EXPRESSION OF FATTY ACID OXIDATION-RELATED GENES IN *Acsl4* $^{L\,-/-}$ PRIMARY HEPATOCYTES

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Abstract

Long-chain acyl-CoA synthetases are a family of enzymes responsible for catalyzing the reaction converting long chain fatty acids to fatty acyl-CoA. ACSL4, a specific member of this family, plays a role in eicosanoid metabolism and has been found to be associated with liver diseases including non-alcoholic fatty liver disease and hepatocellular carcinoma. In order to study ACSL4 in the context of hepatic lipid metabolism, a murine liver-specific Acsl4 knockout (*Acsl4* $^{L -/-}$) was developed and characterized in our lab. Based on a previous experiment in our lab, we hypothesized that *Acsl4* $^{L -/-}$ would cause a defect in beta adrenergic signaling. In order to test this hypothesis, we conducted experiments to determine the effects of various treatments on the expression of fatty acid oxidation-related genes in both knockout and control hepatocytes. Treatments with fatty acids caused increases in expression of several fatty acid oxidation-related genes in both knockout and control hepatocytes. Hormone treatments did not cause anticipated increases in expression of fatty acid oxidation-related genes in several of the control groups. Because of this we were not able to make conclusions about the effects of the knockout. These results indicated that *Acsl4* $^{L -/-}$ did not cause a defect in beta adrenergic signaling.

Acknowledgements

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Chapter I. Introduction

1.1 Liver and Lipid Metabolism

The liver is perhaps the most important organ when it comes to lipid metabolism, acting as the main site for fatty acid (FA) synthesis, fatty acid oxidation, complex lipid synthesis, and very low-density lipoprotein (VLDL) production. The liver acts as a mediator for fat metabolism to meet the energy requirements of the rest of the body. These metabolic pathways are controlled by various enzyme interactions and regulated by the liver under different hormonal and physiological conditions.

Lipid metabolism starts with the digestion and absorption of dietary fats. In the mouth, lipids are first broken down by lingual lipases and further processed in the stomach and duodenum where dietary fats are emulsified with help from bile acids secreted from the gallbladder. Lipases secreted from the pancreas further digest lipids into free FA and monoacylglycerol to be absorbed by enterocytes. Within the enterocyte, triacylglerol (TAG) molecules are resynthesized and repackaged along with cholesterol esters (CE), phospholipids (PL), lipoproteins, and fat-soluble vitamins into chylomicron lipoproteins that pass into lacteals that eventually enter circulation. Peripheral tissues hydrolyze TAG from chylomicrons via lipoprotein lipase to obtain FA while remnant chylomicrons and other lipoproteins are taken up by the liver. The liver incorporates the components of remnant lipoproteins particles into various metabolic pathways.

Long-chain FAs are activated by the long chain acyl-CoA synthetase (ACSL) enzyme family, which converts long chain FAs into long chain fatty acyl-CoAs and shuttles them into different metabolic pathways.[1] Depending on energy status, activated FAs can enter synthetic pathways such as *de novo* lipogenesis, TAG synthesis, and phospholipid synthesis. TAG synthesis is initiated by ACSLs, with glycerol-3-phosphate acyltransferase (GPAT) catalyzing the formation of TAG by esterifying glycerol-3-phosphate with acyl-CoA. Acetyl-CoA carboxylase (ACC) catalyzes the conversion of acetyl-CoA to malonyl-CoA and is a rate-limiting enzyme of *de novo* lipogenesis. ACC activity is inhibited under low energy conditions. Elongation of malonyl-CoA for FA synthesis is catalyzed by fatty acid synthetase (FAS). These processes are highly regulated by insulin and glucagon signals that regulate appropriate pathways during fed and fasting conditions respectively. As the major lipid metabolism center of the body,

the liver also serves as the main distribution center. It exports lipids, TAG, CE, and phospholipids by producing and secreting VLDL for peripheral utilization of lipids and FAs. Hepatic concentrations of lipid and apolipoprotein B100 directly affect VLDL production and secretion.

Beta-oxidation is the degradative pathway of FAs activated by ACSLs, which produces acetyl-CoA from fatty acyl-CoA to be used as energy production via the Krebs cycle and oxidative phosphorylation. The main regulated enzyme of beta-oxidation is carnitine palmitoyltransferase I (CPT-1), which catalyzes the entry of fatty acyl CoA into the mitochondria via conversion to acyl-carnitine. CPT-1 is inhibited by malonyl CoA, which is produced by ACC during de novo lipogenesis. Beta-oxidation is also regulated by the transcription factor peroxisome proliferator-activated receptor alpha (PPARa). PPARa ligand activation induces several genes controlling FA import including CPT-1 and several major enzymes within the beta-oxidation pathway including acyl-CoA dehydrogenases.[2]

1.2 ACSL4 Gene and Protein

While all enzymes in the ACSL family serve the same enzymatic function, the ACSL isoforms vary in substrate specificity, subcellular location, and potential impact on downstream metabolic functions. With the exception of ACSL4, which is localized to both the plasma membrane and cytosol, most of the ACSL isoforms are membrane associated. [3] Accurate methods for measuring enzymatic activity for individual ACSL isoforms have not been well established due to non-specificity of ACSL inhibitors. It is still unclear how each isoform's distinct structure and subcellular location affect function and activity. This study aims to investigate the effects of a liver-specific ACSL4 knock out (*ACSL4* $^{L-/-}$) mouse model to study the role of ACSL4 in hepatic lipid metabolism.

