臺北醫學大學 醫學院 臨床醫學研究所碩士論文

TAIPEI MEDICAL UNIVERSITY

COLLEGE OF MEDICINE

GRADUATE INSTITUTE OF CLINICAL MEDICINE

MASTER THESIS

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備勞喘吸入劑抑制脂多醣刺激巨噬細胞產生間白素-六基因分子機制研究

MOLECULAR MECHANISM OF FENOTEROL INHALANT SOLUTION-INDUCED SUPPRESSION OF *INTERLEUKIN-6* GENE EXPRESSION IN LIPOPOLYSACCHARIDE-ACTIVATED MACROPHAGES

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中　華　民　國　九　十　七　年　一　月

誌　　謝

埋首臨床工作至今，已近十五個年頭了，非常珍惜再有此機會回到學校，探索研究生命科學相關知識。在這兩年多的求學過程中，獲得了許多新知及突破，更從老師及學長姐身上學到實事求是的求知精神及研究態度，令我感激不已。

能夠完成這本論文，首先要感謝我的指導教授陳瑞明老師及陳大樑老師，不斷地在邏輯思考、實驗方法及設計方面諄諄教導。也感謝口試委員台大毒理所翁祖輝老師、台大麻醉部范守仁老師以及北醫解剖學科馮琮涵老師撥允參加我的口試，並對我的論文細心斧正。還要感謝實驗室的學長莊淇源醫師、吳鋼治醫師、張懷嘉醫師、吳茲端醫師、呂志成醫師、李元文醫師、陸怡伶、林雍偉醫師在各方面的協助與指導，尤其是同學嘉瑱、皓耘及佳紋，謝謝你們不斷的鼓勵與支持。另外，還要感謝實驗室的怡伶學姐、怡真、一斌、倩綾、稚瑋、秀瑾、欣怡對我的包容與幫助。

最後要感謝我的家人、妻子以及我的兩個可愛的兒子，無怨無悔地為我付出，且在我 主耶穌基督的引領之下一同攜手走來，使我得以順利完成這份論文。

Abbreviations

RAW 264.7: mouse alveolar macrophage-like cell line

LPS or L: lipopolysaccharide

IL: interleukin

FEN or F: fenoterol or fenoterol hydrobromide

RT-PCR: reverse transcriptase-polymerase chain reaction

JNK: c-Jun N-terminal kinase

p-JNK: phosphorylated c-Jun N-terminal kinase

MEK-4: MAP kinase kinase 4

p-MEK-4: phosphorylated MAP kinase kinase 4

NF-κB: nuclear factor-kappa B

TNF: tumor necrosis factor

TLR: toll-like receptor

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Abstract in Chinese

備勞喘（fenoterol）吸入劑為一種短效且具選擇性之乙二型交感神經刺激劑（β_2-agonist），常被廣泛地使用於阻塞性肺部疾病或是嚴重肺部感染症而導致急性呼吸窘迫症（ARDS）之病患，作為控制其支氣管攣縮之藥物。引起急性呼吸窘迫症（ARDS）最普遍的原因為嚴重感染，而某些乙型交感神經刺激劑，經近期研究發現可被用於早期緩解急性呼吸窘迫症。備勞喘在過去的研究中極少被探討關於抗發炎之效果，對於其作用機制亦未曾被闡明。一些先發研究顯示，在動物或人體實驗中，乙型交感神經刺激劑可抑制細胞激素，例如：腫瘤壞死因子（TNF）以及間白素-六（IL-6）之生成。本研究之目的在評估備勞喘在經脂多醣（LPS）活化之 RAW264.7 類巨噬細胞模式中，是否可以產生 IL-6 mRNA 之免疫抑制效應，並且探討其可能之分子機制。

RAW264.7 巨噬細胞於不同濃度 1, 10 及 100 μM 之備勞喘劑下，經有或無 100 ng/ml LPS 加入，在一、六及二十四小時之時間間隔後，皆對細胞存活率（viability）沒有影響。 以備勞喘及 LPS 處理 RAW264.7，LPS 可促使 IL-6 mRNA 之產生，並在第一及第六小時被備勞喘所抑制。為了探討備勞喘抑制 IL-6 mRNA 之分子機制，我們研究不同的轉錄因子。在 LPS 活化之巨噬細胞中，備勞喘能於兩個小時內顯著地抑制 c-Jun 核蛋白質。在被 LPS 活化之巨噬細胞中，備勞喘也能於一個小時內顯著地抑制 c-Jun 總細胞蛋白，但無法抑制 c-Fos 或 NF-κB 蛋白。為了闡明 c-Jun 的上游路徑，我們也設計研究 p-JNK 以及 p-MEK-4 蛋白。

結果顯示，在兩個小時內被 LPS 活化之 RAW264.7 中，p-JNK 1/2 蛋白，可被備勞喘不顯著地抑制。在活化之 RAW264.7 中且於五十分鐘內，經備勞喘處理後，p-MEK-4 蛋白亦可被備勞喘不顯著地抑制。

本研究結果顯示備勞喘可抑制 IL-6 mRNA，且其機轉可能是透過 c-Jun 或許也可能是透過與 JNK/MEK-4 有關之分子路徑。

Abstract

Fenoterol inhalant solution, a short-acting and selective beta2-agonist, is widely used as aerosal inhalation for management of bronchospasm in patients with obstructive lung disease or severe lung infection. The most common cause of acute respiratory distress syndrome (ARDS) is severe infection, and some beta-adrenergic agents are under studies as novel treatments targeting earlier resolution of ARDS. The studies of fenoterol for the anti-inflammation effect are rare and no known mechanism has been elucidated in previous articles. Previous studies revealed that beta-agonist caused cytokine inhibition (e.g. TNF and IL-6 production) in animal or human models. The aims of this studies were to evaluate the effect of fenoterol on IL-6 mRNA production and its possible molecular mechanisms in the cell model of lipopolysaccharide (LPS)-activated macrophage-like cell line, RAW264.7.

Exposure of RAW264.7 macrophages to 1, 10, 100 μM fenoterol at 1-, 6-, and 24-hour intervals with or without 100 ng/ml LPS did not affect cell viability. While treated with fenoterol and LPS in RAW264.7, the IL-6 mRNA production was induced by LPS and inhibited at the 1st and the 6th hours. To clarify the molecular mechanisms by which fenoterol inhibited the IL-6 mRNA, different transcription factors were investigated. Fenoterol could significantly suppress the nuclear protein levels of c-Jun in LPS-activated macrophages within 2 hours. Fenoterol could also significantly suppress the total cellular protein levels of c-Jun but not c-Fos or NF-κB in LPS-activated macrophages within 1 hour. In order to elucidate the upstream pathways, the p-JNK (phosphorylated c-Jun N-terminal kinase) and p-MEK-4 (phosphorylated MAP

kinase kinase 4) proteins were studied. The results revealed that p-JNK1/2 proteins were insignificantly suppressed by fenoterol within 2 hours after the LPS activation in RAW264.7. The p-MEK-4 proteins levels were also insignificantly suppressed by fenoterol within 50 minutes in LPS activated macrophages.

The results of this study showed that fenoterol could suppress IL-6 mRNA expression through c-Jun and possibly JNK/MEK-4-dependent pathway.

Introduction

I. Lung infection and alveolar macrophages

(I) Pneumonia, acute lung injury and acute respiratory distress syndrome

Pneumonia is a common disease in the world and is defined as infection and inflammation of the lung tissue after invasion of infectious agents. The overall mortality rate is 14% for hospitalized patients but raised up to 20~50% in those who required intensive care (1-3). In Taiwan, pneumonia also caused significant morbidity and mortality, especially in the aged and children population and remained as the sixth leading cause of death during these years (4).

In 1967, Ashbaugh and colleagues (5) defined acute respiratory distress syndrome as an acute lung injury (ALI) syndrome associated with sepsis or trauma. The syndrome is similar to neonatal respiratory distress as the original name, the adult respiratory distress syndrome. Now, it is formally named the acute respiratory distress syndrome (ARDS). ARDS is associated with many clinical risk factors that may cause direct lung injury either by infection or by secondary processes that activate systemic inflammation and subsequently damage the lung. The diagnositic criteriae (6) were: severe respiratory distress and 1 or more risk factors (e.g. Infection or sepsis, pancreatitis, and trauma), impaired arterial oxygenation (hypoxemia), bilateral pulmonary infiltrates on chest radiograph, and should exclude clinical evidence of elevated left atrial pressure (or pulmonary artery wedge pressure of 18 mmHg, if available).

The cardinal feature of ARDS is refractory hypoxemia, which is caused by formation of protein-rich alveolar edema after damage to the

lung's alveolar-capillary barrier. Alveolar-capillary damage in ARDS can be initiated by physical or chemical injuries or by extensive activation of innate inflammatory responses. Such damage causes the decrease of the lung's edema safety factor and edema develops at lower capillary pressures. Widespread alveolar edema in ARDS would impair alveolar ventilation, exclude oxygen, inactivate surfactant and increase dispersion of ventilation and perfusion, produce intrapulmonary shunt and decrease lung compliance (7, 8).

The incidence of ARDS in at-risk populations is not certain, but prospective estimates range from 1.5 to 12.9 cases per 100,000 people per year depending on diagnostic criteria (7). The most common cause of ARDS is severe infection, accounts for approximately 50% of all cases. These infections may involve localized disease (such as pneumonia) or systemic disease, including sepsis or sepsis syndrome. Sepsis-related conditions, particularly severe gram-negative infections, are also associated with multiple organ failure or progressive respiratory failure. The multiple organ failure syndrome is the major cause of death in ARDS, and the mortality rate of the ARDS syndrome is about 40% (8-11). The acute respiratory distress syndrome is distinguished mainly by pulmonary gas exchange with the ratio of PaO_2 to the inspired fraction of oxygen (FiO_2). A PaO_2/FiO_2 ratio of 300 or less defines acute lung injury, and a ratio of 200 or less defines ARDS regardless of the amount of positive end-expiratory pressure (PEEP) needed to support oxygenation. Physiologic indexes of oxygenation are also diagnostically useful, but the PaO_2/FiO_2 ratio and physiologic scoring systems do not correlate with prognosis (11, 12).

(II) Alveolar macrophages in acute lung injury and acute respiratory distress syndrome

The macrophages may play an important role to initiate the innate immune response against affending pathogens in the host (13). After pathogens invasion, the innate immune system would turn on at first, and the macrophage will perform phagocytosis and chemotaxis reaction with subsequent release of inflammatory mediators, including cytokines, complement and free radicals (14). The invading pathogens would stimulate the toll-like receptors (TLR's) on the cell surface and the macrophage would release many inflammatory mediators for subsequent immune cascades to fight against the pathogens (15).

Alveolar macrophages also respond directly to bacterial products such as bacterial lipopolysaccharide (LPS) and gram-positive cell wall products such as leipoteichoic acids. Alveolar macrophages are major source of chemokines (16) in the air spaces and produce cytokines IL-6, 8, GRO-related peptides, and epithelial neutrophilactivating protein (ENA)-78.

Although many factors are involved in the pathogenesis of ARDS, the local cytokines release of alveolar macrophages play a central role for the syndrome (17). During sepsis and ARDS, alveolar macrophages can respond to LPS and release large amounts of cytokines which have been associated with a more adverse outcome in this condition (18).

