Hirsutine, an indole alkaloid of *Uncaria rhynchophylla*, inhibits inflammation-mediated neurotoxicity and microglial activation

HWAN YONG JUNG¹*, KYONG NYON NAM²*, BYUNG-CHOEL WOO¹, KYOO-PIL KIM¹, SUNG-OK KIM³ and EUNJOO H. LEE²

¹Department of Cardiovascular and Neurologic Diseases (Stroke Center), Hospital of Oriental Medicine, Kyung Hee University, Seoul 130-702; ²Graduate School of East-West Medical Science, Kyung Hee University, Yongin-si 446-701; ³Department of Herbal Pharmacology, College of Oriental Medicine, Daegu Haany University, Daegu 706-060, Republic of Korea

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Abstract. Chronic microglial activation endangers neuronal survival through the release of various pro-inflammatory and neurotoxic factors. As such, negative regulators of microglial activation have been considered as potential therapeutic candidates to reduce the risk of neurodegeneration associated with inflammation. *Uncaria rhynchophylla* (*U. rhynchophylla*) is a traditional oriental herb that has been used for treatment of disorders of the cardiovascular and central nervous systems. Hirsutine (HS), one of the major indole alkaloids of *U. rhynchophylla*, has demonstrated neuroprotective potential. The aim of the present study was to examine the efficacy of HS in the repression of inflammation-induced neurotoxicity and microglial cell activation. In organotypic hippocampal slice cultures, HS blocked lipopolysaccharide (LPS)-related hippocampal cell death and production of nitric oxide (NO), prostaglandin (PG) E₂ and interleukin-1β. HS was demonstrated to effectively inhibit LPS-induced NO release from cultured rat brain microglia. The compound reduced the LPS-stimulated production of PGE₂ and intracellular reactive oxygen species. HS significantly decreased LPS-induced phosphorylation of the mitogen-activated protein kinases and Akt signaling proteins. In conclusion, HS reduces the production of various neurotoxic factors in activated microglial cells and possesses neuroprotective activity in a model of inflammation-induced neurotoxicity.

Introduction

Immune and inflammatory responses in the central nervous system (CNS) are principally mediated by microglia. These responses are activated during neuropathological conditions and restore CNS homeostasis (1). The activation of microglia involves proliferation, migration to the injury site, increased expression of immunomodulators and transformation into phagocytes (1,2). Activated microglia also promote neuronal injury through the release of proinflammatory and cytotoxic factors, including cytokines, nitric oxide (NO) and reactive oxygen species (ROS) (2). Chronic microglial activation has been implicated in neuronal destruction associated with various neurodegenerative diseases, including Alzheimer's and Parkinson's (3). Therefore, downregulation of negative-regulatory mechanisms to reduce the activation of microglial cells is essential to avoid excessive CNS inflammatory processes (4). The identification of agents that target over-activated microglial cells is essential for the reduction of neuronal destruction associated with neurodegenerative diseases.

*Uncaria rhynchophylla* is a traditional oriental herb that has been used for treatment of disorders of the cardiovascular and central nervous systems (5). Hirsutine (HS) is a major indole alkaloid of *U. rhynchophylla*. HS has been reported to have antihypertensive and antiarrhythmic activities through its effects on intracellular Ca²⁺ levels in rat aorta and the action potential in cardiac muscle (6,7). In a previous study, HS was demonstrated as effective for the protection of rat cardiomyocytes from hypoxia-induced cell death (8). Studies using animal models have revealed that extracts isolated from *U. rhynchophylla* demonstrate neuroprotective potential against diverse neuronal injuries associated with excitotoxicity, amnesia, epileptic seizures and Parkinson's and Alzheimer's disease (9-13). *In vitro* studies on the neuroprotective roles of HS have demonstrated that the compound attenuates glutamate-induced cell death in PC12 and cerebellar granule cells (14,15). Based on these studies, components of *U. rhynchophylla* have been proposed to act as neuroprotective agents. However, the efficacy of the compounds on neuroinflammation control has been largely unexplored. The purpose of the present study was to examine...
the ability of HS in the control of inflammatory responses of the brain microglia and the protective potential of HS for reducing inflammation-induced neurotoxicity.

Materials and methods

Drug, chemicals and reagents. All cell and tissue culture products were purchased from Invitrogen (Carlsbad, CA, USA). HS (product no. 082-0461) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Escherichia coli lipopolysaccharide (LPS) and other chemicals were purchased from Sigma (St. Louis, MO, USA). Antibodies against phospho-p44/42 MAPK, p44/42 MAPK, phospho-SAPK/JNK, SAPK/JNK, phospho-p38, p38, phospho-Akt and Akt were purchased from Cell Signaling Technology (Beverly, MA, USA).

