Recombinant FSH glycoforms are bioactive in mouse preantral ovarian follicles

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Abstract

Female reproductive aging is characterized by a rise in follicle stimulating hormone (FSH) levels during peri-menopause. N-linked glycans are co-translationally attached to the Asn^7 and Asn^{24} residues on the FSHβ subunit. Differences in the number of N-glycans on the FSHβ subunit result in distinct glycoforms: hypo-glycosylated ($FSH^{21/18}$, glycans on either Asn^{24} or Asn^7, respectively) or fully-glycosylated (FSH^{24}, glycans on both Asn^7 and Asn^{24}). The relative abundance of FSH glycoforms changes with advanced reproductive age, shifting from predominantly $FSH^{21/18}$ in younger women to FSH^{24} in older women. Previous *in vitro* studies in granulosa cell lines and *in vivo* studies using *Fshb* null mice showed these glycoforms elicit differential bioactivities. However, the direct effects of FSH glycoforms on the mouse ovarian follicle have not yet been determined. In this study, we isolated secondary follicles from pre-pubertal mice and treated them with 20- or 100 ng/mL purified recombinant FSH glycoforms for one-hour or 18-20 hours. Analysis of phosphorylated PKA substrates showed that glycoforms were bioactive in follicles following one-hour treatment, although differential bioactivity was only observed with the 100 ng/mL dose. Treatment of follicles with 100 ng/mL of each glycoform also induced distinct expression patterns of FSH-responsive genes as assessed by qPCR, consistent with differential function. Our results, therefore, indicate that FSH glycoforms are bioactive in isolated murine follicles.

Keywords

ovarian follicle; follicle-stimulating hormone; glycosylation; aging

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Introduction

Reproductive aging is universal, affects all females, and occurs decades prior to the decline of any other human organ system (Broekmans et al., 2007, Broekmans et al., 2009, Banerjee et al., 2014). One of the earliest signs of aging is increasing basal levels of circulating follicle stimulating hormone (FSH), associated with a shortened menstrual cycle and follicular phase (McTavish et al., 2007). This sharp rise in FSH is thought to result from decreased inhibin B levels due to the decreasing number of small antral follicles as the ovarian reserve is depleted with age (Klein et al., 1996). FSH is a glycoprotein hormone secreted by the anterior pituitary gonadotrope cells which is essential for ovarian folliculogenesis. In adult females, functional FSH receptors (FSHR) located on granulosa cells respond to FSH, leading to follicle maturation. Similar to other pituitary and placental hormones, FSH consists of two non-covalently associated heterodimeric α- and β-subunits, of which the β-subunit is hormone-specific and confers biological activity (Hunzicker-Dunn and Mayo, 2015, Das and Kumar, 2018). FSH signaling is regulated in part via N-glycosylation. This is a multi-enzyme driven process wherein the multi-subunit oligosaccharyl transferase (OST) enzyme complex co-translationally attaches $GlcNAC_2 Man_9Glc_3$ precursors onto conserved Asn^{24} and/or Asn^7 residues of the human FSH β-subunit (Pierce and Parsons, 1981, Baenziger and Green, 1988, Wang et al., 2016b). Previous studies have established the presence of different N-glycosylated FSH glycoforms in human pituitaries and/or urine based on the presence of one or two N-glycans (Dalpathado et al., 2006, Davis et al., 2014, Bousfield et al., 2014b, Bousfield et al., 2015) in the FSHβ subunit. FSHβ with N-glycans on both Asn^7 and Asn^{24} residues is detected as a 24 kDa band on immunoblots, and the heterodimer consisting of this subunit is designated fully-glycosylated FSH^{24}. Two additional glycoform variants exist that are classified as hypo-glycosylated - FSH^{21} and FSH^{18} based on the absence of Asn^{24} or Asn^7 glycans, resulting in 21 and 18 kDa size immunoreactive bands, respectively. These band sizes correspond to the nomenclature of the glycoforms. Purified recombinant hypo-glycosylated FSH^{21} and FSH^{18} exist as a mixture, which is designated $FSH^{21/18}$ (Wang et al., 2016b, Davis et al., 2014, Bousfield et al., 2007).

Changes in the relative abundance of FSH glycoforms occur with age (Davis et al., 2014, Bousfield et al., 2007, Anobile et al., 1998, Creus et al., 1996, Loreti et al., 2009, Padmanabhan et al., 1999, Jiang et al., 2015). Hypo-glycosylated $FSH^{21/18}$ is the predominant form in younger women with ovulatory cycles, and fully-glycosylated FSH^{24} is predominant in older women (Davis et al., 2014, Bousfield et al., 2007, Bousfield et al., 2014a, Jiang et al., 2015, Bousfield et al., 2014b, Butnev et al., 2015, Green et al., 1986). This change in glycoform abundance as a function of age is significant in the context of female reproductive aging not only due to the overall rise in levels of FSH at peri-menopause but also because of the differences in bioactivity between these glycoforms. *In vitro* studies using radio-ligand and radio-receptor assays (Bousfield et al., 2014b, Butnev et al., 2015, Bousfield et al., 2014a, Bousfield et al., 2007, Dalpathado et al., 2006, Bousfield et al., 2015), and a study conducted on an FSH-responsive KGN granulosa tumor cell line (Jiang et al., 2015) showed that the hypo-glycosylated $FSH^{21/18}$ glycoform preparation was more bioactive than the fully-glycosylated FSH^{24} glycoform. The $FSH^{21/18}$ glycoform

exhibits enhanced receptor occupancy and faster kinetics, and lower doses of FSH[21] are needed for cAMP, estradiol, and progesterone production in granulosa cell lines compared to FSH[24]. *In vivo* studies using immature *Fshb* null mice in a pharmacological rescue approach investigated the biological effects of these glycoforms and further demonstrated that hypo-glycosylated FSH[21/18] and fully-glycosylated FSH[24] glycoforms act via distinct biological pathways to regulate differential effects in target tissues (Wang et al., 2016b). Although these studies suggest unique age-specific actions of FSH glycoforms, the *in vitro* studies were conducted in isolated granulosa cells from a tumor cell line (Jiang et al., 2015), and the *in vivo* studies interrogated the whole ovary, precluding analysis of specific FSH-responsive sub-compartments within the ovary (Wang et al., 2016b). Therefore, the goal of this study was to investigate the effects of recombinant FSH glycoforms directly on the follicle, which is the functional unit of the ovary and primary target of FSH.

