

Dehydrocostus Lactone Down - regulates Lipopolysaccharide-induced SAA3 gene activation in the Microglia.

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Running Title: DDL inhibits the LPS-induced SAA3 gene expression in microglia

Objective: Research on the mechanism of Dehydrocostus lactone (DDL) in microglial cells of the brain needs to be improved, despite its reported anti-inflammatory, anticancer, and anti-proliferation properties as a natural product of Eerdun Wurile. The precise functions of the Serum amyloid A (SAA) genes are not yet defined. However, these genes are suggested to possess antibacterial properties and can attract monocytes and neutrophils. On the other hand, SAA genes can also induce inflammatory cytokines and metalloproteinases in smooth muscle cells and macrophages, thus promoting inflammation. This study aimed to investigate the impact of DDL on SAA3 gene expression in microglial cells stimulated by LPS.

Methods: The anti-inflammatory effects of DDL were studied using LPS-stimulated murine BV2 microglia. BV2 was cultured in DMEM, and then 4µM DDL was added. Then BV2 was treated with one ng/ml LPS for 24 hours to stimulate.

Results: LPS treatment increased the expression of SAA3 mRNA in BV2 microglial cells, while DDL pre-treatment inhibited LPS-induced SAA3 mRNA expression.

Conclusion: Microglia cells treated with LPS display a significant reduction in the expression of SAA3 gene transcripts upon treatment with DDL.

Keywords: Microglia, Dehydrocostus Lactone, Lipopolysaccharide, Serum amyloid A, SAA3 gene

Introduction

Neuroinflammation is a typical response to infection, brain injury, and neurodegenerative diseases. It is a pathological

feature that occurs when the immune system reacts to damage in the nervous system. The resident macrophages in the brain,

known as microglia, play a vital role in detecting foreign pathogens and neuronal injuries and initiate primary host defense mechanisms in response [1]. When neuroinflammation is initiated, microglia become activated, migrate, and produce immune mediators that can be cytotoxic and cytotropic [2-6]. Prolonged activation of microglia can harm neurons by releasing cytotoxic molecules, such as proinflammatory mediators, reactive oxygen intermediates, nitric oxide, nerve growth factor, and chemotactic cytokines [7]. Microglial cells can become activated by injury, infection, and specific stimulators such as amyloid beta (Ab) and serum amyloid A (SAA). In mice, SAA is encoded by a family of three inducible genes: SAA1, SAA2, SAA3, and SAA4. In humans, SAA3 is not translated. The liver is the primary site of SAA synthesis; SAA3 is also expressed in extrahepatic tissues in response to lipopolysaccharide (LPS). SAA induces the migration of monocytes and neutrophils and stimulates the production of cytokines, chemokines, and matrix metalloproteinases (MMPs). Although the inducible SAA proteins are barely detectable in normal brains, SAA has been found in the brains of patients with AD [8-12]. Jin Liu et al. suggested that targeting SAA could be a potential therapeutic intervention for AD [13].

Dehydrocostus lactone (DDL) is a major component found in the dried root of *Saussurea Lappa Clarke*, which is commonly used in Asian traditional medicine. Additionally, DDL is a crucial element of Eerdun Wurile, a Mongolian medicine frequently used to treat neurological diseases [14, 15]. DDL has been reported to have anticancer, anti-proliferation, and anti-inflammatory activity by inducing cells to undergo cell cycle arrest, apoptosis, differentiation, and inhibition of overproduction of inflammatory mediators [16-18]. However, the mechanism of DDL in neural inflammation has yet to be fully explained.

This study aims to explore the mechanism of the neuroinflammatory effect of traditional medicine, Eerdun Wurile. Additionally, it will demonstrate how this medicine can significantly contribute to the treatment of neuroinflammatory diseases in the future. Specifically, the study investigates the effects of DDL on SAA3 gene expression in lipopolysaccharide-stimulated microglial cells of mice.

Materials and Methods

Study design

In our study, we employed basic methods of cell culture molecular biology and in vitro screenings to compare the anti-inflammatory effect of DDL in three independent study groups with three repeats. We analyzed the expression level of the SAA3 gene in microglial cells of mice that were not stimulated, stimulated with LPS, or pretreated with DDL and then stimulated with LPS.

