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Targeting nitric oxide in the subacute restorative treatment of ischemic stroke

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Abstract

Introduction—Stroke remains the leading cause of adult disability. Thus, it is imperative to develop restorative therapies for ischemic stroke designed specifically to treat the intact brain tissue to stimulate functional benefit. Therapies targeting amplification of brain repair processes with nitric oxide (NO) donors and phosphodiesterase type 5 (PDE5) inhibitors in preclinical studies are emerging and showing improvement of functional recovery after stroke.

Area covered—This review will mainly cover the effect of NO donors, which produce NO, and PDE5 inhibitors, which elevate cyclic guanosine 3′,5′-monophosphate (cGMP), on neural restorative events in ischemic brain and highlight mechanisms underlying their restorative therapeutic activity .

Expert opinion—During stroke recovery, interwoven restorative events occur in ischemic brain, which include angiogenesis, neurogenesis, oligodendrogenesis, astrogliosis, and neurite outgrowth. Emerging preclinical data indicate that restorative therapies targeting multiple parenchymal cells including neural stem cells, cerebral endothelial cells, astrocytes, oligodendrocytes, neurons would be more effective than agents with a single cell target. Preclinical data suggest that elevated cGMP levels induced by NO donors and PDE5 inhibitors act on cerebral endothelial cells, neural stem cells, and oligodendrocyte progenitor cells to enhance stroke-induced angiogenesis, neurogenesis and oligodendrogenesis, respectively. These interacting remodeling events collectively improve neurological function after stroke.

Keywords

Nitric oxide; cGMP; PDE5 inhibitor; stroke; angiogenesis; neurogenesis

1. Introduction

Ischemic stroke triggered by blockage of blood flow within an artery in the brain by a clot constitutes more than 80% of stroke that is third leading cause of death and remains the leading cause of adult disability [1–6]. There have been substantial advances in understanding ischemic neuronal injury. However, although neuroprotection has been validated in experimental stroke, clinical trials show that none of neuroprotective drugs achieve clinical benefit for treatment of acute stroke [1–6]. The only Food and Drug Administration (FDA) approved treatment for acute stroke (within 4.5h) is thrombolysis with tissue plasminogen activator (tPA) that is applied to approximately 3% to 8.5% of stroke patients [1, 2, 7]. Thus, it is imperative to develop therapies for ischemic stroke

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designed specifically to reduce neurological deficits, which can be employed to treat the vast majority of patients.

Studies in animal models of stroke and patients with stroke show that brain repair processes take place, which are associated with limited neurological improvement. Brain repair includes cerebral angiogenesis, axonal and dendritic sprouting, oligodendrogenesis and neurogenesis. Emerging data from preclinical experiments indicate that pharmacological and cell-based therapies targeting amplification of brain repair processes after stroke substantially improve functional recovery [8–16].

Nitric oxide (NO) is an activator of soluble guanylate cyclase that causes cyclic guanosine 3′,5′-monophosphate (cGMP) formation in target cells [17, 18]. Stimulation of natriuretic peptide also generates cGMP [17, 18]. Phosphodiesterase type 5 (PDE5) enzyme is highly specific for hydrolysis of cGMP [19, 20]. The effect of NO and NO synthase (NOS) on acute stroke has been extensively investigated (please see review [21]). NO reacts with excessive superoxide generated immediately after stroke and forms peroxynitrite, which leads to cell death [22, 23]. Additional neurotoxicity of NO is mediated by protein S-nitrosylation in which NO reacts with specific cysteine thiols to regulate protein activities [24, 25]. In general, preclinical studies demonstrate that NO generated by the neuronal and inducible NOS (nNOS and iNOS) after stroke is detrimental to neuronal survival, whereas endothelial NOS (eNOS) and endothelial NO are neuroprotective [21]. A clinical trial is currently under way to determine if an NO donor is effective for treatment of stroke (Efficacy of Nitric Oxide in Stroke study)[26]. Readers can find comprehensive information of the effects of NO and reactive oxygen species on acute stroke in recently published excellent review articles [21, 23, 27]. In this review, we will mainly cover the effect of NO donors, which produce NO, and PDE5 inhibitors, which elevate cGMP, on neural restorative events in ischemic brain and highlight mechanisms underlying their restorative therapeutic effects.