Experimental animals. Rats were maintained in accordance with the Institutional Animal Care and Use Committee guidelines of Kyung Hee University. All animal protocols were approved by the Animal Ethics Committee of Kyung Hee University in accordance with the 14th article of the Korean Animal Protection Law.

Organotypic hippocampal slice culture. Organotypic hippocampal slice cultures were prepared from male Sprague-Dawley rats (seven days old; Orient, Kyunggido, Korea) using the methods previously described by Stoppini and others (16,17). Briefly, the hippocampus was isolated and cut transversely at a thickness of 350 µm with a McIlwain Tissue Chopper (Mickle Laboratory Engineering, Surrey, UK). The slices were placed on membrane inserts (Millicell-CM; Millipore, Bedford, MA, USA) in six-well plates. Each well contained 1 ml of culture medium composed of 50% MEM, 25% Hank's Balanced Salt Solution and 25% horse serum. The slices were cultured at 36°C in an incubator in the presence of 5% CO₂ for 12-14 days and the medium was changed every 2-3 days.

LPS treatment and assessment of neuronal damage. Neurotoxicity was evaluated by the uptake of the fluorescent dye propidium iodide (PI) as previously described (17,18). Briefly, LPS (10 µg/ml) was applied to hippocampal cultures with or without pretreatment with HS. Following LPS treatment, the culture medium was collected and subjected to the nitrite assay prior to being replaced with fresh serum-free medium containing 5 µg/ml PI. Neuronal death was observed within 30-60 min of PI addition. PI-stained images were captured using a laser scanning microscope (LSM 510; Carl Zeiss, Cambridge, UK) and the observed PI-uptake areas were measured using confocal microscopy with LSM 510 software (release 3.2; Carl Zeiss). All the data were background subtracted using the fluorescence emission originated from a region on the insert containing no tissue. For immunofluorescent staining of neurons, hippocampal slices were fixed in 4% paraformaldehyde and stained with Alexa fluor 488-conjugated mouse anti-NeuN monoclonal antibody (Chemicon International, Temecula, CA, USA). The immunostained images were observed under a Carl Zeiss LSM 510 microscope.

Primary microglia culture. Primary microglial cells were prepared from cerebral cortices of one-day-old rat pups (Orient) as described previously (19,20). Cells reached confluence at 12-14 days and flasks were agitated to remove the microglia. The detached cells were incubated for 1 h and the non-adherent cells were removed. The adherent microglial cells were cultured for 24 h and the purity of the cultures was routinely >95%, as judged by immunostaining with an anti-CD11b antibody (Chemicon). The cells were pretreated with HS in fresh medium containing 0.1% fetal bovine serum for 30 min prior to the addition of LPS.

Nitrite assay. Nitrite in culture supernatants was measured as an indicator of NO production. An aliquot of the culture supernatant was mixed with a volume of Griess reagent (Molecular Probes, Eugene, OR, USA) and the absorbance at 570 nm was determined using a microplate reader. Sodium nitrate (0-100 µM) was used as a standard to assess nitrite concentrations.

Cell viability assay. For the cell viability assay, cultures were incubated in MTT solution (1 mg/ml; Sigma) in two volumes of culture medium for 1 h at 37°C. The MTT solution was then removed, the cells were dissolved in dimethyl sulfoxide (150 µl) and optical density of the samples was measured at 570 nm using a microplate reader.

IL-1β and prostaglandin (PG) E₂ assays. Following each treatment, culture medium was collected in microcentrifuge tubes and centrifuged at 10,000 x g for 10 min. The supernatants were assayed for secreted mediators using PGE₂ and rat IL-1β immunoassay kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

Intracellular ROS assay. Presence of intracellular ROS was measured using a non-fluorescent 2’,7’-dichlorofluorescein (DCFH-DA; Molecular Probes) dye as described previously (21). DCF fluorescence was measured using a Wallac 1420 fluorometer (Perkin Elmer, Waltham, MA, USA) at 485 nm for excitation and 530 nm for emission.

