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MULTI-AUTHOR REVIEW

NADPH oxidases as therapeutic targets in ischemic stroke

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Abstract Reactive oxygen species (ROS) act physiologically as signaling molecules. In pathological conditions, such as ischemic stroke, ROS are released in excessive amounts and upon reperfusion exceed the body's antioxidant detoxifying capacity. This process leads to brain tissue damage during reoxygenation. Consequently, antioxidant strategies have long been suggested as a therapy for experimental stroke, but clinical trials have not yet been able to promote the translation of this concept into patient treatment regimens. As an evolution of this concept, recent studies have targeted the sources of ROS generation—rather than ROS themselves. In this context, NADPH oxidases have been identified as important generators of ROS in the cerebral vasculature under both physiological conditions in general and during ischemia/reoxygenation in particular. Inhibition of NADPH oxidases or genetic deletion of certain NADPH oxidase isoforms has been found to considerably reduce ischemic injury in experimental stroke. This review focuses on recent advances in the understanding of NADPH oxidase-mediated tissue injury in the cerebral vasculature, particularly at the level of the blood–brain barrier, and highlights promising inhibitory strategies that target the NADPH oxidases.

Keywords NADPH oxidase · Reactive oxygen species · Ischemia · Reperfusion · Stroke · Brain

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Introduction

Stroke is an acute cerebrovascular event associated with brain tissue damage due to insufficient regional cerebral perfusion caused by a sudden block of an artery supplying blood to the brain. In most cases, the cause of stroke is the formation of a thrombotic or embolic clot (ischemic stroke accounts for approx. 87 % of cases), whereas rupture of a blood vessel only occurs in every eighth to ninth patient (intracerebral hemorrhage, approx. 10 %; subarachnoidal hemorrhage, approx. 3 %) [1]. After heart diseases and cancer, stroke is the third leading cause of death in industrialized nations, and the World Health Organization estimates that about 15 million people experience a stroke each year. Stroke mortality is as high as 5.5 million/year, with another 5 million patients being permanently disabled [2]. With declining mortality rates in the last decades and increasing stroke incidence, the socioeconomic impact of cerebrovascular diseases is considerable, with an estimated total cost of $40.9 billion in 2007 [1], which will presumably further increase in upcoming years.

In ischemic stroke, early restoration of cerebral blood flow is crucial to prevent persistent brain damage. Thrombolysis with the serine protease tissue plasminogen activator (tPA) is only effective within the first 4.5 h after the onset of stroke symptoms [3] and has been for more than a decade the only Federal Drug Administration approved medical reperfusion therapy. More recently, the use of mechanical reperfusion devices, particularly in patients with contraindications against tPA or failed reperfusion with medical thrombolysis, has increased. Unfortunately, despite high success rates of recanalization in these patients, the clinical outcome of these therapies is still not favorable [4]. An important factor for all therapeutic considerations is that delayed or missing reperfusion

leads to irreversible tissue damage. Loss of neurons and blood–brain barrier (BBB) disruption followed by potentially space-occupying and life-threatening brain edema and intracerebral hemorrhage all promote the expansion of the brain infarct; consequently, with each successive treatment, the success of the reperfusion therapy is naturally limited.

Cerebral ischemia/reperfusion (I/R) has been studied extensively in animal models. Re-establishment of the cerebral blood flow results in restoration of the nutrient supply of oxygen and glucose, which is indispensable for neuronal survival. However, mitochondria and oxidation-promoting enzymes also utilize oxygen, and activation of these generator systems leads to the release of a large amount of reactive oxygen species (ROS) during reperfusion. In physiological conditions, free radical formation is sufficiently counterbalanced by antioxidative enzymes, such as superoxide dismutase (SOD) or catalase. In contrast, oxidative stress results from an imbalance between ROS production and their elimination, with subsequent cellular ROS accumulation [5]. Although there is also ample evidence that low amounts of ROS contribute to physiological signaling in the brain [6, 7], the classical oxidative stress model, in which ROS are direct mediators of tissue damage [8–11], has been shown to be operative. Additionally, the brain apparently possesses only limited antioxidant defense mechanisms and seems to be especially vulnerable to a free radical attack [12]. Similar to other organs, depending on the disease situation and the model studied, ROS formation occurs from different enzymatic sources. The traditional view of pathology-associated ROS is that they are produced by mitochondria, cyclooxygenase, monoaminooxygenase, and xanthine oxidase, and these sources have indeed been linked to a number of diseases of the central nervous system (CNS) [12–16].

With the growing interest in vascular NADPH oxidases, the role of this family of enzymes in the physiological function and pathophysiological dysfunction of cerebral cells has attracted considerable interest. Studies have suggested that NADPH oxidases are key enzymes in a broad spectrum of diseases of the CNS, such as Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson disease, and stroke (reviewed by [6, 17]).

ROS formation has been demonstrated to be of particular pathophysiological significance in ischemic stroke followed by reperfusion [18, 19]. However, our knowledge of the role of NADPH oxidases in this regard is still limited. Moreover, due to the complex multicellular and heterogeneous composition of brain tissue, we need to expand our knowledge in terms of NADPH oxidase expression, NADPH oxidase activation, and signaling of the individual NADPH oxidases in cerebral tissue.

In this review, we summarize the recent findings on ROS-mediated brain injury following cerebral I/R with a particular focus on the role of vascular NADPH oxidases. We also discuss potential therapeutic strategies targeting NADPH oxidases in ischemic stroke.

ROS in ischemic brain injury

Cerebral ischemia characterized by interrupted cerebral blood flow and decreased substrate delivery initiates a cascade of molecular events starting with progressive energy depletion (ATP stores), anaerobic glycolysis, lactate acidosis, loss of the ability to maintain the membrane potential with depolarization, release of excitatory amino acids (glutamate), cellular influx of calcium/sodium and water followed by cell swelling (cytotoxic edema), mitochondrial failure, free radical production, inflammatory processes, and activation of gene expression, ultimately resulting in brain tissue damage [20, 21]. During this time the free radical detoxifying mechanisms, such as SOD, catalase, glutathione peroxidase, ascorbic acid, or tocopherol, are perturbed, most likely due to excessive generation of free radicals, inactivation of the scavenger systems, consumption of radical scavengers, insufficient replenishment of antioxidants, and/or failure of detoxifying agents to appropriately distribute to the target tissue.

