Post-Stroke Inhibition of Induced NADPH Oxidase Type 4 Prevents Oxidative Stress and Neurodegeneration

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Abstract

Ischemic stroke is the second leading cause of death worldwide. Only one moderately effective therapy exists, albeit with contraindications that exclude 90% of the patients. This medical need contrasts with a high failure rate of more than 1,000 pre-clinical drug candidates for stroke therapies. Thus, there is a need for translatable mechanisms of neuroprotection and more rigid thresholds of relevance in pre-clinical stroke models. One such candidate mechanism is oxidative stress. However, antioxidant approaches have failed in clinical trials, and the significant sources of oxidative stress in stroke are unknown. We here identify NADPH oxidase type 4 (NOX4) as a major source of oxidative stress and an effective therapeutic target in acute stroke. Upon ischemia, NOX4 was induced in human and mouse brain. Mice deficient in NOX4 (Nox4⁻/⁻) of either sex, but not those deficient for NOX1 or NOX2, were largely protected from oxidative stress, blood-brain-barrier leakage, and neuronal apoptosis, after both transient and permanent cerebral ischemia. This effect was independent of age, as elderly mice were equally protected. Restoration of oxidative stress reversed the stroke-protective phenotype in Nox4⁻/⁻ mice. Application of the only validated low-molecular-weight pharmacological NADPH oxidase inhibitor, VAS2870, several hours after ischemia was as protective as deleting NOX4. The extent of neuroprotection was exceptional, resulting in significantly improved long-term neurological functions and reduced mortality. NOX4 therefore represents a major source of oxidative stress and novel class of drug target for stroke therapy.


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Abbreviations: C5SS, constructive interference in steady state; KO, knock out; pMCAO, permanent middle cerebral artery occlusion; ROS, reactive oxygen species; rt-PA, recombinant tissue plasminogen activator; tMCAO, transient middle cerebral artery occlusion; TTC, 2,3,5-triphenyltetrazolium chloride; WT, wild type.

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Introduction

Ischemic stroke has outstanding medical relevance as it is the second leading cause of death in industrialized countries [1]. Due to the aging of the population, the incidence of stroke is projected to rise even further in the future [2]. Despite tremendous research activity, with more than 100 clinical trials in human stroke patients [3], only one therapy approved by the United States Food and Drug Administration is available, i.e., thrombolysis using recombinant tissue plasminogen activator (rt-PA). However, the efficacy of rt-PA on functional outcomes is moderate at best, and more than 90% of all stroke patients must be excluded from rt-PA treatment because of over 25 labeled contraindications. Therefore, there is an unmet need for more effective therapies in acute stroke.
Author Summary

Stroke is the second leading cause of death worldwide. Today, only one approved therapy exists—a drug that breaks down blood clots—the effectiveness of which is moderate, and it can only be used in about 10% of patients because of contraindications. New therapeutic strategies that are translatable to humans and more rigid thresholds of relevance in pre-clinical stroke models are needed. One candidate mechanism is oxidative stress, which is the damage caused by reactive oxygen species (ROS). Antioxidant approaches that specifically target ROS have thus far failed in clinical trials. For a more effective approach, we focus here on targeting ROS at its source by investigating an enzyme involved in generating ROS, known as NADPH oxidase type 4, or NOX4. We found that NOX4 causes oxidative stress and death of nerve cells after a stroke. Deletion of the NOX4-coding gene in mice, as well as inhibiting the ROS-generating activity of NOX4 with a pharmacological inhibitor, reduces brain damage and improves neurological function, even when given hours after a stroke. Importantly, neuroprotection was preserved in old male and female Nox4−/− mice as well as in Nox4+/− mice subjected to permanent ischemia. NOX4 thus represents a most promising new therapeutic target for reducing oxidative stress in general, and in brain injury due to stroke in particular.

Although a plethora of drugs for the treatment of acute stroke are effective in animal models, their translation into clinical practice has completely failed [3,4]. As a result, many pharmaceutical companies have withdrawn from drug discovery in this area. To overcome this lack of clinically effective neuroprotective drugs, innovative strategies are urgently needed to identify pathways that can be targeted with innovative therapies [5]. Higher quality study designs are also required [6,7].

One such high-potential pathway in ischemic stroke may be the occurrence of oxidative stress, i.e., the increased occurrence of reactive oxygen species (ROS) above physiological levels. Oxidative stress has been suggested for many years to cause tissue damage and neuronal death. The toxicity of ROS can be further increased by nitric oxide to produce reactive nitrogen species such as peroxynitrite (ONOO−), a molecule that causes oxidation and nitration of tyrosine residues on proteins [8]. Disappointingly, there is no conclusive evidence of a causal link between oxidative stress and the development of disease, and there is no successful therapeutic application targeting oxidative stress. To date, clinical attempts to scavenge ROS by applying antioxidants did not result in clinical benefit [9] or even caused harm [10,11]. However, the characterization of the relevant enzymatic sources of oxidative stress may allow therapeutic targeting of oxidative stress by preventing the formation of ROS in the first place, instead of scavenging ROS after they have been formed.

