CALPAIN INHIBITOR REDUCES CYOTOTOXITY INDUCED BY LPS IN C2C12 CULTURED CELLS

Ke Shang[1,2], Junfeng Zhang[1,2], Touseef Amna[1], Jieun Yang[1], Xiangchao Cheng[2] and Inho Hwang[1*]

[1]*Department of Animal Science and Biotechnology, Chonbuk National University, Jeonju, 561-756, Republic of Korea*

[2]*Department of Animal Science and Technology, Henan University of Science and Technology, Luoyang, 471003, China*

Abstract –The aim of the present study was to examine the probable mechanisms and signal pathways involved in cyototoxity induced by bacterial lipopolysaccharide (LPS). Herein, we selected muscle precursor C2C12 cells as a model cell line to test the effect of selective calpain inhibitor PD150606 on LPS induced inflammation and apoptosis. To examine the *in vitro* cytotoxicity, mouse myoblast C2C12 cells were treated with different concentrations of LPS and viability of cells was analyzed by cell counting Kit-8 assay and simple microscopy. Apoptotic cell death was examined by CLSM at regular time intervals. Additionally LPS induced apoptosis in C2C12 cells were determined by mRNA expression of caspase-3 and μ-calpain. Moreover, the mRNA expression of Tumor necrosis factor alpha (TNF-α) and was also assayed by RT-PCR. Our results point out that LPS treatment produced dose dependent toxicity. Pre-treatment with calpain inhibitor PD150606 that can significantly attenuated LPS-induced inflammation /apoptosis. The findings of the study indicate that the mRNA expression of μ-calpain, caspase-3, and TNF-α are significantly ($p<0.05$) increased in LPS induced C2C12 cells, whereas a significant decrease ($p<0.05$) in mRNA expressions of aforementioned genes were observed when pretreated with calpain inhibitor PD150606. Our study has outlined the current understanding regarding the role of caspase-3 and μ-calpain in skeletal muscle wasting and thus helps in the development of novel therapy/chemotherapeutic system for skeletal muscle diseases in future.

Key Words – Skeletal muscle, C2C12 cells, PD150606, LPS, Inflammation, Apoptosis

I. INTRODUCTION

Skeletal muscle atrophy is a serious problem leading multiple disorders/or diseases such as chronic obstructive pulmonary disease, acquired immune deficiency syndrome (AIDS), muscular dystrophy, chronic heart failure, sepsis, cancer, aging and so on. The loss of lean body mass is one of the most important consequences of severe infection and sepsis[1]. Generally speaking the mechanisms leading to sepsis-induced changes in skeletal muscle are linked to amplification of proinflammatory cytokines, free-radical and activation of proteolytic pathways including caspase and calpain activation respectively. It is also given to understand that calpain plays significant roles in induction of apoptosis by cleaving caspase-3 into its active form. Caspase-3 degrades calpastatin through proteolysis and thereby amplifies calpain activity. It is reported in the literature that lipopolysaccharide (LPS) of cell wall of Gram negative bacteria is a strong inducer of many cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6. In addition, LPS also stimulates NO synthase-2 (NOS2) mRNA expression in C2C12 myoblasts and primary cultures of human myocytes[2]. On the basis of previous reports it is well established now that LPS stimulates the expression of cytokines in both skeletal muscle *in vivo* and myoblasts *in vitro*. In the present study we have selected muscle precursor C2C12 cells as a model cell line to test the effect of cell permeable selective calpain inhibitor [3-(4-iodophenyl)-2-mercapto-(Z)-2-propenoic acid] (PD150606) on LPS induced inflammation. In previous report its is described that PD150606 is capable to reduce calpain activation and reduce cell death in rat and rabbit renal proximal tubules subjected to hypoxia or nephrotoxins[3]. The objective of the present study was to examine the probable mechanisms and signal pathways involved in apoptosis induced by LPS in C2C12 cells under *in vitro* conditions. To best of our knowledge there is no published report about the possible mechanism about the inhibition of LPS induced damage of muscle

precursor C2C12 cells with PD150606. Understanding about the PD150606 inhibition mechanism in induced LPS inflammation in C2C12 cells will help in the development of novel chemotherapeutic system for skeletal muscle diseases in near future.

II. MATERIALS AND METHODS

Cell culture and morphology analysis

C2C12 cells were cultured in growth medium (Dulbecco's modified Eagle's medium, DMEM (pH 7.4) containing 10% fetal bovine serum, 1% penicillin-streptomycin-neomycin Solution) in a humidified incubator at 37°C with 5% CO_2 and 95 % air environment as describe elsewhere [4]. Morphological alterations and cell damage were qualitatively investigated [5] using a light phase-contrast microscope (Olympus CK×41).

