Targeting oxidative stress for the treatment of ischemic stroke: Upstream and downstream therapeutic strategies

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Abstract:
Excessive oxygen and its chemical derivatives, namely reactive oxygen species (ROS), produce oxidative stress that has been known to lead to cell injury in ischemic stroke. ROS can damage macromolecules such as proteins and lipids and leads to cell autophagy, apoptosis, and necrosis to the cells. This review describes studies on the generation of ROS, its role in the pathogenesis of ischemic stroke, and recent development in therapeutic strategies in reducing oxidative stress after ischemic stroke.

Key words:
Ischemic stroke, mitochondria, oxidative stress, reactive oxygen species

Introduction

Reactive oxygen species (ROS) are a group of reactive oxygen-containing molecules that readily react with macromolecules resulting in irreversible functional alterations or even complete destruction. While ROS play crucial roles in human physiological processes, ROS overproduction is a noteworthy feature of ischemic stroke and ROS is an important mediator of ischemic damage. Significant progress has been made in understanding the mechanisms underlying ROS-induced brain damage after ischemic stroke. Accordingly, anti-ROS approaches have been extensively explored for the treatment of ischemic stroke, including both upstream and downstream strategies. The upstream strategies focus on attenuating the ROS production from different sources after ischemic stroke while the downstream strategies target neutralizing ROS and/or disabling the subsequent detrimental actions. Although the protective effects of antioxidants against ischemic stroke have been demonstrated in experimental ischemic stroke models in numerous studies, all antioxidant treatments have failed to provide therapeutic effects in clinical trials. Despite their detrimental effects, ROS play very important roles in normal physiological process and homeostasis, such as synaptic activity, vascular tone regulation, and inflammatory response. Further studies on the mechanism of ROS in ischemic damage should lead to more specific targeting or combination treatments that may reduce their detrimental effects without interfering their normal functions.

Functions of Reactive Oxygen Species under Normal Physiological Conditions

Under normal physiological conditions, ROS play important roles in many biological processes including cell signaling, gene transcription regulation, immune response, and apoptosis. ROS work as the second messengers during signal transduction of many growth factors, such as epidermal growth factor, platelet-derived growth factor, and NK1.

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How to cite this article: Li W, Yang S. Targeting oxidative stress for the treatment of ischemic stroke: Upstream and downstream therapeutic strategies. Brain Circ 2016;2:153-63.
Xanthine oxidase (XO) is also a source of ROS during ischemic stroke. XO is a molybdo-flavin enzyme that catalyzes the conversion of hypoxanthine to xanthine and xanthine to urate. This enzyme exists in two interconvertible forms: an NAD-dependent dehydrogenase (xanthine dehydrogenase) and oxygen-dependent superoxide production oxidase (XO). XO has higher affinity to O₂ than NAD+ and hydrogen peroxide is the major product of XO. Ischemia increased the activity of XO in rat brain. XO is an important source of superoxide anion radicals in blood after forebrain ischemia/reperfusion in rats and hydrogen peroxide derived from XO contributed brain edema induced by ischemia/reperfusion in gerbils.

Other intracellular enzymes that catalyze the production of ROS include cyclooxygenases (COXs), lipoxygenases (LOXs), and cytochrome P450 enzymes. These enzymes are involved in the metabolism of free arachidonic acid released from cell membrane phospholipids during ischemia. COX metabolism of arachidonic acid has been proposed as a major source of superoxide generation during reperfusion in ischemic piglet brain.

**Functions of Reactive Oxygen Species in Ischemic Stroke**

Under normal conditions, ROS production in the brain is balanced by the endogenous enzymatic and nonenzymatic antioxidative mechanisms. The enzymes include superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT). SOD catalyzes dismutation of superoxide to hydrogen peroxide, providing the first line against ROS damage. GPX and CAT further metabolize hydrogen peroxide to water and oxygen. Nonenzymatic endogenous antioxidative small molecules also play very important roles in defending against oxidative stress, especially in extracellular spaces where the enzymes are absent or in very low levels. Small-molecule antioxidants can be water-soluble or lipid-soluble, and these molecules include glutathione (GSH), Vitamins E and C (inhibits oxidation of membrane lipid), N-acetylcysteine (NAC), and melatonin. In humans, levels of most antioxidants (Vitamins A, E, and C) were reduced immediately after an acute ischemic stroke, probably due to the larger amount of ROS produced that cannot be balanced by endogenous antioxidants. In normal conditions, ROS play beneficial roles in regulating many important cellular processes, such as gene expression, cell proliferation and migration, and immune response. However, when ROS produced during ischemic stroke exceed the need for maintaining normal functions and cannot be balanced by endogenous antioxidants, they can cause excessive damage.

