Research Paper

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Anti-Inflammatory Effects of Aqueous Extract of Withania somnifera on LPS-Stimulated Pro-inflammatory Mediators in J774 Murine Macrophages

Biochemistry

KEYWORDS : Withania somnifera, murine macrophages-J774, anti-inflammatroy activity, Plant extract, IL-1, TNF- α and NO

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ABSTRACT

Withania somnifera (Linn.) (Solanacea) is an Indian popular herb used in the treatment of many chronic diseases. Historically, the plant has been used as an anti-inflammatory, liver tonic, astringent and to cure ulcers, insomnia and more. Recently it is also being used in the treatment of immunological disorders such as bronchitis, asthma, and many others. Clinical trials and animal research support the use of Ashwaganda in the management of IgE mediated allergies, inflammation, anxiety, cognitive and neurological disorders. Previously, we reported that the dried roots of this plant are found to associate with immunosuppressive activity. The present study includes anti-inflammatory effects of Withania somnifera aqueous fraction (WSAF) on the production of pro-inflammatory molecules from the lipopolysaccharide (LPS) stimulated J774 murine macrophage cell lines. The findings indicated that WSAF exerted a dose dependent inhibition of LPS induced production of interleukin-1 (IL-1), tumor necrosis factor (TNF- α) and nitric oxide (NO). This data supports the potential use of WS as herbal medicine in the treatment of inflammatory diseases.

Introduction

Withania somnifera (WS) is an honored herb that occupied a prominent position in the Indian Ayurvedic medicine and other traditional medical systems for over 3000 years. It is compared with Chinese Ginseng in the potency and efficacy and described as Indian Ginseng. It is a perennial plant belonging to family *Solanaceae*. It is commonly known as *Aswagandha* or Winter Cherry and is widely distributed in Asia, Africa and Southern Europe. In India it is cosmopolitan in distribution and grows throughout the tropical and sub-tropical regions. Morphologically WS is a small woody shrub usually erect, branched, unarmed and grows up to 1-2.5 meters in length. Chemical and pharmacological complexity of the plant, especially the roots ascribes the diverse medicinal properties (1). Withanolides, a group of steroidal lactones are potential pharmacologically active compounds (2). The leaves of WS are used in treatment of tumors and tubercular glands and withanolides isolated from leaves are reported to exhibit antibacterial, anti-fungal and antitumor properties. Alkaloids, withanolides and sitoindosides isolated from WS are known for anti-aging, anti-inflammatory, antioxidant, anti-tumour, anti-stress, Anti-ulcerogenic, anti-arthritic effect and immunomodulatory properties (1-4). So far many active compounds have been isolated from the herb. Withaferin A, sitoindosides VII-X, 5-dehydroxy withanolide-R, Withasomniferin-A, 1-oxo-5beta,6beta-epoxy-witha-2-ene-27-ethoxy-olide,4 - (1 – hydroxyl - 2, 2 - ethyl cyclo propanone) -2,3-dihydrowithaferin A, 2,3-dihydrowithaferin A, 24,25-dihydro-27-desoxywithaferin A, 27-O-beta-D-gluco pyranosyl viscose lactone B, 4,16-dihydroxy-beta,6beta-epoxyphysagulin D, Viscosa lactone B and Diacetyl withaferin A (5,6). WS is also reported to relieve weakness and nervous exhaustion, build sexual energy and promote healthy sleep. It is used to promote intellect and memory. Most of the work reported is in relation to physagulin D (1-6)-beta-D-glucopyranosyl-(1- 4)-beta-D-glucopyranoside, 27-O-beta-D-glucopyranosylphysagulin D, physagulin D, Withanoside I-VII(7-9) . Majority of the pharmacological constituents were reported to be isolated from non-polar solvent extracts of the herb.

Inflammation is a complex immunological mechanism mediated by pro-inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF), and anti-inflammatory cytokines such as IL-10 and reactive oxygen and nitrogen species from activated macrophages (10 - 12). Down regulation of excessive production of pro-inflammatory cytokines suppresses an array of inflammatory diseases (13). Many herbal preparations are known to exhibit anti-inflammatory activities (14, 15). Herbal compounds that are capable of inhibiting production of inflammatory mediators from activated macrophages act as potential anti-inflammatory agents (16, 17). The present study was designed to investigate the effect of WS aqueous extract on the production of pro-inflammatory molecules from the LPS-stimulated J774 murine macrophage cells.

