MS.ID-IJPBS-3005
International Journal of Pharmacy and Biological Sciences-IJPBS™ (2023) 13 (2): 223-230
Online ISSN: 2230-7605, Print ISSN: 2321-3272
Research Article | Pharmaceutical Sciences | OA Journal | MCI Approved | Index Copernicus

Differential Effects of 2 to16 Carbon Saturated Fatty Acids on Autophagy and Cell Viability of HepG2 Cells

J. Kamishikiryo* and A. Shima
Department of Biochemistry, Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, 985-1, Sanzo, Higashimura-Cho, Fukuyama, Hiroshima 729-0292, Japan.

Received: 10 Jan 2023 / Accepted: 8 March 2023/ Published online: 01 April 2023
***Corresponding Author Email:** j-kamishi@fukuyama-u.ac.jp
Phone: +81-84-936-2111; Fax: +81-84-936-2024

Abstract
Dietary fat increases levels of saturated fatty acids (SFAs) in plasma, which exacerbate metabolic syndrome and non-alcoholic fatty liver disease (NAFLD). Palmitic acid, a long-chain SFA (LCFA), inhibits autophagy and causes apoptosis of hepatocytes. However, the effects of other SFAs on hepatocytes remain unknown. In this study, we explored how SFAs whose chain lengths range from 2- to 16-carbon atoms affect autophagy and cell viability of hepatocytes in vitro. Our results showed that only 14- and 16-carbon LCFAs suppress autophagy and reduce cell viability; 2- to 12-carbon SFAs, which are found in foods and have garnered attention for their health benefits, did not affect autophagy and did not reduce cell viability. Thus, replacing LCFAs with short-chain fatty acids and medium-chain fatty acids in foods may reduce the incidence of NAFLD.

Keywords
Autophagy, Cell viability, Long-chain fatty acid, Middle-chain fatty acid, Non-alcoholic fatty liver disease, Short-chain fatty acid

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1. INTRODUCTION
Elevated plasma levels of free fatty acids (FFAs) trigger and exacerbate metabolic syndrome. FFA-induced lipotoxicity in hepatocytes contributes to the pathogenesis of non-alcoholic fatty liver disease (NAFLD), the most common liver disease whose incidence increases as the obese population grows worldwide [1, 2]. NAFLD can range from simple steatosis to non-alcoholic steatohepatitis (NASH). If the disease does not improve, NASH can progress to cirrhosis and liver cancer.

Macroautophagy (hereafter referred as autophagy) is an intracellular degradation system that delivers cytoplasmic components to lysosomes. Autophagy maintains cell and tissue homeostasis by degrading unwanted or damaged components. The disturbance of autophagy is implicated in the pathogenesis of NAFLD [3], namely in hepatocytes [4], which promotes not only steatosis but also NASH.

To date, more than 30 autophagy-related (Atg) genes have been identified that regulate autophagy or autophagy-related processes [5, 6]. Rubicon is an autophagy-related protein that suppresses autophagy, especially during the autophagosome-lysosome fusion process [7, 8]. Unlike most autophagy-related proteins, Rubicon suppresses autophagy and has therefore garnered attention as a potential therapeutic target.

Palmitic acid is a 16-carbon fatty acid that belongs to long-chain fatty acids (LCFAs) and promotes insulin resistance and NAFLD [2, 9- 11]. Palmitic acid inhibits autophagy and induces cell apoptosis in hepatocytes. In 2016, Tanaka et al. showed that palmitic acid upregulates Rubicon protein levels by suppressing

proteasomal degradation and therefore stabilizing the protein [12].

Short- and medium-chain fatty acids (SCFAs and MCFAs) regulate cell metabolism in plants and animals. In humans, SCFAs are primarily generated by intestinal bacteria and are metabolized by enterocytes and the liver [13]. MCFAs are found in coconut milk and animal milk, which are often consumed as dietary supplements [14]. Although both SCFAs and MCFAs have garnered recent attention, their effects on autophagy and cell viability remain unknown.

To complement studies on palmitic acid, we examined the effects of SCFAs, MCFAs, and LCFAs on autophagy and cell viability of HepG2 cell line.

