INVESTIGATION OF 1ALPHA,25-DIHYDROXY VITAMIN D3 MEMBRANE RECEPTOR ERP60 IN ADIPOCYTES FROM MALE AND FEMALE LEAN AND OBESE MICE

A Thesis
Presented to
The Academic Faculty

by

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In Partial Fulfillment
of the Requirements for the Degree
Masters in Science in the
School of Biomedical Engineering

Georgia Institute of Technology
December 2009

INVESTIGATION OF 1ALPHA,25-DIHYDROXY VITAMIN D3 MEMBRANE RECEPTOR ERP60 IN ADIPOCYTES FROM MALE AND FEMALE LEAN AND OBESE MICE

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Date Approved: October 2009

ACKNOWLEDGEMENTS

I would like to thank both Dr. Barbara Boyan and Dr. Zvi Schwartz for their endless support during these past years. My experience in their lab was superb in every way. I miss the work, my co-workers and the stimulating conversations spurred by the desire to further scientific knowledge. I would also like to thank Dr. Hanjoong Jo and Dr. Clifton Baile for their advisement and expertise in the subject matter included herein. Lastly, I would like to thank my parents and husband for without their love and support I may not have had the strength to finish this work.

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LIST OF SYMBOLS AND ABBREVIATIONS

$1\alpha,25(OH)_2D_3$	Vitamin D metabolite 1alpha,25-dihydroxy Vitamin D3
BM	Bone Marrow
BMS	Bone Marrow Stromal
FBS	Fetal Bovine Serum
PBS	Phosphate Buffered Solution
PKC	Protein Kinase C
PM	Plasma Membrane
RIPA Buffer	RadioImmuno Precipitation Assay Buffer
qPCR	Quantitative Polymerase Chain Reaction
RT-PCR	Real Time Reverse Transcription Polymerase Chain Reaction
UV	Ultra Violet
VDR	Vitamin D Receptor

SUMMARY

The purpose of this study is to determine whether or not adipocytes harvested directly from fat pads or induced from bone marrow in lean and obese mice exhibit a sex-dependent rapid response to vitamin D metabolite $1\alpha,25(OH)_2D_3$ and if so to elucidate if it is via an ERp60 receptor mediated signaling pathway. The role of $1\alpha,25(OH)_2D_3$ and specifically the membrane effect will be examined in two genetically distinct mice to see if their cells have a differing sensitivity. The results indicate that there are differing responses in adipocytes that are induced from bone marrow versus differentiated fat pad adipocytes, and the function of $1\alpha,25(OH)_2D_3$ is sex-specific in some cases. Additionally, all the adipocytes tested demonstrated a rapid response to $1\alpha,25(OH)_2D_3$; mRNA for nVDR and ERp60 were found in all cells however the only functional protein found in the plasma membrane was ERp60 indicating that it may be necessary for the rapid response whereas nVDR is not required.

CHAPTER 1

BACKGROUND

It has been seen that cells isolated from human bone marrow have the ability to differentiate into cells from many types of tissues (1). Osteoblasts and adipocytes are thought to derive from the same mesenchymal stem cell precursor (2) . Since patients with deteriorating bone quality show increased adipogenesis in the bone marrow there is great interest in factors that cause differentiation to shift from fatty tissue cells toward cells contributing to bone formation (3) (4).

Dietary vitamin D, in combination with UV sun exposure, experiences 25-hydroxylation in the liver and 1alpha-hydroxylation in the kidney, before it becomes its known active form 1α,25-dihydroxy vitamin D3. 1α,25$(OH)_2D_3$ is a steroid hormone that induces differentiation via a nuclear receptor in osteoblasts (5) as well as through a plasma membrane associated receptor, ERp60 (6) (7) (8) (9). There have been reports that male and female osteoblasts respond differently to steroid hormones, but it is not known whether this is the case for adipocytes and additionally if sex-specific effects are found only in fully differentiated cells.

Nuclear vitamin D receptors (VDR) are present in adipocytes nuclei; however it is currently unknown whether these receptors are present in the plasma membrane where it is possible that they contribute to rapid responses within the cell. It is also unknown whether functional ERp60 is present in adipocytes, whether levels of this receptor are different in adipocytes that are induced from bone marrow versus differentiated fat pad adipocytes, and whether its function is sex-specific.

1.1 Known Effects of 1, 25$(OH)_2D_3$ in Osteoblast

1α,25-dihydroxy vitamin D3 is a steroid hormone that has been shown to induced differentiation in osteoblasts by activating PKC (10), stimulating alkaline phosphatase production (11), stimulating phospholipase A2 activity and prostaglandin E2 synthesis (12). Studies indicate that these responses occur via both a nuclear receptor in osteoblasts as well as through a membrane associated receptor, ERp60, which has been shown to activate rapid phospholipase A2-dependent signaling cascades resulting in increases in activity of protein kinase C (PKC) and PKC-dependent ERK1/2 mitogen activated protein kinase (13).

1.2 Known Effects of 1α,25$(OH)_2D_3$ in Adipocytes

In adipocytes 1α,25-dihydroxy vitamin D3 has been shown to inhibit adipogenesis, increase apoptosis and decrease lipid accumulation (14). One known mechanism by which 1α,25$(OH)_2D_3$ inhibits adipogenesis is by decreasing expression of peroxisome proliferator activated receptor gamma (PPARγ) (15), a transcription factor activated by polyunsaturated fats which is essential for adipogenesis and adipocytes differentiation (16) (17). Additionally, 1α,25$(OH)_2D_3$ has been shown to increase expression of nuclear vitamin D receptor in adipocytes, while decreasing expression of AP2, adipose specific gene 422 (14). What is currently unknown is whether or not 1α,25$(OH)_2D_3$ effects adipocytes via a non-genomic rapid response and if the membrane receptor ERP60 is present and functional.

CHAPTER 2

METHODS

In collaboration with Dr. Baile's lab a mouse animal model was used to obtain tissue samples for the following experimental design. Male and female mice of the ob/ob strains plus their controls were used; the control mice are referred to throughout this work as lean mice, whereas the ob/ob mice are referred to as obese mice. Methods are also detailed in the grant supporting this work: "Investigation of Vitamin D Membrane Receptors in Adipocytes from Bone Marrow and Fat Pads"; study number 600-UGA-MIC-MAD-05-006; AUP number A2006-10047-m2.

2.1 Experimental Design

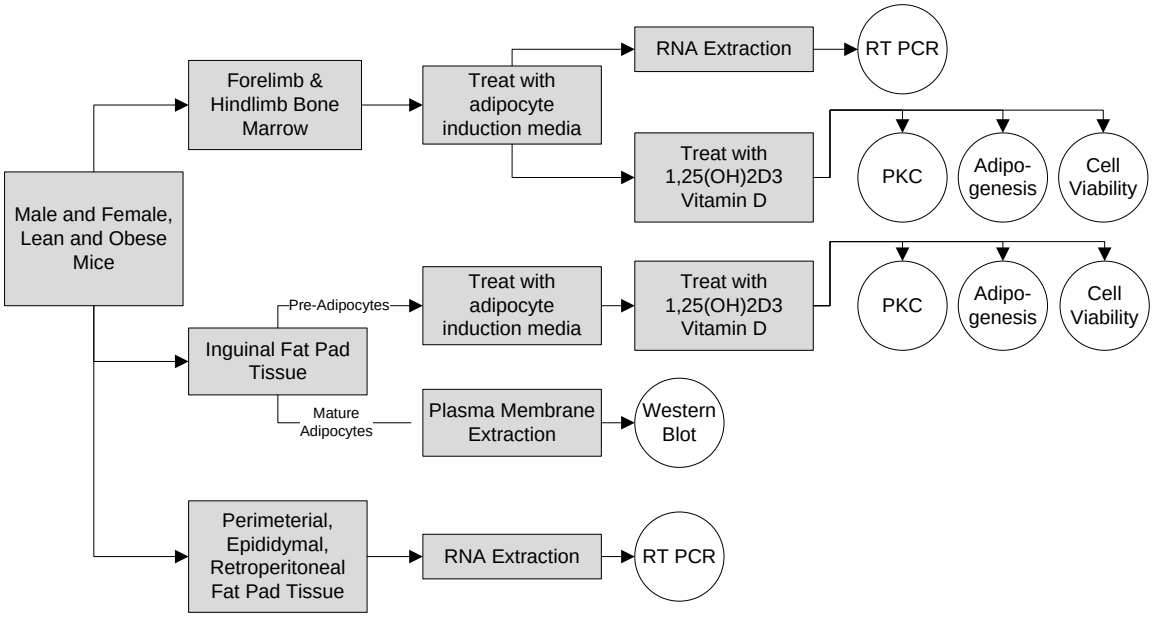


Figure 2.1.1 Experimental design summarizing events from the harvest of the cells to the results presented herein. Four mice of each type lean, obese and male, female were to be used, except one lean male which was lost prior to harvest.

2.2 Primary Harvest and Treatments

2.2.1 Tissue Harvest

On January 12, 2006 fifteen mice were sacrificed to obtain inguinal fat pad tissue, perimeterial/epididymal fat pad tissue, retroperitoneal fat pad tissue, left and right forelimbs as well as the left and right hindlimbs. Four 18 week old mice of each type (male and female, lean and obese) were to be sacrificed, however one lean male was lost prior to harvest. They were caged by sex, fed standard mouse chow, and given water ad libitum. Mice were euthanized with CO_2 and the lower portion of the body shaved using Nair^{TM}. After 5 minutes the area was rinsed with water. The mice were secured onto acrilymide boards with rubber bands. Betadine was applied liberally over the shaved areas and the underlying board before rinsing with 50%:50% Methanol:ETOH. A mid-line incision was made through the skin, taking care not to cut into the peritoneal cavity. The inguinal pads were dissected in situ from each mouse and placed into 50 ml conical vials filled DMEM/F12 media (~10 ml). Samples from mice of the same sex and genotype were pooled. The abdominal cavity was opened and retroperitoneal and epididymal or perimetrial fat removed and placed on ice in RNAse free tubes for mRNA extraction. The left and right femur and tibia were removed from the mice; the proximal and distal ends of the bones were removed and the bones placed upright in sterile 0.5ml RNAse free microcentrifuge tube. The tubes had a punctured hole in the bottom but were contained within a larger 2 ml microcentrifuge tube.

