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NLRP3 INFLAMMASOME ACTIVATION IN MACROPHAGE (RAW 264.7) CELLS BY LIPOPOLISACCARIDE/NIGERICIN: REGULATORY EFFECT OF PSORALIDIN
LİPOPOLİSAKKARİT/NİGERİSİN İLE MAKROFAJ (RAW 264.7) HÜCRELERİNDE NLRP3 İNFLAMAZOM AKTİVASYONU: PSORALİDİN'İN REGÜLATÖR ETKİSİ

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ABSTRACT

Psoralidin, a prenylated coumestan, has been reported its anti-inflammatory effect, but the regulatory effects on inflammasome activation and pyroptosis-related cytokines are not clear. The aim of this study was to investigate the regulatory role of psoralidin on inflammasome activation and expression of pyroptosis-related cytokines in Lipopolysaccharide (LPS)/Nigericin-stimulated RAW 264.7 macrophages. Regulatory mechanism of psoralidin on inflammasome activation was evaluated using Western blotting for NLRP3, adaptor protein apoptosis-associated speckle-like protein (ASC) and Caspase-1. Pyroptosis-related cytokines were evaluated using RT-PCR for interleukin-1β (IL-1β)and interleukin-18 (IL18). Non-cytotoxic concentration of Psoralidin (5.0μM) significantly inhibited LPS/Nigericin induced inflamasome activation. In addition, pretreatment with psoralidin suppressed the LPS/Nigericin-induced production of IL-1β and IL18. Our results indicate that the regulatory effects of psoralidin on inflammasome activation and pyroptosis related cytokine production in RAW 264.7 macrophages are associated with suppression of NLRP3inflammasome activation and inhibition of the release of pyroptosis related cytokines. According to these results, Psoralidine may be considered as a potential therapeutic candidate for the prevention of inflammatory diseases or to support the treatment of inflammation.

Keywords: Inflammasomes, Lipopolysaccharides, Pyroptosis, Cytokines

ÖZ

Prenile edilmiş bir komestan olan psoralidin'in anti-inflamatuvar etkisi bildirilmiştir, ancak inflamazom aktivasyonu ve piroptozis ile ilişkili sitokinler üzerindeki düzenleyici etkileri açık değildir. Bu çalışmanın amacı, psoralidinin Lipopolisakkarit (LPS)/Nigerisinile uyarılan RAW 264.7 makrofajlarında inflamazom aktivasyonu ve piroptozis ile ilişkili sitokinlerin ifadelenmesi üzerindeki düzenleyici rolünü araştırmaktı. Psoralidinin inflamazom aktivasyonu üzerindeki düzenleyici mekanizması, NOD (Nükleotid bağlama ve oligomerizasyon alanı) benzeri reseptör proteini 3 (NLRP3), adaptör protein apoptozla ilişkili benek benzeri protein (ASC) ve Kaspaz-1 için Western blot kullanılarak değerlendirildi. Piroptozis ile ilişkili sitokinler, interlökin-1β(IL-1β)ve interlökin-18 (IL-18) için RT-PCR kullanılarak değerlendirildi. Psoralidin'in sitotoksik olmayan konsantrasyonu (5.0μM) LPS-Nigerisin ile uyarılan inflamazom aktivasyonunu önemli ölçüde inhibe etti. Ek olarak, psoralidin ile ön tedavi, LPS/Nigerisin kaynaklı IL-1β ve IL18 üretimini bastırdı. Sonuçlarımız, Psoralidin'in RAW 264.7 makrofajlarında inflamazom aktivasyonu ve piroptozis ile ilişkili sitokin üretimi üzerindeki düzenleyici etkilerinin, NLRP3 inflamazom aktivasyonunun baskılanması ve piroptoz ile ilgili sitokinlerin salınımının inhibisyonu ile ilişkili olduğunu göstermektedir. Bu sonuçlara göre, Psoralidin, inflamatuvar hastalıkların önlenmesi veya inflamasyon tedavisinin desteklenmesi için potansiyel bir terapötik aday olarak düşünülebilir.

