Novel insights into the mechanism of reactive oxygen species-mediated neurodegeneration

Shuji Wakatsuki*, Toshiyuki Araki*

Abstract
Neurite degeneration, a major component of many neurodegenerative diseases, such as Parkinson’s disease, Alzheimer’s disease, and amyotrophic lateral sclerosis, is not part of the typical apoptosis signaling mechanism, but rather it appears that a self-destructive process is in action. Oxidative stress is a well-known inducer of neurodegenerative pathways: neuronal cell death and neurite degeneration. Although oxidative stress exerts cytotoxic effects leading to neuronal loss, the pathogenic mechanisms and precise signaling pathways by which oxidative stress causes neurite degeneration have remained entirely unknown. We previously reported that reactive oxygen species generated by NADPH oxidases induce activation of the E3 ubiquitin ligase ZNRF1 in neurons, which promotes neurite degeneration. In this process, the phosphorylation of an NADPH oxidase subunit p47-phox at the 345th serine residue serves as an important checkpoint to initiate the ZNRF1-dependent neurite degeneration. Evidence provides new insights into the mechanism of reactive oxygen species-mediated neurodegeneration. In this review, we focus specifically on reactive oxygen species-induced neurite degeneration by highlighting a phosphorylation-dependent regulation of the molecular interaction between ZNRF1 and the NADPH oxidase complex.

Key Words: neurite degeneration; oxidative stress; phosphorylation; reactive oxygen species; ubiquitin ligase

Introduction
Neurons, the main component of our brain, have long processes called axons and dendrites. By communicating with other cells through these neurites, they form a neural network that is the basis of all brain function. In many neurologic disorders, including Parkinson’s disease, Alzheimer’s disease, and amyotrophic lateral sclerosis, neurites are gradually lost and neurons die causing the brain and spinal cord to malfunction (Gan et al., 2018; Soto and Pritzkow, 2018). A study has shown that cell death often requires activation of an intracellular reaction that actively kills cells (Moujalled et al., 2021). On the other hand, it has been known that neurite degeneration which precedes neuronal cell death is controlled by a mechanism other than cell death, but the details remain unknown (DiAntonio, 2019; Coleman and Høke, 2020).

Neurite degeneration observed in the area of neurites distal to the site of physical injury is known as Wallerian degeneration, which is a prototypical neurite degeneration (Waller, 1851). It has been demonstrated that Wallerian degeneration is not a passive phenomenon caused by the disruption of transport from the cell body due to damaged neurites, resulting in depletion of nutrients and materials; but rather an active one that requires enzymatic reactions within the neurites (Coleman and Høke, 2020; Krauss et al., 2020). For example, in neurons lacking the NAD-degrading enzyme sterile-α and armadillo-motif-containing protein 1 (SARM1), Wallerian degradation is markedly delayed in a variety of animal models, including mammals and insects (Figley and DiAntonio, 2020; Krauss et al., 2020). Thus, it has become clear that Wallerian degeneration is robustly regulated by self-destructive reactions, whereas it is still unknown how physical damage to neurites translates into destructive reactions.

The human brain consumes about 20% of the body’s energy (Watts et al., 2018). Oxygen metabolism produces reactive oxygen species (ROS) as a byproduct. When environmental stressors exacerbate ROS generation, detoxification mechanisms fail to remove excess ROS, ROS serves as oxidative stress and damages cells (Shields et al., 2021). Neurons have high-energetic activities that pose significant challenges in detoxifying ROS, particularly in highly specialized cell compartments such as axons and dendrites. Oxidative stress has long been associated with neurologic disorders. For example, mutations in known Parkinson’s disease-related genes such as PARK1, PARK2, SNCA, and LRRK2 are thought to impair mitochondrial function, resulting in increased ROS levels and vulnerability to oxidative stress (Dias et al., 2013; Kolodkin et al., 2020). However, the pathogenic mechanisms and precise signaling pathways by which oxidative stress causes neurite degeneration are unknown.

In this review, we focus specifically on ROS-induced neurite degeneration, highlighting a phosphorylation-dependent regulation of molecular interaction between the E3 ubiquitin ligase zinc and ring finger 1 (ZNRF1) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Wakatsuki et al., 2015, 2022; Figure 1). We then discuss the putative target of the ROS-induced neurite degeneration as a therapeutic strategy for treating neurologic diseases.

