

In Vitro Impact of FSH Glycosylation Variants on FSH Receptor-stimulated Signal Transduction and Functional Selectivity

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FSH exists as different glycoforms that differ in glycosylation of the hormone-specific β -subunit. Tetra-glycosylated FSH (FSH²⁴) and hypo-glycosylated FSH (FSH^{18/21}) are the most abundant glycoforms found in humans. Employing distinct readouts in HEK293 cells expressing the FSH receptor, we compared signaling triggered by human pituitary FSH preparations (FSH^{18/21} and FSH²⁴) as well as by equine FSH (eFSH), and human recombinant FSH (recFSH), each exhibiting distinct glycosylation patterns. The potency in eliciting cAMP production was greater for eFSH than for FSH^{18/21}, FSH²⁴, and recFSH, whereas in the ERK1/2 activation readout, potency was highest for FSH^{18/21} followed by eFSH, recFSH, and FSH²⁴. In β -arrestin1/2 CRISPR/Cas9 HEK293-KO cells, FSH^{18/21} exhibited a preference toward β -arrestin-mediated ERK1/2 activation as revealed by a drastic decrease in pERK during the first 15-minute exposure to this glycoform. Exposure of β -arrestin1/2 KO cells to H89 additionally decreased pERK1/2, albeit to a significantly lower extent in response to FSH^{18/21}. Concurrent silencing of β -arrestin and PKA signaling, incompletely suppressed pERK response to FSH glycoforms, suggesting that pathways other than those dependent on Gs-protein and β -arrestins also contribute to FSH-stimulated pERK1/2. All FSH glycoforms stimulated intracellular Ca²⁺ (iCa²⁺) accumulation through both influx from Ca²⁺ channels and release from intracellular stores; however, iCa²⁺ in response to FSH^{18/21} depended more on the latter, suggesting differences in mechanisms through which glycoforms promote iCa²⁺ accumulation. These data indicate that FSH glycosylation plays an important role in defining not only the intensity but also the functional selectivity for the mechanisms leading to activation of distinct signaling cascades.

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FSH, or follitropin, is synthesized and secreted by the anterior pituitary gland in multiple molecular forms that vary in oligosaccharide number and type of carbohydrate moieties attached to the protein core [1, 2]. This gonadotropin is composed of 2 subunits associated through noncovalent interactions: an α -subunit, which is common to all glycoprotein

Abbreviations: AUC, area under the curve; eFSH, equine FSH; Emax, dose necessary to reach maximal cAMP production; FSHR, FSH receptor; hFSH, human FSH; hFSHR, human FSH receptor; IBMX, 3-isobutyl-methyl-xantine; iCa²⁺, intracellular Ca²⁺; KD, equilibrium dissociation constant; MAb, monoclonal antibody; PMSF, phenylmethanesulfonyl fluoride; recFSH, recombinant FSH; WB, Western blot

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hormones (LH, chorionic gonadotropin, and TSH) and a FSH β -subunit, which confers specificity to the gonadotropin [3]. In the human FSH (hFSH) heterodimer, 4 Asn residues, α N52, α N78, β N7, and β N24 are targets for N-linked glycosylation (Fig. 1) [4]. Oligosaccharide heterogeneity in glycoprotein hormones comes in 2 forms, macroheterogeneity, which results from the absence of 1 or more oligosaccharide chains from a hormone variant [5–8], and microheterogeneity resulting from variations in the type of carbohydrates comprising the oligosaccharide populations attached to the protein core of the hormone [9–14]. In general, oligosaccharides on the β -subunit play a major role in determining the circulatory half-life

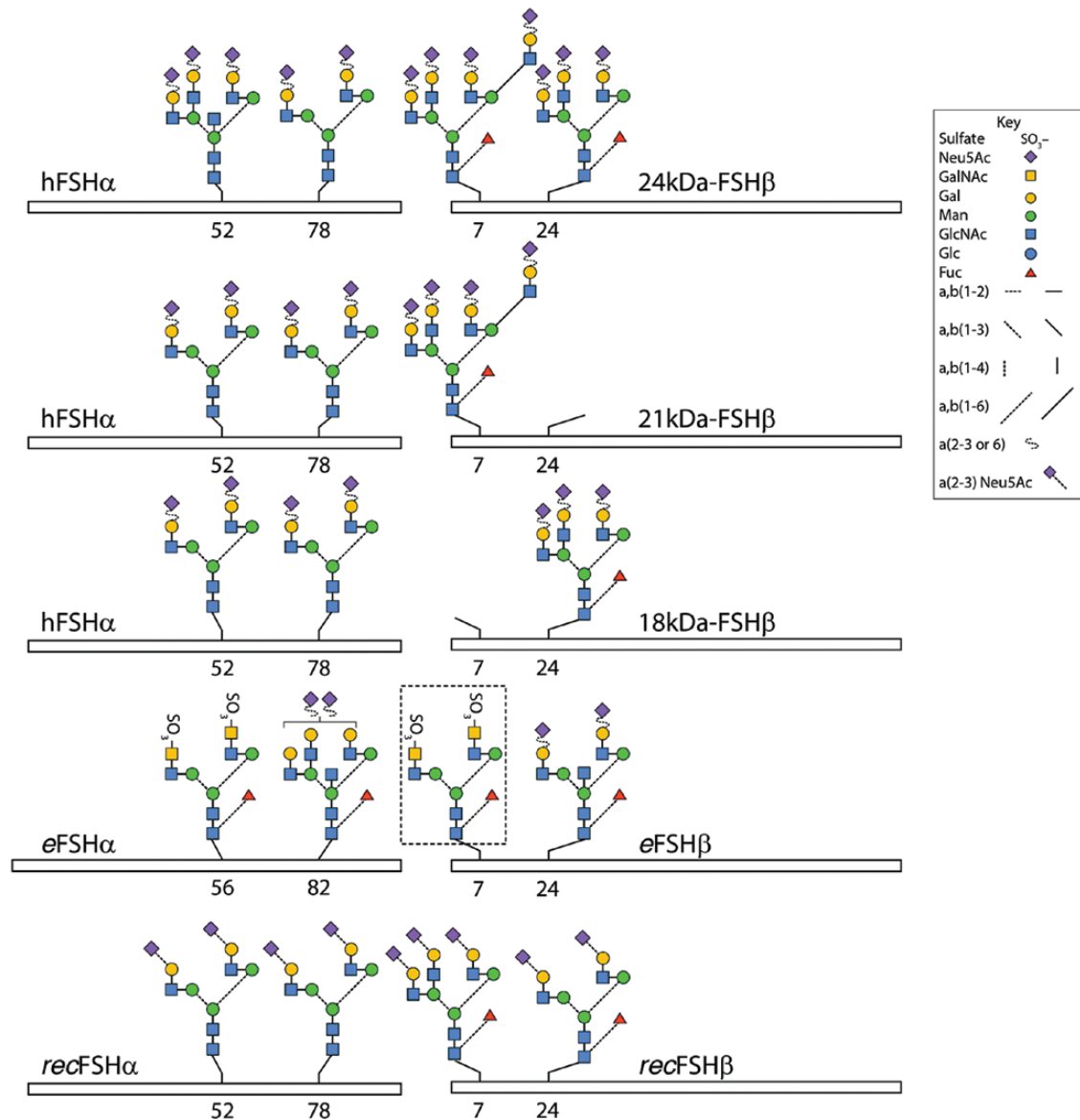


Figure 1. Typical glycans attached to human pituitary FSH, human recombinant FSH produced by Chinese hamster ovary cells (*recFSH*) glycoforms, and equine FSH (*eFSH*). The bars indicate the common- α and hormone-specific FSH β subunits. N-glycosylation sites are indicated by the numbers below the bars. The *eFSH* α subunit has 4 additional residues at the N-terminus, accounting for the difference in numbering. The glycan at position β 7 in *eFSH* (dotted area) is absent from 90% of the molecules present in highly purified preparations [34]. Glycans attached to *recFSH* were taken from the report by Mastrangeli et al [104]. Note that glycans at the α subunit of FSH^{18/21} are in fact smaller (*i.e.* biantennary) than those present in FSH²⁴, which account for the different migration profiles of FSH^{18/21} shown in Fig. 2D.

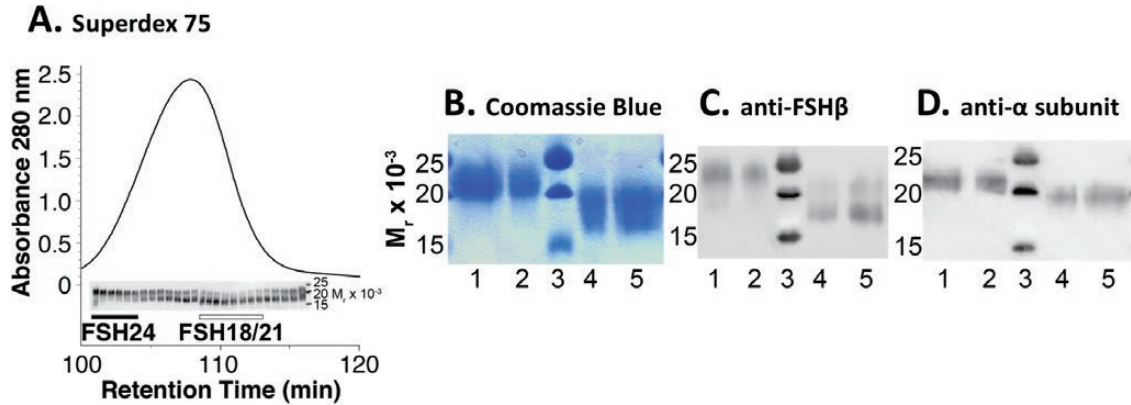


Figure 2. (A) Superdex 75 chromatography of immunopurified pituitary hFSH. Three 1×30 cm Superdex 75 columns were connected in series and equilibrated with 0.2 M ammonium bicarbonate containing 20% acetonitrile. The chromatogram was developed with the same buffer using a flow rate of 0.4 mL/min at 25°C. Fractions under the FSH heterodimer peak were collected and 1- μ g samples evaluated by Western blot using anti-FSH β antibody 15-1.E3.E5 [45] diluted 1:2000 (inset). The solid bar indicates fractions pooled to obtain FSH²⁴ and the open bar indicates fractions pooled to obtain FSH^{18/21}. (B-D) FSH glycoform preparations. Samples of FSH²⁴ (VB-II-277A) and FSH^{18/21} (VB-II-277B) were subjected to SDS-PAGE under reducing conditions and FSH was detected by (B) Coomassie Blue staining, (C) FSH β Western blot, or (D) FSH α Western blot. (B) Lane 1, 5 μ g FSH²⁴; lane 2, 2.5 μ g FSH²⁴; lane 3, BioRad Precision Plus MW markers; lane 4, 2.5 μ g FSH^{18/21}; lane 5, 5 μ g FSH^{18/21}. (C,D) Western blots with anti-FSH β antibody 15-1.E3.E5 [45] diluted 1:2000 and anti-FSH α antibody 15-2.C3.C5 [36, 105] diluted 1:1000, respectively. Lane 1, 1 μ g FSH²⁴; lane 2, 0.5 μ g FSH²⁴; lane 3, BioRad Precision Plus MW markers; lane 4, 0.5 μ g FSH^{18/21}; lane 5, 1 μ g FSH^{18/21}. Differences in migration between the α -subunits of FSH²⁴ (lanes 1 and 2) and FSH^{18/21} (lanes 4 and 5) in panel D are due to the higher content of smaller, biantennary glycans at both Asn⁵² and Asn⁷⁸ in the latter, which led to a more rapid migration in the blot.