Anahtar kelimeler: Inflamazomlar, Lipopolisakkaritler, Piroptozis, Sitokinler

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INTRODUCTION

Interleukin-1β (IL-1β) and interleukin-18 (IL-18) have a central role in the inflammatory process with their pleiotropic effects (1). Expression of several proinflammatory cytokines is regulated at the transcriptional level, but additional proteolytic regulation is required for the secretion of IL-1β and IL-18 cytokines. Multimeric protein platforms called the inflammasomes are critical for activation of this proinflammatory cytokines (2). Among the identified inflammasomes, The NOD (nucleotide-binding and oligomerization domain)-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome is the one that has been frequently studied by researchers and is known to be initially activated by ATP and some bacterial toxins (3). Subsequently, several stimuli triggering NLRP3 inflammasome activation were identified, including multiple microbial products, endogenous molecules, and particulate matter (4-6). The essential components of the functional NLRP3 inflammasome are NLRP3, adapter protein ASC (apoptosis-associated speckle-like protein) and caspase-1(7). Increasing evidence in animal models and *in vitro* studies supported by human data has implied that inflammasome activation is among the effective mechanisms in the pathogenesis of inflammatory diseases (8). Therefore, suppression of proteolytic maturation of IL-1β and IL-18 through regulation of NLRP3 inflammasome activation may be an effective target in the treatment of inflammatory disorders.

Psoralidin, isolated for the first time in the seeds of *Psoraleacorylifolia* (Leguminosae) has numerous pharmacological bioactivities including anti-inflammatory (9), anti-oxidant (10), anti-microbial (11), anti-osteoporosis (12), and anti-tumor activity (13). Although its anti-inflammatory effect has been evaluated through mechanisms such as suppression of cyclooxygenase-2 (COX-2) and regulation of IL-6-induced STAT-3 activation, the effect of psoralidin on inflammasome activation and IL-1β/IL-18 expression have not been reported. In the present study, we aimed to demonstrate the modulatory effect of psoralidin on the regulation of inflammation and inflammasome activation by the way of IL-1β/IL-18 secretion in macrophages stimulated with LPS/Nigericin.

MATHERIAL AND METHODS

Cell culture

Mouse macrophage cells RAW 264.7 were cultured in DMEM medium supplemented with % 10 Fetal Bovine Serum and antibiotics Penicillin+Streptomycin (100 U/ml+10 µg/ml and grown in a standard cell culture incubator.

Cytotoxicity assay (MTT)

Cytotoxicity of psoralidin (Sigma-Aldrich cat no: SML0932) was measured with3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT)assay (14). RAW 264.7cells (20000cell/well) were treated with 0-40 µmol/Lpsoralidin in DMEM for 24 h. MTT dissolved in dH_2O and added tothe well with final concentration of 0,5 mg/mL.MTT was incubated in the well for 1 h.After incubation period, DMSO was added for solubilization of formazan crystals. In the wells where the purple color was observed, absorbances were recorded at 570 nm using a microplate reader (Bio-Tek ELX800, BioTek Instruments Inc., Winooski, VT).

Western Blot

RAW264.7 cells were pretreated with 2.5 and 5.0 µM psoralidin for 4 hours, followed by incubation with LPS/ Nigericin (1 µg/mL/20 µM) for 1 hour. After treatment procedure, total protein was isolated using RIPA lysis reagent containing protease inhibitor cocktail. Protein content measured with BCA kit (Pierce, USA). Each protein was loaded and separated by standard polyacrylamide gel electrophoresis procedure. To detect protein expression, proteins transferred to the PVDF membrane were probed with the specific antibodies against NLRP3, ASC, Caspase-1 and β-Actin (YL Biont, Shanghai, China) followed by the secondary antibodies coupled to horseradish peroxidase. Antibody labeled proteins on the membrane were detected using chemiluminescent kit (Pierce, USA) using imaging system (ChemiDoc MP Bio-Rad).

Quantitative real-time PCR

RNAzol total RNA isolation kit (*Sigma-Aldrich*, St. Louis, MO) was used according to the manufacturer's instructions. WizScript™ cDNA Synthesis Kit was used for revers transcription in a total volume20 µL. To quantify cDNA, qPCR was performed using *WizPure™ PCR 2X Master*.Primer sequences were as follows: IL-1β F: 5'-ACCTAGCTGTCAACGTGTGG-3' R: 5'-TCAAAGCAATGTGCTGGTGC-3', IL-18 F:5'-GACAGCCTGTGTTCGAGGAT-3' R: 5'-TTCACAGAGAGGGTCACAGC-3'. Light Cycler 480 (Roche Diagnostics GmbH, Mannheim, Germany) was utilized for Quantative Real-time PCR. Target gene transcripts was normalized to β-actin.

Statistical Analysis

All data were presented as mean±SD. analyzed by Sigma Plot 12 software. Comparison among groups was analyzed with student-t test and $P<0,05$ was considered as significant difference. The conformity of variances to normal distribution was evaluated through the Shapiro-Wilk test.

