科技部補助專題研究計畫成果報告
期末報告

探討Gamma次亞麻油酸調控細菌內毒素Lipopolysaccharide誘發骨骼肌肉耗損之功效

計 畫 類 別 ：個別型計畫
計 畫 編 號 ：NSC 102-2320-B-040-001-
執 行 期 間 ：102年08月01日至103年07月31日
執 行 單 位 ：中山醫學大學營養學系（所）

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中 文 摘 要 ： Abstract

The ubiquitin-proteasome pathway (UPP) and autophagy-lysosomal pathway (ALP) are major protein degradation pathways that are activated during inflammatory conditions and have been proposed as a therapeutic target for preventing skeletal muscle loss. Recent reports indicate that lipopolysaccharide (LPS) may induce both UPP and ALP pathways through the activation of toll-like receptor4 (TLR4)/ nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs), suggesting that multiple signaling pathways mediate muscle wasting in inflammatory diseases. In this study, we tested the hypothesis that the anti-inflammatory 18-carbon polyunsaturated fatty acids (18-carbon PUFAs), including gamma-linolenic acid (GLA), linoleic acid (LA), and linolenic acid (LNA) would have the opposite effects of LPS-induced muscle wasting in C2C12myotubes and C57BL/6 mice. The results show that LA and LNA were less effective than GLA in inhibiting LPS-induced muscle wasting. The 18-carbon PUFAs inhibited LPS-induced LC3Ⅱ protein expression. Both GLA and LNA treatments significantly inhibited LPS-induced MuRF1 expression as well as ubiquitinated protein accumulation to prevent MyHC protein loss in C2C12 myotubes and gastrocnemius (GA) muscle. The acute stimulation of LPS-induced TLR4/NF-κB signaling transduction and c-Jun N-terminal Kinase (JNK) phosphorylation were significantly suppressed by 18-carbon PUFAs. Constitutively active IKKβ/NF-κB pathway could abolish the inhibitory effect of GLA on LPS-induced muscle wasting. Moreover, GLA also inhibited c-Src and JNK phosphorylation in chronic LPS-stimulation. In conclusion, our data suggested that in vivo and in vitro, GLA avoids LPS-induced muscle loss through inhibited TLR4/NF-κB/JNK signaling in the acute inflammation and decreased c-Src/JNK phosphorylation in the chronic inflammation.

中文關鍵詞： gamma 次亞麻油酸,肌肉耗損, lipopolysaccharide,

nuclear factor-κB

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英文關鍵詞： gamma linolenic acid, muscle wasting,

lipopolysaccharide, nuclear factor-κB

Title: Gamma-Linolenic Acid Prevents Lipopolysaccharide-Induced Muscle Wasting in both Acute and Chronic Inflammatory Responses

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Abstract

The ubiquitin-proteasome pathway (UPP) and autophagy-lysosomal pathway (ALP) are major protein degradation pathways that are activated during inflammatory conditions and have been proposed as a therapeutic target for preventing skeletal muscle loss. Recent reports indicate that lipopolysaccharide (LPS) may induce both UPP and ALP pathways through the activation of toll-like receptor4 (TLR4)/ nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs), suggesting that multiple signaling pathways mediate muscle wasting in inflammatory diseases. In this study, we tested the hypothesis that the anti-inflammatory 18-carbon polyunsaturated fatty acids (18-carbon PUFAs), including gamma-linolenic acid (GLA), linoleic acid (LA), and linolenic acid (LNA) would have the opposite effects of LPS-induced muscle wasting in C2C12myotubes and C57BL/6 mice. The results show that LA and LNA were less effective than GLA in inhibiting LPS-induced muscle wasting. The 18-carbon PUFAs inhibited LPS-induced LC3 II protein expression. Both GLA and LNA treatments significantly inhibited LPS-induced MuRF1 expression as well as ubiquitinated protein accumulation to prevent MyHC protein loss in C2C12 myotubes and gastrocnemius (GA) muscle. The acute stimulation of LPS-induced TLR4/NF-κB signaling transduction and c-Jun N-terminal Kinase (JNK) phosphorylation were significantly suppressed by 18-carbon PUFAs. Constitutively active IKKβ/NF-κB pathway could abolish the inhibitory effect of GLA on LPS-induced muscle wasting. Moreover, GLA also inhibited c-Src and JNK phosphorylation in chronic LPS-stimulation. In conclusion, our data suggested that *in vivo* and *in vitro*, GLA avoids LPS-induced muscle loss through inhibited TLR4/NF-κB/JNK signaling in the acute inflammation and decreased c-Src/JNK phosphorylation in the chronic inflammation.

Key words: gamma linolenic acid, muscle wasting, lipopolysaccharide, nuclear factor-κB, c-Jun N-terminal Kinase

1. Introduction

Maintenance of muscle mass is an important health issue. In several biological processes, such as cancer, cachexia (mainly due to the loss of lean body mass) is one of the crucial factors to cause the death. Muscle homeostasis is

controlled by the balance between protein synthesis and degradation [1].
(Muscle mass is controlled by the balance between protein synthesis and degradation)
In addition to cancer, muscle wasting is a major feature of the cachexia associated with inflammatory diseases such as sepsis, infection, cancer, diabetes, inflammatory bowel disease, arthritis, congestive heart failure (CHF), and chronic obstructive pulmonary disease (COPD), resulting in promoted degradation of muscle protein [2, 3]. Progressive of skeletal muscle wasting causes reduced physical activities which increase patient morbidity and mortality through disability, injury, and osteoporosis as well as impaired respiratory function [4, 5]. Therefore, prevention of muscle wasting is an important event to improve the quality of life.