A potential source of ROS are NADPH oxidases, the only known enzyme family that is only dedicated to ROS production [12]. Four rodent genes of the catalytic subunit NOX, Nox1, Nox2, Nox3, and Nox4, have been identified, of which Nox1, Nox2, and Nox4 are expressed in the vasculature. NOX4 is the most abundant vascular isoform; its expression is even higher in cerebral than in peripheral blood vessels [13] and, further, induced in stroke [14]. Therefore, we hypothesized that NOX4 is the most relevant source of ROS in stroke.

To test this hypothesis, we generated constitutively NOX4-deficient (Nox4−/−) mice and directly compared them to NOX1-deficient (Nox1−/−) and NOX2-deficient (Nox2−/−) mice. NOX4 has been implicated in the regulation of systemic and hypoxic vascular responses. Therefore, we had to exclude systemic vascular effects of NOX4 deletion on blood pressure, which may affect stroke outcome independent of a specific neuronal or neurovascular mechanism. Finally, to examine the therapeutic potential of NOX4 as a drug target, we infused the specific NADPH oxidase inhibitor VAS2870 [15] after ischemia, thus mirroring the clinical scenario.

Results

NOX4 Is Induced during Ischemic Stroke in Mice and Humans

Because NOX4 mRNA is expressed at higher levels in cerebral than in peripheral blood vessels [13] and is induced in stroke [14], we first sought to validate these data not only at the mRNA but also at the protein level. In all experiments, we followed current guidelines defining methodological standards for experimental stroke studies [4,6,7,16,17]. Here we chose a model of acute ischemic stroke in which mice are subjected to transient middle cerebral artery occlusion (tMCAO). This disease model is thought to involve oxidative stress and an induction of Nox4 expression [18]. Indeed, expression of NOX4 mRNA was significantly higher 12 h and 24 h after tMCAO in the basal ganglia and neocortex of wild-type mice than in sham-operated controls, in which basal NOX4 expression was low (Figure 1A). This result was validated by immunohistochemistry using a specific NOX4 antibody. We detected a stronger staining in neurons and cerebral blood vessels in wild-type mice subjected to tMCAO than in sham-operated controls. Although immunohistochemistry is not quantitative, this finding suggests higher levels of NOX4 protein (Figure 1B). Importantly, NOX4 staining was also stronger in brain samples from stroke patients. Although NOX4 was barely detectable in healthy brain regions, clear positive labeling of NOX4 was seen in neurons and vascular endothelial cells from the forebrain cortex of stroke patients. This finding was confirmed by double labeling for NeuN (a neuronal marker) or von Willebrand factor (an endothelial marker) and NOX4 in brain tissue (Figure 1B). These data indicate that NOX4 protein is induced during brain ischemia in mice, and this observation would be in agreement with a major functional role for NOX4 in ischemic stroke. Our limited observations in a small number of human cases provide some support to the hypothesis that these processes are also important in human stroke.

Nox4−/− but Neither Nox1−/− nor Nox2−/− Mice Are Protected in Both Transient and Permanent Ischemic Stroke

We first subjected 6- to 8-wk-old male Nox4−/− mice to tMCAO and, after 24 h, assessed infarct volumes by staining brain sections with 2,3,5-triphenyltetrazolium chloride (TTC) (Figure 2A). Infarct volumes were significantly smaller, by approximately 75%, in male Nox4−/− mice than in sex-matched wild-type controls (25.5±14.8 mm³ versus 78.7±19.5 mm³, respectively). The smaller infarct volume was functionally relevant: compared with wild-type mice, Nox4−/− mice had significantly better overall neurological function (Bederson score 1.2±0.7 in Nox4−/− mice versus 3.7±1.1 in wild-type mice) as well as better basal motor function and coordination (grip test score 4.3±0.7 in Nox4−/− mice versus 1.7±1.3 in wild-type mice) 24 h after tMCAO (Figure 2B). Gender can significantly influence stroke outcome in rodents [4,16,17]. Therefore, we also subjected female Nox4−/− mice to 60 min of tMCAO. In line with the results in male mice, Nox4-deficient female mice also developed significantly smaller infarctions.
Role of NOX4 in Stroke

Figure 1. Induction of NOX4 expression after ischemic stroke in mice and humans. (A) Relative gene expression of Nox4 in the ischemic basal ganglia (left) and cortex (right) of wild-type mice after sham operation and 4 h, 12 h, and 24 h after tMCAO (n = 5), *p < 0.05, one-way ANOVA, Bonferroni post-hoc test, compared with sham-treated controls. (B) Immunohistochemical detection of NOX4 protein in ischemic brains of wild-type mice (sham operation or tMCAO, day 1) and humans (samples from stroke patients, after routine autopsy). We compared NOX4 immunolabeling in the ischemic forebrain cortex and the unaffected contralesional side. In ischemic samples, NOX4 was predominately expressed in neurons (arrowheads) and endothelial cells (arrows). This distribution was confirmed by visualization of NOX4 and NeuN or NOX4 and von Willebrand Factor in the same structures. All scale bars represent 100 μm.

