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Quantification of HFSHR Signaling to Determine LIPID Raft Residency

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QUANTIFICATION OF HFSHR SIGNALING TO DETERMINE LIPID RAFT RESIDENCY

By

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ABSTRACT

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Human follicle stimulating hormone (hFSH) is a gonadotropin responsible for regulating reproductive systems by stimulation of Sertoli cells in males and granulosa cells in females. The hFSH receptor (hFSHR) is a seven transmembrane receptor that belongs to the G protein coupled receptor family. The receptor is functionally connected to a G protein on the inside of the cell. Once FSH activates its receptor, a cascade of signaling begins, resulting in the activation of adenylyl cyclase, which increases the intracellular levels of cAMP. In addition, hFSHR stimulation also activates the p44/42 MAP kinase. The spike in cAMP activates the enzyme protein kinase A (PKA), which triggers a series of downstream effectors resulting in follicular stimulation and gametogenesis.

Previous work in the Cohen Lab has shown that hFSHR is located in cholesterol-rich, detergent-resistant microdomains known as lipid rafts. In an HEK293 cell line stably expressing hFSHR, disruption of lipid rafts by the cholesterol chelator methyl beta-cyclodextrin (MβCD) interferes with PKA activation. Current research is focused on the relevance of hFSHR lipid raft residency in the human granulosa cell line hGrC1; focusing in particular on the activation of signal transduction pathways by hFSHR. The goal was to develop an enzyme-based, quantitative, non-radioactive assay for cAMP stimulation that could be used to study the effects of lipid raft disruption by MβCD on hFSHR signaling in hGrC1 cells. The β-

galactosidase assay showed quantitative dose-dependent responses to hFSH, which indicated that it should be useful for testing MβCD to further determine lipid raft dependence of hFSHR signaling. Studying the regulation of signaling by hFSHR provides more insight into the receptor function and potentially represents new approaches to contraception or treatment of infertility.

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INTRODUCTION

The endocrine system is responsible for chemical messaging through the circulatory system by which regulation and maintenance of homeostasis is controlled through paracrine signaling. These chemical messengers are referred to as hormones, which are synthesized and released by glands into the blood stream where they can travel to the target tissue to elicit the desired response (Griffin and Ojeda, 1988). Regulation of the release of such hormones is controlled on multiple levels by which many signals promoting the activation of each pathway originate in the neurons of the hypothalamus which then signal to the pituitary via a hormone to secrete another hormone into the circulatory system to in turn promote the secretion of a third hormone from some other gland present in the body, such as the adrenal or thyroid glands which act to control the body's response to stress and metabolism (Hadley, 2000).

The endocrine system also works to control the human reproductive system. Figure 1 gives a schematic of the hormone pathways involved in both the female and male reproductive system.

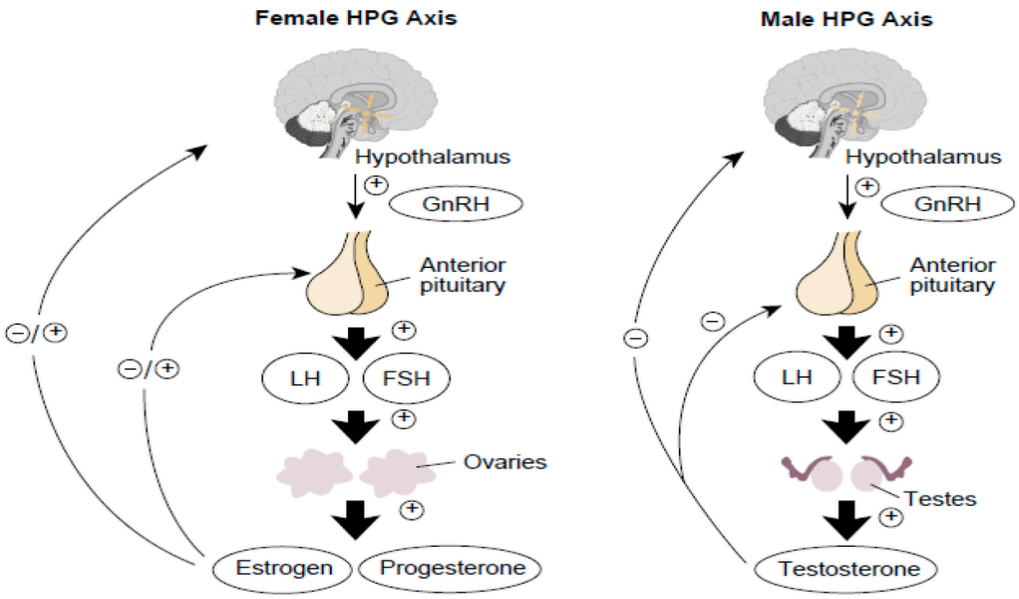

Figure 1. Hypothalamic-pituitary-gonadal axis for females and males.

This pathway is referred to as the hypothalamic-pituitary-gonadal (HPG) axis, meaning the hormone response originates in the hypothalamus then signals the anterior pituitary and then finally the gonads, ovaries in females and testes in males, stimulating the secretion of the androgens estrogen, progesterone, and testosterone (Figure 1) (Gharib, *et al*. 1990).