Protein degradation contains at least two major cellular proteolytic systems in skeletal muscle, the ubiquitin-proteasome pathway (UPP) and the autophagy-lysosomal pathway (ALP), which involved in various forms of muscle wasting [6]. The UPP participate a large part of the degradation of myofibrillar proteins and most soluble proteins in skeletal muscle [7, 8].
(The UPP is thought to degrade myofibrillar proteins and most soluble proteins)
In acute quadriplegic myopathy, UPP system expressed muscle-specific E3 ligases muscle atrophy F-box (MAFbx) and muscle ring finger protein -1 (MuRF1), and caused a subacute muscle disorder characterized by generalized progressive muscle weakness and wasting that is mainly associated with a patient history of sepsis, multiple organ failure, surgery and intensive care admission [9].
(increased levels of MAFbx/atrogin-1 mRNA has been observed following acute quadriplegic myopathy, a subacute muscle disorder characterised by generalised progressive muscle weakness and atrophy that is predominantly associated with a patient history of sepsis, multiple organ failure, surgery and intensive care admission)
Moreover, in MuRF1 and MAFbx knockout mice, it appeared resistant to the effect of denervation-induced muscle wasting suggested that MuRF1 and MAFbx are critical to muscle protein loss [10]. Recent study showed that TGFβ and myostatin can bind to its cognate receptor, ActRⅡB, which induced the transmembrane activation of Smad (phosphorylation) and FoxO 1 and 3 (dephorsphorylation), as a consequence to increase the expression levels of MuRF1 and MAFbx, eventually cause the loss of muscle mass [11]. In addition to the UPP, the ALP has also shown to mediate physiological muscle wasting, including starvation and denervation, with the formation of autophagosome for removing cellular masses, damaged organelles, and/or proteins [12]. Although the UPP and ALP were firstly considered as independent pathways serving different function, accumulating evidence indicates that these two proteolytic systems can work in a cooperative manner to promote muscle wasting [13, 14]. (Although the ubiquitin-proteasome and autophagy were

initially considered as independent pathways serving distinct functions, accumulating evidence suggests that these two proteolytic systems can function in a cooperative manner to stimulate muscle wasting)

As a consequent, regulation and control of these two proteolytic systems activities is considered as a key target for therapeutic intervention in muscle wasting.

Lipopolysaccharide (LPS) is used to serve as a model system to induce acute and chronic inflammatory condition that causes a severe and rapid loss of body protein, much of which is originated from skeletal muscle [6].

(LPS is known to rapidly induce muscle protein loss within 24 h [Premer et al., 2002])

In acute inflammation, LPS rapidly stimulated the activation of toll-like receptor4 (TLR4), and its downstream signal nuclear factor-κB (NF-κB). NF-κB is a major coordinator of these two proteolytic systems, it increases the expression levels of muscle-specific E3 ligases as well as several autophagy-related genes and contributes to muscle weakness and damage in myositis [6, 15]. The supplement of eicosapentaenoic acid (EPA), but not linoleic acid (LA), inhibits the IκBα/NF-κB/MuRF1 pathway in C2C12 myotubes [16]. Moreover, the expression of the IκBα superrepressor (MISR) and IκB kinase β (IKKβ) knockout mice have been shown to ameliorate muscle wasting in response to denervation, unloading, and LPS-induced acute lung injury [17-19]. Conversely, overexpression of constitutively active IKKβ caused significant muscle wasting; it demonstrated that IKKβ is sufficient to induce an atrophy phenotype in rodent skeletal muscle [17]. Collectively, these evidences demonstrate that inhibition the TLR4/NF-κB signaling transduction in acute inflammation is necessary for the recovery of muscle wasting from disuse and illness.

The mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinase1/2 (ERK1/2) and c-Jun N-terminal kinase (JNK), and p38MAPK, which is involved in muscle catabolism and wasting, were also activated in inflammation [36]. Docosahexaenoic acid (DHA) can reduce MuRF1 expression in differentiated myotubes during co-culture with macrophages and diminish soleus muscle wasting in rat via the inhibition of JNK/AKT signaling pathway [21, 22]. In addition, IKK complex can activate autophagy in response to cellular starvation through JNK pathway [23]. Moreover, the expression of MuRF1 and MAFbx, which induced by LPS, TNF-α, and cigarette smoke were blunted by the treatment of curcumin, SB203580 (both are p38 MAPK inhibitors), and PD98059 (ERK inhibitor). It shows that LPS, TNF-α, and cigarette smoke can enhance MuRF1 and MAFbx expression by ERK/p38 MAPK dependent mechanism and lead to muscle wasting [24-26].

In addition to acute inflammation, LPS can also induce the chronic inflammatory response through the activation of JNK and cause muscle wasting and weakness. And this chronic inflammatory can be inhibited by the mono- and polyunsaturated FA [27, 28]. Recent evidence indicates that following the TLR4 receptor-mediated responses, prolonged LPS or saturated FA treatment, can also activate c-Src and JNK cascades [27]. Using LPS-induced inflammatory response in C2C12 myotubes and C57BL/6 mice can serve as an acute and chronic model system to test whether 18-carbon polyunsaturated fatty acids (18-carbon PUFAs) such as gamma-linolenic acid (GLA), linoleic acid (LA), and linolenic acid (LNA) (in addition to DHA and EPA) can also inhibit the inflammation response in both acute and chronic situation.

Gamma-linolenic acid (GLA) is an 18-carbon PUFA containing three double bonds, which is found mostly in the plant seed oils of borage, evening primrose, black currant, and hemp. GLA can be synthesized from LA by the catalization of delta-6-desaturase enzyme. However, lacking sufficient delta-6-desaturase activity to synthesize GLA, has been reported in various physiologic and pathophysiologic states, including aging, nutrient deficiency, smoking, and diabetes. Works from several laboratories have shown that supplementation with GLA can overcome the decreased enzymatic activity of delta-6-desaturase and lesen the signs and symptoms of cancer and inflammatory diseases such as sepsis, atherosclerosis, rheumatoid arthritis, acute respiratory distress syndrome [29]. Our previous study found that GLA reduced LPS-induced the pro-inflammatory mediator production via MAPKs/NF-κB signaling pathway in RAW264.7 macrophages, but the effect of GLA in preventing muscle wasting remains unclear [30]. In this study, we show that GLA effectively against LPS-induced TLR4/NF-κB/JNK and c-Src/JNK pathway, blocks the upregulation of MuRF1 and LC3 Ⅱ, and reverse the loss of muscle mass *in vitro* and *in vivo*.

