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Nerve growth factor alleviates cerebral infarction and neurologic deficits by regulating VEGF, SDF-1 and S100A12 expression through PI3K pathway

Zheng LI[1§]; Zhongle ZHAO[2§]; Peng ZHANG[3]

[1] Department of Neurosurgery, People's Hospital of Deyang City, Deyang, China
[2] Department of Emergency, the Forth People's Hospital of Shaanxi, Xi'an, China
[3] Department of Emergency, First People's Hospital of Nantong, Nantong, China
[§] These authors contributed equally to this work

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Abstract: Stroke remains the leading cause of death and disability worldwide, which destroys the quality of patients' lives and thus is becoming a heavy burden to the society. However, the current therapeutic approaches are far from satisfaction. The objective of this study is to elucidate the impact of nerve growth factor (NGF) on the brain damage induced by cerebral ischemia and its potential molecular mechanism. Middle cerebral artery occlusion (MCAO) rats were used as animal models and neurological functions were evaluated by modified Neurological Severity Score (NSS). Brain cell apoptosis was analyzed by TUNEL-positive staining while brain infarct size was determined according to 2% 2,3,5-triphenyltetrazolium chloride (TCC) staining volume. Rats receiving NGF demonstrated significantly alleviated brain damage, reflected by a substantial improvement in the neurobehavioral outcome, a decrease in brain cell apoptosis and shrinkage of brain infarct volume. Further analysis revealed a markedly elevated circulating vascular endothelial growth factor (VEGF) and stromal cell-derived factor 1 (SDF-1) levels as well as a significant downregulation of SA10012 expression in NGF treated group compared with the untreated group. Strikingly, the protective effect of NGF on cerebral ischemic injury was abolished in rats treated with both NGF and PI3K inhibitors, indicating that phosphoinositide-3-kinase (PI3K) signaling is essential for NGF function. In conclusion, NGF treatment might be a potential therapeutic approach against cerebral infarction by downregulating SA10012 expression and upregulating VEGF, SDF-1 in a PI3K signaling dependent manner.

Introduction

Despite the advances achieved in recent years, cerebral infarction still has high disability and mortality rates, which continues to be a severe threat to the human health (Bustamante *et al*., 2016). More than half of the surviving patents from stroke develop profound complications are frequently associated with brain damage and the subsequent impairment of motion and cognitive functions (Bernhardt *et al*., 2017).

Nerve growth factor (NGF), produced by both neuron and microglia, is a neurotrophin that plays a pivotal role in the differentiation, maturation, and survival of neurons during development (Sofroniew *et al*., 2001). In adulthood, the acute survival of neurons is not dependent on NGF. However, deprivation of NGF still impacts the structural and functional plasticity of neurons in both peripheral and central nervous system (Nguyen *et al.,* 2009; Palmer *et al*., 2000). In response to local tissue injury, inflammation, ischemia, and hypoglycemia, NGF expression is markedly upregulated, which is believed to promote regrowth and repairing of the damaged neurons (Nguyen *et al.,* 2009; Sun *et al*., 2009). Indeed, in a rat sciatic nerve crush injury model, treatment of NGF-β fused with a collagen-binding domain facilitates peripheral nerve repair and enhances functional recovery following nerve damage (Emanueli *et al*., 2002). NGF function is mediated by the activation of the two receptors: high affinity receptor tyrosine kinase receptor (TrkA) and a low affinity p75 neurotrophin receptor ($p75^{NTR}$). The interaction of NGF and TrkA further activates the downstream signaling mediators phosphoinositide-3-kinase (PI3K) and protein kinase B (AKT), which had been shown to exert striking anti-apoptotic and neurotrophic effects in PC12 cells (Nguyen *et al.,* 2009; Karatzas *et al*., 2013). NGF has been considered as a major therapeutic interventions against Alzheimer's disease, spinal cord injury and more recently, ischemic brain injuries (Tuszynski and Blesch, 2004).

