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EXPRESSION OF IL6 mRNA IS INDEPENDENT WITH EXPRESSION OF TLRS mRNA IN LIPOPOLYSACCHARIDE-TREATED MYOTUBES

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Abstract. Obesity-related metabolic disorders such as insulin resistance and type 2 diabetes are raising as critical health problems of the modern world. Obesity induces an increased plasma level of lipopolysaccharide (LPS) that contributing to system chronic inflammation. This response has been shown as a marked factor linking obesity and its related metabolic disorders. In the present study, we use LPS to treat C2C12 skeletal muscle cells and observe the expression of several inflammatory makers. Our results show that the expression of inflammatory cytokine IL6 mRNA is strongly induced in the LPS-treated C2C12 cells compared to the control cells. Surprisingly, the expression of the upper controller of inflammation TLR2 mRNA in the LPS-treated C2C12 cells is similar to that in the control cells. Consistent with this expression mRNA level of TLR4, another upper regulator of inflammation does not differ between the two group cells. Taken together, our current data suggest that the LPS induced expression of IL6 mRNA in C2C12 cells is not dependent on the regulation of neither TLR2 nor TLR4.

Keywords: Lipopolysaccharide, myotubes, inflammation.

1. Introduction

Raising of metabolic disorders such as insulin resistance, type 2 diabetes, liver hepatitis, and heart diseases is a crucial health issue in the world [1]. Interestingly, these metabolic dysfunctions are shown to be closely related to overweight and obesity which is usually induced by chronic high-calorie intake [2-3]. Several studies have indicated that obesity-related chronic inflammatory responses lead to many metabolic diseases. Notably, inflammation is characterized by an increased level of inflammatory cytokine interleukin 6 (IL6) which in turn induced glucose and lipid metabolic dysfunctions. These changes are linking to the aforementioned disorders [4-5]. Thus, an increased level of IL6 has been considered as a key sign of obesity and inflammation in both the *in vivo* and *in vitro* experimental models.

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Lipopolysaccharide (LPS), the intermediate metabolite, is strongly increased in the plasma of obese individuals [6]. It is worthy to note that, LPS has been used to induced chronic inflammatory models in mouse and cell lines (e.g., adipocytes). We, here, thus use LPS to treat C2C12 skeletal muscle cells to observe whether LPS induces skeletal muscle inflammation. Since obesity-related skeletal muscle inflammation is an important factor leading to system metabolic dysfunctions. Additionally, IL6 expression and its signaling in obese and inflammatory skeletal muscle cells have not been well elucidated yet. Therefore, the present study should be significant to reveal the mechanism linking obesity, inflammation, and metabolic diseases.

2. Content

2.1. Materials and methods

**** Cell Culture***

The primary myoblast cell line C2C12 was purchased from the American Type Culture Collection (ATCC, Manassas, USA). The C2C12 myoblasts (2.5×10^5cells/mL) were cultured 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, 100μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA), and 20 μg/mL gentamicin (Gibco). When the cells reached almost 100% confluence, the medium was switched to the differentiation medium consisting of DMEM plus 2% horse serum (Gibco), which then was changed after 2 days.

**** Treated cell with lipopolysaccharide (LPS)***

Lipopolysaccharide (LPS) was purchased from Sigma (USA). LPS was dissolved in water. After 4 days of differentiation, the matured muscle cells (myotubes) were incubated with 100 ng/mL LPS in serum-free DMEM for 24 h. The medium with no treatment was used as control of LPS-treated groups (Figure 1). After 24 h of the treatment, 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 shown as mean (X) ± standard error of the mean (SE).

