Neurochemistry International 99 (2016) 158–168

Contents lists available at ScienceDirect

Neurochemistry International

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

The hypnotic bromovalerylurea ameliorates 6-hydroxydopamine-induced dopaminergic neuron loss while suppressing expression of interferon regulatory factors by microglia

Hiromi Higaki [a], Mohammed Emamussalehin Choudhury [a], Chisato Kawamoto [a], Keisuke Miyamoto [a], Afsana Islam [a], Yurika Ishii [a], Kazuya Miyanishi [a], Haruna Takeda [a], Naoto Seo [a], Kana Sugimoto [a, b], Hisaaki Takahashi [a, c], Hajime Yano [a], Junya Tanaka [a, *]

[a] Department of Molecular and Cellular Physiology, Graduate School of Medicine, Ehime University, Japan
[b] Department of Legal Medicine, Graduate School of Medicine/Faculty of Medicine, Osaka University, Japan
[c] Division of Pathophysiology, Faculty of Pharmaceutical Sciences, Hokuriku University, Japan

ARTICLE INFO

Article history:
Received 4 April 2016
Received in revised form
2 June 2016
Accepted 28 June 2016
Available online 5 July 2016

Keywords:
IRF
Parkinson
Proinflammatory
Neuroprotective factors
NO
Cytokine

ABSTRACT

The low molecular weight organic compound bromovalerylurea (BU) has long been used as a hypnotic/sedative. In the present study, we found that BU suppressed mRNA expression of proinflammatory factors and nitric oxide release in lipopolysaccharide (LPS)-treated rat primary microglial cell cultures. BU prevented neuronal degeneration in LPS-treated neuron-microglia cocultures. The anti-inflammatory effects of BU were as strong as those of a synthetic glucocorticoid, dexamethasone. A rat hemi-Parkinsonian model was prepared by injecting 6-hydroxydopamine into the right striatum. BU was orally administered to these rats for 7 days, which ameliorated the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and alleviated motor deficits. BU suppressed the expression of mRNAs for interferon regulatory factors (IRFs) 1, 7 and 8 in the right (lesioned) ventral midbrain as well as those for proinflammatory mediators. BU increased mRNA expression of various neuroprotective factors, including platelet-derived growth factor and hepatocyte growth factor, but it did not increase expression of alternative activation (M2) markers. In microglial culture, BU suppressed the LPS-induced increase in expression of IRFs 1 and 8, and it reduced LPS-induced phosphorylation of JAK1 and STATs 1 and 3. Knockdown of IRFs 1 and 8 suppressed LPS-induced NO release by microglial cells. These results suggest that suppression of microglial IRF expression by BU prevents neuronal cell death in the injured brain region, where microglial activation occurs. Because many Parkinsonian patients suffer from sleep disorders, BU administration before sleep may effectively ameliorate neurological symptoms and alleviate sleep dysfunction.

1. Introduction

Microglia are the sole mesoderm-derived cell type in the brain parenchyma, and have functions similar to those of macrophages and monocytes. In the normal adult brain, microglia are characterized by small somata and numerous long ramified processes that continuously move (Nimmerjahn et al., 2005). Through these fine movements, microglia survey the microenvironment of the brain as well as the functional state of synapses (Schafer et al., 2012; Kettenmann et al., 2013; Wake et al., 2013). Microglia can rapidly activate in response to various pathologic changes in the brain. Upon activation, microglia release a number of substances that can potentially damage neurons, such as reactive oxygen/nitrogen species, proinflammatory cytokines and glutamate (Takeuchi et al., 2008; Marinova-Mutafchieva et al., 2009; Neher et al., 2013; Patel et al., 2013). However, microglial cells are also known to support neuronal cell survival by releasing a number of neuroprotective factors and growth factors (Choudhury et al., 2011), as well as by detoxifying free radicals (Toku et al., 1998; Tanaka et al., 1999).

