NADPH oxidases promote apoptosis by activating ZNRF1 ubiquitin ligase in neurons treated with an exogenously applied oxidant

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ABSTRACT

Reactive oxygen species (ROS) play an important role in causing neuronal death in a number of neurological disorders. We recently reported that ROS serve as a signal to activate neuronal apoptosis and axonal degeneration by activating ZNRF1 (zinc- and RING-finger 1), a ubiquitin ligase that targets AKT for proteasomal degradation in neurons. In the present study, we showed that the NADPH oxidase family of molecules is required for ZNRF1 activation by epidermal growth factor receptor (EGFR)-dependent phosphorylation in response to axonal injury. We herein demonstrate that NADPH oxidases promote apoptosis by activating ZNRF1, even in neurons treated with an exogenously applied oxidant. These results suggest an important role for NADPH oxidase in the initiation/promotion of neuronal degeneration by increasing ROS in close proximity to protein machineries, including those for ZNRF1 and EGFR, thereby promoting neuronal degeneration.

ARTICLE HISTORY

Received 30 December 2015
Revised 12 January 2016
Accepted 13 January 2016

KEYWORDS
6-hydroxydopamine; apoptosis; caspase; neurodegenerative diseases; oxidative stress; Parkinson’s disease; phosphorylation; superoxide; ubiquitin-proteasome system; ZNRF1

Reactive oxygen species (ROS) are a major inducer of neuronal death, and have been associated with a number of human neurological disorders such as Parkinson’s disease, Alzheimer’s disease, Huntington’s disease, and amyotrophic lateral sclerosis. An important cellular source of ROS is the NADPH oxidase family of molecules, the only known enzyme family to produce ROS. Previous studies reported that a genetic deficiency in or the pharmacological inhibition of NADPH oxidase activity prevents neuronal apoptosis. These findings suggest that NADPH oxidases contribute to neuronal apoptosis.

We recently reported that ROS serve as a signal to activate 2 pathways that induce neuronal degeneration; neuronal apoptosis and axonal degeneration, by activating ZNRF1, a ubiquitin ligase that targets AKT in neurons. We found that, in response to increases in ROS, the epidermal growth factor receptor (EGFR)-dependent phosphorylation of ZNRF1 at the 103rd tyrosine residue activated the ubiquitin ligase activity of ZNRF1 in order to target AKT for proteasomal degradation. In a recent study, we employed a brain ischemia model (middle cerebral artery occlusion) and oxidant-induced Parkinson’s disease model as models of ROS-induced neuronal cell death in vivo. While the presence/production of excess ROS due to the significant changes in energy metabolism caused by ischemia is easily estimated in the brain ischemia model, previous studies have shown that the inhibition of NADPH oxidases significantly reduces neuronal damage, suggesting that NADPH oxidase-dependent ROS production beneath the neuronal membrane induced by ROS produced elsewhere plays a significant role in causing neuronal death in this model. This finding prompted us to examine the contribution of NADPH oxidase activity in the oxidant-induced neuronal apoptosis model employed in our recent study.

Five genes of the catalytic subunit of NADPH oxidases, NOX2-4 and DUOX 1 and 2 have been identified in rodents. In order to assess the contribution of NADPH oxidase activity to the induction of neuronal apoptosis in 6OHDA-treated neurons, we compared the degree of inhibition of ZNRF1 phosphorylation and neuronal apoptosis in 6OHDA-treated cultured cortical neurons expressing siRNA for the catalytic subunit of NADPH oxidases expressed in cortical neurons. We found that ZNRF1 phosphorylation was significantly inhibited by the down-regulation of NOX2-4 and DUOX2, but not by that of DUOX1. Decreased levels of neuronal apoptosis were also consistently observed in neurons expressing siRNA for NOX2-4 and DUOX2 (Fig. 1). These results suggest that the inhibition of NADPH oxidase activity and resultant reduction in ZNRF1 activation down-regulates oxidative stress-induced neuronal apoptosis.

