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# Reperfusion and Neurovascular Dysfunction in Stroke: from Basic Mechanisms to Potential Strategies for Neuroprotection

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**Abstract** 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, which result from excess production of reactive oxygen species in mitochondria, significantly limit the benefits of stroke therapies. We have developed a focal stroke model using mice deficient in mitochondrial manganese-superoxide dismutase (SOD2−/+) to investigate neurovascular endothelial damage that occurs during reperfusion. Following focal stroke and reperfusion, SOD2−/+ mice had delayed blood-brain barrier breakdown, associated with activation of matrix metalloproteinase and high brain hemorrhage rates, whereas a decrease in apoptosis and hemorrhage was observed in SOD2 overexpressors. Thus, induction and activation of SOD2 is a novel strategy for neurovascular protection after ischemia/reperfusion. Our recent study identified the signal transducer and activator of transcription 3 (STAT3) as a transcription factor of the mouse SOD2 gene. During reperfusion, activation of STAT3 and its recruitment

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into the SOD2 gene were blocked, resulting in increased oxidative stress and neuronal apoptosis. In contrast, pharmacological activation of STAT3 induced SOD2 expression, which limits ischemic neuronal death. Our studies point to antioxidant-based neurovascular protective strategies as potential treatments to expand the therapeutic window of currently approved therapies.

**Keywords** Cerebral ischemia · Oxidative stress · Reactive oxygen species · Mitochondria · Mn-SOD · STAT3 · NADPH oxidase · CK2 · Neuroprotective signaling

## Introduction

Nearly 80% of strokes are caused by occlusion of a cerebral artery by a thrombus. Thus, effective stroke therapies require recanalization of occluded cerebral blood vessels. Reperfusion strategies have proven to be the most effective therapies for stroke treatment. However, reperfusion of ischemic brain tissue can have harmful consequences, including the breakdown of the blood-brain barrier, which can lead to cerebral edema and/or brain hemorrhage, as well as neurovascular injury and neuronal death. During cerebral ischemia, cerebral blood flow is reduced by occlusion of vessels in brain tissues that are supplied with oxygen [1]. Reperfusion after ischemia causes oxidative stress, which is a result of overproduction of reactive oxygen species (ROS) in mitochondria. This overproduction significantly limits the benefits of stroke therapies, and these ROS trigger many cellular and molecular events, including protein oxidation/nitrosylation/nitration, lipid peroxidation, and DNA damage, which can induce cell death following cerebral ischemia and reperfusion [2].

Manganese-containing superoxide dismutase (Mn-SOD or SOD2), a mitochondrial antioxidant enzyme for superoxide, is a primary cellular defense enzyme involved in protecting cells from oxidative stress [3]. Homozygous mutant mice that are deficient in SOD2 die within the first 10 days of life with dilated cardiomyopathy, accumulation of lipid in liver and skeletal muscle, and metabolic acidosis [4]. We have shown that superoxide radicals and infarction volumes increase after cerebral ischemia and reperfusion in Mn-SOD-deficient mice (SOD2−/+) [5]. SOD2 knockout (KO) mice are more susceptible to hemorrhage, but the incidence of post-ischemic hemorrhage is reduced in SOD2 transgenic (TG) mice, which have reduced vascular endothelial cell death [6]. Our recent study demonstrated that Mn-SOD is a direct target of signal transducer and activator of transcription 3 (STAT3) in ischemia reperfusion-induced neuronal cell death and that the loss of STAT3 activity reduced Mn-SOD expression after cerebral ischemia [7]. STAT3 is a transcription factor and an intracellular signal transducer that is activated by cytokines, growth factors, and receptor- or nonreceptor-tyrosine kinases [8, 9]. Tyrosine phosphorylation of STAT3 at Y705 is required for STAT3 activity. After phosphorylation at Y705, dimerization, and nuclear translocation, STAT3 binds to the promoters of target genes and finally induces gene expression [10]. In this review, we will discuss antioxidant-based neuroprotective strategies as potential treatments that may expand the molecular therapeutic window targeting STAT3 activity.

## Cellular and Molecular Events Following Cerebral Ischemia

In mitochondria of brain tissue, approximately 2–5% of electron flow produces superoxide anion radicals ($O_2^{\bullet-}$) and hydrogen peroxide ($H_2O_2$) [11]. These ROS are scavenged by SOD, glutathione peroxidase, and catalase. Other antioxidants, such as glutathione, ascorbic acid, and α-tocopherol, are also involved in the detoxification of free radicals [12].