Parkinson's disease (PD), the second most prevalent neurodegenerative disorder after Alzheimer's disease, is a leading cause of

* Corresponding author. Department of Molecular and Cellular Physiology, Graduate School of Medicine, Ehime University, Toon, Ehime 791-0295, Japan.
E-mail address: jtanaka@m.ehime-u.ac.jp (J. Tanaka).

http://dx.doi.org/10.1016/j.neuint.2016.06.013
0197-0186/

neurological disability in the elderly (Hirsch and Hunot, 2009). The main pathological feature is progressive loss of dopaminergic neurons in the substantia nigra (SN). Along with the neuronal degeneration, activated microglia release a variety of harmful neurotoxic substances (Gerhard et al., 2006; Choudhury et al., 2011). Among the factors in the PD brain causing microglial activation, high mobility group box 1 (HMGB1) is important as an endogenous potential ligand for Toll-like receptor (TLR) 2 or 4 (Fang et al., 2012). Microglial cells express TLR4 at a significant level and respond to a typical TLR4 ligand, lipopolysaccharide (LPS), leading to the expression of inducible nitric oxide synthase (iNOS) or proinflammatory cytokines such as interleukin-1β. Similarly, HMGB1 can cause microglial activation, thereby aggravating neuronal degeneration in the SN of animal models of PD (Zhang et al., 2013; Sasaki et al., 2015).

Given the major role of microglia in neuronal degeneration in PD, novel therapeutic strategies are needed for inhibiting microglial proinflammatory activation. Numerous drugs have been tested for their ability to suppress microglial proinflammatory activation (Franco and Fernandez-Suarez, 2015), including glucocorticoids (Tanaka et al., 1997; Golde et al., 2003), antidepressants (Hashioka et al., 2007; Hwang et al., 2008; Lehnardt, 2010), non-steroidal anti-inflammatory drugs (NSAIDs) (Melton et al., 2003; Strohmeyer et al., 2005), adrenergic agonists (Mori et al., 2002; Ishii et al., 2015), minocycline (Wang et al., 2005), cytokines (Choudhury et al., 2011) and antibodies (Sasaki et al., 2015).

We recently found marked anti-inflammatory effects of the hypnotic bromovalerylurea (BU; $C_6H_{11}BrN_2O_2$, CAS: 496-67-3) on LPS-treated macrophages (Kikuchi et al., 2015). BU was originally patented in the US over 100 years ago, and is still used as a sedative/hypnotic, mainly in Japan (Tanaka, 2011). BU should be able to cross the blood–brain barrier easily, and to suppress the activation of microglial cells. In this study, we found that BU effectively suppressed LPS-induced activation of microglial cells in culture, and it ameliorated neurodegeneration and motor deficits in the 6-hydroxydopamine (6-OHDA)-induced rat model of Parkinsonism. Although BU did not affect microglial morphology in the 6-OHDA-injected brain, it suppressed expression of proinflammatory cytokines. Furthermore, BU suppressed the expression of interferon regulatory factors (IRFs), which have been implicated in the activation of microglial cells (Masuda et al., 2012, 2015; Yoshida et al., 2014; Tanaka et al., 2015). Our findings provide insight into the mechanisms underlying the anti-inflammatory actions of BU.

2. Materials and methods

2.1. Primary cultures

All animal experiments were carried out in accordance with the Guidelines for Animal Experimentation of Ehime University Graduate School of Medicine. Rat primary microglial cultures were prepared as described previously (Tanaka et al., 1998). Briefly, whole forebrains from neonatal rats were dissected out and dissociated into individual cells that were cultured as a mixed glial cell culture in 75-cm^2 flasks containing 10% fetal bovine serum-supplemented Dulbecco's modified Eagle's medium (DMEM) for 11 or 14 days. Microglial cells were obtained from the mixed glial culture. The purity of the microglial culture was >99% (Yokoyama et al., 2004). Purified microglial cells were seeded onto poly-L-lysine (PLL)-coated glass coverslips placed in 4-well culture plates. Cortical neurons dissociated from the cerebral cortices of 17-day-old rat embryos were seeded on PLL-coated 4-well culture plates at a density of 1×10^5 cells/cm^2 (Tanaka et al., 1999). Microglial cells (5×10^4 cells/cm^2) were seeded onto the neurons on the second day.