Cell viability assay

The cell viability was evaluated using a CCK-8 assay. Briefly, cell density of 1×10^4 cells/well was seeded in 96-well tissue culture plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA). After incubation the cells were treated with different concentrations (1, 10, 50, 100, 150, 200 µg/mL) of LPS for further 10 h. Untreated cells served as controls. After specific exposure of LPS the incubated cells were treated with 10 µl of water-soluble tetrazolium-8 (CCK-8) solution in each well (100µl medium) was added and incubated for 4 h at 37 °C according to the manufacturer's instructions. At the end of the experiment, absorbance was measured at 450 nm for each well by a microplate spectrophotometer (model 680; Bio-Rad Laboratories, Hercules, CA). The viability of the treated samples was expressed as a percentage of non-treated control samples, which was assumed to be 100 %.

Immunofluorescence microscopy

Cell death was further confirmed from confocal microscopy. The C2C12 cells were seeded in coated dishes and allowed 4 h to attach and grow. After proper attachment the cells were treated with LPS and calpain inhibitor for specific time duration as described above in the present study. Acridine orange/ethidium bromide (AO/EB) staining was used to visualize nuclear changes and apoptotic body formation that are characteristic of apoptosis. The Images were acquired on a CLSM equipped with laser (VIS) and scanning (META) module in an epifluorescence mode. Images were collected and saved using the Software LSM (Zeiss image Confocal Software) and exported to Adobe PhotoShop for digital processing [5].

RNA isolation and Real-time RT-PCR

The C2C12 cells were seeded in 24-well plate treated with LPS and calpain inhibitor for specific time duration as described above in the present study. Cells were lysed in Trizol reagent and total RNA was extracted from both treated and untreated samples strictly following the manufacturer's information. Briefly the first-strand cDNA was synthesized from 1µg of total RNA using the MMLV reverse transcriptase with the anchored oligo (dT) 12–18 primer (Gene Link). Real-time PCR was performed using a cDNA equivalent of 10ng of total RNA from each sample with primers specific for µ-calpain, caspase 3, TNF-α and a housekeeping gene encoding GAPDH. The reaction was carried out in 20µl using SsoFast^{TM} EvaGreen Supermix (Bio-Rad) according to the manufacturers' instructions. Relative ratios were calculated based on the $2^{-\Delta\Delta CT}$ method [6]. PCR was monitored using the CFX96TM Real-Time PCR Detection Systems (Bio-Rad).

III. RESULTS AND DISCUSSION

Effect of LPS on cell viability

It was observed in the present study that the inflammation increases with increasing concentration and maximum 17.26 % and 22.27% inhibition was found at 150µg/mL and 200µg/mL respectively after 12 h of incubation period. No significant toxicity was found at 1µg/mL and 10 µg/mL concentrations respectively (Fig. 1). The data lead to the conclusion that the growth inhibition is concentration dependent. Morphological alterations induced by LPS in C2C12 cells were also tested. Untreated cells were thin and elongated with two tapering ends (Fig.

2a). After incubation with a high concentration (150µg/mL) of LPS, few rounded cells and some areas devoid of cells were also noticed in the same culture medium. The treatment led to the aggregation of dense irregular cellular debris (Fig. 2b).

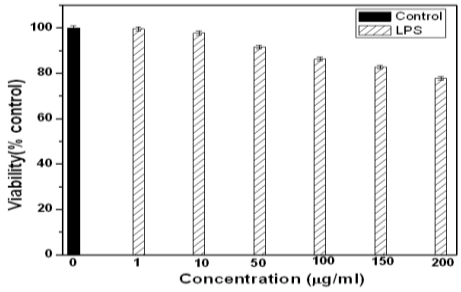

Fig. 1 In vitro cytotoxicity of different concentration (1, 10, 50, 100, 150, 200 µg/mL) of LPS after incubation for 12 h. Untreated C2C12 cells were used as a control.

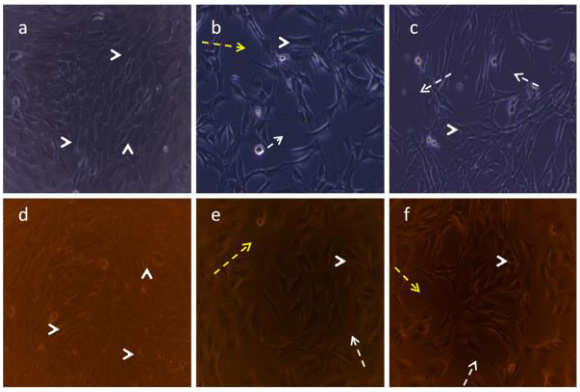

Fig. 2 Representative phase contrast images of C2C12 cells (a) unexposed cells, (b) exposed to 200 µg/ml LPS, and (c) exposed to LPS and 50 µM PD150606.
Note: Magnification 40×.