The ovarian follicle provides a unique organotypic system in which to investigate the impact of FSH glycoforms without any systemic side effects of the body or of surrounding ovarian cells (Xu et al., 2016). In the follicle, FSH drives granulosa cell proliferation and production of estrogen, which in turn promotes follicle growth, follicle and oocyte maturation and ovulation (Hunzicker-Dunn and Maizels, 2006). Here we examined the biological activity of these age-specific glycoforms on known FSH responsive protein and gene pathways. We hypothesized that individual FSH glycoforms would induce downstream signaling pathways differentially in the ovarian follicle. To this end, we isolated murine secondary follicles and first validated FSH dose responsiveness using a commercially available recombinant human FSH (rec. hFSH) preparation. We then treated isolated follicles with recombinant hypo-glycosylated FSH[21] and fully-glycosylated FSH[24] at two concentrations at one-hour and 18-20 hour exposure time-points. In response to these FSH glycoforms, we specifically examined the activation of the PKA signaling pathway and several known FSH-responsive genes in the ovarian follicle. Our data confirm that age-specific FSH glycoforms are bioactive *in vitro* and stimulate different downstream signaling pathways in the follicle.

Materials and Methods

Mice

Pre-pubertal 12-14-day-old CD1 female mice were obtained from a breeding colony. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Northwestern University and were performed in accordance with the guidelines and requirements of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were housed in a controlled barrier facility in the Center for Comparative Medicine at Northwestern University (Chicago, IL). Food and water were provided ad libitum. Temperature, humidity, and photo-period (14L:10D) were kept constant. Ovaries were harvested from mice and removed from the bursa according to standard procedure.

Follicle isolation

Two-layered secondary follicles (mean size 129.56 μm ± 10.48 SD) were mechanically isolated using insulin-gauge needles in dissection medium (Leibovitz 15 [L15] media (Life Technologies Corporation, Grand Island, NY) supplemented with 1% fetal bovine serum

(FBS, Life Technologies Corporation) and 50 U/mL Pen Strep (Thermo Fisher Scientific, Waltham, MA). Healthy two-layered secondary follicles were identified based on their diameter (measured using ImageJ) and morphology (immature oocyte surrounded by two layers of granulosa cells) as previously described (Xu et al., 2006). After isolation, the follicles were allowed to recover from the isolation procedure for a minimum of one hour in maintenance medium (minimum essential medium [α-MEM] (Life Technologies Corporation) supplemented with 1% FBS and 50 U/mL Pen Strep at 37°C in a humidified atmosphere of 5% CO_2 in air.

Recombinant FSH preparations and treatment

Recombinant hFSH was obtained from Organon (Oss, The Netherlands, a gift from Dr. Irving Boime), and predominantly consists of fully-glycosylated FSH[24]. Individual recombinant human FSH glycoforms – fully-glycosylated FSH[24] and hypo-glycosylated FSH[21] – were purified from rat pituitary GH_3 cells co-expressing fully-glycosylated FSH[24] and hypo-glycosylated FSH[21] and FSH[18] as a 1:1 mixture. The purification and characterization of the hormones from GH_3-conditioned media was described in detail elsewhere (Butnev et al., 2015, Bousfield et al., 2014a, Jiang et al., 2015). Secondary follicles in groups of 10 were treated with rec. hFSH at concentrations of 50, 100 and 250 ng/mL concentration diluted in growth media for one-hour and 18-20 hour time-points. These doses correspond to ~ 5, 10 and 25 mIU/mL respectively of the standard reference human FSH preparation (Wang et al., 2016b). Growth medium was composed of α-MEM supplemented with 3 mg/ml bovine serum albumin (MP Biomedicals, Santa Ana, California), 1 mg/ml bovine fetuin (Sigma-Aldrich, St. Louis, MO), and 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml selenium (Thermo Fisher Scientific). Follicles maintained in growth media alone served as controls. All cultures were performed in 96-well Ultra-Low Attachment plates (Corning, Corning, New York). Secondary follicles were also treated with of 20- and 100 ng/mL of purified recombinant human FSH glycoforms FSH[24] and FSH[21] in growth medium for one-hour and 18-20 hours. These concentrations and times were chosen based on *in vitro* studies where differences in glycoform bioactivity were documented (Jiang et al., 2015). After treatment, follicles were imaged using 10X and 20X long working distance objectives on an EVOS FL Auto microscope (Life Technologies Corporation). Follicles were then either snap-frozen on dry ice for Western blot and qPCR or fixed for immunofluorescence analysis. For Western blots, sets of 10 follicles of uniform sizes were rinsed through L15 supplemented with 50 U/mL Pen Strep and 3 mg/ml polyvinylpyrolidine (Sigma-Aldrich) (L15/PVP/PS) to remove exogenous protein supplements and snap-frozen in minimal volume.

Western blot analysis

The snap-frozen follicles were thawed on ice and lysates were prepared in the presence of 1X phosphatase inhibitor cocktail (Thermo Fisher Scientific) to preserve protein phosphorylation. The follicle lysates were then separated on 4-15% SDS-PAGE polyacrylamide gels (Bio-Rad, Hercules, CA) according to standard procedure and transferred onto Amersham Hybond P 0.45 PVDF membranes (GE Healthcare Life Sciences, Pittsburg, PA). The membranes were blocked in 3% Amersham ECL Blocking Agent (GE Healthcare Life Sciences) diluted in Tris buffered saline containing 0.1% v/v

