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NADPH Oxidase-2: Linking Glucose, Acidosis, and Excitotoxicity in Stroke

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Abstract

Significance: Neuronal superoxide production contributes to cell death in both glutamate excitotoxicity and brain ischemia (stroke). NADPH oxidase-2 (NOX2) is the major source of neuronal superoxide production in these settings, and regulation of NOX2 activity can thereby influence outcome in stroke. ***Recent Advances:*** Reduced NOX2 activity can rescue cells from oxidative stress and cell death that otherwise occur in excitotoxicity and ischemia. NOX2 activity is regulated by several factors previously shown to affect outcome in stroke, including glucose availability, intracellular pH, protein kinase ζ/δ, casein kinase 2, phosphoinositide-3-kinase, Rac1/2, and phospholipase A2. The newly identified functions of these factors as regulators of NOX2 activity suggest alternative mechanisms for their effects on ischemic brain injury. ***Critical Issues:*** Key aspects of these regulatory influences remain unresolved, including the mechanisms by which rac1 and phospholipase activities are coupled to N-methyl-D-aspartate (NMDA) receptors, and whether superoxide production by NOX2 triggers subsequent superoxide production by mitochondria. ***Future Directions:*** It will be important to establish whether interventions targeting the signaling pathways linking NMDA receptors to NOX2 in brain ischemia can provide a greater neuroprotective efficacy or a longer time window to treatment than provided by NMDA receptor blockade alone. It will likewise be important to determine whether dissociating superoxide production from the other signaling events initiated by NMDA receptors can mitigate the deleterious effects of NMDA receptor blockade. *Antioxid. Redox Signal*. 22, 161–174.

Introduction

ISCHEMIC STROKE RESULTS from critically reduced blood flow in one or more arteries of the brain or spinal cord. If blood flow is not promptly restored, cells in the ischemic territory die of energy failure. If blood flow is restored (ischemia–reperfusion), cells may nevertheless go on to die from excitotoxicity, effects of inflammation, or programmed cell death. These processes may also kill cells that are not themselves subjected to critical ischemia, but are in the vicinity of ischemic tissues. Consequently, these processes—excitotoxicity, inflammation, and programmed cell death—are a primary focus of current stroke research (48, 59).

Of these injury mechanisms, neuronal excitotoxicity is the one triggered most rapidly after ischemia. Excitotoxicity results from sustained action of glutamate at neuronal glutamate receptors, primarily N-methyl-D-aspartate (NMDA)-type glutamate receptors (31, 105). In stroke, this is caused by the combined effects of neurotransmitter glutamate release, impaired glutamate reuptake, and reversed action of glutamate uptake transporters (62, 153). The importance of excitotoxicity as a cell death mechanism in stroke has been firmly established in a variety of stroke models and animal species, using pharmacological, genetic, and other approaches to block NMDA receptor activation (1, 47, 58, 126, 135, 137, 141, 162, 166, 168). Excitotoxicity is a particularly important cell death mechanism in stroke involving ischemia–reperfusion (43, 57, 141). However, the beneficial effects of NMDA receptor blockade in stroke require intervention at relatively short time points after onset of ischemia or reperfusion, typically within 3 h (167, 174). Clinical studies corroborate this result; interventions targeting excitotoxic injury have had no demonstrable efficacy when

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initiated many hours after ischemia onset, but more rapid initiation has shown salutary effects (74).

Glutamate excitotoxicity was first identified as a cell death mechanism in 1957 (113), and has since become recognized as a pathogenic mechanism in many acute and chronic neurological disorders (30, 53, 109, 122, 154). Despite this long history, our understanding of the events linking receptor activation to cell death continues to evolve. Early studies identified Ca^{2+} influx as a requisite event in this process (28) and correlated the magnitude of this Ca^{2+} influx (52, 69) and the subsequent mitochondrial Ca^{2+} uptake (170) with neuronal death. It was later established that the gaseous signaling molecules, nitric oxide and superoxide, were both produced during sustained NMDA receptor activation by Ca^{2+}-dependent processes, and that production of both was required for excitotoxic death (17, 138, 158). Neither of these signaling molecules is intrinsically highly reactive or toxic, but in combination they form the highly reactive species peroxynitrite, which in turn can form additional reactive species such as hydroxyl radical and carboxyl radical, all of which readily and irreversibly react with DNA, proteins, lipids, and other crucial cell constituents (138). Subsequent events, notably poly(ADP-ribose) polymerase-1 activation and mitochondrial failure, lead to secondary energy depletion and cell death (6, 55, 97, 110, 116, 128, 188).

Nitric oxide is nonpolar, lipid permeable, and has a relatively long half-life and diffusion distance in the brain (138). Superoxide, by contrast, is polar, largely lipid impermeable, and has a relatively short half-life and diffusion distance, due in part to the ubiquitous distribution of extracellular and intracellular superoxide dismutase (138). Nitric oxide is produced by neuronal nitric oxide synthase in response to Ca^{2+} entry through NMDA receptors (158). The source and regulation of NMDA-induced superoxide production have been less clear, but accruing evidence points to neuronal NADPH oxidase (NOX) as the dominant source in this setting. In this study, we review this evidence, the regulatory mechanisms linking NMDA receptor activation to NOX2 activation, and the relevance of these factors to stroke pathogenesis.

