6-2022

Lipid Raft Disruption Alters Human Follicle Stimulating Hormone Receptor Signaling

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Sumbitted in partial fulfillment
of the requirements for
Honors in the Biochemistry Program

UNION COLLEGE
2022

Table of Contents

Abstract

Over 6.7 million people struggle with infertility each year. Studying signaling by reproductive hormones in fertility can allow us to gain a better understanding of the signaling pathways that must function correctly for proper fertility. Some infertility is due to incorrect human follicle stimulating hormone receptor (hFSHR) function. When follicle stimulating hormone (FSH) binds to hFSHR this begins a signaling cascade where the end product is the maturation of sperm by Sertoli cells in men, and egg development and production of estrogen through stimulation of granulosa cells in women. It has been determined that hFSHR is localized to microdomains of the cell membrane called lipid rafts which are characterized by higher concentrations of sphingolipids and cholesterol. This composition makes them less fluid than non-raft membranes, and it is believed that lipid rafts regulate signaling of proteins that reside in the rafts, including hFSHR. It was hypothesized that if the lipid rafts in cell membranes were removed then hFSHR signaling would be altered. To remove lipid rafts HEK293 cells stably expressing hFSHR were treated with sphingolipid synthesis inhibitors (Myriocin or Fumonisin B1) or a cholesterol withdrawing drug (Methyl-β-cyclodextrin). All treatments result in disruption of lipid rafts. hFSHR signaling from the cells with disrupted lipid rafts were compared to wild type hFSHR signaling by western blot. Two pathways were investigated: cAMP production and the activation of p44/42 MAP kinase (ERK1/2). cAMP production was measured indirectly by detecting phosphorylated cAMP Response Element Binding protein (p-CREB). p44/42 MAPK signaling was measured by directly detecting the phosphorylation of the kinase (p-p44/42). It was found that treatment with the lipid raft disrupting agents resulted in increased basal cAMP production (as measured by pCREB activation). However, time-dependent hFSH stimulation was decreased compared to cells with intact lipid rafts. In contrast, p-p44 signaling in the drug treated cells was not altered compared to untreated cells. These results suggest that G protein mediated activation of adenylyl cyclase to produce cAMP is lipid raft dependent while p44/42 MAPK activation is not. Further understanding of how lipid raft residency allows for functional hFSHR signaling would allow for the development of new treatments and pharmaceuticals for men and women struggling with infertility.

Introduction

Globally, infertility affects over 48 million couples.[1] In the United States alone one in eight couples will struggle with infertility. Infertility is defined as being unable to conceive after one year, or more, of unprotected intercourse.[2] Both men and women can struggle with infertility, in fact in 35% of couples both a male and female factor are recognized.[2] Currently, infertility is treated using either medicines, surgery and assisted reproductive technology (ART) such as intrauterine insemination and *in vitro* fertilization (IVF). However, treatments can be costly and success rates can vary for each couple and some couples are successful at becoming pregnant.

Medicines used to treat infertility are focused on stimulating ovulation, or regulating it.[3] Common medications used to treat infertility include Clomiphene citrate, gonadotropins, and letrozole.[3] Clomiphene citrate causes a women's body to believe it has lower estrogen levels, which causes the pituitary gland to release more follicle stimulating hormone (FSH) which is necessary for pregnancy.[3] Gonadotropins are hormones that stimulate the gonads who control endocrine function. FSH is a gonadotropin and by directly injecting FSH it stimulates egg development in woment.[3] Letrozole is an aromatase inhibitor and aromatase is an enzyme necessary to produce estrogen. By inhibiting aromatase, estrogen levels decrease causing the pituitary to release more FSH to stimulate egg development. However, there are many risks associated with fertility drugs including high risk pregnancies with multiples fetuses.[3] Other symptoms and risk associated with fertility drugs are depression, anxiety and physical symptoms, such as nausea, headaches and hot flashes.[3] If fertility drugs are taken for over a year there is a risk of developing ovarian cancer as well.[3]

Women struggling with infertility often turn to assisted reproductive technology (ART), such as intrauterine insemination (IUI) and *in vitro* fertilization (IVF). The most successful form

of assisted reproductive technology is *in vitro* fertilization (IVF).[2] However, IVF is not a treatment that is widely available or accessible to all women suffering from infertility and it is an incredibly invasive procedure. To begin, women are injected with FSH, as mentioned before, to stimulate egg development. The more eggs that are developed means more eggs can be retrieved to turn into potential pregnancies, increasing the success rate. An aspirating needle is used to retrieve the eggs from the follicles (Figure 1).[4] This step puts the women at risk of infection or injury to major blood vessels.[4] Once the eggs are retrieved they are combined with a sperm sample *in vitro* and grown in medium (Figure 1). When the egg and sperm are combined and incubated it can potentially become an embryo. Once there are one or more embryos from this procedure they are implanted into the uterus using a catheter (Figure 1).[4] Then the couple has to wait two weeks before taking a pregnancy test to determine if the procedure was successful. One cycle takes three weeks to complete.[4]

However, cycles can be unsuccessful and hard to predict. Most doctors state three cycles of IVF are needed to increase the chance of pregnancy and success. On top of multiple cycles needed, not every state allows insurance to cover IVF. Most of the states and companies deem the procedure "medically unnecessary" so insurance companies don't have to cover it.[5] Each IVF cycle costs from $12,000 to $17,000, not including the medications needed for the procedure.[5] Including the medication needed for this procedure the cost can accumulate to $25,000 for only one round, and as mentioned before most couples need more than one round.[6] The cost of IVF makes this procedure unavailable to many couples, and even if the couple can afford it the success rate of IVF is only 37.8%.[5] To add to this, as the women's age increases the chance of success decreases. The success rate for IVF for women ages 35 to 37 is 24% and it decreases to 16% for women ages 38 to 40.[2]

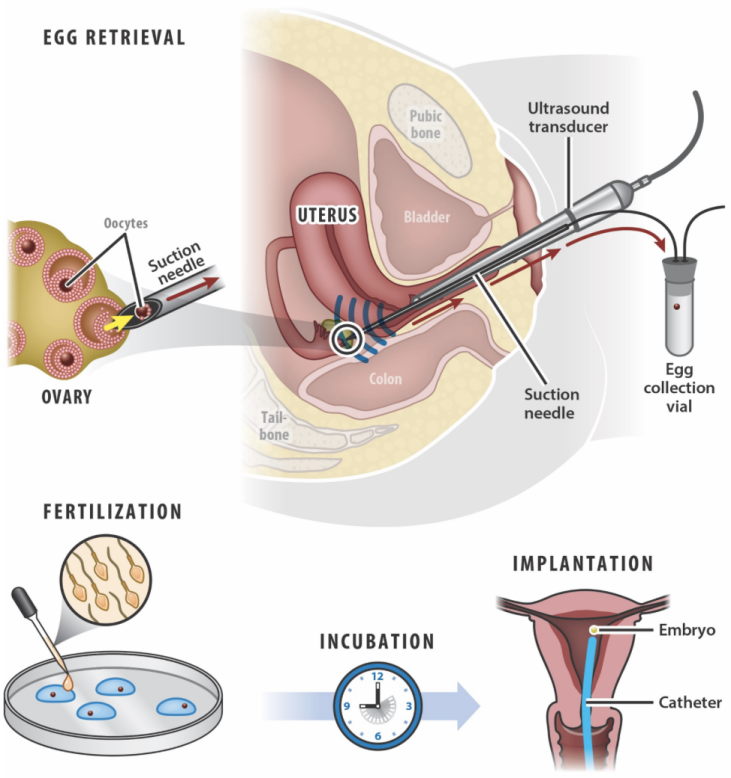

Figure 1. Diagram of *in vitro* fertilization process. From Palm Beach Fertility Center. (2022).In-VitroFertilization.https://www.palmbeachfertility.com/fertility-services/fertility-treatments/in-vitro-fertilization/

