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Research Paper

Complement receptor 3 mediates NADPH oxidase activation and dopaminergic neurodegeneration through a Src-Erk-dependent pathway

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ABSTRACT

Microglial NADPH oxidase (**Nox2**) plays a key role in chronic neuroinflammation and related dopaminergic neurodegeneration in Parkinson's disease (PD). However, the mechanisms behind **Nox2** activation remain unclear. Here, we revealed the critical role of complement receptor 3 (CR3), a microglia-specific pattern recognition receptor, in **Nox2** activation and subsequent dopaminergic neurodegeneration by using paraquat and maneb-induced PD model. Suppression or genetic deletion of CR3 impeded paraquat and maneb-induced activation of microglial **Nox2**, which was associated with attenuation of dopaminergic neurodegeneration. Mechanistic inquiry revealed that blocking CR3 reduced paraquat and maneb-induced membrane translocation of **Nox2** cytosolic subunit $p47^{phox}$, an essential step for **Nox2** activation. Src and Erk (extracellular regulated protein kinases) were subsequently recognized as the downstream signals of CR3. Moreover, inhibition of Src or Erk impaired **Nox2** activation in response to paraquat and maneb co-exposure. Finally, we found that CR3-deficient mice were more resistant to paraquat and maneb-induced **Nox2** activation and nigral dopaminergic neurodegeneration as well as motor dysfunction than the wild type controls. Taken together, our results showed that CR3 regulated **Nox2** activation and dopaminergic neurodegeneration through a Src-Erk-dependent pathway in a two pesticide-induced PD model, providing novel insights into the immune pathogenesis of PD.

1. Introduction

Parkinson's disease (PD) is the most common neurodegenerative movement disorder and affects more than 1.7% population over 65 years [1]. The pathological hallmark of PD is the progressive dopaminergic neurodegeneration in the substantia nigra (SN) coupled with inclusions known as Lewy bodies and Lewy neuritis [2]. Dopamine replacement intervention is still the gold standard therapy for PD, which provides temporary symptomatic relief but fails to stop disease progression. The therapeutic strategies aimed at arresting dopaminergic neurodegeneration are lacking due to the obscure of the mechanisms of PD. Therefore, elucidating the potential mechanisms of dopaminergic neurodegeneration and developing related therapeutic interventions are urgently needed.

Neuroinflammation mediated by glia cells, especially microglia, is a common feature shared by multiple neurodegenerative disorders including PD [3]. Activation of glial cells including microglia and astroglia and accumulation of proinflammatory factors are observed in the area of SN and striatum in PD patients and animal models [4,5]. Experimental animals treated with inflammogen lipopolysaccharide (LPS)

Abbreviations: Aβ, β-amyloid; CNS, central nervous system; CR3, complement receptor 3; DAMPs, damage associated molecular patterns; DEPs, diesel exhaust particles; DHE, dihydroethidium; Erk, extracellular regulated protein kinases; HMGB1, high-mobility group box 1; Iba-1, ionized calcium binding adaptor molecule 1; iC3b, complement C3 fragment; CAM-1, intercellular adhesion molecule-1; LPS, lipopolysaccharide; MPP^+, 1-methyl-4-phenyl-pyridium iodide; Nox2, NADPH oxidase; oxLDL, oxidized low-density lipoprotein; PAMPs, pathogen associated molecular patterns; PD, Parkinson's disease; PRRs, pattern recognition receptors (PRRs); SFKs, Src family kinases; SN, subsantia nigra; SOD, superoxide dismutase; TH, tyrosine hydroxylase

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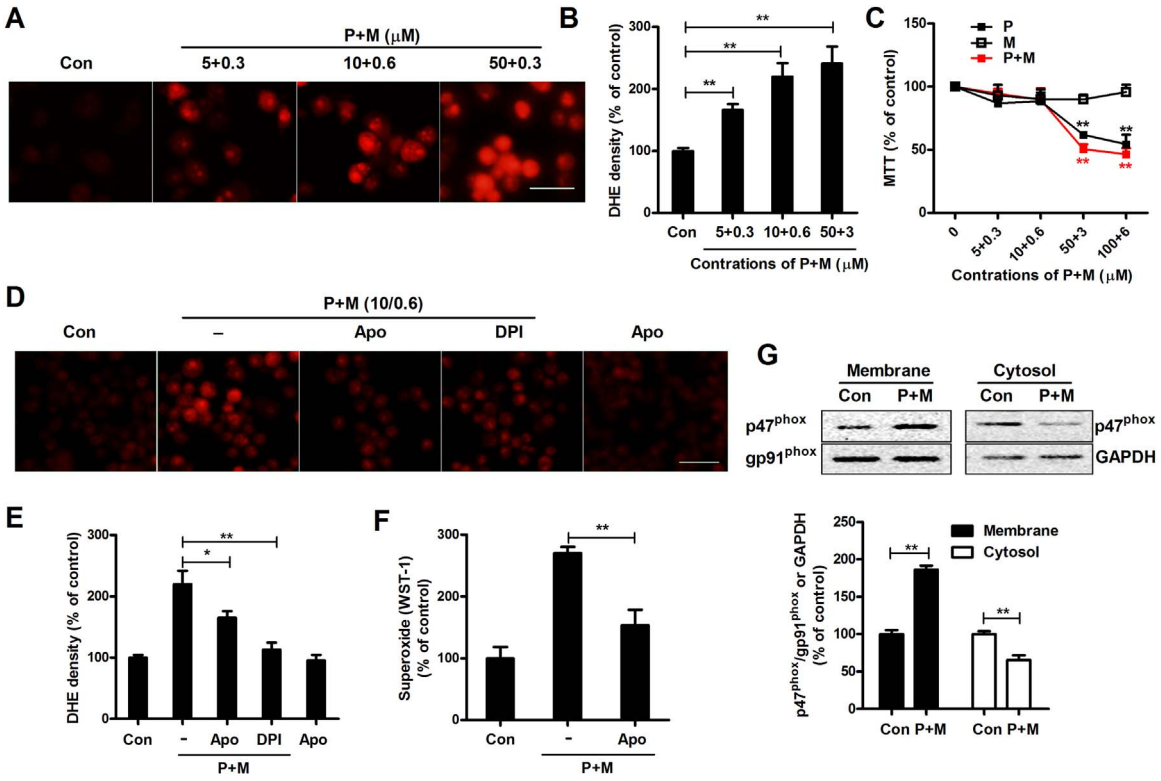

