Oxidative Stress and Mitochondrial Dysfunction in Neurovascular Injury after Stroke

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Effective stroke therapies require recanalization of occluded cerebral blood vessels. However, reperfusion can cause neurovascular injury, leading to cerebral edema, brain hemorrhage, and neuronal death by apoptosis/necrosis. These complications of reperfusion, which result from excess production of reactive oxygen species (ROS), significantly limit the benefits of stroke therapies. We recently found two novel targets for neurovascular protection after ischemia/reperfusion: the signal transducer and activator of transcription 3 (STAT3) pathway and NADPH oxidase (NOX). Manganese-superoxide dismutase (SOD2) plays a critical role in neurovascular injury as a first-line defense against ROS produced in mitochondria, and STAT3 is a major transcriptional factor of the SOD2 gene. During reperfusion, activation of STAT3 and its recruitment into the SOD2 gene are blocked, resulting in increased oxidative stress and neuronal apoptosis. Pharmacological activation of STAT3 with interleukin-6 induces SOD2 expression, which limits ischemic neuronal death. In contrast, NOX is a pro-oxidant multi-subunit enzyme. After ischemia/reperfusion, NOX in the neurovascular unit forms an activated complex to generate ROS, which induce oxidative injury in the neurovascular unit. Pharmacological and genetic inhibition of NOX is neuroprotective against cerebral ischemia/reperfusion. Superoxide dismutase and NOX regulate ROS production in the ischemic brain, interacting with each other in a Yin and Yang relationship. Our neurovascular protective strategies targeting these two enzymes may expand the therapeutic window of the currently approved therapies.

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Introduction

Reperfusion strategies have proven to be the most effective therapies for ischemic stroke treatment; however, recanalization can also induce reperfusion injury, which causes progression of vasogenic edema, hemorrhagic transformation, and an increase in infarction volume. During ischemia, cerebral blood flow is significantly reduced in the brain regions that are supplied with oxygen by the occluded vessel. Reoxygenation during reperfusion provides oxygen to sustain neuronal viability. Furthermore, mitochondria and pro-oxidant enzymes also utilize replenished oxygen as a substrate, and overproduce reactive oxygen species (ROS). Under normal physiological conditions, antioxidant enzymes, including superoxide dismutase (SOD), scavenge ROS, and other...
small molecular antioxidants are involved in detoxification of ROS. During reperfusion, however, these endogenous antioxidant defenses are likely to be perturbed as a result of overproduction of ROS, inactivation of detoxification systems, consumption of antioxidants, and a failure to adequately replenish antioxidants in ischemic brain tissue. Excessive ROS are crucial for the pathophysiology of reperfusion injury.

Although considerable efforts have been expended for many years to develop neuroprotective reagents, most such reagents have failed to show any beneficial activity in patients with stroke. Therefore, much attention has recently shifted from neurons alone to the neurovascular unit as a more realistic therapeutic target in ischemic injury. The neurovascular unit, which consists of microvessels (endothelial cells, basal lamina matrix, astrocyte end-feet, and pericytes), astrocytes, neurons, axons, and supporting cells (microglia and oligodendroglia), maintains the homeostatic milieu necessary for normal brain activities. Protection of this unit seems essential to reduce brain damage and neurological deficits after ischemia.

We recently found two novel targets for protection of the neurovascular unit after ischemia/reperfusion: the signal transducer and activator of transcription 3 (STAT3)–manganese–SOD (SOD2) pathway and NADPH oxidase (NOX). In this review, we discuss these pathways as a therapeutic strategy for ischemic stroke.

Cellular and Molecular Events Following Cerebral Ischemia

Several oxygen radicals are generated after ischemia/reperfusion, including superoxide anions (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radicals (·OH). A major source of O$_2^-$ are mitochondria, where approximately 2% to 5% of the molecular oxygen consumed during normal physiological respiration is converted into O$_2^-$ and H$_2$O$_2$. Pro-oxidant enzymes such as xanthine oxidase and NOX also generate O$_2^-$, O$_2^-$ reacts with nitric oxide (NO), which is generated from NO synthase, and produce peroxynitrite, a strong oxidative radical. Highly reactive ·OH are produced by H$_2$O$_2$ through the Fenton reaction and the Haber–Weiss reaction.

Although cells possess a ROS detoxification system, such as SOD, glutathione peroxidase, and catalase, ischemia/reperfusion induces overproduction of ROS that is beyond the capacity of the ROS detoxification system. These excessive ROS cause macromolecular damage, such as lipid peroxidation, protein oxidation, protein nitrosylation/nitration, and nucleic acid damage in ischemic tissues, leading to cell death. Moreover, overproduced ROS initiate the intrinsic apoptosis pathway and activate numerous signaling pathways, including the phosphatidylinositol-3 kinases–Akt pathway, mitogen-activated protein kinase pathway, nuclear factor-κB pathway, and the p53 pathway.