Intrauterine insemination (IUI) is another method of ART that is more accessible to couples financially, however the success rate is lower. For (IUI) sperm is injected into the uterus using a catheter (Figure 2).[7] This method is done when the woman is ovulating to increase the number of sperm that reach the woman's fallopian tubes to fertilize the egg (Figure 2).[7] Typically IUI is $300 to $1,000 without insurance coverage.[8] This is significantly cheaper than IVF. Also it only takes around ten to fifteen minutes and doesn't require anesthesia, so it is also much less invasive than IVF.[8] While IUI is more accessible and less invasive than IVF, the success rate is much lower. The success rate of IUI is only 11.7%.[8]

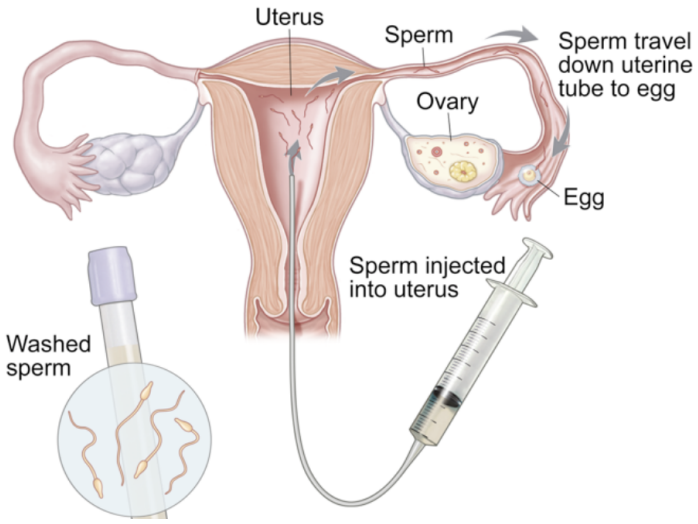

Figure 2. Diagram of intrauterine insemination (IUI). From Johns Hopkins Medicine.(2022).IUI.https://www.hopkinsmedicine.org/gynecology_obstetrics/specialty_areas/fertility-center/infertility-services/intrauterine-insemination.html

Alternatively, in 2017 the CDC reported that 64.9% of women ages 15 to 49 were using some form of contraception to prevent pregnancy.[9] There are many methods of birth control that are not very effective such as the withdrawal method which is only 78% effective, or tracking ovulation which is 76% effective.[10] The most common kind of contraceptive used by women is an oral contraceptive, which is a pill taken daily. Some oral contraceptives contain a mix of estrogen and progestin, while some only contain progestin. The pill is 99% effective if taken correctly, however many people report missing a pill or forgetting to take one. When a pill is forgotten the effectiveness of the contraceptive decreases to 91%.[11] Followed by oral contraceptives, another popular method of contraceptive is a hormonal IUD which is placed into the uterus and releases a small amount of progestin to prevent pregnancy. The IUD is 99% effective, but some people have severe cramping and heavy menstruation with the IUD which

causes it to need to be removed.[10] Both oral contraceptives and IUDs have reported side effects including weight gain, mood changes, depression, back pain, headaches, nausea, irregular bleeding, acne, pelvic pain, and many other symptoms.[11] The oral contraceptive and IUD also have reported long term effects such as stroke, blood clots and liver tumors.[11]

Fertility is regulated by the hypothalamic-pituitary-gonadal (HPG) axis (Figure 3).[12] The HPG axis is a mechanism which connects the hypothalamus, pituitary glands and gonads in order to regulate the reproductive system. The hypothalamus releases gonadotropin releasing hormone (GnRH) which travels to the anterior pituitary and binds to gonadotrope cells. When GnRH binds to the receptor it stimulates the gonadotropes to release follicle-stimulating hormone (FSH) which controls fertility in both men and women, and luteinizing hormone (LH) (Figure 3).[12] FSH goes to the Sertoli cells in males and binds to a receptor to promote spermatogenesis. In women FSH goes to granulosa cells and binds to a receptor to stimulate egg development and the production of estrogen.[12] The receptor that FSH binds to in both men and women is the human follicle stimulating hormone receptor (hFSHR). Problems in fertility in both men and women can be from the FSH itself, or it can be from improper hFSHR signaling.

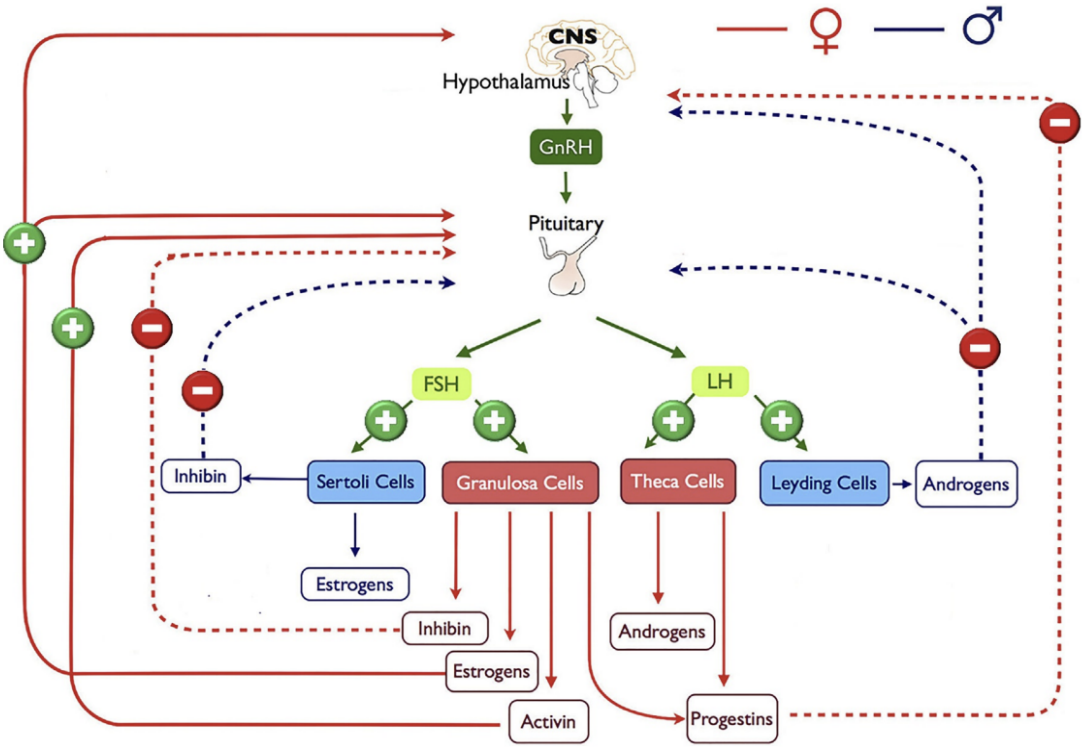

Figure 3. HPG axis which links the hypothalamus, pituitary and gonads to regulate reproduction in men and women. From Cohen Brian D., and Dias James A. (2019) Follitropin. Reference Modulein Biomedical Sciences. Elsevier. 11-Mar-19 doi: 10.1016/B978-0-12-801238-3.99542-4.