Tween-20 (TBS-T) (block) for 3-4 hours at room temperature. Blots were probed with primary antibody diluted in block at 4°C 18-20 hours. The primary antibody used was phospho-PKA substrates (RRXS*/T*) (1:1000 dilution) (Cell Signaling Technologies, Danvers, MA) (catalog number 9624S). Blots were rinsed three times in TBS-T and incubated in horse radish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:10,000) (GE Healthcare Life Sciences) diluted in block for 1 hour at room temperature. Following secondary antibody incubation, the blots were washed three times in TBS-T and developed with a chemiluminescent substrate, ECL Prime (GE Healthcare Life Sciences) according to the manufacturer's instructions. Blots were stripped using Restore Western Blot Stripping Buffer according to manufacturer's instructions (Thermo Fisher Scientific), blocked for one hour and re-probed with 1:2500 dilution of an antibody against MSY2 (generous gift of R. Schultz, University of Pennsylvania), 18-20 hours at 4°C. MSY2 is a highly abundant oocyte-specific protein (Yu et al., 2001) and was used as a loading control to ensure equal numbers of follicles were loaded onto the gel. The amount of total protein in small numbers of isolated follicles is very low, and thus there is no reliable way to accurately measure total protein levels. Therefore, we loaded equal number of follicles per lane, included follicles only of similar diameters and morphology and normalized the data based on the expression of MSY2. Each follicle contains a single oocyte and if the follicles are at the same developmental stage, the expression of MSY2 should be constant across groups. By normalizing to the follicle number (and keeping follicle number and starting sizes consistent) our analysis assessed PKA phosphorylation directly in the follicle as a response to FSH. The secondary antibody used and detection methods were the same as for phospho-PKA substrates. Densitometry analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD). For the phospho-PKA substrate antibody, the most distinct band at ~70 KDa was used for quantification purposes. Mean pixel intensities for p-PKA substrate and MSY2 bands were determined following background subtraction. Because equal numbers of follicles were loaded per lane, the mean pixel intensity values of the band of interest were normalized to MSY2 mean pixel intensity values. The fold change value was determined by dividing the normalized phospho-PKA substrate intensities of FSH-treated follicles over normalized phospho-PKA substrate intensities of control follicles.

Immunofluorescence analysis

Follicles were fixed in 3.8% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 1 hour at room temperature and then washed in PBS (Life Technology Corporation) containing 0.3% BSA (Sigma-Aldrich), 0.01% Tween-20 (Sigma-Aldrich) and 0.02% NaN$_3$ (Sigma-Aldrich) (blocking buffer). Follicles were permeabilized for 30 minutes in buffer containing 1X PBS, 0.3% BSA, 0.1% Triton X-100 (Sigma-Aldrich), and 0.02% sodium azide. Following permeabilization, follicles were incubated in 20 μg/mL Proteinase K (Sigma-Aldrich) for 5 minutes at 37°C. Proteinase K has been used previously for unmasking antigens in histological sections (Svistunova et al., 2012). Following two more 5-minute washes in blocking buffer, follicles were incubated in the phospho-PKA substrate antibody diluted in blocking buffer (0.717μg/μL) at 4°C 18-20 hours. Non-immune rabbit IgG used at the same concentration (Vector Laboratories, Burlingame, CA) was used as an isotype control to show that staining was specific even following Proteinase K treatment (Supplemental Figure 1). Follicles were rinsed in blocking buffer three times and then

incubated in an Alexa-Fluor-488 conjugated goat anti-rabbit secondary antibody diluted in blocking buffer (1:100) (Life Technologies) for 1-hour at room temperature. Staining for F-actin was performed simultaneously using rhodamine-phalloidin (R415, Life Technologies) at a 1:50 dilution in blocking buffer. Follicles were rinsed again in blocking buffer three times and mounted in Vectashield containing DAPI (4',6-diamidino-2-phenylindole; Vector Laboratories) to detect DNA. Follicles were imaged with a Leica SP5 inverted laser-scanning confocal microscope using a 40x objective (Leica Microsystems). Z-stacks of 1μm optical section thickness were taken. Images were processed and quantified using ImageJ. For quantification of phospho-PKA substrates fluorescence intensity, the optical section containing the middle of the follicle (including the granulosa cells and the oocyte with a visible nucleus) was used. The ratio of fluorescence intensity of the follicle relative to the total area of the follicle based on pixel intensity was calculated and reported for follicles within each treatment group.

RNA isolation, cDNA synthesis and Taqman Real-time qPCR assays

Isolated murine follicles were treated as above with 100 ng/mL FSH glycoforms and then snap-frozen in groups of 50. Total RNA was isolated from 100 snap-frozen mouse ovarian follicles treated with 100 ng/mL FSH glycoforms using RNEasy micro columns (Qiagen), and the RNA was quantified using a NanoDrop spectrophotometer (ThermoScientific) at 260 nm. Total RNA was reverse transcribed by the oligo dT method using the SuperScript III kit (ThermoScientific) as described (Wang et al., 2014, Wang et al., 2015, Wang et al., 2016a). Taqman real time qPCR assays were performed on the cDNA samples in triplicate using custom-made or pre-inventoried primer/combo mixes (ThermoScientific or IDT). Expression of *Ppil1* was used as an internal control and the relative amounts of mRNA expression were calculated as described (Wang et al., 2014, Wang et al., 2015, Wang et al., 2016a). A list of genes interrogated is provided in Supplemental Table 1.

Statistical analysis

All experiments were performed with a minimum of two or three biological and technical replicates unless specified. Graphs were created and data were analyzed using GraphPad version 7 for Mac OS X (GraphPad Software, La Jolla, CA, www.graphpad.com). A one-way ANOVA with Tukey's post-hoc test was used to compare responses across treatment groups. A P-value of <0.05 was considered significant.

Results

Isolated murine secondary follicles are responsive to rec. hFSH in a dose-responsive fashion at the one-hour time point

To validate our model system, we first confirmed that isolated secondary follicles were responsive to rec. hFSH following one-hour and 18-20 hours of treatment using phosphorylated PKA substrates as an endpoint because PKA is a well-known target of FSH in the ovarian follicle (Figure 1). FSHR, expressed on granulosa cells is a G-protein-coupled receptor (GPCR), to which FSH binds and signals through multiple downstream signaling pathways, including the canonical Gsα/cAMP/PKA pathway. FSHR couples to Gsα and produces cyclic AMP (cAMP) which binds and activates Protein Kinase A (PKA). The

catalytic subunits of PKA phosphorylate other downstream proteins (Das and Kumar, 2018, Hunzicker-Dunn and Mayo, 2015). The blots for phosphorylated PKA substrates in isolated follicles therefore show multiple bands (Figure 1 A, B). Although we do not know the specific protein identity of these bands, immunoblot analysis to assess PKA substrate phosphorylation is standard practice that has been utilized previously to assess the effects of FSH glycoforms (Jiang et al., 2015, Wang et al., 2016b). Known PKA downstream targets include CREB, PI3K/Akt, Histone H3 and p38 MAPK (Hunzicker-Dunn and Maizels, 2006).

