

## Review Article

# Nitric oxide: considerations for the treatment of ischemic stroke

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**Some 40 years ago it was recognized by Furchgott and colleagues that the endothelium releases a vasodilator, endothelium-derived relaxing factor (EDRF). Later on, several groups identified EDRF to be a gas, nitric oxide (NO). Since then, NO was identified as one of the most versatile and unique molecules in animal and human biology. Nitric oxide mediates a plethora of physiological functions, for example, maintenance of vascular tone and inflammation. Apart from these physiological functions, NO is also involved in the pathophysiology of various disorders, specifically those in which regulation of blood flow and inflammation has a key role. The aim of the current review is to summarize the role of NO in cerebral ischemia, the most common cause of stroke.**

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## Introduction

In the late 1970s, it was recognized that the endothelium releases a factor that relaxes vascular smooth muscle cells thereby causing vasodilatation (Furchgott and Zawadzki, 1980). Since the chemical structure of this factor was unknown at the time, it was named EDRF (endothelium-derived relaxing factor). Later on, EDRF was identified to be a color and odorless gas, nitric oxide (NO) (Furchgott *et al*, 1987; Ignarro *et al*, 1987*a, b*). Since then, NO, the most convincing gaseous signaling molecule in vertebrates, was increasingly recognized as one of the most versatile and unique molecules mediating such diverse physiological functions as the maintenance of vascular tone, thrombotic-thrombolytic homeostasis, cell growth, and inflammation. Apart from its physiological functions, NO has also an important role in the pathophysiology of various disorders, specifically those in which regulation of blood flow and inflammatory reactions are key pathophysiological events.

## Nitric Oxide—Physiology

Nitric oxide is an uncharged gas that can easily cross biological membranes. It is, however, quickly deactivated by oxidation with a biological half-life of a few seconds thereby limiting its spatial extent in biological tissues (Gibson and Roughton, 1957; Stamler *et al*, 1992). Nitric oxide is synthesized by a group of three NO synthases (NOS) from L-arginine (Furchgott and Zawadzki, 1980; Palmer *et al*, 1988), a reaction that requires the presence of molecular oxygen, nicotinamide adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin (BH<sub>4</sub>), heme, Flavin mononucleotide (FMN), and calmodulin (Bredt and Snyder, 1990). Two constitutively expressed isoforms, mainly located in endothelial cells and in neurons (eNOS and nNOS, respectively) generate low NO levels, and are constitutively expressed and calcium dependent (Knowles *et al*, 1989). The third isoform, inducible NOS (iNOS), is expressed in many different cell types, for example, macrophages, astrocytes, and microglia, and is calcium independent (Fleming *et al*, 1991; Cho *et al*, 1992). Once upregulated, iNOS produces large amounts of NO, which can damage or even destroy cells, for example, microorganisms or the brain tissue.

Nitric oxide toxicity usually occurs through a direct interaction with various protein moieties. This may result in the reversible formation of mixed

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disulfides between cysteines and glutathione, termed S-glutathiolation (Cohen and Adachi, 2006), the formation of nitrosothiols (Stamler, 1994), or protein nitrosylation (Xu *et al*, 1998; Matsushita *et al*, 2003). Toxicity may also result from formation of peroxynitrite anion (ONOO<sup>-</sup>), a strong oxidant and mediator of tissue injury after its combination with superoxide anion (Dawson and Dawson, 1996). Nitric oxide also can trigger apoptosis directly or via ONOO<sup>-</sup> (Leist *et al*, 1997*a, b*).

Apart from these direct actions, NO can also promote intercellular signaling via activation of soluble guanylate cyclase and subsequent formation of cyclic guanosine monophosphate (cGMP). The best documented signaling activity using this mechanism is the migration of NO generated by the endothelium into neighboring smooth muscle cells whereupon NO relaxes vascular smooth cell through Ca<sup>2+</sup>-mediated formation of cGMP and phosphorylation of downstream kinases that ultimately impact calcium availability (Murad *et al*, 1987).

Endothelial NOS-derived NO has furthermore been demonstrated to have a key role in vascular remodeling and angiogenesis (Papapetropoulos *et al*, 1997; Rudic *et al*, 1998; Murohara *et al*, 1998).

In the brain, NO is mainly formed by nNOS and eNOS. Accordingly, it serves as a neurotransmitter and neuromodulator (Garthwaite *et al*, 1988; Dawson and Snyder, 1994; Dawson, 1995) and—among many other functions—is concerned with the maintenance of basal cerebral blood flow (CBF) (Tanaka, 1996). Specifically, NO has been implicated in cerebral autoregulation (Dirnagl *et al*, 1994; Richards *et al*, 1997; White *et al*, 2000), chemoregulation of CBF (Iadecola, 1992; Thompson *et al*, 1996; Lavi *et al*, 2003), and neurovascular coupling (Dirnagl *et al*, 1994).

