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FREE FATTY ACID ENHANCES EXPRESSION OF INFLAMMATORY MARKERS IL6, TLR2, TLR4 IN C2C12 MUSCLE CELLS

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Abstract: It is well known that obesity is increasing as worldwide epidemic and it is often accompanied by increased metabolic disorders. Having an insight awareness about obesity-related conditions will be a strategy to protect against obesity-related dysfunctions. In the present study, we differentiate C2C12 myocytes into mature myotubes. Then the cells were treated with free fatty acid (FFA) to mimic the obese microenvironment.The result showed that the level of IL6 mRNA, a key inflammatory cytokine, was significantly higher in the myotubes treated with FFA compared to the myotubes treated with the control medium. Consistent with this, the level of TLR2 mRNA, an important regulator of inflammatory responses, was markedly increased in the cells treated with FFA compared to the control cells. Additionally, mRNA level of TLR4, another key regulator of the inflammatory response, was highly expressed in the FFA treated cells compared with the control cells. These data demonstrate that the C2C12 myotubes cultured with FFA can be an *in vitro* obese model for further researches.

Keywords: Free fatty acid, myotubes, inflammation.

1. INTRODUCTION

Chronic inflammatory response has been considered as an important characteristic of obesity. This response is also reported to be a pivotal factor that links obesity with metabolic disorders such as type 2 diabetes, fatty liver diseases, and cardiovascular diseases (Pedersen, 2013). Related to the inflammatory response in obesity is increased expression level of many regulators of inflammatory molecules such as TLRs (Toll like receptors) as well as inflammatory cytokines such as tumor necrosis factor alpha/TNF-α, interleukin 6/IL6, monocyte chemoattractant protein 1/MCP-1(Rogero et al., 2018).

Inflammatory response in obesity is occurring in many tissues including adipose tissues, liver tissue, and skeletal muscle tissues. Several recent studies have indicated that skeletal muscle inflammation plays a key role in obesity-related metabolic dysfunctions such as insulin resistant and type 2 diabetes(Leet et al., 2013). Increased inflammatory response in skeletal muscle of obesity is associated with the increases in levels of triglycerides as well as free fatty acids (FFA)(Kitessa &Ableywardena, 2016). Thus, in the present study, we established an *in vitro* model of obese skeletal muscle microenvironment by culturing C2C12 myotubes with FFA and examining the inflammatory response in the cultured cells.

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2. MATERIALS AND METHODS

2.1. Cell Culture

The mouse myoblast cell line C2C12 was purchased from the American Type Culture Collection (ATCC, Manassas, USA). The C2C12 myoblasts (2.5×10^5cells/mL) were grown at 37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Gibco, NY, USA) containing 10% fetal bovine serum (FBS) (Gibco), 100 units/mL penicillin, 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA), and 20 μg/mL gentamicin (Gibco). When the cells reached 100% confluence, the medium was replaced with the differentiation medium consisting of DMEM plus 2% horse serum (Gibco), which was changed after 2 days.

2.2. Treated cell with free fatty acid

Free fatty acid, palmitic acid, was purchased from Sigma. This free fatty acid (FFA) was dissolved in ethanol and conjugated with BSAat a 10 : 1 molar ratio before use. After 3 days of differentiation, myotubes were incubated with 500 μM palmitic acid (FFA) in the serum-free DMEM containing 50 μM BSA for 24 h. The equal amount of ethanol in the serum-free DMEM containing 50 μM BSA was used as a control group (Figure 1). 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 refered from the previous study of Le et al., 2013.

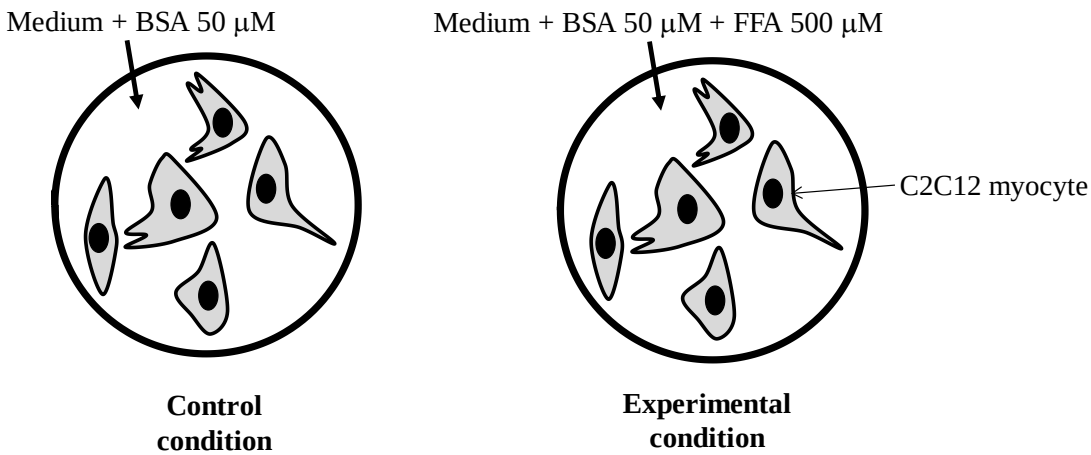

Figure 1. *Design of experiment*

2.3. Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from the lysed cells with TrizolReagent (Invitrogen). Two microgram aliquots of total RNAwere reverse transcribed to cDNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA). The qRT-PCR amplification of the cDNA was performed in duplicate with a SYBR premix ExTaq kit (TaKaRa Bionc., Forster, CA, USA) using a Thermal Cycler Dice (TaKaRa Bio Inc., Japan). 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 levelsof the house-keeping gene *β-actin*. The primers used in the analysis are listed in Table 1.

