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The Effects of Chronic AMPK Activation on Hepatic Triglyceride Accumulation and Glycerol-3-Phosphate Acyltransferase Activity with High Fat Feeding

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The Effects of Chronic AMPK Activation on Hepatic Triglyceride
Accumulation and Glycerol-3-Phosphate Acyltransferase
Activity with High Fat Feeding

Mary Elizabeth Curtis

A thesis submitted to the faculty of Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

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William Winder
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of a thesis submitted by

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The dissertation of Mary Elizabeth Curtis is acceptable in its final form including (1) its format, citations, and bibliographical style are consistent and acceptable and fulfill university and department style requirements; (2) its illustrative materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory and ready for submission.

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ABSTRACT

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High fat feeding increases hepatic fat accumulation and is associated with hepatic insulin resistance. AMP Activated Protein Kinase (AMPK) is thought to inhibit lipid synthesis by the acute inhibition of glycerol-3-phosphate acyltransferase (GPAT) activity and transcriptional regulation via SREBP-1c. The purpose of this study was to determine if chronic activation of AMPK prevented an increase in GPAT1 activity in rats fed a high fat diet. Rats were fed a control (C), or a high fat (HF) diet (60% fat) for 6 weeks and injected with saline or a daily AICAR dose of 0.5 mg/g body weight. Chronic AMPK activation by AICAR injections resulted in a significant reduction in hepatic triglyceride accumulation in both the C and HF fed animals (C, 5.5 ± 0.7 ; C+AICAR, 2.7 ± 0.3 ; HF, 21.8 ± 3.3 ; and HF+AICAR, 8.0 ± 1.8 mg/g liver). HF feeding caused an increase in total GPAT and GPAT1 activity which was not affected by chronic AMPK activation (GPAT1 activity vs. C, C+AICAR, $92 \pm 19\%$; HF, $186 \pm 43\%$; HF+AICAR, $234 \pm 62\%$). Markers of oxidative capacity, including citrate synthase activity and cytochrome c abundance, were not affected by chronic AICAR treatment. Interestingly, HF feeding caused a significant increase in LCAD (up 66% from C), a marker of fatty acid oxidation capacity. These results suggest that chronic AMPK activation limits hepatic triglyceride accumulation independent of a reduction in total GPAT1 activity.

Keywords: AMPK, GPAT1, SREBP-1c, mTOR, LCAD

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Manuscript

The Effects of Chronic AMPK Activation on Hepatic Triglyceride Accumulation and Glycerol-3-Phosphate Acyltransferase activity with High Fat Feeding

Introduction

AMP-activated protein kinase (AMPK) is a major regulator of energy homeostasis and nutrient metabolism. AMPK is known to regulate fatty acid metabolism, protein synthesis, and glucose uptake (5, 18). Furthermore, the activation of AMPK occurs by allosteric and covalent modification of the enzyme in response to an energy deficit (23). AMPK exerts its effects on energy metabolism by acutely regulating various enzymes and proteins as well as influencing transcription and translation of genes involved in energy metabolism (1, 41, 74). For these reasons, AMPK is of tremendous interest in understanding the mechanisms involved in hepatic lipid accumulation.

Hepatic lipid accumulation occurs in conditions of elevated dietary fat, obesity and decreased metabolic function and is associated with decreased liver function. There are a number of mechanisms that could lead to increased hepatic triglyceride accumulation. Simply put, hepatic triglyceride accumulation is the result of a greater amount of lipid uptake and/or synthesis relative to lipid oxidation and release into the circulation (9, 69). Non-alcoholic fatty liver disease (NAFLD) is defined as hepatic fat accumulation greater than five percent of liver weight in the absence of excessive alcoholic intake (27). This disease has been reported to affect from 10-30 percent (about 90 million people) of the adult population in the United States, making it the most common chronic liver condition among adults (42, 44, 46, 50, 52, 53, 55, 63, 66). It has extended to adolescents with one study reporting approximately 61 percent of

overweight and obese adolescent subjects having elevated liver enzymes and hepatic steatosis (64). NAFLD is strongly associated with insulin resistance and metabolic syndrome and if not corrected, results in cirrhosis of the liver (7). In fact, it has been previously reported that non-alcoholic fatty liver disease is the hepatic representation of metabolic syndrome (22, 27, 36, 49, 53).

Consistent with AMPK's demonstrated role in energy metabolism, AMPK has been reported to have an effect on increasing lipid oxidation and inhibiting lipid synthesis. One such proposed influence of AMPK on lipid regulation is in the acute inhibition of glycerol-3-phosphate acyltransferase (GPAT), an integral enzyme in the process of triglyceride accumulation. GPAT is the rate limiting enzyme that catalyzes the first committed step in triglyceride synthesis (6, 8). Of the four known isoforms, mitochondrial *N*-Ethylmaleimide (NEM)-resistant GPAT (GPAT1) only makes up 10 percent of the total GPAT activity in all tissues but the liver. In the liver, GPAT1 makes up to 30 to 50 percent of the total GPAT activity, making it a significant contributor to hepatic triglyceride regulation (15, 47). NEM is an organic compound inhibits all isoforms of GPAT except GPAT1 allowing for isolation of GPAT1 activity. Chemical activation of AMPK by Aminoimidazole Carboxamide Ribonucleotide (AICAR) reduces fat accumulation in the hepatocyte by decreasing GPAT1 activity by 30 to 40 percent (8, 29, 41). It is also likely that AMPK may limit the fatty acids available for triglyceride synthesis by causing an increase in fat oxidation rates. AMPK inhibits acetyl-CoA carboxylase (ACC), an enzyme that catalyzes the formation of malonyl-CoA. Malonyl-CoA inhibits carnitine palmitoyltransferase I (CPT1) resulting in decreased beta oxidation and increased fat synthesis. Decreasing malonyl-CoA production will increase CPT1 activity (38, 72, 74, 76). Therefore,

through AMPK's acute role of inhibiting GPAT1 and increasing CPT1, there is an overall increase in oxidation relative to triglyceride synthesis.

In addition to acute regulation of the enzymes of triglyceride synthesis, recent evidence points to a role in which AMPK influences the transcription and translation of enzymes for lipid synthesis (74) (68). Sterol regulatory element binding protein-1c (SREBP-1c) increases the transcription of lipid synthesis enzymes such as ACC, fatty acid synthase (FAS), GPAT, and stearoyl-CoA desaturase (SCD1) (10, 35, 44, 58, 60). Previous work suggests that activation of AMPK decreases promoter activity of SREBP-1c in the liver as well as the transcriptional activity of an upstream transcription factor and regulator of SREBP-1c expression, liver X receptors (LXR α) thus decreasing SREBP-1c and LXR (74). Further, AMPK decreases SREBP-1c by interfering with the mammalian target of rapamycin complex (mTOR) activity. This proposed mTOR-dependent mechanism is thought to be by cleavage and activation of SREBP-1c (51). Chronic AMPK activation then inhibits mitochondrial GPAT1 abundance by decreasing SREBP-1c activity (11, 74). Thus, in addition to AMPK's role as an acute regulator, AMPK further inhibits hepatic lipid accumulation by inhibiting SREBP-1c through transcriptional regulation reduction of mTOR activity.

There is evidence for chronic AMPK activation dependent inhibition of lipogenic enzymes (20, 68). Therefore, our purpose for this study was to examine the chronic activation of AMPK on enzymes critical for hepatic triglyceride accumulation and enzymes of lipid synthesis, particularly ACC and GPAT1. This study was designed to provide further understanding of the role of chronic activation of AMPK on hepatic triglyceride synthesis and storage.

Materials and Methods

Animal Care

All procedures related to animal care and use were approved by the Institutional Animal Care and Use Committee of Brigham Young University.

Diet

Wistar Rats were divided into 4 groups of 7-10 animals each. Two groups consumed laboratory chow diet, 5053 PicoLab® Rodent Diet 20, and two groups consumed the high fat diet (12, 14, 17). Food and water were provided *ad libitum*.

High Fat diet (g/kg of food):

116.3 g olive oil, 232.7 g flax seed oil, 87.2 g sugar, 174.6 g starch, 226.6 g casein, 4.5 g methionine, 30.7 g gelatin, 51.2 g bran, 22.5 g vitamin mix (Harlan Teklad, AIN76A), 52.2 g mineral mix (Harlan Teklad, AIN76), 1.4 g choline chloride.

Table 1. Macronutrient composition of the two diets used in these studies.

	Chow Diet (% of calories)	High Fat Diet (% of calories)
Protein	23.6	20
Carbohydrate	64.5	20
Fat	12	60

Experimental design

Table 2. A two-factor research design was used to examine the effects of high fat feeding as well as AMPK activation on factors important for the determination of fat accumulation in liver tissue.

Chow Groups	High Fat Groups
Chow + Daily Saline Injections	High Fat (HF) + Daily Saline Injections
Chow + Daily AICAR Injections	High Fat (HF) + Daily AICAR Injections

AICAR injections

AICAR was dissolved in 0.9% NaCl and administered subcutaneously every morning for six weeks at a dose of 0.5 mg AICAR/g body weight. Rats were sacrificed 24 hours after the last injection was given. Throughout the six weeks, rats from Control and High Fat groups were handled daily and injected with a comparable amount of saline at the time the AICAR groups received AICAR injections.

Dissections

Rats were anesthetized with pentobarbital sodium (65 mg/kg body weight.) Liver tissue was extracted once rats were completely sedated. Liver was quickly removed and clamp frozen with liquid nitrogen chilled metal tongs then wrapped in aluminum foil and stored at -90 degrees Celsius.

Citrate Synthase Activity

As a measure of mitochondrial oxidative capacity, whole tissue homogenate was used to measure citrate synthase activity by the method of Srere (62). Briefly, homogenates were prepared in 175 mM KCl, 10 mM GSH, 2 mM EDTA, pH 7.4 (100 mg of tissue per ml.) Homogenates were

further diluted in a solution of 100 mM Tris pH 8.0. Citrate synthase activity was measured and recorded for each sample.

Measurement of Protein/Enzyme Abundance and Phosphorylation

Standard BCA protein assays were performed to determine protein concentration in the whole tissue or membrane fraction homogenate. The results were used to normalize protein content for Western Blots and activity assays.

