2015

Effects of Conjugated Linoleic Acid (CLA) on Skeletal Muscle Metabolism

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EFFECTS OF CONJUGATED LINOLEIC ACID (CLA) ON SKELETAL MUSCLE METABOLISM

A Dissertation Presented

by

YOO KIM

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

September 2015

The Department of Food Science
EFFECTS OF CONJUGATED LINOLEIC ACID (CLA) ON SKELETAL MUSCLE METABOLISM

A Dissertation Presented

by

YOO KIM

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Eric A. Decker, Department Head
Department of Food Science
DEDICATION

Dedicated to my beautiful and committed wife, wonderful sons,

beloved parents, and parents-in-law
ACKNOWLEDGMENTS

First of all, I would like to express my sincere gratitude to my advisor, Dr. Yeonhwa Park, for giving me a great opportunity to successfully do a doctoral program in her lab and for instilling true inspiration. Indeed, she has provided me with the academic and financial support throughout my doctoral studies. I would also like to thank my committee members, Dr. McClements, Dr. Zhang and Dr. Kim, for serving on my committee and for sharing the tremendous knowledge in their own field. They give me all great values I aspire to emulate and integrate in my future career.

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ABSTRACT

EFFECTS OF CONJUGATED LINOLEIC ACID (CLA) ON SKELETAL MUSCLE METABOLISM

SEPTEMBER 2015

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Although it is well known that increased physical activity can help reduce incidences of obesity, physical activity can be influenced by complex issues, making it difficult to use universal means to control obesity. Recent discoveries of the effects of exercise at the cellular level opened up the unique opportunity to develop compounds with ‘exercise-like’ effects. In fact, CLA has been shown to promote voluntary activity and endurance capacity in mice. Thus, the purpose of these studies is to determine the mechanisms of conjugated linoleic acid on muscle metabolism via the modulation of biochemical events including mitochondrial biogenesis and the alternation of fiber composition in skeletal muscle in order to develop preventive strategies for obesity. Based on in vitro fundamental mechanism studies, we observed that CLA stimulated mitochondrial biogenesis by signaling the up-regulation of peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) and related biomarkers involved in muscle metabolism. These new findings provided the opportunity to consider CLA as a candidate for bioactive compound mimicking exercises. We conducted additional animal studies, which were organized into three parts. The first part was an adult obesity study with the nescent basic helix loop helix 2 (Nlh2) gene knockout mice, which are the genetically induced inactivity adult-onset obesity model. Moreover, the study was designed for childhood obesity with post-weaning Nlh2 mice under the pre-obese state. Lastly, this was an exercise model, which divided normal mice into four groups
according to diet and training, such as sedentary or exercise regimes. Consequently, these three studies have shown that CLA enhances voluntary activity and endurance capacity. Additionally, there were increases of muscle mass and decreases of body fat mass through modulating muscle metabolism by stimulating mitochondrial biogenesis, inducing genes of muscle fibers, and enhancing lipid metabolism based on the AMP-activated protein kinase (AMPK) signaling pathway. Particularly, the PPARδ-mediated pathway plays a central role in overall skeletal muscle metabolism. The current results suggest that CLA acts as a potential exercise-mimetic, resulting in improved physical activity, which can also support its function of regulating body fat.
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CHAPTER 1
INTRODUCTION

Around 40-60% of the world population suffers from obesity and related chronic diseases including type II diabetes, cardiovascular disease, metabolic syndrome and certain types of cancer. Calorie restriction accompanied with physical activity leading to increased energy expenditure is elucidated as the most effective strategy to reduce obesity.\textsuperscript{1,2} Although increased physical activity contributes to various health benefits for attenuating an incidence of obesity as well as its associated pathologies, complex social, environmental, physiological and psychological factors can affect the subject’s physical activity. Thus, it is difficult to simply use physical activity as universal means to control obesity. However, over the last decade, a growing body of evidence has shed light on the unique importance to intervene and develop drugs or dietary components as an exercise mimetic to demonstrate the alteration of molecular targets by exercise.\textsuperscript{3-6}

On a weight basis, skeletal muscle tissue is the largest portion of total body mass in the normal human body, excluding obese individuals. Skeletal muscle is one of the most metabolically demanding tissues, which accounts for almost half of all energy expenditure, as well as three-fourths of all glucose disposal. In addition, skeletal muscle has a unique metabolic flexibility in energy metabolism, responding to physiological and pathophysiological conditions. Particularly, it is possible for skeletal muscle to selectively use fuel sources such as glycogen, glucose and fatty acids for energy production depending on aerobic or anaerobic metabolism in response to exercise or chronic contractile activity.\textsuperscript{7-9} Although skeletal muscle as a whole acts as a contributor to glucose and fatty acid metabolism, the composition of fibers in individual muscle plays a critical role in muscle energy metabolism. Specifically, the metabolic plasticity of fibers in skeletal muscle relies on mitochondrial content and composition. Mitochondrial biogenesis—which is generally referred to as mitochondrial adoptions responding to external stimuli such as
exercise—increases mitochondrial volume and changes its composition, contributing to an alteration of metabolic preference from glucose to fatty acids.\textsuperscript{10-12}

Conjugated linoleic acid (CLA) is a mixture of geometric and positional conjugated isomers of linoleic acid. The effects of CLA have been previously demonstrated to influence the development of cancer and atherosclerosis, in addition to preventing osteoporosis and modulating immune responses.\textsuperscript{13,14} Moreover, CLA has garnered special attention in the last two decades for its anti-obesity effects through various mechanisms, such as the regulation of energy and lipid metabolism, adipogenesis, inflammation and adipocyte apoptosis.\textsuperscript{15,16} Furthermore, in animal models, it was reported that CLA improved voluntary activity and endurance capacity and modulated energy metabolism.\textsuperscript{17,18}

Although further studies are needed to demonstrate the role of CLA in skeletal muscle, the accumulated evidence to date of increased lean body mass and improved physical activity provide clues on how CLA might modulate skeletal muscle metabolism. Thus, we expect that the regulating mechanisms of CLA in skeletal muscle can elucidate the preventive effects on obesity along with currently known effects on adipose tissue. In addition, we expect that the information obtained from the successful completion of this research will expand our understanding of the use of bioactive food compounds and physical exercise to control obesity.
2.1 Overview of CLA

Since the discovery of CLA in beef, it is known that the primary dietary sources of CLA are meats and dairy products from ruminants. It is reported that in dairy products, CLA contents range between 0.34 and 1.07% of the total fat, and in raw or processed beef range between 0.12 and 0.68%.\textsuperscript{19} Although we continuously consume CLA daily in foods originated from ruminant, concentrations of CLA in food (less than 10 mg CLA/g fat) are not substantial.\textsuperscript{20} The average daily CLA intake in the U.S. from food sources is 104-151 mg and 176-212 mg for women and men, respectively.\textsuperscript{21,22}

CLA has two active isomers (cis-9,trans-11 and trans-10,cis-12 isomers), which produce different biological effects.\textsuperscript{14} CLA isomers mostly originate from either biosynthesis or chemical synthesis. The cis-9,trans-11 is a natural predominant isomer which accounts for over 80% of CLA isomers and is derived from the biohydrogenation of rumen bacteria.\textsuperscript{23,24} In addition to cis-9,trans-11, the most notable CLA isomer is the trans-10,cis-12. However, in natural foods the amount of trans-10,cis-12 is very low. When chemical synthesis is used, this isomer forms in significant amounts.\textsuperscript{20} Although CLA has at least 28 different isomers, cis-9,trans-11 and trans-10,cis-12 isomers have demonstrated the most favorable health benefits. Currently, most commercial CLA preparations are composed of almost equal amounts of cis-9,trans-11 and trans-10,cis-12 isomers (also referred to as a CLA mixture or 50:50 mix).

Over the last two decades, studies have uncovered some negative health issues of trans fat, and it is therefore necessary to define the trans fatty acids in CLA.\textsuperscript{25} The definition of trans fat labeling by U.S. Food and Drug Administration (FDA) is “all unsaturated fatty acids that contain one or more isolated double bonds in a trans configuration”.\textsuperscript{26} This definition means that
conjugated linoleic acid (CLA) is ruled out from this regulation to label *trans* fats, since non-conjugated *trans* fatty acids are distinguished from conjugated forms. Further, in July 2008, the FDA approved CLA for a GRAS (Generally Recognized as Safe) status for specific food categories such as fluid milk, yogurt, meal-replacement shakes, nutritional bars, fruit juices and soy milk. Thus, it is expected that there will be an increase in applications of CLA for human health benefits.

2.2 CLA and Body Composition Change

The human body is typically composed of fat, muscle (protein or lean body mass), water and bone, and measuring body composition is an efficient way to assess obesity. Most of the initial CLA studies in animals used this method to determine CLA’s anti-obesity effects. Since the first finding of the effect of CLA on body composition changes in mice in 1997, numerous studies have reported that CLA supplementation contributed to modulate body composition by reducing body fat and/or increasing lean body mass in various mammalian models. Herein, the previous studies in mice and rats on the changes in body composition including both body fat mass and lean body mass are described in Tables 2.1 and 2.2. Among about 100 human studies on body fat regulation, Table 2.3 summarizes the clinical trials measured changes of both body fat reduction and lean body mass.

2.2.1 Effects of CLA on Body Fat Reduction

One of the most attractive aspects of CLA is to reduce body fat mass through various mechanisms. According to the experimental purpose, previous studies have adopted different strains, genders, forms and concentrations of CLA and varying durations in mouse studies (Table 2.1). The effect of CLA on body fat reduction is likely to be the most responsive in mice, where previous studies at levels of 0.5-2.2% of dietary CLA mixture or 0.2% of single isomer have shown a decrease in body fat mass by 40-80%. In the same context, 0.5-3.0% CLA-fed rats
Table 2.1. Effects of conjugated linoleic acid (CLA) on body composition in mice

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<th>Form&lt;sup&gt;c&lt;/sup&gt;</th>
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<th>Results&lt;sup&gt;e&lt;/sup&gt;</th>
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<td>↓</td>
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<td>ApoE KO</td>
<td>M</td>
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<td>-</td>
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<td>8 weeks</td>
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Table 2.1. Continued

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<th>LBM</th>
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<th>Energy Expendituref</th>
<th>Biomarkersg</th>
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<td>N2KO</td>
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<td>12 weeks</td>
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<td>-</td>
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<td></td>
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<td>CF-1</td>
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<td>↓</td>
<td>↑ EE / ↓ RQ</td>
<td>↑ CPT-1 / ↑ UCP2</td>
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<td>↓</td>
<td>↓</td>
<td>↑</td>
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<td>↑</td>
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<td>4 weeks</td>
<td>↓</td>
<td>↓</td>
<td>-</td>
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<td>Scalerandi et al., 201468</td>
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<td>Mixture</td>
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<td>4 weeks</td>
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<td>↓</td>
<td>-</td>
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**a** ApoE KO, apolipoprotein E knockout; ddY, Deutschland, Denken, and Yoken; MH, metabolic rate high; ML, metabolic rate low; N2KO, Nescient helix-loop-helix 2 gene knockout; PPARα-KO, peroxisome proliferator-activated receptor α knockout; SENCAR, sensitive to carcinogenesis.

**b** F, female; M, male.

**c** Mixture, a mixed isomer of cis-9,trans-11 and trans-10,cis-12; c9t11, cis-9,trans-11 CLA isomer; t10c12,trans-10,cis-12 CLA isomer.

**d** Dosage (%) means a designated weight percentage of CLA in diet.

**f** EE, energy expenditure; RER, respiratory energy ratio; RQ, respiratory quotient; VA, voluntary activity.

**g** CPT, carnitine palmitoyltransferase; GLUT4, glucose transporter type 4; IL-6, interleukin 6; TNF-α, tumor necrosis factor alpha; UCP2, uncoupling protein 2; ↔, no change.
Table 2.2. Effects of conjugated linoleic acid (CLA) on body composition in rats

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<th>Rat</th>
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<td>Gender&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Form&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Dosage (%)&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>de Almeida et al., 2013&lt;sup&gt;79&lt;/sup&gt;</td>
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<sup>a</sup>F, female; M, male.

<sup>b</sup>Mixture, a mixed isomer of cis-9,trans-11 and trans-10,cis-12; c9t11, cis-9,trans-11 CLA isomer; t10c12,trans-10,cis-12 CLA isomer.

<sup>c</sup>Dosage (%) means a designated weight percentage of CLA in diet.

<sup>d</sup>BW, body weight; BFM, Body fat mass; LBM, lean body mass; -, no change; ↑, increase; ↓, decrease.

<sup>e</sup>TG, triglyceride.
<table>
<thead>
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<th>Characteristic</th>
<th>Gender</th>
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<th>Results</th>
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<td>12 weeks</td>
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<td>Mixture</td>
<td>1.7/3.5/ 5.1/6.8</td>
<td>6 and 12 weeks</td>
<td>-</td>
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<td>↑ 6.8 at 12 wk</td>
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<td>Mixture / t10c12</td>
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<td>↑ RMR</td>
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<td>Mixture (TG/Free form)</td>
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<td>↓</td>
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<td>1 year extension open study / ↓ food intake</td>
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<td>Colakoglu et al., 2006</td>
<td>Normal</td>
<td>F</td>
<td>Mixture</td>
<td>3.6</td>
<td>6 weeks</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>Larsen et al., 2006</td>
<td>Overweight / Obese</td>
<td>F+M</td>
<td>Mixture</td>
<td>3.4</td>
<td>1 year</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Weight regain / hypocaloric diet</td>
</tr>
</tbody>
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(Continued)
<table>
<thead>
<tr>
<th>References</th>
<th>Characteristic</th>
<th>Gender</th>
<th>CLA Supplementation</th>
<th>Results</th>
<th>Energy Expenditure</th>
<th>Other comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pinkoski et al., 2006&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Unknown</td>
<td>F+M</td>
<td>Mixture</td>
<td>5.0</td>
<td>↓</td>
<td>↔ RMR</td>
</tr>
<tr>
<td>Gaullier et al., 2007&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Overweight / Obese</td>
<td>F+M</td>
<td>Mixture</td>
<td>3.4</td>
<td>↓</td>
<td>↔ Calorie intake</td>
</tr>
<tr>
<td>Lambert et al., 2007&lt;sup&gt;9&lt;/sup&gt;</td>
<td>Normal / Overweight</td>
<td>F+M</td>
<td>Mixture</td>
<td>2.6</td>
<td>-</td>
<td>↔ RMR</td>
</tr>
<tr>
<td>Laso et al., 2007&lt;sup&gt;9&lt;/sup&gt;</td>
<td>Overweight / Obese</td>
<td>F+M</td>
<td>Mixture</td>
<td>3.0</td>
<td>↓</td>
<td>↔ Appetite</td>
</tr>
<tr>
<td>Nazare et al., 2007&lt;sup&gt;9&lt;/sup&gt;</td>
<td>Normal / Overweight</td>
<td>F+M</td>
<td>Mixture</td>
<td>2.8</td>
<td>-</td>
<td>↑ RMR</td>
</tr>
<tr>
<td>Steck et al., 2007&lt;sup&gt;9&lt;/sup&gt;</td>
<td>Obese</td>
<td>F+M</td>
<td>Mixture</td>
<td>3.2/6.4</td>
<td>-</td>
<td>↔ RMR / ↔ RQ</td>
</tr>
<tr>
<td>Tarnopolsky et al., 2007&lt;sup&gt;9&lt;/sup&gt;</td>
<td>Normal / Overweight</td>
<td>F+M</td>
<td>Mixture</td>
<td>5.4 + 5 g creatine monohydrate</td>
<td>6 months</td>
<td>-</td>
</tr>
<tr>
<td>Watras et al., 2007&lt;sup&gt;10&lt;/sup&gt;</td>
<td>Overweight</td>
<td>F+M</td>
<td>Mixture</td>
<td>3.2</td>
<td>↓</td>
<td>↑ RMR</td>
</tr>
<tr>
<td>Diaz et al., 2008&lt;sup&gt;10&lt;/sup&gt;</td>
<td>Overweight / Obese</td>
<td>F</td>
<td>Mixture</td>
<td>1.8 + 0.4 mg creatine picolinate</td>
<td>12 weeks</td>
<td>-</td>
</tr>
<tr>
<td>Park et al., 2008&lt;sup&gt;10&lt;/sup&gt;</td>
<td>Overweight / Obese</td>
<td>F+M</td>
<td>Mixture</td>
<td>2.4</td>
<td>↓</td>
<td>↓ Food intake</td>
</tr>
<tr>
<td>Sneddon et al., 2008&lt;sup&gt;10&lt;/sup&gt;</td>
<td>Normal / Obese</td>
<td>M</td>
<td>Mixture</td>
<td>2.3 + 1.3 g ω-3 fatty acid</td>
<td>12 weeks</td>
<td>↑</td>
</tr>
<tr>
<td>Norris et al., 2009&lt;sup&gt;10&lt;/sup&gt;</td>
<td>Obese</td>
<td>F</td>
<td>Mixture</td>
<td>6.4</td>
<td>↓</td>
<td>Type 2 diabetes / postmenopausal</td>
</tr>
<tr>
<td>Raff et al., 2009&lt;sup&gt;10&lt;/sup&gt;</td>
<td>Normal / Overweight / Obese</td>
<td>F</td>
<td>Mixture / c9t11</td>
<td>5.5/4.7</td>
<td>-</td>
<td>↓ by Mixture</td>
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(Continued)
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<tr>
<th>References</th>
<th>Characteristic</th>
<th>Gender</th>
<th>Form</th>
<th>Dose (g/day)</th>
<th>Duration</th>
<th>BW</th>
<th>BFM</th>
<th>LBM</th>
<th>Energy Expenditure</th>
<th>Other comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornish et al., 2009&lt;sup&gt;106&lt;/sup&gt;</td>
<td>Obese</td>
<td>F+M</td>
<td>Mixture</td>
<td>4.3 + 9 g creatine monohydrate + 36 g whey protein</td>
<td>5 weeks</td>
<td>-</td>
<td>-</td>
<td>↑</td>
<td></td>
<td>↔ Energy intake</td>
</tr>
<tr>
<td>Racine et al., 2010&lt;sup&gt;107&lt;/sup&gt;</td>
<td>Overweight / Obese</td>
<td>F+M</td>
<td>Mixture (TG)</td>
<td>2.4</td>
<td>7 months</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
<td></td>
<td>Childhood model</td>
</tr>
<tr>
<td>Joseph et al., 2011&lt;sup&gt;108&lt;/sup&gt;</td>
<td>Overweight / Obese</td>
<td>M</td>
<td>Mixture / c9t11</td>
<td>2.8/2.7</td>
<td>8 weeks</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>Crossover design</td>
</tr>
<tr>
<td>Chen et al., 2012&lt;sup&gt;109&lt;/sup&gt;</td>
<td>Overweight / Obese</td>
<td>F+M</td>
<td>Mixture</td>
<td>1.7</td>
<td>12 weeks</td>
<td>↓</td>
<td>↓</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macaluso et al., 2012&lt;sup&gt;110&lt;/sup&gt;</td>
<td>Normal / Overweight</td>
<td>M</td>
<td>Mixture</td>
<td>4.8</td>
<td>3 weeks</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>Crossover design / serum testosterone ↑</td>
</tr>
<tr>
<td>Lopez-Plaza et al., 2013&lt;sup&gt;111&lt;/sup&gt;</td>
<td>Overweight</td>
<td>F+M</td>
<td>Mixture</td>
<td>3.0</td>
<td>24 weeks</td>
<td>↓</td>
<td>↓</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shadman et al., 2013&lt;sup&gt;112&lt;/sup&gt;</td>
<td>Overweight</td>
<td>F+M</td>
<td>Mixture</td>
<td>2.4 + 100 IU/day Vitamine E</td>
<td>8 weeks</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>Cosupplementation/ Type 2 diabetes</td>
</tr>
<tr>
<td>Ormsbee et al., 2014&lt;sup&gt;113&lt;/sup&gt;</td>
<td>Overweight / Obese</td>
<td>F+M</td>
<td>Mixture</td>
<td>CLA + Green tea + BCAA (branched chain amino acid)</td>
<td>8 weeks</td>
<td>↓</td>
<td>-</td>
<td>-</td>
<td></td>
<td>Cosupplementation</td>
</tr>
</tbody>
</table>

<sup>a</sup>F, female; M, male.

<sup>b</sup>Mixture, a mixed isomer of cis-9, trans-11 and trans-10, cis-12; c9t11, cis-9, trans-11 CLA isomer; t10c12, trans-10, cis-12 CLA isomer; TG, triglyceride form; Free form, free fatty acid form.

<sup>c</sup>BW, body weight; BFM, Body fat mass; LBM, lean body mass; -, no change; ↑, increase; ↓, decrease.

<sup>d</sup>EE, energy expenditure; RER, respiratory energy ratio; RMR, resting metabolic rate; RQ, respiratory quotient; ↔, no change.
accumulated less body fat, although the magnitude of the reduced body fat was dependent on the strain of rats and the site of the adipose tissue (Table 2.2). Specifically, CLA supplementation in Sprague-Dawley rats showed the reduced peritoneal and parametrial fat mass by 25-30%.\(^70\) Additionally, 0.5% CLA diets in OLETF rats induced 14% and 44% less fat accumulation in omental and epididymal adipose tissues, respectively.\(^114\) Along with the availability of purified or enriched CLA isomer, further studies have demonstrated the specific CLA isomers’ effects on body fat accretion. With respect to body fat reduction, the \textit{trans-10,cis-12} CLA isomer was identified as the primary active form in mice, not the \textit{cis-9,trans-11} isomer.\(^18,31,58,62,115\) In congruence with the mice study, the \textit{trans-10,cis-12} CLA isomer remarkably induced a reduction of body weight and fat mass in OLETF rats.\(^116\) Also, 1.5% CLA mixture fed Zucker diabetic fatty (ZDF) rats had significantly lower body weight gain and fat mass, while it was observed that the \textit{cis-9,trans-11}-fed group had no effect on changes in body composition, especially body fat reduction.\(^117\) In another isomer-specific study, the eight week feeding \textit{trans-10,cis-12} isomer did not achieve significant changes in body weight and adipose mass in the same rat strain.\(^118\)

On the basis of evident results from animal studies, a number of clinical trials have been carried out to determine the effects of CLA on body fat accretion.\(^14,22,119\) Human intervention studies of supplemented CLA mostly used the CLA mixture form (\textit{cis-9,trans-11} and \textit{trans-10,cis-12} isomers). Although accumulating evidence elicits the effect of CLA on the reduction of body fat mass in humans,\(^120\) it is less responsive to animal studies, especially compared to the mouse model.