Search Strategy and Selection Criteria
The references cited in this review have been obtained from the following databases for studies regarding the roles of ROS in neurodegeneration: PubMed, Google Scholar, and Science Direct. No restriction on publication dates was applied for search strategy.

Neuritic Self-Destructive Pathways
During development, excess or unnecessary axons, dendrites, and synapses are removed to establish a mature neural network. This process of selective removal of such structures, called neurite pruning, occurs without the death of the neuron that owns them (Riccomagno and Kolodkin, 2015). Genetic and biochemical studies in several models provide evidence that caspase activity is required for this developmental pruning (Unsain and Barker, 2015; Dehkordi et al., 2022; Figure 2). The involvement of death receptor 6 has also been shown in mammalian neurite pruning (Nikolaev et al., 2009).

In a series of genetic screens in flies and mice, mitogen activated protein kinase (MAPK) and its upstream kinase, dual leucine zipper kinase (DLK), are identified to be required for axonal degeneration (Miller et al., 2009). Mutant mice lacking DLK show a significant delay in degeneration of the distal axon of the severed sciatic nerve compared with wild-type animals. DLK acts through another kinase c-Jun N-terminus kinase (JNK). Inhibition of this pathway delays axonal fragmentation induced by a microtubule-destabilizing drug vincristine. These results suggest that the DLK/JNK pathway functions in a variety of injury-induced axonal self-destruction. In addition to the DLK/JNK pathway, several kinases that promote axonal degeneration have recently been identified (Yang et al., 2015; Figure 2).
High levels of oxidative stress are commonly observed in the brains of patients with neurodegenerative conditions (Zuo et al., 2015). Many diseases (Ryan and Pimplikar, 2005; Cole et al., 2007; Hou et al., 2009; Williamson et al., 2011). Our results showing ZNRF1-dependent AKT suppression of any of the axonal degeneration pathways previously described. These results suggest that mtDNA damage induces axonal degeneration through a response different from the one by nuclear DNA damage and that ROS may play a key role in the progression of degeneration (Geden et al., 2021). However, the details of how ROS generated in axons promotes axonal degeneration remain unclear. We previously reported that ROS generated by NADPH oxidases induces activation of the E3 ubiquitin ligase ZNRF1 in neurons, which promotes neurite degeneration. We first summarize the mechanism of ZNRF1-dependent neurite degeneration.

**ZNRF1: A Key Molecule in the Regulation of Neurite Degeneration**

We have previously developed an “in vitro Wallerian degeneration model”, in which radially extended neurites from primary cultured murine dorsal root ganglia neurons are injured to analyze the progression of injury-induced degeneration (Wakatsuki et al., 2011). Not only is this model simple and reproducible, but the morphological features of neurites observed during degeneration are very similar to those caused by toxic stimuli or in neurodegenerative diseases. Using this model, we analyzed the intracellular signaling mechanisms that regulate neurite degeneration in a variety of pathophysiological situations and found that the E3 ubiquitin ligase ZNRF1 promotes Wallerian degeneration by targeting AKT, which is degraded by NADPH oxidases inducing activation of the E3 ubiquitin ligase ZNRF1 in neurons, thereby activating cytoskeletal integrity is lost, which promotes neurite degeneration. Thus, ZNRF1 is a key molecule in the regulation of Wallerian degeneration.

**Activation of ZNRF1 by Oxidative Stress**

Phosphorylated CRMP2 is often observed in the neurons of animal models and patients with brain ischemia, as well as in other neurodegenerative diseases (Ryan and Pimplikar, 2005; Cole et al., 2007; Hou et al., 2009; Williamson et al., 2011). Our results showing ZNRF1-dependent AKT suppression of any of the axonal degeneration pathways previously described. These results suggest that mtDNA damage induces axonal degeneration through a response different from the one by nuclear DNA damage and that ROS may play a key role in the progression of degeneration (Geden et al., 2021). However, the details of how ROS generated in axons promotes axonal degeneration remain unclear. We previously reported that ROS generated by NADPH oxidases induces activation of the E3 ubiquitin ligase ZNRF1 in neurons, which promotes neurite degeneration. We first summarize the mechanism of ZNRF1-dependent neurite degeneration.

