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Article

Investigating the impact of short-chain fatty acids on myofiber dynamics and insulin sensitivity

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SUMMARY

Sarcopenia is a condition characterized by age-associated decline in skeletal muscle health. This condition, especially given the increasing prevalence of metabolic dysfunction with aging and recent recognition of the pivotal role of metabolism in sarcopenia, has become an urgent health concern. The rising incidence of obesity and metabolic disorders further underscores the need to explore metabolites as potential therapeutic targets. Short-Chain fatty acids (SCFAs), a group of metabolites abundant in the human body, are promising modulators of muscle physiology. However, their individual roles in muscle fiber dynamics and insulin sensitivity require further exploration. We hypothesized that all SCFAs would exert similar effects on muscle fiber formation and insulin sensitivity in a concentration-dependent manner. To test our hypothesis, we investigated the impacts of SCFAs on myofiber differentiation and degradation using C2C12 mouse myoblasts as a model. We found that SCFAs, particularly acetate and butyrate, exerted concentration-dependent effects in promoting muscle fiber formation. In contrast to our hypothesis, propionate promoted myofiber degradation and inhibited myofibroblast differentiation. The inhibition of myofibroblast differentiation by propionate correlated with increased lipid accumulation in muscle fibers and decreased insulin sensitivity. The insights derived from this study contribute to a deeper understanding of nutrient-mediated muscle health. Moreover, they offer potential avenues for targeted interventions aimed at alleviating age-related muscle dysfunction and metabolic challenges.

INTRODUCTION

Aging, recognized as a pervasive and gradual process, impacts a staggering 900 million individuals globally (1). Estimates from a 2015 United Nations report suggested a remarkable surge in the population of those aged sixty and above, soaring to over 2.1 billion individuals. This projection is ascribed to escalating birth rates and the advancements in both living standards and healthcare systems (2). The complexities of aging and its association with various diseases have recently undergone extensive examination. Within the spectrum of age-associated maladies, metabolic dysfunction emerges as a pivotal concern, notably in muscle tissue (3).

Muscle tissue is the largest tissue in human body---it is a big pool for energy storage and its health can affect the progression of many diseases. Muscle tissue undergoes constant dynamic changes, encompassing myotube formation and muscle tissue degradation. The process of muscle cell differentiation initiates with the transformation of mesodermal precursor cells into single-cell myoblasts, which subsequently fuse to generate myocytes (4). Further fusion of myocytes leads to the development of multinucleate myotubes. This intricate process can be compromised or decelerated with aging, contributing to muscle dysfunction (5). This is seen in conditions like sarcopenia a persistent and wide-ranging disorder that primarily impacts skeletal muscles marked by reductions in skeletal muscle mass and functional capacity, ultimately resulting in diminished physical ability (6, 7). In the United States, 36.5% of adults, with an average age of 70.1 years, are afflicted by sarcopenia (8). The prevalence of sarcopenia exhibits significant variability, ranging from 2.5% to 28% in men and 2.3% to 11.7% in women within different communities (9). Additionally, in a large cohort community study conducted in Taiwan, the prevalence of sarcopenia reached as high as 13.6% in men aged 75 years and above (10). Consequently, the urgency to address sarcopenia is clear.

Short-chain fatty acids (SCFA), including acetate, propionate, and butyrate, constitute a subset of fatty acids synthesized through the fermentation of indigestible polysaccharides by the gut microbiota (11). This group of compounds is critically involved in maintaining homeostasis, fostering micronutrient synthesis, orchestrating immune system responses, and balancing energy metabolism (12). Moreover, the far-reaching impact of SCFAs is emphasized by their capacity to mitigate the phenomenon of “inflamm-aging” in aged mice (13). SCFAs not only increase skeletal muscle glucose content but also stimulate fatty acid oxidation, this promotes a metabolic shift that culminates in heightened skeletal muscle function and exercise capacity in rodent subjects treated with SCFAs (14). Despite these findings, there remains a noticeable gap in the understanding of the effects exerted by individual SCFA species on muscle tissue.