2. Materials and Methods

2.1 **Materials.**

The mouse C2C12 myoblast was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Fetal bovine serum (FBS) was from HyClone (Logan, UT, USA). DMEM, penicillin–streptomycin solution, and M-MMLV reverse transcriptase were from Gibco Laboratory (Grand Island, NY, USA). TRIzol reagent, OPTI media and lipofetamine^{TM2000} were from Invitrogen (Carlsbad, CA, USA); and GLA, LA and LNA were from NuChek Prep, Inc. (Elysian, MN, USA); borage oil and lipopolysaccharide were obtained from Sigma (St. Louis, MO, USA). The antibodies against p65, GAPDH, IκB-α, phospho-IκB-α (Ser32/36),

phospho-IKKα/β (Ser176), IKKα/IKKβ, MuRF1, MAFbx, Ub, c-Src, and phosphor-c-Src were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); antibody against LC3 was from MBL (San Diego, CA, USA); antibody against MF20 was from eBioscience (San Diego, CA, USA); antibody against p65 was from GeneTex (Alton Pkwy Irvine, CA, USA) ; and antibody against PARP was from Roche (Basel, Switzerland, Switzerland). Oligonucleotide primer sequences of MuRF1and MAFbx for real-time quantitative reverse transcriptase-PCR and biotin-labeled and unlabeled double-stranded NF-κB consensus oligonucleotides and a mutant double-stranded NF-κB oligonucleotide for electrophoretic mobility shift assay (EMSA) were synthesised by MDBio Inc. (Taipei, Taiwan). The probe#31 was selected by using Roche Diagnostics (Basel, Switzerland, Switzerland). The IKK-2 WT (plasmid # 11103) and IKK-2 S177E S181E (IKK-2 SE) (plasmid # 11105) were from Addgene (Cambridge, MA); and pNFkB-Luciferase plasmid was from Stratagene Inc. (La Jolla, CA, USA). Luciferase Assay System, β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer, and pSV-β-galactosidase control vector were from Promega (Dane County, Wisconsin, USA). All other chemicals and reagents were analytical grade and were obtained commercially.

2.2 **Cell Cultures and Treatments**

The mouse C2C12 myoblasts were cultured in growth medium (DMEM supplemented with 10% FBS) at 37°C under 5% CO2. At 80% confluence, the myoblasts were switched to differentiation medium (DMEM supplemented with 2% horse serum; 2%HS-DMEM) for 6 days to differentiate into myotubes, as described previously [25]. Differentiated C2C12 myotubes were incubated in DMEM with the supplement of 5% HS in the presence of 100 μM GLA, LA, or LNA for 12 h followed by incubation with/without 100 ng/mL LPS as indicated in the Fig. legends.

2.3 **Animals and Treatments**

Five-week-old male C57BL/6 mice were obtained from the National Health Research Institute (Tainan, Taiwan). The animals were fed a standard mice diet and were reached to about 20g body weight, they were divided into normal (N), LPS-treated (LPS), LPS plus coconuts oil-treated (LCO), LPS plus soybean oil-treated (LSO), and LPS plus borage oil-treated (LBO) groups (n = 5). Mice were housed in plastic cages with the thermal control at 23 ± 1∘C and 60 ± 5% relative humidity in a 12-hour light and dark cycle. Food and drinking water were available ad libitum. A dose of 150 μl per mice of borage oil (BO), soybean oil (SO), and coconuts oil (CO) was orally administered to each mice every other day for 28 days. After 28 days, intraperitoneal (i.p.) injection of LPS (1 mg/kg in 150 μl) or an equal volume of vehicle (PBS) was administered. At the indicated times, gastrocnemius (GA) muscles were collected from the mice immediately after rapid euthanization. Above procedure has received

ethical approval from the institutional animal ethics committee of Chung Shan Medical University, Taichung, Taiwan.

2.4 **Cell Viability Assay**

Cell viability was assessed by the 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl-tetrazolium bromide assay. C2C12 myoblasts were incubated in 2% HS-DMEM to differentiate into myotubes and supplement with GLA, LA, or LNA to a final concentration of 100 μM for 12 h. The cells were further incubated in DMEM supplemented with 5% horse serum (5% HS-DMEM) with/without LPS (100 ng/ml) for another 24 h. The cell viability assay was performed according to our previous report [31].

2.5 **RNA Isolation and Quantitative Real-Time PCR**

RNA isolation was performed according to our previous study [31]. Target genes were amplified by specific primers for mMuRF1 (forward: 5'-GTGTACGGCCTGCAGAGG-3', reverse: 5'-CTTCGTGTTCCTTGCACATC-3') or mMAFbx (forward: 5'-GGTGGCACTGGTTTAGAGGA-3', reverse: 5'-ATCGGCTCTTCCGTTGAAA-3') and probe #31were mixed with universal master mix to a final volume of 10 μl and were amplification of DNA by using ABI StepOne system (Foster City, CA, USA). The amplification of β-actin was used as an internal control to adjust the threshold cycles (Ct) and transformed with△△Ct method to calculate the relative quantification of target gene expressions [32].