**ZNRF1: A Key Molecule in the Regulation of Neurite Degeneration**

We have previously developed an “in vitro Wallerian degeneration model”, in which radially extended neurites from primary cultured murine dorsal root ganglia neurons are injured to analyze the progression of injury-induced degeneration (Wakatsuki et al., 2011). Not only is this model simple and reproducible, but the morphological features of neurites observed during degeneration are very similar to those caused by toxic stimuli or in neurodegenerative diseases. Using this model, we analyzed the intracellular signaling mechanisms that regulate neurite degeneration in a variety of pathophysiological situations and found that the E3 ubiquitin ligase ZNRF1 promotes Wallerian degeneration by targeting AKT, which is degraded by NADPH oxidases inducing activation of the E3 ubiquitin ligase ZNRF1 in neurons, thereby activating cytoskeletal integrity is lost, which promotes neurite degeneration. Thus, ZNRF1 is a key molecule in the regulation of Wallerian degeneration.
Phosphorylation of p47 at S345: An Initial Checkpoint for Reactive Oxygen Species-Induced Neurite Degeneration

Does the transient interaction of p47 pS345 with ZNRF1 contribute to the induction of ROS generation and the activation of ZNRF1? To answer this, we examined whether overexpression of p47 and its mutants in cultured dorsal root ganglia neurons might affect ROS generation and neurite integrity. Overexpression of p47 WT did not affect ROS generation and neurite integrity. Overexpression of a constitutively active form of p47 (p47 S303, 304, 328D, or p47 35D) (Roepstorff et al., 2008; De Virgiliis et al., 2020) weakly increased ROS levels (the level was much weaker than that seen in injured neure 303 but did not induce neurite degeneration). Interestingly, overexpression of a dominant negative form of p47 (p47 S303, 304, and 328A, or p47 35A) (Roepstorff et al., 2008) decreased ROS generation and suppressed neurite degeneration. Conversely, overexpression of p47 S345A significantly suppressed ZNRF1 phosphorylation by EGFR and AKT ubiquitination, thereby protecting neurites from injury-induced degeneration. Detailed examination with pull-down experiments revealed that in injured neurites, p47 pS345A can associate with NOX2, but not with ZNRF1; whereas p47 S35A can bind to ZNRF1, but not with NOX2. Thus, phosphorylation of S35 is considered critical for p47 to bind and activate ZNRF1, whereas phosphorylation of S303, 304, and 328 is considered necessary for its association with NOX2 to activate NADPH oxidase. What about the association with EGFR? In injured neurites, EGFR can bind p47 S345A, but not p47 35A. These results suggested that p47 S345A can associate with the NADPH oxidase complex and bind to EGFR, whereas p47 35A cannot associate with and activate ZNRF1 (Figure 2). Thus, p47 S345A could behave like a “dominant-negative” that inhibits ZNRF1 activation. Our findings imply that phosphorylation at S345 may serve as an essential checkpoint to initiate ROS-mediated neurite degeneration (Wakatsuki et al., 2022).

Conclusion and Future Directions

There are two phosphorylation-dependent regulations in p47 activation: one through protein kinase C phosphorylation at S303, 304, 328 in ROS generation, and the other through tumor necrosis factor-induced phosphorylation at S345. The MAPK cascade controls tumor necrosis factor-induced phosphorylation of S345, whereas S345 phosphorylation by p38 MAPK controls tumor necrosis factor-induced phosphorylation of S345. Thus, S345 phosphorylation is regulated by different pathways. Future studies should consider that pharmacological or genetic interventions that target each step of ZNRF1 activation, i.e., phosphorylation by EGFR and/or its interaction with p47 pS345, may lead to improvement of disease symptoms and suppression of neurite degeneration.

Dang et al. (2006) reported that in neutrophils, proinflammatory cytokines such as granulocyte-macrophage colony-stimulating factor and tumor necrosis factor induce phosphorylation of p47 at S345 and regulate oxidative bursts in the “priming” process. Extracellular signal-regulated kinases (ERKs) are the protein kinases involved in granulocyte-macrophage colony-stimulating factor-induced phosphorylation of S345, while p38 MAPK controls tumor necrosis factor-induced phosphorylation of the same site. Neutrophils engulf invading foreign microorganisms, generate ROS, and degrade to release various enzymes. This process also controls the immune response against microbial invasion, and the ability to generate ROS is maximized through prior exposure to microbes or endogenous substances (Vogt et al., 2019). Thus, S345 is a point of convergence used by different MAPK activities to induce priming of ROS generation. The molecular mechanism to activate p38 MAPK by nerve injury is still unknown. Detailed analysis of how a variety of degenerating stimuli could induce S345 phosphorylation may lead to a potential answer to this question.

