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EFFECT OF OBESE FACTORS FREE FATTY ACID AND LIPOPOLYSACCHARIDE ON EXPRESSION OF METABOLIC AND MYOGENIC GENES *CPT1b* AND *Myogenin* IN MUSCLE CELLS

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Abstract. Carnitine palmitoyl transferase 1b (CPT1b) and Myogenin are important markers of metabolism and myogenesis, respectively. The present study aimed to research the expression of *CPT1b* and *Myogenin* mRNAs in skeletal muscle C2C12 cells incubated with obese factors, free fatty acid (FFA), or lipopolysaccharide (LPS), to support the mechanism related to obesity and metabolic disorders. The result indicated that FFA significantly induced upregulation of *CPT1b* and *Myogenin* mRNAs in the skeletal muscle cells. In contrast, LPS treatment did not affect on expression of *CPT1* mRNA and tendency reduced expression of *Myogenin* mRNA in the skeletal muscle cells. These data support the evidence FFA is an energy and nutrient source for metabolism and myogenesis in the skeletal muscle cells whereas LPS seemingly suppresses myogenesis. Therefore, the mechanisms linking FFA or LPS to obesity induced skeletal muscle metabolic dysfunctions could be different.

***Keywords*:** free fatty acid, lipopolysaccharide, skeletal muscle cells, CPT1b, Myogenin.

1. Introduction

Obesity-related metabolic disorders such as type 2 diabetes, cardiovascular and liver diseases are typical issues of modern life. Especially, obesity is rapidly increasing in the world, for example, the rate of obesity in the United States is raising more than doubled in 10 years and about 61% of adult people of this country is overweight or obese [1]. Several factors in obese objects are linked to metabolic changes. One of those is chronic inflammatory responses such as an increase in levels of proinflammatory cytokines, including tumor necrosis factor-alpha (TNFα), interleukin 6 (IL6) which disturb normal metabolic functions [2]. Carnitine palmitoyl transferase 1b (CPT1b) plays an important role in the regulation of lipid metabolism in the cells. CPT1b acts as an enzyme that catalyzes a form of long-chain fatty acyl-CoAs to long-chain acylcarnitines which are uptaken into the mitochondrial for β-oxidation. CPT1 has three isoforms (a, b, and c), among them, CPT1b has been reported to be highly expressed in skeletal muscle tissue [3].

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Interestingly, a recent study has indicated that CPT1b inhibiting leads to increased insulin signaling, this thus supporting that regulation of CPT1b expression or activity will be a target for the treatment of metabolic dysfunctions [4].

Myogenin is a transcription factor specific for muscle cells. This factor is involved in myogenesis, muscle development, and mass [5]. Since skeletal muscle is the biggest tissue in the body and a typical site of free fatty acids and sugar deposition, changes in this tissue mass can lead to alteration of system metabolic homeostasis [6]. Free fatty acid (FFA) and lipopolysaccharide (LPS) are often considered obese factors whose levels are increased in obese animals and people. Moreover, increases in FFA and LPS levels are accompanied by increased chances of metabolic disorders, including insulin resistance and type 2 diabetes [7]. Therefore, in the current study, the skeletal muscle C2C12 cells were differentiated to myotubes and then treated with obese factors, FFA or LPS, to examine the expression of *CPT1b* and *Myogenin* mRNAs which are the markers of skeletal muscle lipid metabolism and myogenesis, respectively. The result showed that FFA markedly induced upregulation of *CPT1b* and *Myogenin* mRNAs in the skeletal muscle cells. On the other hand, LPS treatment had no effect on the expression of *CPT1* mRNA and mildly suppressed expression of *Myogenin* mRNA in the muscle cells. These data demonstrate that FFA affects skeletal muscle metabolism that may be involved in the upregulation of CPT1b and myogenesis whereas LPS seemingly suppresses myogenesis and contributing to alters skeletal muscle metabolic homeostasis.

2. Content

2.1. Materials and methods

2.1.1. Skeletal muscle cell culture

The cell culture protocol is following the protocol mentioned in the latest study [8]. The mouse primary muscle cell line C2C12 (2.5×10^5 cells/mL) were incubated at 37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 μg/mL gentamicin (Gibco). When the cells reached about 100% confluence, the medium was changed with the differentiation medium consisting of DMEM plus 2% horse serum, which was changed every 2 days.