2.2.2 Stromal Vascular Primary Culture of Mouse Preadipocytes

Inguinal fat pads were transferred from 50ml conical tubes into sterile 30ml beakers. There they were minced into a homogenous product which was incubated in a sterile solution of 0.1M Hepes (4ml) and Type 1 collagenase (1ml) for 45 minutes in a shaking water bath (37°C, 115RPM). The digested fat was then poured into a 10ml

syringe and filtered (240μm) into a 50ml centrifuge tube. This solution was then centrifuged at 300-500rpm for 5 minutes to sediment debris. The floating adipocyte layer was removed for plasma membrane extraction (see section 2.2.3). The infranatent was put into a second sterile tube and then centrifuged at 1100rpm for 10 minutes at room temperature. After decanting, the cell clumps were disrupted with 5ml of DMEM F-12 Ham(Sigma-Aldrich, D-8900) containing 10% FBS. The solution was filtered (20μm) to remove endothelial cell aggregates and centrifuged at 1100rpm for 5 minutes. The cells were pooled by gender and genotype, counted in a hemocytometer, diluted in full media (DMEM/F12, 10%FBS, antibiotics) and seeded at a density of 25,000 cells/cm^2 in 24-well plates for PKC assay, and in 96-well plates for adipogenesis and viability assay.

One day after plating, the media was replaced with DMEM/F12 with 5% FBS. Every two days the media was replaced until confluency (5 days). At confluence, differentiation was induced by feeding with adipocyte induction media. The induction media consisted of 250μM methylisobutylxanthine, 0.1μM dexamethasone, 17nM insulin, 100μM indomethacin in a DMEM/F12 10% FBS solution. For adipogenesis and cell viability assay 10^{-9}, 10^{-8} M 1α,25$(OH)_2D_3$ is included in the induction media. After 48 hours the induction media was replaced with DMEM/F12, 10% FBS and 17nM insulin. After 48 hours the insulin containing media was removed and the cultures fed every other day with DMEM/F12 and 10% FBS.

Day 0 cells seeded

Day 5 cells treated with induction media

Day 11 cells harvested for adipogenesis and cell viability assays.

PKC activation samples were serum starved for 24 hours.

Day 12 cells were treated with 10^{-10}, 10^{-9}, 10^{-8} M 1α,25$(OH)_2D_3$ for 9, 90, or 270 minutes for PKC activation assay in ob/ob male, lean male, ob/ob

female, lean female. After treatment, samples were washed twice with PBS and incubated with 100µl RIPA (Sigma, R-0278) for 30 minutes on ice then stored at -20°C.

2.2.3 Plasma Membrane Preparation from Adipocytes

Plasma membranes were isolated from inguinal fat pads adipocytes. All steps were carried out at 4°C. The isolated adipocytes were washed with 1ml homogenizing buffer and centrifuged briefly at 5,000 rpm. Homogenizing buffer consists of 10mM Tris-HCl, 1mM EDTA and 0.25M sucrose at an overall pH of 7.4. After decanting the supernatant, 600µl of homogenizing buffer was added to each tube and the cells were manually disrupted by moving a Teflon pestle up and down five times, turning 360 degrees between each stroke. Homogenized samples were then centrifuged for 15 minutes at 12,000 rpm; the pellet was re-suspend with 500µl of 2M sucrose. Samples were centrifuged again at 11,750 rpm for 10 minutes. The supernatant was poured into a disposable Sorvall centrifuge tube and diluted 7-fold with ice cold ultra pure water; samples were centrifuged at 16,500 rpm for 15 minutes using rotor SA600. About 2ml of the supernatant was discarded with the remaining infranatant being transferred into an EP tube; samples were centrifuged at 13,000rpm for 20 minutes on a table top centrifuge. A strong pellet was obtained, washed once with 1ml of PBS, centrifuged and ultimately re-suspend in 50µl of PBS and stored at -70°C. Samples were used for Western Blot (see section 2.3.6).

2.2.4 Primary Culture of Mouse Bone Marrow Stromal (BMS) Cells

The harvested limbs, in microcentrifuge tubes, were centrifuged for one minute at 13,500rpm. The bones were removed and the cells were re-suspended in 200µl of media with FBS and antibiotics, pooled, and transferred into a 15ml conical tube with 3ml of warm media. Bone marrow cells from the two femur and two tibia per animal were

pooled. The stromal cells were disrupted by aspirating through 18 and 20 gauge needles. Cells were centrifuged at 1200rpm for 5 minutes, supernatant discarded, re-suspended in 2ml of media and counted in a hemocytometer. The cells were plated at an initial seeding density of 5 million cells per 100mm plate. Approximately 8 million cells were obtained from each lean mouse and around 5 million cells were obtained from each obese mouse After two hours of incubation at 37°C and 5% CO_2, the media was changed to remove any non-adherent cells; culture media was changed every 3 to 4 days. After two weeks cells were washed with 1X PBS and released from plate by treatment with trypsin (.25% in 1mM EDTA) for 10minutes at 37°C. Reaction was stopped by adding 2.5ml calf serum, centrifuging for 5 minutes at 12,000rpm, discarding the supernatant and re-suspending in fresh media. Cells were seeded at a density of 20,000 cells/cm^2 in 6-well plates for RNA extraction, 24-well plates for PKC assay, and 96-well plates for adipogenesis and cell viability assay. After 2 weeks, cells reached confluence and were treated with adipogenic induction media for 3 days. Adipogenic media contains 1% antibiotic-antimycotic, 0.25mM methylisobutylxantheine, 0.1μM dexamethasone, 10μg/ml insulin, 60μM indomethacin, 5μM Ciglitizone (PPARg agonist), and 4.5mg/ml glucose all in DMEM 10% FBS. Following the three days, the cells were treated with maintenance media for 1 day. Adipogenic maintenance media contains 10μg/ml insulin, 1% antibiotic-antimycotic, and 4.5mg/ml glucose all in DMEM 10% FBS. For adipogenesis assays cells were treated with 10^{-10}, 10^{-9}, 10^{-8} $1\alpha,25(OH)_2D_3$ throughout differentiation in the adipogenic media. The feeding cycle, 3 days induction media, 1 day maintenance media, was repeated three times culminating in 7 days of incubation with maintenance media. At this point cells were (1) harvested for RNA extraction; (2) treated with 10^{-10}, 10^{-9}, 10^{-8} M $1\alpha,25(OH)_2D_3$ for 9, 90, 270 minutes, washed twice with PBS, then harvested with RIPA buffer and stored at -80°C for PKC activation assay; (3) assayed for adipogenesis.

2.2.5 RNA Extraction

RNA is isolated from perimeterial, epididymal, and retroperitoneal fat pad tissue via a modified protocol using zirconia beads and a TRIZOL reagent (Invitrogen, Cat. No. 15596-026), followed by homogenization, extraction with chloroform and isopropanol, washed with ethanol and finally eluted into 15µl of DEPC water.

RNA was isolated from adipocyte induced bone marrow stromal cells by using RNeasy Mini kit (QiagenTM) following manufacturer's instructions.

2.3 Assays

2.3.1 Adipogenesis Using AdipoRed™

Postconfluent preadipocytes were incubated with control or $1\alpha,25(OH)_2D_3$ during the induction period. When the cells filled the plate (day 6-8), the plate was removed from the cell culture incubator and allowed to cool to room temperature. The supernatant was removed and each well carefully rinsed with 200µl of PBS. Each well was then filled with 200µl PBS. AdipoRed reagent (5µl) was added with a multi-channel pipette. The plates were mixed by tapping the edge of the plate against the lab bench several times. After 10 minutes, the fluorescence was read (excitation 485nm, emission 572nm).

2.3.2 Cell Viability Using ApoStrand™ ELISA Apoptosis Detection Kit AK-120

Adipocytes were incubated with control or $1\alpha,25(OH)_2D_3$ for 24 and 48h. Apoptosis was measured by ssDNA ELISA kit which in principle is based on the ability of a monoclonal antibody to detect denatured DNA and the fact that apoptotic cells are specifically prone to denaturing when exposed to formamide.

2.3.3 Protein Kinase C Activity

Cells were plated on 24-well plates, treated with 10^{-10}, 10^{-9}, 10^{-8} M $1\alpha,25(OH)_2D_3$ for 9, 90, or 270 minutes, aspirated and washed twice with 0.5ml cold PBS. RIPA

(100µl) was added to each well and stored on ice for 30 minutes before storing at -20°C. Using "Protein Kinase C Biotrak Enzyme Assay" from Amersham Biosciences (PC RPN77) PKC activity was measured. In brief, peptides are combined with the cell lysate and adenosine-5'-[γ-32P]ATP (Perkin Elmer, BLU002A500UC). If there is active PKC in the sample, the radioactive phosphate isotope will be transferred onto the peptide which is then transferred to binding papers where all of the peptides are captured. The un-bound ^{32}P is washed away. The binding papers are put into vials and the radioactive ^{32}P is counted in a scintillation counter.

2.3.4 Real Time Reverse Transcription Polymerase Chain Reaction (RTPCR)

The following primers were used within the RT-PCR method.

ERP60 primers:

Sense (forward): 5'GATGGCAACTTGAAGAGATACC3'

Antisense (reverse): 5'CTCTGCTACCACCACCTT3'.

It is important to note that another set of ERP60 primers was investigated, however did not result in any visible or measurable data for the samples nor the controls:

5'GTGCTAGAACTCACGGAC3' and 5'AGCCTCACTGAATGAATC3'.

The nVDR primer **(18)**:

Sense (forward): 5'GAGGTGTCTGAAGCCTGGAG3'

Antisense (reverse): 5'ACCTGCTTTCCTGGGTAGGT3'.

It is important to note that another set of nVDR primers was investigated, however did not result in any visible or measurable data for the samples:

5'TCAAGGGAGGCAGGCAGAAG3' and 5'TAGGCTTTGGGCAGGTAGGG3'.

The GapDH primers:

Sense (forward): 5'TGCACCACCAACTGCTTAG3'

Antisense (reverse): 5'GATGCAGGGATGATGTTC3'.

The first step in RT-PCR was reverse transcription. The RNA samples were diluted and placed into a .2mL thin walled eppendorf tube; 1µgRNA with DEPC water added to achieve a total volume of 13µL. PCR machine was used to control the temperature of the samples; 65˚C for 10 minutes, 4˚C for 5 minutes. To each tube the following was added: 2µL 10X buffer RT, 2µL 5mM dNTP, 1µL Omniscript RT, 2µL antisense primer. PCR machine was again used to control the temperature of the samples; 37˚C for 60 minutes. At this point the stock cDNA samples were completed. Any unused portion was frozen at -80˚C.