2.6 **Western Blot Analysis**

GA muscle was homogenized (1 : 5, w/v) in an ice-cold potassium phosphate buffer (pH 7.4) containing 7.4 mM K_2HPO_4, 2.6 mMKH_2PO_4, 1.15%KCl, and 1mM phenylmethylsulfonyl fluoride (PMSF). After each experiment, cultured cells were washed twice with PBS and were harvested with 150 μL of lysis buffer containing 10 mM Tris-HCl, 5 mM EDTA, 0.2 mM PMSF, and 20 μg/mL aprotinin, pH 7.4. Protein contents in each sample were quantified by Lowry assay [33]. Equal amounts of proteins were denatured and separated on SDS polyacrylamide gels and then transferred to polyvinylidene difluoride membranes (PVDF) (millipore, Boston, MA, USA). The membranes were blocked with 5% nonfat dry milk in a buffer containing 10 mM Tris-HCl and 100 mM NaCl, pH 7.5, at 4°C overnight. The blots were then incubated sequentially with primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Immunoreactive protein bands were developed by using enhanced chemiluminescence kits (millipore, Boston, MA, USA) and were then quantitated with an AlphaImager 2000 (Alpha Innotech Corporation).

2.7 **Extraction of Nuclear Proteins and Electrophoretic Mobility Shift Assay (EMSA)**

Nuclear proteins of GA muscle were extracted as described by Chen et al. [34]. The

C2C12 myotubes were scraped with ice-cold PBS and centrifuged. The LightShift Chemiluminescent EMSA kit (Pierce Chemical) and synthetic biotin-labeled, double-stranded consensus oligonucleotides of NF-κB consensus oligonucleotides (forward: 50-AGTTGAGGGGACTTTCCCAGGC-30; reverse: 50-GCCTGGGAAAGTCCCCTCAACT-30) was used to measure NF-κB nuclear protein DNA-binding activity. Nuclear proteins, poly (dI-dC), and biotin-labeled double-stranded oligonucleotides of NF-κB were mixed with binding buffer to a final volume of 20 μl and were incubated at room temperature for 30 min. The competitions between nonlabeled double-stranded oligonucleotides of NF-κB as well as a mutant double-stranded NF-κB oligonucleotide (5′-AGTTGAGGCGACTTTCCCAGGC-3′) were also performed to confirm the protein binding specificity, respectively. The nuclear protein-DNA complex was separated by electrophoresis on a 8% Tris/Boric acid/EDTA-polyacrylamide gel and was then electrotransferred to a nylon membrane (Hybond™-N+, Amersham Pharmacia Biotech Inc, Piscataway, NJ). The membranes were treated with streptavidin-horseradish peroxidase, and the nuclear protein-DNA bands were exposed by using a SuperSignal West Pico kit (Pierce Chemical Co.).

2.8 **Plasmids and Transiently Transfection**

At 50-60% confluence, C2C12 myoblasts were transiently transfected with IKK-2 WT or an IKK-2 SE vector using a lipofectamine reagent for 24 h. The transfected myoblasts were differentiated in 2% HS-DMEM for 6 days and were then treated as shown in the Fig. legends.

2.9 Reporter Gene Assay

C2C12 myotube were transiently transfected with a 3X NF-κB luciferase reporter vector or were transiently cotransfected with a 3X NF-κB luciferase reporter vector and either an IKK-2 WT or an IKK-2 SE vector as indicated. Twenty-four hours after transfection, cells were differentiated in 2% HS-DMEM for 6 days and were then treated as indicated in the Fig. legends. Supernatants of the cell lysates were applied to measure the luciferase and b-galactosidase activities by Luciferase Assay System and b-Galactosidase Enzyme Assay System with Reporter Lysis Buffer, respectively.

2.10 **Measurement of Myotubes Diameter**

Myotubes diameter was measured as described in Menconi et al. [35] with modifications. Briefly, myotube cultures were photographed under a phase-contrast microscope with 100 fold amplification after indicated treatment. The diameters were measured in a total of 100 myotubes over 10 random choice fields by using AlphaImager 2000 (Alpha Innotech Corporation). The myotubes were measured at the both ends and middle along the cell or tissues. The measurements were conducted in a masked fashion, and the results were expressed as a fold of the diameter in the

control/normal group.

2.11 **Histology**

The mice were killed and the muscle tissues were removed and fixed in 10%formalin solution for 24 h at room temperature (RT). The samples were processed by tissue processor (Leica, ASP300 S). The tissues were series dehydrated by alcohol, cleared in xylene, and impregnated with liquid paraffin wax at 56 °C. Tissue blocks were sectioned with microtome to the thickness of 5 μm (Leica, 2235). The sections were floated on a water bath with a temperature of 40 °C, and picked up on special coated glass slides (Superfrost plus, Menzel Glasser, Germany). Sections were deparaffinized and rehydrated prior for hematoxylin and eosin (H&E) staining.

2.12 **Immunohistochemistry (IHC)**

Immunohistochemistry was performed on 3 μm-thick sections of formalin-fixed paraffin-embedded tissue. The slide was washed with xylene for 3 times, each time for 5 minutes to remove the paraffin, then washed with graded series of ethanol (100%, 100%, 95%) three times, each time for 5 minutes to rehydrate again and finally dH2O. IHC was performed with a BenchMark IHC staining system (Vision BioSystems, San Francisco, CA). Sections were stained by an antibody against the p50 subunit of NF-κB (sc-114), a rabbit polyclonal antibody against the active form of the p50 subunit of NF-κB (specifically against the NLS) (Santa Cruz, CA, USA). The titer of p50 NLS antibody was 1:200 dilutions. The human breast cancer was used as positive control. Anti-rabbit Envision (DAKO, Santa Barbara, CA) was used as secondary antibody. The slides were counterstained with hematoxylin (Ven- tana Medical Systems). Immunohistochemical staining was analyzed by using an upright fluorescence microscope in the Instrument Center of Chung Shan Medical University, which is supported by National Science Council, Ministry of Education and Chung Shan Medical University.