At the one-hour timepoint, there was a dose-dependent increase in the fold change of PKA substrate phosphorylation to rec. hFSH treatment which was significant relative to untreated control at the 25 mIU/mL dose ($P<0.05$) (Figure 1C). This trend was not observed at the 18-20-hour time point (Figure 1D). These findings indicate that the canonical PKA signaling pathway is activated in a dose-responsive manner to rec. hFSH in the follicle, but the signaling activation is time-dependent and occurs rapidly. These results also confirm that isolated ovarian follicles provide us with a reliable system to study the direct effects of individual FSH glycoforms.

Recombinant FSH glycoforms induce phosphorylation of PKA substrates in secondary follicles

To determine whether recombinant human fully-glycosylated FSH[21] and hypo-glycosylated FSH[24] glycoforms activate the PKA signaling pathway, we treated isolated secondary follicles with 20- and 100 ng/mL FSH glycoforms at one-hour and 18-20 hour timepoints and examined the phosphorylation of PKA substrates. Treatment of follicles with 20 ng/mL of either purified FSH glycoform did not affect their morphology at any time point (Figure 2 A-F). By western blot, each glycoform tended to stimulate PKA substrate phosphorylation relative to the untreated control at both time points but this did not reach statistical significance (Figure 2 G-J). In addition to assessing PKA substrate phosphorylation by Western blot, we also performed wholemount immunocytochemistry on intact follicles. Within the follicle, phosphorylated PKA substrates localized primarily to the outer granulosa cells but were also observed within the oocyte with particular enrichment in the nucleus (Figure 2K-Q). The staining was specific as it was not observed in the non-immune IgG isotype control (Supplemental Figure 1). Based on immunofluorescence, phospho-PKA substrate expression increased significantly following the one-hour treatment with 20 ng/mL FSH[21] and FSH[24] ($P<0.05$) (Figure 2 K-N). No increase was observed at the 18-20-hour time point (Figure 2 O-R). These results indicate that follicles respond to FSH glycoform concentrations as low as 20 ng/mL, but this bioactivity is significant only at the one-hour time point.

Treatment with the 100 ng/mL dose of purified FSH glycoforms was similar to the treatment with 20 ng/mL in that follicle morphology was unaffected at both the one-hour and 18-20 hour time points (Figure 3 A-F), and Western blot analysis indicated a consistent but not significant trend of increased PKA substrate phosphorylation relative to the untreated control at both time points (Figure 3 G-J). However, by immunofluorescence we detected a significant increase in phospho-PKA substrate expression following one-hour treatment with

100 ng/mL FSH[24] relative to untreated control (P<0.05). Unlike treatment with 20 ng/mL, there was a significant difference between FSH[21] and FSH[24] action following treatment with 100 ng/ml of the FSH glycoforms at the one-hour time point (P<0.05) (Figure 3 K-N). No differences were observed at the 18-20 hour time point (Figure 3 O-R). These results indicate that treatment of follicles with 100 ng/mL of FSH glycoforms activates differential patterns of PKA signaling in the ovarian follicle, and this activation is time-dependent and most robust at the one-hour time point.

Hypo- and fully-glycosylated FSH glycoforms elicit time-dependent changes in gene expression in isolated follicles

Phosphorylation of PKA substrates, while a well-accepted readout of FSH action, is only a single endpoint which occurs at the protein and post-translational level. Our findings indicate that the response to the FSH glycoforms occurs rapidly within one hour. Therefore, we wanted to take a broader approach by examining the expression of well-known FSH-responsive genes – *Apaf1*, *Cyp19a1*, *Caps3*, *Cepbp*, *Esr1*, *Ereg*, *Fshr*, *Inha*, *Inhbb*, *Kcnj8*, *Ki67*, *S100a6* and *Tagln*. (Table 1) (Wang et al., 2016b). These genes were shown previously to be differentially regulated by FSH glycoforms *in vivo* in whole ovaries (Wang et al., 2016b). To test the *in vitro* bioactivity of recombinant human hypo-glycosylated FSH[21] and fully-glycosylated FSH[24] glycoforms in regulating gene expression, isolated secondary follicles were treated with 100 ng/mL glycoforms. At one-hour and 18-20 hour time points, RNA was isolated and reverse-transcribed, and Taqman real-time qPCR assays were performed for these particular genes of interest. We specifically focused on the 100 ng/mL doses because this concentration elicited a differential response in PKA signaling within the follicle as assessed by immunofluorescence (Figure 3 K-N).

Expression of *Esr1*, *Inha* and *Kcnj* did not differ between untreated controls or between glycoforms at either timepoints (Supplemental Figure 2). At the one-hour time point, seven genes (*Apaf1*, *Casp3*, *Cebpb*, *Ereg*, *Fshr*, *Inhbb* and *Tagln*) had expression patterns in which one of the glycoforms caused a significant up- or down-regulation relative to control (Figure 4). Only one gene, *Cyp19a1*, showed upregulation relative to the untreated control when treated with both FSH[21] and FSH[24] (Figure 4D). Three genes, (*Casp3*, *Cebpb* and *Cyp19a1*) were differentially regulated by the two glycoforms, with FSH[24] inducing higher expression compared to FSH[21] for all three genes (Figure 4).

At the 18-20-hour timepoint, four genes (*Ereg, Fshr, S100a6* and *Tagln*) were significantly down-regulated following treatment with both glycoforms relative to control (Figure 5 D, E, G, H). Two genes showed a significant up-regulation (*Casp3*) (Figure 5A) or down-regulation (*Ki67*) (Figure 5F) by one of the glycoforms relative to control. Similar to the one-hour response, *Cyp19a1* showed upregulation by both FSH[21] and FSH[24] relative to the untreated control (Figure 5C). *Cebpb* was also the only gene at the 18-20-hour time point to have differential regulation between glycoforms with FSH[21] treatment resulting in higher expression relative to FSH[24] (Figure 5B). These data demonstrate that treatment of follicles with 100 ng/mL of FSH glycoforms results in significant up-regulation as well as differential regulation at the one-hour time point compared to the 18-20-hour timepoint.