II. Beta2-agonists in acute lung injury

Although some therapies such as surfactant and nitric oxide, demonstrated benefits of improved oxygenation, these benefits could not reduce the duration of mechanical ventilation or mortality. Inflammatory

mediator-targeted therapies were promising; however, larger trials have found therapies such as cytokine modulation, platelet-activating factor inhibition and neutrophil elastase inhibitors to be ineffective in the treatment of ALI/ARDS. Earlier studies have established that elevated endogenous catecholamine levels can help remove alveolar edema fluid in a septic shock insult (19, 20) and it is till 2006, Su et al. (21) also found protective effect of endogenous β-adrenergic tone on lung fluid balance in mice with acute pneumonia. Novel therapies in development for treatment of ALI/ARDS are exogenous surfactant, modulating neutrophil activity therapies, such as prostaglandin and complement inhibitors. Some treatments targeting earlier resolution of ARDS, such as β-agonists and granulocyte macrophage colony-stimulating factor were under aggressive studies (22). Preclinical studies with β_2-agonists and granulocyte macrophage colony-stimulating factor have shown promise for restoring alveolar capillary barrier integrity or reducing pulmonary oedema, and further studies are being conducted to test for true clinical benefit (23).

III. Immunosuppression of beta agonists and fenoterol

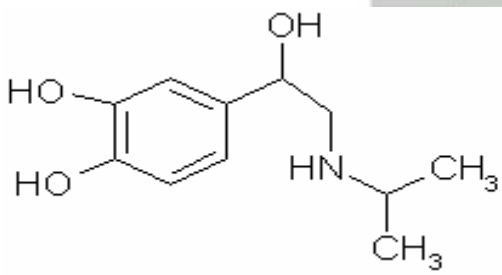
(I) Immunosuppression of β-agonists

Severn et al. (24) firstly demonstrated a dose-dependent suppression of TNF production by adrenaline in THP-1 cells and human whole blood, which is dependent on β-receptor stimulation and is mediated by increased intracellular cAMP levels. It was also noted that noradrenaline caused a dose-dependent inhibition of TNF and IL-6 production in human whole blood cells (25). Straub et al. (26) demonstrated that isoproterenol inhibited IL-6 secretion in the spleen. Isoproterenol was found to be a nonspecific β-agonist exerts anti-inflammation effects through the cAMP/IκB/NF-κB pathway (27). In

2005, Maris et al. (28) even found that salmeterol, a long acting β-agonist, could exhibit anti-inflammatory effects in healthy volunteers after LPS inhalation.

(II) Fenoterol

Fenoterol is a direct acting sympathomimetic agent, selectively stimulating β_2-receptors in the therapeutic dose range. It contains 1-(3,5-dihydroxy-phenyl)-2-((1-(4-hydroxy-benzyl)-ethyl)-amino)-ethanol hydrobromide (also named as fenoterol hydrobromide), has good water solubility and a molecular weight of 384 (chemical structure figure shown as below).



In clinical studies fenoterol was shown to be highly efficacious in manifest bronchospasm. It prevents bronchoconstriction following exposure to various stimuli such as exercise, cold air, and the early response following allergen exposure. Occupation of β_2-receptors activates adenyl cyclase via a stimulatory Gs-protein. The increase in cyclic AMP activates protein kinase A which then phosphorylates target proteins in smooth muscle cells. This in turn leads to the phosphorylation of myosin light chain kinase, inhibition of phosphoinositide hydrolysis, and the opening of large-conductance calcium-activated potassium channels.

Fenoterol relaxes bronchial and vascular smooth muscle and protects against bronchoconstricting stimuli such as histamine,

methacholine, cold air, and allergen (early response). After acute administration the release of bronchoconstricting and proinflammatory mediators from mast cells is inhibited. Further, an increase in mucociliary clearance has been demonstrated after administration of higher doses of fenoterol. Tremor is a more frequently observed effect of β-agonists. Unlike the effects on the bronchial smooth muscle, the systemic effects of β-agonists are subject to the development of tolerance.

Pharmacokinetics of fenoterol: following inhalation of fenoterol in obstructive lung diseases, bronchodilatation occurs within a few minutes. The bronchodilator effect lasts 3-5 hours. After inhalation, depending upon the method of inhalation and the system used, about 10-30% of the active ingredient released from the aerosol preparation reaches the lower respiratory tract, whereas the remainder is deposited in the upper respiratory tract and in the mouth. As a result, some of the fenoterol which has been administered by inhalation enters the gastro-intestinal tract. After inhalation of one puff from a BEROTEC metered aerosol an absorption rate of 17% of the dose has been determined. Absorption then follows a biphasic course, 30% of fenoterol being rapidly absorbed with a half-life of 11 minutes, and 70% being slowly absorbed with a half-life of 120 minutes.

There is no correlation between plasma levels and the pharmacodynamic time response curve following inhalation. The long bronchodilator action following inhalation compared with that following intravenous administration is not supported by the systemic plasma levels (29).

In literature, there is rare evidence of similar immunosuppressive effects reported for fenoterol. Studies (30) showed stimulation effects of

cyclic-AMP and inhibition effects of leukotriene B4 production caused by different β_2-agonists (including fenoterol, salbutamol and reproterol). So far, no molecular mechanism or pathway of anti-inflammation was ever elucidated for such a common and short acting β_2-agonist.

IV. Lipopolysaccharides, inflammatory cytokines and lung injuries

(I) Lipopolysaccharides and inflammatory cytokines

The lipopolysaccharide (LPS) component of the outer membrane of gram-negative bacteria is considered responsible for the pathological role of gram-negative sepsis (31). LPS triggers the production of cytokines, which in turn mediate most of the biological effects. There is evidence that tumor necrosis factor alpha (TNF) is the first cytokine in a cascade of these proteins that is induced by LPS (32). The essential role of TNF in the toxicity evoked by bacteremia has ever been documented in animal studies showing that neutralization of TNF activity prevents mortality in otherwise lethal sepsis (33). Interleukin 6 (IL-6) release follows shortly after the appearance of TNF in experimental endotoxemia and sepsis (34, 35) and is likely to be the major stimulus for acute-phase protein synthesis in systemic infection (36). In sepsis, high concentrations of TNF and IL-6 in serum are paralleled by markedly elevated levels of stress hormones in the circulation.

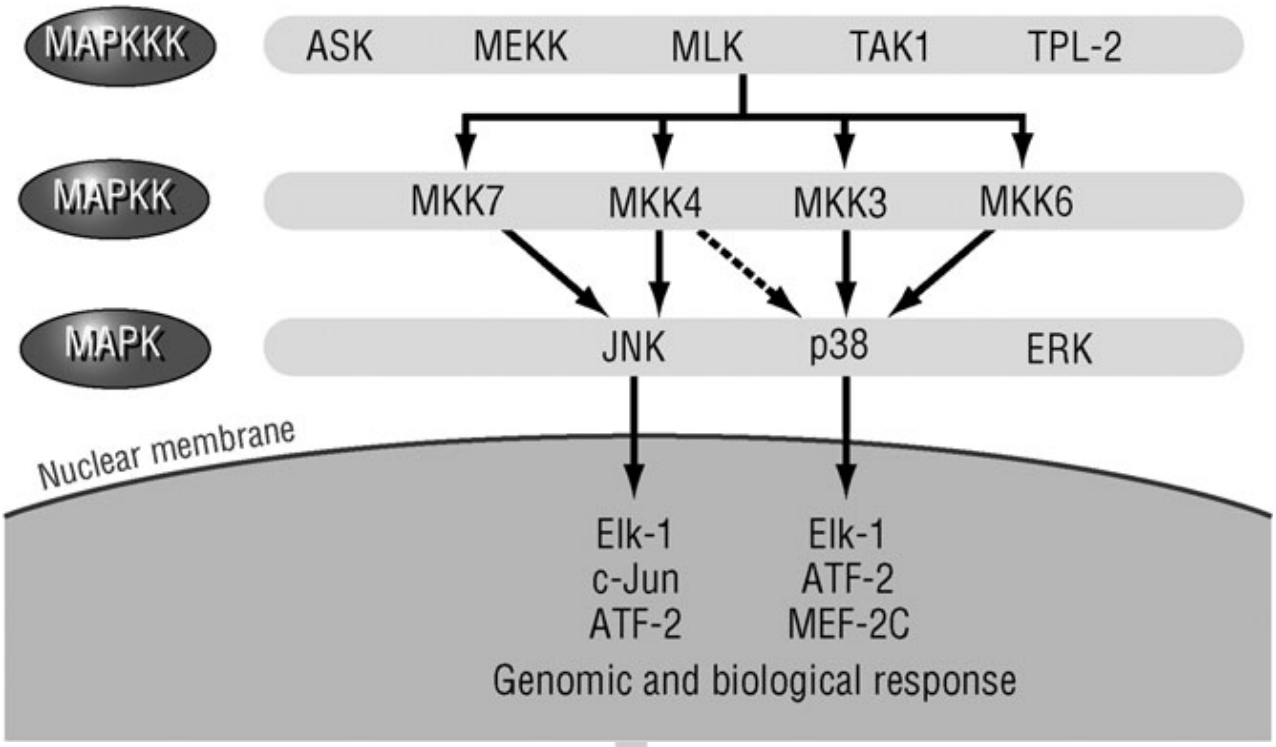
(II) Inflammatory cytokines and acute respiratory distress syndrome

The inflammatory mediators play an important role in the pathophysiology of inflammation in ARDS. These mediators include tumor necrosis factor (TNF)-α; interleukin (IL)-1, -4, -6, -8, -10, and -13; substance P; platelet activating factor (PAF); complement component (C5a); adhesion molecules (e.g. vascular adhesion molecule-1,

intercellular adhesion molecule-1); E- and P-selectins; L-selectin; and vasoactive mediators (e.g. nitric oxide). The transcription factor NF-κB plays a central role in the regulation of many genes responsible for the mediators generation in inflammation. Investigations of the interactions between various cell populations have led to the concept of cytokine networking with chemokines playing a central role (37). These events are mediated via the generation of early response cytokines, the expression of cell surface adhesion molecules, and the production of chemotactic molecules, chemokines (38), which are a specific class of inflammatory mediators that play a key role in the pathogenesis of ARDS.

V. MAPK cascades in the signaling pathways

A classic MAPK cascade (39) is composed of an MAPK, the kinase that activates the MAPK through phosphorylation on serine and tyrosine residues (called a MAPK kinase, MKK, MAPKK, or MAP2K), and the kinase that activates the MKK (called a MKK kinase, MEKK, MAPKKK, or MAP3K, see as below). Members of the activator protein (AP)-1 family (40) are dependent on their phosphorylation by mitogen-activated protein kinases (MAPKs) or MAPK-activated kinases.



Nature 2001;410:37–40

VI. MAPK and inflammatory cytokines

Chen et al. (41) also found ectopic expression of mitogen-activated protein kinase phosphatase-1 (MKP-1) could inactivate c-Jun N-terminal kinase (JNK) and P38 and further inhibit TNF-α and IL-6 production in LPS-activated-macrophges. There was also a number of transcription factors playing a critical role for regulating *IL-6* gene expression, and this included NF-κB, NF-IL6, CREB/ATF, Jun-Fos, STAT and NT-AT families (42). Nakamura et al. (43) studied LPS-stimulated renal macrophages and further found terbutaline, a short-acting β_2- agonist, could induce down regulation of *IL-6* gene production through mitogen-activated protein kinase (MAPK) and cAMP pathways. The MAPK family includes extracellular signal-regulated protein kinase (ERK-1/2), stress-activated protein kinases (SAPK), P38, and JNK. Sanchez-Tillo (44) further showed JNK1 was required for MKP1 induction in LPS-activated macrophages.