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Protection from Ischemic Stroke in Nox4−/− Mice Is a Result of Reduced Oxidative Stress, Neuronal Apoptosis, and Blood-Brain-Barrier Leakage

Next we sought to elucidate the underlying mechanisms of this NOX4-specific neurotoxicity in stroke. NOX4 can form superoxide or H₂O₂, which can interact with nitric oxide to form reactive nitrogen species. Therefore, we stained brain sections with broad-spectrum indicators of oxidative/nitritative stress, i.e., dihydroethidium [28] and nitrotyrosine [8]. At 12 h and 24 h after tMCAO, brains from wild-type mice exhibited a significantly larger amount (by a factor of 2.5–3.5) of ROS in neurons than brains from sham-operated animals, as quantified by dihydroethidium staining (Figure 3A). Neurons from Nox4−/− mice, in contrast, showed only very small ischemia-induced increases in ROS relative to those in sham-operated controls (p > 0.05). ROS formation from neurons after 24 h was also significantly reduced in Nox4−/− mice subjected to pMCAO (Figure S6). Because the dihydroethidium stain may also indicate oxidative chemistry events, including formation of ONOO− and nitration of protein tyrosine residues [8], we analyzed the extent of protein nitration in Nox4−/− and wild-type mice subjected to tMCAO. In agreement with our findings on the generation of ROS, tissue nitration occurred to a lesser extent in ischemic brains from Nox4−/− mice than in those from wild-type controls (Figure 3B). Oxidative chemistry events such as the formation of ROS and peroxynitrite, as detected by...
Role of NOX4 in Stroke

Transient Ischemia: tMCAO

6 - 8 weeks

18 - 20 weeks

Infarct volume mm³

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Long-term outcome: tMCAO

Day 1

Day 6

Permanent Ischemia: pMCAO

Infarct volume mm³

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Permanent Ischemia: PT

Infarct volume mm³

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Figure 2. Nox4 deficiency confers long-term neuroprotection and reduces mortality after acute ischemic stroke in young adult and aged mice of either sex. (A) Upper panel shows representative TTC staining of three corresponding coronal brain sections of 6- to 8-wk-old male and female wild-type (WT) mice, male Nox4−/− mice, and male and female Nox2−/− mice, as well as 18- to 20-wk-old male wild-type and Nox4−/− mice on day 1 after tMCAO. The ischemic infarcts (white) appear smallest in the Nox4−/− mice of either age or sex (arrows), and this result was confirmed by infarct volumetry (lower panel). **, p<0.0001, and *, p<0.001, one-way ANOVA, Bonferroni post-hoc test compared with wild-type mice (n=8–19 per group). (B) Neurological Bederson score (upper panel) and motor score (lower panel) on day 1 after tMCAO in the eight mouse groups indicated above. (C) Serial magnetic resonance images of cerebral infarcts 1 d and 6 d after tMCAO in wild-type and Nox4−/− mice (lower panel). The broken white lines show hyperintense ischemic lesions on day 1 after tMCAO in wild-type and Nox4−/− mice. Infarcts on day 1 are smaller in Nox4−/− mice than in wild-type mice and remain restricted to the basal ganglia on day 6. Hematoxylin and eosin staining confirmed neuronal damage in the cortex of wild-type mice 24 h after tMCAO (top panel, left), whereas cortical integrity was preserved in Nox4−/− mice (top panel, right). (D) Mortality (upper panel) and long-term functional outcome (Bederson score, lower panel) in 6- to 8-wk-old male Nox4−/− mice and wild-type controls. Survival curve (upper panel): **, p<0.001, and *, p<0.05, one-way ANOVA, Bonferroni post-hoc test compared with wild-type mice (n=10–15 per group). Long-term outcome (lower panel): ***, p<0.0001, and *, p<0.05, one-way ANOVA, Bonferroni post-hoc test compared with wild-type mice (n=10–15 per group). (E) Upper panel shows representative TTC staining of three corresponding coronal brain sections of 6- to 8-wk-old male wild-type mice (left) and matching Nox4−/− mice (right) on day 1 after pMCAO. Lower panel: Infarct volumes as measured by infarct volumetry (left) and Neurological Bederson score (right). Nox4 deficiency also protects the brain from permanent ischemia. **, p<0.001, and *, p<0.05, two-tailed Student’s t-test compared with wild-type mice (n=7–11 per group). (F) Representative coronal brain sections of wild-type and Nox4−/− mice stained with TTC on day 1 after permanent cortical phototrombosis (PT) (upper panel). Cortical infarctions are smaller in the absence of NOX4 (arrow). The lower panel shows infarct volumes in wild-type and Nox4−/− mice on day 1 after cortical phototrombosis. **, p<0.001, two-tailed Student’s t-test compared with wild-type mice (n=7 per group). All scale bars represent 100 μm.

dihydroethidium staining and nitrotyrosine immunolabeling, can induce neuronal apoptosis, which is a well-established mechanism of tissue damage in ischemic stroke [29,30]. Indeed, superimposed TUNEL and NeuN immunolabeling revealed widespread apoptosis of neurons in wild-type mice 24 h after stroke onset (Figure 3C). In contrast, the number of apoptotic neurons in Nox4−/− mice subjected to tMCAO was significantly lower, and the basal apoptotic turnover rate in Nox4−/− mice fell within the range found in sham-operated mice (p>0.05) (Figure 3C).