Discussion

We tested the effects of individual purified hypo-glycosylated FSH[21] and fully-glycosylated FSH[24] glycoforms on murine secondary follicles. Recombinant GH_3 cell-derived FSH glycoform preparations were biologically active at the level of the ovarian follicle and directly regulated signaling responses within the follicle. Previously, hypo-glycosylated FSH[21/18] and fully-glycosylated FSH[24] glycoform preparations were tested *in vitro* in radio-ligand and radio-receptor assays (Bousfield et al., 2014a, Bousfield et al., 2014b, Butnev et al., 2015) and in immortalized granulosa cell tumor lines (Jiang et al., 2015). In these studies, hypo-glycosylated FSH[21/18] was more active than the fully-glycosylated FSH[24]. Pharmacological rescue studies testing the recombinant GH_3 cell-derived FSH glycoform preparations using an immature *Fshb* null mouse model indicated that these preparations were biologically active *in vivo* (Wang et al., 2016b) and acted via different downstream signaling pathways. We used the unique features of the isolated ovarian follicle as an organotypic system consisting of the female gamete and the supporting somatic cells to investigate the effects of these glycoforms without contributions from other ovarian somatic tissue within the stroma. Additionally, the responses to FSH seen in this study are innate to the follicle as the mice used were prepubertal (sexually immature) and were not primed prior to follicle isolation. Therefore, no external source of FSH action was introduced into the system, confirming that the follicle is indeed directly responsive to the glycoforms.

Our findings demonstrate that the ovarian follicle responds to FSH glycoforms and activates signaling pathways in a time-dependent fashion, as seen by the increase in PKA substrate phosphorylation following one-hour treatment. These results are consistent across lower (20 ng/mL) and higher (100 ng/mL) concentrations of fully- and hypo-glycosylated FSH glycoforms. The PKA signaling pathway is known to act rapidly and transiently within the ovarian follicle. For example, phosphorylation of CREB, a transcription factor regulated by PKA, can be detected in granulosa cells within one minute of FSH addition (Cottom et al., 2003). Our results at the one-hour timepoint confirm the rapid nature by which the PKA signaling pathway responds to FSH stimulation to induce downstream targets. Although previously published *in vivo* data have demonstrated responses to FSH glycoforms in whole ovarian extracts at 30 minutes (Wang et al., 2016b), we chose one-hour as our earliest time point to examine due to the technical challenges of manipulating multiple groups of follicles within a shorter time interval. We expect, however, that we would likely also observe effects at shorter time points because seven genes were upregulated in response to either glycoform at the 1 hour timepoint compared to the 18-20-hour timepoint. For example, at the one-hour time point FSH[24] increased *Fshr* mRNA expression relative to control, but at the 18-20 hour time point both FSH[21] and FSH[24] decreased *Fshr* mRNA expression relative to control. There are several explanations as to why there is attenuation of PKA substrate phosphorylation and gene expression changes following 18-20-hour treatment with the glycoforms. First, the hormones may not be stable over the course of the longer-term treatment. Second, the FSHR, like most other GPCRs, is known to exhibit desensitization with prolonged exposure to an agonist (Hunzicker-Dunn and Mayo, 2015).

Although increased trends of PKA substrate phosphorylation were observed following FSH glycoform treatment relative to untreated control, only the one-hour time point following

glycoform treatment indicated a differential response between hypo- and fully-glycosylated glycoforms. These results support the *in vivo* studies on *Fshb* null mice (Wang et al., 2016b) that showed hypo- and fully-glycosylated FSH glycoforms differentially regulate FSH-responsive gene expression within the whole ovary following one-hour of hormone injection. FSH-responsive genes in the ovary were differentially regulated and therefore implicated in distinct cell signaling events (cell cycle, apoptosis and cell adhesion events for FSH[21] compared to cell differentiation pathways and transcription factor-mediated events for FSH[24]) (Wang et al., 2016b). There are several key differences when comparing our gene expression findings to those obtained *in vivo* from whole ovarian extracts (Wang et al., 2016b) (Table 1). *In vivo* both FSH[21] and FSH[24] treatment resulted in upregulation of six genes (*Apaf1*, *Casp3*, *Cyp19a1*, *Ereg*, *Fshr*, *Ki67*). Differential responses between glycoforms coincided *in vivo* and *in vitro* only for the *Casp3* gene, but FSH[21] resulted in upregulation compared to FSH[24]. This was also seen in *Apaf1* and *Fshr* where differential responses were observed *in vivo*. Overall, although the glycoforms elicited differential patterns of gene expression in the follicle, the general patterns were different from those observed *in vivo*. These differences are important because they suggest that the glycoforms activate unique downstream signaling pathways in individual compartments of the ovary (for example, the stroma, theca cells, and granulosa cells) either directly or indirectly. The microenvironment is different between the *in vivo* and *in vitro* studies in which follicles were isolated and directly treated with FSH glycoforms. Thus the glycoforms may have indirect effects on cells besides the granulosa cells *in vivo*. It is important to note that our conclusions are based on analysis of a select signaling pathway and group of FSH-responsive genes. An unbiased large-scale gene expression profiling by RNASeq analysis will be required in the future to further identify the downstream effects of FSH glycoforms on the ovarian follicle.