ROS can interact with amino acids in protein molecules and cause protein modification or degradation. It can also react with the side chains and the backbone of protein, which can lead to protein oxidation, peptide bond cleavage, and
This compound has been proposed to target the pathways from upstream ROS production to their downstream effects on macromolecules.

**Reactive oxygen species scavenger**

ROS scavenger, such as vitamins, NAC, and lipoic acid (LA), are the most commonly used antioxidants. Vitamin E is a potent, lipid-soluble antioxidant. It can interrupt the chain reaction of free radical production during lipid peroxidation by ROS. Vitamin E has been reported to be protective in rodent ischemic stroke models as shown by reduced lesion volume and lessened behavioral impairments. A phase III clinical trial published in 2006 reported that NXY-059 administered within 6 h after acute ischemic stroke reduced disability at 90 days. NXY-059 also improved motor function of monkeys after ischemic stroke. NXY-059 is (Disufenton sodium) a broad-spectrum radical scavenger that has been approved for the treatment of stroke in Asia since 2002. Edaravone (5-methyl-2-phenyl-4H-pyrazol-3-one) is a free radical scavenger that has been approved for the treatment of stroke in rats since 2004. Edaravone is lipophilic and can readily cross the BBB. Several clinical trials in Japan have shown that Edaravone treatment was beneficial for a subset of stroke patients. Decreased lesion size, attenuated MMP-9 activation, and reduced BBB damage after ischemia were reported in rodent stroke models treated with Edaravone. Edaravone also reduced recombinant tissue plasminogen activator (rtPA)-induced BBB damage in rodents, suggesting Edaravone as a promising candidate to expand the time window of rtPA treatment. Future clinical trials may expand the use of Edaravone for the treatment of ischemic stroke in other countries.

**Therapeutic Strategies to Reduce Oxidative Stress for Treatment of Ischemic Stroke**

In ischemic stroke, oxidative stress is created by the excessive ROS, whose effects cannot be balanced by endogenous antioxidants, resulting in wide-spread damages by oxidation of lipid acid, protein, and DNA, which lead to cell death. To counteract this oxidative stress, different strategies have been proposed.

Lipid peroxidation, which is the oxidative degradation of lipids, by ROS is more damaging than protein oxidant to cells during ischemic stroke. Lipid peroxidation by ROS leads to a self-propagation of free radical reaction. ROS attack lipids containing carbon-carbon double bonds, especially polyunsaturated fatty acids, producing lipid radicals. Lipid radical is not stable and can react with oxygen and form lipid peroxyl radical; lipid peroxyl radical can react with other lipid acids to generate another lipid radical and lipid peroxide. Two lipid radicals react to form end products of lipid peroxidation-reactive aldehydes, such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). MDA and HNE have been used as markers for lipid peroxidation. MDA can react with amino acids in proteins and other molecules to form its adducts such as malondialdehyde-acetyledehyde and advanced lipid peroxidation end-products, which can produce secondary deleterious effects by promoting intra- or inter-molecular protein or DNA crosslinking to cause protein modification and DNA damage/mutation.

4-HNE is a very reactive compound with three reactive groups: an aldehyde, a double bond, and a hydroxyl group. 4-HNE is a second messenger that can regulate several transcription factors such as nuclear factor erythroid 2-related factor 2, activating protein-1, NF-kB, and peroxisome-proliferator-activated receptors. 4-HNE also regulates major cell signaling pathways, such as MAPK and PI3K / AKT. Lower nontoxic concentrations of 4-HNE are beneficial to cells by promoting cell proliferation, differentiation, antioxidant defense, and anti-inflammation while high concentrations of 4-HNE induce cell apoptosis. Increased lipid peroxidation has been found in human stroke patients as well as rat cerebral ischemia models and has been proposed to play an important role in cell death by ischemic stroke.

ROS break DNA double strands, cause intra- and inter-strand crosslinks, protein-DNA crosslinks, DNA mutations, and DNA structural changes. 8-hydroxy-2-deoxyguanosine (8OHdG) is one of the most common products of oxidative damage of DNA and increased levels of 8OHdG suggested extensive DNA oxidation, which precedes DNA fragmentation, in ischemic stroke in rats.

**NXY-059** is (Disufenton sodium) a broad-spectrum nitroline-based free radical scavenger. This compound reduced lesion volume in rats after permanent MCAO. NXY-059 also improved motor function of monkeys after permanent MCAO. A Phase III clinical trial published in 2006 reported that NXY-059 administered within 6 h after acute ischemic stroke reduced disability at 90 days. However, a following larger clinical trial failed to support the efficacy of NXY-059 for acute ischemic stroke.