ACSL4 is a member of the ACS family that is encoded by the *Acsl4* gene located on the X-chromosome adjacent to the *Col4a5* gene at Xq23 [4]. The ACSL4 protein contains 711 amino acids with an AMP-dependent synthetase domain and an AMP-binding domain. Alternative splicing produces two possible forms of the ACSL4 isoform. ACSL4 variant 1 is a 75 kDa protein located within the cytosol. ACSL4 variant 2 is a 79 kDa protein that contains additional hydrophobic amino acids at the N-terminus that contribute to membrane localization in both the outer mitochondrial membrane and the endoplasmic reticulum.[5]

1.3 ACSL4 Abnormalities and Human Disease

Sequence abnormalities and sequence deletions surrounding the *Acsl4* gene have been associated with several conditions including elliptocytosis, mental retardation, and Alport syndrome. Alport syndrome is an X-linked condition that is characterized by a large deletion of the Xq22.3-23 (including the *Acsl4* gene) region of the X-chromosome. Its symptoms include progressive renal failure, hearing loss, and vision loss. High *Acsl4* expression in specific regions of the brain, including the cerebellum and hippocampus, and its deficiencies associated with mental retardation suggest that the enzyme may play an important role in brain development.[6] Investigations of *Acsl4* expression during mouse development showed high levels of expression in mice embryos and newborn brains.[7]

ACSL4 dysregulation has also been studied in the context of hepatic lipid dysfunction associated with non-alcoholic fatty liver disease (NAFLD) and hepatocellular carcinoma. Studies using CLOCK-deficient mouse models found that reduced *Acsl4* and *Fabp1* expression was associated with reduced steatosis on a high fat diet.[8] This suggests that ACSL4 may play a role in the development of hepatosteatosis and circadian controlled lipid metabolism. In human studies, researchers identified polymorphisms in *Acsl4* that were related to liver fat content. Higher hepatic lipid content of NAFLD patients, along with hyperinsulinemia and obesity, were associated with the expression of a rare ACSL4 rs7887981 allele.[9] ACSL4 was also found to be involved in hepatocellular carcinoma development.[10] Several human hepatoma cell lines, including Hep3B and HepG2, have exhibited high ACSL4 expression.[11] In patients, ACSL4 has been found to be up-regulated (between 2.3 and 27.5-fold) in hepatocellular carcinoma compared to adjacent non-cancerous tissue.[11] These findings suggest that ACSL4 expression may contribute to dysregulation in hepatic lipid metabolism associated with both cancer and steatosis.

1.4 ACSL4 Tissue and Substrate Preferences

As mentioned before, each ACS isoform has a specific FA substrate preference and a unique distribution across tissues in the body. Kinetic analyses have shown that ACSL4 has a preference for polyunsaturated FAs, with lower affinities for saturated and mono, di-, and tri-unsaturated FAs. The enzyme preferentially utilizes arachidonate and eicosapentaenoate as substrates over the other 8-12 carbon saturated FAs and 14-22 carbon unsaturated FAs examined in the analyses.[6] ACSL4 is present in the brain and steroidogenic tissues. RNA analyses have

revealed the presence of *Acsl4* transcripts in the adrenal cortex, brain, lung, ovaries, testes, and liver.[6] Because of its preference for polyunsaturated FAs, ACSL4 may play an important role in eicosanoid metabolism. Arachidonate has potential to generate inflammatory products as a precursor to several clinically important eicosanoids and metabolites of eicosanoids. Cyclooxygenases metabolize arachidonate to prostaglandins and thromboxanes that mediate inflammation and platelet aggregation. To prevent excessive eicosanoid synthesis, free arachidonate is converted to arachidonoyl-CoA by ACS enzymes and re-esterified into phospholipids.

1.5 Study Aims and Hypothesis:

Liver-specific ACSL4 knock out mice *(ACSL4* $^{L\,-/-}$) have been developed to further investigate the role of ACSL4 in hepatic lipid metabolism. Under fasting and ketogenic conditions, *ACSL4* $^{L\,-/-}$ mice and control mice do not exhibit differences in ACS activity, serum TAG, or serum glucose. Ongoing studies have suggested that *ACSL4* $^{L\,-/-}$ may affect transcriptional regulation of beta-oxidation by disrupting adrenergic signaling. Hepatic lipid metabolism is tightly controlled by hormone responses. Glucagon and catecholamines, such as norepinephrine, stimulate G-protein coupled receptors and initiate signaling cascades that regulate both glucose and lipid homeostasis via transcriptional regulation. Stimulation of adrenergic and glucagon receptors increases production of cAMP and activates protein kinase A (PKA). PKA phosphorylates transcription factor CREB, which induces PGC-1α gene expression. Acting as a coactivator, PGC-1α is recruited by transcription factors such as PPARα that bind to the promoter regions of FA oxidation enzyme genes to increase expression.[12]