Western blot analysis. Cells were lysed on ice in lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail; Sigma]. Following centrifugation, the supernatant was collected and assayed for protein concentration using a DC Protein Assay kit (Bio-Rad, Hercules, CA, USA). Lysate samples containing 30 µg of protein were fractionated by SDS-10% polyacrylamide gel electrophoresis and then electroblotted onto nitrocellulose membranes. The membranes were probed with primary antibodies and immunoreactivity was detected with ECL Reagent (Amersham Biosciences, Piscataway, NJ, USA).

Statistical analysis. For statistical analysis, data were expressed as the mean ± SEM from three independent experiments. The Student's paired t-test was used for statistical analyses which were performed using SPSS software (version 13.0, SPSS Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.
Results

Protection against LPS-mediated neuronal damage. In an effort to develop neuroprotective drugs, strategies to ameliorate the inflammatory microenvironment, which indirectly damages neurons via glial cell mediators, are promising (4). We previously established an experimental condition to determine the effect of LPS exposure on neuronal damage in organotypic hippocampal slice cultures (20). In the present study, slice cultures exposed to LPS for 72 h exhibited marked PI uptake in the hippocampus in comparison to untreated control slices (Fig. 1A). The increased PI uptake was markedly blocked by treatment with HS (Fig. 1A and B), in parallel with the inhibition of LPS-induced production of various proinflammatory mediators, including NO, PGE\textsubscript{2} and IL-1β (Fig. 1D-F). Reduced immunoreactivity of NeuN, a neuronal-specific marker, accompanied with elevated PI fluorescence indicated that loss of neurons resulted from the LPS insult (Fig. 1C). Treatment with HS restored immunoreactivity of NeuN and simultaneously decreased PI fluorescence (Fig. 1C). Together, these results indicate that HS has protective effects against inflammation-induced neurotoxicity.

Suppression of LPS-stimulated microglial inflammatory responses. To determine the mechanisms of the anti-inflammatory effects of HS in more detail, various mediators of microglial activation were measured. The effects of treatment with HS on secretion of proinflammatory mediators from microglial cells were tested. HS suppressed LPS-induced nitrite release from microglial cells in a dose-dependent manner (Fig. 2A). Cell viability, as measured using the MTT assay, was often reduced by LPS. HS, hirsutine; LPS, lipopolysaccharide; PI, propidium iodide; PGE\textsubscript{2}, prostaglandin E\textsubscript{2}; IL-1β, interleukin-1β.

Figure 1. Effect of HS on LPS-induced hippocampal cell death. Organotypic hippocampal slice cultures were pretreated with HS at the indicated concentrations for 30 min prior to the addition of 10 µg/ml LPS. Following stimulation with LPS for 72 h, the culture medium was replaced with fresh serum-free medium containing PI. (A) PI fluorescence images. Scale bar, 500 µm. (B) Quantification of PI images. Data are expressed as percentage of the LPS value (mean±SEM, n=10-15 each). (C) The same slices were immunostained with NeuN that marked neuronal nuclei (NeuN, green; PI uptake, red). A magnified image from an outlined area in the region of the LPS-treated hippocampal slices is demonstrated in the right upper panel to reveal the substantial colocalization of NeuN immunoreactivity and PI fluorescence. Scale bar, 500 µm. Determinations of (D) nitrite, (E) PGE\textsubscript{2} and (F) IL-1β in culture supernatants of hippocampal slices. **P<0.001 vs. control group; *P<0.001, P<0.05 vs. LPS-only treated group. HS, hirsutine; LPS, lipopolysaccharide; PI, propidium iodide; PGE\textsubscript{2}, prostaglandin E\textsubscript{2}; IL-1β, interleukin-1β.

Figure 2. Effect of HS on nitrite production and cell viability in microglial cells. Primary microglial cells were incubated in the absence (control) or presence of LPS. The cells were pretreated with the indicated amounts of HS for 30 min prior to the addition of LPS. Twenty-four hours following LPS treatment, the cultures were subjected to (A) nitrite and (B) cell viability assays. (C) As a reference, cells were treated with HS only for 24 h and the cultures were subjected to nitrite quantification. Data are expressed as the mean±SEM from triplicate assays. **P<0.001 vs. control group; *P<0.001, P<0.05 vs. LPS-only treated group. HS, hirsutine; LPS, lipopolysaccharide; PI, propidium iodide; PGE\textsubscript{2}, prostaglandin E\textsubscript{2}; IL-1β, interleukin-1β.
Effect of HS on secretion of PGE2 and production of ROS. Primary microglial cultures were prepared in triplicate and stimulated with LPS (100 ng/ml) with or without pretreatment with indicated amounts of HS. (A) Following 24 h incubation, culture supernatants were assayed for PGE2 using ELISA. (B) Following 6 h LPS incubation with or without pretreatment with HS, levels of intracellular ROS in microglia were determined using DCFH-DA. Data are expressed as the mean ± SEM from triplicate assays. *P<0.01 vs. control group; **P<0.001, *P<0.05 vs. LPS-only treated group. HS, hirsutine; PGE2, prostaglandin E2; ROS, reactive oxygen species; LPS, lipopolysaccharide.