The hormone of study in this research is follicle stimulating hormone (FSH), a glycoprotein hormone released from the anterior pituitary in response to stimulation of the gland by gonadotropin releasing hormone (GnRH). Luteinizing hormone (LH) is another glycoprotein released by the anterior pituitary in response to GnRH. FSH and LH are secreted by the same method in both males and females, however, the actions these two hormones elicit differ between genders. In males, FSH stimulates testicular growth and promotes increased levels of testosterone in the testes through production of androgen binding protein in Sertoli cells aiding in spermatogenesis, while LH is responsible for the production of testosterone in

Leydig cells (Dias et al., 2002, Hadley, 2000). Both hormones are required for the maturation of spermatozoa in males (Griffin and Ojeda, 1988).

In a similar fashion, FSH acts in cooperation with LH to induce hormonal responses in females. LH stimulates the production of estrogen and progesterone in the ovaries, whereas a spike in LH induces ovulation. FSH is responsible for the development of the ovarian follicle, and in combination with LH responsible for estrogen secretion from the follicle (Gardner & Shoback, 2007).

In order for FSH to elicit a response out of the cells in which it acts on, it must somehow be received and its signal transduced by these cells. This process is carried out by its specific receptor, follicle stimulating hormone receptor (FSHR). FSHR is a seven transmembrane G protein coupled receptor (GPCR). Figure 2 displays the typical structure of a GPCR embedded in the lipid bilayer of a cell.

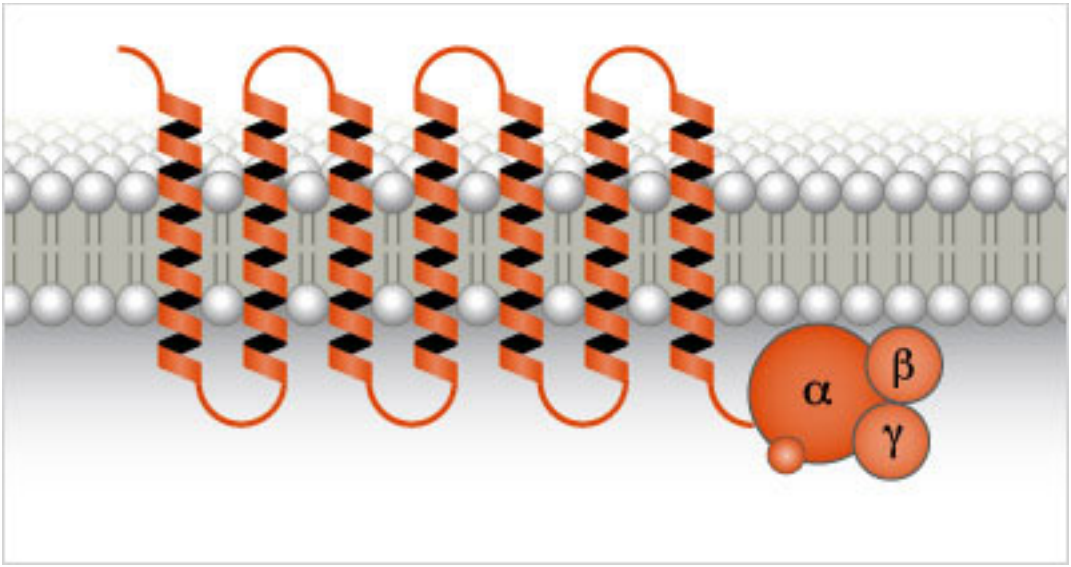

Figure 2. General structure of a G-protein coupled receptor (Mayo Clinic, 2017).

GPCRs are amongst some of the most common receptors in cells, totaling about 80% of all signals transduced across lipid bilayers (Millar & Newton. 2010). In order for

these GPCRs to carry out their functions, they must consist of an extracellular ligand-binding domain, a transmembrane domain consisting of seven alpha helices, and an intracellular domain responsible for interaction with its coupled G proteins, shown in Figure 2 as the alpha (α), beta (β), and gamma (γ) heterotrimeric subunits (Voet et al., 2006).

The G-proteins, more technically known as guanine-nucleotide-binding signal transduction proteins, are the part of the GPCR actually responsible for passing on the chemical signal received from the receptor. This process occurs as a result of a conformation change in the receptor as a result of its ligand binding, inducing the activation of its coupled G-proteins. Figure 3 displays the process of G-protein activation through receptor binding.

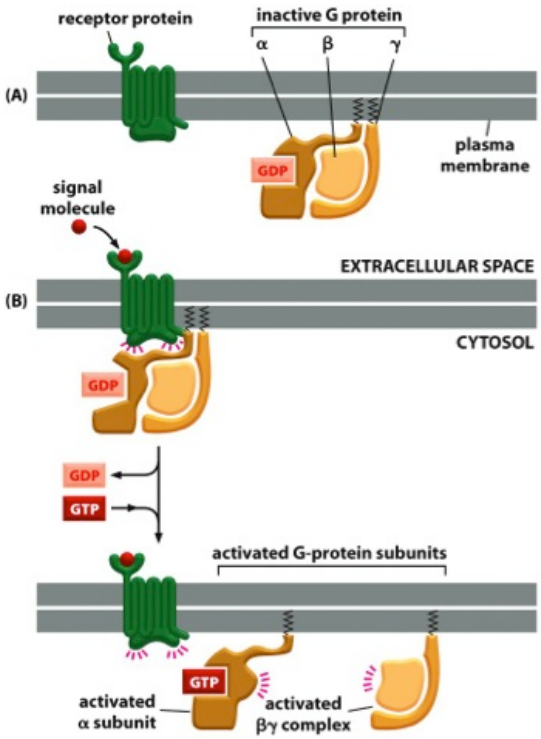

Figure 3. Signal activation and transduction of a G-protein from a GPCR (Oregon State, 2011).