Although our data clearly indicate that FSH glycoforms are directly active on the murine follicle, isolated ovarian follicles exposed to short term treatments may not accurately reflect the *in vivo* physiology of a normally cycling ovary. During follicular maturation, a cohort of immature follicles is recruited by FSH but only a subset of these follicles survive to full maturity with most becoming atretic during the cycle (Hunzicker-Dunn and Maizels, 2006, Hunzicker-Dunn and Mayo, 2015). The follicles that were selected for these experiments were morphologically healthy, but their quality as it would be *in vivo* is unknown. *In vivo* studies also showed that estradiol production is increased within ovaries from *Fshb* null mice injected with hypo-glycosylated FSH[21/18] (Wang et al., 2016b). Although we measured steroid hormone production of follicles following FSH glycoform treatment, the levels were too low to detect in the culture media via standard ELISA techniques likely due to the small number of isolated follicles combined with the short nature of our cultures. We also do not know whether treatment using these glycoforms have downstream effects on follicle growth dynamics and oogenesis outcomes. *In vitro* follicle growth studies testing the efficacy of these glycoforms on follicle growth, maturation and survival in addition to investigating oocyte outcomes will, therefore, be important in validating the current results (Kreeger et al., 2006, Xu et al., 2006). However, the amount of hormone required to sustain these long-term cultures is substantial and beyond the capacity of the volumes used in the present study.

The hypo-glycosylated human FSH variant and its changing abundance with age is well documented (Bousfield et al., 2014a, Bousfield et al., 2014b). Younger women undergoing ovulatory cycles require rapid hormone action and clearance rates (Wide and Eriksson, 2013) and FSH[21] (more abundant in younger women) has been shown to possess these characteristics (Bousfield et al., 2014a, Jiang et al., 2015, Butnev et al., 2015). Because the abundance of FSH[24] increases with aging when ovarian responses are diminished and based on *in vitro* biochemical data which demonstrated that FSH[24] exhibits less affinity for FSHRs in terms of binding and other kinetic parameters (Bousfield et al., 2014a, Jiang et al., 2015, Butnev et al., 2015), it has been speculated that FSH[24] is ineffective in maintaining ovarian function in old age. However, changes in FSHR number or FSH ligand-receptor recycling dynamics cannot be ruled out as other potential reasons for defective follicle maturation and oocyte development in old age. This issue has been long debated and newer technological advances in reliably measuring FSHRs will hopefully resolve this issue in the future. Defective follicle maturation and oocyte development could possibly be associated with the loss of hypo-glycosylated FSH glycoforms due to aging, manifesting in reduced ovarian function (Wang et al., 2016b, Jiang et al., 2015, Bousfield et al., 2014a). This does not exclude the ability of FSH[24] to elicit responses when directly injected or added to follicle cultures. Our published *in vivo* data (Wang et al., 2016b) and the present *vitro* studies indicate that FSH[24] and FSH[21] have distinct gene expression signatures. Because these glycoforms elicit distinct pathways both in *in vitro* and *in vivo* models, it is possible that these glycoforms or the ratio of glycoforms will have significant physiological effects, and this warrants further investigation. Additional studies including longer cultures and use of human tissue samples to analyze folliculogenesis and oogenesis outcomes specifically will be required to draw decisive conclusions regarding clinical implications of these glycoforms. Additionally, this study was conducted only on follicles from young, prepubertal mice. Further analysis of these glycoforms on follicles from older mice would provide necessary insights into FSH glycoform responsiveness on ovarian follicles with age.

In conclusion, our studies show that FSH glycoform treatment for one-hour elicits bioactivity within the follicle as measured by an induction of PKA signaling and modulating gene expression. Future studies investigating the effects of these glycoforms in human ovarian follicles will be of considerable interest in understanding clinical benefits of the hormone.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

List of abbreviations

ART	Assisted reproductive technology
cAMP	Cyclic adenosine 5′-monophosphate
CREB	Cyclic AMP-responsive element binding protein
FSH	Follicle stimulating hormone
FSHR	FSH receptor
GPCR	G-protein coupled receptor
MAPK	Mitogen-activated protein kinases
PKA	Protein kinase-A
PVDF	Polyvinylidine difluoride
rec. hFSH	Recombinant human FSH

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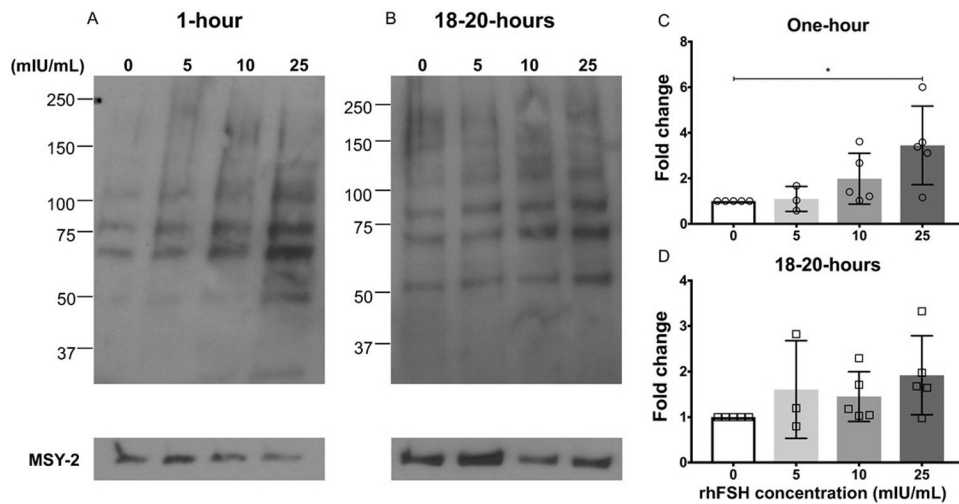
Figure 1. Rec. hFSH treatment induces phosphorylated PKA substrates in a dose-dependent manner in secondary follicles.