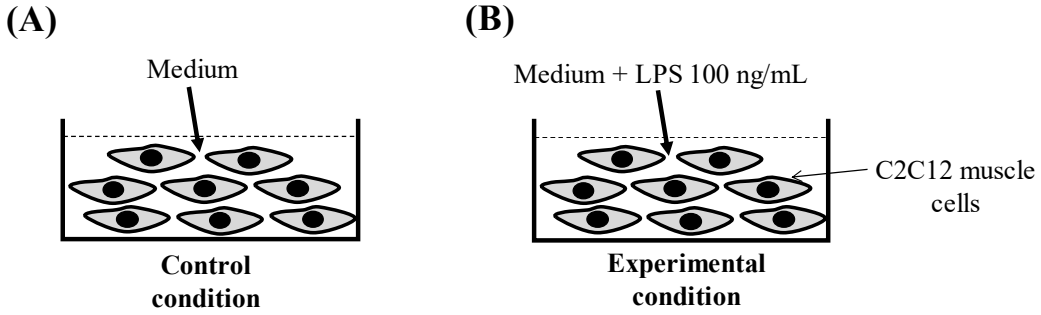

Figure 1. The experiment designs

C2C12 myotubes were established for 4 days in the dishes, then treated with or without lipopolysaccharide (LPS) at 100 ng/mL for 24 h. (A) the cells treated with the medium only using as the control group. (B) the cells treated with the medium supplemented with LPS 100 ng/mL using as the experimental group

**** Quantitative Real-Time PCR (qRT-PCR)***

Total RNA was extracted from the lysed cells with Trizol Reagent (Invitrogen). Two microgram aliquots of total RNA were reverse transcribed to cDNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA). The qRT-PCR amplification of the cDNA was done in duplicate with an SYBR premix Ex Taq kit (TaKaRa Bio Inc., Forster, CA, USA) using a Thermal Cycler Dice (TaKaRa Bio Inc., Japan). All reactions were carried out 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 RealTime System TP800 software (Takara Bio Inc.) and all values were normalized to the levels of the control gene *β-actin*. The primers used in the qRT-PCR amplification are listed in Table 1.

Table 1. List of mouse primers used for qRT-PCR analysis

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

**** Statistical Analysis***

The results were shown as means ± standard error of the mean (*SE*). Comparisons of variables were performed by using Student's t-test. Comparisons were considered to have differedsignificant when $P < 0.05$. The statistical analysis was conducted using Microsoft Excel Software.

2.2. Results and discussion

2.2.1. Lipopolysaccharide (LPS) increased expression level of IL6 mRNA in C2C12 myotubes

In the current study, the matured C2C12 skeletal muscle cells (myotubes) were cultured with LPS for 24 hours to observe the inflammatory response. As a result, the mRNA level of IL6, a key inflammatory cytokine, was significantly higher in the LPS-treated myotubes than that in the control medium-treated myotubes (Figure 2A and 2B). It is interesting to note that expression levels of IL6 mRNA and protein are markedly increased in the LPS-treated mice and this is accompanied by metabolic disorders [6]. However, previous studies have not shown the sources of IL6 induced by LPS treatments [6-7]. Thus, our current data demonstrate that increased expression of IL6 in skeletal muscle cells can contribute to obesity-related inflammation.

(A)

Exp	Medium	Medium + LPS 100 ng/mL
1	0.56	22.15
2	0.64	21.87
3	0.46	16.79
X	***0.55***	***20.27***
SE	***0.05***	***1.74***
P	***0.0003***	

(B)

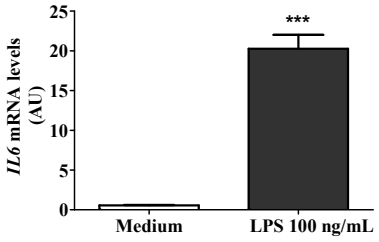

Figure 2. LPS increased expression of IL6 mRNA

*C2C12 myotubes were established for 4 days, then treated with or without lipopolysaccharide (LPS) at 100 ng/mL for 24 h. 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.*

2.2.2. LPS mildly affected the expression of TLR2 mRNA in C2C12 myotubes

To examine the signaling related to LPS-induced skeletal muscle inflammation, we tested the expression of toll like receptor 2 (TLR2) which is a key upper regulator of IL6. Unfortunately, the expression level of TLR2 mRNA was not significantly increased in the LPS-treated myotubes compared with that in the control cells (Figure 3A and 3B). However, recent researches have reported that LPS treatments enhance the expression of TLR2 and IL6 in mouse fat cells/adipocytes and that suppression of TLR2 led to reduced IL6 expression [8]. Thus, increased expression of IL6 in the LPS-treated myotubes is regulated by another regulator(s).