1. SOD2 Activity and STAT3 Signaling

1. The Antioxidant Enzyme SOD

SODs are specific antioxidant enzymes that detoxify O$_2^-$ to H$_2$O$_2$ and molecular oxygen. SOD has three isoforms: copper–zinc SOD (SOD1), SOD2, and extracellular SOD (SOD3). Numerous studies using knockout (KO) and transgenic (Tg) animals have proven that SOD1 and SOD2 are actively involved in neuroprotection after cerebral ischemia (see Nizuma et al. for a review). Although only a few studies have used SOD3 KO or Tg animals in cerebral ischemia research, they have shown that SOD3 also has neuroprotective roles.

2. Neurovascular Injury and SOD2

SOD2 is located in the mitochondrial matrix and plays a critical role as a first-line defense against mitochondrial ROS. We investigated neurovascular injury after transient focal ischemia using SOD2 KO mice and SOD2 overexpressing Tg mice and found that the level of SOD2 expression greatly affected neurovascular injury after ischemia. Following focal ischemia/reperfusion, SOD2 KO mice demonstrated delayed (72 hr) blood–brain barrier breakdown associated with activation of matrix metalloproteinase and high brain hemorrhage rates, whereas a decrease in vascular endothelial cell apoptosis and hemorrhage rates was observed in SOD2 overexpressors. Expression of the SOD2 gene is highly regulated and is inducible by numerous stimuli in various cells and tissue, in contrast to the constitutively expressed SOD1 gene. Thus, induction of the SOD2 gene can be a novel strategy for neurovascular protection after ischemia/reperfusion. Our recent study identified STAT3 as a good candidate for this strategy.
3. STAT3–SOD2 Pathway

STAT3 is a transcription factor as well as an intracellular signal transducer. Tyrosine phosphorylation of STAT3 at Y705 is necessary for STAT3 activation. Phosphorylated STAT3 forms dimers, translocates to the nucleus, binds to the specific promoters of target genes, and induces gene expression (Fig. 1).

The SOD2 gene is one of the target genes of STAT3. Following an extensive promoter analysis, we found that there are multiple putative binding motifs of STAT3 in the mouse SOD2 promoter and that STAT3 indeed binds to some of the SOD2 promoter. Under physiological conditions, phosphorylated STAT3 was recruited into the SOD2 promoter and upregulated transcription of the SOD2 gene. However, its recruitment into the SOD2 promoter was completely blocked and transcription of the SOD2 gene was significantly reduced after cerebral ischemia. Pharmacological (AG490) and genetic (small interfering RNA) inhibition of STAT3 aggravated oxidative and neuronal injury after ischemia by reducing SOD2 expression.

Many studies have reported that cytokines, growth factors, and hormones activate STAT3, which then acts as a neuroprotectant against brain injury. Among them, interleukin-6 is a well-known cytokine that activates STAT3 via activation of Janus kinase. We recently found that treatment with interleukin-6 increased phosphorylated STAT3 and SOD2 expression, and reduced infarction size after transient focal ischemia in mice (Jung et al., unpublished data). Therefore, cytokines (including growth factors and hormones) could be candidates for molecular therapeutic reagents via the STAT3–SOD2 pathway. Fig. 1 summarizes the STAT3–SOD2 pathway after ischemia/reperfusion.

2. NOX

1. General Characteristics of NOX

Another target is a pro-oxidant enzyme, NOX, which is a multi-subunit enzyme that transfers an electron from NADPH to molecular oxygen to generate $O_2^{•−}$. Originally discovered in phagocytic cells, this protein was first shown to provide host defense against bacteria via a rapid respiratory burst of $O_2^{•−}$. However, a vast number of recent studies have redefined NOX in a wide array of cells and tissues in the context of a diverse set of functions: mediation of normal intracellular signaling, modulation of inflammatory response, and regulation of cell growth and death.

Seven NOX isoforms have been identified: NOX1 to 5 and Dual Oxidase 1 and 2. Among them, NOX1, 2 and 4 have been implicated in cerebral ischemia. In this
paper, we focus on NOX2, which is the prototype of NOX and is the most extensively studied.

2. NOX2 in the Central Nervous System

The transmembrane NOX2 protein, also known as gp91phox (phox: phagocyte oxidase), produces ROS on activation with interaction with another transmembrane protein, p22phox, as well as multiple cytosolic subunits (p47phox, p67phox, and p40phox), and the Rho GTP-binding protein Rac1. Upon stimulation, activation of Rac1 and phosphorylation of p47phox by protein kinase c initiates migration of the cytosolic subunits to the membrane, whereby a functional complex is formed and generates O$_2^-$ (Fig. 2)\(^2\).