Given that age-related muscle decline is fundamentally linked with metabolic dysfunction, it is logical to approach the study of sarcopenia from a nutritional and metabolic perspective. In this study, we hypothesized that all SCFAs exert similar effects on muscle fiber formation and insulin sensitivity and that the impact may be concentration-dependent. We found that SCFAs, especially acetate and butyrate, enhanced muscle fiber formation in a concentration-dependent manner. In contrast, propionate promoted myofiber degradation and inhibited myofibroblast differentiation, which correlated with

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increased lipid accumulation and reduced insulin sensitivity. Ultimately, we aimed to unravel the functions of each SCFA within muscle fibers. Our results hold the potential to offer tangible guidance, shedding light on the optimal strategies for dietary approaches that can positively influence the trajectory of aging-related conditions.

RESULTS

Effect of SCFS on the differentiation of C2C12 cells

We first sought to determine how different SCFAs affect myoblast differentiation. This is crucial because this process is impaired with aging and sarcopenia, resulting in reduced skeletal muscle mass and physical ability (4, 7). Therefore, increasing the differentiation of myoblasts holds promise for mitigating aging-induced muscle atrophy.

We treated C2C12 myoblast cells with various SCFAs to induce differentiation and assessed differentiation extent of the cells based on the density and thickness of myotubes, with myosin serving as a marker protein indicative of myotube formation (15). We found that acetate and butyrate significantly increased the formation of myotubes relative to controls (PBS-only), and propionate treatment inhibited the formation of myotubes (**Figure 1a-c**). Acetate promotes myotube formation at a low concentration (100 μM), while butyrate demonstrates this effect at a higher concentration (200 μM). Propionate treatment shows no significant difference in myosin expression relative to controls (**Figure 1b,c**). These findings suggest that acetate and butyrate, at specific concentrations, can enhance muscle genesis.

Effect of SCFAs on the lipid accumulation of C2C12 cells

Intermuscular adipose tissue (IMAT) describes adipocytes located within muscle bundles (16). As people age, adipose tissue is potentially to harmful ectopic sites like IMAT (17). This redistribution is of adipose tissue leading to lipid accumulation is also correlated with muscle formation and insulin sensitivity loss (17). To measure this factor, we quantified lipid content following SCFA treatment. We found that propionate treatment increases lipid accumulation in C2C12 cells during the induction of differentiation (**Figure 2**). This observation may offer a partial explanation for the diminished formation of myotubes with propionate treatment. There was no significant difference observed after butyrate or acetate treatment (**Figure 2**).

Effect of SCFAs on the insulin sensitivity of myoblasts

SCFAs have been recognized for their potential in enhancing the metabolic health of adipose tissue, muscle, and the liver, ultimately leading to improved insulin sensitivity within these vital tissues (18). As part of our investigation, we quantified insulin sensitivity in response to SCFAs treatment, focusing on the phosphorylation of AKT in response to insulin stimulation. To specifically measure insulin responsiveness rather than baseline p-AKT levels, we subjected cells to a 12-hour serum-free culture period followed by a 100nM insulin pulse for 30 minutes. When examining the impact of acetate at high concentrations, a slight increase in p-AKT levels was observed, demonstrating a marginal elevation compared to both the control group and the propionate-treated groups (**Figure 3**). Conversely, cells subjected to butyrate treatment exhibit notably higher p-AKT level relative to all other groups, indicating a robust elevation in insulin sensitivity in response to butyrate. Intriguingly, no noticeable difference is discernible between the propionate-treated group and the control group in terms of p-AKT expression, suggesting that, within the scope of this specific analysis, propionate does

