

The role of NADPH oxidase in brain ischemia

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Abstracts

Oxygen free radicals or oxidants that arise from molecular oxygen by successive single-electron reduction reactions, include the superoxide anion (O_2^-), the hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\cdot OH$) (Chan 1996). Physiological levels of free radicals are needed for redox sensitive signal pathways, cell development and cell apoptosis (Chan 2001). During brain ischemia/reperfusion (I/R), oxygen is not well used; and robust oxygen radicals are generated in the brain. Excessive free radicals cause the cellular macromolecular damages of lipids, proteins, and nucleotide acids; thus, they play a crucial role in ischemic/reperfusion brain damage. NADPH oxidase (NOX) is a pro-oxidant enzyme that is expressed in various brain regions and its level is regulated by ischemia. This review is focused on the role of NOX in brain ischemia. Results from both *in vivo* and *in vitro* studies suggested that NOX plays a role in ischemic brain damage through producing oxygen radicals, contributing to excitotoxicity, and exacerbating post-ischemic inflammation process.

Keywords: NADPH oxidase, free radicals, excitotoxicity, brain ischemia.

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1. Introduction

Cerebral blood flow is significantly reduced in ischemic brain regions. During reperfusion, oxygen is replenished. This is important for sustaining neuronal viability. However, the reflow after occlusion often causes an increase in oxygen to levels that cannot be fully utilized by mitochondria even under normal physiological flow conditions (Chan 2001). It has been demonstrated that about 2-5% of the electron flow in isolated brain mitochondria produces superoxide radicals (Boveris and Chance 1973). In addition to mitochondria, many prooxidant enzymes are known to participate in oxygen radicals generation in cerebral ischemia, including xanthine oxidase (XO), nitric oxide synthase (NOS), cyclooxygenase (COX) and NADPH oxidase (NOX) (Chan 2001). Antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GSHPx), and catalase, can remove oxygen radicals by working with some other small molecular antioxidants, including glutathione, ascorbic acid, and vitamin E (Chan 2001). During reperfusion, the antioxidative defense mechanism is compromised when oxygen radicals are over-produced, in addition to the inactivation of detoxification systems and

consumption of antioxidants in post-ischemic tissue (Chan 1996). Robust oxygen radicals are generated and they are directly involved in cellular macromolecules damage such as lipids, proteins and nucleic acids, which will eventually lead to cell death (Chan 2001). The copper-zinc SOD (SOD1) transgenic mice, which have a 2.3-fold overexpression of SOD1 in brain tissue, exhibited 40% less brain infarct and edema, as well as less oxygen radicals generation in the brain than the wild-type mice after ischemia reperfusion (I/R) (Epstein et al. 1987; Kinouchi et al. 1991; Noshita et al. 2002). On the contrary, after global ischemia more oxygen radicals were generated and higher neuronal death rate and mortality were detected in the SOD1-/- mice with a 50% decrease in SOD1 activity (Kawase et al. 1999). Furthermore, SOD1 knock out mice with no SOD1 activity exhibited 100% mortality after 1 h focal ischemia and 24 h reperfusion, which is significantly higher than in wild-type mice (11.1%) (Kondo et al. 1997). These results suggested that free oxygen radicals play an important role in ischemic brain damage.

2. General characteristics of NADPH oxidase (NOX)

Structure of NOX

NOX is a multisubunit complex composed of membrane associated subunits of gp91^{phox} and p22^{phox} and cytosolic subunits of p47^{phox}, p67^{phox}, and p40^{phox}. The catalytic subunit of the enzyme gp91^{phox}, also termed as NOX2, is present with several homologs including NOX1, NOX3, NOX4, NOX5 (Bedard and Krause 2007). As NOX is being activated, p47^{phox} phosphorylation first occurs which

subsequently causes cytosolic subunits p47^{phox}, p67^{phox}, and p40^{phox} translocate into membranes and fuse with catalytic subunit gp91^{phox} (Bedard and Krause 2007). The activated enzyme complex transports electrons to oxygen, thus producing superoxide anion, which is a member of oxygen radicals (Bedard and Krause 2007). Currently several genetic mutation of subunits such as gp91^{phox} knock out (gp91^{phox}-/-) and gp47^{phox} knock out (gp47^{phox}-/-) mice have been produced and they have been widely used in NOX studies (Bedard and Krause 2007).

