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Which NADPH Oxidase Isoform Is Relevant for Ischemic Stroke? The Case for Nox 2

Timo Kahles and Ralf P. Brandes

Abstract

Significance and Recent Advances: Ischemic stroke is the leading cause of disability and third in mortality in industrialized nations. Immediate restoration of cerebral blood flow is crucial to salvage brain tissue, but only few patients are eligible for recanalization therapy. Thus, the need for alternative neuroprotective strategies is huge, and antioxidant interventions have long been studied in this context. Reactive oxygen species (ROS) physiologically serve as signaling molecules, but excessive amounts of ROS, as generated during ischemia/reperfusion (I/R), contribute to tissue injury. ***Critical Issues:*** Nevertheless and despite a strong rational of ROS being a pharmacological target, all antioxidant interventions failed to improve functional outcome in human clinical trials. Antioxidants may interfere with physiological functions of ROS or do not reach the crucial target structures of ROS-induced injury effectively. ***Future Directions:*** Thus, a potentially more promising approach is the inhibition of the source of disease-promoting ROS. Within recent years, NADPH oxidases (Nox) of the Nox family have been identified as mediators of neuronal pathology. As, however, several Nox homologs are expressed in neuronal tissue, and as many of the pharmacological inhibitors employed are rather unspecific, the concept of Nox as mediators of brain damage is far from being settled. In this review, we will discuss the contribution of Nox homologs to I/R injury at large as well as to neuronal damage in particular. We will illustrate that the current data provide evidence for Nox2 as the most important NADPH oxidase mediating cerebral injury. *Antioxid. Redox Signal*. 18, 1400–1417.

Introduction

FOLLOWING HEART DISEASE and cancer, stroke is the third leading cause of death in industrialized nations. The World Health Organization reported that estimated 15 million people suffer from an acute cerebrovascular event each year, taking the life of 5.5 million/year and accounting for 5 million permanently disabled patients/year (176).

Ischemic stroke is caused by a sudden cessation of cerebral perfusion due to cerebral artery occlusion, whereas rupture of an intracranial vessel results in hemorrhagic stroke or subarachnoidal hemorrhage. Ischemic stroke accounts for more than 80% of overall strokes. Obviously, rapid and early restoration of cerebral blood flow is pivotal to minimize persistent brain damage in this situation. Currently, reperfusion is mostly achieved by thrombolytic therapy with recombinant tissue plasminogen activator, which has proved to be beneficial within the first 4.5 h after the onset of symptoms (72). Alternatively, mechanical recanalization strategies have been developed, but it is currently open whether this approach is superior to thrombolysis (119). No matter which method is used, rapid reperfusion is the goal of all therapies, and a lack of reperfusion results in bad outcome by all means. It is however assumed that supportive neuroprotective measures might help to extend the narrow time window for clinical beneficial reperfusion therapy (141).

With the restoration of cerebral blood flow and thus supply of glucose and oxygen (O_2), oxidation-promoting enzymes and mitochondria are activated and generate an extensive surge of reactive oxygen species (ROS). At low concentrations, ROS act as signaling molecules and contribute to physiological cell functions (42, 92, 101). The excessive ROS formation during reperfusion after ischemic injury, however, leads to overt oxidative stress and contributes to tissue damage (23, 108, 146). Interestingly, the brain appears to be exceptionally vulnerable to oxidative stress due to its already-restricted antioxidant capacity (3). Thus, lowering ROS seemed to be a promising supportive approach in stroke therapy (71). In patients suffering from acute ischemic stroke, however, not a single substance has proved to be effective in improving functional outcome in clinical trials so far (67, 91). Just recently, NXY-059, a spin-trap agent, even though promising in

Institut für Kardiovaskuläre Physiologie, Fachbereich Medizin der Goethe-Universität, Frankfurt am Main, Germany.

preclinical experiments and in a smaller pilot study, failed to be protective in the confirmatory phase III clinical trial (45, 151). Potential reasons for the failure of antioxidant therapy include interferences with physiological, required functions of ROS, lack of access to target regions and cells of the brain, and failure to reach sufficient concentrations at the site of ROS-mediated damage in cellular subcompartments, the latter being the consequence of low reactivity of the antioxidant or insufficient accumulation within cells as reviewed by (177).

Most of these problems could be overcome if ROS-scavenging therapies would be replaced by approaches lowering ROS generation with specific and effective inhibitors of ROS-generating enzymes. Pathology-associated ROS potentially derive from several enzymatic sources, including xanthine oxidase, cyclooxygenases, or mitochondria (165). ROS-producing NADPH oxidases of the Nox family have been studied extensively in vascular physiological and pathophysiological processes, but also other organs, including the brain. More and more evidence suggests that NADPH oxidases of the Nox family play a major role in function and also in dysfunction of various cell types of the brain (cf. 5.2) (101, 153). In particular, during reoxygenation, NADPH oxidases (Nox) appear to be a main source of ROS production (2).

This review highlights the findings of NADPH oxidase of the Nox family as mediators of cerebral disease in general and in particular of cerebral ischemia/reperfusion (I/R) injury. We particularly will focus on the contribution of individual Nox homologs in this context and analyze the data on pharmacological Nox inhibition regarding their isoform-specific actions.

NADPH Oxidase-Mediated ROS Production

Nox structure and regulation

The mammalian Nox family of NADPH oxidases comprises seven members, denominated Nox1 to Nox5, dual oxidase (Duox)-1, and Duox-2. Duox-1 and 2 appear to be expressed predominantly in epithelial cells (15), whereupon disease processes, they might also be induced in other cells. Tissue-specific expression was mainly found for Nox3, which is almost exclusively expressed in the inner ear. The least *in vivo* studied Nox homolog is Nox5, which due to a gene deletion is missing in mice or rats (63), the working horses of today's animal research. Similar as the Duox enzymes, Nox5 is activated by calcium, and several recent studies suggest that in human cells, Nox5 might be an important source of ROS, for instance, in vascular cells [(121), reviewed by (13)]. As however almost nothing is known regarding possible functions of Duox enzymes, Nox3, and Nox5 in the brain, we will focus this review on Nox1, Nox2, and Nox4 (Fig. 1), which are all expressed in the cardiovascular system and in the brain, and which have all been linked to cerebral disease processes (36, 83, 89). As stroke is also to some extent a vascular disease, analysis of vascular NADPH oxidases is essential for its understanding.

The Nox homologs are named by the large catalytic Nox protein. To achieve catalytic activity, Nox1, Nox2 as well as Nox4 have to form an integral membrane complex with p22phox. Nox2 (formerly known as gp91phox) and Nox1 require additional cytosolic subunits for their activation: the small GTPase Rac, $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$ as well as NoxA1 and NoxO1, respectively. Activation of Nox1 and Nox2 usually occurs by agonist stimulation, for instance, with tumor necrosis factor α or angiotensin II (Ang II) through assembly and translocation of the regulatory cytosolic subunits to the membrane. The prototype NADPH oxidase is Nox2, which was first identified in phagocytes as a mediator of host defense. Although data on cell-specific distributions of Nox homologs have become more and more diverse and conflicting, most evidence supports that Nox2 is the predominant Nox homolog of endothelial cells (ECs), cardiomyocytes, and fibroblasts and cells derived from hematopoietic linages, whereas Nox1 dominates in epithelial cells and cancer, but is also expressed in smooth muscle cells (SMCs). In contrast, Nox4, which to our current knowledge does not require cytosolic subunits, is constitutively active and ubiquitously expressed in differentiated cells of all embryonic germ layers. Induction of Nox4 has been demonstrated during differentiation, healing, and in response to hypoxia and transforming growth factor $\beta 1$ in several cell types, but also after application of cannabidiol in human leukemia cells [reviewed by (92, 120)]. While Nox1 and Nox2 are expressed in the plasma membrane, the subcellular localization of Nox4 has been a matter of longstanding debate. In addition to the nucleus, the plasma membrane, focal adhesions, the cytoskeleton, and the endoplasmic reticulum, Nox4 was also spotted in mitochondria (69, 99), and suggested to crosstalk with ROS production of the respiratory chain (46, 179).