We also detected NOX4 in cerebral blood vessels (Figure 1B, white arrow indicates endothelial cells). Therefore, we hypothesized that Nox4 deficiency also influences the disruption of the blood–brain barrier and edema formation mediated by ROS [31]. Integrity of the blood–brain barrier was preserved in Nox4−/− mice on day 1 after tMCAO. This finding correlated with significantly less brain edema in Nox4−/− mice than in wild-type controls, as assessed by the extent of extravasation of Evans blue stain (0.0±5.9 mm3 in Nox4−/− mice versus 96.2±5.9 mm3 in wild-type mice). Importantly, almost no brain edema was seen in the brain regions where infarcts were regularly present in Nox4−/− mice (basal ganglia; Figure 3D, area delineated by the broken white line). This result indicates that the lesser edema seen in the Nox4−/− mice was a specific phenomenon and mechanistically relevant but was not due to smaller infarct volumes.

Treatment with the NOX Inhibitor VAS2870 Effectively Protects Ischemic Brain Damage Even When Applied After Stroke

Finally, we wanted to examine whether these genetic insights into the biology of oxidative stress in stroke and the role of NOX4 in general can be translated into a therapeutic intervention. Importantly, this intervention would have to be effective post-stroke and ideally it would be pharmacological. Therefore, we examined the efficacy of a validated, low-molecular-weight NADPH oxidase inhibitor, VAS2870 [15,32–34], in vital brain slices and in vivo. VAS2870 equally inhibits the ROS-generating activity of all NOX subunits, i.e., NOX1, NOX2, and NOX4. Vital brain slices [35] taken from wild-type mice 12 h after tMCAO produced significantly less ROS after pretreatment with 10 μM VAS2870, as did brain slices from untreated Nox4−/− mice (Figure 4A). Importantly, incubating ischemic slices from Nox4−/− mice with VAS2870 had no additional inhibitory effect on superoxide formation (Figure 4A). This finding further underlines the extraordinary role of NOX4 in generating oxidative stress during the course of ischemic stroke, while other NOX isoforms such as NOX1 or NOX2 are obviously less relevant.

To determine whether VAS2870 is also active when applied in vivo, we administered 2 mg of VAS2870 intrathecally to wild-type mice 2 h and 12 h after tMCAO. This experimental therapeutic approach significantly reduced brain infarct volumes (20.7±4.0 mm3 in VAS2870-treated mice versus 82.4±6.4 mm3 in vehicle-treated controls) and significantly improved neurological function, to the same extent as observed for the deletion of Nox4 in mice (Figure 4B and 4C). Moreover, less oxidative stress was detected in ischemic brains from VAS2870-treated animals than in those from vehicle-treated controls (Figure 4D). Again, post-stroke application of VAS2870 to Nox4−/− mice had no additive neuroprotective or superoxide-lowering effect compared to the outcome in wild-type animals treated with VAS2870 or untreated Nox4−/− mice (Figure 4B–4D). This observation is consistent with our ex vivo findings in ischemic brain slices and reaffirms that NOX4 rather than NOX1 or NOX2 is critically involved in the pathophysiology of ischemic stroke. Another, less specific inhibitor that also targets molecules other than NADPH oxidases [36,37], apocynin, had no effect on infarct size or functional outcome when given post-stroke and did not reduce the formation of ROS in vivo (Figure 4B and 4C).

To further examine whether the neuroprotective effect observed in Nox4−/− mice is specifically related to reduced ROS formation and not due to other nonspecific or developmental defects, we performed a rescue experiment by restoring cerebral ROS levels in Nox4−/− mice during the course of ischemic stroke by applying exogenous H2O2 (Figure 4B–4D). Indeed, intrathecal administration of H2O2 rescued the phenotype in Nox4−/− mice (Figure 4B–4D).

Discussion

Here we identify NOX4 as a relevant molecular source of oxidative stress in cerebral ischemia, including some cases of human stroke. Our data suggest that NOX4-mediated oxidative stress leads to neuronal damage via leakage of the blood–brain barrier and neuronal apoptosis—two pathophysiological hallmarks of ischemic stroke. The extent of neuroprotection conferred by the absence of NOX4 in male and female Nox4−/− mice was exceptional and preserved in old animals. Importantly, the outcomes of these genetic experiments were mimicked when we pharmacologically inhibited NADPH oxidases within a clinically relevant time after induction of stroke. We consider this a key
Role of NOX4 in Stroke