VII. C-Jun nuclear protein regulation

The activation of c-Jun is classically associated with JNK, but p38 has also been shown to be involved in LPS-induced c-Jun expression that may influence c-Jun related transcription activities. Inhibition of MEK or p38 also has been shown to reduce stress-induced c-Jun and c-Fos transcription in NIH 3T3 and HeLa TK cells (45, 46).

Leppä et al. (47) found that MEK-induced ERK activation in PC12 cells induced c-Jun expression, while JNK signalling did not. Therefore, dual input of expression and phosphorylation of c-Jun provided by the ERK pathway was required to direct neuronal differentiation in PC12 cells.

Carter et al. (48) found even that LPS activates both the extracellular signal–regulated kinase (Erk) and p38 kinases, and that this activation is augmented when the cells are cultured in serum. Inhibition of either the Erk (with PD98059) or p38 (with SB203580) kinase pathway resulted in only a partial reduction in cytokine (interleukin-6 and tumor necrosis factor) messenger RNA accumulation and cytokine release, whereas inhibition of both pathways simultaneously resulted in a decrease in cytokine gene expression to near-control levels.

VIII. Study hypothesis and specific aims

(I) Study hypothesis

1. Hypothesis

In LPS-activated macrophages, fenoterol can suppress IL-6 mRNA production. The major mechanism of suppression is to inhibit the activation of cytoplasmic proteins p-MEK-4 and p-JNK, then to inhibit the formation and translocation of transcription factor c-Jun, and finally to

inhibit the IL-6 mRNA production.

2. Description

Rare was known about the immunosuppressive effects of fenoterol and till now no molecular mechanism of anti-inflammation effects for fenoterol was ever elucidated for this β_2-agonist. In the preliminary data of this study, fenoterol could suppress IL-6 mRNA production in LPS-activated macrophages. Fenoterol could also inhibit the downstream transcription factor c-Jun protein, but not the NF-κB and c-Fos proteins. So in this study, it was proposed that fenoterol could suppress *IL-6* gene expression through c-Jun/p-JNK/p-MEK-4 dependent pathway.

(II) Specific aims

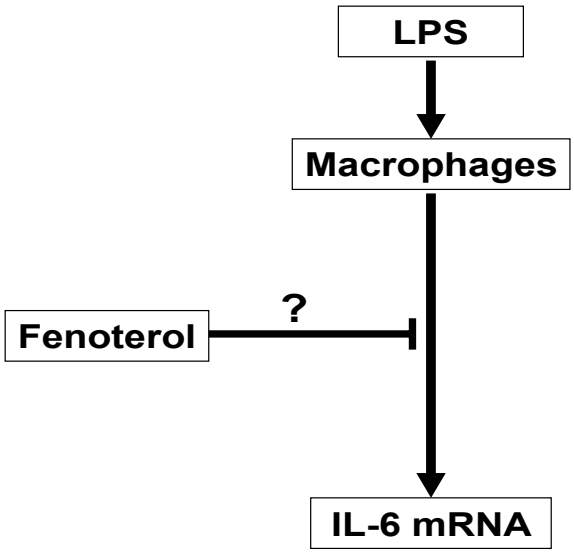
In order to test the study hypothesis, a series of specific study aims were set up as the followings:

1. Fenoterol of different concentrations and time durations are not cell-toxic on MTT viability test.
2. At 24 hours, fenoterol of different concentrations with or without LPS is not cell-toxic on MTT viability test.
3. Fenoterol can suppress IL-6 mRNA production in LPS-activated macrophages.
4. Fenoterol can suppress nuclear protein c-Jun production and translocation.
5. Fenoterol can suppress p-JNK proteins in LPS-activated macrophages.
6. Fenoterol can suppress p-MEK-4 proteins in LPS-activated macrophages.

IX. Study design

(I) The effects of fenoterol on IL-6 mRNA in LPS-activated macrophages

1. Flow chart

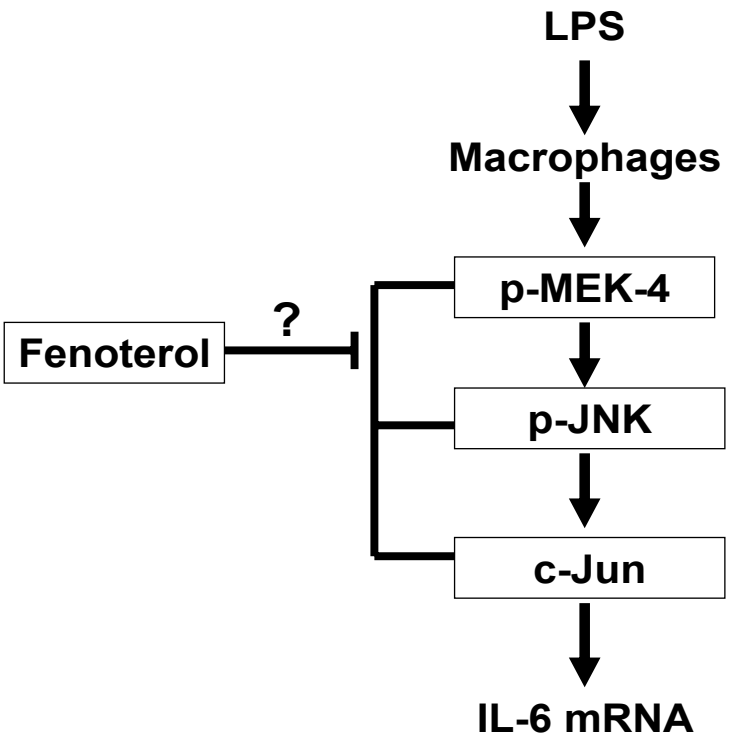


2. Description:

By MTT cell viability assay, fenoterol of different concentrations and time durations with or without LPS is not cell-toxic. Fixed concentrations of fenoterol (100 μM) and LPS (100 ng/ml) were chosen according the the previous studies. After administration of LPS for 6 hours, and after different time intervals (1 and 6 hours), fenoterol was added in each 6 cm culture plates. mRNAs from different plates were all extracted, and by RT-PCR method, IL-6 mRNA was quantitated and analyzed between different groups.

(II) The effects of fenoterol on the of c-Jun nuclear protein and the activation of p-JNK and p-MEK-4 cytoplasmic protein in LPS-activated macrophages

1. Flow chart



2. Description:

After administration of LPS to 6 cm culture plates at different time durations (usually ≤ 2hrs), fenoterol was also added for another shorter durations of time. At different reaction durations, nuclear protein and total cell proteins from different groups were all extracted. These proteins were quantitated and analyzed for the expression of different c-Jun nuclear protein and total proteins (c-Jun, NF-κB, c-Fos, p-JNK and p-MEK-4) by immunoblotting method.

Materials and Methods

I. Experimental materials

Dulbeco's modifications of eagle's medium (DMEM) with 10% FBS (fetal bovine serum), 200 units/ml penicillin, 200 μg/ml streptomycin and 0.584 mg/ml glutamine was purchased from Life Technologies (Grand Island, NY, USA); fenoterol hydrobromide inhalant solution was donated by Boehringer Ingelheim (Bracknell, Berkshire, UK) with a stock concentration of 1.63 mM; PCR primers of mouse IL-6 and β-actin were synthesized according to designs in previous literature (49, 50).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Amersham Life Science (Piscataway, NJ, USA); bicinchonic acid (BCA) protein analysis system and ExpressDirect mRNA Capture & RT system were purchased from Pierce (Rockford, IL, USA); monoclonal antibodies for PCNA, c-Jun , NF-κB, c-Fos and MEK-4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); monoclonal antibodies for JNK, p-JNK and p-MEK-4 were purchased from Cell Signaling Technology (Danvers, MA, USA) . LPS and anti-mouse β-actin monoclonal antibody were purchased from Sigma (St. Louis, MO, USA).

II. Cell culture and drug treatment

Murine macrophage-like RAW 264.7 cells (American Type Tissue Collection, Rockville, MD, USA) were used in this study as the experimental model. The cells were cultured in culture plate with DMEM as culture medium. The incubator was 37℃ with 5% CO_2 in wet condition. After cells growing up

to near full condition in 10 cm plate, they were removed for cell counting. They were divided under subculture into 96-wells clusters or 6 cm plates, then ready with culture for drugs administration till the next day. Fenoterol inhalant solution was stored in room air & protected from light, and prepared by dissolving it in PBS solvent with a stock concentration of 1 mM.

III. Determination of cell viability

Liu et al. (1999) introduced a colorimetric 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) method to determine the appropriate cell viability. Five thousand macrophages were cultured in 96-well tissue culture clusters for overnight. 100 ng/ml LPS and different concentrations of fenoterol (1, 10, and 100 μM) at different reaction durations (1, 6, and 24 hours) were added individually into each cultured wells. The cells were then cultured with fresh medium containing 0.5 mg/ml MTT for another 3 hours. The blue formazan product in cells was dissolved in DMSO and measured spectrophotometrically at a wavelength of 550 nm.

IV. Reverse-transcription polymerase chain reaction (RT-PCR) assay

Total RNA from control and LPS-treated macrophages was prepared for RT-PCR analyses. The primers for IL-6 and β-actin mRNA were synthesized according to previously described designs (49, 50). The oligonucleotide sequences of upstream and downstream primers for IL-6 mRNA analyses were

Mouse IL-6:

5’-ATG AAG TTC CTC TCT GCA AGA GAC T-3’

5’-CAC TAG GTT TGC CGA GTA GAT CTC-3’

Mouse β-actin:

5’-GTG GGC CGC TCT AGG CAC CAA-3’

3’-CTT TAG CAC GCA CTG TAG TTT CTC-5’,

respectively. The PCR products were loaded and separated using 1.8% agarose gels containing 0.1 mg/ml ethidium bromide.

V. Preparation of nuclear and total cell proteins and immunodetection analyses

After drug treatment, nuclear and total extracts of macrophages were prepared. Nuclear proteins extracts were prepared with buffer A (pH 7.4, 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 2 mM PMSF, 10 μg/ml apotenin, 10 μg/ml leupeptin) and buffer B (5 mM DTT, 10 μg/ml leupeptin in lysis buffer) solutions. Total cell proteins were prepared with radio-immunoprecipitation assay (RIPA) buffer (pH 7.2, 25 mM Tris-HCl, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, 1 mM ethylenediaminetetraacetic acid with anti-proteinase 0.2 mM PMSF, 1 μg/ml apotenin, 1 μg/ml leupeptin). Protein concentrations were quantified by a BCA protein assay kit (Pierce). Different proteins (100 μg/well) were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. After blocking, nuclear factors and total cell proteins were immunodetected using polyclonal antibodies against mouse c-Jun, c-Fos and NF-κB (Santa Cruz Biotechnology, Santa Cruz, CA). Proliferating cell nuclear antigen (PCNA) by a mouse monoclonal

antibody against rat PCNA protein (Santa Cruz Biotechnology) and anti-mouse β-actin monoclonal antibody (Sigma, St. Louis, MO, USA) were used as the internal control standard. Intensities of the immunoreactive bands were determined using a digital imaging system (UVtec).

VI. Immunodetection of JNK/MEK-4 and phosphorylated JNK/MEK-4

Cell lysates were prepared in ice-cold radioimmunoprecipitation assay (RIPA) buffer (pH7.2, 25 mM Tris-HCl, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, 1 mM ethylenediaminetetraacetic acid with anti-proteinase 0.2 mM PMSF, 1 μg/ml apotenin, 1 μg/ml leupeptin). Protein concentrations were quantified using a bicinchonic acid protein assay kit (Pierce, Rockford, IL). The proteins (100 μg per well) were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. After blocking, phosphorylated JNK was immunodetected using a rabbit polyclonal antibody corresponding to residues Thr183/Tyr185 of human JNK (Cell Signaling, Danvers, MA). JNK was detected using a mouse monoclonal antibody against human JNK as the internal standard. P-MEK-4/MEK-4 goat polyclonal antibodies were also used. These protein bands were quantified using a digital imaging system (UVtec, Cambridge, UK).