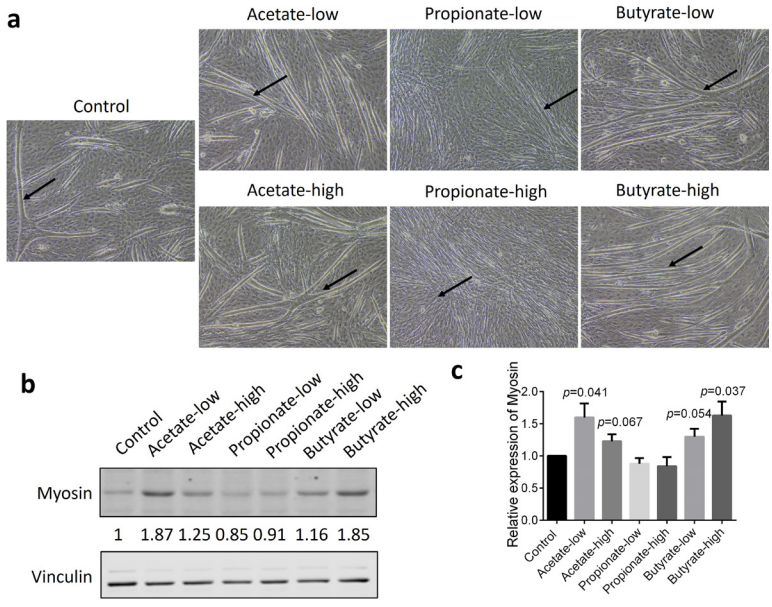

Figure 1: Impact of SCFAs on myotube formation. C2C12 cells were treated with high or low concentrations: 100 μM (low) and 500 μM (high) for acetate, and 50 μM (low) and 200 μM (high) for both propionate and butyrate, of SCFAs during differentiation into myotubes. The cells were treated for 5 days. **a)** Representative images depicting C2C12 cells post differentiation induction and SCFA treatment. Arrows indicate myofiber. **b)** Western blots showing myosin protein levels in C2C12 cells after treatment, with representative blots. Numbers below the myosin band indicate the quantification of band intensity normalized to control (PBS-only). Vinculin expression is also shown as a reference protein. **c)** Quantification of myosin protein levels, represented as mean ± SEM relative to control (n=3). Student's t-test was used to analyze significance compared to the control group.

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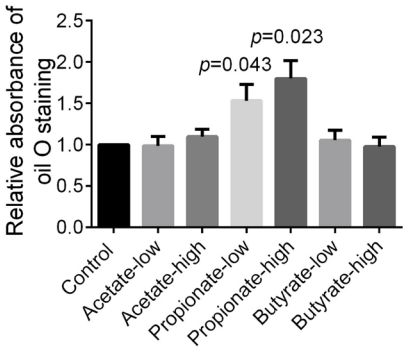

Figure 2: Influence of SCFAs on lipid accumulation in C2C12 cells. C2C12 cells were induced to differentiate and treated with acetate, butyrate, and propionate, with concentrations: 100 μM (low) and 500 μM (high) for acetate, and 50 μM (low) and 200 μM (high) for both propionate and butyrate for 5 days. The lipid content in muscle cells was quantified using Oil Red O staining. Absorbance is presented as mean ± SEM relative to control (PBS-only) (n=4). Student's t-test was performed to assess significance of treatment groups compared to the control group.

not significantly influence insulin sensitivity when assessed through the phosphorylation of AKT (**Figure 3**). Research proposes that lipotoxicity, stemming from the accumulation of lipids compounds in obesity, may result in insulin resistance (19). The observed outcomes in insulin sensitivity are also align with our observed impacts on myotube formation.

Effects of SCFA treatment on myofiber degradation

Muscle mass is regulated by the balance between muscle fiber formation and degradation (20). Therefore, measuring muscle fiber degradation is critical in determining overall muscle mass. We studied the influence of SCFAs on muscle degradation by treating myotubes with SCFAs for five days. Following this treatment period, cells treated with acetate or butyrate, at both low and high concentrations (100 μM and 500 μM for acetate, and 50 μM and 200 μM for butyrate), showed no significant difference in terms of muscle fiber thickness and density (**Figure 4a**). In the propionate group, cells subjected to both concentrations of propionate (50 μM and 200 μM) displayed a notable decrease in muscle fiber thickness and density **(Figure 4a**).