Distribution and expression of NOX

NOX was initially discovered in neutrophils. It functions to generate superoxide anions for killing bacteria, thus, plays an important role in host defense (Lambeth 2004). *In vivo* studies have also demonstrated the expression of NOX subunits p47^{phox}, p67^{phox} and gp91^{phox} in various brain regions including cortex, medulla, hypothalamus, pons and cerebellum (Bedard and Krause 2007; Infanger et al. 2006; Kim et al. 2005). *In vitro* studies have also revealed that NOX subunits are expressed in cultured neurons, glia (including astrocytes and microglia) and brain vascular endothelia (Hong et al. 2006). The abundant expression of NOX in different brain regions indicates an important role of NOX in the CNS, such as microglia normal function and long-term potentiation and learning (Knapp and Klann 2002). Indeed, microglia from p47^{phox}-/- mice are unable to initiate immune response to phorbol ester. Both learning and memory are impaired in gp91^{phox}-/- and p47^{phox}-/- mice (Kishida et al. 2006; Lavigne et al. 2001).

Interestingly, the expression of NOX in the central nerve system (CNS) is also regulated by ischemia-reperfusion (I/R). In addition to the increase of gp91^{phox} subunit expression, more p47^{phox} subunits were found translocated to membrane in the penumbra area of rat brain after 2 h focal ischemia followed by 2 h reperfusion (Hong et al. 2006). Being consistent with the protein changes, both the NOX activity and superoxide anion levels started to increase after ischemia and peaked within 2 h of reperfusion in the penumbra area after focal ischemia (Vallet et al. 2005). This suggests that NOX plays a role in oxidant generation in the post-ischemic brain. Another NOX isoform NOX4 has also been investigated in a mouse permanent focal brain ischemia. Focal ischemia resulted in a dramatic increase in cortical NOX4 mRNA levels. It was detectable as early as 24 h after the onset of ischemia and persisted throughout the 30 days of follow-up period, reaching a maximum between days 7 and 15

(Abramov et al. 2007). The early onset and distribution pattern of mRNA increase, which is mostly detected in neurons near the ischemic area, indicates the neuronal reaction to the ischemic insult being the main underlying mechanism. However, the peak period corresponds to the time of neoangiogenesis, as it occurs mainly in the peri-infarct region and its prominent expression is detected in newly formed capillaries during peak period. Thus various NOX isoforms not only contribute the oxidative injury after brain ischemia, but also involve in other pathophysiological process, such as angiogenesis after ischemia (Abramov et al. 2007).

3. NOX in ischemic brain damage *in vitro*.

In cultured hippocampal and cortical neurons which are subjected to oxygen glucose deprivation (OGD) treatment, three distinct mechanisms contributed to generating oxygen radicals and causing oxidative injury to neurons, with each mechanism operating at a different stage of ischemia and reperfusion (Abramov et al. 2007). At the early phase, during hypoxia, oxygen radicals were produced by mitochondria and the xanthine oxidase (XO). A late phase oxygen radicals generation appeared during reoxygenation and was absent in cells from gp91^{phox}-/- mice. Therefore, NOX is the main contributor to the late phase oxygen radicals generation (Abramov et al. 2007). Furthermore, the inhibition of the NOX by diphenyleneiodonium (DPI) or genetic knock out of NOX subunit gp91^{phox} reduced cortical or hippocampus neuronal death by 30-40% (Suh et al. 2007). Those data further support that NOX is involved in ischemic neuronal damage. In consistence with the findings in OGD model, 2 h glucose deprivation (GD) and 1 h glucose reperfusion (GR) treatment were found to cause significant oxygen radicals generation. This process can be abolished by NOX inhibitor apocynin or genetic knock out p47^{phox} (Suh et al. 2007). Moreover, wild-type neurons exhibited 92% cell death while only 8-10% cell death was observed in p47^{phox}-/- and apocynin treatment neurons (Suh et al. 2007).