2. MATERIALS AND METHODS

2-1. materials

Sodium acetate (2:0), 30 w/v% albumin solution (fatty acid-free), chloroquine diphosphate, and Immunostar Zeta chemiluminescence reagents were purchased from FUJIFILM Wako Chemicals (Osaka, Japan). Sodium butyrate (4:0), sodium hexanoate (6:0), sodium n-octanoate (8:0), sodium decanoate (10:0), sodium laurate (12:0), sodium myristate (14:0), sodium palmitate (16:0), and BODIPY 493/503 were purchased from Tokyo Chemical Industry (Tokyo, Japan). ProLong Diamond Antifade Mountant and complete protease inhibitor cocktail were obtained from ThermoFisher Scientific (Waltham, MA, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. Cell Counting Kit-8 was purchased from Dojindo (Kumamoto, Japan). Anti-LC3 (Cat. No. PM036), anti-p62 (SQSTM1) (Cat. No. PM045), anti-Beclin 1 (Cat. No. PD017), and anti-b-Actin (Cat. No. M177-3) antibodies were purchased from MBL (Nagoya, Japan). Anti-Rubicon (Cat. No. D9F7), anti-SAPK/JNK (Cat. No. 9252), anti-Phospho-SAPK/JNK (Thr183/Tyr185) (Cat. No. 4668), anti-mTOR (Cat. No. 2983), anti-p70 S6 Kinase (Cat. No. 2708), anti-Phospho-p70 S6 Kinase (Cat. No. 9234) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). HRP-conjugated goat anti-rabbit IgG (H + L) polyclonal antibody (Cat. No. 611-1302) was purchased from Rockland Immunochemicals (Gilbertsville, PA, USA). HRP-conjugated goat anti-mouse IgG (H + L) polyclonal antibody (Cat. No. A90-116P) was purchased from Bethyl (Montgomery, TX, USA). The human hepatoma cell line, HepG2 (RCB1648), was provided by the RIKEN Bio Resource Research Center through the National Bioresource Project of the Japan Agency for Medical Resource and Development.

2-2. fatty acid-BSA conjugation

Fatty acid-BSA conjugates were prepared as described previously [9, 12]. In brief, each fatty acid sodium salt was dissolved in 150 mM NaCl and then mixed with prewarmed 24% BSA solution to yield a stock concentration of 7.5 mM fatty acid/12% BSA. The solubilized fatty acid stock solutions were filtered through a 0.22 µm filter and stored at −20℃ until use.

2-3. cell culture

HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 units/ml penicillin, 100 µg/ml streptomycin, and non-essential amino acids at 37 ℃ in 5% CO_2.

2-4. cell viability assay

Cell viability was measured using a Cell Counting Kit-8 (CCK-8) according to the manufacturer's instructions. In brief, HepG2 cells were seeded at 6 x 10^3 cells per well in 96-well plates. The next day, the cells were treated with fatty acids in the 10% FBS/DMEM for 16 hr. After the treatment, CCK-8 solution was added to the medium before further incubation for 3 h at 37℃ in 5% CO_2. After incubation, absorbance was read at 450 nm.

2-5. western blotting

HepG2 cells were seeded at 3 x 10^5 cells per well in 12-well plates. The next day, the cells were treated with fatty acids in 10% FBS/DMEM for 16 h. After the treatment, cells were washed with cold PBS and lysed with lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% Glycerol, 5% 2-mercaptoethanol, 0.02% Bromophenol blue, 1 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 2 mM Na_3VO_4, 2 mM NaF, 1 mM sodium molybdate, complete protease inhibitor cocktail) and transferred to microcentrifuge tubes. Cell lysates were then sonicated and boiled for 5 min. The lysates were subjected to SDS-PAGE, and the proteins were transferred to a polyvinylidene membrane. The membrane was blocked in TBST (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) containing 1% skim milk. The membrane was probed with primary antibody overnight at 4°C followed by incubation with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Signals were visualized with Immunostar Zeta chemiluminescence reagents. Signal intensities were analyzed with a LuminoGraph II imaging analyzer (ATTO, Tokyo, Japan).

2-6. autophagic flux assay

Cells were treated with 20 µM chloroquine diphosphate 2 h before harvest and were subjected to western blotting. The autophagic flux index was calculated as follows [12]:

Autophagic flux index = (LC3-II expression levels with Chloroquine)/ (LC3-II expression levels without Chloroquine)

Each LC3-II expression level was normalized by its expression of beta-actin. In each experiment, the autophagic flux index of the control group was normalized to 1.

