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FORUM REVIEW ARTICLE

Pathophysiology and Treatments of Oxidative Injury in Ischemic Stroke: Focus on the Phagocytic NADPH Oxidase 2

Federico Carbone,[1,2,*] Priscila Camillo Teixeira,[3,*] Vincent Braunersreuther,[1] François Mach,[1] Nicolas Vuilleumier,[3,†] and Fabrizio Montecucco[1–3,†]

Abstract

Significance: Phagocytes play a key role in promoting the oxidative stress after ischemic stroke occurrence. The phagocytic NADPH oxidase (NOX) 2 is a membrane-bound enzyme complex involved in the antimicrobial respiratory burst and free radical production in these cells. ***Recent Advances:*** Different oxidants have been shown to induce opposite effects on neuronal homeostasis after a stroke. However, several experimental models support the detrimental effects of NOX activity (especially the phagocytic isoform) on brain recovery after stroke. Therapeutic strategies selectively targeting the neurotoxic ROS and increasing neuroprotective oxidants have recently produced promising results. ***Critical Issues:*** NOX2 might promote carotid plaque rupture and stroke occurrence. In addition, NOX2-derived reactive oxygen species (ROS) released by resident and recruited phagocytes enhance cerebral ischemic injury, activating the inflammatory apoptotic pathways. The aim of this review is to update evidence on phagocyte-related oxidative stress, focusing on the role of NOX2 as a potential therapeutic target to reduce ROS-related cerebral injury after stroke. ***Future Directions:*** Radical scavenger compounds (such as Ebselen and Edaravone) are under clinical investigation as a therapeutic approach against stroke. On the other hand, NOX inhibition might represent a promising strategy to prevent the stroke-related injury. Although selective NOX inhibitors are not yet available, nonselective compounds (such as apocynin and fasudil) provided encouraging results in preclinical studies. Whereas additional studies are needed to better evaluate this therapeutic potential in human beings, the development of specific NOX inhibitors (such as monoclonal antibodies, small-molecule inhibitors, or aptamers) might further improve brain recovery after stroke. *Antioxid. Redox Signal.* 23, 460–489.

Introduction

REACTIVE OXYGEN SPECIES (ROS) have been shown as critical mediators in cell homeostasis (40) as well as in inflammatory responses (114). Typically, ROS generation is triggered by the transfer of an electron to oxygen, forming superoxides ($O_2^{\bullet -}$). Hence, many cellular respiration and metabolic processes generate ROS. The escape of single electrons may occur in mitochondria, but it might also involve peroxisomes and a large amount of enzymes (xanthine oxidase, nitric oxide synthase, and P450 cytochromes). However, in the reaction catalyzed by NADPH oxidases (NOX), NADPH is the main electron donor according to the following reaction:

[1]Division of Cardiology, Foundation for Medical Researches, Department of Medical Specialties, University of Geneva, Geneva, Switzerland.
[2]Department of Internal Medicine, University of Genoa School of Medicine, IRCCS Azienda Ospedaliera Universitaria San Martino–IST Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy.
[3]Division of Laboratory Medicine, Department of Genetics and Laboratory Medicine, Geneva University Hospitals, Geneva, Switzerland.
*These authors equally contributed as first authors to this work.
†These authors equally contributed as last authors to this work.

$$NADPH + H^+ + 2O_2 \rightarrow NADP^+ + 2H^+ + 2O_2^{\bullet -}$$

Accordingly, the NADPH activity has been shown to play an active role in cellular homeostasis, especially in the central nervous system, where a tight balance between oxygen supply and oxidative stress is required to maintain structural and functional integrity.

NOX is a membrane-bound enzyme complex whose components are separated between the cytosol ($p47^{phox}$/$p67^{phox}$/$p40^{phox}$ and the GTPase Rac1/Rac2) and the plasma membrane (flavocytochrome subunits $gp91^{phox}$ and $p22^{phox}$). Seven isoforms of NOX (NOX1-5 and dual oxidase (DUOX) 1–2 recently termed as NOX6-7) have been identified with different tissue distribution, structure, subunit requirement, and trigger (203). Classically, NOX1 has been detected in the colon epithelium and vascular endothelium, smooth muscle cells, fibroblasts, and microglia. NOX2 (also known as the phagocytic NOX) is mainly involved in antimicrobial respiratory burst, but is also synthesized by cardiomyocytes, endothelial cells, fibroblasts, neurons, and pancreatic beta cells (50). NOX3 expression is limited to the inner ear, whereas NOX4 was recognized in the kidney, vascular cells, osteoclasts, and neurons (7). NOX5 is mostly retrieved in the lymphoid tissue and testis (14). Finally, DUOX are mainly synthesized in the thyroid gland, but they were found also in airway epithelial cells (DUOX1) and the gastrointestinal tract (DUOX2) (155).

On the other hand, ROS show also different biological properties. Usually, NADPH-generated $O_2^{\bullet -}$ does not spread readily across membranes and is short lived, resulting in a local effect. However, superoxide dismutase (SOD) may accelerate the nonenzymatic dismutation of $O_2^{\bullet -}$, generating hydrogen peroxide (H_2O_2), a nonradical compound that readily diffuses across membranes (18):

$$2O_2^{\bullet -} + 2H^+ \rightarrow 2H_2O_2 + 2O_2$$

In the presence of a reduced transition metal, H_2O_2 may, in turn, react to produce the hydroxyl radical ($^{\bullet}OH$). In certain conditions, peroxidases generate hypochlorous acid (HOCl) and singlet oxygen ($^1O_2$) from H_2O_2, whereas peroxynitrite is the result of an interaction between superoxide and nitric oxide. Since the high reactivity of ROS induces a direct cellular damage (by enhancing mitochondrial permeability, lipid peroxidation, matrix metalloproteinase [MMP] activation, and DNA oxidation), a fine tuning of redox balance is required to maintain cell homeostasis (54). Antioxidant enzymes (*e.g.*, SOD, catalase, glutathione [GSH/GSSG], and the thioredoxin system) restore the redox equilibrium, providing an additional control on intracellular signal transduction.

Initially, ROS have been recognized as mediators of inflammation. Their signaling is ubiquitous and modulates several intracellular pathways regulating cellular growth, proliferation, differentiation, apoptosis, cytoskeletal regulation, migration, and contraction.

More recently, the role of oxidative stress has been pointed out also in stroke pathophysiology as a pivotal player of the signaling cascade triggered by ischemia/reperfusion (I/R) injury (86). The aim of this review is to update the role of oxidative stress in stroke pathophysiology, including carotid atherosclerotic plaque vulnerability as a condition favoring this disease. The role of phagocytic ROS and NOX2 will be discussed, also with respect to clinical and therapeutic perspectives.

The Intracellular ROS Signaling in Phagocytes

Phagocytes are the best-known source of ROS in both physiology and pathophysiology (48). An enzyme stored in the primary granules of neutrophils (*e.g.*, myeloperoxidase [MPO]) generates HOCl from H_2O_2, according to the following reaction:

$$Cl^- + H_2O_2 \rightarrow OCl^- + H_2O$$

MPO also enhances phagosome toxicity by promoting generation of chloramines, aldehydes, $^1O_2$, ozone (O^3), and especially $^{\bullet}OH$ (the most reactive ROS) (166). In addition, respiratory burst plays a role as second messenger in several signaling pathways (55). As summarized in Figure 1, an important target for ROS is represented by the mitogen-activated protein kinase (MAPK), a Ser/thr kinase family that converts extracellular stimuli into a wide range of cellular responses.

Through their upstream signaling cascade molecules (including transmembrane receptors, phosphatases, and tyrosine kinases) (211), ROS activate MAPK signaling, mainly through the inhibition of phosphatases of Jun amino (N)-terminal kinases 1/2/3 (JNK1/2/3), p38, or extracellular signal-regulated kinase 1/2 (ERK1/2) (84, 97, 225).

In turn, tyrosine kinase receptors represent not only a target for ROS but also a potential signaling pathway that promotes NOX activation *via* c-Src-mediated mechanisms (63).

Other ROS signaling pathways leading to MAPK activation include apoptosis signal-regulated kinase-1, Raf-independent activation of c-JUN and p39 (33), and protein kinase C (PKC), activated by rise of intracellular Ca^{++} concentrations induced by oxidative stress (182) (Fig. 1). The oxidization of phosphatases and tensin homolog also promotes phosphoinositide-3-kinase (PI3K) activation, involved in phagocyte spreading, chemotaxis, NOX assembling, and pathogen killing (74) through Akt signaling (115).

The activation of MAPK is also regulated by the intracellular amount of ROS. A low intracellular concentration of H_2O_2 triggers antiapoptotic pathways MEK 1/2 and Erk2, whereas higher levels promote oxidization of cysteine residues in JNK and p38 MAPK, ultimately leading to cell apoptosis (58).

Ultimately, ROS might also upregulate a large amount of nuclear transcription factors, including hypoxia-inducible factor (HIF)-1, activation protein (AP)-1, NF-κB, and p53.

In particular, HIF-1α acts as a feedback mechanism in ischemic-induced oxidative stress, which generates reducing equivalents in the form of NADH and NADPH *via* glycolysis or the pentose phosphate pathway, as well as synthesis of the antioxidant pyruvate.

The AP-1 transcription program upregulates the synthesis of CXCL2 and IL-6 (32) in addition to synergizing with NF-κB (197, 228) in promoting the transcription of genes encoding for other cytokines, chemokines, adhesion molecules, and apoptosis (198).