2.1.2. Free fatty acid (FFA) and *lipopolysaccharide* (LPS) treatment

Palmitic acid, a typical type of free fatty acid, and lipopolysaccharide (LPS) were purchased from Sigma (USA). The free fatty acid (FFA) was dissolved in ethanol and combined with BSA at a 10:1 molar ratio and LPS was dissolved in water. After 3 days of differentiation, myotubes were incubated with 500 μM FFA in the serum-free DMEM containing 50 μM BSA for 24 h or with 100 ng/mL LPS in serum-free DMEM for 24 h. The same amount of ethanol in the serum-free DMEM containing 50 μM BSA and the medium with no treatment were used as the controls of FFA and LPS-treated cells, respectively. After incubation time, the cells were washed twice with PBS and lysed in Trizol Reagent (Invitrogen) for quantitative real-time PCR analysis. The experiment was done in triplicate and the data are expressed as mean (X) ± standard error of the mean (SE).

2.1.3. Quantitative real-time PCR

Two microgram aliquots of total RNA extracted from the lysed cells of each experimental group were reverse transcribed to cDNA using M-MLV reverse transcriptase (Promega, USA). The quantitative RT-PCR (qRT-PCR) amplification of the cDNA was performed in duplicate with an SYBR premix ExTaq kit (TaKaRa Bio Inc., USA) using a Thermal Cycler Dice (TaKaRa Bio Inc.). Reactions were performed with the same schedule: 95 °C for 10 s and 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Results were analyzed with Real-Time System TP800 software (Takara Bio Inc.) and the values were normalized to the levels of the housekeeping gene *β-actin*. The primers are shown in Table 1.

Table 1. Mouse primers used for qRT-PCR tests

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
β-actin	CATCCGTAAAGACCTCTATGCCAAC	ATGGAGCCACCGATCCACA
CPT1b	GAGACAGGACACTGTGTGGGTGA	AGTGCCTTGGCTACTTGGTACGAG
Myogenin	TGCCCAGTGAATGCAACTCC	TCCACCGTGATGCTGTCCA

2.1.4. Location and time of the study

All experiments were carried out in 2012 at the Laboratory of Food Science and Nutrition, University of Ulsan, South Korea. Data analysis was performed in 2021 at the Department of Human and Animal Physiology, Hanoi University of Education.

2.1.5. Statistical analysis

The results were displayed as means ± standard error of the mean (*SE*). Comparisons of variables were performed by using Student's *t*-test. The *P values* < 0.05 were named as significant differences in comparisons.

2.2. Results and discussions

2.2.1. Free fatty acid (FFA) enhanced expression of *CPT1b* mRNA in skeletal muscle C2C12 cells

Increased plasma FFA level is characteristic of obesity and this is accompanied by skeletal muscle metabolic dysfunctions especially insulin resistance and type 2 diabetes [7]. However, the mechanism(s) linking obesity-related increased blood FFA levels and metabolic malfunction has not been well elucidated. Thus, the present study aimed to examine whether FFA treatment affects the expression of the molecule(s) related metabolic function in the cultured skeletal muscle C2C12 cells. The result showed that expression of *CPT1b* mRNA was significantly increased in the FFA treated skeletal muscle cells compared to that in the control cells (Figure 1A and 1B). A recent study has revealed that the mouse with skeletal muscle-specific knock out CPT1b has lower inflammatory responses compared to the wild-type animal. Moreover, mRNA expression of pro-inflammatory cytokines (TNFα, IL6) is strongly suppressed in the FFA-treated CPT1 knock-out (KO) primary skeletal muscle cells compared to the FFA-treated wildtype muscle cells [9]. Since increased inflammatory responses in FFA-treated skeletal muscle cells and the HFD-fed mice are closely associated with insulin resistance

and type 2 diabetes [10, 11], therefore, the increased expression of *CPT1b* mRNA in the FFA-treated C2C12 muscle cells could link fatty acid oxidation with inflamed-metabolic dysfunction in skeletal muscles.

(A)

Exp	BSA 50 μM	FFA 500 μM
1	0.84	2.25
2	0.76	2.16
3	0.91	2.46
X	***0.84***	***2.29***
SE	***0.04***	***0.09***
P	***0.0001***	

(B)

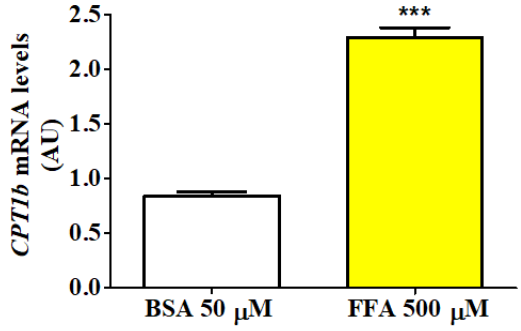

Figure 1. Expression of CPT1b mRNA in the FFA-treated cells

C2C12 myotubes were established for 3 days, then treated with free fatty acid (FFA) at 500 μM for 24 h. Free fatty acid (palmitate) was prepared in ethanol containing bovine serum albumin (BSA, 10% w/v). Real time RT-PCR analysis for expression of CPT1b mRNA. Levels of mRNA were normalized to levels of β-actin mRNA. (A) data analysis of CPT1b mRNA levels. (B) comparison of CPT1b mRNA levels. Data represent the results of three independent experiments (Exp). Values are means (X) ± standard error (SE).