The hFSHR is a G protein-coupled receptor (GPCR), which is a class of proteins that contain seven transmembrane alpha helices, connected by three intracellular loops and three extracellular loops, that stimulate intracellular signaling pathways (Figure 4).[13] GPCRs are the largest family of transmembrane proteins, and there are over 800 GPCRs in humans.[14] The hFSHR contains an extracellular domain which consists of twelve leucine rich repeats (LRR) and connects to the N-terminus of the protein (Figure 4).[15]A LRR is defined as a 20-30 long amino acid residue that contains mostly leucines which gives it an arc shape (Figure 4).[16] FSH binds to this extracellular domain of hFSHR, but more specifically FSH binds to the orthosteric site. The orthosteric site consists of nine of the twelve LRRs that make up the whole extracellular

domain.[16] When FSH binds to the hFSHR it undergoes a conformational change that stabilizes the receptor to stimulate the signal transduction pathways necessary for fertility.[17] There are three known proteins that can be involved in the different downstream signaling pathways, the heterotrimeric G protein, G-protein-coupled receptor kinases (GRKs) and arrestin proteins.[14]

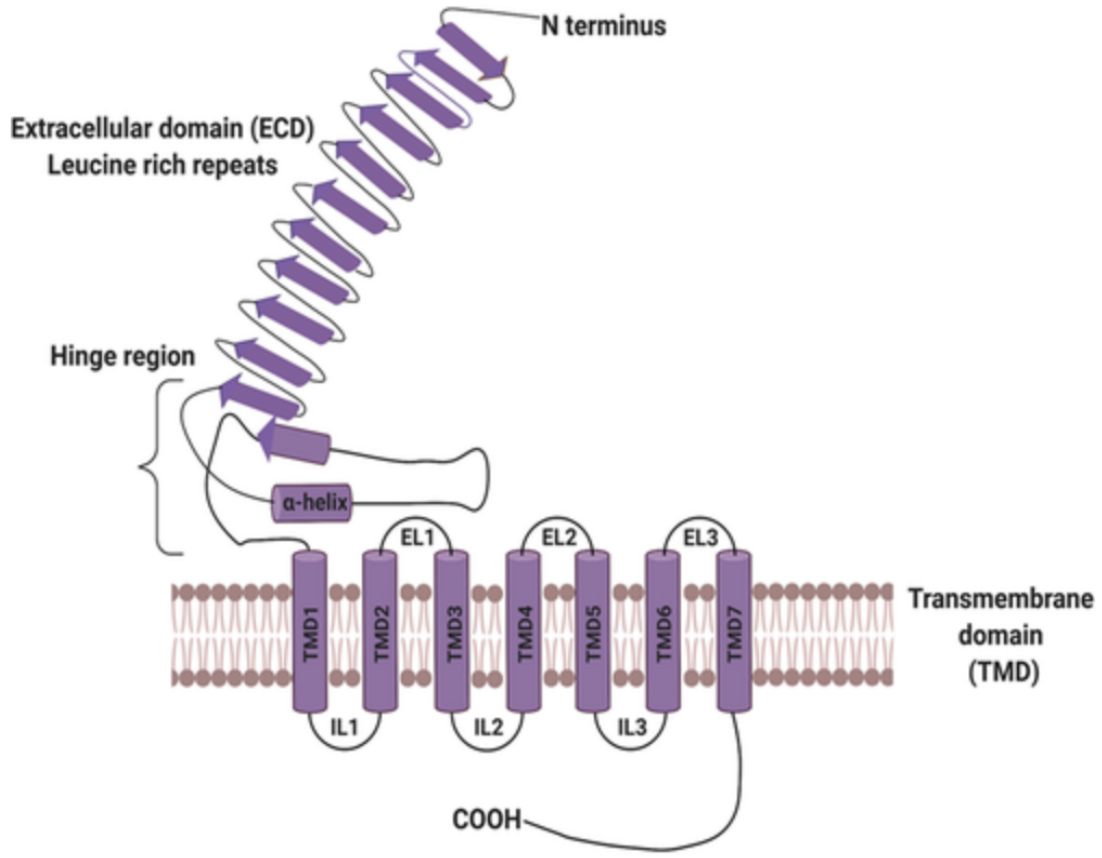

Figure 4. hFSHR structure showing the seven transmembrane domains connected by extracellular and intracellular loops. From Landomiel, F., De Pascali, F., Raynaud, P., Jean-Alphonse, F., Yvinec, R., Pellissier, L. P., Bozon, V., Bruneau, G., Crépieux, P., Poupon, A., & Reiter, E. (2019). Biased Signaling and Allosteric Modulation at the FSHR. *Frontiers in endocrinology*, *10*, 148. https://doi.org/10.3389/fendo.2019.00148

A G protein is a heterotrimeric protein complex consisting of Gα, Gβ and Gγ subunits.[18] The G protein is inactive when guanosine diphosphate (GDP) is bound to the Gα domain.[18] The

activation of the G protein by hFSHR causes GDP on the Gα to exchange with a guanosine triphosphate (GTP). This exchange causes the Gα subunit to separate from the Gβγ subunit. The Gα and Gβγ subunits activate different signaling cascades within the cells. When the signaling is finished, the Gα subunit causes the hydrolysis of GTP to GDP and a monophosphate.[18] GDP is then again bound to the Gα subunit, which results in the rejoining of the Gβγ to Gα causing the G protein to be inactive again. When the Gα subunit is active, it activates adenylyl cyclase, which converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cyclic AMP) (Figure 5). Cyclic AMP then activates protein kinase A (PKA) (Figure 5). PKA goes on to phosphorylate the cAMP response element binding protein (CREB), also known as p-CREB when it is phosphorylated (Figure 5).

As well as the Gα/cAMP/PKA pathway, another important pathway involved in hFSHR signaling is the p-p44/42 MAPK pathway, which begins with the activation of β-arrestin. The activation of hFSHR results in G-protein-coupled receptor kinases (GRKs) phosphorylating the C-terminal of the hFSHR (Figure 5).[14] When hFSHR is phosphorylated it signals for β-arrestin to bind to the receptor (Figure 5).[14] hFSHR is then internalized by endocytosis using β-arrestin as a scaffold protein.[14] hFSHR is internalized in an endosome at the end of this process.[14] The endocytosis of hFSHR into endosomes inside of the cell activates ERK1/2 (Figure 5).[14] This process is independent of cAMP signaling, whereas p-CREB signaling is cAMP dependent. Yet both p-CREB and ERK 1/2 measure the signaling from hFSHR.

