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A self-propelling cycle mediated by reactive oxide species and nitric oxide exists in LPS-activated microglia

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ABSTRACT

It has been widely accepted that microglia, the innate immune cells in the brain, can be chronically activated in response to neuron death, fuelling a self-renewing cycle of microglial activation followed by further neuron damage (reactive microgliosis), which has been considered as the main reason responsible for the progressive nature of neurodegenerative diseases. In the present study, it was found that LPS (lipopolysaccharide) significantly induced the activation of N9 microglia, and the increase of NO level induced by pretreatment of LPS could last after the removal of LPS. The culture medium of activated microglia significantly decreased the viability of rat primary cortical neuron. These results can be blocked by the antioxidant *N*-acetylcysteine (NAC) and nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase inhibitor diphenyleneiodonium sulfate (DPI), suggesting that intracellular reactive oxide species (iROS) released from the activated microglial cells may continue to further activate microglia. Next, it was shown that the iROS level increased rapidly after the LPS treatment in microglia cells followed by the NO production through the regulation of iNOS (inducible nitric oxide synthase) expression. The increase of iROS could be reversed by gp91phox (the critical and catalytic subunit of NADPH oxidase) siRNA. Moreover, NO released from sodium nitroprusside (SNP) was able to increase the iROS production of N9 microglia by regulating of the activity and the expression of NADPH oxidase. In conclusion, our research suggests for the first time that there may exist a self-propelling cycle in microglial cells possibly mediated by iROS and NO when they become activated by LPS. It may be responsible partially for the ongoing microglial activation and the progressive nature of neurodegenerative diseases.

1. Introduction

Microglia are resident monocytes in the central nervous system (CNS), functionally similar to macrophages (Vallejo et al., 2010). Under normal physiological conditions, microglia are involved in immune surveillance and host defense against infectious agents. However, microglia readily become activated in respond to a variety of stimuli, including ischemia, inflammation, injury or immunological challenges. Unregulated response or over-activation of microglia has a disastrous neurotoxic consequence which is considered as a common mechanism of neurodegenerative diseases. Thus, inhibition of microglial activation and subsequent inflammatory process in numerous CNS diseases is regarded as an important therapeutic target (Zhang et al., 2010; Hensley, 2010; Chakraborty et al., 2010).

Cytokines, reactive oxygen species (ROS) and nitric oxide (NO) have emerged as important mediators of inflammatory processes during microglia activation (Miller et al., 2009; Whitton, 2007; Owens, 2002). It is well known that the intracellular ROS (iROS) derived from nicotinamide adenine dinucleotide phosphate (NADPH) oxidase play special roles in neurodegeneration by serving dual functions (Arora et al., 2010; Choi et al., 2012). First, high concentrations of ROS released extracellularly may exert direct toxicity to dopaminergic neurons, and second, the increase in intracellular concentrations of ROS may modify the signaling events leading to the activation of microglia (Sedeek et al., 2009; Muller and Morawietz, 2009; Robinson, 2009; Huh et al., 2011). As an important bioactive molecule, NO plays vital roles in various physiological

Abbreviations: CAT, catalase; CNS, central nervous system; DCFH-DA, 2′7′-dichlorodihydrofluororescein diacetate; DMSO, dimethylsulfoxide; DPI, diphenyleneiodonium sulfate; FBS, fetal bovine serum; HBSS, Hank's balanced salt solution; IFN-γ, interferon-γ; IL-1β, Interleukin-1β; MDM, Iscove's modified Dulbecco's medium; iNOS, inducible nitric oxide synthase; iROS, intracellular reactive oxide species; L-NAME, *N*-nitro-L-arginine methyl ester; LPS, lipopolysaccharide; NAC, *N*-acetylcysteine; NADPH, nicotinamide adenine dinucleotide phosphate oxidase; MAPKs, mitogenactivated protein kinases); MTT, methyl thiazolyl tetrazolium; NBT, nitro blue tetrazolium chloride; NF-κB, nuclear factor-κB; NO, nitric oxide; POD, peroxidase; siRNA, small interfering RNA; SNP, sodium nitroprusside; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α.

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and pathological processes in many organ systems, including the brain (Ritt et al., 2011; Souza et al., 2010; Sato et al., 2011). NO is enzymatically formed from arginine by the enzyme NO synthase (NOS). The inducible form (iNOS), which is expressed in various cell types including microglial cells in response to a wide variety of stimuli, is regulated mainly at the transcriptional level, and does not require calcium for its activity (Ferreira et al., 2010; Wang et al., 2010).

It is well known that most neurodegenerative diseases are delayed and progressive in nature. For example, the time period between the exposure to environmental neurotoxins and the manifestation of the Parkinson disease (PD) symptoms is assumed to be approximately 8–10 years. In absence of repeated and continual exposure, it is unlikely that these toxins remain present in the brain at the time of PD diagnosis years later, suggesting that the microglial activation initiated by early toxic insult is propagated and potentially amplified throughout the disease. This phenomenon is hypothesized that a self-propelling cycle may exist between microglial activation and neuronal damage, which is commonly referred to as reactive microgliosis (Streit, 2001, 2000; Streit et al., 1999).

