European Journal of Pharmacology 760 (2015) 129–135

Contents lists available at ScienceDirect

European Journal of Pharmacology

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

Neuropharmacology and analgesia

A natural compound macelignan protects midbrain dopaminergic neurons from inflammatory degeneration via microglial arginase-1 expression

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ARTICLE INFO

Article history:
Received 4 December 2014
Received in revised form
5 April 2015
Accepted 8 April 2015
Available online 25 April 2015

Chemical compounds studied in this article:
Nitric oxide (PubChem:145068)

Keywords:
Parkinson disease
Microglia
Alternative activation
Arginase
Peroxisome proliferator-activated receptor
Nitric oxide

ABSTRACT

Inflammatory events involving activated microglia have been recognized to play an important role in pathogenesis of various neurodegenerative disorders including Parkinson disease. Compounds regulating activation profiles of microglia may provide therapeutic benefits for Parkinson disease characterized by degeneration of midbrain dopaminergic neurons. Here we examined the effect of macelignan, a compound derived from nutmeg, on inflammatory degeneration of midbrain dopaminergic neurons. Treatment of midbrain slice cultures with interferon (IFN)-γ and lipopolysaccharide (LPS) caused a substantial decrease in viable dopaminergic neurons and an increase in nitric oxide (NO) production indicated by extracellular nitrite accumulation. Application of macelignan (10 μM) concomitantly with LPS prevented the loss of dopaminergic neurons. Besides nitrite accumulation, up-regulation of inducible NO synthase protein expression in response to IFN-γ/LPS was confirmed by Western blotting, and immunohistochemical examination revealed expression of inducible NO synthase in a subpopulation of Iba-1-poitive microglia. However, macelignan did not affect any of these NO-related parameters. On the other hand, macelignan promoted expression of arginase-1 in midbrain slice cultures irrespective of the presence or the absence of IFN-γ/LPS treatment. Arginase-1 expression was mainly localized in a subpopulation of Iba-1-positive cells. Importantly, the neuroprotective effect of macelignan was antagonized by N^{ω}-hydroxy-nor-L-arginine, a specific arginase inhibitor. The neuroprotective effect of macelignan was also prevented by GW9662, a peroxisome proliferator-activated receptor γ (PPARγ) antagonist. Overall, these results indicate that macelignan, a compound with PPARγ agonist activity, can provide neuroprotective effect on dopaminergic neurons in an arginase-dependent but NO-independent manner.

1. Introduction

Neurodegenerative disorders are characterized by progressive degeneration and loss of a particular population of central nervous system neurons. In the case of Parkinson disease, dopaminergic neurons in the substantia nigra are preferentially affected by progression of the disease. The causes of dopaminergic neurodegeneration are multifactorial, including cytotoxic actions of endogenous/exogenous neurotoxins and several genetic factors linked to familial forms of the disease (Jenner et al., 2013). On the other hand, accumulating lines of evidence indicate that pathogenic mechanisms of Parkinson disease involve neuroinflammatory events characterized by activation of microglia, a brain macrophage-like cell population (Taylor et al., 2013). Irrespective of the primary causes triggering neurodegeneration, activation of microglia occurs in response to appearance of deteriorated cells and cell debris, and plays an important role in progression of pathological events in the substantia nigra. This may be related to the fact that the substantia nigra contains much larger populations of microglia than the other brain regions (Kim et al., 2000).

A growing number of evidence shows that microglia can undergo activation into distinct phenotypes, reminiscent of peripheral macrophages (David and Kroner, 2011). One is M1 or classical activation, which is generally thought to produce cytotoxic influences in their surrounding tissues by releasing pro-inflammatory cytokines and nitric oxide (NO). The other one is M2 or alternative activation, which is

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http://dx.doi.org/10.1016/j.ejphar.2015.04.021
0014-2999/

thought to produce reparative and cytoprotective influences on their surroundings. M2 phenotype is characterized by expression of several specific markers including arginase-1. Theoretically, inhibition of classical activation and promotion of alternative activation of microglia should provide therapeutic benefits in neurodegenerative disorders. In this respect, a number of naturally-occurring low-molecular-weight compounds have been reported to suppress M1 activation of microglia, thereby prevents neuronal injury under various situations (D.K. Choi et al., 2011). However, the potential effect of these compounds on M2 activation of microglia and its contribution to neuroprotection remain unexplored.

Macelignan, a compound isolated from nutmeg, has the ability to produce several pharmacological actions (Paul et al., 2013). A study using primary cultures of microglia demonstrated that macelignan inhibited lipopolysaccharide (LPS)-induced production of NO as well as inflammatory cytokines such as tumor necrosis factor-α and interleukin-1β (Jin et al., 2005). Therefore, the primary actions of this compound under neuroinflammatory conditions may be related to suppression of M1 activation of microglia. On the other hand, macelignan has also been reported to possess agonist activity at peroxisome proliferator-activated receptors (PPARs; Han et al., 2008). Notably, activation of PPARγ has been shown to increase expression of M2 activation markers such as arginase-1 in peripheral macrophages (Odegaard et al., 2007; Pourcet and Pineda-Torra, 2013). Therefore, macelignan might promote M2 activation of microglia, which may contribute to its neuroprotective effect. However, potential neuroprotective effect of macelignan has not been investigated in detail, except in the case with hippocampal HT22 cell line challenged with oxidative stress (Jin et al., 2005). Accordingly, we examined pharmacological effect of macelignan with reference to neuroprotective properties in midbrain slice cultures under inflammatory conditions.