and *in vivo* bioactivity of the gonadotropin [15], whereas the oligosaccharide in position α Asn⁵² is primarily involved in activation of the receptor/signal transducer (G protein) system and the ensuing biological response [16–19], an effect probably mediated through stabilizing the structure and/or conformation of the hFSH dimer particularly upon binding to its cognate receptor [20–22]. Nevertheless, previous and more recent studies have shown that glycans at α Asn⁷⁸ and oligosaccharides in the β -subunit also play an important role in hFSH-mediated signal transduction [23–26].

hFSH macroheterogeneity occurs at either 1 or both FSH β N-glycosylation sites [5–7] (Fig. 1). Western blots of hFSH revealed the presence of 2 FSH β bands, a 24-kDa band that possesses both Asn⁷ and Asn²⁴ N-linked glycans (designated as fully/tetra-glycosylated, 24 kDa-FSH β) and a 21-kDa band that lacks the Asn²⁴ glycan (hypo-glycosylated, 21 kDa-FSH β) [5, 8]. The corresponding hFSH heterodimers are designated FSH²⁴ and FSH²¹, respectively. Purified FSH²¹ preparations include a second hypo-glycosylated variant, 18 kDa-FSH β , which lacks the Asn⁷ glycan. The corresponding heterodimer is designated as FSH¹⁸. In addition, both α FSH²¹ and α FSH¹⁸ bear more biantennary glycans than FSH²⁴. A third hypo-glycosylated 15 kDa-FSH β variant has been isolated from human pituitaries; however, studies in transgenic mice indicate poor assembly with FSH α and very low levels of secretion of the FSH¹⁵ glycoform [27]. Thus, there appear to be 3 physiologically relevant hFSH variants, FSH²⁴, FSH²¹, and FSH¹⁸.

In vitro and *in vivo* studies have shown that naturally occurring pituitary- and GH3 cell-derived recombinant hFSH glycoforms exhibit distinct FSH receptor (FSHR) binding kinetics and bioactivity as compared to fully glycosylated hFSH [25, 26, 28, 29]. Thus, similar to the influence of microheterogeneity on the ability of the gonadotropins to activate and trigger intracellular signaling [12, 13, 30], FSH β macroheterogeneity may also contribute to its bioactivity.

It is currently accepted that in many cell systems FSH stimulation triggers activation of a complex array of nonlinear signaling cascades mediated not only by the canonical Gs/cAMP/protein kinase A pathway but also by other G proteins and FSHR receptor interacting proteins [31, 32]. Considering that activation of this complex signaling network and, in particular, of distinct signaling modules probably occurs through stabilization of distinct FSHR conformations in response to binding of particular ligands [29], we here analyzed the in vitro functional effects of several tetra- and hypo-glycosylated FSH glycoforms employing multiple readouts to facilitate identification of glycosylation-promoted preferential activation of one or more signaling pathways in the same ligand/receptor system (i.e., biased agonism [33]) among the different FSH glycoforms. For this purpose, we analyzed the effects of four distinct highly purified FSH preparations, tetra- and hypo-glycosylated pituitary hFSH (FSH²⁴ and FSH^{18/21}, respectively), equine FSH (*e*FSH, 90% hypo-glycosylated (Fig. 1) [34]), and Chinese-hamster ovary cell-produced human recombinant FSH (*rec*FSH or follitropin- α ; tetra-glycosylated), using cultured human embryonic kidney-293 (HEK293) cells stably expressing the human FSHR (hFSHR) as the target for FSH action. These four preparations also differ, to varying extents, in microheterogeneity, including differences in oligosaccharide complexity determined by the number of antennas attached to the N-linked oligosaccharide core, degree of sialylation, fucosylation and sulfation, position of terminal sialic acid residues (α 2-6-linked, α 2-3-linked, or both), and presence of bisecting GlcNAc residues (Fig. 1) [5, 34–43].

1. Material and Methods

A. Hormones

Human pituitary FSH glycoforms were purified from lyophilized pituitary tissue (a generous gift of Dr. James A. Dias, The University at Albany, Albany, NY). *e*FSH (batch VB-I-171) was purified from horse pituitaries obtained from Animal Technologies, Inc., (Tyler, TX). Recombinant human FSH produced in Chinese hamster ovary cells (batches AU012310 and BA024393) was a kind gift of Merck Serono (Mexico City, Mexico); according to the manufacturer, the reported batch-to-batch consistency for this particular preparation is relatively high as disclosed by the coefficients of variation in the range of 7% to 15% for the most and least abundant isoforms of the gonadotropin [38].

B. Human FSH Glycoform and *e*FSH Purification

A 600-g batch of lyophilized human pituitaries was homogenized using 3, 15-second bursts at a setting of 10 with a Brinkman PT-10 Polytron in water containing 1 mM phenylmethanesulfonyl fluoride (PMSF) at 4°C. The extract was adjusted to pH 5.5 with HCl and extraction continued for a total of 15 minutes [44]. The extract was centrifuged for 30 minutes at 30,000 $\times g$ at 4°C, the supernatant was decanted, its volume measured, and adjusted to 75% saturated ammonium sulfate. After overnight precipitation, centrifugation, dialysis, and lyophilization, the dried protein was fractionated on a 5.0 cm \times 200 cm Sephacryl S-100 column in 0.126 M ammonium bicarbonate buffer, pH 7.8. The column fractions were tested for hFSH by radioimmunoassay using reagents obtained from the National Hormone and Pituitary Program, and immunopositive fractions were pooled and lyophilized. hFSH was immunopurified from this fraction by 4 rounds of immunoaffinity chromatography using 2 anti-human FSH β monoclonal antibody (MAb) columns, 1 prepared with mAb 15-1.E3.E5 [45] (a mouse monoclonal recognizing the FSH β subunit that is influenced by N-glycosylation) produced in our laboratory, and another with mAb 4882 [46] (mouse monoclonal raised against hFSH recognizing FSH via the α -subunit) (a generous gift of SP Development Co., Ltd, Bedford, UK), connected in series [36]. The hFSH preparations recovered after 4 cycles of affinity chromatography were combined and fractionated on 3, 1 \times 30 cm Superdex 75 columns (GE Healthcare Life Sciences,

Marlborough, MA) connected in series, to separate FSH²⁴ and FSH^{18/21} glycoforms as previously described [36]. All Superdex 75 fractions were characterized separately. Glycoform abundance in each fraction was determined by Western blot analysis using anti-human FSH β monoclonal antibody 15-1.E3.E5 [45]. The fractions possessing largely 24 kDa-FSH β were pooled to generate FSH²⁴ and those possessing largely 18kDa- and 21 kDa-FSH β were pooled to obtain FSH^{18/21}.

eFSH was purified by our previously reported method, modified by 75% ammonium sulfate precipitation of the 50% ethanol extract before CM-Sephadex ion exchange chromatography [47].

C. Cell Culture

Authenticated human embryonic 293 kidney (HEK293) cells stably expressing the recombinant hFSHR (HEK293-hFSHR⁺) driven by the cytomegalovirus promoter [48], with less than 5 consecutive passages, were maintained in a humidified atmosphere of 5% CO₂ at 37°C in high-glucose DMEM (Life Technologies Inc., Grand Island, NY) supplemented with 10% fetal calf serum (Sigma Aldrich, St. Louis, MO), 5 μ g/mL geneticin (Life Technologies), and antibiotic (penicillin plus streptomycin) reagent (Life Technologies). Expression of the hFSHR protein at the cell surface plasma membrane was verified by immunoblotting as described later. Cells were grown to 70% to 80% confluence in 100-mm-diameter cell culture dishes (Corning, Corning, NY) at 37°C and an initial density of 1×10^6 cells. Twenty-four hours before the experiment, cells were mechanically dispersed and plated at a density of 75 or 200×10^3 cells/500 μ L in 12- or 24-well culture dishes (Corning), depending on the particular experiment (see the following section). On the day of the experiment, cells were starved for 4 hours in DMEM-HEPES 10 mM medium (Life Technologies) supplemented with antibiotic and 0.1% BSA (Sigma). All experiments were performed in triplicate incubations, unless specified, and repeated at least 3 times.

2. Surface Plasmon Resonance

A. Membrane Preparation and Biacore Kinetic Analysis

Cultured HEK293-hFSHR⁺ cells were collected from confluent 100×15 mm culture dishes (Corning). Cells were centrifuged twice at $1000 \times g$ for 10 minutes with PBS to finally obtain a pellet that was resuspended in 1 mL ice-cold 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM EGTA, 0.1 mM PMSF, 2 mg/mL aprotinin, and 10 mg/mL leupeptin homogenization solution. Cells were then lysed with 10 strokes in a Dounce homogenizer, and then centrifuged at $30,000 \times g$ for 20 minutes. The pellet was redissolved in 1 mL of 20 mM Tris-HCl, pH 8.0, 3 mM MgCl₂, 10 mg/mL DNAase I plus PMSF, 2 mg/mL aprotinin, and 10 mg/mL leupeptin resuspension solution (Sigma), and homogenized again. A final centrifugation at $30,000 \times g$ for 20 minutes was performed and the pellet containing the membrane fraction was redissolved in 0.5 mL of resuspending solution, homogenized, and stored at 4°C for immediate use.