Reagents

LPS and DDL were purchased from Sigma-Aldrich Co. LL (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Thermo Fisher Scientific, USA). RNAiso Plus was obtained from Takara Biomedical Technology Co., Ltd. (Beijing, China), and a QIAGEN RNeasy Mini kit was obtained from QIAGEN (Hilden, Germany). Oligo dT and Super-Script III RT were purchased from Invitrogen (Carlsbad, CA, USA) and SYBR Green from Applied Biosystems (Foster City, CA, USA).

Cell culture and treatment

BV2 microglial cells were obtained from ATCC (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a humidified incubator with 5% CO₂. Cells were cultured in a 75 cm² cell culture flask (Corning, Action, MA, USA) and were split twice a week. For the experiments, cells were plated on 5×10⁵ cells/35 mm dishes and cultured for 24 hours before treatments.

The BV2 cells were cultured in three different conditions, respectively. 1) The negative control group (n=3) cells were cultured only with a culture medium. 2) The positive control group (n=3) cells were only treated with 1 g/ml LPS for 24 hours to stimulate. 3) the treatment group (n=3) cells were pretreated with DDL at 4µM for 30 minutes and then cultured with 1 µg/ml LPS for 24 hours.

RNA isolation and quantification real-time polymerase chain reaction (qRT-PCR)

According to the manufacturer's instructions, total RNA was extracted using RNA iso Plus and a QIAGEN RNeasy Mini kit. The expression levels of *SAA3* mRNA in three group BV2 cells were assessed by quantitative real-time PCR. In brief, the total RNA from each sample was reverse transcribed using oligo dT and Super-Script III-RT. qRT-PCR was conducted by ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA) using SYBR Green.

Statistical analysis

The data for each group were expressed as mean± standard deviation. Because of the small sample sizes, examination for differences among the three groups was conducted using the Kruskal-Wallis test with a critical p-value of p<0.05. Multiple post-hoc comparisons were performed using the Wilcoxon sign rank test. The crucial p-value for the post-hoc tests was adjusted using the Bonferroni method. Since there were 3 groups, there

were 3 possible pairwise comparisons. Consequently, the critical p-value for the post-hoc tests was $p < 0.05/6 = 0.016$. All analyses were performed using SPSS 25.0.

Ethical Statement

The study was approved by the Research Ethics Committee of the Mongolian National University of Medical Sciences on 18th January 2019 (No.2018/3-01).

Results

QRT-PCR examined the effect of DDL on LPS-induced *SAA3* mRNA expression level in BV2 microglial cells. In the non-stimulated negative control group, the *SAA3* mRNA expression level was 1.006 ± 0.126 . The *SAA3* mRNA expression level increased to 8.890 ± 0.955 in the LPS-stimulated positive control group. In contrast, the *SAA3* mRNA expression level significantly decreased to 2.283 ± 0.335 in the pre-treated with DDL and LPS stimulated group (Table 1, figure 1).

Table 1. The *SAA3* mRNA expression level in three study groups

Sample	n	Mean±SD	CI 95%	P-value
Negative control	3	1.006 ± 0.126 ^a	0.90-1.09	-
Positive control	3	8.890±0.955	1.52-2.36	0.004
Treatment group	3	2.283 ± 0.335 ^a	1.01-1.55	0.003

RT-PCR analyzed the expression level of the *SAA3* gene. *Kruskal-Wallis test with $p < 0.05$, ^apairwise comparison using Wilcoxon signed rank test with $p < 0.016 (=0.05/3)$

The result of qRT-PCR showed that *SAA3* mRNA expression level significantly increased in the LPS-stimulated positive control compared ($p < 0.05$) to the negative control. The LPS-stimulated *SAA3* mRNA expression level was significantly suppressed in the

DDL pre-treatment group compared ($p < 0.05$) to the positive control group (Figure 1). This data suggests DDL inhibits LPS-induced *SAA3* gene expression in *in vitro* microglia cell lines.

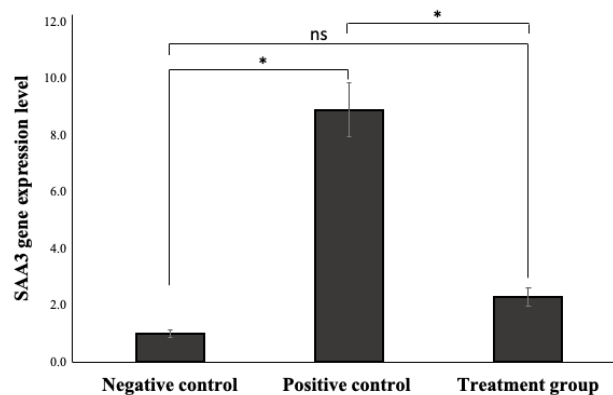


Figure 1. The effect of dehydrocostus lactone (DDL) on lipopolysaccharides (LPS) induced *SAA3* gene expression. *The GAPDH* gene is used for internal control in the assay. *Kruskal-Wallis test with $p < 0.05$