2-7. BODIPY 493/503 staining and confocal imaging

HepG2 cells were seeded on cover glass and treated with 0.5 mM fatty acid for 16 h. Cells were washed in PBS, fixed with 4% paraformaldehyde, and stained with 2 µM BODIPY 493/503 solution for 15 min in the dark. The cells were then washed in PBS and mounted with ProLong Diamond Antifade Mountant. Confocal images were collected using a laser-scanning microscope (Leica DM2500).

2-8. statistical analysis

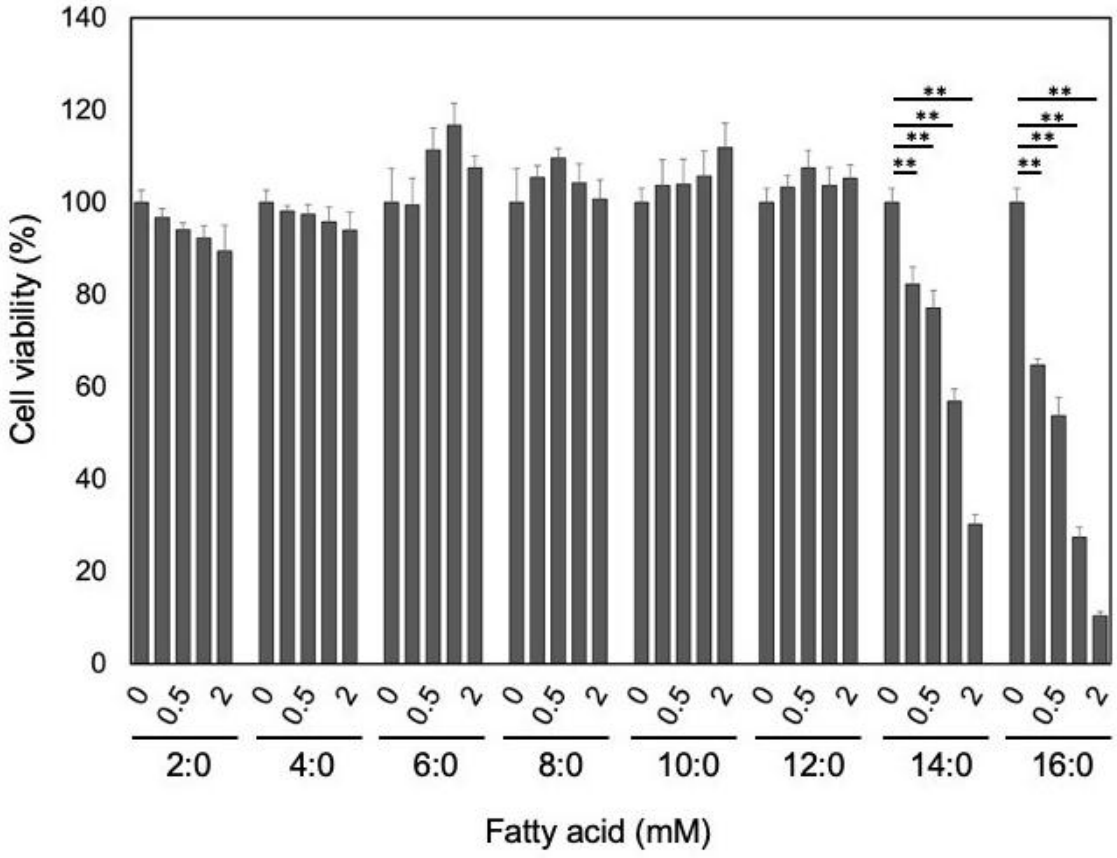
Data are expressed as mean ± standard deviation (S.D.). Statistical analyses were performed using one-way analysis of variance followed by a post-hoc Tukey's test. The sample size is indicated in each respective figure legend. $p < 0.05$ was considered statistically significant.

3. RESULTS

3-1. effects of saturated fatty acids of different chain lengths on HepG2 cell viability

We first investigated the effect of SCFAs (acetic acid (2:0), butyric acid (4:0), and caproic acid (6:0)), MCFAs (caprylic acid (8:0), capric acid (10:0), and lauric acid (12:0)), and LCFAs (myristic acid (14:0) and palmitic acid (16:0)) on the cell viability of the human hepatoma cell line, HepG2, which was supplemented with FFA-BSA complex and assessed using the CCK-8 assay. We tested different concentrations of FFA over a time frame of 16 h. LCFAs (14:0 and 16:0) significantly reduced cell viability in a concentration-dependent manner (Figure 1). SCFAs (2:0, 4:0, and 6:0) and MCFAs (8:0, 10:0, and 12:0), however, did not impact cell viability even at 2 mM.