In general, Nox enzymes of the NADPH oxidase family facilitate transport of electrons from NADPH onto O_2,

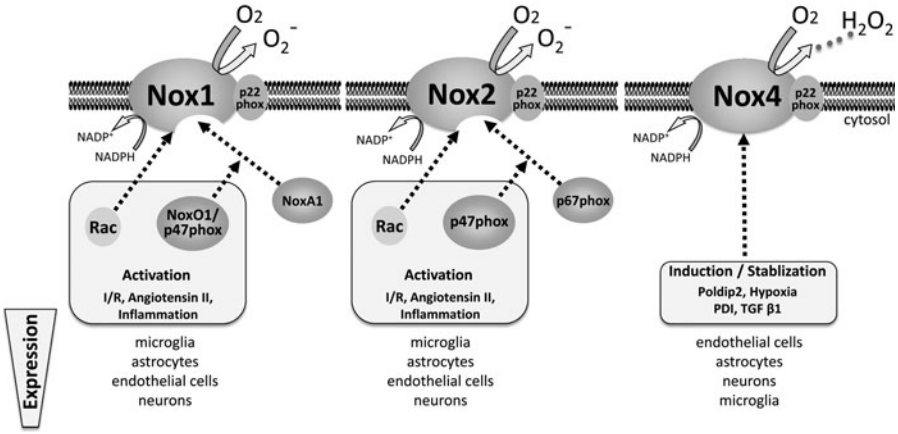

FIG. 1. NADPH oxidases in the brain: Structure, activation, and expression. Only data for Nox1, Nox2, and Nox4 are shown, as the other Nox protein have basically not been studied in nervous tissue. H_2O_2, hydrogen peroxide; I/R, ischemia/reperfusion; Nox, NADPH oxidases; O_2, oxygen; O_2^-, superoxide; PDI, protein disulfide isomerase; TGF $\beta 1$, transforming growth factor $\beta 1$.

resulting in the generation of superoxide (O_2^-). Duox enzymes and Nox4, however, predominantly produce hydrogen peroxide (H_2O_2) (47, 109, 148, 158, 160). Although the mechanisms underlying the potentially functional important difference are still unclear, these different products might hint at different functions of Nox1/2 compared to Nox4. For instance, only O_2^-, but not H_2O_2, reacts with nitric oxide (NO) to form peroxynitrite ($ONOO^-$), which is a much more powerful oxidant than O_2^- or H_2O_2. In contrast, H_2O_2 was reported to even increase the expression and activity of endothelial nitric oxide synthase (28, 53), and thus some recent data support a protective function of Nox4 in the vascular system (40, 138, 186).

Physiological Functions of Nox Proteins Outside the Brain

Physiologically, Nox-derived ROS either serve as signaling modulators and second messengers [for review see (14, 25)] or fulfill specific functions as reactive intermediates in highly defined specific reactions. The latter comprised Nox2 in host defense (136, 155), Nox3 in otoconia formation of the inner ear (127), or Duox-2 in thyroid hormone synthesis (113). Nox proteins have also been implicated in O_2 sensing, but this aspect has not been settled sufficiently, and is a matter of ongoing debate (9, 62, 175, 184). In any case, a link between O_2 and Nox proteins has long been sought, in particular, due to the fact that the enzyme itself requires O_2, and it is still debated whether only hyperoxia or also hypoxia is accompanied by an increased formation of ROS (62). Biochemically, hypoxia is a rather reducing situation, but not only the redox potential but also in the case of NADPH oxidases, and the pathways leading to ROS formation are subject to alterations under this condition (137). Nevertheless, the evidence that Nox activation occurs at the onset of reperfusion is much stronger than that in response to hypoxia (2).

Nox Proteins and I/R Outside the Brain

A large number of studies demonstrated increased ROS formation during hypoxia/ischemia in general, and there is ample evidence for a surge in ROS generation with reoxygenation/reperfusion. Traditionally, mitochondria have been considered to be the major source of ROS. In addition, a continuously growing number of studies identified Nox proteins as contributors of ROS generation during I/R.

Lung

Several studies reported a role for Nox in I/R injury to the lungs. Al-Mehdi *et al.* (6) found that I/R-mediated ROS increase in lungs involves the activation of Nox2 in the pulmonary endothelium, and bone marrow transplant experiments and tissue-specific Nox2-deficient mice have meanwhile established a central role of endothelial Nox2 in I/R injury in this organ (174), also obviously and expected, leukocytic Nox2 is also not irrelevant. The fact that I/R lung injury was attenuated, and that the induction of proinflammatory cytokines was reduced in wild-type (WT) mice transplanted with bone marrow from $p47^{phox}$-deficient mice, but not vice versa, supported this view (180). Furthermore, in the isolated saline-perfused lung, ischemia led to membrane depolarization and ROS generation in pulmonary microvascular ECs mediated by the phosphoinositide-3-kinase/protein kinase B, protein kinase C (PKC) pathway, which is known to activate Nox2 (30). Accordingly, apocynin, an antioxidant and relatively unspecific Nox2 inhibitor, prevented I/R lung injury (133) and decreased vascular permeability in a sheep model of pulmonary injury (50).

Heart

Potentially as the ratio of endothelium to nonendothelial tissue is much smaller, a role of Nox proteins in myocardial I/R injury is less well established than for the lung and rather controversial. Hoffmeyer *et al.* (78) did not detect a significant difference in the myocardial area at risk or left ventricular function in acute coronary artery occlusion and reperfusion in mice with a mutation of the $p47^{phox}$ subunit of Nox as compared to heterozygous controls. Additional data were derived from models of permanent coronary artery occlusion. Deletion of Nox2 did not affect left ventricular remodeling. In fact, lipid peroxidation products as a marker of oxidative stress and mortality increased in mice lacking a functional Nox2 (61). On the contrary, a significant contribution to adverse cardiac remodeling and contractile dysfunction was found for Nox2 in the murine coronary artery ligation model (106). Moreover, another study suggested a critical role for $p47^{phox}$ after experimental myocardial infarction for left ventricular remodeling/dysfunction and survival (51). Data on the contribution of other Nox isoforms to myocardial dysfunction after infarction or I/R are missing. Moreover, Nox proteins appear to be involved in ischemic preconditioning, which is in itself a situation of I/R: similar as in the brain (98), Nox2 is required to elicit protection by preconditioning in experimental models of myocardial I/R (19, 164). However, without preconditioning, Nox2 deficiency alone failed to affect the infarct size.

Gastrointestinal tract

Data on knockout (KO) mice are still spare for this anatomical region. Pharmacological Nox inhibition with the unspecific apocynin attenuated necrotic and apoptotic cell death, O_2^- production, and lipid peroxidation in the murine and rat models of hepatic I/R (104, 150). Moreover, apocynin and the Nox2-inhibitory peptide gp91ds-tat (41) prevented remote liver damage in a murine model of bilateral hind limb I/R (52). Similarly, inactivation of Rac-1, a mandatory subunit for Nox2 and Nox1 activation, protected the liver from I/R injury in rats (74). Application of dominant negative Rac in $Nox2^{-/-}$ mice, however, demonstrated that the beneficial effect of Rac inhibition extends beyond the blockade of Nox activation (126), which is conceivable, giving the central role of Rac for cytoskeletal functions. Finally, in the splanchnic artery occlusion and reperfusion model in rats, apocynin attenuated tissue injury, proinflammatory cytokine production, adhesion molecule expression, nitrotyrosine formation, nuclear factor kappa B expression, and apoptosis (132).