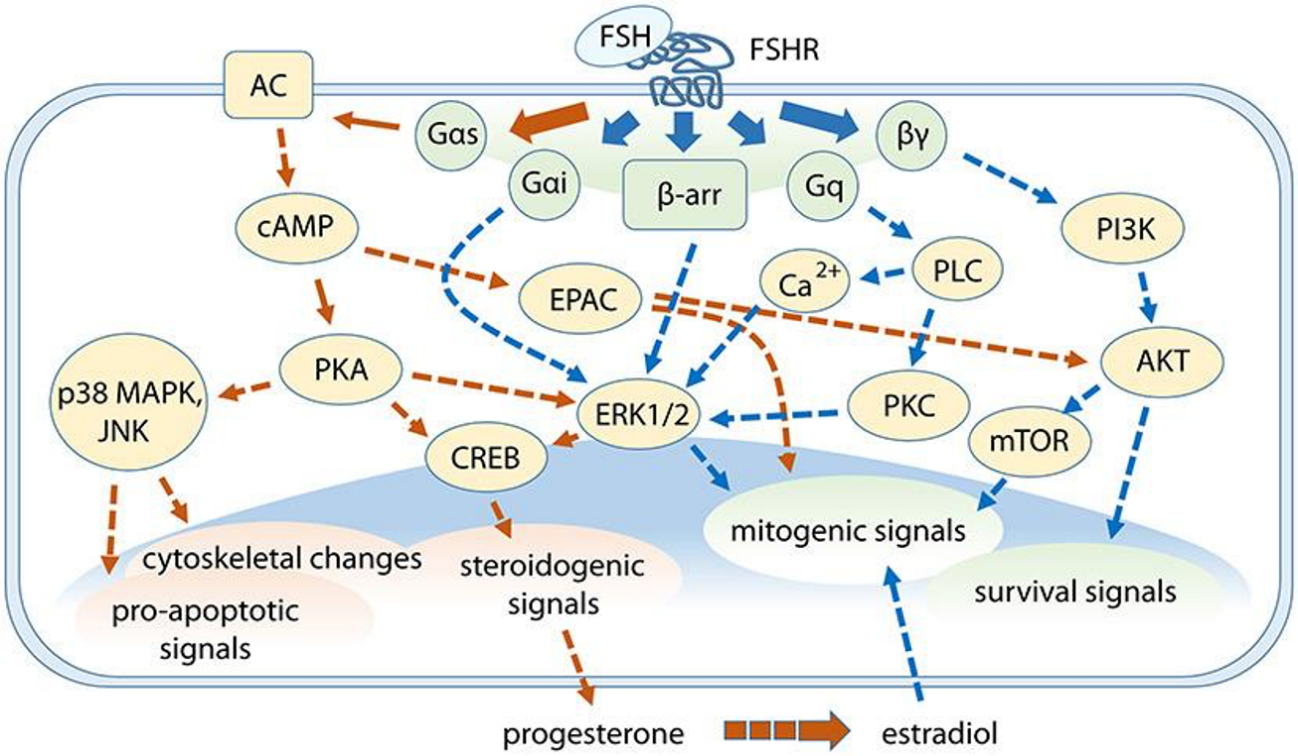

Figure 5. Signal transduction pathways that are stimulated when FSH binds to hFSHR which result in fertility. From Casarini, L., & Crépieux, P. (2019). Molecular Mechanisms of Action of FSH. *Frontiers in Endocrinology.* 10:305. doi:10.3389/fendo.2019.00305.

Previous work in our lab has determined that hFSHR is localized to microdomains of the cell membrane called lipid rafts, similar to many other GPCRs. Lipid rafts are composed of higher concentrations of sphingolipids and cholesterol compared to non-raft membranes, and are also taller compared to non-raft membranes (Figure 6). Lipid rafts are found on both the inner and outer leafs of the cell membrane (Figure 6). Sphingolipids are amphipathic molecules, consisting of long saturated fatty acid chains which are hydrophobic, and a "head" which is hydrophilic.[19] The sphingolipid "heads" contain phosphates, alcohols and amines. The long saturated fatty acid chains of the sphingolipids allow them to pack tightly together which make lipid rafts more rigid than the rest of the cell membrane (Figure 6).[19] Cholesterol fills in between

the sphingolipid heads, which holds the lipid raft together and fills in all of the gaps (Figure 6). This composition makes them less fluid than non-raft membranes, therefore research shows that the roll of lipid rafts in the cell membrane include ligand binding, specificity, membrane sorting, signal transduction and receptor targeting and recycling once signaling is complete.[20] It is believed that lipid rafts regulate the signaling of many GPCRs that are localized in them, including hFSHR.

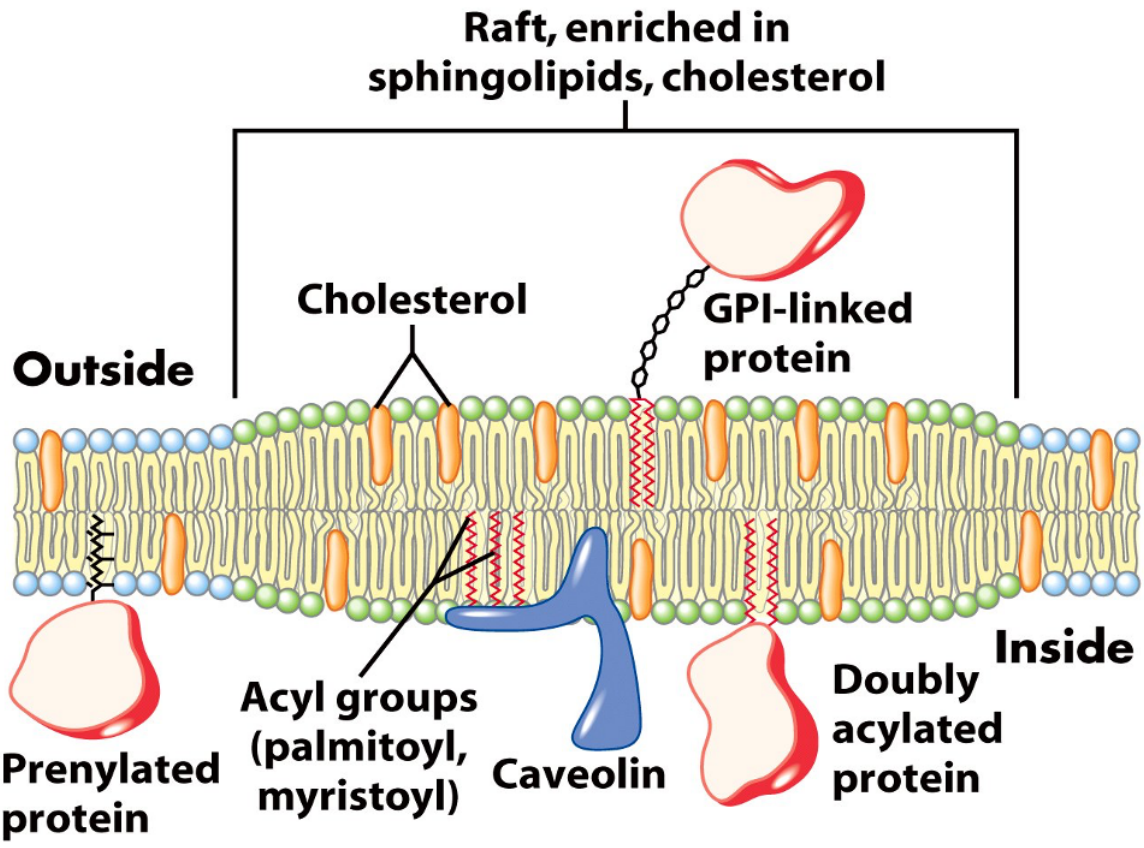

Figure 6. Depiction of a lipid raft microdomain of the cell membrane containing cholesterol and sphingolipids, as well as specific proteins that reside in lipid rafts. From Nelson, D. L., & Cox, M. M. (2017). Lehninger Principles of Biochemistry (7th ed.). W.H. Freeman.

Two specific GPCRs that are located in lipid rafts that have been studied are the human serotonin$_{1A}$ receptor (5-HT$_{1A}$) and human β-adrenergic receptors (β1AR). 5-HT$_{1A}$ has been shown to interact with lipid rafts, and a study was conducted to disrupt the lipid rafts using Fumonisin B1 to determine if it altered signaling.[21] There are fourteen types of Fumonisins, which are natural mycotoxins, with Fumonisin B1 being the most common.[21] Fumonisin B1 is a sphingolipid inhibitor, specifically it inhibits the enzyme, sphingosine N-acetyltransferase, which catalyzes the acylation of sphingoanine during sphingolipid synthesis.[21] When cells containing rafts are treated with Fumonisin B1 it stops the metabolism of new sphingolipids, and without new sphingolipids replacing the old ones the lipid rafts fall apart because once of the major characteristics of lipid rafts is the high concentration of sphingolipids. This study treated cells containing 5-HT$_{1A}$ receptors with Fumonisin B1 and found that using almost 80% of sphingolipids are depleted, therefore Fumonisin B1 is successful at disrupting lipid rafts.[21] One the cells were treated with Fumonisin B1 for 24 hours the study found that cAMP production was increased when lipid rafts were disrupted.[21]

The other study focused on β1AR and how lipid raft residency regulated the downstream signaling.[22] However, this study used methyl-β-cyclodextrin (MβCD) to disrupt lipid rafts. MβCD which is a drug that withdraws cholesterol.[23] MβCD structure gives it a hydrophobic pocket which allows it to trap cholesterol in it, which removes it from the lipid raft.[23] High concentrations of cholesterol are necessary to hold lipid rafts together, so removing cholesterol causes the rafts to fall apart. This study found that treating cells with MβCD caused over 50% of cholesterol to be depleted from the membrane.[23] This study found that when cells containing the β1AR were treated with MβCD there was increased Protein Kinase A (PKA) signaling, and also increased cAMP production. Both studies, looking at different GPCRs that reside in lipid rafts

and using two different methods of lipid raft disruption, saw altered signaling from the receptor when it was no longer present in the rafts.