NAC is an antioxidant that has a free thiol group capable of reacting with ROS; it is a GSH precursor which can exert an indirect antioxidant effect. NAC has been reported to reduce lesion volume and improve neurological score in rat MCAO models. It also increased hippocampal neuron survival in a transient forebrain ischemia model in rats. GSH
monoethyl ester, which can be effectively transported into cells and converted to GSH, has been reported to be protective in a rat ischemic stroke model.\[163\]

LA can react with ROS and it also recycles Vitamin E and Vitamin C.\[144\] LA reduced mortality rate of rats after cerebral ischemia.\[145\] LA pretreatment reduced lesion volume in rat MCAO model when administered 30 min before the collision.\[145\] A recent study further indicated that infusion of LA through the jugular vein immediately after reperfusion reduced lesion volume and promoted functional recovery in rat MCAO model.\[146\]

Tirilazad (U-74006F) is a synthetic lipid-soluble nonglucocorticoid. It is an inhibitor of lipid peroxidation as well as an antioxidant and free radical scavenger.\[147\] Tirilazad reduced lesion volume and attenuated neurologic deficits in a rat permanent MCAO model.\[148\] Another study reported that tirilazad reduced lesion volume after transient but not permanent focal cerebral ischemia in rats.\[149\] A meta-analysis of the efficacy of tirilazad in experimental stroke concluded from 18 studies that tirilazad reduced infarct volume by 29.2% and improved neurobehavioral score by 48.1%.\[150\] A clinical trial (RANTTAS) with 660 patients found that 6 mg/kg per day for 3 days dose did not improve overall functional outcome.\[151\] A following clinical trial of higher dose (12–15 mg/kg per day) in acute ischemic stroke found that tirilazad treatment reduced mortality and increased functional recovery in both men and women.\[152\] However, a meta-analysis of 4 published and 2 unpublished clinical trials concluded that tirilazad increased death and disability in acute ischemic stroke patients, which precludes future trials of the drug.\[153\]

Citicoline is a natural compound that is an intermediate in the generation of phosphatidylcholine from choline.\[154\] Citicoline can stabilize cell membranes and reduce free fatty acid release caused by lipid peroxidation during ischemia.\[155\] Many studies have examined its protective effects in animal models of stroke. A meta-analysis of fourteen studies concluded that citicoline reduced lesion volume by 27.8%.\[156\] Several clinical trials of citicoline for the treatment of stroke have been conducted.\[157-159\] One of the trials reported that citicoline improved functional outcome and reduced neurologic deficit.\[157\] Although the other two studies concluded that citicoline was ineffective in improving functional outcomes,\[158,159\] a pooled analysis of data from four clinical trials found that citicoline was safe and promoted recovery after acute ischemic stroke.\[160\] In 2012, the International Citicoline Trial on Acute Stroke reported that citicoline was not efficacious for moderate-to-severe acute ischemic stroke.\[161\] GM1-ganglioside, which may also stabilize membranes, was tested in clinical trials; however, the results did not support improved outcome after treatment.\[162-164\]

Reactive oxygen species degradation

ROS can be degraded by SOD and CAT, which makes them candidates for stroke treatment. Intravenous administration of polyethylene glycol-conjugated SOD (PEG-SOD) and CAT (PEG-CAT) reduced infarct volume in rats.\[165\] PEG-SOD or recombinant human SOD alone also reduced ischemic damage in animals.\[166-167\] Synthetic combined superoxide dismutase/CAT mimetics EUK-134 and EUK-8 reduced infarct volume when administered 3 h after MCAO.\[141\] A SOD mimic, M40401, generated a protective effect in gerbil ischemic stroke models;\[168\] it also reduced infarct size and improved neurological score when administered either before or after MCAO in rat.\[169\]

Reducing reactive oxygen species generation in ischemic stroke

Failure of ROS scavengers in ischemic stroke clinical trials suggests that it may be very difficult to eliminate the detrimental effects of ROS when it is already generated. Attenuating excessive ROS production after onset of ischemic stroke might provide a more effective strategy for treatment of ischemic stroke. In animal stroke models, excessive ROS generation persisted through occlusion and there is even a second perk of ROS generation after reperfusion, suggesting that there could be a time window for treatment to reducing ROS generation after occurrence of occlusion. NOX inhibition has been proposed as a strategy to reduce oxidative stress in ischemic stroke by reducing ROS generation.\[170-173\] NOX inhibitor apocynin has been extensively studied for stroke treatment, and several studies have reported its protective effect against ischemic stroke.\[172-175\] NOX inhibitor diphenyleneiodonium (DPI) was protective in a rat MCAO model when administered with dimethyl sulfoxide;\[176\] however, DPI is not a specific NOX inhibitor.\[177\] A more specific NOX inhibitor, VAS2870, has been found to reduce stroke lesion volume and improve long-term neurological functions in mice.\[178\] However, recent study indicated that VAS2870 has significant off-target effects.\[178\] NOX inhibition is an important strategy to reduce ROS production in ischemic stroke; however, it is still not clear which NOX isoform and what cell types play a major role in NOX ROS production during ischemic stroke.\[45,170\] Further studies are warranted to examine the underlying mechanism and develop/test more specific NOX inhibitors, such as gp91ds-tat\[179\] and GKT136901,\[180\] for the treatment of ischemic stroke.