Evidence of contribution of Nox2 to human I/R

Although plenty of data on Nox-mediated I/R alterations in various organ systems in rodents or in cultured cells are available, it was just recently that Loukogeorgakis *et al.* (107) demonstrated that I/R-induced endothelial dysfunction of

the brachial circulation is absent in patients carrying inactivating mutations of the Nox2 or $p47^{phox}$. Accordingly, *in vitro* anoxia and reoxygenation of human platelets promoted the production of isoprostanes and thromboxane B2 *via* Nox2-dependent ROS formation and vascular function, as well as NO production by platelets was higher in patients with inactive NADPH oxidase due to mutations (12, 171). These data provided the needed strong support for a role of Nox2 in human I/R damage.

Nox Function in the Brain

Nox expression in the nervous system

Nox homolog expression was observed in all parts of the central and peripheral nervous system. Interestingly, several Nox isoforms appear to be coexpressed in the same cell, but execute different functions. Nox1, Nox2, Nox3, and Nox4 messenger ribonucleic acids (mRNAs) were detected in whole-brain extracts by PCR (10, 83), which obviously provides little information regarding functional significant protein levels. To our knowledge, no data on Nox5 expression in the brain have been published so far, but this might be a consequence of the limited availability of nonrodent brain preparations. A few studies determined Nox1, Nox2, and Nox4 protein and/or mRNA expression in defined cerebral regions, of which most of them focused on Nox2 [reviewed by (153)]; these analyses, however, are far from being exhaustive, and are rather erratic than systemic.

Interestingly, cerebral arteries have been studied in detail, and Nox1, Nox2, Nox4, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, NoxA1 and NoxO1 were observed to be expressed at the mRNA level, as well as Nox1, Nox2, Nox4 and $p22^{phox}$ on the protein level in mice and rats. Interestingly, Nox4 expression was 10-times higher in the rat basilar artery compared to the aorta, and likewise, Nox2 content as well as Nox activity of cerebral arteries were higher than in systemic arteries. Given that these arteries, however, have a different tissue composition and ratio of endothelium to SMCs and fibroblasts, it is still open whether the difference is functionally important or just a consequence of the different cellularity. Nox4 expression in the basilar artery of male rats exceeded that of female rats (116), and gender differences have previously been noted for the expression of other Nox homologs, too. Thus, care should be taken to study male and female mice separately.

Endothelial cells. Cerebral vascular ECs exhibit high Nox expression: Nox1, Nox2, Nox4, $p47^{phox}$, $p67^{phox}$, NoxO1, and NoxA1 transcripts were all observed in cerebral ECs of the rodent brain. Ischemia or Ang II induced Nox2 protein in these cells, whereas endothelial Nox4 protein expression increased in cerebral ECs after stroke or in response to angiogenesis (Table 1) [reviewed by (88)].

Pericytes. In small cerebral arterioles and capillaries, endothelial arteries are surrounded by pericytes. The cells contribute to the control of vascular tone and contract during stroke and resemble many features of SMCs. Pericytes are thought to mediate small-vessel contraction after reperfusion as a consequence of oxidative/nitrosative stress, leading to calcium overload and Rho kinase activation (181). Cerebral pericytes have, however, not been characterized regarding Nox expression, but data on retina pericytes are available,

Table 1. NADPH Oxidase Subunit Expression in Cerebral Arteries

Condition	*mRNA/ protein*	*Tissue*	*Species*	*Nox subunits*	*Ref.*
Basal	mRNA				
		Basilar artery	R	Nox1, Nox2, Nox4 $p22^{phox}$, $p47^{phox}$	(128)
		Cerebral arteries	R	Nox2, Nox4 $p22^{phox}$, $p47^{phox}$, $p67^{phox}$	(58)
		Microvessels	M	Nox1, Nox2, Nox4 $p22^{phox}$, $p47^{phox}$, $p67^{phox}$	(68)
		ECs	R	Nox1, Nox2, Nox4 $p22^{phox}$, $p47^{phox}$, $p67^{phox}$ NoxA1, NoxO1	(5)
		EC	M	Nox1, Nox2, Nox4	(89)
Basal	Protein				
		Basilar artery	R	Nox1, Nox2, Nox4	(116)
		Basilar artery	R	Nox4	(117)
		Cerebral arteries	M	Nox2, $p47^{phox}$	(114)
		Cerebral arteries	M	Nox2	(115)
		Microvessel	R	Nox2, $p22^{phox}$	(95)
		ECs	R	Nox2	(93)
		ECs	M	Nox2	(93)
Stimulation	Protein				
I/R		Microvessel	R	Nox2	(105)
I/R		Microvessel	M	Nox4	(96)
I/R		Newly formed capillaries	M	Nox4	(167)
I/R		ECs	M	Nox2, $p47^{phox}$	(183)
I/R		ECs	H	Nox4	(96)

ECs, endothelial cells; I/R, cerebral ischemia/reperfusion; NADPH, nicotinamide adenine dinucleotide phosphate; Nox, NADPH oxidases; M, mouse; R, rat; H, human; Ref., references

which potentially could be translated to the brain. Retinal pericytes express Nox2 and $p47^{phox}$ (123, 159), and inhibition of NADPH oxidases reduces ROS formation in these cells (43, 66). Stimulation of isolated rat retinal microvessels with endothelin-1 (ET-1) increased the ROS formation in the surrounding vascular pericytes, an effect, which was blocked by apocynin or PKC inhibition, but not by the mitochondrial complex I inhibitor rotenone, the xanthine oxidase inhibitor allopurinol, or the NO synthase inhibitor N-nitro-L-arginine methyl ester (110). In cultured bovine retinal pericytes, the saturated free fatty acid palmitate, which is frequently elevated in diabetes, increased oxidative stress and apoptosis. Overexpression of dominant negative forms of $p47^{phox}$, $p67^{phox}$, or Rac-1 under these conditions attenuated ROS formation and caspase-3 activity, whereas overexpression of a constitutively active Rac-1 enhanced caspase-3 activity (27). Moreover, hyperglycemia increases Nox2 protein levels in pericytes, and apocynin decreased Nox activity, ROS production, and caspase-3 activity in these cells (123). Similarly, cyclic stretch in porcine retinal vascular pericytes, a model for hypertension, induced an apocynin- and diphenylene iodonium (DPI)-sensitive ROS generation. These inhibitors also decreased the stretch-induced phosphorylation of the stress activity kinase c-Jun N-terminal kinase, caspase-3 activity, and apoptosis.

Astrocytes. Astrocytes are attached to the basal vascular membrane in close vicinity to ECs, and data on cultured astrocytes suggest that also these cells express Nox proteins: cultured rat astrocytes contain transcripts of Nox1, Nox2, Nox4, and $p47^{phox}$, and upon hypo-osmotic stimulation, rapidly produced large amounts of ROS, by a mechanism sensitive to apocynin (140). Also, murine astrocytes express Nox1, Nox2, and Nox4 mRNA (89). On the protein level, Abramov *et al.* (1) demonstrated the presence of Nox2 and $p22^{phox}$ in membrane fractions of whole-cell lysates from rat primary astrocytes and $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and Rac in the cytosolic fraction by Western blots. In addition, the presence of $p67^{phox}$ and Nox2 protein in astrocytes was illustrated by immunohistochemistry in primary cultured rat astrocytes and *in situ* in freshly prepared tissue sections of mice by co-localization staining with the astroglial marker glial fibrillary acidic protein (1). After stimulation with the PKC activator phorbol ester, astrocytic ROS production increased notably by a pathway involving intracellular calcium. Importantly, phorbol ester-induced responses were almost absent in astrocyte cultures of mice lacking a functional Nox2 homolog.

