Brain Research Bulletin 120 (2016) 151–158

Contents lists available at ScienceDirect

Brain Research Bulletin

journal homepage: www.elsevier.com/locate/brainresbull

Anti-inflammatory effects and antioxidant activity of dihydroasparagusic acid in lipopolysaccharide-activated microglial cells

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ARTICLE INFO

Article history:
Received 2 September 2015
Received in revised form
17 November 2015
Accepted 18 November 2015
Available online 22 November 2015

Keywords:
Dihydroasparagusic acid
Microglia
Inflammation
Oxidative stress
Neurodegenerative disease
Lipopolysaccharide

ABSTRACT

The activation of microglia and subsequent release of toxic pro-inflammatory factors are crucially associated with neurodegenerative disease, characterized by increased oxidative stress and neuroinflammation, including Alzheimer and Parkinson diseases and multiple sclerosis. Dihydroasparagusic acid is the reduced form of asparagusic acid, a sulfur-containing flavor component produced by *Asparagus* plants. It has two thiolic functions able to coordinate the metal ions, and a carboxylic moiety, a polar function, which may enhance excretion of the complexes. Thiol functions are also present in several biomolecules with important physiological antioxidant role as glutathione. The aim of this study is to evaluate the anti-inflammatory and antioxidant potential effect of dihydroasparagusic acid on microglial activation in an *in vitro* model of neuroinflammation. We have used lipopolysaccharide to induce an inflammatory response in primary rat microglial cultures. Our results suggest that dihydroasparagusic acid significantly prevented lipopolysaccharide-induced production of pro-inflammatory and neurotoxic mediators such as nitric oxide, tumor necrosis factor-α, prostaglandin E_2, as well as inducible nitric oxide synthase and cyclooxygenase-2 protein expression and lipoxygenase activity in microglia cells. Moreover it effectively suppressed the level of reactive oxygen species and affected lipopolysaccharide-stimulated activation of mitogen activated protein kinase, including p38, and nuclear factor-kB pathway. These results suggest that dihydroasparagusic acid's neuroprotective properties may be due to its ability to dampen induction of microglial activation. It is a compound that can effectively inhibit inflammatory and oxidative processes that are important factors of the etiopathogenesis of neurodegenerative diseases.

1. Introduction

Neurodegenerative diseases are becoming more common due to the increase in life expectancy (Wong, 2013). It is recognized that microglial activation-mediated neuroinflammation plays an important role in the process of several neuroinflammation-associated diseases, including Alzheimer and Parkinson diseases, and multiple sclerosis (Amor et al., 2010). Over-activation of microglial cells contributes the demise of neurons (Lim et al., 2015).

Microglia, the key innate immune cells in the central nervous system (Zhu et al., 2014), provide beneficial functions for neuron cells, including cellular maintenance and innate immunity. In physiological conditions, microglial cells display a ramified morphology but, when activated in response to various immunological stimuli and neuronal injuries, turn into an amoeboid type and release neurotoxic factors, including nitric oxide (NO), prostaglandin E_2 (PGE_2), inflammatory cytokines, such tumor necrosis factor-α (TNF-α) and reactive oxygen species (ROS) (Block et al., 2007; Tao et al., 2014;

Abbreviations: AA, arachidonic acid; COX-2, cyclooxygenase-2; DHAA, dihydroasparagusic acid; iNOS, inducible nitric oxide synthase; LOX, lipoxygenase; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase; NF-κB, nuclear factor-kB; NO, nitric oxide; PGE_2, prostaglandin E_2; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α.

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http://dx.doi.org/10.1016/j.brainresbull.2015.11.014
0361-9230/

Su et al., 2014). Uncontrolled activation of microglia leads to neurotoxic effects.

Neuroinflammation can be both a cause and a result of chronic oxidative stress (Pontiki et al., 2012). The role of the oxidative stress and free radicals in the development of neurological disease is currently focus of many studies. ROS act as secondary messengers capable of modifying pro-inflammatory gene expression in microglia-associated neurodegenerative diseases by altering kinase cascades and activating transcription factors, including mitogen activated protein kinase (MAPKs), and nuclear factor-kB (NF-κB) (Kim et al., 2008; Pawate et al., 2004). Various studies have demonstrated that suppressing the exaggerated inflammatory response by activated microglial cells helps to attenuate the severity of neurodegenerative diseases (Hirsch et al., 2012; Maccioni et al., 2009).

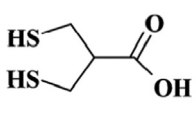
Dihydroasparagusic acid (DHAA) is a natural dimercaptanic acid; it is the reduced form of asparagusic acid, a sulfur-containing flavor component produced by *Asparagus* plants, and was firstly isolated from concentrated *Asparagus* juice in 1948 (Jansen, 1948). It is present in small quantities in its natural source but it was recently synthesized by an improved procedure (Venditti et al., 2013).

Only a few studies have been carried out on the biological activities of this substance. In particular they were demonstrated to act as growth inhibitors for various plants and nematodes (Yanagawa et al., 1972; Takasugi et al., 1975) while stimulating growth and pyruvate oxidation in *Streptococcus faecalis* and *Asparagus* mitochondria (Yanagawa et al., 1973a,b). Moreover, DHAA has been recently reported to exert several interesting biological activities, including a good radical scavenging capacity and antioxidant activity and an inhibitory activity on mushroom tyrosinase (Venditti et al., 2013).