A previous study tested the effects of norepinephrine treatments on cultured primary hepatocytes from both control and *ACSL4* $^{L\,-/-}$ mice. Results showed lowered gene expression of beta-oxidation genes among *ACSL4* $^{L\,-/-}$ hepatocytes after treatment compared to control. Treatments with PKA activator 8-Br-cAMP (a long-acting derivative of cAMP) induced similar levels of gene expression of beta-oxidation genes in both *ACSL4* $^{L\,-/-}$ and control hepatocytes suggesting a possible role of ACSL4 in adrenergic signaling pathway before PKA activation. Oleic acid, but not long chain saturated or polyunsaturated FAs, has been found to stimulate cAMP production and PKA activity. PKA phosphorylates SIRT1, which then deacetylates and activates PGC-1α to subsequently increase expression of beta-oxidation genes.[13]

Therefore, our study aimed to examine the effects of *Acsl4* deletion on the expression of FA oxidation genes after treatments with several hormones and metabolites that promote oxidation and to validate the findings of the previous study. We hypothesize that after treatments that activate the adrenergic receptor, *ACSL4* $^{L -/-}$ hepatocytes will exhibit lower FA oxidation gene expression compared to control. We also hypothesize that after oleic acid treatment, *ACSL4* $^{L -/-}$ hepatocytes will exhibit greater FA oxidation gene expression compared to control. Lastly, we hypothesize that after treatments that directly elevate intracellular cAMP levels, *ACSL4* $^{L -/-}$ and control hepatocytes will have similar FA oxidation gene expression.

Chapter II. Methods and Materials

2.1 Primary hepatocyte isolation and preparation

Primary hepatocytes were isolated from the livers of 3-month-old *ACSL4* $^{L-/-}$ mice and floxed control littermates. Because *Acsl4* is X-linked, we used male knockout mice for our experiments. Hepatocytes were plated at 4 X 10^5 cells per well in 6-well plates with William's E. media (5% serum). After two hours, William's E. media was replaced with low glucose DMEM (1g/L glucose, 10% fetal bovine serum, penicillin-streptomycin). Before treatments, primary hepatocytes of both genotypes were serum starved in low glucose DMEM (5mM glucose, 1mM sodium pyruvate, 1mM L-Glutamine, serum free) media using 1 ml per well for 4 hours.

2.2 Primary hepatocyte hormone treatments

All treatments were prepared using the same glucose (5mM) DMEM media at 1 mL per well. Hormone solutions were added to three wells of each plate and the corresponding vehicle control solutions were added to the remaining three wells of each plate. The norepinephrine solution was prepared at 10μM from a 10mM norepinephrine stock (dissolved in water) diluted in media. The 8-Br-cAMP treatment solution was prepared at 1 mM from an 80mM stock (dissolved in water) diluted in media. The forskolin treatment solution was prepared at 40 μM from a 4mM stock (dissolved in DMSO) diluted in media. The glucagon treatment solution was prepared at 100mM from a 1mg/mL stock (dissolved in phosphate buffered saline) diluted in media. All vehicle control solutions were prepared by diluting the corresponding vehicle (Table 1) in media by the same dilution factor used for diluting the stock hormone solution. After incubating the hepatocytes for 1 hour, the cells were washed twice with chilled PBS and frozen at -80° C.

2.3 Primary hepatocyte lipid treatments

All treatments were prepared using the same glucose (5mM) DMEM media at 1 mL per well. Lipid solutions were added to three wells of each plate and the corresponding vehicle control solutions were added to the remaining three wells of each plate. The oleate treatment was prepared at 500μM from a 100mM stock (in 50% ethanol) diluted in media. The palmitate treatment was prepared at 500μM from a 200mM stock (in 50% ethanol) diluted in media. Both

stock solutions were first diluted with fatty acid free bovine serum albumin (1:25 for oleate and 1:50 for palmitate) and allowed to complex at 37° C for 1 hour. Carnitine and serum free media was added to both solutions for final concentrations of 1mM carnitine and 500µM of FA. After treating the primary hepatocytes for 3.5 hours, the cells were washed twice with chilled PBS and stored at -80°C.

2.4 Gene expression in *ACSL4* $^{L-/-}$ and control primary hepatocytes

Before testing expression of FA oxidation genes, the liver-specific knockout of *Acsl4* was confirmed. RNA was extracted from both *ACSL4* $^{L-/-}$ hepatocytes and littermate control hepatocytes using phenol chloroform extraction (Invitrogen TRIzol reagent). RNA concentration was measured by spectrophotometry and cDNA was made by reverse transcription (iScript cDNA synthesis kit) using 1µg of RNA. The mRNA levels of *Acsl4* were quantified with real time PCR using ACSL4 and β-actin primers (Table 1) to determine the relative expression of *Acsl4* compared to *Actin* (Biorad iTaq universal SYBR green supermix). *Acsl4* expression in *ACSL4* $^{L-/-}$ mice was less than 1% of control littermates, confirming the knockout. The same process was carried out to determine the gene expression levels of the other genes of interest using the corresponding forward and reverse primers (Table 2) and normalizing to *Actin* expression. For each data set generated, cycle threshold values were normalized to *Actin* and treatment control values.