ROS, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and their derivatives, are excessively produced during I/R. In addition, reactive nitrogen species and peroxynitrite ($ONOO^-$) also accumulate during I/R, being formed from O_2 and neuronal nitric oxide synthase-derived nitric oxide (NO). O_2^- molecules are mainly derived from xanthine oxidase (XO), NADPH oxidases, and mitochondria. Mitochondria, whose main physiological function is the production of adenosine triphosphate by oxidative phosphorylation through the electron transport chain, are usually accepted as a principal source of ROS in this regard [22], but due to the critical function of these organelles, it is difficult to investigate mitochondria-dependent ROS formation with pharmacological inhibitors of the electron transport chain in vivo or with knockout strategies. Other ROS-producing enzymes have also been implicated in I/R. Cyclooxygenases are capable of promoting ROS generation via the oxidative transformation of arachidonic acid, with subsequent O_2^- production, a step which requires enzyme induction in cerebral tissue. However, although cyclooxygenase II is constitutively expressed in the endothelium, it is not considered to be a prime candidate mediator of early postischemic injury [23]. Xanthine oxidase, which is formed from xanthine dehydrogenase by oxidation of the enzyme and proteolytic cleavage, has been shown to contribute to I/R injury in many non-neural

tissues, particularly the heart, in rodent models. Pharmacological inhibition of the enzyme with, for example, allopurinol limits the increase in inflammatory markers [24], attenuates vascular ROS formation [25], and improves endothelial function [26]. However, the importance of xanthine oxidase for cerebral ROS formation remains controversial, with a few investigations reporting beneficial effects [27, 28], and other studies, including a recent clinical trial in patients with subcortical infarcts, failing to detect improvements [29, 30].

At the present time, NADPH oxidases are the best characterized sources of O_2^-. Although almost all cells posses NADPH oxidase activity, quantitatively neutrophils, with their high NADPH oxidase expression, are of greatest importance for stimulated ROS formation. Neutropenia attenuates ROS formation after stroke [31]. However, the NADPH oxidase activity of other cells is substantial and, therefore, NADPH oxidases have attracted progressively more interest as key mediators of reperfusion-associated brain damage [32] (as reviewed in [33]). Interplay between the aforementioned ROS generating systems appears to occur, and the concept of NADPH oxidases being triggers of mitochondrial radical release has been developed [34, 35].

As a result of the great impact of ROS as mediators of tissue damage in ischemic stroke, a vast number of interventional experimental studies have focused on the relevance of antioxidants, on the inhibitors of ROS generation, and on other agents that potentially interfere with the redox-environment in ischemic stroke in animal models. Most importantly, the vast majority of publications in this field have determined their relevance and reported a beneficial effect [36–38]. The results of several observational studies in humans focusing on biomarkers of ROS damage support the concept of radical formation during I/R and demonstrate a reciprocal relation between the blood levels of antioxidant biomarkers and cardiovascular diseases or death [39–41]. Unlike in animal models, the results of the interventional translational studies in humans were disappointing [42]. In fact, traditional antioxidants, such as beta-carotene and vitamin A or E supplements, may even increase mortality [43–45]. These rather unexpected results are usually explained by a potential interference of the treatment with the numerous physiological actions of ROS as a signaling molecule, such as the regulation of cellular redox potential or matrix metalloproteinases or the reduction of metal ions (reviewed in [46]), and a particular unfavorable chemistry. Indeed, low reaction rate constants of vitamins and many antioxidants require excessive concentrations of these compounds to yield significant alterations in the ROS level. Also, secondary ROS are frequently formed from antioxidants, and thus radical chain reaction may not be effectively terminated by the compounds. Furthermore, several other mechanisms could account for the failure of these larger clinical trials, including (1) supplementation of inefficient amounts of specific antioxidants, (2) supplementation of only one antioxidant, leading to an imbalance in the detoxifying system, (3) reduced affinity of ROS to the antioxidant compared to cellular structures with subsequent tissue damage, (4) failure to reach the appropriate site of action or even (5) adverse pleiotropic effects of the supplemented antioxidant. It should also at least be considered that experimental data are subject to a significant positive selection bias. Finally, the experimental stroke models usually select modes of application of the test compounds which favor therapeutic effects, whereas in the clinical stroke scenario the opposite situation predominates.

Despite the current failure to translate the findings of basic research and animal experiments into clinical practice in humans, the oxidative stress concept in cerebrovascular diseases has not been rejected. Rather, the study of oxidative stress in this context demands advanced approaches, such as targeting the sources of ROS generation—not their products. Moreover, dosage, route, and time and duration of the administration of the agent are all critical determinants of the therapeutic effect. For example, the timing of the inhibition of the matrix-degrading gelatinase matrix metalloproteinase 9 (MMP9) results in a considerable difference in its effect, varying from being beneficial in the acute period of stroke [47] to harmful during repair [48]. Similarly, NOX2, a subunit of NADH oxidase (gp91phox), and nNOS-derived NO may be beneficial to some extent in stroke as they are required for preconditioning before experimental cerebral ischemia [49] or in a mouse model of excitotoxic brain injury [50]. On the other hand, their role in acute, ongoing stroke is apparently rather detrimental [32, 51].

Role of I/R-mediated ROS increase for BBB integrity

All vascular cells are able of producing ROS. Many animal models of neurological diseases with involvement of the cerebral vasculature, such as Alzheimer's disease [52–54] or ischemic stroke (reviewed by [55]), as well as models of stroke-related risk factors [56–58] have shown a disease-associated increase in ROS formation and signs of attenuated endothelial function (reviewed by [59]). Indeed, various effects of ROS on the vasculature have been demonstrated, including inhibition of the regulation of vascular tone and cerebral blood flow (reviewed by [59, 60]), impairment of thromboresistance by altering platelet activation [61], and alterations of the BBB integrity (reviewed by [62, 63]).

Despite different pathogenic mechanisms, oxidative stress and increased BBB permeability are common

findings in several diseases of the CNS (further discussed in [63]), leading to discussions on whether BBB disruption simply coincides with oxidative stress or whether it is a consequence of increased ROS formation. Many experimental neuroprotection studies on antioxidant approaches have found that antioxidants do have beneficial effects on the maintenance of BBB integrity during I/R, pointing to a causal relationship between oxidative stress and BBB disturbances.

BBB integrity is crucial for the maintenance of intracerebral homeostasis and for the prevention of potentially life-threatening postischemic intracerebral hemorrhages [64] or brain edema [65]. Brain edema makes a considerable contribution to brain tissue injury [66]. Both fluid extravasation into the interstitial space and cytotoxic cellular edema lead to brain swelling. However, the bony skull limits brain expansion, resulting in increases in intracerebral pressure and subsequently reducing perfusion of the tissue in proximity to the infarct which per se was not hypoperfused. Due to this process, the 'tissue at risk' is extended beyond the area primarily affected by the vascular occlusion initiating the stroke. Local edema formation basically consists of two principal components [67]. The first is an early, so-called cellular edema that occurs within minutes of stroke onset, typically with cell swelling as a consequence of ceased regional blood flow, ATP depletion, breakdown of ionic gradients, depolarization, and the influx of cations and water. The second component reflects BBB breakdown and is characterized with interstitial fluid accumulation following a biphasic profile and denominated vasogenic edema [68]. The vasogenic edema in turn comprises two phases. The initial phase is an early contraction of endothelial cells and disassembly of tight junction structures [62], accompanied by transient opening of the BBB and extravasation of intravascular fluids. The second phase occurs hours later and is characterized by activation of matrix-degrading proteases [69], a process that is further promoted by thrombolytic therapy and reperfusion. In particular, this second phase serves to explain the increased incidence of intracerebral hemorrhages following thrombolysis in acute stroke [70]. It should be mentioned, however, that whether the opening of the BBB in the postischemic clinical setting actually follows a biphasic pattern with a transient improvement [68] or whether it remains leaky once open [71, 72] is still controversial.