In addition, the subjects’ weight status may contribute to CLA’s efficacy on body fat control. Normal weight subjects supplemented with 0.7-5.5 g of CLA mixture for 4-16 weeks had a decreased body fat mass by 4-20\%,\(^91,93,105,121-123\) whereas overweight and obese individuals treated with 1.7-6.8 g of CLA mixture for 4-104 weeks showed reductions of 3-15\%.\(^81,85,88,89,98,108,124-127\) However, other studies observed no effect of CLA on body fat mass in both subject groups (normal and overweight/obese).\(^22\) Interestingly, the study in 6-10 year old
overweight and obese children at levels of 3 g of CLA mixture for 7 months significantly reduced peripheral and abdominal body fat accumulation (\%).\textsuperscript{107} Moreover, Ha et al.\textsuperscript{128} reported that 15-year-old obese volunteers supplemented with 6 g of CLA mixture for 6 months had decreased body weight and fat content, as well as improved physical activity. To date, there are two meta-analyses focused on both body weight and body fat mass to establish a significant dose and duration for CLA’s efficacy.\textsuperscript{129,130} Whigham et al.\textsuperscript{129} reported that 3.2 g/day CLA supplementation led to significantly reduce body fat mass in humans, although it was a modest loss. A study by Blankson et al.\textsuperscript{82} supported this finding, demonstrating that CLA’s effective dose of body fat reduction plateaued at 3.4 g/day between the range of 1.7 and 6.8 g/day. Along with CLA’s dose efficacy, they reported that supplementation with CLA up to 6 months linearly reduced body fat, then attenuated response and eventually showed no additional effect at approximately 2 years. This is consistent with a meta-analysis in overweight and obese individuals based on fifteen previous clinical trials,\textsuperscript{130} indicating long-term CLA intake significantly induces loss in body weight and fat mass.

Combining all these results together, CLA likely reduces body fat mass. However, it is not mirrored in body weight decrease, since CLA might be involved in other composition changes in the body. In fact, the previous clinical studies showed no changes in body weight by CLA supplementation even though body fat reduction occurred.\textsuperscript{82,86,94,96,99,105,113} In support of these observations, body fat mass and lean body mass were simultaneously influenced by CLA supplementation. Particularly, seven human intervention studies\textsuperscript{82,87,91,93,94,99,105} along with eight publications in rodents\textsuperscript{27,29-31,54,62,63,69} demonstrated reduced body fat accretion as well as increased lean body mass by CLA supplementation. Thus, it is important to further investigate the effects of CLA on lean body mass.

### 2.2.2 Effects of CLA on Increased Lean Body Mass

In terms of the effects of CLA on body composition change, most studies have been
focused on the decreased body fat mass. However, a growing body of evidence elucidates the importance of increased lean body mass, body proteins (%), or specific skeletal muscle tissue (gastrocnemius and quadriceps muscle) weights on anti-obesity effects of CLA, as lean body mass is highly associated with energy expenditure.\textsuperscript{14-16,120}

To elicit CLA’s effects on the change of lean body mass, various studies either including or excluding exercise regimens in animals and humans were conducted (Table 2.1, 2.2 and 2.3). Along with reduced body fat mass, 0.25-0.5% CLA mixture supplementation significantly increased body protein (%) in body composition in mice.\textsuperscript{27,29,30,54,63,131,132} The \textit{trans-10,cis-12} CLA isomer significantly increased protein accretion in various mouse models.\textsuperscript{18,31,58,62} These results suggest that the \textit{trans-10,cis-12} CLA isomer plays a critical role in body composition changes, since in most CLA supplementation studies on body fat reduction as well as increased lean body mass, the effect of the \textit{cis-9,trans-11} CLA isomer was not observed. Interestingly, in over 12-month-old C57BL/6J, 0.5% of the \textit{trans-10,cis-12} isomers and CLA mixture for a 6 month intervention showed prevention of age-associated skeletal muscle loss without a training regimen.\textsuperscript{58,60} In Sprague-Dawley rats, 3.0% CLA mixture supplementation increased lean body mass.\textsuperscript{69} This change was also shown with 0.5-1.0% CLA treatment in Wistar rats for 6-10 weeks.\textsuperscript{133,134} It was suggested that CLA supplementation caused repartitioning of body composition, less adipose depots and more lean mass.\textsuperscript{69} There are consistent reports that CLA contributes to increased lean mass in different cross-bred pigs. The CLA mixture between 0.25 and 2% acted as a repartitioning agent to induce decreased back fat and increased lean body mass in pigs.\textsuperscript{135-139}

The intervention study of CLA in humans yielded less substantial and partially contrary results compared to those observed in the animal models (Table 2.3). In fact, the results in human studies varied with regard to the changes in body weight, body fat mass and lean body mass (Table 2.4). In terms of the prevention of obesity, group I and II in Table 2.4 are the ideal outcomes after CLA supplementation. While increased lean body mass may group a perceived
body weight gain like group II and/or III, this change in body composition would also provide additional health benefits beyond simply a decreased value in body weight.

Among clinical trials with CLA supplementation independent of exercise training, five of them reported that the body composition change of not only reduced body fat but also of increased lean body mass. Specifically, the duration of supplementation with CLA in these studies were 3-12 months, indicating the long-term intervention might be more helpful to increase lean body mass even without exercise. Moreover, two studies with no exercise regimen reported that supplemented CLA showed no body fat reduction but showed an increase of lean body mass. Schoeller et al. demonstrated the overall effects of CLA on lean body mass using a meta-analysis in 18 independent clinical studies. These studies made the conclusion that CLA supplementation led to a relatively rapid onset of increased lean body mass, however the total increase was not drastic (less than 1%). This result supports the previous mouse study, implying CLA affects body composition through modulating lean muscle mass, prior to reducing fat mass. It suggests that CLA may target muscle metabolism to reduce body fat through metabolic alteration in skeletal muscle.

Table 2.4. The grouping dependent on the changes in body weight (BW), body fat mass (BFM) and lean body mass (LBM) after CLA supplementation in human studies

<table>
<thead>
<tr>
<th>Group</th>
<th>BW</th>
<th>BFM</th>
<th>LBM</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
<td>87,91,109</td>
</tr>
<tr>
<td>II</td>
<td>-</td>
<td>↓</td>
<td>↑</td>
<td>82,86,93,94,99,105</td>
</tr>
<tr>
<td>III</td>
<td>↑</td>
<td>-</td>
<td>↑</td>
<td>103</td>
</tr>
<tr>
<td>IV</td>
<td>↓</td>
<td>↓</td>
<td>-</td>
<td>85,90,100,104,109,111</td>
</tr>
<tr>
<td>V</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>83,84,88,92,95,97,101,108,110,112,141</td>
</tr>
</tbody>
</table>
2.2.3 Mechanism of Body Composition Change by CLA

The source of energy intake is simply from consumed food, while source of energy expenditure comes from several forms such as basal metabolic rate (BMR), adaptive thermogenesis and physical activity. The regulation of body composition is relevant to energy balance, since energy intake over energy expenditure leads to the storage of excess energy as fat in the body. Multiple mechanisms to better understand the CLA effects on body composition change have been demonstrated. These approaches are mostly associated with CLA-mediated energy modulation including lowered energy intake and enhanced energy expenditure along with the inhibition of fat accumulation in adipose tissue.

2.2.3.1 Regulation of Energy Balance by CLA

On the basis of previous observations demonstrating energy balance changes by CLA supplementation in animals and humans, the putative mechanisms of CLA on the regulation of energy balance are the reduction of food intake and the increase of energy expenditure.

Early studies demonstrated CLA-fed mice showed a reduced food intake as a function of body fat reduction, whereas others have reported inconsistent results in comparison with the former (Table 2.1). Similarly, this discrepancy on food intake was reported in rat studies. In fact, two studies of CLA supplementation independent to exercise regimens reported decreased food intake in rats, while others observed no change in food intake by CLA. In theory, reduced food intake causes fewer gains of body weight and body fat, which may affect overall body composition changes. Herein, it is questionable whether body fat reduction by CLA is derived from decreased food intake or other causes. With regard to this question, the subsequent study clearly answered that CLA’s effects on body composition change were independent of lowered food intake by using a pair feeding comparison. This conclusion supports human clinical trials, since they did not support the correlation between CLA supplementation and reduced food intake. Thus, it is unlikely that food intake reduction by CLA is a
Next, we should consider energy expenditure, as enhanced energy expenditure is a primary function that influences body composition, including body fat reduction. CLA supplementation has provided various pieces of evidence to show enhanced energy expenditure with increased oxygen consumption in animal models. In clinical trials CLA supplementation increased BMR (same as resting metabolic rate, RMR), whereas other studies did not report an influence of CLA on BMR regardless of changes to body composition. Thus, further human studies are needed to provide a more clear interpretation of the effect of CLA on basal metabolic rate.

CLA supplementation is likely involved in the activation of thermogenesis, as early studies demonstrated CLA upregulates uncoupling proteins (UCPs) expressed in various tissues such as adipose, liver and skeletal muscle in mice and rats. Adaptive thermogenesis is responsible in mitochondria-mediated biochemical processes, especially in combusting stored or excess energy into heat, where UCPs play a crucial role. UCP1 to UCP5 are expressed in distinct tissues in body, although their basic role to generate heat by using ATP is almost same.

Along with the internal heat produced by BMR and thermogenesis, physical activity by contractile skeletal muscle works to expend energy, thus contributing to body fat reduction. Although the magnitude of energy expended in physical activity varies depending on the type (resistance or endurance training), intensity, duration and frequency of exercise, CLA supplementation may affect physical activity and subsequently regulate energy expenditure. In fact, previous studies in rodents reported that a combination of CLA supplementation with endurance training enhanced energy consumption, while the results of clinical studies were inconsistent.

At the cellular level, energy expenditure may be explained by biochemical events. To convert stored or excess fat in the body into energy, fatty acid β-oxidation plays a pivotal role,
resulting in reduced body fat mass and subsequently leads to even or negative energy balance. Although increased fat oxidation in the CLA-fed animals might be estimated by the alteration of energy sources based on measurements of the respiratory quotient (RQ, the representative marker for assessment of a primary energy source either carbohydrate or fat), the upregulation of carnitine palmitoyltransferase 1 (CPT-1) in skeletal muscle as the direct marker for fatty acid β-oxidation provided more reliable evidence of enhanced lipid metabolism. Intriguingly, Close et al. reported supplemented groups with 4 g of CLA mixture for 6 months had significantly increased fat oxidation and energy expenditure during sleep in human subjects.

Herein, we discussed the effects of CLA on the regulation of energy balance by food intake and energy expenditure, suggesting CLA might lead to the negative energy balance (energy intake less than consumption) in the whole body. Thus, we need to look into the tissue-specific functions of CLA.

### 2.2.3.2 Effect of CLA on Adipocyte Metabolism

Adipose tissue undergoes two physiological and morphological events to grow: hyperplasia (increase in cell number) and hypertrophy (increase in cell size). While hyperplasia plays a critical role in proliferation of preadipocytes involved in adipogenesis, hypertrophy is responsible for the continuous storage of excess energy in adipose tissue, directly leading to adiposity. The mechanisms of CLA on adipocytes are elucidated to in vitro and in vivo experimental models. The suggested mechanisms to date are (1) suppression of lipogenesis; (2) stimulation of lipolysis; (3) inhibition of adipogenesis; and (4) modulation of cytokines / adipokines. The trans-10, cis-12 CLA isomer, not the cis-9, trans-11 CLA isomer, is the active form in these mechanisms. Of particular importance, CLA mostly interacts with peroxisome proliferator-activated receptor γ (PPARγ), which is a key regulator of lipogenesis, lipolysis, adipogenesis and inflammation in adipocyte metabolism.
First, the putative mechanism to reduce body fat by CLA is the suppression of lipogenesis, since several studies reported that CLA supplementation attenuated the expression levels of stearoyl-CoA desaturase (SCD), acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS) and sterol regulatory element-binding protein-1 (SREBP1). Second, CLA stimulates lipolysis via the activation of hormone sensitive lipase (HSL), inducing the mobilization of stored triglyceride and the subsequent release of free fatty acid and glycerol cleavage. However, there is still a debate regarding the effects of CLA on lipolysis.

Third, CLA inhibits adipogenesis through reduced PPARγ expression levels. The key transcription factors to induce adipogenesis are PPARγ and CAAT/enhancer binding proteins (C/EBPs). For differentiation from preadipocytes to adipocytes, C/EBPβ and C/EBPδ are primarily increased, activating the transcription of C/EBPα and PPARγ. Particularly, the trans-10,cis-12 CLA isomer suppressed the activity of PPARγ and C/EBPs as well as related target genes such as acyl-CoA binding protein (ACBP) and perilipin on adipogenesis.

Last, CLA may modulate cytokines such as nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) through IκB and IκB kinase (IKK), subsequently inducing an increase of tumor necrosis factor α (TNFα), interleukin (IL) 6 and 8 and IL-1β, associated with inflammatory mechanisms. In addition, CLA may induce adipokines like leptine and adiponectin, representing the total amount of adipose tissue, resulting in a decrease of TG synthesis and an increase of lipolysis.

Collectively, CLA may have the potential to regulate body composition change, although the magnitude of efficiency is dependent upon several variables. While the effects of CLA on adipocyte metabolism are supported by strong evidence, the role of CLA on skeletal muscle metabolism and its main organization of energy expenditure is not fully known. However, the previous studies showed that increased lean body mass and enhanced thermogenesis and fatty acid β-oxidation in muscle with CLA might be associated with skeletal muscle metabolism. Particularly, the interaction of CLA with exercise might be able to provide critical evidence of
CLA’s effects on metabolism in skeletal muscle.

2.3 CLA and Skeletal Muscle Metabolism

The potential effects of CLA on skeletal muscle metabolism have drawn relatively less interest, even though CLA also plays a central role in the regulation of energy balance in the body. Skeletal muscle typically accounts for nearly 40% of the total body mass and acts as a significant regulator in overall energy metabolism.\(^{177}\) Generally, muscle metabolism is a term used to describe the complex biochemical reactions associated with the function and development in muscle. These reactions modulate energy metabolism in muscle, thereby influencing energy expenditure by the alteration of exercise ability and adaptive thermogenesis. The discovery of increased lean body mass and upregulated molecular markers predominately expressed in skeletal muscle, such as CPT1 and UCP2 and 3, by CLA supplementation in the previous studies provide the opportunity to uncover the correlation between CLA intake and muscle metabolism.

2.3.1 Energy Metabolism in Skeletal Muscle

In general, energy is derived from glucose and fatty acids in skeletal muscle. In the muscle fibers, glucose and fatty acids are stored as modified structures, glycogen and triglycerides, respectively. Although energy substrates used by muscle depend on the type, intensity and duration of muscle work of the subject, skeletal muscle predominantly prefers to use energy sources from fatty acids at rest. On the other hand, during high-intensity performance, anaerobic metabolic pathways such as anaerobic glycolysis and the creatine kinase reaction provide energy as primary sources when aerobic metabolism alone is not possible to cope with energy demands in exercising muscle fibers.\(^{7,9}\) Low-intensity endurance exercise corresponding to less than 60% of maximal oxygen uptake, like jogging and swimming, consumes glucose and fatty acids as the primary energy sources during the first hour. Subsequently, prolonged endurance exercise during the following one to four hours causes the substantial uptake of free
fatty acids from intramuscular fat and stored triglycerides in adipose tissue as an energy source in skeletal muscle. Thus, prolonged endurance exercise is the better way to use up stored fat in the body through enhanced aerobic metabolism.

Skeletal muscle integrates a number of biochemical processes for energy homeostasis between demand and supply. Cells in skeletal muscle store chemical energy as adenosine triphosphate (ATP) and phosphocreatine, which are immediate sources of energy. In terms of energy metabolism, skeletal muscle continuously produces energy derived from the hydrolysis of ATP in response to energy demand by skeletal muscle contraction and relaxation. During skeletal muscle work, ATPases break down ATPs and release 7.3 kcal per ATP of energy. This process is divided into anaerobic (absence of oxygen) and aerobic (presence of oxygen) metabolic pathways.

Glycolysis is the catabolic pathway for glucose in the cytosol under both anaerobic and aerobic conditions. In skeletal muscle, anaerobic glycolysis (breakdown of glycogen) permits energy supply under certain circumstances like high-intensity muscular performance when aerobic metabolism alone cannot provide sufficient levels of ATP. However, its net yield is only 2 ATP by the oxidation of 1 mole of glucose. In addition to anaerobic glycolysis, the degradation of phosphocreatine by creatine kinase is another metabolic pathway that generates ATP under anaerobic conditions in skeletal muscle.

Aerobic metabolism, especially aerobic glycolysis, in skeletal muscle takes place in the mitochondria, which is an efficient way to produce ATP through mitochondrial oxidative phosphorylation. Subsequently, a total of 38 ATP are produced from 1 mole of glucose under aerobic conditions. Along with oxidative phosphorylation in mitochondria, the citric acid cycle (Krebs or tricarboxylic acid (TCA) cycle) plays a critical role in energy generation in aerobic metabolism. In fact, the process of ATP production is tightly regulated among cytosolic glycolysis, mitochondrial β-oxidation and the citric acid cycle in skeletal muscle.
2.3.2 Intracellular Signaling Pathways Regulating Muscle Metabolism

Skeletal muscle is a tissue to show metabolic plasticity in response to alterations of external and internal conditions such as nutrient deprivation during fasting or calorie restriction and contractile activity including exercise. Upon a decrease of nutrient availability, muscle switches its mode from glucose to fatty acid utilization as a main energy substrate. In response to endurance exercise, skeletal muscle triggers a remodeling fiber-type from oxidative slow-twitch to glycolytic fast-twitch fibers. These adaptations in skeletal muscle are accompanied by an increase in mitochondrial biogenesis, which are phenotypic alterations: increased volume (content per gram of tissue) and changed composition (protein-to-lipid ratio in inner mitochondrial membrane) in mitochondria. Moreover, metabolic reactions including aerobic glycolysis, fatty acid β-oxidation, TCA cycle and oxidative phosphorylation rely on mitochondria, as these metabolic events take place in mitochondria, suggesting that enhanced mitochondrial biogenesis contributes to increased energy expenditure. The purpose of these metabolic alterations is to enhance energy production. Consequently, in order to properly regulate energy homeostasis, these adaptations positively regulate various molecular biomarkers involved in energy metabolism in skeletal muscle.

A number of regulators participate in these adaptive responses in skeletal muscle, among them AMP-activated protein kinase (AMPK) is the prime initial sensor of fuel and energy status in skeletal muscle. Particularly, AMPK controls a regulatory pathway in fatty acid β-oxidation. AMPK negatively activates acetyl-CoA carboxylase (ACC), inducing the inhibition of malonyl-CoA, which is the suppressor of CPT1 (a rate-limiting enzyme for fatty acids β-oxidation in mitochondria). As a consequence of reduced susceptibility to this shuttling system, fatty acid β-oxidation is increased. Similar to AMPK, sirtuin 1 (SIRT1, a conserved nicotinamide adenine dinucleotide (NAD)$^+$-dependent deacetylase) acts as a sensor in response to the metabolic stimuli such as stress, starvation or calorie restriction. SIRT1 regulates several transcriptional factors such as protein 53 (p53), forkhead box O (FOXO) and NFκB. SIRT1 has drawn special attention
Indeed, AMPK and SIRT1 may coherently mediate the response at the cellular level to stress and the reduced nutrient availability in skeletal muscle. Peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), a downstream marker regulated by AMPK and SIRT1, is an inducible coactivator and a master regulator for mitochondrial biogenesis, which orchestrates several transcription factors such as PPARs, nuclear respiratory factor (NRF) 1 and 2, estrogen-related receptor α (ERRα) and myocyte enhancer factor 2 (MEF2), to initiate mitochondrial biogenesis and induce the change in fiber characteristics in skeletal muscle.

Skeletal muscle is classified into three different fiber types in humans and four different fiber types in rodents that have different physiological and metabolic characteristics. Typically, skeletal muscle fibers are divided into two subcategories, slow-twitch and fast-twitch, and are correlated with contractile properties. The fiber in human is composed of three myosin heavy chain (MHC) isoforms: MHC I, MHC IIa, and MHC IIx/d. MHC I is slow twitch type I fibers, which has a greater mitochondrial content, oxidative capacity and resistance to fatigue by using fatty acids as a main energy source; compared to MHC IIx/d, a fast twitch type II fiber as known glycolytic fiber that uses glucose and phosphocreatine as a primary energy provider; the peculiarity of MHC IIa is intermediate between MHC I and IIx/d.