However, ROS may also directly interact with DNA. The inhibition of histone deacetylase and the generation of

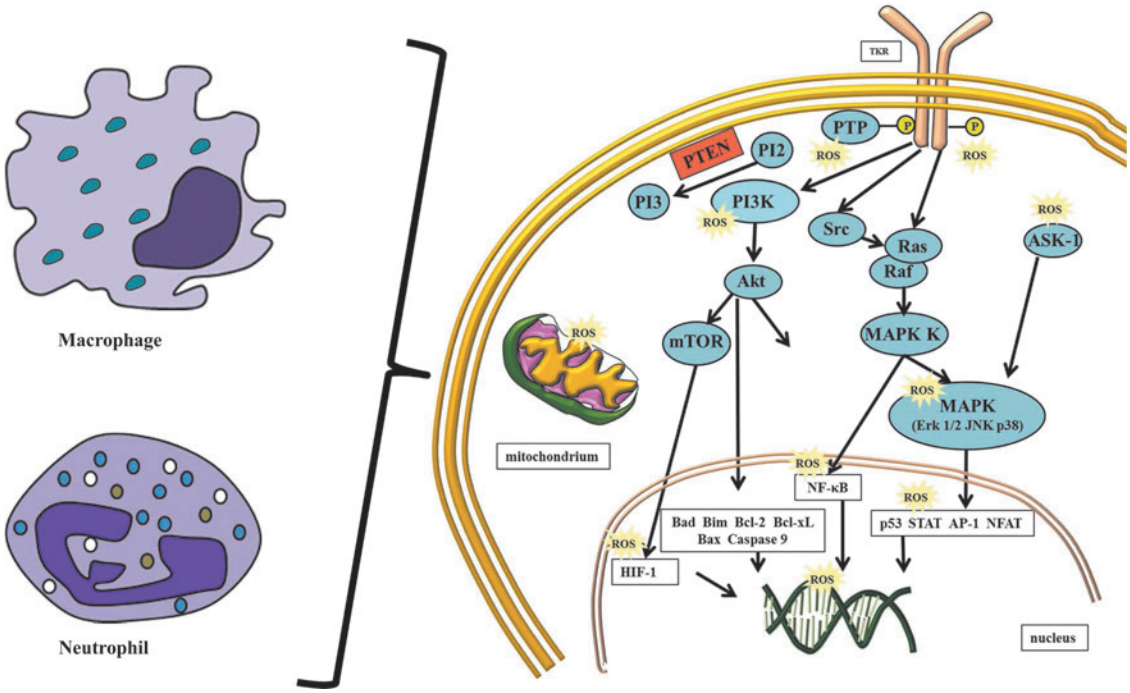

FIG. 1. Summary of ROS-activated metabolic pathways within activated phagocytes. ROS regulate several signaling pathways in phagocytes. In particular, both apoptotic and signaling intracellular pathways inducing the expression of a variety of inflammatory genes have been shown to be activated by ROS within phagocytes. Akt, protein kinase B; AP-1, activating protein-1; HIF-1, hypoxia-inducible factor-1; JNK, c-jun N-terminal kinase; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; NFAT, nuclear factor of activated T cell; PI3K, phosphatidylinositol-3-kinase; PTEN, phosphatase and tensin homolog; PTP, protein tyrosine phosphatase; ROS, reactive oxygen species; TKR, tyrosine kinase receptor. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

adducts between DNA and electrophilic products arising from other oxidative reactions involve proteins, carbohydrates, polyunsaturated fatty acids, and even nucleic acids themselves (126).

On the other hand, a defective ROS generation (a condition also recognized in chronic granulomatous disease) was shown to play an immunomodulatory effect in autoimmune diseases. ROS might act as anti-inflammatory molecules enhancing the regulatory T-cell-mediated responses (108) and suppressing effector T cells (61).

The Contribution of Oxidative Stress to I/R Injury

Oxidants largely contribute to the cerebral I/R injury. After an ischemic insult, a strong increase in NOX2 (synthesized by microglial cells and recruited phagocytes) (71) and NOX4 (upregulated in neuronal cells and brain microvascular endothelial cells [BMECs]) (96, 217) was reported.

Several experimental models of stroke investigated the specific role of NOX isoforms in cerebral I/R injury (Table 1). Walder *et al.* showed that NOX2 deletion was associated with reduction in infarct size. Furthermore, this effect disappeared after transplantation of wild-type bone marrow (221). The authors concluded that the detrimental effect of NOX2 activation did not concern only circulating neutrophils, but it might also involve pathophysiological processes of resident cells (*i.e.*, microglia, endothelial cells, and neurons) (221). Improvements in blood–brain barrier (BBB) permeability and reduction in poststroke brain swelling were also recognized in $NOX2^{-/-}$ mice after experimental ischemic stroke (29, 96).

A similar strong neurological protection after cerebral ischemia was also reported in mice deficient for NOX4 (107). Radermacher *et al.* have recently suggested an accessory role for NOX2, pointing out the role of NOX4 as the most promising target against stroke injury (167). Conversely, NOX1 deletion was shown to have no impact on poststroke ROS production, stroke size, and neurological outcome (89, 107). Despite several differences in ischemic protocols (intravascular occlusion or artery suture) and time of ischemia (ranging from 25 to 120 min), these results strengthened the critical relevance of certain NOX isoforms in short-term cerebral I/R injury.

Unfortunately, only few investigated the potential delayed effect of oxidants on ischemic stroke sequelae. Since ROS may enhance antiapoptotic pathways (such as PI3K/Akt and ERK 1/2) through the phosphorylation of vascular endothelial growth factor receptor-2 (216), further studies are needed to evaluate the molecular role of oxidative stress in brain recovery after prolonged reperfusion timing and chronic ischemia that might influence residual poststroke disabilities.

Phagocytic Oxidants in the Pathophysiology of Ischemic Stroke

Stroke is a leading cause of morbidity and mortality in Western countries (175). Cardiac (*i.e.*, atrial fibrillation), vascular, and coagulation disorders may promote stroke

Table 1. Animal Studies on NOX Genetic Deletion

Author	*Year*	*Animal model*	*Model*	*Time of euthanasia from stroke onset*	*Outcome*
Walder *et al.* (221)	1997	$NOX2^{-/-}$ mouse	Focal cerebral ischemia by intravascular occlusion of right CCA and MCA for 120 min	24 h	KO mice developed smaller infarcts ($p < 0.05$)
Kahles *et al.* (96)	2007	Male $NOX2^{-/-}$ mouse	Focal cerebral ischemia by intravascular occlusion of right MCA for 120 min	24 h	KO mice developed smaller infarcts ($p < 0.05$). They also present reduced BBB permeability and then less brain swelling ($p < 0.05$)
Kunz *et al.* (111)	2007	Male $NOX2^{-/-}$ and $COX2^{-/-}$ mouse	Focal cerebral ischemia by intravascular occlusion of MCA for 25 min	2 and 72 h	After 72 h, $NOX2^{-/-}$ mice exhibited smaller infarcts ($p < 0.05$) according to reduced ROS production ($p < 0.05$) compared to WT and $COX2^{-/-}$ mice After 2 and 72 h, $COX2^{-/-}$ mice developed smaller infarcts ($p < 0.05$) compared to WT mice, without reduction in ROS production compared to $NOX2^{-/-}$ mice
Chen *et al.* (29)	2009	Male $NOX2^{-/-}$ mouse	Focal cerebral ischemia by suture of left MCA for 75 min	24 and 72 h	KO mice presented smaller infarcts ($p < 0.05$) and a better neurologic-deficit score ($p < 0.05$). Moreover, peroxidation products (HNE, MDA, 8-OHdG) are less than in WT mice. KO mice also presented less ICAM-1 and MPO expression
Jackman *et al.* (90)	2009	Male $NOX1^{-/-}$ mice	Focal cerebral ischemia by intravascular occlusion of MCA for 30 min	24 h	KO mice developed smaller cortical infarcts ($p < 0.05$), without affecting brain swelling ($p < 0.05$), total infarct volume, or neurological outcome
Kahles *et al.* (95)	2010	$NOX1^{-/-}$ mouse	Focal cerebral ischemia by intravascular occlusion of MCA for 60 min	24 h	In less than 2 h of cerebral occlusion, KO mice developed smaller cortical infarcts ($p < 0.05$), prevent BBB disruption, and then brain swelling ($p < 0.05$). This results in a better neurological outcome according to the Benderson score ($p < 0.05$). Oxidative stress markers (protein carbonyls) are not increased compared to WT mice

(*continued*)

Table 1. (Continued)

Author	*Year*	*Animal model*	*Model*	*Time of euthanasia from stroke onset*	*Outcome*
Kleinschnitz *et al.* (107)	2010	Male $NOX1^{-/-}$, $NOX2^{-/-}$, and $NOX4^{-/-}$ mouse	Focal cerebral ischemia by intravascular occlusion of MCA for 60 min	Up to 24 h	$NOX4^{-/-}$ mice developed smaller infarcts ($p < 0.0001$) with better neurological function, according to the Benderson score ($p < 0.01$), and also with increased survival ($p = 0.003$). Serial MRI showed a sustained protection against stroke in KO mice $NOX1^{-/-}$ and $NOX2^{-/-}$ mice, compared to WT, showed differences neither for infarct size nor for neurological outcome
			Permanent focal cerebral ischemia by intravascular occlusion of MCA	Up to 24 h	$NOX4^{-/-}$ mice developed smaller infarcts ($p < 0.001$) with better neurological function, according to the Benderson score ($p < 0.05$), and also with increased survival ($p = 0.003$)
Chen *et al.* (28)	2011	Male $NOX2^{-/-}$ mouse	Focal cerebral ischemia by suture of left MCA for 60 min	24 and 72 h	KO mice presented decreased infarct volume both at 24 and at 72 h ($p < 0.05$). They also showed less microglial activation, evaluated by length microglia process assay ($p < 0.05$). At 24 and 72 h, there was also a reduction in proinflammatory factor transcription (TNF-α, CCL2, CCL3, and iNOS) compared to WT

8-OHdG, 8-hydroxy-2-deoxyguanosine; BBB, blood–brain barrier; CCA, common carotid artery; CCL, CC chemokine ligands; COX2, cyclooxygenase2; HNE, 4-hydroxy-2-nonenal; ICAM-1, intercellular adhesion molecule 1; iNOS, inducible nitric oxide synthase; KO, knockout; MCA, middle cerebral artery; MDA, malondialdehyde; MPO, myeloperoxidase; MRI, magnetic resonance imaging; NOX, NADPH oxidase; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; WT, wild type.

occurrence, but the relevant cause of ischemic stroke is also represented by severe carotid stenosis due to an atherosclerotic plaque that, when it ruptures, might generate arterial occlusion or thromboembolism (129). Accordingly, the recognition of different patterns of carotid atherosclerosis has emerged as a pivotal feature in stroke pathophysiology (162). The role of oxidative stress has been well established and represents the link between several proatherosclerotic conditions (such as hypertension, insulin resistance, and dyslipidemia) (158) and intraplaque inflammation. In addition, the potential relationship between oxidative stress and atrial fibrillation (195) strengthens the role of ROS in the pathophysiology of stroke of cardiovascular origin. The following paragraphs will update evidence on the role of phagocyte-generated ROS *via* the NOX2 activity.