DHAA was also studied as a chelating ligand for a series of iron and nickel complexes (Volkers et al., 2006) and, recently, a protective effect toward mercury poisoning has been demonstrated in an *in vitro* study (Bianco et al., 2013). Indeed, from a structural point of view, DHAA is quite similar to chelating antidotes currently used in therapy of heavy metals poisoning as dimercaptosuccinic acid, dimercaptopropanesulphonic acid sodium salt and dimercaptopropanol, having two thiolic functions able to coordinate the metal ions and a carboxylic moiety, a polar function, which may enhance excretion of the complexes. Thiol functions are also present in several biomolecules with important physiological role as in glutathione, one of the most effective antioxidant, and also in lipoic acid which is a cofactor for several enzymatic systems.

So it is interesting to explore the potential activity of DHAA toward enzymatic systems involved in several pathological processes which inflammation cascade is an important target.

The present study was designed to evaluate the anti-inflammatory and anti-oxidant potential effect of dihydroasparagusic acid on the suppression of microglial activation in an *in vitro* model of neuroinflammation. Lipopolysaccharide (LPS) is a most common toxic agent to induce neuroinfammation in the central nervous system (Song et al., 2014). It was used as a stimulus of microglial activation being capable of causing a wide variety of pathophysiological effects, inducing the production and release of several pro-inflammatory cytokines and mediators, especially though the increased production of reactive oxygen intermediates such as superoxide radical ($O_2^{\bullet -}$), lipid peroxides, and NO, which cause oxidative stress (Hou et al., 2014).



Cell viability (MTT test)	
Control	100%
DHAA 1 μM	114,9%±7,70
DHAA 10 μM	98,7%±3,90
DHAA 100 μM	105,1%±9,71

Fig. 1. Structure of dihydroasparagusic acid (DHAA) and its effect on microglial cell viability. Primary rat microglial cells were treated with DHAA (1–10–100 μM) for 24 h. The cell viability was assessed by MTT reduction assay. Values are expressed as mean ± S.D. ($n = 3$).

2. Materials and methods

2.1. Chemicals

DHAA was synthesized and characterized as described earlier (Venditti et al., 2013). Briefly β-β′-diiodoisobutyric acid (II)/iodomethylacrylic acid (II') was subjected to a nucleophilic substitution by reaction with a mixture of thioacetic acid/potassium thioacetate, leading to the formation of the dithioacetylated derivative, which, after hydrolysis, formed DHAA (Fig. 1). Dulbecco's MEM and D-MEM/F12 media, penicillin/streptomycin, fetal calf serum (FCS) were purchased from Invitrogen (Paisley, Scotland). Trypsin, DNAse I, LPS, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), *N*-(1-napthyl)-ethylenediamine dihydrocloride, *o*-phosphoric acid (H_3PO_4), sodium nitrite and sulfamilamide, antibody against β-actin, were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Citotoxicity detection kit (LDH) was from Roche (Roche Diagnostics, Indianapolis, IN, USA). 2′,7′-dichlorofluorescein diacetate ($DCFH_2$-DA) was from Fluka (Fluka AG, Basel, Switzerland). PGE_2 and TNF-α kits were from R&D Systems (USA & Canada). Polyclonal antibody anti-Iba1 was from Wako (Wako Pure Chemical Industries Ltd., Osaka, Japan). Polyclonal anti-inducible nitric oxide synthase (iNOS) and anti-cyclooxygenase-2 (COX-2) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal anti-phosphoERK1/2, anti-phospho-p38 and anti-phospho-IkBα (Ser32) antibodies were from Cell Signaling (Cell Signaling, MA, USA). Western blot enhanced chemiluminescence detection system was from Bio-Rad Laboratory (Hercules, CA, U.S.A.).

2.2. Primary rat microglial cell cultures and treatment

All the animal-related procedures were conducted in accordance with European Communities Council Directive n° 86/609/EEC. Microglial cells were obtained from the cerebral cortex of 1- or 2- day old decapitated Wistar rats as previously described (Togna et al., 2013). Briefly the cortices were dissected and digested for 20 min at 37 °C in 0.25% trypsin and for further 5 min in presence of 50 KU/ml of DNAse I. Cells were plated at a density of 3,3 × 106 cells/cm^2 in T75 flasks, in 10 ml D-MEM supplementing with 10% FCS and antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin). After 10 and 14 days from dissection, microglia was detached from the astrocyte monolayer by shaking, and the cells re-suspended in D-MEM/F12 (10% FCS and antibiotics). Thereafter the cells were placed in 24-well plastic plates at a density of 5 × 105 cells/ml, incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Purity of microglial cell populations (>98%) was verified by staining with Iba-1 (1:1000) antibody. DHAA, dissolved in Tris–HCl buffer pH 7.0, was added at different concentrations (1, 10 and 100 μM) to microglial cultures 30 min before stimulation by LPS 10 ng/mL (from *Escherichia coli* serotype 026:B6) for different incubation times. The control group was treated with Tris–HCl buffer diluted in the culture medium at the same final concentration used for DHAA. The supernatants were collected after 24 h incubation and kept at −80 °C for measurements of lactate dehydrogenase (LDH), NO, PGE_2 and TNF-α production. Cells were used for subsequent protein measurement, ROS determination, MTT assay, lipoxygenase (LOX) activity and