NGF induces angiogenesis (Greenberg and Jin, 2005) and prevents endothelial cell apoptosis in a ischemic wound

*Address correspondence to: Zhongle Zhao, zhonglezhao3060@163.com

healing mouse model (Bernhardt *et al*., 2004), indicating that NGF might play an essential role in reparative angiogenesis during cerebral ischemia injury. However, the impact of NGF in the reparative neovascularization of the cerebral ischemic injury remains unexplored. In this study, the neuroprotective role of NGF in ischemic stroke rat model was investigated. We found that NGF treatment limited the cerebral injury by upregulating the expression of the angiogenesis mediators, such as vascular endothelial growth factor (VEGF) and stromal cell-derived factor 1 (SDF-1). Inhibition of PI3K signaling almost completely abolished the protective effect of NGF against ischemic brain injury, which suggested that the NGF might function through PI3K pathway.

Material and Methods

Animals

SPF grade male Wistar rats (Henan Experimental Animal Center, Zhengzhou, China), weighing 250-350 g, were used throughout this study. Rats were housed in specific-pathogen-free (SPF) facilities with a 12:12 dark/light cycle and fed ad libitum. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of People's Hospital of Deyang City, which were performed in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals (National Academy Press).

Animals were randomized to 3 groups: NGF (Staidson Pharmaceuticals Co., Ltd, Beijing, China) treated group, untreated group (saline) and NGF + PI3K inhibitor (LY294002, Shanghai Sheng Long Biotechnology Co. Ltd, Shanghai, China) treated group (N = 18). Within each group, rats were further randomized into 3 subgroups: day 1, day 3, and day 7 post-surgery, with 6 rats in each subgroup. Additional animals were included to exclude unqualified rats to ensure enough animals in each group. To reduce bias, investigators remained blinded to the experimental group for induction and assessment of ischemia. Animals undergoing ischemia were further randomized to each experimental group.

Rat MCAO model and treatment

Cerebral ischemia was established using medial cerebral artery occlusion model (MCAO) following the methodology in a previous publication by Longa with slight modifications (Longa *et al.,* 1989; Belayev *et al*., 1996). Animals were fasted for 12 h prior to surgery. Anesthesia was induced by 10% chloral hydrate (300 mg/kg) through intraperitoneal injection. The left common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were exposed through a midline incision of the neck. ECA and CCA were ligated near bifurcation. A silicone coated 4-0 nylon suture with dimensions 0.35 mm diameter; 2 mm coating length was inserted through the common carotid artery to the internal carotid artery (approximately 18-22 mm from the external-internal carotid artery bifurcation) until mild resistance was met. This indicated the tip was lodged properly in the anterior cerebral artery, and thus, occluded the MCA. The suture thread was then secured and the CCA was released. During surgery, rats were maintained at constant temperature by using a heating pad.

Neurological evaluation was performed on the experimental animals according to Longa's 5-point scale score system (0, normal, no neurological sign; 1, cannot completely stretch contralateral forelimbs; 2, contralateral circling when walking; 3, contralateral fall over when walking; 4, cannot walk, and had a depressed level of consciousness). Animals were excluded from this study if dyspnea, moribund, unstable vital sign or intracerebral hemorrhage were present. Qualified animals received either NGF (10 IU/day), NGF (10 IU/day) + PI3K inhibitor (LY294002, 100 mg/kg) or saline by intraperitoneally injection for 7 days, which was started after 2.5 h of MCAO establishment.

All animals were housed individually following surgery to ensure a consistent post-surgical environment. A post-operative care plan was employed to help prevent weight loss and dehydration after surgery. Weight, basic behavior and general health were monitored daily. Neurological functions of these animals were determined by modified Neurological Severity Score at days 1, 3, and 7 post-surgery. The score was measured on an 18 points scale: 0, no neurological deficit; 1-6 mild ischemic injury; 7-12 moderate ischemic injury; 13-18 severe ischemic injury.

Blood and tissue collection and analysis

At the designated endpoints, blood was first harvested from the rats into heparin coated collection tubes by retro-orbital bleeding. Animals were then deeply anaesthetized with isoflurane and perfused with 90 mL normal saline followed by 270 mL 4% paraformaldehyde. The brain was removed quickly, sliced into 2 mm thick coronal sections and immersed in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C for 20 min. Normal brain tissues were stained red while the unstained areas of the fixed brain slices were defined as infarcted. Images of these brain sections were captured through a computer scanner (600 dpi). The percentage of hemispheric lesion volume (% HLV) was calculated by measuring both lesion area and hemisphere area using image analysis software (Image J; National Institutes of Health, USA) (Berti *et al*., 2002).