Figure 3. Nox4 deficiency confers neuroprotection by reducing oxidative stress, neuronal apoptosis, and disruption of the blood-brain barrier. (A and B) Left panels show representative brain sections from sham-operated wild-type (WT) mice and wild-type and Nox4−/− mice 24 h after tMCAO. Sections were stained for ROS and oxidative chemistry using dihydroethidium (DHE) (A), or stained for reactive nitrogen species by using nitrotyrosine (B). Right panels show the number of cells per square millimeter that are positive for ROS or oxidative stress (A) or reactive nitrogen species (B) in the ischemic hemispheres of sham-operated wild-type mice and wild-type and Nox4−/− mice 12 h and 24 h after tMCAO (n = 4 per group). (C) Left panels show representative brain sections from sham-operated wild-type mice and wild-type and Nox4−/− mice 24 h after tMCAO, immunolabeled for the neuronal marker NeuN and subjected to TUNEL to show apoptosis. Right panel shows the number of TUNEL-positive neurons per square millimeter in the ischemic hemispheres of sham-operated wild-type mice and wild-type and Nox4−/− mice 24 h after tMCAO (n = 4 per group). (D) Left panels show corresponding coronal brain sections of wild-type and Nox4−/− mice on day 1 after tMCAO and injection of Evans blue. Extravasation of Evans blue was reduced in areas where infarcts were regularly present in Nox4−/− mice (basal ganglia, broken white line). The right panel shows the extent of extravasation (i.e., edema volume) as determined by planimetry in the wild-type and Nox4−/− mice 24 h after tMCAO (n = 6 per group). For (A–C), ***p < 0.0001, and ***, p < 0.001, compared with sham-treated mice; ***, p < 0.0001, and **, p < 0.001, compared with wild-type mice by two-way ANOVA, Bonferroni post-hoc test. For (D), **, p < 0.001, Two-tailed Student’s t-test, compared with wild-type mice. All scale bars represent 100 μm.

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finding for the wider concept of oxidative stress, which might also be of relevance for other disease states, such as neurotrauma and neuroinflammation, where oxidative stress, blood-brain barrier damage, and neurotoxicity are involved. Rather than focusing on antioxidants and the disappointing outcomes of their application, the identification of the relevant source of oxidative stress and preventing its formation may represent an approach with clinical potential.

The hypothesis that free radicals are involved in acute ischemic stroke and account for secondary infarct growth dates back to the 1970s [38] but has remained unproven [39,39]. The extent of neuroprotection that we observed is exceptional compared with that seen in many other pre-clinical stroke studies, in which the reduction of infarct size usually does not exceed 30%–40% [40]. Such moderate reductions of infarct volume have not translated into improvement of neurological status [3]. Most notably, continuous assessment of functional deficits until 7 d after stroke revealed that Nox4-null mice indeed showed a better amplitude rather than simply altered kinetics of recovery. This protection in Nox4−/− mice was further underlined by a significantly reduced post-stroke long-term mortality.

Secondary infarct growth mediated for example by edema formation or hemorrhagic transformation is common during the course of brain ischemia and can lead to worsening of neurological symptoms [39]. Serial magnetic resonance imaging revealed that infarcts in Nox4−/− mice remain small, even at later stages of infarct development, and signs of intracerebral hemorrhage were consistently absent, thus indicating that NOX4 inhibition is likely to be safe and persistently effective.

A plethora of compounds have provided neuroprotection in animal models of brain ischemia, but they all failed in human clinical trials [4]. This translational roadblock has been attributed mainly to inadequate pre-clinical study design and severe methodological shortcomings. Important confounding factors are a lack of randomization or rater-blinded evaluation of study results, and use of only one stroke model [16]. Strictly adhering to current expert recommendations for basic stroke trials, we here demonstrate that in the absence of NOX4, brain tissue can be salvaged after ischemia or reperfusion injury (as occurs in the tMCAO model). Most importantly, neuroprotection was preserved in old male and female Nox4−/− mice as well as in Nox2−/− mice subjected to permanent ischemia (i.e., cortical photothermosis or pMCAO). Compared to in the tMCAO model, however, the reduction of infarct size in the pMCAO model was less pronounced though still significant. Distinct pathomechanisms that can be positively influenced only in the presence of tissue reperfusion, i.e., after tMCAO but not pMCAO, such as progressive thrombus formation in the cerebral microvasculature [41], might account for this quantitative difference. Indeed, preliminary results suggest that clotting is attenuated in the cerebral vessels of Nox4−/− mice subjected to tMCAO but not pMCAO (unpublished data). Clearly, elimination of NOX4 remains beneficial in the absence of arterial recanalization, a condition frequently observed in human stroke.

In our experiments, deficiency of NOX1 or NOX2 had no impact on infarct size or functional outcome after tMCAO. Although others have described protective effects of NOX2 deficiency after experimental stroke [42–44], we could not reproduce those findings. The exact reasons for this discrepancy are unclear at present. Differences in the experimental protocols and middle cerebral artery occlusion times, which varied between 30 min and 120 min in previous investigations, might play a role here [42–44]. In contrast to these previous studies, however, we used especially high numbers (n = 19) of Nox2−/− mice to verify our findings. Moreover, type-II (beta) error of the differences between infarct volumes in Nox2−/− mice and wild-type controls was only 7% in our study (93% power; respectively) (Tables S3 and S4), which is a very powerful result compared to the positive reports on Nox2 deficiency in cerebral ischemia [42–44] as well as to many other experimental stroke studies in general [4,45]. Moreover, the fact that VAS2870, which specifically inhibits NADPH oxidases, could not further decrease infarct size and ROS formation in Nox4−/− mice ex vivo and in vivo (Figure 4) clearly argues against a major role of NOX1 or NOX2 in the pathophysiology of acute ischemic stroke. Finally, protein expression levels of NOX1 and NOX2 were almost unchanged in the brains of Nox4−/− mice (Figure 3C), underlining that the profound neuroprotection we observed is mediated by deficiency or blockade of NOX4 itself and not by secondary effects.