SOD processes a dismutation reaction of $O_2^{\bullet-}$ and produces $H_2O_2$, which is then detoxified by catalase or glutathione peroxidase, and finally converted to water and oxygen ($O_2^{\bullet-}+O_2^{\bullet-}+2H^+ \rightarrow H_2O_2+O_2$) [1]. $H_2O_2$ breaks down easily if transition metal ions are present, producing hydroxyl radical ($^{\bullet}OH$) through the superoxide-driven Fenton reaction ($H_2O_2+Fe^{2+} \rightarrow HO+Fe^{3+}+{}^{\bullet}OH$) [13]. This hydroxyl radical is a very strong oxidizer and can attack various kinds of organic structures, such as phospholipids and nucleic acids [14]. Peroxynitrite is formed by the nonenzymatic reaction with nitric oxide and $O_2^{\bullet-}$ at a rate constantly close to diffusion [15]. It is also a potent oxidant/nitrating agent and can inhibit the function of SOD [15], as well as the mitochondrial respiratory chain [16], thus leading to increased $O_2^{\bullet-}$ and peroxynitrite formation [15]. Superoxide can lead to the inactivation of a variety of enzymes [13] and is involved in vascular dysfunction, cerebral ischemic injury [17–19], vasospasm after subarachnoid hemorrhage [20, 21], atherosclerosis [22], and meningitis [23].

In neurological disorders like cerebral ischemia and reperfusion, cerebral blood flow is reduced by occlusion of vessels, causing a reduction in the oxygen supply in the affected region of the brain. Reperfusion after ischemia causes oxidative stress in neurovascular units and affected brain regions. It also causes overproduction of ROS in mitochondria and these overproduced ROS consume antioxidants, inactivate the detoxification system, disturb the endogenous antioxidative defense system, and finally, could lead to a dramatic rise in intracellular ROS.

Numerous studies of cellular macromolecules have demonstrated that ROS are directly involved in oxidative damage such as lipid peroxidation, protein oxidation, protein nitrosylation/nitration, and nucleic acid damage in ischemic tissues, leading to cell death [3, 24]. Within the first several minutes of cerebral ischemia, reduced cerebral blood flow induces a series of biochemical events that cause metabolic dysfunction such as reduction in ATP formation, failure of the Na-K-ATPase pump, a decrease in tissue pH, membrane depolarization, $Ca^{2+}$ influx through activation of voltage-operated calcium channels, and excessive release of excitatory amino acids (glutamate) [25]. Activation of glutamate receptors leads to a further increase in intracellular calcium, activation of intracellular enzymes such as phospholipase, nitric oxide synthase, protease, endonuclease, and oxidase during cerebral ischemia within several hours [25]. Recent studies have demonstrated the production of superoxide radicals by *N*-methyl-D-aspartate (NMDA) receptor-mediated nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation [26]. These events amplify ROS production, mitochondrial dysfunction, proapoptotic protein activation, cytochrome c release, and caspase activation, and these cascades finally result in apoptotic cell death within several days/weeks after cerebral ischemia. Production of ROS after ischemia also causes DNA damage and this damage induces energy depletion of cells. Membrane damage caused by protease activation, protein malfolding by $Ca^{2+}$ influx, and DNA damage results in necrotic cell death after cerebral ischemia. Finally, within several days/weeks after cerebral ischemia, brain damage such as blood-brain barrier disruption and intracerebral hemorrhage occurs.

## Neuroprotective Strategies and Molecular Targets

### Targeting Mn-SOD Activity and STAT3 Signaling

Superoxide dismutases are specific antioxidant enzymes that detoxify $O_2^{\bullet-}$ and produce $H_2O_2$. Three SODs have been identified, copper/zinc SOD (SOD1), SOD2, and extracellular SOD (SOD3) [1]. SOD1 is a copper- and zinc-containing homodimer that is distributed exclusively in intracellular cytoplasmic spaces [27]. SOD2 is a manganese-containing enzyme that localizes to the mitochondria after encoding from nuclei [28]. SOD3, the most recently identified, is an extracellular protein that exists as a copper- and zinc-containing tetramer with the capacity to remove superoxide [29].