One slice of each brain (2 mm), fixed in 10% phosphate buffered formalin, dehydrated in ascending grades of ethyl alcohol, cleared in xylene and embedded in molten paraplast (58-62°C). Five-micron thick histological sections were cut in the hippocampal CA1 area. Apoptotic cells were determined by Roche in situ cell apoptosis assay kit (Sigma, St. Louis, USA) and apoptotic cells were identified as TUNEL staining positive cells via electron microscope (H-7500; Hitachi, Japan), which showed brown particles inside nucleus.

Plasma was isolated by centrifugation at 4000 rpm/min for 15 min. Plasma VEGF, SDF-1, and S100A12 were determined by ELISA kits according to manufacturer's instruction (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China).

Statistics

All data were presented as mean ± SD. Statistical analysis was carried out by SPSS 21.0 software. The difference between two groups was analyzed by student's *t* test. The differences among three groups were compared with one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test. $P < 0.05$ was considered statistically significant.

Results

Neurobehavioral outcomes

Successful induction of the ischemia stroke model by MCAO was confirmed by the behavioral and histological measurement. Extra rats were recruited to ensure minimum 6 animals in each treatment group. Brain damage was evaluated by the neurological score. As listed on Tab. 2, animals in all groups demonstrated similar levels of behavior deficit 1 day after MCAO. Interestingly, according to the neurological function score measured at day 3 day post-surgery, rats receiving NGF treatment displayed significantly improvement in recovery compared to the untreated MCAO rats ($p < 0.05$). Continued improvement was further confirmed by evaluation of behavior score at day 7 post-surgery ($p < 0.05$).

Accumulating evidence suggests that PI3K signaling pathway is required for the recovery after brain damage (Noh *et al.,* 2013; Zhang *et al*., 2014), we hypothesize NGF might function through activation of PI3K/AKT to exert its neuroprotection effects post ischemia stroke. To test our hypothesis, rats were treated with NGF combined with PI3K inhibitor, LY294002. Interestingly, the inhibition of the PI3K pathway almost completely abolished the neuroprotective effects of NGF. As shown in Tab. 1, rats in NGF+PI3K inhibitor treated group demonstrated significantly worse neurobehavioral outcome compared to rats in NGF treated group. This was reflected in both days 3 and 7 post-surgery evaluation ($p < 0.05$). These results suggest that NGF might promote the cerebral repair after stroke in a PI3K dependent manner.

Brain infarct volume

To obtain direct evidence on the effect of NGF and PI3K signaling pathway in the actual brain damage, we determined the brain infarct volume by measuring the white areas on TTC stained sections. The brain infarct sizes were similar among all three groups of rats in day 1 post ischemia injury, indicating that consistent brain damage (Tab. 1). While at 3 days and 7 days post MCAO surgery, NGF treated rats showed significantly reduced infarct lesion size compared to the untreated, surgery only group ($p < 0.05$). In line with what we have found with the behavior neurological score, rats receiving NGF + PI3K inhibitor showed similar infarct volume to the untreated group, which was significantly bigger than those from the NGF only group ($p < 0.05$).

Neuronal apoptosis assessment

The extent of neuron apoptosis following ischemia stroke directly impacts the severity of the injury and the following recovery. To further elucidate the neuroprotective effects of NGF, we analyzed the apoptosis status in brain sections by counting the TUNEL staining positive cells (brown) as shown in Fig. 1 and Tab. 3. As expected, a substantial decrease in TUNEL positive apoptotic cells was observed in NGF treated group compared to untreated group ($p < 0.05$), such protective effect was also dependent on PI3K signaling pathway. Of particular note, NGF showed significant protective effects against apoptosis as early as day 1 post MCAO, while the significant neuroprotective effects of NGF on behavioral and cerebral infarction were observed from day 3 post-MCAO.