Standard Western blotting procedures were performed. Briefly, aliquots of the liver homogenates were subjected to SDS-PAGE and then transferred to a nitrocellulose membrane. The membrane was blocked with 5% milk solution followed by incubation with the primary antibody for the protein of choice overnight at 4 degrees Celsius. The next day, the membranes were rinsed with Tris-Buffered Saline Tween-20 (TBST) and incubated with the appropriate secondary antibody dissolved in 1% milk in TBST for 1 hour. This was followed again by rinses in TBST followed by one rinse in TBS. Protein content was detected by chemiluminescence. Protein band densitometry was quantified and analyzed with Image J and Alpha Ease software. (74)

Primary antibodies for the following proteins were used: Total AMPK (Cell Signaling; cat. No. 2532L), pAMPK (Cell Signaling, Beverly, MA; cat. No. 2535L), total ACC (GE Healthcare; cat. No. RPN1231V), SREBP-1c (Santa Cruz, Santa Cruz, CA; cat. No. SC13551), mTOR (Cell Signaling Technology Beverly, MA; cat. No. 2972).

In addition, we looked at 4E-BP (Cell Signaling Technology Beverly, MA; cat no. 9452) for an indication of mTOR activity and Cytochrome C (Santa Cruz, Santa Cruz, CA; cat. No. sc-13156) and LCAD (a gift from Daniel P. Kelly) for an indication of oxidative capacity.

Triglyceride Assay

Liver triglycerides were measured according to the Folch method. Forty to fifty milligrams of frozen liver were homogenized with a 2:1 concentration of chloroform:methanol and agitated in a cold room overnight at 4 degrees C. One ml of 0.9% NaCl was added to each sample solution, vortexed and centrifuged for 1 hour at 1000 x g at 4 degrees C. The organic phase was removed and dried down in a lyophilizer. Samples were reconstituted with 100 µl of Tert-Butanol Triton X solution. Triglyceride content was then analyzed in each sample with the WAKO triglyceride assay kit as per manufacturer's instructions (13).

GPAT Activity Assay

The activity of microsomal and mitochondrial GPAT was measured using the method described previously (41, 57, 70). Briefly, membrane fractions of liver tissue were formed using sample homogenates prepared with 25 mg of liver and homogenization buffer (250 mM Sucrose, 10 mM TrisHCl, 1 mM DTT, 1 mM EDTA, pH 7.4). Homogenate was centrifuged at 100,000 x g for 1 hour, after which the supernatant was removed and the pellet reconstituted with 400 µl of the same homogenization buffer, aliquoted and frozen in -90 degree Celsius freezer. Total GPAT activity was measured using a cocktail of 800 µM [¹⁴C]glycerol 3-phosphate (G-3-P), 60 µM palmitoyl Co-A, 75 mM TrisHCl, 4mM MgCl, 2mg/ml BSA, 8mM NaF, 1Mm DTT, and non-labeled G3P. Sample (40 µg protein) was added to the solution incubated with and without 2 mM N-ethylmaleimide (NEM), an inhibitor of microsomal GPAT to isolate and measure the remaining activity, GPAT1 activity. The reaction was run for 10 min at 37°C and stopped with 0.6 ml 1% HClO₄ and chloroform-methanol (2:1). After 5 minutes on ice, another 1 ml of 1% perchloric acid and 1 ml of chloroform was added to the solution. Samples were centrifuged for 1 hour at 1000 x g and washed 3 times with 1% perchloric acid after which 1 ml of organic phase

containing the labeled G-3-P incorporated into lysophosphatidic acid was dried down using a lyophilizer. After reconstituting the samples with 2:1 tert-butanol triton x solution, the sample with scintillation fluid was placed into scintillation tubes and counts measured with a scintillation counter. Subtracting the mitochondrial GPAT (NEM-resistant) activity from the total activity allowed for determining GPAT1's activity for each sample.

Statistical Analysis

Significant differences between groups were determined using two way ANOVA and Bonferonni post hoc test for multiple comparisons. The statistical software SigmaStat (Systat Software Inc, San Jose, CA) was used. Statistical significance is defined at $p < 0.05$. Results are presented as means \pm standard error of mean (SEM).

Results

Chronic Activation of AMPK Limits Hepatic Triglyceride Accumulation.

As expected, chronic activation of AMPK using daily AICAR injections caused a reduction in hepatic triglyceride content. We verified that the subcutaneous AICAR injections at the dose we used (0.5 mg/g body weight) were sufficient to cause activation of AMPK in the liver by measuring AMPK phosphorylation one hour after acute subcutaneous injection in rats (see Figure 1). Chronic activation of AMPK limited the normal increase in triglyceride accumulation that occurs with high fat feeding such that it was not significantly different from the control group (See Figure 2). This finding is consistent with what others have reported as the effect of AMPK activation on fat accumulation in the liver (19, 48, 79). The mechanism by which AMPK causes this reduction in a prolonged treatment of six weeks has yet to be fully characterized.

Lipid synthesis

Chronic Activation of AMPK Decreased SREBP-1c in Livers of Rats Fed a High Fat Diet.

Based on previous findings, chronic AMPK activation would be expected to reduce transcription of GPAT through an inhibition of mTOR and SREBP-1c (51, 74). AMPK is known to inhibit mTOR activity therefore we examined mTOR and a downstream target of mTOR (4EBP) as an indication of mTOR activity (54). Western blots on total mTOR complex protein in each group did not indicate a significant difference between the groups (See Figure 3). The total abundance of 4E-BP was significantly increased with chronic AMPK activation with both the chow and high fat feeding (See Figure 4a.) The phosphorylation state was determined by the shift in molecular weight of total 4E-BP protein (percentage of the total protein in the two hypophosphorylated bands migrated down 1-2 kDa compared to the total at molecular weight of 20 kDa as previously documented) (54, 67). Considering the shift of 4EBP, our results indicate that there a was lower amount of phosphorylated 4EBP following chronic AICAR treatment compared to the control group, which is consistent with the known inhibitory effect of AMPK on mTOR activity (See Figure 4b).

AMPK plays a major role in the activity of SREBP-1c in the liver by inhibiting mTOR complex activity (20). SREBP-1c is positively regulated by mTOR and therefore lipogenesis is upregulated with increased mTOR activity (51). SREBP-1c has a full length (inactive) and cleaved (active) form so we wanted to look at both forms in our study (56). High fat feeding caused a marked increase in total full length, uncleaved SREBP-1c abundance. Consistent with the pattern observed in hepatic triglyceride accumulation, chronic activation of AMPK caused a reduction in the total full length, uncleaved SREBP-1c abundance in rats fed either chow or high fat diet (see Figure 5a). The cleaved SREBP-1c showed increases with high fat feeding and

decreases with chronic AMPK activation as well but the differences were not as pronounced (See Figure 5b). Therefore, our data indicates that chronic activation of AMPK inhibits both full length, uncleaved and cleaved SREBP-1c protein abundance; this was consistent with what we observed with the mTOR dependent response as seen with 4EBP phosphorylation.

Chronic activation of AMPK had no effect on GPAT1 activity but a high fat feeding effect was present.

Lipid synthesis enzymes increased by SREBP-1c include ACC and GPAT. We first examined the abundance of total ACC in response to high fat feeding and chronic AMPK activation and found that AMPK activation caused a significant reduction in total ACC protein in the chow group. Interestingly, high fat feeding did not produce a significant increase in total ACC protein (See Figure 6). These results are consistent with cleaved SREBP1-c total content.

GPAT1 activity was measured because it is another lipogenic target of SREBP-1C and is a rate limiting enzyme for triglyceride synthesis (11). High fat feeding caused an increase in total and NEM sensitive GPAT activity in the liver (Figure 7a). Surprisingly, chronic activation of AMPK in either control or high fat fed animals did not cause a reduction in total or NEM sensitive (GPAT1) activity (Figure 7b). Our results present the novel finding that there is not a direct correlation of chronic activation of AMPK with GPAT1 activity We expected to see a reduction in GPAT1 activity based on previous results in hepatocytes regarding the effect of AMPK on GPAT activity (29, 41). These findings prompted further exploration of the mechanisms and regulation of fatty acid oxidation.

Lipid oxidation

Long chain acyl-CoA dehydrogenase (LCAD) was not influenced by chronic AMPK activation but was increased with high fat feeding.

Hepatic lipid accumulation is a balance between the lipid synthesis and oxidation so two markers of mitochondrial oxidative capacity in the liver were measured. Neither high fat feeding nor chronic activation of AMPK showed statistically significant differences between groups for citrate synthase activity (See Figure 8) or cytochrome c content (data not shown) with either high fat feeding or chronic activation of AMPK in the liver. Long chain acyl-CoA dehydrogenase (LCAD), a key enzyme responsible for the first step of oxidation of the long-chain fatty acyl-CoAs was measured (78). A significant increase in LCAD was observed in response to high fat feeding suggesting greater capacity for fat oxidation (See Figure 9a). Interestingly no effect was seen in the animals treated with AICAR (See Figure 9b). These results suggest that chronic AMPK activation does not play a significant role in changing the oxidative capacity of liver content contrary to what has been found previously in skeletal muscle (12, 17, 73). Similar to the GPAT1 data, these results do not account for the observed difference in hepatic triglyceride levels in response to chronic AMPK activation.

Discussion

The purpose of this study was to examine a potential mechanism by which chronic AMPK activation limits fat accumulation in the liver. We hypothesized that AMPK would cause a reduction in GPAT1, a rate limiting enzyme for triglyceride synthesis. GPAT1 has been shown to be influential in the development of NAFLD through its role in triglyceride synthesis in the liver and when overexpressed leads to excess triglyceride synthesis (6, 29, 33, 34, 43). Neschen et al. and Hammond et al. demonstrated that GPAT1 knockout mice had less triglyceride accumulation when compared to wild type mice fed a high fat diet (16, 45). AMPK is thought to

inhibit the transcription of GPAT by reducing the activation of SREBP-1c. SREBP-1c is the primary transcription factor for GPAT1 and other lipogenic enzymes (10, 58, 60). In contrast to our hypothesis, we found that chronic AMPK activation did not cause a reduction in GPAT1 activity in either the control group or the animals receiving a high fat diet. Therefore, results from this study suggest that chronic AMPK activation limits triglyceride accumulation in the liver by a mechanism other than a reduction in triglyceride synthesis capacity.