Chapter III. Results

3.1 Oleate and palmitate treatment effects on gene expression

To determine whether *ACSL4* $^{L-/-}$ disrupts oleate's signaling effects on the expression of beta-oxidation genes, *ACSL4* $^{L-/-}$ and control hepatocytes were treated with oleate and palmitate. Two experiments were conducted using a different set of hepatocytes. RT-PCR results from Experiment 1 showed increased *Cpt1a, Acox1, Pgc1a*, and *Ppara* expression (relative to *Actin*) following both oleate and palmitate treatments (Figure 1). These increases in gene expression occurred in both *ACSL4* $^{L-/-}$ and control hepatocytes. Experiment 2 results, with palmitate treatment, showed increased *Cpt1a, Acox1*, *Ppara* and *Creb* expression in control hepatocytes along with increased *Acox1* and decrease *Ppara* expression in *ACSL4* $^{L-/-}$ hepatocytes (Figure 2). Experiment 2 results, with oleate treatment, showed increased Creb expression and decreased Pgc-1α and Pparα expression in control hepatocytes. In *ACSL4* $^{L-/-}$ hepatocytes, oleate treatment resulted in increased Creb and Acox1 expression and decreased Pgc-1α and Pparα expression.

3.2 Hormone treatment effects on gene expression

To investigate whether *ACSL4* $^{L-/-}$ disrupts adrenergic signaling effects on beta oxidation gene expression, *ACSL4* $^{L-/-}$ and control hepatocytes were treated with norepinephrine, forskolin, glucagon, and 8-Br-cAMP. All of these hormone treatments increase expression of fatty acid oxidation-related genes by increasing intracellular cAMP (Figure 5). RT-PCR results from Experiment 1 showed increased *Pgc1a, Cpt1a*, and *Ppara* expression following norepinephrine treatment only in control hepatocytes (Figure 3A). With 8-Br-cAMP treatment, *Pgc1a* expression increased only in *ACSL4* $^{L-/-}$ hepatocytes. There was also lowered *Cpt1a* and *Ppara* expression among all *ACSL4* $^{L-/-}$ hepatocytes in the 8-Br-cAMP study compared to control (Figure 3B). After forskolin treatment, *Ppar* expression decreased only in *ACSL4* $^{L-/-}$ hepatocytes (Figure 3C). After glucagon treatment, *Pepck* expression increased only in control hepatocytes (Figure 3C). There was also lowered *Pdk4* expression in *ACSL4* $^{L-/-}$ hepatocytes compared to control following glucagon treatment.

After correcting the defects of Experiment 1, the experiment was repeated. Experiment 2 results showed different patterns of gene expression after treating hepatocytes with

norepinephrine and 8-Br-cAMP treatments. Unlike in Experiment 1, norepinephrine treatment increased *Pgc1a* expression in both *ACSL4* $^{L-/-}$ and control hepatocytes and increased *Cpt1a* expression in only *ACSL4* $^{L-/-}$ hepatocytes. *Cpt1a* and *Ppara* expression decreased among control hepatocytes following norepinephrine treatment (Figure 4A). Treatment with 8-Br-cAMP only affected *Pgc1a* expression, with increased expression in both control and *ACSL4* $^{L-/-}$ hepatocytes (Figure 4B). Following forskolin treatment, only *Pgc1a* expression increased in control hepatocytes while only *Cpt1a* expression decreased in *ACSL4* $^{L-/-}$ hepatocytes (Figure 4C). Following glucagon treatment, both *Pepck* and *G6pase* expression increased in control hepatocytes (Figure 4D). Unlike in Experiment 1, *G6pase* expression also increased in *ACSL4* $^{L-/-}$ hepatocytes following glucagon treatment.

Chapter IV. Discussion

4.1 Adjustments to Methods and Materials

Experiment 1 produced gene expression data that were inconsistent with the expected treatment effects. The data also exhibited high variability between technical replicates within treatment groups. This was likely due to the poor quality of the extracted RNA and reagents used for treatments. Further analysis of A260/A230 ratios revealed poor RNA purity of Experiment 1 extracted RNA. Prior to performing Experiment 2 with new cells, several adjustments were made to improve experimental conditions. In order to improve the quality and purity of the extracted RNA, the extraction was performed with smaller batches of cells at a time to prevent thawing/degradation that would disturb expression patterns. RNA was also eluted using spin columns to improve purity and yield for extraction. RNAase free micro-volume tubes were also used in place of standard micro-volume tubes during cDNA synthesis. New reagents were purchased for norepinephrine, 8-Br-cAMP, and glucagon treatments prior to the second experiment to replace expired reagents used for the first experiment.

4.2 Hormone treatment effects on gene expression

The main objective of this study was to validate the findings of a previous experiment in our lab that suggested a possible role for ACSL4 in beta adrenergic signaling. While Experiment 2 was conducted using higher quality RNA resulting in less apparent variability, the hormone treatments did not induce the gene expression patterns that were expected in control hepatocytes. All hormone treatments used for this study should have induced a similar response in control cells by raising intracellular levels of cAMP. Subsequent PKA activation results in CREB phosphorylation and downstream induction of PGC1a to upregulate genes associated with fatty acid oxidation and gluconeogenesis. Norepinephrine interacts with beta adrenergic GPCRs and causes the alpha subunit to activate adenylyl cyclase to convert ATP to cAMP. Glucagon works similarly to increase cAMP production by interacting with a different GPCR. Forskolin directly interacts with adenylyl cyclase at its catalytic domain to increase cAMP production. 8-Br-cAMP is a lipophilic synthetic analog that can enter cells, is resistant to cellular phosphodiesterases, and can directly activate PKA.