Microglia. As microglial cells are basically tissue-resident macrophages, at least the expression of Nox2 can be inferred. Indeed, microglial cells of adult mice express Nox1, Nox2, and Nox4 mRNA. As compared to ECs, Nox2 expression was greater, whereas Nox4 expression was clearly lower in these myeloid-derived cells (89), and similar observations were made in rat microglia (75). In isolated microglia of newborn mice, Cheret *et al.* (34) identified Nox1, Nox2, and $p22^{phox}$ mRNA expression as well as that of the cytosolic subunits NoxO1, NoxA1, Rac-1, Rac-2, $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$. Interestingly, Nox4 transcripts were not detectable in microglia of newborn mice. Similar as to rodents, microglial cells isolated from the human fetal brain expressed Nox2, $p22^{phox}$, $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$ transcripts (70).

Not surprisingly, Nox expression, which is controlled by multiple factors, including the tissue environment and the differentiation state of the cell and the organ, differs considerably between primary brain microglial cells and microglia cell lines. For example, the human microglial cell line clone 3 (HMC3) exhibits high expression of Nox4, but basically no Nox2 expression, which is truly the opposite to native microglia (102). Thus, data on Nox in cell lines should be handled with caution.

Neurons. Nox1, Nox2, and Nox4 are also contained in neurons. While investigating the role of NADPH oxidases in apoptotic death of cerebellar granule neurons of rats, Coyoy *et al.* (39) reported protein expression of $p22^{phox}$, Nox1, Nox2, and Nox4 and the cytosolic $p47^{phox}$ and $p67^{phox}$. At the mRNA level, also the expression of p40phox was detected. In primary cultures of murine neurons, at least mRNA expression for Nox1, Nox2, and Nox4 is apparent (89). By immunohistology, Nox2 and $p22^{phox}$ as well as the cytosolic subunits p40, $p47^{phox}$, and $p67^{phox}$ were detected throughout the brain with particularly high expression in the cortex, the hippocampus, the amygdala, striatum, and the thalamus (149). In resting primary embryonic cortical neurons, the Nox organizer $p47^{phox}$ resided in the cytosol and translocates to the membrane upon N-methyl D-aspartic acid (NMDA) stimulation (24). Also in human neurons, Nox homologs and associated proteins are expressed, as shown for human neuron-enriched hippocampal cultures (129) and human primary cortical cultures of fetal brain tissue (73).

Nox1 and Nox4 were less well studied in these cells: Nox1 transcripts are found in neuronal tissue adjacent to the central nervous system (CNS) like in dorsal nerve roots (82) or in pheochromocytoma PC-12 cell lines (81). Investigating various types of murine CNS tissue, Nox4 expression on the transcriptional and protein level was demonstrated in cortical and hippocampal neurons and cerebellar Purkinje cells. Of note is the fact that not all cortical neurons exhibit colabeling with a Nox4 antibody pointing to differential expression levels in the same cell type possibly due to different functions within the subpopulations (167). In total, however, Nox4 protein expression appears to be low in the healthy human cortex (96).

Collectively, these data demonstrate that all cell types of the brain express Nox homologs and cytosolic activator proteins. Many of the data, however, were obtained by PCR or with the help of human antibodies in rodent tissue, which frequently yield false-positive stainings. The true level of cerebral expression of most Nox-associated proteins and the Nox homologs themselves is therefore still under debate. Eventually, only functional analyses will help understanding whether the detected elements are of physiological importance.

Nox and neuronal dys-/function in diseases of the nervous system

Given the wide distribution of Nox homologs in the brain and the multitude of effects elicited by ROS, it is not surprising that Nox enzymes have been implicated not only in cerebral I/R injury but also in a broad spectrum of very diverse neurological afflictions, which range from physiological signaling of, for example, pain to neurodegeneration like in amyotrophic lateral sclerosis (ALS) or in Alzheimer's disease (AD). Exemplarily, we describe the role of NADPH oxidases

in the psychiatric disorder schizophrenia and in the neurodegenerative AD, the latter being associated with neurovascular dysregulation.

Nox and excitation: schizophrenia. In an experimental model of schizophrenia, the repetitive application of ketamine, an NMDA receptor antagonist, leads to dysfunction of a subpopulation of cortical fast-spiking inhibitory interneurons with a loss of parvalbumin and the γ-aminobutyric acid (GABA)-producing enzyme glutamic acid decarboxylase 67 (GAD67) and an increase in O_2^- production along with augmented $p22^{phox}$ and Nox2 protein. Cotreatment with apocynin prevented these effects and significantly reduced Nox activity (16). The pathophysiological relevance of these findings was further supported by a postmortem study of patients suffering from schizophrenia showing a decrease in GAD67 and NMDA receptor subunit 2A (NR2A), the latter being a regulator of the physiological activity of the NMDA receptor, in a subset of GABAergic interneurons (178). Ketamine-mediated Nox activation and O_2^- production in the brain with a subsequent loss of interneurons require neuronal production of interleukin-6 (IL-6). Genetic deletion of IL-6 in mice or the removal of IL-6 from the culture medium abolished O_2^- production and preserved the interneurons (17). The effect elicited by the IL-6/Nox/interneuron pathway is reversible in the adult brain, but not in the developing cortex. Thus, it can alter the postnatal formation of neuronal circuits and might increase the vulnerability to permanent maturational process deficits, resulting in the evolution of schizophrenic symptoms in adolescence [reviewed by (18)]. Recently, Sorce *et al.* (154) contributed data on the relevance of NADPH oxidases after repetitive ketamine application using Nox2-deficient mice. The KO animals were resistant to ketamine with respect to behavioral alterations and increase in tissue markers of stress. They preserved normal extracellular levels of glutamate and dopamine and did not lose the functionally relevant NR2A subunit of the NMDA receptor in the prefrontal cortex. Thus, Nox2 promotes the development of psychotic symptoms after ketamine application by increasing the excitatory mediators glutamate and dopamine.

Nox and neurodegenerative diseases. Protein aggregation is an important pathomechanism implicated in AD, ALS, and Parkinson's disease (143). As oxidation alters the solubility of proteins, oxidative stress has almost traditionally been linked to neurodegenerative diseases, although the source of ROS has been a matter of longstanding debates. Conversely, some of the oxidized proteins themselves activate and induce Nox proteins as observed in the brains of AD patients (8, 26) or in response to the amyloid β (Aβ) and amyloid precursor protein (APP). In these cases, Nox-derived ROS mediate cerebrovascular dysfunction, activate microglia, and damage neurons [reviewed by (21)]. In mouse models of AD, genetic deletion of Nox2 prevents oxidative stress, improves behavioral deficits and outcome, but does not affect Aβ accumulation. These observations suggest Nox-derived ROS as enhancers of AD plaque toxicity, but not mediators of plaque formation [reviewed by (153)]. Accordingly, in WT, but not Nox2 KO mice, the Nox2 inhibitory peptide gp91dstat prevented Aβ-induced vascular ROS production and reversed vascular dysregulation (130, 131). A final aspect leading to neurodegeneration is cerebrovascular dysfunction. Pharmacological inhibition or genetic deletion of Nox2 reduced cerebrovascular oxidative stress and rescued endothelium-dependent relaxation or functional hyperemia in models of Ang II, Aβ, APP, or aging-induced cerebrovascular dysfunction [reviewed by (80, 131)]. Furthermore, Nox2 deficiency restored cerebrovascular reactivity and cognitive function in mice overexpressing APP.

Collectively, all these data suggest that Nox proteins are involved in oxidative stress during neurodegeneration. The data obtained largely in mice may even suggest that Nox proteins contribute to disease progression, but whether this is also the case in humans is currently undetermined.

