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# EFFECT OF FREE FATTY ACID ON EXPRESION OF LIGHT AND HVEM mRNA LEVELS IN CULTURED SKELETAL MUSCLE CELLS

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**Abstract:** Light, tumor necrosis factor superfamily member 14 (TNFSF14), is a secreted peptide of the TNF superfamily. It binds to the herpesvirus entry mediator (HVEM). Light/HVEM signaling is well reported as an important factor linking inflammation and obesity. Here, in the current study, we manipulated skeletal muscle cells incubated in media containing free fatty acid (FFA) to mimic an obesity-related inflammatory environment. Our result revealed that FFA strongly induced expression of mRNA levels of light and interleukin 6 (IL6) - an inflammatory cytokine in the skeletal muscle cells. Surprisingly, expression of HVEM mRNA levels was not significantly different between the FFA-treated skeletal muscle cells and the control cells. These data suggest that FFA increases light expression in skeletal muscle cells that in turn, can bind to HVEM as an autocrine effect inducing inflammatory responses in skeletal muscle cells.

***Keywords*****:** Free fatty acid, skeletal muscle cells, light/HVEM.

## 1. Introduction

Several tumor necrosis factor superfamily (TNFSF) members have been reported to be involved in linking inflammation and obesity-related metabolic disorders [1]. Among them, light, (TNFSF14, a ligand of herpesvirus entry mediator (*HVEM*), plays an important role in the regulation of obesity-related inflammatory responses. The *light/HVEM pathway* is an important co-signaling pathway for immune cells such as T lymphocytes. Light-mediated HVEM signaling activates NF-kB transcriptional programs in T cells [2]. Since NF-kB is a key regulator of inflammation, thus, activation of NF-kB gives risen production of inflammatory cytokines including tumor necrosis factor-alpha (TNFα), interleukin 6 (IL6), and monocyte chemoattractant protein 1 (MCP1) [3].

Interestingly, a recent study has reported that light/HVEM signaling is obviously involved in cross-talk between adipocytes – metabolic cells and macrophages – immune cells and this leads to risen protein levels of the critical inflammatory cytokines including TNFα, IL6, and MCP1 in the cultured media of those cells [4]. Among metabolic tissues

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such as adipose tissue, liver tissue, and skeletal l tissue, the latest tissue has a crucial role in the regulation of body metabolic homeostasis. It is clear elucidated that skeletal muscle tissue is the biggest tissue in the body accounting for about 40% - 50% of the dry body weight and a major site of free fatty acid and glucose consumption [5]. Thus, changing of skeletal muscle physiology and/or morphology can alter whole-body metabolic regulation. Additionally, obesity-related skeletal muscle inflammation is accompanied by metabolic complications (e.g., insulin resistance, type 2 diabetes) [6]. Therefore, study the mechanism(s) linking obesity and skeletal muscle dysfunction would be helpful in the prevention of obesity-related chronic metabolic disorders. Consequently, in the current study, we cultured C2C12 skeletal muscle cells in media containing free fatty acid (palmitic acid) and examined inflammatory response related to light/HVEM signaling that has not been well-known in skeletal muscle yet.

# 2. Content

## 2.1. Materials and methods

**** Skeletal muscle cell culture***

The cell culture protocol is following the protocol mentioned in the previous study [7]. The mouse myoblast cell line C2C12 myoblasts ($2.5\times10^5$ cells/mL) were cultured 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$\mu$g/mL streptomycin, and 20$\mu$g/mL gentamicin (Gibco). When the cells got about 100% confluence, the medium was changed with the differentiation medium consisting of DMEM plus 2% horse serum, which was changed every 2 days. Cell culture was carried out in the laboratory of the Department of Food Science and Nutrition, University of Ulsan, South Korea.

**** Free fatty acid treatment***

Free fatty acid palmitic acid was purchased from Sigma (Sigma-Aldrich, Missouri, USA). The free fatty acid (FFA) was dissolved in ethanol and combined with BSA at a 10:1 molar ratio. After 3 days of differentiation, myotubes were incubated with 500$\mu$M FFA in the serum-free DMEM containing 50$\mu$M BSA for24h. Thesameamountofethanol in the serum-free DMEM containing 50 $\mu$M BSA was used as the control. 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 ($SE$). The protocols of cell culture and treating cells are referred from a previous study [8].

**** Quantitative real-time PCR***

Total RNA was extracted from the lysed cells. Two microgram aliquots of total RNA were reverse transcribed to cDNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA). The quantitative RT-PCR (qRT-PCR) amplification of the cDNA was performed in duplicate with an SYBR premix ExTaq kit (TaKaRa Bio Inc., CA, USA) using a Thermal Cycler Dice (TaKaRa Bio Inc.). All 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 all values

were normalized to the levels of the control gene $\beta$-actin. The used primers are shown in Table 1.