2.13 Statistical Analysis

Data are expressed as means ± S.D. from at least three independent experiments. Data were evaluated for statistical significance by one-way ANOVA and Tukey's multiple-range test by Statistical Analysis System. A value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1 18-carbon PUFAs inhibit LPS-induced muscle wasting of C2C12 myotubes

In previously reported that LPS mainly reduces the protein levels of myosin heavy chain (MyHC), and induces wasting in cultured C2C12 myotubes [6]. To investigate whether LPS-induced MyHC loss in C2C12 myotubes can be inhibited by 18-carbon PUFAs. C2C12 myotubes were pre-incubated with 100 μM GLA, LA,

and LNA for 12 h followed by incubation with/without 100 ng/mL LPS for further 24 h. Following incubation, myotubes were photographed and diameters were measured as described in Materials and Methods. The results as shown in Fig. 1A, 18-carbon PUFAs could reverse LPS-induced muscle wasting by 1.8-, 1.7-, and 1.9- fold, respectively. Compared with methanol vehicle control, exposure of C2C12 myotubes to LPS or to LPS with 18-carbon PUFAs did not have adverse effect on the cell viability at the test concentrations for 24 h (P<.05, Fig. 1B). Western blot analysis reveals that the level of MyHC was decreased by 100 ng/ml LPS treatment, which was recovered from 18-carbon PUFAs treatments (P<.05, Fig. 1C). These results demonstrate that 18-carbon PUFAs can inhibit LPS-induced muscle wasting of C2C12 by preventing the loss of MyHC.

3.2 GLA and LNA reversed LPS-induced MuRF1 and LC3 Ⅱ expression, but LA only inhibited LPS-induced LC3 Ⅱ expression in C2C12 myotubes

Two systems including UPP and ALP may involve in MyHC degradation. The proteasome activity has no different under the treatment of 18-carbon PUFAs (data not shown). In UPP system, the mRNA and protein level of MuRF1 was increase by LPS treatment, which was suppressed by GLA and LNA but not LA treatment. Although GLA and LNA inhibit LPS-induced MuRF1 expression, the MAFbx expression was unaffected (P<.05, Fig. 2A and B). Autophagsome marker LC3 Ⅱ was also determined by western bolt analysis. LPS treatment increase LC3 Ⅱ protein level, which was repressed by 18-carbon PUFAs pretreatments (P<.05, Fig. 2C). Ubiquitination was examined by anti-Ub antibody and shows in LPS induced the accumulation of ubiquitinated protein, which was reduced by 18-carbon PUFAs pretreatments (P<.05, Fig. 2D). These results represented that the recovery of MyHC may due to the inhibition of UPP and ALP by 18-carbon PUFAs.

3.3 18-carbon PUFAs reduced LPS stimulated TLR4/NF-κB/ JNK and c-Src/JNK signaling in C2C12 myotubes

To investigated the link between PUFAs and proteolytic systems. Cellular transduction signal of TLR4/NF-κB/JNK (within 4 hours) and c-Src/JNK (8 hours) activation were examined. The results show that LPS induced-TLR4 as well as IKKβ and IκB-α phosphorylation were downregulated by 18-carbon PUFAs pretreatments compared with control (P<.05, Fig. 3A and B). JNK phosphorylation was also reduced by the pretreatment of GLA, LA, or LNA (P<.05, Fig. 3B). Translocation of p65 into nuclear was determined by protein analysis with nuclear extract, which reveals p65 was translocated into nuclear under the treatment of LPS and largely resided on the cytoplasm by GLA, LA, and LNA pretreatment (P<.05, Fig.

3C). To investigate the binding efficiency of p65 to its target gene, EMSA analysis was performed. The results also show 18-carbon PUFAs pretreatments will prevent the LPS induced NF-κB binding activity to its response element (P<.05, Fig. 3D). The GLA, LA, or LNA pretreatments also declined the LPS induced NF-κB reporter gene expression (P<.05, Fig. 3E).

Under the pretreatments of 18-carbon PUFAs, we also examined the role of c-Src in LPS-induced JNK activation. C2C12 myotubes were treated with LPS for 8 hr, and total and phosphorylation of c-Src and JNK were assessed by Western blot with an antibody that recognizes Tyr418 and Thr183/Tyr185 phosphorylation. As shown in Fig 3F, pretreatment with 100 μM GLA, LA, or LNA prevent LPS-induced c-Src and JNK phosphorylation. In brief, these results demonstrated that LPS may induce proteolytic systems activity by TLR4/NF-κB/JNK (within 4 hours) and c-Src/JNK (within 8 hours), and the activation can be suppressed by the pretreatments of 18-carbon PUFAs in short and long time.

3.4 The inhibition effects of GLA were abolished by constitutively activated NF-κB

To further confirm that the inhibition of the UPP and ALP by 18-carbon PUFAs are mediated via the suppression of IKK/NF-κB. A constitutively activated IKK-2 SE, which mimic the phosphorylation state of IKK, were expressed by introducing IKK-2 SE vector into C2C12 myotubes. The overexpression of IKK-2 SE in C2C12 myotubes the LPS-induced in protein phosphorylation levels of endogenous p65 and NF-κB binding activity to its response element were further increased (3- and 4-fold, respectively) (P<.05, Fig. 4A and B), as compared with control IKK-2 WT (P<.05, Fig. 4A and B). As transected with IKK-2 SE, pretreatment with GLA shows no reduction in the levels of MuRF1 and LC3 II protein as well as reversed MyHC loss in C2C12 myotubes (P<.05, Fig. 4C and D). The effect of LA and LNA are less clear, it still maintained the degredation of LCIII and MyHC loss under the expression of IKK-2 SE. These results demonstrated that constitutively activated mutant IKKβ abolished inhibitory effect GLA in the MuRF1as well as LC3 II protein expression on LPS-induced C2C12 myotubes, which proved that IKKβ/NF-κB-dependent pathway served as one of the possible mechanism of muscle wasting in C2C12 myotubes.