NOX in the Central Nervous System

NOX has been best characterized in phagocytes and other cells of the immune system, where it plays a crucial role in bacterial killing. It has more recently been established that NOX is also expressed by many other cell types, in which it serves a variety of signaling functions. NOX is a multisubunit protein complex distinguished on the basis of their catalytic and associated subunits: NOX1–NOX5, DUOX 1, and DUOX2 (10). Of these, NOX2 is the dominant form expressed by phagocytes, and it is also the dominant form expressed by central nervous system (CNS) neurons, astrocytes, and microglia. NOX2 is composed of three cytosolic subunits, $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$, which when activated bind with two membrane-bound subunits, $p22^{phox}$ and $gp91^{phox}$ (the catalytic unit) and the small GTPase Rac1 to form an active transmembrane enzyme complex. NOX2 generates superoxide by the transfer of electrons to molecular oxygen on one side of the membrane, while oxidizing NADPH to NADP and H^+ on the other side (Fig. 1). Once activated, the continued function of NOX2 in phagocytes requires a continuous source of NADPH and a rapid excretion of H^+ to prevent intracellular acidification and membrane depolarization.

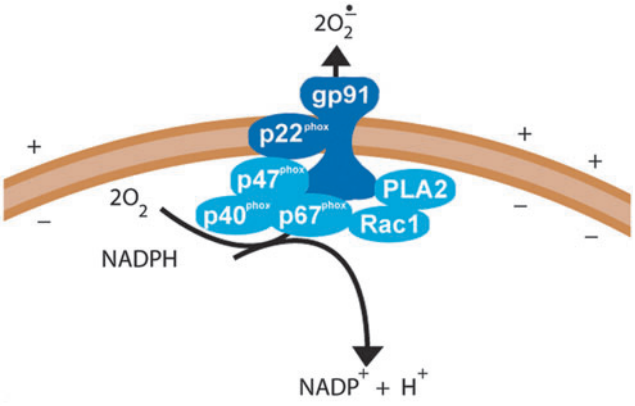

FIG. 1. NADPH oxidase (NOX). The NADPH oxidase-2 (NOX2) complex generates superoxide on one side of the membrane, while oxidizing NADPH to NADP and H^+ on the other side. Activation of the enzymatic complex requires translocation of the cytosolic subunits (light blue) $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ to the membrane along with Rac1 and phospholipase A2 (PLA2), where the membrane-bound subunits (dark blue) gp91 and $p22^{phox}$ are located. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Other NOX isoforms are also found in the CNS, most notably NOX4 in endothelial cells (5, 10). The different NOX isoforms utilize different (but somewhat overlapping) organizing subunits and have differing regulation (10). Furthermore, some NOX isoforms localize to subcellular compartments, as determined, in part, by differences in isoform dependence on specific organizing subunits (71). NOX4 may be unique in producing hydrogen peroxide, rather than superoxide, as its final product (10, 120), and NOX4 was recently reported to colocalize with mitochondria in endothelial cells (99) and neurons (26).

NOX2 as a Source of Superoxide in Excitotoxicity and Stroke

Potential superoxide sources in neurons include mitochondria, cytosolic dehydrogenases, phospholipases, cyclooxygenases, and other oxidases, in addition to NOX. Of these, mitochondria were long thought to be the primary source of superoxide production in excitotoxicity. Support for this idea was based, in part, on the mitochondrial localization of oxidant-sensitive fluorescent indicators during neuronal glutamate exposure and a reduction in this signal with mitochondrial inhibitors (11, 50, 51, 151). However, the localization of oxidized dyes to the mitochondria does not itself establish mitochondria to be the source of oxidant production, as demonstrated by oxidation of mitoSOX in neuronal mitochondria by xanthine/xanthine oxidase added to the culture medium (82). A reduced fluorescent signal in response to mitochondrial inhibitors is likewise insufficient to establish mitochondria as the oxidant source, because these inhibitors also cause mitochondrial and plasma membrane depolarization, with resultant dye efflux (133). A quantitative assessment of mitochondrial superoxide production in excitotoxicity has been difficult because there are at least

seven potential sources of superoxide production within mitochondria (9), and it is not feasible to block all of these or to make viable neurons devoid of mitochondria altogether. In addition, mitochondria normally act as sinks for extramitochondrial sources of reactive oxygen species (9), such that impaired mitochondrial function can artificially mimic superoxide production by eliminating this scavenging capacity.

Several lines of evidence point to the NOX2 isoform of NOX as the primary source of excitotoxic neuronal superoxide production. First, inhibition of NOX2 with either apocynin (139, 169, 172), diphenylene iodonium (7), or a tat-conjugated peptide inhibitor of NOX2 assembly (16, 17, 150) all block superoxide formation and neuronal death that otherwise result from NMDA receptor activation. Second, $p47^{phox-/-}$ neurons, which cannot form a functional NOX2 complex, likewise show markedly attenuated superoxide formation and cell death in response to NMDA (17, 60, 65, 150), and these are restored in $p47^{phox-/-}$ neurons transfected to express $p47^{phox}$ (17, 150). A third line of evidence arises from the specific requirement for glucose to fuel NADPH regeneration through the pentose phosphate pathway (45, 67). When flux through this pathway is blocked with glucose-free medium, 2-deoxyglucose, or 6-aminonicotinamide, there is again reduced superoxide formation and neuronal death (17, 171, 172). Fourth, neurons treated with a peptide inhibitor of protein kinase C (zeta) (PKCζ), which is required for neuronal $p47^{phox}$ phosphorylation (41, 54), show reduced superoxide formation and reduced cell death after NMDA exposures (16, 17). These findings were corroborated by studies using NMDA injections into mouse hippocampus *in situ*: NMDA injections induced neuronal superoxide production and subsequent neuronal death, and these were prevented by apocynin and by $p47^{phox}$ gene deficiency (17).