2. The effect of NO donors and PDE5 inhibitors on stroke-induced angiogenesis

Stroke induces angiogenesis in the adult human and rodent brains [28, 29]. Angiogenesis, the sprouting of new capillaries from pre-existing vessels, is initiated at peri-infarct areas during the first few weeks and persists for several months after the onset of stroke [30–32]. Experimental studies indicate that the NO/cGMP signaling pathway amplifies angiogenesis in the ischemic brain. Systemic administration of the NO donor, DETANONOate, to rats 24 h after stroke substantially increases angiogenesis in the peri-infarct region [8]. The effect of an NO donor on cerebral angiogenesis likely occurs through the NO/cGMP signaling pathway, because blockage of soluble guanylate cyclase by a pharmacological inhibitor abolishes the NO donor induced angiogenesis [8]. Furthermore, treatment with PDE5 inhibitors, sildenafil and tadalafil, initiated 24h after stroke elevates brain cGMP levels and markedly increases angiogenesis [8, 33]. Elevation of cGMP levels in the ischemic brain by PDE5 inhibitors is likely specific, because administration of tadalafil to ischemic rats does not substantially change brain cAMP levels [33]. Advanced age is a major risk factor and a leading cause of severe disability for stroke patients [1, 2, 7, 34–36]. The PDE5 enzyme is present in the brain and aging decreases the basal brain levels of cGMP [37]. Aging also reduces angiogenesis [38, 39]. However, treatment of stroke with DETANONOate and sildenafil in animals at the age of 18 months considerably augments brain cGMP levels and angiogenesis to a similar level, respectively, observed in young adult ischemic rats treated with these compounds[40]. These data indicate that even in aged animals, angiogenic potential is present in cerebral endothelial cells in response to these treatments. In addition to cerebral endothelial cells, circulating endothelial progenitor cells also contribute to the

formation of new vessels in the ischemic brain [41]. In a mouse model of hindlimb ischemia, vardenafil, another PDE5 inhibitor, promotes angiogenesis through enhancement of mobilization of endothelial progenitor cells [42]. However, whether endothelial progenitor cells contribute to ischemia-induced cerebral angiogenesis by the NO/cGMP pathway remains to be determined.

Newly generated vessels are permeable, which could exacerbate ischemic damage [43]. Thus, it is critical to examine whether angiogenesis observed in the peri-infarct region generates functional vessels. Longitudinal MRI measurements of ischemic brain show that the angiogenic peri-infarct region exhibits a transient increase in vascular permeability 2 to 3 weeks after stroke. After that, vascular leakage subsides, whereas CBF is elevated 6 weeks after stroke [44]. However, treatment of stroke with sildenafil leads to early (3 to 4 weeks after stroke) augmentation of CBF in the angiogenic peri-infarct region and elevated CBF persists for at least 6 weeks after stroke [40, 45, 46]. These data suggest that angiogenic vessels increased by sildenafil have biological function. More importantly, the enhanced angiogenesis is correlated with improvement of neurological outcome during stroke recovery in ischemic animals treated with DETANONOate, sildenafil and tadalafil [8, 33]. Patients with stroke who have higher vascular density exhibit better neurological outcomes [29, 47, 48]. Ablation of stroke-induced angiogenesis with endostatin exacerbates neurological outcome in experimental stroke [49]. Collectively, these data suggest that angiogenesis improves neurological outcome. However, the effect of angiogenesis on improved neurological function could be either direct or indirect, as detailed further below.