Nevertheless, we cannot completely rule out contributions of other sources of ROS. Referring to this, Block et al. recently reported that a functional NOX4 is present and regulated in mitochondria, indicating the existence of a hitherto undescribed source of mitochondrial ROS [46].

An unprecedented need exists for more effective therapies for acute stroke, the second leading cause of death worldwide [1]. We have demonstrated that pharmacological inhibition of NADPH oxidases using the specific NADPH oxidase inhibitor VAS2870 [15,32–34] protects mice from brain ischemia within a clinically meaningful 2-h time window. In contrast, the commonly used organic compound apocynin may not be a NOX inhibitor in vascular cells but rather acts as a nonspecific antioxidant [36]. It also inhibits Rho kinase inhibitor [37], an activity that increases its nonspecific actions. If apocynin inhibits NADPH oxidases at all, it supposedly blocks the migration of the cellular NADPH oxidase complex subunit p47phox to the membrane, thus interfering with assembly of the functional NOX complex [47]. Therefore, it is unlikely to inhibit the NOX4-containing NADPH oxidase, which acts independently of any cytosolic subunits [12]. Indeed, in our experiments, application of apocynin had no effect on the...
formation of ROS or of functional outcome after experimental stroke in vivo.

In summary, we have demonstrated that NOX4-derived oxidative stress is a crucial player in the pathophysiology of acute ischemic stroke, while Nox4 deletion does not affect basal vascular or renal function. Nox4 gene reconstitution experiments in Nox4−/− mice and studies of the effects of different, structurally unrelated NOX inhibitors—should they become available—would be desirable to further substantiate the causality between NOX4 deficiency and protection from cerebral ischemia. Pharmacological inhibition of NADPH oxidases using specific compounds may also pave new avenues for the treatment of ischemic brain injury in humans. Because NADPH oxidase-mediated production of ROS may represent a general mechanism of neurotoxicity, our findings may extend to other ischemic disorders and neurodegenerative or inflammatory diseases. Further studies in relevant disease models are warranted.

Materials and Methods

Refer to the Text S1 for more detailed methodology. The generation of the Nox4-null mice is described in Figure S3.

Human Specimens

Specimens from patients who had experienced a stroke were collected during routine autopsy at the Department of Neuropathology, University of Würzburg, Germany.

Stroke Study Design

Detailed study characteristics are provided in Table S2. We strictly followed the recent international expert recommendations for conducting research in mechanism-driven basic stroke studies [4,6,7,16,17,40].

Stroke Models

If not otherwise mentioned, we performed 60 min of tMCAO in 6- to 8-wk-old male mice weighing 20–25 g, as described previously [48,49]. To exclude age- and gender-specific effects, 18- to 20-wk-old male and 6- to 8-wk-old female mice were used in some subgroups. For pMCAO the occluding filament was left in situ until sacrificing the animals [41].

At 2 h and 12 h after the induction of tMCAO, subgroups of wild-type mice or Nox4−/− mice were randomly selected to receive either 2 mg of the NOX-specific inhibitor VAS2870 (Vasopharm GmbH [32,33]) or carrier solution (10% dimethyl sulfoxide, Sigma) intrathecally, as described previously [50]. In another group, wild-type mice were injected intravenously with 100 μg of apocynin 1 h after the occlusion of the middle cerebral artery. In order to restore ROS levels in Nox4−/− mice, animals received repetitive intrathecal injections of H2O2 (15 mg/kg) immediately after the occlusion of the middle cerebral artery and then every hour until 6 h after stroke induction.

Cortical photothermolysis was induced in 6- to 8-wk-old wild-type or Nox4−/− mice as described previously [51,52].

Stroke Analysis

Stroke analysis was performed as described previously [53,54]. To determine infarct size, mice were killed 24 h after tMCAO, pmCAO, or cortical photothermolysis. Brains were cut in 2-mm-thick coronal sections using a mouse brain slice matrix (Harvard Apparatus). The slices were stained with 2% TTC (Sigma-Aldrich) to visualize the infarcts. Planimetric measurements (ImageJ software, United States National Institutes of Health), calculating lesion volumes, were corrected for brain edema as described previously [55].

Determination of brain edema using Evans blue dye was performed as described previously [19].

Magnetic resonance imaging was performed repeatedly at 24 h and 6 d after stroke on a 1.5-T magnetic resonance unit (Vision Siemens) as described previously [56]. We used a custom-made dual channel surface coil designed for examining mice (A063HACG; Rapid Biomedical). The imaging protocol comprised a coronal T2-weighted sequence (slice thickness 2 mm) and a blood-sensitive coronal three-dimensional T2-weighted gradient echo CISS (slice thickness 1 mm) sequence. Magnetic resonance images were assessed with respect to infarct morphology and the occurrence of intracerebral bleeding.