Surface plasmon resonance was performed using the Biacore system (Biacore T200, GE Healthcare Life Sciences). The capture surface was prepared to achieve a target immobilization level of 10,000 RU with the membrane fraction preparation. Conditioning and baseline stabilization were accomplished with a short pulse of NaOH (10 mM) injected at a flow rate of 10 μ L/min for 60 seconds before BSA (0.1 mg/mL) was injected during 1 minute to ensure complete blocking of nonspecific binding sites. Surface regeneration was accomplished using a 2-step process: 15 seconds of HBS-N (0.1 M HEPES, 1.5 M NaCl) at 20 μ L/min for 60 seconds, followed by 2 injections of OGP 40 mM (Octyl β -D glucopyranoside) detergent solution at 10 μ L/min for 60 seconds. Measurement of the equilibrium dissociation constant (KD) was performed at 25°C in HBS-EN buffer. Binding curves were generated in duplicate by injecting the hFSH glycoforms for 120-second association followed by 600-second

dissociation at 30 $\mu\text{L}/\text{min}$ in individual runs. Kinetic coefficients were calculated with the Biacore T200 Evaluation software using the 1:1 Langmuir binding model with global fit parameters and the bulk refractive index value held constant at zero [49]. Based on the dissociation rate constant (K_d) (s^{-1}) and association rate constant (K_a) ($\text{M}^{-1} \text{s}^{-1}$) obtained, the equilibrium dissociation or affinity constant KD (M) was calculated as the ratio of the rate constant k_a and k_d ($\text{KD} = k_d / k_a$).

B. cAMP Production

HEK293-hFSHR⁺ cells cultured in 24-well plates at a density of 75×10^3 cells/500 μL , were starved for 4 hours and then stimulated with increasing doses (0 to 150 ng/mL of FSH dissolved in DMEM) of each FSH glycoform preparation (FSH^{18/21}, FSH²⁴, eFSH, and recFSH) in DMEM supplemented with 0.125 mM 3-isobutyl-methyl-xantine (IBMX) (Sigma). At the end of the 3-hour incubation period, cells and media were removed from triplicate wells and total (extra- plus intracellular) cAMP accumulation was measured in acetylated samples by radioimmunoassay as described previously [50], using 2-O-monosuccinyl cAMP tyrosyl methyl ester iodinated by the chloramine T method as the labeled ligand and the CV-125 anti-cAMP antibody [51] (provided by Dr. Albert Parlow and the National Hormone and Peptide Program, Harbor-UCLA Medical Center, Los Angeles, CA) at a 1:70,000 final dilution. For the kinetics of cAMP production, cells were exposed to 50 ng/mL of FSH²⁴, FSH^{18/21}, and eFSH, or 100 ng/mL recFSH (which corresponds to the dose necessary to reach maximal cAMP production [E_{max}] for cAMP production of each glycoform; see Results) during 0 to 120 minutes. At each time period (every 5 minutes for the first 20 minutes and then every 10 minutes during the ensuing 100 minutes) during the incubation time, cells and media were collected and submitted to total cAMP measurement as described previously.

C. Reporter Gene Assay

For the reporter gene assay, cultured HEK293-hFSHR⁺ cells (75×10^3 cells/500 μL) were transiently transfected with the cAMP-sensitive pSOMLuc reporter plasmid (kindly donated by Dr. Eric Reiter, Institut National de la Recherche Agronomique, Nouzilly, France) using a previously described procedure [52]. After a 6-hour incubation period in the presence or absence of increasing doses (0–100 ng/mL) of each FSH glycoform preparation dissolved in equal volumes of DMEM, cells were lysed and luciferase activity measured using a luciferase assay system (Promega Co., Madison, WI). The light produced was measured in a luminescence counter and expressed as fold increase over basal.

D. Western Blots of the hFSHR and β -arrestins 1 and 2

After sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (7.5%), Western blotting of whole cell lysates from HEK293-hFSHR⁺ cells was performed using as primary antibody the highly specific anti-human FSHR monoclonal antibody mAb106.105 [53, 54] (kindly donated by Dr. James A. Dias, University at Albany, Albany, NY) and the secondary anti-mouse IgG horseradish peroxidase conjugate (Jackson ImmunoResearch Laboratories Inc, West Grove, PA) [55], as previously described [56]. Development of the signal was performed using the Clarity Western ECL substrate kit (BIORAD, Hercules, CA). A reprobed membrane with a 1:3000 anti-mouse glyceraldehyde-3-phosphate dehydrogenase monoclonal antibody (Merck Millipore, Burlington, MA) [57] and 1:10,000 goat-anti-mouse IgG conjugated with horseradish peroxidase (Jackson ImmunoResearch) [55] was used to confirm equal protein gel loading.

Western blotting of β -arrestins 1 and 2 (β -arr1/2) was performed in cell lysates from control and CRISPR β -arr1/2 HEK293-hFSHR⁺ KO cells. After SDS-PAGE (10%), blocked membranes were probed with the monoclonal anti- β -arrestin 1 antibody D803J [58] or with anti- β -arrestin 2 antibody C16D9 [59] (Cell Signaling Technology, Danvers, MA) at a 1:3000

dilution, and then with donkey anti-rabbit IgG-horseradish peroxidase conjugate (GE Healthcare Life Sciences, Chicago, IL) [60] at a 1:4500 dilution. Development of the signal and verification of protein gel loading were performed as above.

E. ERK1/2 Phosphorylation

HEK293-FSHR⁺ cells were replated at a density of 200,000 cells/500 μ L in 12-well culture plates (Corning) and tested for FSH-stimulated ERK1/2 phosphorylation [52, 61, 62]. Briefly, after a 4-hour preincubation period in serum-free medium (DMEM-10 mM HEPES plus 0.1% BSA and 100 IU/mL penicillin-streptomycin reagent), 0 to 300 ng/mL or 50 ng of each FSH glycoform were added to cells and incubated for 5 or 120 minutes. At the end of the incubation period, cells were lysed in 2X Laemmli buffer and analyzed by Western blot (WB). The membranes were incubated overnight at 4°C with rabbit anti-human phospho-ERK1/2 (1:3000) antibody (Cell Signaling) [63] and then with secondary anti-rabbit IgG horseradish peroxidase conjugate (GE Healthcare) [60]. Equal protein loading was confirmed in a membrane reprobed with primary polyclonal antibody against total ERK1/2 (1:10,000) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) [64]. Development of the signal was performed using the Clarity Western ECL substrate kit (BIORAD). In a second set of studies, HEK293-hFSHR⁺ control cells and CRISPR β -arr1/2 HEK293-hFSHR⁺ KO cells (see the following section), were incubated with 50 ng/mL of each FSH preparation for 0 to 120 minutes in the presence or absence of 20 μ M H89 (PKA inhibitor) (Sigma) or vehicle. At the end of each incubation period (0, 5, 15, 30, 60, 120 minutes), cells were collected and processed for ERK phosphorylation measurement by WB as described previously. Results are expressed as the pERK/total ERK ratio calculated by densitometric analysis of the blots using the Chemidoc MP detection system (BIORAD).

F. β -arr1/2 Silencing by CRISPR/Cas9

After corroborating the identity of the HEK293-hFSHR⁺ cell line using the AuthentiFiler PCR Amplification Kit (Thermo Fisher Scientific, Waltham, MA, USA), cells were co-transfected with the CRISPR/Cas9 ARR1 (KN201279G2) and ARR2 (KN201168G2) (ORIGENE, Rockville, MD) gRNA constructs in the pCas-Guide vector using liposome-mediated endocytosis (TurboFectin, ORIGENE) in OPTIMEM (Life Technologies), following the instructions of the manufacturer. Cells also were cotransfected with donor template DNAs provided by the manufacturer to introduce a cassette containing the green fluorescent protein and puromycin resistance genes to the genome of the target cell by homologous recombination. Control hFSHR⁺ cells were simultaneously transfected with a negative control CRISPR/Cas9 plasmid (without a specific target) (Scramble, ORIGENE). Forty-eight hours after transfection, cells were treated with 1 μ g/mL puromycin (Sigma) and after 3 weeks of treatment, the puromycin-resistant, green fluorescent protein expressing cells were cloned by serial dilution in a 96-well plate. Cloned, puromycin-resistant CRISPR β -arr1/2 HEK293-hFSHR⁺ KO cells were grown and extracts from confluent cells were then analyzed for β -arrestin 1 and β -arrestin 2 protein expression by WB as described.

G. Measurement of FSH-stimulated Intracellular Ca²⁺ Accumulation by Flow Cytometry

HEK293-hFSHR⁺ cells grown in 100-mm-diameter cell culture dishes were detached after a brief exposure to trypsin-EDTA (Life Technologies), washed 3 times in PBS, and then transferred to flow cytometry tubes (BD Biosciences, San José, CA) (500×10^3 cells/tube). Cells were then incubated for 30 minutes at 37°C with 3 μ M Fura Red (Thermo Fisher Scientific Inc., Waltham, MA), washed 3 times with PBS, resuspended in 500 μ L warm (37°C) PBS and fluorescence measured under different conditions [presence (2 mM) or absence of Ca²⁺, pretreatment with 3 mM EGTA (for 15 minutes before adding FSH, to chelate extracellular Ca²⁺) or 1 μ M thapsigargin (for 20 minutes before FSH exposure, to deplete

intracellular calcium stores and prevent their refilling) in a BD LSRFortessa cytometer (BD Biosciences, Franklin Lakes, NJ) at 480 nm, in the presence or absence of each FSH glycoform at a final concentration of 500 ng/mL. Sample acquisition was performed at low speed. Data extractions were performed using the FlowJo software (BD Biosciences). Data are presented as the relative changes in fluorescence intensity (which reflects intracellular Ca^{2+} [iCa^{2+}] accumulation in response to FSH stimulation) over 300 seconds, taking as 100% the maximal change occurring by exposure to *e*FSH.