(A)

Exp	Medium	Medium + LPS 100 ng/mL
1	0.98	1.16
2	0.91	1.03
3	0.89	1.01
X	***0.93***	***1.07***
SE	***0.03***	***0.05***
P	***0.0616***	

(B)

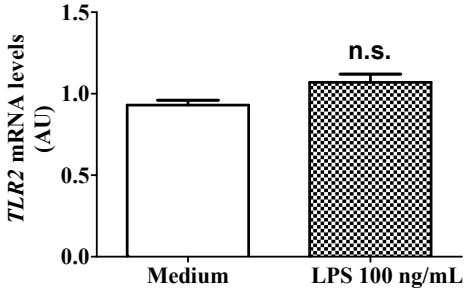

Figure 3. LPS had a mild effect on the expression of TLR2 mRNA

C2C12 myotubes were established for 4 days, then treated with or without lipopolysaccharide (LPS) at 100 ng/mL for 24 h. 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 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.

2.2.3. LPS had no effect in increased expression TLR4 mRNA levels in C2C12 myotubes

We then examined the expression of toll like receptor 4 (TLR4), another upper regulator of inflammatory cytokine IL6 [9], in the LPS-treated cells. Consistent with the above data, the expression level of TLR4 mRNA was similar between the LPS-treated myotubes and the control cells (Figure 4A and 4B). This data provides additional evidence to confirm that LPS-induced inflammation in skeletal muscle cells is independent on TLRs-related signaling.

(A)

Exp	Medium	Medium + LPS 100 ng/mL
1	0.95	0.93
2	0.92	0.89
3	0.97	0.94
X	***0.95***	***0.92***
SE	***0.01***	***0.02***
P	***0.2746***	

(B)

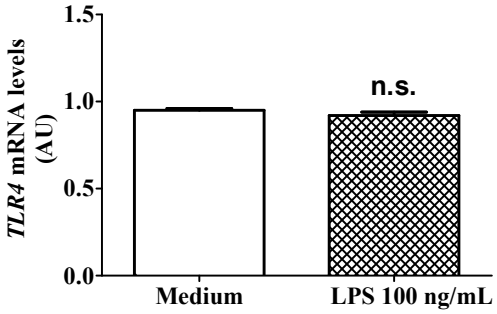

Figure 4. LPS had no effect on increased expression of TLR4 mRNA

C2C12 myotubes were established for 4 days, then treated with or without lipopolysaccharide (LPS) at 100 ng/mL for 24 h. 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 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.

List of abbreviations: *LPS: lipopolysaccharide; IL6: interleukin 6; TLR2: toll like receptor 2; TLR4: toll like receptor 4; β-actin: beta-actin; DMEM: Dulbecco's modified eagle medium*; *ATCC: American type culture collection*.

3. Conclusions

As a consequence, the present study used LPS to treat skeletal muscle cells to establish an obese inflammatory condition in skeletal muscle in vitro and observed that mRNA expression of inflammatory cytokine IL6 was significantly upregulated in the LPS-treated myotubes compared with that in the control medium-treated cells. However, mRNA expression of TLR2 and TLR4, key upper regulators of IL6, was not significantly differed between the experimental and control groups. Taken together, data of the current study demonstrated that LPS, a critical metabolite of obese individuals, strongly induces skeletal muscle cell inflammation and this effect is independent of TLRs signaling.

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