****P < 0.001 compared between the experimental group and the control group.*

2.2.2. FFA increased expression of *Myogenin* mRNA in skeletal muscle C2C12 cells

The skeletal muscle is the biggest tissue in the body, it accounts for about 40 - 50% of body mass making it is an important tissue of free fatty acid, glucose consumption, and energy metabolism [12]. Consistent with this, changes in skeletal muscle mass will lead to increases in the risk of insulin resistance and diabetes [6]. Hence, the current study aimed to examine if FFA treatment alters the expression of a key myogenic gene *Myogenin*. Interestingly, expression of *Myogenin* mRNA was markedly upregulated in the FFA-treated C2C12 muscle cells compared with that in the control-treated cells (Figures 2A and 2B). The previous study has demonstrated that linoleic acid, another free fatty acid, has a trophic effect when its supplementation significantly increased the strength of the gastrocnemius muscle of the dystrophic mice. Additionally, the saturated fatty acid and linoleic acid-rich diet resulted in impaired insulin sensitivity and oxidative stress in skeletal muscle [13]. As a consequence, the present study's data suggest that FFA treatment induces increases in mRNA expression of both *CPT1b* and *Myogenin* which may contribute to a high risk of skeletal muscle insulin resistance by increases in inflammatory responses and fat oxidative stress rather than by increased myogenesis.

(A)

Exp	BSA 50 μM	FFA 500 μM
1	0.71	2.53
2	0.65	2.41
3	0.86	2.92
X	*0.74*	*2.62*
SE	*0.06*	*0.15*
P	***0.0003***	

(B)

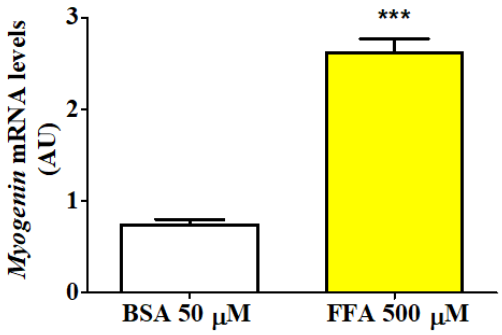

Figure 2. Expression of Myogenin mRNA in the FFA-treated cells

*C2C12 myotubes were established for 3 days, then treated with FFA at 500 μM for 24 h. Free fatty acid (palmitate) was prepared in ethanol containing bovine serum albumin (BSA, 10% w/v). Real time RT-PCR analysis for expression of Myogenin mRNA. Levels of mRNA were normalized to levels of β-actin mRNA. (A) data analysis of Myogenin mRNA levels. (B) comparison of Myogenin mRNA levels. Data represent the results of three independent experiments (Exp). Values are means (X) ± standard error (SE). ***P < 0.001 compared between the experimental group and the control group.*

2.2.3. *Lipopolysaccharide* (LPS) did not increase expression of *CPT1b* mRNA in skeletal muscle C2C12 cells

It is well known that LPS is strongly induced by skeletal muscle inflammation and insulin resistance [7]. Thus, the present study manipulated the experiment to examine LPS treatment alters *CPT1b* mRNA expression in skeletal muscle cells. The result showed that the expression of *CPT1b* mRNA did not differed between the LPS-treated skeletal muscle cells and the medium-treated cells (Figure 3A and 3B). Our previous studies have indicated that FFA supplementation significantly induced increases in expression of inflammatory cytokine IL6 together with the upregulation of Toll Like Receptors TLR2 and TLR4, whereas, increased IL6 expression in LPS treatment was not associated with the changes in expression of TLR2 and TLR4 in the skeletal muscle cells [8, 14]. FFA activates TLRs leading to activation of Nuclear Factor kappa B (NF-kB) that in turn, upregulates expression of the inflammatory cytokine genes [15]. On the other hand, LPS can enhance Reactive Oxidation Species (ROS) in the cells which then increases expression of Mitogen-Activated Protein Kinase (MAPK) leading to activation of NF-kB [16]. Because FFA and LPS can induce activation of different inflammatory pathways they, thus, have differed effects on the expression of *CPT1b* mRNA in the skeletal muscle cells.