The possibility remains that superoxide produced by NOX2 might induce a secondary, possibly greater production of superoxide in mitochondria by disrupting sensitive iron–sulfur domains of mitochondrial electron transport complexes. Evidence for this has been reported in cardiac myocytes (14, 39, 94), and there is also evidence that superoxide generated in one mitochondrion can induce superoxide production in the neighboring mitochondria in this cell type (196). However, cardiac myocytes have a uniquely high mitochondrial density and stacked mitochondrial organization, and it is currently unknown if a similar superoxide-induced mitochondrial superoxide production occurs in neurons. Weighing against this idea are the observations that NMDA-induced superoxide production is not reduced in neurons that overexpress mitochondrial superoxide dismutase or in wild-type neurons treated with inhibitors of mitochondrial electron transport (17).

As noted above, several reports suggest that some NOX isoforms, including NOX2, can be localized intracellularly (26, 61, 99). The role of these intracellular NOX isoforms in excitotoxic injury remains uncertain, but the phenomenon of cell-to-cell transmission of excitotoxic injury (excitotoxic spread) further supports NOX2 at the plasma membrane to be the primary source of NMDA-induced neuronal superoxide production. Excitotoxic spread would be favored by superoxide released into the extracellular space, whereas superoxide released into the intracellular space would more likely affect the cell in which it was released. These alternatives were evaluated using neuronal cultures in which only a small subset of neurons expressed functional NOX2. The addition of NMDA to these cultures produced oxidative damage and death primarily in neurons nearby to NOX2-expressing neurons, and much less frequently in the NOX2-expressing neurons themselves (Fig. 2). Moreover, the cell-to-cell transmission of oxidative stress was blocked by the addition of superoxide dismutase to the medium, thereby confirming the extracellular oxidant species to be superoxide (150).

As in excitotoxicity, a crucial role for NOX in cell death has been identified in animal models of stroke. Ischemia–reperfusion increases the NOX2 activity in the brain, and both inhibitors of NOX2 activity and genetic downregulation or deficiency of NOX2 components reduce oxidative stress and infarct size in focal cerebral ischemia (81, 89, 90, 178, 186). These manipulations likewise reduce oxidative stress and neuronal death after transient forebrain ischemia (77, 172, 193, 195). The preponderance of evidence points to NOX2 as the dominant NOX isoform generating superoxide in ischemia [as reviewed by Kahles and Brandes (83, 84)], but evidence suggesting a role of NOX4 in ischemic injury

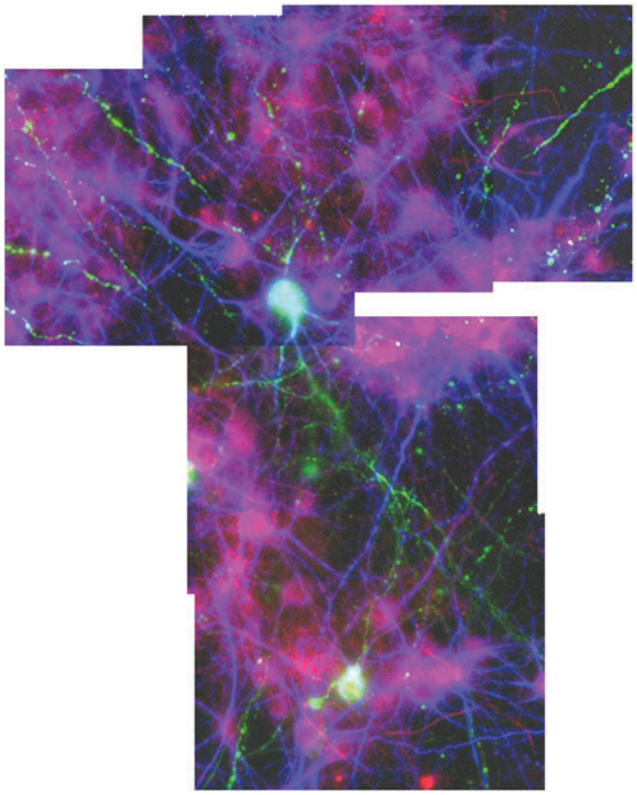

FIG. 2. Neuronal NOX2 activation leads to oxidative stress in neighboring neurons. $p47^{phox-/-}$ neurons, which cannot assemble a functional NOX2 complex, were transfected at low efficiency with GFP-labeled $p47^{phox}$ (*green*) to reconstitute the NOX2 activity in a small fraction of cultured neurons. The cultures were treated with 100 μM N-methyl-D-aspartate (NMDA) for 30 min. 4-hydroxynonenal (4HNE) formation (red) identifies oxidative stress in many nontransfected neurons contiguous with processes of the two NOX2-competent neurons in the field (*green*). Neuronal soma and processes are labeled *blue* by immunostaining for MAP2. Reprinted from Reyes *et al.* (150). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

has also been presented (96). Responses in neonatal brain may differ; however, one study found that NOX2 inhibition and downregulation reduced neonatal hypoxic/ischemic injury (112), but an earlier study did not (49).