VII. Statistical analysis

Student's *t*-test was used as statistical method. We decided on $\alpha = 0.1$ as the significant level for Student's *t*-test, and had performed Bonferroni's adjustment for each individual analysis. Statistical differences between the

control and drug-treated groups and the one-drug and two-drugs-treated groups were considered significant when the *P*-value of the test was less than or equal to the adjusted α level.

Results

I. Effects of fenoterol and LPS on cell viability of macrophages

In order to examine the macrophage cell toxicity at different concentrations and time durations of fenoterol with or without LPS, MTT assay was used for studies.

Macrophages were treated with 1, 10, and 100 μM fenoterol for 1 and 6 hours, respectively. The cell viability test showed no significant difference between each group (Table 1).

Another group of macrophages was treated with 1, 10, and 100 μM fenoterol (FEN) with or without LPS 100ng/mL for 24 hours. Cell viability test showed no significant difference between each group (Table 2, *P*-value < 0.05 was significant).

From the above data, 100 μM fenoterol (FEN) with or without LPS 100 ng/ml for as long as 24 hours would be non cell-toxic in macrophages.

II. Effects of fenoterol on IL-6 mRNA production in LPS-activated macrophages

In order to evaluate the effects of fenoterol on IL-6 mRNA production in LPS-activated macrophages, RT-PCR method was used.

RAW264.7 macrophages were exposed to 100 ng/ml LPS for 6 hours, or combinations of 100 μM fenoterol respectively for 1 and 6 hours adding to the above 6-hr LPS. Total RNA was prepared for RT-PCR analysis of IL-6. β–actin mRNA was used as internal control for data adjustment (Figure 1A, bottom panel), and the cDNA bands were analyzed and quantified. The data showed

a trend of IL-6 mRNA inhibition for 1-hr L+F (Figure 1A, top panel, lane 6) and 6-hr L+F groups(Figure 1A, top panel, lane 5), but it didn't reach statistically significant between groups (*P*-value < 0.033 was significant).

From the above data, fenoterol had suppressive effect on *IL-6* gene expression in LPS-activated macrophages.

III. Effects of fenoterol on nuclear and total protein c-Jun production in LPS-activated macrophages

In order to study different transcription factors involving in the pathways in LPS-activated macrophages, specific anti c-Jun antibody was used for detection of translocation or production of this protein.

RAW264.7 macrophages were exposed to 100 ng/ml LPS, or a co-treatment of LPS and 100 μM fenoterol, respectively for 1 and 2 hours. Nuclear protein was prepared for immunoblotting analysis of c-Jun protein, and these protein bands were analyzed and quantified. The results revealed that after co-treatment of LPS and fenoterol for 2 hours, the translocation of c-Jun nuclear protein was more significant at 1st hour then attenuated at 2nd hour (Figure 2A, top panel, lanes 3 and 5). And, fenoterol could suppress the c-Jun nuclear protein translocation significantly at 2nd hours (Figure 2A, top panel, lane 6) with *P*-value= 0.016 compared with the LPS 2-hr treated group (Figure 2, top panel, lane 5).

In order to study the effects of fenoterol on c-Jun nuclear protein expression at 6th hours, RAW264.7 macrophages were exposed to 100 ng/ml LPS or a combination of LPS and 100 μM fenoterol for 6 hours. The data showed a trend of c-Jun protein suppression in the 6-hr L+F group (Figure 3A,

lane 3), but it didn't reach statistically significant between groups (Figure 3, *P*-value < 0.05 was significant).

For the effects of LPS and fenoterol on c-Jun total protein production within 1 hour, RAW264.7 macrophages were exposed to 100 ng/ml LPS or a co-treatment of LPS and 100 μM fenoterol for 1 hour. Total cellular protein was prepared for immunoblotting analysis of c-Jun protein. The results revealed that fenoterol could also suppress the c-Jun total protein production significantly (Figure 4A, top panel, lane 4) with *P*-value= 0.0005 (*P*-value < 0.05 was significant) compared with the 1-hr LPS treated group (Figure 4A, top panel, lane 2).

From the above data, fenoterol could suppress the translocation and production of c-Jun protein expression in LPS-activated macrophages within 1 to 2 hours.

IV. Effects of fenoterol on other total proteins (NF-κB and c-Fos) production in LPS-activated macrophages

In order to study different transcription factors involving in the pathways in LPS-activated macrophages, specific anti NF-κB and c-Fos antibodies were used for detection of these proteins production.

For the effects of fenoterol on total NF-κB and c-Fos proteins within 1hour. RAW264.7 macrophages were exposed to 100 ng/ml LPS, or a co-treatment of LPS and 100 μM fenoterol for 1 hour. Total cellular proteins were prepared for immunoblotting analysis of NF-κB and c-Fos proteins. The results revealed that fenoterol couldn't suppress the NF-κB and c-Fos proteins production significantly (Figures 5A and 6A).

From the above data, fenoterol couldn't suppress the production of NF-κB and c-Fos proteins in LPS-activated macrophages within 1 hour.

V. Effects of fenoterol on p-JNK proteins production in LPS-activated macrophages

In order to define the upstream pathway involving in the c-Jun regulation for fenoterol in LPS-activated macrophages, specific anti p-JNK antibody was used for detection of protein activation.

In the beginning, we determined the effects of LPS concentration and timing on p-JNK total proteins expression within 40 minutes. RAW264.7 macrophages were exposed to high dose- (1 μg/ml) or low dose- (100 ng/ml) LPS, respectively at different time durations of 10, 15, 20 and 40 min. Total cellular protein was prepared for immunoblotting analysis of p-JNK and JNK proteins, and these protein bands were analyzed and quantified. The results revealed that high dose-LPS (1 μg/ml) could induce more p-JNK proteins at 10^{th} min. and low dose-LPS (100 ng/ml) could also induce p-JNK proteins production at time-dependent manner within 40 minutes (Figure 7A, top panel, no statistics done).

For the effects of LPS and fenoterol on JNK total protein production within 2 hours. RAW264.7 macrophages were exposed to 100 ng/ml LPS or a co-treatment of LPS and 100 μM fenoterol respectively, for 1 and 2 hours. Total cellular protein was prepared for immunoblotting analysis of p-JNK and JNK protein. The results revealed that LPS could insignificantly induce p-JNK protein in a time-dependent and a decreasing manner within 2 hours (Figure 8B). In 2 hours, fenoterol could insignificantly suppress the p-JNK total protein production (Figure 8A, top panel, lanes 3 and 5).

From the above data, fenoterol could insignificantly suppress the p-JNK protein activation in LPS-activated macrophages within 1 to 2 hours.

VI. Effects of fenoterol on p-MEK-4 proteins production in LPS-activated macrophages

In order to define more upstream pathway involving in the c-Jun regulation for fenoterol in LPS-activated macrophages, specific anti p-MEK-4 antibody was used for detection of protein activation.

As for the effects of LPS and fenoterol on p-MEK-4 total protein production within 50 min, RAW264.7 macrophages were exposed to 100 ng/ml LPS or a co-treatment of LPS and 100 μM fenoterol at different time intervals for 10, 20 and 50 min. Total cellular protein was prepared for immunoblotting analysis of p-MEK-4 and MEK-4 protein. The results revealed fenoterol could insignificantly suppress the p-MEK-4 total protein production within 50 minutes (Figure 9, no statistics done).

From the above data, fenoterol could insignificantly suppress the p-MEK-4 protein activation in LPS-activated macrophages within 50 min.

Discussion

Previous studies have revealed that β-agonist caused cytokine inhibition (e.g. TNF and IL-6 production) in different septic models, and some β-adrenergic agents are studied as treatments targeting earlier resolution of ARDS (22). By the literature review till now, no known mechanism is ever elucidated in previous articles for the anti-inflammation effect of fenoterol. The purposes of this study were to evaluate the effect and the mechanism for fenoterol on IL-6 mRNA inhibition in LPS-activated macrophage-like cell line RAW264.7. The results of this study revealed that fenoterol could suppress IL-6 mRNA expression through c-Jun-dependent and possibly MEK-4/JNK-dependent pathway. This was a novel study which elucidated the relationship of fenoterol, c-Jun protein and IL-6 expression.

However, in spite of this novel study, there were some limitations. First, we chose a macrophage-like cell line, RAW264.7, as an in vitro model of acute lung injury. This couldn't really represent the whole condition in diseased lungs, and might ignore the relationship between different cells and systems. Second, in this study, we measured the IL-6 mRNA as an end point of the pathway but didn't survey the IL-6 protein levels which could probably limit its clinical applications. Third, we designed a simple RT-PCR method for evaluation of *IL-6* gene expression and this might not reflect all the aspects of IL-6 expression. In literature, many other methodologies were ever introduced (51) for the studies of *IL-6* gene expression, such as IL-6 genomic DNA isolation by hybridization, IL-6 promoter-containing plasmids transfection and electrophoretic mobility shift assay for the binding of the transcription factor and IL-6 promoter in wild type and mutant type.

In this study, we used Student's *t*-test and α = 0.1 as the significance level and performed Bonferroni's adjustment for α in each statistical analysis. However, small sample size would make the standard error of mean (SEM) levels relatively high and limit statistical significance in this study. These will be adjusted if larger sample sizes were completed in the future.

There was also a discrepancy between this study and other articles. Our study demonstrated that fenoterol could inhibit IL-6 mRNA through c-Jun -related mechanism, while the role of NF-κB was unclear. Libermann and Baltimore found NF-κB could activate *IL-6* gene expression in human monocytic cell line U-937 (51). Farmer and Pugin reported the IκB/NF-κB pathway involving the anti-inflammatory effects of TNF-α and IL-8 in the human promonocyte THP-1 cells (27). While in different studies, Erk and p38 kinase pathways were found necessary for *IL-6* gene expression in LPS stimulated human alveolar macrophages (48) and JNK1 was also reported (44) to be required for pro-inflammatory cytokines biosynthesis (TNF, IL-1 , IL-6). These different pathways might be due to different cell models or different β-agonists used, and further study designs will be required for clarifying the differences.

I. Effects of fenoterol and LPS on cell viability of macrophages

In this study, the cell viability test by MTT showed no significant concentration and time duration effects of fenoterol with or without LPS in macrophage (Tables 1 and 2). In that, we chose a fixed concentration of 100 ng/ml LPS reasonably according to the same concentration in the previous studies in our laboratory. The time duration of MTT was extended up to 24 hour in order to meet the following experimental designs (maximally, 6 hours

of studies observation). Because the exact alveolar concentration of fenoterol in human studies is unreliable (29), 100 μM fenoterol was used in this study according to the previous experimental concentration range of 10^{-4} to100 μM (27, 30).

II. Effects of fenoterol on IL-6 mRNA production in LPS-activated macrophages

"Fenoterol could suppress IL-6 mRNA production" was one of our major specific aims in this study. But by our results, fenoterol could only insignificantly suppress IL-6mRNA within 6 hours in LPS-activated macrophages (Figure 1A, B). This was because the sample size was only two in number and the standard error of mean (SEM) value was relatively large in each groups, and this would subsequently make the statistics insignificant. But it is still reasonable that fenoterol could inhibit cytokines according to the pervious studies (27). Further studies would be performed with more sample number in future design.