(A)

Exp	Medium	Medium + LPS 100 ng/mL
1	1.00	0.97
2	0.86	0.83
3	0.95	0.93
X	***0.94***	***0.91***
SE	***0.04***	***0.04***
P	***0.6717***	

(B)

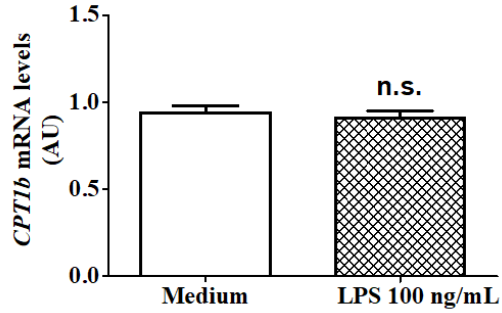

Figure 3. Expression of CPT1b mRNA in the LPS-treated cells

C2C12 myotubes were established for 3 days, then treated with or without lipopolysaccharide (LPS) at 100 ng/mL for 24 h. Real time RT-PCR analysis for expression of CPT1b mRNA. Levels of mRNA were normalized to levels of β-actin mRNA. (A) data analysis of CPT1b mRNA levels. (B) comparison of CPT1b mRNA levels. Data represent the results of three independent experiments. Values are means (X) ± standard error (SE). n.s. is not significant between the experimental group and the control group.

2.2.4. LPS did not affect on the expression of *Myogenin* mRNA in skeletal muscle C2C12 cells

LPS is a strong factor that induces skeletal muscle inflammation. Because inflammation is associated with skeletal muscle atrophy [17], thus, the next experiment is aimed to test if LPS treatment suppresses the expression of myogenic genes. The current study's data showed that the expression level of *Myogenin* mRNA was tendency lower in the LPS-treated skeletal muscle C2C12 cells than that was in the control cells. However, this comparison was not significantly differed (Figure 4A and 4B).

(A)

Exp	Medium	Medium + LPS 100 ng/mL
1	0.96	0.89
2	1.01	0.96
3	0.91	0.87
X	***0.96***	***0.91***
SE	***0.03***	***0.03***
P	***0.2505***	

(B)

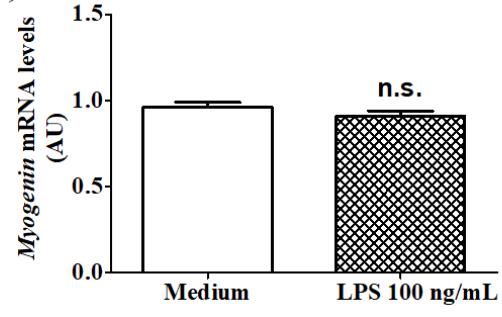

Figure 4. Expression of Myogenin mRNA in the LPS-treated cells

C2C12 myotubes were established for 3 days, then treated with or without LPS at 100 ng/mL for 24 h. Real time RT-PCR analysis for expression of Myogenin mRNA. Levels of mRNA were normalized to levels of β-actin mRNA. (A) data analysis of Myogenin mRNA levels. (B) comparison of Myogenin mRNA levels. Data represent the results of three independent experiments. Values are means (X) ± standard error (SE). n.s. is not significant between the experimental group and the control group.

The previous study has demonstrated that LPS treatment significantly inhibits the expression of *Myogenin* protein in C2C12 myoblast cells with doses of 100 ng/mL and 1000 ng/mL of LPS [18]. The data here showed no significant suppression of *Myogenin* mRNA expression by LPS treatment in C2C12 myotube cells. This difference in our study and the aforementioned one is attributed to different cell developed stages of treatments, the current treatment at myotube-differentiated stage whereas the previous treatment at the myoblast-undifferentiated stage. Consequently, these data suggest that inflammatory factor LPS can inhibit myogenesis with a strong effect at a very early stage of skeletal muscle cell development.

3. Conclusions

As a result, the present study demonstrates a different effect of FFA and LPS on the expression of *CPT1b* and *Myogenin* mRNAs which are markers of fat metabolism and myogenesis in skeletal muscle, respectively. Expression of both *CPT1b* and *Myogenin* mRNAs was strongly increased in the C2C12 myotubes treated with FFA while expression of them was not significantly differed in the LPS treated myotubes compared with the control cells. FFA induced higher expression of CPT1b contributing to oxidative stress that leading to inflammatory responses rather than increased myogenesis. LPS related skeletal muscle inflammation may not be attributed to oxidative stress but could be affected ROS signaling and LPS inhibits skeletal muscle myogenesis at the very early stage of muscle cell development. Further studies should elucidate the relation among FFA, inflammatory cytokines, and CPT1b as well as insulin signaling molecules in skeletal muscle cells to clearly show the mechanism(s) that links obesity, inflammation, and metabolic disorders in the skeletal muscle.

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