3.5 Borage oil (rich in GLA) reduced LPS-induced muscle wasting in C57BL/6 mice model

To examine LPS-induced muscle wasting was inhibited by fatty acids in animal model. C57BL/6 mice were administrated of borage oil (150 μl/mice, rich

in GLA) in diet every other day for 28 days prior to LPS injection. Fig. 5A is the H&E staining of GA muscle sections, its shows the muscle wasting in response to LPS. The muscle diameters decrease 1.93-fold as compared to control, which was prevented from the Supplement of borage oil (BO) but not from coconuts oil (CO) and soybean oil (SO). In cells, the LPS is known to induce muscle protein loss by TLR4/NF-κB/JNK and c-Src/JNK signaling transduction. To determine whether CO, SO, and BO also prevented theLPS-induced- TLR4/NF-κB/JNK and c-Src/JNK activation in animal model, the western analysis were performed. In Fig. 5B, it demonstrated that the upregulation of TLR4, phsphorylation of IKK-β, IκB-α and reducing of total IκB-α protein levels, which leading to the enhancement of p65 translocated into nuclear by LPS, were totally diminished by BO and SO but not CO. Similarly, the BO pretreatment also blocked LPS-induced c-Src and JNK phosphorylation (P<.05, Fig. 5B).

Immunohistochemistry for NF-κB p50 shows LPS induced the level of NF-κB p50 translocated into nucleus as compared to control (P<.05, Fig. 5C). Supplement with BO will decrease the translocation effect, while CO and SO groups were not (P<.05, Fig. 5C). The real-time PCR analysis of MuRF1 and MAFbx mRNA expression shows that LPS administration induced a 23- and 23-fold in GA muscle of C57BL/6 mice, respectively (P<.05, Fig. 5D). Pretreatment with BO (but not CO and SO), the MuRF1 and MAFbx mRNA expression were reduced to 11- and 10-fold, respectively, preventing the mouse muscle protein degradation induced by LPS (P<.05, Fig. 5D).

Western blot analysis is also consistent with the results of previous RNA studies (P<.05, Fig. 5E). Since LPS has been shown to upregulate LC3 Ⅱ protein level and ubiquitinated protein accumulation in the GA muscle [4, 6], we conducted Western blot analysis to evaluate the effect of CO, SO, and BO to inhibit the LPS upregulated LC3 Ⅱ protein level and LPS activated ubiquitinated protein accumulation at 18 h. The results shows that BO can decrease both LPS stimulation of LC3 Ⅱ protein level and ubiquitinated protein accumulation; while CO and SO groups can only inhibit LC3 Ⅱ protein level, without reducing ubiquitinated protein accumulation (P<.05, Fig. 5E). In conclusion, these data suggest that BO ameliorated LPS-induced C57BL/6 mice muscle wasting through the inhibition of TLR4/NF-κB/JNK and c-Src/JNK signaling transduction to inactivation of UPP and ALP system.

4. Discussion

Inflammation is a critical factor in many human diseases, including sepsis, poliomyelitis, diabetes, cancer, renal failure, or pulmonary obstruction, that can increase protein degradation eventually leading to muscle wasting [36]. LPS, a

component of bacterial wall and commonly used inducer of the myotubes, acts via TLR4 leading to the activation of NF-κB and MAP kinase cascades. These events triggered its downstream proteolytic systems, the UPP and ALP, to enhance muscle wasting. In the TLR4 knockout mice, these effect were abolished, which confirms that TLR4 is indeed the key mediator of LPS-induced muscle degradation in acute inflammatory response [6]. In our data, 18-carbon PUFAs prevented LPS-induced TLR4 protein expression with a short time and downregulated MuRF1 and LC3 Ⅱ expression in C2C12 myotubes (Fig. 2A, B, C, and 3A). Moreover, saturated FA has been demonstrated to elicit TLR4-dependent responses in multiple cell types [37-39]. For example, under the treatment of coconut oil (CO, rich in lauric acid, C12:0), the LPS induced inflammation via TLR4 will be further activated through NF-κB in RAW264.7 macrophages [40, 41]. By contrast, several omega-3 and omega-6 PUFAs attenuate the lauric acid-stimulated LPS response via effects at the expression level of TLR4 and inflammatory mediator in RAW246.7 macrophages and 293T cells [40, 42]. Similarity, our data also confirm that LPS-induced TLR4 and LC3 Ⅱ protein expression was reduced in GA muscle of mice fed a SO and BO diet. Moreover, BO treatment prevents LPS-induced MuRF1 and MFAbx expression in GA muscle, demonstrating that GLA can reduce LPS-induced TLR4, E3 ligases and LC3 Ⅱ protein expression (Fig. 5B, D, and E).

The essential role of the IKKβ/NF-κB pathway in the muscle wasting has been convincingly demonstrated by a previous study, and disruption of IKKβ/NF-κB activation has been shown to delay or prevent muscle wasting [17]. Recent studies showed that activation of NF-κB in muscle-specific transgenic expression of activated IKK (MIKK) mice have been shown to induce significant wasting via expression of the muscle specific E3 ligase MuRF1, but not MAFbx [17]. Huang et al. [16] recently reported that with EPA inhibited LPS-stimulated NF-κB activation by blocking the activity of IKKβ kinase and IκBα degradation to prevent muscle wasting. Similarly with our data, the treatment of 18-carbon PUFAs can inhibit the upstream IKK and IκBα phosphorylation, and prevent the IκBα degradation under LPS-induced condition (Fig. 3B and 5B). In addition, we found that LPS-induced nuclear translocation of p65 and its transcription activity for target gene were attenuated in response to GLA, LA, and LNA pretreatment (Fig. 3C, D, and E). Furthermore, as shown in Fig. 5A and 5B, in the experiment of constitutively activation of IKK-β, it shows that GLA cannot inhibit LPS-induced MuRF1 expression in C2C12 myotubes. In addition, GLA also cannot reduce LPS-stimulated LC3 Ⅱ expression in C2C12 myotubes. This provides evidence that GLA antagonizes muscle wasting in LPS-activated C2C12 myotubes and GA muscle by attenuating the upstream of IKK-β in the acute inflammatory condition.

