Biomedicine & Pharmacotherapy 69 (2015) 42–46

Available online at
ScienceDirect
www.sciencedirect.com

Elsevier Masson France
EM|consulte
www.em-consulte.com/en

Short communication

Ligustilide inhibits tumour necrosis factor-alpha-induced autophagy during C2C12 cells differentiation

Ying Shi [a], Liming Xiao [b], Yi Yin [a], Lianbo Wei [a,*]

[a] Department of Traditional Chinese Medicine, Zhujiang Hospital of Southern Medical University, Guangzhou 510282, China
[b] The First Affiliated Hospital, Nanchang University, Nanchang 330006, China

ARTICLE INFO

Article history:
Received 8 October 2014
Accepted 5 November 2014

Keywords:
Ligustilide
Myoblast
Myotube
Autophagy

ABSTRACT

Ligustilide is widely thought to be the most potent bioactive constituent of *Angelica sinensis*. We have previously reported the role of ligustilide in preventing TNF-α-induced apoptosis and identified the presence of autophagosome clusters. Then, we hypothesised that autophagy may contribute to muscle loss and that ligustilide could protect cell fibres by regulating the autophagic process. The aim of this study was to identify the effects of ligustilide on autophagy regulation during cell differentiation in the presence of TNF-α. We then observed intracellular morphologic changes and autophagosome formation using transmission electron microscopy. LC3B expression was assessed by immunofluorescence and Atg-7, Atg-5, Atg-12 and LC3B expression levels were detected by western blot. The results revealed a reduction in the number of TNF-α-induced autophagosomes after ligustilide treatment accompanied by a decrease in Atg-7, Atg-5, Atg-12 and LC3B expression, as well as a reduction of the LC3BII/I ratio in a concentration-dependent manner. Our findings provide evidence supporting a protective effect of ligustilide against TNF-α-induced autophagy during myotubes formation.

1. Introduction

Autophagy is a process of cellular self-digestion essential in many aspects. An increasing number of studies show the complex roles of autophagy in human health and diseases. Indeed, a basal level of autophagy is required for cellular survival and homeostasis and too little or too much autophagy may result in diseases. Skeletal muscle, which represents 40–50% of the human body mass, constitutes one of the most important organs involved in metabolic regulation. Excessive protein degradation within the skeletal muscle tissue is detrimental for the whole body energy balance and can lead to death. The autophagic and ubiquitin-proteasome apoptotic systems are closely interrelated and their coordinated activation contributes to muscle loss [1–3]. Therefore, studying the autophagic and ubiquitin-proteasome systems is of significant importance for the prevention of muscle atrophy.

Our group has previously reported that ligustilide could prevent TNF-α-induced apoptosis during C2C12 cell differentiation [4]. Ligustilide exerted a protective effect against the effects of TNF-α by promoting cell proliferation and reducing apoptosis in C2C12 cells. To demonstrate the existence of TNF-α-induced apoptosis, cells were observed under transmission electron microscopy, which revealed the presence of autophagosomes in both mock- and TNF-α-treated groups. TNF-α-mediated upregulation of macroautophagy had been demonstrated previously [5,6]. We then hypothesised that autophagy may contribute to muscle loss and that ligustilide could protect the cells by regulating the autophagic process via a mechanism still not fully understood.

The aims of this study were to investigate whether ligustilide could regulate TNF-α-induced cellular autophagy during C2C12 differentiation and to explore the possible mechanisms regulating autophagy-related pathways.

2. Materials and methods

2.1. Reagents

FBS, horse serum and DMEM/F-12 were purchased from HyClone Laboratories (Logan, UT, USA). DMSO was purchased

Abbreviations: TNF-α, Tumour necrosis factor-alpha; FBS, Foetal bovine serum; DMEM/F-12, Dulbecco's modified eagle's medium F-12 Liquid; DMSO, Dimethyl sulfoxide; GM, Growth medium; DM, Differentiation medium; PBS, Phosphate buffered saline; DAPI, 4′,6-Diamidino-2-phenylindole; Atg, Autophagy associated gene.

? Corresponding author. Tel./fax: +86 20 6164 3456.
E-mail addresses: shiying3646@163.com (Y. Shi), 891564786@qq.com (L. Xiao), 806982591@qq.com (Y. Yin), weilianbo@163.com (L. Wei).

http://dx.doi.org/10.1016/j.biopha.2014.11.002
0753-3322/

from Sigma (Saint-Louis, MO, USA). Ligustilide was purchased from the National Institute for Food and Drug Control (HPLC ≥ 98%). RapidStep™ ECL reagent used for western blotting was purchased from Millipore (Bedford, USA). RIPA lysis buffer and BCA protein assay kit were purchased from Shanghai Beyotime Biological Corporation. Atg-7, Atg-5, Atg-12 and LC3B antibodies were purchased from Cell Signaling Technologies (Danvers, USA). Mouse anti-GAPDH, anti-rabbit and anti-mouse antibodies were purchased from EarthOx Biotechnology (EarthOx, CA, USA). The other chemicals and reagents used were of analytical grade.

2.2. Cell culture

C2C12 cells were purchased from the Chinese Academy of Sciences (Shanghai, China) and were maintained in growth medium (GM), composed of DMEM/F-12 supplemented with 10% FBS and incubated at 37 °C in a water-saturated atmosphere of 5% CO2.