Yang et al. (2015) showed that the mitogen-activated protein kinase kinase 4 (MEK4), which plays an important role in the extravascular proinflammatory cytokine signaling pathway, increased oxidative stress in neurites initiated within a few hours of injury and lasts for more than one day (Wakatsuki et al., 2011; Araki and Wakatsuki, 2012). MEK4 activates p38 MAPK and kinase-related MAPK (p38β and p38γ) and stimulates cytokine expression. MEK4 activates p38 MAPK and kinase-related MAPK (p38β and p38γ) and stimulates cytokine expression. This process is the front line of defense against microbial invasion, and the ability to generate ROS is maximized through prior exposure to microbes or endogenous substances (Vogt et al., 2019). Thus, S345 is a point of convergence used by different MAPK activities to induce priming of ROS generation. The molecular mechanism to activate p38 MAPK by nerve injury is still unknown. Detailed analysis of how a variety of degenerating stimuli could induce S345 phosphorylation may lead to a potential answer to this question. [298x633]
genes after injury and promote regeneration of injured axons. Loss of function of phosphate and tensin homolog deleted from chromosome 10 (PTEN) is known to promote axonal regeneration in the mammalian central nervous system. ROS generated by axonal NOX2 oxidizes PTEN in an injury-dependent manner, which activates the phosphatidylinositol-3 kinase (PI3K) pathway. This indicates that there might be a positive feedback loop between the NOX complex and phosphatidylinositol-3 kinase, and continuous activation of NOX2 in the injured neurite may allow the regeneration program to be maintained over time. Thus, the molecular processes under ROS-mediated control in neuronal cells, especially in neurite degeneration and/or regeneration, are diverse and plenty of unresolved issues remain. It is also worthwhile to discuss that mitochondria have crucial roles in neurite degeneration and/or regeneration. The exciting fields of ROS-mediated signaling and neuropathic pathology will continue to advance, revealing more remarkable and unexpected regulatory targets.

Author contributions: SW drafted the manuscript, conducted literature search and prepared figures. TA provided constructive suggestions and edited the manuscript. Both authors approved the final version of the manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

Open access statement: This is an open access journal, and articles are distributed under the terms of the Creative Commons AttributionNon-Commercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new licenses are issued under the identical terms.

References

Araki T, Sasaki Y, Milbrandt J (2004) Increased nuclear NAD biosynthesis and Sirt1 activation prevent axonal degeneration. Science 305:1010-1013.

Araki T, Wakatsuki S (2016) Regulation of neuronal axonal degeneration by NDRG1 ubiquitin ligase. Neurosci Res 139:21-25.

Bedard K, Krause KH (2007) The NOX family of ROS-generating NADPH oxidases: pathway, physiology and pathology. Physiol Rev 87:245-313.

Broughton BR, Reuters DC, Sobey CG (2009) Apoptotic mechanisms after cerebral ischemia. Stroke; a journal of cerebral circulation 40:e331-339.

Cole AR, Noble W, van Aalten L, Plattner F, Meimaridou R, Hogan D, Taylor M, LaFrancois Broughton BR, Reuters DC, Sobey CG (2009) Apoptotic mechanisms after cerebral ischemia. Stroke; a journal of cerebral circulation 40:e331-339.

Krauss R, Bosanac T, Devraj R, Engber T, Hughes RO (2020) Axons matter: the promise of treating neurodegenerative disorders by targeting SARM1-mediated axonal degeneration. Trends Pharmacol Sci 31:281-293.

Lassègue B, Griendling KK (2010) NADPH oxidases: functions and pathologies in the vasculature. Arterioscler Thromb Vasc Biol 30:653-661.

Lennicke C, Cocheren HM (2021) Redox metabolism: ROS as specific molecular regulators of cell signaling and function. Mol Cell 81:3691-3707.

Miller BR, Press C, Daniels RW, Sasaki Y, Milbrandt J, DiAntonio A (2009) A dual leucine kinase-dependent axon self-destruction program promotes Wallerian degeneration. Nat Neurosci 12:387-389.

Moualled J, Strasser A, Liddell JR (2021) Molecular mechanisms of cell death in neurodegenerative diseases. Cell Death Differ 28:2029-2044.