We recently showed that ROS generated by NADPH oxidases serve as an intracellular signaling mediator after...
axonal injury in order to promote Wallerian degeneration by inducing EGFR-dependent ZNRF1 phosphorylation. These findings combined with the present results suggest that the NADPH oxidase family of molecules play a more significant role in initiating neuronal degeneration than previously expected. NADPH oxidases accelerate apoptosis even when cell death is induced by an exogenously applied oxidant, suggesting that ROS generated by NADPH oxidases, which are closely located to the protein machineries that promote apoptosis, including those of ZNRF1 and EGFR, are more relevant for initiating neuronal degeneration than ROS generated elsewhere. NADPH oxidase expression protects primary cultured cortical neurons from 6OHDA-induced apoptosis. 6OHDA-induced neuronal apoptosis is suppressed when the catalytic subunit of NADPH oxidase NOX2, 3, 4 or DUOX1, 2 is down-regulated in cultured primary cortical neurons. Cultured cortical neurons were transfected with siRNA for the indicated NADPH oxidase, and then treated with 25 μM 6OHDA and maintained for 24 h. Control siRNA-transfected neurons served as a negative control. (a, b) Representative photomicrographs for cleaved caspase 3 immunostaining are shown in (A). The nucleus and cell body were counterstained with DAPI and βIII-tubulin, respectively. The arrowhead indicates one of the apoptotic nuclei. (B) The ratios of the cleaved caspase 3-positive cell number to total number of βIII-tubulin-positive cells for each condition are shown (mean ± SEM, 5 independent experiments). The asterisks indicate a significant difference (One-way ANOVA with Tukey's post hoc test, *P < 0.05, **P < 0.01) from the control (open bar). (C–E) An immunoblot analysis was also performed for caspase 3, cleaved caspase 3, ZNRF1 pY103, and ZNRF1. Representative immunoblots (C), quantified expression levels for ZNRF1 pY103 normalized to ZNRF1 relative to the level in 6OHDA-treated controls (D), and quantified expression levels for cleaved caspase 3 p20 normalized to actin relative to the level in 6OHDA-treated controls (D) are shown (mean ± SEM, 5 independent experiments). The asterisks indicate a significant difference (One-way ANOVA with Tukey’s post hoc test, *P < 0.05, **P < 0.01) from the control (open bar). β-actin served as a loading control.
oxidases may also play a role in enhancing ROS levels in order to affect the protein machineries promoting apoptosis. Interestingly, we found that downregulation of NOX3, 4, and DUOX2 are similarly effective in inhibiting 6OHDA-induced apoptosis, but not as effective as downregulation of ZNRF1-AKT-GSK3B signaling that we previously reported. These results may suggest that more than 2 functionally redundant NADPH family molecules are located in the close proximity of the protein complex for promoting apoptosis. Elucidating the mechanisms underlying the activation of NADPH oxidases in neurons in more detail will provide an insight into the initiation mechanism of neurodegeneration.

Methods

Cerebral hemispheres were removed from embryonic day 14 C56BL/6J mice and dissociated by papain, and equal numbers of cells were seeded at a density of 2 × 10^5 cells/well on 24-well plates coated with poly-L-lysine (Sigma, St. Louis, MO) and laminin (Sigma, St. Louis, MO) in culture media. Cultures were maintained in Neuro-medium (Miltenyi Biotec) containing 2% Neuro-Brew-21 and 1 mM glutamine from the third day in vitro. Regarding the transfection of siRNA, control or targeting siRNAs for the gene of interest were transfected into cultured cortical neurons using DharmaFECT1 transfection reagent according to the manufacturer’s protocol (Thermo scientific). The siRNAs used in the present study were purchased from Qiagen (negative control siRNA, Cat. No. 1033076; DUOX1 targeting siRNAs, Cat. No. SI00984795 and SI00984802; DUOX2 targeting siRNAs, Cat. No. SI04393543 and SI04393550) or Bioneer (NOX2 targeting siRNAs, Cat. No. 1344848; NOX3 targeting siRNAs, Cat. No. 1392770 and 1392772; NOX4 targeting siRNAs, Cat. No. 1392780 and 1392781). Cells were treated with 25 μM 6OHDA (Sigma, St. Louis, MO) for 24 hr. They were then washed, cultured in Neuro-medium containing 2% Neuro-Brew-21 and 1 mM glutamine for 24 h, and used in immunoblotting or immunostaining experiments. The antibodies used were as follows: anti-ZNRF1 antiserum; antiserum for phosphorylated ZNRF1 at Y103; rabbit anti-caspase 3 and anti-cleaved caspase 3 antibodies (9662, 9661, Cell Signaling Technology); rabbit polyclonal anti-βIII-tubulin antibody (Poly18020, BioLegend); anti-β-actin antibody (622101, BioLegend).

Abbreviations

EGFR epidermal growth factor receptor
NAC N-acetyl-L-cysteine
NADPH nicotinamide adenine dinucleotide phosphate, reduced form
ROS reactive oxygen species
ZNRF1 zinc- and RING-finger 1

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Funding

This work was supported in part by a Grant-in-Aid for Scientific Research on Innovative Areas (“Brain Environment” Kakenhi 24111559 and 2611731) from the Ministry of Education, Culture, Sports, Science and Technology (S. W.); a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (S. W., Kakenhi 24500398); Intramural Research Grant for Neurological and Psychiatric Disorders of NCNP (T.A.); grants from Takeda Science Foundation (S. W., and T. A.), Suzuken Memorial Foundation (S. W.), and Pfizer academic contributions (T.A.).

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