Upon binding of the ligand to its complementary extracellular binding domain of the GPCR, the α subunit, bound to a GDP molecule yielding it inactive, is able to exchange GDP for a GTP, rendering the α subunit active and dissociating it from the βγ subunit (Figure 3). These active α and βγ subunits then go on to transduce the desired signal through different pathways. The signal pathway is turned off upon hydrolysis of GTP to GDP by the GTPase activity of the α subunit, returning the G-proteins to their coupled inactive conformation shown in step (A) of Figure 3.

In this study, hFSHR contains a specific G-protein α subunit known as $G_s\alpha$, which is responsible for the activation of adenylyl cyclase, an important step within the FSH signal transduction pathway (Alberts et al., 2004). Figure 4 displays two of the FSHR signaling pathways focused on in this study.

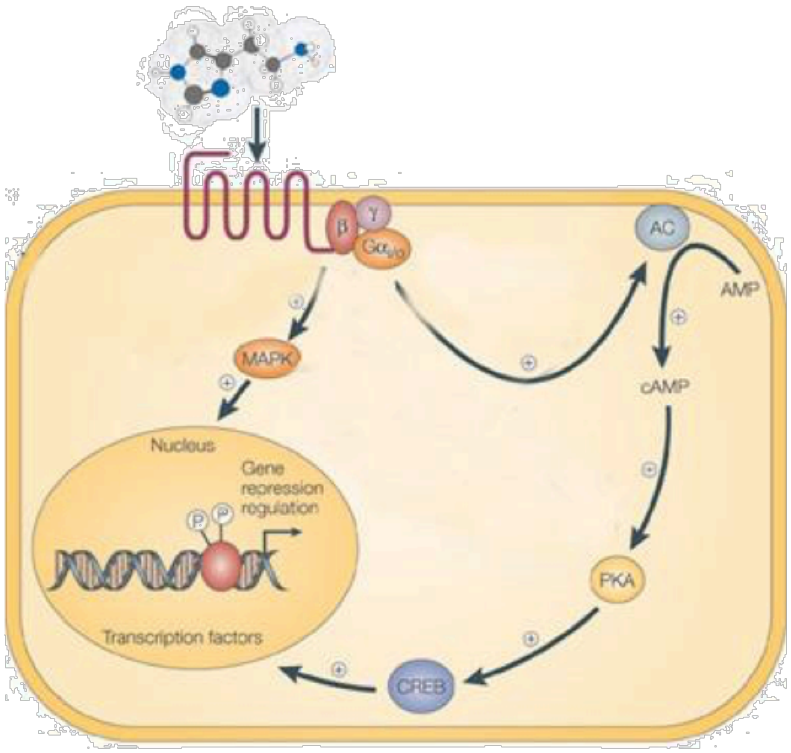

Figure 4. Brief outline of two FSHR signaling pathways used in this study.

The first pathway as previously mentioned operates through the activation of adenylyl cyclase by $G_s\alpha$, which in turn synthesizes cyclic adenosine monophosphate (cAMP), a cellular secondary messenger, from adenosine triphosphate (ATP). From here, cAMP activates protein kinase A (PKA) which then phosphorylates the cAMP response element binding protein (CREB), allowing it to alter gene expression resulting in stimulation of pathways involved in spermatogenesis, oogenesis, and estrogen production. The other pathway involved operates through signal transduction via the mitogen activated protein kinase (MAPK) pathway, which is stimulated upon activation of the protein β-arrestin as a result of FSH binding to FSHR resulting in further gene regulation coding for reuptake of the receptor.

The location of such FSH signal pathway components depend upon the composition of the cell membrane in which some, such as FSHR, adenylyl cyclase, and $G_s\alpha$ are, are embedded in. The cell membrane is composed of lipids, ampithatic molecules with polar heads and nonpolar hydrocarbon tails, conformed into a bilayer with the polar heads on the outside and nonpolar tails on the inside, which can be seen in Figure 5 (Voet et al., 2006). Figure 5 displays a simple conformation of a lipid bilayer and the effect of saturated versus unsaturated nonpolar tail regions of the lipids on overall membrane structure.

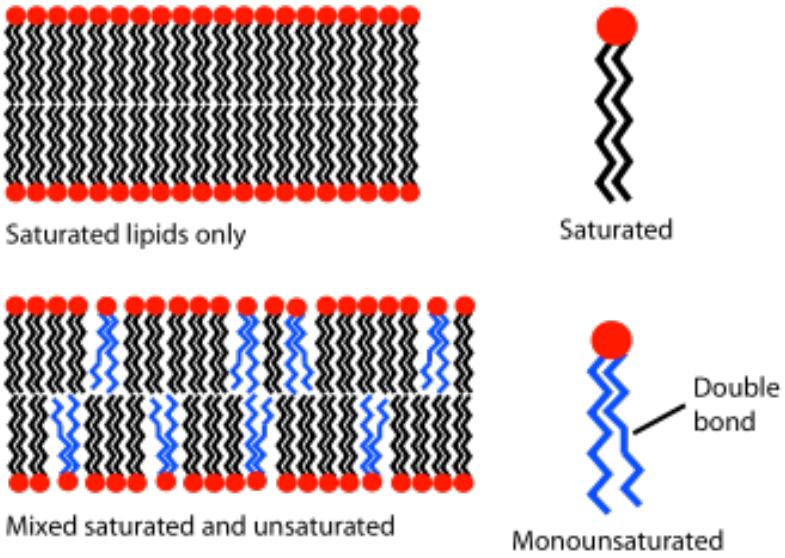

Figure 5. The effect of saturated and unsaturated hydrocarbons on plasma membrane structure.