3. The effect of NO donors and PDE5 inhibitors on stroke-induced neurogenesis

During brain development, neural stem cells in the ventricular zone (VZ) generate neuroblasts that migrate to the cortex to populate cortical neurons [50]. Several studies have suggested that the NO/cGMP pathway is involved in cortical neurogenesis [51–53]. Treatment of pregnant rats with nitroarginine-methylester (L-NAME), an inhibitor of NOS, reduces cGMP levels by approximately 50% and cortical neurons by approximately 40% in fetal brain [52]. However, administration of sildenafil along with L-NAME completely reverses the effect of L-NAME on cGMP and cortical neurogenesis [52]. In addition, incubation of neural stem cells harvested from the subventricular zone (SVZ) of postnatal mice with an NO donor (NOC-18) for 24h substantially promotes proliferation of these cells in a cGMP-dependent manner [51]. More importantly, incubation of human neural progenitor cells with the NOC-18 considerably increases neuronal cell motility by elevating cGMP levels [53]. Together, these studies suggest that the NO/cGMP pathway acts on neural stem cell proliferation and/or neuroblast migration to mediate the cortical neurogenesis. In the adult rodent, the VZ is replaced by an ependymal layer, while the SVZ of the lateral ventricle shrinks and persists [54]. There are at least two regions of adult rodent brain containing neural stem cells, one is the SVZ of the lateral ventricle and the other is the subgranular zone of the dentate gyrus [50]. Under physiological conditions, neural stem cells in the SVZ generate neuroblasts that migrate to the olfactory bulb where they differentiate into interneurons [50]. After stroke, neuroblasts generated in the SVZ migrate to peri-infarct regions and some of them express phenotypes of mature neurons in young and aged animals [55]. In addition, stroke induced neurogenesis has been detected in patients [56–59]. These data provide a compelling argument for further research to develop therapies by amplifying endogenous neurogenesis in the injured brain [55, 60]. Compounds that target the NO/cGMP pathway in neural progenitor cells have been investigated in non-ischemic and ischemic brains of the adult rodent [8, 61, 62]. Under non-ischemic conditions, inhibition of nNOS by either pharmacological inhibitors including L-NAME and 7-nitroindazole(7NI), or genetic ablation of nNOS promotes proliferation of neural progenitor

cells in the SVZ and the subgranular zone of the dentate gyrus [63, 64]. Immunohistochemistry analysis reveals that SVZ neural progenitor cells do not express nNOS [63]. In vitro, co-culture of SVZ neural progenitor cells with neurons derived from wild-type mice suppresses progenitor cell proliferation, while this effect is abolished when the progenitor cells are co-cultured with neurons harvested from nNOS−/− mice [65]. Together, these data indicate that under physiological condition endogenous NO from nNOS exerts negative control of neural progenitor cell proliferation. The negative effect of nNOS on ischemia-induced neurogenesis has also been reported [66, 67] (Table 1). Adult nNOS−/− mice subjected to stroke exhibit substantial enhancement of neurogenesis compared to wild-type mice [66]. Blockage of nNOS activity by 7NI also further increases stroke-induced neurogenesis [66]. In contrast to nNOS, ablation of eNOS and iNOS genes or inhibition of iNOS by aminoguanidine blocks stroke-induced neurogenesis [67–69] (Table 1). Further studies indicate that augmentation of stroke-induced neurogenesis by blockage of nNOS is mediated by upregulation of iNOS, because knockdown of iNOS suppresses 7NI-enhanced neurogenesis in ischemic brain [67]. Under physiological conditions, nNOS and eNOS primarily contribute to NO production in the brain [21]. However, in ischemic brain, iNOS upregulated by stroke also forms NO [21, 67, 69]. Together, these studies suggest that elevation of NO after stroke regardless of its source could enhance neurogenesis. Indeed, administration of NO donors initiated 24 h after stroke significantly increases neurogenesis in the ischemic brain [8, 61, 62]. Although NO exerts neurotoxicity by formation of peroxynitrite and S-nitrosylation, NO donors given 24h after stroke do not exacerbate ischemic cell damage, suggesting that elevation of cGMP through activation of soluble guanylate cyclase by NO donors could contribute to augmentation of neurogenesis in ischemic brain. Several studies have investigated this possibility. Adult SVZ neural progenitor cells express PDE5 [37]. Inhibition of neural progenitor cell PDE5 by sildenafil considerably augments cGMP levels and increases progenitor cell proliferation and neuronal differentiation [37]. Later treatments (either 1 or 7 days after stroke) of ischemic animals with sildenafil substantially increase newly generated neurons in the peri-infarct regions and these new neurons exhibit phenotypes of mature neuronal markers [9, 37, 70] (Table 1). Stroke-induced neurogenesis enhanced by DETANONOate, sildenafil and tadalafil is observed not only in young adult, but also in aged rats, although the absolute numbers of newly generated neurons are less in aged rats than that in young adult rats [8, 33, 61, 62] (Table 1). In vitro data indicate that sildenafil can directly act on neural stem cells to enhance neurogenesis by increasing stem cell proliferation and differentiation [37]. This is further supported by a recent in vivo study [71]. By tracking progeny of SVZ nestin lineage neural stem cells after stroke, administration of sildenafil to ischemic mice at age of 12 months robustly increases new neurons in peri-infarct striatum and new oligodendrocytes in peri-infarct corpus callosum [71]. Ablation of newly generated neurons in the ischemic exacerbates neurological function [72, 73]. Oligodendrocytes myelinate axons and stroke induces axonal outgrowth in the peri-infarct areas [74–76]. Thus, in addition to angiogenesis, amplification of endogenous neurogenesis and oligodendrogenesis in the ischemic brain by the NO donors and PDE5 inhibitors could contribute to improvement of neurological outcome after stroke.