While all of these hormones have different mechanisms of action, they were expected to elicit similar increases in the expression of fatty acid oxidation genes in control hepatocytes. Not only did all of the treatments have inconsistent effects on increasing fatty acid oxidation gene expression, norepinephrine treatment actually caused a decrease in *Cpt1a* and *Ppara* expression in control hepatocytes. The *Acsl4* $^{L-/-}$ hepatocytes did exhibit different expression patterns compared to control, however results were inconclusive due to the inconsistent effects observed in the control samples. Overall, the hormone treatments did not produce the anticipated effects and did not validate the findings of our previous study. Because of this, we were unable to form conclusions about whether the liver specific ACSL4 knockout decreased beta adrenergic signaling or other exogenous mechanisms of FA oxidation gene regulation.

4.3 Fatty acid treatment effects on gene expression

This study also explored the role of fatty acids as intracellular signaling molecules to modulate fatty acid oxidation. Oleate increases intracellular levels of cAMP and expression of fatty acid oxidation genes in C_2C_{12} myotube cells.[14] While the mechanism behind how oleate elicits this cellular response is unknown, this finding illustrates the potential for therapeutic manipulation of intracellular lipid signaling to alter fatty acid oxidation through transcriptional regulation. Similar to the results from hormone treatment experiments, the gene expression changes following the lipid treatments in our study were not consistent with the findings of the published study. Oleate treatments were expected to increase expression of fatty acid oxidation genes while palmitate was not expected to affect expression. In control hepatocytes palmitate treatment increased gene expression of all genes of interest except *Pgc-1a* while oleate treatment only increased *Creb* expression. Similar to what was observed in the hormone treatment experiment, the ACSL4 knockout seemed to alter the cellular responses to treatments. Because the published study observed this signaling effect of oleate in C_2C_{12} myotubes, it is possible that there may be a different effect in primary hepatocytes. This experiment must be repeated in order to draw conclusions on how different long chain fatty acids modulate fatty acid oxidation in hepatocytes and how the absence of ACSL4 alters this process.

4.4 Future Directions

In an attempt to validate the findings of the previous study, most of the results from the hormone treatment experiment were inconclusive. Several steps can be taken to optimize treatment conditions to improve consistency of gene expression changes. Due to different mechanisms of action, each treatment could induce changes in gene expression at different rates. Time course experiments would allow us to optimize hormone treatment times for our study and better determine the effects of the ACSL4 knock out on gene expression. Concentration of the hormone treatments were based on the previous study and prior literature but could also be optimized in the context of our experiment. The efficacy of each treatment could also be validated by using immunoassays to measure intracellular cAMP concentrations. To further explore how each individual treatment or ACSL4 knockout affected gene expression, covalent modifications of CREB, PGC-1a, and SIRT-1 could be measured using western blot. We also plan to repeat the fatty acid treatment experiments to determine the role of fatty acid signaling in modulating gene expression in primary hepatocytes.

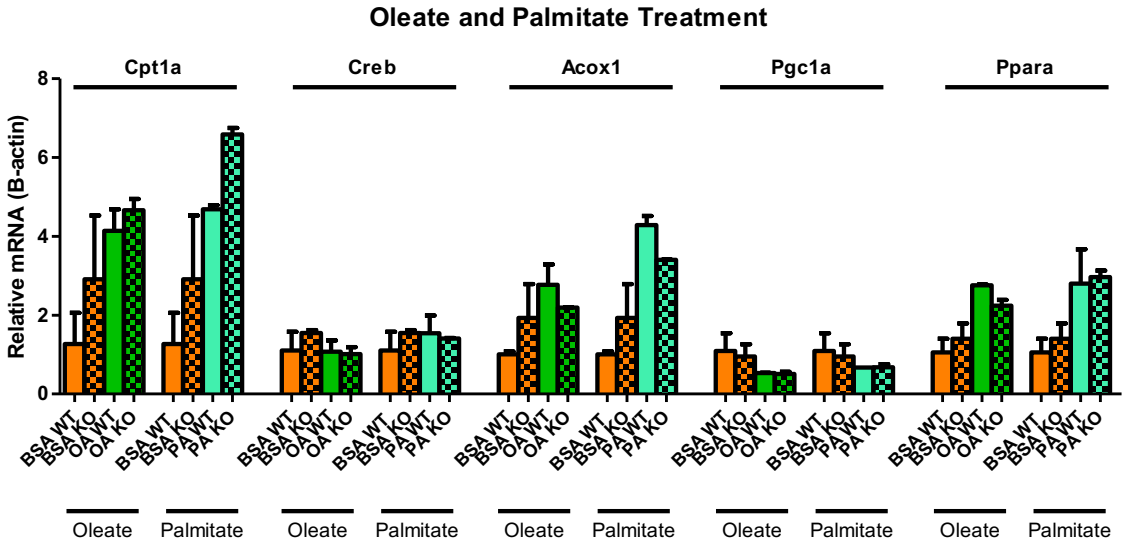

Figure 1. Lipid Treatments Gene Expression (Experiment 1)

Relative gene expression (Actin) of *Cpt1a, Creb, Acox1, Pgc1a*, and *Ppara* in ACSL4 $^{L -/-}$ and WT hepatocytes following 3.5-hour oleate or palmitate treatment.