The role of NOX2 in cell death following permanent focal ischemia may be less important than in ischemia–reperfusion (91), a point that will be addressed below. It should also be noted that superoxide is additionally produced by astrocytes and microglia in the brain at later time points (hours to days) after ischemia as part of the innate immune response to injury (13, 27, 111, 191, 195). This inflammatory oxidative stress may be a significant cause of secondary ischemic brain injury.

Regulation of NOX2 During NMDA Receptor Stimulation

NOX2 activation in phagocytes requires several independent events, including phosphorylation and membrane translocation of the cytosolic $p47^{phox}$ subunit, activation and translocation of the small GTPase Rac (Rac1 and/or Rac2, depending upon cell type), the activation of phosphoinositide-3-kinase (PI3K), and activation of phospholipase A2 (PLA2) (10, 33). Under resting conditions, $p47^{phox}$ assumes an autoinhibitory conformation, which prevents binding to the membrane-bound $p22^{phox}$ subunit (Fig. 3). Extensive phosphorylation of serine residues on $p47^{phox}$ leads to unfolding and unmasking of key binding domains, including a PX (phox) binding domain, which is required for binding to $p22^{phox}$ and membrane lipids, and SH3 (Src homology) binding domains, which target $p47^{phox}$ to a proline-rich region located in the C-terminus of gp91phox (63, 104). In phagocytes, these serine residues are phosphorylated by PKC, which is in turn activated by changes in intracellular calcium following receptor-mediated influx of extracellular calcium or release from internal stores. Neurons, unlike phagocytes and other cell types, uniquely activate NOX2 in response to NMDA receptor stimulation, and consequently, $p47^{phox}$ phosphorylation is achieved in a somewhat different way.

Calcium influx through NR2B-containing NMDA receptors

Calcium influx has long been established as a requisite event in excitotoxicity (29), but it has been difficult to resolve whether the route of calcium influx is important in this process (23, 118, 165). Tymianski and colleagues showed that NMDA-induced nitric oxide production requires calcium influx specifically through NMDA receptors (157, 158). The pathway leading to NOX2 activation similarly requires calcium influx selectively *via* NMDA receptors.

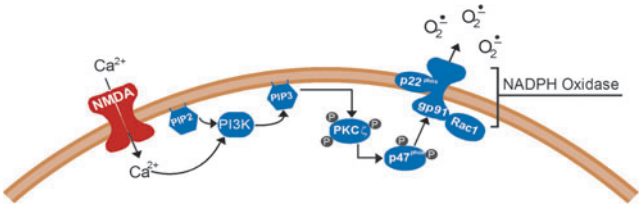

FIG. 3. Coupling of NOX2 activation to NMDA receptor activation in neurons. Calcium influx *via* NR2B-containing NMDA-type glutamate receptors induces phosphoinositide-3-kinase (PI3K) to form phosphatidylinositol (3,4,5) trisphosphate (PIP3). PIP3 activates protein kinase C (zeta) (PKCζ), which in turn phosphorylates the $p47^{phox}$ organizing subunit of NOX2. Phosphorylated $p47^{phox}$ induces assembly of the NOX2 complex at the cell surface. The active NOX2 complex additionally requires binding to an activated GTPase, Rac1. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

NMDA receptors are usually composed of two NR1 subunits and two NR2 subunits, of which four subtypes have been described: NR2A, NR2B, NR2C, and NR2D (127, 181). Neuronal NMDA-induced NOX2 activation is blocked by selective antagonists of NR2B subunits (16), by NR2B deletion, or by replacing the NR2B C-terminus with the NR2A C-terminus (Brennan-Minnella, unpublished results). This specific requirement for NR2B in NMDA-induced NOX2 activation is consistent with recent studies identifying NR2B-containing NMDA receptors as responsible for excitotoxic neuronal death (118). Specificity for the route of Ca^{2+} influx in this process has additionally been shown by studies in which, Ca^{2+} elevations of comparable magnitude induced by ionomycin, as measured with both high- and low-affinity calcium-sensitive dyes, do not induce $p47^{phox}$ phosphorylation or NOX2 activation in neurons (16). However, ionomycin-induced calcium elevations, to levels much higher than occur with NMDA receptor activation, can trigger neuronal superoxide production by mechanisms independent of NOX2 (16, 165).

Roles of PKCζ and PI3K

Neurons, unlike phagocytes, activate $p47^{phox}$ primarily *via* the PKC isoforms, PKCζ and PKCδ. Peptide inhibitors of both PKCζ and PKCδ have been shown to block neuronal death induced by NMDA receptor activation and ischemia (18, 35, 98), and the role of these PKC isoforms in activating NOX2 thus provides a potential mechanism for this neuroprotective effect. PKCζ and PKCδ are not directly activated by cytosolic Ca^{2+} elevations (36), consistent with the failure of ionomycin-gated calcium to activate neuronal NOX2 (16). Instead, PKCζ (and possibly PKCδ) is activated by a pathway involving PI3K (Fig. 3).