Proatherosclerotic role of phagocytic oxidants in carotid plaque vulnerability: focus on intraplaque phagocytes and autoantibodies

The incidence of moderate- to high-grade extracranial carotid artery stenosis (>50%) is 1%–3% in all adults but increases up to 6.9% with aging (49). Since embolism of thrombotic material or sudden acute arterial lumen occlusion following plaque rupture is often responsible for cerebral ischemia starting ischemic stroke (137), several intraplaque and circulating mediators of inflammation are currently under investigation as promising pathophysiological targets (135). Among these molecules, the role of phagocytic ROS in atherogenesis has been recently emphasized (56, 87, 103, 220) by investigating atherosclerosis acceleration in autoimmune diseases (98, 157). Common inflammatory mediators might favor the development of these pathological conditions *via* the activation of immune cells, and a consequent increase in ROS production and amplification of the inflammatory response (131, 199). Potential mechanisms by which ROS might favor autoimmune responses have been indicated in the modification of a broad range of endogenous molecules generating self-derived neoantigens. As already known, in case of sufficient homology between the neoantigens or foreign antigens and host native epitopes (often referred to as ''molecular mimicry''), autoimmune reactions may occur. In addition, the active immunization with modified autoantigens has been shown to be an alternative way of eliciting an autoimmune response through a mechanism known as epitope spreading. This consists in the extension of the immune response to other less dominant epitopes because of molecular mimicry (200). For instance, lipid peroxidation may form adducts with free amino groups of lysine and other amino acids. These aldehyde-modified proteins are highly immunogenic, so that both the ROS-generated neoantigens and their specific antibodies may act as pro- or anti-inflammatory molecules by modulating different innate immune receptors (112). As suggested by Miller and coworkers, these oxidation-specific epitopes (including modification of lipids, carbohydrates, proteins, and DNA) might represent damage-associated molecular patterns (132) recognized by receptors of innate immunity (including CD36, scavenger receptor-A, and Toll-like receptors) (121) and also an autoantibody response (25). The most reported self-derived neoantigens susceptible to induce autoimmune reactions in the context of atherogenesis are not only oxidized low-density lipoproteins (oxLDLs) (160) but also heat shock proteins (232), β2-glycoprotein 1 (β2-GPI), (127) cardiolipins (72), and more recently, high-density lipoproteins (44). Actually, oxLDLs are a product of different ROS-mediated modifications involving both lipid and protein components of LDL. Especially, the two more immunogenic aldehydes (*i.e.*, malondialdehyde and 4-hydroxynonenal) (10) were shown to promote all stages of atherogenesis, including endothelial dysfunction, transendothelial migration of immune cell, and foal cell formation (69, 73). On the other hand, the autoimmune response against oxLDLs remains quite controversial (174, 192). This is mainly due to the high heterogeneous nature of anti-oxLDL autoantibodies. In fact, oxLDL autoantibodies were shown to inhibit foam cell formation by preventing the oxLDL uptake, but they may also have a potential proatherogenic effect by promoting immune complex deposits within tissues and complement-mediated cell damage (192). Instead, the binding between oxLDL and β2-GPI generates proatherogenic antibodies that are positively associated with increased cardiovascular risk (70). The uptake into the macrophages of this complex results in the upregulation of both scavenger (CD36) and Fcγ receptor I and the activation of β2-GPI-specific T cells (113, 235).

Furthermore, the intraplaque infiltration of autoantibodies may act, in turn, as a potential oxidant enhancer. Anticardiolipin antibodies may increase the substrates for ROS production by upregulating the expression of inducible nitric oxide synthase, whereas an intrinsic ability to induce ROS was attributed to the Fc portion (226).

Considering that oxidative stress might increase the affinity of autoantibodies for their targets, the intraplaque autoimmune response could be a pivotal trigger for intraplaque oxidant production in atherogenesis (Fig. 2).

Neutrophil-generated ROS in cerebral ischemia: protection or injury?

Although cerebral ischemia triggers local and systemic inflammatory responses, the reperfusion (restoration of blood flow) was shown to induce detrimental effects on brain cells, potentially mediated by an increase in ROS levels. These mediators are especially produced in the peripheral region of the necrotic area (where the ischemic injury quickly evolves to necrosis) that is named ''ischemic penumbra'' (86). This area is characterized by preserved ion homeostasis and transmembrane electrical potentials, but absent electrical activity (12). In the hyperacute phase of stroke, the shift of mitochondrial function toward anaerobic glycolysis decreases NAD^+ to NADPH within neuronal cells, resulting in an increased amount of $O_2^{\bullet}$ (150).

Since the upregulation of antioxidant enzymes is not sufficient to compensate this marked increase in ROS generation (4), a redox unbalance establishes and contributes to trigger the early inflammatory response, characterized by rapid resident cell activation (mainly microglial cells) followed by the intracerebral recruitment of circulating leukocytes.

The infiltration of phagocytes occurs early after stroke onset (within the first 30 min and reaching the peak in the first hours after reperfusion) (109). These cells represent a main source of ROS in the subacute phase of stroke (hours to days). This phagocytic burst also increases BBB permeability by releasing proinflammatory cytokines, chemokines, elastases,

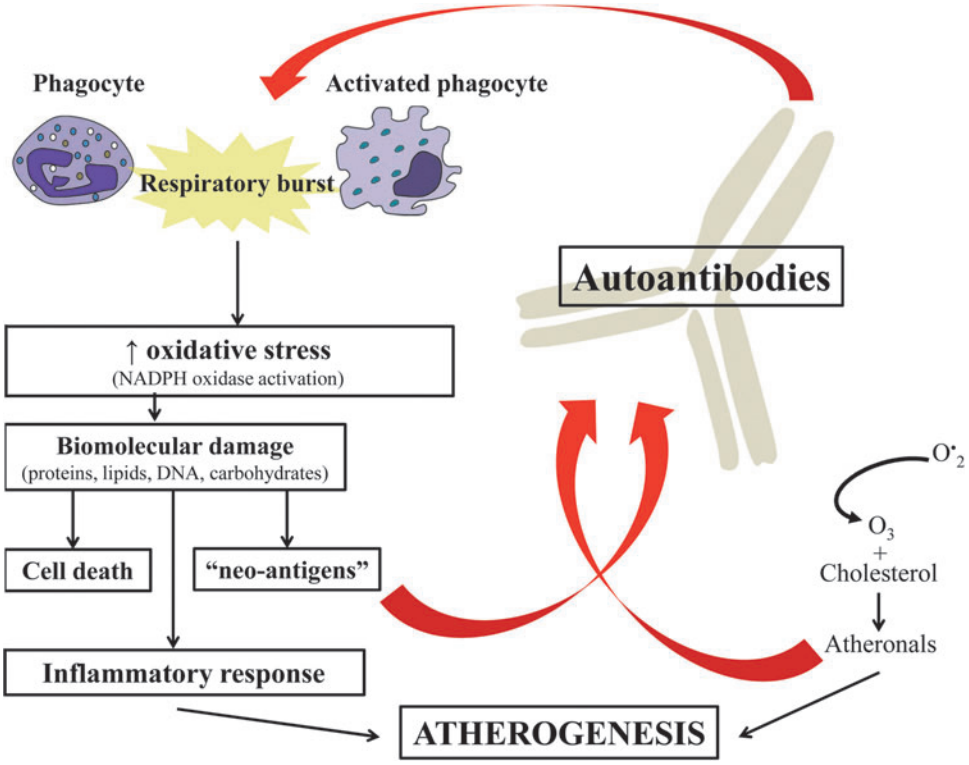

FIG. 2. Respiratory burst and autoimmunity: a vicious circle. Oxidant-mediated modifications on lipids, DNA, proteins, and carbohydrates have not only functional effects. In fact, the oxidization of these compounds and atheronal allows the exposition of epitopes acting as self-antigens. Thus, the consequent antibody-mediated immune responses (a recently identified pivotal mechanism in atherogenesis) might potentially contribute to phagocyte activation and plaque progression. This represents an indirect proatherosclerotic mechanism triggered by ROS. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

and MMPs, as well as by promoting adhesion molecule expressions (184). BBB derangement is critical since it promotes brain edema, neuronal death, and hemorrhagic transformation of the ischemic brain (92). Accordingly, several mouse models of stroke showed that inhibition of neutrophil recruitment reduces infarct volume, brain swelling, and mortality (39, 60, 83, 164). Partially confirming these results, human studies demonstrated that neutrophil cerebral infiltration after stroke and the circulating ratio of neutrophils/lymphocytes were directly associated with a poor neurological outcome correlated with neutrophil recruitment (5, 21, 64, 165, 210).

As potent phagocytes, neutrophils can release a variety of ROS. For instance, $^{\bullet}OH$ (the most reactive ROS) has been described as a typical product of phagocytic H_2O_2 metabolism. Initially recognized through an increase of cerebral hydroxylated salicylate (23, 136), several experimental models of ischemic stroke later confirmed this finding (124, 145, 146, 201). Accordingly, a decrease of $^{\bullet}OH$ was reported after protective hyperbaric oxygenation (218, 237) or treatment with oxidant scavengers (118, 214).

Considering other activities of ROS of neutrophil origin, these reactive molecules were shown to be directly induced by I/R, thus amplifying the ischemic damage on neuronal cells. On the one hand, the sensitization of mitochondrial permeability transition pore activity by ROS (*i.e.*, $^{\bullet}OH$) enhances the intracellular Ca^{++} overload (46, 68) that ultimately leads to mitochondrial respiratory chain inhibition and then neuronal cell death (219).

On the other hand, ROS may directly promote neuronal cell apoptosis by activating p53- (81), JNK-, and p38 MAPK (246), as well as by inhibiting PI3K/Akt pathways (152). Other mechanisms of injury include lipid peroxidation, protein denaturation, and DNA modifications (30).

Oxidant release by resident brain phagocytes (microglial cells)

Alongside neutrophil activation, also microglial cells (the resident macrophages of the brain) were shown to enhance oxidative stress in response to brain injury (239). Similarly to other phagocytes, microglial cells generate ROS *via* NOX1 and NOX2 activation (31) as well as trigger the release of cytokines (such as IL-1β, IL-6, and tumor necrosis factor-α) and gelatinases (such as MMP-9) (67, 104). However, microglia exert also delayed neuroprotective effects by expression of neurotophins, fibroblast growth factor, and transforming growth factor-β (147). As reported by Lalancette and coworkers, selective ablation of proliferating resident microglia provided deep alteration in the temporal dynamics of proinflammatory cytokine expression, resulting in a significant increase of the infarct size (117).

Likewise, resident astrocytes may reduce brain oxidative stress activating the nuclear factor erythroid 2-related factor 2 (Nrf2)-dependent pathway. In response to oxidative stress, nuclear translocation of Nrf2 promotes the expression of specific antioxidant genes, including HO-1 and NADPH-quinone oxidoreductase (in turn involved in GSH/GSSG biosynthesis, use, and export) (100, 188). Thus, the upregulation of Nrf2 (recognized within the penumbra region of ischemic rat brain) (41) might represent a promising therapeutic target (6).