Nox in Cerebral Ischemia

The role of Nox in cerebral I/R has been studied in several model systems ranging from *ex vivo* cell culture studies to a broad spectrum of animal experiments. In rodents, the vast majority of researchers use the middle cerebral artery occlusion (MCAO) model, which allows an effective reperfusion and timely as well as locally very defined ischemia.

Nox expression in the course of cerebral I/R

With the aid of the MCAO model, it was observed that Nox2 protein increases from day 1 to 3 after stroke in the penumbra in ECs (183) and microglia (70). Also, the total vascular Nox activity increased in arteries of the penumbra (118). In an analysis of whole hemispheres, rapid changes in Nox expression were noted: ischemia of 2 h increased Nox2 and $p22^{phox}$ mRNA and protein expression in the ischemic hemisphere in rats, an effect which was enhanced in diabetic animals (100). Also, Nox4 expression changes in stroke models: Nox4 mRNA increased early after ET-1 induced stroke in the cortex of rats (112) and in neurons and in newly formed capillaries surrounding the ischemic core (167). Also in vessels, a dramatic induction of Nox4 occurs after stroke (118). Accordingly, Nox4 protein gradually increases in the ischemic cortex and basal ganglia in the hours after stroke in mice and was also found to be elevated in human cerebral vessels after stroke (96) (Table 1). Collectively, these data suggest that Nox2 and Nox4 expression increases after stroke. No data are currently available for Nox1, Nox3, Nox5, and the Duox enzymes, and thus potential expression changes in these homologs remain to be determined.

Nox homologs in stroke: Nox2 is the bad guy!

Fifteen years ago, Walder *et al.* (172) first studied Nox KO mice in a model of transient focal cerebral ischemia, and these animals were genetically deficient of Nox2 (Table 2). The KO mice were largely protected against the loss of cerebral tissue in an MCAO protocol employing 2 h of ischemia, followed by 22 h of reperfusion. This effect was partially dependent on Nox2 contained in tissue-resident cells, but also in circulating leukocytes, as revealed by bone marrow transplant experiments. Indeed, neutrophils, which contain the highest level of Nox2, are of special importance for O_2^- production and I/R injury: inhibition of leukocyte adhesion (31, 187) or depletion of neutrophils (111) resulted in smaller lesion volumes after experimental stroke (76). In a recent experiment using the bone marrow transplant approach and Nox2 KO animals, similar results were obtained, although transplantation of

Table 2. Knockout/Knockdown of Specific Nox Isoforms in Experimental Ischemic Stroke

Genotype	Ischemic period [min]	Assessment of lesion volume after MCAO [h]	Stain	KO protective?	Ref.
siRNA Nox1 (rat)	90	24	N.D.	Yes	(35)
$Nox1^{y/-}$	30	24	Thionin	No	(86)
	60	24	TTC	No	(96)
	60	24	TTC	Yes	(89)
	120	24	TTC	No	(89)
	Permanent	24	TTC	No	(89)
$Nox2^{y/-}$	25	72	CV	Yes	(97)
	30	24	Thionin	Yes	(44)
	60	24	TTC	No	(96)
	75	24	TTC	Yes	(33)
	120	24	TTC	Yes	(172)
	120	24	TTC	Yes	(90)
	120	24	HE	Yes	(163)
$Nox4^{-/-}$	60	24	TTC	Yes	(96)
	Permanent	24	TTC	Yes	(96)

MCAO, middle cerebral artery occlusion; KO, knockout; CV, cresyl violet; HE, hematoxylin and eosin; N.D., not determined; TTC, 2,3,5-triphenyl-tetrazolium chloride.

Nox2 KO bone marrow into WT mice yielded a much greater protection than in the previous study (163).

Several subsequent studies confirmed the protective effect of genetic deletion of Nox2 in cerebral I/R injury and strongly support the importance of Nox2 in stroke. In the 2-h ischemia/22-h reperfusion protocol of MCAO, lesion volume, edema formation, as well as early blood–brain barrier (BBB) leakage (Fig. 2) were dramatically attenuated in mice lacking a functional Nox2 protein, the latter most likely due to attenuated Rho kinase activation, stress fiber formation, and subsequent EC contraction (90). In line with these experiments, Chen *et al.* (33) also found a reduction in the lesion volume at 24 h in Nox2 KO as compared to WT mice, accompanied by an improved neurological function in a protocol using 75 min of cerebral ischemia. Furthermore, the authors also detected a reduction in oxidation products and an early increase in a 150-kDa cleaved spectrin fragment compatible with calcium homeostasis disturbances and calpain-mediated cell necrosis during early reperfusion (3 h). After 24 h of reperfusion, 150-kDa and 120-kDa cleaved spectrin fragments became apparent pointing to calpain-mediated necrosis and caspase-mediated apoptosis, also late after reperfusion (Fig. 3). Nox2 deficiency attenuated the postischemic inflammation demonstrated by an attenuated induction of intercellular adhesion molecule-1 and a reduced neutrophil infiltration into the brain as reflected by the neutrophil-specific marker myeloperoxidase (MPO). A subsequent study from the same group confirmed the neuroprotection in the MCAO model at 24 h and 72 h after 1 h of brain ischemia in Nox2 KO mice and also observed an attenuation of postischemic inflammatory gene expression and a decrease in the abundance of products of oxidative stress (32). The protective effect of Nox2 deficiency was also present with shorter ischemic periods. Similarly, a recent report stated significantly smaller infarcts at 24 h after a 30 min ischemic period in Nox2 KO mice in comparison to WT animals, and also this was associated with attenuated O_2^- generation, increased NO availability, and reduced nitrosative stress (44). Comparing WT and Nox2 KO mice 3 days after 25 min MCAO, Kunz *et al.* (97) observed a 44% reduction in ROS production and 61% smaller lesion volume in animals deficient of Nox2 compared to WT controls. Accordingly, genetic deletion of $p47^{phox}$, which is required for the assembly and activation of Nox2, was neuroprotective *in vivo* and *in vitro* (157). The importance of Nox2 in the context of stroke is strongly supported by *in vitro* data from murine hippocampal neurons lacking a functional Nox2, which exhibit a substantial attenuated ROS generation when exposed to oxygen/glucose deprivation (OGD). Genetic deletion of Nox2 also attenuated the intermittent hypoxia-induced cognitive deficits and markers of oxidative stress in mice (125). Finally, Nox2 deficiency reduced neuronal death, oxidative injury, and microglial activation in a model of murine transient global cerebral ischemia (183) and attenuated neuronal cell death within the ganglion cell layer in a mouse model of retinal I/R (182).

Interestingly, despite these findings, Nox2 is also required for preconditioning as shown for ethanol- and lipopolysaccharide-preconditioning protocols (98, 173). As however preconditioning is at least in part mediated by ROS, these data do not contradict the detrimental action of Nox2 in stroke.

