Apocynin in the Treatment of Ischemic Stroke

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Abstract: Apocynin has been used as an efficient inhibitor of the NADPH oxidase complex in experimental studies. NADPH oxidase was originally identified immune cells as playing an important microbicidal role. In cerebral ischemia, inflammation is increasingly being recognized as contributing negatively to neurological outcome, with NADPH-oxidase as an important source of superoxide. Recently, several forms of this oxidase have been found in a variety of non-immune cells. Neuronal NADPH oxidase is thought to participate in long-term potentiation and intercellular signaling. However, excessive superoxide production is damaging and has been shown to play an important role in the progression of brain injury. NADPH oxidase is a multisubunit complex composed of membrane-associated $gp91^{phox}$ and $p22^{phox}$ subunits and cytosolic subunits, $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ and Rac. When NADPH oxidase is activated through phosphorylatoin of $p47^{phox}$, cytosolic subunits translocate to the cell membrane and fuse with the catalytic subunit, $gp91^{phox}$. The activated enzyme complex transports electrons to oxygen, thus producing the superoxide anion ($O_2^{\bullet -}$), a precursor of reactive oxygen species. An NADPH oxidase assembly inhibitor, apocynin, has been shown alleviate oxidative stress and improves neurological outcome after cerebral ischemia. There is recent interest in the role of NADPH oxidase and apocynin as neuroprotective strategies against ischemia. This review will focus on therapeutic effects of NADPH oxidase assembly and its inhibitor apocynin in stroke and other brain injuries.

Keywords: Apocynin, ischemia, superoxide, NADPH oxidase.

1. INTRODUCTION

Stroke is a significant cause of death and disability in industrialized nations, yet, there are few treatments. The major therapeutic strategy for treatment of acute ischemic stroke is rapid recanalization, either by pharmacological means through thrombolytic agents, or mechanical thrombectomy [1, 2]. However, the time window for intervention limits these therapies to a small number of patients, and their inappropriate use can actually worsen outcome. This worsened outcome has been blamed on complications of delayed recanalization such as worsened brain edema or symptomatic brain hemorrhage, a phenomenon commonly referred to as 'reperfusion injury' [3, 4]. Thus, therapies to minimize reperfusion injury might expand populations of stroke patients eligible for treatment. Reactive species, radicals derived from oxygen or nitric oxide are thought to be major contributors to this damage. Upon reperfusion, the brain is quickly exposed to oxygenated blood, and injured mitochondria of the ischemic brain are rendered incapable of detoxifying free radical [5]. Further, immune cells which infiltrate ischemic tissue or plug ischemic microvasculature can also generate reactive species through several enzyme systems [6]. Recent studies have focused on the role of superoxide generating systems in immune cells and their consequences on reperfusion injury. One enzyme system is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, or NOX, originally found on leukocytes, but now recognized in several types of cells in the brain. Inhibition of NOX can potentially reduce the amount of superoxide generated during reperfusion, and thus limit reperfusion injury. Such a strategy has the potential to not only treat acute ischemic stroke, but also reduce complications of recanalizing strategies by using it in combination with thrombolytics or mechanical thrombectomy devices.

2. NADPH OXIDASE

NOX is a membrane-bound enzyme complex. It can be found in the plasma membrane as well as in the membrane of phagosome. NOX was originally found in leukocytes and is a major source of reactive oxygen species generation [7]. The complex is normally latent in neutrophils and is activated to assemble in the membranes during respiratory burst. Among many enzymes utilizing molecular oxygen as substrate, NOX can serve as a source of ROS in CNS. NOX is a multi-component enzyme comprising a cytoplasmic subunits ($p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ and Rac2) and upon phosphorylation, these subunits can form a complex and translocate to the plasma membrane to dock with the plasma membrane subunits ($p91^{phox}$, $p22^{phox}$) [8]. The catalytic core of the enzyme is thought comprise $gp91^{phox}$ and $p22^{phox}$ [9]. Catalysis of NOX occurs in the $p91^{phox}$ subunit (Nox2) and is initiated by transferring of electrons from molecular oxygen through redox coupling with NADPH, FAD and heme to produce superoxide anion ($O_2^{\bullet -}$) [10] (Fig. **1**).