To quantitatively assess muscle fiber degradation, we examined myostatin expression, a marker protein for myotube degradation. Cells treated with both acetate and butyrate expressed comparable levels of myostatin relative to the control group (PBS-only) (**Figure 4b-c**). However, the propionate-high group (200 μM) exhibited a noticeable elevation in myostatin expression, surpassing all other groups (**Figure 4b-c**).

Next, we asked how propionate induces myotube degradation. Myotube degradation involves numerous molecular mechanisms, with NF-κB identified as a key transcriptional regulator for inflammatory cytokines and implicated in various aging-related diseases (21). NF-κB has been found to regulate the expression of myostatin (22). To ascertain whether propionate affects the activity of NF-κB to influence myostatin expression, we measured the phosphorylation of p65, a key subunit of the NF-κB complex,

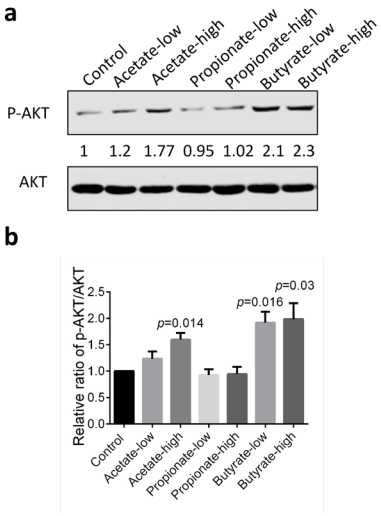

Figure 3: Impact of SCFAs on insulin sensitivity. C2C12 cells were induced to differentiate and treated with acetate, butyrate, and propionate, with concentrations: 100 μM (low) and 500 μM (high) for acetate, and 50 μM (low) and 200 μM (high) for both propionate and butyrate or 5 days. To specifically measure insulin responsiveness rather than baseline p-AKT levels, cells were subjected to a 12-hour serum-free culture period followed by a 100nM insulin pulse for 30 minutes. Phosphorylation of AKT, serving as an indicator of insulin receptor activation, was measured to assess the effect of SCFAs on insulin sensitivity. **a)** Representative blots illustrating the levels of phosphorylated AKT and total AKT protein. **b)** Relative ratio of p-AKT to AKT. Data shown as mean ± SEM (n=3). Student's t-test was performed to assess significance compared to the control group (PBS – only).

where phosphorylation indicates the activation of NF-κB (23). Both low and high concentrations of propionate increased the phosphorylation of p65, suggesting that propionate might promote the expression of myostatin through the activation of NF-κB signaling (**Figure 4d-e)**.

In summary, the findings suggest that propionate tends to promote muscle fiber degradation, while acetate and butyrate appear to support muscle fiber formation. Moreover, these effects seem to be concentration-dependent, highlighting the variable impact of specific types of SCFAs on skeletal muscle health.

DISCUSSION

Sarcopenia, a prevalent and pervasive condition, significantly impacts the functionality, mass, and physical performance of skeletal muscles (24). Extensive research efforts have sought to devise interventions aimed at enhancing muscle function (25). Given the crucial metabolic role of muscles, their performance is influenced by dietary factors, nutrients, and overall metabolic dynamics (26). In this study, we investigated the effects of individual SCFA treatment on fiber formation, degradation, and insulin sensitivity using C2C12 myoblasts. Consistently, propionate treatment inhibited muscle fiber formation and promoted degradation, potentially having adverse effects on muscle mass maintenance. Acetate and butyrate, on the other hand, promoted myofibroblast differentiation and muscle fiber formation. These findings indicate varying potentials for applying SCFAs to maintain muscle mass.