Saturated fatty acids contain completely hydrogenated tails, whereas unsaturated fatty acids contain double bonds resulting in kinks within the tails. These kinks in the tails alter the structure of the lipid bilayer in which they are embedded in giving a more disordered domain.

The degree of order within the membrane determines the fluidity of that portion of the lipid bilayer as more saturated regions pack more closely together limiting the degree of transverse diffusion in which components of the membrane can travel throughout the domain (Voet et al., 2006). On the other hand, regions that contain a higher concentration of unsaturated lipids will give that portion of the membrane a more fluid environment (Alberts et al., 2004). The ways in which these domains are distributed are not random and usually contain proteins and lipids specific to these regions, known as microdomains (Voet et al, 2006). The membrane microdomains most important to this study are referred to as lipid rafts. Figure 6

displays the common structure of a lipid raft microdomain within the cell membrane.

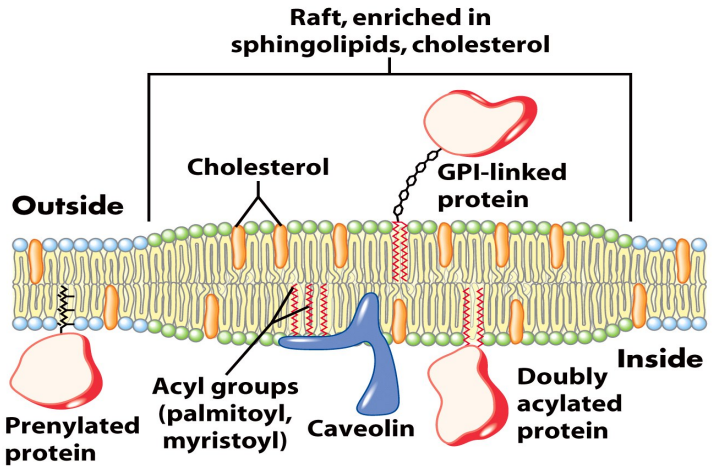

Figure 6. General structure of a lipid raft microdomain (University of Alberta, 2011).

Lipid rafts are defined as highly ordered, dense, detergent resistant regions of the cell membrane containing high concentrations sphingolipids and cholesterol (Figure 6). Although resistant to detergents such as Triton-X 100, these microdomains have been found to be disrupted by the removal of cholesterol via reagents such as methyl-β-cyclodextrin (MβCD) (Chini & Parenti. 2004). These membranes are important to facilitation of cell signal transduction pathways as they co-localize receptors with their effective signaling components. Providing a close proximity of such components allows for the signal to be transduced in a much quicker and efficient manner (Inset et al., 2005). Lipid rafts allow for certain signaling molecules to co-localize, others to travel into and out of the raft upon activation or inactivation, and also function to keep different signaling pathways separate from one another (Voet et al, 2006).

In many cases the receptors localizing to lipid rafts are GPCRs and their functions depend on localization within these lipid rafts, specifically a subset of lipid rafts known as caveolae (Chini & Parenti. 2004). Caveolae are unique from other lipid rafts in that they contain the scaffolding protein caveolin on the inner leaflet of the membrane aiding to the invaginated membrane structure typical of these domains (Quest et al., 2004). Previous research in the Team Cohen lab has provided evidence that FSHR is one of these GPCRs that reside within lipid rafts, specifically caveolae.

The goal of this study is to determine the dependence of FSHR signaling on its lipid raft residency and to quantify the results. Very little research has been done on the effect of disrupting lipid rafts on the signaling pathway of FSHR and the magnitude of such processes. As previously mentioned, one way in which to disrupt lipid rafts is by removal of cholesterol by used of MβCD, which is the primary lipid raft disruptor used in this study. The FSHR signaling pathway is a crucial part of the reproductive pathway in both males and females. In determining the effect of receptor residency, it can aid in the search for innovative methods of treatment for infertility and also novel approaches to contraception.

METHODS

Cell Culture

HEK293 cells that stably express hFSHR (HEK293R) were grown and maintained as monolayer cultures at 37°C and 5% CO_2 in Minimum essential Medium (MEM) supplemented with 10% fetal bovine serum albumin (BSA), penicillin, streptomycin, glutamate, and gentamicin. HEK293R cells used for β-galactosidase assay were transfected with CRE-β-galactosidase reporter plasmid using lipofectamine 3000 reagent.

FSH Stimulation, Cell Harvesting, and Protein Quantification for Immunoblotting

Upon confluency, 2 mL of serum free MEM was added to each control well 1 hour before hormone treatment. Lipid raft disrupted cells received 2 mL 5mM MβCD in serum free MEM. Dosages of high, medium, low, and 0 FSH were added to the wells. The dosages consisted of 40, 13.3, 4, and 0 ng FSH/1 mL serum free MEM per well. The cells were incubated at 37°C for 30 minutes upon addition of FSH. Media was aspirated, and cells were washed with 1 mL of 4˚C PBS. 250 µL Igepal DOC lysis buffer was added with supplemental protease inhibitor and phenylmethylsulphonyl fluoride (PMSF) and incubated at 4˚C for 20 minutes. Cells were scraped and Dounce homogenized with 10 strokes and subsequently centrifuged at 13,000 x g for 10 minutes at 4˚C, saving the supernatant. Subsequent protein concentration was determined by BCA assay using a 96 well plate reader by measuring absorbance at 430 nm.