4. The effect the NO/cGMP pathway on coupling of neurogenesis and angiogenesis in the ischemic brain

Under physiological conditions, neurogenesis in the SVZ of the lateral ventricle and the subgranular zone of the dentate gyrus occurs within an angiogenic niche [77–79]. Angiogenesis and neurogenesis induced by stroke are coupled [28, 31, 32, 43, 49, 80–84]. Cerebral endothelial cells harvested from the ischemic boundary augment generation of neurons from neural progenitor cells derived from the non-ischemic SVZ, whereas ischemic

neural progenitor cells promote naïve cerebral endothelial cells to enhance in vitro angiogenesis [84, 85]. Transplantation of neural progenitor cells into ischemic brain promotes angiogenesis [86]. Migration of neuroblasts generated in the SVZ to the ischemic boundary is closely associated with cerebral vessels [32, 44, 49]. Suppressing angiogenesis substantially attenuates migration of neuroblasts in the SVZ to the ischemic region and exacerbates neurological deficits [49]. Thus, enhancement of angiogenesis and neurogenesis in ischemic brain by the NO/cGMP pathway are likely coupled.

Vascular endothelial growth factor (VEGF) and its receptor, VEGF receptor 2 (VEGFR2), appear to play an important role in mediating coupling of angiogenesis and neurogenesis. DETANONOate and sildenafil upregulate VEGF expression in cerebral endothelial cells through elevation of cGMP and blockage of VEGFR2 abolishes cGMP-enhanced angiogenesis [8]. In addition to angiogenesis, VEGF secreted by cerebral endothelial cells can further amplify neurogenesis [84] (Fig. 1). On the other hand, SVZ neural progenitor cells express an array of angiogenic factors, including angiopoietin 2, VEGFR2 and fibroblast growth factor [87]. VEGF generated by SVZ neural progenitor cells facilities angiogenesis [84]. Accordingly, neurogenesis enhanced by NO donors and PDE5 inhibitors could likely magnify angiogenesis in ischemic brain.

The phosphatidylinositol 3 kinase (PI3K)/Akt pathway affects multiple cellular functions such as cell survival, proliferation, differentiation and migration [88, 89]. NO/cGMP activates the PI3K/Akt signaling pathway in neural progenitor cells and endothelial cells [10, 13, 90] (Fig. 1). Activation of the PI3K/Akt pathway is required for VEGF induced angiogenesis [90]. Elevation of cGMP by sildenafil increases Akt activity in SVZ neural stem cells, whereas blockage of the PI3K/Akt by the PI3K inhibitor, LY 294002, suppresses cGMP-induced Akt activity [37]. Pro-neuronal basic helix-loop-helix (bHLH) transcription factors, such as mammalian achaete-scute homolog 1 (Mash1) and neurogenin 1 (Ngn1) mediate neural stem cell differentiation into neurons [91, 92]. Akt activity regulates the assembly and activity of bHLH–coactivator complexes to promote neural stem cell differentiation into neurons [89]. Inhibition of the PI3K/Akt pathway in neural stem cells suppresses Mash1 and Ngn1 expression [89]. Therefore, NO/cGMP increases Akt activity, which consequently upregulates pro-neuronal transcription factors, leading to amplification of neurogenesis in ischemic brain (Fig. 1). Collectively, these data suggest that the PI3K/Akt signaling pathway plays an important role in coupling of angiogenesis and neurogenesis boosted by NO/cGMP and that neurogenesis and angiogenesis in ischemic brain act in concert to promote improvement of neurological function after treatment of stroke with NO donors and PDE5 inhibitors.