(A, B) Immunoblot analysis of phosphorylated PKA substrates in murine follicles following rec. hFSH treatment for one-hour (A) and 18-20 hours (B). Densitometry analysis of phospho-PKA substrate protein levels were performed and values indicated (C, D) were normalized to MSY-2 levels. The experiment was repeated 5 times (3 times for 5mIU/mL concentration) with 10 follicles in each group. Representative images are shown. The arrow indicates the phospho-PKA substrate band that was chosen for densitometry. Data are expressed as mean ± S.D. * P<0.05.
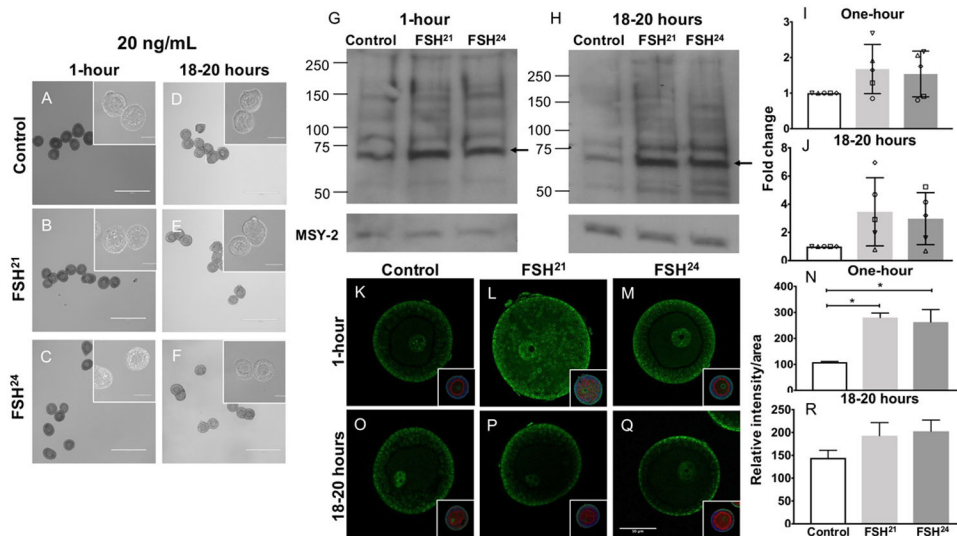
Figure 2. Treatment of follicles with 20ng/mL individual purified FSH glycoforms for one-hour results in upregulation of phospho-PKA substrates.

(A-F) Follicles were morphologically intact with healthy oocytes following treatment with 20 ng/mL FSH glycoforms at the one-hour and 18-20 hour timepoints. Scale bar = 400μm (100μm for insets). (G, H) Immunoblot analysis of phosphorylated PKA substrates in murine follicles following FSH glycoform treatment for one-hour (G) and 18-20 hours (H). (I, J) Densitometry analysis of phospho-PKA substrate protein levels were performed and values were normalized to MSY-2 levels. Experiment was repeated 5 times with 10 follicles per group. (K-R) Immunofluorescence analysis of phosphorylated PKA substrates in murine follicles following FSH glycoform treatment for one-hour (K-M) and 18-20 hours (O-Q). Single optical sections are shown. Insets show DNA in blue and F-actin in red. Images corrected to +20% brightness and −20% contrast. (N, R) Relative intensity per area of all follicles examined is plotted for each time point. Between 10-21 follicles were examined at each time point. Data expressed as mean ± S.D. Scale bar = 50μm. * P<0.05.
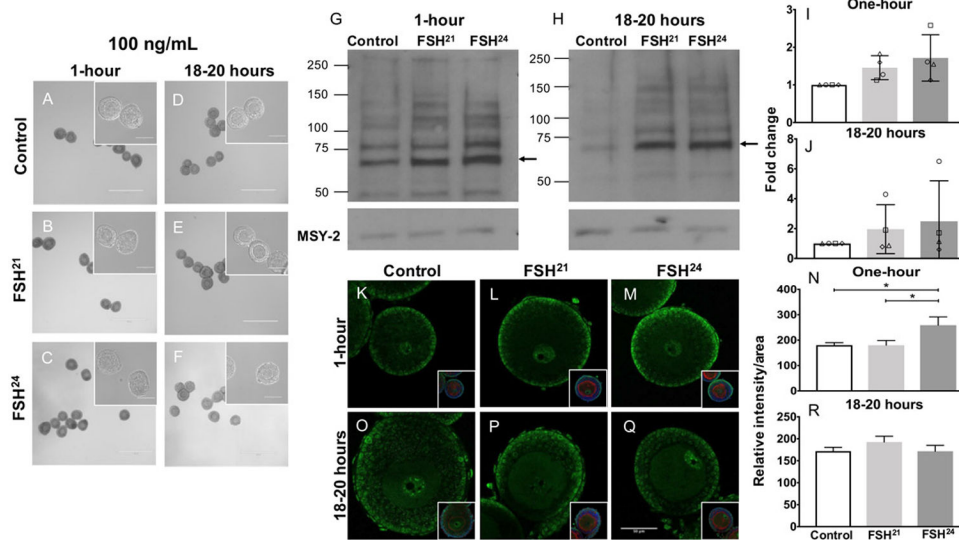
Figure 3. Treatment of follicles with 100ng/mL individual purified FSH glycoforms for one-hour results in differential regulation of phospho-PKA substrates.

(A-F) Follicles were morphologically intact with healthy oocytes following treatment with 100 ng/mL FSH glycoforms at the one-hour and 18-20-hour time points. Scale bar = 400μm (100μm for insets). (G, H) Immunoblot analysis of phosphorylated PKA substrates in murine follicles following FSH glycoform treatment for one-hour (G) and 18-20 hours (H). (I, J) Densitometry analysis of phospho-PKA substrate protein levels were performed and values were normalized to MSY-2 levels. Experiment was repeated 4 times with 10 follicles per group. (K-R) Immunofluorescence analysis of phosphorylated PKA substrates in murine follicles following FSH glycoform treatment for one-hour (K-M) and 18-20 hours (O-Q). Single optical sections are shown. Insets show DNA in blue and F-actin in red. Images corrected to +20% brightness and −20% contrast. (N, R) Relative intensity per area of all follicles examined is plotted for each time point. Between 13-20 follicles were examined at each time point. Data expressed as mean ± S.D. Scale bar = 50μm. * $P<0.05$.
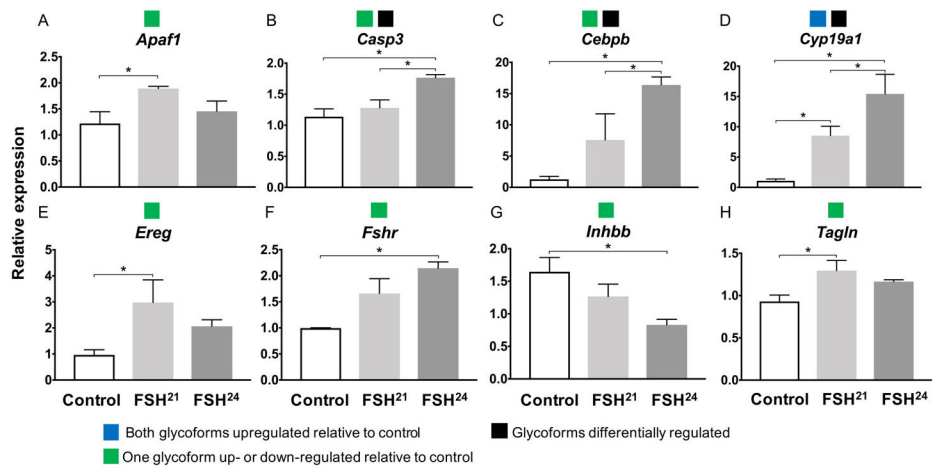
Figure 4. One-hour treatment with 100ng/mL FSH glycoforms shows gene expression changes in secondary follicles.