Figure 1: Effect of FFA on HepG2 cell viability.

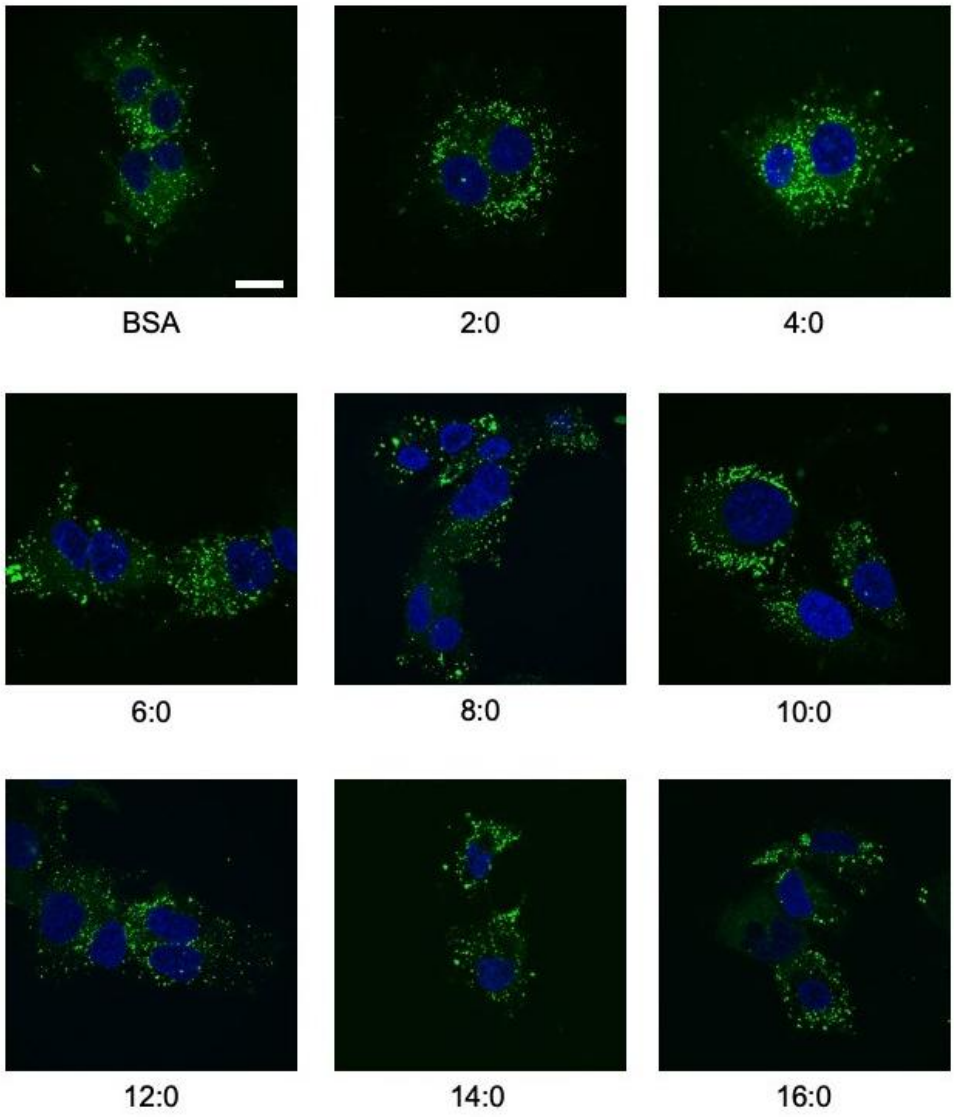


HepG2 cells were incubated with indicated concentrations of FFA for 16 h. Cell viability was calculated as a percentage of BSA-treated cells. Data are expressed as the mean ± S.D. (n =4). Significant differences compared with the BSA-treated cells are shown *$p < 0.05$ or **$p < 0.01$ using Tukey's post-hoc test.

3-2. effect of FFA of different chain lengths on HepG2 cell lipid accumulation

Next, we assessed the effect of FFA on HepG2 cell lipid accumulation. HepG2 cells were exposed to 0.5 mM FFA for 16 h and intracellular lipids were stained using BODIPY 493/503. No differences between the FFAs of different chain lengths were detected (Figure 2).