The prolonged treatment of LPS, in both cell and animal model will create a chronic inflammation situation. It is cause by the activation of c-Src and its downstream signal JNK. The JNK belong to the MAPKs group and is activated by physical stress and receptor-mediated mechanisms such as TNF receptor 1 (TNFR1) and TLR 2 and 4 [43]. A recent study by Holzer et al. [27] described that long-chain SFA induced c-Src clustering within membrane subdomains, leading to the long-term JNK activation, whereas unsaturated FA such as palmitoleic acid (MUFA) and EPA (PUFA) are the suppressor of the JNK activation. For example, it is reported that DHA improved muscle wasting via inhibiting of JNK/AKT signaling pathway to reduce MuRF1 expression in differentiation myotubes during co-culture with macrophages and atrophy soleus in rat [21, 22], that is indicated by down-regulating of JNK activation is a key role to prevent and delay muscle wasting in chronic inflammation. Our findings are in agreement with prevention of LPS-induced c-Src and JNK phosphorylation by GLA (BO) treatment in C2C12 myotubes and GA muscle (Fig 3F and 5B), but not CO and LA (SO) treatment in chronic inflammation. These results strongly suggest that the GLA inhibition of LPS-induced muscle wasting is through the c-Src/JNK mediated signaling pathway.

In the present study, our findings are schematically presented in Fig. 6. In C2C12 myotubes and GA muscle, GLA, more potent than LA and LNA, diminished LPS-induced MuRF1 and LC3 Ⅱ expression via TLR4/NF-κB /JNK signaling in the acute inflammation. In addition, LA and LNA were less efficient in inhibiting LPS-induced long-term c-Src activated as well as JNK phosphorylation in the chronic inflammation to prevent MyHC protein loss. Taken together, these results support the role of GLA in blocking LPS-induced muscle degradation and suggest its potential usefulness in the clinical intervention of LPS-stimulated muscle wasting.

5. Legends

Fig 1. The 18-carbonPUFAs prevents LPS-induced C2C12 myotubes wasting. Cells were pretreated with 100 μM GLA, LA, or LNA for 12 h and then were treated with either vehicle control or 100 ng/ml LPS for 24 h. (A) Myotube diameter was observed by using a phase-contrast microscope, at 100 × magnification to evaluate myotube wasting. Scale bars = 100μm. (B) Cell viability was measured by using the MTT assay. (C) MyHC content in cell lysate was evaluated by Western blot analysis. Values are the mean ± SD, n = 3. Values not sharing the same letter are significantly different ($P < 0.05$).

Fig 2. Involvement of UPP and ALP in 18-carbon PUFAs prevents LPS-induced C2C12 myotubes wasting. C2C12 myotubes were pretreated with 100 μM GLA, LA,

or LNA for 12 h and then were treated with either vehicle control or 100 ng/ml LPS for 8 h. The mRNA and protein levels of MuRF1 and MAFbx were measured by Real-time PCR (A) and Western blot (B), respectively. Cells were pretreated with 100 μM GLA, LA, or LNA for 12 h follow by stimulation with either vehicle control or 100 ng/mL LPS for LC3 Ⅱ protein level at 8h (C) and ubiquitin protein level at 24h (D) were identified by Western blot. Values are the mean ± SD, n = 3. Values not sharing the same letter are significantly different ($P < 0.05$).

Fig 3. Role of TLR4/NF-κB/JNK and c-Src/JNK signaling in 18-carbon PUFAs prevents LPS-induced C2C12 myotubes wasting.
C2C12 myotubes were preincubated with 100 μM GLA, LA, or LNA for 12 h and then were treated with either vehicle control or 100 ng/ml for (A) TLR4 protein at 1 h and (B) IKKβ, IκB-α as well as JNK protein in the cytosolic factions at 2 h. Western blot analysis was used to measure the both for recognized native and phosphorylated form. (C) Nuclear extracts were used for Western blot analysis of p65. (D) Aliquots of nuclear extracts were used for EMSA to determine the interaction between NF-κB and its response element. (E) C2C12 myotubes were treated with GLA, LA, or LNA (100 μM) for 12 h follow by stimulation with LPS (100 ng/ml) for 4 h. Cells were harvested and assayed the luciferase and β-galactosidase activity, respectively. (F) Cell were pretreated with 100 μM GLA, LA, or LNA for 12 h and then incubated with either vehicle control or 100 ng/ml LPS for 8 h. Aliquots of total protein were used for Western blot analysis by specific antibodies included anti-c-Src and JNK antibodies, both for recognized native and phosphorylated form. Protein expression is shown as a decrease in the normalized phosphorylation in the treated cells relative to the LPS. Values are the mean ± SD, n = 3. Values not sharing the same letter are significantly different ($P < 0.05$).

Fig 4. GLA prevents LPS-induced muscle wasting depends on IKKβ/NF-κB pathway in C2C12 myotubes. Cells were transiently transfected with IKK-2 WT and IKK-2 SE for 24 h and switched to 2% HS-DMEM for 6 days. Cells were pretreated with 100 μM GLA, LA, or LNA for 12 h and incubated with either vehicle control or 100 ng/ml LPS for the next 2 h. (A) Aliquots of total protein and nuclear extracts were used for protein analysis of IKK, IκB-α, and p65, antibodies both for recognized native and phosphorylated form were used. Protein expression is shown as an increase in the normalized phosphorylation in the treated cells relative to the IKK-2 WT control. (B) The method is the same as (A), but the treatment of LPS was for 4h. The cells were harvested and determined by Luciferase Assay System. Cells were pretreated with 100 μM GLA, LA, or LNA for 12 h, then incubated with/without 100

ng/ml LPS for MuRF1, MAFbx and LC3 Ⅱ protein levels at 8 h (C) and MyHC protein level at 24 h (D). Values are the mean ± SD, n = 3. Values not sharing the same letter are significantly different ($P < 0.05$).