III. Effects of fenoterol on nuclear and total proteins (c-Jun, NF-κB and c-Fos) production in LPS-activated macrophages

(I) C-Jun nuclear and total protein expression treated with LPS and fenoterol

In our study, fenoterol could significantly (*P*-value = 0.0016 vs. 2-hr LPS group) suppress c-Jun nuclear protein at 2nd hour in LPS-activated macrophages (Figure 2). This is a novel finding that fenoterol could

mediate the translocation of c-Jun nuclear protein in the pathways of *IL-6* gene expression.

Also in this study, fenoterol could significantly suppress c-Jun total protein at 1st hour in LPS-activated macrophages (Figure 4). This maybe related to the effect of fenoterol on the c-Jun total protein production not only via the protein translocaton in the pathway.

(II) Other total protein expression treated with LPS and fenoterol

In our studies, fenoterol could not significantly suppress NF-κB or c-Fos total proteins within 1 hour in LPS-activated macrophages (Figures 5 and 6). This is different from other study that isoproterenol as a nonspecific β-agonist exerts anti-inflammation effects through the cAMP/IκB/NF-κB pathway (27). This might be due to different concentrations or kinds of β-agonists and different cell models used and thus it presented in a different signaling pathway.

IV. Effects of fenoterol on p-JNK proteins production in LPS-activated macrophages

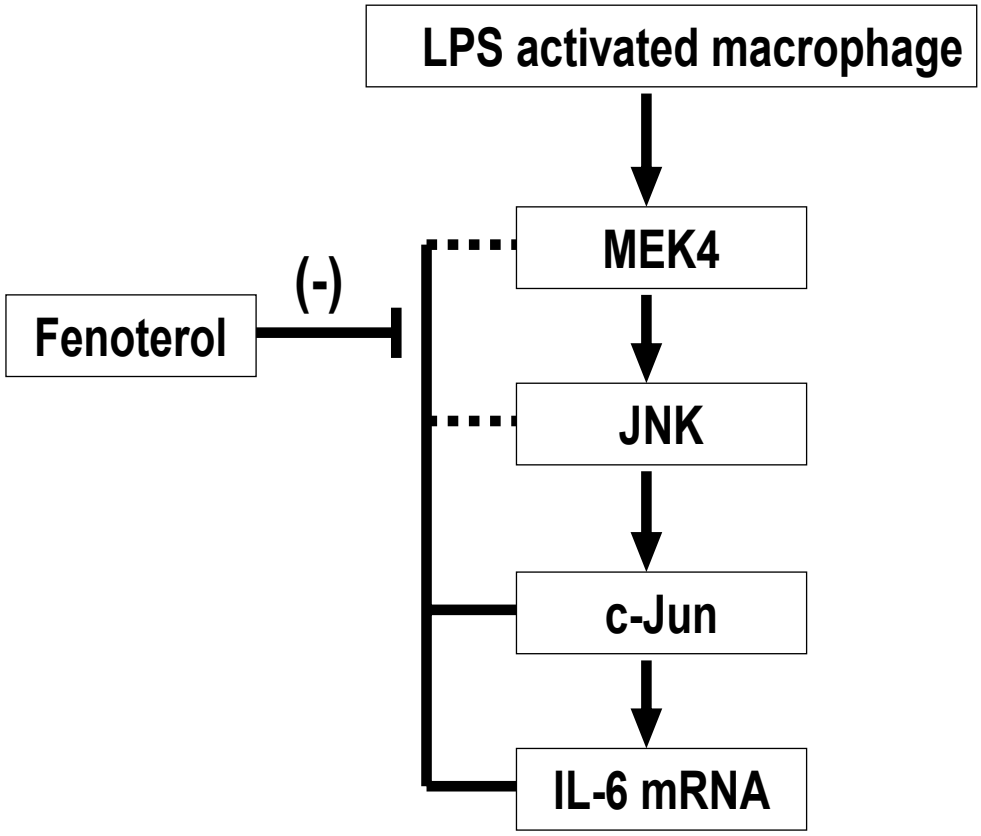
By our results, fenoterol could only insignificantly suppress p-JNK protein within 1 to 2 hours (Figure 8) in LPS-activated macrophages. This might due to a smaller sample size thus making the statistics insignificant or might relate to other upstream signaling (e.g. P38 or ERK) in the pathways. Further planning would be performed with larger sample size or different upstream signaling proteins (e.g. P38 or ERK proteins) in the future.

V. Effects of fenoterol on p-MEK4 proteins production in LPS-activated macrophages

In our study, the suppression of fenoterol on p-MEK-4 protein within 50 min (Figure 9) was insignificant in LPS-activated macrophages, and the sample size was small so we didn’t make a mean comparison statistical analysis for the data. The other upstream signaling proteins might be involved in the pathways. Future studies with larger sample sizes or with different upstream signaling proteins will be strongly suggested.

Conclusion

In conclusion, the results of this study revealed that fenoterol could suppress IL-6 mRNA production. The pathway was c-Jun-dependent and possibly related to JNK/MEK-4. A scheme for the summary of our results was shown as below:



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Tables and Figures

Table 1. Effects of concentration and time duration of fenoterol treatment on macrophage viability

	Cell viability, O.D. values at 550nm	
FEN, μM	1 hr	6 hr
0	1.62 ± 0.09	1.59 ± 0.07
1	1.64 ± 0.06	1.52 ± 0.09
10	1.65 ± 0.05	1.46 ± 0.09
100	1.68 ± 0.07	1.54 ± 0.05

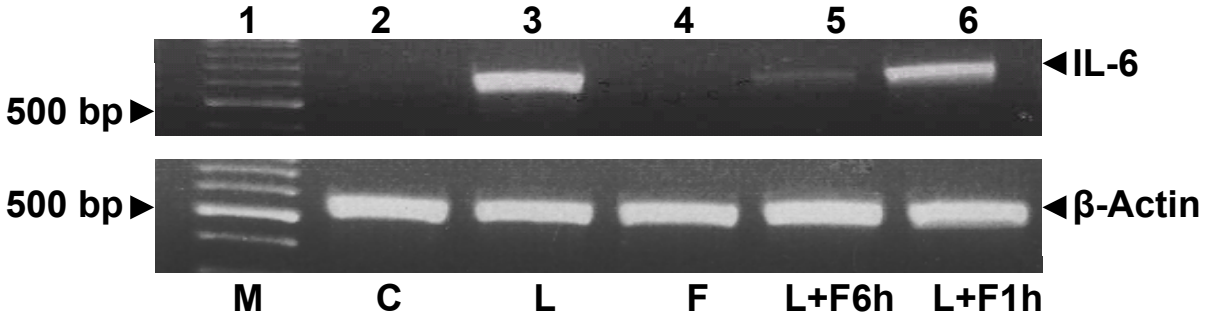
Macrophages were treated with 1, 10, and 100 μM fenoterol (FEN) for 1 and 6 hours, respectively. Cell viability was determined by the MTT assay. Each value represented the mean ± SEM for n = 6 and showed no significant difference between each group (P-value < 0.05 was significant).

Table 2. Effects of LPS and fenoterol on macrophages viability

	Cell viability, O.D. values at 550nm in 24 hours	
FEN, μM	FEN	LPS+ FEN
0	0.63 ± 0.03	0.82 ± 0.06
1	0.69 ± 0.04	0.75 ± 0.04
10	0.80 ± 0.10	0.80 ± 0.04
100	0.78 ± 0.05	0.72 ± 0.07

Macrophages were treated with 1, 10, and 100 μM fenoterol (FEN) with or without LPS 100 ng/ml for 24 hours. Cell viability was determined by the MTT assay. Each value represented the mean ± SEM for n = 6 and showed no significant difference between each group (P-value < 0.05 was significant).

A



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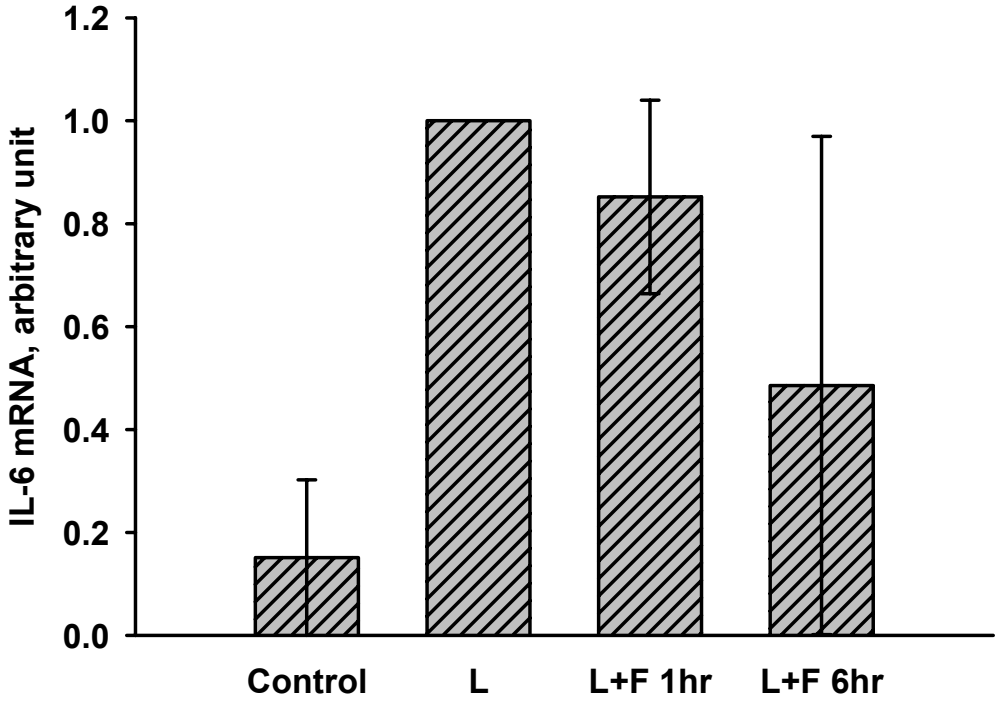
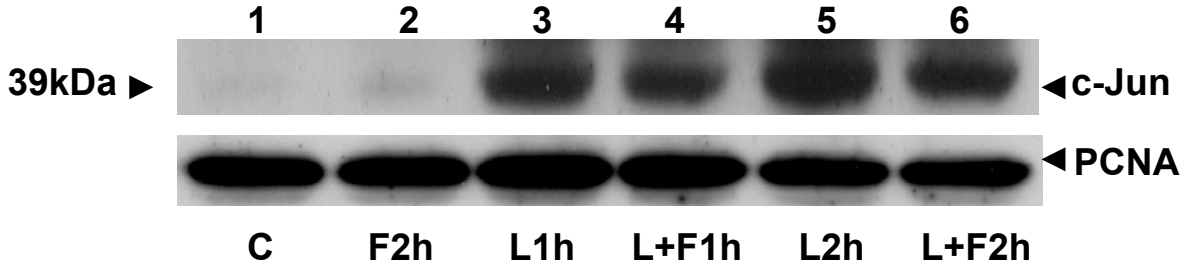


Figure 1. Effects of LPS and fenoterol on IL-6 mRNA production. RAW264.7 macrophages were exposed to 100 ng/ml LPS for 6 hours, or combinations of 100 μM fenoterol respectively for 1 and 6 hours adding to 6 hours-LPS. Total RNA was prepared for RT-PCR analysis of IL-6 and β–actin mRNA. (A) These cDNA bands were analyzed and quantified. (B) Each value represents the mean ± SEM for n = 2. The data showed a trend of IL-6 mRNA inhibition in L+F 1hr and L+F 6hr groups, but it didn't reach statistically significant between groups (P-value < 0.033 was significant).