Unlike conventional PKCs, which are activated by calcium and/or diacyl glycerol, PKCζ requires binding to phospholipids generated by PI3K, specifically phosphatidylinositol (3,4,5) trisphosphate (32). PI3K phosphorylates the 3 position of hydroxyl groups on the inositol ring of specific phosphatidylinositol membrane phospholipids (25), which are concentrated at the cytosolic surface of plasma membranes. These phosphorylated lipid substrates in turn recruit signaling proteins with lipid binding domains, the most common being pleckstrin homology (PH) domains, FYVE domains, and importantly for NOX2 activation, PX homology domains (107).

Both the PI3K inhibitor wortmannin and a PKCζ inhibitory peptide prevent $p47^{phox}$ phosphorylation, superoxide formation, and cell death, without blocking NMDA-induced calcium influx or mitochondrial depolarization (16). These factors suggest that PI3K activation is upstream of PKCζ in the pathway linking neuronal NMDA receptors to NOX2. Moreover, the wortmannin-dependent inhibition of NMDA-induced superoxide production can be overcome by either transfection with a constitutively active form of PKCζ or by exogenous supply of phosphatidylinositol (3,4,5) trisphosphate (16).

NMDA receptor activation is known to induce phosphorylation of Akt, an established downstream target of PI3K (185); however, the mechanism by which NMDA-gated Ca^{2+} influx triggers PI3K activation remains unresolved. Early reports suggested that the SH2 binding domain of the p85 subunit of PI3K could bind directly to phosphorylated tyrosine residues on the NR2B subunit of NMDA receptors. More recently, however, it has been shown that the PI3K activation by NMDA receptors may require the adaptor protein APPL1 (75, 131, 189).

The role of PI3K in excitotoxicity and oxidative stress contrasts with the well-established, prosurvival effects of PI3K mediated by the Akt pathway (19). Previous work has demonstrated that the prosurvival pathways required for the survival of developing neurons are dependent on NMDA-induced activation of PI3K (102, 140), and that selective disruption of NMDA-induced Akt signaling results in widespread neuronal apoptosis (189). The contrasting toxic and trophic roles of NMDA-induced PI3K activity may be reconciled by the observations that PI3K and superoxide are both mediators of intercellular signaling and synaptic plasticity in the nervous system (37, 93, 121). The cytotoxic, PI3K-dependent superoxide production that occurs during sustained NMDA receptor activation may thus be viewed as an exaggerated, pathological manifestation of a process that normally functions in neuronal plasticity.

In addition to their role in physiological signaling, there is evidence that PI3K and NOX-dependent superoxide are required for ischemic preconditioning. Ischemic preconditioning is a process whereby cells exposed to sublethal ischemia acquire resistance to subsequent otherwise lethal insults (56). In both an *in vitro* model using oxygen glucose deprivation in cultured astrocytes (155) or with transient global ischemia in mice (124), the protective effects of ischemic preconditioning were abolished when the selective inhibitor of PI3K, LY294002 was administered. Intriguingly, NAPDH oxidase activity is also required for ischemic preconditioning, at least in certain settings. The protective preconditioning effect achieved with ethanol is reversed by the NOX inhibitor apocynin (187), and similarly, lipopolysaccharide (LPS) administration fails to elicit ischemic preconditioning in NOX2-deficient animals (80, 88, 101). These observations suggest that the protective effects of ischemic preconditioning may require both PI3K activation and NOX superoxide production.

Regulation of NOX2 by Rac and protein kinase CK2

Rac1 (or in some cells, Rac2) is an additional requisite component of functional NOX2 oxidase, and it may also be activated by PI3K (25). Rac1 is a Rho GTPase signaling protein and functions as a guanine nucleotide exchange factor to catalyze the conversion of inactive GDP-bound GTPases to active GTP-bound complexes. Rac1 contains lipid binding domains, and PI3K activity increases GTP-bound Rac1 (70, 78). Ligand-dependent activation of NOX in fibroblasts and astroglioma cells is prevented by a dominant negative Rac1, (106, 173), and conversely, the NOX activity is increased by a constitutively active form of Rac1 (173). Evidence suggests that Rac1 is required for translocation of the $p67^{phox}$ cytosolic subunit (123), and may also act upstream of other required lipid mediators of NOX activation such as PLA2 (106, 144). In a stroke model, Brann and colleagues reported that the Rac inhibitor NSC23766 attenuated NOX activation and reduced oxidative stress and neuronal death (149). Interestingly, 17 beta-estradiol leads to phosphorylation/inactivation of Rac1 by an extranuclear signaling mechanism, resulting in a profound attenuation of NOX activation and superoxide production in ischemia–reperfusion (15, 195); a factor that may contribute to the significant gender differences observed in ischemic brain injury (79).

Rac1 is additionally regulated by protein kinase CK2 (also known as casein kinase 2) a serine/threonine kinase found in neurons and most other cell types (164). CK2 consists of three different subunits; two catalytic (α and α') and one regulatory subunit (β). Recent work showed that neuronal CK2 protein expression and kinase activity are significantly reduced in the ischemic cortex (90). Moreover, pharmacological or siRNA-mediated suppression of CK2 activity exacerbates neuronal death in both cell culture and animal models of brain ischemia (89, 90). Decreased levels of CK2 correlated with increased NOX assembly and NOX-dependent superoxide production. Coimmunoprecipitation studies showed a direct physical interaction between CK2 and Rac1, and the authors proposed a model in which, under basal conditions, CK2 binds Rac1, preventing activation of NOX (90). Following ischemia, proteasome-dependent degradation of CK2 allows for Rac1 release and facilitates NOX complex assembly and superoxide formation (90).