Immunoblotting

Samples were subjected to SDS-PAGE. Protein was transferred to nitrocellulose membranes using a semi-dry transfer apparatus at 15 volts for 20 minutes and incubated in TBST with 5% milk for 2 hours at 4˚C. Membranes were subsequently washed 3 times for 5 minutes in 50 mL TBST incubated with primary antibody overnight at 4˚C. On the following day, the membranes were subjected to 3, 5 minute washes in TBST and subsequent incubation with secondary antibody conjugated with horseradish peroxidase and 3 additional 5 minute washes in TBST. Immunoglobulins were detected using a chemiluminescent substrate (Supersignal PicoWest, Thermo Scientific).

FSH Stimulation, Cell Harvesting, and Protein Isolation for β-Galactosidase Assay

Upon confluency, 2 mL of serum free MEM was added to each control well 2 hours before hormone treatment. Lipid raft disrupted cells received 2 mL 5mM MβCD in serum free MEM. Dosages of high, medium, low, and 0 FSH were added to the wells. The dosages consisted of 40, 20, 10, 7.5, 5, 2.5 and 0 ng of FSH/1 mL serum free MEM per well. The cells were incubated at 37°C for 4 hours upon addition of FSH. Media was aspirated, and cells were washed with 1 mL of 4˚C PBS. Cells were then scraped into 1 mL PBS/EDTA and loaded into microfuge tubes. After centrifuging for 5 minutes at 250 x g, the supernatant was aspirated. The pellet was resuspended in 200 uL of 0.25 M Tris at pH 8.0, otherwise known as the lysis buffer. The sample was frozen at -80°C and thawed in a 37°C water bath and then repeated twice more. Next, the sample was spun at 13,000 x g for 5 min to pellet the

insoluble material, and the supernatant was transferred to new tubes for use in the β-galactosidase assay.

CRE β-Galactosidase Reporter Plasmid

The CRE β-Gal Plasmid used in this study was constructed and sent to us courtesy of Dr. Patricia Hinkle at the University of Rochester. This plasmid was used as a way to quantify signaling as a result of cAMP production due to adenylyl cyclase activation from the FSHR signaling pathway. In response, the lacZ gene will be turned on by the transcription factor CREB binding to the promoter region via CRE, in turn producing cAMP concentration dependant β-galactosidase levels within the cell that can be quantified by the β-galactosidase assay by use of ONPG, a substrate of β-galactosidase, which is converted to a chromophore that absorbs light at 420nm. Figure 7 gives a visual of how the β-gal plasmid will work with respect to the FSH pathway in which this study is focused on.

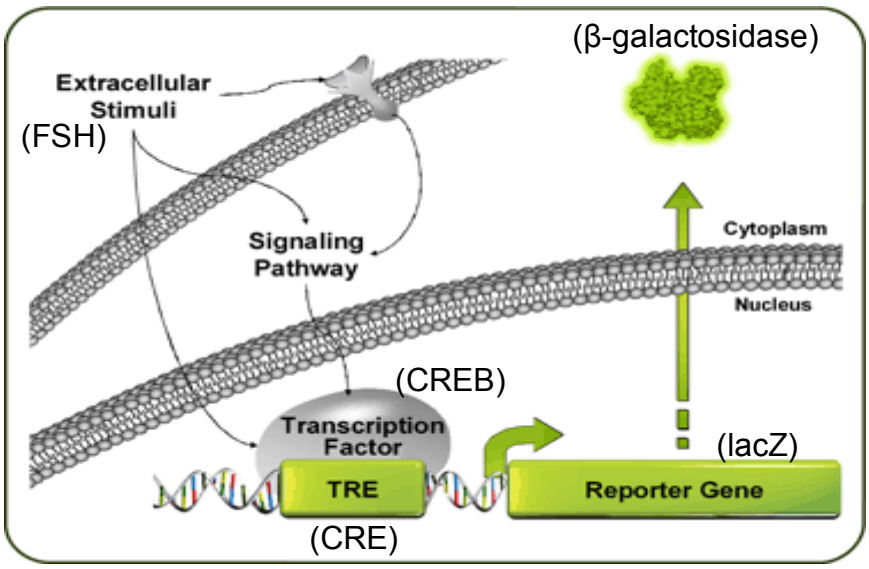

Figure 7. General overview of how a reporter plasmid functions in response to an external stimuli. Parenthes indicate specific components of the CRE β-galactosidase reporter plasmid response pathway in this study (Qiagen, 2011).

β-Galactosidase Assay

In a new microfuge tube, 100 uL sample was loaded in addition to 100 uL of 4 mg/mL ONPG. Next, 200 uL of cleavage buffer with β-mercaptoethanol was added to the tube. Cleavage buffer was made according to the formula given by the Invitrogen β-Gal Assay Kit Version F instruction manual. The blank for this assay consisted of the same concentrations but deionized water was used in place of the sample. Upon mixture of the components in the microfuge tubes, they were incubated in a 37°C water bath for 2 hours, allowing for the reaction to carry out and turn the solution yellow. The solutions were then loaded into a 90-well dish in triplicate and subjected to absorbance readings at 420 nm. An average of the three wells was obtained for each hormone concentration in absorption units to compare FSHR signaling.