More recent evidence suggests a role of NO in neuronal proliferation and differentiation. Tanaka *et al* (1994) showed that NO inhibits neural proliferation and differentiation in the developing brain; *in vitro* data further supported a role for NO in the differentiation of developing as well as adult neuronal cells (Peunova and Enikolopov, 1995; Viani *et al*, 1997). *In vivo*, NO negatively regulates cell proliferation in the developing (Tegenge and Bicker, 2009) as well as adult (Oh *et al*, 2010) brains (see Gibbs, 2003; Contestabile and Ciani, 2004 for reviews).

Neurogenesis, which in the adult brain seems restricted to the subventricular zone, the olfactory bulb, and the subgranular zone of the hippocampus (Zhao *et al*, 2008), persists in the adult brain and can be induced after cerebral insults (Zhao *et al*, 2008; Imayoshi *et al*, 2009). Nitric oxide exerts a dual effect on neurogenesis: While nNOS-derived NO decreases neurogenesis (Packer *et al*, 2003; Moreno-Lopez *et al*, 2004; Zhu *et al*, 2006), NO produced by endothelial (Reif *et al*, 2004) or inducible (Luo *et al*, 2007; Bechade *et al*, 2011) NOS seems to stimulate it.

## Nitric Oxide—Role in Cerebral Ischemic Preconditioning

It was discovered in the early 1990s (Kitagawa *et al*, 1990) that exposing the brain to sublethal injury increases its tolerance for subsequent insults (Gidday, 2006). Among the stimuli investigated for induction of tolerance are sublethal ischemia and proinflammatory mediators (Stagliano *et al*, 1999; Dawson and Dawson, 2000; Dirnagl *et al*, 2003; Kunz *et al*, 2007). Nitric oxide seems to have a major role in mediating ischemic tolerance induced by preconditioning. While there is mounting evidence that iNOS-derived NO is a key effector of ischemic preconditioning (Park *et al*, 2003; Cho *et al*, 2005), the contribution of constitutive NOS is not fully elucidated yet (Gidday *et al*, 1999; Gonzalez-Zulueta *et al*, 2000; Atochin *et al*, 2003).

## Nitric Oxide—Pathophysiology in Stroke

Cerebral ischemia induces multiple and distinct changes in cerebral NO content and signaling. Occlusion of the middle cerebral artery (MCAo) results in an increased production of NO by 20-fold for up to 30 minutes (Malinski *et al*, 1993; Kader *et al*, 1993) most likely through increased calcium availability and activation of nNOS (Huang *et al*, 1994). Thereafter, the brain tissue NO is reduced below detectable levels for up to 7 days (Malinski *et al*, 1993; Sugimura *et al*, 1998), indicating a long-lasting NO deficiency in the ischemic brain. If reperfusion occurs, NO concentration may transiently increase by 50% for about 30 minutes (Fassbender *et al*, 2000; Uetsuka *et al*, 2002). Concomitant with changes in NO levels, the activities of eNOS and nNOS increases within the first few minutes after MCAo, but decrease significantly thereafter (Kader *et al*, 1993). In contrast to the constitutive NOS isoforms, iNO becomes upregulated from 12 hours after MCAo for up to 7 days (Niwa *et al*, 2001).

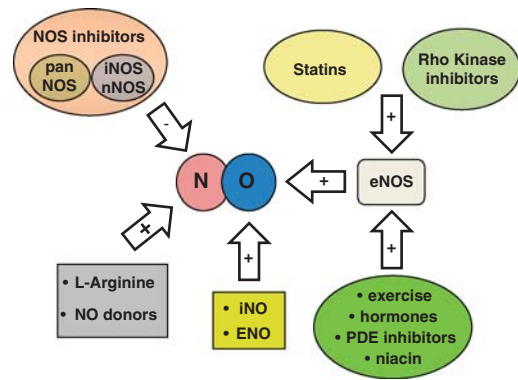
In the chronic phase after cerebral ischemia, angiogenesis has been shown to occur (Beck and Plate, 2009); the extent of collateralization is a predictor for neurological outcome after stroke (Christoforidis *et al*, 2005). Nitric oxide has a crucial role in angiogenesis after ischemic stroke: NO donor DETA-NONOate increased angiogenesis after experimental cerebral ischemia (Zhang *et al*, 2003; Chen *et al*, 2004). Endothelial NOS-deficient mice revealed significantly impaired neovascularization after stroke, indicating that endothelial-derived NO mediates this effect (Cui *et al*, 2009).

Based on the time course of ischemia-induced changes in NO levels and NOS regulation in the brain and cerebral blood vessels, several strategies were suggested to manipulate the NO system for the treatment of stroke.