Vital Brain Slices

Vital brain slices from infarcted mouse brains (between −2 mm and −4 mm from bregma) were prepared as described previously [57].

Quantitative PCR Analysis

After RNA isolation, we quantified NOX4 mRNA expression using real-time PCR and the TaqMan system (TaqMan Gene Expression Arrays for murine NOX4, assay ID Mm00479246_m1, Applied Biosystems), using 16s rRNA (TaqMan Predesigned Assay Reagents, part number 4319413E, Applied Biosystems) to normalize the amount of sample RNA.

Histology and Immunohistochemistry

Histology was performed by using formalin-fixed mouse brains on day 1 after tMCAO. Samples were embedded in paraffin and
Oxidative Chemistry Biomarkers

The presence of ROS and other oxidants such as ONOO− was visualized on frozen mouse brain sections 12 h and 24 h after tMCAO or 24 h after pMCAO using dihydroethidium (Sigma; visualized on frozen mouse brain sections 12 h and 24 h after Nox4 sham-operated controls, wild-type and

(A and B) Radiotelemetry recordings of basal mean arterial pressure (PAP) in isolated perfused lungs during normoxic (21% O2) ventilation. (E) Strength of hypoxic pulmonary vasoconstriction (HPV) as indicated by the maximum increase in PAP (APAP) upon acute hypoxic ventilation (10 min, 1% O2) in isolated perfused lungs. No significant differences were observed between wild-type and Nox4−/− mice. Data are derived from six mice in each case. (F) Renal hypertrophy as assessed by kidney weight per body surface area (BSA) (g/m²). There was no significant difference in terms of renal mass between wild-type and Nox4−/− mice at 17 wk of age. (G) Albuminuria at 17 wk of age (µg/24 h). There was no significant difference in 24-h urinary albumin excretion between wild-type and Nox4−/− mice at 17 wk of age.

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Figure S2 Cerebral blood flow, cerebral vasculature, and brain structure are normal in Nox4−/− mice. (A) Regional cerebral blood flow (rCBF) in the right territory of the middle cerebral artery as measured by laser Doppler flowmetry in wild-type (WT) mice and in Nox4−/− mice (n = 3 each). Representative data were selected at baseline levels and after insertion of the thread (ischemia) and again 10 min after removal of the thread (reperfusion). No significant differences were observed between the groups at any time point. p > 0.05, two-way ANOVA. (B) Assessment of the cerebral vasculature in wild-type and Nox4−/− mice. A complete circle of Willis (white arrows) was identified in all animals studied, and the distribution of the trunk and branch of the middle cerebral artery appeared to be anatomically identical among the genotypes. (C) Normal brain structure in Nox4−/− mice. Representative Nissl-stained 5-µm coronal paraffin-wax-embedded brain sections of 3-mo-old wild-type and Nox4−/− mice (n = 3 each), showing a macroscopic view (uppermost panel), formation of the hippocampus formation (center panel), and somatomotor areas of the neocortex (lowermost panel).

Found at: doi:10.1371/journal.pbio.1000479.s002 (1.64 MB TIF)

Figure S3 Generation of Nox4 knockout mice and counter-regulation of Nox1 and Nox2. (A) Construct development for Nox4 knockout mice. Exons 14 and 15 are flanked by loxP sites and followed by a floxed neomycin resistance gene (neo) and a negative-selection cassette coding for diphtheria toxin A (dta) as described in the Text S1. Embryonic stem cell clones were generated by homologous recombination with the targeting vector. Transient expression of Cre recombinase results in three different recombination events. Y1 results in deletion of the neo cassette and thus floxed exons 14 and 15. These cells can be used to generate conditional Nox4 knockout. Y2 results in deletion of the floxed exons, and type 3 results in the deletion of exons 14 and 15 and the neo cassette. These cells were used to generate the Nox4 knockout mice. (B) Western blot demonstrating the absence of the 64-kDa NOX4 band in the aorta, lung, and kidney of Nox4−/− mice. (C) Expression of NOX1 and NOX2 is not upregulated in Nox4−/− mice. The uppermost left panel shows results of densitometric analysis of the NOX1 134-kDa band in brain samples of the cortex and basal ganglia from Nox4−/− (pale bar) and wild-type mice (black bar). Data are presented as the relative amount of the NOX1 band normalized to GAPDH and represent the mean ± standard error of three samples. The right panel shows a Western blot comparison of brain and aorta samples from wild-type mice demonstrating the presence of the 134-kDa band in both samples. The center and lowest panels show results of densitometric analysis of the 91- and 53-kDa NOX2 bands seen in brain samples from the cortex and basal ganglia of Nox4−/− (pale bar) and wild-type mice (black bar). Data are presented as the

