensitization and internalization either when coexpressed with the empty plasmid or with the WT receptor, which may contribute to and/or amplify the persistent activation of the receptor in both absence and presence of agonist, as it has been postulated to explain the effects of the F278C mutation at the melanocortin 2 receptor (Swords et al., 2002) and the Del301-303 variant of the α_{2B} adrenergic receptor (Nguyen et al., 2011). In this scenario, it may well be possible that mutations in GPCRs may bear altered activities conferred not only by constitutive signaling but also by disengagement from the mechanisms which dampen the prolonged response to agonist.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mce.2012.08.011.

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