Biochemical and Biophysical Research Communications 495 (2018) 2165–2170

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Involvement of nitric oxide/reactive oxygen species signaling via 8-nitro-cGMP formation in 1-methyl-4-phenylpyridinium ion-induced neurotoxicity in PC12 cells and rat cerebellar granule neurons

Kumiko Masuda [a, b], Hiroyasu Tsutsuki [c], Shingo Kasamatsu [d], Tomoaki Ida [d], Tsuyoshi Takata [e], Kikuya Sugiura [f], Motohiro Nishida [g], Yasuo Watanabe [e], Tomohiro Sawa [c], Takaaki Akaike [d], Hideshi Ihara [a, *]

[a] Department of Biological Science, Graduate School of Science, Osaka Prefecture University, Osaka, Japan
[b] Project Management Department, SHIONOGI & CO., LTD., Osaka, Japan
[c] Department of Microbiology, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan
[d] Department of Environmental Health Sciences and Molecular Toxicology, Tohoku University Graduate School of Medicine, Sendai, Japan
[e] Department of Pharmacology, Showa Pharmaceutical University, Machida, Tokyo, Japan
[f] Department of Veterinary Science, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Osaka, Japan
[g] Division of Cardiocirculatory Signaling, National Institute for Physiological Sciences (Okazaki Institute for Integrative Bioscience), National Institutes of Natural Sciences, Aichi, Japan

ARTICLE INFO

Article history:
Received 8 December 2017
Accepted 15 December 2017
Available online 16 December 2017

Keywords:
nNOS
NO/ROS redox signaling
8-nitro-cGMP
H-Ras/ERK signaling
MPP^+
Neurotoxicity

ABSTRACT

To investigate the role of nitric oxide (NO)/reactive oxygen species (ROS) redox signaling in Parkinson's disease-like neurotoxicity, we used 1-methyl-4-phenylpyridinium (MPP^+) treatment (a model of Parkinson's disease). We show that MPP^+-induced neurotoxicity was dependent on ROS from neuronal NO synthase (nNOS) in nNOS-expressing PC12 cells (NPC12 cells) and rat cerebellar granule neurons (CGNs). Following MPP^+ treatment, we found production of 8-nitroguanosine 3′,5′-cyclic monophosphate (8-nitro-cGMP), a second messenger in the NO/ROS redox signaling pathway, in NPC12 cells and rat CGNs, that subsequently induced *S*-guanylation and activation of H-Ras. Additionally, following MPP^+ treatment, extracellular signal-related kinase (ERK) phosphorylation was enhanced. Treatment with a mitogen-activated protein kinase (MAPK)/ERK kinase (MEK) inhibitor attenuated MPP^+-induced ERK phosphorylation and neurotoxicity. In conclusion, we demonstrate for the first time that NO/ROS redox signaling via 8-nitro-cGMP is involved in MPP^+-induced neurotoxicity and that 8-nitro-cGMP activates H-Ras/ERK signaling. Our results indicate a novel mechanism underlying MPP^+-induced neurotoxicity, and therefore contribute novel insights to the mechanisms underlying Parkinson's disease.

1. Introduction

Parkinson's disease (PD) is caused by depletion of dopamine from the striatum accompanied by degradation of dopaminergic neurons in the substantia nigra [1]. Although the etiology of PD is not fully understood, it is well known that PD is influenced by several factors including environmental conditions and genetics [1]. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is recognized as causing traits seen in sporadic PD [2]. MPTP absorbed by an animal reaches the brain because it can penetrate the blood–brain barrier. In the brain, monoamine oxidase B converts MPTP to 1-methyl-4-phenylpyridinium (MPP^+) which is a toxic metabolite. MPP^+ is then taken up into neurons via the dopamine transporter and induces neuronal cell death by inhibition of complex I activity in the mitochondrial respiratory chain [3]. Although

Abbreviations: MPP^+, 1-methyl-4-phenylpyridinium; NPC12 cells, nNOS-expressing PC12 cells; CGNs, cerebellar granule neurons; 8-nitro-cGMP, 8-nitroguanosine 3′,5′-cyclic monophosphate; GST, glutathione *S*-transferase; L-NAME, *N*G-nitro-L-arginine methyl ester; DHE, dihydroethidium.