RESULTS

Effects of psoralidin on cell viability

In our study, a significant decrease in cell viability was observed starting with 10 µmol/L psoralidin (82.7±2.7 % of control) concentration as assessed by MTT was obtained after 24 h treatment period (Figure I).

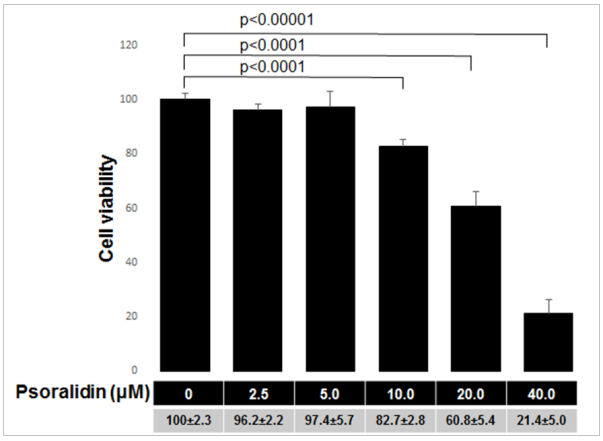

Figure I. The effect of psoralidin (0-40 µM) administration on RAW 264.7 mouse macrophage cell viability, n=4.

Psoralidin was non-toxic at concentrations 2.5 and 5.0 µmol/L. The non-toxic concentrations were used for the further experiments

Effects of Psoralidin in NLRP3 inflammasome activation

The NLRP3 inflammasome complex is a highly important protein organization in the inflammatory response, and its activation triggers pyroptosis through secretion of IL-1β and IL-18.To investigate the mechanism of psoralidin-mediated inhibition of IL-1β and IL-18 secretion, expression levels of inflammasome component proteins such as NLPR3, caspase-1 and ASC were examined by western blotting. Combination of LPS and Nigericin was significantly induced NLRP3, ASC and Caspase-1 expressions in RAW 264.7 cells. As shown in (Figure II), psoralidin reduced the expression of NLRP3 inflammasome components. Only the high concentration of psoralidin was able to down-regulate the expression of NLRP3 inflammasome components.

Effects of Psoralidin in IL-1β and IL-18 mRNA expressions.

We examined the effect of psoralidin on IL-1β and IL-18 mRNA expression in RAW 264.7 macrophages. Firstly, cells pre-treated with psoralidin (2.5 and 5.0 µmol/L) for 4 h and then treated with LPS-Nigericin combination (1 µg/ml-20 µM) for 1 h to induce IL-1β and IL-18 cytokine expression. According to the quantitative real-time PCR analysis results, psoralidin pretreatment significantly reduced the mRNA expression of IL-1β and IL-18. (Figure III).

Psoralidin was significantly able to down-regulate the expression of IL-1β and IL-18 cytokines in LPS/ Nigericin-stimulated cells.

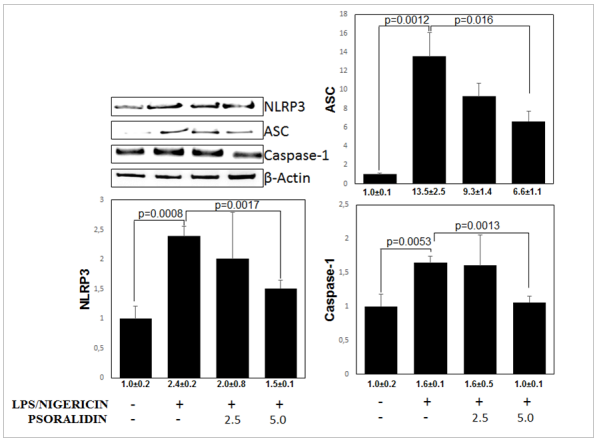

Figure II. Regulatory effect of psoralidin (2.5 µM and 5.0 µM) on LPS/Nigericin (1 µg/mL)/Nigericin 20 µM) induced inflammasome activation. n=3