Finally, also BMECs are involved in the oxidative signaling following ischemic stroke. These cells are physiological

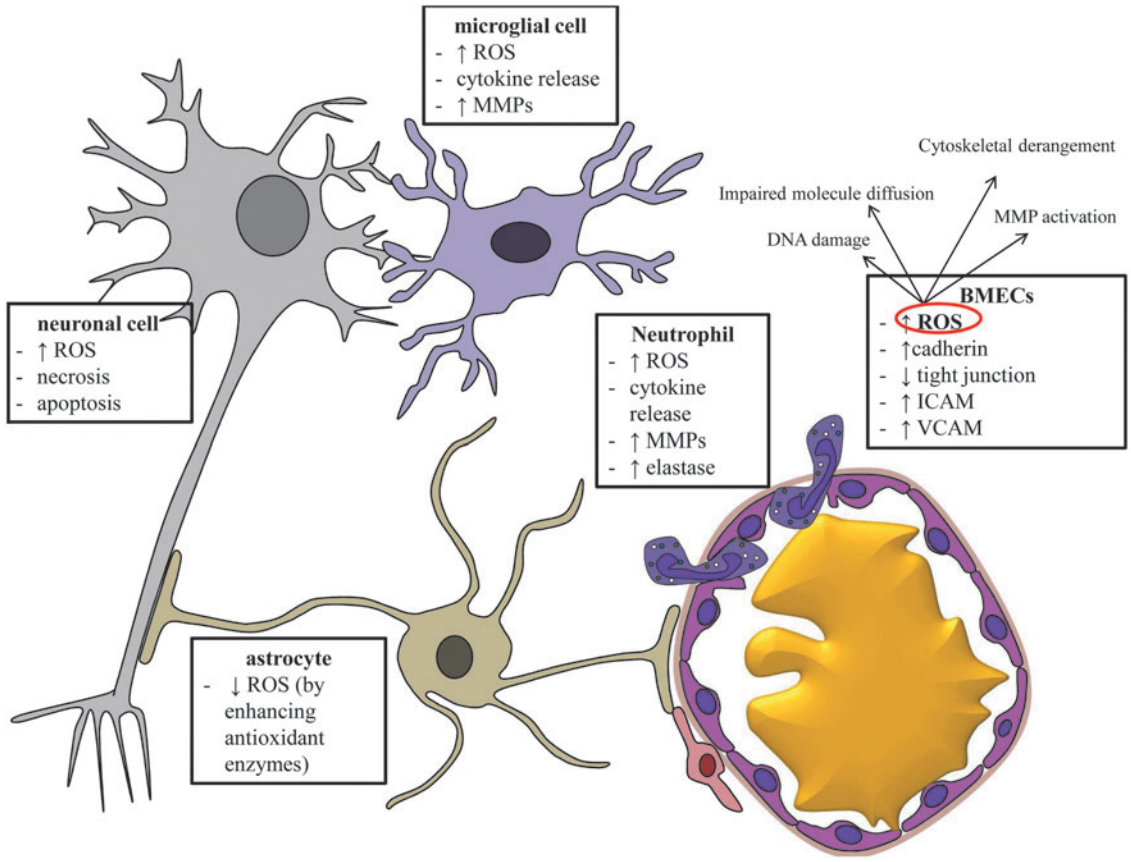

FIG. 3. Neurovascular microenvironment in ischemic stroke. After an ischemic stroke, neuronal, microglial, and immune cells, astrocytes as well as BMECs have been shown to play various protective and detrimental activities at the same time. All cell subsets have been shown to release ROS. Whereas neurons are the first cell subset able to generate ROS after stroke, resident microglial cells and brain microvascular endothelial cells have been shown to release also chemokines and cytokines recruiting circulating immune cells. Once infiltrated within the brain, monocytes and neutrophils (together with microglial cells) can release great amounts of ROS, influencing brain injury *via* the activation of MMPs and direct detrimental effects (*i.e.*, DNA damage, impaired molecule diffusion, and cytoskeletal derangement). BMECs, brain microvascular cells; MMPs, matrix metalloproteases. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

sensors and effectors of blood flow and constitute a cellular network with neuronal, glial, and other accessory cells, referred to as the neurovascular unit (43). Although BMECs themselves release ROS, they represent a target of oxidative stress that ultimately leads to BBB dysfunction. In particular, ROS-induced lipid peroxidation in BMECs results in (i) impairment of membrane molecule diffusion, (ii) alteration of DNA, (iii) downregulation of tight junction, and (iv) alteration in cytoskeletal organization and MMP activation (172) (Fig. 3).

Treatments Targeting Phagocytic ROS in Ischemic Stroke Injury

Rationale for downregulating ROS in stroke

Taking into account the limited availability of endogenous antioxidant molecules and enzymes within the brain (82), the administration of antioxidant molecules has been proposed as the first promising therapeutic approach. However, this strategy has so far failed to provide a clinically relevant reduction of oxidative injury (204). At present, there is no single convincing explanation for these results. However, the different sources of ROS, the timing of the release, and their involvement in physiological cell function may potentially explain this poor efficacy. Another therapeutic approach preventing ROS formation (rather than scavenging them) was also investigated. Selective and indirect nonspecific NOX inhibitors (*i.e.*, lipid-lowering HMG-CoA inhibitors also called statins) have been tested to regulate also downstream pathways of ROS signaling. Finally, a critical challenge in ROS inhibition is represented by the development of highly selective NOX inhibitors and their downstream pathways. Although not yet available in clinical practice, several NOX inhibitors are under preclinical and first clinical phases of development. As recently reviewed by Streeter, many outcomes targeting NOX function have been selected for evaluating these inhibitors: (i) NOX expression; (ii) signal transduction downstream of NOX-induced pathway; (iii) trafficking of NOX to the appropriate subcellular compartments; (iv) assembly of NOX complex; (v) electron transfer from NADPH to oxygen; and (vi) oxidation of downstream targets (202). In the following paragraphs, we will update evidence of these different therapeutic approaches with a

critical overview of the current evidence from basic and clinical researches.

Specific NOX inhibition vs. *inhibition of pathways leading to NOX inhibition*

The direct inhibition of NOX activity has been indicated as a very promising therapeutic approach. However, the specific inhibition of NOX isoforms still presents relevant limitations due to the lack of selective compounds and the poor clarification of their individual role in stroke. Therefore, due to the high homology among the different NOX isoforms, the development of isoform-selective compounds remains the main challenge in the field of direct NOX inhibition. Several compounds with a low specificity to inhibit NOX have been investigated. In several experimental models of cerebral I/R injury, diphenylene iodonium (DPI) has been shown to be a potent flavoprotein inhibitor, which inhibits unspecific electron transfer chains, including NOX enzymes (13, 190). This effect on phagocytes induces NOX2 inhibition (88), MMP activity (141), inflammatory microglial response (223), and leukocyte recruitment within the ischemic brain (142). However, recent comparative pharmacological analysis confirmed the poor selectivity of DPI in NOX2 inhibition. Similar results were shown by the serine protease inhibitor 4(2-aminoethyl) benzenesulfonylfluoride (230). Also, additional compounds that were believed as selective for NOX inhibition disappointed expectations. Among these, Celastrol failed to show selective activity on NOX1-2 as expected by its affinity for $p22^{phox}$ (91). On the other hand, $p47^{phox}$ inhibitors might be really selective ligands of NOX2 and potentially suitable for clinical use, especially the chimeric 18-amino acid peptide gp91ds-tat (169). On the other hand, the analogous natural peptide PR-39 was burdened by a poor bioavailability (229).

As summarized in Table 2, different treatments investigating a direct NOX inhibition have been investigated in animal models of cerebral ischemia and *in vitro*. Among these, a large numbers of studies used the 4-hydroxy-3-methoxy-acetophenone (apocynin), a natural, powerful antioxidant compound used in traditional Ayurvedic medicine (1). Although its molecular mechanism is not fully understood, apocynin would impair NOX2 assembly and activation (242), thus resulting as a NOX2-targeted therapeutic strategy against stroke (227). Experimental models of either focal or global cerebral ischemia have supported the neuroprotective role of apocynin. A block of lipid peroxidation in the brain following global cerebral ischemia was first reported by Wang and coworkers (222). Other indirect evidences of antioxidant properties of apocynin were obtained by assaying $O_2^{\bullet -}$, malonaldehyde, 8-OHdG/dG, carbonyl group, and H_2O_2 levels (29, 35, 36, 89, 102, 138, 187, 207, 227, 241, 248, 249). More specifically, Yoshioka *et al.* reported that apocynin attenuated the translocation to the cell membrane of NOX cytosolic subunits ($p57^{phox}$ and $p67^{phox}$) (241).

As reported by Genovese *et al.*, apocynin was able to inhibit the NF-κB pathway by preventing IκB-α degradation. As result, the shift of the Bcl-2/Bax balance toward an antiapoptotic state enhanced survival of neuronal cells (62). In addition, apocynin promoted neuronal death by apoptosis rather than necrosis (36). Overall, this nonselective activity of apocynin contributes to maintain the BBB function (96, 102, 208), reduce inflammatory response (29, 62, 241), and promote neuronal cell survival (36, 62, 241, 249). However, some paradoxical effects and limitations made apocynin are not suitable for clinical testing against cerebral injury. They include a higher efficacy when administered before the onset of ischemia (89), a narrow therapeutic range (208), as well as a possible exacerbation of stroke-induced bran injury (102). Additionally, H_2O_2 and MPO (and thus neutrophils) are required to convert apocynin in the active form (42). Conversely, the native form is a prodrug highly effective in scavenging (93) nonradical oxidant species (such as HOCl and H_2O_2), but has only a weak effect toward free radicals (163), including the $O_2^{\bullet -}$ (78).

The inhibition of NOX downstream activities has been reported also for Rho kinase inhibitors. The experimental drug Fasudil reduced angiotensin II-stimulated $O_2^{\bullet -}$ production as well as NOX1, NOX2, NOX4, and $p22^{phox}$ expression on endothelial cells (177). All available studies agreed to show both brain and neurological beneficial recovery following Fasudil administration (170, 178–181, 191, 206, 212, 233, 236). However, Fasudil was associated with severe hypotension and vasodilatation, thus raising some concerns on its safety. Thus, Rho kinase inhibitors are currently registered only in Japan for arterial spasm prevention in patients with subarachnoid hemorrhage and not used against stroke.

Considering that the phosphorylation of $p47^{phox}$ is also a target of PKC activation, specific inhibitors of this pathway were investigated (19, 26). Preliminary studies would confirm the effectiveness of this therapeutic approach (11, 34, 77), but the wide involvement of PKC in many cellular pathways might limit its specificity and potentially increase the risk of adverse events. Among the several ongoing studies (204), the pharmacological inhibition of Toll-like receptor 4 with resatorvid has been recently associated with reduced infarct volume and an improved neurological disability score after transient focal ischemia in mice. This treatment was associated with NOX4 inhibition and suppression of p38 MAPK, NFκB, and MMP-9 expression (205). In addition, the recent discovery of histone deacetylases as downstream signaling pathways of NOX might provide new insights for targeted drug discovery (76).

Finally, nonselective NOX inhibition has been reported for the small-molecule triazolopyrimidine VAS2870 (209) both *in vitro* and *in vivo* in a mouse model of transient focal cerebral ischemia (107). Its effects were similar to those observed in $NOX4^{-/-}$ mice, but whether VAS2870 is a selective NOX4 inhibitor or rather a pan-inhibitor of NOX enzymes is still matter of debate (94, 231).

Overall, despite these promising insights, some concerns remain about the clinical translation of these basic research findings. The majority of these experimental studies was designed to assess the effectiveness of these compounds in a very early setting (or even a pretreatment), thus representing proofs of principle rather than preclinical studies. In addition, considering that the time of ischemia in these animal models ranges around 30 min (35–37, 59, 89, 187, 222, 227, 241), we believe that these beneficial results might be difficult to be applied in the clinical settings. On the other hand, several compounds between NOX inhibitors were able to induce some benefits in different models and times of administration, supporting a therapeutic potential for these compounds (75, 89, 143, 179, 180).