NOX2 and its subunits are expressed in microglia, vascular systems, and neurons in the central nervous system (CNS). Microglia are macrophage-like cells and consequently produce large amount of ROS through NOX\(^2\)\(^5\)\(^6\). In the vascular system of the CNS, NOX2 is expressed in endothelial cells and smooth muscle cells, although NOX4 is the most abundant isoform in these cells\(^3\)\(^4\)\(^9\). NOX expression in neurons was considered unlikely for a long time; however, recent studies have demonstrated that NOX2 is expressed and has functions in neurons of the hippocampus, cortex, amygdala, striatum, and thalamus\(^4\)\(^3\).

Under physiological conditions, NOX2 in the CNS is required for processes such as neuronal signaling, memory, and central cardiovascular homeostasis; however, overactivation of NOX2 is implicated in neurodegenerative disease including stroke and Alzheimer’s disease\(^1\)\(^6\).

3. Role of NOX2 in Cerebral Ischemia

Recent studies have revealed that neuronal NOX2 has an important role in O$_2^-$ production after cerebral ischemia/reperfusion. NOX2 was the primary source of O$_2^-$ induced by N-methyl-D-aspartate (NMDA) receptor activation in primary cortical neurons\(^5\). It is well known that cerebral ischemia elicits a massive increase in extracellular glutamate, which activates the NMDA receptor and triggers neuronal cell death\(^6\). Therefore, neuronal NOX might be one of the major sources of O$_2^-$, even after ischemia/reperfusion. Indeed, in neurons treated with oxygen-glucose deprivation (OGD), which mimics ischemia/reperfusion stress, a primary source of O$_2^-$ during reoxygenation was NOX2, while O$_2^-$ were produced by mitochondria and xanthine oxidase during hypoxia\(^7\). In addition to neurons, the roles of NOX2 in endothelial cells have also been studied using cerebral vascular endothelial cell cultures. Treatment with apocynin, a NOX2 inhibitor, reduced expression of p47phox and cell death in endothelial cells subjected to OGD\(^8\).

The in vivo roles of NOX2 in cerebral ischemia have been studied using animal models. In ischemic brains, NOX2 subunits were upregulated\(^7\)\(^2\)\(^3\)\(^4\), and the cytosolic subunits translocated to the membrane\(^1\)\(^5\)\(^2\)\(^3\)\(^4\). NOX enzymatic activity was also shown to increase after ische-
vascular delayed after kinase-mediated cerebral rovascular al,2i) and inhibition significantly increased NOX2 activity via Rac1 activation.

In focal ischemia models, NOX2 inhibition using pharmacological (apocynin) or genetic (gp91phox KO) manipulation alleviated oxidative damage in the ischemic brain and reduced infarction volume54(47,51). Moreover, Kahles et al.21 reported direct evidence of the role of NOX2 in neurovascular injury. NOX2 promoted oxidant formation in cerebral vascular endothelial cells, which then led to Rho kinase-mediated endothelial cell contraction and BBB disruption after ischemia. Genetic deletion of gp91phox or apocynin alleviated post-ischemic BBB disruption.

Several studies have presented the roles of NOX2 after global cerebral ischemia. Oxidative damage and delayed neuronal cell death in the hippocampal CA1 subregion were reduced by genetic ablation of p47phox or treatment with apocynin65(52). Recently, we reported that NOX2 was activated in neurons, endothelial cells, and reactive microglia in the striatum after relatively prolonged global ischemia. Both pharmacological (apocynin) and genetic (gp91phox KO) inhibition of NOX2 attenuated oxidative injury, microglial activation, and death of medium spiny neurons in the striatum after ischemia54.

These findings, obtained from focal and global ischemia models, demonstrate that NOX2 is activated in the neurovascular units, and that ROS produced via NOX2 have extremely important roles in neurovascular injury after stroke.

Interestingly, NOX2 expression after ischemia was affected by endogenous levels of SOD. In SOD1 Tg mice, which have a 3.1-fold overexpression of SOD1 in brain tissue and showed reduction of brain infarction volume and edema23, the upregulation of gp91phox after ischemia was less pronounced compared with wild-type mice. In addition, SOD1 KO mice showed more significant NOX upregulation, which generated more endogenous O2·-, thereby amplifying the progress of brain injury after ischemia7. These results indicate that NOX2 expression is regulated by the redox state of the brain tissue and that there is elaborate crosstalk between NOX2 and SOD.

Conclusion

In this review, we present two novel treatment strategies against neurovascular injury after ischemic stroke: induction of SOD2 via the STAT3 pathway, and inhibition of NOX. SOD and NOX regulate ROS production in the ischemic brain, interacting with each other in a Yin and Yang relationship. Further studies of these pro- and antioxidant enzymes may lead to the development of a new stroke treatment in the future.

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