It is well documented that AMPK activation reduces hepatic triglyceride accumulation (32, 48, 79). However the mechanisms responsible for this reduced triglyceride content in response to high fat feeding are not fully understood. AMPK has been best characterized as a regulator of fatty acid oxidation (39). AMPK effects an increase in oxidation by inhibition of ACC (79). Inhibition of ACC results in less malonyl-CoA synthesis and there is a greater activity of CPT1 due to reduced inhibition by malonyl-CoA (37-39). Recently, a greater appreciation of AMPK as a regulator of triglyceride synthesis is developing. Sterol regulatory element binding protein-1c (SREBP-1c) is a major regulator of lipogenic enzymes and AMPK reduces SREBP-1c and downstream lipogenic enzymes through an mTOR-dependent mechanism (74, 79). GPAT1 is one lipogenic enzyme that has been clearly associated with an increase in triglyceride synthesis and accumulation. The regulation of GPAT1 by SREBP-1c is evidenced by the seven fold increase in GPAT1 by an overexpression of SREBP-1c in adipocytes (11). Further, an expected increase in GPAT1 with refeeding does not occur in liver in the absence of SREBP-1c (61). Results from our study confirm that AMPK activation leads to a reduction in SREBP-1c abundance. Consistent with this reduction in SREBP-1c content, we observed an AMPK dependent reduction in total ACC, one of the enzymes positively regulated by SREBP-1c. GPAT1 activity assay results were unexpected and differed from the pattern observed with

triglycerides, SREBP-1c and ACC. Total and NEM-sensitive (GPAT1) GPAT activity was increased with high fat feeding but chronic AMPK activation did not appear to have an inhibitory effect. Therefore our results in intact liver with chronic AMPK activation suggest an alternative regulation of total triglyceride synthesis capacity via GPAT1 than has previously been proposed in isolated hepatocytes with acute AMPK activation.

The regulation of SREBP-1c by AMPK is thought to be dependent upon inhibition of mammalian target of rapamycin (mTOR) and transcriptional activity of LXR and SREBP-1c (25, 51, 74). SREBP-1c is significantly decreased by inhibitors of mTOR such as rapamycin. This indicates that through AMPK's inhibition of mTOR activity, AMPK has the effect of reducing SREBP-1c activity (31). Further, AMPK's role in reducing mTOR activity results in decreased protein synthesis in liver tissue (54). The mechanism by which AMPK decreases mTOR was proposed by Inoki et al. to be by phosphorylation and activation of an upstream protein in the signaling cascade, tuberous sclerosis complex 1/2 (TSC1/2) (21, 28). mTOR phosphorylates downstream proteins such as eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) and ribosomal protein p70 S6 (S6K1) thereby increasing translation of various proteins and overall protein synthesis (26, 54). Therefore, we can get an indication of the effect of chronic AMPK activation on mTOR activity by measuring the phosphorylation state (or shift) of 4E-BP (54, 67). Our study validated the effect of AMPK activation in the liver by showing a decrease in phosphorylated 4E-BP (increased hypophosphorylation) in the AICAR treated groups (67). This suggests an inhibition of mTOR activity and explanation for the pattern seen in the SREBP-1c results.

Triglyceride accumulates in the liver particularly with high fat feeding for an extended period of time through an up-regulation of lipogenic enzymes that enhance fatty acid and

triglyceride synthesis and greater inhibition of CPT-1, a major regulator of beta-oxidation. This is evidenced by a marked decrease in beta oxidation when GPAT1 is overexpressed in hepatocytes (30, 33, 34) and increased beta oxidation markers when GPAT1 is knocked out in myocytes of mice (45). There is clear evidence that a high fat diet results in significantly higher weights and triglyceride levels in the liver after prolonged high fat feeding (24, 69, 75). Our study duplicated such results in that there was an increase in triglycerides after prolonged high fat feeding. Further, confirming results of AMPK activation in cultured hepatocyte models (19, 59, 77), the chronic AICAR treated intact liver tissue in our study had reduced levels of triglycerides in the liver to nearly the level reported in the control group. AMPK activation inhibits triglyceride accumulation by increasing beta oxidation in the cell (71, 79) as well as in its proposed inhibition of mTOR and downstream targets such as SREBP-1c as noted above (3, 28). These mechanisms could explain the fat accumulation with high fat feeding and reductions with chronic AICAR treatment in the livers that was seen in our triglyceride assay results. Therefore, the reduction seen in triglyceride accumulation with chronic AMPK activation was consistent with what was expected.

Increased fat oxidation with high fat feeding could be another contributing factor to explain the conflicting findings of triglyceride content and GPAT1 data in our study. High fat feeding has been shown to increase oxidative capacity with a simultaneous increase in fatty acid oxidation (4, 40). This high fat effect on fatty acid oxidative capacity gave reason for measuring LCAD, a marker of fatty acid oxidative capacity. Further, AMPK activation is known to influence mitochondrial biogenesis in both skeletal muscle (12, 17, 73) and in adipose tissue (65). Interestingly, we did not see an increase in either citrate synthase activity or cytochrome c content with either high fat feeding or chronic activation of AMPK in the liver. However, a

significant increase of LCAD with high fat feeding was observed. The increase seen in LCAD is consistent with the high fat effect expected but the chronic AMPK activation effect was not apparent from the data. Therefore, the issue of how the chronic effects of AMPK activation lead to a decrease in hepatic triglyceride accumulation remains to be resolved.

It is important to note some of the limitations in our study. First, our study did not investigate the acute regulation of GPAT by AMPK on GPAT noted in other studies. AMPK has been shown to have an acute inhibitory effect on GPAT1 activity as shown in previous studies which is likely due to phosphorylation of GPAT1 (2, 29, 41, 47). This acute effect is not the focus of our study and it is not known to what degree this factor played in overall triglyceride accumulation. The reduction in triglycerides could be explained solely by the acute inhibition of GPAT by AMPK. Second, the fat in the high fat diet used in this study was composed of olive oil and flaxseed oil and was not a typical composition for a high diet (see methods) due to use of tissues from animals in a companion study. This may influence fat accumulation patterns seen in our study and or responsiveness to AMPK. Therefore more work could be done to see if our results in the chronic setting were unique to the type of fat used in our study.

Conclusion

In conclusion, the chronic activation of AMPK in livers of rats fed a high fat diet demonstrates a main high fat effect in the absence of an AICAR effect on GPAT1 activity over time despite the effect on triglyceride content and transcriptional regulation in the liver. Further areas of interest to help explain our findings include research in the role of AMPK activation and possible additional lipid regulatory mechanisms such as cellular uptake or release of fatty acids and other lipid molecules.

Tables and Figures

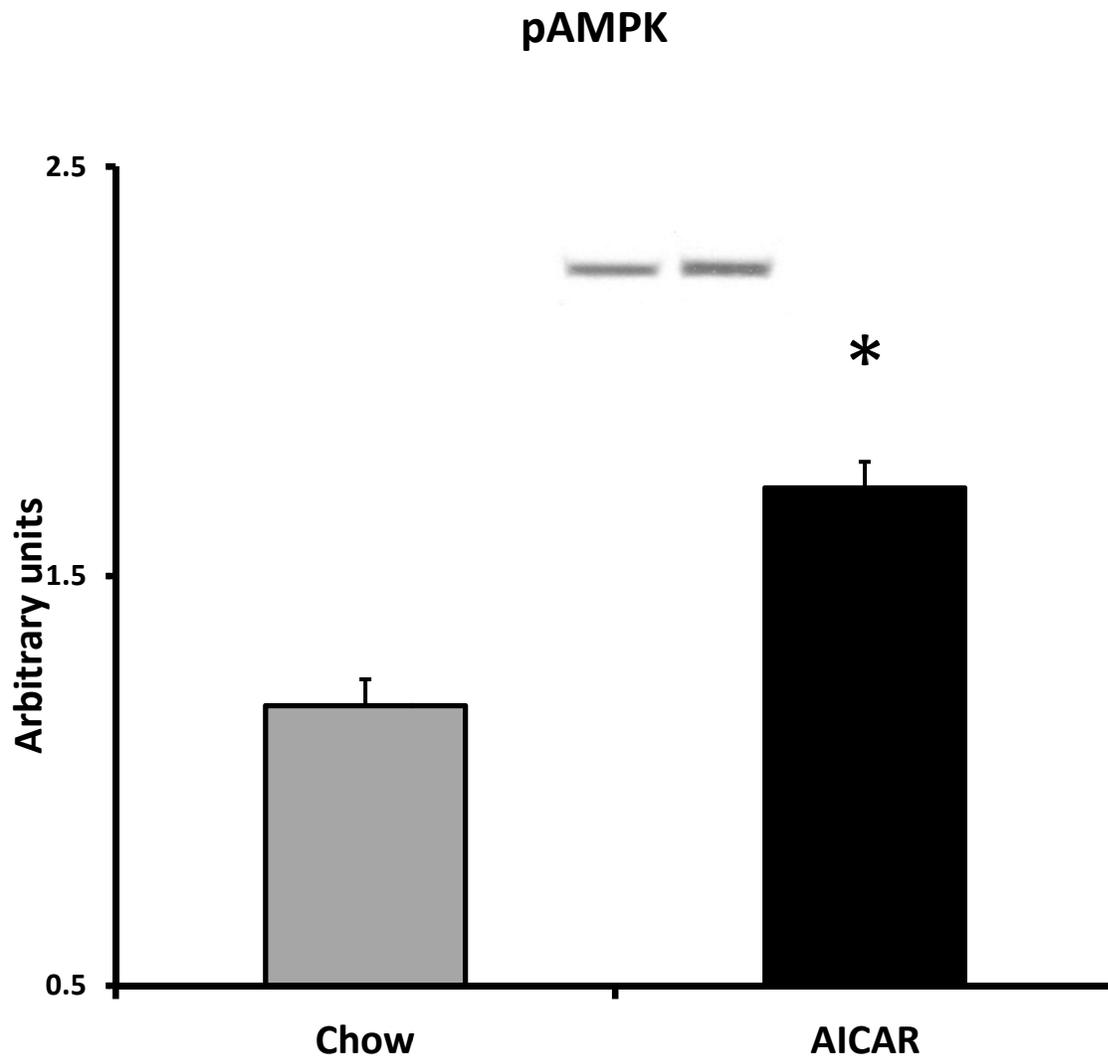


Figure 1. Phospho-AMP-activated protein kinase (pAMPK) content in the liver was increased with acute 5-aminoimidazole-4carboxamide riboside (AICAR) treatment (n=5-7). Livers from AICAR-treated rats were removed 1 hr after injection. Asterisk (*) denotes a main effect of AICAR ($p < 0.05$). Graph represents means \pm SE.