In past decades, experimental brain research focused on pathophysiological mechanisms of cerebral I/R and predomonantly on neuroprotection of neurons. Recently, a more holistic approach of addressing the neurovascular unit as a whole has attracted increased attention. This unit comprises neuronal, glial, and vascular components and provides a basis for investigating an integrated differential

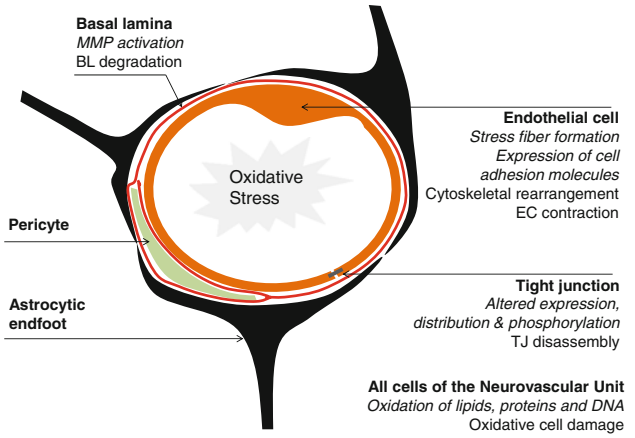

Fig. 1 Targets of reperfusion injury at the neurovascular unit: anatomical structure (*bold*), mechanism (*italic*), and effect (*regular*). *EC* Endothelial cell, *BL* basal lamina, *MMP* matrix metalloproteinases, *TJ* tight junction

response of the brain to exogenous threats. The multi-cellular type of approach facilitates analysis of the cross-talk between cells in the brain and seems to be a more promising way to understand cerebrovascular diseases (Fig. 1) [73].

The BBB, an important target of vascular ROS, as mentioned above, represents a significant part of the neurovascular unit. It is basically formed by intraluminal endothelial cells (EC), pericytes, the surrounding basement membrane, and attached astrocytic endfeet and constitutes the interface between structures of the CNS and the systemic circulation. Cerebral endothelial cells, which are connected to each other by tight and adherent junctions, lack fenestrations and exhibit low pinocytic activity [62]; therefore, they have a low permeability. These cells establish a physical and metabolic barrier for the maintenance of a firmly controlled intracerebral microenviroment which is thought to be mandatory for ideal neuronal function. Adjacent pericytes have contractile elements that modulate the vascular perfusion pressure and thereby the capillary blood flow [74]. The basement membrane or basal lamina (BL) consists of various matrix proteins (collagen IV, fibronectin, heparan sulphate proteoglycans, laminin, among others) and encompasses the EC layer, providing the structural framework of the BBB. To further seal the BBB, the BL is connected to the EC by endothelial integrins. Astrocytes and their endfeet are linked to the outer face of the BL by dystrophin–dystroglycan complexes and regulate the blood flow through cerebral capillaries. Astrocytes are also involved in the control of BBB maturation and in the development and maintenance of barrier properties [75]. Excitotoxicity, as it occurs in ischemic stroke, negatively affects astrocytic function, by inhibiting the repair mechanism and leading a detachment of endfeet; both effects lead to the opening of the BBB [76].

Numerous studies suggest that I/R-induced BBB opening is mediated at least in part by ROS, but different enzymatic sources of ROS production have been considered important. Mitochondria are abundant in cerebral tissue, and mitochondrial complex I is a major source of cerebral intracellular ROS [77]. I/R promotes secondary mitochondrial dysfunction [78] from energy depletion and calcium overload, with a subsequent release of excessive amounts of ROS [65].

Microglia and macrophages are activated early in the course of I/R (reviewed by [79]). Microglia are the resident macrophages of the brain and are derived from mesenchymal progenitors that migrate into the brain during postnatal development [80]. In the healthy brain, microglial cells comprise a stable population and have only negligible exchange with blood-derived monocytes. During brain injury, large numbers of blood monocytes/macrophages enter the brain, but the peak occurs relatively late, between days 3 and 7 (reviewed by [79, 80]). In contrast, microglial activation occurs within minutes after ischemia and precedes BBB disruption. Once activated, microglia, which contain a Nox2-dependent leukocyte-type NADPH oxidase, generate large amounts of O_2^- [81, 82]. At later timepoints, microglia also generate NO by inducible NO synthase (iNOS) [82], which is upregulated after cerebral ischemia [83, 84], and the subsequent formation of peroxynitrite may further damage the injured tissue [85]. It is therefore not surprising that inhibition of microglial activation by minocyclin or the iNOS inhibitor W1400 preserves BBB integrity in the rodent transient middle cerebral artery occlusion model or following intrahippocampal injection of Aβ1–42 [86, 87].

Mast cells have recently been identified as a potential contributor to early BBB opening in cerebral ischemia. Mast cells are potent inflammatory cells that are also present in the wall of cerebral vessels and which can release vast amounts of vasoactive mediators and proteases, thereby promoting BBB disruption. To what extent there is an interaction between postischemic ROS formation and mast cell-mediated BBB leakage is currently unclear. The close temporal association of early ROS-induced BBB opening [32] and mast cell-mediated increase in BBB permeability [88] is consistent with such a link.

Based on these data, antioxidant therapies which target postischemic vascular leakage have been shown to attenuate BBB dysfunction after I/R [89]. ROS affect the BBB on many levels. For example, they can directly oxidize proteins and lipids, activate membrane-degrading enzymes [90], trigger inflammatory responses, modify the structure and function of tight junctions, and initiate cytoskeletal reorganization [62].

In resting endothelial cells, subunits of microfilaments (G-actin) are distributed throughout the cytosol and function to stabilize the cells. Upon activation, G-actin subunits assemble into long F-actin polymers, known as stress fibers, and contract the cell, resulting in the opening of the BBB. Notably, small GTPases of the Rho family which include RhoA, cdc42, and Rac1, are involved in the control of these processes, and ROS promotes their activation [91]. Moreover, Rac1 takes part in the activation of the NADPH oxidases Nox1 and Nox2 [92]. Experimental studies have demonstrated stress fiber formation in brain microvascular endothelial cells (BEC) following exposure to O_2^- [93], such as through Rho-mediated phosphorylation of ROCK. This kinase, via its action on myosin light chain phosphatase, subsequently increases myosin light chain (MLC) phosphorylation, which ultimately results in cytoskeletal reorganization. MLC kinase, which also phosphorylates the MLC, also acts on tight junction proteins, which normally seal the paracellular routes, further disturbing endothelial barrier integrity [94].