The second step was the polymerase chain reaction which amplified the cDNA. In new tubes the following was combined: 2.5µL 10X PCR buffer, 17.375µL DEPC water, 1µL 5mM dNTP, 1µL sense primer, 1µL antisense primer, 1µL MgCl2, 1µL cDNA sample. PCR machine was used to control the temperature of the samples: hot start was selected to begin at 94˚C at which point the program paused. Once the plate reached this temperature for 30 seconds 0.125µL Taq polymerase (Fisher Brand – 250u) was added to each tube. The PCR machine was set to resume, for the remainder of the program the temperature returned to 54.0˚C for ERP60 and GapDH samples and 60.4˚C for nVDR samples. At this point the cDNA samples were amplified and were ready to be run on the gel for imaging. Ready Gel (5% TBE, 10 wells, 30µL) was placed into the chamber with 500mL 1x TBE. For each sample, 2µL of loading dye and 10µL of the amplified cDNA sample was combined and loaded into the gel. The ladder was loaded into the gel as well. The samples were run at 100 Volts for 55mminutes. The gel was

removed from the chamber and placed into a flat container with 35mL dH2O and 5µL ethidium bromide where it was rocked gently for 10 minutes. The ethidium bromide solution was removed and replace with dH2O which was rocked gently for 25 minutes. Using the Versadoc an image of the gel was taken.

In order to run the analytical portion of real time PCR the following was done using ERP60 primers, nVDR primers and GapDH primers. GapDH was used to normalize the quantitative data. The cDNA samples resulting from the reverse transcription PCR were diluted 1.5µ of sample into 43.5µL of DEPC water. RC library cDNA was used as the standard. The standard was purified using a Qiagen Miniprep Kit, following the manufacturer's instructions. Six 1:3 serial dilutions were made from the standard. The standards (6µL each) and the samples (3µL each) were loaded into the 96-well plates. A master mixture was created containing the following per reaction: 12.5µL SYBR green, 0.5µL 10µM reverse primer, 0.5µL 10µM forward primer, .011µL 25mM MgCl2, 8.489µL DEPC water. Into each sample well, 22µL of the master mix was added; into each standard well, 19µL of the master mix was added. The RT-PCR program had the following cycles:

Table 2.3.4.1 Description of the real time PCR program protocol. Note that cycle 2 step 2 has two set points listed. 54.0˚C was used for ERP60 and GapDH samples; 60.4˚C was used for nVDR samples.

Cycle Number	Repeats	Step	Dwell Time (minutes)	Set Point (˚C)
1	1	1	03:00	95,0
2	40	1	00:10	95.0
		2	00:45	54.0 / 60.4
3	1	1	01:00	95.0
4	1	1	01:00	55.0
5	80	1	00:10	55.0

2.3.5 Western Blot

The following antibodies were used within this western blot method:

ERP60 antibodies: 1˚ Ab101 ERP60 antibody Rabbit, 2˚ Jackson IR, AffiniPure Donkey Anti-Rabbit, HRP conjugated.

nVDR antibody: 1˚ VDR (C-20):sc-1008, 2˚ Jackson IR, AffiniPure Donkey Anti-Rabbit, HRP conjugated.

Protein samples were diluted one part sample to four parts Laemmli sample buffer. Samples were boiled for 3 minutes at 85˚C, then frozen at -80˚C. Ready-Made gel was set up in a container filled with running buffer: 6.06g tris, 28.82g glycene, 400mL methanol, and add DI water for a total of 2L. Samples (7.5µg protein each) and ladder (5µL) were loaded into the gel. The gel was run at 80Volts for 15 minutes, then at 120Volts for 50 minutes. The protein was transferred onto a nitrocellulose membrane using a transfer case covered in transfer buffer. The layering within the case was as follows: sponge, filter paper (BioRad Mini Trans-Blot), gel, transfer membrane (nitrocellulose), filter paper, sponge. The transfer was run at 100Volts for 2 hours. The

transfer membrane was removed from the case and submerged for 2 hours in blocking solution: 20mL PBS/Tween and 1g powdered Carnation instant non-fat milk. The transfer membrane was removed from the blocking solution an incubated with the 1˚antibody overnight t 4˚C. Note that the ERP60 1˚ was diluted 1:5000 with blocking solution; nVDR 1˚ was diluted 1:500 with blocking solution. The transfer membrane was washed three times for five minutes with PBS/Tween buffer, totaling in 15 minutes, and then incubated with 2˚ antibody for 1 hour at room temperature. The 2˚ antibody was diluted 1:5000 with blocking solution. The transfer membrane was washed three times for five minutes with PBS/Tween buffer, totaling in 15 minutes, and then placed on a transparency sheet with 750µL of Super Signal West Pico kit developing solution for 15 minutes. The transfer membrane was dried for 10 minutes and imaged on a Versadoc system.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Adipogenesis

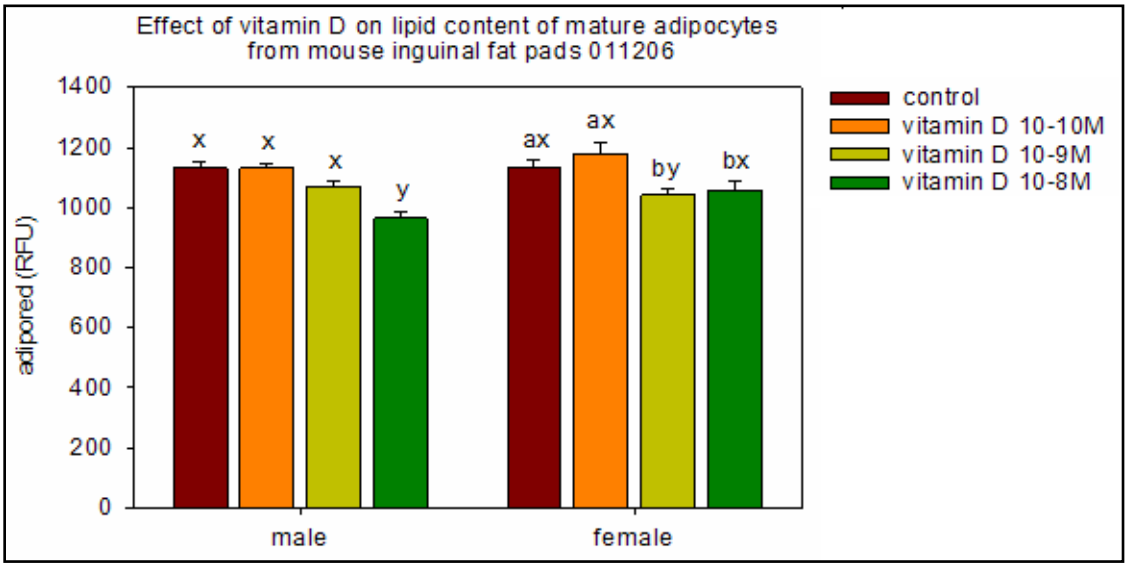


Figure 3.1.1 Effects of $1\alpha,25(OH)_2D_3$ on adipogenesis in male and female adipocytes.

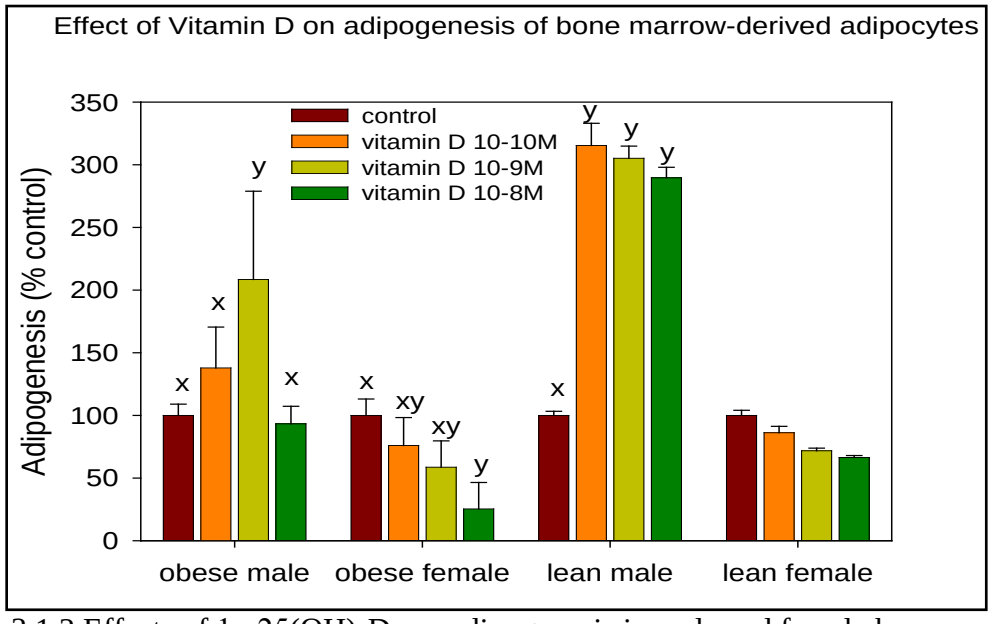


Figure 3.1.2 Effects of $1\alpha,25(OH)_2D_3$ on adipogenesis in male and female bone marrow induced adipocytes in lean and obese mice, percent of control.

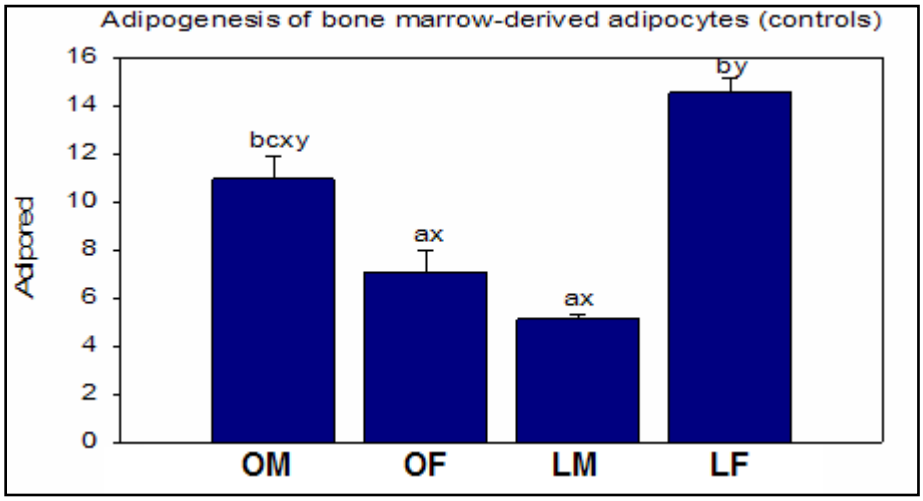


Figure 3.1.3 Control adipogenesis values for male and female bone marrow induced adipocytes in lean and obese mice.