Gene expression was assayed by Taqman real time qPCR assays following one-hour of glycoform treatment. Groups of 100 follicles were analyzed in triplicate. * $P<0.05$. Colored boxes in legend indicates the four gene expression patterns observed for both one-hour and 18-20-hour exposure time points.

Figure 5. 18-20-hour treatment with 100ng/mL FSH glycoforms shows a decreased trend in gene expression in secondary follicles.

Gene expression was assayed by Taqman real time qPCR assays following 18-20-hour glycoform treatment. Groups of 100 follicles were analyzed in triplicate. * P<0.05. Colored boxes in legend indicates the four gene expression patterns observed for both one-hour and 18-20-hour exposure time points.
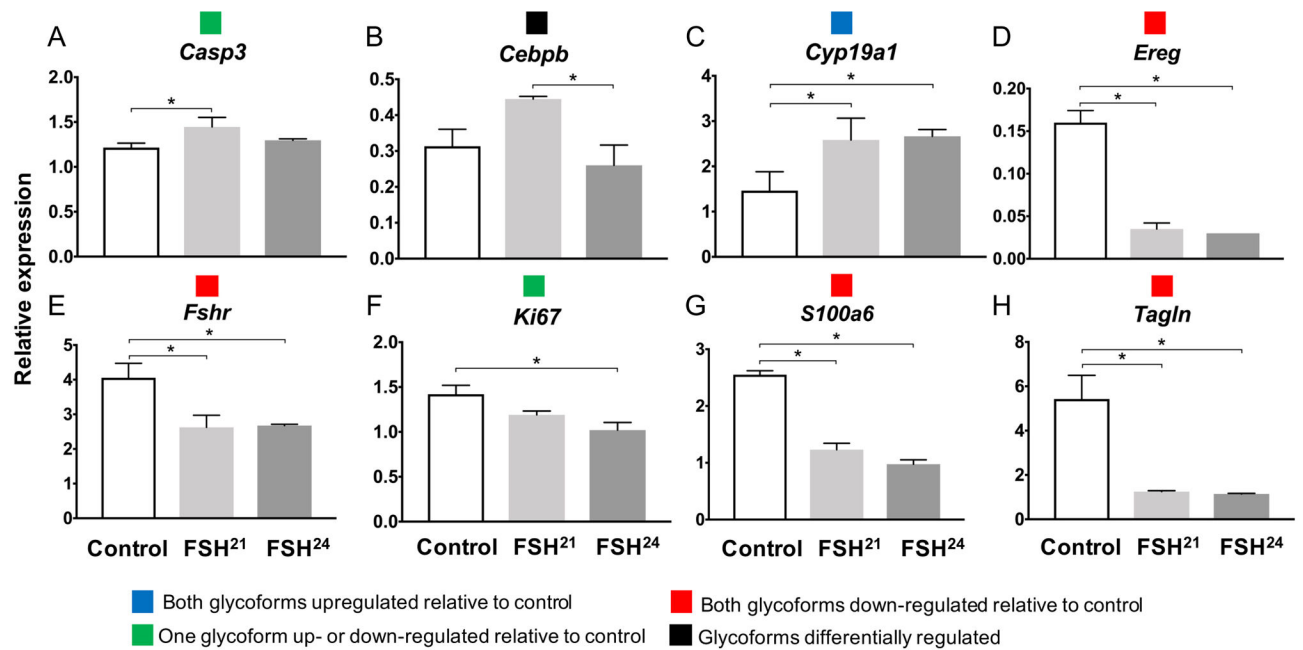
Table 1.

Summary of gene expression in ovarian follicles following treatment with FSH glycoforms (100 ng/ml) and comparison to *in vivo* gene expression results in whole ovaries.

Gene	One-hour (*in vitro*)		One-hour (*in vivo*) [1]		18-20 hours (*in vitro*)	
	Up or down regulated relative to control	Up or down regulated between glycoforms	Up or down regulated relative to control	Up or down regulated between glycoforms	Up or down regulated relative to control	Up or down regulated between glycoforms
Apaf1	**21**	NS	**21, 24**	21 > 24	NS	NS
Casp3	**24**	24 > 21	**21, 24**	21 > 24	**21**	NS
Cebpb	**24**	24 > 21	NS	NS	NS	21 > 24
Cyp19a1	**21, 24**	24 > 21	**21, 24**	NS	**21, 24**	NS
Ereg	**21**	NS	**21, 24**	NS	21, 24	NS
Fshr	**24**	NS	**21, 24**	21 > 24	21, 24	NS
Inhbb	24	NS	**24**	NS	NS	NS
Ki67	NS	NS	**21, 24**	NS	24	NS
S100a6	NS	NS	NS	NS	21, 24	NS
Tagln	**21**	NS	**21**	NS	21, 24	NS

[1] In vivo data from WANG, H., MAY, J., BUTNEV, V., SHUAI, B., MAY, J. V., BOUSFIELD, G. R. & KUMAR, T. R. 2016. Evaluation of in vivo bioactivities of recombinant hypo- (FSH(21/18)) and fully- (FSH(24)) glycosylated human FSH glycoforms in Fshb null mice. *Mol Cell Endocrinol,* 437, 224-236.

Bolded text indicates upregulation of gene expression and text not bolded denotes downregulation of gene expression. Data indicated as "NS" was not significant.