H. FSH-stimulated hFSHR Desensitization

For hFSHR desensitization experiments, cultured HEK293-hFSHR⁺ cells were plated on poly-D-lysine-coated 24-well plates (75×10^3 cells/500 μL) and preincubated for 2 hours in the absence or presence (12 to 100 ng/mL) of each FSH preparation. After the preincubation period, media were removed for secreted (extracellular) cAMP measurement and cells were washed twice with warm PBS, which was replaced with fresh culture medium (with IBMX) containing 1200 ng/mL of each FSH glycoform, and cells returned to the incubator for 30 additional minutes. Thereafter, plates were put on ice and the medium and cells collected for measurement of total cAMP content as described previously.

I. Statistical Analysis

Between-group comparisons to determine statistically significant differences between KDs, as well as ED_{50} , E_{max} , and slope values (kinetic experiments) in the cAMP readouts, and pERK1/2/total ERK ratios at 5 minutes and areas under the curve (AUC), were performed using a least significance difference test after ANOVA followed by the post hoc Duncan's multiple comparison test. Differences in areas under the FSH-stimulated curve in the iCa^{2+} studies were determined using ANOVA or Kruskal-Wallis test followed by either Duncan or Tukey test as appropriate. The Shapiro-Wilk test was used for testing the normal distribution of the data and the Bartlett test for testing homogeneity of variances (particularly when data from multiple replicate experiments were considered). To analyze the simultaneous effects of FSH glycoforms at different concentrations (pERK1/2, desensitization, and dose-response pSOMLuc curves), an analysis of deviance using the doses "en bloc" was used, when only differences between the FSH glycoforms curves were of interest. Experiments were repeated 3 to 8 times in duplicate or triplicate incubations.

3. Results

A. Human FSH Glycoform Extraction and Purification

After extraction of the pituitaries with water, ammonium sulfate precipitation, dialysis, and lyophilization, 16.7 g total protein containing 97.2 mg hFSH immunoreactivity was recovered. FSH immunoreactive fractions emerging from a Sephacryl S-100 column were pooled and lyophilized, yielding 91 mg hFSH immunoreactivity in 3.74 g total protein. Four rounds of dual immunoaffinity column chromatography with 15-1.E3.E5 and 4882 mAbs [45, 46] produced purified hFSH as indicated by SDS-PAGE and WB analysis (data not shown). Pooled immunoaffinity chromatography fractions were separated into glycoform fractions by Superdex 75 chromatography (Fig. 2A). Because the 15-1.E3.E5 antibody [45] is a low-affinity antibody, we did not observe any free subunit peak usually generated after elution of hFSH with pH 2.7 buffer. All individual fractions from each hFSH heterodimer peak were analyzed by WB with 15-1.E3.E5 antibody [45] (Fig. 2A, inset). The fractions eluted at the beginning of the heterodimer peak comprised largely the FSH²⁴ glycoform, whereas the fractions collected at the end of the peak were mostly the FSH^{18/21} glycoforms [36].

Human FSH glycoform preparations were characterized by SDS-PAGE and FSH subunit Western blots. The Coomassie Blue-stained gels confirmed chemical purity because only the

FSH subunit bands were detected (Fig. 2B). Anti-FSH β Western blot revealed the presence of ~13% 21 kDa- and ~87% 24kDa-FSH β in the FSH²⁴ preparation (Fig. 2C), whereas the FSH^{18/21} preparation included a significant amount (~73%) of 18kDa-FSH β . The mobility of the FSH²⁴ α band was retarded relative to that of the FSH^{18/21} α band, reflecting smaller N-glycans in the latter (Fig. 2D).

4. Functional Studies

A. KD of the FSH Glycoforms Determined by Surface Plasmon Resonance

The KDs resulting from the interaction of each FSH glycoform with the FSHR embedded in liposomes and determined by surface plasmon resonance are shown in Fig. 3A. Although all KDs were in the nanomolar range, the KD value corresponding to *rec*FSH was significantly ($P < 0.01$) higher (6.4 ± 0.8 nM) than those exhibited by the other glycoforms, with the lowest value being 1.4 ± 0.2 nM for *e*FSH, followed by FSH²⁴ and FSH^{18/21} (2.9 ± 0.7 and 3.2 ± 0.6 nM, respectively).

B. cAMP Production

Fig. 3B shows total (intra- plus extracellular) cAMP production from HEK293-hFSHR⁺ cells after 3 hours' incubation in the presence of increasing doses of each FSH glycoform. Both *e*FSH and *rec*FSH evoked the highest total cAMP production levels followed by FSH^{18/21} and FSH²⁴ (lower inset in Fig. 3B). The dose necessary to reach E_{\max} by *rec*FSH was 100 ng/mL, which was twice the amount required by the other glycoforms. In fact, the highest potency (ED_{50}) for stimulating total cAMP production was exhibited by *e*FSH, followed by FSH^{18/21}, FSH²⁴, and *rec*FSH (Fig. 3B, upper inset). The kinetics of FSH glycoform-stimulated maximal cAMP production by HEK293-hFSHR⁺ cells are shown in Fig. 3C. Maximum cAMP production was reached earlier (40 minutes after FSH exposure) in the presence of *e*FSH, FSH^{18/21}, and FSH²⁴ than *rec*FSH (which occurred at 60 minutes). The slope of the regression line for the 8 time points analyzed (until maximal cAMP levels were reached), was steeper for *e*FSH (16 ± 1.0) than for the other glycoforms (FSH^{18/21}, 12.0 ± 0.6 ; FSH²⁴, 11.4 ± 0.5 ; and *rec*FSH, 10.5 ± 0.5) (Fig. 3C inset), confirming the higher potency of the former glycoform over the others.

To compare the cAMP/PKA transcriptional-associated response elicited by the different FSH glycoforms at the hFSHR, HEK293-hFSHR+ (+ supraindex) cells were transfected with the cAMP-sensitive reporter plasmid pSOMLuc. The results showed that all FSH glycoform preparations induced a robust dose-dependent response in luciferase activity, with *e*FSH presenting the highest and FSH²⁴ the lowest response (Fig. 3D). In terms of maximal effect, these results were very similar to those observed for total cAMP production.

C. FSH Glycoform-stimulated ERK 1/2 Phosphorylation

We next examined whether different FSH glycoforms exhibited differential effects at the FSHR to trigger ERK1/2 MAPK phosphorylation, a well-known FSHR-mediated effect previously shown to be triggered by both the cAMP/PKA and β -arr1/2 pathways [61, 65–67]. Dose–response curves after 5 minutes' incubation (Fig. 4A) showed unequivocal dose-dependent increases in ERK1/2 phosphorylation resulting from exposure of HEK293-hFSHR⁺ cells to FSH^{18/21}, *e*FSH, and *rec*FSH, whereas the increase in pERK stimulated by FSH²⁴ was low at all doses tested (Fig. 4A). In this particular readout for FSH action, FSH^{18/21} evoked the highest response, followed by *e*FSH, although differences between their AUCs did not reach statistical significance; meanwhile, *rec*FSH exhibited an intermediate response and FSH²⁴ the lowest one (Fig. 4A). A time-course of ERK1/2 phosphorylation provoked by each FSH glycoform (at a fixed dose of 50 ng/mL) was next performed in HEK293-hFSHR⁺ cells, and pERK1/2 was analyzed by WB in whole cell