How do ROS open the BBB? Direct exposure of BEC to H_2O_2 leads to stress fiber formation (Fig. 1) [32, 93]. In addition, H_2O_2 also induces a redistribution and degradation of junction proteins, such as occludin and zonula occludens 1 and 2. On the molecular level, H_2O_2 activates phospholipase C, which in turn increases intracellular calcium via IP_3 formation. The process, also via MEK, phosphorylates p44/42 MAP kinases, and through the stimulation of the PI3 kinase/protein kinase B/Akt pathway via Rho kinase leads to the disruption of tight junction integrity (occludin, claudin-5) [93]. H_2O_2 also actives the multifunction tyrosine kinase Src [95], which is able to phosphorylate Tiam1, a Rac1-GEF. This process results in the disassembly of adherens junctions [96].

In summary, this section highlights that the BBB is a target of ROS signaling under experimental conditions and during stroke. The relative contribution of the different sources of ROS to ROS-mediated effects in the cerebral vasculature remains to be established, but there is growing evidence for a pivotal role of NADPH oxidases in this context.

NADPH oxidases in the cerebral vasculature

Nox isoforms

NADPH oxidases comprise a family of ROS-producing enzymes. The seven established homologs, Nox1–5 and the dual oxidases Duox 1 and 2, are named after their membrane-bound catalytic subunit. Nox1, Nox2, Nox4, and Nox5 have all been identified as important sources of ROS in the vasculature. Nox2, also known as gp91phox (glycoprotein 91 phagocytic oxidase), represents the prototype Nox and has been intensively studied. It consists of the

membrane-bound heterodimer Nox2 and p22phox, also referred to as cytochrome b558, plus the regulatory cytosolic subunits p47phox, p67phox, p40phox and the small Rac GTPase. Nox2 is activated after phosphorylation of p47phox through one of several possible kinases, such as protein kinase C, protein kinase B/Akt, or p21-activated kinase: after phosphorylation, p47phox translocates to the membrane, and by this process it brings the activator protein p67phox in contact with Nox2. After this assembly step, the activated multiunit complex transfers electrons from NADPH to oxygen, leading to O_2^- generation. Similar to Nox2, Nox1 is composed of the membrane-bound dimer Nox1 and p22phox and of the cytosolic subunits NOXO1, NOXA1, and Rac and is also activated by the interaction of the cytosolic subunits with the membrane-integrated ones. In contrast to Nox1 and Nox2, Nox4 does not require cytosolic subunits for its activation, although it does interact with p22phox [46, 59, 97]. Furthermore, enzymatic activity of Nox4 gives rise to H_2O_2 rather than O_2^- [98–101]. Nox5 activity is regulated by calcium, and as a homodimer it does not require other subunits [102]. Interestingly, unlike in humans, Nox5 is missing in the rodent genome [103]. Finally, Nox3, which shows structural similarities, especially with Nox1 [104], is important for otoconial formation. Mutations of Nox3 alter gravity perception of the inner ear [105], but as yet no function has been attributed to Nox3 in the vasculature.

Expression of NADPH oxidases in cerebral vessels

The different NADPH oxidase isoforms are widely expressed throughout the brain [17, 106]. Although our knowledge of Nox expression in the cerebral vasculature is rather limited (Table 1), several studies have characterized cerebral Nox expression and localization (reviewed in [17]). As there is no neuro- or vessel-specific Nox protein, such characterization has usually revealed differences in the expression patterns of the individual Nox proteins. Of these, Nox4 is remarkable as it exhibits a particular high expression in endothelial cells of the brain [107], and in total it appears to have the highest mRNA expression in the body [108].

In rodent cerebral blood vessels, Nox1, Nox2, Nox4, p22, p47, p67, NOXO1, and NOXA1 [56, 107, 109–111] have been identified at the mRNA level, and Nox1, Nox2, Nox4, p22, and p47 proteins [112–122] have been described. Remarkably, Nox4 protein was reported to be expressed tenfold higher in the basilar artery than in the aorta [116]. Nox4 is also induced in hypertension [111], and basilar arteries of male rats have higher Nox4 expression than those of female rats [115]. Nox2 has also been reported to be more prevalent in cerebral arteries than in peripheral ones [114]. Nox1, Nox2, and Nox4 mRNA has been identified in brain endothelial cells [107, 109], where Nox4 content was higher than Nox1 content which in turn was higher than Nox2 content [107]. The particularly high

Table 1 Expression of NADPH oxidases in the cerebral vasculature

Species	Nox subunits	mRNA/protein	Tissue	Remarks	Reference
Mouse	Nox1, Nox2, Nox4	mRNA	EC		[109]
Mouse	Nox1, Nox2, Nox4 p22, p47, p67	mRNA	Microvessel		[56]
Rat	Nox2, Nox4 p22, p47, p67	mRNA	Arteries	Zucker obese and lean rats	[110]
Rat	Nox1, Nox2, Nox4 Noxo1, Noxa1 p47, p67	mRNA	EC		[107]
Rat	Nox1, Nox2, Nox4 p22, p47	mRNA	Basilar artery	WKY and SHR	[111]
Rat	Nox2 p22	Protein	Vessel		[112]
Mouse and Rat	Nox2	Protein	EC		[113]
Rat	Nox4	Protein	Basilar artery		[116]
Rat	Nox1, Nox2, Nox4	Protein	Basilar artery		[115]
Mouse	Nox2	Protein	Arteries		[114]
Mouse	Nox2 p47	Protein	Arteries		[117]
Mouse	Nox2 p47	Protein	EC	Following CI	[118]
Rat	Nox2	Protein	Microvessel	Following CI	[120]
Mouse	Nox2	Protein	EC	Following AngII	[121]
Mouse	Nox4	Protein	Newly formed capillaries	Following CI	[122]
Mouse	Nox4	Protein	Vessel	Following CI	[119]
Human	Nox4	Protein	EC	Following CI	[119]

EC Brain endothelial cells, *WKY* Wistar–Kyoto rats, *SHR* spontaneously hypertensive rats, *CI* cerebral ischemia, *AngII* angiotensin II

expression of NADPH oxidases in the brain and the vasodilatory response upon their activation suggest an important role for these oxidases in the control of vascular tone [116]. This should be taken into account for any Nox inhibitory strategy. For a more detailed review on cerebral Nox expression, see [17].

The role of NADPH oxidases in ischemic stroke

In a model of permanent middle cerebral artery occlusion (MCAO), Nox4 mRNA levels in neurons increased within the first days, peaked between days 7–15 and slowly declined until day 30. This peak was paralleled by Nox4 mRNA detection in newly formed capillaries, suggesting a role for Nox4 in stroke repair [122]. In the transient MCAO model, Nox4 protein content increased in the ischemic hemisphere within hours of reperfusion and was elevated in murine cerebral vessels and human brain endothelial cells following ischemic stroke. Likewise, Nox2 protein was found to increase after cerebral ischemia in rodents, with significant amounts observed in microvessels [120] and endothelial cells at 24 and 72 h following stroke onset [118]. At the same time Nox activity was enhanced in cerebral arteries of the penumbra for 3 days [123].