3.2 Cell Viability

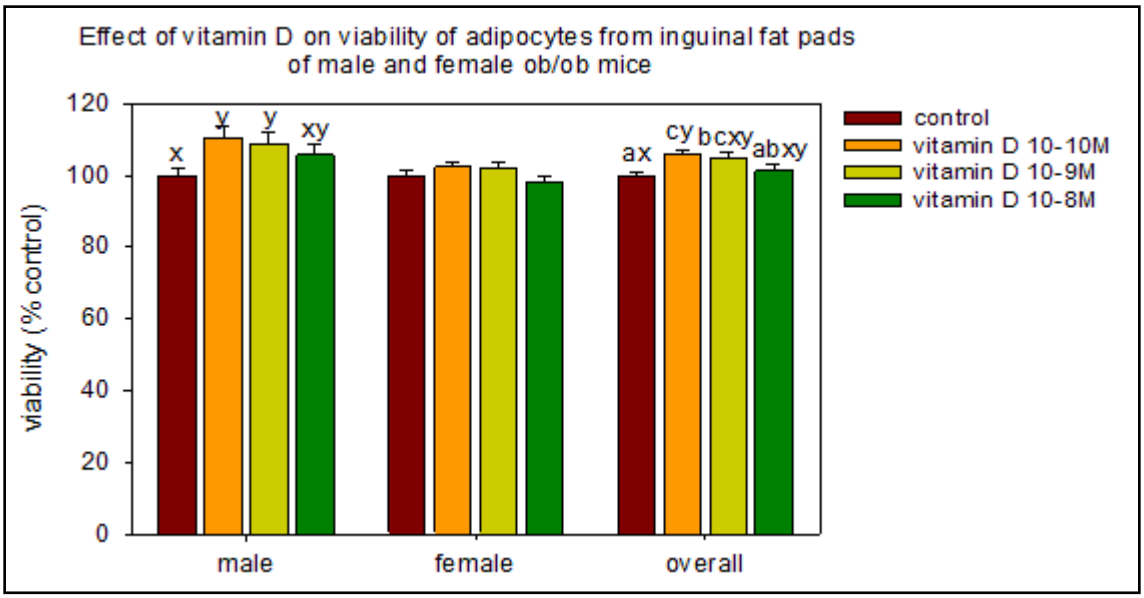


Figure 3.2.1 Effects of $1\alpha,25(OH)_2D_3$ on cell viability in male and female adipocytes.

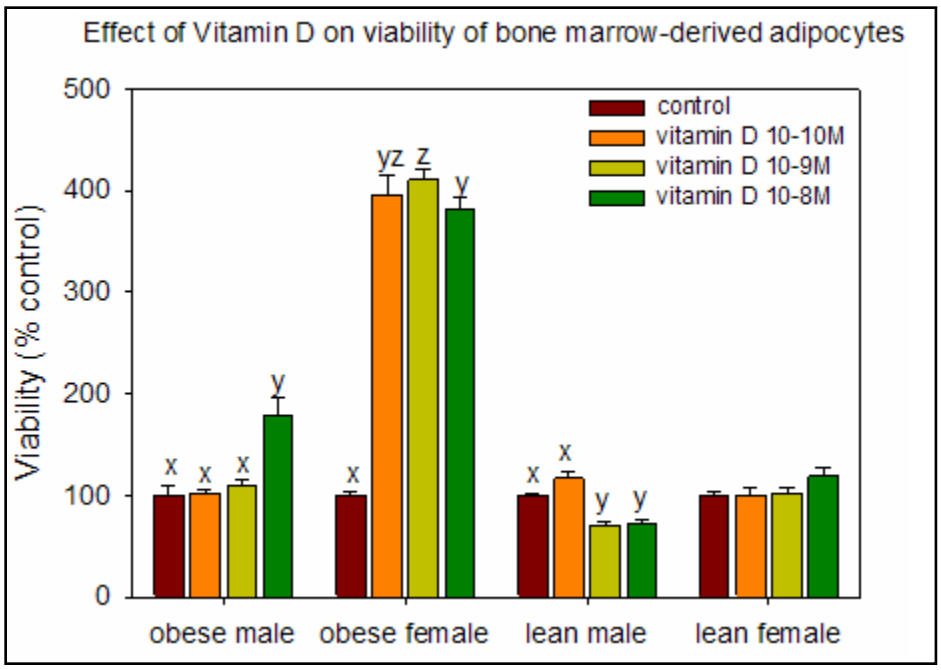


Figure 3.2.2 Effects of $1\alpha,25(OH)_2D_3$ on cell viability male and female bone marrow induced adipocytes in lean and obese mice, percent of control.

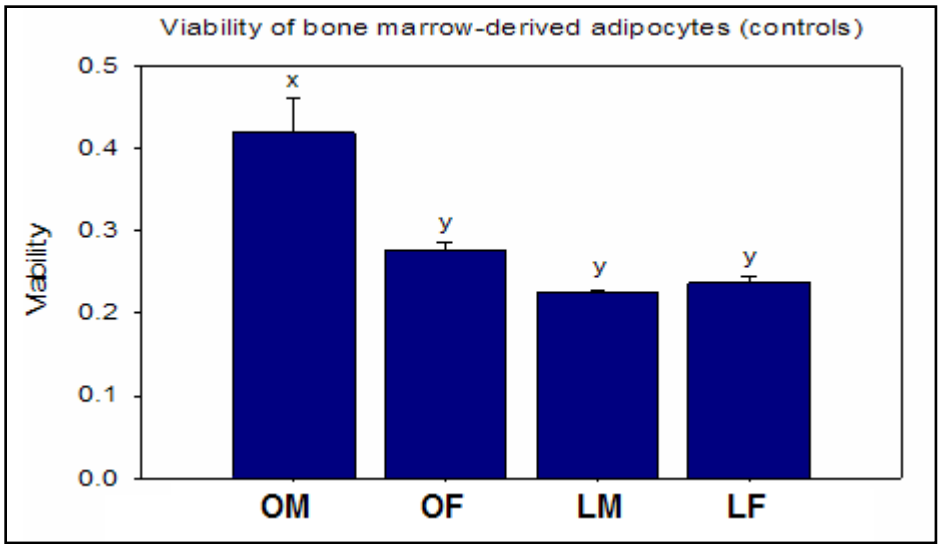


Figure 3.2.3 Control cell viability values for male and female bone marrow induced adipocytes in lean and obese mice.

3.3 Effect of 1α,25(OH)$_2$D$_3$ on PKC Signaling

Due to the large sample quantity, each group was treated with vitamin D metabolite 1α,25(OH)$_2$D$_3$ at different times; comparing between groups is possible by using the control as a baseline to which the data is normalized all. All statistical significance is compared to control and was calculated by 2 sample t-test ($p \leq 0.05$) performed on raw data. Raw data is included on Appendix A.

3.3.1 PKC Assay in Bone Marrow Induced Adipocytes

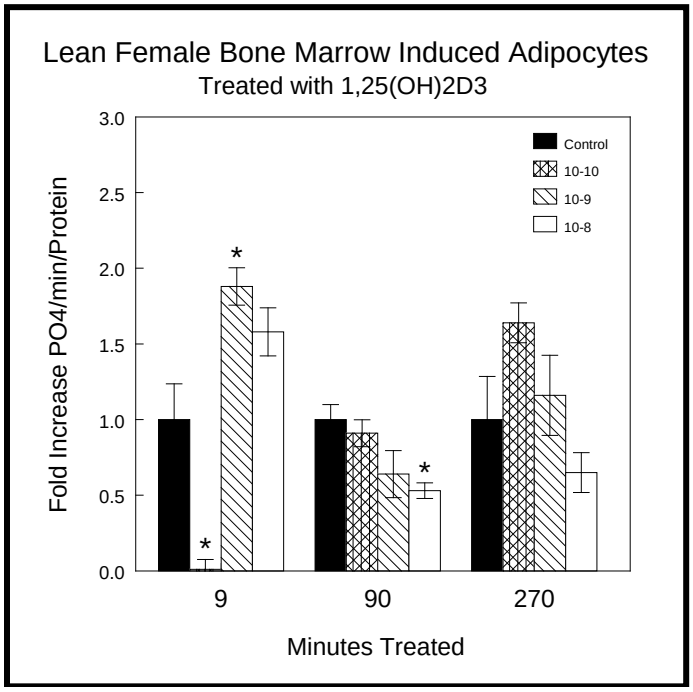


Figure 3.3.1.1 PKC activity after 1α,25(OH)$_2$D$_3$ treatment for 9, 90, and 270 minutes on bone marrow induced adipocytes from lean female mice.

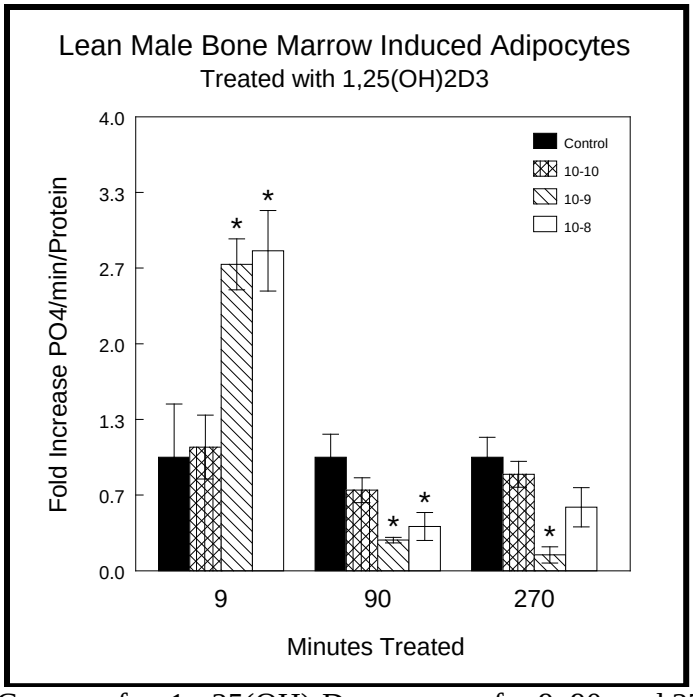


Figure 3.3.1.2 PKC assay after 1α,25$(OH)_2D_3$ treatment for 9, 90, and 270 minutes on bone marrow induced adipocytes from lean male mice.

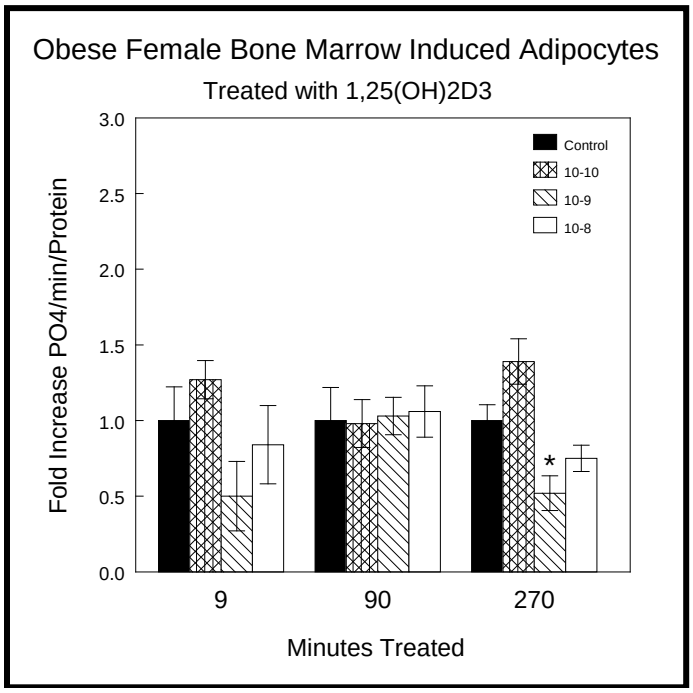


Figure 3.3.1.3 PKC assay after 1α,25$(OH)_2D_3$ treatment for 9, 90, and 270 minutes on bone marrow induced adipocytes from obese female mice.