DISCUSSION AND CONCLUSION

Inflammation, which is considered a beneficial immunological response to various stimuli, is highly effective in the pathogenesis of many diseases when it reaches uncontrollable levels. As innate immune cells, macrophages regulate inflammatory responses, release of cytokines, and inflammatory mediators when activated by sterile or non-sterile stimuli, via stimulation of Toll like receptor 4 (TLR4) or other pattern recognition receptors (15). Stimulation of TLR4 or other pattern recognition receptors not only affects cytokine release, but also stimulates multi-protein inflammasome organization, which is another inflammatory response mechanism in the cell. Of course, over activation of macrophages is associated with disruption of inflammatory responses, which causes expression, maturation and secretion of cytokines and proinflammatory mediators, resulting in the development of inflammatory disease (16). Therefore, suppressing the uncontrolled inflammatory response is a potential target for the treatment of inflammatory diseases. In the previous studies, anti-inflammatory effects of psoralidin have been demonstrated, but its effect on inflammation through NLRP3 inflammasome activation is not clear. In fact, studies showing the anti-inflammatory activity of psoralidin are limited. Yang et. al. have been reported that, Psoralidin showed anti-inflammatory effect by reducing the increased COX-2 expression and prostaglandin E2 (PGE2) production by irradiation through the regulation of PI3K/Akt and NF-KB pathways in lung fibroblast cells. In the same study, psoralidin directly interacted with the 5-lipoxygenase activating protein (FLAP)/5-LOX pathway and blocked IR-induced Leukotriene B4 (LTB4) production (17). NLRP3 inflammation is well

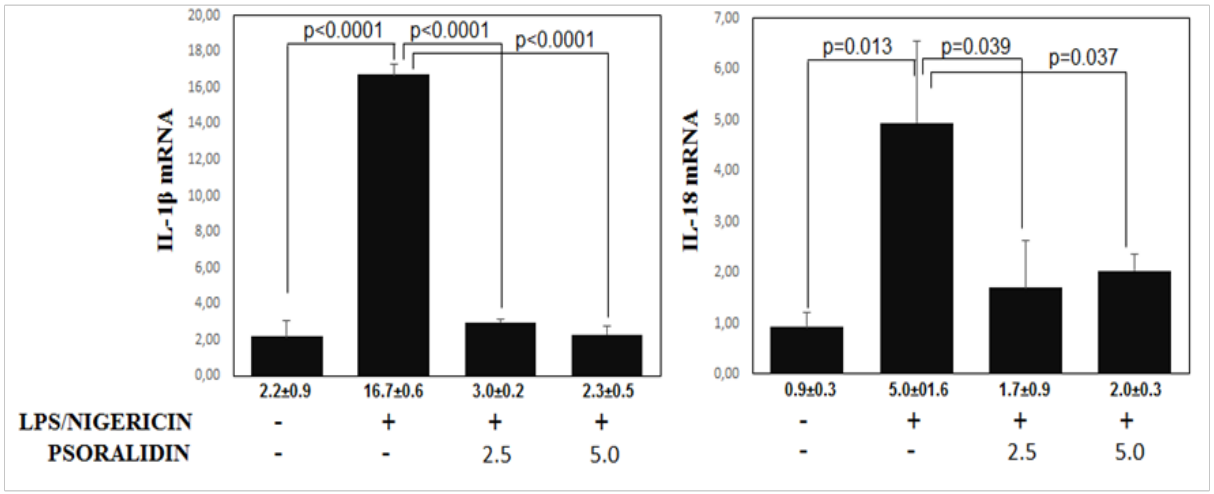

Figure III. Effect of psoralidin (2.5 µM and 5.0 µM) on LPS/Nigericin (1 µg/mL)/Nigericin 20 µM)-induced pyroptotic cytokine (IL-1β and IL-18) expression.

characterized in the inflammatory response, and its over activation is very important in the pathogenesis of various inflammatory diseases and disorders (18). Therefore, inhibiting the aberrant activation of NLRP3 inflammation is a potential therapeutic strategy for chronic inflammation-related human diseases (19-22). In this study, psoralidin suppressed excessive NLRP3 inflammasome activation by attenuating NLRP3, ASC and caspase-1 protein expressions.

The NLRP3 inflammasomeis responsible for the maturation of cytokines interleukin-1β and IL-18 (23). This result might be explained by the observations that psoralidin not only reduced NLRP3 inflammasome activation, but also inhibited expression of proinflammatory cytokines IL-1β and IL-18 in LPS/Nigericinsitumulated macrophage cells.

Psoralidine (5.0μM) exerted its anti-inflammatory effects by reducing the LPS/Nigericin-induced NLRP3, ASC and Caspase-1 expressions. Psoralidin also exhibited anti-inflammatory properties by inhibiting NLRP3 inflammasome activated IL-1β and IL-18 production. Psoralidin may be a potential treatment option for diseases and disorders associated with inflammation.

Conflict of interest

The authors declared that there is no conflict of interest.

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