2. Materials and methods

2.1. Culture preparation and drug treatment

Organotypic midbrain slice cultures were prepared according to the methods described previously (Katsuki et al., 2009; Kurauchi et al., 2009). All procedures were approved by Kumamoto University ethics committee on animal experiments, and were conformed to the Guidelines of the United States National Institutes of Health regarding the care and use of animals for experimental procedures. Briefly, coronal midbrain slices (350-μm thick) were prepared from 2 to 3 d-old neonatal Wistar rats (Nihon SLC, Shizuoka, Japan) under sterile conditions with a tissue chopper. Slices were transferred onto microporous membranes (Millicell-CM, Merck Millipore, Darmstadt, Germany) in six-well plates. Culture medium, consisting of 50% minimum essential medium/HEPES, 25% Hanks' balanced salt solution and 25% heat-inactivated horse serum (Life Technologies, Tokyo, Japan) supplemented with 6.5 mg/ml glucose, 2 mM L-glutamine and 10 U/ml penicillin-G/10 μg/ml streptomycin, was supplied at a volume of 0.7 ml per well. The culture medium was exchanged with fresh medium on the next day of culture preparation, and thereafter, every 2 d. Slices were maintained in a 34 °C, 5% CO_2 humidified atmosphere. At 17 d in vitro, treatment was initiated by transfer of culture inserts to culture wells filled with 0.7 ml of drug-containing serum-free medium. Serum-free medium consisted of 75% minimum essential medium and 25% Hanks' balanced salt solution supplemented with 6.5 mg/ml glucose, 2 mM L-glutamine and 10 U/ml penicillin-G/10 μg/ml streptomycin.

2.2. Drugs

Rat interferon (IFN)-γ (PeproTech, Rocky Hill, NJ, USA) at 50 ng/ml and LPS (from *Escherichia coli*, serotype 0111; B4; Sigma, St Louis, MO, USA) at 10 μg/ml were used to activate microglia in slice cultures (Kurauchi et al., 2009). Macelignan, (8*R*,8′*S*)- 7-(3,4-methylenedioxyphenyl)-7′-(4-hydroxy-3-methoxyphenyl)-8,8′-dimethylbutane, was isolated from *Myristica fragrans* Houtt. (nutmeg) as described previously (E.J. Choi et al., 2011), dissolved in dimethylsulfoxide at 3–30 mM, and applied to cultures at final concentrations of 3–30 μM. The final concentration of dimethylsulfoxide in culture medium was 0.1%, which was non-toxic to dopaminergic neurons in slice cultures. N^{ω}-hydroxy-nor-l-arginine (nor-NOHA, Enzo Life Sciences, Farmingdale, NY, USA) was dissolved in distilled water at 100 mM and applied at a final concentration of 100 μM. GW9662 (Sigma) was dissolved in ethanol at 1 mM and used at a final concentration of 2 μM.

2.3. Immunohistochemistry

Slices were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer containing 4% sucrose at 4 °C for 2.5 h, and processed for tyrosine hydroxylase (TH) immunohistochemistry with avidin-biotinylated horseradish peroxidase method as described previously (Katsuki et al., 2009). Rabbit anti-TH polyclonal antibody (1:500, Merck Millipore) and biotinylated anti-rabbit IgG from goat (1:200, Vector Laboratories, Burlingame, CA, USA) were used as a primary and a secondary antibody, respectively. Positively stained cells, bearing developed dendrites that were at least more than twice as long as cell diameter, were considered as viable dopaminergic neurons. Dopaminergic neurons resided in a restricted area within each slice, reflecting their localization in the ventral part of the midbrain in vivo. In our microscopic observations, the size of the grid for cell counting ($450 \times 670\ \mu m^2$) was roughly equal to the area containing dopaminergic neurons in each slice. The investigator was not blinded to the treatments, but the position of the grid could be set in an objective manner to contain the maximal number of dopaminergic neurons in individual slices, and the number of viable dopaminergic neurons was counted.

Expression of Iba-1, inducible nitric oxide synthase (iNOS) and arginase-1 was determined by double immunofluorescence histochemistry. Rabbit anti-Iba-1 antibody (1:100; Wako Chemicals, Osaka, Japan), mouse anti-iNOS (1:100; BD Transduction Laboratories, San Diego, CA, USA) and goat anti-arginase-1 (N-20, 1:100; Santa Cruz Biotech. Inc., Dallas, TX, USA) were used as primary antibodies. Alexa Fluor 594-conjugated donkey anti-rabbit IgG (H+L) (1:500; Life Technologies), Alexa Fluor 488-conjugated donkey anti-mouse IgG(H+L) (1:200, Life Technologies), and Alexa Fluor 488-conjugated donkey anti-goat IgG(H+L) (1:500, Life Technologies) were used as secondary antibodies. For iNOS/arginase-1 double staining, Alexa Fluor 594-conjugated donkey anti-mouse IgG(H+L) (1:200, Life Technologies) was used. Fluorescence images were acquired with the use of confocal microscopy system (Olympus, Tokyo, Japan), from the ventral portion of midbrain slices where dopaminergic neurons were located. For quantitative evaluation of arginase-1 expression, a field of $200 \times 200\ \mu m^2$ was chosen blindly, and the percentage of arginase-1-positive cells within Iba-1-positive cells was obtained.

2.4. Nitrite quantification

Colorimetric Griess assay was employed to determine the concentration of nitrite in culture medium that reflected the amount of NO released from cultured tissues. Culture supernatants (50 μl) were reacted with an equal volume of Griess reagent (Sigma) for 10 min at 22–25 °C, and absorbance of diazonium compound was obtained at a wavelength of 560 nm.