Based on previous research performed in the Cohen lab, and the results from the studies mentioned above, this project had two goals. One goal being to determine how lipid raft residency of the hFSHR is determined. The second goal being how hFSHR signaling is altered when the lipid rafts are disrupted. For the first goal of the project it was hypothesized that hFSHR lipid raft residency is hormone dependent. For the second goal of the project it was hypothesized that the disruption of lipids rafts in the cell membrane of HEK293-hFSHR cells will alter hFSHR signaling.

Methods

Cell Culture

All experiments performed used HEK293-hFSHR cells. HEK293-hFSHR cells are human embryonic kidney cells that stably express hFSHR. The cells were cultured into 6 well dishes for the lipid raft disruption experiments, and T25 flasks for the density centrifugation experiments, both in DMEM containing the G418 antibiotic for the lipid raft disruption experiments. Cells that contain the FSHR gene also have G418 resistance. If the cells lose resistance it is likely they lose the FSHR causing the cells to die. This ensures that all of the cells in the experiments contain FSHR since those are the only ones resistant to G418, making sure the cells are pure. Cells for both types of experiments were grown at 37℃ with 5% CO_2 flow until they were at 75% or greater confluency.

Density Centrifugation

Sucrose density centrifugation was performed by putting 45% sucrose containing the cells treated with hormone on the bottom, and layering 35% sucrose and 5% sucrose on top (Figure 7). The gradients were spun in the ultracentrifuge for 24 hours at 240,000xg. This allows the components of the cell membrane to separate based on their densities. The gradient is then separated into fractions to determine where hFSHR resides. Lipid rafts are more buoyant than the rest of the cell membrane so they are found in the early fractions where the 5% sucrose is. The rest of the non-raft membrane is heavier so it sinks to the bottom and is found in the bottom fractions where the 45% sucrose is. The fractions were loaded onto an SDS-PAGE and a western blot was performed to determine where hFSHR is located.

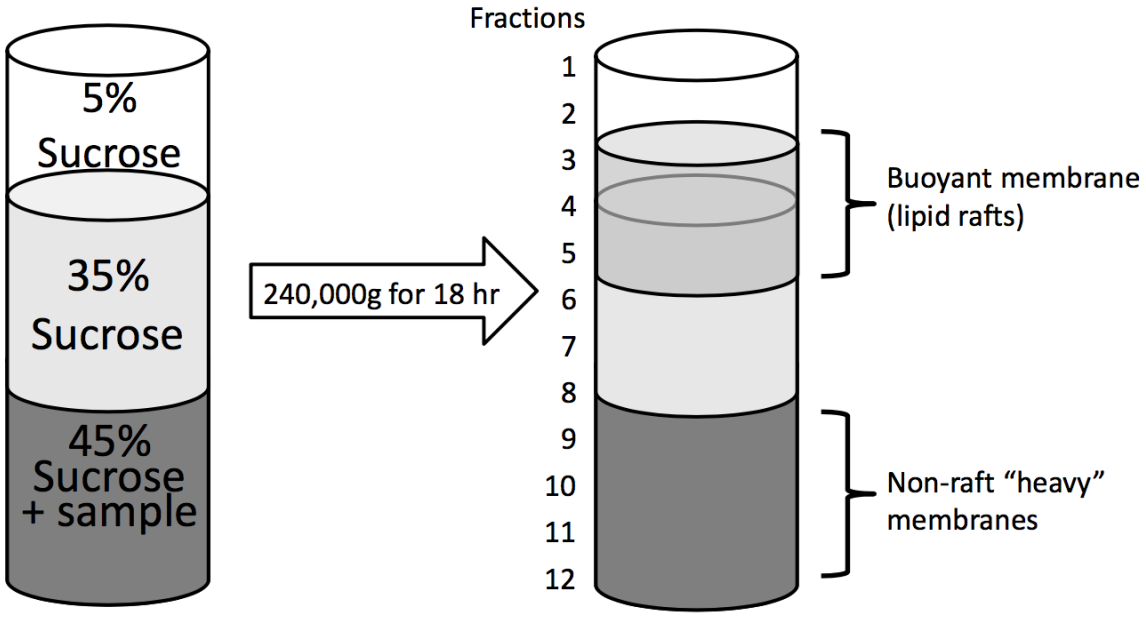

Figure 7. Model of sucrose density centrifugation with the corresponding fractions.

Treatment with Fumonisin B1 and MβCD

For the cells treated with Fumonisin B1, the media in the 6 well dishes was aspirated and replaced with DMEM medium containing G418 and 10μM of Fumonisin B1. The cells were treated with 10μM Fumonisin B1 for 24 hours at 37℃ with 5% CO_2. Control cells were treated with 10μM of DMSO for 24 hours at 37℃ with 5% CO_2. After 24 hours of treatment the medium containing Fumonisin B1 was aspirated and replaced with serum free media for one hour. For the MβCD treated cells, the media in the 6 well dishes was aspirated and replaced with serum free medium containing 5mM of MβCD and 10μM of DMSO . The cells were treated with MβCD for one hour at 37℃ with 5% CO_2. Control cells were treated with 10μM DMSO for one hour at the same conditions. Two different drug treatments were used to ensure any difference seen in signaling was due to lipid raft disruption, and not from removing just one of the components of a lipid raft. If only one drug treatment was used, such as Fumonisin B1 to remove sphingolipids, it could not be determined if a difference in signaling was from removing the lipid rafts or removing just the sphingolipids. By using two different drug treatments the results could be compared.

hFSH Treatment, Protein Extraction, BCA Assay, and SDS-PAGE

After lipid raft disruption was complete, using either drug, both experiments followed the same methods. The cells were treated with a hFSH time course. Urinary hFSH was used and it was diluted to 80ng/100 μL in serum free media. Each well in the dish containing cells was treated with 100 μL of diluted urinary hFSH in serum free media for either 0, 5, 15 or 30 minutes of incubation. After hFSH treatment the cells were put on ice before they were lysed using

Igepal-DOC lysis buffer. A dounce homogenizer was used and the lysate was harvested. Once the samples were obtained a BCA Assay was run following the protocol from Thermofisher to determine the protein amount in each sample. Samples were then diluted using a 2X sample buffer to ensure each sample had the same concentration of protein. The samples were heated at 75℃ for 3 minutes and spun down using a centrifuge. Following this, the samples were loaded into a 10% sodium dodecyl-sulfate polyacrylamide gel to separate the proteins by size so the hFSHR could be identified.