As inflammatory cells, particularly neutrophils, accumulate in the ischemic brain parenchyma [124, 125] and as neutrophils contain large amounts of the phagocytic oxidase Nox2, which is a potential source of superoxide in this context, NADPH oxidase has come into focus as mediators of reperfusion injury. Indeed, depleting neutrophils or targeting the endothelium–leukocyte interaction with specific antibodies has yielded smaller brain infarcts [126–129]. However, whether the latter is the result of ROS-mediated brain damage or other actions of leukocytes was not determined in those studies.

Much insight into a possible role for NADPH oxidases in stroke came from Nox knockout mice in break-through work pioneered by Walder et al. [130], who were the first to use Nox2 knockout mice in this setting. These researchers found that following experimental cerebral ischemia, Nox2 knockout mice develop smaller infarcts than their wild-type (WT) littermates. In a series of bone marrow transplant experiments aimed at determining the source of NADPH oxidase-derived superoxide, these same authors observed that bone marrow from Nox2 knockout mice transplanted into WT animals did not reduce lesion volume, as would be expected if leukocytes were the main source of Nox2-derived ROS. The fact that bone marrow from WT mice in Nox2 knockouts resulted in similar infarcts than those obtained in WT animals, however, points to a notable contribution of leukocyte-derived Nox2 in this process. Thus, neuroprotection was only achieved in animals with a general, not leukocyte-specific deletion of Nox2. From these data it has to be concluded that although leukocytes might be important in stroke development, tissue resident Nox2, as present in microglia, endothelial, cells, and neurons, is at least of equal functional importance as the Nox2 contained in leukocytes. In line with this observation, NADPH oxidases have been identified as the major source of ROS during early reoxygenation in neuronal cultures in vitro [131]. In addition, hippocampal neurons lacking a functional Nox2 demonstrate a dramatically attenuated ROS production in ex vivo models of hypoxia/reoxygenation or the oxygen–glucose deprivation (OGD) model [131].

In recent years, much additional work has been devoted to the characterization of different Nox knockout mice in experimental stroke [32, 49, 119, 132–136] (Table 2). With the exception of [119], the results of these studies largely confirm the neuroprotective effect elicited by genetic deletion of Nox2. Accordingly, the genetic deletion of p47phox, which is required for Nox2 (and in some cases perhaps Nox1) activation, also elicits neuroprotective effects in vivo and cultured cells [137]. Interestingly, it was also noted that BBB dysfunction after stroke is largely attenuated in Nox2 knockout mice [32]. The mechanisms by which NADPH oxidases promote early or delayed BBB opening are incompletely understood (see above), and it is likely that different systems are activated in parallel. In a co-culture model of brain endothelial cells and astrocytes that was subjected to hypoxia/reoxygenation, an initial increase in calcium activated O_2^- production, which in turn stimulated the phosphorylation of the MLC in endothelial cells [94]. We were able to demonstrate stress fiber formation (Fig. 1) with the contraction of brain endothelial cells after exposure to ROS with subsequent barrier dysfunction in vitro. Inhibition of Rho-kinase or PI3-kinase prevents endothelial barrier disruption in response to H_2O_2 [32]. The PI3 kinase pathway has been studied in detail in the model of transient MCAO utilizing PI3 kinase knockout mice. Knockout of this important signaling molecule is associated with a decreased expression of Nox1, 2, and 4 mRNA and an attenuated proinflammatory response: both the expression of the adhesion molecules ICAM1, E-selectin, and P-selectin and the activation of the upstream transcription factor NFκB are reduced, as demonstrated by the detection of altered levels of its p65 subunit [138]. The consequences of all these alterations are significantly smaller cerebral infarct volumes and attenuated BBB leakage with a simultaneous maintenance of the expression of the tight junction structural protein claudin5 and the basal laminar protein collagen IV [138].

The higher expression of Nox1 and Nox4 compared to Nox2 in endothelial cells of cerebral arteries [107] might suggest a prominent function for these enzymes in the cerebral vasculature. Only a few studies have addressed the

Table 2 Selected animal studies on genetic deletion and/or pharmacological inhibition of NADPH oxidases in ischemic stroke

Species	Stroke model	Genetic approach	Pharmacological approach	Lack of functional NOX confers protection?	Reference
Mouse	tMCAO	Nox1 −/−	–	No	[139]
Mouse	pMCAO	Nox 1 −/−	–	No	[109]
Mouse	tMCAO	Nox 1 −/−	–	No	[119]
Mouse	tMCAO,	Nox 1 −/−	–	Yes	[109]
Mouse	tMCAO	Nox 2 −/−	–	Yes	[130]
Mouse	tMCAO	Nox 2 −/−	Apocynin, Atorvastatin, TcdB	Yes	[32]
Mouse	tMCAO	Nox 2 −/−	–	Yes	[203]
Mouse, newborn	tHypoxia and pCCAO	Nox 2 −/−	–	No	[132]
Mouse	tMCAO	Nox 2 −/−	Apocynin	Yes	[133]
Mouse	tMCAO	Nox 2 −/−	–	Yes	[135]
Mouse	tMCAO	Nox 2 −/−	Apocynin (after stroke)	No	[119]
Mouse	tMCAO	Nox 2 −/−	–	Yes	[134]
Mouse	tMCAO, pMCAO	Nox 4 −/−	VAS 2870	Yes	[119]
Mouse	tBCCAO	p47phox −/−	Apocynin	Yes	[137]
Rat	tMCAO	–	Atorvastatin	Yes	[149]
Gerbil	tBCCAO	–	Apocynin	Yes	[204]
Rat	tMCAO	–	Apocynin	Yes	[143]
Mouse	tMCAO	–	Apocynin	Yes	[142]
Mouse	tMCAO	–	Apocynin	Yes	[136]
Rat female, young	tMCAO (clot + tPA)	–	Apocynin	Yes	[144]
Rat, female, aged	tMCAO (clot + tPA)	–	Apocynin	No	[144]
Mouse	tBCCAO	–	Apocynin	Yes	[118]
Rat	tMCAO	–	Apocynin	Yes	[146]
Rat	t4VO	–	NSC23766 (Rac GTPase inhibitor)	Yes	[152]
Mouse	tMCAO	–	Normobaric hyperoxia	Yes	[192]
Mouse	tBCCAO	–	Ischemic postconditioning	Yes	[202]

tMCAO Transient middle cerebral artery occlusion, *pMCAO* permanent MCAO, *BCCAO* bilateral common carotid artery occlusion, *tPA* tissue plasminogen activator, *4VO* four vessel occlusion, *TcdB Clostridium difficile* lethal toxin B