There are two types of cGMP-dependent protein kinases (PKGs), type I (PKG-1) and II (PKG-II)[93, 94]. Sildenafil suppresses cardiac hypertrophy after heart failure and regulates angiogenesis through PKG1 [95, 96] (Fig. 1). However, the role of PKG-1 and PKG-2 in mediating therapeutic effects of NO donors and PDE5 inhibitors remain poorly documented in ischemic brain.

5. Expert opinion and conclusion

Mechanisms underlying neurotoxicity of NO include formation of peroxynitrite, the reaction product from NO and excessive superoxide, and protein S-nitrosylation [22–25]. Based on these mechanisms, inhibition of nNOS and protein S-nitrosylation and antioxidant therapies by inhibition of reactive oxygen species formation have been demonstrated to reduce ischemic neuronal damage in experimental stroke [23, 24, 66, 67]. However, a clinical trial of the free radical trapping agent NXY-059 failed to achieve clinical benefit for treatment of patients with acute stroke [27, 97], whereas the clinical application of agents targeting

inhibition of nNOS and protein S-nitrosylation is unknown. Analysis of preclinical studies and failed clinical trials suggests that neuroprotection alone without restoration of tissue perfusion and vascular integrity may not be effective for the treatment of acute stroke [2, 3, 5, 6]. Without adequate tissue perfusion, neuroprotective agents may not reach or will be present at a suboptimal concentration in the ischemic penumbra where damaged neurons still can be rescued within limited time period. NO increases CBF by dilating cerebral blood vessels and inhibiting platelet aggregation [98]. Preclinical studies in acute stroke show that inhalation of NO improves CBF and reduces ischemic cell damage, suggesting that prompt restoration of tissue perfusion by NO diminishes the neurotoxicity of NO in ischemic brain. A recent review of patients with ischemia/reperfusion injury induced by cardiopulmonary bypass, organ transplant, or myocardial infarction indicates that administration of NO donors is safe and reduces ischemia/reperfusion injury [99]. A clinical trial, Efficacy of Nitric Oxide in Stroke study, is currently under way to determine safety and efficacy of transdermal glyceryl trinitrate, an NO donor, in patients with acute stroke [26].

In contrast to acute stroke, during stroke recovery, interwoven restorative events are present in ischemic brain, which include angiogenesis, neurogenesis, oligodendrogenesis, astrogliosis, and neurite outgrowth. Agents used for restorative therapies given days after the onset of stroke have accesses to the target tissues via CBF. Emerging preclinical data indicate that restorative therapies targeting multiple parenchymal cells including neural stem cells, cerebral endothelial cells, astrocytes, oligodendrocytes, neurons would be more effective than agents with a single pharmacological target. Preclinical data reviewed above suggest that elevated cGMP levels by NO donors and PDE5 inhibitors act on cerebral endothelial cells and neural stem cells to enhance stroke-induced angiogenesis and neurogenesis, respectively. These interacting remodeling events collectively improve neurological function after stroke.

A case report shows that in a compassionate use application, sildenafil has evoked remarkable recovery in a locked-in patient [100]. A safety study of sildenafil (25 mg daily for 2 weeks) shortly after ischemic stroke onset has been conducted in 12 patients [101]. The study shows that sildenafil at this dose given days 2 to 9 after symptom onset is safe in patients with mild to moderately severe stroke [101]. However, due to patient recruitment issues, a dose-tiered of sildenafil clinical Phase I safety trial in stroke patients was terminated (www.clinicaltrials.gov). Based on the experimental studies showing promising restorative therapies of NO donors and PDE5 inhibitors after stroke, additional clinical trails are warranted.