When the cationic amino-acid substrate L-arginine was administered intravenously after MCAo, CBF

and spike activity were restored within the ischemic penumbra (Dalkara *et al*, 1994), and infarct size reduced (Morikawa *et al*, 1992, 1994). L-arginine's effect was enantiomer specific and not observed in eNOS-deficient mice. By contrast, experiments using NOS inhibitors and selective NOS isoform-deficient mice revealed that NO generated by the neuronal isoform is detrimental to tissue survival and neurological outcome (Huang *et al*, 1994; Hara *et al*, 1996; Zaharchuk *et al*, 1997). This also applies to the generation of NO by the inducible isoform following ischemic challenge (Iadecola *et al*, 1995, 1997; Zhang *et al*, 1996a; Zhao *et al*, 2003). In contrast to the detrimental functions of nNOS and iNOS enzymatic activity, eNOS and eNOS-derived NO is neuroprotective under most conditions of ischemia–reperfusion, although oxygen radical generation and enhanced injury can occur with eNOS uncoupling in the presence of BH<sub>4</sub> deficiency, a mechanism implied in the pathophysiology of vascular diseases (Landmesser *et al*, 2003; Alp and Channon, 2004). Endothelial NOS knockout mice have lower post-ischemic CBF levels and larger infarcts than their wild-type littermates following MCAo (Lo *et al*, 1996; Huang *et al*, 1996). Interestingly, eNOS-deficient mice not only exhibit larger infarcts, they also show smaller penumbral regions (Lo *et al*, 1996), indicating that eNOS may be critical for maintaining and restoring penumbral and collateral blood flow. Increases in infarct size in mice deficient in eNOS phosphorylation (Atochin *et al*, 2007) or deficient in the expression of the  $\alpha$ -1 subunit of the obligate heterodimer, soluble guanylate cyclase, are consistent with these findings (Atochin *et al*, 2010). Furthermore, deficient angiogenesis after stroke might contribute to the increased postischemic damage in eNOS null mice (Cui *et al*, 2009).

Another restorative mechanism after stroke that is NO dependent is neurogenesis (see Zhang *et al*, 2005, 2008 for reviews). While iNOS-derived NO was shown to be neurotoxic in the acute phase after stroke, several studies suggest an important role of the inducible isoform for cell proliferation after experimental ischemia: iNOS-positive cells increased significantly in the periinfarct zone 1 and 3 days after focal ischemia (Sehara *et al*, 2006), the number of iNOS-positive cells increased with increasing survival time (Corsani *et al*, 2008). Treatment with iNOS inhibitor aminoguanidine prevented postischemic neurogenesis (Zhu *et al*, 2003); the phenomenon could not be detected in iNOS null mice, either (Zhu *et al*, 2003). Nitric oxide produced by nNOS exerts negative effects on neurogenesis (Packer *et al*, 2003; Moreno-Lopez *et al*, 2004; Luo *et al*, 2007), nNOS inhibition has been shown to increase neurogenesis after experimental ischemia (Sun *et al*, 2005). Neuronal NOS and iNOS therefore seem to have opposite roles in postischemic neurogenesis. There are hints that there is crosstalk between the neuronal and inducible NOS isoform: genetic deletion of nNOS or



**Figure 1** Summary of therapeutical strategies influencing nitric oxide signaling in ischemic stroke. eNOS, endothelial nitric oxide synthases; iNOS, inducible NOS; nNOS, neuron NOS; PDE, phosphodiesterase.

nNOS downregulation after cerebral ischemia-induced iNOS upregulation and—thus—neurogenesis while the protective effect of pharmacological nNOS inhibition was abolished in iNOS-deficient mice (Luo *et al*, 2007).

Given the ample evidence for NO-mediated neuroprotection after cerebral ischemia, several treatment strategies have been examined that increase postischemic NO content specifically generated by eNOS activity to augment CBF to the penumbra. One putative strategy is to replenish the early post-ischemic NO deficit by application of NO or NO precursors; another is to increase or modulate endogenous NO production/bioavailability by enhancing eNOS activity (Endres *et al*, 2004); for example, via the phosphatidylinositol 3-kinase (PI3-kinase)/protein B kinase (Akt) pathway (Dimmeler *et al*, 1999). Another potential mechanism is to directly or indirectly inhibit NOS, preferably nNOS and/or iNOS. Putative treatment strategies are summarized in Figure 1.

## Therapeutic Approaches

### L-Arginine and Nitric Oxide Donors

After encouraging preclinical data following application of the NO precursor L-arginine (Morikawa *et al*, 1992, 1994; Dalkara *et al*, 1994), other NO donors, for example, nitrite, were reported to be neuroprotective in various models of transient and permanent cerebral ischemia (Willmot *et al*, 2005b; Jung *et al*, 2006). Several clinical trials were initiated. Application of L-arginine, however, failed to significantly improve outcome in stroke in preclinical and clinical trials (see Bath *et al*, 2002 for a meta-analysis). The main reason for the failure of translation was probably the reduction of mean arterial pressure following systemically administered NO/NO precursors. The impact on cerebral perfusion pressure was probably compensated to a greater extent in normally perfused brain tissues as compared with the area

distal to occlusion. It seems likely that CBF in normally perfused brain tissue increased at the expense of blood flow in ischemic tissue, thereby further reducing the already critically low penumbral perfusion (Vorstrup *et al*, 1986; Lassen, 1990). By contrast, the only successful strategy delivering NO without affecting CBF was the transdermal application of glyceryl trinitrate. Glyceryl trinitrate delivered in this way even improved systemic blood flow; however, cerebral infarct size following MCAO was unchanged (Willmot *et al*, 2006). Transdermal application of glyceryl trinitrate is currently evaluated in a clinical trial (ENOS, efficacy of nitric oxide in stroke trial, start date July 2001 (ENOS Trial Investigators, 2006)).