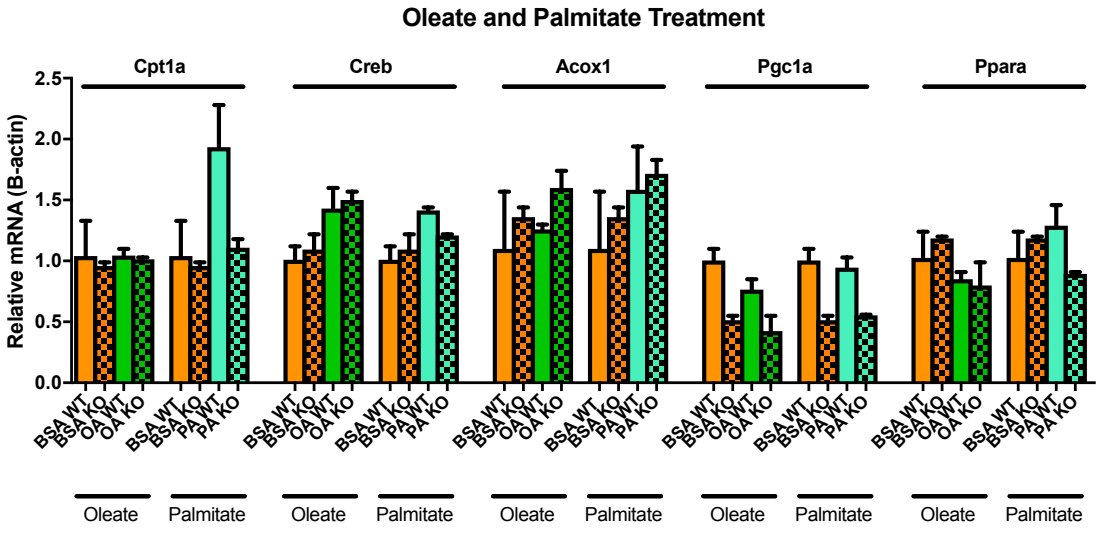

Figure 2. Lipid Treatments Gene Expression (Experiment 2)

Relative gene expression (*Actin*) of *Cpt1a, Creb, Acox1, Pgc1a*, and *Ppara* in *ACSL4* $^{L -/-}$ and WT hepatocytes following 3.5-hour oleate or palmitate treatment.