A



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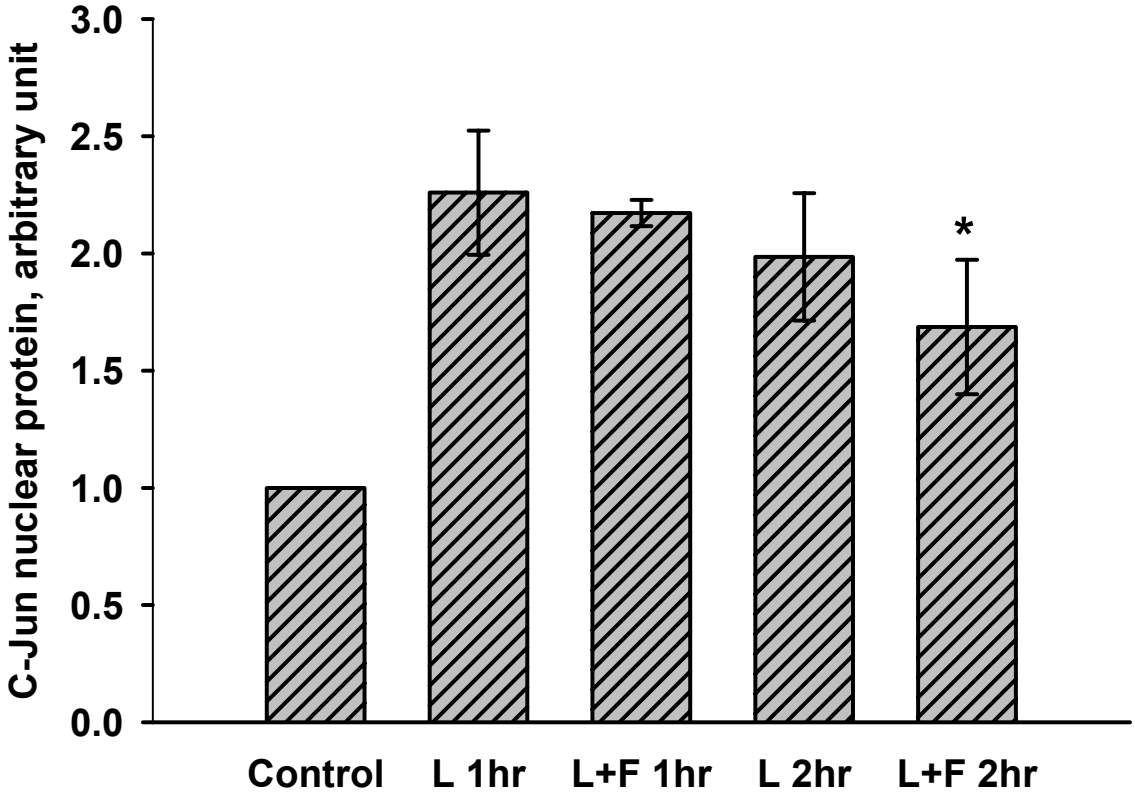


Figure 2. Effects of LPS and fenoterol on c-Jun nuclear protein production within 2 hours. RAW264.7 macrophages were exposed to 100 ng/ml LPS, or a co-treatment of LPS and 100 μM fenoterol, respectively for 1 and 2 hours. Nuclear protein was prepared for immunoblotting analysis of c-Jun protein.

(A) These protein bands were analyzed and quantified. (B) Each value represents the mean ± SEM for n = 2.

* Value significantly differed from the 2-hr LPS treated group, P-value < 0.025.

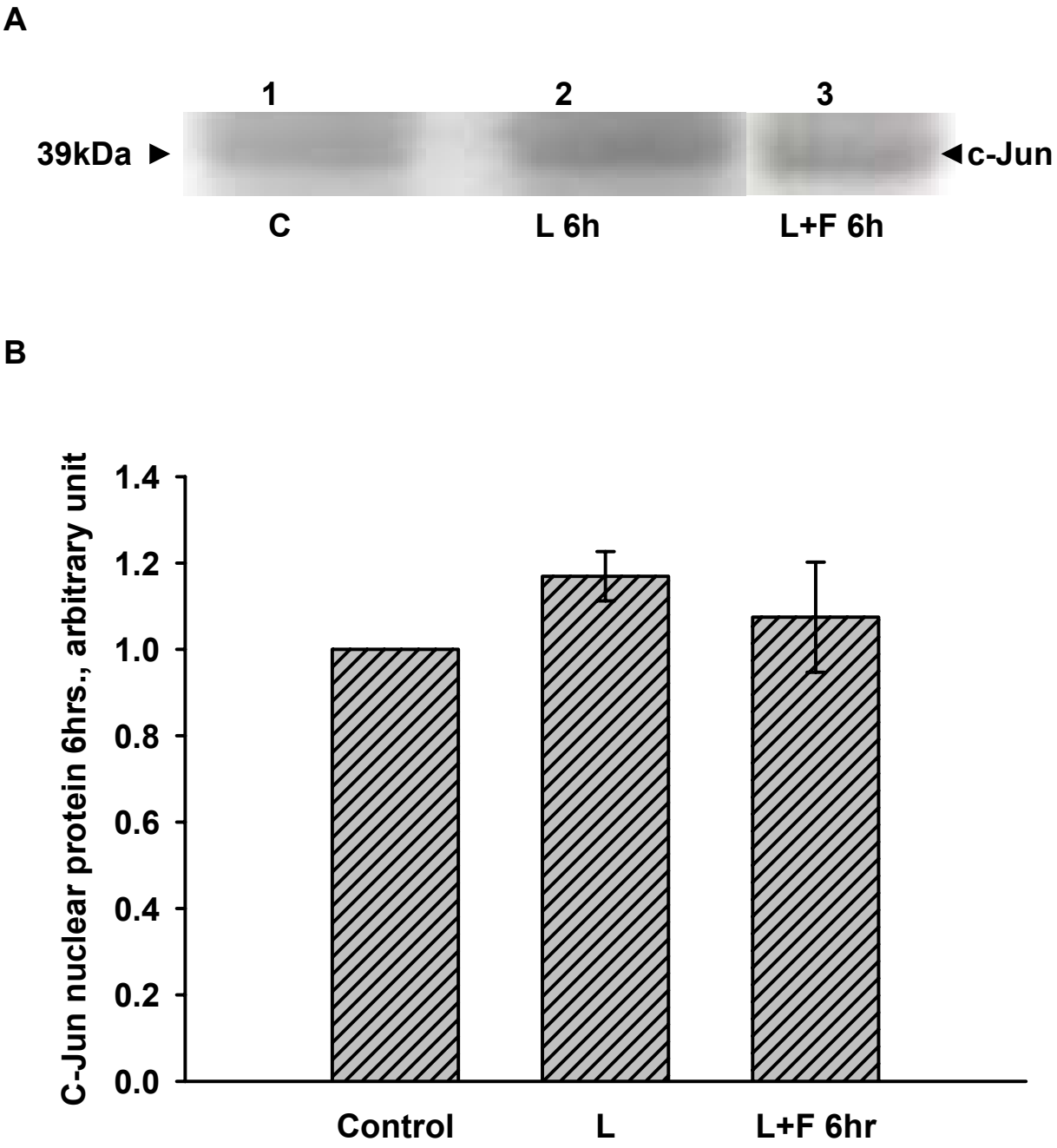
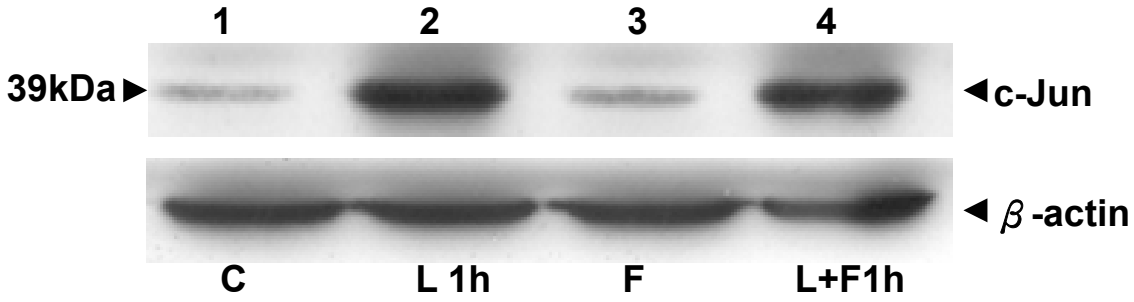


Figure 3. Effects of LPS and fenoterol on c-Jun nuclear protein production at the 6th hour. RAW264.7 macrophages were exposed to 100 ng/ml LPS or a combination of LPS and 100 μM fenoterol for 6 hours. Nuclear protein was prepared for immunoblotting analysis of c-Jun protein. (A) These protein bands were analyzed and quantified. (B) Each value represents the mean ± SEM for n = 2. The data showed a trend of suppression of c-Jun protein for L+F 6hr group, but it didn't reach statistically significant between groups (P-value < 0.05 was significant).

A



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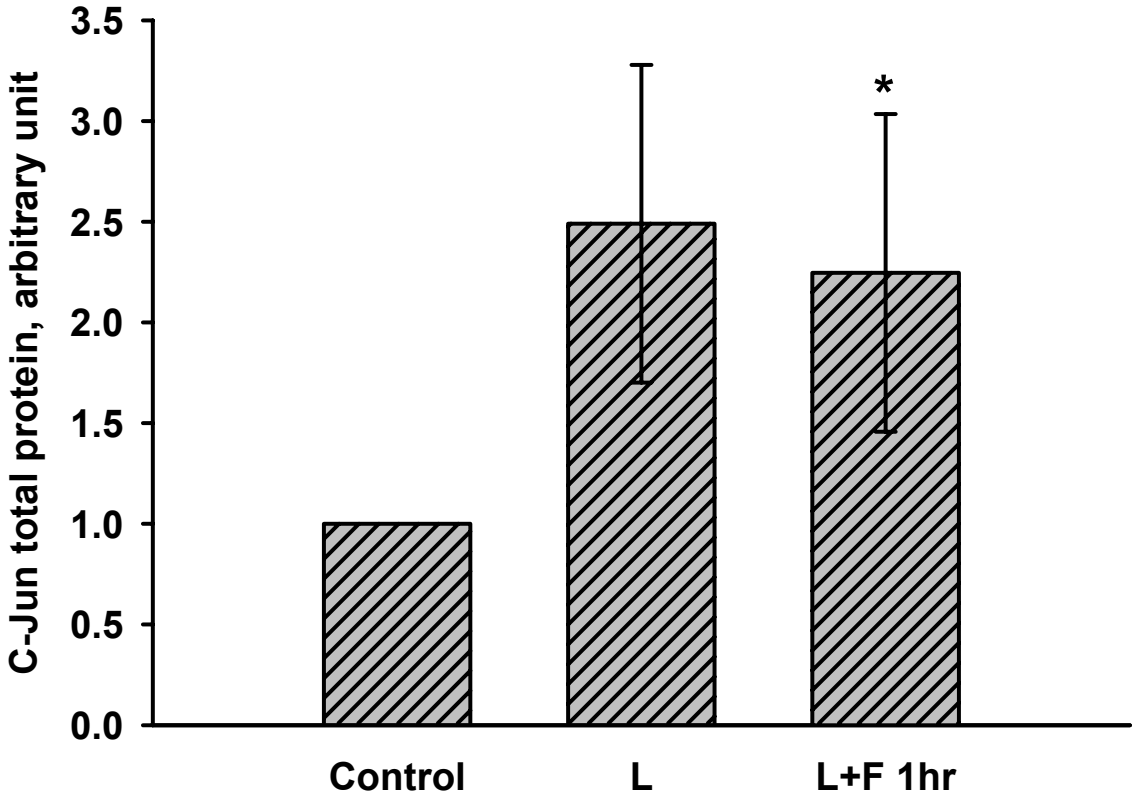
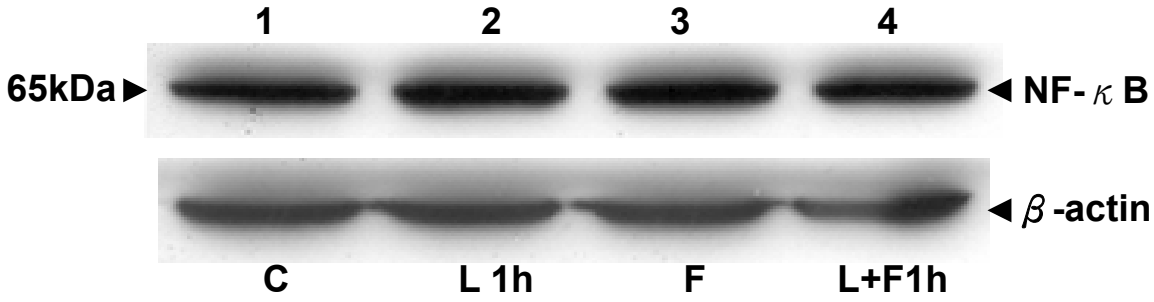