Brain injuries such as ischemia reperfusion and stroke increase $O_2^{\bullet-}$ and ROS in mitochondria [3]. The important protective role of SOD2 as a first-line defense against mitochondrial ROS production after cerebral ischemia is supported by studies using SOD2 mutant mice. SOD2 KO mice showed exacerbated infarct volume [30], increased cytochrome c release, and DNA fragmentation after permanent focal cerebral ischemia [31]. Several transgenic studies have also shown that SOD2 protects against oxidative stress-induced cellular apoptosis and injuries. SOD2 TG mice or cells that overexpress SOD2 showed reduced infarct volume after cerebral ischemia or reduced neural apoptosis against oxidative stress [32]. In cell culture models, SOD2 overexpression significantly reduced cell death mediated by the toxic effects [32–34]. Expression of human SOD2 in mitochondria protects TG mice from oxygen-induced lung injury [35]. Therefore, targeting SOD2 activity provides a potent neuroprotective advantage during brain damage such as ischemia reperfusion and stroke.

We have recently reported that targeting SOD2 expression by STAT3 activity is neuroprotective in mouse cerebral ischemia [7] (Fig. 1). STAT3 upregulates transcription of the SOD2 gene in the mouse brain, and expression of SOD2 is significantly reduced by ischemic reperfusion in a mouse model of middle cerebral artery occlusion (MCAO). Expression of the SOD2 gene is highly regulated and inducible by numerous stimuli in various cells and tissue [36–38]. The mRNA and protein levels of SOD2 were significantly decreased after reperfusion, and phosphorylation of STAT3 at Y705 was also rapidly decreased at early post-reperfusion periods after transient focal ischemia [7]. Phosphorylated STAT3 is usually recruited into the mouse SOD2 promoter and upregulates transcription of the mouse SOD2 gene in the normal brain. Following an extensive promoter analysis, we found that there are multiple putative binding motifs of STAT3 in the mouse SOD2 promoter and that STAT3 binds to some of the SOD2 promoter. However, its recruitment into the SOD2 promoter was completely blocked and transcription of the mouse SOD2 gene was significantly reduced [7]. This finding was confirmed by a pharmacological approach for STAT3 inhibition using AG490 and by a molecular approach for STAT3 knockdown using siRNA transfection in mouse brain tissue or mouse primary cortical neurons. SOD2 was revealed to be a gene that is a specific target of STAT3. The loss of STAT3 activity by ischemic reperfusion increases the generation of ROS and protein nitration, as well as neuronal cell death by reducing SOD2 expression. Also, more inhibition of STAT3 using AG490 under ischemia reperfusion enhances infarct volume [7] and this effect was reversed by treatment with interleukin-6 (IL-6) via recovery of SOD2 expression (Jung et al., unpublished data). IL-6 is a well-known cytokine that activates STAT3 in various cells and tissues and recovers the levels of SOD2 mRNA and protein, which were reduced by cerebral ischemia (Jung et al., unpublished data). This indicates that targeting SOD2 expression by