RESULTS

Western Blot results demonstrate evidence that lipid raft disruption alters FSHR signaling.

In order to determine the effect of lipid raft residency of FSHR on signaling was determined by the use of MβCD to disrupt the lipid raft microdomains home to FSHR. Two pathways were analyzed to determine this signaling phenomenon by the use of immunoblotting. The first pathway examined was through activated protein kinase A (PKA) as a result of increased cAMP concentrations due to adenylyl cyclase activation by $G_s\alpha$. Because various PKA substrates are activated by the FSHR signaling pathway, it is possible to measure the effect of lipid raft disruption for at different FSH concentrations using antibodies that probe for these phosphorylates substrates. The results of this procedure can be seen in Figure 8.

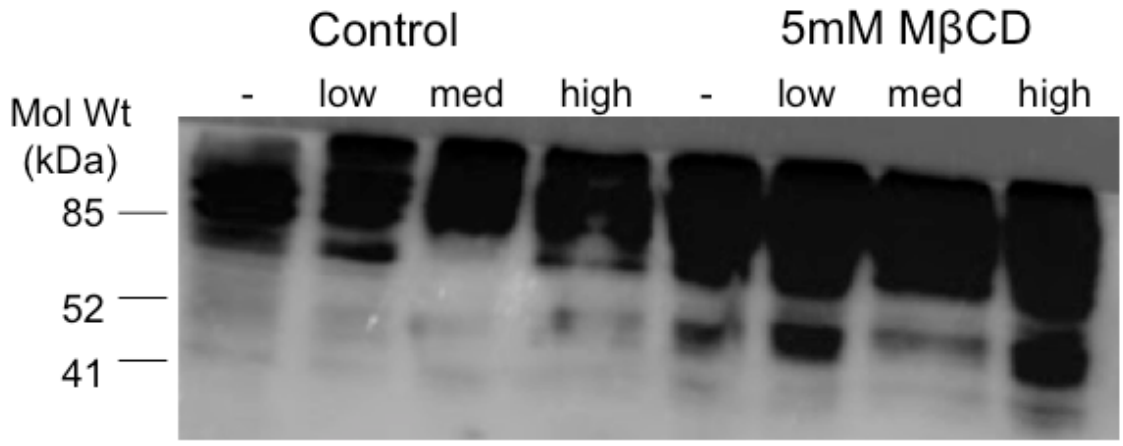

Figure 8: Western Blot of HEK293R cells treated with MβCD for 1 hour and then treated with varying amounts of FSH for 30 minutes versus control cells treated with equivalent concentrations of FSH, no lipid raft disrupted, HEK293R cells. Cell extracts were probed for phosphorylated substrates of protein kinase A (PKA).

Upon comparison of various FSH concentrations from the MβCD treated cells it is clear that the banding pattern is in fact altered due to the lipid raft disruption (Figure 8). The most noticeable difference is the presence of the more intense bands weighing approximately 50 kDa in the MβCD lanes, where as there are very faint bands comparable in the control lanes (Figure 8). However, it is unclear to determine the difference amongst the heavier bands between FSH dosages and treatment versus control.

The second FSHR signaling pathway examined by western blot analysis was the p44/MAPK pathway, which is activated by phosphorylation upon signal transduction via β-arrestin as a result of receptor stimulation by FSH. Probing for phospho-p44 MAPK would therefore provide the effects different FSH dose responses on MβCD versus control cells in a pathway separate from that of the previously examined PKA. The results for this procedure can be seen in Figure 9.

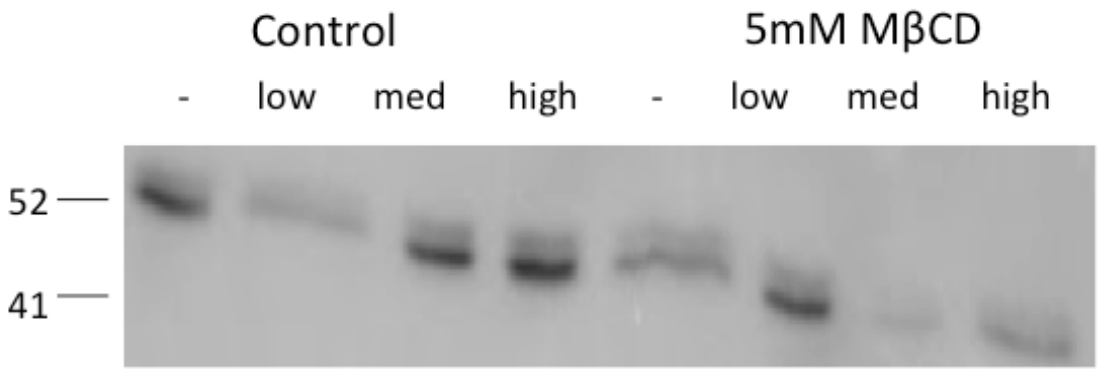

Figure 9: Western Blot of HEK293R cells treated with MβCD for 1 hour and then treated with varying amounts of FSH versus control, no lipid raft disrupted, HEK293R cells. Cell extracts were probed for phospho-p44 MAP kinase.