Preparation of the root extract

Withania somnifera roots were procured from the authorized medicinal plant distributor and systematically authenticated with the help of taxonomist. The roots were properly cleaned and coarsely powdered to prepare 30% aqueous extract. The extract was prepared by soaking 300gms of material in 1li of double distilled water for 72 hours with intermittent shaking at room temperature. Extract was separated by vacuum filtration and the residue was re-extracted twice with distilled water and the pooled extract was lyophilized. Finally, partially non polar solutes from the lyophilized material were removed by washing with absolute alcohol. The resulting material was hydrated and lyophilized to remove the traces of alcohol and stored in capped glass container. This extract is designated as *withania somnifera* aqueous fraction (WSAF).

Cell Line

The murine macrophage cell line J774 was obtained from the ATCC, Rockville, MD, U.S.A. The cells were sub-cultured and maintained in DMEM, supplemented with 10% FCS, 200 U/ml penicillin and 200 µg/ml streptomycin, for 3-5 days, at 37^{0}C in a humidified 5% CO_2 modulator chamber. The viability of cells was determined by trypan blue exclusion.

Culture of macrophages

Murine macrophage cell line J774 was cultured in 96 well tissue culture plate in triplicate by placing 1x10^5 cells in RPMI-1640 medium supplemented with 2mM glutamine, gentamycin (40ug/ml), penicillin (50IU/ml) and streptomycin (50ug/ml), and 10% heat inactivated FCS at 37^{o}C in a humidified 5% CO_2 modulator chamber.

Measurement of Pro-inflammatory Cytokines (TNF- α & IL-1)

Murine macrophage cell line J774 was pretreated with different concentrations (002, 0.02, 0.2, 2.0 and 20.0µg/well) of WSAF for 2h. The pretreated cells were stimulated with 5.0ng/well LPS and incubated for 24h. The effect of WSAF on the production of pro-inflammatory cytokines (IL-1 and TNF- α) was determined in the culture supernatants. Cytokines were measured using mouse ELISA kits (Life Technologies).

Measurement of NO production

The influence of WSAF was assessed on nitrite production, as a sign of NO synthesis. The NO level was determined by Griess method (18). Briefly, 100 µl of culture supernatant was mixed with 100 µl of Griess reagent (1% sulphanilamide, 0.1% NEDD, and 5% orthophosphoric acid) and incubated at room temperature for 5min. The optical density of the azo dye was measured spectrophotometrically at 548nm. The amount of nitrite in the sample was determined using a sodium nitrite standard curve.

Results

Effect of WSAF on pro-inflammatory cytokines

Culture supernatants collected from the LPS induced murine macrophages J774 treated with different concentrations of WSAF were immunoassayed for IL-1 and TNF- α levels by ELISA. Untreated Macrophages produced appreciable amounts of IL-1 and TNF- α while macrophages treated with WSAF prior to LPS exerted a dose dependent inhibition on the production of both IL-1 and TNF- α. This data also reveals that 2.0μg/well is sufficient to inhibit the production of both IL-1 and TNF- α from the LPS induced macrophages as shown in Figure-1. (Figure 1 about here)

Effects of WSAF on LPS-induced NO Production

Influence of WSAF was assessed on LPS induced NO production from murine macrophages J774. The WSAF inhibited NO production by 0, 10.5, 50.9, 75.0 and 75.0% respectively. Significant difference in NO level was found between WSAF treated and untreated macrophages. Maximum inhibition was observed at 2.0 and 20.0 μ g/well (Figure-2). (Figure 2 about here)

Discussion

Many experimental studies proved *W. somnifera* to be a good natural herbal source of strong and safe chemotherapeutic agent. It is the medicinal plant used traditionally to treat various types of diseases. Therapeutic potentiality rewarded to this plant is mainly because of its diverse quality and quantity of secondary metabolites (6, 8). Previously, we have reported that the pharmacologically active compounds extracted into aqueous media to suppress OVA induced IgE antibodies in murine model (19). In this study, we have examined the effect of WSAF on the pro-inflammatory cytokines and NO production from the LPS induced murine macrophages J774.