In contrast to the reported beneficial effects of Nox2 deficiency in multiple studies of experimental stroke, a recent study was not able to reproduce the neuroprotection in mice lacking a functional Nox2 homolog after 1-h MCAO and 23 h of reperfusion (96). The reason for this discrepant result is unknown, as it would require in detail comparison of the experimental protocols side by side and a careful analysis of the health status and the genetic background of the mice, which has not been carried out. Although representing a revolutionary approach to study mechanisms *in vivo*, engineered mice are not without problems. As reconstitution experiments are rarely performed, it often remains unclear, whether an observed effect in such an animal is truly the consequence of the altered gene, but not of additional changes introduced into the genome (22). Moreover, the genetic background has an often underestimated influence on experimental readouts and may influence experiments even if backcrossing was performed for more than 10 generations. If a genetically altered line is bred in a homozygous fashion for a long time, genetic and epigenetic drift occurs, which influences the results. This aspect is of great importance for the $Nox2^{y/-}$ mice, as the immune deficit in these animals renders them susceptible to infection and thus increases the selection pressure. Indeed, we observed that the susceptibility of our $Nox2^{y/-}$ line to some bacteria decreased in the course of breeding and so switched our colony from a homozygous to heterozygous breeding pattern to prevent such a drift. The aspect of infection in $Nox2^{y/-}$ mice might also strongly influence the outcome in stroke models. Infections may have a preconditioning effect, and thus may potentially decrease the stroke size. Also, with respect to the selection bias mentioned above, the fitter animals will be selected in an unclear environment and may also experience smaller strokes. Thus, approaches that rescue the phenotype such as exogenous application of the protein produced or bone marrow

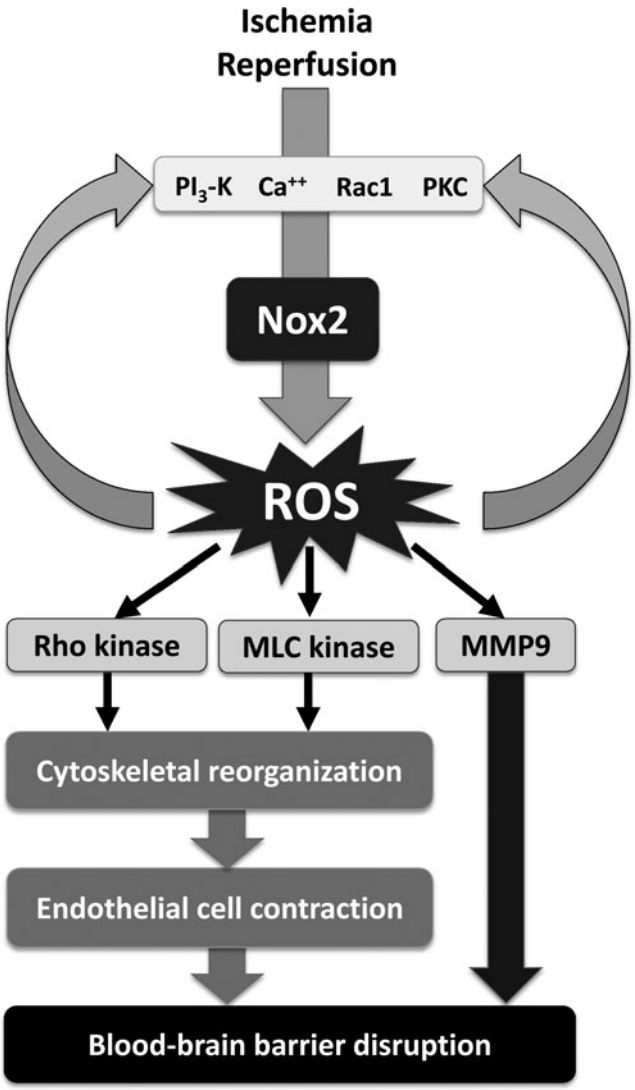

FIG. 2. Nox-mediated blood–brain barrier disruption in cerebral ischemia/reperfusion. Ischemia/reperfusion activates elements upstream of Nox2, including PI3-kinase, PKC, and calcium. The subsequent ROS formation not only activates downstream targets involved blood brain–barrier opening but also promotes further stimulation of the elements upstream of Nox2: ROS are known to increase calcium and to activate PKC, PI3-kinase, and Rac. MLC kinase, myosin light-chain kinase; MMP, matrix metalloproteinase; PKC, protein kinase C; ROS, reactive oxygen species.

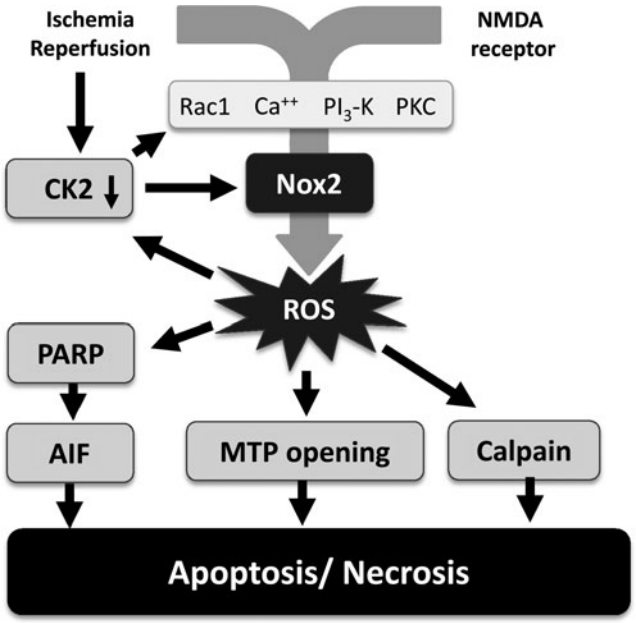

FIG. 3. Nox-mediated neuronal cell death in cerebral ischemia/reperfusion. Ischemia/reperfusion as well as NMDA receptor activation increase intracellular calcium and other elements involved in Nox2 activation, which leads to subsequent ROS formation. As illustrated for the ROS-dependent inactivation of casein kinase 2, several positive feedback loops further increase ROS formation in a vicious circle entertained by an oxidation of target molecules. Apoptosis is eventually initiated by energy breakdown through PARP and activation of apoptosis-inducing factors as well as MTP opening. Necrosis can also occur, for example, as a consequence of the calcium- and ROS-mediated activation of the protease calpain by proteolysis, which subsequently cleaves a large number of cellular proteins with high activity. MTP, mitochondrial transition pore; NMDA, N-methyl D-aspartic acid; PARP, poly-ADP ribose polymerase.

transplants would provide a strong aid to control these aspects. Moreover, it is desirable to confirm data from genetically altered animals by pharmacological inhibitors or biologicals [further recommended reading: (54, 55)]. The discovery of specific Nox inhibitors in the future will substantially complement and further clarify the current, occasionally inconsistent results obtained by the use of KO mice.

Even though expression levels of Nox1 and Nox4 have been detected in ECs of cerebral arteries (4) and the brain at large, only few studies investigated the role of Nox1 in experimental stroke. Two studies in Nox1-deficient mice failed to show neuroprotection in models of 0.5 h and 1 h of MCAO and reperfusion at 24 h after induction of ischemia (86, 96), whereas in a rat transient MCAO (1 h) model, knockdown of Nox1 by adenovirus-mediated transduction of Nox1 small hairpin RNA significantly reduced the lesion size and attenuated neuronal cell death. Likewise, ROS generation, Nox1 expression, 8-hydroxy-2′-deoxyguanosine immunoreactivity, and cell death were reduced when applying the same knockdown approach to primary rat cortical neuron cultures exposed to OGD and reoxygenation (35). In our hands, 1 h MCAO followed by 23 h of reperfusion resulted in an better neurological functional outcome and in a moderate reduction in lesion volume, cerebral edema, and BBB leakage in Nox1 KO compared to WT mice. Whether this neuroprotection also remains stable in the days following the stroke, or whether the lesion volume of KO and WT controls converges later, needs to be established. However, the neuroprotective effect of Nox1 deficiency disappears if ischemia extends beyond 1 h (89). Given the narrow window for a potential pharmacological intervention, Nox1 therefore does not appear to be a promising target for stroke treatment.