TABLE 1

Neurological score evaluation after MCAO (Mean ± SD, N = 6)

Groups	Day 1 Post-surgery	Day 3 Post-surgery	Day 7 Post-surgery
Untreated	11.83 ± 0.75	8.00 ± 0.89	6.00 ± 0.63
NGF	11.33 ± 0.52	5.83 ± 0.75*	4.17 ± 1.17*
NGF + PI3K inhibitor	11.67 ± 0.82	7.83 ± 0.75	6.33 ± 0.52

TABLE 2

Infarct volume after MCAO (mm^3) (Mean ± SD, N = 6)

Group	Day 1 Post-surgery	Day 3 Post-surgery	Day 7 Post-surgery
Untreated	103.41 ± 6.02	138.00 ± 3.89	125.62 ± 4.26
NGF	101.31 ± 4.28	124.55 ± 8.37*	110.17 ± 5.92*
NGF + PI3K inhibitor	102.76 ± 4.96	136.36 ± 4.02	126.27 ± 3.64

*NGF *vs.* untreated; NGF *vs.* NGF + PI3K inhibitor; $p < 0.05$

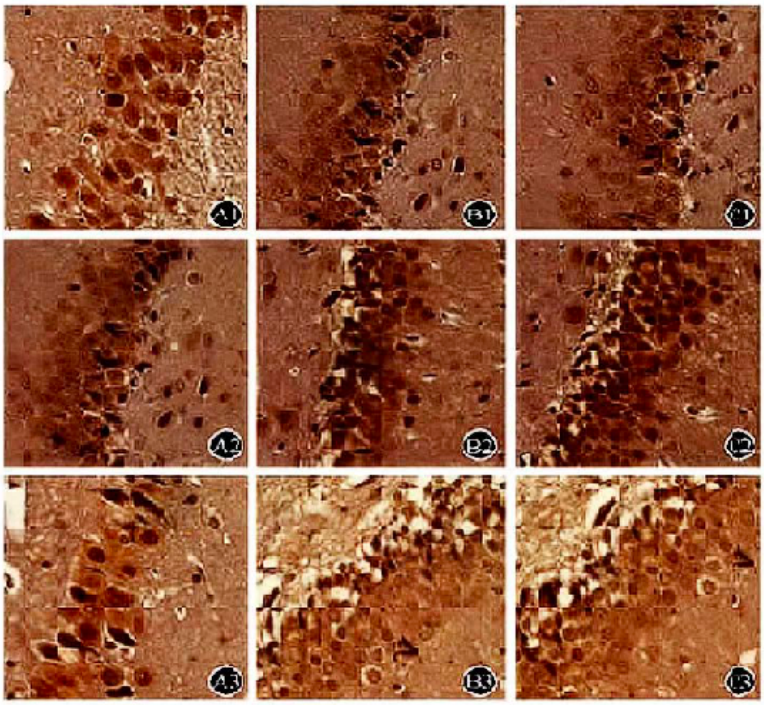

FIGURE 1. NGF treatment prevented brain cells from apoptosis in MCAO rats. TUNEL staining was performed on paraffin-embedded hippocampus sections of rats taken down at different time point (1, 2, 3 refer to days 1, 3, and 7 post-surgery). Apoptotic cells were determined by Roche in situ cell apoptosis assay kit. TUNEL positive apoptotic cells are stained brown. A. Untreated group. B. NGF treatment group. C. NGF + PI3K inhibitor treated group.

TABLE 3

TUNEL positive cell after MCAO (%) (Mean ± SD, N = 6)

Groups	Day 1 Post-surgery	Day 3 Post-surgery	Day 7 Post-surgery
Untreated	10.21 ± 0.36	16.80 ± 0.78	14.42 ± 0.75
NGF	8.56 ± 0.92*	10.46 ± 0.49*	9.15 ± 0.52*
NGF + PI3K inhibitor	10.53 ± 0.47	17.14 ± 0.62	13.81 ± 0.46

*NGF *vs.* untreated; NGF *vs.* NGF + PI3K inhibitor; $p < 0.05$

TABLE 4

Serum VEGF level after MCAO (μg/L) (Mean ± SD, N = 6)