Western Blotting

Following the SDS–PAGE, the gel was transferred onto a membrane and blocked using 5% BSA in TBST. Once blocked, the membranes were put into primary. For both lipid raft disruption experiments, the membranes were probed with an antibody to measure phospho-CREB signaling, and an antibody to measure ERK1/2 signaling. All membranes in the lipid raft disruption experiments were probed with either an AKT primary antibody, or an APPL1 antibody as a loading control. In the density centrifugation experiments the membranes were probed with an anti-hFSHR mAb106.105 antibody to probe for the presence of hFSHR. All primary antibodies were diluted 5uL of the antibody in 10mL of 5% BSA in TBST. All experiments followed the same protocol for secondary, however different secondary antibodies were used based on whether it was anti-mouse or anti-rabbit. The secondary antibodies were diluted 2uL of the antibody in 10mL of 5% milk in TBST. All membranes were developed for 5 minutes using a Thermo Scientific Supersignal West Femto Maximum Sensitivity Substrate.

Results

Western blot analysis of a sucrose density gradient from cells treated with FSH for either 0, 5, 15 or 30 minutes show that hFSHR residency in the lipid raft is hormone dependent (Figure 8). At 0 minutes and 5 minutes of hormone treatment the hFSHR is present in fractions 2, 3 and 4 which corresponds to samples taken from the 5% sucrose (Figure 8). Since lipid rafts are more buoyant, their density corresponds to the density of the 5% sucrose so the rafts float to the top fractions during centrifugation. From this it can be concluded that hFSHR is found in lipid rafts at 0 and 5 minutes of hormone treatment. This can be compared to cells treated with FSH for 15 and 30 minutes, which are found in fractions 9, 10, 11 and 12 (Figure 8). These fractions correspond to fractions taken from the 45% sucrose. Non-raft membranes are heavier, and their density corresponds to the density of the 45% sucrose, so during centrifugation they sink to the bottom and are found in the bottom fractions. Therefore, at 15 and 30 minutes of hormone treatment the hFSHR is no longer residing in lipid rafts, rather it is found in the non-raft membrane. The amount of time of the hormone treatment determines whether or not the hFSHR is present in lipid rafts.

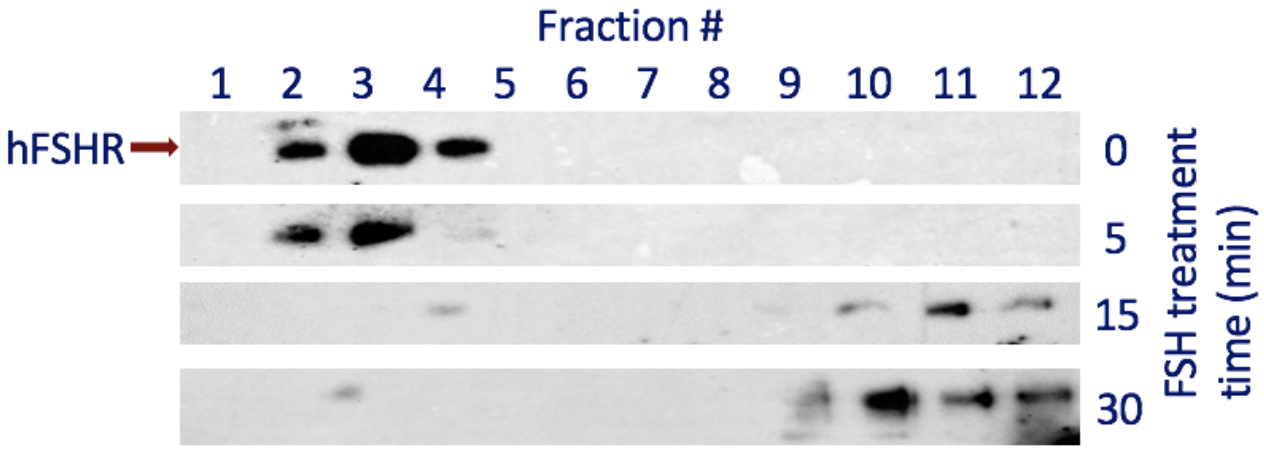

Figure 8. Western blot of a sucrose density centrifugation from a hFSH time course interval. The cell extracts were probed with an anti-hFSHR monoclonal antibody. At 0 and 5 minutes of hormone treatment hFSHR was found in lipid rafts. At 15 and 30 minutes of hormone treatment hFSHR moved out of the lipid raft.

Western blot analysis of a density centrifugation of cells treated with Fumonsin B1 was used to determine if this treatment , sphingolipid synthesis inhibition, alters hFSHR lipid raft residency (Figure 9). hFSHR was found in fractions 8, 9, 10, 11 and 12 (Figure 9). These are fractions taken from the 45% sucrose, where the non-raft membrane sinks based on densities. Since hFSHR is found in the bottom fractions, it can be concluded that Fumonsin B1 treatment disrupts the lipid rafts, causing hFSHR to reside in the non-raft membrane. Once it was determined that the drug treatments disrupt lipid rafts and cause the hFSHR to reside in the non-raft membrane, signaling experiments were performed.

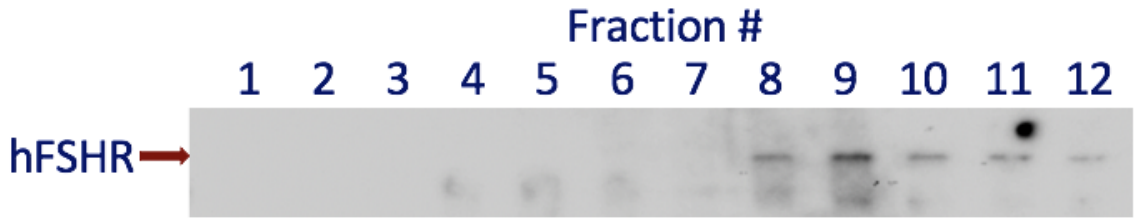

Figure 9. Western blot of fractions from sucrose density centrifugation of cells treated with Fumonisin B1. hFSHR was found in the bottom fractions, consistent with Fumonisin B1 disrupting lipid rafts.

CREB activation in cells treated with Fumonsin B1 to disrupt the lipid rafts was analyzed via western blot analysis (Figure 10). The control cells, treated with DMSO, show a typical FSH response (Figure 10). A typical FSH response is a small amount of signaling at 0 minutes of FSH and then at 5, 15 and 30 minutes of hormone treatment there is an increase in signaling that stays consistent between the times. The Fumonisin B1 cells have decreased CREB activation at 0 and 5 minutes compared to the control cells (Figure 10). CREB is an indirect measurement of cAMP production, therefore cAMP production in cells treated with Fumonsin B1 is delayed compared to the control cells. This membrane was re-probed with an anti-AKT antibody as a loading control, which showed consistent protein amounts between samples (Figure 10).

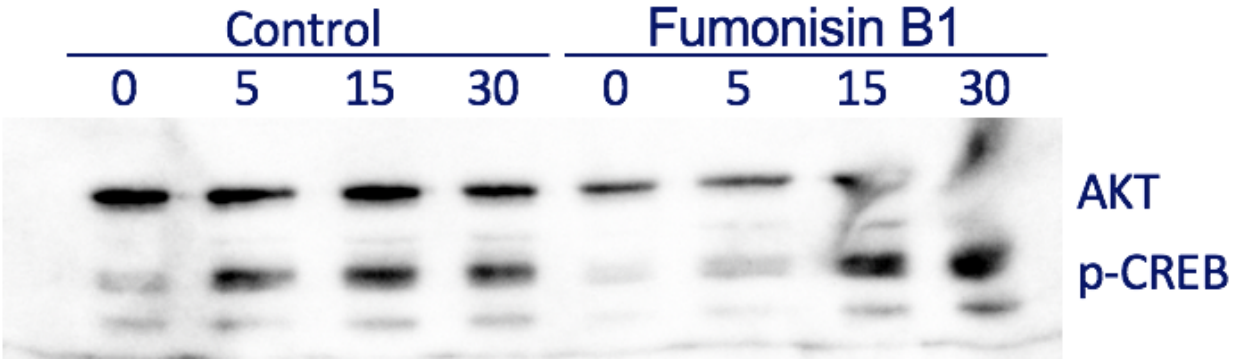

Figure 10. hFSHR signaling after disruption of the lipid rafts with fumonisin B1 shows delayed CREB activation. Western blots of extracts from cells treated with fumonisin B1 and FSH as indicated were probed with an anti-phospho-CREB antibody as an indirect measurement of cAMP production and an anti-AKT antibody as a loading control.