The first genes to be implicated as a fiber type transformation are PGC-1α and PPARδ. In transgenic mice, ectopically expressing PGC-1α in skeletal muscle induces muscle fiber conversion of fast-twitch type II fibers into slow-twitch type I fibers. In a similar manner, the overexpression of PPARδ (the most abundant form of PPARs in skeletal muscle) interacts with PGC-1α and is highly involved in the development of mitochondrial oxidative type I fibers in skeletal muscle. Taken together, a signaling cascade from AMPK to PPARδ is a relevant metabolic pathway to regulate metabolism in skeletal muscle. Thus, we primarily target this pathway to decipher the mechanism of CLA on skeletal muscle metabolism.
2.3.3 Effects of CLA on Skeletal Muscle Metabolism

It has been revealed that long-chain fatty acids (LCFAs) with over 14 carbons, including CLA, are primary endogenous ligands of the nuclear receptor family of transcription factors PPARs (PPARα, γ and δ). Indeed, it is known that LCFAs regulate energy metabolism in skeletal muscle.\(^{193}\)

With regard to the regulation of skeletal muscle metabolism, the previous studies reported the effects of CLA isomers and mixture on AMPK expression levels in murine skeletal muscle cells. Both the cis-9,trans-11 and trans-10,cis-12 isomers stimulated the phosphorylation of AMPK (active form), resulting in improved muscle energy metabolism.\(^{194-196}\) In addition, each CLA isomer positively regulates ACC expression levels, indicating CLA treatment may affect enhanced fatty acid β-oxidation.\(^{194,196}\) The cis-9,trans-11 CLA isomer showed elevated AMPK phosphorylation with lower concentrations (~50 µM), while the trans-10,cis-12 isomer gradually activated phosphorylated AMPK up to 120 µM in a dose-dependent manner, and then plateaued.\(^{195}\) Compared to several studies of CLA on AMPK, it is limited on the effect of CLA on SIRT1 activity in skeletal muscle, even though AMPK and SIRT1 both mutually regulate and share many common target molecules.\(^{185}\)

Although sufficient evidence demonstrating the role of CLA in mitochondria in skeletal muscle has not yet been provided, recent studies reported direct and/or indirect effects of CLA on the biochemical alteration of several molecular markers associated with mitochondrial biogenesis and metabolism.\(^{18,58,152,197-199}\) In fact, CLA treatment in human skeletal muscle cells did not affect the activity of PGC-1α, a primary regulator in mitochondrial biogenesis, even when mitochondrial content was increased by CLA.\(^{197}\) In a similar context, CLA fed mice and rats demonstrated no significant differences in PGC-1α compared to controls.\(^{152,198}\) On the other hand, CLA supplementation significantly upregulated molecular biomarkers such as succinate dehydrogenase, cytochrome c oxidase, superoxide dismutase 2, catalase and glutathione peroxidase in skeletal muscle, resulting in increased ATP production and thermogenesis via
improved oxidative phosphorylation and anti-oxidative capacity in rodent models.\textsuperscript{58,152}

In regards to the effect of CLA on PPAR\(\delta\), one of key regulators in muscle fiber type transformation, 50 \(\mu\)M of CLA mixture added to murine muscle cells showed a significant increase in PPAR\(\delta\) expression levels.\textsuperscript{199} Supporting this result, Kim et al.\textsuperscript{18} reported the trans-10,cis-12 CLA isomer supplementation significantly upregulated PPAR\(\delta\) gene expression level in mouse skeletal muscle, while Parra et al.\textsuperscript{198} observed no CLA effect on PPAR\(\delta\) and muscle fiber change in mice. Although further investigation is required in \textit{in vivo} studies for a better understanding on the correlation between CLA supplementation and muscle fiber type switch, CLA fed pigs showed a remarkably altered gene expression of muscle fiber types.\textsuperscript{200,201} In these studies, 1.2-2.0\% of CLA supplementation significantly increased the expression levels of oxidative type I fiber, but not the expression levels of glycolytic type IIb and IIx fibers in skeletal muscle.

### 2.3.4 Effect of CLA on Physical Activity

On the basis of the effects of CLA on increased muscle mass relative to fat mass gains, a number of studies in animals and humans were carried out to evaluate the interaction of CLA supplementation with exercise (Table 2.5 and 2.6). Exercise is typically divided into two distinct trainings, resistance and endurance, relying on the metabolic system to produce ATP by anaerobic and aerobic conditions, respectively. The previous animal studies of supplemented CLA along with exercise provided a better understanding of improved endurance capacity via mitochondrial fatty acid \(\beta\)-oxidation than resistance capacity (Table 2.5). In fact, 0.5-1.0\% CLA supplementation combined with endurance training showed a significantly greater fat oxidation in skeletal muscle, resulting in an increased maximum swimming or running capacity in mice.\textsuperscript{149,202-204} Specifically, Kim et al.\textsuperscript{18} reported that the trans-10,cis-12 isomer increased the maximum running time and distance significantly over the cis-9,trans-11 treated animals in the trained-mice. Interestingly, the effect of CLA on physical activity is also supported by animal studies that
measured voluntary activity (non-exercise physical activity), showing increased voluntary movement along with the upregulation of CPT1 and UCP2.\textsuperscript{17,205} In rat models, no synergistic effects of CLA treatment and exercise training on endurance capacity and lean body mass were observed.\textsuperscript{133,134}

Currently, there have been seventeen CLA intervention studies reported in humans associated with exercise (Table 2.6). Among them, ten studies have tested exercise outcomes. Overall, the outcomes in physical activity of CLA supplementation dependent on exercise were inconsistent. Five clinical trials demonstrated the positive results of combined CLA with a resistance-training regimen.\textsuperscript{82,93,99,106,110} Only Colakoglu et al.\textsuperscript{91} reported a synergistic effect of CLA with endurance training. Furthermore, there were four clinical trials that evaluated the effect of CLA supplementation without exercise on physical activity.\textsuperscript{94,100,128,206} Among them, two studies showed improved physical activity by CLA treatment for 3 or 6 months.\textsuperscript{100,128}

Four studies in humans evaluated the effects of cosupplementation of CLA with other supplements such as creatine monohydrate, chromium picolinate, whey protein or amino acids under physical training.\textsuperscript{99,101,106,207} Two cosupplementation studies of CLA and creatine monohydrate for short- (5 weeks) or long-term (6 months) interventions, accompanied with resistance training, showed increased lean body mass and improved strength compared to the control.\textsuperscript{99,106} Interestingly, Macaluso et al.\textsuperscript{110} carried out a clinical trial to investigate the effect of CLA with resistance training on testosterone levels in serum. This study designed a short-term intervention (3 weeks), showing no change of body weight, fat mass and lean body mass. However, serum testosterone levels were significantly increased by CLA supplementation and resistance exercise. Subsequently, Barone et al.\textsuperscript{204} demonstrated that CLA supplementation promoted endurance capacity in mice trained on the rotarod via the upregulation of testosterone biosynthesis. Recently, Usui et al.\textsuperscript{208} suggested that enhanced mitochondrial biogenesis in skeletal muscle led to the testosterone-induced increase in energy expenditure. This may provide another clue to help explain how CLA can enhance energy expenditure and improve physical activity via
Table 2.5. Effects of conjugated linoleic acid (CLA) with exercise regimes on physical activity in animals

<table>
<thead>
<tr>
<th>References</th>
<th>Animal</th>
<th>CLA Supplementation</th>
<th>Results&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Exercise Type</th>
<th>Muscle Metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>BW</td>
<td>BFM</td>
<td>LBM</td>
</tr>
<tr>
<td>Mizunoya et al., 2005&lt;sup&gt;149&lt;/sup&gt;</td>
<td>BALB/c Mice</td>
<td>M Mixture 0.5% 1 week</td>
<td>↓</td>
<td>↓</td>
<td>-</td>
</tr>
<tr>
<td>Bhattacharya et al., 2005&lt;sup&gt;131&lt;/sup&gt;</td>
<td>BALB/c Mice</td>
<td>M Mixture 0.4% 14 weeks</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Di Felice et al., 2007&lt;sup&gt;132&lt;/sup&gt;</td>
<td>ICR Mice</td>
<td>M Mixture 0.425 mg/day 6 weeks</td>
<td>↓</td>
<td>↔</td>
<td>↑</td>
</tr>
<tr>
<td>Banu et al., 2008&lt;sup&gt;209&lt;/sup&gt;</td>
<td>C57BL/6 Mice</td>
<td>F Mixture 0.5% 10 weeks</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Zhang et al., 2009&lt;sup&gt;210&lt;/sup&gt;</td>
<td>ICR Mice</td>
<td>M Mixture 0.5% 18 weeks</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Kim et al., 2010&lt;sup&gt;146&lt;/sup&gt;</td>
<td>BALB/c Mice</td>
<td>M Mixture 1.0% 10 weeks</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Kim et al., 2012&lt;sup&gt;138&lt;/sup&gt;</td>
<td>129 Sv/J Mice</td>
<td>M c9t11/t10c12 0.5% 6 weeks</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Hur et al., 2013&lt;sup&gt;203&lt;/sup&gt;</td>
<td>ICR Mice</td>
<td>F Mixture 1.0% 6 weeks</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Barone et al., 2013&lt;sup&gt;204&lt;/sup&gt;</td>
<td>BALB/c Mice</td>
<td>M Mixture 0.5% 6 weeks</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Shen et al., 2015&lt;sup&gt;411&lt;/sup&gt;</td>
<td>129 Sv/J Mice</td>
<td>M t10c12 0.1% 7 weeks</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Mirand et al., 2004&lt;sup&gt;212&lt;/sup&gt;</td>
<td>Wistar Rats</td>
<td>M Mixture / c9t11/t10c12 1.0% 6 weeks</td>
<td>↓</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Faulconnier et al., 2006&lt;sup&gt;213&lt;/sup&gt;</td>
<td>Wistar Rats</td>
<td>M Mixture / c9t11/t10c12 1.0% 6 weeks</td>
<td>↓</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td>Mirand et al., 2006&lt;sup&gt;133&lt;/sup&gt;</td>
<td>Wistar Rats</td>
<td>M Mixture / c9t11/t10c12 1.0% 6 weeks</td>
<td>↓</td>
<td>↑</td>
<td>↑ by Mixture</td>
</tr>
<tr>
<td>Salgado et al., 2012&lt;sup&gt;214&lt;/sup&gt;</td>
<td>Wistar Rats</td>
<td>F+M Mixture 0.5% 10 weeks</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
</tr>
</tbody>
</table>

<sup>a</sup>F, female; M, male.

<sup>b</sup>Mixture, a mixed isomer of cis-9,trans-11 and trans-10,cis-12; c9t11, cis-9,trans-11 CLA isomer; t10c12,trans-10,cis-12 CLA isomer.

<sup>c</sup>Dosage (%) means a designated weight percentage of CLA in diet.

<sup>d</sup>BW, body weight; BFM, Body fat mass; LBM, lean body mass; -, no change; ↑, increase; ↓, decrease.

<sup>e</sup>EE, energy expenditure; RER, respiratory energy ratio; ↔, no change.

<sup>f</sup>CPT1, carnitine palmitoyltransferase 1; LPL, lipoprotein lipase; PPARδ, peroxisome proliferator activated receptor δ; UCP2, uncoupling protein 2.
Table 2.6. Effects of conjugated linoleic acid (CLA) with exercise regimes on physical activity in humans

<table>
<thead>
<tr>
<th>References</th>
<th>Characteristic</th>
<th>Gender</th>
<th>CLA Supplementation</th>
<th>Results&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Exercise Type</th>
<th>Muscle Metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blankson et al., 2000&lt;sup&gt;12&lt;/sup&gt;</td>
<td>Overweight / Obese</td>
<td>F+M</td>
<td>Mixture 1.7/3.5/5.1/6.8</td>
<td>↓ 1.7, 3.4 and 6.8 at 12 wk</td>
<td>↓ 1.7, 6.8 at 12 wk</td>
<td>Standardized training</td>
</tr>
<tr>
<td>Thom et al., 2001&lt;sup&gt;23&lt;/sup&gt;</td>
<td>Normal</td>
<td>F+M</td>
<td>Mixture 1.8</td>
<td>↓</td>
<td>-</td>
<td>Strenuous</td>
</tr>
<tr>
<td>Kreider et al., 2002&lt;sup&gt;24&lt;/sup&gt;</td>
<td>Normal / Overweight</td>
<td>M</td>
<td>Mixture 6.0</td>
<td>-</td>
<td>-</td>
<td>Resistance</td>
</tr>
<tr>
<td>Loeffelholz et al., 2003</td>
<td>Overweight</td>
<td>F+M</td>
<td>Mixture 3.8</td>
<td>↓</td>
<td>-</td>
<td>Resistance</td>
</tr>
<tr>
<td>Colakoglu et al., 2006&lt;sup&gt;93&lt;/sup&gt;</td>
<td>Normal</td>
<td>F</td>
<td>Mixture 3.6</td>
<td>↓</td>
<td>↓</td>
<td>Endurance</td>
</tr>
<tr>
<td>Pinkoski et al., 2006&lt;sup&gt;93&lt;/sup&gt;</td>
<td>Unknown</td>
<td>F+M</td>
<td>Mixture 5.0</td>
<td>↓</td>
<td>↑</td>
<td>Resistance ↔ RMR</td>
</tr>
<tr>
<td>Adams et al., 2006&lt;sup&gt;14&lt;/sup&gt;</td>
<td>Overweight / Obese</td>
<td>M</td>
<td>Mixture 3.2</td>
<td>-</td>
<td>-</td>
<td>Resistance</td>
</tr>
<tr>
<td>Nazare et al., 2007&lt;sup&gt;77&lt;/sup&gt;</td>
<td>Normal / Overweight</td>
<td>F+M</td>
<td>Mixture (TG)</td>
<td>-</td>
<td>-</td>
<td>Regular training</td>
</tr>
<tr>
<td>Tarnopolsky et al., 2007&lt;sup&gt;99&lt;/sup&gt;</td>
<td>Normal / Overweight</td>
<td>F+M</td>
<td>Mixture 5.4+5 mg creatine monohydrate</td>
<td>-</td>
<td>↓</td>
<td>Resistance</td>
</tr>
<tr>
<td>Diaz et al., 2008&lt;sup&gt;101&lt;/sup&gt;</td>
<td>Overweight / Obese</td>
<td>F</td>
<td>Mixture 1.8+0.4 mg creatine picolinate</td>
<td>-</td>
<td>-</td>
<td>Endurance</td>
</tr>
</tbody>
</table>

(Continued)
<table>
<thead>
<tr>
<th>References</th>
<th>Characteristic</th>
<th>Gender</th>
<th>Form</th>
<th>Dosage (^b)</th>
<th>Duration</th>
<th>BW</th>
<th>BFM</th>
<th>LBM</th>
<th>Exercise Type</th>
<th>Energy Expenditure (^c)</th>
<th>Biomarkers (^f)</th>
<th>Exercise Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornish et al., 2009(^{106})</td>
<td>Obese</td>
<td>F+M</td>
<td>Mixture</td>
<td>4.3+9g creatine mono-hydrate+36g whey protein</td>
<td>5 weeks</td>
<td>-</td>
<td>-</td>
<td>↑</td>
<td>Resistance</td>
<td></td>
<td></td>
<td>↑</td>
</tr>
<tr>
<td>Michishita et al., 2010(^{107})</td>
<td>Normal / Overweight</td>
<td>F+M</td>
<td>Mixture</td>
<td>1.6+1.52g amino acids</td>
<td>12 weeks</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Resistance</td>
<td></td>
<td></td>
<td>↔</td>
</tr>
<tr>
<td>Chen et al., 2012(^{109})</td>
<td>Overweight / Obese</td>
<td>F+M</td>
<td>Mixture</td>
<td>1.7</td>
<td>12 weeks</td>
<td>↓</td>
<td>↓</td>
<td>-</td>
<td>Resistance</td>
<td></td>
<td></td>
<td>↔</td>
</tr>
<tr>
<td>Macaluso et al., 2012(^{110})</td>
<td>Normal / Overweight</td>
<td>M</td>
<td>Mixture</td>
<td>4.8</td>
<td>3 weeks</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Resistance</td>
<td>↑ Testosterone in serum</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>Bulut et al., 2013(^{111})</td>
<td>Overweight</td>
<td>M</td>
<td>Mixture</td>
<td>3.0</td>
<td>4 weeks</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Endurance</td>
<td>↔ PWC(<em>{FT}) ↔ VO(</em>{2\text{ max}}) ↔ GET ↔ RCP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jenkins et al., 2014(^{115,116})</td>
<td>Normal / Overweight</td>
<td>M</td>
<td>Mixture</td>
<td>5.63</td>
<td>6 weeks</td>
<td></td>
<td></td>
<td>Endurance</td>
<td>↔ RMR</td>
<td>↑ Glycogen resynthesis / ↑ GLUT4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tsao et al., 2015(^{114})</td>
<td>Normal</td>
<td>M</td>
<td>Mixture</td>
<td>3.8</td>
<td>8 weeks</td>
<td>-</td>
<td></td>
<td>Endurance</td>
<td>↔ RMR</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

\(^{a}\) F, female; M, male.

\(^{b}\) Mixture, a mixed isomer of cis-9,trans-11 and trans-10,cis-12; c9t11, cis-9,trans-11 CLA isomer; t10c12,trans-10,cis-12 CLA isomer.

\(^{c}\) Dosage (%) means a designated weight percentage of CLA in diet.

\(^{d}\) BW, body weight; BFM, Body fat mass; LBM, lean body mass; -, no change; ↑, increase; ↓, decrease.

\(^{e}\) GET, gas exchange threshold; PWC\(_{FT}\), physical working capacity at the fatigue threshold; RCP, respiratory compensation point; RMR, resting metabolic rate; VO\(_{2\text{ max}}\), maximal oxygen uptake; ↔, no change.

\(^{f}\) GLUT4, glucose transporter type 4.
skeletal muscle energy metabolism.

2.4 Literature Review Conclusion and Project Rationale

The literature review has discussed that CLA induces a decrease of body fat and an increase of lean body mass via various regulatory mechanisms. To date, most mechanistic studies of CLA on body composition change have been focused on adipocyte metabolism in adipose tissue. However, a growing number of studies highlighting the importance of CLA’s effect on skeletal muscle metabolism indicate that CLA supplementation may affect skeletal muscle metabolism representative to increased energy expenditure and enhanced physical activity. However, the underlying mechanism of how CLA modulates skeletal muscle metabolism has not been fully discovered. Thus, it is important to investigate the mechanistic study of CLA on skeletal muscle metabolism including previously well-established signaling pathways such as mitochondrial biogenesis and muscle fiber type transformation via the AMPK/SIRT1-PGC-1α-PPARδ signaling cascade.
CHAPTER 3

OBJECTIVES OF THE PROJECT

The long-term goal is to develop prevention and/or treatment strategies for obesity and its related pathologies. The objective of this project is to uncover the mechanisms of CLA on muscle metabolism. The central hypothesis is that CLA targets the AMP-activated protein kinase (AMPK) signaling pathway and subsequently the underlying downstream signaling cascades associated with skeletal muscle metabolism. The rationale of the proposed research is that by understanding factors contributing to skeletal muscle and physical activity, we will be able to direct more efficient prevention strategies for obesity and associated chronic diseases in the future.

The project aims are as follows:

Specific Aim 1: Determine molecular targets of CLA’s effects on muscle metabolism in in vitro model: The objective of this project is to determine the key molecular mediators of CLA’s effects on muscle metabolism. The hypothesis to be tested is that CLA modulates AMPK, which can affect PPARγ coactivator 1α (PGC-1α), and activates the underlying downstream signaling cascades including mitochondrial biogenesis.

Specific Aim 2: Determine the effects of CLA on physical activity and physiological changes in skeletal muscle in in vivo models: The objective of this is to determine if CLA influences voluntary activity and/or endurance exercise capacity in mice through the modulation of molecular markers in skeletal muscle metabolism. Two hypotheses to be tested are [1] CLA elicits improved voluntary activity by promoting mitochondrial biogenesis via the modulation of AMPK signaling pathway and [2] CLA and endurance exercise synergistically improves muscle metabolism by activating mitochondrial biogenesis and muscle fiber type transformation, leading to the enhancement of endurance capacity. Specifically, the first hypothesis is tested with two experimental designs targeting adulthood and childhood obesity.
4.1 Introduction

Skeletal muscle and the corresponding metabolic contributions play a critical role in maintaining homeostasis in the human body. Skeletal muscle metabolism includes adaptive responses to physical activity on glucose uptake, fatty acid oxidation, mitochondrial biogenesis and muscle fiber type shift.\(^\text{177}\) It is known that these adaptive responses of skeletal muscle involve a number of regulators, among them AMP-activated protein kinase (AMPK) is the most well-known molecular target of exercise in skeletal muscle.\(^\text{217}\) During muscular adaptive responses, AMPK increases ATP production by controlling a number of transcriptional genes or proteins, including peroxisome proliferator-activated receptor \(\gamma\) coactivator 1-\(\alpha\) (PGC-1\(\alpha\)), peroxisome proliferator-activated receptors (PPARs) and mitochondria-related proteins. These signaling cascades play an important role in improving muscle metabolism.\(^5\)

Mitochondrial biogenesis is one of the adaptive responses in skeletal muscle and is the process of new mitochondrial formation resulting in direct improvement of energy production through increasing capacity of respiratory components.\(^\text{218,219}\) It has been reported that impaired mitochondrial biogenesis contributes to obesity and related diseases, such as diabetes and cardiovascular disease through mitochondrial dysfunction and resulting lipid accumulation in skeletal muscle.\(^\text{220}\) PGC-1\(\alpha\) is known as the primary simulator of mitochondrial biogenesis. Activated PGC-1\(\alpha\) subsequently triggers upregulation of nuclear respiratory factors (NRF-1 and -2) and mitochondrial transcription factor A (Tfam), wherein NRF-1 and -2 play roles in the expression of a number of nuclear-encoding mitochondrial proteins and Tfam controls mitochondrial DNA replication and transcription. In turn, these nuclear-encoded markers activate
mitochondrial proteins and antioxidant enzymes in mitochondria, resulting in mitochondrial biogenesis.\textsuperscript{221-224}

Conjugated linoleic acid (CLA) is a mixture of geometric and positional conjugated isomers of linoleic acid (LNA). CLA has shown a variety of beneficial effects such as anti-cancer, anti-atherosclerosis, antioxidant, and anti-obesity.\textsuperscript{14} Previously it was reported that CLA improved voluntary activity and endurance capacity in mice.\textsuperscript{17,18} There was further support that modulation of skeletal muscle metabolism by CLA was mediated by increased PPAR\(\delta\) gene expression \textsuperscript{225}. In addition, it was previously reported that the c9,t11 CLA isomer induced the activation of AMPK\(\alpha\) in C2C12 skeletal muscle cells,\textsuperscript{194} however, it was not clear whether CLA isomers control PGC-1\(\alpha\) and subsequent mitochondrial biogenesis. Thus we investigated the role of CLA isomers on PGC-1\(\alpha\) based on AMPK signaling pathway and mitochondrial biogenesis-related biomarkers to determine its role on mitochondrial biogenesis using C2C12 cells, an established murine muscle cell line.