### Nitric Oxide Synthase Inhibitors

The excessive production of NO by brain parenchyma is a characteristic of ischemic stroke. Hence, inhibition of NOS was viewed as an alternative strategy to reducing NO availability in cells expressing the neuronal isoform. In a first approach, nonselective inhibition of NOS by *N* $\omega$ -nitro-L-arginine methyl ester hydrochloride (L-NAME) reduced stroke volume after MCAO (Wei *et al*, 1994; Margaille *et al*, 1997). Further approaches using the same strategy, that is, pan-NOS inhibitors, yielded conflicting results (Willmot *et al*, 2005a) most likely due to their differential activity on the three different NOS isoforms (eNOS: protective, nNOS/iNOS: destructive).

More selective approaches using nNOS and iNOS inhibitors seem to be more promising. Pharmacological inhibition of nNOS with 7-nitroindazole given 5 minutes after MCAO reduced infarct volume in rats by up to 27% (Yoshida *et al*, 1994; Zhang *et al*, 1996b) and the application of aminoguanidine (Zhang *et al*, 1996a; Cash *et al*, 2001), *N*-3-(amino-methyl-benzyl)-acetamide (Parmentier *et al*, 1999; Perez-Asensio *et al*, 2005), or 7-nitroindazole adduct 3-bromo-7-nitroindazole (Srinivasan and Sharma, 2012), all regarded as fairly selective iNOS inhibitors significantly reduced postischemic brain damage after transient and permanent cerebral ischemia. Similarly, noncompetitive NOS inhibitors, like the BH4 analog 4-amino-tetrahydro-L-biopterine, which putatively inhibit only newly formed NOS isoforms *in vivo*, that is, iNOS, reduced brain edema when applied after traumatic brain injury (Terpolilli *et al*, 2009). Another study reduced postischemic brain damage effectively by using antisense oligodeoxynucleotide to iNOS (Parmentier-Batteur *et al*, 2001).

However, NOS inhibitors currently are not in use clinically and are not evaluated in any ongoing clinical stroke trial because most are nonselective and may thus cause deleterious side effects due to their actions on eNOS. The development of highly specific inhibitors of NOS subtypes might make this therapeutic approach feasible (Salerno *et al*, 2002).

### Statins (HMG-CoA-Reductase Inhibitors)

HMG-CoA-reductase inhibitors, so called statins, completely inhibit the conversion of HMG coenzyme A to mevalonate thereby blocking cholesterol and isoprenoid intermediate synthesis (Liao and Laufs, 2005). Isoprenoid intermediates such as farnesylpyrophosphate and geranylgeranylpyrophosphate are responsible for modulation of activity of several small GTPases such as Ras, Rho, and Rac, which are blocked by statins.

Originally developed to treat and prevent coronary heart disease, statins were found to be effective also in primary and secondary prevention of ischemic stroke in several randomized clinical trials (Amaranco *et al*, 2004, 2006; Kennedy *et al*, 2007; Ridker *et al*, 2008). Initially designed to treat hypercholesterolemia, it soon became evident that the beneficial effect of statins in stroke patients was independent of the serum cholesterol lowering effect of the compounds (Treasure *et al*, 1995; Laufs *et al*, 2001). Statins have a number of pleiotropic effects that contribute to vascular integrity and health of the blood vessel wall. Experimental stroke studies revealed that long-term treatment with HMG-CoA-reductase inhibitors induced eNOS upregulation (Endres *et al*, 1998; Laufs *et al*, 2000b, 2002; Amin-Hanjani *et al*, 2001) and blocked hypoxia induced downregulation of eNOS in rodents (Laufs *et al*, 1997), thus increasing NO bioavailability and CBF (Endres *et al*, 1998) and saving brain tissue from ischemic injury (Endres *et al*, 1998; Laufs *et al*, 2000b, 2002; Amin-Hanjani *et al*, 2001). The effects were dose dependent and observed with a number of HMG-CoA-reductase inhibitors. That statins mediate their neuroprotective effect through the upregulation of eNOS and consecutive improvement of penumbral blood flow was further validated by experiments showing that treatment was ineffective in mutant mice deficient in eNOS expression (Endres *et al*, 1998; Laufs *et al*, 2000b, 2002).

In addition to the eNOS-dependent effect of statins *in vivo*, an eNOS-independent neuroprotective effect of statins *in vitro* has been reported. This effect is believed to be mediated by alterations in the cholesterol containing neuronal membrane subdomains thus protecting neuronal cultures against NMDA (*N*-methyl-D-aspartate)-mediated excitotoxicity (Zacco *et al*, 2003; Bosel *et al*, 2005; Ponce *et al*, 2008). However, like all so far mentioned *in vivo* studies, these studies applied statins as a pretreatment underlining the role of statins treatment in the prevention rather than the treatment of stroke.