Table 2. Antioxidants: *In Vivo* Research Studies

Author	*Year*	*Animal*	*Model*	*a. Drug, dose, administration way b. Timing of drug administration from stroke onset c. Time of euthanasia from stroke onset*	*Outcome measured*
Apocynin					
Wang *et al.* (222)	2006	Male gerbil	Permanent global cerebral ischemia by 5-min-long bilateral clamping of CCAs	a. Apocynin 5 mg/kg intraperitoneal b. 30 min before ischemia c. 3 h	Treatment blocked lipid peroxidation ($p < 0.01$)
				a. Apocynin 5 mg/kg intraperitoneal b. 5 min after reperfusion c. 3 h	Treated animals showed less delayed neuronal death, reactive astrocyte, and microglial cell infiltrate ($p < 0.001$)
Kahles *et al.* (96)	2007	Male mouse	Focal cerebral ischemia by intravascular occlusion of right MCA for 120 min	a. Apocynin 40 mg/kg intraperitoneal b. 1 h before ischemia 24 h	Apocynin prevented increase in BBB permeability ($p < 0.05$)
Tang *et al.* (207)	2007	Male rat	Focal cerebral ischemia by ligation of left CCA for 90 min	a. Apocynin 50 mg/kg intraperitoneal b. 30 min before ischemia c. 2 and 24 h	Treated animals had reduced NADPH oxidase activity, superoxide level, and infarct volume ($p < 0.05$)
Tang *et al.* (208)	2008	Male mouse	Focal cerebral ischemia by 120-min-long intravascular occlusion of MCA	a. Apocynin 2.5 mg/kg intraperitoneal b. 30 min before ischemia c. 24 h	Penetrating the intact BBB, apocynin reduce infarct size, and BBB disruption ($p < 0.05$). Moreover, treated animals improved neurological outcome assessed by modified Benderson's score ($p < 0.01$) and showed a trend toward decreased mortality. Also, superoxide was reduced by apocynin to levels even below that of sham ($p < 0.05$)
Jackman *et al.* (89)	2009	NOX2-deficient male mouse	Focal cerebral ischemia by intravascular occlusion of MCA for 30 min	a. Apocynin 2.5 or 5 mg/kg intraperitoneal b. 30 min before ischemia Or a. Apocynin 2.5 mg/kg intraperitoneal b. 1 h after reperfusion c. 24 h	Pretreatment reduced total infarct volume ($p < 0.05$), but not mortality or neurological impairment. 5 mg/kg or postreperfusion treatment did not show significant results. Apocynin inhibits superoxide and H_2O_2 production in wild-type mice, not in $NOX2^{-/-}$ mice
Zia *et al.* (249)	2009	Premature rabbit	Glycerol-induced intraventricular hemorrhage	a. Apocynin 2.5 mg/kg intravenous b. 3 and 12 h following the birth c. 6 and 24 h	Treatment reduced cell apoptosis ($p = 0.012$) and ROS production ($p < 0.01$)
Chen *et al.* (29)	2009	Male mouse	Focal cerebral ischemia by suture of left MCA for 75 min	a. Apocynin 4 mg/kg intraperitoneal b. 5 min before reperfusion c. 24 and 72 h	Treated mice showed less infarct volume and improved neurological outcome ($p < 0.05$). Treatment also reduced oxidative stress, evaluated by MDA and 8-OHdG, and inflammation assessed by ICAM-1, MPO, and COX-2 ($p < 0.05$)

(*continued*)

TABLE 2. (CONTINUED)

Author	*Year*	*Animal*	*Model*	*a. Drug, dose, administration way b. Timing of drug administration from stroke onset c. Time of euthanasia from stroke onset*	*Outcome measured*
Kelly *et al.* (102)	2009	Female aged rat	Focal cerebral ischemia by 90-min-long intravascular occlusion of MCA	a. Apocynin 5 mg/kg intraperitoneal b. 30 min before ischemia c. 24 h	Treatment was effective to improve neurological outcome only in young rats. Indeed, in aged rats, apocynin increased stroke volume, BBB permeability, and mortality ($p < 0.05$). Aged treated rat showed reduced SOD and GPx activity compared to young rats ($p < 0.05$)
Zhao *et al.* (247)	2010	Male alcohol-fed rat	Focal cerebral ischemia by suture of right MCA for 120 min	a. Apocynin 7.55 mg/kg orally daily b. 1 month before ischemia c. 24 h	Chronic treatment in alcohol-fed rats improves neurological score and reduces both infarct volume and superoxide production ($p < 0.05$)
				a. Apocynin 5 mg/kg intraperitoneal b. 30 min before ischemia c. 24 h	Treatment reduces total infarct volume in both nonalcohol-fed and alcohol-fed rats ($p < 0.05$)
Murotomi *et al.* (138)	2011	Male rat	Focal cerebral ischemia by 90-min-long intravascular occlusion of right MCA	a. Apocynin 5 mg/kg intraperitoneal b. 60 min before ischemia and 5 min after reperfusion onset c. 24 h	Treatment reduced infarct volume and level of oxidized proteins ($p < 0.05$)
Yoshioka *et al.* (241)	2011	Male mouse	Global cerebral ischemia by bilateral ligation of CCAs for 15 or 30 min	a. Apocynin 2.5 mg/kg intravenous b. 15 min before ischemia c. 24 h	Apocynin attenuated recruitment of NADPH oxidase cytosolic subunits (gp57phox and gp 67phox) to the cell membrane and then reduced oxidative protein damage assessed by protein carbonyl groups detection ($p < 0.05$). Treatment also reduces microglial activation ($p < 0.05$) and neuronal injury ($p < 0.05$)
Shen *et al.* (187)	2011	Male mouse	Global cerebral ischemia by 20-min-long bilateral occlusion of CCAs (microvascular clips)	a. Apocynin 40 mg/kg intravenous b. 10 min before ischemia c. 7 days	Treatment improves neurological outcome at 7 days ($p < 0.01$) reducing neuronal death and superoxide production ($p < 0.01$)
Genovese *et al.* (62)	2011	Male rat	Permanent focal cerebral ischemia by intravascular occlusion of MCA	a. Apocynin 5 mg/kg intraperitoneal b. 5 min before reperfusion c. 24 h	Treatment improved neurological score ($p < 0.01$) and the cerebral staining showed a lesser cortical and striatal damage ($p < 0.01$). Apocynin also reduced IL-1β and ICAM-1 expression by NF-κB inhibition. Finally, apocynin inhibits neuronal apoptosis inhibiting NF-κB and Bax pathway and promoting Bcl-2 signaling

(*continued*)

TABLE 2. (CONTINUED)

Author	Year	Animal	Model	a. Drug, dose, administration way b. Timing of drug administration from stroke onset c. Time of euthanasia from stroke onset	Outcome measured
Connell *et al.* (36)	2011	Male rat	Focal cerebral ischemia by suture of right MCA for 30 min	a. Apocynin 1, 5, 10, or 20 mg/kg intravenous b. 30 min before or after reperfusion c. 5.5 h	Treatment before reperfusion results in a dose-dependent neuroprotection (for 10 and 20 mg/kg doses: $p \leq 0.05$) Apocynin did not alter SOD activity, whereas administration before ischemia enhanced GSH, H_2O_2 and HNE-His adduct levels ($p \leq 0.05$). Finally, DNA fragmentation, marker of cell death, increased when apocynin was administrated before ischemia ($p \leq 0.05$)
Connell and Saleh (37)	2012	Male rat	Focal cerebral ischemia by suture of right MCA for 30 min	a. Apocynin 0.1 or 1 mg/kg combined with 0.005 mg/kg of lipoic acid intravenous b. 30 min before ischemia c. 5.5 h	Combined treatment resulted in significant neuroprotection, reducing infarct volume ($p \leq 0.05$)
Connell *et al.* (35)	2012	Male rat	Focal cerebral ischemia by suture of right MCA for 30 min	a. UPEI-100 (apocynin 0.01 *M* and lipoic acid 0.01 *M*) intravenous b. 30 min before ischemia or immediately before reperfusion c. 5.5 h	Preischemia UPEI-100 administration decreased infarct volume ($p \leq 0.05$) and delayed treatment leads to increased infarct size. Treatment also increased GSH and reduced HNE-His adduct levels ($p \leq 0.05$)
Weston *et al.* (227)	2013	Male rat	Focal cerebral ischemia of the right MCA induced by 10-min local endothelin-1 infusion	a. Apocynin 50 mg/kg intraperitoneal b. 1 h before ischemia and then again 24 and 48 h after reperfusion c. 72 h	Treatment did not improve functional outcomes, but reduce infarct area ($p < 0.05$). Apocynin did not reduce total superoxide, but attenuated in microglial cells ($p < 0.01$) and increase in neuronal cells ($p < 0.01$)
Rho kinase inhibitor					
Satoh *et al.* (178)	1996	Male rat	Permanent focal cerebral ischemia through 60-min-long microembolization of left ICA	a. Fasudil 3 or 10 mg/kg intravenous b. 5 min after cerebral embolization c. 24 h	Treatment dose dependently reduced infarct areas both 3 mg/kg ($p < 0.05$) and 10 mg/kg ($p < 0.01$). Fasudil also decreased neuronal cell loss ($p < 0.01$) and at 10 mg/kg improved clinical neurological outcome ($p < 0.05$). Finally, it also reduced brain water content ($p < 0.05$)
Satoh *et al.* (179)	1999	Male rat	Permanent focal cerebral ischemia through 60-min-long microembolization of left MCA	a. Fasudil 3 or 10 mg/kg intravenous b. From 5 to 60 min after cerebral embolization c. 24 h	Fasudil reduced infarct area ($p < 0.01$) and clinical neurological outcome ($p < 0.05$). Moreover, Fasudil impaired neutrophil chemotactic activity

(*continued*)

TABLE 2. (CONTINUED)