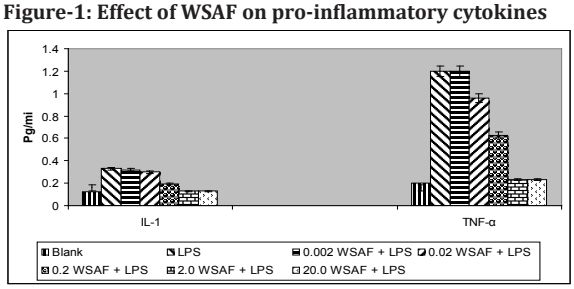
Inflammation is a general physiological and immune reaction observed when human body attempts to counteract against the potentially harmful agents like bacteria, fungi, viruses and other pathogens(20). Macrophages play an important role in the production of inflammatory mediators during pathological conditions (10 – 12). However, excessive production of these molecules induces inflammatory diseases. LPS is known to induce the production of pro-inflammatory cytokines, NO, and prostaglandins (PGs) from activated macrophages. Agents that inhibit the biosynthesis of these mediators may be considered as important therapeutic anti-inflammatory components (21). Cytokines IL-1 and TNF-α and NO are important and relatively easily measurable pro-inflammatory molecules (22). The present experimental data clearly demonstrates that WSAF inhibited the secretion of IL-1β and TNF- α from LPS induced macrophages in a dose dependent manner as shown in the Figure – 1. So far many plant species were reported to be associated with anti-inflammatory properties. However, there is scanty information regarding the mechanism of action of the plant crude extracts or their active compounds on the suppression of the pro inflammatory molecules from macrophages.

NO is produced from L-argenine by the action of NO synthase. It is an important molecule synthesized by activated macrophages and is known to mediate diverse physiological functions such as cGMP-dependent vasodilation, inflammation, regulation of immune response. However, over-production of NO stimulates the synthesis of pro-inflammatory mediators, which in turn boost the symptoms of inflammation (23, 24). Several species are known to suppress the production of NO (25). Our experimental data demonstrated that WSAF down-regulated the NO production significantly in a dose dependent manner from LPS-induced macrophages as shown in the Figure-2. Therefore, crude extracts with bioactivities are considered as a valuable source for the isolation of potential anti-inflammatory compounds.

Conclusion

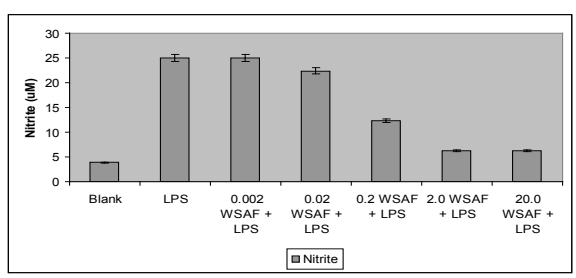
In conclusion the present study demonstrated that the aqueous solubles of *Withania somnifera* contained strong anti-inflammatory compounds that suppressed the production of pro-inflammatory cytokines and NO from the LPS activated murine macrophages. Further studies are in progress to isolate and characterize the putative compounds there in.

Figure-1: Effect of WSAF on pro-inflammatory cytokines



Effect of WSAF on IL-1 and TNF-α production from LPS stimulated-J774 macrophage cells. Cells were treated with 0.002 to 20.0 μg/well and then stimulated with 5.0ng/well LPS. IL-1 and TNF- α in the cell free culture supernatants were measured by ELISA method. Data presented as means ± SD from three sets of independent experiments.

Figure-2: Effects of WSAF on LPS-induced NO Production



Inhibitory effect of WSAF on NO production of LPS stimulated-J774 cells. Cells were treated with 0.002 to 20.0 μg/well and then stimulated with 5.0ng/well LPS. The NO production was determined by Griess reagent. Data presented as means ± SD from three sets of independent experiments.

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