Fig. 1. Paraquat and maneb co-exposure activates NOX2 in microglia. (A) The production of intracellular superoxide was assessed by DHE in BV2 microglial cells treated with different concentrations of P + M. The representative images of DHE oxidation were shown. (B) The density of red fluorescence of DHE oxidation was quantified. (C) Cell viability of BV2 microglial cells treated with different concentrations of P + M was detected using MTT assay. (D) BV2 microglial cells were pre-treated with apocynin or DPI, two widely used NOX2 inhibitors, and then P + M-induced production of intracellular superoxide was measured using DHE. (F) The density of red fluorescence of DHE oxidation was quantified. (F) P + M-induced production of extracellular superoxide was measured as described in "Materials and Methods". (G) The membrane translocation of NOX2 cytosolic subunit, $p47^{phox}$ was detected after 15 mins of P + M stimulation by using Western blot and the density of blots was quantified. Gp91phox and GAPDH were used as internal membrane and cytosolic control, respectively. Results were expressed as a percentage of controls from three experiments performed in duplicate. $^{*}p < 0.05$, $^{**}p < 0.01$. Bar = 30 μm.

display progressive dopaminergic neurodegeneration and L-dopa-reversible motor impairments [6–9]. Humans exposed to LPS also develop parkinsonian syndromes [10], supporting an etiologic role of neuroinflammation in PD. Although the exact mechanisms for neuroinflammation in PD remain unclear, recent studies revealed an essential role of NADPH oxidase (**Nox2**) in the initiation and maintenance of neuroinflammation. **Nox2** is a superoxide-producing enzyme and is highly expressed in microglia [11]. We found that pharmacological inhibition or genetic deletion of **Nox2** markedly reduced microglia-mediated neuroinflammation as well as dopaminergic neurodegeneration in multiple in vivo and in vitro PD models [8,9,12–14]. In addition, **Nox2**-derived H_2O_2 was further identified as a key mediator for the regulatory effects of microglia on astroglial activation in experimental models of PD [15]. Given the high level of **Nox2** in the SN of PD patients compared with control subjects [16], elucidating the mechanisms of **Nox2** activation is particularly important, which may help us to better understand the immune pathogenesis of PD and thus, develop novel therapeutic strategies.

Pattern recognition receptors (PRRs) play important roles in innate immune responses by recognizing and binding to pathogen associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs) [17]. Activation of PRRs induces the release of inflammatory cytokines that help remove pathogens or restore tissue homeostasis. However, chronic activation of these receptors can cause inflammatory disease [18]. Previous studies indicated that scavenger receptors (SRs) and complement receptor 3 (CR3, also called $\alpha_M\beta_2$ or CD11b/CD18), two members of PRR family [19,20], are involved in regulating Nox2 activation. Genetic deletion or antibody neutralization of CD36, one of the extensively studied SRs, significantly decreased **Nox2**-generated superoxide induced by oxidized low-density lipoprotein (oxLDL) [21,22] and β-amyloid (Aβ), the main component of senile plaques in Alzheimer's disease (AD) [23–25]. By contrast, CR3 was essential for LPS [26], high-mobility group box 1 (HMGB1) [27] and diesel exhaust particles [28]-induced **Nox2** activation. However, whether SRs or CR3 is involved in **Nox2** activation in PD remains unknown. In this study, by using a two pesticide (paraquat/maneb)-induced PD model, we investigated the role of SRs and CR3 in **Nox2** activation and related dopaminergic neurodegeneration.

2. Materials and methods

2.1. BV2 microglial cells

The mouse microglia BV2 cell line was maintained as described previously [29]. Briefly, BV2 microglial cells were maintained at 37 °C in DMEM supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin in a humidified incubator with 5% CO_2 and 95% air. The cells were split or harvested every 3–5 days.

2.2. Primary cell cultures

Mesencephalic neuron-glia culture was prepared from the ventral mesencephalon of embryonic day 14 ± 0.5 SD rats according to previously published protocol [30]. The culture was maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air in minimum essential medium (MEM) containing 10% heat-inactivated fetal bovine serum (FBS), 10% heat-inactivated horse serum (HHS), 1 g/L glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μM nonessential amino acids, 50 U/ml penicillin, and 50 μg/ml streptomycin. Seven days after initial seeding, immunocytochemical analysis indicated that neuron-glia