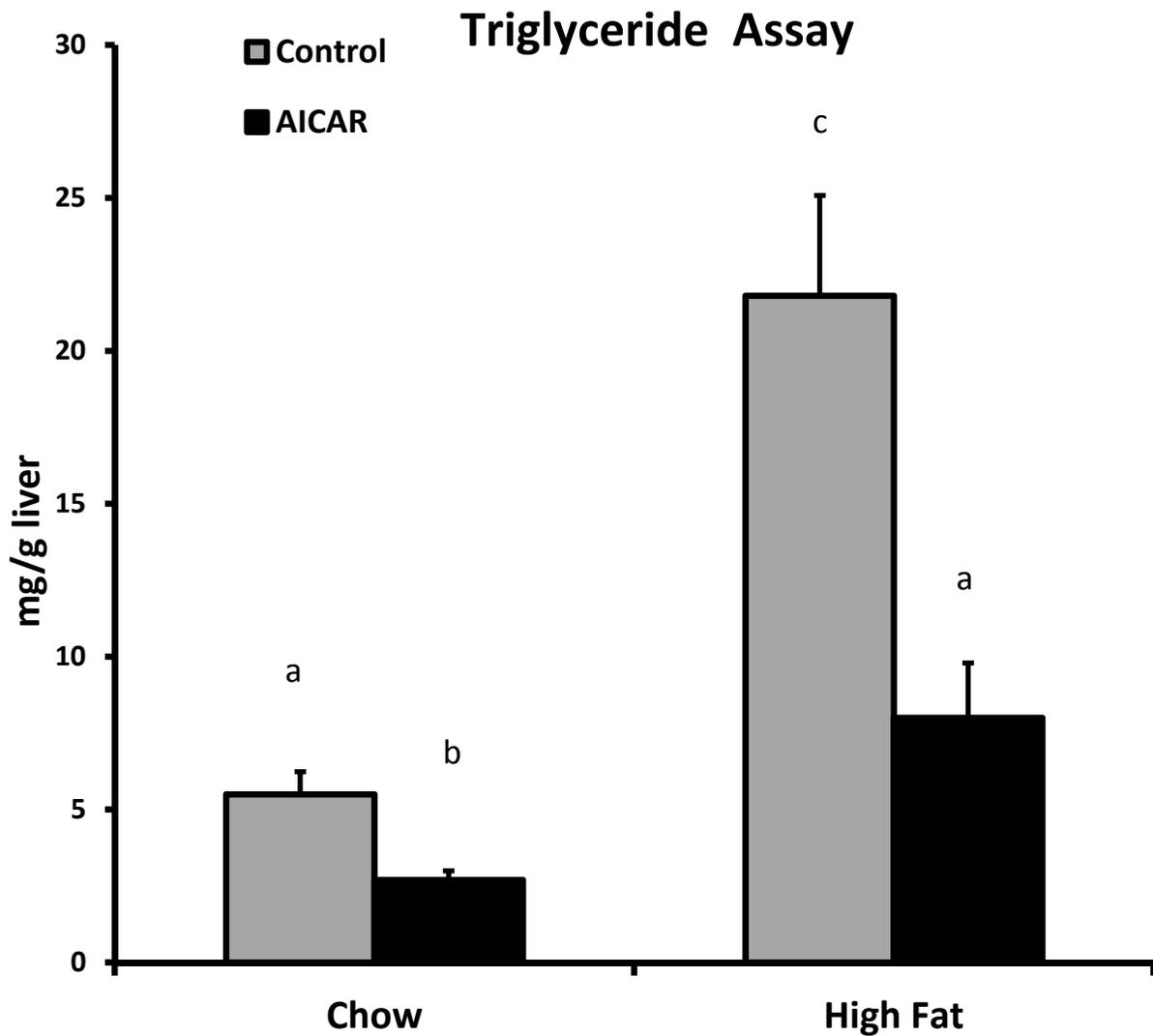


Figure 2. Chronic activation of liver AMPK with daily injections of AICAR limited the normal increase in triglyceride accumulation that occurs with high fat feeding such that it was not significantly different from the control group (n = 7-12). Letters are used to represent significance; same letter means no significant difference (P < 0.05). Graph represents means \pm SE.

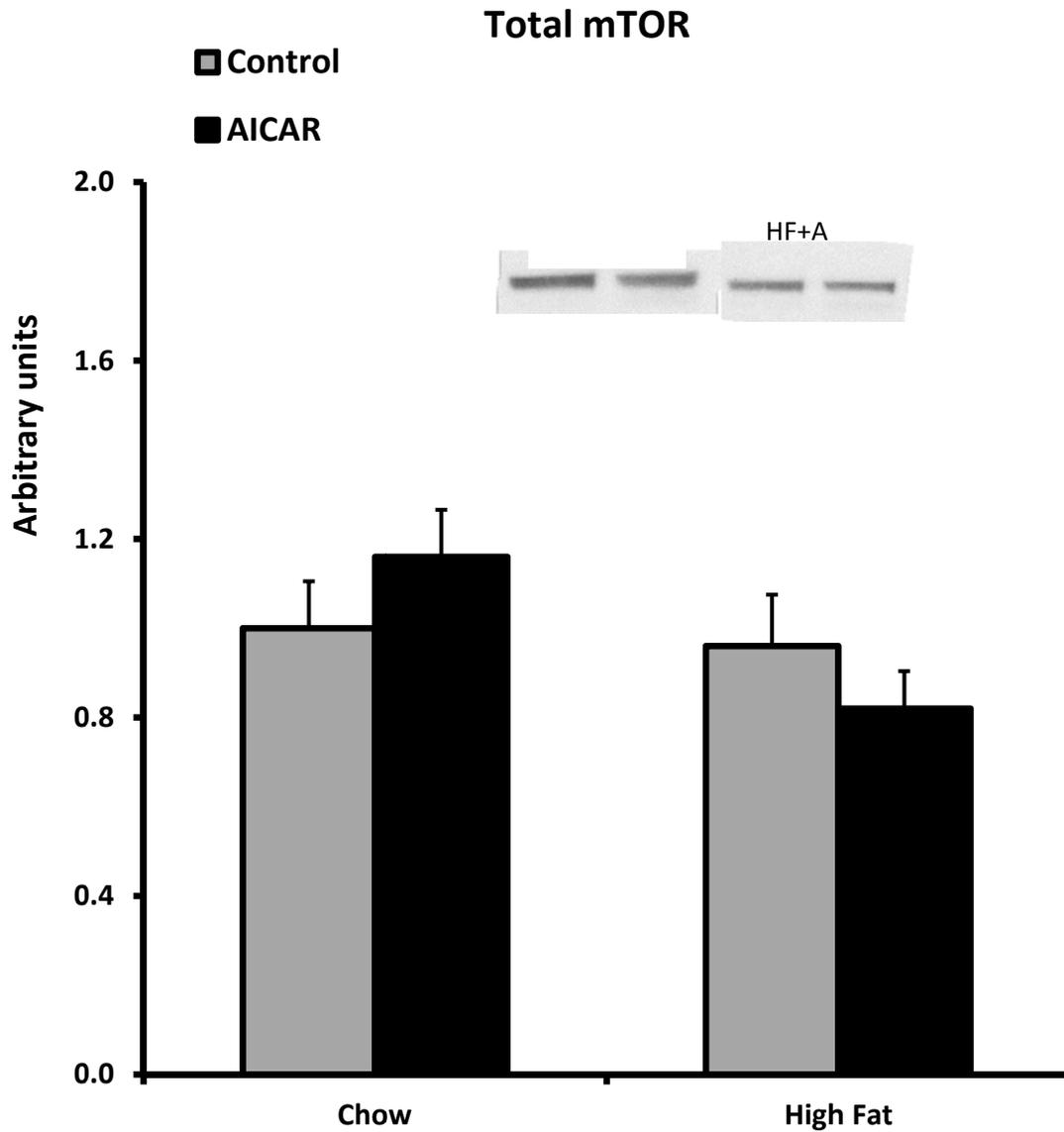


Figure 3. Total mammalian target of rapamycin complex (mTOR) protein content of liver extracts revealed no significant differences with high fat feeding or chronic AMPK activation (n = 4-5). Bands for all 4 groups were taken side by side with no interruption. Graph represents means \pm SE.

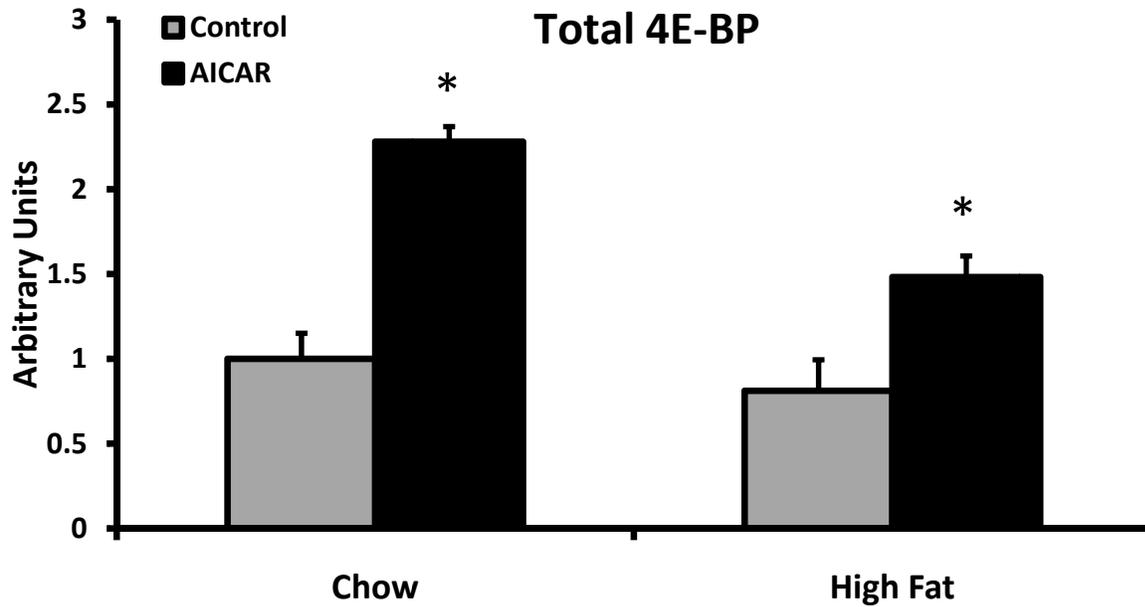


Figure 4a. Total Eukaryotic initiation factor 4E-binding protein (4EBP) results show an increase with the treatment of AICAR (n = 4-5). Bands for all 4 groups were taken side by side with no interruption Asterisk (*) denotes a main effect of AICAR treatment (P<0.05). Graph represents means ± SE.