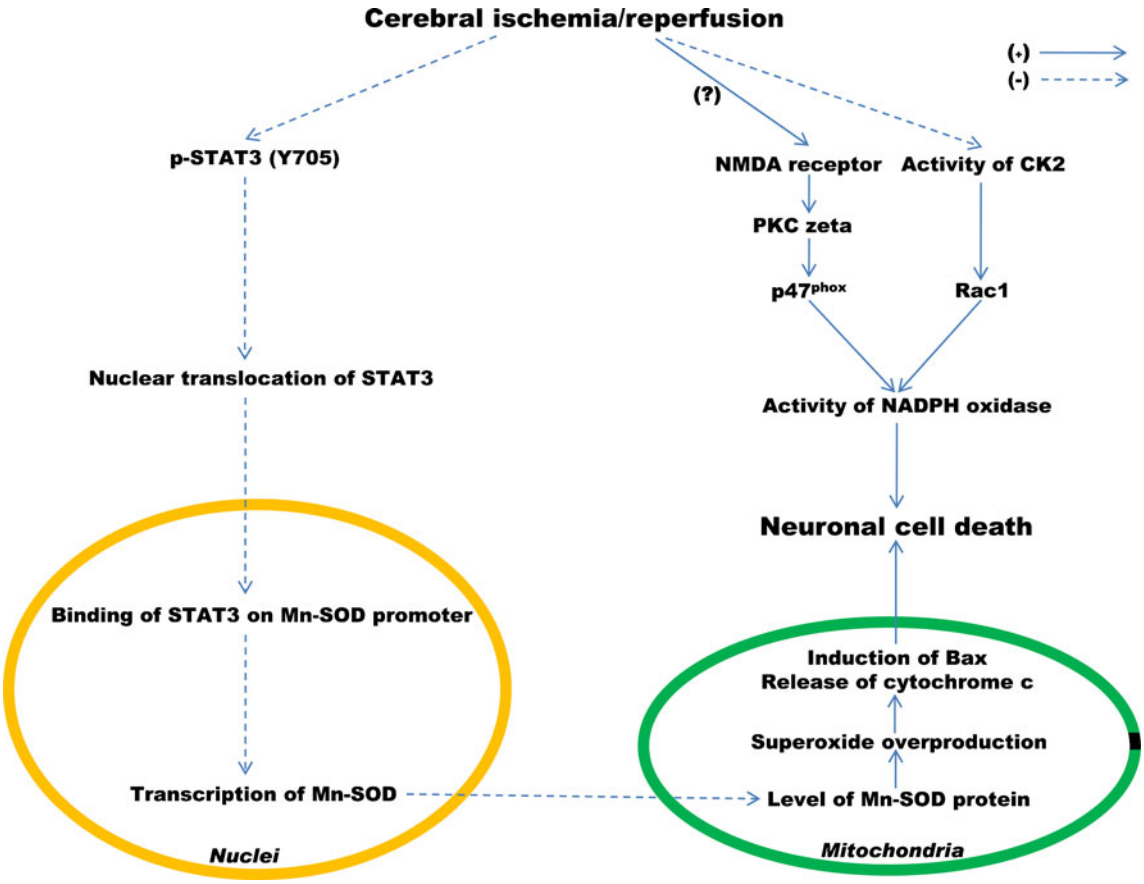


**Fig. 1** Induction of Mn-SOD (SOD2) expression by STAT3 is neuroprotective after cerebral ischemia and reperfusion. *PKC* protein kinase C

STAT3 activation using treatment with cytokines could be a therapeutic candidate for neuroprotection in cerebral ischemic brain injury.

SOD2 synthesis in eukaryotic cells is dramatically upregulated by inflammatory mediators, including lipopolysaccharide, tumor necrosis factor α, IL-1 and IL-6, and interferon–γ [36, 37, 39–42]. This upregulation in SOD2 mRNA in response to some cytokines may result from an increase in the rate of transcription of SOD2 [37–39, 41]. Many studies have reported that cytokines [43–54], growth factors [55–61], and hormones [62, 63] are neuroprotective and activate STAT3. They accomplish neuroprotection through STAT3 activation in response to multiple signaling pathways in the central nervous system following injury (Table 1). Six STATs have been identified [64], but among them, STAT3 is mainly characterized as neuroprotective against various brain injuries, including cerebral ischemia. Secretoneurine reduced infarct size in rats after MCAO via STAT3 activation and protected primary cortical cells against oxygen–glucose deprivation (OGD) via STAT3-induced antiapoptotic protein expression [65]. Estradiol reduced infarct size in rats after MCAO via induction of STAT3 phosphorylation [66]. Administration of an IL-6 receptor antibody increased apoptosis and enlarged infarct size in mice after MCAO via STAT3 dephosphorylation [67]. Therefore, cytokines (including growth factors and hormones) for neuroprotection against cerebral ischemia could be candidates for molecular therapeutic reagents via STAT3-upregulated mRNA synthesis of SOD2 (Fig. 2).

## Targeting NADPH Oxidase and Molecular Signaling

Despite the key role of SOD2 in the first-line defense against mitochondrial oxidative stress during cerebral ischemia and reperfusion, oxidative stress is also generated in cytoplasmic compartments and cytoplasm plays an important role in neuronal death. One such cytoplasmic enzyme is NADPH oxidase, a prooxidant enzyme that generates superoxide through an electron transfer from NADPH to molecular oxygen. NADPH oxidase was originally identified in neutrophils, but its expression and distribution have subsequently been found in many other cell types such as neurons, astrocytes, and microglia in the cortex, hippocampus, and cerebellum [68–71].