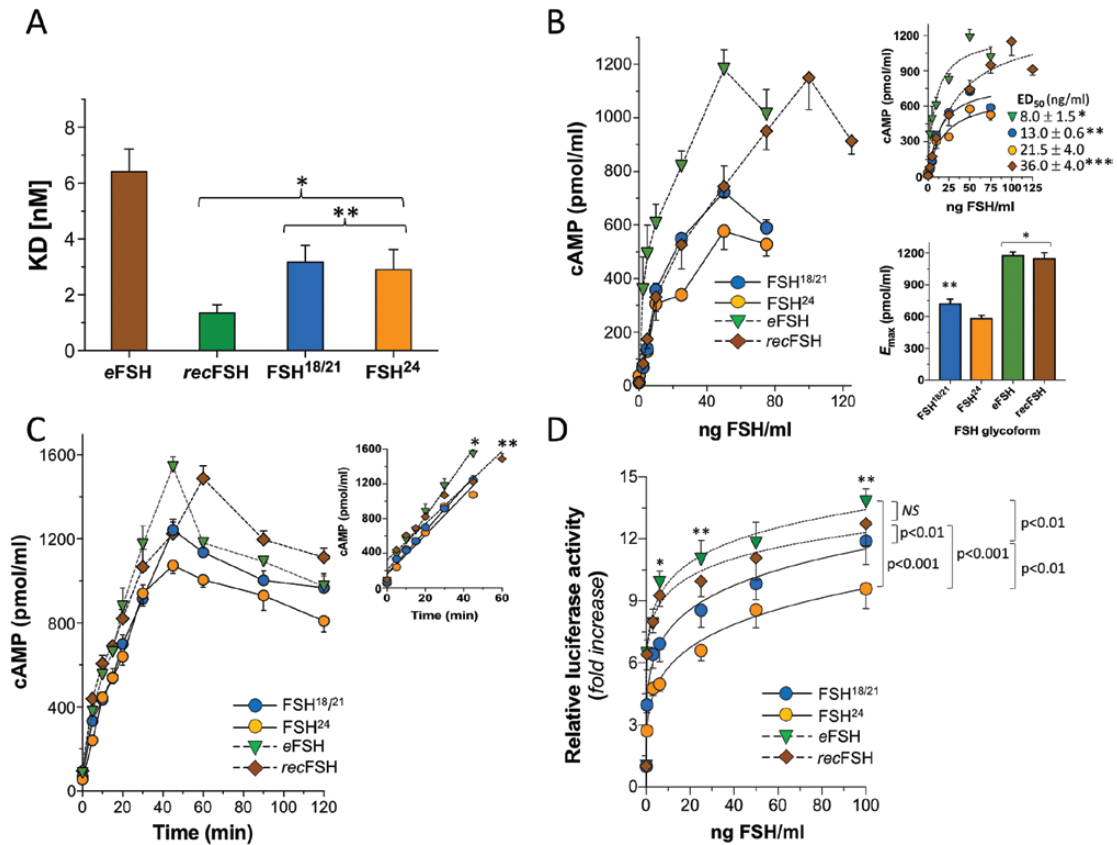


Figure 3. (A) Equilibrium dissociation constant (KD) of FSH glycoforms as assessed by plasmon surface resonance (Biacore). Each bar represents the mean \pm SD from 4 independent experiments. * $P < 0.001$ vs *recFSH*; ** $P < 0.01$ vs *eFSH*. (B) Dose-response curves of total (intra- plus extracellular) cAMP production by HEK293-hFSHR⁺ cells exposed to the different FSH glycoforms for 3 hours. Data are presented as mean \pm SD from 3 independent experiments. (Upper inset) Nonlinear regression curve of data shown in the main graph with the corresponding ED₅₀ values for each glycoform (* $P < 0.01$ *eFSH* vs all others; ** $P < 0.05$ FSH^{18/21} vs FSH²⁴, *** $P \leq 0.02$ *recFSH* vs FSH^{18/21} and FSH²⁴). (Lower inset) E_{max} of each glycoform from the data shown in the main graph (* $P < 0.001$ *eFSH* and *recFSH* vs FSH^{18/21} and FSH²⁴; ** $P = 0.01$ FSH^{18/21} vs FSH²⁴). (C) Kinetic curves of total (intra- plus extracellular) cAMP production by HEK293-hFSHR⁺ cells exposed to 50 ng/mL (*eFSH*, FSH^{18/21}, and FSH²⁴) or 100 ng/mL (*recFSH*) concentrations of FSH glycoforms. (Inset) Linear regression of the curves shown in the main graph, using only points until cAMP production reached maximum values (* $P < 0.01$ *eFSH* vs all other glycoforms; ** $P = 0.03$ *recFSH* vs FSH^{18/21}; $P = N.S.$ *recFSH* vs FSH²⁴, and FSH^{18/21} vs FSH²⁴). (D) Dose-response curves for pSOMLuc expression by HEK293-hFSHR⁺ cells transiently transfected with the cAMP-sensitive pSOMLuc reporter plasmid and exposed to different FSH glycoforms. Each point represents the mean \pm SEM of 3 independent experiments. * $P < 0.02$ *eFSH* vs FSH²⁴ and *recFSH* vs FSH²⁴; ***eFSH* vs FSH²⁴. The levels of significance shown in the right side of the figure correspond to the differences between the FSH glycoform curves considering all doses “en bloc.”

lysates. FSH-induced ERK1/2 phosphorylation responses were detected for all FSH glycoforms; the increase in pERK signal was rapid, reaching its maximum after 5 minutes of FSH exposure, then declining progressively during the ensuing 115 minutes. Both early (mainly, but not exclusively dependent on cAMP/PKA) and late (occurring at or after 15 minutes of FSH exposure, and primarily mediated through the β -arr1/2 pathway) pERK responses [61] were significantly higher for FSH^{18/21} (Fig. 4B and Figs. 5A and B). The lowest pERK response was elicited by FSH²⁴, whereas both responses were similar for *eFSH* and *recFSH*, albeit slightly higher for *eFSH*.

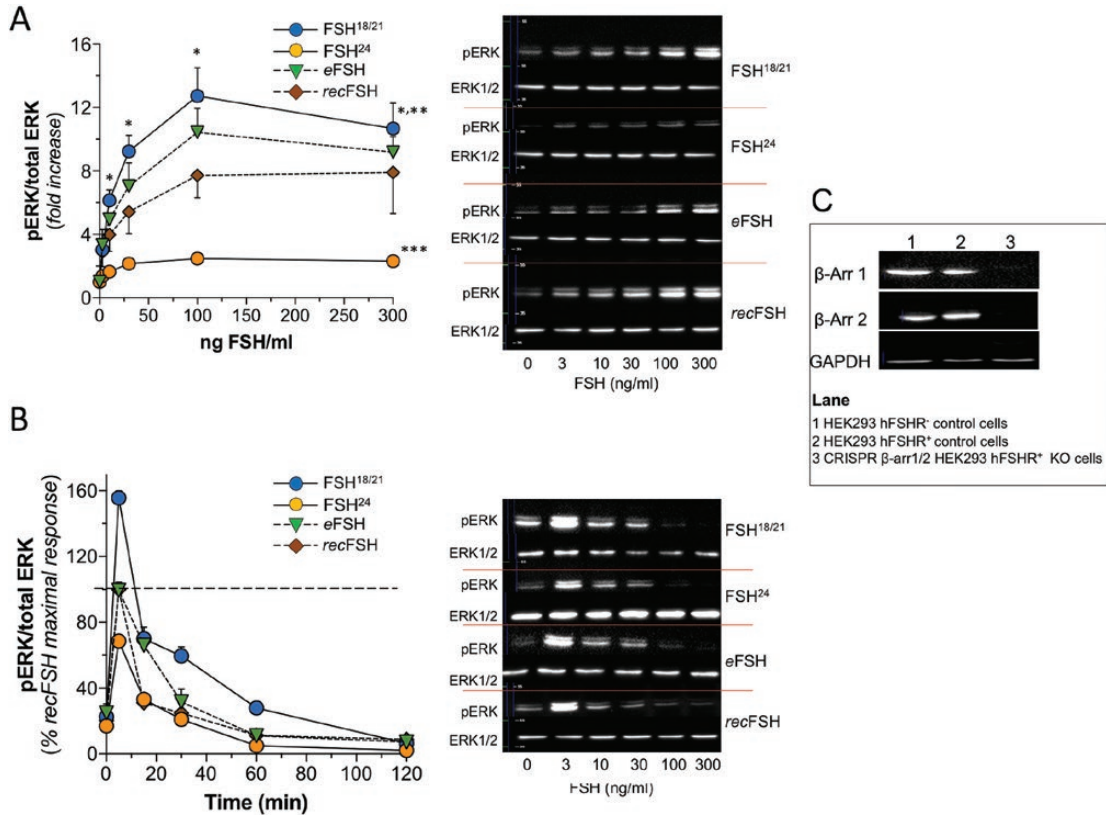


Figure 4. ERK1/2 phosphorylation levels stimulated by exposure of HEK293-hFSHR⁺ cells to FSH glycoforms. (A) Dose-response curves for ERK phosphorylation stimulated by increasing concentrations of each FSH glycoform. Results are expressed as the pERK/total ERK ratio calculated by densitometric analysis of the blots. Representative immunoblots from a single experiment are shown at the right of the graph. Comparisons among doses and dose-response curves: * $P < 0.03$ FSH^{18/21} vs FSH²⁴ at 10, 30, 100, and 300 ng/mL doses; dose-response curves (all doses “en bloc”): ** $P < 0.04$ FSH^{18/21} vs recFSH and FSH²⁴; *** $P < 0.01$ FSH²⁴ vs recFSH and eFSH. (B) ERK1/2 phosphorylation levels during 120 minutes of exposure of HEK293-hFSHR⁺ cells to 50 ng/mL of each FSH glycoform and quantified by densitometric analysis of the corresponding immunoblots (see Figs. 5A and 5B for statistical comparisons among areas under the pERK1/2 curve and pERK levels at 5 minutes). Representative immunoblots are shown to the right of the graph. Data were normalized to the maximal recFSH-stimulated ERK phosphorylation at 5 minutes, arbitrarily chosen as 100%. Graphs shown in (A) and (B) are the means \pm SD of 3 independent experiments. C. Representative immunoblot of β -arrestins 1 and 2 in hFSHR⁺ and hFSHR control HEK293 cells (lanes 1 and 2, respectively) and in CRISPR β -arr1/2 KO cells (lane 3).

D. FSH Glycoform-stimulated ERK Phosphorylation in CRISPR β -arr1/2 HEK293-hFSHR⁺ KO Cells

To determine whether FSH glycosylation differentially affects cAMP/PKA- and β -arr1/2-mediated ERK1/2 phosphorylation, CRISPR β -arr1/2 KO and control (not silenced) HEK293-hFSHR⁺ cells (Fig. 4C) were exposed to 50 ng/mL of each glycoform during 0 to 120 minutes. In all cases, exposure to FSH induced a robust increase in pERK1/2 signal in control cells, whereas in CRISPR β -arr1/2 KO cells a $\leq 40\%$ attenuation at 5 minutes and during the early (0–15 minutes) and, to lesser extent, the late (15–120 minutes) pERK1/2 response was observed (Fig. 6). This decrease in FSH-stimulated ERK1/2 phosphorylation was more evident for FSH^{18/21}, in which the pERK response of CRISPR β -arr1/2 KO cells at 5 minutes was $31 \pm 9\%$ of that observed in control cells (Figs. 6A and 5C), and significantly ($P \leq 0.01$) lower than those elicited by the other glycoforms in these cells (Fig. 5C and