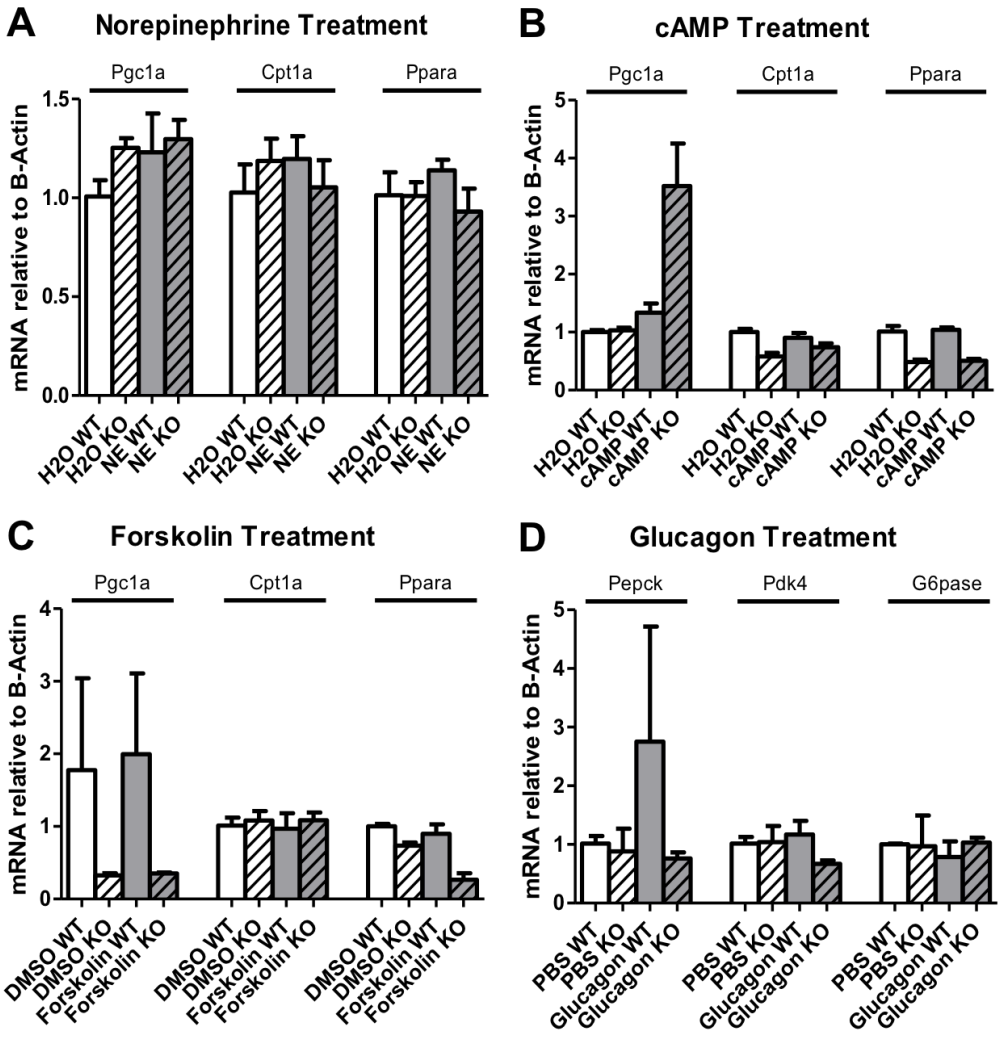

Figure 3. Hormone Treatment Gene Expression (Experiment 1)

Relative gene expression (*Actin*) of *Pgc1a, Cpt1a*, and *Ppara* following 1-hour (A) norepinephrine, (B) 8-Br-cAMP, and (C) forskolin treatments. Relative expression of *Pepck, Pdk4,* and *G6pase* following 1 hour (D) glucagon treatment

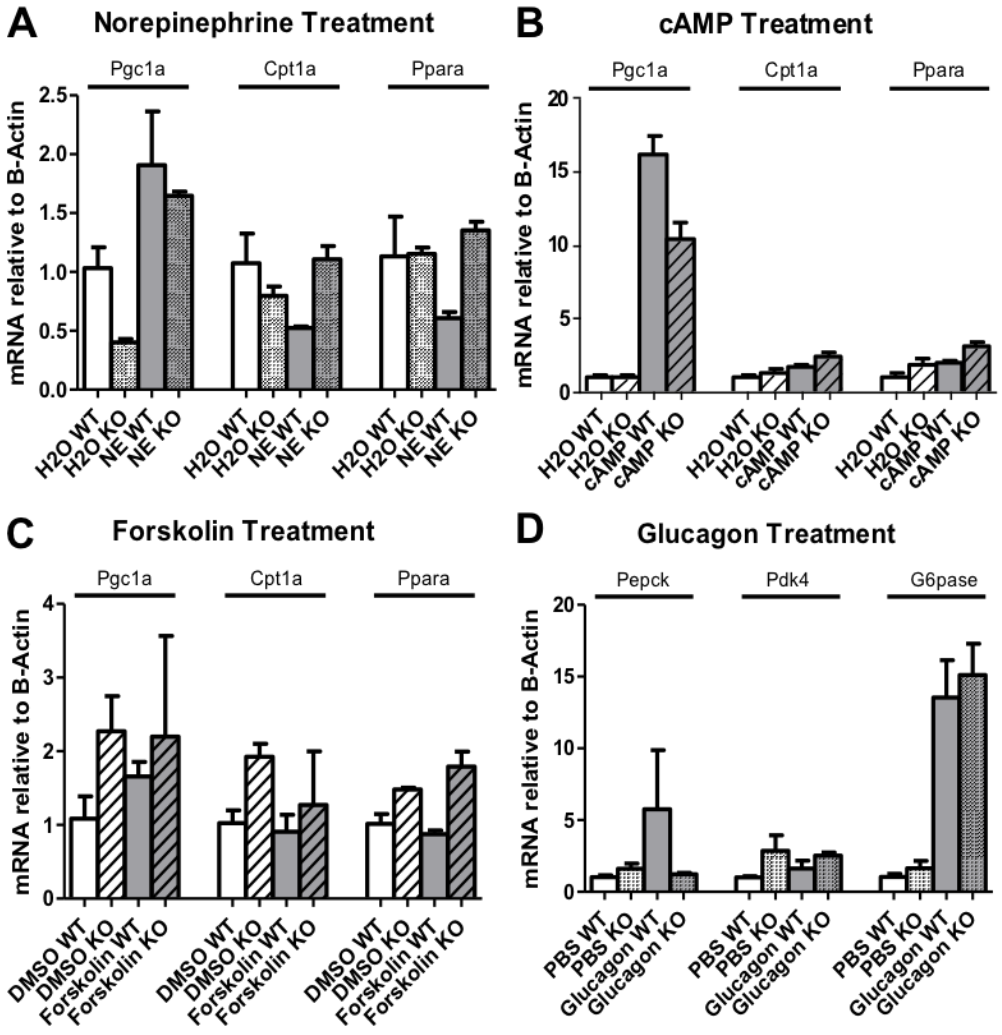

Figure 4. Hormone Treatment Gene Expression (Experiment 2)

Relative gene expression (*Actin*) of *Pgc1a, Cpt1a*, and *Ppara* following 1-hour (A) norepinephrine, (B) 8-Br-cAMP, and (C) forskolin treatments. Relative expression of *Pepck, Pdk4,* and *G6pase* following 1-hour (D) glucagon treatment