Figure 4. Effects of LPS and fenoterol on c-Jun total protein production within 1 hour. RAW264.7 macrophages were exposed to 100 ng/ml LPS or a co-treatment of LPS and 100 μM fenoterol for 1 hour. Total cellular protein was prepared for immunoblotting analysis of c-Jun protein. (A) These protein bands were analyzed and quantified. (B) Each value represents the mean ± SEM for n = 3.

* Values significantly differ from the LPS treated group, P-value< 0.05.

A



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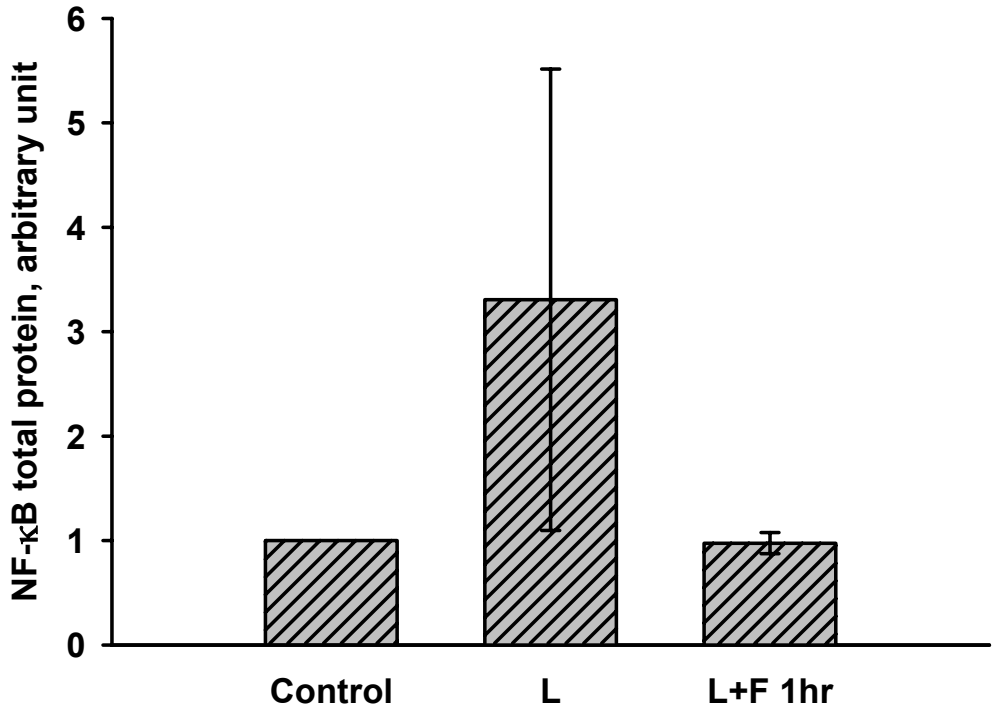
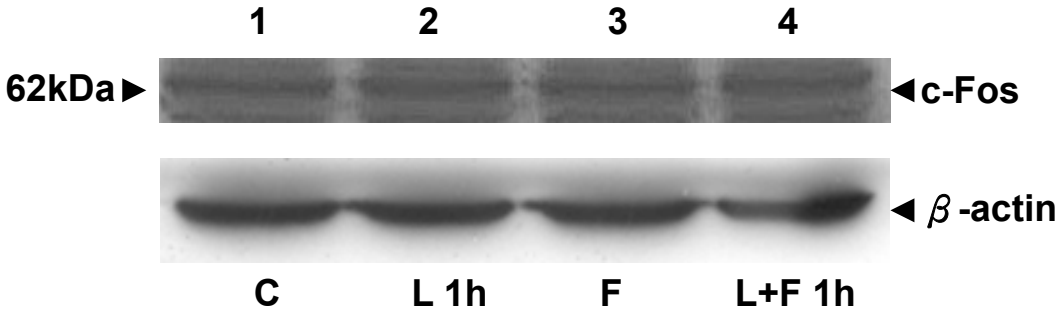


Figure 5. Effects of LPS and fenoterol on NF-κB total protein production within 1 hour. RAW264.7 macrophages were exposed to 100 ng/ml LPS or a co-treatment of LPS and 100 μM fenoterol for 1 hour. Total cellular protein was prepared for immunoblotting analysis of NF-κB protein. (A) These protein bands were analyzed and quantified. (B) Each value represents the mean ± SEM for *n* = 3. The data showed no significant difference between each groups (*P*-value < 0.05 was significant).

A



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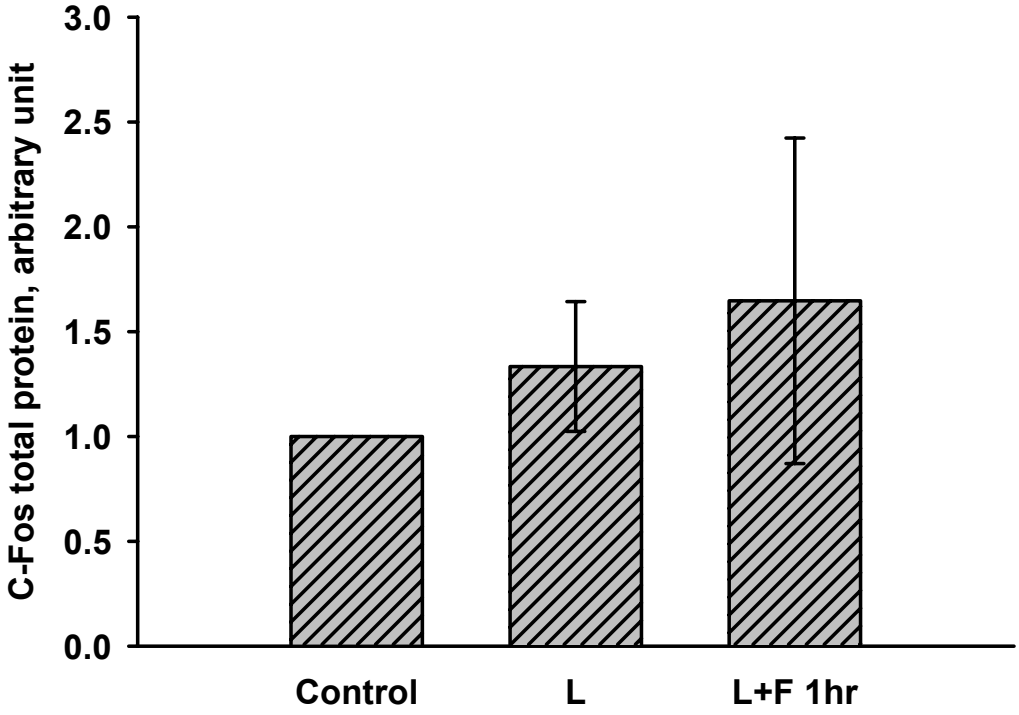
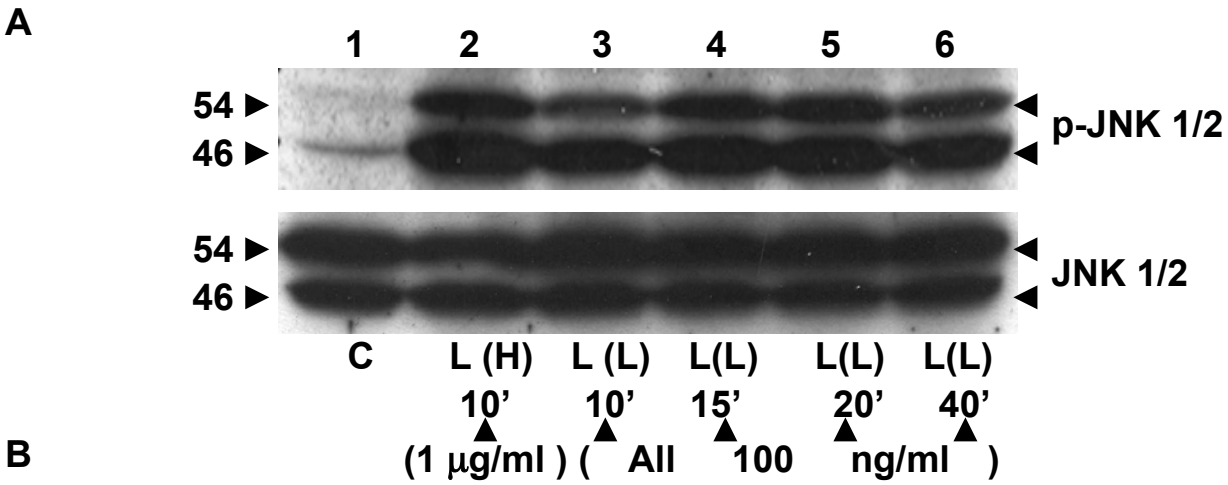


Figure 6. Effects of LPS and fenoterol on c-Fos total protein production within 1 hour. RAW264.7 macrophages were exposed to 100 ng/ml LPS or a co-treatment of LPS and 100 μM fenoterol for 1 hour. Total cellular protein was prepared for immunoblotting analysis of c-Fos protein. (A) These protein bands were analyzed and quantified. (B) Each value represents the mean ± SEM for n = 3. The data showed no significant difference between each groups (P-value < 0.05 was significant).