***Table 1. The primers used for qRT-PCR analysis***

| Gene | Forward primer (5' → 3') | Reverse primer (5' → 3') |
|---|---|---|
| *β-actin* | CATCCGTAAAGACCTCTATGCCAAC | ATGGAGCCACCGATCCACA |
| *IL6* | CCACTTCACAAGTCGGAGGCTTA | GCAAGTGCATCATCGTTGTTCATAC |
| *HVEM* | AACTTGCTGCAGCGCATCTC | TGCTCACTGCAGACCTGCTTC |
| *LIGHT* | CGGTGATGGGCACTTGTGA | CAGGAGCTATCGACTGAACTTGGA |

**** 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. *P values* < 0.05 were considered to be significant differences in comparisons.

## 2.2. Results and discussions

### 2.2.1. Free fatty acid supplementation strongly induced IL6 mRNA expression in skeletal muscle cells

It is well-known that increases in plasma free fatty acids (FFA) are characterized by obesity. High plasma FFA level is considered as a pivotal factor that induces burdens in several metabolic tissues such as adipose tissues, liver tissues, and skeletal muscle tissues in the high fat diet-fed obese mice [9, 10]. FFA induces inflammatory responses in these tissues leading to increased production of inflammatory cytokines including IL6 and TNFα which is associated with metabolic dysregulations [11].

**(A)**

| Exp | BSA 50 µM | FFA 500 µM |
|---|---|---|
| 1 | 0.97 | 19.19 |
| 2 | 0.85 | 21.54 |
| 3 | 0.81 | 19.37 |
| ***X*** | ***0.88*** | ***20.03*** |
| ***SE*** | ***0.05*** | ***0.76*** |
| ***P*** | ***< 0.001*** | |

**(B)**

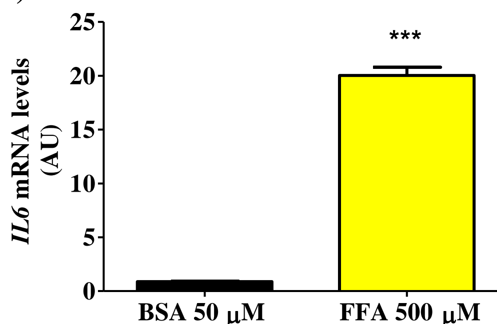



***Figure 1. Expression of IL6 mRNA in myotubes***

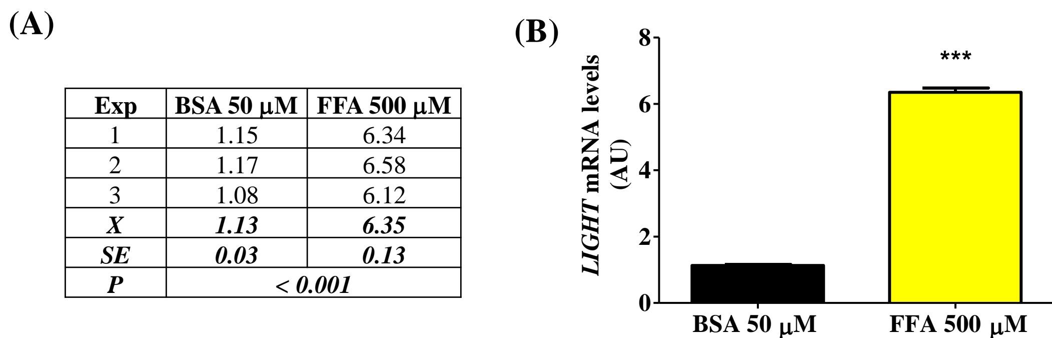
*C2C12 myotubes were established for 4 days, then treated with free fatty acid (FFA) at 500 µM for 24 h. Free fatty acid (palmitate) was prepared in ethanol containing bovine serum albumin (BSA, 10% w/v). Real time RT-PCR analysis for expression of IL6 mRNA. Levels of mRNA were normalized to levels of β-actin mRNA. (A) data analysis of IL6 mRNA levels. (B) comparison of IL6 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. AU is an arbitrary unit.*

Here, we cultured C2C12 skeletal muscle cells in media containing FFA to test inflammatory response in the cells and saw that expression of IL6 mRNA level was significantly upregulated in the FFA-treated skeletal muscle cells compared with that was in the control cells (*P* < 0.001) (Figure 1A and 1B). These data give more evidence to prove that FFA is an important factor that linking obesity and inflammatory responses in skeletal muscle tissues. Thus, study the mechanism(s) involved in FFA-induced skeletal muscle cells' inflammation may usel to treat obesity.