Figure 2: Effect of FFA on HepG2 cell lipid accumulation.

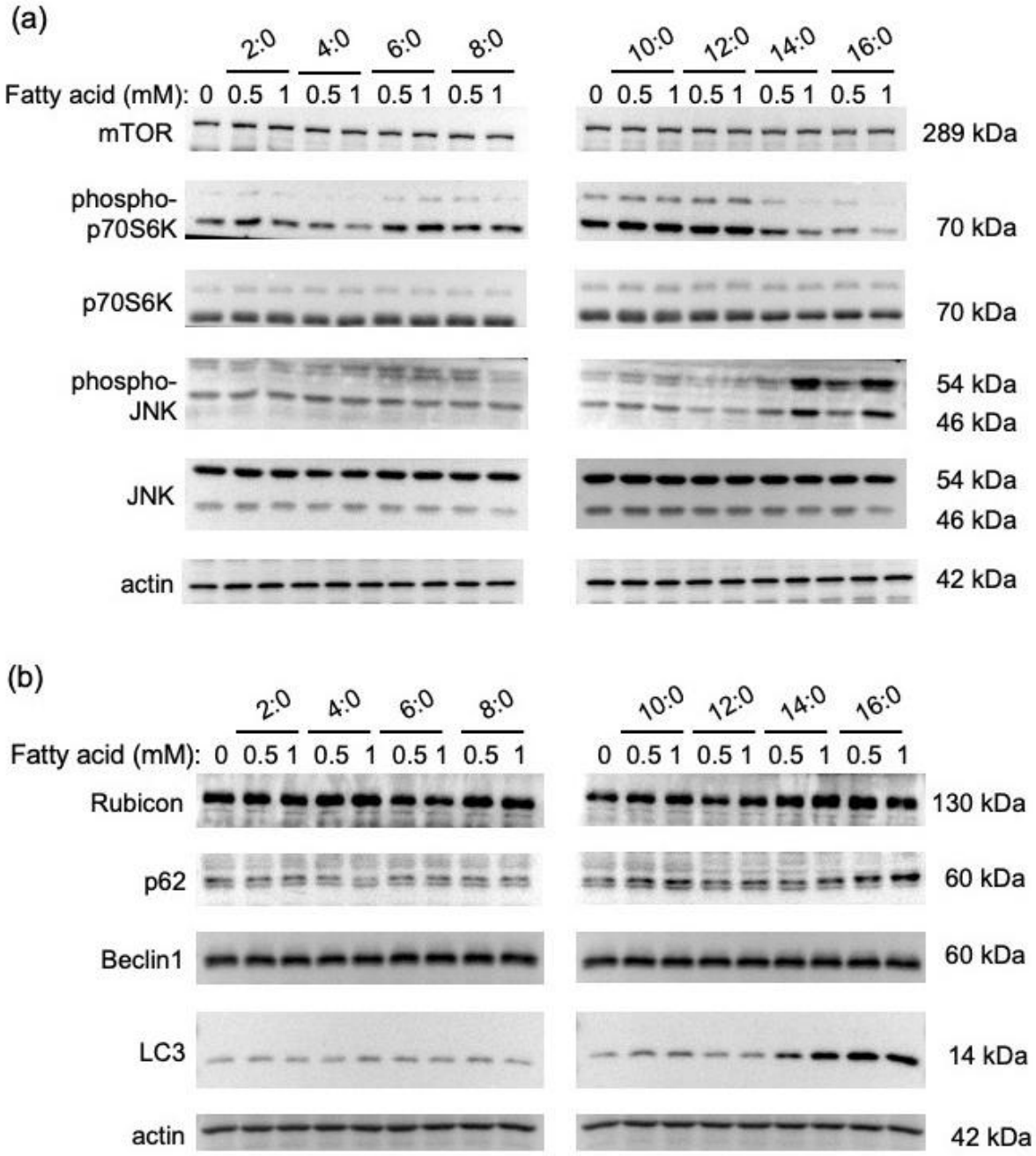


HepG2 cells were treated with 0.5 mM FFA for 16 h. Representative confocal microscopy images show the formation of lipid droplets following BODIPY 493/503 staining. Scale bar = 20 µm.

3-3. LCFAs affect the expression levels of cell viability- and autophagy- related proteins.