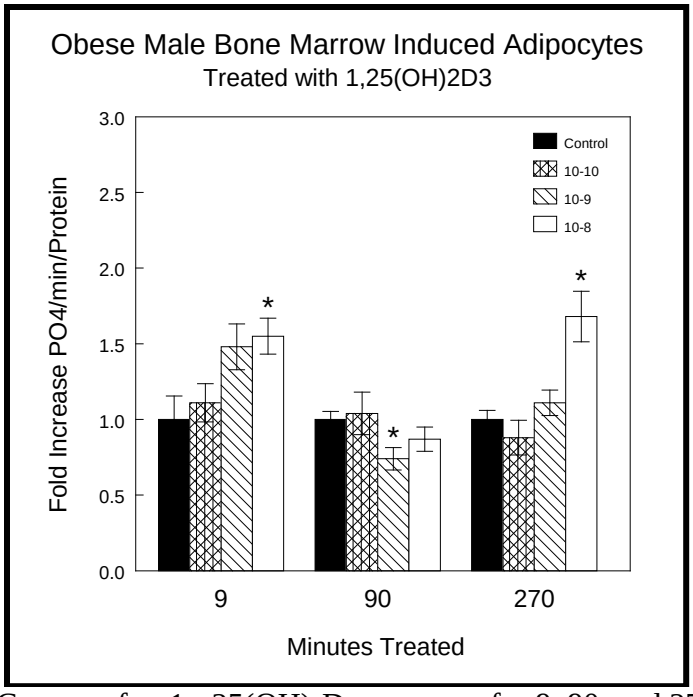


Figure 3.3.1.4 PKC assay after $1\alpha,25(OH)_2D_3$ treatment for 9, 90, and 270 minutes on bone marrow induced adipocytes from obese male mice.

3.3.2 PKC Assay in Fat Pad Adipocytes

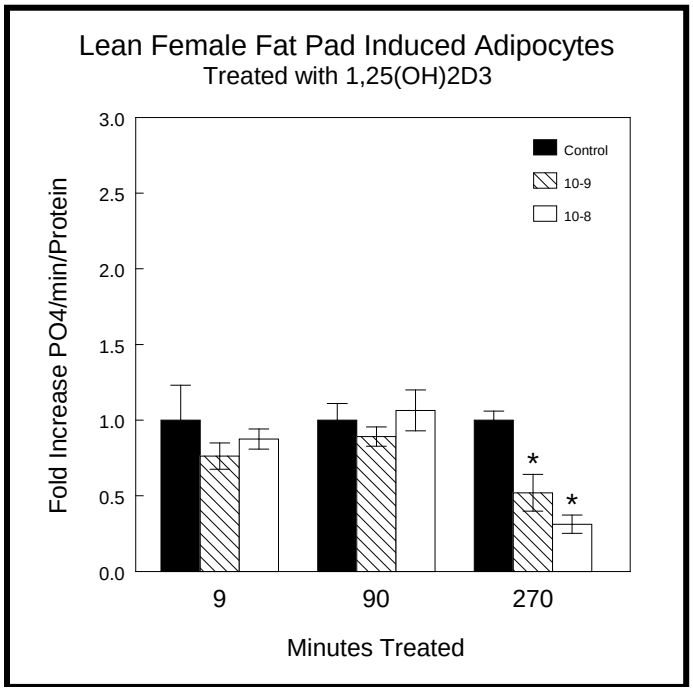


Figure 3.3.2.1 PKC assay after $1\alpha,25(OH)_2D_3$ treatment for 9, 90, and 270 minutes on fat pad adipocytes from lean female mice.

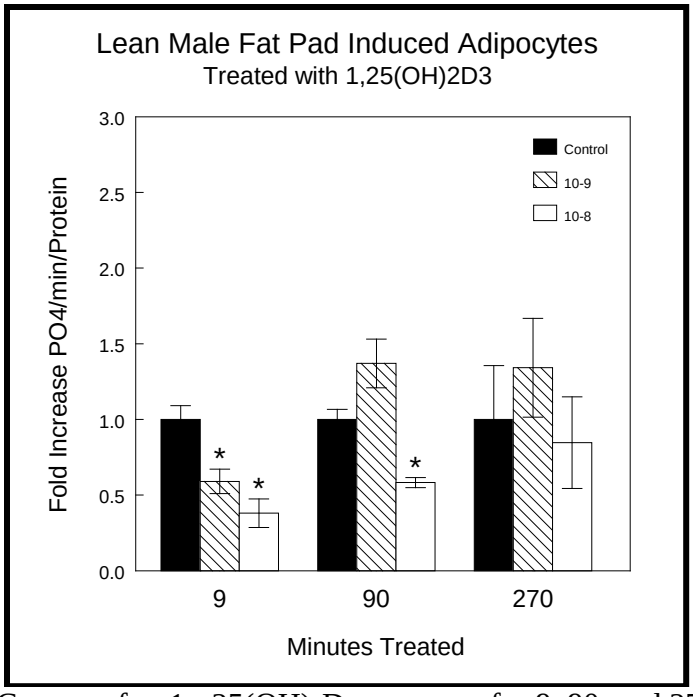


Figure 3.3.2.2 PKC assay after $1\alpha,25(OH)_2D_3$ treatment for 9, 90, and 270 minutes on fat pad adipocytes from lean male mice.

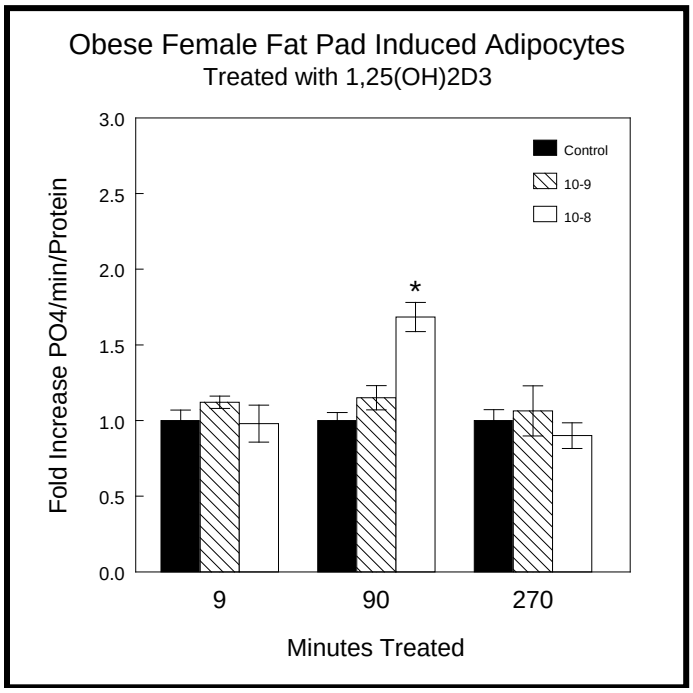


Figure 3.3.2.3 PKC assay after $1\alpha,25(OH)_2D_3$ treatment for 9, 90, and 270 minutes on fat pad adipocytes from obese female mice.

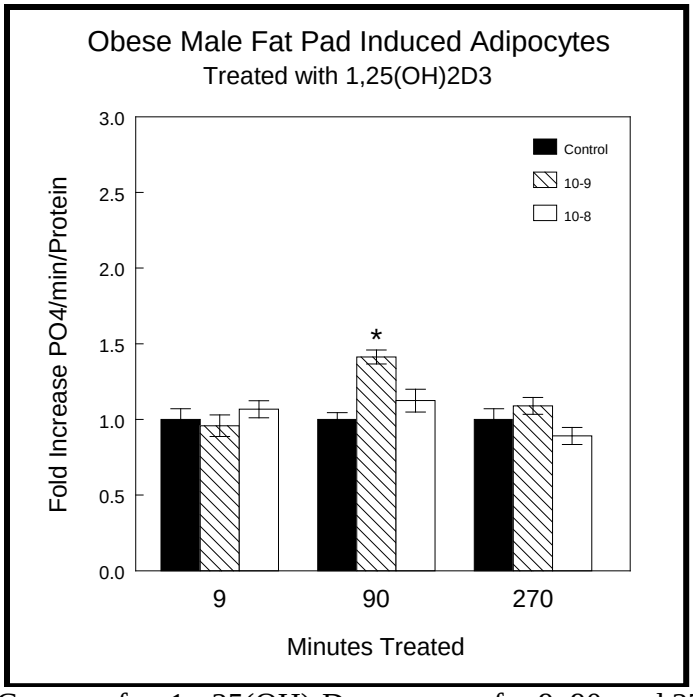
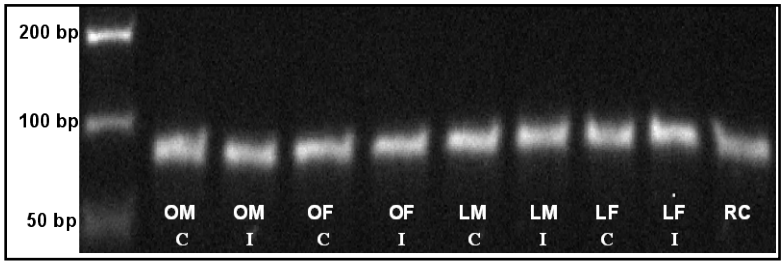


Figure 3.3.2.4 PKC assay after 1α,25$(OH)_2D_3$ treatment for 9, 90, and 270 minutes on fat pad adipocytes from obese male mice.