To evaluate the value of statins for the acute treatment of stroke, several studies applied HMG-CoA-reductase inhibitors after the initiation of cerebral ischemia. Surprisingly, also under these conditions, statins significantly reduced infarct size and brain edema formation (Sironi *et al*, 2003; Kilic *et al*, 2005; Nagaraja *et al*, 2006; Sugiura *et al*, 2007; Prinz *et al*, 2008; Mariucci *et al*, 2011). Interestingly,

neuroprotection could be achieved by statin application as late as 2 days after the onset of MCAo (Sugiura *et al*, 2007). The main neuroprotective mechanisms are thought to be mediated via enhancement of eNOS activity (Mariucci *et al*, 2011), either by phosphorylation of eNOS at the S1177 site via the PI3 k/Akt pathway (Kureishi *et al*, 2000; Dimmeler *et al*, 2001) leading to reduction of inflammation (Sugiura *et al*, 2007) and induction of angiogenesis and neurogenesis (Chen *et al*, 2003) or via mRNA stabilization by inhibition of the Rho Kinase (ROCK) pathway (see below). This was proven by measurements of CBF, which showed that immediate postischemic CBF was not affected by acute statin treatment (Kilic *et al*, 2005; Berger *et al*, 2008), but that 5 days later—consistent with angiogenesis—CBF was improved (Berger *et al*, 2008).

Given their widely proven neuroprotective actions in experimental as well as in clinical trials, statins are recommended for primary as well as for secondary prevention of stroke (Heart Protection Study Collaborative Group, 2002; Adams *et al*, 2007). Despite these clear recommendations, it remains unclear whether HMG-CoA-reductase inhibitors indeed improve cerebral perfusion when given in the acute postischemic phase (Kennedy *et al*, 2007). Furthermore, most recent data suggest that statins exhibit untoward effects such as an increased risk of infection (Becker *et al*, 2011) and a higher incidence of hemorrhagic stroke (Collins *et al*, 2004; Goldstein *et al*, 2008; Vergouwen *et al*, 2008), stressing the need for further clinical evaluation of HMG-CoA-reductase inhibitors in the context of acute ischemic injury.

### Rho Kinase (ROCK)—Inhibitors

There is ample evidence that the neuroprotective effect of statins/statin-induced upregulation of eNOS activity is at least in part mediated by inhibition of rho kinase (Laufs and Liao, 1998; Laufs *et al*, 1999, 2000a). Rho kinase (ROCK) is a serine–threonine kinase whose activity is modulated by isoprenoid intermediate geranylgeranylpyrophosphate (GGPP). Rho kinase is implicated in the pathophysiology of atherosclerosis (Miyata *et al*, 2000), myocardial infarction, and hypertension (Uehata *et al*, 1997). Activation of Rho kinase in endothelial cells under hypoxic conditions (Wolfrum *et al*, 2004) has been related to the mechanism responsible for the down-regulation of eNOS during and after ischemia (Takemoto *et al*, 2002; Ming *et al*, 2002; Wolfrum *et al*, 2004; Jin *et al*, 2006).

Rho kinase inhibitors such as fasudil or hydroxyfasudil rapidly lead to increased eNOS activity by Akt kinase-dependent phosphorylation of eNOS at S1179 (Fulton *et al*, 1999). This results in a significant reduction of ischemic brain damage when given before (Rikitake *et al*, 2005; Shin *et al*,

2007) but also when given after (Shin *et al*, 2007) experimental cerebral ischemia. The observed neuroprotective mechanism was proven to be eNOS dependent, based on the observation that ROCK inhibitors failed to improve CBF or reduce infarct volumes in eNOS null mice (Rikitake *et al*, 2005; Shin *et al*, 2007).

Apart from their well-documented neuroprotective effect in experimental stroke models, Rho kinase inhibitors lower systemic blood pressure potently, which may limit their use for the treatment of acute stroke (Uehata *et al*, 1997; Shimokawa, 2002; Takahara *et al*, 2003). Interestingly, however, the ROCK inhibitor hydroxyfasudil, which is neuroprotective, caused hypotension in wild type as well as in eNOS null mice (Shin *et al*, 2007), suggesting that the blood pressure lowering effect may be mediated by actions independent of eNOS activity. Therefore, the development of ROCK inhibitors with selectivity toward the cerebral circulation or toward ROCK2 (ROCK $\beta$ ), the isoform most mainly expressed in the brain and heart (Nakagawa *et al*, 1996; Wei *et al*, 2001), may increase the potential of these compounds in acute stroke therapy.

### Phosphodiesterase Inhibitors

Emerging evidence suggests that phosphodiesterase (PDE) inhibitors such as dipyridol exhibit neuroprotective effects beyond their well-known antiplatelet properties. It was shown that dipyridol augmented the neuroprotective effects of statins in an eNOS-dependent way (Kim *et al*, 2008). The putative mechanism involves an increase in eNOS S1177 phosphorylation, as demonstrated in spontaneously hypertensive rats (Oyama *et al*, 2011) based on the reduction in postischemic brain damage following pretreatment with PDE inhibitor cilostazol. While these are the first encouraging data suggesting that PDE inhibitors can also increase eNOS activity in stroke patients (Serebruany *et al*, 2011), clinical use of PDE inhibitors is so far restricted to secondary prevention of ischemic stroke: Two large clinical trials (ESPRIT: European/Australasian Stroke Prevention in Reversible Ischaemia Trial (Halkes *et al*, 2007), ESPS-2: European Stroke Prevention Study 2 (Diener *et al*, 1996)) proved that dipyridole in combination with acetylsalicylic acid (ASA) is more effective in preventing secondary ischemic insults than ASA alone. Use of PDE inhibitors in the early postischemic phase is currently not recommended due to a possibly increased bleeding risk by PDE-induced platelet inhibition. However, a recently completed clinical trial with cilostazole (CAIST: Cilostazol in acute ischemic stroke treatment (Lee *et al*, 2011)) showed that cilostazol is comparable to aspirin in terms of safety and bleeding complications so further clinical evaluation of cilostazol mono- or combination-therapy in acute stroke is ongoing (Nakamura *et al*, 2011).