Fig 5. Feeding borage oil prevents LPS-induced muscle wasting through TLR4/NF-κB/JNK and c-Src/JNK signaling in C57BL/6 mice.
After CO, SO, BO (150 μl/mice) or PBS administration for 28 days, mice were i.p. injected with LPS (1mg/kg) for 18h. (A) Graph represented the longitudinal sections of H&E-stained GA muscle (400X magnification, scale bar = 100 μm). (B) Western analysis of GA muscle lysates were performed by anti-TLR4, anti- IKKα/β, anti-IκBα, anti-c-Src, anti-JNK, and anti-p65 antibodies, both antibodies for recognized native and phosphorylated form were used. Protein expression is shown as a decrease in the normalized phosphorylation in the GA muscle relative to the LCO. (C) Immunohistochemistry images presented with the active NF-κB p50 antibody in GA muscle sections. (D) Real-time PCR, total RNA was isolated from g GA muscle with specific MuRF1 and MAFbx primers to examine the RNA expression. The β-actin was used as internal control. (E) Western blot of GA muscle lysates were proceeded with anti-MyHC, anti-MuRF1, anti-MAFbx, anti- LC3 Ⅱ, and anti-Ub antibodies. Values are the mean ± SD, n = 5-10. Values not sharing the same letter are significantly different ($P < 0.05$). Abbreviates is presented as follows: N: normal; LPS: lipopolysaccharide; LCO: lipopolysaccharide + coconuts oil; LSO: lipopolysaccharide + soybean oil; LBO: lipopolysaccharide + borage oil; and PS: positive staining.

Fig. 6. Schematic outline of GLA prevents LPS-induced muscle wasting.
Model showing the pathways that GLA was more potent than LA and LNA to diminish LPS-induced MuRF1 and LC3 Ⅱ expression, TLR4-mediated IKK activation, IκB-α phosphorylation and degradation, p65 nuclear translocation, and DNA binding activity of NF-κB in the acute inflammation. GLA was effective than LA and LNA in blocking LPS-stimulated long-term c-Src activated as well as JNK phosphorylation in the chronic inflammation to prevent MyHC protein loss. (The dash line is represented the indirect effect within the pathway)

6. References

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科技部補助計畫衍生研發成果推廣資料表

日期:2014/10/29

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<tr><td>計畫主持人: 劉凱莉</td></tr>
<tr><td>計畫編號: 102-2320-B-040-001- 學門領域: 保健營養</td></tr>
<tr><td colspan="2">無研發成果推廣資料</td></tr>
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102年度專題研究計畫研究成果彙整表

計畫主持人：劉凱莉			計畫編號：102-2320-B-040-001-				
計畫名稱：探討Gamma次亞麻油酸調控細菌內毒素Lipopolysaccharide誘發骨骼肌肉耗損之功效							
成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	1	0	100%		
		研討會論文	1	1	100%		Gamma-Linolenic Acid Prevents Lipopolysaccharide-Induced Muscle Wasting in both Acute and Chronic Inflammatory Responses
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	4	4	100%	人次	
		博士生	2	2	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	0	1	100%	篇	
		研究報告/技術報告	0	0	100%		Gamma-Linolenic Acid Prevents Lipopolysaccharide-Induced Muscle Wasting in both Acute and Chronic Inflammatory Responses
		研討會論文	0	0	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
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		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	無

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

科技部補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：□已發表 □未發表之文稿 ■撰寫中 □無

專利：□已獲得 □申請中 ■無

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其他：（以 100 字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）

The ubiquitin-proteasome pathway (UPP) and autophagy-lysosomal pathway (ALP) are major protein degradation pathways that are activated during inflammatory conditions and have been proposed as a therapeutic target for preventing skeletal muscle loss. Recent reports indicate that lipopolysaccharide (LPS) may induce both UPP and ALP pathways through the activation of toll-like receptor4 (TLR4)/ nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs), suggesting that multiple signaling pathways mediate muscle wasting in inflammatory diseases. In this study, we tested the hypothesis that the anti-inflammatory 18-carbon polyunsaturated fatty acids (18-carbon PUFAs), including gamma-linolenic acid (GLA), linoleic acid (LA), and linolenic acid (LNA) would have the opposite effects of LPS-induced muscle wasting in C2C12myotubes and C57BL/6 mice. The results show that LA and LNA were less effective than GLA in inhibiting LPS-induced muscle wasting. The 18-carbon PUFAs inhibited LPS-induced LC3Ⅱ protein expression. Both GLA and LNA treatments significantly inhibited LPS-induced MuRF1 expression as well as ubiquitinated protein accumulation to prevent MyHC protein loss in C2C12

myotubes and gastrocnemius (GA) muscle. The acute stimulation of LPS-induced TLR4/NF-κB signaling transduction and c-Jun N-terminal Kinase (JNK) phosphorylation were significantly suppressed by 18-carbon PUFAs. Constitutively active IKKβ/NF-κB pathway could abolish the inhibitory effect of GLA on LPS-induced muscle wasting. Moreover, GLA also inhibited c-Src and JNK phosphorylation in chronic LPS-stimulation. In conclusion, our data suggested that in vivo and in vitro, GLA avoids LPS-induced muscle loss through inhibited TLR4/NF-κB/JNK signaling in the acute inflammation and decreased c-Src/JNK phosphorylation in the chronic inflammation.