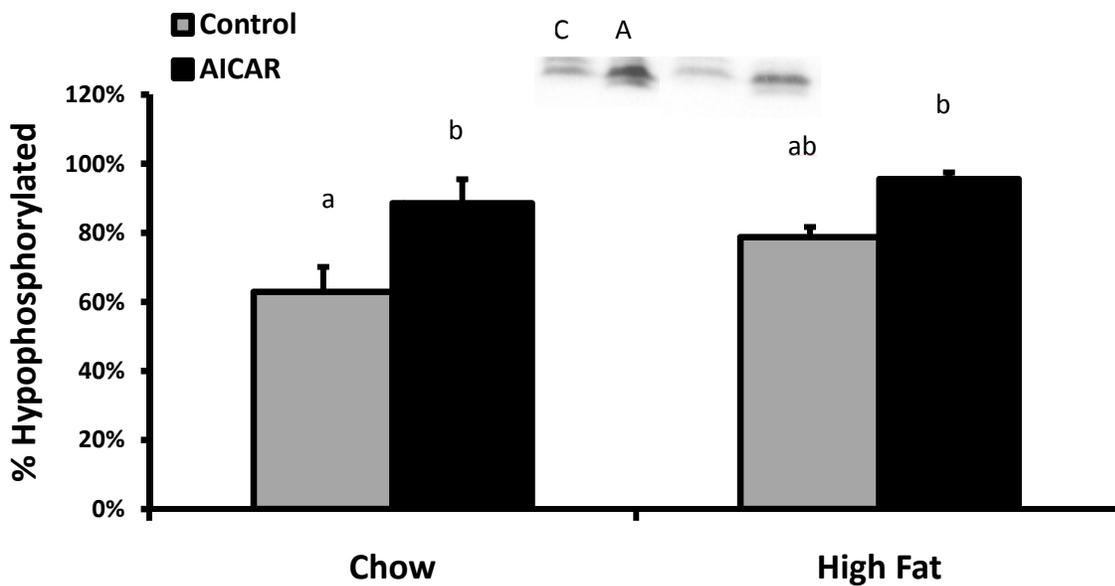


Figure 1b. Eukaryotic initiation factor 4E-binding protein (4EBP) phosphorylation (percentage of the total protein in the 2 hypophosphorylated bands compared to total) showed an increased phosphorylation with AICAR treatment (n = 4-5). Bands for all 4 groups were taken side by side with no interruption. Letters are used to represent significance; same letter means no significant difference (P < 0.05). Graph represents means ± SE.

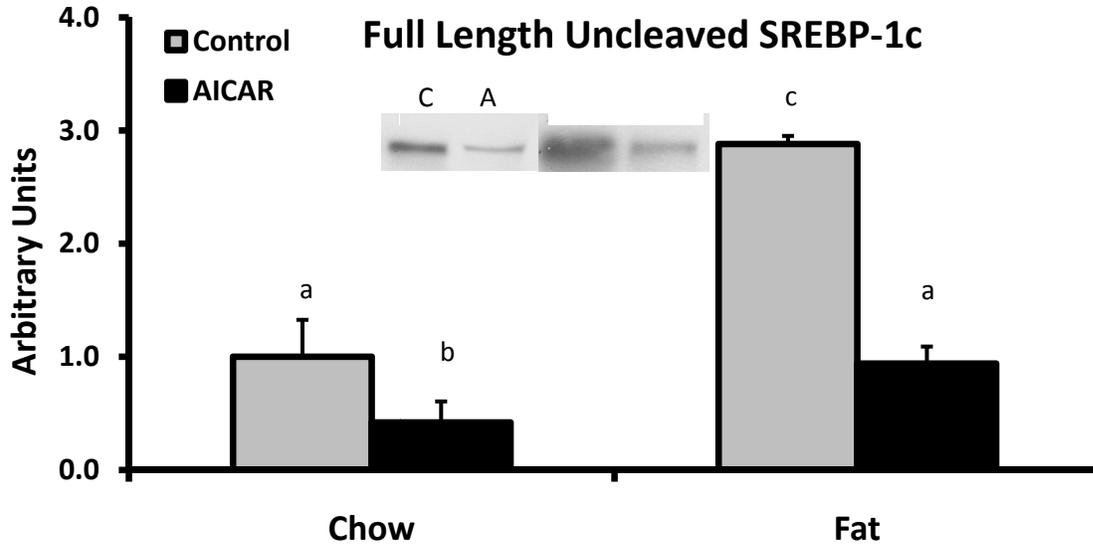


Figure 2a. Chronic activation of AMPK caused a reduction in the total abundance of uncleaved Sterol regulatory element binding protein-1c (SREBP-1c) in rats fed either chow or high fat diet (n = 3-5). Bands for all 4 groups were taken side by side with no interruption. Letters are used to represent significance; Bars that carry the same letter indicate no significant differences were observed ($P < 0.05$). A significant main effect of AICAR was observed ($p < 0.05$). Graph represents means \pm SE.

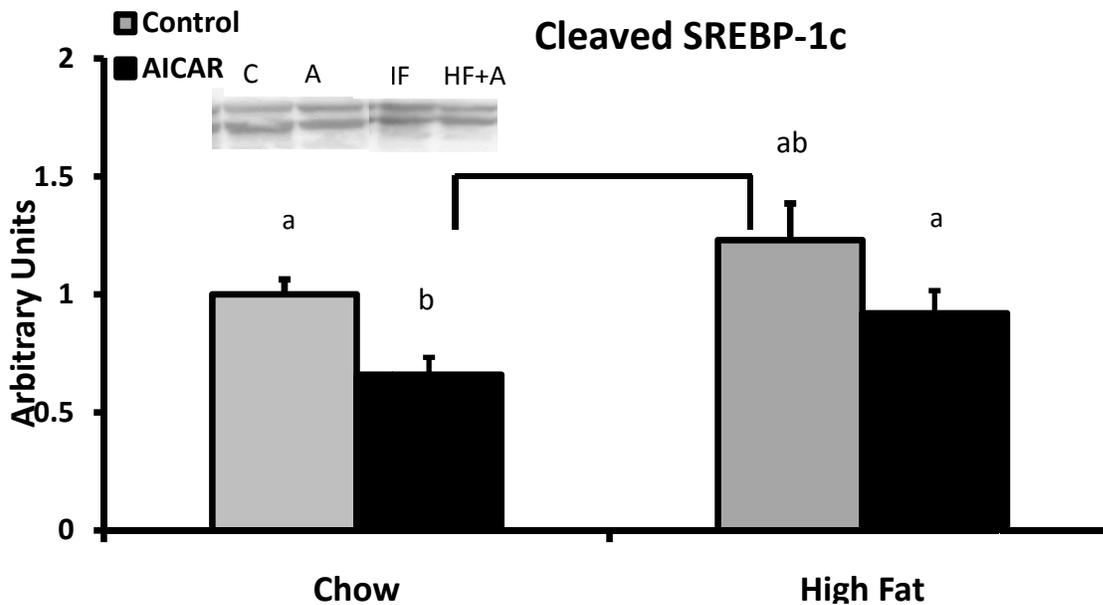


Figure 5b. Chronic activation of AMPK caused a reduction in the total abundance of cleaved (65-68 kDa bands) SREBP-1c in the liver of rats fed either chow or high fat diet (n = 4-5). Bands for all 4 groups were taken side by side with no interruption. Bars that carry the same letter indicate no significant differences were observed ($P < 0.05$). A significant main effect of AICAR and High fat feeding was observed ($p < 0.05$). Graph represents means \pm SE.

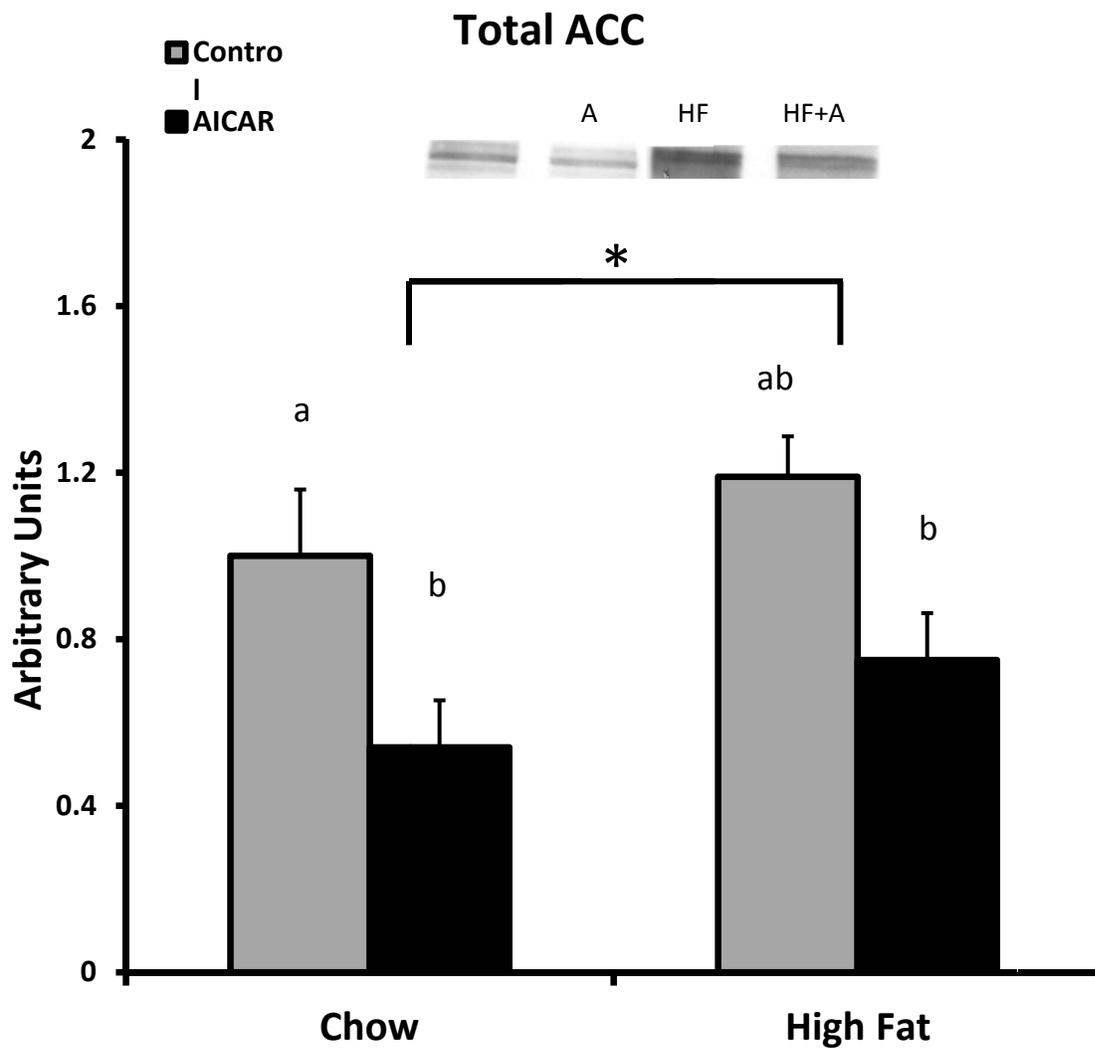


Figure 6. Total acetyl coA carboxylase (ACC) content had a main effect of chronic AMPK activation (n = 7-10). High fat feeding blunted the decrease in total ACC content with the HF + AICAR group. Bands for all 4 groups were taken side by side with no interruption. Asterisk (*) denotes a main effect of AICAR (P < 0.05). Graph represents means ± SE.