Treatment of HepG2 cells with 16:0 LCFA induces apoptosis and suppresses autophagy [12]. Therefore, we investigated how SCFAs, MCFAs, and LCFAs affect the expression levels of cell viability- and autophagy-related proteins in HepG2 cells. As shown in Figure 3a, LCFAs suppressed the phosphorylation of p70S6K and promoted the phosphorylation of c-Jun N-terminal kinase (JNK), while SCFAs and MCFAs did not. Furthermore, LCFAs upregulated expression levels of Rubicon, sequestosome 1 (SQSTM1)/p62, and microtubule-associated protein 1 light chain 3 (LC3)-II proteins, while SCFAs and MCFAs did not affect the Rubicon and LC3-II protein levels (Figure 3b).

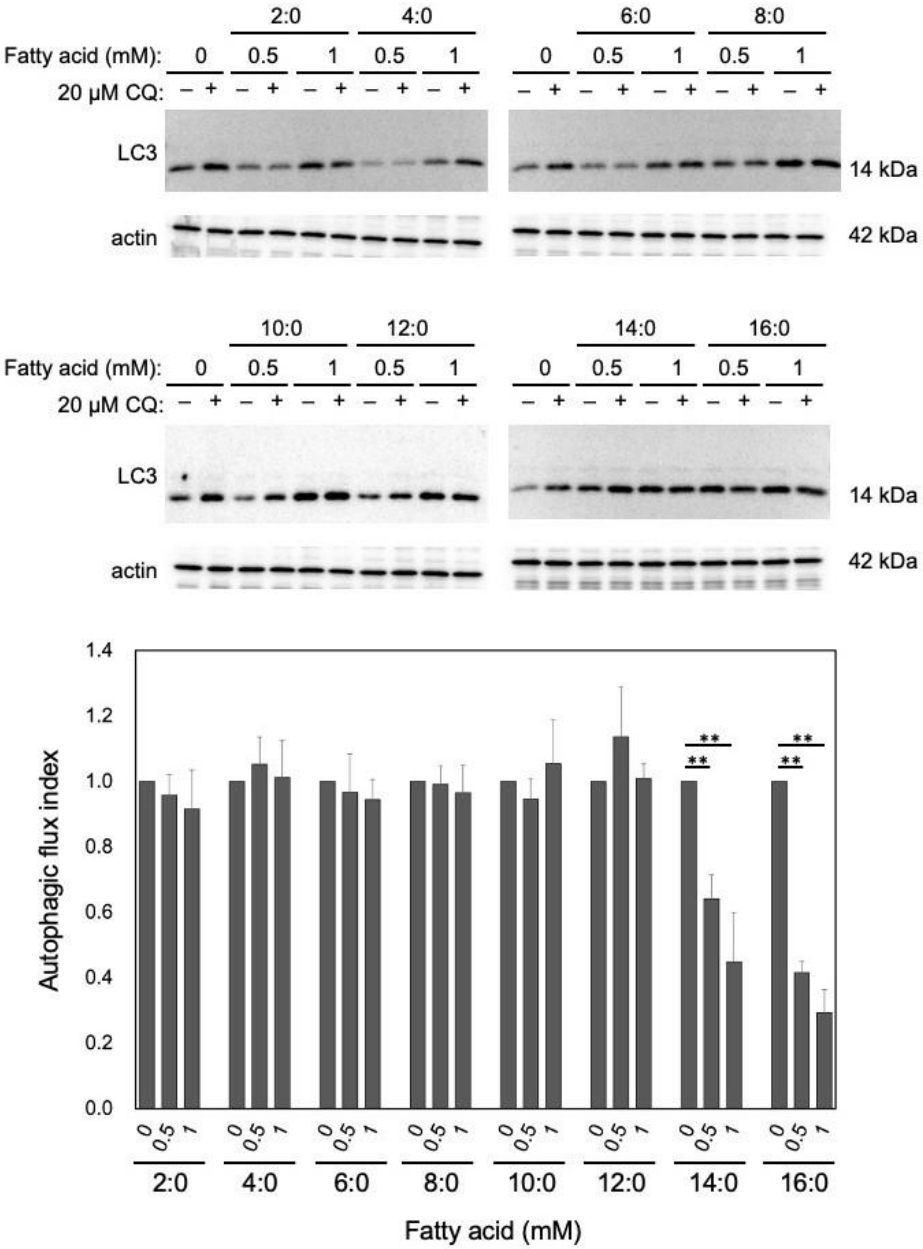
Figure 3: Expression levels of cell viability and autophagy-related proteins by western blotting. (a) Expression levels of cell viability-related proteins. (b) Expression levels of autophagy-related proteins.