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

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
IL6	CCACTTCACAAGTCGGAGGCTTA	GCAAGTGCATCATCGTTGTTCATAC
TLR2	GGACGTTTGCTATGATGCCTTTG	ACGAAGTCCCGCTTGTGGAG
TLR4	GGGCCTAAACCCAGTCTGTTTG	GCCCGGTAAGGTCCATGCTA
β-actin	CATCCGTAAAGACCTCTATGCCAAC	ATGGAGCCACCGATCCACA

2.4. Statistical Analysis

The results were shown as means ± standard error of the mean (*SE*). Comparisons of variables were carried out by using Student's *t* test. Differences were considered to be significant when $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Free fatty acid (FFA) enhanced IL6 mRNA expression in C2C12 myotubes

(A)

Exp	BSA 50 μM	FFA 500 μM
1	0.94	24.14
2	0.89	27.35
3	0.84	17.74
X	***0.89***	***23.08***
SE	***0.03***	***2.82***
P	***0.0014***	

(B)

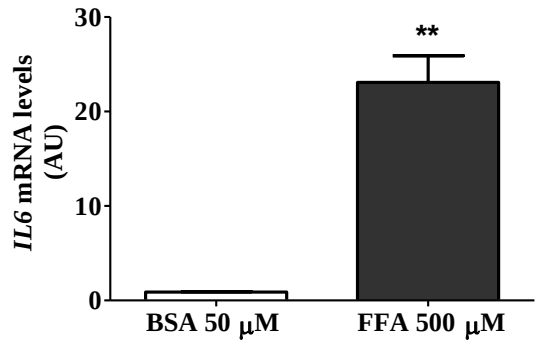

Figure 2. *FFA increased expression of IL6 mRNA. C2C12 myotubes were established for 4 days, and then treated with free fatty acid (FFA) at 500 μM for 24 h. Free fatty acid (palmitate) was prepared in ethanol containing bovine serum albumin (BSA, 10% w/v). Real time RT-PCR analysis for expression of 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 results of three independent experiments (Exp). Values are means (X) ± standard error of the mean (SE). **P < 0.01 compared between the experimental group and the control group.*

To test whether FFA induces an inflammatory response in C2C12 myotubes, we checked level of IL6 mRNA, a key inflammatory cytokine, in the myotubes. As shown in Figure 2A and 2B, the level of IL6 mRNA was markedly higher in the FFA treated myotubes than that in the control medium treated myotubes. Previous study has also

shown that IL6 mRNA level is significantly higher in the high-fat diet (HFD) fed mice than that in the regular diet fed mice (Gil et al., 2015). Since the high-fat diet fed mice is considered as an *in vivo* obese model (Vedova et al., 2016), the increased of IL6 mRNA level in the FFA treated myotubes is also supposed as a sign of obese model in *in vitro*.

3.2. FFA increased TLR2 mRNA levels in C2C12 myotubes

The next is to examine if the increase of mRNA IL6 expression is associated with the upregulation of inflammatory controllers. We, hence, tested the expression level of TLR2 mRNA and saw that TLR2 mRNA was highly expressed in the C2C12 myotubes treated with FFA compared to that in the BSA treated control myotubes (Figure 3A and 3B). TLR2 has been reported as an important regulator of inflammation. The upregulation of TLR2 has been demonstrated in skeletal muscle of the HFD fed mice and this change was accompanied by increased IL6 level (Ahmad et al., 2012). As a consequence, the increase in IL6 mRNA level in the current study may prove the meaning of FFA in inducing skeletal muscle inflammation.

(A)

Exp	BSA 50 μM	FFA 500 μM
1	0.55	1.18
2	0.62	1.07
3	0.46	1.25
X	***0.54***	***1.17***
SE	***0.05***	***0.05***
P	***0.0009***	

(B)

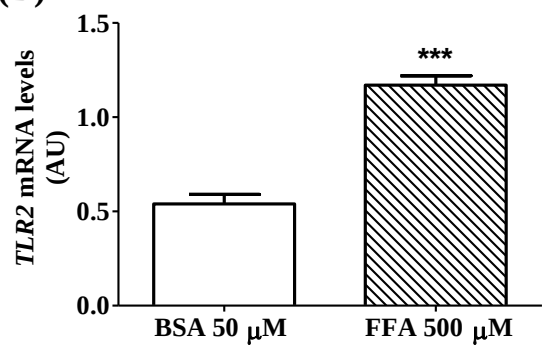

Figure 3. *FFA increased expression of TLR2 mRNA. C2C12 myotubes were established for 4 days, then treated with free fatty acid (FFA) at 500 μM for 24 h. Free fatty acid (palmitate) was prepared in ethanol containing bovine serum albumin (BSA, 10% w/v). Real time RT-PCR analysis for expression of TLR2 mRNA. Levels of mRNA were normalized to levels of β-actin mRNA. (A) data analysis of TLR2 mRNA levels. (B) comparison of TLR2 mRNA levels. Data represent results of three independent experiments. Values are X ± SE. ***P < 0.001 compared between the experimental group and the control group*