Similar to the PKA probed blot, it can be seen that treatment with MβCD does alter FSHR signaling in HEK239R cells (Figure 9). The results of this blot are easier to determine due to the presence of only two visible bands. In the control cells, from low to high the trend appears to be increased signaling, whereas lipid raft disrupted cells display a decrease in signaling in the high and medium dosages compared to the low does (Figure 9). The effect of disrupting lipid rafts and receptor residency as a result are very clear upon analysis of two separate signaling pathways originating from FSHR (Figure 8 & 9), however, the magnitude of these effects cannot be established, as a loading control probe could not be successfully conducted.

B-Galactosidase Assay provides evidence of FSHR responding in a dose dependent manner.

Due to the inability to accurately determine the effects of different dosages of FSH and receptor residency on signaling, the second half of this study aimed to quantify these effects. As shown previously by the PKA blots (Figure 8), western blot analysis presents a trend of dose dependence and signal alteration in HEK293R cells as a result of MβCD treatment, but these results cannot be quantified. By use of a CRE β-galactosidase reporter plasmid, the varying cAMP concentrations as a result of different FSH doses and MβCD treatment can be quantified. This works as an effective measure of quantifying the previous effects of the PKA blots as the two

operate through the same signaling sub-pathway from FSHR. The results of the varying FSH dose β-galactosidase assay can be seen in Figure 10.

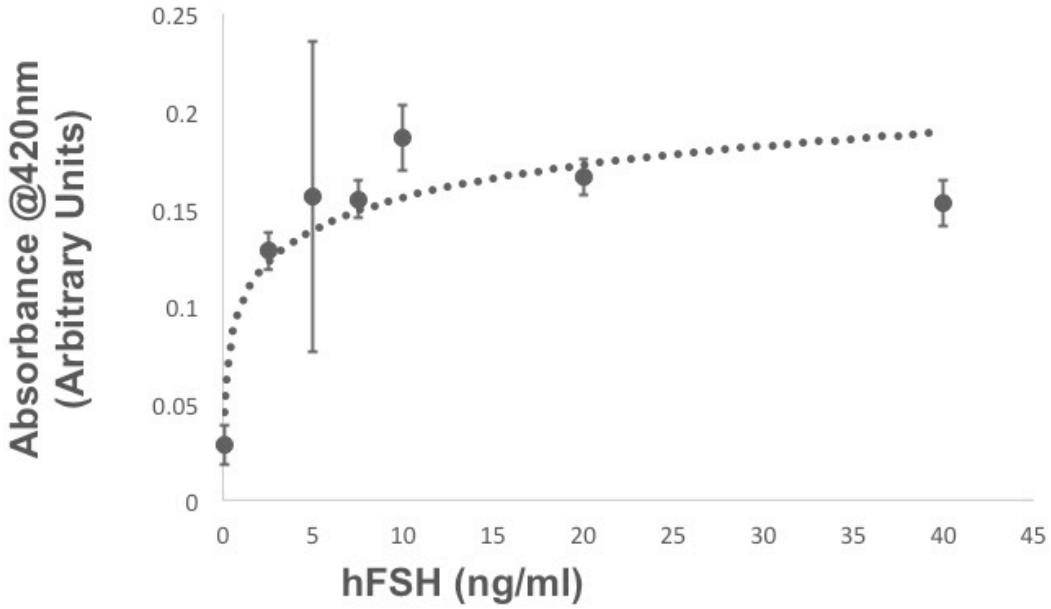

Figure 10: Dose response to FSH of β-galactosidase reporter in HEK293R cells. HEK293R cells were transiently transfected with a CRE-β-galactosidase reporter plasmid then were treated with varying doses of hFSH for 4 hours before being lysed. To determine relative amounts of enzyme present the extracts were incubated with ONPG, a substrate of β-galactosidase, which is converted to a chromophore that absorbs light at 420nm.

A clear dose response pattern can be seen in untreated HEK293R cells of increased signaling at a decreasing rate (Figure 10). The assay could not be completed for lipid raft disrupted cells as after two hours of MβCD treatment

followed by four hours of FSH treatment, the cells appeared dead/floating and aspiration of the media left no quantifiable sample concentrations.

DISCUSSION

Previous research in this lab has shown that FSHR resides within lipid rafts and that its signaling is dependent upon this residency, as determined by MβCD, fillipin, and sphingomyelinase as determined by immune blotting. This study further supports this claim by analysis of the MAPK and PKA signal transduction pathway from FSHR with similar immunoblotting techniques. It was found in this study by western blotting that HEK293R cells responded to MβCD treatments in a dose dependent manner showing decreased signaling with higher dosages of FSH, as opposed to the control cells, which responded with increased signaling amongst the MAPK pathway. However, the PKA blots were more difficult to compare apart from some lone isolated bands present in the MβCD treated cells as there were many phosphorylated PKA substrates probed for. In addition, in both blots there was no successful probe for a control such as the receptor itself. Although the protein concentrations were normalized across each sample, the loading control makes it hard to accurately depict the results.

In an attempt to quantify the results seen from the western blot, specifically the PKA pathway, a β-gal assay was used. The reporter plasmid itself was a CRE β-galactosidase plasmid, making it a good measure of signaling via the PKA pathway attempting to be quantified due to the fact that both tests are in essence an indirect measure of cAMP levels in the cell due to adenylyl cyclase activation as a result of FSHR stimulation. The results of this experiment provided supporting evidence from the western blot analysis that HEK293R cells respond to FSH in a dose dependent manner. Due to the quantifiable approach from this assay, the manner of

such response can be proposed in which signaling increases at a decreasing marginal rate. This mechanism was expected for the control cells because there is only so much receptor present on the cell surface and at some point, the FSH concentration will be so great that all receptors will be bound and an increase in concentration will not yield more signaling. This pattern is similar to that proven of Michaelis-Menton kinetics, shown in Figure 11.