Also, the role of Nox4 is still obscure. An observational study in a mouse model of permanent focal cerebral ischemia identified a dramatic increase in cortical neuronal Nox4 expression detectable at 24 h and persisting throughout the

30-day follow-up period with maximum days ranging 7–15. Nox4 protein was detected in newly formed capillaries corresponding to the peak around day 7 (167) pointing to a potential role for Nox4 in stroke repair. The only experimental stroke study in Nox4-deficient mice to date (96) revealed an impressive protection at 24 h after stroke induction in the 1-h and permanent MCAO model. This effect was independent of age or gender. Restoring oxidative stress by adding H_2O_2 abolished the stroke protective phenotype in Nox4 KO mice. In a different Nox4 KO strain, we failed to observe any protection in the MCAO model (Kahles 2008 unpublished observation), and indeed, the rational for Nox4 being an important mediator of acute stroke is weak: on the protein level, Nox4 is basically undetectable in the brain and has to be induced in response to stroke first (96). Thus, at time of reperfusion when the vast of the damage occurs, Nox2 expression largely dominates over that of Nox4. In vascular and cardiac injury models, more and more studies suggest that Nox4 can be protective (40, 96, 138) as it is unable of generating $ONOO^-$ (160), an important mediator of tissue damage and rather promotes NO formation. Thus, additional studies have to be carried out to address the potential of Nox4 as a mediator of cerebral injury in stroke.

Collectively, these data provide a strong basis of an important role of Nox2 in I/R damage of the brain. Although Nox1 and Nox4 might not be unimportant under certain experimental situations, their overall impact appears to be much less, and confirmatory data supporting the functional importance of these Nox homologs still have to be sought.

Nox inhibition in stroke: a quest of specificity?

Although genetically modified mice considerably broadened our understanding of the contribution of NADPH oxidases to stroke pathology, specific pharmacological inhibition of Nox proteins would not only confirm and complement the findings, but it would also open up the field to translational studies. Obviously, specific Nox inhibitors, once developed, would be potential candidates for stroke therapy. Several drugs, which are used to treat common cardiovascular/stroke risk factors like hypertension or hyperlipidemia, interfere with NADPH oxidase induction or activation. Apart from their primary blood pressure- or lipid-lowering effects, these agents appear to elicit beneficial, pleiotropic effects in stroke possibly linked to inhibition of NADPH oxidases and have some effect in cerebrovascular diseases.

Lipid-lowering HMG-CoA reductase inhibitors, also termed statins, act on small GTPases like Rac-1. Statin treatment abolishes the geranylgeranylation of Rac-1 and prevents the translocation of Rac-1 from the cytosol to the membrane, which is an essential step in Nox activation. A multitude of experimental stroke studies demonstrated a neuroprotective effect of statins independent of whether treatment is initiated before (57, 79, 90, 166) or after the cerebrovascular event [reviewed by (11)] and also independent of the lipid-lowering potential (pleiotropic effects). In line with the experimental data, statins are beneficial in the primary and secondary prevention of ischemic stroke in humans (7, 37). Their effect, however, is rather modest, and future trials have to investigate whether the protection occurs class wide with all statins or is substance specific. Of note is the fact that withdrawal of pre-existing statin treatment led to impairment of endothelial function (169) and resulted in increased mortality and morbidity in patients with acute coronary syndrome and ischemic stroke (20, 56). Furthermore, statin use early in stroke hospitalization was strongly associated with improved survival after stroke (60). Despite these positive observations, statins act on multiple targets, and thus the observed effects are probably not just a consequence of NADPH oxidase inhibition.

The renin-angiotensin-aldosterone-system (RAAS) also is a potential pharmacological target for the treatment of ischemic stroke with a link to NADPH oxidases: Ang II and aldosterone induce and activate Nox proteins in vessels and even in defined regions of the brain (29, 134, 189). In a model of cerebral I/R in rats, candesartan, a representative of the angiotensin II type-1 receptor blockers, reduced products of oxidative stress, $p22^{phox}$, and Nox2 mRNA induction as well as tissue injury and concomitantly improved functional outcome (100). Furthermore, candesartan attenuated the prothrombotic platelet-leukocyte-EC interaction in cerebral vessels after bilateral common carotid artery occlusion and reperfusion and attenuated the I/R-associated increase in ROS (84). Aliskiren, a new renin inhibitor, appears to be as effective as candesartan in cerebral I/R in rats, as it significantly reduced mortality, improving functional outcome as compared to WT controls. This neuroprotective effect was independent of blood pressure and might be related to a decrease in proinflammatory gene expression in the ischemic core (147). So far, the clinical data on RAAS inhibition yielded conflicting results ranging from beneficial to no effect [reviewed by (144), (145, 185)]. Additional studies are therefore needed to further evaluate the concept of RAAS inhibition in stroke and to better establish the link to Nox proteins.

DPI is a widely used potent, yet unspecific, NADPH oxidase inhibitor for *in vitro* experiments. Data of DPI in animal models of stroke are scarce due to its toxicity (*e.g.*, hypoglycemia and cardiomyopathy), irreversible binding to the flavin adenine nucleotide of Nox, broad range of unspecific effects, and its low solubility (87). In a rat model of focal cerebral I/R (124), dimethyl sulfoxide (DMSO) was therefore used as a solvent for DPI, and structural and functional outcome was assessed up to 48 h after stroke in DMSO-treated, DMSO/DPI-treated, and untreated animals. Lesion volume and functional outcome were significantly reduced in all the treated animals. I/R injury at 24 h and 48 h following stroke, however, was not different between DMSO- and DMSO/DPI-treated animals, pointing to the well-known protective effect of the antioxidant DMSO, which was obviously not further outperformed by DPI.

A large number of Nox inhibitor studies in experimental stroke have been undertaken with the natural organic compound apocynin. Experimental settings differ considerably in terms of time and route of administration, dose, model of focal I/R, ischemic period, species, and age of animals (Table 3). Overall, apocynin showed to be protective if applied before reperfusion or at the latest with reperfusion. Post-treatment revealed no beneficial effect. Even with pretreatment, some authors observed an increase in mortality when higher doses of apocynin were used (85), or when specific experimental conditions different from the conventional MCAO model were applied: in a pharmacological recanalization model, where external tissue plasminogen activator dissolves an intravascular clot, apocynin had no positive effect when aged female rats were studied, whereas in young female animals,

Table 3. Pharmacological Nox Inhibition with Apocynin in Focal Cerebral Ischemia/Reperfusion

Time of admin.[a]	*Route of admin.*	*Dose [mg/kg]*	*Ischemic period [min]*	*Spec.*	*Geno-type*	*Time of lesion assessment [h]*	*Prot.?*	*Ref.*
−1 month period	po	7.5 daily	120	R[b]	n/a	24	No	(188)
−3 h	iv	0.4/4.0	120	M	WT	24[c]	No	(90)
−3 h	iv	40.0	120	M	WT	24[c]	Yes	(90)
≤−2.5 h	ip	5.0	≥120[d]	R	Young	24	Yes	(94)
≤−2.5 h	ip	5.0	≥120[d]	R	Aged	24	No	(94)
−2.5 h	ip	30.0	90	R	N/A	24[c]	Yes	(105)
−2.5 h	ip	5.0[b]	120	R	N/A	24	Yes	(188)
−2 h & +5 min	ip	5.0	90	R	N/A	24	Yes	(122)
−1 h	ip	2.5	30	M	WT	24	Yes	(85)
−1 h	ip	5.0	30	M	WT	24	No	(85)
−1 h	ip	2.5	30	M	$Nox2^{y/-}$	24	No	(85)
−1 h	iv	1.0/5.0	30[e]	R	N/A	6	No	(38)
−1 h	iv	10.0/20.0	30[e]	R	N/A	6	Yes	(38)
−0.5 h	ip	50.0	90	R	N/A	24	Yes	(161)
−0.5 h	iv	2.5	120	M	WT	24	Yes	(162)
−0.5 h	iv	3.75/5.0	120	M	WT	24	No	(162)
−5 min	ip	5.0	120	R	N/A	24	Yes	(65)
−5 min	ip	4.0	75	M	WT	24 & 72	Yes	(33)
0	iv	2.5	120	M	WT	24	Yes	(163)
0	iv	2.5	120	M	$Nox2^{y/-}$	24	No	(163)
0	iv	20.0	30[e]	R	N/A	6	Yes	(38)
0	iv	0.1mg	60	M	WT	24	No	(96)
+0.5 h	iv	20	30[e]	R	N/A	6	No	(38)
+1 h	ip	2.5	30	M	WT	24	No	(85)