NADPH oxidase is a multicomponent enzyme composed of membrane-bound subunits (Nox2 and $p22^{phox}$) and

**Table 1** Neuroprotective factors that activate STAT3

| STAT3 activators | Effects of neuroprotection | References |
|---|---|---|
| Growth factors | | |
| EGF | Reduced infarct size after cerebral ischemia | [55] |
| | Induced neurogenesis after cerebral ischemia | [57] |
| IGF | Increased neuronal survival | [59] |
| | Increased neuritic length | [60] |
| NGF | Induced neuronal differentiation | [57] |
| | Increased STAT3-inducible GAP-43 gene | [58] |
| BDNF | Increased neurite outgrowth | [56] |
| | Reduced infarct size after permanent MCAO | [61] |
| Cytokines | | |
| IL-6 | Decreased infarct size after cerebral ischemia | [46, 51] |
| | Increased neuronal survival | [45] |
| | Promoted axon outgrowth, neuronal differentiation | [50] |
| LIF | Reduced infarct size after cerebral ischemia | [54] |
| CNTF | Protected ganglion cells | [52] |
| G-CSF | Increased STAT3-inducible Bcl-2 and Pim-1 in neurons | [43, 47] |
| | Increased STAT3-inducible cIAP2 in glia | [43] |
| EPO | Decreased ischemia-induced apoptosis | [44, 53] |
| | Promoted regeneration of CNS neurons | [48] |
| | Increased STAT3-inducible Bcl-2 | [49] |
| Hormone | | |
| Estradiol | Reduced neuronal death after cerebral ischemia | [62] |
| | Sustained smaller infarcts after MCAO | [63] |

*EGF* epidermal growth factor, *IGF* insulin-like growth factor, *NGF* nerve growth factor, *BDNF* brain-derived neurotrophic factor, *LIF* leukemia inhibitory factor, *CNTF* ciliary neurotrophic factor, *G-CSF* granulocyte-colony stimulating factor, *EPO* erythropoietin