Effect of HS on secretion of PGE2 and production of ROS. HS was found to reduce LPS-induced production of PGE2 (Fig. 3A). Intracellular ROS act as second messengers in the regulation of the LPS-stimulated production of neurotoxic factors in microglia (22). The ROS levels measured using DCFH-DA revealed that pretreatment with HS decreased LPS-induced ROS production in microglia (Fig. 3B).

HS inhibits multiple signaling pathways. Multiple signaling pathways, including those involving mitogen-activated protein kinases (MAPKs) and Akt, have been reported to be involved in LPS-induced signal transduction, which results in the induction of proinflammatory gene expression (23-26). The present study demonstrates that HS markedly inhibited the LPS-enhanced phosphorylation of p44/42 MAPK, p38, SAPK/JNK (Fig. 4). Among the kinases, phosphorylation of SAPK/JNK was reduced to the greatest extent by HS (Fig. 4). Taken together, the present data indicate that the anti-inflammatory action of HS in microglia is, at least in part, mediated by the inhibition of these signaling pathways.

Discussion

In the present study, HS, one of the major alkaloids of *U. rhynchophylla*, effectively repressed diverse inflammatory mediators induced by LPS, including NO, PGE2, intracellular ROS and phosphorylation of MAPKs and Akt in primary microglial cell culture. Beyond the control of microglial activation, more direct efficacy of HS against inflammation-induced neurotoxicity was observed in hippocampal slice cultures. As such, our results demonstrate that HS may be useful in ameliorating brain disorders associated with uncontrolled microglia-mediated inflammatory responses. Taken together with previous studies on anti-inflammatory actions of rhynchophylline and isorhynchophylline (27,28), the present study supports a pharmacological potential for *U. rhynchophylla* and its active components in manipulating neuroinflammation associated with diverse neuropathologies.

Among the diverse neuroprotective activities of *U. rhynchophylla*, antioxidant properties are well established (12,14,29). In previous studies, *U. rhynchophylla* exhibited the ability to reduce levels of free radicals in rat brain and increase glutathione levels in PC12 cells (12,29). *U. rhynchophylla* inhibits the NMDA receptor-activated ion current in hippocampal neurons (10). Additionally, *U. rhynchophylla* inhibits the aggregation of amyloid β protein, a pathological hallmark of Alzheimer's disease (9). Neuroprotective potential conferred by HS has also been explained with its inhibitory capacities on oxidative stress, ion channels and Ca2+ influx (14,15,30). However, little is known regarding the effects of *U. rhynchophylla* and its active compounds on microglia.

According to a previous study, *U. rhynchophylla* reduces microglial activation in the region of neuronal damage caused by kainic acid administration (13). In the present study, *U. rhynchophylla* reduced ED1- and inducible NO synthase-immunoreactive cell counts in rat brain, demonstrating that *U. rhynchophylla* may suppress microglia activation in vivo. Numerous studies have demonstrated an inhibitory function of rhynchophylline-type alkaloids of *U. rhynchophylla* in microglial activation in vitro (27,28). Rhynchophylline and isorhynchophylline have been shown to suppress the release of NO and proinflammatory cytokines and the phosphorylation of p44/42 and p38 MAPKs in LPS-activated N9 microglial cell lines (28).
A detailed study on alkaloids from *U. rhynchophylla* previously identified that geissoschizine methyl ether, a corynanthean-type indole alkaloid, is a potent acetylcholinesterase inhibitor and may be a suitable candidate for Alzheimer’s disease (31). Matsumoto et al demonstrated that isorhynchophylline regulates neurotransmission by suppressing the serotonin 5-hydroxytryptamine 2A receptor function in the brain by competitive antagonism (32). Studies of alkaloids, including the present study, may broaden understanding of the pharmacological value of these compounds of *U. rhynchophylla* in the treatment of neuropathologies, by highlighting various beneficial roles in neuronal survival, synaptic plasticity and microglial activation in the CNS. In this sense, the present results may stimulate further investigation to confirm novel neuropharmacological roles of each individual compound of *U. rhynchophylla*.

References