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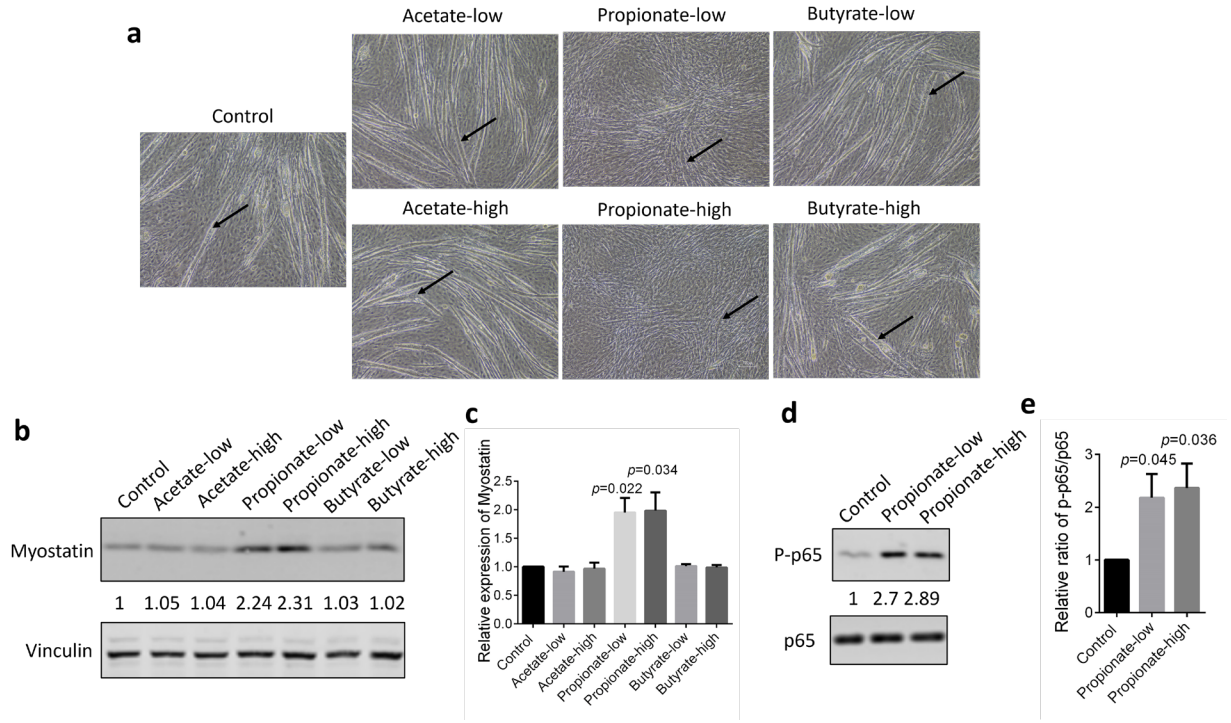

Figure 4: Influence of SCFAs on muscle fiber degradation. C2C12 cells were induced to differentiate into myotubes and then treated with acetate, butyrate, and propionate, with concentrations: 100 μM (low) and 500 μM (high) for acetate, and 50 μM (low) and 200 μM (high) for both propionate and butyrate for 5 days. **a)** Representative images of C2C12 cells after differentiation and treatment with SCFAs. Arrows indicate myofiber. **b)** Myostatin protein levels were measured via western blot to assess myofiber degradation at the molecular level. Numbers below the myostatin band indicate the quantification of band intensity normalized to control (PBS-only). Vinculin expression is also shown as a reference protein. Representative blots of myostatin are shown. **c)** Quantification of myosin protein levels is presented as mean ± SEM relative to control (n=3). Student's t-test was used to assess significance compared to the control group. **d)** Representative blots of phosphorylated p65 and total p65 protein levels after propionate treatment, indicating NF-κB activity. **e)** Quantification of the ratio of p-p65/p65 is presented as mean ± SEM relative to control (n=3). Student's t-test was used to assess significance compared to the control group.