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Effect of PD15000 on LPS-induced cytotoxicity

LPS-induced cell death was significantly decreased in dose dependent manner when the cells were pretreated with PD150606 (50 µM and 100 µM) (Fig. 3). Thus from the morphological observations (Fig. 3c) and CCk-8 data it is clear that the PD150606 is very effective.

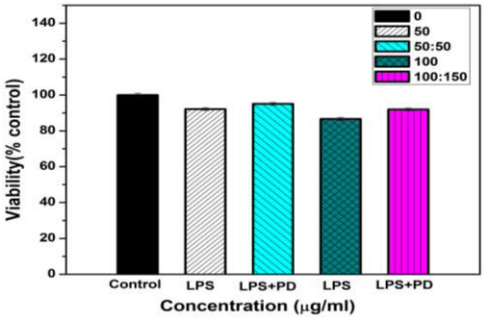

Fig. 3 *In vitro* cytotoxicity of LPS (50 and 100µg/mL) on C2C12 myoblasts pretreated (4h) with PD150606 (50 µM) after 12h incubation.

Apoptotic morphology by Acridine orange–Ethidium bromide staining

In order to get insights into cell death we further performed Immunofluorescence staining. From the attained CLSM images (Fig. 4), it is obvious that the proportion of living and dead cells corresponds to both time and the concentration of LPS. Nevertheless the number of apoptotic cells was significantly decreased when the cells were pre-exposed to PD150606 (Fig. 4e).

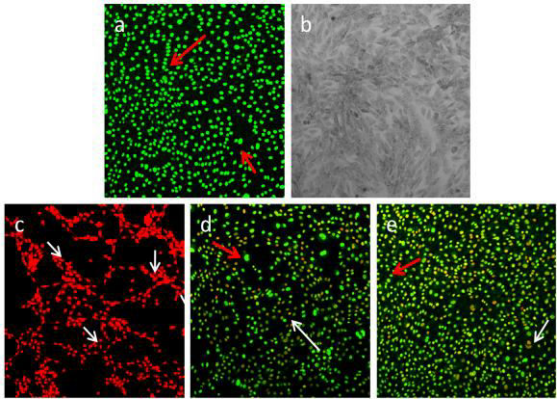

Fig. 4 Representative confocal images of C2C12 cells: unexposed control (a) unexposed control without staining (b) exposed to 300 µg/ml LPS after 12 h (c), exposed to 200 µg/ml LPS after 12 h (d), exposed to 200 µg/ml LPS and 50 µM PD150606 (e).

RNA expression

The mRNA expression of µ-calpain, caspase-3, and TNF-α are significantly ($p<0.05$) increased in LPS induced C2C12 cells, whereas a significant decrease ($p<0.05$) in mRNA expressions of

aforementioned genes were observed when pretreated with PD150606 (Fig. 5).

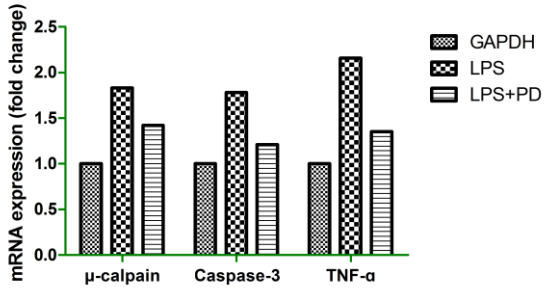

Fig. 5 mRNA expression of LPS (50μg/mL) on C2C12 myoblasts pretreated (4h) with PD150606 (50μM) after 12h incubation.

Conclusion

In conclusion, it was found that the calpain inhibitor PD150606 prevents the μ-calpain and caspase-3 activation, decreased mRNA expression of μ-calpain , caspase-3 and TNF-α and attenuated inflammation/apoptosis in LPS-stimulated C2C12 cells. Our study has outlined the current understanding regarding the role of caspase-3 and μ-calpain in muscle precursor C2C12 cells and thus will help in the development of novel therapeutic procedures for skeletal muscle diseases in future.

Statistical analysis

Statistical evaluation was carried out by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT). A value of $p < 0.05$ was considered statistically significant.

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