Superoxide can be produced in phagosomes, which contain ingested bacteria and fungi, or it can be produced outside of the cell. In a phagosome, superoxide can spontaneously form hydrogen peroxide that will undergo

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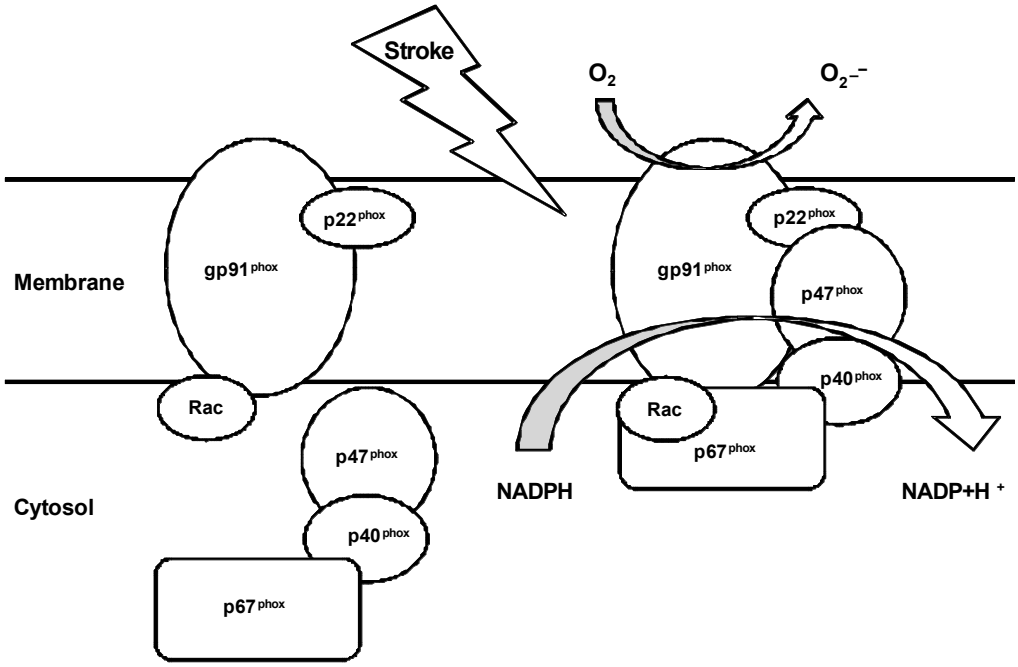

Fig. (1). NADPH oxidase activation in stroke. NADPH oxidase comprises cytosolic ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and Rac) and membrane subunits ($gp91^{phox}$ and $p22^{phox}$) which associate to make an activated enzyme. The NADPH-binding domain is predicted to be the cytosolic side of the membrane, whereas $O_2^{\bullet -}$ generation is predicted to occur on the extracellular side. The exact mechanism by which stroke triggers this activation is not fully clear, and there may actually be several.

further reactions to generate reactive oxygen species (ROS). Vascular ROS are produced in endothelial, adventitial, and VSMCs and derived primarily from NOX, a multisubunit enzyme catalyzing a superoxide anion ($O_2^{\bullet -}$) production by the 1 electron reduction of oxygen using NADPH as the electron donor: $2O_2 + NADPH \rightarrow 2O_2^{-} + NADP + H^{+}$ [11]. Superoxide is capable of killing bacteria and fungi by mechanisms that are not yet fully understood, but may inactivate critical metabolic enzymes, initiate lipid peroxidation and liberate redox active iron, which allows the generation of indiscriminate oxidants such as the hydroxyl radical. Superoxide probably kills bacteria directly as the virulence of many pathogens is dramatically attenuated when their superoxide dismutase (SOD) genes are deleted. However, downstream products of superoxide also include hydrogen peroxide and hypochlorous acid, the reactive agent in bleach.