In our daily lives, SCFA consumption may be a possible method of enhancing muscle health. Acetate can be found in dairy, grain-based foods, and vinegar (4-6% acetic acid) (27). Oral vinegar intake has been shown to increase serum acetate levels in healthy patients by 230μmol/L after 15 minutes of ingestion (28). Vinegar has also shown beneficial effects on glucose and lipid metabolism (29). Butyrate is often found in high fiber foods, like legumes and whole grains. A diet high in fiber is also shown to decrease risks of colorectal cancer (30). Propionate is commonly found in the form of sodium propionate as an additive and preservative against mold, yeast, and bacteria. Therefore, it may be found in preserved meat, fruits, and flour-based foods, such as bread (31). In relation to the results of this study, including a higher percentage of acetate and butyrate-containing foods and limiting consumption of propionate-containing processed foods may allow for a diet more beneficial to muscle health in old age. These insights deepen our understanding of the intricate dynamics of SCFAs on muscle fibers, providing valuable guidance for further exploration and potential therapeutic strategies.

This study encountered several limitations that warrant consideration. First, the use of 2% horse serum during the treatment phase introduces the possibility of adding metabolites that could lead to slightly higher actual concentrations than originally calculated. However, it is crucial to note that all experimental groups were exposed to the same serum conditions, suggesting that the observed outcomes are likely not significantly influenced by serum presence. Furthermore, while three replicates were used for imaging purposes and representative images were presented, we did not conduct quantitative analyses of fiber size and quantity. In future studies, incorporating immunofluorescent staining targeting myosin could enhance the accuracy of fiber quantification. Moreover, this study was conducted at a single time point, which may limit the comprehensive understanding of the results. Further investigations including multiple time points that reflect stages of muscle formation and degradation with and without SCFA treatment would provide more comprehensive insights into the temporal dynamics and potential variations in the observed effects. Finally, the use of SCFA concentrations higher than physiological conditions may lead to more pronounced effects. However, these concentrations do not directly reflect in vivo conditions. Future studies using physiologically relevant SCFA levels would be necessary to confirm the application of these findings.

These findings from our study provide a valuable glimpse into the dynamic interplay between SCFAs and insulin sensitivity, highlighting the differential effects of acetate and butyrate, while underscoring the minimal impact of propionate in this specific context. Further exploration of these outcomes could pave the way for a deeper understanding of SCFA-mediated metabolic improvements and their implications for overall health.

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MATERIALS AND METHODS

Cell culture and treatment

C2C12 mouse myoblast cells were purchased from the American Type Culture Collection (ATCC) and cultured in DMEM (High-glucose, 10-013-CV, Corning), supplemented with 10% FBS (Sigma-Aldrich) and 1% penicillin-streptomycin (Gibco). Cell cultures were maintained at a constant temperature of 37°C with a 5% CO_2 environment, and the medium was refreshed daily. To induce myoblast differentiation, media with 2% horse serum replaced the initial media with 10% FBS. To determine the effect of SCFAs on the differentiation of myofibroblasts, the C2C12 cells were seeded in 6-well plates, and the metabolite treatments commenced on the third day following the differentiation induction.

To measure the effects of SCFAs on muscle fiber degradation, the cells were induced to fully differentiate for 7 days and then maintained in DMEM medium supplemented with 10% FBS. To determine the effects on myofiber degradation, metabolites were added 7 days after induction of differentiation.

SCFAs were dissolved in phosphate buffered saline (PBS), and PBS without SCFAs was used as the control treatment. The tested concentrations for SCFAs were: 100 µM and 500 µM for acetate, and 50 µM and 200 µM for both propionate and butyrate. These concentrations were based on the physiological SCFA levels observed in human blood: 100 to 150 µM for acetate, 4 to 5 µM for propionate, and 1 to 3 µM for butyrate (32). The chosen treatment concentrations were deliberately set higher than the physiological levels to detect significant outcomes. The concentration of propionyl-carnitine for cell treatment is 100 and 200 µM.