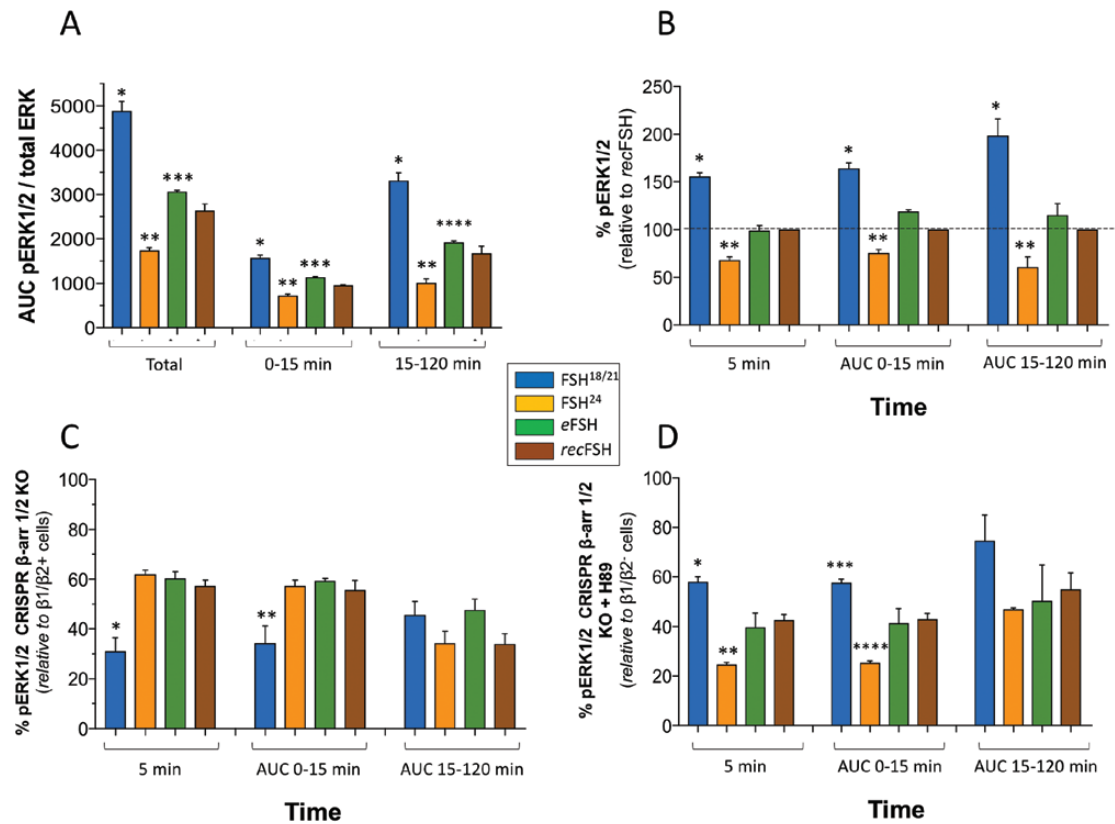


Figure 5. pERK levels at 5 min and areas under the curves (AUC) for the initial (0–15 minutes) and late (15–120) pERK responses to FSH glycoforms. (A) AUC of pERK (total area corresponding to the 0 to 120 minute period as well as to the early (0–15 minutes) and late (15–120 minutes) pERK responses in lysates of HEK293-hFSH⁺ cells exposed to 50 ng/mL of FSH glycoforms (see Fig. 4B). * $P < 0.01$ vs all others; ** $P < 0.01$ vs eFSH and recFSH; *** $P < 0.01$ vs recFSH; **** $P = 0.05$ vs recFSH. (B) Percent of pERK1/2 normalized to the maximal response of recFSH at 5 minutes, arbitrarily chosen as 100%. * $P < 0.01$ vs all others; ** $P < 0.01$ vs eFSH. (C) Percent of pERK1/2 in CRISPR β -arr1/2 HEK293-hFSHR⁺ KO cells, relative to control β -arr1/2 non-KO HEK293-hFSHR⁺ cells. * $P < 0.01$ vs all others; ** $P \leq 0.05$ vs all others. (D) Percent of pERK1/2 in CRISPR β -arr1/2 HEK293-hFSHR⁺ KO cells in the presence of H89, relative to CRISPR β -arr1/2 HEK293-hFSHR⁺ KO cells in the absence of H89. * $P < 0.01$ vs FSH²⁴ and recFSH, and $P < 0.05$ vs eFSH; ** $P < 0.01$ vs recFSH; *** $P < 0.01$ vs FSH²⁴ and recFSH, and $P = 0.05$ vs eFSH; **** $P \leq 0.05$ vs recFSH and eFSH.

Figs. 6B-D). Furthermore, the percent decrease in the area under the pERK1/2 curve in CRISPR β -arr1/2 KO cells during the first 0 to 15 minutes of FSH exposure (i.e., the early phase), also was greater for FSH^{18/21} than for FSH²⁴, recFSH, and eFSH (Fig. 5C).

To further dissect the contribution of the β -arrestin signaling pathway to FSH glycoform-stimulated ERK1/2 phosphorylation, CRISPR β -arr1/2 HEK293-hFSHR⁺ KO cells were exposed to the PKA inhibitor H89 during FSH stimulation. As shown in digital research materials repository [68] and Fig. 5D, the response of β -arr1/2 KO cells to the FSH glycoforms in the presence of H89 further decreased ERK 1/2 phosphorylation. Nevertheless, the attenuation in FSH-stimulated pERK1/2 response in the presence of H89 was significantly ($P < 0.01$) lower when cells were exposed to FSH^{18/21} than to the other glycoforms (Fig. 5D). Interestingly, concurrent silencing of both β -arrestins (via CRISPR) and PKA signaling (via H89), did not completely suppress pERK phosphorylation to basal levels in response to FSH glycoform stimulation, supporting the view that pathways other than those mediated by PKA and β -arrestin also contribute to FSH-stimulated ERK1/2 phosphorylation, particularly during the 0- to 15-minute initial phase [32, 69].

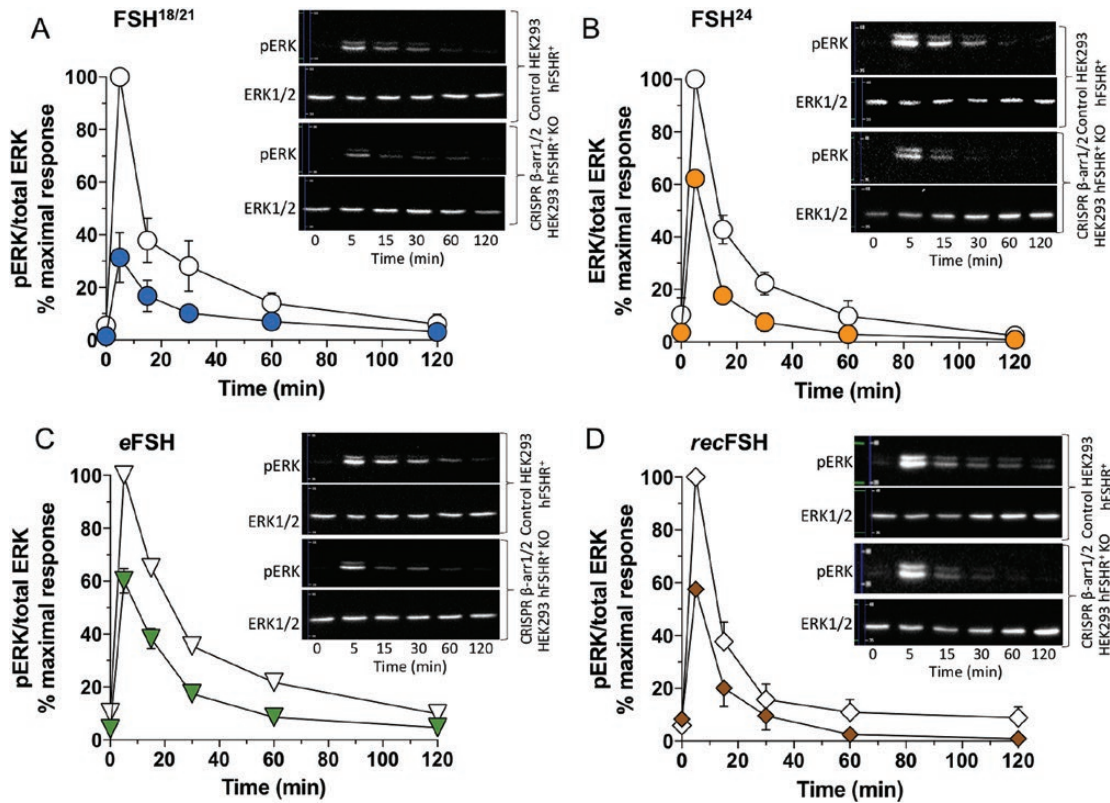


Figure 6. ERK1/2 phosphorylation in control (open symbols) and CRISPR β -arr1/2 HEK293-hFSHR⁺ KO cells (colored symbols). Each panel (A–D) shows the kinetics of pERK response to 50 ng of each glycoform. Results are expressed as the pERK/total ERK ratio calculated by densitometric analysis of the blots; data were normalized to the maximal ERK phosphorylation response in control cells at 5 minutes, arbitrarily chosen as 100%. The insets show representative immunoblots for each glycoform in control and KO HEK293 cells. Because the pERK signal for FSH²⁴ was weak (see Fig. 4A), the blot shown in the inset was overexposed to better appreciate the differences between control and β -arr1/2 KO HEK293-hFSHR⁺ cells. See Fig. 5C for statistical differences between the glycoforms.

E. FSH-stimulated Intracellular Ca²⁺ Accumulation

To determine whether FSH glycoforms provoke Ca²⁺ mobilization through similar or distinct mechanisms (T-type Ca²⁺ channels and/or Ca²⁺ mobilization from intracellular stores [70–78]), HEK293-hFSHR⁺ cells were exposed to 500 ng/mL of each FSH glycoform under different conditions (presence/absence of extracellular calcium or depletion of iCa²⁺ stores) for 240 seconds, and iCa²⁺ accumulation was detected by flow cytometry. As shown in the digital research materials repository [68], exposure of HEK293-hFSHR⁺ cells to high-dose FSH led to a rapid decrease in Fura Red dye fluorescence (expressed as changes in mean fluorescence intensity), starting at ~70 to 80 seconds and returning to baseline levels at ~160 to 180 seconds after addition of FSH. Nevertheless, the effect of each FSH glycoform on Ca²⁺ mobilization varied depending on the particular experimental condition. In the presence of extracellular Ca²⁺ in the PBS incubation buffer, exposure to eFSH provoked the highest effect on iCa²⁺ accumulation, followed by recFSH, and FSH²⁴ and FSH^{18/21}, with the effect of the latter 2 overlapping (Fig. 7A and digital research materials repository [68]). Omission of Ca²⁺ from PBS, decreased the iCa²⁺ response by 25 ± 6% (eFSH), 29 ± 6% (recFSH), 10 ± 3% (FSH^{18/21}), and 33 ± 7% (FSH²⁴) (Figs. 7B and digital research materials repository [68]), with the addition of EGTA in the incubation buffer yielding similar results (Figs. 7C and S2C). Meanwhile, pretreatment with thapsigargin further decreased iCa²⁺ accumulation stimulated by all glycoforms (>50% decrease in iCa²⁺ response): Figs. 7D and

digital research materials repository [68]. As shown, the iCa^{2+} response to $FSH^{18/21}$ was the most profoundly affected by thapsigargin pretreatment (Fig. 7D and digital research materials repository [68]), with levels ~80% below those observed in the absence of this Ca^{2+} ATPase inhibitor and the presence of extracellular Ca^{2+} . These data indicate that all FSH glycoforms stimulate Ca^{2+} mobilization in HEK293-hFSHR⁺ cells through both T-type Ca^{2+} channels and intracellular stores, but predominantly through the latter when stimulated by $FSH^{18/21}$.