NOX activation depends on phosphorylation, especially of the $p47^{phox}$ subunit [9]. While other subunits can be phosphorylated, $p47^{phox}$ phosphorylation appears key in the membrane translocation of other subunits as well. Kinases known to phosphorylate p47 include several of protein kinase C isoforms (β, δ and ζ) as well as p38 and p21 mitogen activated kinases (MAPK) and protein kinase B. Further, it appears that NOX can be regulated by the inflammatory transcription factor, nuclear factor kappa B (NFκB). NFκB can induce $gp91^{phox}$ expression, as cells deficient in NFκB's p65 subunit express less $gp91^{phox}$ in response to lipopolysaccharide (LPS) stimulation [12].

3. APOCYNIN

Apocynin (4-hydorxoy-3-methoxyacetopheone), also known as acetovanillone, is a natural organic compound. It has been isolated from a variety of plant sources and is being studied for its variety of pharmacological properties. Apocynin was first described by Oswald Schmiedeberg, a German pharmacologist, in 1883 and was first isolated from the root of Canadian hemp (Apocynum cannabinum). At the time, this plant was already being used for its effectiveness against edema and heart problems. In 1971, apocynin was also isolated from Picrorhiza kurroa, a small plant that grows at high altitudes in the western Himalayas. P. kurroa was used for ages by the indigenous peoples as a treatment for liver and heart problems, jaundice, and asthma. In 1990, Simons *et al.* isolated apocynin to a pharmacologically useful level using an actively guided isolation procedure [13]. Apocynin has a smell of vanilla and a melting point of 115C. It is an acetophenone with a molecular weight of 166.17 [14, 15] (Fig. **2**).

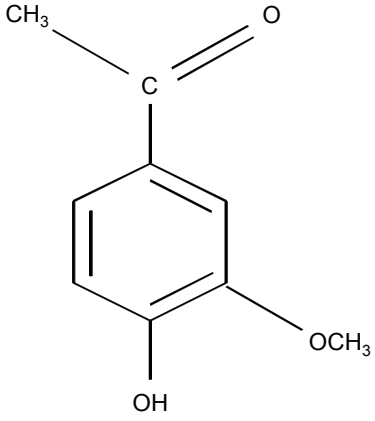

Fig. (2). Chemical structure of apocynin.

Apocynin has anti-oxidant and anti-inflammatory capabilities, specifically to block the activity of NOX by interfering with the assembly of the cytosolic and membrane components of the enzyme. Apocynin has been studied in many experiment models involving phagocytic and nonphagocytic cells [16, 17].

NOX is an enzyme that effectively reduces O_2 to superoxide ($O_2^{\bullet-}$), which can be used by the immune system to kill bacteria and fungi. Apocynin is an inhibitor of NOX activity and thus is effective in preventing the production of the superoxide in human white blood cells or neutrophilic granulocytes. It does not however obstruct the phagocytic or other defense roles of Granulocytes. Due to the selectivity of its inhibition, apocynin can be widely used as an inhibitor of NOX without interfering in other aspects of the immune system.

Apocynin was used to determine whether ionic activation due to proton flux across the membrane of renal medulla cells was coupled to NOX production of superoxide. Apoycnin was introduced to the cells and completely blocked the production of superoxide. This was a key component in determining that the proton outflow was responsible for the activation of NADPH oxidase.

4. NADPH OXIDASE ACTIVATION INDUCES SUPEROXIDE PRODUCTION AFTER STROKE

Reactive oxygen species are an important underlying factor in delayed neuronal death induced by cerebral ischemia–reperfusion (I/R). During reperfusion, robust oxidants are generated and are directly involved in the damage to cellular macromolecules, such as lipids, proteins, and nucleic acids, eventually leading to cell death [5]. Many prooxidant enzymes, including nitric oxide synthase, cyclooxygenase (COX), and xanthine oxidase, participate in oxidative injury in cerebral ischemia [18]. Superoxide produced from NOX may interact with nitric oxide from inducible nitric oxide synthase (iNOS) to form peroxynitrite, which is an especially reactive species that can contribute to neuronal death [19].