Western blot analysis was performed to look at the activation of CREB in cells treated with MβCD (Figure 11). The control cells, treated with DMSO, showed the typical FSH response. The cells treated with MβCD showed decreased CREB activation at 0, 5, 15 and 30 minutes of FSH treatment compared to the control cells (Figure 11). Again, since CREB activation is an indirect measurement of cAMP production, the cells treated with MβCD showed delayed cAMP production. An anti-AKT antibody was used again as a loading control and revealed consistent sized bands, concluding that each sample contained the same amount of protein. Therefore differences in signaling can be attributed to disruption of the lipid rafts, and not a difference in protein amount.

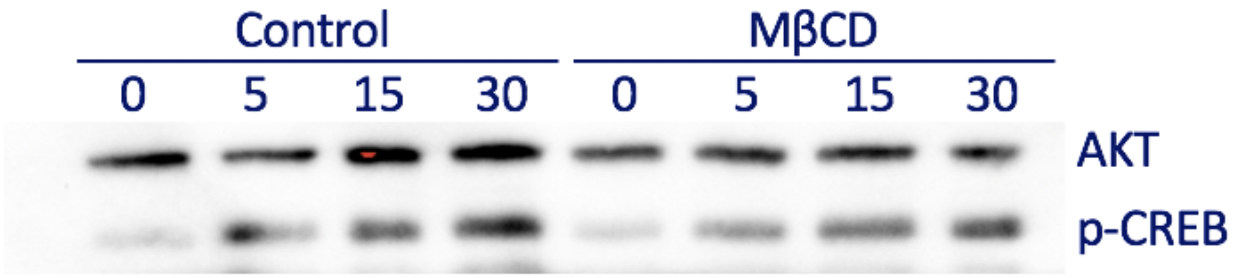

Figure 11. hFSHR signaling after disruption of the lipid rafts with MβCD shows delayed CREB activation. Western blots of extracts from cells treated with MβCD and FSH as indicated were probed with an anti-phospho-CREB antibody as an indirect measurement of cAMP production and an anti-AKT antibody as a loading control.

The activation of ERK1/2 in cells treated with Fumonsin B1 was investigated via western blot analysis (Figure 12). The control cells, treated with DMSO, show the typical FSH response, which shows increased signaling at 5, 15 and 30 minutes of hormone treatment (Figure 12). Cells treated with Fumonsin B1 showed an increase in ERK1/2 activation at 5 and 15 of hormone treatment compared to control cells. The membrane was re-probed with an anti-APPL antibody as a loading control which showed consistent bands for both control and Fumonsin B1 treated cells at all times.

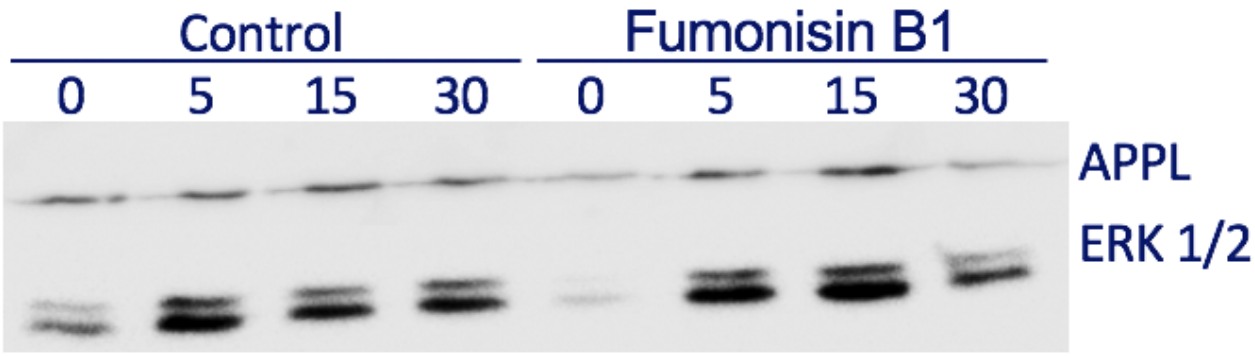

Figure 12. hFSHR signaling after disruption of the lipid rafts via Fumonisin B1 shows increased ERK1/2 activation. Western blots of extracts from cells treated with Fumonisin B1 and FSH as indicated were probed with an anti-phospho-ERK1/2 antibody and an anti-APPL antibody as a loading control.

Cells treated with MβCD were investigated for altered ERK1/2 signaling via western blot analysis (Figure 13). The control cells, treated with DMSO, had an FSH response (Figure 13). The cells treated with MβCD showed increased ERK1/2 activation at 5, 15 and 30 minutes of hormone treatment compared to the control cells (Figure 13). The membrane was re-probed with an anti-APPL antibody for a loading control and the bands were consistent for each sample (Figure 13).

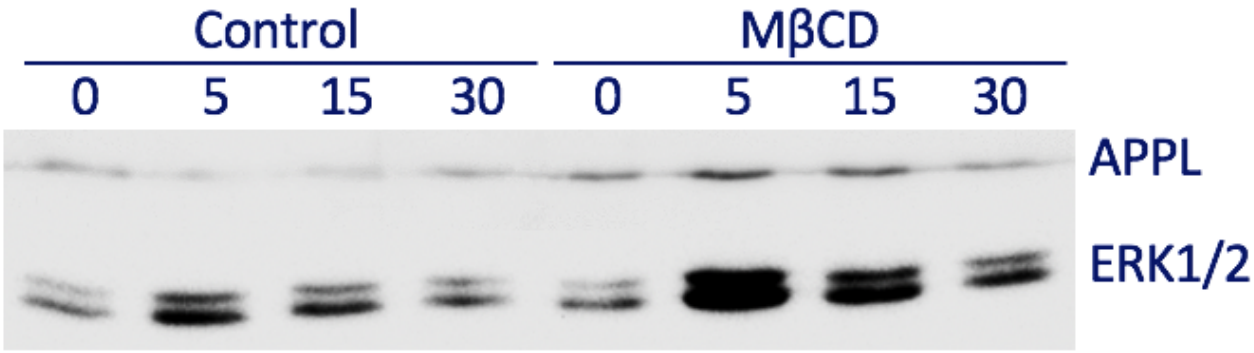

Figure 13. hFSHR signaling after disruption of the lipid rafts via MβCD shows increased ERK1/2 activation. Western blots of extracts from cells treated with MβCD and FSH as indicated were probed with an anti-phospho-ERK1/2 antibody and an anti-APPL antibody as a loading control.

Discussion

hFSHR is a GPCR that is known to interact with lipid rafts in order to signal properly to allow fertility in men and women. The overall goal of this study was to investigate how lipid rafts control signal transduction. Specifically, the first goal was to investigate what determines hFSHR lipid raft residency. Through sucrose density centrifugation we found that hFSHR lipid raft residency is hormone dependent. In the beginning hFSHR is found in the lipid rafts, and after 15 minutes of hormone treatment hFSHR moves out of the lipid rafts (Figure 8). Without hormone, hFSHR resides in the lipid raft, and at certain time points of hormone incubation it moves out of the lipid rafts, therefore it is hormone dependent. Seeing that hFSHR moves out of the lipid rafts after hormone treatment makes sense based on what we know about β-arrestin. When hormone binds to hFSHR it begins signaling and the GRKs phosphorylate the receptor to trigger β-arrestin to bind to the receptor to internalize it, which would move it out of the lipid raft which agrees with the results.