F. FSH-stimulated hFSHR Desensitization

Desensitization of the FSHR to FSH glycoform stimulation in HEK293-hFSHR⁺ cells was next examined (Fig. 8). Initially, cells were incubated with increasing doses (0-100 ng/mL) of FSH glycoforms for 2 hours and then reexposed to a high (1200 ng/mL) concentration of the corresponding glycoform for 30 additional minutes. During the first 2 hours of incubation, all glycoforms elicited a robust increase in extracellular cAMP accumulation (Fig. 8A). Upon reexposure to the high agonist dose, the FSHR displayed desensitization regardless of the FSH glycoform and dose used during pretreatment (Fig. 8B). Nevertheless, receptor desensitization to the high FSH glycoform dose was significantly ($P < 0.01$) attenuated for *e*FSH at all preexposure doses and for *rec*FSH at the 10 to 50 ng/mL doses, compared with reexposure to $FSH^{18/21}$ and FSH^{24} , which exhibited a more profound level of FSHR desensitization. In the latter glycoforms, desensitization provoked by exposure to the high FSH glycoform dose for 30 minutes was very similar, except in cells prestimulated with the highest dose (100 ng/mL) of $FSH^{18/21}$, in which the cAMP response upon FSH re-exposure was significantly ($P < 0.01$) lower.

5. DISCUSSION

Despite the fact that FSH binding has been considered to exclusively be a protein-protein interaction, variations in glycosylation (either as micro- or macroheterogeneity) has been known for a long time to influence the response of the target cell to the FSH stimulus [8, 11, 28, 62, 79, 80]. To analyze the impact of oligosaccharide heterogeneity on hFSH activity, we here studied the *in vitro* bioactivities of the 2 major, naturally occurring pituitary hFSH glycoforms $FSH^{18/21}$ and FSH^{24} at the hFSHR stably expressed in HEK293 cells and compared these activities with those exhibited by 2 other FSH preparations exhibiting distinct glycosylation patterns. Although earlier studies have shown that FSHR density in target cells plays a role in determining the preference of signaling cascades used [52, 81], this was not of concern as apparently the density of FSHR in our stably transfected HEK293-hFSHR⁺ cells was kept constant. The finding that the FSH^{24} preparation contained a small amount of FSH^{21} in FSH^{24} was not a major concern because we have observed that when 1 glycoform contains $\geq 80\%$ of the preparation, it behaves as if it is 100% (unpublished observations).

Keeping in mind the possibility that these FSH glycoforms might exhibit some degree of functional selectivity or biased agonism at the hFSHR [67], the *in vitro* bioassays used addressed different biochemical readouts stimulated through distinct downstream pathways. The results showed that the ability of hFSH glycoforms to stimulate each of these endpoints varied depending on the particular glycoform tested. In the cAMP accumulation, cAMP/PKA-mediated transcriptional response, and ERK1/2 phosphorylation bioassays, hypo-glycosylated $FSH^{18/21}$ always was more potent/active than the fully glycosylated FSH^{24} glycoform, whereas *e*FSH showed the greatest potency/activity in the cAMP and pSOM-Luc assays but not in the pERK1/2 dose-response assay, in which $FSH^{18/21}$ was the most active glycoform. In all of these assays, and particularly in the pERK1/2 assay, FSH^{24} was the least potent/active glycoform among all preparations tested. These observations suggested biased agonism of *e*FSH for the Gs-cAMP-PKA-CREB pathway and of $FSH^{18/21}$ for the ERK1/2 activation path. The differences in potency between $FSH^{18/21}$ and FSH^{24}

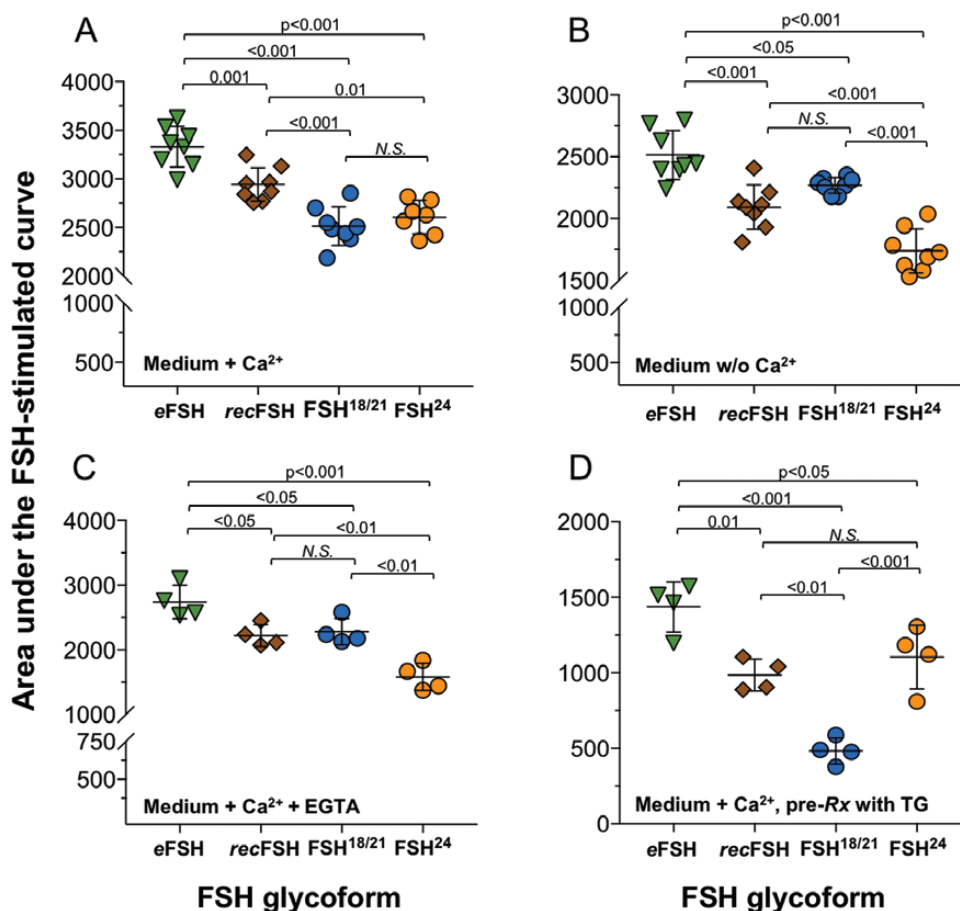


Figure 7. Intracellular iCa^{2+} accumulation in HEK293-hFSHR + cells expressed as the area under the FSH-stimulated curve calculated from the data presented in the digital research materials repository [68]. Areas under the curve were calculated considering the first significant change in fluorescence ($1/n$ mean fluorescence intensity) following addition of FSH and the last value of the fluorescence descending curve before reaching baseline levels (see curves in the digital research materials repository [68]). Each panel corresponds to different conditions (A and B) presence or absence of Ca^{2+} in the incubation buffer, (C) addition of EGTA, (D) or pretreatment with thapsigargin (TG) before FSH addition. Each symbol represents the normalized result of an independent experiment (see Fig. S2); horizontal and vertical lines indicate the mean \pm SD from 4 to 8 independent experiments, respectively. Differences among FSH glycoform responses are shown on the top of each graph. N.S.: not significant. Different scales in the Y-axes were used to allow better appreciation of the differences among glycoforms.

on the Gs-cAMP-PKA signaling pathway observed in the present study follow the pattern previously reported for recombinant GH_3 -expressed hFSH glycoform preparations in stimulating KGN cells [26]. Although both pituitary hFSH and recombinant GH_3 -derived hFSH show many similarities in oligosaccharide structure (i.e., in microheterogeneity), there are some differences that mainly include variations in the relative abundance/frequency of triantennary and biantennary glycans as well as in glycan size and flexibility [36], which apparently are not relevant for binding at the FSHR as both pituitary and GH_3 -derived hypo- and tetra-glycosylated FSH glycoforms displayed similar differences in FSHR binding when tested in heterologous and homologous radioreceptor binding assays, with FSH^{18/21} showing higher binding activity than FSH²⁴ [25, 36], albeit they share similar Kds at the hFSHR, as was observed in the present study [36]. Further, it has been reported that for both pituitary and recombinant GH_3 -derived FSH^{18/21} glycoforms, more FSH binding sites are available than for their corresponding FSH²⁴ counterparts [25, 36], which may be one of the

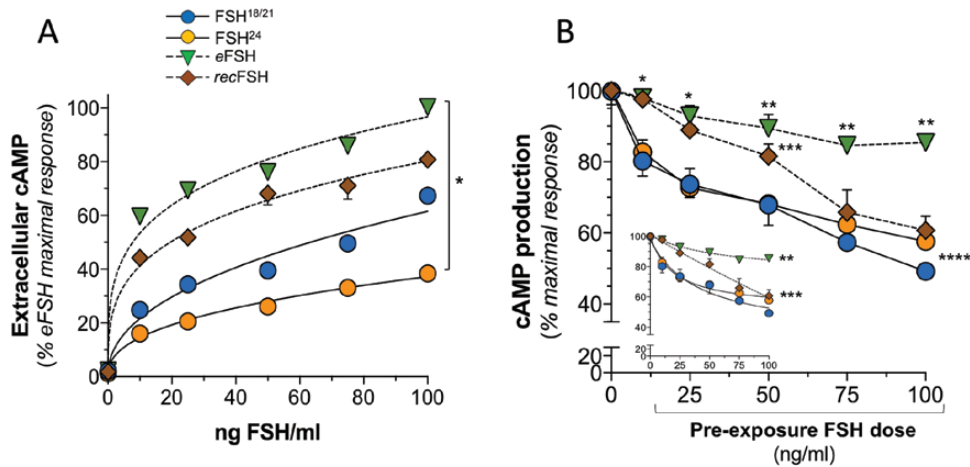


Figure 8. Desensitization of the hFSHR in HEK293-hFSHR⁺ cells exposed to different FSH glycoforms. (A) Cells were stimulated for 2 hours with increasing amounts of each glycoform in the presence of IBMX, and cAMP in the incubation media was measured by RIA. *All treatments were significantly different from each other at $P < 0.01$. (B) After stimulation with FSH, cells were washed twice and then rechallenged with a saturating (1200 ng/mL) dose of each FSH glycoform in the presence of IBMX; total (intra- and extracellular) cAMP was then determined. * $P < 0.01$ eFSH and recFSH vs FSH^{18/21} and FSH²⁴ in cells preexposed to 10 and 25 ng/mL FSH; ** $P < 0.01$ eFSH vs all other glycoforms in cells preexposed to 50, 75, and 100 ng/mL FSH; *** $P < 0.01$ recFSH vs FSH^{18/21} and FSH²⁴ in cells preexposed to 50 ng/mL; **** $P < 0.01$ FSH^{18/21} vs FSH²⁴ and recFSH at the 100 ng/mL preexposed FSH dose. Inset: Nonlinear regression of the data shown in the main graph. Level of significant differences among the glycoform curves in the inset graph (all doses “en bloc”): ** $P < 0.01$ eFSH vs all other glycoforms; *** $P < 0.01$ recFSH vs FSH^{18/21} and FSH²⁴.

mechanisms through which activation of the Gs-cAMP-PKA pathway is higher for FSH^{18/21}. The high potency of eFSH in the cAMP accumulation and cAMP-PKA transcriptional activation assays is not surprising given that this particular preparation has been shown to be highly active when its binding is compared with its counterparts in other species, including humans [4, 82, 83]. In this vein, besides being predominantly hypo-glycosylated (Fig. 1), oligosaccharides in eFSH α -subunit (at Asn⁵⁶) are predominantly biantennary and smaller than in pituitary hFSH and the structure of the determinant loop of its β -subunit differs from those of other FSH preparations [4, 34], which may facilitate binding to and activation of the FSHR. In the case of recFSH the most outstanding differences lie in that its oligosaccharides are predominantly biantennary and lack sialic acid linked α 2-6, which may also facilitate receptor activation and Gs-cAMP-PKA signaling, by as-yet-unknown mechanisms. Finally, an additional mechanism through which eFSH and recFSH may have exhibited higher efficacy than hypo- and fully glycosylated hFSH could be the attenuated desensitization of the hFSHR when stimulated by the former compounds versus the latter as shown in the present study.