There is increasing evidence that inflammation accompanying ischemic stroke accounts for some of its progression, at least acutely [6, 20, 21]. A robust inflammatory reaction characterized by peripheral leukocyte influx into the cerebral parenchyma and activation of endogenous microglia follows focal cerebral ischemia. This leads to the generation of reactive oxygen species (ROS) which can then stimulate ischemic cells, even ischemic neurons, to secrete inflammatory factors. Generation of ROS by inflammatory cells occurs *via* several enzyme systems, but NOX is the major enzyme that generates superoxide. How NOX is activated in stroke is not entirely clear, but phosphorylation of the $p47^{phox}$ subunit appears important. $p47^{phox}$ phosphorylation can occur through several kinases also upregulated and activated by brain ischemia, including several protein kinase C isoforms and the p38 and p21 MAPKs. While numerous forms of the enzyme have now been described [7], phagocytic NOX, also referred to as NOX2, is associated with immune cells.

Recent studies have also suggested that NOX is also expressed in the central nervous system. *In vitro* studies have shown NOX expression in neurons, astrocytes, and in microglia [10]. Immunohistochemistry studies have shown that NOX subunits are widely distributed in the cortex, the hippocampus, and in the cerebellum *in vivo* [22-25]. NOX has also been documented to increase in the brain after experimental stroke [26]. Mice deficient in the $gp91^{phox}$ subunit had smaller infarcts than mice with an intact enzyme in models of focal cerebral ischemia followed by reperfusion [27-29]. Further, NOX appears to play a significant role in reperfusion injury, as reperfusion permits the restoration of glucose to the ischemic brain. The restoration of glucose (rather than oxygen, which is traditionally thought to be a source of ROS in this setting) appears to 'fuel' NOX by serving as an electron donor to produce damaging levels of superoxide [30]. Interestingly, reperfusion in the presence of glucose appears to increase neuronal NOX activity and NOX deficiency or inhibition prevents this. NOX also appears to be a primary souce of ROS generated by NMDA receptor activation [31].

The importance of vascular NOX has also been demonstrated. Aged mice have dysregulated cerebrovascular responses compared to similar aged mice deficient in NOX [32]. Vascular NOX also appears to contribute to cerebrovascular dysfunction and behavioral deficits in models of Alzheimer's disease [33, 34].

5. APOCYNIN INHIBITS NADPH OXIDASE - INDUCED SUPEROXIDE PRODUCTION

Apocynin is a commonly used NOX inhibitor with relatively low affinity (IC50 ~10 μmol/L) in neutrophils [35]. It does not seem to interfere with the PMNs other defense mechanisms, as it does not affect phagocytosis or intracellular killing [36]. Apocynin inhibits the release of $O_2^{\bullet-}$ through NOX by blocking migration of $p47^{phox}$ to the membrane, thus interfering with assembly of the functional NOX complex [37]. The inhibitory action of the compound is not entirely specific to NOX, however. Some of its inhibitory activity at least initially may involve myeloperoxidase (MPO) because apocynin does not inhibit NOX in cells deficient in MPO [38]. MPO together with hydrogen peroxide can facilitate APO dimerization (Fig **3a**), and these dimers can prevent assembly of an active enzyme complex (Fig. **3b**). Furthermore, agents such as zymosan that promote the release of MPO also enhance the efficacy of apocynin [39]. In cells that are not rich in MPO, apocynin can reduce oxidant stress through a nonspecific oxidative scavenger effect instead of NOX inhibition [40]. However, besides MPO, other peroxidases, such as horseradish peroxidase, can also induce apocynin dimer formation with a consequent NOX inhibitory effect [37, 41]. In addition, *in vivo* studies showed that MPO secreted by neutrophils can be taken up by endothelial cells, in which apocynin can then be metabolized to active dimers, thus inhibiting vascular NOX [37]. In line with this concept, it was observed that supplementation with thiol provided either as glutathione or cysteine prevents the inhibitory effect of apocynin on the NAPDH oxidase. Apocynin dimer formation may be responsible for its delayed inhibitory property [42], and it has been suggested that this dimer is what blocks NOX activity [39].