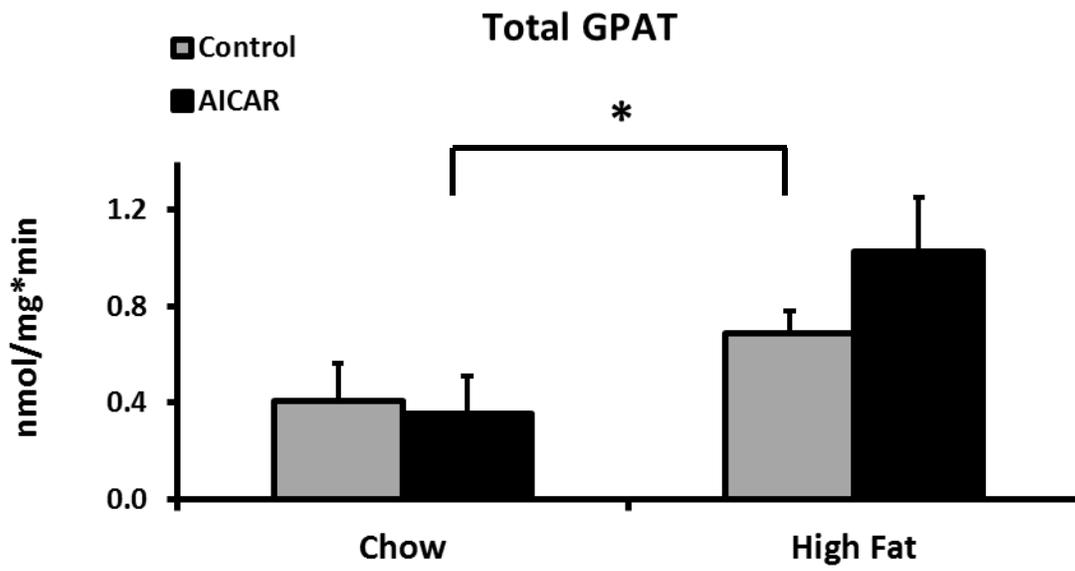


Figure 7a. High fat feeding increased total glycerol-3-phosphate acyl-transferase (GPAT) activity (n = 5-8) in liver. A main AICAR effect on total GPAT activity was absent. Asterisk (*) denotes a main effect of high fat feeding (P < 0.05). Graph represents means ± SE.

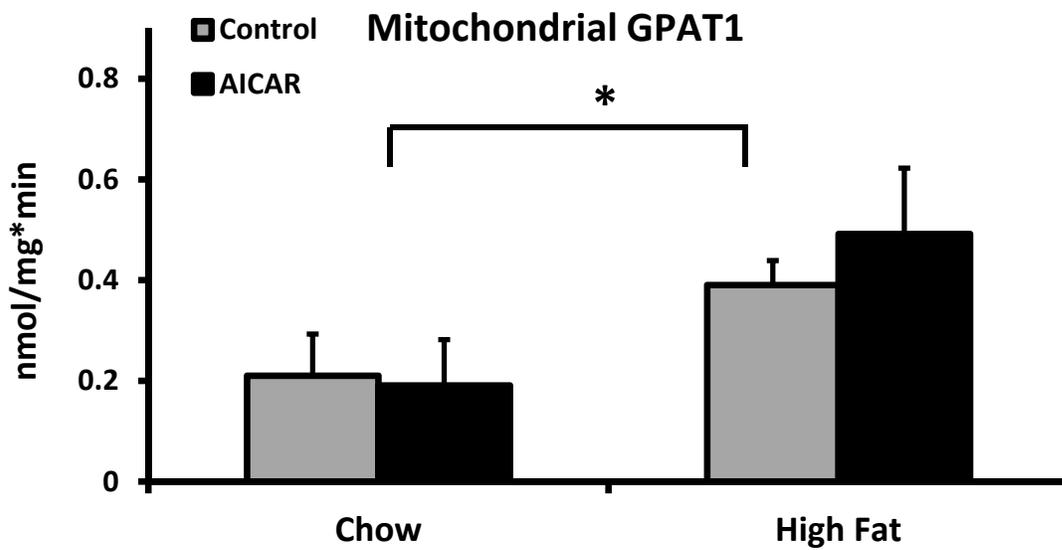


Figure 7b. High fat feeding increased NEM-sensitive glycerol-3-phosphate acyl-transferase (GPAT1) activity in liver (n = 5-8). A main AICAR effect on total GPAT activity was absent. *Main treatment effect (P < 0.05). Graph represents means ± SE.

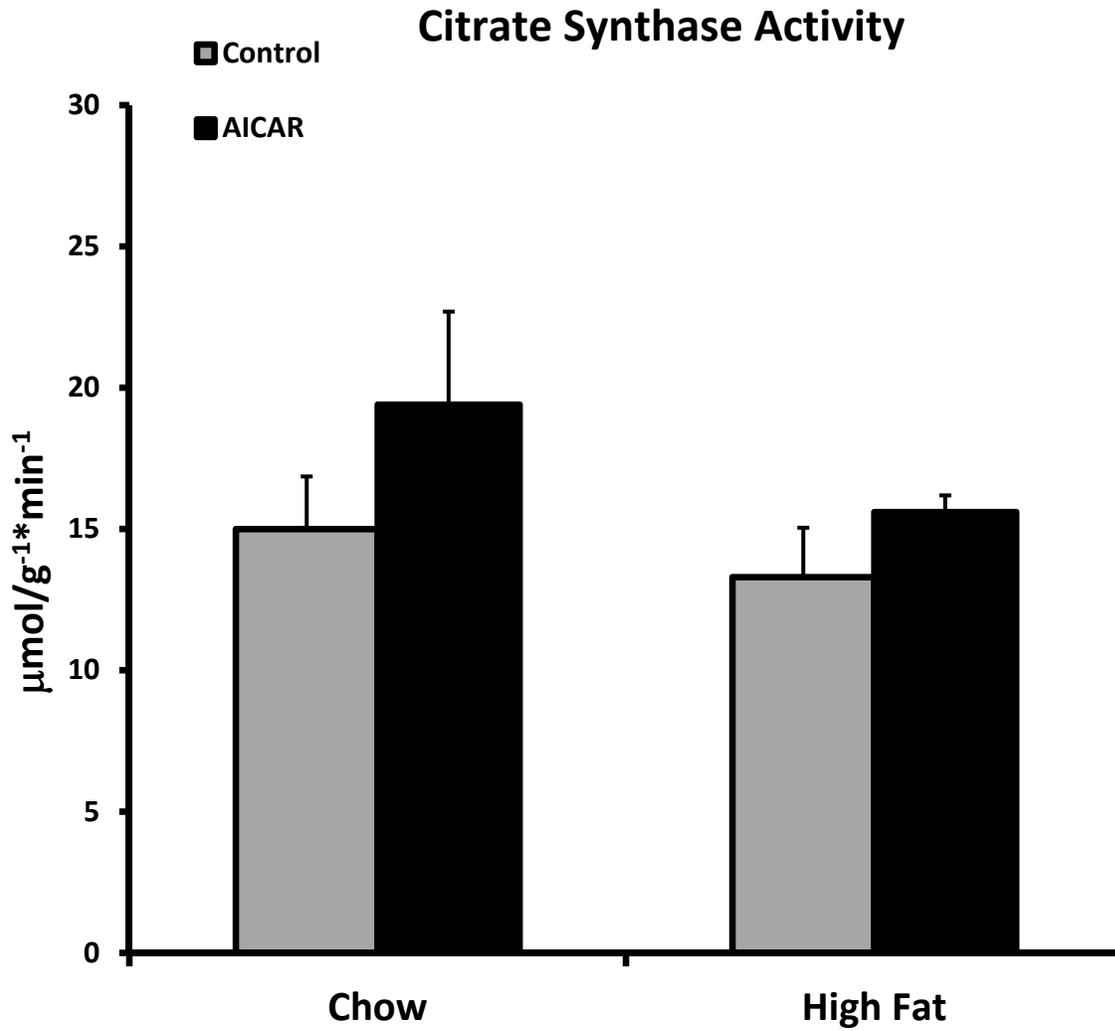


Figure 8. Citrate Synthase activity in the liver did not increase with either high fat feeding or chronic AMPK activation (n = 4-5). Graph represents means \pm SE.

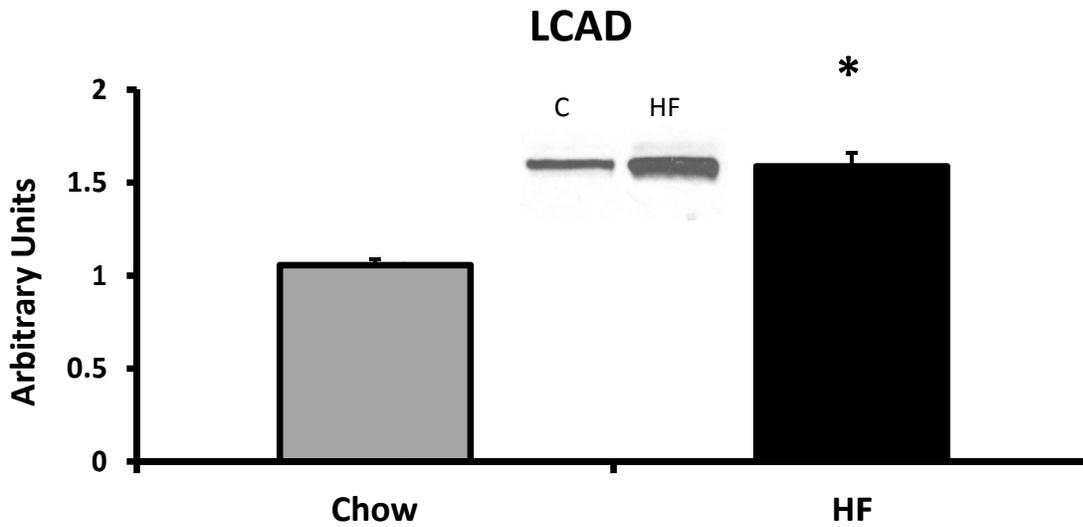


Figure 9a. There was a main high fat effect on total long chain acyl CoA dehydrogenase (LCAD) content in the liver (n = 8-10). Asterisk (*) indicates a main effect of high fat diet (P < 0.05). Graph represents means ± SE.