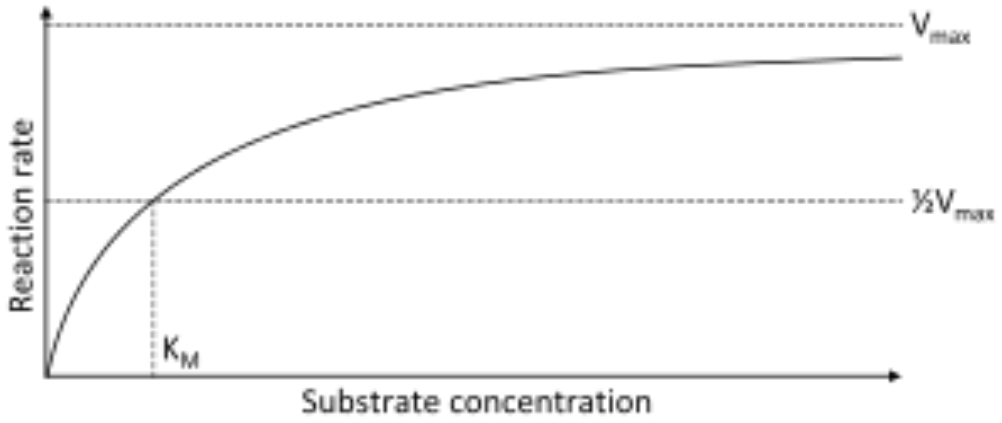

Figure 11. Typical Michaleis-Menton relationship for substrate concentration versus reaction rate.

As a result of this assay, it can be said that doses between 10 and 20 ng/ml do not yield any greater cAMP production and therefore signaling. In addition, it is possible that a negative feedback loop may exist, therefore, causing the decrease in signaling observed at 20 and 40 ng/ml in comparison to that of the 10 ng/ml dose. However, more data points are necessary to determine both of these claims.

Unfortunately, a dose response curve using the β-gal assay was unattainable for the HEK293R cells treated with MβCD, so the signaling quantification as a result of raft disruption could not be determined. Because FSHR signaling does vary depending on lipid raft residency as shown by previous studies in this lab and in this

study, it was hypothesized that MβCD treated cells would show a different pattern than that of the control cells. This curve could not be obtained due to the small amount of protein sample obtained from the cell lysates, as most of the cells post six hour treatment were floating and were aspirated away with the media. This left minimal cells to be scraped off of the wells and therefore a protein quantity under the limit of detection for the β-gal assay used in this study.

The six-hour time incubation time of this procedure, although consequently toxic to the HEK293R cells, was necessary for the production of comparable levels of β-galactosidase. Four hours was the length of time it took the cells to respond first to the FSH treatment by protein phosphorylation and other cytosolic stimulatory results from FSHR stimulation and then to undergo the necessary gene regulatory responses to such cAMP concentration increases through the activation of the transcription factor CREB. The receptor to nucleus signal transduction pathways such as those studies in the western blots occur fast, over the course of seconds to minutes, whereas the ensuing gene expression occurs over hours. Upon development of the protocol for this specific β-gal assay, treatment times with FSH two hours and under yielded protein levels, specifically β-galactosidase levels, under the limit of detection of the assay.

It is proposed that the cells detached from the bottom of the well as a result of the long incubation time in the MβCD. The length of time treated with MβCD must have been the result of this cellular response due to the fact that the cells treated with MβCD for only an hour and a half for the western blots had no trouble maintaining adherence throughout the incubation. It is possible that in addition to

altering FSHR signaling as a result of lipid raft disruption, other important cell components were disturbed, such as the integrins responsible for maintaining that adherence to the well (Yanagisawa et al., 2004). If this is true then perhaps the MβCD treated HEK293R cells were not actually dead but simply lost the ability to maintain adherence to the bottom of the well.

CONCLUSION

The goal of this study was to provide quantitative results of the effect of disrupting lipid rafts on FSHR signaling. The phospho-p44 MAPK and phosphor-PKA western blots provide quality evidence that lipid raft residency does in fact impact the signaling pathway of FSHR, although the magnitude of these effects could not be quantified from this technique. The β-gal assay was used to investigate this issue and to provide the quantitative data necessary to produce the precise effects of lipid raft disruption on signaling via FSHR. The results of this assay displayed dose dependence in control HEK293R cells but no absorbance could be obtained for the MβCD treated cells due to the massively diminished quantity of cells post six-hour incubation. It can be concluded from this study that HEK293R cells respond in a dose dependent manner through the fast signal transduction as seen by western blotting and slow gene regulation as seen by the β-gal assay. However, it can also be concluded that in studying the effects of any membrane destabilization, the incubation time has to be taken into account with regards to cell toxicity and the effect of other plasma membrane components. Therefore, future experiments should focus on using quantifiable techniques that focus on the fast pathway of signal transduction. One possible technique that can be used to measure cAMP levels within the cell directly as opposed to gene expression is the use of FRET-EPAC or ELISA. Future studies could also use other lipid raft disruptors such as sphingomyelinase or a statin drug as the toxicity could have been a result specific to MβCD so a different reagent may evoke a different response in the cells. Further investigation on the dependence of FSHR lipid raft residency is important as it could

aid to future treatments for infertility and innovative drug interactions for contraception.

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