role of Nox1 in experimental stroke. In general, the lack of Nox1 was found not to confer protection in transient [109, 119, 139] and in permanent cerebral ischemia [109]. In contrast, we recently reported a moderate reduction in lesion volume and cerebral edema and an ameliorated BBB leakage with relatively mild ischemic damage (60 min ischemia followed by 23 h of reperfusion) after the knockout of Nox1. We also found that the neuroprotective effect of the genetic deletion of Nox1 was lost in more aggressive models of cerebral ischemia. Whether this protection persists or whether it is compensated for during the course of infarct development needs to be established. Overall, given this narrow window for a potential pharmacological intervention, Nox1 does not appear to be a relevant therapeutic target in stroke. The situation for Nox4 is also still unclear. The single published study utilizing Nox4 knockout mice reported an overwhelming protective effect, which was achieved even after permanent vascular occlusion [119]. The mechanism of the potential protective effect elicited by Nox4 deletion is still obscure, and confirmatory data are lacking for any function of Nox4 in the brain. Thus, additional studies will be required to ultimately define the role of this Nox protein in the brain.

Stroke therapy targeting NADPH oxidases

Pharmacological Nox inhibitors

A relatively large number of studies have investigated the importance of NADPH oxidases in ischemic stroke with the aid of pharmacological Nox-inhibitors, such as diphenylene iodonium (DPI) or apocynin (Fig. 2). A major drawback of these approaches is, however, that the compounds are not sufficiently specific for NADPH oxidases. Furthermore, they are not isoform selective, and it is not

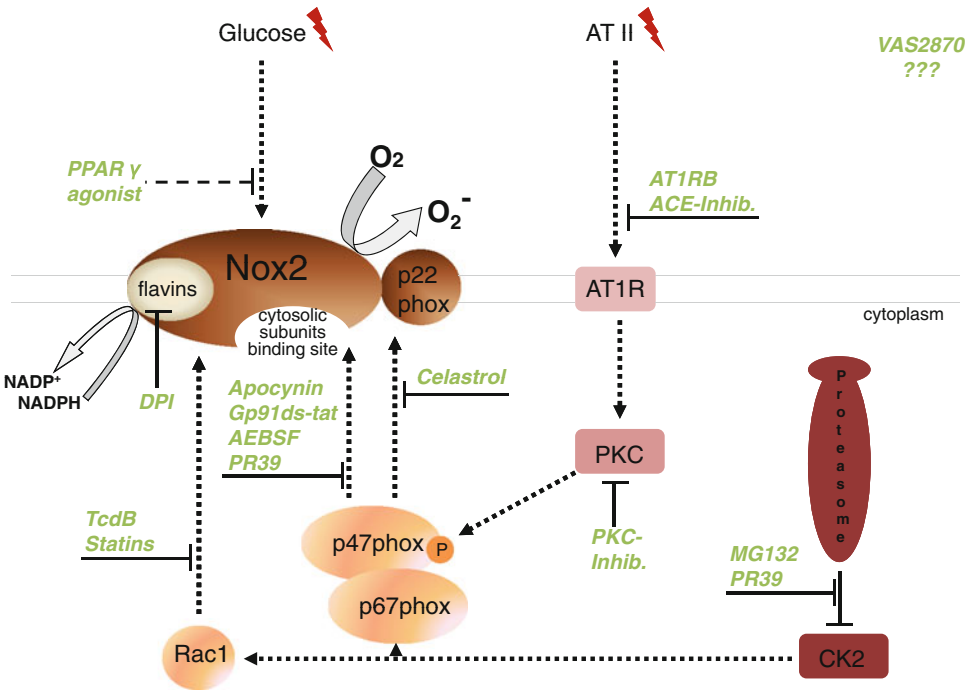

Fig. 2 Strategies of NADPH oxidase (Nox) inhibition in ischemic stroke. *PPAR* Peroxisome proliferator-activated receptor, *AT1RB* angiotensin II 1 receptor blocker (e.g., candesartan), *ACE-Inhib* angiotensin-converting enzyme inhibitor, *AT1R* angiotensin II 1 receptor, *PKC* protein kinase C, *CK2* casein kinase 2, *TcdB Clostridium difficile* lethal toxin B, *Statins* HMG-CoA-reductase inhibitor (e.g., atorvastatin), *DPI* diphenylene iodonium

even clear for most of them whether they actually penetrate the BBB.

Diphenylene iodonium

Diphenylene iodonium acts as an nonspecific inhibitor of flavoproteins that in high concentrations can inhibit other sources of vascular ROS, including mitochondria or NOS. Thus, DPI rather overestimates the disease relevance attributable to NADPH oxidases [140]. Due to its apparent inhibitory effects on mitochondria, cytochrome P450 monoxygenase and all NOS isoforms, the systemic use of DPI is clearly very limited [141].

Apocynin

Apocynin, a natural compound from traditional Chinese medicine, is thought to prevent the assembly of Nox2 with p47phox and is therefore considered to act as a Nox activation inhibitor. As apocynin is non-toxic, cheap, available, and easy to administer, the suitability of the compound for the treatment of experimental stroke has been extensively studied in mice, rats, and gerbils. Almost without exception, apocynin treatment has resulted in a significant reduction in lesion volume, in markers of oxidative cell damage, or in ROS production, and even in Nox subunit expression [32, 118, 120, 133, 136, 137, 142–146] (Table 2). Following hypoxia/reoxygenation of the isolated posterior cerebral arteries of rats, apocynin and the SOD mimetic/antioxidant tempol attenuated O_2^- formation and enhanced endothelium-dependent vasoreactivity, the latter being unaffected by the xanthine oxidase inhibitor allopurinol. These observations also identify NADPH oxidase-derived O_2^- as a mediator of posthypoxic endothelial dysfunction [147]. Indeed, apocynin prevents the translocation of the cytosolic subunit p47phox to the membrane-bound subunit Nox2 and so impedes the assembly and the activation of the enzyme.

Despite the positive effects of apocynin, the true mechanism of action of the compound is still controversial. In addition to blocking p47phox translocation, the compound has been identified as an antioxidant, particular in vascular cells [140], and can elicit a complex of effects on gene expression. Finally, apocynin alters the cellular glutathione level and can interfere with many cellular signaling cascades. Despite these considerations, it has been reported that apocynin does not confer additional stroke protection in Nox2 knockout mice [136], which illustrates that apocynin and Nox2 deletion act on the same signaling pathway. The results in experimental stroke studies promote apocynin as a promising agent for acute stroke treatment. Based on its low toxicity, a study in humans appears feasible, but to the best of our knowledge, such a study has not been carried out. A potential limitation of apocynin is that the compound is a pro-drug which requires activation by myeloperoxidase [140]. Whether this aspect represents a limitation for therapy in humans is unclear.