LPS (from *Escherichia coli* serotype 055:B5; Sigma-Aldrich, St. Louis, MO) was added at 1 μg/ml to serum-free culture medium [E2 medium; DMEM containing 10 mM HEPES (pH 7.3; Gibco, Grand Island, NY), 4.5 mg/ml glucose, 5 μg/mL insulin, 5 nM sodium selenite, 5 μg/mL transferrin (Gibco) and 0.2 mg/mL bovine serum albumin (Sigma-Aldrich, St. Louis, MO)] to activate microglial cells. BU was dissolved in DMSO at 100 mg/ml, and then diluted to 1–100 μg/ml in culture medium.

2.2. Determination of nitrite release

Conditioned media were obtained from pure microglial cultures or neuron-microglia cocultures maintained in 4-well PLL-coated plates for 24 h in serum-free medium containing 1 μg/ml LPS, with or without BU (1–100 μg/ml). The conditioned media were used for measurement of nitrite to evaluate LPS-induced NO release. The nitrite assay, based on the Griess reaction, was performed as described elsewhere (Zhang et al., 2002). To normalize the released NO level with the cellular protein contents in pure microglial culture, LPS-treated microglial cells were solubilized with RIPA buffer [50 mM Tris-HCl, pH8.0, 150 mM sodium chloride, 0.5 w/v% sodium deoxycholate, 0.1 w/v% sodium dodecyl sulfate, 1.0 w/v % NP-40 substitute] and the protein contents were determined by Pierce BCA protein assay reagents (Thermo Scientific, Rockford, IL).

2.3. 6-OHDA treatment and BU administration

Adult male Wistar rats, weighing 220–250 g, were housed under standard laboratory conditions. The animals were allowed free access to food and water throughout the experiments. The rats were kept under a 12/12-h dark/light cycle. 6-OHDA (10 μg/μl; Sigma-Aldrich) was dissolved in saline containing ascorbic acid (Wako, Osaka, Japan) and then administered into the right striatum as described elsewhere (Choudhury et al., 2011). In brief, 5 μL of 6-OHDA solution in a Hamilton syringe was injected into the right striatum through a hole on the skull at 1 mm anterior to bregma and 3 mm lateral from the midline. The depth of the needle tip was 5 mm from the skull surface. BU dissolved in water at a concentration of 500 mg/L was administered as drinking water. Because each rat drank ~25 ml/day, the total daily dose was ~50 mg/kg, corresponding to 3 g/60 kg body weight/day, which is the maximum clinical dose for humans. The oral BU administration was started shortly after the 6-OHDA injection into the striatum and it was maintained until sacrifice of the animals.

2.4. Immunoblotting

Cultured cells and ventral midbrain tissues were used for immunoblotting analysis. Pure microglial cultures or neuron-microglia cocultures were incubated with either nothing, LPS (1 μg/ml) or LPS + BU (100 μg/ml) for 150 min or 48 h, and lysates were prepared using Laemmli sample buffer containing phosphatase inhibitor cocktail solution II (Wako). These lysates were then subjected to immunoblotting to evaluate iNOS expression and the neuronal viability. The left and the right sides of the ventral midbrain from 6-OHDA-treated animals were dissected 7 days after the administration, and the tissues were immediately homogenized in 10 volumes of Laemmli sample buffer containing 3% SDS. The cell and tissue lysates were electrophoresed, transferred to nitrocellulose membranes, and immunoblotted with the antibodies listed in Table 1. The immunoreaction was visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Tanaka et al., 1998). Immunoreactive bands were analyzed by densitometry using ImageJ 1.43u (Wayne Rasband, National