In addition to energy deprivation, it was also well established that glutamate-mediated excitotoxicity significantly contributes to ischemic brain damage (Lipton 1999). In brain tissue, normal levels of extracellular glutamate measured by microdialysis are 1-5 μM . It rises to between 50-90 μM in the striatal core and rises to 30-50 μM in the cortical core and/or penumbra after focal ischemia (Lipton 1999). Excessive glutamate activates N-methyl-D-aspartic acid (NMDA) and α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors, thus, causes the massive anoxic depolarization and cellular ion

homeostasis disturbance. This further aggravates ischemic brain damage (Lipton 1999; Nikolova et al. 2005). Previous reports have also demonstrated that glutamate application results in NOX activation in neuronal cells (Nikolova et al. 2005). Using *in vivo* hydroethidine (Het) injection method, it was revealed that glutamate treatment induced significant increase in oxygen radical level, which was abolished by NOX inhibitors, DPI, apocynin, and neopterin. Apocynin pretreatment reduced cell death rate from 40% to 15% in cells subjected to glutamate treatment (Nikolova et al. 2005). Taken together, these findings indicate that NOX plays a role in the pathological process of excitotoxicity. In addition to causing the oxidative stress, NOX is also involved in the signal pathways activation after glutamate application. Functioning as a NOX inhibitor, DPI or genetic p47^{phox} knock out blocks the extracellular signal-regulated kinase (ERK) pathways in hippocampal CA1 neurons (Kishida et al. 2005). However, it still needs further elucidated whether NOX dependent signal pathways activation contributes to the protection effect against excitotoxicity.

4. The role of NOX in brain ischemia *in vivo*

NOX inhibition alleviates oxidative stress and brain damage in focal ischemia

The role of NOX in focal ischemic brain damage was first demonstrated by comparing the brain damage between wild-type and gp91^{phox}^{-/-} mice after focal ischemia (Walder et al. 1997). Focal ischemia was induced by 2 h middle cerebral artery occlusion (MCAO). The infarct volume after 2 h MCAO and 22 h reperfusion was significantly less in gp91^{phox}^{-/-} mice (29.1mm³) than their wild-type littermates (54.0mm³). In addition, the elimination of a functional NOX from the circulation by radiation did not reduce the infarct size induced by MCAO. This suggests that the protection effect of NOX inactivation against brain ischemia is not due to neutrophils inactivation (Walder et al. 1997). The role of NOX was also examined using genetic disruption of another NOX subunit p47^{phox} and with the pharmacological inhibitor apocynin. Inactivating NOX with either apocynin or deletion of the p47^{phox} or gp91^{phox} subunit blocked neuronal death *in vivo* after focal ischemia (Tang et al. 2008). Furthermore, apocynin treatment also significantly reduced neurological deficit score in mice after focal ischemia, thus the mice with NOX inhibiting not only showed less brain damage but also maintained better behavior functions (Chen et al. 2009; Jackman et al. 2009; Tang et al. 2008). After NOX inhibition, the *in vivo* protection against focal ischemia is at least partially due to the alleviated

oxidative stress. Significantly less superoxide was found in neurons and microglia after NOX inhibition by *in vivo* Het injection methods (Tang et al. 2008). In addition, apocynin treatment or genetic mutation of gp91^{phox} protected cell lipid or DNA from post-ischemic oxidative damage (Chen et al. 2009)