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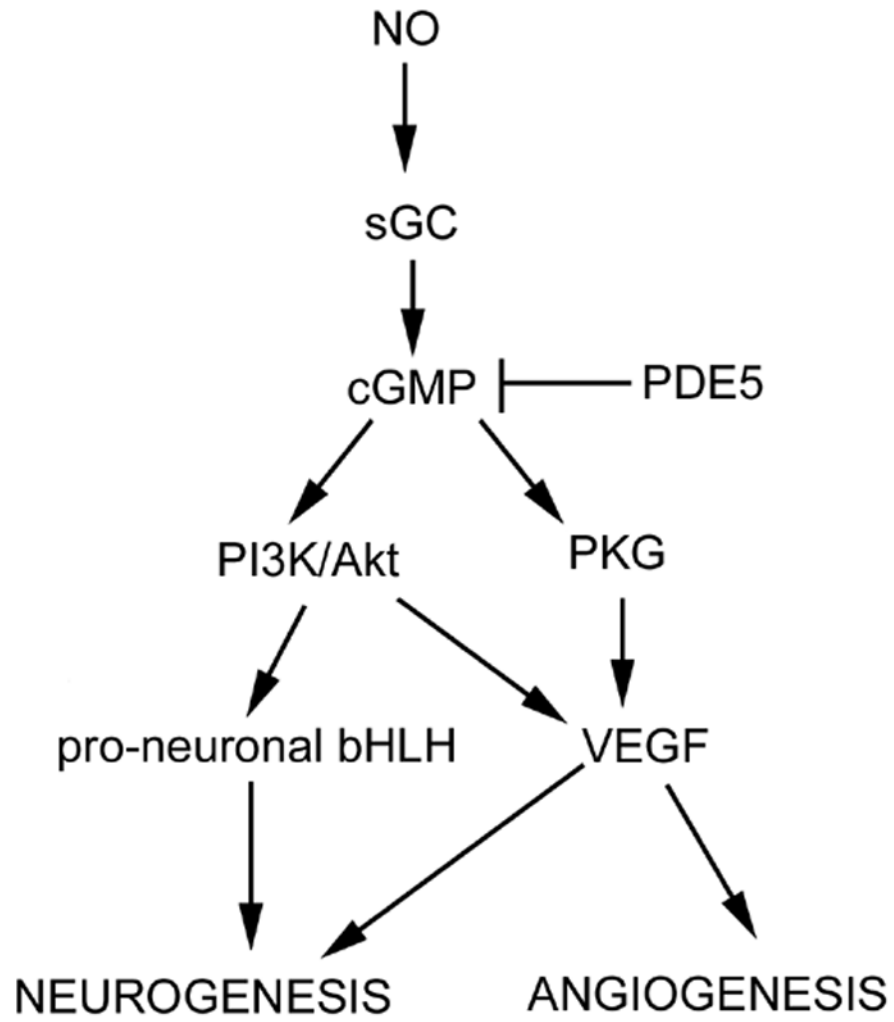

Figure 1.
Schematic representation of signaling pathways that may be involved in NO donors and PDE5 inhibitors-induced angiogenesis and neurogenesis in the ischemic brain. sGC = soluble guanylate cyclase, PKG = cGMP-dependent protein kinases.

Table 1

The effect of NO and PDE5 inhibitors on neurogenesis in ischemic brain.

	Species	Ischemic lesion	neurogenesis	references
NO donors				
DETANONOate	rat	No change*	increase	61, 62
NOS inhibitors				
7NI	mouse	reduction	increase	66, 67
aminoguanidine	mouse	reduction	reduction	67, 69
Transgenic mice				
eNOS−/−	mouse	increase	reduction	68
iNOS−/−	mouse	reduction	reduction	67, 69
nNOS−/−	mouse	reduction	increase	66, 67
PDE5 inhibitors				
sildenafil	rat, mouse	No change*	increase	61, 62, 71
tadalafil	rat	No change*	increase	33

*compounds were given 24h after the onset of stroke. Data of ischemic lesion and neurogenesis presented in the cited studies are statistically significant ($p<0.05$).