Author	*Year*	*Animal*	*Model*	*a. Drug, dose, administration way b. Timing of drug administration from stroke onset c. Time of euthanasia from stroke onset*	*Outcome measured*
Toshima *et al.* (212)	2000	Male rat	Permanent focal cerebral ischemia by left CCA ligation	a. Fasudil 1 or 10 mg/kg intraperitoneal b. 5 min after first cerebral embolization and daily in the following 2 days c. 48 and 96 h	Fasudil improved neurological deficits both at 1 mg/kg ($p < 0.05$) and at 10 mg/kg ($p < 0.01$)
Takanashi *et al.* (206)	2001	Male rat	Permanent focal cerebral ischemia by left MCA microembolization for 60 min	a. Fasudil 0.01 or 2.5 mg intrathecal b. Immediately before ischemia c. 4, 24, and 72 h	Treatment improved functional neurological outcome ($p < 0.001$) and reduced the infarcted area ($p < 0.001$)
Satoh *et al.* (181)*I*	2001	Male rat	Global cerebral ischemia induced through cerebral microembolism	a. Fasudil 10 mg/kg intravenous b. Begun 5 min after cerebral embolization and maintained for 60 min c. 24 h	Treatment reduced neutrophil recruitment ($p < 0.05$) and infarcted area ($p < 0.05$). Fasudil also improved neurological outcome ($p < 0.05$)
Rikitake *et al.* (170)	2005	Male mouse	Focal cerebral ischemia by intravascular occlusion of MCA for 120 min	a. Fasudil 1, 3, or 10 mg/kg intraperitoneal b. Daily for 2 days before ischemia c. 24 h	Fasudil decreased in a dose-dependent manner infarct volume ($p < 0.05$) and this correlated with improved neurologic deficit score ($p < 0.05$). Fasludil increased eNOS activity and stimulated NO generation in a concentration-dependent manner
Yagita *et al.* (233)	2007	Male rat	Permanent focal cerebral ischemia by ligation of right CCA	a. Fasudil 10 mg/kg intraperitoneal b. 5 min after ischemia c. 48 h	Treatment improved functional neurological outcome ($p < 0.05$) and reduced the infarcted area ($p < 0.05$)
Yamashita *et al.* (236)	2007	Male mouse	Permanent focal cerebral ischemia by intravascular suture of left MCA	a. Fasudil 10 mg/kg intraperitoneal b. 30 min before ischemia c. 24 h	Treatment improved functional neurological outcome ($p < 0.01$) and reduced the infarcted area ($p < 0.05$)
Shin *et al.* (191)	2007	Mouse	Permanent focal cerebral ischemia by occlusion of MCA (microvascular clips)	a. Fasudil 10 mg/kg intraperitoneal b. 60 min before or 5 min after ischemia c. 24 h	Preischemic more than postischemic treatment reduced the infarcted area ($p < 0.05$)
Satoh *et al.* (180)	2008	Male rat	Focal cerebral ischemia (cerebral thrombosis induced by Sodium Laurate infusion in left ICA)	a. Fasudil 10 mg/kg intraperitoneal b. Start 6 h after ischemia and continued until day 4 c. 5 days	Fasudil reduced neutrophil recruitment ($p < 0.05$) and then infarcted area ($p < 0.05$) and neurological outcome ($p < 0.01$)
Statins					
Nagotani *et al.* (143)	2005	Hypertensive stroke prone rat	Global cerebral ischemia	a. Atorvastatin 20 mg/kg or simvastatin 20 mg/kg daily b. Start 14 days before euthanasia c. 4 and 24 h	Atorvastatin ($p < 0.05$) and simvastatin ($p < 0.01$) reduced infarct volume

(*continued*)

TABLE 2. (CONTINUED)

Author	*Year*	*Animal*	*Model*	*a. Drug, dose, administration way b. Timing of drug administration from stroke onset c. Time of euthanasia from stroke onset*	*Outcome measured*
Hayashi *et al.* (75)	2005	Male rat	Focal cerebral ischemia by right MCA ligation for 90 min	a. Atorvastatin 20 mg/kg or simvastatin 20 mg/kg daily b. Start 14 days before ischemia c. 14 days	Simvastatin treatment, but not atorvastatin, improved neurological score after 24 h ($p < 0.05$). After 14 days, simvastatin reduced infarct size ($p < 0.01$) and also oxidative stress evaluated by HNE-His and 8-OHdG assay ($p < 0.01$)
Hong *et al.* (80)	2006	Male rat	Focal cerebral ischemia by left MCA ligation for 120 min	a. Atorvastatin 10 mg/kg or simvastatin 10 mg/kg b. 48, 24, and 2 h before reperfusion c. 24 h	Atorvastatin reduced infarct size ($p < 0.01$) and prevents the peak of NADPH oxidase activity ($p < 0.05$) and the increased superoxide levels ($p < 0.001$) during reperfusion. In particular, atorvastatin inhibits overexpression of $gp91^{phox}$ ($p < 0.01$) and membrane-translocated $p47^{phox}$ ($p < 0.05$)
Kawai *et al.* (101)	2011	Male rat	Focal cerebral ischemia by intravascular occlusion of right MCA for 90 min	a. Atorvastatin 10 mg/kg and/or amlodipine 3 mg/kg daily b. For 28 days before ischemia c. 24 h	Atorvastatin treatment reduced infarct volume both alone ($p < 0.05$) and in combination therapy ($p < 0.01$). Atorvastatin reduced also oxidative stress markers such as HNE-His and 8-OHdG ($p < 0.01$). Finally, atorvastatin reduced inflammation markers TNF-α and MCP-1 ($p < 0.01$)
Gaur and Kumar (59)	2011	Male rat	Global cerebral ischemia by occlusion of CCA (microvascular clips) for 30 min	a. Atorvastatin 10 or 20 mg/kg and/or candesartan 0.1 mg/kg daily b. For 7 days before ischemia c. 24 h	Both combination therapy and atorvastatin alone improved neurological outcomes ($p < 0.05$). Treatments also improve mitochondrial enzyme complex activity: NADPH dehydrogenase, succinate dehydrogenase, cytochrome oxidase, and MMT ($p < 0.05$). Moreover, atorvastatin and or candesartan attenuated brain MDA and nitrite concentration ($p < 0.05$) as well as restored SOD activity ($p < 0.05$)

COX-2, cyclooxygenase-2; eNOS, endothelial nitric oxide synthase; GPx, glutathione peroxidase; GSH, reduced glutathione; IL, interleukin; MCP, monocyte chemoattractant protein; MDA, malondialdehyde; MMT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO, nitric oxide; NOX, NADPH oxidase; SOD, superoxide dismutase.

Antioxidant treatments

A nonspecific inhibition of NOX-mediated transduction signaling was reported for statins. These drugs are known to induce pleiotropic effects independent of their lipid-lowering properties that might be associated with infarct volume reduction and improvement in the neurological score in the experimental model of I/R injury. In addition, these results were consistent with the reduction of oxidative stress markers and further confirmed by clinical studies (59, 75, 80, 101). Statins abolished the geranylgeranylation of Rac1, preventing its translocation from the cytosol to the membrane. This suppresses the catalytic subunit of NOX2, and the translocation of p47phox leads to reduced $O_2^{\bullet -}$ generation (81). Overall, the other pleiotropic effects of statins, also including antiapoptotic, antithrombotic, and anti-inflammatory mechanisms, may promote cerebral recovery after stroke (219).

Similarly, also, peroxisome proliferator-activated receptor-γ agonists were able to reduce brain injury, but only in animal models of transient middle cerebral artery occlusion (189). The nonspecific inhibition of these compounds is based on the increase of CuZn-SOD (27, 106) as well as the suppression of cerebral expression of NOX4 (243).

In addition, the inhibitors of the renin-angiotensin-aldosterone system (*i.e.*, candesartan) have shown to induce antioxidant activities. Counteracting the pro-oxidant effect of angiotensin II, candesartan reduced oxidative stress impairing $gp91^{phox}/p22^{phox}$ mRNA expression and lipid peroxidation products. These results were associated with a smaller infarct volume, decreased edema, and improved neurological recovery either alone or in combination with atorvastatin (59).

Potential treatments for humans

As reported in Table 3, nonspecific NOX inhibitors (*i.e.*, free radical scavenger and antioxidants) have been clinically tested for reducing the oxidative stress after stroke.

The first compound tested was the nitrone NXY-059 (disodium 4-[(tert-butylimino)methyllbenzene-1,3-disulfonate N-oxide). By promoting the formation of nitroxide, these compounds were shown to stabilize reactive free radicals. For this reason, they have been investigated as a potential treatment reducing oxidant-mediated effects in ischemic stroke. Despite some encouraging results in animal models of stroke (53), in a large randomized placebo-controlled trial, NXY-059 substantially failed to improve neurological outcomes in stroke patients (45, 193). On the other hand, two other free radical scavengers are currently under clinical evaluation. Ebselen, is currently undergoing a phase III clinical trial (159) to confirm the preliminary clinical results on patients with acute ischemic stroke (154, 176, 234). Originally, Ebselen was used as a free radical scavenger (mimic of GSH peroxidase). However, this drug was also shown to react with peroxynitrite and to inhibit lipoxygenases, NO synthases, PKC, and H^+/K^+-ATPase (161). Recently, NOX2 has been also recognized as a target of this Ebselen. As showed by Smith *et al.*, Ebselen and their analogs may inhibit the assembly of the NOX2 subunit $p47^{phox}$ to $p22^{phox}$ (196). Finally, beneficial results were shown using Edaravone (a hydroxyl radical scavenger), already registered in Japan for the treatment of ischemic stroke.

On the other hand, relevant expectancies have been raised by some compounds developed by GenKyoTex (Geneva, Switzerland). By screening the biological activity of original pyrazolo-pyrido-diazepine, -pyrazine, and -oxazine dione derivatives, two molecules with potential activity as the dual NOX1/4 inhibitor were identified: GKT136901 and GKT137831 (58). Their oral bioavailability and favorable pharmacokinetic/pharmacodynamics profile make these compounds the most suitable for a clinical translation. Accordingly, a phase II clinical trial as antioxidant therapy in patients with diabetic nephropathy has been approved by the U.S. Food and Drug Administration for GKT137831 (1).

Treatments with general antioxidants (such as statins) are currently shown to be effective in both primary prevention (9, 17, 57, 149, 194) and recurrence of stroke (8, 17, 24, 65, 66, 149). Clinical studies investigating ROS and NOX expression levels in circulating phagocytes from stroke patients might provide additional knowledge on the role of the molecules in stroke pathophysiology and solve some controversies (15, 123, 139).

Limitations of antioxidant therapies

Many limitations have been suggested concerning general antioxidants as valuable therapeutic approaches against ischemic stroke. In fact, clinical trials have so far reported conflicting results and a marked mismatch with preclinical findings. Clinical studies are characterized for the moment by major limitations in the study design [such as a small sample size (15, 134, 139) and the recruitment of high-risk or high-selected patients]; thus, they are not representative of the general population. Furthermore, the simultaneous administration of various and nonselective antioxidants might result in unpredictable biological effects, thus reducing the scientific relevance of the results (2, 38, 185). As already observed in cross-sectional studies, the dose of 100 mg/day of vitamin C might be associated with a very slight increase in plasma concentrations (110, 140, 240). Likewise, antioxidant supplementation studies demonstrated that the intake of vitamin E might be associated with an increased risk of hemorrhagic stroke (183). Potentially due to these concerns, as well as to genetic factors (52), antioxidant vitamin supplementation was also shown as ineffective in reducing the incidence of major cardiovascular events, including ischemic stroke in a recent meta-analysis (238).

On the other hand, the results of preclinical studies could be also affected by major limitations. Animal models of stroke (Tables 1 and 2) are generally performed using young, male, and healthy animals. Thus, they are very different from a human stroke population that is usually older and with additional comorbidities and risk factors (such as overweight, hypertension, and diabetes), as well as includes the female gender.

Old age has been also shown to play a main role as risk factor for stroke. The aged brain is prone to enhance the inflammatory response (and thus oxidative stress) after neurological injury, leading to worse poststroke outcomes (128). These features are especially related with an enhanced activation of microglial cells in the aged brain (151). On the other hand, the role of cardiovascular comorbidities has been well established to be associated with worse poststroke outcomes as suggested by an experimental model of stroke induced in obese (120), diabetic (51), and hypertensive (122) animals.