3.4 Vitamin D Receptor Expression



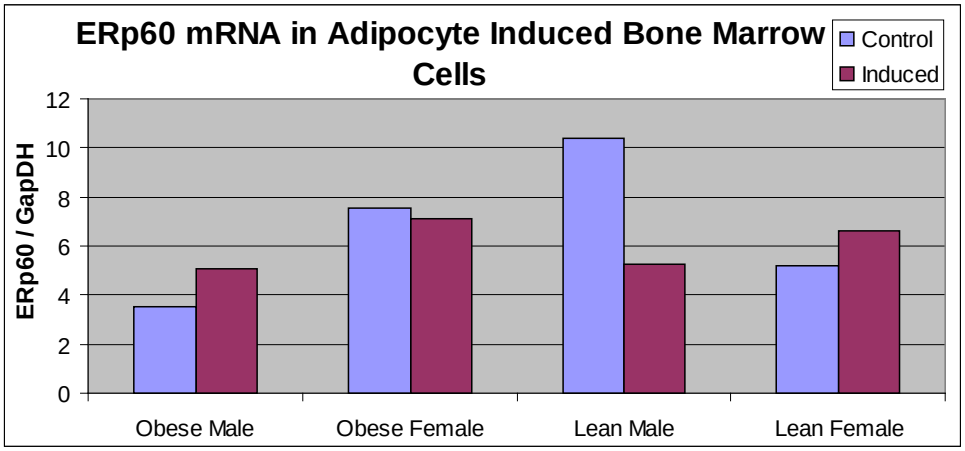


Figure 3.4.1 RT-PCR and qPCR for ERp60 in BM adipocytes; control, C, and induced, I. Positive control used in the gel is a whole tissue mRNA extraction from rat chondrocytes.

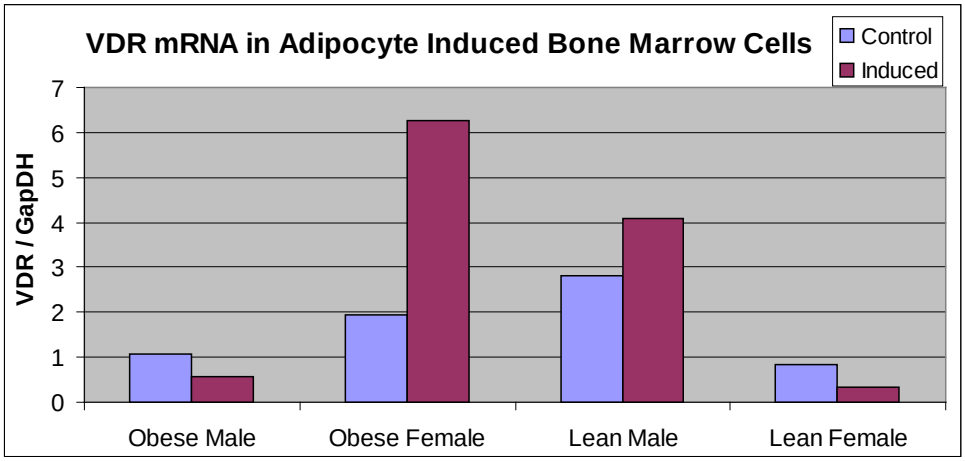


Figure 3.4.2 qPCR for VDR in BM adipocytes: control, C, and induced, I. (Gel image not shown).

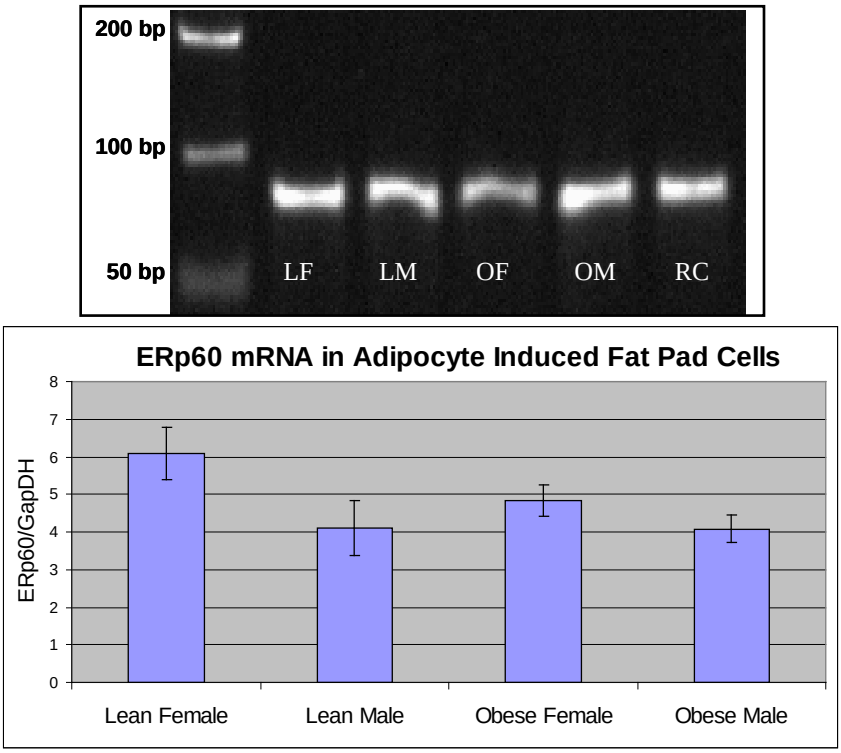


Figure 3.4.3 RT-PCR and qPCR for ERp60 in adipocyte fat pad cells. Positive control used in the gel is a whole tissue mRNA extraction from rat chondrocytes.

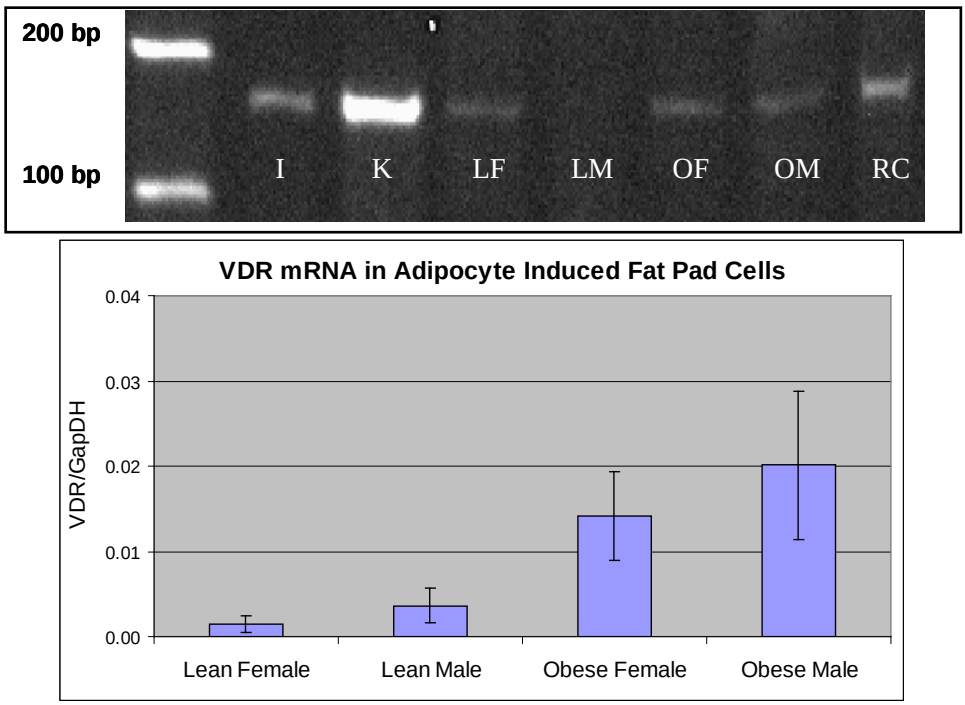


Figure 3.4.4 RT-PCR and qPCR for VDR in adipocyte fat pad cells. Positive controls used in the gel image are whole tissue mRNA extractions from: I=Mouse Intestine, K=Mouse Kidney, RC= Rat Chondrocytes.

3.5 Vitamin D Receptor Protein in Plasma Membrane

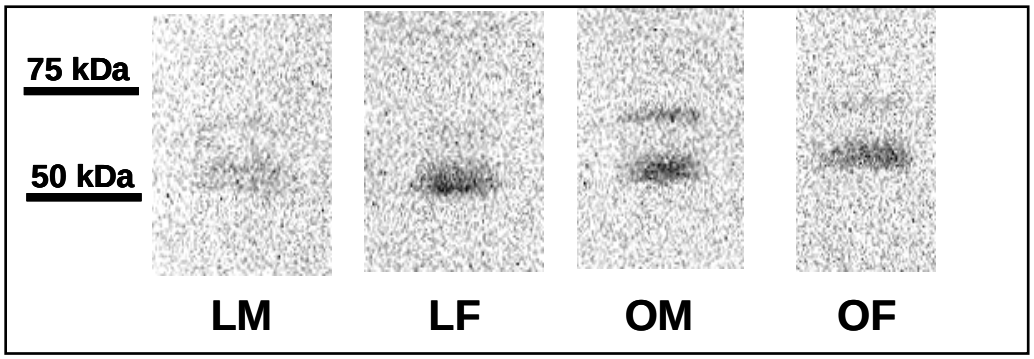


Figure 3.5.1 Western blot for ERp60 in fat pad adipocyte plasma membrane fractions harvested from lean and obese male and female mice.

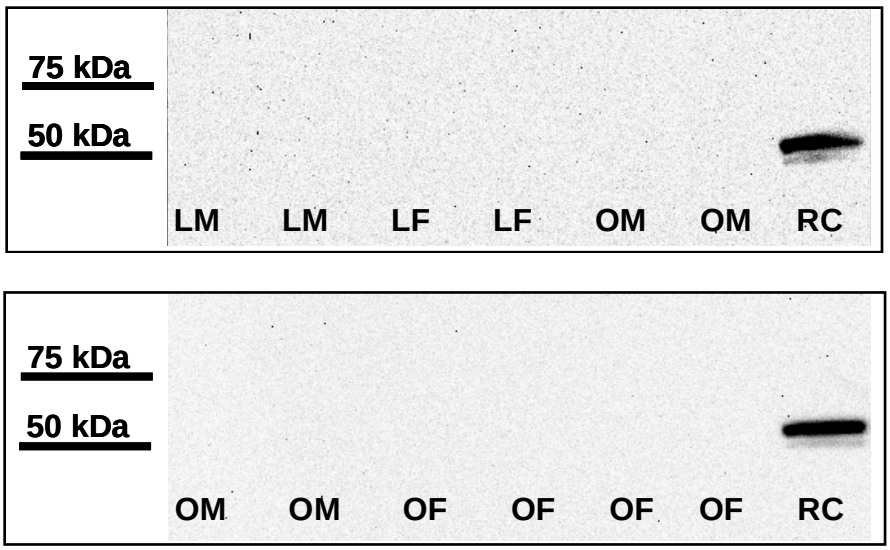


Figure 3.5.2 Western blot for nVDR in fat pad adipocyte plasma membrane fractions harvested from lean and obese male and female mice. Positive control used is a whole cell lysate from rat chondrocytes.