6. APOCYNIN PROTECTS THE BRAIN FROM ISCHEMIC DAMAGE

NOX is the one of the major sources of super oxide generation, and reperfusion injury can further worsen

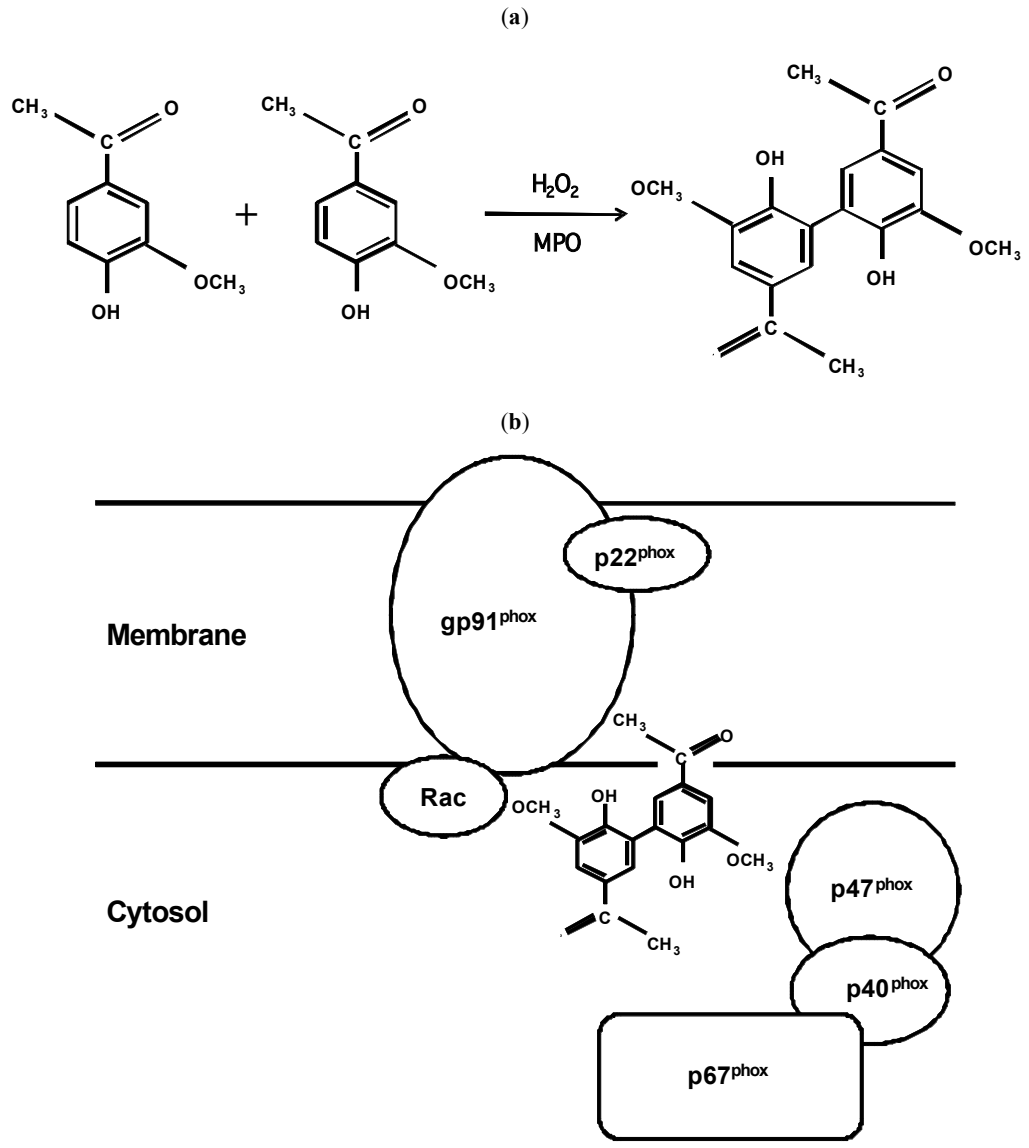

Fig. (3). Mechanism of NOX inhibition by apocynin. (**a**) Apocynin dimerization by myeloperoxidase (MPO) and hydrogen peroxide (H_2O_2), (**b**) The mechanism of inhibition of apocynin on NADPH oxidase-induced superoxide production. Apocynin prevents assembly of an intact enzyme complex by preventing the association of cytosolic subunit p47 with the membrane subunit gp91.