Cell imaging

After a five-day treatment period with SCFAs, the cells were imaged using light microscopy, allowing for the clear visualization of muscle fibers. A total of three distinct views were chosen from each well, and the representative image for each view was showcased. Each experimental group featured three replicate wells, and the entire experiment was replicated three times.

Protein extraction and quantification

Protein samples were extracted using a RIPA buffer supplemented with protease inhibitors (250 mM PMSF, 5 mg/ml pepstatin A, 10 mg/ml leupeptin, and 5 mg/ml aprotinin). After adding the RIPA buffer, the cells were left on ice for 15 minutes. The protein content of the extracted samples was determined utilizing the DC Protein Assay kit II (BioRad), following which 30 µg of total protein was mixed with 4×Laemmli sample buffer and subsequently incubated at a temperature of 95 °C for a period of 5 minutes to denature protein for western blotting.

Western blotting

The protein samples were separated via SDS-PAGE under reducing conditions. Subsequently, the separated proteins were electrophoretically transferred onto a nitrocellulose membrane (GE Healthcare). This membrane was blocked using TBS-based Odyssey Blocking buffer (LI-COR). To target proteins of interest, specific antibodies were employed, including myosin (Sigma-Aldrich, M4276, 1:2000), myostatin (Proteintech, 19142, 1:2000), vinculin (Sigma-Aldrich, V9264, 1:5000), phosphor-AKT (S308) (Cell Signaling Technology, 13038, 1:2000), pan AKT (Cell signaling technology, 4691, 1:2000), phosphor-p65 (Ser536) (Cell signaling technology, 3033, 1:2000), p65 (Cell signaling technology, 3242, 1:2000). Primary antibodies were incubated overnight at 4 °C, after which the membrane underwent triple washing with TBST buffer. Following this, the membrane was incubated with the appropriate donkey anti-rabbit Alexa Fluor 488 (A-21206—Thermo Fisher Scientific, 1:10,000) or donkey anti-mouse Alexa Fluor 555 (A31570—Thermo Fisher Scientific, 1:10,000) immunoglobulin for a duration of 1 hour at room temperature. The ensuing signals were developed using the LI-COR Odyssey CLx Imaging System.

Quantification of lipid content

To quantify total lipid content in myofibers, Oil Red O staining was employed. Briefly, C2C12 cells were induced to differentiate into myotubes, and SCFA treatment commenced on the second day post-induction, continuing for 5 days. Oil Red O staining was initiated by removal of the medium, followed by washing the cells twice with PBS. Subsequently, 4% formalin was added, and cells were incubated for 30 minutes. After formalin removal, cells were washed with dH_2O twice. A 60% isopropanol solution was used to incubate the cells for 5 minutes. Following removal of isopropanol, Oil Red O solution (Sigma) was applied to the cells for 15 minutes. Post-washing with dH_2O, pure isopropanol was added to extract Oil Red O from the cells, and absorbance was measured at 518 nm by Perkin Elmer EnVision 2103 Microplate Reader to quantify the amount of Oil Red O in the cells.

Measurement of insulin sensitivity

C2C12 cells underwent differentiation induction, and the treatment with SCFAs commenced on the second day, lasting for 5 days. The cells were subjected to a 12-hour serum-free culture period. Subsequently, 100nM insulin was introduced to activate the insulin receptor and downstream signaling. Thirty minutes post-insulin treatment, the cells were lysed, and total protein was collected. Phosphorylation of AKT was utilized as an indicator of insulin receptor activity.

Statistical analyses

Statistical analyses for individual experiments were conducted on distinct samples, and all measurements were obtained independently. Data analyses were carried out using Microsoft Excel 2013 and GraphPad Prism 7. Unless explicitly stated otherwise, results are presented as mean ± SEM. Significance was determined using the two-tailed Student's t-test, and corresponding p-values indicating significant differences between groups are provided in the results. It is important to note that multiple t-test were performed without correction for multiple comparisons. Western blot experiments were independently replicated (n = 3), and representative images are included.

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