In the literature, there was a study done on the estrogen receptor β (ERβ) which also resides in lipid rafts at some point during signaling.[24] The study found that before hormone is added, at time 0 of hormone treatment, ERβ is found in the non-raft membrane.[24] However, when the cells containing ERβ were treated with estrogen, the receptor moved into the lipid rafts immediately to begin signaling.[24] These results don't directly agree with our results, since hFSHR moved out of the lipid raft, however it does show that receptors utilize lipid rafts to regulate signaling. There are different theories as to how lipid rafts regulate signaling, one being what we saw with hFSHR where the receptor moves out of the lipid raft to signal (Figure 13C).[25] Another theory agrees with the study done on ERβ where the receptor starts outside of the lipid

raft and upon ligand binding it moves into the lipid raft to signal (Figure 13B).[25] Some receptors reside in the lipid rafts and when the ligand binds the receptor stays in the lipid raft to signal (Figure 13A).[25] The last theory is that a receptor does not reside in the lipid raft, but when ligand binds to the receptor and the receptor signals to something else located in the lipid raft (Figure 13D).[25] Different receptors localize to lipid rafts at different times and in different methods.

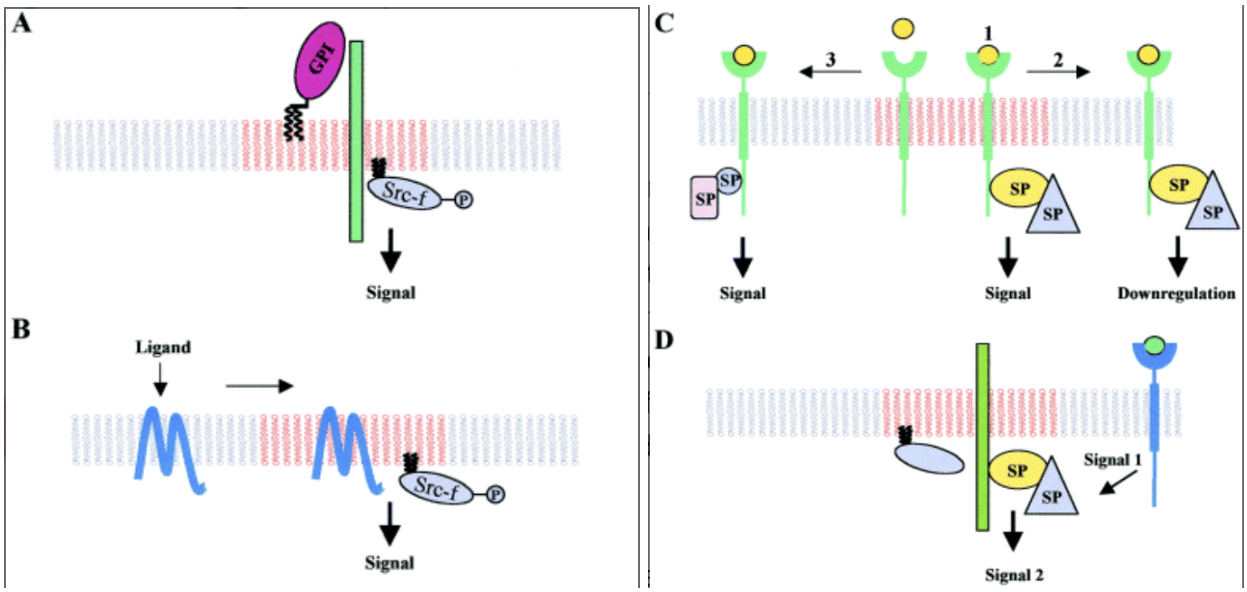

Figure 14. Different ways lipid rafts regulate signaling of receptors. (A) The receptor resides in the lipid raft and upon ligand binding the receptors stay in the lipid raft to signal. (B) The receptor is found in the non-raft membrane and upon ligand binding the receptor moves into the lipid raft to signal. (C) The receptor resides within the lipid raft and upon ligand binding the receptor moves out of the lipid raft to signal. (D) The receptor is found outside of the lipid raft but upon ligand it activates something else inside the lipid raft to begin a signal. From Zajchowski LD, Robbins SM. Lipid rafts and little caves. Compartmentalized signalling in membrane microdomains. Eur J Biochem. 2002 Feb;269(3):737-52.

The second goal of this project was to determine how lipid raft disruption alters hFSHR signaling. We consistently found that disrupting the lipid rafts, using either MBCD or Fumonisin B1, decreased cAMP production, as seen through the delayed CREB activation (Figure 10, 11). Therefore cAMP production is lipid raft dependent. Alternatively, we consistently found that

disrupting the lipid rafts, again using either MBCD or Fumonisin B1, increased ERK1/2 activation. Therefore, ERK1/2 activation is lipid raft independent. Our lab hypothesized that when hFSHR is out of the lipid rafts, due to the disruption from either drug, it allows β-arrestin to internalize the receptor quicker which activates ERK1/2 sooner, which is why we are seeing an increase in only ERK1/2 activation, and not in cAMP production.

Based on these results our lab will be investigating the following question: Does β-arrestin cause hFSHR to leave the lipid raft, or does hFSHR leaving the lipid raft make the receptor accessible to β-arrestin? In order to investigate this question and siRNA-mediated knockdown of β-arrestin will be created.[26] When the knockdown is created, if the results show that the receptor doesn't leave the raft, then β-arrestin is responsible for causing hFSHR to leave the raft. If the results show the receptor still leaves the lipid raft, even though β-arrestin is knocked down, then hFSHR leaving the raft allows the receptor to be accessible to β-arrestin so it can bind.

This experiment also brings up the question of whether ERK1/2 is activated when β-arrestin binds to hFSHR, or if β-arrestin has to internalize hFSHR through endocytosis to activate ERK1/2. To answer this question a dynamin k 44a mutant plasmid will be established. Dynamin polymerizes the membrane of the newly formed endosome, to pinch it off from the membrane, to allow for endocytosis.[27] By using this mutant it prevents endocytosis of hFSHR after β-arrestin binds and ERK1/2 activation can be measured. If there is still ERK1/2 activation then the binding of β-arrestin to hFSHR causes activation. If there is no ERK1/2 activation then β-arrestin must bind and trigger endocytosis of hFSHR in order to activate hFSHR.

Investigating the signaling in fertility will allow us to understand more about the signaling pathways necessary for correct fertility in men and women. Understanding when the

hFSHR localizes to lipid rafts, and how that alters signaling, will allow us to gain significant understanding of what needs to be functioning properly for fertility signaling. Once these pathways and localization of the hFSHR are understood, new treatments can be discovered that are more accessible and cost significantly less than current fertility treatments. Understanding the signaling behind proper fertility can also allow new contraceptives to be created which block some part of the signaling pathway to prevent pregnancy that have less side effects than current contraceptives.

Acknowledgments

I would like to thank Professor Brian Cohen for all his support, advice and guidance throughout my summer research fellowship, as well as during the year on my senior thesis. I would also like to thank him for not only my thesis, but also for all of his help throughout my graduate school admission process. I would like to thank all the members in the Team Cohen lab this year for their support and joy throughout the year, and also past Team Cohen members for all of the research they completed which this work is based off of. And lastly, I would like to thank the Union College Undergraduate Research Summer Fellowship Grant, Student Research Grant, and Faculty Grant which helped fund this project.

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