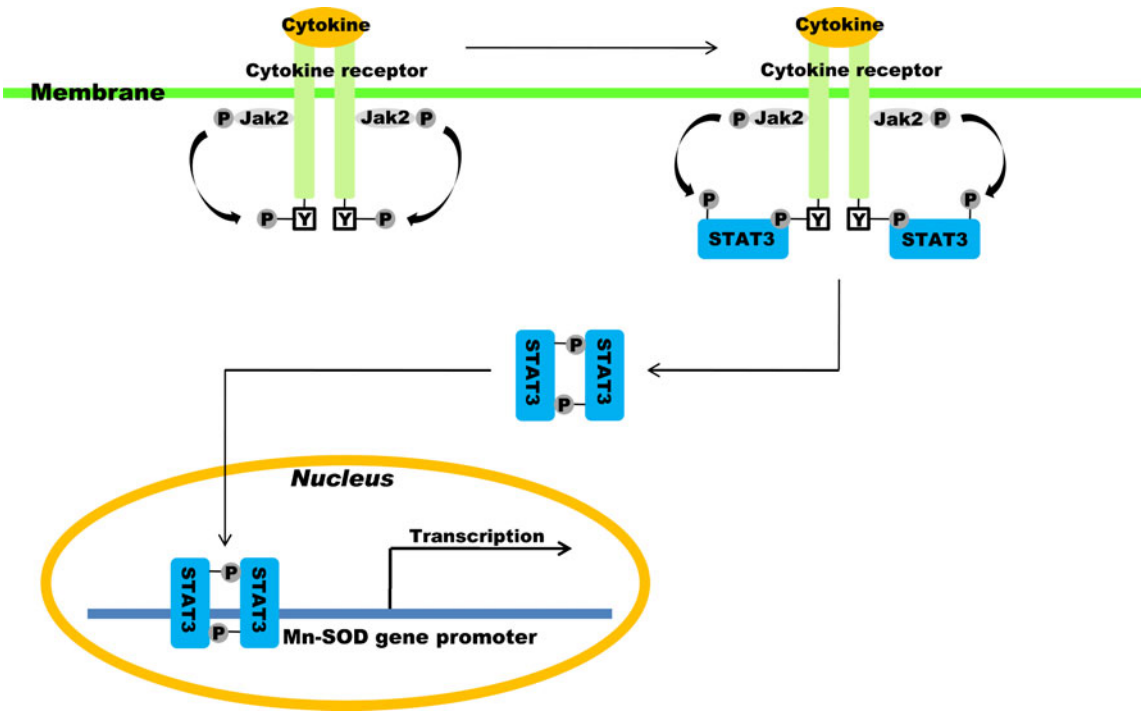


**Fig. 2** Neuroprotective strategy. Targeting Mn-SOD activity via STAT3 activation. *P* phosphorylation, *Jak2* Janus activating kinase 2, *Y* tyrosine residue

cytosolic subunits ($p47^{phox}$, $p67^{phox}$, and $p40^{phox}$) [68, 72]. It is activated through migration of cytosolic subunits ($p47^{phox}$, $p67^{phox}$, guanosine 5'-triphosphate-Rac1) from cytoplasm to the membrane forming the active NADPH oxidase complex [68]. Rac1 participates in the multicomponent assembly in active NADPH oxidase as a membrane-bound subunit [73] and is a key activator of Nox2 [74, 75]. Expression and activity of NADPH oxidase are regulated by various stresses such as ischemic injury [76, 77] and redox stress in amyotrophic lateral sclerosis [78, 79] and are upregulated in Alzheimer's disease [80–82]. Several studies have shown that NADPH oxidase plays a role in oxidative stress, contributing to ischemic brain injury. NADPH oxidase is the major source of NMDA-induced superoxide formation in neurons [26]. NMDA induces a rapid increase in neuronal superoxide production, followed by neuronal death. This increase was completely blocked by apocynin, a NADPH oxidase inhibitor. However, $p47^{phox-/-}$ neurons showed a much attenuated NMDA-induced superoxide production, as well as cell death relative to wild-type (WT) neurons [26]. Superoxide production and membrane translocation of $p47^{phox}$ were also blocked by inhibiting protein kinase C ζ, which activates the NADPH oxidase complex [26] (Fig. 1). In apocynin-treated mice and gp91 KO mice, infarction volume after MCAO was significantly less than in the WT mice [76]. This indicates that inhibition of NADPH oxidase is neuroprotective after ischemia reperfusion. Crosstalk between NADPH oxidase and SOD after cerebral ischemia has also been reported. The $gp91^{phox}$ protein significantly increased at 1 h of reperfusion, however, the amount of $gp91^{phox}$ in SOD1 TG mice was much less than in WT mice after focal ischemia. In SOD1 KO mice, $gp91^{phox}$ was significantly more upregulated than in WT mice after focal ischemia at 1 and 4 h of reperfusion [76]. Enhanced expression of $gp91^{phox}$ in SOD2−/+ mice after focal cerebral ischemia and reperfusion has also been examined [83]. Death of endothelial cells in SOD1 TG mice subjected to 6 h of OGD/24 h of reoxygenation was less than in SOD1 WT mice [84], and treatment with apocynin reduced expression of p47 and death in endothelial cells subjected to OGD/reoxygenation [83]. These reports suggest that downregulation of NADPH oxidase activity can be a molecular target for neuroprotection against cerebral ischemic injury.

We recently examined casein kinase 2 (CK2), a novel negative regulator of NADPH oxidase, after cerebral ischemia in mice [85] (Fig. 1). CK2 activity was rapidly reduced in mouse brains after cerebral ischemia, and this CK2 inhibition significantly increased NADPH oxidase activity via Rac1 activation. We also examined the molecular targeting mechanism of NADPH oxidase activation by CK2 activity under ischemia reperfusion. CK2 interacted directly with Rac1 in mouse brains and this interaction was significantly diminished after reperfusion in a mouse MCAO model [85]. CK2 inhibition after MCAO enhanced ischemia infarction and neuronal cell death via NADPH oxidase activation, but these effects were reversed by inhibition of NADPH oxidase activity using apocynin [85]. These results support the idea that targeting NADPH downregulation by enhancing CK2 activity after cerebral ischemia at an early post-reperfusion period can be an alternative to SOD2 as a molecular therapeutic target for neuroprotection against oxidative stress in brain injury.

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