NOX inhibition maintains post-ischemic BBB integrity

Blood-brain-barrier (BBB) which is composed by endothelial cells and astrocytes, strictly controls the exchanges between the blood and the brain compartments by limiting passive diffusion of blood-borne solutes while actively transporting nutrients to the brain (Risau and Wolburg 1990). Damage to the BBB often results in hemorrhage and edema, thus further aggregating neuronal cell death. Extracellular matrix (ECM) is a multifunctional complex of proteins and proteoglycans such as type IV collagen, laminin, and fibronectin. ECM is assembled in a highly organized manner and plays a crucial role in endothelium cells structural integrity. Matrix metalloproteinases (MMPs) target critical ECM; and the activation of MMPs result in compromised integrity of BBB (Romanic et al. 1998; Rosenberg 1995). Previous studies have demonstrated that post-ischemic oxygen radicals can activate MMP, cause BBB compromise, and finally exacerbate brain edema and intracerebral hemorrhage (Pun et al. 2009). In post-ischemic SOD1 overexpression transgenic mice, in addition to less oxygen radicals generation, significantly less MMP-9 activation was observed compared to wild type mice (Kamada et al. 2007). Moreover, SOD1 overexpression transgenic mice exhibited significant less Evan's blue leakage compared to wild-type mice, which indicates better preserved BBB integrity in SOD1 transgenic mice (Kamada et al. 2007). Recent studies showed that the activation of MMP-9 in post-ischemic brain attributed to NOX-generated oxygen radicals. NOX2 expression is significantly increased in rat microvascular structure after 90 min focal ischemia and 22.5 h reperfusion (Liu et al. 2008). I/R caused activated MMP9 increasing by 7-fold and brain swelling by 20%. Pretreatment with apocynin reduced MMP9 upregulation and brain edema by 50% (Liu et al. 2008). In addition to pharmacological approaches, NOX transgenic mice were also used to study the role of NOX in BBB damage after focal ischemia. In wild-type mice, 2 h MCAO and 22 h reperfusion induced significant Evans blue extravasation, indicating increased BBB permeability (Kahles et al. 2007). In consistence with the results from rat, gp91^{phox}^{-/-} mice exhibited less post-ischemic Evans blue extravasation. Furthermore, gp91^{phox}^{-/-} mice exhibited 40% less infarction and 50% less edema compared to vehicle treated animals (Kahles

et al. 2007). Thus, in addition to reducing the oxidative stress in neurons and glia, selective inhibition of NOX protects the endothelium, prevents BBB disruption, and reduces brain damage in experimental stroke.

NOX inhibition reduces brain damage after global ischemia

In addition to focal ischemia, the role of NOX was also studied in global ischemia. Global ischemia was induced by occlusion of bilateral common carotid arteries (CCA) for 5 mins in gerbils. After global ischemia and 4 d reperfusion, ~90% cell death was observed in hippocampus area. In addition, strong 4-hydroxynonenal (HNE) immunoactivity was found after ischemia, indicating lipid peroxidation due to excessive production of oxygen radicals. Eight-hydroxyl-deoxyguanosine (8-OHdG), a biomarker for DNA oxidative damage, also increased after ischemia in hippocampal neurons (Wang et al. 2006). Apocynin (5mg/kg, intraperitoneal injection) administered prior to ischemia significantly attenuated the increase in HNE and 8-OHdG. Furthermore, apocynin increased CA1 survival neurons from ~10% to ~60% (Wang et al. 2006). Thus, NOX contributed to brain damage in global ischemia as well as in focal ischemia.

In addition to causing neuron death, global ischemia also resulted in a substantial increase in microglial cells, especially over the hippocampal CA1 area where extensive neuronal death was detected after global ischemia (Wang et al. 2006). Ischemic animals that were pretreated with apocynin demonstrated 50% less microglial cells in CA1 area as compared to those without apocynin treatment (Hailer 2008). Thus apocynin administration not only offered neuroprotection against I/R-induced oxidative stress, but also diminished glial cell activation. It is demonstrated that activated microglia can secrete proinflammatory cytokines including IL-1 β , IL-6 and TNF, which exert negative effects on neuronal integrity and survival after ischemia (Hailer 2008). In addition, activated microglia express inducible nitric oxide synthase (iNOS) that synthesizes nitric oxide (NO \cdot), thus, causes subsequently neurotoxicity in neurons (Choi et al. 2005). NOX inhibitor apocynin reduced microglia secretion of IL-1 β by 80% after lipopolysaccharide (LPS) application. In addition, apocynin or DPI abolished iNOS upregulation in microglia while gp91^{phox} -/- microglia generated 60% less NO \cdot compared to wild-type microglia after LPS treatment (Cheret et al. 2008). Moreover, we also found that gp91^{phox} -/- mice showed less microglia activation and less protein nitration after 1 h ischemia and 24 h or 72 h reperfusion (unpublished data).

This further suggests that NOX plays a role in inflammatory process; and that inhibition of NOX could inhibit microglia activation and be neuroprotective against brain ischemia.

5. Summary

In summary, results from both *in vitro* and *in vivo* experimental studies suggest that NOX play a role in ischemic brain damage. In addition to generating oxygen radicals and causing direct oxidative injury to the brain, NOX also plays a role in post-ischemic excitotoxicity or inflammatory process. Thus, NOX can be a potential pharmacological target for reducing ischemic brain damage.

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