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NITRIC OXIDE–CYCLIC GMP SIGNALING PATHWAY LIMITS INFLAMMATORY DEGENERATION OF MIDBRAIN DOPAMINERGIC NEURONS: CELL TYPE-SPECIFIC REGULATION OF HEME OXYGENASE-1 EXPRESSION

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Abstract—Excessive production of nitric oxide (NO) by microglia is at least in part responsible for the pathogenesis of various neurodegenerative disorders including Parkinson disease, but at the same time NO may also play a distinct role as a signaling molecule such as an activator of soluble guanylyl cyclase. Here we investigated potential roles of the NO-soluble guanylyl cyclase–cyclic GMP signaling pathway in the regulation of dopaminergic neurodegeneration. Activation of microglia by interferon-γ (IFN-γ) followed by lipopolysaccharide (LPS) caused dopaminergic cell death in rat midbrain slice cultures, which was dependent on NO production. 1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), a soluble guanylyl cyclase inhibitor, as well as KT5823, an inhibitor of cyclic GMP-dependent protein kinase, exacerbated dopaminergic cell death induced by IFN-γ/LPS. Conversely, 8-bromo-cyclic GMP attenuated IFN-γ/LPS cytotoxicity on dopaminergic neurons. Notably, although heme oxygenase-1 (HO-1) was expressed prominently in cells other than dopaminergic neurons in control cultures, robust expression of HO-1 was induced in surviving dopaminergic neurons challenged with IFN-γ/LPS. ODQ and KT5823 decreased, whereas 8-bromo-cyclic GMP increased, the number of dopaminergic neurons expressing HO-1 after IFN-γ/LPS challenge, without parallel changes in HO-1 expression in other cell populations. An NO donor 3-(4-morpholinyl)sydnonimine hydrochloride also induced HO-1 expression in dopaminergic neurons, which was abolished by ODQ and augmented by 8-bromo-cyclic GMP. Moreover, IFN-γ/LPS-induced dopaminergic cell death was augmented by zinc protoporphyrin IX, an HO-1 inhibitor. The NO donor cytotoxicity on dopaminergic neurons was also augmented by ODQ and zinc protoporphyrin IX. These results indicate that the NO-cyclic GMP signaling pathway promotes the induction of HO-1 specifically in dopaminergic neurons, which acts as an endogenous protective system to limit inflammatory degeneration of this cell population.

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Abbreviations: CoPPIX, cobalt(III) protoporphyrin IX; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HO-1, heme oxygenase-1; IFN-γ, interferon-γ; iNOS, inducible isoform of nitric oxide synthase; LDH, lactate dehydrogenase; L-NAME, N^{ω}-nitro-L-arginine methyl ester; LPS, lipopolysaccharide; NO, nitric oxide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; PD, Parkinson disease; PKG, cyclic GMP-dependent protein kinase; RT, reverse transcriptase; sGC, soluble guanylyl cyclase; SIN-1, 3-(4-morpholinyl)sydnonimine hydrochloride; TH, tyrosine hydroxylase; ZnPPIX, zinc protoporphyrin IX; 8-Br-cGMP, 8-bromoguanosine-3′,5′-cyclic-monophosphate.

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Parkinson disease (PD) is a progressive neurodegenerative disorder, pathologically characterized by selective loss of dopaminergic neurons in the midbrain substantia nigra pars compacta (Dauer and Przedborski, 2003; Fahn and Sulzer, 2004). Diverse sets of environmental and genetic factors, as well as intrinsic characteristics of dopaminergic neurons, may be involved in the pathogenesis of PD as primary causes of neurodegeneration (Dodson and Guo, 2007; Thomas and Beal, 2007).