### Other Neuroprotective Strategies Influencing the Endothelial Nitric Oxide Synthases Pathway

**PI3 K/Akt pathway—S1177 phosphorylation—drugs:** Niacin (vitamin D3) was shown to increase the levels of high-density lipoprotein (HDL), thus increasing levels of HDL-cholesterol (HDL-C) and lowering levels of serum triglycerides (Guyton, 1998; Elam *et al*, 2000). High-density lipoprotein promotes reendothelialization and stimulates migration of endothelial cells (Seetharam *et al*, 2006) by increasing NO concentration by activation of eNOS activity via the PI3/Akt pathway (Mineo *et al*, 2003; Drew *et al*, 2004).

After experimental ischemic stroke niacin (vitamin D3) and niacin derivative niaspan significantly improved functional outcome independently of HDL-C levels due to improved angiogenesis (Chen *et al*, 2007; Shehadah *et al*, 2010); again, this is thought to be conferred mainly by eNOS via the PI3 K/Akt pathway.

Niacin and niacin derivative niaspan are potent drugs to increase levels of HDL-cholesterin and thus help to restore vascular function and reduce occurrence of subsequent cardiovascular events without obvious side effect (Guyton *et al*, 2000; Grundy *et al*, 2002). However, a recently completed study (AIM-HIGH (Boden *et al*, 2011)) could not detect significant benefit of niacin treatment in addition to statins compared with statins alone in primary prevention of cardiovascular events; ischemic stroke was recorded as a secondary parameter.

Currently, a randomized double-blinded phase II study is investigating whether niaspan can improve recovery after ischemic stroke (Randomized, Controlled Trial of Extended-Release Niacin (Niaspan, Abbott Laboratories, Abbott Park, IL, USA) to Augment Subacute Ischemic Stroke Recovery, start date: April 2009, NCT00796887).

Another group of compounds known to increase levels of HDL-cholesterol are liver X receptor agonists (Brunham *et al*, 2006). TO901317, a synthetic liver X receptor agonist, was shown to improve neurological function after MCA occlusion in healthy but not in eNOS-deficient mice (Chen *et al*, 2009). The suggested neuroprotective mechanism is promotion of angiogenesis via increased eNOS phosphorylation, but also antiinflammatory effects are discussed (Morales *et al*, 2008). Further evaluation of the mechanisms involved are necessary before clinical evaluation.

**PI3 K/Akt pathway—S1177 phosphorylation—hormones:** Steroids hormones when used in pharmacological doses increase CBF and improve outcome after experimental ischemic stroke (de Court *et al*, 1994; Bertorelli *et al*, 1998). This effect is due to a rapid onset of transcription-independent eNOS activity via the PI3/Akt pathway (Limbourg *et al*, 2002; Hafezi-Moghadam *et al*, 2002). In an animal model of transient cerebral ischemia, dexamethasone increased CBF and reduced infarct volume for up

to 3 days after MCAO in wild type but not in eNOS knockout mice (Limbourg *et al*, 2002). Despite these promising data, clinical trials yielded mixed results (Patten *et al*, 1972; Bauer and Tellez, 1973); corticosteroids are therefore not recommended for the treatment of acute stroke (Adams *et al*, 2007).

Other hormones are proposed to exert their neuroprotective effect by an increase in eNOS activity: Thyroid hormone-mediated neuroprotection is mediated by the PI3/Akt pathway and eNOS phosphorylation after acute stroke (Hiroi *et al*, 2006). Furthermore, estrogen confers increased eNOS activity via the same mechanism (Haynes *et al*, 2000; Simoncini *et al*, 2000). While these results might add to our understanding of gender related differences in stroke incidence and outcome, it remains unclear whether hormone replacement or treatment might be a tool to acutely increase CBF after ischemic stroke. Adiponectin, an adipokine generated by visceral fat cells was shown to increase eNOS phosphorylation. Following MCAO, infarct size was larger in adiponectin-deficient mice, an effect reversed by increasing adiponectin expression in its knockout mouse (Nishimura *et al*, 2008). Low plasma levels of adiponectin have been implicated in obesity, hypertension, and diabetes.

Although there is ample evidence that eNOS phosphorylation is an important mechanism of action for the hormones mentioned, one has to keep in mind that given the multifaceted and sometimes contrasting effects of hormones eNOS modulation might not be the sole mechanism of action.