* Corresponding author. Department of Biological Science, Graduate School of Science, Osaka Prefecture University, 1-1 Gakuen-cho, Sakai, Osaka 599-8531, Japan. Fax: +81 72 254 9163.
E-mail address: ihara@b.s.osakafu-u.ac.jp (H. Ihara).

https://doi.org/10.1016/j.bbrc.2017.12.088
0006-291X/

the mechanism of neurotoxicity induced by MPP^+ has not yet been fully elucidated, some evidence supports the relevance of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [1,4].

When nitric oxide synthases (NOSs) produce nitric oxide (NO), NOSs also produce superoxide [5] by utilizing the electron released by nicotinamide adenine dinucleotide phosphate (NADPH) in an uncoupling reaction (switching nitric oxide to superoxide formation). The ROS generation from NOSs appears to contribute to the functional diversity of NOSs. Actually, it has been reported that superoxide produced by the uncoupling reaction of neuronal NOS (nNOS) is an important factor in MPP^+-induced neurotoxicity in studies using rat cerebellar granule neurons (CGNs) and human neuroblastoma cells [6,7]. Furthermore, in the MPTP mouse model, neurotoxicity induced by MPTP in nNOS knockout mice is less than induced in wild type mice [8]. Taken together, these observations suggest that nNOS may exacerbate MPTP-induced neurotoxicity. However, the detailed molecular mechanism of the involvement of nNOS in MPTP-induced neurotoxicity is not clear.

To date, two mechanisms underlying NO activity in the nervous system have been examined—a cyclic guanosine monophosphate (cGMP)-dependent pathway and chemical modification, including nitrosylation and nitration, of proteins and lipids shown to participate in the inflammatory response [9,10]. A recent study of the NO/ROS redox signal is noteworthy, and we previously found that 8-nitroguanosine 3′,5′-cyclic monophosphate (8-nitro-cGMP) is a second messenger in NO/ROS signaling and is formed endogenously under both the production of NO and ROS [11]. 8-Nitro-cGMP provides an electrophilic signal for the formation of a protein-*S*-cGMP adduct (*S*-guanylation). In fact, some studies have shown that 8-nitro-cGMP takes on critical roles, such as having an antioxidant effect or inducing autophagy by modification of proteins related to the signaling pathway, under various pathophysiological conditions [11–14]. Additionally, 8-nitro-cGMP induced electrophilic H-Ras activation via *S*-guanylation and cellular senescence via the mitogen-activated protein kinase (MAPK) signaling pathway in myocardial cells [14]. Our previous studies have demonstrated that ROS derived from nNOS modulate a NO/ROS redox signal via production of 8-nitro-cGMP [15,16]. Consequently, it may be surmised that 8-nitro-cGMP would be involved in MPP^+-induced neurotoxicity. However, there is no evidence of a relationship between 8-nitro-cGMP and MPP^+-induced neurotoxicity.

In this study, we investigated the involvement of the NO/ROS redox signaling pathway via 8-nitro-cGMP in MPP^+-induced neurotoxicity to clarify the mechanism of neurotoxicity in PD.