After initiation of neurodegeneration by the primary causes, neuroinflammatory processes are considered to take place as secondary events to accelerate and exacerbate neurodegeneration. Indeed, accumulation of reactive microglia is found in the substantia nigra of PD patients (McGeer et al., 1988; Hirsch et al., 1998) and experimental animal models of PD (McGeer et al., 2003). Nitric oxide (NO) is among the most important molecular species produced by activated microglia that can affect neuronal viability (Block et al., 2007). Dopaminergic neurons in mice lacking inducible isoform of nitric oxide synthase (iNOS) are resistant to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine toxicity (Liberatore et al., 1999), and pharmacological inhibition of iNOS *in vitro* and *in vivo* rescues dopaminergic neurons from degeneration induced by activated microglia (Iravani et al., 2002; Shibata et al., 2003).

On the other hand, NO is also considered an important signaling molecule in the CNS, as a regulator of synaptic plasticity, neurosecretion and neurotransmission (Calabrese et al., 2007). These functions of NO are at least in part mediated by the activation of soluble guanylyl cyclase (sGC) and consequent recruitment of cyclic GMP-dependent intracellular signaling (Calabrese et al., 2007; Zayas and Trimmer, 2007). In the substantia nigra, the NO–sGC–cyclic GMP signaling pathway has been shown to stimulate phosphorylation of DARPP-32, a key regulator of information processing in dopaminoceptive neurons (Tsou et al., 1993).

Few studies have addressed the roles of cyclic GMP signaling during the pathogenic events with relation to PD. Midbrain guanylyl cyclase activity and cyclic GMP production are enhanced in an NO-dependent manner in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mice model of PD, although the pathological impact of these changes is unknown

(Chalimoniuk et al., 2006). In primary midbrain cultures with decreased glutathione, NO triggers cell death in an sGC- and cyclic GMP–dependent manner (Canals et al., 2001), which suggests that the sGC–cyclic GMP signaling can be deleterious to neurons. By contrast, cytoprotective actions of the NO–sGC–cyclic GMP signaling, via induction of heme oxygenase-1 (HO-1), have been reported in non-neural cells (Polte et al., 2000). Expression of HO-1 is also induced under various pathological conditions in the CNS, and this enzyme can exert cytoprotective actions through multiple mechanisms (Huang et al., 2005; Scott et al., 2007).

To gain insight into the roles of the cyclic GMP signaling in NO-mediated degeneration of dopaminergic neurons, we examined the effects of several drugs that affect the NO–sGC–cyclic GMP signaling cascade in interferon-γ (IFN-γ)/lipopolysaccharide (LPS)-treated midbrain slice cultures. We have previously reported in this IFN-γ/LPS neuroinflammation model that excessive production of NO by iNOS in microglia is primarily responsible for the induction of dopaminergic neurodegeneration (Shibata et al., 2003). Here we find that, contrary to the deleterious role reported in primary midbrain cultures (Canals et al., 2001), the sGC–cyclic GMP signaling downstream of NO production affords a cytoprotective effect on dopaminergic neurons, via regulation of HO-1 expression.

EXPERIMENTAL PROCEDURES

Culture preparation

Organotypic midbrain slice cultures were prepared according to the methods described previously (Katsuki et al., 2006). All procedures were approved by our institutional animal experimentation committee, and animals were treated in accordance with the Guidelines of the United States National Institutes of Health regarding the care and use of animals for experimental procedures. All efforts were made to minimize the number of animals used and their suffering. Briefly, 2 to 3-day-old neonatal Wistar rats (Nihon SLC, Shizuoka, Japan) were anesthetized by hypothermia and decapitated, and the brain was removed from the skull and separated into two hemispheres. Coronal midbrain slices (350-μm thick) were prepared under sterile conditions with a tissue chopper (Narishige, Tokyo, Japan), and transferred onto microporous membranes (Millicell-CM, Millipore, Bedford, MA, USA) in six-well plates. Six slices from one hemisphere were placed together in one membrane. Culture medium, consisting of 50% minimal essential medium/Hepes, 25% Hanks' balanced salt solution and 25% heat-inactivated horse serum (Invitrogen Japan, 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 each well. The culture medium was exchanged with fresh medium on the next day of culture preparation, and thereafter, every 2 days. Slices were maintained in a 34 °C, 5% CO_2 humidified atmosphere.