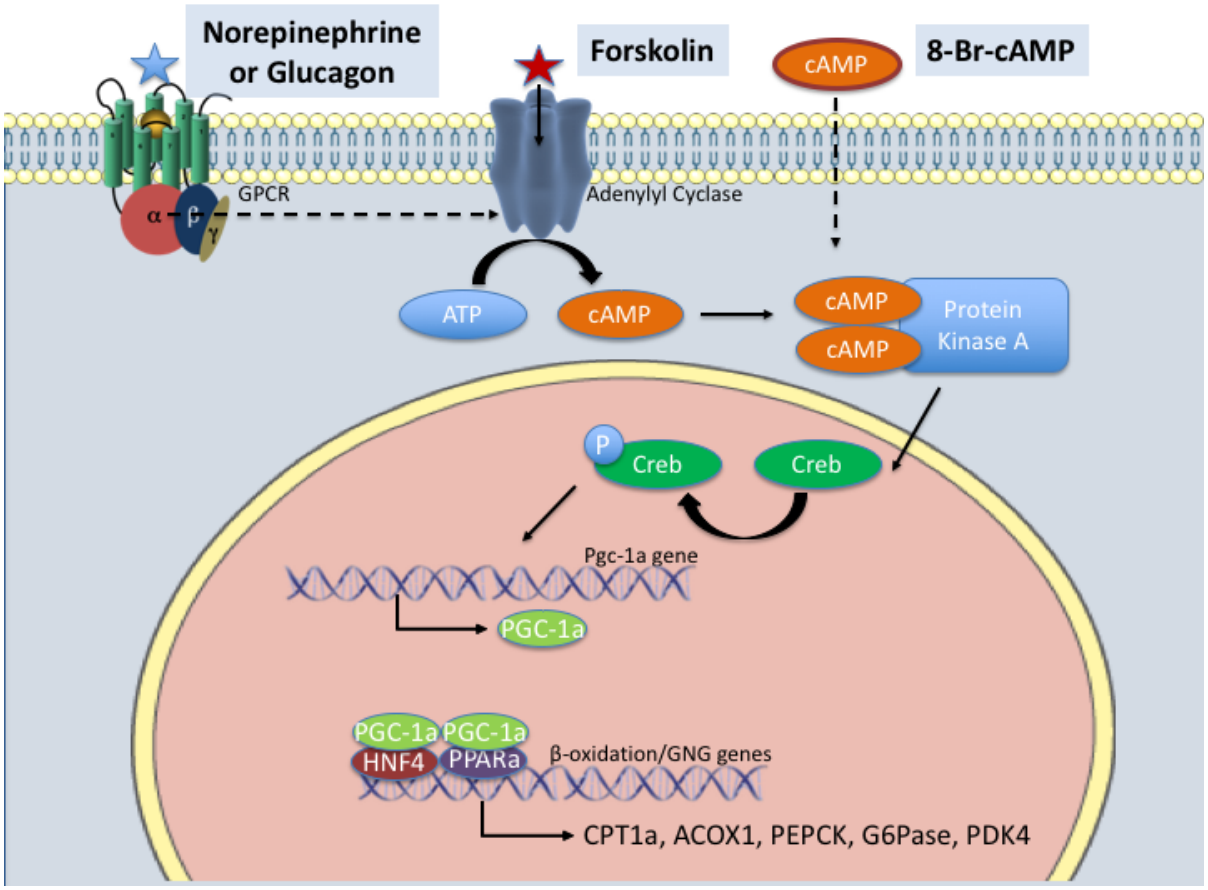

Figure 5. Hormone Treatments and their Signaling Mechanisms

Treatment	Vehicle	Concentration	Time (Hr)
Norepinephrine	H_2O	10μM	1
8-Br-cAMP	H_2O	1mM	1
Forskolin	DMSO	40μM	1
Glucagon	PBS	100mM	1
Oleate	BSA	500μM	3.5
Palmitate	BSA	500μM	3.5

Table 1. Treatment and Corresponding Vehicle Conditions

Target	Direction	Sequence
Acsl4	Forward	ACAGCCTTGGAACCCGGGAGA
	Reverse	CCCAATGCAGTGAGGCCACTTCC
β-Actin	Forward	GGCTCCTAGCACCATGAAGA
	Reverse	GAAAGGGTGTAAAACGCAGC
Pgc-1α	Forward	CAACATGCTCAAGCCAAACCAACA
	Reverse	CGCTCAATAGTCTTGTTCTCAAATGGG
Pparα	Forward	CCTCAGGGTACCACTACGGAGTT
	Reverse	TCGCCGAAAGAAGCCCTTA
Cpt1a	Forward	CAACTATTATGCCATGGATTTTGTGCTT
	Reverse	CGATACATGATCATGGCGTGAACG
Creb	Forward	TCAGCCGGGTACTACCATTC
	Reverse	TTCAGCAGGCTGTGTAGGAA
Acox1	Forward	GGTGGACCTCTGTCTTGTTCA
	Reverse	AAACCTTCAGGCCCAAGTGAG
Pepck	Forward	TCAACACCGACCTCCCTTAC
	Reverse	CATTGTGCCGCTATCTCAAA
G6pase	Forward	TCTGTCCCGGATCTACCTTG
	Reverse	TGGCAAAGGGTGTAGTGTCA
Pdk4	Forward	CCGCTGTCCATGAAGCA
	Reverse	GCAGAAAAGCAAAGGACGTT

Table 2. RT-PCR Primer Sequences

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