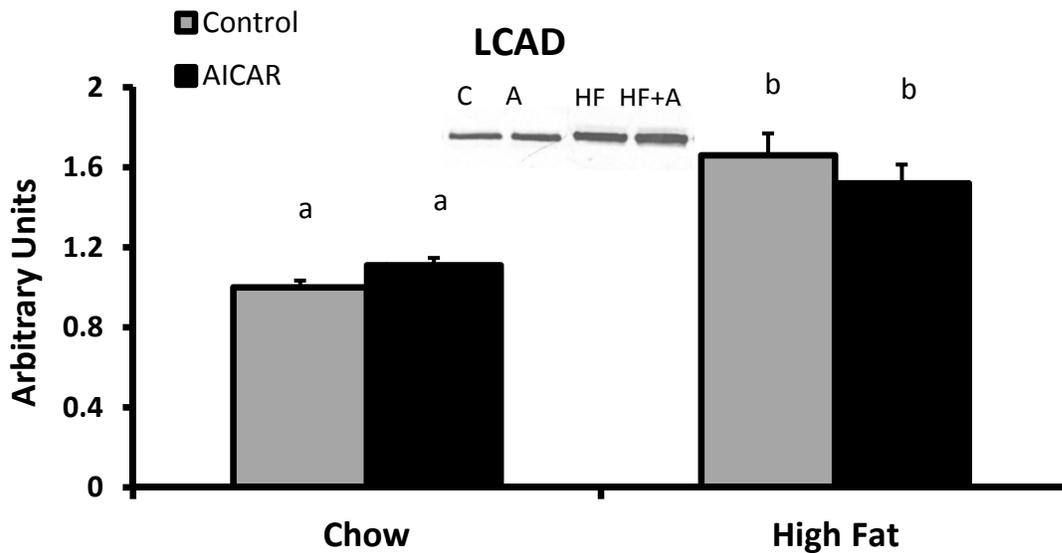


Figure 9b. Chronic activation of AMPK did not have an effect on total long chain acyl CoA dehydrogenase (LCAD) content in the liver (n = 4-5). Bands for all 4 groups were taken side by side with no interruption. Letters are used to represent significance; same letter means no significant difference (P < 0.05). Graph represents means ± SE.

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Appendix A: Literature Review

Non-alcoholic Fatty Liver Disease

Non-alcoholic fatty liver disease (NAFLD) is defined as hepatic fat infiltration greater than five percent of liver weight in the absence of excessive alcoholic intake (39). Such a disease has a significant impact as it can lead to cirrhosis and diminished liver function (4, 39, 84). Hepatic triglyceride accumulation occurs when there is a greater lipid uptake and/or synthesis relative to lipid oxidation and release into the circulation (16, 87). This disease affects many Americans, especially with the increase in the obesity rates across the nation and the impact obesity has on the development of NAFLD (18). Both adults and children are at increasing risk for NAFLD. There are as many as 30 percent of adults in the western world population and up to 61 percent of obese adolescents diagnosed with NAFLD (15, 59, 70, 81, 84). Metabolic syndrome and NAFLD are closely related, in fact NAFLD has been described as the hepatic representation of metabolic syndrome (35, 39, 61, 70). For example almost 90 percent of those with NAFLD have at least one risk factor for metabolic syndrome (51, 70). The study of the mechanism behind NAFLD is clearly of importance due to the impact on our nation's health.

Insulin resistance is one of the diagnostic features of metabolic syndrome and is clearly a significant factor in the development of NAFLD (9, 61, 75). Interestingly, insulin resistance is a cause of the triglyceride accumulation in the liver (75) and triglyceride accumulation increases the risk for insulin resistance. Each has a negative contribution to feed off the other to increase insulin resistance and triglyceride accumulation (60, 67). The proposed mechanism by which insulin resistance increases triglyceride accumulation is explained by the rising levels of hormone sensitive lipase in adipose tissue that occurs in insulin resistance. Hormone sensitive lipase is an enzyme that hydrolyzes triglycerides with the result of increased free fatty acids in the blood (19, 88). These fatty acids are taken to the liver to be absorbed, reformed into

triglycerides and deposited if there are excess hepatic triglycerides (61). Lipid intermediates that interfere with insulin signaling such as diacylglycerols (DAG) may be increased with an elevated level of the enzyme glycerol-3-phosphate acyltransferase (GPAT) that is observed in NAFLD. This is another factor impairing insulin signaling (60). This impairment in insulin signaling leads to an increased risk for hepatic triglyceride accumulation and thus, insulin resistance causes one to be at a greater risk for hepatic steatosis.

Limiting hepatic fat accumulation is a balance of lipid synthesis and oxidation and is important for overall metabolic function in the liver. Thus, the objective of this review is to describe metabolic processes involved in the regulation of hepatic fat accumulation.

Triglyceride Synthesis and Oxidation

AMPK

AMPK (5' adenosine monophosphate (AMP)-activated protein kinase) is a major regulator of energy homeostasis having a major role in fatty acid metabolism, protein synthesis, and glucose uptake (6, 26). The structure of AMPK consists of a heterotrimeric serine/threonine protein kinase made up of one catalytic and two regulatory subunits (28). Different isoforms have greater expression in the various tissues with the liver having a significant expression of the $\beta 1$ isoform of the β subunit and less expression of both $\alpha 1$ and $\alpha 2$ (79, 89, 91). AMPK activation occurs by allosteric and covalent modification of the enzyme in response to a challenge to the energy state by increasing the AMP level (AMP/ATP ratio elevated) in the cell (12, 14). AMPK is also activated when phosphorylated by an upstream kinase such as LKB1, calmodulin-dependent protein kinase kinase or transforming growth factor- β (29, 57, 77). Furthermore, chemical activation of AMPK used in numerous studies is done through the nucleoside AICA

riboside (5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside), otherwise known as AICAR (3, 13, 22). AICAR is an analog of adenosine and when phosphorylated, forms ZMP, thus increasing the amount of ZMP in the cell. The ZMP/ATP ratio is similar to the AMP/ATP ratio in that as it increases, activation of AMPK is increased. However, it is important to note that ZMP is not specific to effects on AMPK activation alone (13, 89). Thus, in response to a change in energy metabolism, AMPK is activated by allosteric and covalent regulation.

AMPK has the effect of decreasing lipid and protein synthesis, and increasing lipid oxidation and insulin sensitivity (50, 56, 83). The role of AMPK activation in glucose homeostasis is a well-known beneficial effect. In fact, the insulin sensitizing drug Metformin, used for type 2 diabetics for lowering blood glucose in the blood, works largely by an AMPK dependent mechanism in peripheral tissues (97). Muscle contraction, has also been shown to increase AMPK activation and induce translocation of the GLUT4 vesicle to the membrane resulting in increased insulin sensitivity (30). Muscle contraction and AICAR injection will stimulate GLUT4 vesicles to move to the membrane to increase glucose uptake into the cell in a similar fashion (89). LKB1 knockout studies have shown decreased AMPK activity and as a result, elevated blood glucose levels with excessive insulin secretion by the pancreas (77). Additionally, preventing the activation of AMPK has been shown to upregulate the gluconeogenic enzyme glucose-6-phosphatase in the liver (77). This has a potential for increasing insulin resistance. Furthermore, AMPK regulates protein synthesis by inhibiting mammalian target of rapamycin (mTOR), part of a complex that phosphorylates proteins that influence mRNA translation (71, 85). Such proteins downstream of and phosphorylated by mTOR include 4E-BP1 and S6K1 (43, 71). The result of inhibition of mTOR by AMPK is

decreased protein synthesis. Having a significant role in improving insulin sensitivity and decreasing protein synthesis, AMPK proves to be significant regulator of energy metabolism.

AMPK has a role in lipid metabolism by decreasing triglyceride synthesis and increasing fatty acid oxidation in the cell. More specifically, one study suggests that it is the isoform AMPK α 2 that phosphorylates and inhibits enzymes of lipid synthesis in the hepatocyte (96). Previous work has shown that activation of AMPK inhibits the lipogenic enzymes fatty acid synthase (FAS), glycerol-3-phosphate (GPAT), and acetyl CoA carboxylase (ACC) (1, 90). Inhibition of ACC by AMPK results in decreased malonyl CoA synthesis and an increased carnitine palmitoyltransferase 1 activity (CPT1). Decreasing malonyl CoA production then increases beta oxidation in the cell (54, 90, 92, 93). Therefore, the result of AMPK activation is increased fatty acid oxidation and decreased lipid production and accumulation. This effect of acute activation of AMPK reduces lipid accumulation has been shown in each the liver and skeletal tissue by inhibition of GPAT1 (58). This inhibition of GPAT1 by AMPK is thought to be through phosphorylation of either GPAT1 or an inhibitor of GPAT1 by AMPK (1, 58, 82). This acute AMPK effect has been demonstrated by a 30 to 40 percent decrease in GPAT1 in hepatocytes (58) with AICAR administration or even up to 50% in liver and adipose tissue (66) upon exercise induced AMPK activation. Therefore, based on these findings and AMPK's acute inhibition of lipogenic enzymes, there is an overall increase in oxidation relative to triglyceride synthesis upon AMPK activation in the liver.

GPAT

The enzyme reaction catalyzed by glycerol-3-phosphate acyltransferase (GPAT) was characterized in the liver in 1953 and is the rate limiting step in triglyceride synthesis (40). Triglycerides are synthesized through GPAT catalyzing the esterification of a fatty acyl CoA and

to glycerol-3-phosphate forming lysophosphatidic acid (LPA), a monoacylglyceride (10, 41).