Effect of intracellular acidification on NOX2

Cerebral ischemia produces acidosis to varying degrees. Severe acidosis, below pH 6.4, exacerbates cell ischemic injury (100, 145, 146, 192), but lesser degrees of acidosis, in the range of pH 7.0–6.5, reduce both ischemic injury (161) and glutamate-induced neuronal death (86, 179). These effects may be attributable, in part, to an inhibitory effect of hydrogen ions on calcium influx through NMDA receptor channels, but this effect is minimal at pH values above 6.6, (176, 180, 184).

NOX2 activity is highly pH sensitive (68, 125, 160, 182), with a 50% reduction in activity observed with a 0.2 pH unit reduction in intracellular pH. In many cell types, H^+ generated by NOX2 is released to the extracellular space through voltage-dependent hydrogen ion channels or by Na^+/H^+ exchangers, and inhibition of this release causes intracellular acidification and resultant cessation of NOX2 activity (111, 148, 159). The mechanism of this effect is unlikely to be a simple mass action, given its steep pH dependence; instead, evidence suggests that the H^+ concentration influences the phosphorylation status of $p47^{phox}$ (159).

Given the pH sensitivity of NOX2 and the role of this enzyme in NMDA receptor-mediated cell death, modest reductions in pH might limit neurotoxicity by dissociating NMDA receptor activation from superoxide production (Fig. 4). Studies confirm that both the superoxide production and cell death resulting from neuronal NMDA receptor activation are extremely pH sensitive, with a near complete reduction observed at intracellular pH below 6.7 (normal is pH 7.0). Acidification induced by Na^+/H^+ exchange inhibition or NHE1 gene deficiency also blocks superoxide production and cell death induced by NMDA injected into the mouse striatum, and reduces neuronal injury after ischemia–reperfusion (103, 114).

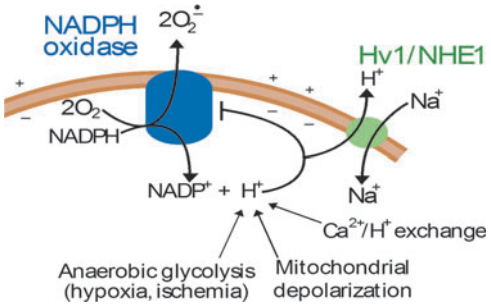

FIG. 4. Intracellular H^+ inhibits NOX2 activity. The oxidation of NADPH during the enzymatic activation of NOX also produces H^+, and removal of these H^+ is required for sustained NOX activity. In most cells types, this is accomplished by the proton channel, Hv1, and/or the Na^+/H^+ exchanger, NHE1. In addition, to production of H^+ by NOX, several other pathways may contribute to intracellular acidification during ischemia, and thus indirectly inhibit NOX2 activity. These include anaerobic metabolism of glucose to lactic acid, mitochondrial depolarization, and extrusion of Ca^{2+} by Ca^{2+}/H^+ exchange. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Excessive superoxide production may be deleterious in ischemia and other settings in which sustained NMDA receptor stimulation, and thus, it is possible that intracellular acidosis may function in a teleologically advantageous way to limit superoxide production. Neurons acidify during ischemia as a result of anaerobic lactic acidosis and other processes (Fig. 4), and the reduced blood flow during ischemia prevents removal of accumulated H^+. Importantly, reperfusion restores aerobic metabolism and clears accumulated H^+, thereby restoring the NOX2 activity. This effect of reperfusion may thereby provide an explanation for why superoxide production is greater in reperfused than nonreperfused ischemic brain, and why both NOX2 inhibitors and NMDA receptor antagonists are more effective as neuroprotective agents in reperfused than nonreperfused brain (22, 91, 175).

PLA2 in stroke and NOX2 activation

PLA2 is a family of enzymes that catalyze the removal of fatty acids from membrane phospholipids at the sn-2 position to produce lysophospholipids and free fatty acids, such as arachidonic acid (2). Several lines of evidence implicate PLA2 in the pathogenesis of ischemic brain injury. PLA2 expression and activity are increased in the injured region of the brain after ischemia (3, 4, 34, 134), and both genetic (12) and pharmacological (76, 194) reductions in PLA2 activity reduce injury in animal models of stroke. Increased PLA2 expression is associated with increased oxidative stress after ischemic injury, and the increase in oxidative stress is attenuated in PLA2-deficient mice (95).

Phagocytes deficient in PLA2 exhibit reduced NOX2 activity in response to phorbol ester and other stimuli. Furthermore, exogenous addition of free arachidonic acid, but not other free fatty acids or metabolites of arachidonic acid, is sufficient to restore NOX function (40, 117). Studies using pharmacological inhibitors or antisense downregulation of PLA2 also show that arachidonic acid production is required for NOX superoxide production (108). The mechanism by which arachidonic acid regulates NOX function remains uncertain, but there is evidence in phagocytic cells that localized arachidonic acid production is required for the release of H^+ formed by NOX (72). In addition to these direct effects, arachidonic acid can be further metabolized by cyclooxygenases and lipoxygenases to form bioactive eicosanoids that indirectly lead to oxidative stress (2) and facilitate NOX2 activation by an as-yet unresolved mechanism (42, 108).