4.2 Materials and Methods

4.2.1 Materials

Dulbecco’s Modified Eagle’s Medium (DMEM) and horse serum (HS) were obtained from Invitrogen (Grand Island, NY). Penicillin/streptomycin mixture, fetal bovine serum (FBS), and bovine serum albumin (BSA) were purchased from Sigma Aldrich (St. Louis, MO). The linoleic acid with 99% purity was purchased from Nu-Chek Prep (Elysian, MN). The t10,c12 CLA; c9,t11 CLA; and CLA mixed isomer (Mixture) were obtained from Natural Lipids (Hovdebygda, Norway). The purity of the t10,c12 CLA preparation was 94% with 2% c9,t11 isomer and 3% other conjugated linoleic acid isomers. The c9,t11 CLA preparation was 90% pure and consisted of 4% t10,c12 isomer, 2% other conjugated linoleic acid isomers and 3% oleic acid. The CLA mixture was 80.7% CLA composed of 37.8% c9,t11, 37.6% t10,c12 and 5.3% other CLA isomers and 13.7% oleic acid, 3.2% stearic acid, 0.4% palmitic acid and 0.2% linoleic acid.
Rabbit anti-phosphorylated AMPKα and rabbit anti-AMPK, voltage-dependent anion channel (VDAC) and superoxide dismutase 2 (SOD2) antibodies were obtained from Cell Signaling (Berberly, MA). Rabbit anti-PGC-1α, sirtuin 1 (SIRT1), PPARδ, NRF-1, Tfam, cytochrome c (Cyt C), subunit IV of cytochrome c oxidase (COX IV), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-rabbit IgG antibody was obtained from Boston Bioproducts Inc. (Ashland, MA). Other chemicals were purchased from either Fisher Scientific (Pittsburg, PA) or Sigma Aldrich (St. Louis, MO).

4.2.2 C2C12 Cell Culture

C2C12 mouse myoblasts (CRL-1772™) were obtained from American Type Culture Collection (Manassas, VA). C2C12 myoblasts were maintained in DMEM containing 10% (v/v) FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in humidified 95% air and 5% CO₂ atmosphere. For differentiation the culture medium was switched to DMEM with 2% (v/v) heat-inactivated horse serum when myoblasts were 90% confluent. The differentiation medium was subsequently replaced with fresh medium every two days. After 6 days of differentiation, myotubes were treated with fatty acids as fatty acid-albumin complexes as previously described. Doses and time of treatment are indicated in each figure legend. All treatments included 50 µM fatty acid free albumin. Concentrations of CLA used reflect the serum levels of CLA after supplementing 0.5% CLA for 4 weeks in rats or supplementing 0.8-3.2 g per day for 2 months in humans, which ranged from 23 to 200 µM. Effects of these fatty acids on cell viability were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) based assay. No significant differences in cell viability were observed in any treatments (data not shown).
4.2.3 Western Blot Analysis

The adherent cells were harvested and then lysed with lysis radioimmuno precipitation assay (RIPA) (50 mM Tris-HCl, 10 Mm NaCl, 5 Mm MgCl₂, and 0.5% NP-40, pH 8.0) buffer (Boston Bioproducts Inc., Ashland, MA) containing protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO) and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL) on ice for 30 min, and centrifuged at 12,000g for 20 min at 4°C. Protein concentration of cell lysate was determined using the DC protein assay kit (Bio-Rad, Hercules, CA). The lysates were separated by 6-15% (w/v) sodium dodecyl sulfate-polyacrylamide gel, transferred to a polyvinylidene fluoride (Millipore, Bedford, MA). After blocking for 1 h at room temperature (RT), the membrane was incubated with the primary antibody in TBS-T containing 5% BSA at 4°C overnight. The membrane was treated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody for 1 h at RT. Detection was performed using the enhanced chemiluminescence solution (Bio-Rad, Hercules, CA) with an Image Station 4000MM (Carestream Health, New Heaven, CT). GAPDH or β-actin intensity was used for an internal control to normalize protein content of each sample. Image J software provided by NIH was used to quantify the results.

4.2.4 Quantitative Analysis of Mitochondrial DNA (mtDNA)

Mitochondrial DNA copy number was determined based on method previously described. Total genomic DNA (gDNA) including mtDNA was purified using Puregene® core kit (Qiagne, Gaithersburg, MD) as described in the manufacturer’s protocol. NADH dehydrogenase subunit 1 (ND1, Mm04225274_s1) and 18S rRNA (Rn18s, Mm03928990_g1) with predesigned TaqMan gene expression assay kits were used to represent the mtDNA and the nuclear DNA (nDNA), respectively. The DNA concentration was diluted to 100 ng/µl, and 1 µl of DNA was used as a template for the real-time PCR. All reactions were carried out in triplicate with the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster, CA). The program of reaction was 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min
at 60°C. Quantification of relative mtDNA copy number was assessed by a comparative Ct method based on analysis of the difference in threshold amplification between mtDNA and nDNA.

4.2.5 Statistical Analyses

All data in Figures 4.1-4.5 from three independent experiments were collected in triplicates. Data in Fig. 4.6 are from one experiment. Data in Figures 4.1-4.5 were analyzed by Two-way analysis of variance (ANOVA) as treatment and experiment as variables, and data in Figure 4.6 were analyzed by one-way ANOVA, followed by PROC MIXED with Least Square Means options of the SAS software for Windows release 9.2 (SAS Institute Inc., Cary, NC) on the W32_VSHOME platform. The experimental groups were compared by multiple comparisons based on the Dunnett’s test. Data are presented as mean ± S.E. *P* values < 0.05 are reported as statistically significant.

4.3 Results

4.3.1 Effect of CLA Isomers on AMPK Signaling Pathway in C2C12 Skeletal Muscle Cells

Based on previous reports that CLA upregulated PPARδ and AMPK expressions, we first determined the effect of CLA isomers on AMPK signaling pathway (Fig. 4.1). The relative expression levels of total AMPKα were not different in all treatments compared to control (Fig. 4.1B). However, treatments with the t10,c12 CLA isomer or the CLA mixture significantly upregulated phosphorylation of AMPKα compared to control (Fig. 4.1C). As a result, increased ratio of phosphorylated AMPKα to total AMPKα by the t10,c12 isomer and the CLA mixture were observed (Fig.4.1D). However, no differences were observed by either linoleic acid or the c9,t11 CLA isomer compared to control in these parameters.

Along with AMPK, SIRT1 is also known to be involved in activation of PGC-1α by deacetylation independent to AMPK. There were no differences in the expressions of SIRT1 by
all treatments (Fig. 4.2). These data suggest that CLA, particularly the t10,c12 isomer, controls AMPKα to regulate PGC-1α, but not via SIRT1.

4.3.2 Effect of CLA Isomers on PGC-1α Expression

We next examined the effects of CLA isomers on the expressions of PGC-1α, which is one of the downstream target proteins of AMPK and also plays a central role in regulation of mitochondrial biogenesis. Both CLA isomers, c9,t11 and t10,c12, and the CLA mixture significantly increased the protein expression levels of PGC-1α compared to control (Fig. 4.3). Linoleic acid itself did not significantly increase expression of PGC-1α over control. These results suggest that CLA may stimulate mitochondrial biogenesis via PGC-1α-mediated signaling cascades.

Figure 4.1 Effect of conjugated linoleic acid (CLA) isomers on protein expression levels of AMP-activated protein kinase α (AMPKα) in C2C12 cells.
4.3.3 Effect of CLA Isomers on Mitochondrial Biogenesis-related Biomarkers

The effects of CLA on mitochondrial biogenesis were measured through PPARδ, NRF-1 and Tфam primarily controlled by PGC-1α. The CLA mixture significantly increased expression of PPARδ compared to control, while the c9,t11 or t10,c12 isomer or linoleic acid did not show any significant effect compared to control (Fig. 4.4B). Among nuclear respiratory
factors (NRF-1 and -2), NRF-1 is more specifically related to mouse Tfam, we thus analyzed the
eexpression level of NRF-1 instead of NRF-2. Both c9,t11 and t10,c12 CLA isomers
significantly upregulated NRF-1 expression compared to control (Fig. 4.4C). Furthermore,
expression of Tfam was significantly increased after treatments of all individual CLA isomers
compared to control, while linoleic acid itself did not have any effect compared to control (Fig.
4.4D). Collectively, these data from mitochondrial biogenesis-related biomarkers suggest that
c9,t11 and t10,c12 CLA isomers may have a positive effect on mitochondrial biogenesis through
modulating NRF-1 and Tfam.

Figure 4.4 Effect of conjugated linoleic acid (CLA) isomers on mitochondria-related factors in
C2C12 cells.

4.3.4 Effect of CLA Isomers on Mitochondrial Content

Overall mitochondrial relative content can be indirectly estimated by determining the
expression levels of cytochrome c and COX IV located in the inner membrane of mitochondria
and VDAC localized in the mitochondrial outer membrane. The c9,t11 CLA isomer
significantly increased the expression of cytochrome c (Fig. 4.5B). On the other hand, the t10,c12 CLA isomer showed significantly increased VDAC expression compared to control (Fig. 4.5D).

No significant effects by linoleic acid were observed on cytochrome c or VDAC. To assess mitochondrial function, we also measured the expression level of SOD2, manganese superoxide dismutase (MnSOD), which is one of the genes encoding mitochondrial protein in the inner mitochondrial matrix and protect mitochondria from reactive oxygen species. In this study, no significant effects were observed by any treatment on expression level of SOD2 (Fig. 4.5E).

Figure 4.5 Effect of conjugated linoleic acid (CLA) isomers on mitochondrial content in C2C12 cells.
We further determined the ratio between mtDNA and nDNA as an indicator of mitochondrial replication. Both CLA isomers, c9,t11 and t10,c12, as well as the CLA mixture significantly increased the ratio mtDNA/ nDNA compared to control, whereas linoleic acid showed no significant difference over control (Fig. 4.6). Taken together, our observations indicate that both CLA isomers induce the increase of mitochondrial DNA replication, although mitochondrial components may be increased in isomer-dependent pathways.

Figure 4.6 Effect of conjugated linoleic acid (CLA) isomers on mitochondrial DNA (mtDNA) copy number in C2C12 cells.

### 4.4 Discussion

In this study, we demonstrated that CLA activated several key biomarkers in mitochondrial biogenesis signaling cascade in C2C12 murine skeletal muscle cells. Of particular importance the c9,t11 and t10,c12 CLA isomers targeted PGC-1α and triggered the activation of downstream proteins such as NRF-1 and Tfam, as well as upregulating PPARδ (the CLA mixture), resulting in modulation in mitochondrial biogenesis. These results suggest that both CLA isomers stimulate mitochondrial biogenesis and this can help explain improved activity and endurance capacity in mice fed CLA reported in previous publications. 17,18

Several studies have demonstrated the effect of polyunsaturated fatty acids such as docosahexaenoic acid (DHA) and the t10,c12 CLA isomer on enhanced glucose uptake and fatty
acid β-oxidation through AMPK upregulation in murine skeletal cells. However, mechanisms of these polyunsaturated fatty acids on downstream signaling cascade from AMPK are not clear yet. It is reported that AMPK is required for regulation of PGC-1α in skeletal muscle and PGC-1α is the most promising target to regulate mitochondrial biogenesis. Moreover, PGC-1α-NRF-1/-2-Tfam cascade is generally accepted as mitochondriogenic pathway, even though there are various PGC-1α-dependent downstream cascades. Our results here support that CLA modulates PGC-1α via AMPK signaling cascades and resulting mitochondrial biogenesis-related molecular markers through PGC-1α-NRF-1-Tfam. In addition, lack of effects on SIRT1 by CLA is consistent with the previous reports that SIRT1 was not required for mitochondrial biogenesis through PGC-1α activation. However, we cannot conclude whether CLA directly targeted AMPK or acted indirectly via upstream regulators of AMPK such as liver kinase B1 (LKB1) and Ca²⁺/calmodulin-dependent protein kinase (CaMK) with current results. Therefore, additional studies are needed to confirm the primary targets of CLA for this activity, particularly upstream regulators of AMPK.

The c9,t11 CLA is the predominant natural CLA form comprising approximately 80% of the total isomers in dairy and meat products from ruminants, while synthetic CLA for commercial preparations is typically a 50:50 mixture of c9,t11 and t10,c12 CLA isomers. Most biological effects of CLA are known to be results of independent or additive effects, however, there are few reports that these two CLA isomers interact. For muscle metabolism, previous studies report on the effects of c9,t11, t10,c12 CLA and CLA mixed isomers on AMPK in skeletal muscle cells. Our data illustrated that the t10,c12 isomer and the CLA mixture upregulated AMPK activity independent of the c9,t11 isomer. This is inconsistent with observations by Qin et al. that the c9,t11 isomer increased the expression of AMPK in a dose and time dependent manner. This discrepancy may be in part due to methodological differences; they examined only c9,t11 isomer treatment (without t10,c12 or CLA mixed isomers) compared to control and all their treatments were combined with palmitate. In addition, our observations were consistent with the
results of Mohankumar et al.\textsuperscript{195} that t10,c12 but not c9,t11 CLA stimulated phosphorylation of AMPK in a time-dependent manner, although they used a different cell line (L6 myotube) and shorter incubation time (30 min).

In the current results, we observed both CLA isomers increased expression of PGC-1\textsubscript{α}, although only the t10,c12 CLA isomer, but not the c9,t11 isomer, activated AMPK (Fig 4.1). Previously Parra et al.\textsuperscript{198} reported that a commercial CLA mixture upregulated PGC-1\textsubscript{α} gene expression in C57BL/6J gastrocnemius muscle although it is not clear if both isomers have effects on PGC-1\textsubscript{α} in this study. Thus, it is possible that there is an additional mechanism contributing to the c9,t11 isomer’s effects on PGC-1\textsubscript{α}, possibly via LKB1 and CaMK.\textsuperscript{183}

PPAR\textsubscript{δ} is abundant in skeletal muscle and is a primary target gene for preventing obesity and improving physical activity through modifying muscle morphology and enhancing oxidative capacity.\textsuperscript{192} Previously it was reported that the t10,c12 CLA isomer upregulated mRNA level of PPAR\textsubscript{δ} in trained 129 Sv/J mice gastrocnemius muscle.\textsuperscript{18} In the current study, the CLA mixture, but not the c9,t11 or t10,c12 isomer, showed significant differences in PPAR\textsubscript{δ} expression compared to control. This observation is similar to a study by Oraldi et al.\textsuperscript{199} that the CLA mixed isomers increased both transcriptional and translational levels of PPAR\textsubscript{δ} in C2C12 cells. It is not clear why the individual CLA did not significantly influence expression of PPAR\textsubscript{δ} in the current study. These discrepancies may derive from differences in experiments, \textit{in vivo} vs. \textit{in vitro}, and the presence or absence of an exercise regime for the \textit{in vivo} study. Although a few methods mimicking exercise training, for example electrical pulse stimulation, are used for \textit{in vitro} testing, this model still has unavoidable limitations.

We also tested effects of CLA isomers on the expression levels of NRF-1 and Tfam (both known to modulate mitochondrial biogenesis\textsuperscript{221}) as downstream regulators of PGC-1\textsubscript{α}. Tfam activation regulates the expression of mitochondrial DNA encoded oxidative respiratory phosphorylation proteins, resulting in the induction of increased mitochondrial contents and functions.\textsuperscript{239} In this study, CLA isomers induced NRF-1 activation as well as Tfam, a
downstream marker from NRF-1, which was significantly activated by both c9,t11 and t10,c12 CLA isomers. This suggests that CLA may increase mitochondrial biogenesis via the universal signaling cascade, PGC-1α-NRF-1-Tfam, and is further supported by the finding that mitochondrial DNA copy number significantly increased after CLA treatments (Fig. 4.6).

In the current study, we tested effects of CLA isomers on several mitochondrial markers, cytochrome c, COX IV and VDAC. The expression levels of cytochrome c, COX IV and VDAC indirectly represents whether tissues or cells are undergoing mitochondrial biogenesis. In particular, cytochrome c is involved in oxidative capacity through phosphorylation, while VDAC is known to be linked to mitochondrial dysfunction and aging as well. Our results suggest that the c9,t11 isomer increased expression of cytochrome c, while t10,c12 isomer increased expression of VDAC. The former observation is consistent with Mollica et al. that the c9,t11 isomer, but not the t10,c12 isomer, induced higher cytochrome c activity in Wistar rats. There are currently no reports of CLA’s effect on VDAC, however, it was reported that other polyunsaturated fatty acids, DHA and eicosapentaenoic acid (EPA), did not increase VDAC expression in male Wistar rats. This is the first report that t10,12 CLA significantly increase VDAC expression in C2C12 cells, which may provide evidence to have more mitochondria. Also our results suggest that individual CLA isomers might selectively activate mitochondrial proteins associated with mitochondrial biogenesis, resulting in stimulation of mitochondriogenic signaling. In addition, although cytochrome c, COX IV and VDAC represent mitochondrial components as an indirect marker for mitochondrial biogenesis, we cannot rule out the possibility that CLA may influence other mitochondrial-related factors, such as COX I, II and III subunits and ATP synthase subunits in C2C12 myotubes.

PGC-1α is also involved in the induction of ROS-detoxifying enzymes such as SOD2 in mitochondria. The c9,t11 CLA isomer increased the mRNA level of SOD2 in earlier studies of CLA-treated in Sprague-Dawley rats and THP-1 macrophages, although we did not observe any differences due to individual CLA isomers on SOD2. These discrepancies may result from
differences in experimental models used, i.e. dietary supplement to animals with exercise and cells with an oxidative stressor.

In conclusion, both CLA isomers were shown to activate PGC-1α, which was linked with upregulation of mitochondrial biogenesis. It is likely that the t10,c12 isomer activates PGC-1α through AMPK phosphorylation and the c9,t11 isomer stimulates PGC-1α via another undetermined pathway as c9,t11 isomer does not activate phosphorylated AMPK. Both c9,t11 and t10c12 CLA isomers enhance the expression of NRF-1 and Tfam. Also the CLA mixture improves PPARδ expression. It is supporting that all CLA treatments significantly increase mitochondrial DNA replication. Taken together, CLA engages the mitochondrial biogenesis process through modulating PGC-1α-NRF-1-Tfam cascade without any isomer interaction. Overall, CLA may play a role as a stimulator of mitochondrial biogenesis.
CHAPTER 5

CONJUGATED LINOLEIC ACID (CLA) IMPROVES MUSCLE METABOLISM VIA STIMULATING MITOCHONDRIAL BIOGENESIS SIGNALING IN GENETICALLY INDUCED INACTIVE ADULT-ONSET OBESE MICE

5.1 Introduction

Reduced energy expenditure, particularly associated with lack of physical activity, plays a significant role in weight gain.\(^1\) However, overall physical activity can be influenced by complex issues, and thus make it difficult to use as universal means to control/prevent obesity.\(^2\) Previously nescient helix-loop-helix 2 wild type (N2KO) mouse model has been established, where these animals exhibit reduced activity at a certain age, 7-10 weeks old, without hyperphagia during preobese period. Targeted deletion of the Nhlh2 transcription factor expressed in the hypothalamus precedes adult-onset obesity in mice, contributing to a disruption of the hypothalamic-pituitary axis.\(^3,4\) Thus, N2KO mice represent an animal model that mimics induction of adult-onset obesity, making it particularly useful to investigate any compounds that target activity in these animals.

Conjugated linoleic acid (CLA) is a mixture of geometric and positional conjugated isomers of linoleic acid. The effects of CLA have been previously demonstrated as to influence on development of cancer and atherosclerosis, as well as preventing osteoporosis and modulating immune responses.\(^5,6\) Moreover, CLA has drawn special attention in the last two decades for its anti-obesity effect through various mechanisms, such as regulation of energy and lipid metabolism, adipogenesis, inflammation and adipocyte apoptosis.\(^7,8\) In addition, it was reported that CLA improved voluntary activity and endurance capacity in animal models, including N2KO animals, and CLA modulated energy metabolism, especially uncoupling protein 2 (UCP2) and peroxisome proliferator-activated receptor δ (PPARδ) in skeletal muscle.\(^9,10\) However, it is
currently not fully understood how CLA influences skeletal muscle metabolism, including mitochondrial biogenesis.

Mitochondrial biogenesis is composed of complex long-term adaptive responses in skeletal muscle.\(^{249}\) It is known that mitochondrial biogenesis is important for endurance capacity but also plays a significant role in proper weight management.\(^{10,220}\) In fact, CLA isomers, \textit{cis-9,trans-11} and \textit{trans-10,cis-12}, stimulated the key molecular markers involved in mitochondrial biogenesis and increased mitochondrial DNA copy number.\(^{250}\) Also, it was previously reported that CLA increased voluntary activity and endurance capacity as well as reduced obesity. Thus, we investigated the role of CLA on skeletal muscle energy metabolism and mitochondrial biogenesis using N2KO mice to understand its underlying mechanisms on weight control.

5.2 Materials and Methods

5.2.1 Materials

CLA was provided by Natural Lipids Ltd. AS (Hovdebygda, Norway). The CLA content of the preparation used was 80.7%. CLA consisted of 37.8\% \textit{cis-9,trans-11}, 37.6\% \textit{trans-10,cis-12} and 5.3\% other CLA isomers and the remainder of the preparation was 13.7\% oleic acid, 3.2\% stearic acid, 0.4\% palmitic acid and 0.2\% linoleic acid. Semi-purified powdered diet (TD07518, 95\% basal mix) was purchased from Harlan Laboratories (Madison, WI). Serum triacylglyceride (TG), glucose, and total cholesterol assay kits were purchased from Genzyme Diagnostics (Charlottetown, PE, Canada). DC protein assay kit was from Bio-Rad (Hercules, CA). Rabbit antibodies for phosphorylated AMP-activated protein kinase \(\alpha\) (AMPK\(\alpha\)), AMPK\(\alpha\), voltage-dependent anion channel (VDAC) and superoxide dismutase 2 (SOD2) were obtained from Cell Signaling (Berberly, MA). Rabbit antibodies for peroxisome proliferator-activated receptor \(\gamma\) coactivator 1\(\alpha\) (PGC-1\(\alpha\)), sirtuin 1 (SIRT1), PPAR\(\delta\), nuclear respiratory factor 1 (NRF-1), mitochondrial transcription factor A (Tfam), cytochrome c (Cyt C), cytochrome c oxidase subunit 4 (COX IV), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and \(\beta\)-actin were purchased
from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-rabbit IgG antibody was from Boston Bioproducts Inc. (Ashland, MA). Other chemicals used were purchased from either Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburg, PA).