Discussion

In this study, we investigated the impact of DDL on the expression of SAA3 mRNA levels in BV2 microglia stimulated with LPS. As a result, the DDL treatment decreases SAA3 mRNA levels in LPS-stimulated BV2 microglia (Figure 1). Murine serum amyloid A3 gene is an apolipoprotein secreted by macrophages [19], and it is stimulated by high glucose in adipose tissue and adipocyte cell line [20]; NF-kappa B transcription factor is a stimulator for SAA3 gene transcription [21]. NF-kappa B transcription factor has been linked to the immune response to infection and autoimmune disease, septic shock, viral infection synaptic plasticity, and memory [22-27]. On the other hand, SAA treatment increased the mRNA levels of IL-6, TNF- α , IL12p40, and IL23p19 with higher potency via the JNK pathway in microglia cells. Additionally, SAA inhibited the apoptosis of microglia, increasing their viability [28]. The SAA gene expression at high levels in cerebrospinal fluid and co-localized with amyloid peptide deposits in AD brain [29].

Microglia are a type of immune cells that reside in the central nervous system (CNS). They are essential in maintaining CNS homeostasis and helping the brain recover from injuries. Based on their functions, microglia are classified into two categories: pro-inflammatory (M1) and anti-inflammatory (M2) phenotypes [30].

The M1 phenotype is activated by LPS or interferon (IFN)- γ . It promotes the transcriptional activation of nuclear factor-kB (NF-kB). It leads to the overproduction of pro-inflammatory cytokines and oxidative metabolites such as IL-1 β , IL-6, TNF- α , chemokines, nitric oxide, and reactive oxygen species (ROS) [31-33].

Furthermore, increased expression of pro-inflammatory cytokines from activated microglia exacerbates ischemia-like injury to blood-brain-barrier components [34].

M2 phenotype is induced by anti-inflammatory cytokines such as IL-4 or IL-1 stimulation. It inhibits neuroinflammation and promotes tissue regeneration and wound healing [32, 33, 35-37].

LPS is a potent stimulator of the innate immune response and plays a critical role in the pathogenesis of inflammatory reactions. It's a significant molecule found in the outer membrane of Gram-negative bacteria [38, 39]. LPS is a valuable model for studying neuronal injury by activated microglia in infection [40, 41].

Our study utilized a specific model demonstrating microglial activation induced by LPS (Figure 1). One of the critical

components we analyzed was DDL, a natural product derived from the root of *Saussurea lappa*, a well-known traditional medicine in Asia. DDL possesses various beneficial properties, such as anti-inflammatory, immunomodulatory, anti-ulcer, and anti-tumor effects. It has shown promise in treating certain neurodegenerative disorders and tumors.

Compound Ancklandia and Berberine Tablets, clinically available drugs containing DDL, are utilized for treating digestive tract diseases due to their anti-inflammatory and anti-microbial properties [42-44].

Previous studies have demonstrated that DDL can hinder cell growth by inducing cells to undergo cycle arrest, apoptosis, migration, and differentiation [46]. Some studies have shown that DDL can penetrate the blood-brain barrier and inhibit neoplastic weight and volume by inhibiting the IKK β /NF-k β /COX-2 signaling pathway [41, 44].

DDL inhibits the activation of NF-kappaB by decreasing the levels of TNF-alpha. Additionally, it suppresses nitric oxide production by repressing the expression of the inducible nitric oxide synthase enzyme in macrophage cells activated by LPS. Furthermore, DDL acts as a promoter of apoptosis by increasing the activation of caspase-3 and caspase-8 [15, 44].

In recent years, researchers have been extensively interested in understanding the mechanism of DDL due to its potential anti-inflammatory and immunomodulatory activities in CNS disorders. However, the effects of DDL in microglial cells and its mechanisms of action have yet to be fully understood. This study was performed in an in vitro system. Additionally, we will validate these findings in an in vivo system and explore other gene expression correlations.

Conclusion: Expression of SAA3 gene transcripts linked to inflammation, activated by LPS in microglia cells and inhibited by DDL. This inhibition is correlated with inactivation transcription factor NF kappa B.

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