Groups	Day 1 Post-surgery	Day 3 Post-surgery	Day 7 Post-surgery
Untreated	67.08 ± 3.08	74.68 ± 4.06	84.14 ± 6.67
NGF	68.71 ± 5.30	104.07 ± 9.04*	122.26 ± 12.03*
NGF + PI3K inhibitor	68.42 ± 4.94	76.20 ± 3.13	86.29 ± 4.84

TABLE 5

Serum SDF-1 level after MCAO (μg/L) (Mean ± SD, N = 6)

Groups	Day 1 Post-surgery	Day 3 Post-surgery	Day 7 Post-surgery
Untreated	123.21 ± 5.43	137.42 ± 6.48	164.29 ± 7.22
NGF	128.70 ± 3.06	224.97 ± 8.48*	262.48 ± 8.91*
NGF + PI3K inhibitor	126.96 ± 3.52	143.81 ± 5.77	168.78 ± 6.28

TABLE 6

Serum S100A12 level after MCAO (μg/L) (Mean ± SD, N = 6)

Groups	Day 1 Post-surgery	Day 3 Post-surgery	Day 7 Post-surgery
Untreated	6.06 ± 0.23	4.77 ± 0.37	3.99 ± 0.31
NGF	5.85 ± 0.63	3.01 ± 0.35*	1.60 ± 0.29*
NGF + PI3K inhibitor	5.97 ± 0.36	4.63 ± 0.47	3.72 ± 0.26

VEGF and SDF-1 secretion

VEGF and SDF-1 are two mediators for angiogenesis and neurogenesis, which are essential in the recovery of cerebral injury. We determined the plasma VEGF and SDF-1 level by ELISA. As shown in Tabs. 4 and 5, the plasma VEGF and SDF-1 levels were similar in all three groups at day 1 post-MCAO surgery. Interestingly, a marked increase of both plasma VEGF and SDF-1 level (~2 fold) was observed in NGF treated group compared with untreated group and NGF+ PI3K inhibitor treated group at day 3 and day 7 post-surgery measurement ($p < 0.05$). No significant difference was seen between untreated group and NGF + PI3K inhibitor group, further confirmed that the neuroprotective effect of NGF was dependent on PI3K signaling pathway.

Plasma S100A12 level

As an inflammation marker, S100A12 (calgranulin C) is correlated with the prognosis of stroke in that a high level of S100A12 is associated with poor outcome of recovery from stroke patients (Stone *et al.*, 2016). At day 1 post surgery, plasma S100A12 levels were similar among three groups of rats. A continuous decrease in S100A12 levels from day 1 to day 7 post surgery was observed in all 3 groups of animals, as detected by ELISA kit. Starting from 3 days and 7 days after MCAO, a significantly lower S100A12 level was observed in NGF treated group compared with either the untreated group or NGF+PI3K inhibitor treated group (Table 6) ($P < 0.05$). No significant difference was seen between untreated group and NGF + PI3K inhibitor.

Discussion

Neurotrophins are essential to regulating neural survival, development and plasticity in nervous system. NGF, as one of the most important neurotrophins, is pivotal for survival and repair of damaged sensory and sympathetic neurons (Sisman *et al.*, 2014). In animal models, NGF administration effectively promotes peripheral nerve growth and restores the functional activity of damaged peripheral neurons (Lee *et al.*, 2016). However, the molecular mechanism of NGF function is rather complex. In line with the earlier findings, our results demonstrate that administration of NGF substantially reduces the cerebral infarct size and brain cell apoptosis in the MCAO rat model. Such neuroprotective effects are mediated through the PI3K signaling pathway. This study also extends the current understanding on the potential mechanism of NGF by identifying the VEGF, SDF-1, and S100A12 as targeting molecules.