Table 3. Clinical Trials Investigating Antioxidant Drugs

Author	Year	Number of patient	Study design (follow-up)	Treatment	Correlation with stroke	Adverse side effects
Ebselen						
Yamaguchi *et al.* (234)	1998	300 stroke patients (151 Ebselen + ST and 149 ST)	Prospective randomized trial (14 days)	Ebselen 150 mg twice a day within 48 h from admission	Treatment provided a better outcome according to GOS score at 1 month ($p = 0.023$), but not at 3 months ($p = 0.056$). Ebselen also improved Barthel Index ($p = 0.01$) and modified Matthew Scale ($p = 0.03$)	Not observed in the treated group
Saito *et al.* (176)	1998	286 ASH patients (145 Ebselen + ST and 141 ST)	Prospective randomized trial (2 weeks)	Ebselen 150 mg twice a day	Treatment did not prevent delayed neurological effects, but provided a significantly better outcome at GOS score ($p = 0.005$) and decreased low-density areas ($p = 0.032$)	The incidence of meningitis or respiratory infection did not differ between the two groups. Instead, the occurrence of hydrocephalus was significantly ($p = 0.042$) reduced in the Ebselen-treated group
Ogawa *et al.* (154)	1999	105 stroke patients (48 Ebselen + ST and 57 ST)	Prospective randomized trial (14 days)	Ebselen 150 mg twice a day within 12 h from admission	In not fully reanalyzed patients Ebselen provided significant reduction in the infarcted volume ($p < 0.05$). Ebselen failed to improve outcome	Liver dysfunction and abdominal bloating were observed in treated group, but the incidence was similar within the two groups
Edaravone						
Edaravone Acute Infarction Study Group (3)	2003	250 stroke patients (125 ST + Edaravone and 125 ST)	Prospective randomized trial (14 days)	From 72 h after stroke, Edaravone 30 mg twice a day	Improved functional outcome in Edaravone group evaluated by Rankin Scale ($p = 0.03$) 3 months after stroke	Not available
Toyoda *et al.* (213)	2004	61 stroke patients (30 ST + Edaravone and 31 ST)	Prospective randomized trial (14 days)	From 6 h after stroke, Edaravone 30 twice a day	Edaravone improved infarct volume and edema at CT control on day 2 ($p < 0.02$) and 5 ($p < 0.07$). Better Rankin Scale in treated group after 8 weeks ($p < 0.03$)	Not investigated
Ogasawara *et al.* (153)	2005	147 patients undergoing CEA (55 ST + Edaravone and 92 ST)	Prospective randomized trial	Edaravone 60 mg 30 min before ICA clamping	Edaravone treatment prevents postoperative cognitive impairment ($p = 0.04$)	Not investigated
Uno *et al.* (215)	205	51 stroke patients (27 ST + Edaravone and 24 ST) and 19 age-matched healthy controls	Prospective randomized trial (14 days)	From 24 h after stroke, Edaravone 30 mg twice a day	In cortical stroke, within first 3 days, Edaravone reduced circulating oxidative stress markers (oxLDL and MnSOD ($p < 0.05$)	Not investigated
Imai *et al.* (87)	2006	38 stroke patients (19 ST + Edaravone and 19 ST)	Prospective randomized trial (7 days)	Within 48 h after stroke, Edaravone 30 mg associated to HBO for	Combined treatment provided short-term outcome (7 days) improvement according to NIHSS score ($p < 0.01$) and also long-term outcome (90 days) according to Rankin Scale ($p = 0.04$)	Minor complications were observed in treated group (otalgia, hypertension, low-back pain, and tachycardia). In either group was recognized the occurrence of parenchymal hematoma (no significant difference)
Nakase *et al.* (144)	2011	176 stroke patients (93 ST + Edaravone and 83 ST)	Prospective randomized trial (14 days)	Edaravone 30 mg twice a day	CT control showed that Edaravone reduced stroke lesion more quick, already in 1–2 months ($p = 0.006$). In small-vessel occlusion stroke subtype, Edaravone improved NIHSS ($p = 0.04$)	Not investigated

(*continued*)

TABLE 3. (CONTINUED)

Author	Year	Number of patient	Study design (follow-up)	Treatment	Correlation with stroke	Adverse side effects
Sharma *et al.* (186)	2011	50 stroke patients (25 ST + Edaravone and 25 ST)	Prospective randomized trial (14 days)	Edaravone 30 mg twice a day	Edaravone showed improving trend in Rankin Scale ($p=0.059$) and Barthel Index increase ($p=0.01$) after 90 days	The incidence of adverse reaction was higher in placebo group (skin rash, abnormal renal function, and fever) compared to Edaravone group (skin rush, fever, abnormal liver, and renal function)
Kimura *et al.* (105)	2012	90 stroke patients (Edaravone + t-PA and Edaravone without t-PA)	Prospective randomized trial (7 days)	Edaravone 30 mg twice a day for 7 days at the same time of t-PA infusion or after follow-up MRI (within 1h after t-PA infusion)	At MRI follow-up, after 1h, simultaneous therapy provided early recanalization ($p=0.007$)	The occurrence of hemorrhagic transformation did not differ between the two groups ($p=0.99$). However, symptomatic intracranial hemorrhage was observed in patients not treated with Edaravone
Statins						
Amarenco *et al.*(9)	2006	4731 patient with previous TIA or stroke (2365treated and 2366 placebo)	Prospective randomized trial (6 months)	Atorvastatin 80 mg daily	Atorvastatin prevented and TIA occurrence stroke (HR 0.77 [CI 95% 0.67–0.88]; $p<0.001$)	Overall, safety assessment was similar between the groups. ALT/AST levels were significantly higher ($p<0.001$) in treatment group as compared to placebo
Blanco *et al.*(17)	2007	89 patients receiving statin before stroke onset (43 statin treated and 46 withdraw statins)	prospective randomized trial (83 months)	Atorvastatin 20 mg daily for 3 months	Treatment reduced death or self-sufficiency occurrence (OR 4.66 [CI 95% 1.46–14.91]; $p<0.05$) and also early neurologic deterioration (OR 8.67 [CI 95% 3.05–24.63]; $p<0.05$) in adjusted analysis. Treatment decreased infarct volume ($p<0.001$)	Not investigated
Montaner *et al.* (134)	2008	60 cortical ischemic stroke patients (30 treated and 30placebo)	Prospective randomized trial (90 days)	Simvastatin 40 mg daily for the first week and after 20 mg daily until day 90	Treatment did not improve mortality risk in multivariate analysis, but improved NIHSS score ($p=0.02$)	Nonsignificant increase in mortality and greater proportion of infections were the main side effects
Goldstein *et al.* (65)	2008	4730 patients within 1–6 months after stroke or TIA (2365 treated and 2365 placebo)	Prospective randomized trial (4.9 years)	Atorvastatin 80 mg daily	Treatment reduced fatal or nonfatal ischemic stroke recurrence (HR 0.79 [CI 95% 0.66–0.95]; $p=0.02$). Increased incidence of hemorrhagic ischemic stroke (HR 1.68 [CI 95% 1.09–2.59]) attenuated overall outcome (adjusted HR 0.84 [CI 95% 0.71–0.99]; $p=0.05$)	Hemorrhagic stroke was more frequent in patients treated with atorvastatin (HR 1.68 [CI 95% 1.09–2.59]; $p=0.02$)
Amarenco *et al.* (8) SPARCL trial	2009	4730 patients within 1–6 months after stroke or TIA (2365 treated and 2365 placebo)	Prospective randomized trial (6 months)	Atorvastatin 80 mg daily	Treatment was effective in reducing stroke recurrence for all entry event stroke subtype (overall $p=0.04$) as well as major cardiovascular events	The rates of adverse events such as creatinine elevation, myalgia, myopathy, or rhabdomyolysis were similar between the two groups. Only liver enzyme elevation was significantly higher in treated group
Goldstein *et al.* (66) SPARCL trial	2009	492 patients of SPARCL trial having an outcome of ischemic stroke (218 statin treated and 274 placebo treated)	Prospective randomized trial (4.9 years)	Atorvastatin 80 mg daily	After 90 days, treated group showed only a trend to better outcome in ischemic stroke subgroup according to Rankin Scale ($p=0.06$)	Not investigated

(*continued*)

Table 3. (Continued)

Author	Year	Number of patient	Study design (follow-up)	Treatment	Correlation with stroke	Adverse side effects
Lingsma *et al.* (123)	2010	780 stroke patients (280 treated and 500 controls)	Prospective randomized trial (3 year)	Not provided	In adjusted analysis, treatment failed to reduce stroke and MI risk (OR 0.8 [CI 95% 0.8–1.2])	Nonsignificant increase in mortality and infections
Fu *et al.* (57)	2010	227 subjects from ROCAS study (133) (33 SBI and 194 non-SBI)	Prospective randomized trial (2 years)	Simvastatin 20 mg daily	Treatment reduced brain infarct (both symptomatic and not) risk (OR 0.09 [CI 95% 0.01–0.82]; $p<0.05$)	Not investigated
Ni Chroinin *et al.* (149)	2011	418 subject (117 continued prestroke statin-treated, 189 started statin after stroke, and 112 nonstatin treated)	Prospective randomized trial (1 year)	Not already treated started within 72 h from stroke onset (different drugs and doses)	On multivariate analysis, new acute poststroke statin treatment independently correlated with survival at 7 days ($p<0.001$), 90 days ($p<0.001$), and 1 year ($p=0.003$) after stroke. Prestroke treatment improved only shorter outcome ($p=0.003$). Prestroke treatment improved outcome compared to nontreated patients both 7 day ($p=0.003$) and 90 days ($p=0.002$ and 1 year ($p=0.05$)	Not investigated
Muscari *et al.* (139)	2011	62 ischemic stroke patients (31 treated and 31 placebo)	Prospective randomized trial (30 to 45 days)	Atorvastatin 80 mg daily	Treatment failed to show a short-term (7 days) benefit according to NIHSS score	There were no significant differences between the two groups in the few subjective complaints occurring during hospitalization and follow-up (nausea, vomiting, constipation, diarrhea, cough, asthma, rash, itching, abdominal pain, muscular pain, infective disease, or hemorrhagic stroke)
Beer *et al.* (15)	2012	40 ischemic stroke patients (17 statin-treated and 23 placebo)	Prospective randomized trial (days 3 and 30)	Within 96 h to stroke onset, atorvastatin 80 mg	Treatment failed to show any radiological or functional improved outcome	Creatinine and ALT concentrations were not significantly influenced by randomization atorvastatin therapy rather than placebo
Sicras-Mainar *et al.* (194)	2012	601 stroke patients (192 statin treated and 409 nonstatin treated)	Retrospective study (6 years)	Different statins and doses	Treatment lowered cumulative hazard off all-causes mortality ($p=0.007$) and also for stroke (adjusted cumulative HR 0.35 [CI 95% 0.19–0.64]; $p=0.001$)	Not investigated
Cappellari *et al.* (24) THRaST study	2013	2072 stroke patients (542 started therapy, 203 continued therapy, 94 switched therapy, and 1233 not treated)	Retrospective study (7 days and 3 months)	Different statins and doses	Statin treatment in the acute phase correlated both with short-term outcome neurologic improvement, according to the NIHSS score (adjusted OR 1.68 [CI 95% 1.26–2.25]; $p<0.001$), and with long-term endpoint death (adjusted OR 0.48[0.28–0.82]; $p=0.007$)	Not investigated

(*continued*)

TABLE 3. (CONTINUED)