5.2.2 Animal and Diet

All animal work was done in compliance with the Institutional Animal Care and Use Committee at the University of Massachusetts, Amherst. 129Sv/J heterozygous for Nhlh2 male and female mice between 8 and 52 weeks of age were used as breeders to prepare for wild type and Nhlh2 knock-out offspring (N2KO). Based on the results from genotyping at weaning, eight female wild type and six N2KO mice were placed in wire-bottomed cages with a 12:12 h light:dark cycle. During a 2-week acclimation period, mice were fed a control diet (20 w/w% fat) and were subjected to baseline test for glucose tolerance and voluntary movement. At the end of the adaptation period, animals were divided into two groups per each genotype based on their body weight. Either control (high-fat, 20 w/w% fat) or 0.5% CLA containing high-fat diet was provided for 10 weeks. Diets and water were available ad libitum. The diet composition is shown in Table 5.1. Body weight and food intake were monitored weekly. At the end of study, the mice were fasted for 4 h, and sacrificed by CO₂ asphyxiation. Blood was collected by cardiac puncture and internal organs (liver, heart, kidney, spleen, adipose tissues including parametrial, retroperitoneal and mesenteric fats and gastrocnemius muscle) were also weighed.

5.2.3 Voluntary Movement Measurement (Non-exercise Physical Activity Test)

Voluntary movement was recorded using LoliTrack Quatro Video Tracking Software Version 1.0 (Loligo Systems, Tjele, Denmark) with a high-resolution camera supported to monitor under dim light. Animals were individually placed into a cage during the dark cycle
Table 5.1. Composition of experimental diet for control and conjugated linoleic acid (CLA) groups.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/kg</th>
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</thead>
<tbody>
<tr>
<td>Casein, ‘vitamin-free’ tested</td>
<td>169.1</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>2.2</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>288.5</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>132</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>195</td>
</tr>
<tr>
<td>CLA or Soybean oil</td>
<td>5</td>
</tr>
<tr>
<td>Mineral mix, AIN-93M-MX (TD 94049)</td>
<td>42.8</td>
</tr>
<tr>
<td>Vitamin mix, AIN-93-VX (TD 94047)</td>
<td>12.4</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>3</td>
</tr>
<tr>
<td>tert-Butylhydroquinone</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>1,000</strong></td>
</tr>
</tbody>
</table>

(5:00pm-5:00am) once biweekly with free access to diet and water (provided as HydroGel®, Clear H₂O, Portland, ME). The cage (30 × 46 × 40 cm) used for activity monitoring is larger than the typical cage to measure proper movement, since the small cage is known to limit voluntary movements. Movement data for 8 h (7:00pm-3:00am) was analyzed excluding the first 2 h (5:00pm-7:00pm) of early phase and 2 h (3:00am-5:00am) of late phase, due to early adaption and due to lack of movement, respectively.

5.2.4 Serum Parameters

Serum was separated by centrifugation at 3,000 g for 20 min at 4°C. Serum samples were used for determination of TG, glucose and total cholesterol using commercial kits following by the manufacturer’s instruction.
5.2.5 Glucose Tolerance Test

Intraperitoneal glucose tolerance test (IGTT) was performed every four weeks as described previously with some modification. Glucose levels were measured using a blood glucose meter, Advocate Redi-Code (Advocate Meters Inc., Dorado, Puerto rico). All mice were fasted for 6 h before IGTT, and glucose levels were monitored from the tail vein at 0 min for baseline measurement. Then a 30% glucose solution (2.0g glucose/kg body weight) was administered by intraperitoneal injection. Blood glucose levels were monitored at 15, 30, 60, 90 and 120 min post glucose injection. The areas under the curve (AUC) were calculated using SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA).

5.2.6 Western Blot Analysis

Gastrocnemius skeletal muscle tissue was ground with liquid nitrogen, prepared for 50 mg aliquots and stored at -80°C until analysis. Each sample was homogenized in lysis radioimmune precipitation assay (RIPA) (50 mM Tris-HCl, 10 mM NaCl, 5 mM MgCl₂, and 0.5% NP-40, pH 8.0) buffer (Boston Bioproducts Inc., Ashland, MA) containing protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO) and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL) and then lysed on ice for 2 h, and centrifuged at 12,000g for 20 min at 4°C. Protein concentration of tissue lysate was determined using the DC protein assay kit. The lysates were separated by an 8-15% (w/v) sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA). After blocking for 1 h at room temperature (RT), membranes were incubated with appropriate primary rabbit antibodies in 0.05% Tween-20 in tris-buffered saline (TBS-T) containing 5% bovine serum albumin at 4°C overnight. After washing, membranes were treated horseradish peroxidase-conjugated goat anti-rabbit IgG antibody for 1 h at RT. Detection was performed using the Enhanced Chemiluminescence solution (Bio-Rad, Hercules, CA) with an Image Station 4000MM (Carestream Health, New Heaven, CT). GAPDH or β-actin intensity was used for an internal
control to normalize protein content of each sample. Image J software was used to quantify the results.

5.2.7 Statistical Analyses

Data were analyzed by two-way analysis of variance (ANOVA) followed by PROC MIXED with Least Square Means options of the SAS software (Version 9.3, SAS Institute Inc., Cary, NC, USA). For the body weight, food intake, voluntary movement and glucose tolerance test, two-way ANOVA and repeated measures with PROC MIXED were performed. In the repeated measures using PROC MIXED, we found that the assumption of constant variance did not appear to hold for the trend of voluntary movement. Thus, data were converted into logarithm-scale and used for analysis. For internal organ, adipose tissue weight, serum parameters and the log-transformed relative protein expression level data two-way ANOVA with PROC MIXED were used. The Tukey-Kramer's method in SAS was used for the multiple comparisons among the experimental groups. Data are shown as the mean ± S.E. P values less than 0.05 are reported as statistically significant.

5.3 Results

5.3.1 Body Weights and Food Intake

Body weight and food intake data are shown in Figure 5.1A and 1B. N2KO mice started to show greater body weight than wild type mice at week 5 (10-week-old) and maintained this trend to the end of the experiment (overall genotype effect, $P < 0.0001$). Furthermore, CLA fed animals showed significant reduction in body weight (overall CLA effect, $P = 0.042$). Greater effects of CLA on body weight were observed in N2KO animals compared to wild type animals, when significance was observed starting at week 6 between N2KO control and N2KO CLA groups (Fig. 5.1A). No significant differences were observed in food intakes between any of groups (Fig. 5.1B).
5.3.2 Tissue Weights

Total adipose tissue weight including parametrial, mesenteric, and retroperitoneal adipose tissue, liver, heart, kidney, spleen and gastrocnemius skeletal muscle tissue weights are shown in Table 5.2. N2KO control animals had the most adipose depots among all treatment groups. Adipose tissue weights except mesenteric fat were significantly different by genotype, while significant interaction between diet and genotypes were observed in mesenteric adipose tissue weights. Only retroperitoneal fat weights but not others showed significantly different by CLA supplementation \( (P = 0.0055) \).
The gastrocnemius skeletal muscle weights were significantly increased with CLA (\(P = 0.0282\)), along with significant genotype effects (\(P < 0.0001\)). No interactions were observed for diets and genotypes in muscle weights. Both genotype and CLA had significant effects on kidney weights. Liver, heart and spleen weight were influenced by CLA significantly, but not by genotypes.

Table 5.2. Effects of conjugated linoleic acid (CLA) on organ weights in wild and N2KO mice.

<table>
<thead>
<tr>
<th></th>
<th>Wild Type</th>
<th>Treatment</th>
<th>N2KO</th>
<th>Effects ((F)-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>CLA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Body Weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adipose tissue</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parametrial fat</td>
<td>3.47 ± 0.92 (^a)</td>
<td>3.47 ± 0.99 (^b)</td>
<td>3.47 ± 0.99 (^b)</td>
<td>0.0444</td>
</tr>
<tr>
<td>Retroperitoneal fat</td>
<td>0.34 ± 0.26 (^a)</td>
<td>0.09 ± 0.03 (^b)</td>
<td>0.12 ± 0.13 (^a)</td>
<td>0.0099</td>
</tr>
<tr>
<td>Mesentric fat</td>
<td>2.74 ± 0.26 (^a)</td>
<td>4.01 ± 0.35 (^b)</td>
<td>4.19 ± 0.27 (^a)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Total</td>
<td>8.32 ± 1.77 (^a)</td>
<td>7.57 ± 0.77 (^b)</td>
<td>15.7 ± 0.29 (^a)</td>
<td>0.0048</td>
</tr>
<tr>
<td>Skeletal muscle tissue</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>1.03 ± 0.05 (^a)</td>
<td>1.15 ± 0.01 (^b)</td>
<td>0.45 ± 0.03 (^a)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Organ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>4.22 ± 0.37 (^a)</td>
<td>5.51 ± 0.21 (^a)</td>
<td>3.33 ± 0.16 (^a)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Heart</td>
<td>0.50 ± 0.04 (^a)</td>
<td>0.58 ± 0.04 (^b)</td>
<td>0.35 ± 0.03 (^a)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.33 ± 0.10 (^a)</td>
<td>1.48 ± 0.03 (^b)</td>
<td>1.00 ± 0.14 (^a)</td>
<td>0.0097</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.20 ± 0.03 (^a)</td>
<td>0.45 ± 0.09 (^b)</td>
<td>0.20 ± 0.03 (^a)</td>
<td>0.0278</td>
</tr>
</tbody>
</table>

Values represent means ± S.E. (\(n = 3-4\)). \(^a-b\)Means with different superscripts within the same row are significantly different (\(P < 0.05\)). \(F\)-values in table are from two-way ANOVA. N2KO, nescient helix-loop-helix knock-out mice; CLA, conjugated linoleic acid; n.s., not significant.

5.3.3 Voluntary Movement Measurement (Non-exercise Physical Activity Test)

Effects of CLA on physical activity were determined by measuring voluntary movement (non-exercise physical activity) biweekly (Fig. 5.2A-F). Data in Figures 5.2A-F were plotted over time (Fig. 5.2G-I). Data were represented as total (19:00-03:00) as well as two different time periods, early (19:00-23:00) and late (23:00-03:00). As reported previously, N2KO mice showed significantly decreased travel distances compared to wild type animals over time (overall genotype effects, \(P < 0.0001\)), meanwhile CLA treatment significantly increased total
Figure 5.2 Effect of conjugated linoleic acid (CLA) on voluntary movement (non-exercise physical activity) in wild type and N2KO mice.

travel distance ($P = 0.0172$). No interactions among genotype, diet and time were observed.

Intriguingly, voluntary movements during the late phase in control diet-fed mice were significantly reduced compared to CLA-diet groups ($P = 0.0022$), whereas during the early phase there was no significant CLA effect on travel distances in all treatment groups. In Figures 5.2G-I, it was evident that control N2KO mice showed declining activity in total as well as early and late phase over time, while CLA-fed N2KO mice either did not display reduced activity or slightly increased voluntary movement during the same time period.
5.3.4 Glucose Tolerance Test

To evaluate whether CLA treatment affects glucose homeostasis, we completed glucose tolerance tests every four weeks (Fig. 5.3). There were no significant differences among all treatment groups at each time point. However, there were significant overall time effects ($P < 0.0001$), which suggests potential impaired glucose tolerance due to high-fat diet over ageing in these animals regardless of diet or genotype effects.

![Figure 5.3 Effect of conjugated linoleic acid (CLA) on glucose tolerance in wild type and N2KO mice.](image)

5.3.5 Serum Parameters

Results of serum parameters are shown in Table 5.3. N2KO animals had higher levels of serum TG than wild type mice ($P = 0.0003$), with overall CLA effects on TG observed ($P = 0.0408$). No interactions were observed for TG on genotypes and CLA effects. Consistent with glucose tolerance results during the experimental period, no difference in the levels of glucose was observed among all experimental groups. Significant interaction between genotypes and CLA was observed in total cholesterol level ($P=0.0080$).
Table 5.3. Effects of conjugated linoleic acid (CLA) on serum parameters in wild and N2KO mice.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild Type</th>
<th>Treatment</th>
<th>N2KO</th>
<th>Effects (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>CLA</td>
<td>Control</td>
<td>CLA</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>36.1 ± 5.8a</td>
<td>28.9 ± 5.4a</td>
<td>97.4 ± 15.4a</td>
<td>63.2 ± 8.9ab</td>
</tr>
<tr>
<td>Glucose</td>
<td>143.8 ± 5.27</td>
<td>175.8 ± 21.69</td>
<td>230.1 ± 33.1</td>
<td>187.0 ± 61.5</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>163.3 ± 18.5a</td>
<td>198.5 ± 17.8ab</td>
<td>292.8 ± 44.1a</td>
<td>165.2 ± 14.1b</td>
</tr>
</tbody>
</table>

Values represent means ± S.E. (n = 3-4). *b* Means with different superscripts within the same row are significantly different (P < 0.05). P-values in table are from two-way ANOVA. N2KO, nescient helix-loop-helix knock-out mice; CLA, conjugated linoleic acid; n.s., not significant.

5.3.6 Effects on Muscle Metabolism

To determine whether CLA treatment influences skeletal muscle metabolism, we investigated the effect of CLA on AMPK signaling pathway (Fig. 5.4A and B). There were no differences in all treatments in the relative expression levels of total AMPKa, a master sensor to maintain energy homeostasis. However, significant effects of CLA on phosphorylation of AMPKa (the active form of AMPKa) compared to the control groups were observed (P = 0.0291 for overall CLA effect). As a result, increased ratio of phosphorylated AMPKa to AMPKa was shown in CLA-fed animals (P = 0.024 for overall CLA effect). Another key biomarker, SIRT1, a NAD-dependent deacetylase inducing PGC-1α activation, was not significantly different in all treatment groups. However, expression of PGC-1α, a master regulator of mitochondrial biogenesis, was increased slightly in N2KO CLA animals compared to respective control, although it did not reach significance (Fig. 5.4B).

5.3.7 Effects on Mitochondrial Biogenesis-related Factors

Based on the evidence that CLA potentiates activation of AMPKa and PGC-1α in N2KO mice in Fig. 5.4, we evaluated whether CLA treatment was involved in mitochondrial biogenesis in the gastrocnemius muscle. Mitochondrial biogenesis plays a key role in increasing oxidative capacity, thus reduces muscle fatigue, resulting in prolonged endurance capacity. We
Figure 5.4 Effect of conjugated linoleic acid (CLA) on protein expression of AMP-activated protein kinase (AMPK), SIRT1, and PGC-1α from the gastrocnemius muscle in wild type and N2KO mice.

have tested PPARδ, NRF-1 and Tfam in the gastrocnemius muscle, all of them are well-known markers for mitochondrial biogenesis that are primarily controlled by PGC-1α. As expected, supplementation with CLA significantly increased the expression levels of PPARδ compared to control-fed animals ($P = 0.0215$ for overall CLA effect), which is a marker associated in mitochondrial biogenesis as well as muscle type transformation. The expression levels of NRF-1, a regulator to mediate nuclear-encoding mitochondrial proteins, were not different in all experimental groups. Expression of Tfam, an important regulator for mitochondrial DNA replication and transcription, was upregulated by CLA-supplementation in comparison to controls ($P = 0.0225$ for overall CLA effect) in both genotype animals (Fig. 5.5A and B).

We further investigated expression levels of mitochondrial proteins, cytochrome c and COX IV located in the inner membrane of mitochondria and VDAC localized in the outer mitochondrial membrane. Also, we measured expression of manganese superoxide dismutase (SOD2), which encodes a mitochondrial matrix protein and protects mitochondria from reactive oxygen species, to assess mitochondrial function. Among them cytochrome c protein levels
Figure 5.5 Effect of conjugated linoleic acid (CLA) on mitochondrial biogenesis-related factors from the gastrocnemius muscle in wild type and N2KO mice.

were significantly increased in wild type animals ($P = 0.0069$ for overall genotype effect). The expression levels of VDAC slightly increased in CLA-fed mice compared to control groups. No significant differences were observed in COX IV and SOD2 (Fig. 5.6A and B).

Figure 5.6 Effect of conjugated linoleic acid (CLA) on mitochondrial content and function from the gastrocnemius muscle in wild type and N2KO mice.
5.4. Discussion

In the current study, we have demonstrated that CLA treatment prevents body weight gain and improves voluntary movements through modulating the key molecular markers related to muscle metabolism via stimulating mitochondrial biogenesis signaling in adult-onset inactivity induced obese mice. Of particular importance CLA-fed mice were shown to activate AMPKα, PGC-1α, and subsequently upregulate mitochondria-related molecular markers, such as PPARδ and Tfam in both wild type and N2KO mice. These observations further support that CLA supplementation helps improve physical activity, and change body composition including increased lean body mass in previous animal studies.\textsuperscript{15,17,27,31,63,205} This is the first report that CLA treatment may lead to preventing the development of obesity in adult-onset inactivity induced obese mice by stimulating mitochondrial biogenesis.

Mitochondria is one of the key cellular compartments generating energy requires for cells and may play a significant role in maintaining energy balance and contribute to attenuating the development of obesity. The process of formation of mitochondria, mitochondrial biogenesis, is a complicated process and regulated by numerous different upstream signals, however, it is well-established that PGC-1α is a primary master regulator of mitochondrial biogenesis.\textsuperscript{218,219,222} Activated PGC-1α subsequently triggers upregulation of nuclear and mitochondrial genes and proteins such as PPARδ, NRF-1 and -2, and Tfam, which lead to mitochondrial biogenesis.\textsuperscript{229} It is further supported that PGC-1α is involved in physical activity and promotes the formation of oxidative slow-twitch muscle fibers resulting in enhanced endurance capacity and improved exercise tolerance.\textsuperscript{190,255} The current results suggest that CLA may potentiate mitochondrial biogenesis via PGC-1α, particularly in N2KO animals. Also increased voluntary activity levels resulting from CLA supplementation may have modulated muscle metabolism through the upregulation of PGC-1α expression. However, further confirmatory studies are needed, including changes of morphological and histological properties of skeletal muscles with regard to fiber type transformation from fast (glycolytic) to slow-twitch (oxidative).
To further understand whether increased PGC-1α activity with CLA was directly influenced or not, we further investigated the upstream activators of PGC-1α; phosphorylation by AMPKα and deacetylation by SIRT1. CLA treatment significantly upregulated the expression of phosphorylated AMPKα (active form of AMPKα) but not SIRT1 activity, suggesting that the influence of CLA on AMPKα activation is more important than that of SIRT1.194-196,238

Although PGC-1α facilitates various molecular events, PGC-1α-NRF-1/-2-Tfam and PGC-1α-PPARδ signaling cascade are important mitochondriogenic pathways.186,256 PPARδ plays a critical role in regulation of muscle fiber type transformation and improvement of endurance capacity by stimulating mitochondrial functions including mitochondrial biogenesis.192,257 It was previously reported that the trans-10,cis-12 CLA isomer increased mRNA expression level of PPARδ in exercise-trained 129 Sv/J mice gastrocnemius skeletal muscle.18 Consistently, the current results support that CLA supplementation upregulated PPARδ expression in both N2KO and wild type mice.

Alternatively, PGC-1α regulates NRF and Tfam. NRF-1 and -2 play significant roles in the expression of nuclear-encoding mitochondrial genes and proteins and Tfam controls the expression of mitochondrial DNA encoded oxidative respiratory phosphorylation proteins. Their activation results in increased mitochondrial mass and functions.239,258,259 In the current study CLA treatment activated Tfam, which is reported to be closely linked with activation of AMPKα.182,260 Taken together, the current results suggest that CLA promotes mitochondrial biogenesis through AMPKα-PGC-1α-PPARδ and/or -Tfam.

Upregulation of cytochrome c, a nuclear-encoded protein of the electron transport chain, has been previously reported in other food bioactive compounds, quercetin and isoflavones.260,261 In this study, CLA supplementation resulted in increased cytochrome c, potentially mediated by increased upstream signaling pathway as described above. However, our current results cannot rule out the possibility that CLA may also influence other regulatory mechanisms for cytochrome
c, such as the early growth response gene-1 (EGR1), specificity protein 1 (Sp1) and thrombospondin-1 receptor CD47.262-264

The targeted deletion of Nhlh2 yields a unique mouse model for inducing obesity, not by increasing food consumption or lowering basic metabolic rate compared to wild type animals but by reducing overall physical activity levels.247,254 Thus, reduced physical activity in N2KO animals plays a crucial role in induction of weight gain and fat accumulation. Consistent with previous publications,205,247,253 we observed no difference in food intake between all treatment groups during the entire experimental period, while N2KO mice reduced voluntary activity and increased body weight gain compared to wild type animals.

Although not significant, we observed CLA treatment marginally contributed to glucose intolerance. It has been suggested that increased fatty acid β-oxidation by CLA might result in glucose intolerance, as proposed “the glucose-fatty acid cycle”.265 In fact, it is known that increased AMPK activity induces acetyl-CoA carboxylase inactivation, and subsequently results in decreasing accumulation of malonyl-CoA, which in turn leads to increased fatty acid β-oxidation in mitochondria through the induction of upregulated CPT-1 expression.266,267 Enhanced fatty acid β-oxidation results in citrate accumulation in the cytosol, which causes inhibition of glucose transporter 4 and 6-phosphofructo-1-kinase.268 In fact, it has been previously shown that CLA increased CPT-1 expression and activities.17,18,63 Moreover, relatively long-term animal and human studies all support that glucose intolerance associated with CLA is an initial response to CLA supplementation, however, there have been no reports of adverse effects of CLA on glucose homeostasis in long-term human studies longer than 6 months.99,100,107,127

Along with glucose intolerance, we observed enlarged liver due to CLA treatment in the current study, which is consistent with other animal studies.17,18,63 It has been suggested that increased liver weight by CLA may be due to tremendous effects of CLA on fat mobilization from adipose tissue and increased hepatic lipogenesis, however, no human studies reported any
changes in markers of liver functions associated with CLA supplementation. Thus, this may be a species-specific response to CLA.\(^{13,269-271}\)

In conclusion, we demonstrated that CLA treatment prevents body weight gain and fat accumulation by increased physical activity in genetically obese N2KO mice. CLA supplementation improved voluntary movement and increased skeletal muscle mass, implying enhanced muscle metabolism through stimulating mitochondrial biogenesis signaling. Although the precise mechanism underlying the stimulation of mitochondria-related molecular markers is not clearly elucidated, it is suggested that CLA exerts its action via the activation of PGC-1\(\alpha\) through AMPK\(\alpha\) phosphorylation, resulting in mitochondriogenic process through regulating PGC-1\(\alpha\)-PPAR\(\delta\) and/or -Tfam signaling cascades in skeletal muscle. This study provides novel insights into the preventive effects of CLA on adult-onset inactivity induced obesity by stimulation of muscle metabolism.
CHAPTER 6
EFFECTS OF POST-WEANING ADMINISTRATION OF CONJUGATED LINOLEIC ACID (CLA) ON DEVELOPMENT OF OBESITY IN NESCIENT HELIX-LOOP-HELIX 2 KNOCKOUT MICE

6.1 Introduction

Since 1980, the prevalence of childhood obesity has almost tripled. Along with overnutrition, lack of physical activity during childhood period is one of the significant contributing factors to development of childhood obesity. Similar to adult obesity, childhood obesity is known to be associated with the increased risk of chronic metabolism diseases. Even though early childhood has been considered to be critical for health later in life, most studies in humans and animals mainly addressed the effects of dietary and exercise regimens rather than focusing on early-onset interventions during critical developmental periods.