VEGF, an angiogenesis mediator, that functions to stimulate endothelial cell regeneration and neovascularization in all type of vessels, has been reported to substantially reduce the infarct lesion size and enhance cerebral neurogenesis and angiogenesis in MCAO induced ischemia injury model (Sun *et al.*, 2003). Neurogenesis is frequently accompanied by angiogenesis to form the neurovascular unit (Greenberg and Jin, 2005). It was first observed in rat that dividing neurons were frequently observed in the vicinity of blood vessels, pointing out that neurogenesis is intimately associated with active vascular recruitment and subsequent remodeling (Palmer *et al.*, 2000). In this study, we find that the neuroprotective effects of NGF are accompanied by elevation of plasma VEGF, suggesting that NGF might indirectly modulate angiogenesis and functional recovery in cerebral infarction through VEGF. Of note, a more recent study indicates that combined therapy of VEGF and NGF in ischemic injury rabbit model demonstrates a more potent neuroprotective effect and longer treatment window post the onset of ischemia than VEGF and NGF alone treatment (Yang *et al.*, 2018).

SDF-1 has been rediscovered recently for its essential role in the neurogenesis and angiogenesis (Shao *et al.*, 2008). SDF-1 promotes the migration, proliferation, and differentiation of neurons, interneurons and granule cells (Cheng *et al.*, 2017). A significant reduction in brain atrophy and improved neurological outcomes were reported in AAV-SDF-1 treated mouse MCAO models (Li *et al.*, 2014). The protective effects of SDF-1 are attributed to the recruitment of endothelial progenitor cells (EPCs) which are required for angiogenesis and proliferation of the neuron cells in ischemia damaged brain (Cheng *et al.*, 2017). The elevation of SDF-1 level in NGF treated group suggests that the protective effect of NGF might also be involved in the recruitment of EPCs to promote neurogenesis and angiogenesis during post cerebral injury.

S100A12 is abundantly expressed and functions as calcium binding protein in granulocytes (Oesterle and Hofmann Bowman, 2015). S100A12 expression is upregulated in patients with acute inflammatory disease, cardiovascular disease and acute ischemic stroke, and serves as an inflammatory marker (Shiotsu *et al.,* 2013; Abbas *et al.*, 2012). S100A12, through its interaction with RAGE and TLRs, stimulates the secretion of pro-inflammatory cytokines (Foell *et al.*, 2007). Several clinical studies have shown that high plasma S100A12 is positively associated with vulnerability of the atherosclerotic plaque and the poor prognosis of stroke (Foel *et al.*, 2007). Interestingly, among other well-known inflammation-related factors, such as C-reactive protein, fibrinogen and leukocyte counts, only S100A12 is associated with the poor functional outcome of stroke (Wakisaka *et al.*, 2014). In this study, a lower level of S100A12 observed in NGF treated group might predict the beneficial effect of NGF in the functional recovery from the ischemic stroke.

We further point out that the protective effect of NGF is dependent on the PI3K pathway. Rats treated with NGF + PI3K inhibitor demonstrate similar neurobehavioral outcome and brain injury to the untreated animals. PI3K pathways are actively involved in a variety of cellular processes, including cell proliferation, differentiation, motility and survival. In the neurological system, PI3K is essential for the survival and differentiation of neurons and nervous system cells (Noh *et al.,* 2013; Zhao, 2009) mainly through the phosphorylation/activation of the downstream AKT and mTOR signaling (Wahane *et al.*, 2014; Zhang *et al.*, 2014). Ischemia and reperfusion both inhibit the PI3K and increase neuron cell apoptosis (Lv *et al.*, 2017) Our finding that the introduction of PI3K inhibitor completely abolishes the elevation of VEGF level is consistent with an early finding that indicates the protective effect of VEGF against brain injury is dependent on PI3K signaling pathway (Kilic *et al.*, 2006).

In summary, the major findings in this study are that

administration of NGF reduces neuron apoptosis and cerebral infarct size in rat ischemic stroke model. NGF treatment also significantly induces the expression of angiogenesis/neurogenesis mediator, such as VEGF and SDF1, indicating that the neuroprotective effect of NGF in the post-ischemia repair/recovery. Meanwhile, a substantial decrease of specific stroke prognosis maker S100A12 has also been observed in NGF treated group, suggesting NGF might have profound effect in the outcome of the stroke in the rehabilitation phase. Further in-depth study identifies that the neuroprotective effects of NGF is likely mediated by PI3K dependent pathway, in that application of PI3K inhibitor completely abolish the neuroprotective effect of NGF. Thus, NGF treatment might still be a potent therapeutic approach against cerebral ischemic diseases.

Conflict of Interest

None.

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