### Other Therapeutic Strategies Conferring Neuroprotection by Endothelial Nitric Oxide Synthases Activation

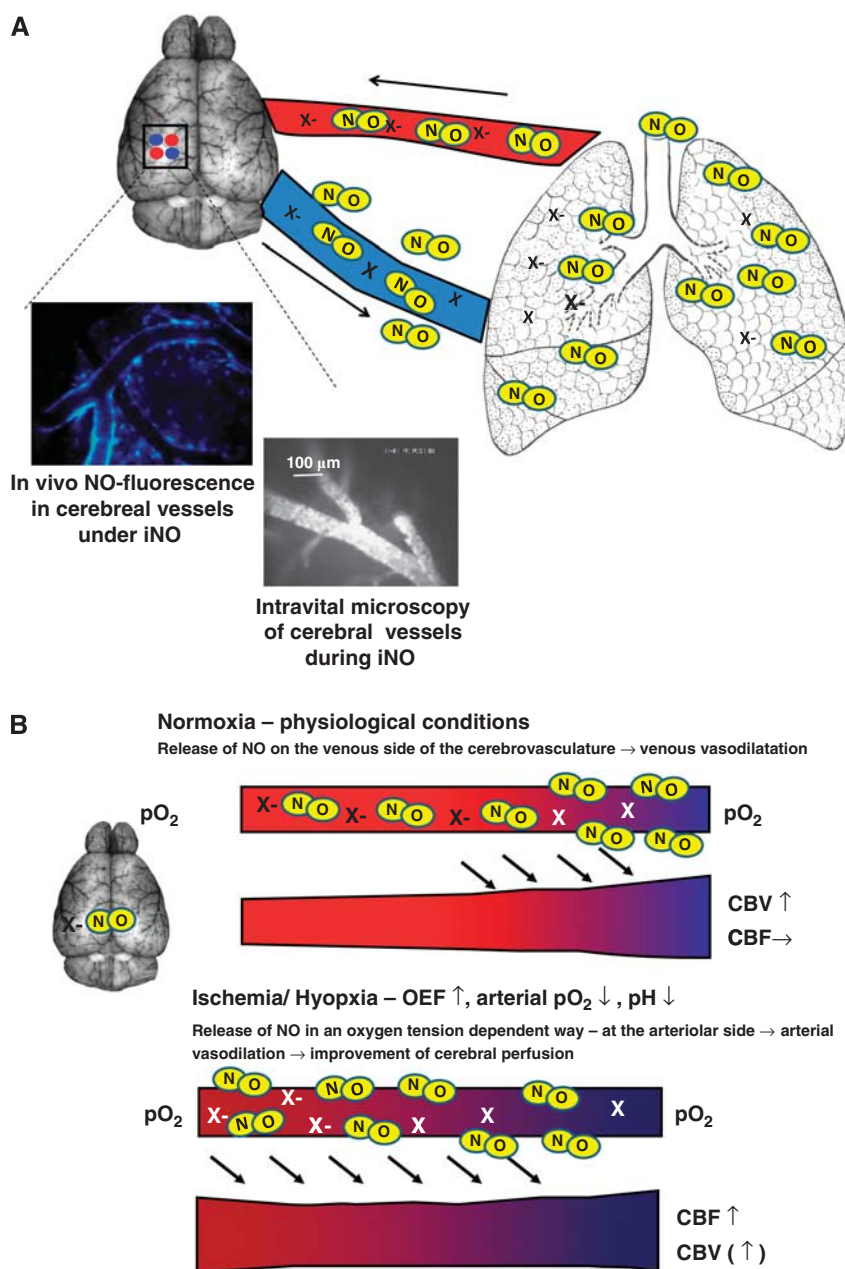
There are a variety of other pharmacological and nonpharmacological strategies that seem to exert neuroprotection mainly via modulation of eNOS activity.

Experimental studies proved that exercise increases eNOS activity, thus improving infarct volume and neurological outcome (Endres *et al*, 2003; Gertz *et al*, 2006). The mechanism may relate to increased shear stress. In line with these findings, the protective effect of prestroke exercise was absent in eNOS null mice (Gertz *et al*, 2006). However, there is no conclusive evidence that physical activity can be neuroprotective when started after stroke (Johansson, 2003; Gertz *et al*, 2006). Nevertheless, the importance of exercise in primary and secondary prevention of ischemic stroke is undoubted.

Among other drugs thought to confer stroke protection via the eNOS/cGMP pathway are the phytoalexine resveratrol, the neuro- and cardioprotectant found in red wine (Tsai *et al*, 2007), peroxisome proliferator-activated receptor- $\gamma$  agonists (Chu *et al*, 2006), and angiotensin II receptor 1 antagonists (Saavedra *et al*, 2006; Oyama *et al*, 2010).

These approaches, however, need to be examined more closely before clinical evaluation is feasible; as with hormones, it remains to be elucidated whether

eNOS phosphorylation is the only important mechanism or whether there are other important modes of action.