4(2-Aminoethyl) benzenesulfonylfluoride

4(2-Aminoethyl) benzenesulfonylfluoride (AEBSF) is a serine protease inhibitor that is not specific for inhibition of

NADPH oxidases. AEBSF interferes with several assays for ROS detection [148]. The compound cannot be used in vivo, and no data on hypoxia/reoxygenation experiments in cultured cells are available.

Atorvastatin

Atorvastatin is a representative of the lipid-lowering class of HMG-CoA reductase inhibitors. The compound reduced cerebral infarct volume and attenuated gp91phox protein accumulation and p47phox translocation as well as O_2^- formation in a rat model of I/R [149] and mitigated BBB opening in a corresponding mouse model [32]. Atorvastatin prevents the geranylgeranylation of Rac1 and thereby prevents the assembly of cytosolic Rac1 with the membrane-bound Nox subunits. This action of the compound belongs to its pleiotropic effects, which are nevertheless relatively important: withdrawal of statin treatment induces endothelial dysfunction through activation of Nox2 by Rac1 [150] and abolishes stroke protection in mice [151]. Obviously, the effects of statins are also by no means specific for NADPH oxidases, and additional studies have tested the role of Rac1 in stroke.

Pretreatment with *lethal toxin B of Clostridium difficile*, which inhibits RhoA and Rac1 preserves BBB integrity after I/R in the mouse [32].

NSC23766, a pharmacological Rac1 GTPase inhibitor, was also able to reduce Nox activation, O_2^- formation, and accumulation of the oxidation products 4-HNE and 8-OHdG in the transient four-vessel occlusion model in the rat. These effects resulted in a partial protection of cognitive spatial abilities and memory function [152].

Candesartan

Candesartan, an angiotensin II AT1 receptor blocker (AT1RB) attenuates infarct volume and edema formation, improves neurological function, and reduces gp91phox/p22phox mRNA expression as well as lipid peroxidation products in transient focal cerebral ischemia in rats [153]. These effects are most likely mediated by preventing the actions of angiotensin II on cerebral vessels. Hence, inhibitors of angiotensin converting enzyme (ACE) have similar effects. Angiotensin II (Ang II) stimulates and induces NADPH oxidases [154] and seems to mediate its hypertensive effect, at least in part via Nox-generated ROS [155, 156]. Angiotensin II also promotes endothelial dysfunction in the cerebral microcirculation through AT1 receptor-mediated cerebrovascular oxidative stress. The source of ROS is a Nox-2-containing NADPH oxidase [56, 113]. However, clinical studies on the efficacy of AT1RB revealed conflicting results: whereas some studies reported beneficial effects (reviewed by [157]), no protection was observed in two recent large trials [158, 159].

PR39

PR39, a natural occurring, peptide-based NADPH oxidase inhibitor, binds to a SH3 domain of p47phox and thus prevents the assembly of NADPH oxidase. PR39 acts nonspecifically in that it binds to multiple SH3-containing proteins and interacts with membrane lipids. Due to its peptide character, biovavailability is limited [141]. In addition, it is a noncompetitive and reversible inhibitor of 20S proteasome [160]. To the best of our knowledge, PR39 has not been tested in stroke.

Gp91ds-tat

Gp91ds-tat is a synthetic peptide which was originally designed to interfere with the assembly of p47phox with gp91phox in intact cells [161]. Gp91ds-tat exerts a relatively large potency in inhibiting vascular O_2^- production. Although it has recently been shown that gp91ds-tat may not inhibit Nox1 and Nox4 [162], the complex interplay between Nox homologs may render the compound unspecific in action [46]. To the best of our knowledge, gp91ds-tat has not been tested in models of cerebral ischemia.

Rho kinase inhibitor

Inhibition of *Rho kinase or the downstream Rho associated kinase* (*ROCK*) was found to reverse H_2O_2-induced endothelial barrier dysfunction in vitro [32] and shown to be effective in many experimental stroke studies (reviewed by [163]). One phase III clinical trial was performed in Japan [164]. This multicenter randomized controlled trial revealed better outcome parameters in patients treated with fasudil, a Rho kinase inhibitor, than in the control group treated with saline. However, this study has several shortcomings (discussed in [163]) and before the experimental data can be translated into common stroke therapy it is mandatory to confirm the results and correct the shortcomings in a future large clinical trial. The mechanisms underlying the neuroprotective effect of Rho kinase/ROCK inhibition in stroke remain largely elusive. Fasudil was found to attenuate angiotensin II-stimulated O_2^- production, decrease Nox1, Nox2, Nox4, and p22phox mRNA expression in systemic arteries, and mitigate the angiotensin II-mediated impairment of endothelium-dependent relaxation [165]. These observations suggest a relevant role for NADPH oxidases in Rho kinase-mediated regulation of vascular tone. Whether there is a similar link between NADPH oxidases and Rho kinase in the cerebral vasculature needs to be established. A potential limitation of the therapeutic administration of Rho kinase/ROCK inhibitors in stroke are hypotension and vasodilation, both of which may further reduce cerebral blood flow to the ischemic area. Vasodilation is a consequence of the reduction in smooth muscle Rho kinase

activity, which directly dilates the artery, and an activation of eNOS, which potentiates this effect through NO release [166]. As Rho kinase/ROCK promotes cardiovascular dysfunction and atherosclerosis, chronic treatment with Rho kinase inhibitors might constitute a promising alternative targeting stroke prevention. Currently, Rho kinase inhibitors are being used in Japan to treat and prevent arterial spasms in patients with subarachnoidal hemorrhage [167].

Peroxisome proliferator-activated receptor gamma agonists

Peroxisome proliferator-activated receptor (PPAR) gamma agonists have proven to be beneficial in the treatment of experimental stroke [168]. Zhang et al. [169] proposed the suppression of the nuclear factor kappa B signaling pathway as the involved mechanism. This observation is in line with a recent study from Lu et al. [170] showing that the PPAR gamma agonist rosiglitazone attenuates hypoxia-induced Nox4 expression and ROS generation in the mouse lung involving the nuclear factor kappa B pathway. Furthermore, PPAR gamma agonists increase anti-inflammatory cytokines and interfere with glial activation after transient cerebral ischemia. This process may lead to a reduction in the delayed neuronal death after stroke [171]. The PPAR gamma agonist reduces O_2^- generation and improves endothelial function in carotid arteries of hypertensive rodents and appears to limit Nox mRNA expression [172, 173]. Moreover, the inhibition of PPAR gamma signaling in cerebral vessels by dominant negative mutants has been found to increase O_2^- production and lead to endothelial dysfunction [174]. In extracerebral vascular cells also, activation of PPAR gamma results in a decreased expression of NADPH oxidases [175]. As glucose and NADPH oxidase promote O_2^- formation and neuronal death in experimental stroke [58] and the PPAR gamma agonist rosiglitazone reduces NADPH oxidase-mediated glucose-induced oxidative stress in cultured endothelial cells [176], a relevant contribution of NADPH oxidases in PPAR gamma signaling in cerebral vessels appears likely. The molecular pathways of PPAR gamma agonist's action in stroke and its specific interaction with NADPH oxidases in this context need further investigation.