Conjugated linoleic acid (CLA) is a term describing a mixture of 18-carbon unsaturated fatty acid isomers with conjugated double bonds. The name, CLA, was first used when its anticancer activities were reported in 1987. Since then, other biological activities of CLA have been reported, including its effects on prevention of atherosclerosis development, modulation of immune responses, promotion of young animal growth, and modulation of body composition, particularly reduction of body fat and improvement of lean and bone masses. It has been suggested that CLA reduces body fat by multiple biochemical mechanisms, including increased energy expenditure, modulated adipogenesis, and/or enhanced fatty acid β-oxidation.

The beneficial effects of CLA supplementation during the obesity onset period in nescient basic helix-loop-helix 2 (Nhlh2) knock-out mice (N2KO) have been previously reported. Targeted deletion of the Nhlh2 transcription factor expressed in the hypothalamus precedes adult-onset obesity in mice, resulting from a disruption of the hypothalamic-pituitary axis. While
N2KO mice have normal levels of food intake, their spontaneous voluntary movement is decreased by more than 50% in both genders.\textsuperscript{247} It was suggested that CLA supplementation in young N2KO animals prevented excessive weight gain by improving voluntary activity.\textsuperscript{205} However, it is not clear if supplementation of CLA during pre-obesity stage would benefit later in life as it was reported in non-obese animals.\textsuperscript{31} Thus the purpose of this study was to determine the effects of CLA supplementation during the early growth period in N2KO animals on development of obesity and symptoms of type 2 diabetes later in life.

6.2 Materials and Methods

6.2.1 Materials

CLA was provided by the Natural Lipids Ltd. AS (Hovdebygda, Norway). The CLA used consisted of 80.7% CLA (37.8\% cis-9,trans-11, 37.6\% trans-10,cis-12 and 5.3\% other CLA isomers) and 13.7\% oleic acid, 3.2\% stearic acid, 0.4\% palmitic acid, 0.2\% linoleic acid and 1.8\% unknown. Semi-purified powdered diet (TD07518) was obtained from Harlan Laboratories (Madison, WI). Serum triacylglyceride (TG), glucose, and total cholesterol assay kits were purchased from Genzyme Diagnostics (Charlottetown, PE, Canada). DC protein assay kit (Bio-Rad, Hercules, CA) was used for protein quantitation. Rabbit antibodies for phosphorylated AMP-activated protein kinase α (p-AMPKα), AMPKα, phosphorylated phosphatase and tensin homolog (p-PTEN), phosphorylated phosphoinositide-dependent kinase (p-PDK), phosphorylated protein kinase B at threonine 308 (p-Akt Thr308) and serine 473 (p-Akt Ser473), Akt and superoxide dismutase 2 (SOD2) were obtained from Cell Signaling (Berberly, MA). Rabbit antibodies for peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), sirtuin 1 (SIRT1), peroxisome proliferator-activated receptor-δ (PPARδ), nuclear respiratory factor 1 (NRF-1), mitochondrial transcription factor A (Tfam), cytochrome c (Cyt C), cytochrome c oxidase (COX IV), insulin receptor substrate 1 (IRS-1), glucose transporter type 4 (GLUT4) and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish
peroxidase-conjugated goat anti-rabbit IgG antibody was from Boston Bioproducts Inc. (Ashland, MA). Other chemicals used were purchased from either Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburg, PA).

6.2.2 Animals and Diets

All animal work was conducted in compliance with the Institutional Animal Care and Use Committee at the University of Massachusetts. As described in a previous report, 247 129Sv/J male offspring mice were obtained through inbreeding between heterozygous male and female breeders. Litters were housed together with dams until weaning. At 3-week-old, all male mice were biopsied (0.5-cm tail) for genotyping. Ten wild type and eight male N2KO mice were selected for this study, and then placed in individual wire-bottomed cages on a 12:12 h light:dark cycle in a temperature and humidity controlled room. During a 1-week acclimatization period, all mice were fed a semi-purified powdered control diet (20 w/w% fat) to induce obesity in rodents 63,205,278 and were subjected to baseline test for voluntary movement and glucose tolerance in serum. Based on body weight, all animals in different genotype groups (wild type and N2KO) were divided into two diet groups and fed either control or 0.5% CLA containing diet for 4 weeks. Then, all animals received the control diet for 8 weeks. Previously it was determined that feeding CLA for 4 weeks in mice was enough to result in changes of body composition as well as changes in activity levels. 17,30 Moreover, it was reported that most CLA remaining in the tissues (liver, adipose tissue and muscle) returned to comparable levels to control animals at 8 weeks post withdrawal. 30 Thus we selected 8 weeks post CLA supplementation in the current study. Dose of CLA was determined based on previous reports that supplementing 0.5% CLA for 2-4 weeks in rats led to serum level range of 23 to 120 µM and serum levels of CLA after supplementation of 0.8-3.2 g per day for 2 months in humans ranged from 23 to 200 µM. 27,226
The compositions of diets are shown in Table 6.1. Diet and water were available *ad libitum* throughout the experiment and provided freshly twice per week. Body weight and food intake were monitored weekly. At the end of the study, mice were fasted for 6 h and sacrificed by CO2 asphyxiation. Blood was immediately collected by cardiac puncture, and internal organs (liver, heart, kidney, spleen, white adipose tissues including epididymal, retroperitoneal and mesenteric fat pads) were weighed at sacrifice.

Table 6.1. Composition of experimental diet for control and conjugated linoleic acid (CLA) groups.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein, ‘vitamin-free’ tested</td>
<td>169.1</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>2.2</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>288.5</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>132</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>195</td>
</tr>
<tr>
<td>CLA or Soybean oil</td>
<td>5</td>
</tr>
<tr>
<td>Mineral mix, AIN-93M-MX (TD 94049)</td>
<td>42.8</td>
</tr>
<tr>
<td>Vitamin mix, AIN-93-VX (TD 94047)</td>
<td>12.4</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>3</td>
</tr>
<tr>
<td>tert-Butylhydroquinone</td>
<td>0.04</td>
</tr>
<tr>
<td>Total</td>
<td>1,000</td>
</tr>
</tbody>
</table>

6.2.3 Voluntary movement measurement (Non-exercise physical activity test)

Non-exercise physical activity (voluntary movement) was recorded using LoliTrack Quatro Video Tracking Software Version 1.0 (Loligo Systems, Tjele, Denmark) with a high-resolution camera under dark condition. This tracking system simultaneously monitored four mice placed in individual cages (30 x 46 x 40 cm) during the dark phase (5:00pm-5:00am) once a month with free access to diet and water (provided as HydroGel®, Clear H2O, Portland, ME).
Movement data for 8 h (7:00pm-3:00am) excluding for the first 2 h (5:00pm-7:00pm) during early phase for adapting to environmental change and 2 h (3:00am-5:00am) during late phase due to lack of movement were analyzed.

6.2.4 Serum Parameters

The levels of glucose, total cholesterol and TG in serum separated by centrifugation at 3,000 g for 20 min at 4°C were determined using commercial kits as specified by the manufacturer’s instruction.

6.2.5 Glucose Tolerance Test

As described previously, intraperitoneal glucose tolerance tests (IGTT) were conducted every four weeks. All mice underwent 6 h fasting and then were subjected to baseline measurement of blood glucose levels at 0 min. Blood was collected from the tail vein using blood glucose meter, Advocate Redi-Code (Advocate Meters Inc., Dorado, Puerto rico). Subsequently, 30% glucose solution (2.0g glucose/kg body weight) was administered through intraperitoneal injection and then blood glucose levels were monitored at 15, 30, 60, 90 and 120 min. Collected data were calculated as the areas under the curve (AUC) with SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA).

6.2.6 Western Blot Analysis

The frozen gastrocnemius skeletal muscle tissue was ground and 50 mg aliquots were prepared and stored at -80°C until analysis. Each sample was homogenized in premixed lysis radioimmune precipitation assay (RIPA) (50 mM Tris-HCl, 10 mM NaCl, 5 mM MgCl₂, and 0.5% NP-40, pH 8.0) buffer (Boston Bioproducts Inc., Ashland, MA), protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO) and phosphatase inhibitor cocktail (Thermo Scientific, Rockford,
IL) and then lysed on ice for 2 h, and centrifuged at 12,000g for 20 min at 4°C. The concentration of protein from tissue lysate was determined using the DC protein assay kit. The normalized lysates (37.5 µg/ml protein) were separated by an 8-15% (w/v) sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA). After incubating with appropriate primary rabbit antibodies, membranes were treated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody for 1 h at room temperature. Detection was performed using the Enhanced Chemiluminescence solution (Bio-Rad, Hercules, CA) with an Image Station 4000MM (Carestream Health, New Heaven, CT). The band density was normalized by β-actin intensity as an internal control. The results were quantified using Image J software (U.S. National Institutes of Health).

6.2.7 Statistical analyses

All data analyses were performed by two-way analysis of variance (ANOVA) followed by PROC MIXED procedure with Least Square (LS) Means statement of the SAS software (Version 9.3, SAS Institute Inc., Cary, NC, USA). For data on body weight, food intake, voluntary movement and glucose tolerance test, two-way repeated measures ANOVA and the slice option in the LS means statement to examine the group differences at each time point with PROC MIXED were used. For internal organ, adipose tissue weight and serum parameters data, two-way ANOVA with PROC MIXED were conducted. The experimental groups were compared by the multiple comparisons basically Tukey-Kramer’ method in SAS. Dunnett’s test in SAS was used for the relative protein expression level. Data are shown as the mean ± S.E. P values less than 0.05 are reported as statistically significant.
6.3 Results

6.3.1 Body Weights and Food Intake

There was significant genotype effect on weight gain; N2KO mice significantly had greater body weight gain over wild type mice \((P = 0.0001, \text{ Fig. 6.1A})\). CLA supplementation during first 4 weeks resulted in no significant different effects on weight gain overall, although wild type CLA-fed group had the least body weight gain among all treatment groups during the whole experimental period (Fig. 6.1A).

![Figure 6.1](image)

Figure 6.1 Effect of post-weaning administration of conjugated linoleic acid (CLA) on body weight (A) and food intake (B) in wild type and N2KO mice.

Overall N2KO mice consumed more food than wild type animals during the experimental period \((P = 0.0252)\). No significant differences in food intake were observed between controls and CLA treatment during pre-obese period, although N2KO CLA group temporarily consumed more food right after withdrawal of CLA supplementation for 2 weeks (N2KO control: \(50.5 \pm 5.6 \text{ g} \) vs N2KO CLA: \(63.0 \pm 2.3 \text{ g}\), the mean \(\pm \text{S.E.}\)). Although three-way interaction among genotype, CLA and time was observed, there was no significant two-way interaction in food intake (Fig. 6.1B).
6.3.2 Tissue and Organ Weights

Total adipose tissue weight including epididymal, mesenteric, and retroperitoneal adipose tissue and internal organ weights (liver, heart, kidney and spleen) are shown in Table 6.2. Epididymal, mesenteric and total adipose tissue weights were significantly increased in N2KO animals compared to wild-types (all $P$ values were less than 0.05), but no significant effects of genotypes was observed for retroperitoneal fat weights. No significant effects of CLA supplementation for first 4 weeks were observed for adipose tissue weights.

No differences of genotype effects were observed in the liver, heart and spleen weights, while significant genotype effects were observed for the kidney weights ($P = 0.0261$). CLA-fed animals had significantly increased liver, heart and kidney weights compared to control animals. No significant interaction between genotypes and CLA was observed in all tissue and organ weights.

Table 6.2. Effects of post-weaning administration of conjugated linoleic acid (CLA) on organ weights in wild and N2KO mice.

<table>
<thead>
<tr>
<th>% Body Weight</th>
<th>Treatment</th>
<th>Effects ($P$-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild Type</td>
<td>N2KO</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>CLA</td>
</tr>
<tr>
<td>Adipose tissue</td>
<td>Epididymal fat</td>
<td>Retropitoneal</td>
</tr>
<tr>
<td></td>
<td>6.89 ± 0.37$^b$</td>
<td>4.56 ± 1.12$^b$</td>
</tr>
<tr>
<td></td>
<td>1.47 ± 0.18</td>
<td>1.05 ± 0.18</td>
</tr>
<tr>
<td>Mesenteric fat</td>
<td>4.12 ± 0.29</td>
<td>3.82 ± 0.42</td>
</tr>
<tr>
<td>Total</td>
<td>12.5 ± 0.30$^b$</td>
<td>9.43 ± 1.68$^b$</td>
</tr>
<tr>
<td>Organ</td>
<td>Liver</td>
<td>3.47 ± 0.24$^b$</td>
</tr>
<tr>
<td></td>
<td>Heart</td>
<td>0.37 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>1.13 ± 0.08$^a$</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>0.26 ± 0.03</td>
</tr>
</tbody>
</table>

Values represent means ± S.E. (n = 3-5). $^a$-$^b$Means with different superscripts within the same row are significantly different ($P < 0.05$). $P$-values in table are from two-way ANOVA. N2KO, nescient helix-loop-helix knock-out mice; CLA, conjugated linoleic acid; n.s., not significant.
6.3.3 Voluntary Movement Measurement (Non-exercise Physical Activity Test)

To evaluate the effects of post-weaning administration of CLA on physical activity, voluntary movement (non-exercise physical activity) was measured every four weeks. Travel distance as total (19:00-03:00), and two time periods, early (19:00-23:00) and late (23:00-03:00) were shown in Fig. 6.2A-C. The changes of total travel distance over time were shown in Fig. 6.2D.

Figure 6.2 Effect of post-weaning conjugated linoleic acid (CLA) treatment on voluntary movement (non-exercise physical activity) in wild type and N2KO mice.
Consistent with the previous reports, N2KO mice showed significant decreasing tendency of activity compared to wild type animals \((P = 0.0492)\). It was evident that all experimental groups except wild type CLA group showed declining activity over time (Fig. 6.2D), with overall CLA effects significant \((P = 0.0444)\), particularly during the late period \((P = 0.0234)\). No difference in activity between N2KO groups was observed, while CLA fed wild types, but not N2KO animals, maintained activity levels throughout experimental. This suggests that supplementation of CLA during pre-obese state prevented reduction of voluntary movement over time in wild types but not in N2KO animals.

### 6.3.4 Glucose Tolerance Test

To determine the effects of post-weaning CLA treatment on glucose homeostasis, glucose tolerance tests were conducted every four weeks (Fig. 6.3A-D). N2KO mice showed glucose intolerance compared to wild type animals \((P = 0.0061)\). In addition, there was significant overall time effect \((P = 0.0148)\), suggesting these animals develop glucose intolerance with ageing along with high-fat diet as used in this study. No significant CLA effect was observed, however, the CLA treated wild type animals improved glucose tolerance at week 12 compared to wild-type controls, even after CLA withdrawal for 8 weeks (Fig. 6.3E, \(P<0.0001\) for the interaction effect between CLA and time). This suggests that post-weaning CLA administration may improve glucose tolerance in wild-types but not in N2KOs.

### 6.3.5 Serum Parameters

The serum levels of TG, glucose, and total cholesterol were analyzed and shown in Table 6.3. There were no genotype or CLA effect on these markers in this study, except glucose levels for CLA \((P = 0.036)\). No interaction between genotype and CLA were observed in all of these parameters.
Figure 6.3 Effect of time-specific post-weaning conjugated linoleic acid (CLA) treatment on glucose levels (A-D), the area under the curve (AUC) change (E) and overall trend of AUC change (F) in wild type and N2KO mice.

6.3.6 Effects on Muscle Metabolism

To assess whether post-weaning administration of CLA attributes to improved skeletal muscle energy metabolism, we investigated the AMPK signaling pathway, a master sensor to
Table 6.3. Effects of post-weaning administration of conjugated linoleic acid (CLA) on serum parameters in wild and N2KO mice.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild Type</th>
<th>Treatment</th>
<th>N2KO</th>
<th>Effects (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>CLA</td>
<td>Control</td>
<td>CLA</td>
</tr>
<tr>
<td>mg/dL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglyceride</td>
<td>120.3 ± 18.3</td>
<td>99.2 ± 10.6</td>
<td>112.9 ± 2.0</td>
<td>128.4 ± 20.8</td>
</tr>
<tr>
<td>Glucose</td>
<td>189.8 ± 11.2</td>
<td>169.8 ± 15.9</td>
<td>286.8 ± 55.3</td>
<td>168.5 ± 35.3</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>267.4 ± 35.0</td>
<td>328.5 ± 25.4</td>
<td>261.9 ± 18.9</td>
<td>232.1 ± 18.2</td>
</tr>
</tbody>
</table>

Values represent means ± S.E. (n = 3-5). P-values in table are from two-way ANOVA. N2KO, nescient helix-loop-helix knock-out mice; CLA, conjugated linoleic acid; n.s., not significant.

Table 6.3. Serum parameters maintain energy homeostasis (Fig. 6.4). Total AMPKα was measured, as it is known that two subunits (α1 and α2) are regulated differentially and overall AMPKα activity is more important. There were no overall effects of any genotype or CLA effects on AMPKα expression, but interaction between them was observed (P = 0.0471) (Fig. 6.4B). For phosphorylated AMPKα (the active form of AMPKα), N2KO animals had significantly lower levels of p-AMPKα compared to wild type (P = 0.0399). The mice supplemented CLA for the first 4 weeks showed significantly higher expression of phosphorylation of AMPKα over the control groups (Fig. 6.4C, P = 0.0264), particularly wild type CLA group compared to the respective control (Fig. 6.4C). In addition, increased ratio of phosphorylated AMPKα to AMPKα by CLA was observed (Fig. 6.4D, P = 0.0471).

Along with AMPKα, SIRT1 (a NAD-dependent deacetylase) is a key activator of PGC-1α (one major downstream marker for AMPKα). There were no significant effects of genotype and CLA on SIRT1 observed, whereas wild type CLA group showed increased SIRT1 expression level over N2KO control (Fig. 6.4E). The expression of PGC-1α, a master regulator of mitochondrial biogenesis, was not influenced by genotype or CLA either (Fig. 6.4F). However, it was evident that CLA treatment had greater effect on PGC-1α than control diet group in wild type animals.
6.3.7 Effects on Mitochondrial Biogenesis-related Factors

We further determined the effect of post-weaning CLA supplementation on mitochondrial biogenesis in the gastrocnemius muscle. Mitochondria are the important organelle to determine the oxidative capacity and fatigue resistance, resulting in prolonged endurance.
For mitochondrial biogenesis several nuclear encoded precursor proteins are involved in this metabolic process\textsuperscript{10,230,282}, among them we selected PPAR\textsubscript{δ}, NRF-1 and Tfam.

PPAR\textsubscript{δ} is a marker involved in mitochondrial biogenesis as well as muscle type transformation from glycolytic to oxidative fiber.\textsuperscript{192} No significant overall effects of genotypes or CLA were observed on PPAR\textsubscript{δ}, however, wild type CLA-fed animals showed significantly greater expression levels of effect of PPAR\textsubscript{δ} compared to both control diet groups (Fig. 6.5B). No significant difference in NRF-1, a regulator to mediate nuclear-encoding mitochondrial proteins, was observed in all experimental groups (Fig. 6.5C). The expression of Tfam, which is the key regulator for mitochondrial DNA replication and transcription, was significantly down-regulated in N2KO mice over wild type mice ($P = 0.0062$). The CLA supplemented group in wild type animals showed significantly greater Tfam expression over control group in N2KO mice (Fig. 6.5D).

Figure 6.5 Effect of post-weaning treatment of conjugated linoleic acid (CLA) on mitochondrial biogenesis-related factors from the gastrocnemius muscle in wild type and N2KO mice.
To estimate overall mitochondrial relative content we used an indirect method to measure mitochondrial proteins including cytochrome c and COX IV located in the inner membrane of mitochondria. Additionally, we tested another mitochondrial protein, superoxide dismutase 2 (SOD2) in the inner mitochondrial matrix, to assess mitochondrial function. The expression levels of cytochrome c were significantly decreased in N2KO mice compared to wild type animals ($P = 0.0496$) but no CLA effects were observed (Fig. 6.6B). No genotype or CLA effects were observed in COX IV (Fig. 6.6C). Overall SOD2 protein levels were significantly reduced in N2KO mice ($P = 0.0224$), while no effects of CLA were observed (Fig. 6.6D).

Figure 6.6 Effect of post-weaning treatment of conjugated linoleic acid (CLA) on mitochondrial content and function from the gastrocnemius muscle in wild type and N2KO mice.
6.3.8 Effects on Markers of Glucose Metabolism

Along with assessment of post-weaning administration of CLA on mitochondrial biogenesis-related factors, we determined the effects of CLA supplementation during first 4 weeks on markers of glucose metabolism in the gastrocnemius muscle. The expression levels of IRS-1, a member of IRS family regulating insulin signaling, were significantly decreased in N2KO animals over wild types, while no CLA effects were observed (Fig. 6.7B, overall genotype effect, $P = 0.0335$).

A downstream biomarker of IRS (PTEN, attributed to negatively regulate the AKT signaling pathway) significantly reduced phosphorylation (inactive form of PTEN) in N2KO mice compared to wild type animals ($P = 0.0094$). No significant effect of post-weaning administration of CLA was observed on PTEN expression. However, CLA-treated wild type animals significantly upregulated phosphorylated PTEN compared to the respective control, while no differences were observed in N2KO animals (Fig. 6.7C).