Like many GPCRs belonging to family A of the GPCR superfamily, the FSHR stimulates ERK1/2 phosphorylation through several mechanisms, mainly activation of the Gs/cAMP/PKA and the β -arr1/2 pathways, but also using the G_i and G_{q/11} signaling cascades [32, 61, 84]. Given this array of pathways, it is thus feasible that upon binding to the FSHR different FSH ligands may provoke distinct conformational rearrangements within the transmembrane domains of the receptor leading to stabilization of a conformation that may facilitate preferential activation of one particular pathway or a subset of pathways leading to differentially mediated signaling (e.g., on ERK1/2 signaling) resulting in an imbalanced or biased response. In the FSHR, the kinetics of Gs/cAMP/PKA- and β -arr1/2-mediated ERK1/2 activation in heterologous cell systems follows a pattern where β -arr1/2-mediated pERK1/2 is delayed and more sustained compared with the Gs/cAMP/PKA-dependent pathway, which is transient but occurs earlier [61]. Nevertheless, it has been shown that in HEK293 cells

expressing the hFSHR, transient silencing of β -arr1/2 by siRNA also may have a variable effect on the early ERK1/2 phosphorylation response to agonist [61, 62, 85]. Further, it was more recently shown that depletion of β -arrestins in 3 different parental HEK293 cell lines by either siRNA or CRISPR/Cas9, equally affected the early phase of ERK1/2 activation in response to FSH exposure [69].

We used HEK293-hFSHR⁺ cells in which β -arr1/2 were knocked out by CRISPR/Cas9 to determine whether macroheterogeneity in the pituitary FSH molecule may promote differential/biased signaling at the hFSHR to stimulate ERK1/2 phosphorylation. These experiments revealed that the ERK1/2 activation response of CRISPR β -arr1/2 hFSHR⁺ KO cells to FSH^{18/21} was more profoundly affected than when cells were exposed to the other glycoforms, which responded in a similar manner (Fig. 5C). These data strongly suggest that the ERK1/2 response to hypo-glycosylated hFSH is, comparatively, more arrestin-dependent (but not G protein-independent) than the other glycoforms. Further, exposure of CRISPR β -arr1/2 KO cells to the PKA inhibitor H89 had less impact on FSH^{18/21}-stimulated ERK1/2 phosphorylation than on the response to the other glycoforms. This result was unexpected given the robust cAMP response of the HEK293-hFSHR⁺ cells to FSH exposure, particularly to *e*FSH and *rec*FSH. The complete lack of suppression of ERK1/2 activation in the presence of both PKA inhibitor and β -arr1/2 silencing, indicates that alternative pathways may be operating in our experimental model to activate FSH-stimulated ERK1/2 signaling. In this vein, it has been shown that activation of the pertussis-sensitive G_{i/o} protein plays an important role on biased ERK1/2 activation in other GPCRs [86] as well as on cell proliferation mediated by the FSHR in maturing mouse Sertoli cells [84]. Signal rewiring in CRISPR/Cas9 genome-edited cells to pathways other than the Gs- and β -arr1/2-mediated pathways also might be contributing to the incomplete suppression of ERK1/2 activation in the presence of PKA inhibition and β -arrestin silencing [69].

The complexity of signaling mediated by the activated FSHR includes Ca²⁺-mediated signaling pathways. In this regard, it has been established that high-dose FSH stimulation induces an increase in iCa²⁺ levels in both rat granulosa cells and Sertoli cells [70, 73, 74, 76, 77]. This increase in FSH-stimulated iCa²⁺ accumulation may be attributed to different mechanisms including influx of extracellular Ca²⁺ through T-type Ca²⁺ channels and release from intracellular stores [70, 71, 76, 78, 87, 88]. Further, it has been shown that in HEK293 and KGN cells transiently expressing the hFSHR, cytosolic Ca²⁺ increase may also occur through interaction between APPL-1 and the first intracellular loop of the hFSHR, involving inositol 1,4,5-trisphosphate (IP₃) accumulation presumably *via* G α_q -PLC β activation [70]. In the present study, we analyzed the effects of different FSH glycoforms on cytosolic Ca²⁺ accumulation under different conditions that may facilitate identification of the mechanisms whereby the glycoforms could preferentially stimulate cytosolic Ca²⁺ accumulation. The results showed that all 4 FSH glycoforms evoked iCa²⁺ accumulation through both of the previously mentioned mechanisms. Remarkably, FSH^{18/21} was the least potent glycoform to stimulate Ca²⁺ mobilization from intracellular stores when the cells were depleted of intracellular Ca²⁺ by preventing their refilling through preexposure to thapsigargin, indicating that this source of Ca²⁺ plays a major role in FSH^{18/21}-stimulated cytosolic Ca²⁺ accumulation. The mechanism(s) subserving this selectivity of FSH^{18/21} towards iCa²⁺ stores might involve IP₃ accumulation *via* G α_q activation of PLC β or other PLC isoenzymes associated with other intracellular regulators [89]. In this vein, it has been previously shown that the closely-related LH/chorionic gonadotropin receptor and TSH receptor also activate G_{q/11}, stimulate phospholipase C, and increase second messengers such as inositol phosphates, calcium, and diacylglycerol [89–95]. Whatever mechanisms are involved in FSH-stimulated mobilization of Ca²⁺ from intracellular stores, the results of the present study indicate that FSH^{18/21} preferentially stimulates the release of Ca²⁺ from this source over that mediated by Ca²⁺ plasma membrane channels, as is the case of the other glycoforms.

Assessment of FSH glycoform-provoked hFSHR desensitization, revealed an interesting pattern, with cells reexposed to *e*FSH and *rec*FSH exhibiting a significantly attenuated refractoriness to further agonist stimulation than those reexposed to FSH^{18/21} and FSH²⁴.

These differences might be due to particular configurations adopted by the hFSHR when activated by the human pituitary glycoforms (see the following section), which might facilitate receptor phosphorylation by receptor kinases (GRK2 and 3) and hence G_{α_s} uncoupling [61, 66, 96], while prolonging this G protein response to eFSH and recFSH.

The molecular mechanisms subserving the differential effects of hypo- and tetra-glycosylated FSH isoforms remain largely unknown, particularly considering that in the most recent crystal structure of the entire hFSHR extracellular domain in complex with FSH revealed that FSH glycans do not participate in binding to the receptor because they appear oriented away from the hormone-receptor interface, rather being sequestered at the periphery of complex [97]. Nevertheless, given the essential role of FSH carbohydrates for full receptor activation and signaling, it is highly possible that they may affect both stability and conformational flexibility of the FSH dimer and thereby affect the association kinetics with the FSHR [29] and secondarily on receptor activation, with hypo-glycosylated pituitary FSH engaging the receptor faster than its tetra-glycosylated counterpart [25]. In addition, hypo-glycosylated FSH also occupies more FSHRs than tetra-glycosylated FSH and exhibits higher in vitro binding activity in some systems [25, 36]. Given that hypo-glycosylated FSH also exhibited a preference for particular signaling pathways (e.g., β -arrestin/ERK1/2 and/or Gi/ERK1/2 vs G_s -cAMP-PKA/ERK1/2 pathways, and Gq/11 vs T-type Ca^{2+} channels to provoke cytosolic Ca^{2+} accumulation), it is also possible that oligosaccharides may additionally influence the final receptor conformation and association with other FSHR molecules and/or partners [20, 21, 97] via distinct association/dissociation kinetics with the receptor that could in turn eventually define the signaling pathways to be selectively or preferentially activated/inhibited from the wide repertoire of associated effectors. In fact, preferential activation of distinct signaling pathways by charge variants of FSH and partially deglycosylated equine LH has been recognized [10, 11, 30, 62, 79, 98] and, more recently, another study showed that the oligosaccharide complexity of recombinant hFSH preparations differentially affected gene expression and steroidogenesis in cultured human granulosa cells [80]. Thus, not only macroheterogeneity but also microheterogeneity in FSH preparations appear to influence the biological responses to gonadotropin stimulation at the target cell level.

It has been widely demonstrated that the relative abundance of FSH glycoforms varies depending on the phase of the menstrual cycle. In serum from normal menstruating women, the abundance of less acidic/more basic isoforms and concentrations of hypo-glycosylated FSH glycoforms is higher during the midcycle than in other cycle phases [99–103], and the content of FSH^{18/21} in the pituitaries of women decreases almost linearly with age throughout reproductive life [5, 43]. Differences in the relative abundance of FSH glycoforms during the menstrual cycle as well as in their distinct effects at the target cell, which are determined by differential glycosylation, offer a potential therapeutic avenue for the design of novel strategies based on the administration of recombinant FSH glycoforms in varying ratios and combinations that may mimic what occurs physiologically during the normal menstrual cycle, and thereby improve the outcomes and minimize side effects of controlled ovarian hyperstimulation with gonadotropins in infertile women.

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