Celastrol

Celastrol is an active biological compound of the medical plant *Tripterygium wilfordii*, which appears to show benefits in cancer and inflammatory conditions. In vitro experiments suggest that Celastrol is a potent inhibitor of NADPH oxidases, particularly Nox1 and Nox2. Inhibition of the functional association of p47phox with p22phox has been found to mediate the inhibition of Nox1 and Nox2 [177]. This novel mode of action cannot explain the inhibitory effect on Nox4 and Nox5, as p22phox is missing in the latter and the p47phox subunit lacks both isoforms. In light of two other studies showing ROS accumulation following celastrol treatment and increased cytotoxicity [178, 179], the potential of celastrol in stroke treatment cannot yet be estimated. Moreover, as expected from its quinoidal structure, celastrol has also been reported to be an antioxidant [180], which might overestimate its protective effects imputable to NADPH oxidases.

Protein kinase C inhibitors

Protein kinase C inhibitors are a group of promising agents which has many effects, including preventing the phosphorylation of p47phox by protein kinase C (PKC), thereby limiting NADPH oxidase activation. In a model of renal hypertension and in rats infused with angiotensin II, the administration of PKC inhibitors showed beneficial effects [181, 182]. Several studies have investigated the role of PKC in stroke. Overall, PKC is activated during ischemia and reperfusion. However, whether the action of PKC is beneficial or harmful depends on the isoform and the time-point (preconditioning, ischemia, or reperfusion) involved (for review see [183]). In addition, PKC is capable of phosphorylating several enzymes which act on gene transcription, cell permeability, and other cellular responses. Therefore, PKC inhibitors are not at all specific, and deleterious effects upon systemic administration should be kept in mind [141].

Casein kinase 2

Casein kinase 2 (CK2) is a critical negative regulator of NADPH oxidase activity. Transient focal cerebral ischemia significantly reduces the protein levels of CK2 subunits as well as CK2 activity. Simultaneously, Nox2 expression increases, and p67phox and Rac1 translocate to the membrane, reflecting steps which are required for NADPH oxidase activation. Accordingly, deactivation of murine CK2 enhances ROS generation and neuronal cell death through an increased NADPH oxidase activity. Inhibition of the degradation of CK2, such as by proteasome inhibitor MG132, preserves CK2 activity in vitro and in vivo and attenuates O_2^- production [184]. CK2 appears to be an interesting target for acute stroke therapy, but further confirmation with specific CK2 activators or genetically modified animals will be needed to further substantiate the findings.

VAS2870

VAS2870, a small-molecule triazolo pyrimidine, belongs to a new class of NADPH oxidase inhibitors [141, 185–187]. It has been found to attenuate phorbol-12-myristate

13-acetate-dependent stimulation of NADPH oxidase in vitro but to have no effect on the translocation of p47phox [188], which points to an interaction downstream of the assembly process. As this effect was observed in phagocytes, it seems likely that VAS2870 inhibits Nox2, but the exact location and mode of action remain to be determined. In endothelial cells, VAS2870 inhibits oxLDL-induced ROS production [189]. Because the compound does not affect basal ROS production, which is generally attributed to Nox4, an interaction with Nox2 or Nox1 is probable. In addition, the inhibition of PDGF-dependent ROS generation in smooth muscle cells favors an interaction with Nox1 rather than Nox2 in these cells [188]. The first evidence for efficacy of VAS2870 was recently obtained in vivo in a mouse model of transient focal cerebral ischemia [119]. Intrathecal injection of VAS2870 significantly reduced tissue damage following experimental stroke. Given the previous data on Nox1 and Nox2 inhibition, it is not clear why the authors conclude that VAS2870 was a Nox4-specific inhibitor based on the fact that the compound had no effect on the small infarcts they produced in Nox4 knockout mice. Additional work will be needed to further dissect the selectively of the isoform and the mode of action of VAS2870 (Table 2).

Further potential candidates for acute stroke treatment due to their inhibitory interaction with NADPH oxidases, with only very little data available to date, are: S17834, GKT136901, ML171, and Fulvene5 (for review [185]). To what extent their interactions are specific and whether they prove to be effective in stroke in vivo should be established in future studies.

Non-pharmacological inhibition of NADPH oxidases

Normobaric oxygen therapy

Normobaric oxygen (NBO) therapy, i.e., normobaric hyperoxia with 95 % O_2/5 % CO_2 or 100 % O_2, during ischemia reduces lesion volume in magnetic resonance imaging (ADC maps) and ameliorates tissue injury at 24, 48 and 72 h after stroke [190–192]. As there are also conflicting results on stroke volume [193], a potential unpredictable vasoconstriction mediated by a high oxygen partial pressure (pO_2), particularly when treated with 100 % O_2, should be considered to be a serious side effect. Also, an overwhelming vasodilation in experiments using a gas mixture of 5 % CO_2/95 % O_2 with a drop in perfusion pressure can occur [194–196]. In terms of mechanisms, an increase in brain tissue pO_2 and decreased ROS generation and attenuated Nox2 induction have been suggest, at least for the rat model of MCAO [191, 192]. NBO treatment during ischemia has the potential to protect against brain edema via inhibition of Nox2-mediated MMP9 induction at the microvascular level [120]

Postconditioning

Postconditioning can be established and is effective in focal cerebral ischemia [197–200]. In the heart, interrupted reperfusion following cardioplegic cold ischemia leads to an improvement of cardiac function associated with reduced O_2^- production and Nox activity [201]. Similarly, interrupted reperfusion preceded by transient focal cerebral ischemia has been observed to confer significant neuroprotection in mice [202]. Mortality, neuronal death, O_2^- production, translocation of p47phox to the membrane, and the amount of activated Rac were significantly reduced in that study, and neurological functional outcome in the experimental group was better than that in the control groups. Interestingly, preconditioning, which describes the phenomenon that sublethal injurious stimuli prior to a massive ischemic insult confers tolerance to subsequent lethal insults, also requires Nox2 (and NO to form peroxynitrite), as demonstrated for excitotoxic brain injury [50] and the mouse model of transient MCAO [49].

Conclusion

Oxidative stress substantially contributes to cerebral reperfusion injury, and NADPH oxidases are a major source of ROS in this context. Inhibition or genetic deletion of functional NADPH oxidases, particularly Nox2 and Nox4, largely mitigate tissue damage following experimental stroke. NADPH oxidase inhibition appears to be a promising strategy for acute stroke therapy. The development of inhibitors targeting specific Nox isoforms will further increase our knowledge on NADPH oxidases in stroke. In addition, an understanding of the relative importance of the different ROS generating systems, their cellular and subcellular distribution, as well as the cross-talk between the participating cells of the neurovascular unit under physiological and pathological conditions will lead to a better stroke therapy in the future.

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