Author	*Year*	*Number of patient*	*Study design (follow-up)*	*Treatment*	*Correlation with stroke*	*Adverse side effects*
Vitamins						
Yokoyama *et al.* Shibata study (240)	2000	2121 healthy subjects (880 men and 1241 women)	Prospective observational (20 years)	Vitamin C concentration at baseline	Serum vitamin C concentration was inversely related to the subsequent incidence of stroke. At multivariate adjusted analysis, p for interquartile range was 0.002	Not investigated
Myint *et al.* *EPIC*-Norfolk population prospective study (140)	2008	20,649 healthy subjects (9449 men and 11,200 women)	Prospective observational (9.5 years)	Vitamin C concentration at baseline	Serum vitamin C concentration was inversely related to the subsequent incidence of stroke. At multivariate adjusted analysis, top quartile had 42% lower RR (0.58 [CI 95% 0.43–0.78]; $p=0.001$) than bottom quartile	Not investigated
Kubota *et al.* JACC study (110)	2011	58,730 healthy subjects (23,119 men and 35,611 women)	Prospective observational (16.5 years)	Dietary intake of vitamin C at baseline	Dietary vitamin C intake was inversely associated with mortality from stroke. At multivariate adjusted analysis, HR for highest versus lowest quintile was 0.70 (CI 95% 0.54–0.92); $p=0.006$) for women and 0.84 (CI 95% 0.62–1.13); $p=0.36$ for men	Not investigated
Heart Protection Study Collaborative Group (2)	2002	20,536 patients with CV disease (10,269 treated and 10,267 nontreated)	Prospective randomized trial (5 years)	Vitamin C supplementation (250 mg daily)	The groups did not show difference for cardiovascular event occurrence, including stroke (RR 0.99 [CI 95% 0.87–1.12]; $p=0.8$)	Not investigated
Cook *et al.* Women Antioxidant Cardiovascular Study (38)	2007	8171 woman with high CV risk or previous CV events (4087 treated and 4084 nontreated)	Prospective randomized trial (9.4 years)	Vitamin C supplementation (500 mg daily)	Vitamin C supplementation did not reduce stroke occurrence (RR 0.86 [CI 95% 0.69–1.08]; $p=0.21$)	Vitamin C supplementation was not associated with adverse effects
Sesso *et al.* Physicians' Health Study (185)	2008	14,641 men (7329 treated and 7312 nontreated)	Prospective randomized trial (8 years)	Vitamin C supplementation (500 mg daily)	Vitamin C supplementation did not reduce stroke occurrence (RR 0.89 [CI 95% 0.74–1.07]; $p>0.05$)	No significant differences were observed in adverse effects, including hematuria, easy bruising, and epistaxis for active vitamin C as compared with placebo
Schurks *et al.* (183)	2010	1,18,765 participants (59,357 treated and 59,408 nontreated)	Meta-analysis of nine studies	Vitamin E different doses	Vitamin E supplementation did not improve stroke risk (RR 0.98 [CI 95% 0.90–1.06]; $p>0.05$)	In the group treated with vitamin E, the incidence rate of hemorrhagic stroke was increased
Bin *et al.* (16)	2011	1,66,282 participants	Meta-analysis of 13 studies	Vitamin E different doses between 50 and 800 mg/day	Meta-analysis showed no significant benefit in the vitamin E group with respect to stroke (RR 1.01 [CI 95% 0.96–1.07]; $p=0.6$)	Not observed

ALT, alanine aminotransferase; ASH, aneurysmal subarachnoid hemorrhage; AST, aspartate transaminase; CEA, carotid endo-arterectomy; CI, confidence interval; CT, computerized tomography; GOS, Glasgow outcome scale; HBO, hyperbaric oxygen; HR, hazard ratio; ICA, internal carotid artery; MI, myocardial infarction; MRI, magnetic resonance imaging; NIHSS, national institutes of health stroke scale; OR, odds ratio; oxLDL, oxidized low-density lipoprotein; ROCAS, Regression of Cerebral Artery Stenosis; RR, relative risk; SBI, subclinical brain infarct; SPARCL, Stroke Prevention by Aggressive Reduction in Cholesterol Levels; ST, standard therapy; TIA, transitory ischemic attack; t-PA, tissue plasminogen activator.

Likewise, gender differences in stroke are increasingly accepted as a main limitation. Experimental models and epidemiological studies agreed in reporting lower incidences of stroke in women until advanced age, when a shift of stroke incidence (and severity) occurs (116, 125). The potential mechanisms involving hormonal activities and signaling is still a matter of debate (130). Estrogens have been recognized to enhance neuronal cell survival (85) and suppress inflammatory response (both circulating and resident within the brain) (171). However, estrogen replacement failed in reducing stroke incidence in postmenopausal women in various clinical trials, including the Women's Health Initiative (WHI) (224). Although some concerns have been raised regarding the study design (especially about the dose and timing of hormone replacement), estrogen did not fully account for the dichotomous response observed between male and female to cerebral ischemia. Indeed, by culturing embryonically derived neuronal cells (thus independent from estrogenic effects), the signaling switch observed among the genders after ischemic injury emerged as a hormone-independent mechanism. In particular, female cells showed longer survival than male ones *in vitro*, consistent with a higher expression of the intracellular proteins ERK1 and Akt (244). In addition, cell death after ischemic injury was triggered by cytochrome C and caspase activation in female cells, whereas ischemic injury in male cells activated nitric oxide synthase and poly(ADP-ribose)polymerase (119).

Therefore, X-chromosome would contribute to a sexual dimorphism in ischemic cell death by X-linked gene expression and unidentified epigenetic modifications (148). Ultimately, a comprehensive knowledge in this field might favor the development of more effective therapeutic strategies (79).

Future Directions: Other Potential Therapeutic Strategies

Despite a strong scientific rationale for their development, the lack of specific NOX inhibitors strongly limits their therapeutic perspectives. These limitations are accompanied by additional concerns, such as the adverse effects induced by an excessive NOX2 suppression as well as the interference on ROS-mediated physiological pathways. The ongoing challenge is to synthetize isoform-specific compounds and exclusively targeting pathophysiological pathways. The current approaches addressing these issues include monoclonal antibodies, small-molecule inhibitors, and aptamers.

A very promising therapeutic strategy is represented by the identification of functional epitopes on NOX, followed by the generation of monoclonal inhibitory antibodies. The first epitope is on $gp91^{phox}$ that allowed the synthesis of the NOX2-specific antibody (22). In addition, more recently, the last extracellular loop of NOX4 has been targeted by a specific monoclonal antibody (245). However, the development of these selective antibodies is only the first step and many obstacles still remain. *In vivo* validation programs are needed, especially considering the difficulties of these antibodies to cross the BBB (245).

Another approach has been proposed by Brown and coworkers. Starting from the chemical composition of diphenylene iodium, they screened large libraries of small molecules with the aim of finding other NOX inhibitors (specifically NOX1) (20). A similar screening project was applied also for other targets in the field of NOX inhibition. Accordingly, to the emerging role of zinc homeostasis in the redox signaling network, zinc ionophores (such as pyrithione) were shown to reverse the expression of NOX2 (99).

Another target for small molecules is the proline-rich domain of $p22^{phox}$ subunit, essential for NOX1 and NOX2 activation. Although other compounds (such as ebselen and celastrol) are known to inhibit this binding, the development of a new drug selectively inhibiting this domain might enhance this therapeutic potential (173).

Finally, the newest approach is represented by the use of aptamers (short-stranded oligonucleotides [15–40 of both DNA and RNA]) first synthetized in the early 1990s. Through a process termed systematic evolution of ligand by exponential enrichment (SELEX), several libraries of either DNA or RNA were screened for sequences with higher affinity toward the target of choice.

The result is the development of interference DNA or RNA (iDNA or iRNA) synthetic compounds, highly specific and further editable to improve their *in vivo* properties (168).Their small dimensions (8–15 kDa) fold into unique three-dimensional structures giving a high specificity to the binding that ultimately leads to the marked inhibition of the enzymatic activity (47). Also, considering the easy and ready reversibility of aptamer binding (156), their clinical applications are potentially promising. However, their use remains to be clinically tested.

Conclusions

In the last decades, from basic research and clinical trials, the detrimental role of phagocytic ROS during cerebral I/R injury has been strongly supported. Many concerns emerged at the same time. First, the role of oxidative stress might promote brain recovery, especially in the late stages of I/R injury. In addition, the timing of different NOX activation plays a pivotal role in the early stages of I/R injury, when different cell types are progressively activated.

These critical issues on ROS and NOX (particularly NOX2) have become the main therapeutic challenge for the development of selective and safe NOX inhibitors.

On the other hand, the low selectivity of available NOX inhibitors hampers the comprehensive knowledge about the involvement of different NOX in stroke pathophysiology.

Alongside with the improvement in detection of penumbral imaging (through diffusion and perfusion magnetic resonance imaging), a fast pharmacological progress is needed to develop more specific and selective NOX inhibitors. In this field, monoclonal antibodies, small-molecule inhibitors, or aptamers are the best and promising examples. However, they remain to be tested in stroke models.

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Address correspondence to:
Dr. Fabrizio Montecucco
Division of Cardiology, Foundation for Medical Researches
Department of Medical Specialties
University of Geneva
Avenue de la Roseraie 64
Geneva 1211
Switzerland

E-mail: fabrizio.montecucco@unige.ch

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Abbreviation Used

8-OHdG = 8-hydroxy-2-deoxyguanosine
β2-GPI = β2-glycoprotein
Akt = protein kinase B
AP-1 = activation protein-1
BBB = blood–brain barrier
BMECs = brain microvascular endothelial cells
CCA = common carotid artery
CCL = CC chemokine ligands
COX2 = cyclooxygenase2
DPI = diphenylene iodonium
DUOX = dual NADPH oxidase
eNOS = endothelial nitric oxide synthase
GPx = glutathione peroxidase
GSH/GSSG = glutathione
H_2O_2 = hydrogen peroxide
HIF-1 = hypoxia-inducible factor
HNE = 4-hydroxy-2-nonenal
HNE-His = 4-hydroxynonenal-histodine
I/R = ischemia/reperfusion
ICAM-1 = Intercellular Adhesion Molecule 1
IL = interleukin
iNOS = inducible nitric oxide synthase
JNK = c-Jun N-terminal kinase
KO = knockout
MAPK = mitogen-activating protein kinase
MCA = middle cerebral artery
MCP = monocyte chemoattractant protein
MDA = malondialdehyde
MMPs = matrix metalloproteinases
MMT = 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MPO = myeloperoxidase

Abbreviations Used (Cont.)

mTOR = mammalian target of rapamycin
NFAT = nuclear factor of activated T cell
NO = nitric oxide
NOX = NADPH oxidase
$O_2^{\bullet-}$ = superoxide
•OH = hydroxyl radical
oxLDL = oxidized low-density lipoprotein
PI3K = phosphoinositide 3 kinase
PKC = protein kinase C
PTP = protein tyrosine phosphatase
ROS = reactive oxygen species
SOD = superoxide dismutase
TKR = tyrosine kinase receptor
TNF = tumor necrosis factor
WT = wild type