Supporting Information

Figure S1 Cerebral blood flow, cerebral vasculature, and brain structure are normal in Nox4−/− mice. (A and B) Radiotelemetry recordings of basal mean arterial pressure (MAP) and heart rate (HR) of wild-type (WT) (open circles, n = 10) and Nox4−/− (filled squares, n = 14) mice. Data are represented as 1-h (A) and 24-h (B) averages of mean arterial pressure (left panels) and heart rate (right panels). Dark and light periods are denoted by black and white bars, respectively. (C) Right ventricular systolic pressure (RVSP) as assessed in vivo in anesthetized Nox4−/− and wild-type mice. (D) Mean pulmonary arterial pressure (PAP) in isolated perfused lungs during normoxic (21% O2) ventilation. (E) Strength of hypoxic pulmonary vasoconstriction (HPV) as indicated by the maximum increase in PAP (APAP) upon acute hypoxic ventilation (10 min, 1% O2) in isolated perfused lungs. No significant differences were observed between wild-type and Nox4−/− mice. Data are derived from six mice in each case. (F) Renal hypertrophy as assessed by kidney weight per body surface area (BSA) (g/m²). There was no significant difference in terms of renal mass between wild-type and Nox4−/− mice at 17 wk of age. (G) Albuminuria at 17 wk of age (µg/24 h). There was no significant difference in 24-h urinary albumin excretion between wild-type and Nox4−/− mice at 17 wk of age.

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Figure S2 Cerebral blood flow, cerebral vasculature, and brain structure are normal in Nox4−/− mice. (A) Regional cerebral blood flow (rCBF) in the right territory of the middle cerebral artery as measured by laser Doppler flowmetry in wild-type (WT) mice and in Nox4−/− mice (n = 4 per group) at baseline levels, after insertion of the thread (ischemia) and again 10 min after removal of the thread (reperfusion). No significant differences were observed between the groups at any time point. p > 0.05, two-way ANOVA. (B) Assessment of the cerebral vasculature in wild-type and Nox4−/− mice. A complete circle of Willis (white arrows) was identified in all animals studied, and the distribution of the trunk and branch of the middle cerebral artery appeared to be anatomically identical among the genotypes. (C) Normal brain structure in Nox4−/− mice. Representative Nissl-stained 5-µm coronal paraffin-wax-embedded brain sections of 3-mo-old wild-type and Nox4−/− mice (n = 3 each), showing a macroscopic view (uppermost panel), formation of the hippocampus formation (center panel), and somatomotor areas of the neocortex (lowermost panel).

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relative amount of either the 91-kDa band or 53-kDa band normalized to GAPDH and represent the mean ± standard error of three samples. The bottom right panel shows a Western blot comparison of NOX2 expression in the brain and aorta of wild-type mice, demonstrating the presence of the 91-kDa and 53-kDa bands in both tissues.

Found at: doi:10.1371/journal.pbio.1000479.s003 (24.75 MB TIF)

**Figure S4** Long-term outcomes are improved in Nox4−/− mice after tMCAO. Long-term outcome of motor function (grip test) in 6- to 8-wk-old male Nox4−/− mice (n = 10) and wild-type (WT) controls (n = 11) 24 h after pMCAO. Two-tailed Student’s t-test compared with wild-type mice. ns, not significant.

Found at: doi:10.1371/journal.pbio.1000479.s005 (0.18 MB TIF)

**Figure S5** Motor function after pMCAO. Motor function was assessed by the grip test in 6- to 8-wk-old male Nox4−/− mice (n = 7) and wild-type (WT) controls (n = 11) 24 h after pMCAO. Two-tailed Student’s t-test compared with wild-type mice. *p < 0.05, **p < 0.01, and ***p < 0.001 compared with sham-treated mice; ***, p < 0.001 compared with sham-treated mice; **p < 0.01 compared with sham-treated mice; *, p < 0.05, one-way ANOVA, Bonferroni post-hoc test compared with wild-type mice.

Found at: doi:10.1371/journal.pbio.1000479.s006 (1.76 MB TIF)

Table S1 Results of blood gas analysis and posterior communicating artery (PComA) score in wild-type and Nox4−/− mice.

Found at: doi:10.1371/journal.pbio.1000479.s007 (0.04 MB PDF)

Table S2 Stroke study population.

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Table S3 Power and type-II (beta) error calculations on infarct volumes depicted in Figure 2A.

Found at: doi:10.1371/journal.pbio.1000479.s009 (0.06 MB PDF)

Table S4 Power and type-II (beta) error calculations on infarct volumes depicted in Figure 2E.

Found at: doi:10.1371/journal.pbio.1000479.s010 (0.05 MB PDF)

Table S5 Power and type-II (beta) error calculations on infarct volumes depicted in Figure 4B.

Found at: doi:10.1371/journal.pbio.1000479.s011 (0.06 MB PDF)

Text S1 Supplementary results, supplementary methods, and supplementary references.

Found at: doi:10.1371/journal.pbio.1000479.s012 (0.32 MB DOC)

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## Author Contributions

The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: CK AS MHdA. Performed the experiments: CK HG MEA EJ MM DB TS CG PK KB MKS AMH SGM GS SM AS LB VGD HF TK. Analyzed the data: CK MEA EM MM DB TS CG PK KB MKS AMH SGM GS SM AS LB VGD HF TK. Contributed reagents/materials/analysis tools: CK HG AMS HHHWS. Wrote the paper: CK KW AMS HHHWS.