Glucose, Hyperglycemia, and NOX2 Activity

The deleterious effect of elevated blood glucose (hyperglycemia) is one of the most robust and reproducible aspects of stroke, particularly in ischemia–reperfusion (119, 130, 143). A widely cited mechanism by which hyperglycemia can exacerbate brain injury is through fueling the accumulation of lactic acid in hypoxic tissues (146); however, elevated glucose concentrations also exacerbate injury in brain slice models of ischemia in which pH is tightly controlled (132, 156), and in animal models of brain ischemia in which tissue acidosis is relieved by reperfusion (38, 142, 183). Moreover, clinical studies suggest that hyperglycemia during reperfusion increases risk of poor outcomes, independent of diabetes or preischemic blood glucose concentrations (20, 152). These observations suggest that a mechanism other than lactic acidosis may contribute to the effect of glucose on reperfusion injury.

In phagocytes and other cell types, the activity of NOX requires the continuous metabolism of glucose by the hexose monophosphate shunt for supply of NADPH substrate (45, 67) (Fig. 5). In cultured neurons, NMDA-induced superoxide production and cell death are both blocked when glucose is omitted from the culture medium, suggesting a similar requirement for glucose in neuronal NOX2 function (17). The same pattern was observed in neuronal cultures exposed

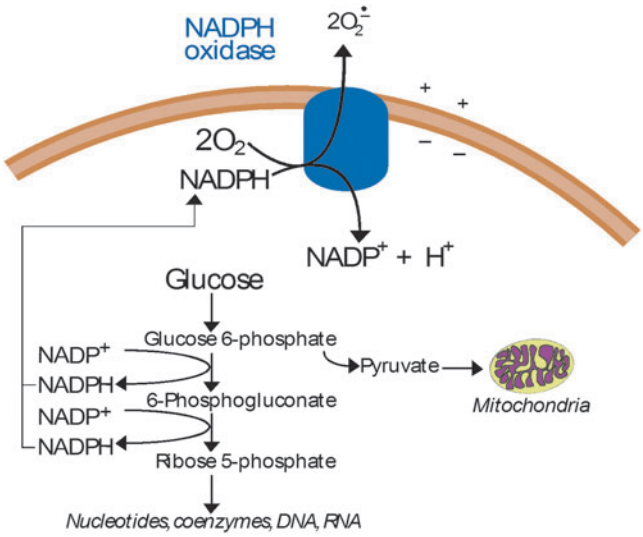

FIG. 5. Glucose is required for NOX2 activity. NOX2 oxidizes NADPH to $NADP^+$, and continuous NOX2 function requires a continuous NADPH supply. NADPH is regenerated by the pentose phosphate pathway, which uses glucose as an obligate substrate. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

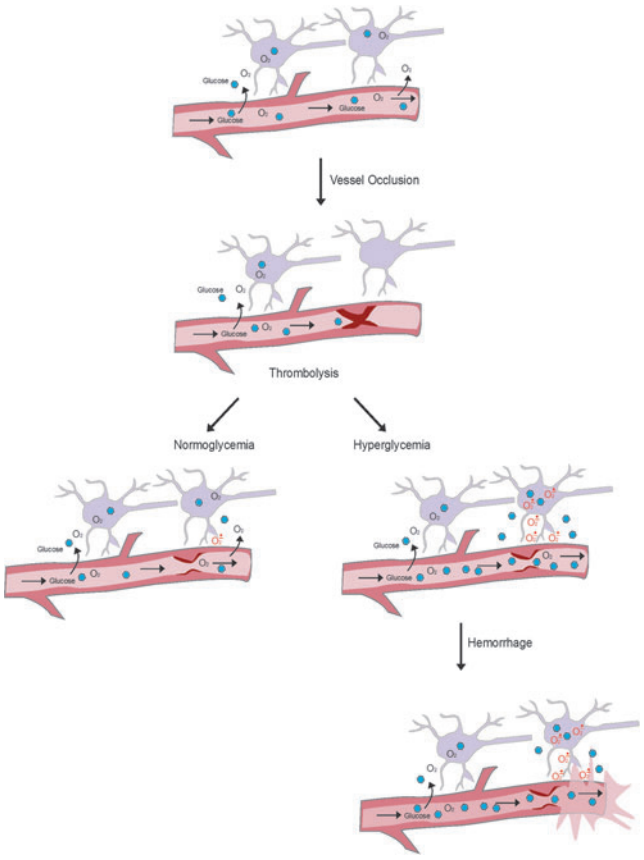

FIG. 6. Hyperglycemia promotes reperfusion injury and hemorrhage by fueling NOX2. Neuronal superoxide production is low under basal conditions. It remains low after arterial occlusion because of limited oxygen supply and lactic acidosis. Upon reperfusion, the oxygen supply is restored and lactic acid is washed out. NOX2 then produces high levels of superoxide in response to extracellular glutamate acting at NMDA-type glutamate receptors. Glucose availability can be rate limiting for NOX2 superoxide production, and under hyperglycemic conditions, the increased glucose supply leads to increased superoxide production. This in turn can increase damage to blood vessel walls and promote reperfusion hemorrhage. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

to transient oxygen/glucose deprivation, a model used to mimic brain ischemia–reperfusion. Neurons in these cultures showed a rapid increase in superoxide production upon return of oxygen and glucose to the medium, and this did not occur if either glucose was omitted or glucose metabolism to NADPH was pharmacologically inhibited (172). Conversely, in a mouse model of ischemia–reperfusion, the superoxide produced during reperfusion was augmented by hyperglycemia, and this effect of hyperglycemia was negated in apocynin-treated and $p47^{phox-/-}$ mice (which cannot form an active NOX2 complex). These reports suggest that hyperglycemia can exacerbate reperfusion injury by supplying increased glucose substrate for superoxide production by NOX (Fig. 6).