CHAPTER 4

CONCLUSIONS

General findings show that all of the adipocytes tested exhibit a PKC response to $1\alpha,25(OH)_2D_3$ treatment, although fat pad adipocytes behave differently from bone marrow induced adipocytes. Nuclear and Membrane Vitamin D Receptors are present in adipocytes; primary and induced from bone marrow. Lean adipocytes have increased sensitivity to Vitamin D when compared to obese adipocytes.

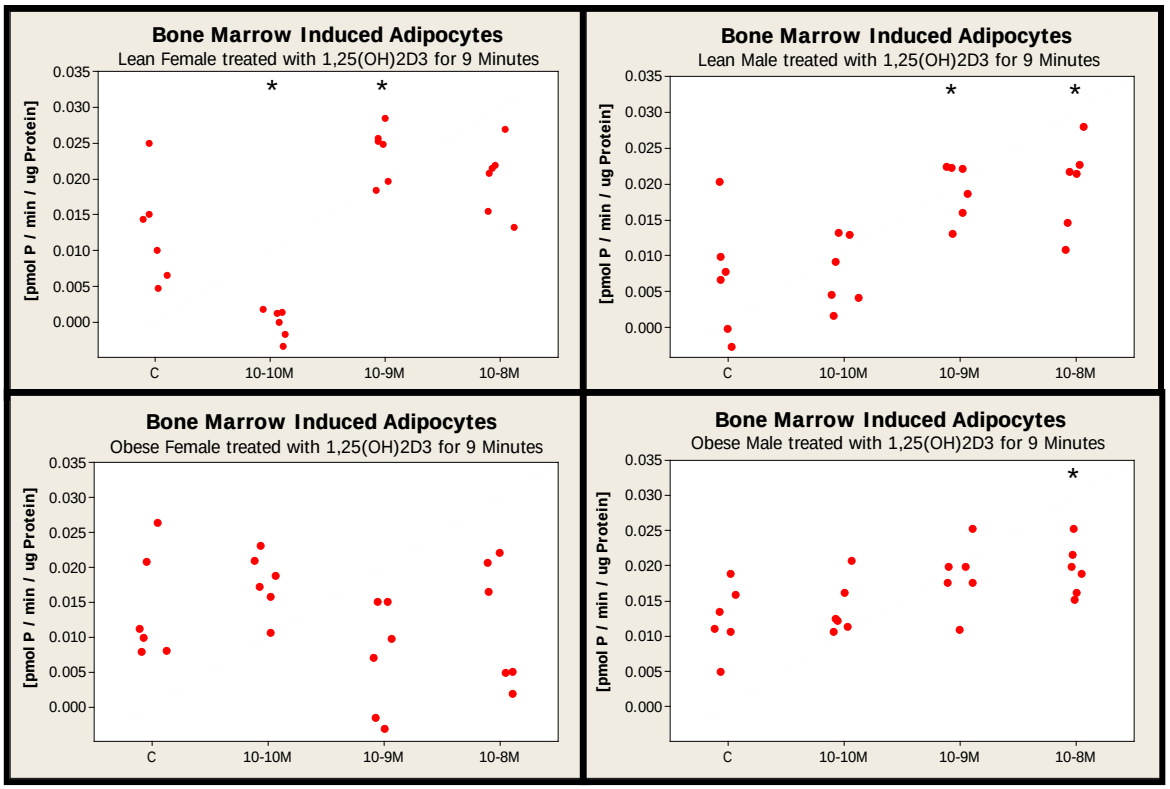
The most notable finding when investigating the fat pad adipocytes was that nVDR protein was not found in the plasma membrane fractions whereas ERP60 protein was found in the plasma membrane fractions of all the samples tested. This leads to the conclusion that the rapid response seen in the fat pad adipocytes could not have been mediated by nVDR protein and also points to ERP60 as a player in rapid PKC activation for these primary cells. Consistent with current literature, the data shows that $1\alpha,25(OH)_2D_3$ decreased adipogenesis in fat pad adipocytes independent of gender. The effect of $1\alpha,25(OH)_2D_3$ on PKC activity in fat pad adipocytes seems to differ for lean mice, showing decreased activity, versus obese mice, showing increased activity.

The bone marrow induced adipocytes had various conflicting responses to $1\alpha,25(OH)_2D_3$ exposure. Adipogenesis increased in males cells after exposure, while decreasing in female cells. Cell viability increased in the obese cells after exposure, while $1\alpha,25(OH)_2D_3$ had no effect or slightly lowered cell viability in lean cells. PKC activity decreased in obese female cells after exposure, while in all other bone marrow induced adipocytes there was an initial increase followed by a decrease.

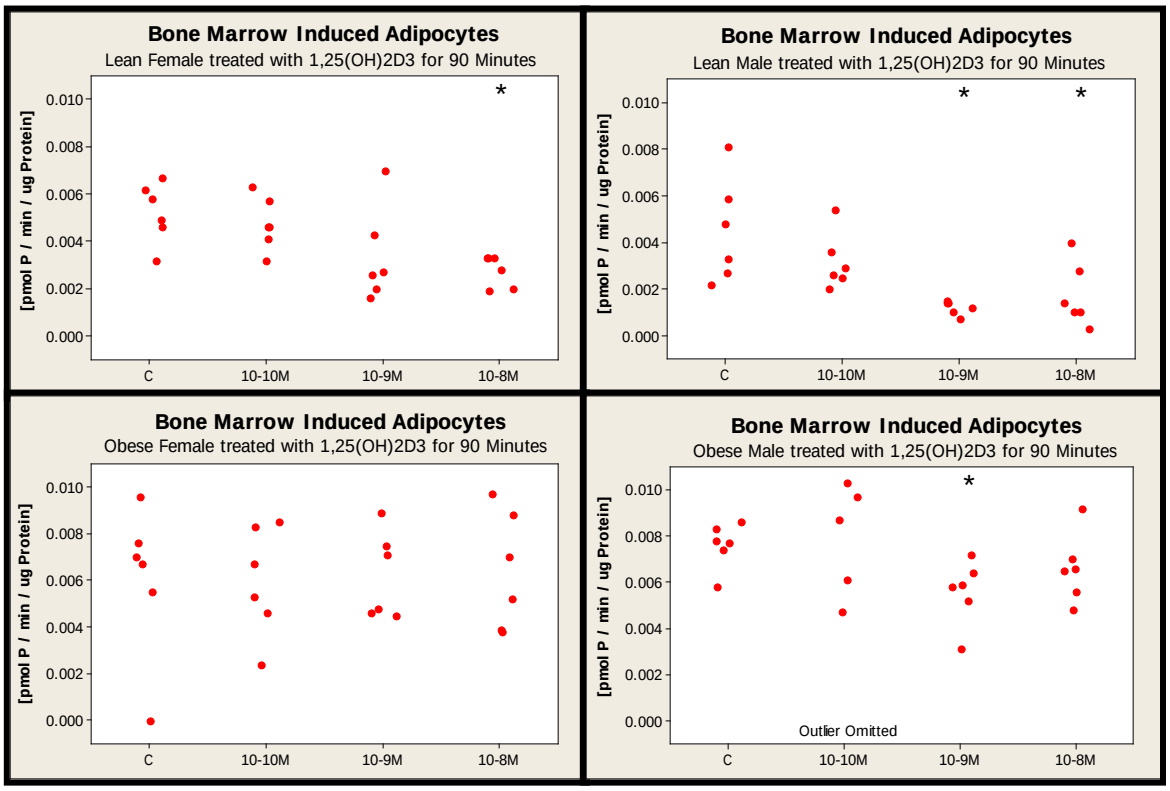
APPENDIX A – RAW DATA

Bone Marrow Induced Adipocytes, PKC Assay

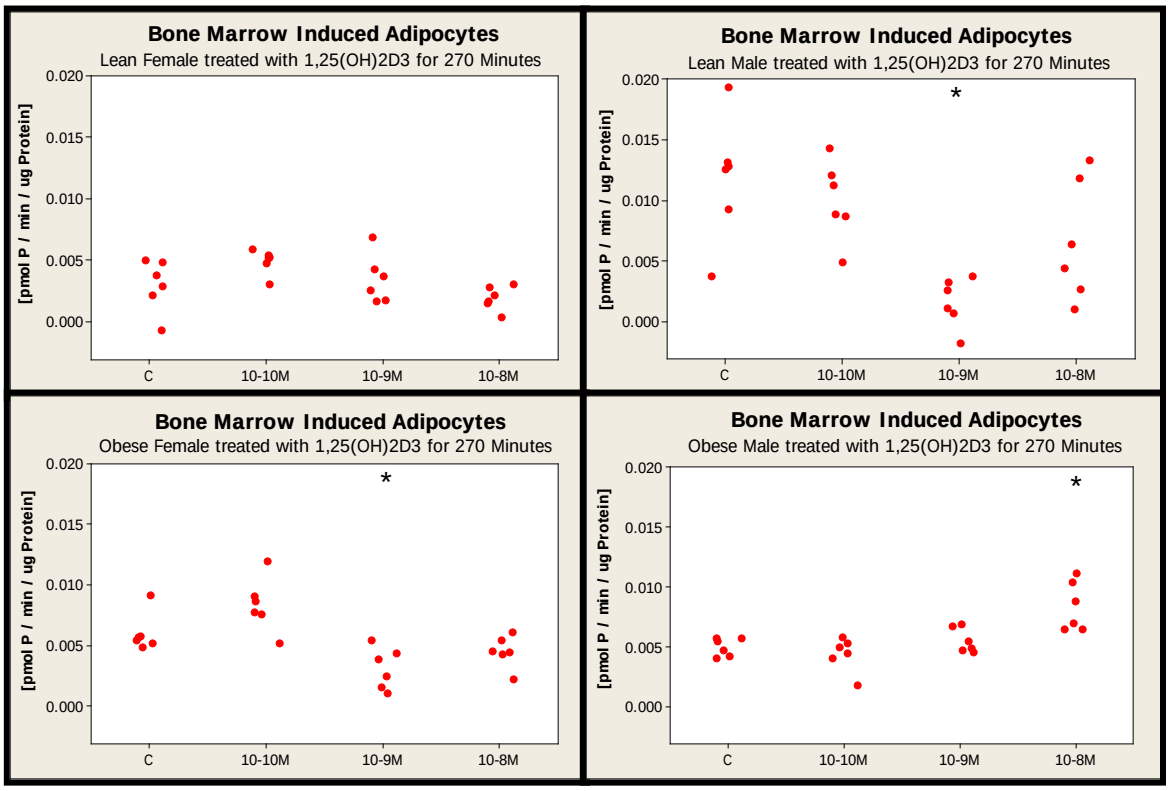
Data sets are grouped based on experimental set-up. Data generated from cell cultures that were treated, harvested, and assayed at the same time are grouped together.