The production of LPA by GPAT1 in the outer membrane of the mitochondria with the enzyme AGAT produces phosphatidic acid (PA). Triglycerides are eventually formed by the addition of a fatty acid to LPA, (a diacylglyceride), facilitated by the enzyme diacylglycerol acyltransferase (DGAT) (25, 94). There are four different isoforms of GPAT that have been characterized. Two of the four isoforms are located at the mitochondria (mitochondrial GPAT1 and GPAT2) (46, 82, 95) and two at the endoplasmic reticulum (microsomal GPAT3 and GPAT4) (7, 62). The mitochondrial isoforms utilize saturated fatty acyl CoA substrates for triglyceride synthesis whereas microsomal isoforms use either saturated or unsaturated fatty acids (2, 10). A sulfhydryl reagent called N-ethylmaleimide (NEM) will inhibit all isoforms but mitochondrial GPAT1, thus is often used when measuring GPAT1 activity (21, 24, 44). Considering the isoform GPAT1 makes up to 30-50 percent of the total GPAT activity in the liver and a significant contributor to triglyceride synthesis, it is the isoform studied most in trying to understand mechanisms behind hepatic triglyceride regulation (23, 64).

GPAT1 is a significant contributor to the development of NAFLD through its role in triglyceride synthesis in the liver and when overexpressed results in excess triglyceride synthesis (7, 45, 48, 49, 60). Further evidence points to GPAT1's role in triglyceride synthesis through use of GPAT1 knockout mice models. Such models result in a significant reduction in hepatic triglyceride accumulation (24, 63). GPAT1 has been shown to increase hepatic insulin resistance as well (60, 63). When GPAT1 expression is increased in the liver, levels of diacylglycerols (DAG), triglycerides (TG) and phospholipids are elevated in the blood, as long as fatty acids are available (49). This rise in DAG is thought to interfere with insulin signaling thereby increasing the risk for insulin resistance (60). Insulin or carbohydrate-rich diets also increase GPAT1 by

upregulating GPAT1 mRNA (45). This elevated level of GPAT1 is attributed to an increase observed in SREBP-1c following said diets (8, 32, 82). However, AMPK aids in reducing triglyceride synthesis. Briefly, Muoio et al demonstrated a reduction GPAT1 activity by 30 to 40 percent with acute activation of AMPK in the liver as described above (58). Thus, triglyceride accumulation through increased GPAT1 activity is partly alleviated by the acute activation of AMPK in the liver.

mTOR

Mammalian target of rapamycin is part of a complex called mammalian target of rapamycin complex 1 (mTORC1) which has 5 parts being: mTOR, regulatory-associated protein of mTOR (Raptor), mammalian lethal with Sec13 protein 8 (mLST8, also known as GβL), proline-rich Akt substrate 40kDa (PRAS40), and DEP-domain-containing mTOR-interacting protein (Deptor) (68). A 289 kDa protein, mTOR is a serine threonine kinase that has several regulatory functions within the cell that includes growth, metabolism, proliferation and survival (43).

Activated by insulin, amino acids and glucose, regulatory actions of mTOR are to increase protein synthesis, lipid synthesis and decrease insulin action (36, 43). Downstream from mTOR includes the proteins ribosomal protein p70 S6 (S6K1) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). mTOR stimulates S6K1 and inhibits 4E-BP1 with the overall effect on these proteins being to increase translation of various proteins resulting in increased protein synthesis (38, 71). In addition, previous studies report insulin signaling to be decreased by mTOR through phosphorylation of S6K1 (73, 83). This is explained by activation of S6K1, phosphorylating and inhibit insulin receptor substrate 1 (IRS1) in muscle. This inhibition of IRS1 negatively affects the insulin signaling cascade and ultimately causes an

increased risk for insulin resistance. However, Li et al reported that pathways involved in gluconeogenesis and lipogenesis split after Akt and before mTORC1 thereby suggesting a role of mTORC1 in insulin stimulated increase of SREBP-1c mRNA but not phosphoenolpyruvate carboxykinase (PEPCK), a gluconeogenic enzyme (47). Therefore, the role of mTOR and insulin resistance has yet to be elucidated and further work needs to be done in that area.

More pertinent to our study, mTOR plays a major role in hepatic triglyceride synthesis by increasing lipid synthesis in the liver (42, 47, 69). Upstream from mTOR, Akt, a member of the insulin signaling cascade that increases glucose uptake, has regulatory effects on mTOR and lipid synthesis. Akt increases mTOR activity by phosphorylating and inactivating tuberous sclerosis complex 1/2 (TSC1/2) and proline-rich Akt substrate 40kDa (PRAS40) (83). The overall effect is increased mTOR activity and thus, increased lipogenesis in the hepatocyte. AMPK inhibits mTOR activity through phosphorylating and increasing TSC1/2 activity. Phosphorylated TSC1/2 has an increased association with mTOR and this association decreases mTOR activity. Thus, AMPK activation decreases lipogenesis through decreased mTOR activity (34, 38, 42, 83). However, mTOR activates SREBP-1c and thereby increases transcription of lipogenic genes such as ACC, FAS, and GPAT1 (69). Li et al found that SREBP-1c is significantly decreased by inhibitors of mTOR such as rapamycin. This indicates that through AMPK's inhibition of mTOR activity, AMPK has the effect of reducing SREBP-1c activity (47). The overall effect of mTOR is increased triglyceride synthesis and therefore contributes to hepatic lipid accumulation.

SREBP-1c

There are three different isoforms of the membrane bound sterol regulatory element binding protein (SREBP) in lipogenic tissues of mammals: SREBP-1a, SREBP-1c, and SREBP-

2 (32). SREBP-2 is transcribed from a gene different from the one that codes for both SREBP-1a and SREBP-1c. SREBP-1a and SREBP-1c differ in regard to the transcription start site on exon 1 (5). SREBP-1a plays a role in the regulation of cholesterol, triglyceride and fatty acid synthesis. Alternatively, SREBP-1c and SREBP-2 have a more focused role in lipid regulation with SREBP-1c regulating enzymes of fatty acid synthesis and SREBP-1 regulating enzymes of cholesterol synthesis (5).

SREBP-1c becomes activated following cleavage and activation which releases SREBP-1c from the membrane and causes nuclear localization. The cleaved form of SREBP-1c then binds to the sterol regulatory element (SRE) and E box sequences in the nucleus on lipogenic genes it regulates (37). In the fed state, the isoform SREBP-1c is expressed significantly more than the other isoforms. Further, SREBP-1c is affected by the energy state, as evidenced by a decrease in SREBP-1c transcription following a fast and significant increase upon refeeding (78).

The SREBP transcription factors regulate genes involved in cholesterol and lipid metabolism such as ACC, FAS, GPAT, and stearoyl-CoA desaturase (SCD1) (5, 31, 37, 42, 69). In fact, increased expression of SREBP-1c is associated with NAFLD (31). SREBP-1c is more specific to fatty acid synthesis than cholesterol synthesis compared to the other isoforms of SREBP. For example, overexpressing SREBP-1c causes a significant increase in triglyceride synthesis and hepatic steatosis whereas there is no change in cholesterol synthesis (78). The promoter region for SREBP-1c has a liver X receptor (LXR) binding site and LXR binding increases the transcription of SREBP-1c (31, 72, 76). This regulatory relationship between LXR and SREBP-1c has been demonstrated by the significant reduction in SREBP-1c has been shown to be significantly decreased in LXR knockout mice (72). Previous work suggests that activation

of AMPK decreases promoter activity of SREBP-1c in the liver as well as the transcriptional activity of the upstream transcription factor and regulator of SREBP-1c expression, LXR α thus decreasing SREBP-1c and LXR (92). Recently it was proposed that mTOR plays a prominent role in activation of SREBP-1c in cell models where mTOR increases cleavage of SREBP-1c to its active form by an unknown mechanism (42, 69). Increasing SREBP-1c activity by an mTOR-dependent mechanism gives one possible explanation for elevated lipogenesis with increased mTOR activity.

Activation of AMPK decreases SREBP-1c in the liver by a couple of different mechanisms. In hepatocytes, activation of AMPK through the drug metformin decreases the mRNA of SREBP-1c and the lipogenic enzymes SREBP-1c regulates. This decrease in SREBP-1c mRNA by AMPK validates the role AMPK has in inhibiting SREBP-1c (97). AMPK decreases SREBP-1c mRNA by phosphorylating the threonine residues of LXR α resulting in inhibition of LXR. S6K1 has the opposite effect by phosphorylating LXR at the serine residues and activates LXR α , thus increasing SREBP-1c content in the liver (27, 33). Thus, AMPK further inhibits hepatic lipid accumulation by inhibiting a major regulator of hepatic lipogenesis, SREBP-1c, through transcriptional regulation.

CPT-1

Localized in the outer membrane of the mitochondria, Carnitine palmitoyltransferase 1 (CPT1) has three different isoforms with CPT1a being the primary isoform found in the liver (53). CPT1, located on the cytosolic face of the outer membrane of the mitochondria, is the key regulator of hepatic metabolic beta oxidation of long chain acyl CoAs in the liver (17, 20, 86). CPT1 regulates not only beta oxidation in liver but in the heart and muscle as well. In fact, there

is a greater sensitivity to malonyl CoA and increased affinity for carnitine in these non-hepatic tissues (55, 74).

Beta oxidation is significantly affected by inhibitors of CPT1 such as malonyl CoA (53). AMPK, as previously described, inhibits ACC therein decreasing the production of malonyl CoA. As a potent inhibitor of CPT1, a reduction in malonyl CoA results in increased CPT1 activity (54, 90, 92, 93). Therefore, with increased lipogenesis from high carbohydrate or fat feeding, malonyl CoA increases and beta oxidation is downregulated (52). Further, Stefanovic-Raci et al found a 60 percent reduction in triglyceride secretion by hepatocytes but no differences in plasma triglycerides with an overexpression of CPT1a (80). An additional regulator of CPT1 is through hormonal control. In a ketotic energy state, CPT1a activity has been shown to increase from a decreased malonyl CoA in such a state (11). On the other hand, insulin has been shown to decrease CPT1a mRNA therefore is a negative regulator of CPT1 (65). There are a number of regulators of CPT1 and beta oxidation and this regulation is a significant factor in lipid homeostasis in the liver.

Conclusion

In summary, hepatic fat accumulation is associated with insulin resistance and a general reduction in liver function. If not controlled, this can lead to cirrhosis of the liver. AMPK is a central regulator of energy homeostasis, thus it is fitting that it is involved in mechanisms responsible for hepatic fat accumulation. AMPK is reported to be involved in the acute regulation of fat oxidation and synthesis. Recent evidence also points to a role in which AMPK influences the transcription/translation of the lipogenic program in hepatic tissue. Therefore, it

was the objective of this review to describe aspects that regulate hepatic lipid accumulation and the way AMPK may be involved.

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