Hyperglycemia and Reperfusion Hemorrhage

The only medical treatment proven clinically effective for acute stroke is thrombolysis with tissue plasminogen activator (tPA). However, the use of tPA carries the risk of reperfusion hemorrhage, and this restricts its use to patients who can be treated within 3–6 h after the onset of symptoms (64). Clinical studies have established a strong association between admission hyperglycemia and the risk of hemorrhage with tPA use, independent of prior diabetes, with the risk of hemorrhage roughly doubling with each 5.5 m*M* (100 mg/dl) increase in blood glucose (8, 21, 44, 46, 73, 87, 163). The reason for this increased risk has not been established, but may also be attributable to an increased NOX2 activity (Fig. 5). Rats undergoing focal ischemia and given tPA at reperfusion were found, as in the clinical studies, to have increased brain hemorrhage if they were rendered hyperglycemic. The hyperglycemia also induced increased superoxide formation in the reperfused brain, and both the increased superoxide and increase hemorrhage rate were prevented by the NOX2 inhibitor apocynin (190). This result is consistent with other studies demonstrating a role of NOX2 in the blood–brain barrier disruption (85, 177).

Conclusions

Studies reviewed here indicate that neuronal superoxide production in stroke and excitotoxicity is not an inevitable physical consequence of mitochondrial calcium overload, but is instead the end result of a complex and regulated signaling pathway linking NMDA receptors and NOX2. Factors and regulatory steps involved in this pathway include glucose supply, intracellular pH, PI3K, Rac1, PLA2, and PKCζ/δ, csk2, and estrogen. Many of these factors have previously been identified as being critical mediators of cell death in stroke, and consequently, their roles as regulators of NOX2 activity provide new or alternative mechanisms for their effects on ischemic brain injury. Conversely, dysregulation of these regulatory factors could in principle amplify superoxide production and cause cell injury at otherwise benign levels of NMDA receptor stimulation.

The key aspects of these signaling pathways remain unresolved. In particular, it remains unclear whether PI3K is directly upstream of the Rac1 activation required for NOX2 activation, or if Rac1 is activated by an independent pathway. Similarly, it is unclear how PLA2 is activated during excitotoxicity and how, at a biochemical level, PLA2 regulates NOX2. The potential interactions between NOX2 and neuronal mitochondria also remain to be established, and it remains possible that superoxide produced by NOX2 could damage mitochondria and thereby induce mitochondrial superoxide release in a feed-forward manner. A fundamental unresolved question is the physiological function of the NMDA/NOX2 signaling pathway in the brain. Superoxide production and NMDA receptor activation have each, independently, been shown to influence synaptic plasticity, the process by which learning and memory occur (115, 121, 129). The cytotoxic superoxide production that occurs during sustained NMDA receptor activation in stroke might thus represent an exaggerated, pathological manifestation of a process that is unique to CNS.

Specific, small molecule inhibitors of NOX2 and other NOX isoforms are currently under development (92), and there is growing enthusiasm for using this approach in the treatment of acute stroke (24, 83, 147). This enthusiasm must be tempered, however, by the experience gained from prior

studies of neuroprotectant therapy in stroke. To be clinically useful, NOX inhibitors must pass readily into the ischemic brain and have tolerable side effects. Most importantly, clinical efficacy with this approach will almost certainly require administration within relatively short periods (hours) of ischemic onset, given the rapid pace of ischemic cell death processes. Studies using NOX inhibitors in animal models of stroke also suggest that rapid administration will be needed (77, 81, 96, 172, 178, 193, 195). This being said, the potential for NOX inhibitors as neuroprotective agents during therapeutic thrombolysis or clot removal is significant, particularly, given the role of reperfusion in NOX2 activation.

Many of the regulatory factors shown to influence the NOX2 activity are pharmacologically accessible, and interventions targeting these factors have been shown to block both excitotoxic and acute ischemic injury. It remains to be established whether these interventions can provide a greater neuroprotective efficacy or a longer time window to treatment than provided by NOX2 or NMDA receptor antagonists, or if dissociating superoxide production from other downstream signaling effects of NMDA receptor activation can mitigate the toxic effects of NMDA receptor blockade (66, 136).

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Abbreviations Used

4HNE = 4 hydroxynonenal
CNS = central nervous system
CK2 = protein kinase CK2 (also known as casein kinase 2)
LPS = lipopolysaccharide
NADPH = nicotinamide adenine dinucleotide phosphate (reduced form)
NMDA = N-methyl-D-aspartate
NOX = NADPH oxidase
PI3K = phosphoinositide-3-kinase
PIP3 = phosphatidylinositol (3,4,5) trisphosphate
PKCδ = protein kinase C (delta)
PKCζ = protein kinase C (zeta)
PLA2 = phospholipase A2
tPA = tissue plasminogen activator