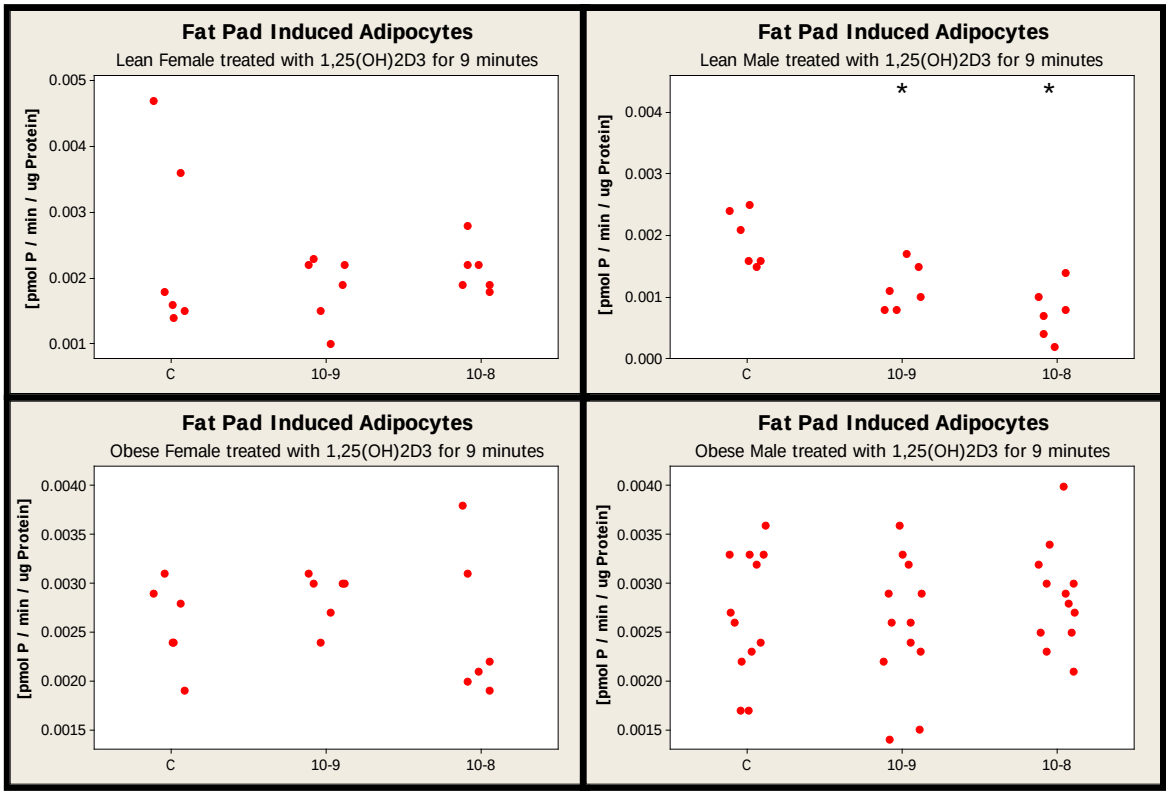
Graph A.1 – PKC assay after 1α,25$(OH)_2D_3$ treatment for 9 minutes on bone marrow induced adipocytes from lean female, lean male, obese female and obese male mice. Statistical significance is compared to control, determined by 2 sample t-test ($p \leq 0.05$)



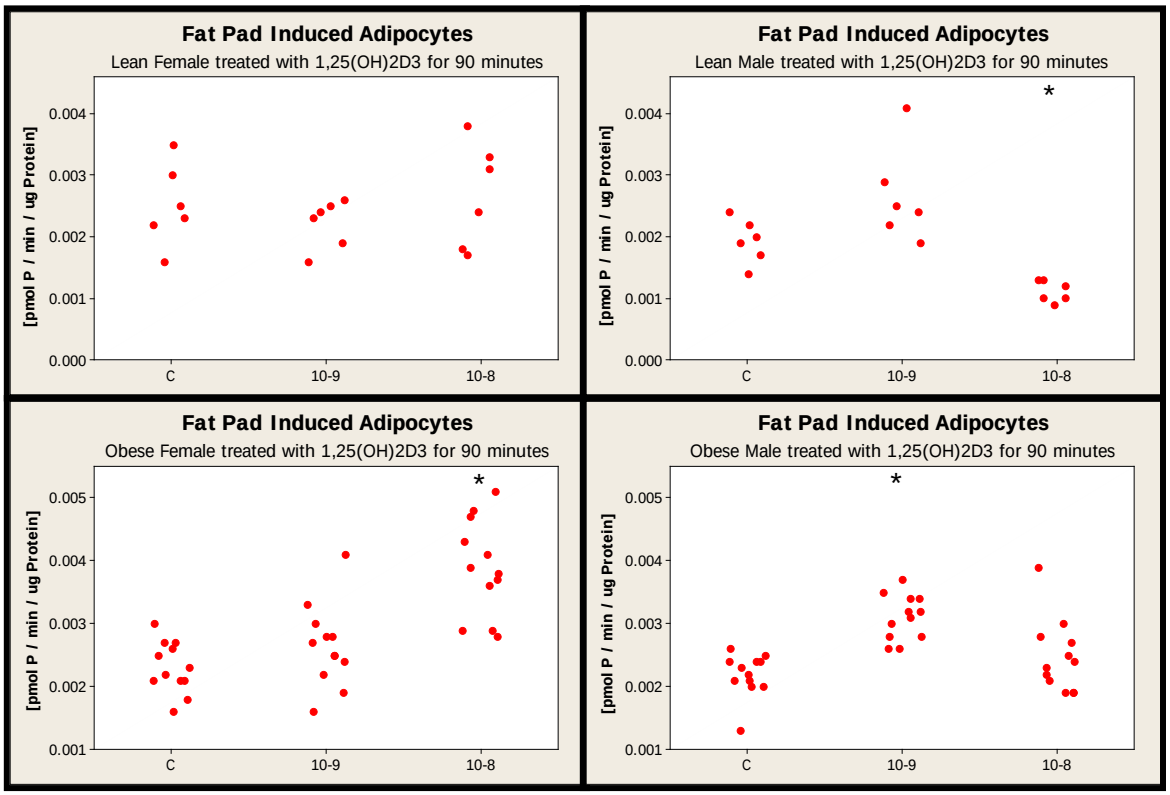
Graph A.2 – PKC assay after $1\alpha,25(OH)_2D_3$ treatment for 90 minutes on bone marrow induced adipocytes from lean female, lean male, obese female and obese male mice. A single outlier was omitted (value = -0.0139) from the Obese Male group treated with 10^{-10}M $1\alpha,25(OH)_2D_3$ due to an error when reading the protein level of the sample. Statistical significance is compared to control, determined by 2 sample t-test ($p \leq 0.05$)



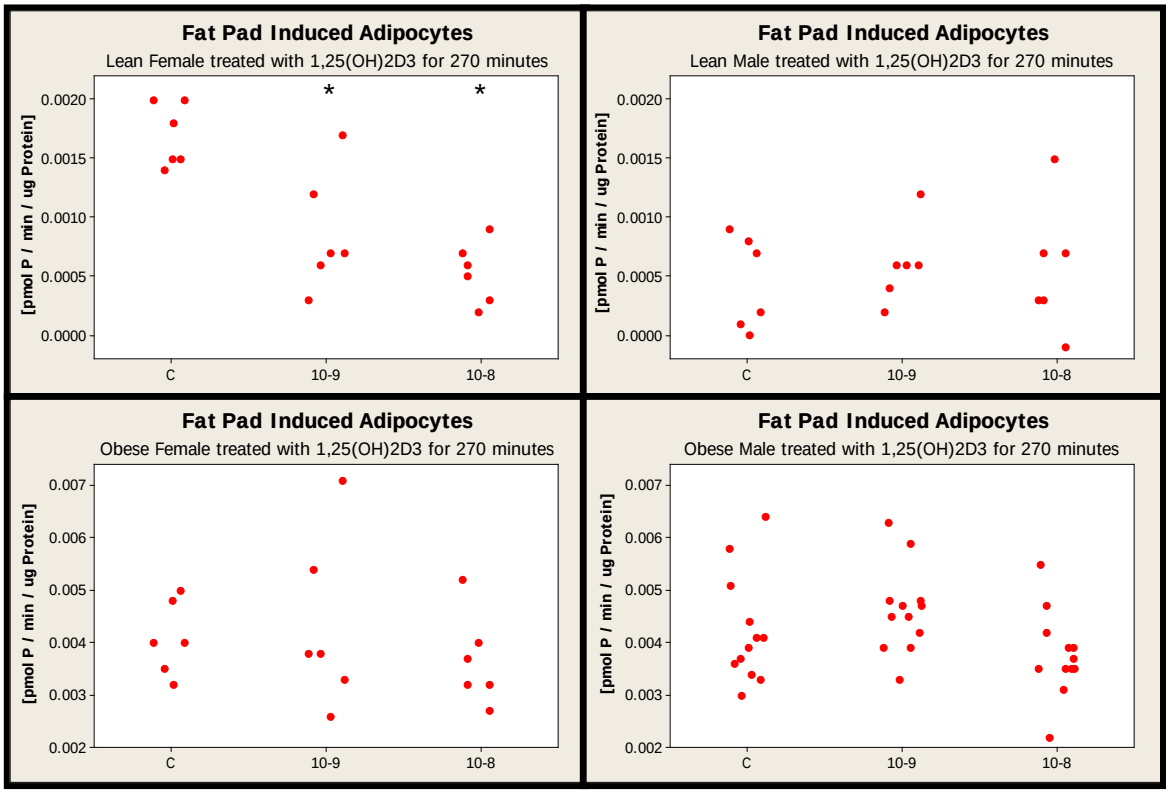
Graph A.3 – PKC assay after 1α,25$(OH)_2D_3$ treatment for 270 minutes on bone marrow induced adipocytes from lean female, lean male, obese female and obese male mice. Statistical significance is compared to control, determined by 2 sample t-tests ($p \leq 0.05$)



Graph A.4 – PKC assay after 1α,25(OH)$_2$D$_3$ treatment for 9 minutes on fat pad adipocytes from lean female, lean male, obese female, and obese male mice. Statistical significance is compared to control, determined by 2 sample t-tests ($p \leq 0.05$)



Graph A.5 – PKC assay after 1α,25(OH)$_2$D$_3$ treatment for 90 minutes on fat pad adipocytes from lean female and lean male mice. Statistical significance is compared to control, determined by 2 sample t-tests ($p \leq 0.05$)



Graph A.6 – PKC assay after 1α,25$(OH)_2D_3$ treatment for 270 minutes on fat pad adipocytes from lean female and lean male mice. Statistical significance is compared to control, determined by 2 sample t-tests ($p \leq 0.05$)

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Leonard J. July 1981, THE JOURNAL OF BIOLOGICAL CHEMISTRY, Vols. Vol. 256, No. 14, pp. 7115-7117.

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