3-4. LCFAs suppress autophagy.

To confirm the effect of FFA on HepG2 cell autophagy, we compared LC3-II expression levels in the presence and absence of chloroquine (CQ). CQ neutralizes the pH of lysosomes and inhibits lysosomal enzyme activity. Chloroquine increased expression levels of LC3-II in BSA-, SCFAs- and MCFAs- treated HepG2 cells (Figure 4). In contrast, the expression levels of LC3-II did not increase in LCFAs-treated HepG2 cells, indicating that LCFAs significantly suppressed autophagic flux.

Figure 4: LC3 turnover assay.



LC3 turnover assay to measure autophagic flux index using chloroquine (CQ) (n = 3). Significant differences compared with the BSA-treated cells are shown as *$p < 0.05$ or **$p < 0.01$ using Tukey's post-hoc test.

4. DISCUSSION

In this study, we investigated the effects of saturated fatty acids with different carbon chain lengths from 2 to 16 on HepG2 cells. Unlike SCFAs and MCFAs, both 14:0 and 16:0 LCFAs suppressed autophagy and reduced cell viability. A previous study has reported that 16:0 fatty acid upregulates Rubicon protein levels and suppresses autophagic flux [12]. Here, we confirmed that 14:0 also upregulates Rubicon protein levels and suppresses autophagic flux. Fatty acids whose carbon chain lengths were lower than 12 did not affect the expression levels of Rubicon protein or cell viability and did not suppress autophagic flux.

The toxicity of fatty acids can be reduced by incorporating them into lipid droplets as triglycerides (TGs) [1, 15, 16]. In this study, we examined intracellular lipid droplets upon the addition of various fatty acids and found no increase in lipid droplets in all cases; previous studies have reported that saturated fatty acids are less readily incorporated into TG than unsaturated fatty acids [2, 17]. Thus, the low toxicity of SCFAs and MCFAs cannot be attributed to their incorporation into lipid

droplets as TGs. We also confirmed that 14:0 and 16:0 LCFAs inhibit p70S6K phosphorylation and promote JNK phosphorylation. Phosphorylated p70S6K promotes protein synthesis and cell proliferation [18]. JNK becomes phosphorylated in response to cellular stress and induces apoptosis [19], which could explain how 14:0 and 16:0 LCFAs decreased cell viability in this study [4, 17, 20].

Defects in autophagy-related genes cause liver inflammation and promote tumorigenesis [21- 23]. Previous studies have shown that the suppression of both early-stage and late-stage autophagy occurs during the development of NAFLD [24- 27]. 16:0 LCFA suppresses late-stage autophagy by increasing Rubicon protein levels; decreasing the amount of LCFAs in foods may suppress Rubicon protein levels and therefore the development of NAFLD.

A limitation of this study is that only HepG2 cells were used; additionally, only their autophagy and cell viability were assessed. In many studies, 16:0 fatty acid causes hepatocellular damage, suggesting a link between NAFLD and NASH. However, few studies have examined the effects of saturated fatty acids other than palmitic acid on autophagy. SCFAs enhance host metabolism [28]; a clinical study has shown that the administration of SCFAs can help treat obesity [29]. Indeed, in vitro studies have shown that MCFAs reduce LCFA-induced fat accumulation, reactive oxygen species production, and apoptosis [30, 31]. Our results reveal the potential for SCFAs and MCFAs to replace LCFAs in foods to maintain normal levels of Rubicon protein as well as the importance of the carbon chain length of saturated fatty acids in maintaining normal autophagy in the liver and preventing the development of NAFLD.

5. CONCLUSIONS

In this study, we found that 14:0 saturated fatty acid inhibits HepG2 autophagy as well as 16:0 saturated fatty acid. Both 14:0 and 16:0 long-chain fatty acids inhibit autophagy by increasing Rubicon protein levels. On the other hand, saturated fatty acids between 2 and 12 carbons have no effect on either autophagy or cell viability.

6. ACKNOWLEDGEMENT

We appreciate Prof. Tetsuo Morita and Dr. Tomoyasu Fujii for valuable discussions. We also thank the students in our laboratory for their technical support.

7. CONFLICT OF INTEREST

There are no conflicts of interest to declare.

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