3.3. FFA increased TLR4 mRNA levels in C2C12 myotubes

It has been shown that expression of TLRs including TLR2 and TLR4 are both induced in skeletal muscle inflammatory response (Ahmad et al., 2012). Consistent with the aforementioned data of TLR2, the expression level of TLR4 mRNA, another regulator of inflammation, was also significantly higher in the myotubes treated with FFA than was that in the control myotubes (Figure 4A and 4B). This result contributed to the confirmation of the FFA potential in inducing an inflammatory response in the cultured skeletal muscle cells.

(A)

Exp	BSA 50 μM	FFA 500 μM
1	0.66	1.1
2	0.59	1.19
3	0.69	1.09
X	***0.65***	***1.13***
SE	***0.03***	***0.03***
P	***0.0004***	

(B)

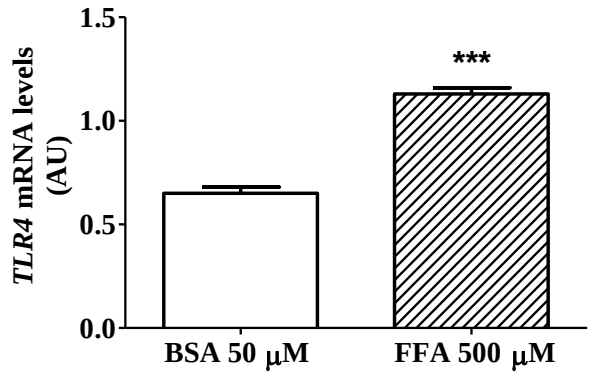

Figure 4. *FFA increased expression of TLR4 mRNA. C2C12 myotubes were established for 4 days, then treated with free fatty acid (FFA) at 500 μM for 24 h. Free fatty acid (palmitate) was prepared in ethanol containing bovine serum albumin (BSA, 10% w/v). Real time RT-PCR analysis for expression of TLR4 mRNA. Levels of mRNA were normalized to levels of β-actin mRNA. (A) data analysis of TLR4 mRNA levels. (B) comparison of TLR4 mRNA levels. Data represent results of three independent experiments. Values are X ± SE. ***P < 0.001 compared between the experimental group and the control group*

4. CONCLUSIONS

In conclusion, the present study manipulated to culture C2C12 myotube cells with FFA to mimic obesity microenvironment and shown that FFA strongly enhanced expression of inflammatory cytokine IL6 mRNA level in the myotubes. This effect was associated with the increases in the expression of inflammatory regulators TLR2 and TLR4 mRNAs. Therefore, these data suggest that the cultured C2C12 myotubes treated with FFA can be an *in vitro* model of obesity-related skeletal muscle dysfunction for further studies.

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AXÍT BÉO TỰ DO LÀM TĂNG SỰ BIỂU HIỆN CỦA CÁC MARKER GÂY VIÊM IL6, TLR2, TLR4 TRONG CÁC TẾ BÀO CƠ C2C12

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Tóm tắt: Các thông tin hiện tại cho thấy rằng tình trạng béo phì đang tăng lên như một đại dịch toàn cầu và nó thường đi kèm với sự tăng lên của nhiều rối loạn chuyển hóa. Có sự hiểu biết sâu sắc về các điều kiện liên quan đến béo phì sẽ là ý nghĩa để bảo vệ chống lại những rối loạn liên quan béo phì. Trong nghiên cứu hiện tại, chúng tôi đã nuôi các tế bào cơ C2C12 đến trưởng thành. Sau đó, các tế bào được nuôi với axít béo tự do (FFA) để mô phỏng lại tiểu môi trường trong béo phì. Kết quả cho thấy rằng mức biểu hiện của IL6 mRNA, một cytokine viêm điển hình, là cao hơn có ý nghĩa thống kê ở các tế bào cơ nuôi với FFA so với các tế bào cơ nuôi trong môi trường đối chứng. Phù hợp với số liệu này, mức mRNA của TLR2, một phân tử điều hòa quan trọng của phản ứng viêm, tăng lên đáng kể ở các tế bào nuôi với FFA so với nhóm đối chứng. Hơn nữa, mức mRNA của TLR4, một phân tử điều hòa chìa khóa khác của phản ứng viêm, cũng biểu hiện rất cao trong các tế bào nuôi với FFA so với các tế bào đối chứng. Những số liệu này minh họa rằng nuôi cấy tế bào cơ C2C12 với FFA có thể là một mô hình béo phì *in vitro* cho các nghiên cứu tiếp theo.

Từ khóa: Axít béo, tế bào cơ trưởng thành, viêm.

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