No difference in protein expression of phosphorylated PDK, a downstream marker of PTEN, was observed for both genotype and CLA (Fig. 6.7C). There were no significant effects of genotypes and CLA in total AKT expressions and phosphorylated AKT (Serine 473) and GLUT4, one of the insulin-regulated glucose transporters (Fig. 6.7D-G). However, we observed marginally increased responses from wild type animals fed CLA on phosphorylated AKT (Threonine 308) compared to the respective control ($P = 0.0547$ and $P = 0.0528$, respectively).

6.4 Discussion

It was previously reported that supplementation of CLA prevents weight gain and body fat accumulation during both the growth period and in aged N2KO mice.$^{57,205,283}$ Particularly, CLA increased voluntary activity when supplemented during growth period.$^{205,283}$ Results from the current study suggest that supplementation of CLA in pre-obese state may reduce weight gain in normal animals but not in the genetically induced inactive adult-onset animal model, although
Figure 6.7 Effect of post-weaning treatment of conjugated linoleic acid (CLA) on glucose metabolism from the gastrocnemius muscle in wild type and N2KO mice.

the stimulation of voluntary activity and improved glucose tolerance remain even after CLA withdrawal in this model.
Previously CLA was reported to be linked with reduced food intake in a number of animal models, although it was not consistent.\textsuperscript{28-30,71} Moreover, it was determined that the reduction of food intake by CLA was independent of its effects on body fat reduction.\textsuperscript{28,30} Others suggested that CLA reduces food intake by decreasing neuropeptides Y (NPY) and agouti-related protein (AgRP), both of which are known to be related to increased appetite\textsuperscript{284} and inducing the storage of glycogen in muscle and liver, which may subsequently serve as satiety signals.\textsuperscript{285,286} In the current study, we did not observe any significant differences in food intake during CLA supplementation periods in both genotypes. However, when CLA was withdrawn, apparent compensation of food intake occurred in N2KO animals fed CLA, but not in wild type animals, coincided with greater weight gain during this period (Week 4-6 in Fig. 6.1A). It is not clear how CLA influenced food intake in these animals, however, these results suggest that CLA may influence food intake differently in obese animals, which has been previously reported in other animal models.\textsuperscript{28,30,32,71}

We observed that N2KO mice had significantly reduced physical activity consistent with previous reports for N2KO animals.\textsuperscript{205,247,248} Overall, post-weaning CLA supplementation significantly prevented reduction of voluntary activity levels compared to controls, particularly in wild type animals. Intriguingly, our results showed that the effects of CLA on activity during the late phase (23:00-03:00) might remain even after termination of CLA treatment up to 8 weeks, whereas during the early phase (19:00-23:00) there was no significant CLA effect in all experimental groups.

Among various molecular mechanisms to affect increased or maintained physical activity levels, AMPK signaling pathway has drawn significant attention since the 1990s.\textsuperscript{183} Upregulated AMPK signaling pathway is involved in skeletal muscle energy metabolism as well as mitochondrial biogenesis, resulting in enhanced endurance capacity and attenuated exercise intolerance.\textsuperscript{3,5,287} Previously, our group demonstrated the effect of CLA on stimulation of mitochondrial biogenesis through AMPK signaling pathway.\textsuperscript{250} In the same context, our data
supported post-weaning administration of CLA might stimulate activation of AMPKα and related factors in wild type but not in N2KO animals.

Wild type animals fed CLA for first 4 weeks showed improved glucose tolerance over time even after no benefits on weight were observed compared to respective control, suggesting CLA may have lasting effects on glucose metabolism later in life. Based on this observation, we assessed effects of CLA treatment during growth period on glucose metabolism through insulin signaling pathway. Among several measured markers involved in insulin signaling cascades, phosphorylated PTEN and phosphorylated AKT at threonine 308 partially provided evidence regarding how to moderate glucose tolerance in the current study. PTEN and AKT are important effectors of insulin responses mediating glucose uptake and glycogen synthesis in skeletal muscle. Our data are consistent with early studies that CLA specific isomers mediate AKT sensitization.

Current the results suggest potential benefits of post-weaning CLA administration to reduce health risk, as suggested previously. This is important since one potential health concern associated with CLA supplementation is glucose intolerance. We observed a non-significant increase in glucose intolerance following CLA supplementation in wild type mice (Fig. 6.3B, week 4), however, when CLA was withdrawn, glucose tolerance improved in the treated groups (week 12 in Fig. 6.3D). As suggested before, this may be due in part to significant shift of energy metabolism by CLA, such as increased fatty acid β-oxidation associated with reduced glucose utilization. However, further studies such as HOMA-IR to better understand glucose and insulin metabolism are needed to determine how post-weaning administration of CLA changes energy sources.

As seen in previous studies, we observed a significant increase in liver weight, as fatty liver, after CLA supplementation. It is unlikely that CLA is directly accumulated in the liver as levels of CLA in the liver and muscle reached maximum 1 week for liver and 2 weeks for muscle after supplementation in mice. Previously it was reported that CLA fed animals
accumulate TG in the liver.\textsuperscript{293,294} It is believed to be associated with CLA’s dramatic effects on lipid metabolism, particularly in the liver and adipose tissues, increased hepatic lipogenesis and decreased fat accumulation in adipose tissues.\textsuperscript{13,15,270,271} It is unlikely that enlarged liver caused by CLA is due to increased glycogen content as it was previously shown that CLA had no effects on enzyme activities involved in gluconeogenesis.\textsuperscript{292} In addition, previous studies reported no pathologically significant observations after 18 months CLA supplementation or no changes in hepatic gene expressions in lipogenesis from N2KO animals.\textsuperscript{57,205,295} Although we provided CLA during pre-obese state only and then withdrew CLA, the significant effect of CLA on liver weight still remains. This is consistent with the previous study that withdrawal of CLA did not completely reverse liver enlargement at the end of a 4 week recovery period.\textsuperscript{269} Our study might not have enough adaptive time to complete this reversal of CLA’s effect. Thus, additional investigation of relationship between short-term CLA supplementation and changes in hepatic genes should be considered to determine if the liver enlargement caused by CLA is completely reversible or not. Clinical trials with CLA reported no changes or increased markers for liver function, and all were within the normal ranges. In addition, it was reported that a high-dose of CLA (14.6 g \textit{cis}-9,\textit{trans}-11 and 4.7g \textit{trans}-10,\textit{cis}-12 CLA per day) for 3 weeks had no changes on markers of liver function in healthy subjects. Thus, it is possible that rodents are more sensitive to CLA’s effect compared than humans.

In conclusion, CLA supplementation during pre-obese state may attenuate reduction of voluntary activity and glucose intolerance after CLA withdrawal in this mouse model. However, it is not sufficient \textit{per se} to maintain health benefits like prevention of weight gain and fat accumulation until later life in genetically obese mice. Wild type animals fed CLA may activate AMPK\textsubscript{\alpha} and PPAR\textsubscript{\delta} as well as promote desensitization of PTEN and sensitization of AKT at threonine 308 in gastrocnemius skeletal muscle, improving voluntary activity and glucose homeostasis. We suggest that post-weaning administration of CLA may partially stimulate the underlying molecular targets involved in muscle energy metabolism in normal animals but not in the
genetically induced inactive adult-onset animal model. The present study would provide a scientific foundation for future trials of targeted application of post-weaning CLA intervention in modulating long-term obesity. If further research on genetically predisposed obese mice discovered an appropriate intervention or combination with an addition regime like exercise, it may be possible to more effectively be used to prevent childhood obesity.
CHAPTER 7
CONJUGATED LINOLEIC ACID (CLA) PROMOTES ENDURANCE CAPACITY VIA PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR (PPAR) δ-MEDIATED METABOLISM IN MICE

7.1 Introduction

There are two distinct types of exercise training: strength and endurance. Strength exercises such as weightlifting and bodybuilding promote protein synthesis and then induce hypertrophy of muscle cells. On the other hand, endurance exercises including jogging, swimming and cycling stimulate mitochondrial biogenesis and then contribute to decreased incidences of obesity through enhanced energy expenditure and switching the energy source from glucose to fat. Also, chronic endurance training triggers a remodeling of the skeletal muscle fiber phenotype from type II (glycolytic fast-twitch fibers) to type I (oxidative slow-twitch fibers). In general, type I fibers in skeletal muscle have a greater density of mitochondria than type II fibers. Thus, oxidative type I fibers promotes fatty acid β-oxidation to spend the stored fat in the body. As a consequence, it induces body fat reduction, leading to the prevention of obesity.

It is known that mitochondrial biogenesis and a shift of fiber type composition are major metabolic events that influence endurance exercise training, and vice versa. The primary biomarkers implicated in mitochondrial biogenesis and fiber type transformation in skeletal muscle are peroxisome proliferator-activated receptor δ (PPARδ) and PPARγ coactivator 1α (PGC-1α). Although it is well established that PGC-1α acts as a master regulator in mitochondrial biogenesis, mice that ectopically overexpressed PGC-1α showed a remarkable conversion of muscle fiber type II to type I. Likewise, muscle specific overexpression of PPARδ in mice simultaneously increased the number of copies of mitochondrial DNA and up-regulated
the expression levels of mitochondrial biogenesis-related genes, inducing a shift of skeletal muscle fibers from type II to type I.

During the last two decades conjugated linoleic acid (CLA) has gained significant attention as a bioactive compound to prevent obesity. Although most of CLA’s anti-obesity effects have been focused on body fat reduction, accumulated evidence demonstrated that CLA increased lean body mass and enhanced physical performances. In fact, the previous studies in rodents reported that CLA improved voluntary activity and endurance capacity through the modulation of molecular targets such as carnitine palmitoyltransferase 1 (CPT1), uncoupling protein 2 (UCP2) and peroxisome proliferator-activated receptor δ (PPARδ) in skeletal muscle. Recently, it was revealed that the specific CLA isomers, cis-9,trans-11 and trans-10,cis-12, induced the up-regulation of key molecular markers involved in mitochondrial biogenesis. This evidence provides the opportunity for future studies to investigate the effects of CLA on skeletal muscle metabolism. Thus, we investigated the regulating mechanisms of CLA in skeletal muscle to elicit the effects of CLA on endurance capacity in mice, and to clarify the interaction of CLA and exercise based on PPARδ-mediated metabolism.

7.2 Materials and methods

7.2.1 Materials

CLA was provided by Natural Lipids Ltd. AS (Hovdebygda, Norway). The purity of CLA was 80.7% (37.8% cis-9,trans-11, 37.6% trans-10,cis-12 and 5.3% other CLA isomers and the remainder of the preparation was 13.7% oleic acid, 3.2% stearic acid, 0.4% palmitic acid, 0.2% linoleic acid and 1.8% was unknown). Semi-purified powdered diets (TD07518, 95% basal mix) were obtained from Harlan Laboratories (Madison, WI). Serum triacylglyceride (TG), glucose, and total cholesterol assay kits were purchased from Genzyme Diagnostics (Charlottetown, PE, Canada). Serum non-esterified fatty acids (NEFA) and L-Lactate were obtained from BioAssay Systems (Hayward, CA). The blood urine nitrogen (BUN) assay kit was
purchased from BioQuant (San Diego, CA). DC protein assay kits for analysis of protein concentration were from Bio-Rad (Hercules, CA). Rabbit antibodies for phosphorylated AMP-activated protein kinase α (p-AMPKα), AMPKα, phosphorylated phosphatase and superoxide dismutase 2 (SOD2) were obtained from Cell Signaling (Berberly, MA). Rabbit antibodies for PGC-1α, sirtuin 1 (SIRT1), PPARδ, nuclear respiratory factor 1 (NRF-1), mitochondrial transcription factor A (Tfam), cytochrome c (Cyt C) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-rabbit IgG antibody was from Boston Bioproducts Inc. (Ashland, MA). Other chemicals used were purchased from either Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburg, PA).

7.2.2 Animals and diet

All work related to animals was done in compliance with the Institutional Animal Care and Use Committee at the University of Massachusetts, Amherst. Twenty 10-week-old male 129Sv/J mice were obtained from breeding colonies. All mice were placed in individual wire-bottom cages with 12h light and dark cycles in a temperature and humidity-controlled room. All mice underwent a 1-week acclimatization period. During that period, a control powder diet with 40% calories from fat was provided to all mice. They were subjected to baseline testing for endurance capacity and voluntary activity. All mice were divided into four different groups according to diet and exercise regimes, either as control or 0.5% CLA with high-fat (40% fat calorie) diets, as well as sedentary or exercise groups. The dose of CLA used for the current study was determined on the basis of previous reports in rats and humans. In rats, 0.5% CLA supplementation for 2–4 weeks caused a range between 23–120 µM in serum and 0.8–3.2 g per day supplemented with CLA for 2 months in humans led to serum levels from 23 to 200 µM. The formulation of diets was shown in Table 7.1. The duration of this study was four weeks. Body weight and food intake were recorded once a week. After a four-week intervention, the
mice fasted for 4 h, exercised for 15 min, run the same way as the training protocol, and then sacrificed by CO₂ asphyxiation. For analysis of serum markers, blood was collected using the cardiac puncture method. Three different parts of adipose tissue (epididymal, retroperitoneal and mesenteric), two designated parts of skeletal muscle (gastrocnemius and quadriceps) and other organs (liver, heart, kidney and spleen) were harvested and weighed.

Table 7.1. Composition of experimental diet for control and conjugated linoleic acid (CLA) groups.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein, ‘vitamin-free’ tested</td>
<td>169.1</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>2.2</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>288.5</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>132</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>195</td>
</tr>
<tr>
<td>CLA or Soybean oil</td>
<td>5</td>
</tr>
<tr>
<td>Mineral mix, AIN-93M-MX (TD 94049)</td>
<td>42.8</td>
</tr>
<tr>
<td>Vitamin mix, AIN-93-VX (TD 94047)</td>
<td>12.4</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>3</td>
</tr>
<tr>
<td>tert-Butylhydroquinone</td>
<td>0.04</td>
</tr>
<tr>
<td>Total</td>
<td>1,000</td>
</tr>
</tbody>
</table>

7.2.3 Endurance capacity test

All mice were subjected to running training on the treadmill (Columbus Instrument, Columbus, OH) during the acclimatization period (week 0). The protocol of adaptation placed all mice on the treadmill to run for 15 min with a speed of 10 m/min on a 0° incline under the control of an electric shock grid. This training for adaptation was repeated three times and then all mice were measured on a baseline of endurance capacity, for a total running time (min) and distance (m) at the end of the adaptation period.
During the whole experimental period, mice in the exercise groups trained with running exercises five times a week on the motorized treadmill. The detailed endurance training protocol performed running training for a total of 15 min (10 m/min for the first 10 min, then an increase of 1 m/min every minute for the following 5 min, with a final speed of 15 m/min for training) on a 10° inclination with an electric shock grid (0.97 mA) to encourage continuous running.

Endurance capacity was evaluated biweekly. The testing protocol used the same initial conditions as the training protocol (10 m/min on 10° inclination), however, the speed was increased by 1 m/min every minute until reaching 25 m/min, and the speed of 25 m/min was maintained until they were fatigued or exhausted. Exhausted means mice stay on the electric shock grid for five continuous seconds or for three times over two seconds. The total running time and distance determined that these variables were representative markers to reflect endurance capacity.

7.2.4 Voluntary movement measurement (Non-exercise physical activity test)

Voluntary movement was recorded using LoliTrack Quatro Video Tracking Software Version 1.0 (Loligo Systems, Tjele, Denmark) with a high-resolution infrared camera during the dark cycle (5:00pm-5:00am) every other week. Animals were individually placed into cages with free access to diet and water (provided as HydroGel®, Clear H2O, Portland, ME). The cage (30 × 46 × 40 cm) used for activity monitoring was larger than the typical cage to avoid limited voluntary movements. Among the monitored movement data for 12 h (5:00pm-5:00am), and 8 h (7:00pm-3:00am) were analyzed with the exception of the first 2 h (5:00pm-7:00pm) and 2 h (3:00am-5:00am) due to early adaption and a lack of movement, respectively.

7.2.5 Serum parameters

The levels of glucose, total cholesterol, TG, NEFA, BUN and L-Lactate in serum were separated by centrifugation at 3,000 g for 20 min at 4°C and were subsequently measured using
7.2.6 Western blot analysis

Each sample from gastrocnemius muscle tissue was homogenized in radioimmune precipitation assay (RIPA) (50 mM Tris-HCl, 10 mM NaCl, 5 mM MgCl₂, and 0.5% NP-40, pH 8.0) buffer (Boston Bioproducts Inc., Ashland, MA), protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO) and a phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL) and was lysed on ice for 2 h with gentle shaking, then centrifuged at 14,000g for 20 min at 4°C. The protein concentration in tissue lysate was normalized at 50 µg/ml of protein. The normalized samples were mixed with loading buffer, and then separated by a 10-15% (w/v) sodium dodecyl sulfate-polyacrylamide gel and were transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA). After blocking, proteins were probed with primary rabbit antibodies, and were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody for 1 h at room temperature. For detection, the Enhanced Chemiluminescence solution (Bio-Rad, Hercules, CA) was used and developed under the Image Station 4000MM (Carestream Health, New Haven, CT) setting. GAPDH was used as a loading control for normalization. The results were quantified using Image J software (U.S. National Institutes of Health).

7.2.7 mRNA analysis

Total RNA was isolated from the gastrocnemius muscle tissue (50 mg) in TRIzol reagent (Invitrogen, Calsbad, CA), and was then reversely transcribed to cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Forster, CA). cDNA with predesigned TaqMan gene expression assay kits was used for the relative quantitation. The target genes were lipoprotein lipase (LPL, Mm00434764_m1), fatty acid binding protein 4 (Fabp4, Mm00445878_m1), PPARα (Mm00440903_m1), CPT1β (Mm00487200_m1) and UCP2 (Mm00627597_m1) for lipid metabolism, and phosphoinositide 3-kinase (PI3K)
(Mm00803160_m1), pyruvate dehydrogenase kinase, isoform 4 (PDK4, Mm01166879_m1), protein kinase B1 (AKT1, Mm01331626_m1) and glucose transporter type 4 (GLUT4, Mm01245502_m1) for glucose metabolism. For analysis of muscle fiber type transformation we used four target genes, myosin heavy chain 1 (MHC1, Mm01332493_m1), MHC2 (Mm01332564_m1), MHC3 (Mm01332463_m1) and MHC7 (Mm01332518_m1). GAPDH (Mm99999915_g1) and 18S ribosomal RNA (18S rRNA, Mm03928990_g1) were used for internal standards. All reactions were triplicated with the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster, CA). The program of reaction was 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Data were calculated using $2^{-\Delta\Delta Ct}$.

7.2.8 Statistical analyses

Data were analyzed by two-way analysis of variance (ANOVA) followed by PROC MIXED with Least Square (LS) Means options of the SAS software (Version 9.3, SAS Institute Inc., Cary, NC, USA). For data on body weight, food intake, endurance capacity and voluntary movement, two-way repeated measure ANOVA and the slice option in the LS means statement were used to examine the group differences at each time point with PROC MIXED. For internal organs, adipose and skeletal muscle tissue weight, serum parameters, the relative protein and mRNA expression level data, two-way ANOVA with PROC MIXED was used. The Tukey-Kramer’s method in SAS was used for the multiple comparisons among the experimental groups. Data were shown as the mean ± S.E. $P$ values less than 0.05 are reported as statistically significant.

7.3 Results

7.3.1 Body weights and food intake

CLA fed mice gained less body weight than control diet groups from week 2, and maintained this trend throughout the experiment (overall CLA effect, $P < 0.0001$). Along with
CLA effects, exercise effects on body weight gain in trained mice were significant (overall exercise effect, \( P = 0.0218 \)). No interaction was observed for diets and exercise in body weight gain (Fig. 7.1A). At week 2, mice supplemented with CLA decreased food intake compared to control diet groups. However, there was no significant difference in food intake at the end of the experiment between any of the groups. Overall CLA and time effects influenced food intake (\( P = 0.0187 \) and \( P < 0.0001 \), respectively) and there was a significant interaction between the two (\( P = 0.0007 \)). Exercise effect was not observed in food intake (Fig. 7.1B).

![Figure 7.1 Effects of conjugated linoleic acid (CLA) on body weight (A) and food intake (B) in sedentary and exercise-trained mice.](image)

<table>
<thead>
<tr>
<th>Effect</th>
<th>Exercise (E)</th>
<th>CLA (D)</th>
<th>Time (T)</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-value</td>
<td>0.0218</td>
<td>&lt; 0.0001</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Effect</th>
<th>Exercise (E)</th>
<th>CLA (D)</th>
<th>Time (T)</th>
<th>( D \times T )</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-value</td>
<td>n.s.</td>
<td>0.0187</td>
<td>&lt; 0.0001</td>
<td>0.0007</td>
</tr>
</tbody>
</table>

### 7.3.2 Tissue weights

Total and individual adipose tissue (epididymal, retroperitoneal, and mesenteric adipose tissue), skeletal muscle (gastrocnemius and quadriceps), liver, heart and spleen weights are shown in Table 7.2. Overall CLA-fed mice showed significantly less adipose tissue accumulation than control diet groups, while no exercise effect was observed in adipose tissue weights. The tissue weights in gastrocnemius muscle were significantly increased by CLA supplementation (\( P = 0.0035 \)). Particularly, CLA-fed mice in the exercise trained group had the most gastrocnemius muscle mass. No differences of exercise effect were observed in the liver, heart and spleen weights, whereas CLA supplementation significantly influenced the liver weight.
Table 7.2. Effects of conjugated linoleic acid (CLA) on organ weights in sedentary and exercise-trained mice.