Drug treatment

At 17–18 days *in vitro*, slices were exposed to indicated concentrations of drugs by transfer of culture inserts to culture plates filled with 0.7 ml of drug-containing serum-free medium. Serum-free medium consisted of 75% minimal essential medium/Hepes 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. Drugs used here were recombinant rat IFN-γ (PeproTech, London, UK), LPS (from *Escherichia coli*, serotype 0111;B4, Sigma, St. Louis, MO, USA), N^{ω}-nitro-L-arginine methyl ester (L-NAME; Sigma), zinc protoporphyrin IX (ZnPPIX; Biomol International, Plymouth Meeting, PA, USA), cobalt(III) protoporphyrin IX chloride (CoPPIX; Frontier Scientific, Logan, UT, USA), 1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ; Wako, Osaka, Japan), 8-bromoguanosine-3′,5′-cyclic-monophosphate sodium salt (8-Br-cGMP; Biomol), KT5823 (Calbiochem-EMD, San Diego, CA, USA) and 3-(4-morpholinyl)sydnonimine hydrochloride (SIN-1; Dojindo, Kumamoto, Japan).

Immunohistochemistry

Slices were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer containing 4% sucrose at 4 °C for 2 h, and processed for tyrosine hydroxylase (TH) immunohistochemistry with avidin-biotinylated horseradish peroxidase method, as described previously (Shibata et al., 2003). Rabbit anti-TH polyclonal antibody (1:500, 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 the cell diameter were considered as viable dopaminergic neurons. The maximal number of viable dopaminergic neurons in an area of 450×670 μm^2 in individual slices was counted. For immunohistochemical examinations of iNOS expression, mouse anti-iNOS polyclonal antibody (1:300, BD Transduction Laboratories, San Diego, CA, USA) and biotinylated anti-mouse IgG from goat (1:200, Vector Laboratories) were used as a primary and a secondary antibody, respectively.

Double immunofluorescence histochemistry was done for TH and HO-1. Primary antibodies for detection of TH and HO-1 were mouse anti-TH (1:250, Affinity BioReagents, Golden, CO, USA) and rabbit anti-heme-oxygenase-1 antibody (1:200, Assay Designs Inc., Ann Arbor, MI, USA), respectively. Alexa Fluor 488–labeled goat anti-rabbit IgG (1:500, Invitrogen) and Alexa Fluor 594–labeled goat anti-mouse IgG (1:500, Invitrogen) were used as respective secondary antibodies. Confocal images were obtained with the usage of Fluoview FV300 system (Olympus, Tokyo, Japan).

Nitrite quantification

The amount of NO released during LPS treatment was quantified as concentrations of nitrite in culture medium by the Griess method. Culture supernatants (100 μl) were collected and reacted with an equal volume of Griess reagent (Sigma) for 10 min at room temperature. Absorbance of diazonium compound was measured at 560 nm with a microplate reader (Immuno Mini NJ-2300, InterMed, Tokyo, Japan). Absolute levels of nitrite were determined with reference to a standard curve obtained from defined concentrations of sodium nitrite.

Lactate dehydrogenase (LDH) release assay

Cytotoxic damage of whole slice cultures was quantified by measurement of LDH release into culture medium. The amount of LDH was determined by a Cytotoxicity Detection LDH kit (Kyokuto Pharmaceutical Industrial Corporation, Tokyo, Japan). In this assay, NAD is reduced to NADH through the conversion of lactate to pyruvate by LDH, and NADH reduces tetrazolium into formazan dyes in the presence of diaphorase. After treatment with LPS or an NO donor for indicated periods (6–72 h), 25 μl of culture supernatants was collected and incubated with 75 μl of the coloring reagent in a 96-well plate at room temperature. After incubation for 1 h, the reaction was stopped by addition of 100 μl of 1 N HCl, and the absorbance at 560 nm (OD 560) was determined with a microplate reader. The reading of background absorbance was subtracted from all values.