However, in addition to the possible role of damaged neuron, whether there exist other factors responsible for the ongoing microglia activation was not known. Thus, two questions were addressed in the present study: (i) exclude the participation of damaged neurons, whether a self-propelling cycle exists in LPS-activated microglia itself. (ii) If such self-propelling circle exists, what is the mechanism? Thus, the present study was designated to study these two issues and the interaction between iROS and NO during the microglia activation. Our results suggest for the first time that a self-propelling cycle mediated by iROS derived from NADPH oxidase and NO produced by iNOS may exist in microglial cells after LPS treatment, which could continue to further activate microglia, in addition to the possible role of damaged neuron. It may be responsible partially for the ongoing microglial activation and the progressive nature of neurodegenerative diseases.

2. Materials and methods

2.1. Regents

Iscove's modified Dulbecco's medium (IMDM), Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Lipopolysaccharide (LPS, E5:055), 2′,7′-dichlorodihydrofluororescein diacetate (DCFH-DA), N-acetylcysteine (NAC), diphenylene iodonium (DPI) and nitroblue tetrazolium (NBT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA); hemoglobin was purchased from Biyuntian Biotech Corporation (Haimen City, Jiangsu Province, China); hydrogen peroxide was from Fuchen Chemical Reagent Factory (Tianjin, China); anti-Iba1 antibody was from Wako Chemicals USA (Richmond, VA, USA); iNOS antibody was from BD Biosciences (San Diego, CA, USA); β-actin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA); gp91phox antibody was from Abcam (Cambridge, UK); Trizol reagent was from Invitrogen Co. (Carlsbad, CA, USA); RevertAidTM First Strand cDNA Synthesis Kit was from Fermentas (Burlington, Ontario, Canada); Taq polymerase was from the Takara Biotechnology (Dalian, China). Superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) assay kits were produced by Naniing Jian Cheng Biotechnology Company (Nanjing, China).

2.2. Cell culture

The murine microglia cell line N9 (kindly provided by Prof. J.M. Wang, Laboratory of Molecular Immunoregulation, Center for Cancer Research, NCI, National Institutes of Health, USA) obtained by immortalization of E13 mouse embryonic cultures with the 3RV retrovirus carrying an activated v-myc oncogene that is similar to primary microglia (Chang et al., 2008). Cells were cultured in IMDM supplemented with 5% FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5×10^{-5} M 2-mercaptoethano at 37 °C in humidified 5% CO_2. Stock cells were passaged 2–3 times/week with 1:4 split ratio and used within 8 passages.

Mouse primary microglia were prepared from the cortex of newborn BALB/c mice (Department of Laboratory Animal Science, Shenyang Pharmaceutical University) (Suzumura et al., 1984) using the shaking method (Giulian and Baker, 1986). All experimental procedures were performed in accordance with the guidelines of the Experimental Animal Care and Use Committee of Shenyang Pharmaceutical University (Shenyang, China). The purity of microglial cells obtained was >95% as determined by immunocytochemistry using anti-Iba1 antibody (Imai et al., 1996). The cells were cultured in DMEM containing 10% heat inactivated FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin.

Cortical neuronal cultures were prepared from 17–19-day-old fetal rats (Sprague–Dawley rats; Animal Resources Center of Shenyang Pharmaceutical University, Shenyang, China) according to the procedures described previously (Yang et al., 2009) Briefly, the cortex was dissected and kept in ice-cold Hank's balanced salt solution (HBSS), then incubated at 37 °C for 30 min in Ca^{2+}/Mg^{2+}-free HBSS containing 0.25% trypsin (Gibco). The cortical tissues were dissociated to single cells by gentle trituration. The cell suspension was mixed with IMDM (Gibco) supplemented with 5% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine. Cells were seeded onto poly-L-lysine coated plates at a density of 1×10^{-6} cells/ml. Cytarabine was added into the medium to inhibit the proliferation of non-neuronal cells. The neurons were allowed to grow for 5 days before further treatment.

2.3. Conditional medium preparation

N9 microglia were allowed to grow for 1 day, then LPS (final concentration is 1 μg/ml), was added to the cultural medium except for the control group. After the treatment for various time periods, cultural medium was removed. N9 microglia was washed three times with PBS and incubated in fresh IMDM culture medium, conditional medium was collected after another 24 h.

2.4. Measurement of cell viability

Cell viability was evaluated by the methyl thiazolyl tetrazolium (MTT) reduction assay (Chang et al., 1998). In brief, N9 microglial cells or cortical neurons were seeded into 96-well microtiter plates. After various treatments, the medium was removed and the cells were incubated with MTT (0.25 mg/ml) for 3 h at 37 °C. The formazan crystals in the cells were solubilized with dimethylsulfoxide (DMSO). The level of MTT formazan was determined by measuring its absorbance at 490 nm with SPECTRA (shell) Reader (TECAN, Grödig, Austria).

2.5. Nitrite assay

Accumulation of nitrite (NO_2^-), an indicator of NO synthase activity, in culture supernatant fluids was measured by the Griess reaction (Barger and Harmon, 1997). Cells were plated into 96-well microtiter plates for various treatments. Fifty microliter culture supernatant fluids were mixed with 50 μl Griess reagent (part I: 1% sulfanilamide; part II: 0.1% naphthylethylene diamide dihydrochloride and 2% phosphoric acid) at room temperature. Fifteen minutes later, the absorbance was determined at 540 nm using the SPECTRA (shell) Reader.