2. Materials and methods

2.1. Materials

The anti-8-nitro-cGMP and anti-S-guanylated protein antibodies were prepared as previously described [11]. MPP^+, RPMI 1640 medium, and HRP-conjugated anti-rabbit secondary antibody were from Sigma-Aldrich (St. Louis, MO). Anti-phospho-ERK and anti-ERK antibodies were from Cell Signaling (Danvers, MA). Anti-H-Ras antibody, glutathione *S*-transferase (GST)-Ras-binding domain (RBD) of Raf-1, MagneGST Glutathione Particles, Protease inhibitor cocktails, *N*G-Nitro-L-arginine methyl ester (L-NAME), PD98059, Horse serum, Fetal bovine serum (FBS), and Dihydroethidium (DHE) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), Millipore (Billerica, MA), Promega (Madison, WI), Nacalai Tesque (Kyoto, Japan), Dojindo Laboratories (Kumamoto, Japan), Cayman Chemical (Ann Arbor, MI), Invitrogen (Carlsbad, CA), Cytosystems (MultiSer; Cytosystems, Castle Hill, NSW, Australia), and ABD Qioquest (Sunnyvale CA), respectively. All other chemicals were from common suppliers and were of the highest grade commercially available.

2.2. Cell culture

PC12 cell stably expressing nNOS (NPC12) was prepared as previously described [17]. PC12 cells were cultured in RPMI 1640 medium containing 5% FBS and 5% horse serum at 37 °C. The cells were seeded at a density of 1×10^4 cells per well in 96-well culture plates, 2×10^7 cells per 100-mm culture dish, and 1×10^5 cells on circular coverslips per well in 4-well culture plates for the cytotoxicity assay, Western blotting analyses, and immunocytochemistry, respectively.

Primary cerebellar granule neurons (CGNs) were prepared from cerebella of 7- to 10-day-old Wistar rats as described previously [18] in compliance with the Guideline for Animal Experimentation at Osaka Prefecture University. Cells (cultured for 12 days *in vitro*) were seeded at a density of 1×10^5 cells per well in 96-well culture plates, 2×10^6 cells per 60-mm culture dish, and 1×10^5 cells on circular coverslips per well in 4-well culture plates for the cytotoxicity assay, Western blotting analyses, and immunocytochemistry, respectively.

2.3. Cytotoxicity assays

Cell viability was assessed with the CellTiter 96 Cell Proliferation Assay kit (Promega). PC12 cells and CGNs seeded in 96-well plates were treated with MPP^+ at various concentrations, and incubated at 37 °C for 24 h. To investigate the effects of ROS, NOS, soluble guanylate cyclase (sGC) and MEK on the cytotoxicity, cells were preincubated with Tiron, L-NAME, NS2028 and PD98059 at 37 °C for 1 h, respectively. After MPP^+-treatment, assay solution was added to the wells. Absorption was measured at 490 nm by a microplate reader (Bio-Rad). Results are expressed as mean cytotoxicity ± standard error (S.E.), determined for triplicate cultures.

2.4. Measurement of intracellular ROS generation

Intracellular ROS generation was detected by using fluorescence microscopy with the oxidant-sensitive dye DHE. PC12 cells treated with MPP^+ were washed, and incubated in 10 μM DHE at 37 °C for 30 min. Then they were washed, fixed with 4% paraformaldehyde at room temperature for 30 min, and observed with an Axioplan microscope (Carl Zeiss, Göttingen, Germany).

2.5. 8-Nitro-cGMP immunocytochemistry

Using PC12 cells and CGNs at 24 and 4 h after MPP^+ treatment, respectively, immunostaining was achieved by using a monoclonal antibody specific for 8-nitro-cGMP, as reported previously [11] and staining was then observed with a fluorescence microscope (ECLIPSE Ti; Nikon, Tokyo, Japan) equipped with an ORCA-R2 camera (Hamamatsu Photonics, Hamamatsu, Japan). Images were processed to reduce noise by using deconvolution software (AutoDeblur; Lexi, Tokyo, Japan). Adobe Photoshop v. 7.0 (Adobe Systems, Waltham, MA) was used for additional image processing and quantification.

2.6. Ras pulldown assay and Western blotting for Ras and its related proteins

The Ras pulldown assay was performed as described previously [14]. Briefly, PC12 cells and CGNs were lysed in Ras pulldown buffer containing 50 mM HEPES (pH 7.4), 1% Triton X-100, 10% glycerol, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM sodium