2.3. Experimental model

C2C12 cells, which are derived from mouse skeletal muscle and can be differentiated into myotubes in culture, were maintained in GM at 37 °C under a humid 5% CO_2/95% O_2 atmosphere. At approximately 60–70% confluency, myoblasts differentiation was initiated by replacing the GM with differentiation medium (DM) composed of DMEM/F-12 supplemented with 2% horse serum. The differentiation medium was changed every 48 h before experimentation.

2.4. Experimental groups and treatments

For the mock group, cells were incubated with DM only. For the TNF-α-control group, cells were incubated with 20 ng/mL recombinant murine TNF-α dissolved in DM. Cells in the ligustilide-treated groups were incubated in DM containing 20 ng/mL TNF-α and ligustilide at diverse concentrations. Ligustilide and TNF-α dissolution procedures were previously described [4].

2.5. Transmission electron microscopy for autophagosome detection

To study the effects of the autophagosomes and morphologic changes in the cells, the cells were treated as described above. Treated cells were fixed in PBS with 2.5% cold glutaraldehyde. After two washes in PBS, the cells were post-fixed in 1% osmium tetroxide in PBS, then dehydrated with a graded series of ethanol and propylene oxide. Then, the samples were infiltrated overnight with a mixture of EPON resin/acetone (1:1) and embedded in EPON resin. The blocks were cut into 0.5 μM sections and stained with uranyl acetate and lead citrate [7]. Finally, cells were observed under a transmission electron microscope.

2.6. Immunofluorescence assay

Treated cells were washed with PBS and fixed in 4% paraformaldehyde for 10 min at room temperature. Then, fixed cells were blocked and permeabilised with 1% FBS containing 0.25% Tween-20 for 30 min at 4 °C and incubated with anti-LC3B (1:200 dilution) overnight at 4 °C. After washing with PBS, the cells were incubated with appropriate secondary antibodies for 1 h at room temperature, then washed with PBS and incubated with DAPI for 15 min. Stained cells were examined by fluorescence microscopy as previously described [8], and images were captured from 6 randomly selected microscopic fields for each treatment (magnification of 400 ×).

2.7. Western blotting

For immunoblotting, 20 μg aliquots of protein lysate were electrophoretically separated using a 12% SDS-polyacrylamide gel and transferred (Bio-Rad, Hercules, California, USA) to PVDF membranes (Pall, USA). After being placed in blocking buffer, the membranes were incubated with the following primary antibodies: anti-Atg-7 (1:1000 dilution), anti-Atg-5 (1:1000 dilution), anti-Atg-12 (1:1000 dilution), anti-LC3B (1:1000 dilution) or anti-GAPDH (1:5000 dilution). GAPDH protein was used as the internal control. After the membranes were washed with TBST, the appropriate HRP-conjugated secondary antibody (1:5000) was added to the preparation, which was incubated at room

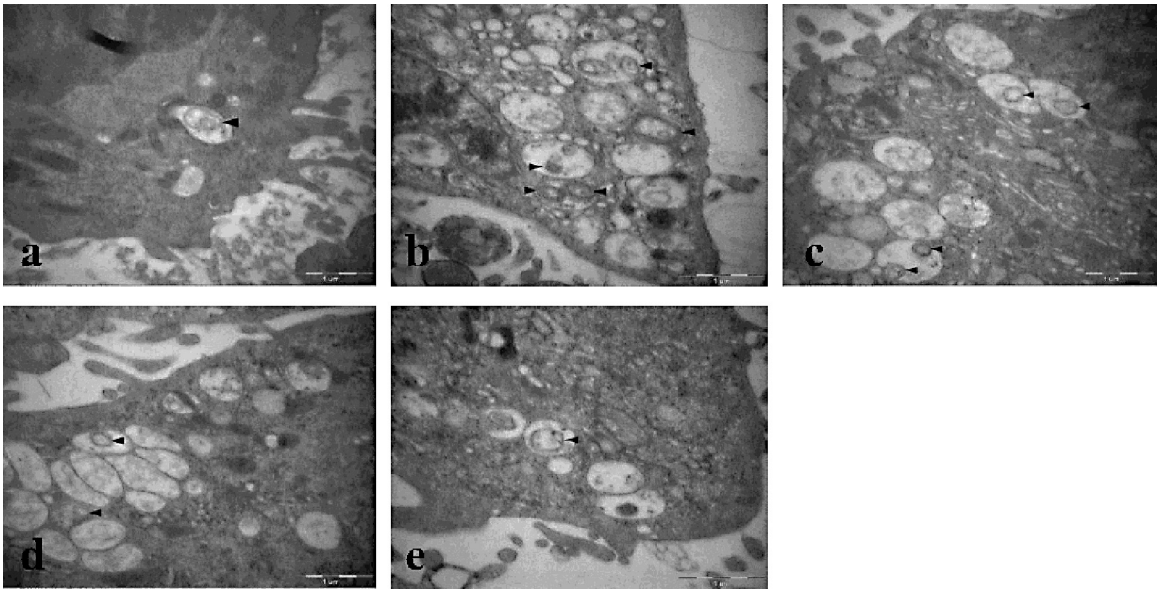

Fig. 1. Ligustilide reduces the number of TNF-α-induced autophagosomes. Ligustilide-mediated effect on autophagy, observed by transmission electron microscopy. The results show cytoplasmic morphology, cellular organelles, and nuclei in C2C12 cells from the mock (a), TNF-α-control (b), 1 μM ligustilide-treated (c), 5 μM ligustilide-treated (d) and 10 μM ligustilide-treated (e) groups. Arrowheads represent autophagic vacuoles.