XO inhibitor allopurinol has shown some beneficial effects on inflammatory indices in ischemic stroke patients in clinical trial\[181\] although a following clinical trial was not able to find any beneficial effect in patients with subcortical stroke.\[182\] A recent clinical trial indicated that allopurinol was well tolerated and improved the 3-month functional status of acute ischemic stroke patients with high levels of serum uric acid.\[183\] Beneficial effects of allopurinol in ischemic stroke have been found in many studies using different animal stroke models.\[35,184-186\] As a drug that has been approved by the Food and Drug Administration and has been used in humans for many years, allopurinol is a very promising candidate for stroke treatment. However, allopurinol can also reduce XO and generate superoxide when inhibiting its activity.\[186\] Many other small molecule XO inhibitors have been developed, such as TEI-6720,\[190\] febuxostat,\[191\] Y-700,\[192\] and BOF-4277.\[193\] It may be interesting to test their effects in ischemic stroke considering their reported improved potency and/or efficacy compared to allopurinol.

COX-2 knockout in mice decreased infarct volume after MCAO\[194\] while COX-2 overexpression increase infarct volume.\[195\] COX-2 inhibitor NS-398 reduced infarct volume and behavioral deficits in mice after MCAO model.\[196,197\] 12/15-LOX knockout mice also exhibited smaller lesion volume after transient MCAO.\[198\]
12/15-LOX inhibitor LOXBlock-1 reduced infarct size in mouse MCAO model; it also reduced rtPA-induced hemorrhage in a distal MCAO clot stroke model.\cite{199} Baicalein, a natural product and specific inhibitor of 12/15-LOX, reduced lesion volume and behavioral deficits in rodent stroke models.\cite{198,200,201}

Mitochondrion is an important source of ROS. CoQ10 is a component of the mitochondrial electron transport chain. When administered, CoQ10 can accumulate in the mitochondria\cite{202} and has been found to be protective against ischemia in various animal models of stroke, which can be attributed to its role as a potent antioxidant and ROS scavenger in mitochondria.\cite{203-205} CoQ10 belongs to the mitochondria-targeted antioxidant (MTA) family.\cite{206} CoQ10 is also an endogenous antioxidant.\cite{207,208}

Therefore, CoQ10 has dual therapeutic benefits by enhancing electron transport chain efficiency and simultaneously acting as an ROS scavenger. However, recent clinical trial for Parkinson’s showed that CoQ10 did not slow disease progression.\cite{209}

Another MTA, MitoQ10, can accumulate in the mitochondria\cite{204} and has been reported to reduce mitochondrial oxidative damage. MitoQ10 has been reported to be effective in many disorders, including Alzheimer’s disease, Parkinson Disease, cardiac ischemia, and hypertension.\cite{211-214} It is an interesting candidate for stroke treatment. Mild uncoupling of mitochondrial respiration and phosphorylation has been proposed as a strategy to reduce mitochondrial ROS production.\cite{215,216} A cationic uncoupler SkQR1 has been shown to reduce lesion volume after ischemic stroke in rat.\cite{217}

We have reported that methylene blue (MB) can shuttle electrons between NADH and cytochrome c and bypass Complex I/III blockage, which reduced electron leakage and ROS generation.\cite{217}

Our study and other studies have indicated that MB is protective against ischemic stroke.\cite{217-220} MB is a small molecular that can easily cross the BBB and can be reoxidized by cytochrome c and reused for electron shuttling. MB and its derivatives as regenerable antioxidants that target mitochondria to reduce ROS production and provide neuroprotection are promising candidates for the treatment for ischemic stroke.\cite{217,222}

### Conclusion

ROS are generated from various sources during ischemia-reperfusion, with mitochondrial electron transport chain as one of the most important sources. While most previous studies and clinical trials for ischemic stroke focused on ROS scavengers, more studies should be conducted to develop and test agents that can reduce ROS generation after onset of stroke, especially from mitochondria. The combination of the upstream and downstream therapeutic strategies should also be considered in the future studies [Figure 1].

### Financial support and sponsorship

This work was partly supported by National Institutes of Health grants R01NS054651 (SY) and R01NS088596 (SY).

### Conflicts of interest

There are no conflicts of interest.

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