Munoz-Requero R, Rasmussen I, Savada M, Cudre-Mauroux C, Salmon P, Bokobza L, de Ursus A, Vilhards F (2008) Stimulus-dependent regulation of the phagocyte NADPH oxidase by a VAV1, Rac1, and Pak1 signaling axis. J Biol Chem 283:7983-7993.

Ross CA, Tabrizi SJ (2011) Huntington’s disease: from molecular pathogenesis to clinical treatment. Lancet Neurol 10:83-98.

Ryan KA, Pimplikar SW (2005) Association of GSK-3 and phosphorylation of CRMP2 in transgenic mice expressing APP intracellular domain. J Cell Biol 171:327-335.

Sambashivan S, Freeman MR (2021) SARM1 signaling mechanisms in the injured nervous system. Curr Opin Neurol 24:69-77.

Shields H, Traa A, Van Raamsdonk JM (2021) Beneficial and detrimental effects of reactive oxygen species on lifespan: a comprehensive review of comparative and experimental studies. Front Cell Dev Biol 9:628157.

Soto C, Pritzков (2018) Protein misfolding, aggregation, and conformational strains in neurodegenerative diseases. Nat Neurosci 21:1332-1340.

Summers DW, Frey E, Waller LJ, Milbrandt J, DiAntonio A (2020) DLK activation synergizes with mitochondrial dysfunction to downregulate axon survival factors and promote SARM1-dependent axon degeneration. Mol Neurobiol 57:1146-1158.

Ueda H, Fujita R (2004) Cell death mode switch from necrosis to apoptosis in brain. Biol Pharm Bull 27:950-955.

Unsin N, Barker PA (2015) New views on the misconstrued: executioner caspases and their diverse non-apoptotic roles. Neuron 88:461-474.

Vogl KG, Summers C, Condiffle AM (2019) The clinical consequences of neutrophil priming. Curr Opin Hematol 26:22-27.

Wakatsuki S, Saitoh F, Araki T (2011) NLR1 promotes Wallerian degeneration by degrading AKT to induce GSK3β-dependent CRMP2 phosphorylation. Nat Cell Biol 13:1415-1425.

Wakatsuki S, Furutani A, Oshimura M, Araki T (2015) Oxidative stress-dependent phosphorylation activates NLR1 to induce neuronal/axonal degeneration. J Cell Biol 211:881-896.

Wakatsuki S, Araki T (2016) NADPH oxidases promote apoptosis by activating NLR1 ubiquitin ligase in neurons treated with an exogenously applied oxidant. Immun Inflamm Biol 9:1143575.

Wakatsuki S, Takahashi Y, Shibata M, Araki T (2022) Selective phosphorylation of serine 345 on p47-phox serves as a priming signal of ROS-mediated axonal degeneration. Exp Neurol 352:114024.

Waller A (1851) Experiments on the section of the glosso-pharyngeal and hypoglossal nerves of the frog, and observations of the alterations produced thereby in the structure of their primitive fibres. Edinb Med Surg J 76:369-376.

Watts ME, Pocock R, Claudianos C (2018) Brain energy and oxygen metabolism: emerging role in normal function and disease. Front Mol Neurosci 11:216.

Williamson R, van Aalten L, Mann DM, Platt B, Plattner F, Bedford L, Mayer I, Howlett D, Usandiz A, Sutherland C, Cole AR (2011) CRMP2 hyperphosphorylation is characteristic of Alzheimer’s disease and not a feature common to other neurodegenerative diseases. J Alzheimers Dis 27:615-625.

Yang J, Yu Z, Renier N, Simon DI, Uruy K, Park DS, Greer PA, Tourcier N, Davis RJ, Tisseur-Lavigne M (2015) Pathological axonal death through a MAPK cascade that triggers a localised actin cytoskeleton deficit. Cell 161:161-176.

Yeung AWK, Tsvetkov NT, Georgieva MG, Ognyanov IV, Kordos K, Jóźwik A, Kühl T, Perry G, Petraitis MC, Mazon E, Atanasov AG (2021) Reactive oxygen species and their impact on neurodegenerative diseases: literature landscape analysis. Antioxid Redox Signal 34:402-420.

Zuo Z, Lavigne M (2015) Pathological axonal death through a MAPK cascade that triggers a localised actin cytoskeleton deficit. Cell 161:161-176.

C-Editors: Zhao M, Liu W, Qi Y, T-Editor: Jia Y