**2.2.2. Expression of light mRNA levels in the FFA-treated C2C12 myotubes**

Raising evidence support that co-stimulators of immune cells are involved in inflammatory responses in metabolic cells such as adipocytes and skeletal muscle cells [1, 8]. Among them, 4-1BB and its ligand 4-1BBL, which are critical lymphocytes' co-stimulators, have been recognized as involving in the induction of obesity-related skeletal muscle inflammation [8]. On the other hand, light/HVEM molecules, other co-stimulatory of T lymphocytes, strongly induce inflammatory responses in adipocytes and adipose tissues [4]. Therefore, in the present study, we examined if light/HVEM signaling is related to FFA-induced muscle cells' inflammation. Interestingly, our results showed that the expression of light mRNA levels in the FFA-treated skeletal muscle cells was severely higher than that in the control-treated cells (*P* < 0.01) (Figure 2A and 2B).

A previous report has shown that FFA strongly induced expression of light mRNA levels in adipocytes this is associated with an increased level of IL6. And blockade of light/HVEM signaling blunted the increases in inflammatory cytokine production [4]. Hence, our data support the hypothesis that light/HVEM signaling not only involves immune cells' activities but also relations to induction of metabolic cells' inflammation.



**(A)**

| Exp | BSA 50 μM | FFA 500 μM |
|---|---|---|
| 1 | 1.15 | 6.34 |
| 2 | 1.17 | 6.58 |
| 3 | 1.08 | 6.12 |
| *X* | *1.13* | *6.35* |
| *SE* | *0.03* | *0.13* |
| *P* | *< 0.001* | |



***Figure 2. Expression of light mRNA in myotubes***

*C2C12 myotubes were established for 4 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 light mRNA. Levels of mRNA were normalized to levels of β-actin mRNA. (A) data analysis of light mRNA levels. (B) comparison of light 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. AU is an arbitrary unit.*

### 2.2.3. Expression of HVEM mRNA levels in the FFA-treated C2C12 myotubes

Light molecule gives its effect via binding to its receptor – HVEM molecule on the target cells [4]. Thus, we next tested the effect of FFA treatment on the expression of HVEM molecule in C2C12 skeletal muscle cells. Unfortunately, our current finding indicated that expression of HVEM mRNA levels was not significantly differed between the FFA-treated C2C12 skeletal muscle cells and the control cells ($P > 0.05$) (Figure 4A and 4B). HVEM is a transmembrane protein which is widely expressed on immune cells such as T cells and macrophages [2]. Consistent with this, a sooner study has not been reported expression data of HVEM molecule in the FFA-treated adipocytes. However, the expression of HVEM mRNA levels was strongly upregulated in adipose tissue of obese mice compared with that in the lean mice [4]. Since obese adipose tissues contain an increased number of immigrated immune cells (e.g., T cells and macrophages) [12], the increased expression level of HVEM mRNA could be derived from those immune cells. As a result, the increased expression of IL6 mRNA in the FFA-treated skeletal muscle cells is concomitant with the upregulation of light rather than HVEM mRNA expression.

**(A)**

| Exp | BSA 50 μM | FFA 500 μM |
|---|---|---|
| 1 | 0.67 | 0.61 |
| 2 | 0.61 | 0.58 |
| 3 | 0.64 | 0.62 |
| ***X*** | ***0.64*** | ***0.60*** |
| ***SE*** | ***0.02*** | ***0.01*** |
| ***P*** | ***0.1570*** | |

**(B)**

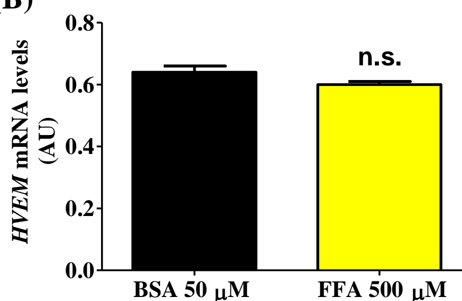



***Figure 3. Expression of HVEM mRNA in myotubes***

*C2C12 myotubes were established for 4 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 HVEM mRNA. Levels of mRNA were normalized to levels of β-actin mRNA. (A) data analysis of HVEM mRNA levels. (B) comparison of HVEM mRNA levels. Data represent the results of three independent experiments (Exp). Values are means (X) ± standard error (SE). n.s. is not significant between the experimental group and the control group.AU is an arbitrary unit.*

## 3. Conclusions

As a consequence, our current study reports the finding that increased expression of IL6 mRNA in free fatty acid (FFA)-treated cultured skeletal muscle cells is paralleled with the upregulation of light mRNA. Expression of HVEM, a specific receptor of light, is not enhanced in the FFA-treated skeletal muscle cells. We thus discussed that FFA induced skeletal muscle cells' inflammation may be attributed to light/HVEM signaling. However, further studies should be carried out in vivo of obese subjects to clearly uncover the mechanism linking between LIGH/HVEM signaling and obese skeletal muscle inflammation.

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