**Figure 2** Nitric oxide (NO) inhalation (iNO). **(A)** Upon inhalation, NO is transported to the brain via the blood in a bioactive form; the nature of this NO carrier (X) is not fully elucidated yet. Among the molecules discussed are S-nitroso-hemoglobin, S-nitrosothiols, and nitrite. In the brain, inhaled NO leads to an increase of NO in the vessel wall (left inlet, *in vivo* NO fluorescence imaged with NO-sensitive dye DAF-FM) and to dilatation of cerebral venules (right inlet, *in vivo* imaging of vessel diameter using FITC-dextran). **(B)** NO inhalation—Putative mode of action: Bioactive NO is released via an oxygen-tension-dependent mechanism; under physiological conditions this happens in the venular compartment of the cerebrovasculature. Release of NO leads to venular dilatation and—thus—to an increase in cerebral blood volume (CBV) without influencing cerebral blood flow (CBF). During cerebral ischemia, oxygen extraction fraction (OEF) increases leading to increased oxygen desaturation, lowering of arteriolar  $pO_2$  and pH on the arteriolar side of the cerebrovasculature. Under these conditions, NO release occurs also on the arteriolar side. This induces arteriolar dilatation and, thereby, increase of CBF. Since the mechanism is restricted to ischemic vessels, normally perfused tissue is not affected. NO inhalation therefore seems to be an ideal tool to counteract regional ischemia by increasing blood flow exclusively to malperfused tissue. DAF-FM, 4-amino-5-(*N*-methylamino)-3',6'-bis(acetyloxy)-2',7'-difluoro-spiro[isobenzofuran-1(3H), 9'-[9H]xanthen]-3-one, diaminofluorescein-FM diacetate; FITC, fluorescein 5-isothiocyanate.

## Inhaled Nitric Oxide Donors

As noted above, many promising strategies that increase the availability of NO to the vessel wall have distinct problems in the context of acute cerebral ischemia. Nevertheless, a rapidly acting, easily accessible treatment is actively being sought that increases the availability of NO to acutely improve CBF—especially within the penumbra.

Nitric oxide inhalation (inhaled nitric oxide, iNO) has been used to treat diseases characterized by pathological pulmonary vasoconstriction, for example, pulmonary hypertension since the early 1990s. Initially, it was hypothesized that NO was rapidly inactivated in pulmonary vessels once in contact with oxyhemoglobin, thus restricting the vasodilatory effect to the lung. This was consistent with the findings that iNO therapy at moderate doses did not alter systemic vascular resistance and—thus—systemic blood pressure in experimental and clinical studies. Based on this notion and the fact that other significant adverse effects could be excluded, iNO was approved for the treatment of persistent pulmonary hypertension in neonates and later used for other pulmonary conditions (Griffiths and Evans, 2005; Kinsella and Abman, 2005). Over the ensuing decade, evidence was presented that strongly suggested that inhaled NO led to distinct extrapulmonary effects, for example, in mesenteric, renal, and cardiac vessels. Recently, there is growing evidence that iNO might be protective in reducing ischemia–reperfusion injury within mesentery (Fox-Robichaud *et al*, 1998), and myocardium (Nagasaka *et al*, 2008), thus reducing tissue damage. While experimental (Rosenberg *et al*, 1995; Lopes Cardozo *et al*, 1996; Kusuda *et al*, 1999) and clinical (Vavilala *et al*, 2001) studies could not detect changes in CBF during iNO therapy, one experimental study revealed that while it did not alter cerebral perfusion, iNO was shown to possess a cerebrovascular effect (Kuebler *et al*, 2003). Nitric oxide inhalation is a clinically approved treatment that has few side effects that might positively influence the outcome following ischemic stroke. Terpolilli *et al* (2011) recently demonstrated an effect of iNO on the cerebrovasculature using *in vivo* microscopy. In their studies under physiological conditions, iNO led to a significant dilatation of cerebral venules, most probably due to an oxygen-tension-dependent mechanism. Under conditions of ischemia, iNO led to arteriolar dilatation and an improvement in cerebral perfusion (see Figure 2). After experimental ischemic stroke, iNO reduced lesion size, improved CBF, cerebral metabolism, and neurological outcome, in part dependent upon improved collateral blood flow to the penumbra. There were no obvious adverse effects of this treatment. Clinical evaluation of these NO inhalation effects is planned in the near future.

The extrapulmonary effects of other inhaled NO donating agents such as nebulized sodium nitroprusside (Fattouch *et al*, 2005), inhaled ethyl nitrite

(Auten *et al*, 2007), and inhaled nitrite (Blood *et al*, 2011) are being investigated in the context of pulmonary pathologies because the observed effects seem at least in part to be mediated by the same pathways as iNO. In the case of inhaled ethyl nitrite, neuroprotection after experimental subarachnoid hemorrhage has recently been demonstrated (Sheng *et al*, 2011), so that this might be another promising inhalative treatment strategy for cerebral ischemia.

## Conclusion

Reduction in NO signaling pathways have been well documented after ischemic stroke, especially within the blood vessel wall. Many strategies have been used to restore these pathways in order to improve CBF and improve histopathological and functional outcome after cerebral ischemia. While some NO-based therapeutical approaches have been implemented into clinical guidelines for stroke prevention, most strategies for acute stroke treatment are still in the experimental stage and more research is needed to explore novel, side effect-free and specific NO-based therapeutic approaches for cerebral ischemia.

## Disclosure/conflict of interest

The authors declare no conflict of interest.

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