<table>
<thead>
<tr>
<th>% Body Weight</th>
<th>Sedentary Treatment</th>
<th>Exercise Treatment</th>
<th>Effects (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>CLA</td>
<td>Control</td>
</tr>
<tr>
<td>Adipose tissue</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epididymal fat</td>
<td>4.75±0.92</td>
<td>2.70±0.34</td>
<td>5.39±0.85</td>
</tr>
<tr>
<td>Retropertoneal fat</td>
<td>1.14±0.35a</td>
<td>0.35±0.04b</td>
<td>1.12±0.14a</td>
</tr>
<tr>
<td>Mesenteric fat</td>
<td>3.35±0.61</td>
<td>2.54±0.20</td>
<td>3.86±0.25</td>
</tr>
<tr>
<td>Total</td>
<td>9.25±1.78ab</td>
<td>5.59±0.60ab</td>
<td>10.4±1.03a</td>
</tr>
<tr>
<td>Skeletal muscle tissue</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>0.96±0.06a</td>
<td>1.13±0.04ab</td>
<td>0.99±0.08ab</td>
</tr>
<tr>
<td>Quadriceps</td>
<td>6.53±0.16</td>
<td>6.68±0.43</td>
<td>6.27±0.31</td>
</tr>
<tr>
<td>Organ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>3.45±0.10ab</td>
<td>5.41±0.14a</td>
<td>3.57±0.11b</td>
</tr>
<tr>
<td>Heart</td>
<td>0.51±0.12</td>
<td>0.59±0.02</td>
<td>0.49±0.07</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.27±0.05</td>
<td>0.38±0.03</td>
<td>0.28±0.06</td>
</tr>
</tbody>
</table>

Values represent means ± S.E. (n = 5). a-bMeans with different superscripts within the same row are significantly different (P < 0.05). P-values in table are from two-way ANOVA. CLA, conjugated linoleic acid; n.s., not significant.

7.3.3 Endurance capacity and voluntary movement (non-exercise physical activity)

To evaluate endurance capacity, we determined the maximum running time and distance on the motorized treadmill. Animals in the CLA groups significantly improved the maximum running time and distance over mice in the control diet groups (P = 0.0166 and P = 0.0174, respectively). Exercise effect failed to reach significant values in endurance capacity. Time effect influenced running time and distance. On the other hand, no interaction among time, diets and exercise were observed. The CLA supplementation and exercise-combined group significantly prolonged the maximum running time (2.4-fold) and distance (3.2-fold) in comparison to the sedentary control diet group at week 4. Furthermore, CLA-fed mice in the sedentary group also increased their running time (1.6-fold) and distance (1.9-fold) compared to the sedentary control diet group at the same time point (Fig. 7.2A and 7.2B). All experimental groups except the sedentary control group steadily increased the maximum running distance over time (Fig. 7.2C).
Effects of CLA on non-exercised physical activity were determined by measuring voluntary movement three times including a baseline test during the entire experimental period (Fig. 7.2D). Overall CLA and exercise effects did not show differences in voluntary movement, although CLA-fed animals showed an increase in voluntary movement compared to control diet-fed mice within the sedentary groups at week 4 (paired test, $P = 0.0157$). On the other hand, in the exercise-trained groups, CLA supplementation led to no significant changes in voluntary movement in comparison to control diet feeding.

![Figure 7.2](image)

Figure 7.2 Effects of conjugated linoleic acid (CLA) on maximum running time (A), distance (B) and trend (C), and voluntary movement (D) in sedentary and exercise-trained mice.

7.3.4 Serum parameters

The concentrations of serum metabolic parameters are shown in Table 7.3. The CLA supplemented animals had significantly lower levels of serum TG than mice in the control diet.
groups (P = 0.0431). No difference of overall CLA and exercise effects were observed for glucose, total cholesterol, NEFA, BUN and L-Lactate in mice.

Table 7.3. Effects of conjugated linoleic acid (CLA) on serum parameters in sedentary and exercise-trained mice.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild Type</th>
<th>N2KO</th>
<th>Effects (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>CLA</td>
<td>Genotype</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>110.3 ± 18.3</td>
<td>99.2 ± 10.6</td>
<td>n.s.</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>189.8 ± 11.2</td>
<td>169.8 ± 15.9</td>
<td>n.s.</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>267.4 ± 35.0</td>
<td>328.5 ± 25.4</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Values represent means ± S.E. (n = 5). P-values in table are from two-way ANOVA. CLA, conjugated linoleic acid; n.s., not significant; NEFA, non-esterified fatty acid; BUN, blood urea nitrogen.

7.3.5 Effects on biomarkers in skeletal muscle energy metabolism

To determine the effects of CLA and/or exercise on skeletal muscle metabolism, we investigated the AMPK-related molecular biomarkers in the regulatory pathway for PPARδ on the basis of the previous study. There were no significant overall effects of CLA or exercise on total AMPKα in all treatments (Fig. 7.3B), while phosphorylated AMPKα (the active form of AMPKα) was significantly up-regulated in the exercise trained mice (P = 0.0342 for overall exercise effect, Fig. 7.3C). As a consequence, the ratio of phosphorylated AMPKα to AMPKα was significantly increased in the exercise trained animals (P = 0.0197 for overall exercise effect, Fig. 7.3D). SIRT1, a NAD-dependent deacetylase cross-talking to AMPK, was significantly influenced by CLA supplementation (P = 0.0009 for overall CLA effect, Fig. 7.3E). However, there was no significant effect of exercise in SIRT1 expression. Similarly, the expression of PGC-1α showed significant differences in the CLA-fed groups (P = 0.0127 for overall CLA effect), but not in the exercise trained mice (Fig. 7.3F).
7.3.6 Effects on biomarkers in mitochondrial biogenesis signaling cascades

On the basis of the evidence that CLA up-regulated SIRT1 and PGC-1α in the CLA-fed mice in Fig. 7.3, we further studied the effects of CLA and/or exercise on mitochondrial biogenesis signaling cascades in the gastrocnemius muscle. It is known that endurance exercise training stimulates mitochondrial biogenesis, resulting in improved oxidative capacity and reduced muscle fatigue. In fact, PGC-1α is a master regulator in mitochondrial biogenesis, which orchestrates several nuclear encoded proteins. Among them, we primarily investigated three major molecular biomarkers, PPARδ, NRF-1 and Tfam. In addition, the activities of mitochondrial proteins, cytochrome c and SOD2, were evaluated for changes of mitochondrial function by nutritional and physical treatments. Overall CLA effects showed significantly increased expression levels of PPARδ compared to control-fed animals (P = 0.0051 for overall CLA effect, Fig. 7.4B), while no exercise effect was observed for PPARδ. The CLA-fed mice significantly increased NRF-1 activity, a marker that regulates nuclear-encoding mitochondrial...
proteins, in comparison to control-fed animals (P = 0.0016 for overall CLA effect). The expression levels of Tfam, a major regulator for mitochondrial DNA replication and transcription, in the CLA supplemented groups were greater than the controls (P = 0.0195 for overall CLA effect). There were no exercise effects in both activities of NRF-1 and Tfam (Fig. 7.4C and 7.4D).

We further determined expression levels of mitochondrial proteins, cytochrome c which is located in the inner membrane of mitochondria, and SOD2 which protects mitochondria from reactive oxygen species. No significant overall effects of CLA or exercise were observed on cytochrome c and SOD2; however, the exercise trained mice showed marginally increased expression levels of both proteins, although the results were not significant (Fig. 7.4E and 7.4F).

![Figure 7.4](image.png)

**Figure 7.4.** Effects of conjugated linoleic acid (CLA) on mitochondrial biogenesis-related molecular markers from the gastrocnemius muscle in sedentary and exercise-trained mice.

### 7.3.7 Effects on muscle fiber type composition

The effects of CLA and/or exercise on changes in the composition of the four different genes in myosin heavy chain (MYH) were evaluated in the gastrocnemius muscle (Fig. 7.5).
These myosin heavy chain genes include MYH7 (slow-twitch fiber type I), MYH3 (embryonic fiber), MYH2 (slow/fast-twitch fiber type IIA) and MYH1 (fast-twitch fiber type IIX/d). The significant differences in MYH7 (P < 0.0001), MYH3 (P = 0.0019) and MYH1 (P = 0.0041) were observed in the CLA-fed animals. On the other hand, the exercise trained mice showed significantly increased gene expression levels of MYH2 (P = 0.0205 for overall exercise effect). Specifically, the CLA-fed mice in the exercise trained group expressed the most mRNA levels of MYH7, MYH3 and MYH2 among all experimental groups.

Figure 7.5. Effects of conjugated linoleic acid (CLA) on skeletal muscle fiber type transformation. Samples were obtained from the gastrocnemius muscle in sedentary and exercise-trained mice.

7.3.8 Effects on lipid and glucose metabolism in skeletal muscle

To determine the effects of CLA and/or exercise on lipid metabolism in skeletal muscle, the selected genes measured the mRNA levels in the gastrocnemius muscle (Fig. 7.6A). The CLA supplementation significantly increased the mRNA expression of LPL (the master regulator of fatty acid uptake, P = 0.00154), PPARα (the key regulator of lipid metabolism, P = 0.00154) and CPT 1β (the key enzyme of fatty acid β-oxidation, P = 0.039), while no effects of CLA were
observed on Fabp4 and UCP2 in muscle. Overall exercise effect was not shown in lipid metabolism.

Figure 7.6. Effects of conjugated linoleic acid (CLA) on mRNA expressions of selected genes representative to lipid metabolism (A) and glucose and insulin metabolism (B) in the gastrocnemius muscle in sedentary and exercise-trained mice.

The relative mRNA expression levels of PI3K, PDK4, AKT1 and GLUT4, markers for glucose and insulin metabolism, were determined in the gastrocnemius muscle (Fig. 7.6B). Among these targets, mRNA levels of GLUT4 (the insulin-regulated glucose transporter) were significantly up-regulated by CLA supplementation (P = 0.0447), but not by exercise training.

7.4 Discussion

Previously, numerous studies with CLA investigated the mechanisms to reduce body weight and body fat mass by modulating metabolism in adipose tissues \(^{15,16}\). However, the effects of CLA on skeletal muscle metabolism have garnered relatively less attention, even though a number of early studies demonstrated that CLA increased lean body mass, including muscle mass and improved physical activities \(^{120}\). In the current study, we have assessed the effects of CLA on
endurance capacity and the metabolic alteration of intracellular molecular markers in skeletal muscle. Of particular importance, up- and down-stream markers of PPARδ regulated mitochondrial biogenesis and muscle fiber type composition. Those markers were primarily investigated as the metabolic events that influence endurance capacity. Results from the present study indicated that CLA supplementation, but not exercise training, stimulated mitochondrial biogenesis and induced genes of myosin heavy chains composed of muscle fiber in mice, resulting in enhancement of lipid metabolism. Thus, CLA supplementation might be a more effective way to control skeletal muscle metabolism rather than exercise training alone.

In this study, the CLA-fed mice had an enhanced endurance capacity compared to the control diet groups. Furthermore, 0.5% CLA supplementation combined with endurance training showed a significant additional effect on the maximum running time and distance (Fig. 7.2). These results are consistent with the previous studies that demonstrate increased maximum swimming or running capacity in mice by CLA treatment \(^{18,149,202-204}\). Indeed, three early studies reported that 0.5% CLA supplemented mice increased voluntary movement (non-exercise physical activity) \(^{17,205,300}\). Although our results did not observe the overall CLA effect in voluntary movement, the CLA-fed mice significantly traveled greater distances compared to control-fed animals within the sedentary group. On the other hand, no CLA effect was observed in the trained mice. This result is supported by a study where the trans-10,cis-12 CLA isomer improved endurance capacity, while there were no changes in voluntary movement in the trained mice \(^{18}\). Ohnuki et al. \(^{148}\) also demonstrated no effect of a single oral administration of CLA on locomotive activity. Thus, further studies with CLA are needed to discover the correlation between voluntary movement and extensive endurance training.

It is known that endurance training activates various signaling cascades in skeletal muscle energy metabolism. In particular, AMPK plays a central role in the adaptive response to exercise, since AMPK senses lowered ATP levels due to increased energy demands by exercise, which in turn leads to the activation of oxidative metabolism for energy homeostasis in the body.
Our results demonstrated that the exercise trained groups had remarkably upregulated AMPK phosphorylation levels in comparison to the sedentary animals, although other markers in the down-stream signaling pathway of AMPK showed no alteration of their activities by overall exercise effect. It might occur due to a bout of exercise with the same intensity and duration as the training protocol before sacrifice. In fact, it has been reported that acute exercise enhanced AMPK activities in skeletal muscle. 

Adaptation in skeletal muscle includes the modulation of several transcriptional factors in response to mitochondrial metabolism to promote lipid consumption as an energy source. Specifically, PGC-1α acts as a key regulator in these transcriptional alterations, which regulates mitochondrial and fatty acid metabolism. In these metabolic events, SIRT1 contributes to the deacetylation of PGC-1α, and in turn controls its activity. In the same context, our data showed that CLA supplementation activated SIRT1 expression in skeletal muscle, and up-regulated PGC-1α (Fig. 7.3E and 7.3F). However, further studies are required to investigate how SIRT1 is activated by CLA.

Mitochondrial biogenesis is a complex process that increases volume and changes mitochondrial composition in response to physical and nutritional stimuli. Activated PGC-1α triggers the modulation of nuclear and mitochondrial regulators such as PPARδ, NRF-1 and -2, and Tfam, which are intimately linked to the stimulation of mitochondrial biogenesis. In the current study, CLA supplementation sequentially facilitated PGC-1α, PPARδ and PGC-1α-NRF-1-Tfam signaling cascades. Therefore, it suggests that CLA may potentiate mitochondrial biogenesis. Although this study demonstrated the alteration in the biochemical markers of mitochondrial biogenesis, it did not include a histological analysis, such as a visualization of fluorescent mitochondria using dyes and microscopy, as well as the number of mitochondrial DNA copies. Thus, further studies are needed in order to better understand the underlying morphological changes of how CLA regulates mitochondrial biogenesis.
Along with PGC-1α, one of the key regulators in mitochondrial biogenesis and muscle fiber type transformation is PPARδ. It is evident that PPARδ in skeletal muscle increases mitochondria-rich oxidative type I fiber. In regards to skeletal muscle fibers, they are briefly categorized into slow-twitch and fast-twitch, depending on their contractile properties. The fiber in humans consists of myosin heavy chains (MHCs), which are segmented into MHC I, MHC IIa and MHC IIX/d. These MHC isoforms have different characteristics: MHC I is an oxidative slow-twitch type I fiber with a high density of mitochondria, which uses fatty acids as the primary energy source; MHC IIX/d is a glycolytic fast-twitch type II fiber, using glucose and phosphocreatine primarily to generate energy; MHC IIa is likely between type I and IIX/d. As shown in the previous study, PPARδ could affect the regulation of muscle fiber type, contributing to prolonged running endurance. In the present study, CLA-fed mice led to an increase of PPARδ expression and the induction of overall MHC genes. Furthermore, CLA supplementation significantly increased the gastrocnemius muscle mass (Table 7.2). Thus, it suggests that CLA supplementation could affect the overall physiology of skeletal muscle based on the PPARδ-driven metabolic events.

In regards to lipid metabolism, free fatty acids in plasma pass through a plasma membrane in skeletal muscle by diffusion, or by transportation using receptor proteins such as cluster of differentiation 36 (CD36), fatty acid binding protein (Fabp) and fatty acid transporter protein 1 (FATP1), which in turn forms fatty acyl-CoA complexes. Subsequently, they enter mitochondria by shuttling through CPT1 and then undergo oxidation. Our data demonstrated that CLA significantly increased three key genes associated with lipid metabolism in skeletal muscle, especially LPL, PPARα and CPT1β, which is consistent with the previous studies in mice. Particularly, two studies among these elucidated to the effects of CLA supplementation combined with endurance training on the alteration of molecular markers in skeletal muscle lipid metabolism. Intriguingly, a number of transcriptional factors to control
lipid metabolism are regulated by PPARδ in skeletal muscle, resulting in increased fatty acid β-oxidation.

Furthermore, PPARδ targets genes in glucose and insulin metabolism. Increased PPARδ induces phosphorylation of PDK, leading to the negative regulation of pyruvate dehydrogenase complex (PDC). As a consequence, carbohydrate oxidation is reduced during fasting. However, results from the current study did not observe the overall effects of CLA or exercise in PDK4 expression, although PPARδ protein levels were increased by CLA supplementation. Parra et al. reported that CLA supplementation did not increase mRNA levels of PDK4, as well as other genes associated with glucose and insulin metabolism. Increased GLUT4 expression in this study is consistent with the previous observations. Therefore, our data suggest that CLA supplementation may not inhibit glucose and insulin metabolism, but may promote lipid metabolism.

Overall, CLA supplementation led to enhanced endurance capacity and positively altered molecular markers in skeletal muscle metabolism, whereas exercise effects were limited in this study. It might be derived from the heterogeneous factors in terms of the exercise protocols such as intensity and duration. Although overall endurance exercise is known to induce mitochondrial biogenesis and muscle fiber type transformation via the modulation of intracellular biomarkers, it is not completely understood how the magnitude of exercise intensity and duration influences these physiological and phenotypic alterations in skeletal muscle. In fact, Tadashi et al. reported that PGC-1α expression levels were significantly correlated with the intensity of running endurance in mice. In our study, we used a mixture of low-intensity and low-volume as an exercise protocol. Thus, it could be suggested that increased endurance capacity under low levels of exercise intensity and duration relies on CLA supplementation rather than exercise.

The potential health concerns related to CLA supplementation to date are glucose intolerance and fatty liver. In the present study, we could not find any evidence in terms of glucose intolerance associated with CLA supplementation. In fact, no significant increases in
serum glucose levels were observed. However, more studies like glucose tolerance tests are needed to confirm the influence of CLA in glucose intolerance. As reported in previous studies, we observed an enlarged liver after CLA supplementation. It can be suggested that CLA dramatically affects lipid metabolism, including reduced adipose tissue and increased hepatic lipogenesis, resulting in the accumulation of TG in the liver. In the same context, the current results showed decreased adipose tissue weight and serum TG levels in the CLA supplemented mice (Table 7.2 and 7.3). Moreover, previous studies in rodents demonstrated no pathological significance in the hepatic markers.

In conclusion, we demonstrated that CLA supplementation, yet not exercise alone, promotes endurance capacity and increases skeletal muscle mass in this mouse model. It implies that CLA supplementation is involved in skeletal muscle metabolism by stimulating mitochondrial biogenesis and inducing muscle fiber gene expression, resulting in increased lipid metabolism. Particularly, enhanced PPARδ-mediated signaling pathways regulate these metabolic events. Although the precise mechanism of whether CLA regulates PPARδ directly or indirectly was not dealt with this study, it is suggested that CLA exerts its action via the activation of PGC-1α through SIRT1, subsequently leading to the up-regulation of PPARδ and its related target markers in skeletal muscle. If further research on CLA supplementation is combined with an appropriate exercise regime, with intensity and duration, it may provide a more effective method to prevent obesity by the modulation of skeletal muscle metabolism.
CHAPTER 8
CONCLUDING REMARKS

To date, calorie restriction and exercise are the most effective strategies to prevent and/or reduce incidences of obesity. Nonetheless, there is an increasing prevalence of obesity due to physical inactivity and sedentary lifestyle. Thus, various studies have been trying to find more efficient prevention and treatment for obesity by understating the determinants contributing to skeletal muscle and physical activity.

It is known that CLA reduces body fat and increases lean mass, resulting in the alteration of body composition. While it is well established that CLA attenuates body fat accumulation through the regulation of metabolism in adipocytes, the mechanisms of CLA on skeletal muscle metabolism are not yet fully known. The objective of this proposed research was to discover the underlying mechanism of how CLA modulates metabolism in skeletal muscle based on the AMPK signaling pathway, and subsequent downstream signaling cascades.

In C2C12 murine skeletal muscle cells, both active CLA isomers (cis-9,trans-11 and trans-10,cis-12) significantly activated PGC-1α, a master regulator of mitochondrial biogenesis, via AMPKα phosphorylation in an isomer-specific manner. Subsequently, CLA isomers enhanced PPARδ, NRF-1 and Tfam, which was needed to regulate mitochondrial biogenesis. Consequently, CLA isomers increased the number of copies of mitochondrial DNA, mirrored by enhanced mitochondrial biogenesis. It was suggested that CLA might act as an activator of mitochondrial biogenesis in skeletal muscle.

In the genetically induced inactivity adult-onset obesity model (Nhlh2 gene knockout mice), CLA supplementation prevented body weight gain and fat accretion by improved physical activity. Specifically, CLA enhanced voluntary movement and increased skeletal muscle mass. It is implied that CLA induced the physiological changes in skeletal muscle, supporting the
activation of AMPKα, PGC-1α and downstream biomarkers related to mitochondrial biogenesis, such as PPARδ and Tfam in CLA-fed mice. Thus, it might indicate that CLA treatment during the developmental period in mice had a preventive effect on obesity caused by physical inactivity.

Next, we demonstrated that post-weaning CLA supplementation contributed to the reduction of voluntary activity and glucose intolerance using the CLA withdrawal model in the high-fat fed Nlhh2 mice. Although the genetically obese mice were not sufficient per se to maintain health benefits by CLA supplementation during the pre-obese state, normal mice showed significantly decreased body weight gain and fat mass along with increased physical activity throughout the experiment. In particular, CLA-fed mice after withdrawal had improved glucose homeostasis. These alterations in normal mice might be derived from the upregulation of AMPKα and PPARδ, as well as the desensitization of PTEN and the sensitization of AKT at threonine 308 in skeletal muscle. Thus, it could be suggested that the post-weaning administration of CLA during the pre-obese state might partially stimulate the underlying molecular targets involved in muscle metabolism in normal mice, yet not in the genetically induced inactive adulthood-onset animal model.

Lastly, we elucidated to the effects of CLA supplementation and/or endurance exercise on skeletal muscle metabolism. Results from this study demonstrated that CLA, but not low-levels of exercise alone, promoted the maximum running time and distance along with increased skeletal muscle mass. In addition, CLA supplementation stimulated mitochondrial biogenesis-related factors through the activation of SIRT1 and PGC-1α in skeletal muscle. Of particular importance, CLA increased the expression levels of PPARδ which is a key regulator of muscle fiber type transformation. Consequently, CLA induced these molecular events such as mitochondrial biogenesis and upregulated genes in muscle fibers, resulting in increased lipid metabolism in skeletal muscle. It implied that CLA exerted its actions via the PPARδ-mediated signaling pathway.

Collectively, CLA potentially acts as an exercise-mimetic, resulting in improved
physical activity, which can support its function of regulating body fat.
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