[a]Relative to start of reperfusion (− is before, + is after, and 0 is at the time of reperfusion).
[b]Fed a liquid diet for 8 weeks.
[c]Blood–brain barrier damage/brain edema.
[d]Clot MCAO model in female rats: tPA at 2 h.
[e]Distal transient MCAO.
Admin., administration; iv, intravenous; ip, intraperitoneal; KO, knockout; MCAO, middle cerebral artery occlusion; Prot., protection; Spec., species; tPA, tissue plasminogen activator; WT, wild type.

neuroprotection occurred (94). In addition, Tang *et al.* (162) reported an increase in intracerebral hemorrhage with higher doses of apocynin. The interpretation of these studies is further complicated by the varying modes of action, application, and dose of apocynin, as well as the specific pathophysiology associated with the disease model used: at low concentrations, apocynin appears to be relatively specific as an inhibitor for the interaction of Nox2 and $p47^{phox}$ in cells containing MPO, thus primarily neutrophils (156, 168). The mode of action of apocynin in other cells is still debated controversially. It is not known whether glycocalix-bound MPO is sufficiently active to activate apocynin in the vascular system in the absence of leukocytes, or whether other peroxidases like cyclooxygenase can activate apocynin. Apocynin also has complex unspecific effects. In high concentrations, it acts as an antioxidant (77), and once activated, can even act as a pro-oxidant and induces oxidative stress (142, 170). With respect to stroke, this could result in a protective preconditioning effect. Importantly, apocynin also lowers intracellular calcium (59, 137), which is a key factor in mediating excitotoxicity (49). Nevertheless, the protective effect of apocynin was not observed in Nox2 KO mice (85), and this at least provides a strong support for a central role of Nox2 in I/R-induced brain damage. Taken together, apocynin is a potent neuroprotecting agent if applied before or at time of reperfusion. Importantly, in relation to stroke onset, apocynin was effective even when given as long as 2 h after induction of ischemia (163). A standardized approach comparing various ischemic periods and doses and addressing the safety issues related to gender and age (94) is mandatory before these experimental data can be translated into the clinical setting.

Other natural compounds like honokiol or plumbagin also inhibited Nox activity or expression and attenuated tissue injury after experimental stroke (48, 103, 152). As these compounds are, however, not well characterized, a link between Nox inhibition and neuroprotection cannot be inferred, so far. Nevertheless, these data suggest that in rodent models of cerebral I/R, salvage of tissue is possible with inhibitors at least acting on the redox status of the cell and potentially on the Nox system.

Due to the tremendous clinical potential of specific Nox inhibitors in several disease indications, including cardiovascular diseases, several initiatives currently develop small-molecule inhibitors. Data on these new, more specific Nox inhibitors, like the compounds from GenKyoTex, are not available for stroke, yet although a peptide inhibitor against Nox2 conferred some protection (139) in a model of global cerebral ischemia. Most of the stroke experimental studies pretreated animals before the onset of ischemia, as this approach overcomes the significant problem of drug delivery during stroke. It also however puts the experimental treatment in a situation more favorable than the clinical scenario. Already in the past, this consideration was used to explain why so many compounds exert protection in animal

experiments, but not in humans. Indeed, in those studies in which Nox inhibitors were applied at time of reperfusion or even later, the protective effect was greatly attenuated as, for example, shown for the beneficial effect of statin treatment (135). Nevertheless, apocynin appears to be an extraordinary promising drug in acute stroke treatment, even when given after stroke onset, but these observations still await confirmation in future standardized trials. In addition, just recently, a strong protective effect against stroke in the MCAO model was observed with intrathecal application of the compound VAS2870 (96). Unfortunately, up to now, it is not known how VAS2870 works. In HEK293 cells overexpressing Nox4, the compound does not inhibit ROS production (Brandes RP 2010, unpublished observation) and in a recent analysis, it was concluded that the compound does not work at all on the enzymatic Nox complex, but rather on upstream signaling (64).

Conclusion

ROS profoundly contribute to tissue damage after I/R in several organs, including the brain. A large number of experimental studies provide evidence that, in particular, Nox2 is a major source of ROS in the brain, although Nox1 and Nox4 are not unimportant. Specific Nox inhibitors and high-quality Nox antibodies are not readily available, and data on Nox function in ischemic stroke are predominantly derived from mouse experiments. Given the small brain of the mouse and therefore the relatively large penumbra in these animals, it is questionable whether these data can be readily translated into the human situation. Most *in vivo* studies investigated the role of Nox2. Overall, genetic deletion of Nox2 elicited strong neuroprotection with a reduced lesion volume, attenuated BBB breakdown, and improved functional outcome. In contrast, only few studies dealt with Nox1 in stroke; the results are heterogeneous and point to an effect restricted to a small time window at the most. Just recently, Nox4 has been suggested to be involved in the pathophysiology of stroke; the particular study however could be criticized for the failure to reproduce previous data on Nox2 and stroke and for the uncertainties regarding the molecular mechanism of action and specificity of VAS2870. Thus, for the time being, any Nox inhibitory strategy for the treatment of stroke should focus on Nox2.

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Address correspondence to:
Prof. Ralf P. Brandes
Institut für Kardiovaskuläre Physiologie
Fachbereich Medizin der Goethe-Universität
Theodor Stern-Kai 7
Frankfurt am Main 60590
Germany

E-mail: brandes@vrc.uni-frankfurt.de

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Abbreviations Used

Aβ = amyloid β
AD = Alzheimer's disease
ALS = amyotrophic lateral sclerosis
Ang II = angiotensin II
APP = amyloid precursor protein
BBB = blood–brain barrier
CNS = central nervous system
CV = cresyl violet
DMSO = dimethyl sulfoxide
DPI = diphenylene iodonium
Duox = dual oxidase
ECs = endothelial cells
ET-1 = endothelin
GABA = γ-aminobutyric acid
GAD67 = glutamic acid decarboxylase 67

Abbreviations Used (Cont.)

HE = hematoxylin eosin
HMC3 = human microglial cell line clone 3
H_2O_2 = hydrogen peroxide
IL-6 = interleukin-6
ip = intraperitoneal
I/R = ischemia/reperfusion
iv = intravenous
KO = knockout
MCAO = middle cerebral artery occlusion
MLC kinase = myosin light-chain kinase
MMP = matrix metalloproteinase
MPO = myeloperoxidase
mRNA = messenger ribonucleic acid
MTP = mitochondrial transition pore
NADPH = nicotinamide adenine dinucleotide phosphate
NMDA = N-methyl D-aspartic acid
NO = nitric oxide
Nox = NADPH oxidases
NR2A = NMDA receptor subunit 2A
O_2 = oxygen
O_2^- = superoxide
OGD = oxygen/glucose deprivation
$ONOO^-$ = peroxynitrite
PARP = poly-ADP ribose-polymerase
PKC = protein kinase C
RAAS = renin-angiotensin-aldosterone-system
ROS = reactive oxygen species
SMCs = smooth muscle cells
TGF-β1 = transforming growth factor-β1
tPA = tissue plasminogen activator
TTC = 2,3,5-triphenyl-tetrazolium chloride
WT = wild type