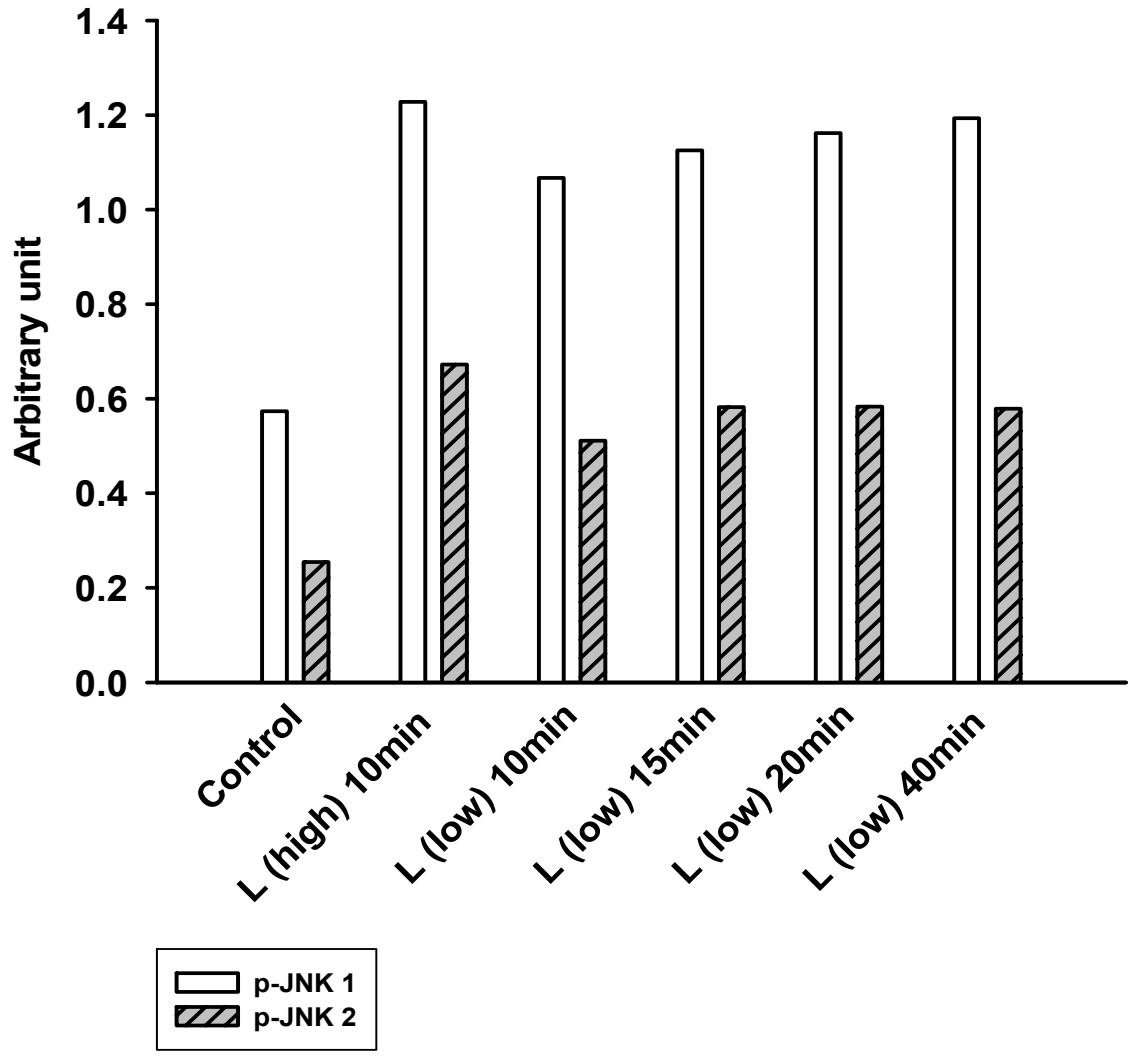
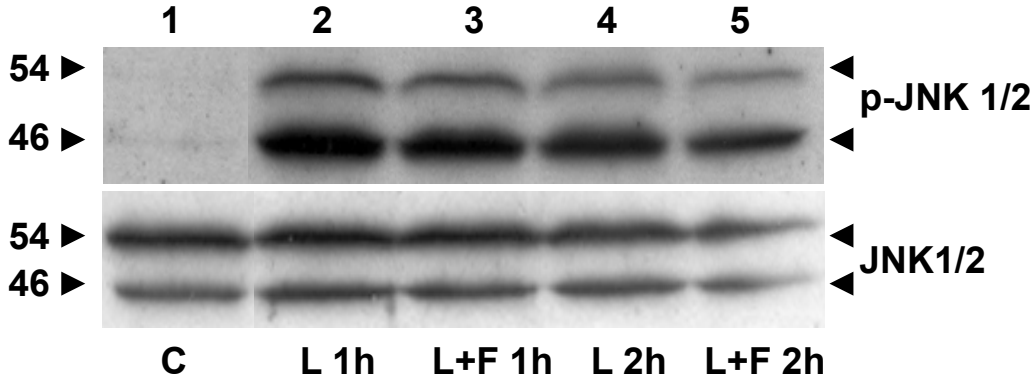


Figure 7. Effects of LPS concentration and timing on p-JNK total proteins production within 40 minutes. High dose-LPS (1 μg/ml) could induce more p-JNK proteins at 10^{th} min. and low dose-LPS (100 ng/ml) could also induce p-JNK proteins production at time-dependent manner within 40 minutes (*n* = 1, no statistics done).

A



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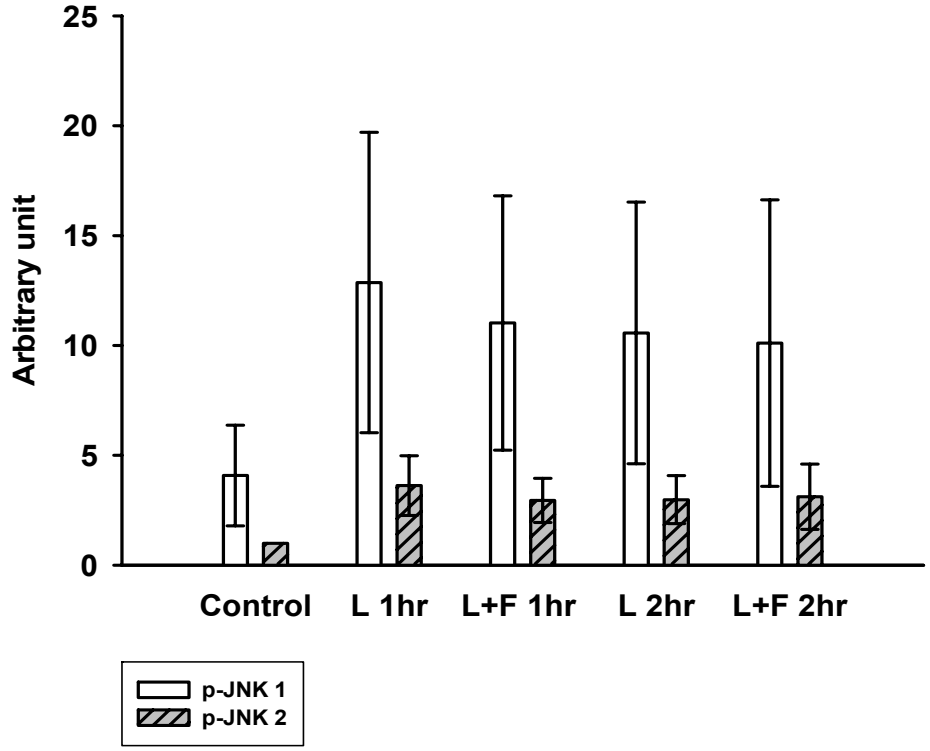
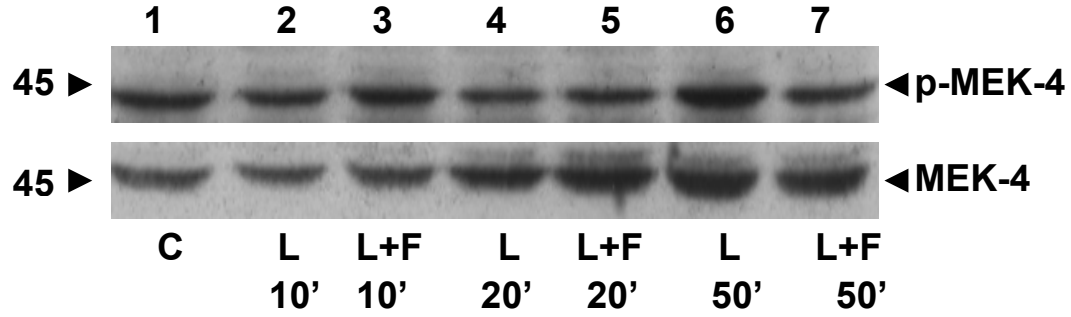


Figure 8. Effects of LPS and fenoterol on p-JNK total proteins production within 2 hours. RAW264.7 macrophages were exposed to 100 ng/ml LPS or a combination of LPS and 100 μM fenoterol, respectively for 1 and 2 hours. Total cellular protein was prepared for immunoblotting analysis of p-JNK1/2 and JNK1/2 protein. (A) These protein bands were analyzed and quantified.
(B) Each value represents the mean ± SEM for *n* = 3. The data showed a trend of suppression of p-JNK1/2 protein for L+F 1hr and L+F 2hr groups, but it didn't reach statistically significant between groups (*P*-value < 0.025 was significant).

A



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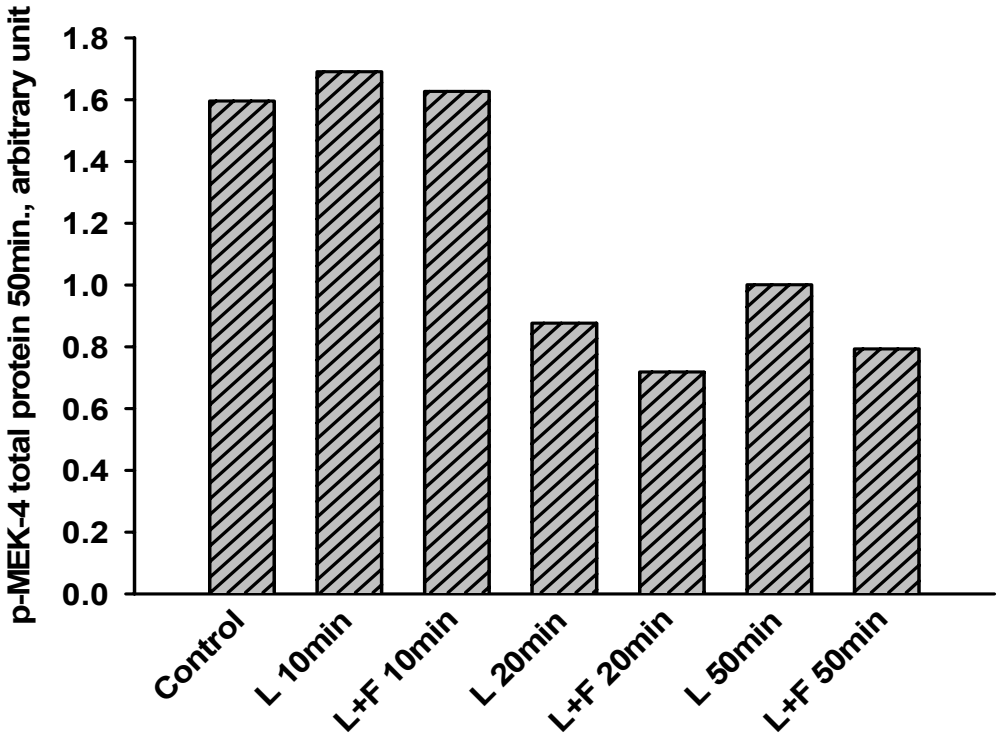

Figure 9. Effects of LPS and fenoterol on p-MEK-4 total protein production within 50 minutes. RAW264.7 macrophages were exposed to 100 ng/ml LPS or a combination of LPS and 100 μM fenoterol, respectively for 10, 20 and 50 minutes. Total cellular protein was prepared for immunoblotting analysis of p-MEK-4 and MEK-4 protein. (A), (B) These protein bands were analyzed and quantified for *n* = 1. The data showed a trend of suppression of p-MEK-4 protein for L+F groups (*n* = 1, no statistics done).