ischemic injury by permitting a massive influx of leukocytes and the generation of ROS in the injured brain [43]. Since apocynin can inhibit NOX, it may have an important role in preventing brain injury due to reperfusion in experimental studies of stroke. Apocynin, by virtue of blocking ROS generation, can inhibit immune responses. In monocytes, it can suppress NF-κB activation and prevent COX-2 expression [44]. It is also effective against abeta-induced microglial proliferation and lipopolysaccharide (LPS) and interferon induced cell death [45, 46]. Altered NADPH oxidase function has been linked to neurological disorders such as Alzheimer's (ALS) and Parkinson's diseases (PD) [47]. Apocynin has been effective in ameliorating neurodegeneration in both *in vivo* and *in vitro* models of PD [48, 49], and retarded disease progression and extended survival in a mouse ALS model [50].

Apocynin has been studied by a few groups in brain ischemia models. From our own lab, we found that a dose of 2.5 mg/kg given parenterally just prior to reperfusion, or 1.5 h after ischemia onset, resulted in reduced infarct volume and improved neurological outcome [51]. We also found that $O_2^{\bullet-}$ is largely generated in neurons and some microglia/monocytes, with no generation in brain vascular endothelial cells. Apocynin markedly reduced $O_2^{\bullet-}$ in the brain. However, apocynin at higher doses (3.75 and 5 mg/kg) failed to show any benefit, and actually increased the severity of brain hemorrhage. Thus, this rather narrow therapeutic dose range may limit its translation to the clinical level. However, other groups have shown salutary effects of apocynin at doses as high as 50 mg/kg [27, 52]. In global cerebral ischemia, 5 mg/kg apocynin attenuated hippocampal injury when given prior to ischemia onset [53]. In safety studies of uninjured mice, apocynin was well tolerated in single oral doses of up to 1000 mg/kg [54].

Immune cell generated NOX also appears important in the maintenance of vascular integrity. The addition of

microglia to endothelial cell and astrocyte cocultures worsens ischemia-like injury, and inhibiting superoxide production with apocynin preserved these BBB constituents *in vitro* [55]. Thus, NOX contributes to BBB disruption downstream events in ischemic stroke. In fact, apocynin attenuated brain edema formation, matrix mettaloproteinase-9 (MMP-9) expression [56], BBB disruption and hemorrhagic transformation [51] as well as inhibiting immune cell responses [29]. The temporal therapeutic window of apocynin has not been systematically studied, and current experimental data are conflicting. Some studies showed that apocynin given at the time of reperfusion (2 h post ischemia onset) protected against experimental stroke [51]. However, a recent study showed that treatment with apocynin 30 min prior to ischemia onset was protective, while treatment begun 1 h after initiation of reperfusion (2h post ischemia onset) had no effect [57]. Reasons for these discrepancies are not clear, but might be due to the importance of inhibiting NOX at the time of reperfusion, and not after.

7. CONCLUSIONS

Taken together, apocynin, a NOX inhibitor, reduces infarct volume, cerebral hemorrhage, and BBB disruption and improves neurological function following experimental stroke by several laboratories. While it may have a relatively narrow therapeutic window, we suggest that apocynin, or similar targets of NOX deserve attention, and studies to develop safe and selective drugs may be useful in the treatment of clinical stroke. Since NOX likely affects damaging effects of stroke during reperfusion, an inhibitor such as apocynin may have great utility in patients at risk for reperfusion injury following thrombolysis or mechanical thrombectomy.

ACKNOWLEDGEMENTS

This work was supported by grants to MAY from: NIH NINDS (R01 NS40516, P50 NS014543), American Heart Association Established Investigator Award and the Department of Defense and the Department of Veterans Affairs.

CONFLICT OF INTEREST STATEMENT

None of the authors have any financial interest relevant to the work presented in this manuscript.

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Received: April 29, 2010 Revised: June 16, 2010 Accepted: September 15, 2010