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Research report

Role of caveolin-1/vascular endothelial growth factor pathway in basic fibroblast growth factor-induced angiogenesis and neurogenesis after treadmill training following focal cerebral ischemia in rats

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ABSTRACT

Exercise is known to aid functional recovery following ischemia, though the mechanisms responsible for the beneficial effects of exercise on recovery from ischemic stroke are not fully understood. Basic fibroblast growth factor (bFGF) contributes to angiogenesis and promotes neurologic functional recovery after stroke. The present study aimed to investigate the possible mechanisms whereby treadmill exercise ameliorated impaired angiogenesis and neurogenesis following transient cerebral ischemia in middle cerebral artery occlusion (MCAO) rats. Treadmill exercise was started 2 days after ischemia-reperfusion in MCAO rats and continued until 7 or 28 days after MCAO, after which the animals were sacrificed. Changes in neurological deficit, infarction volume, neuronal morphology, expression levels of bFGF, caveolin-1, and vascular endothelial growth factor (VEGF), and angiogenesis and neurogenesis in the ischemic penumbra were examined by reverse transcription-polymerase chain reaction, western blots, and/or double immunofluorescence. The results suggested that treadmill exercise promoted the expression of bFGF, improved neurological recovery, and reduced infarct volume compared with non-exercised rats, and also enhanced the expression of caveolin-1, VEGF, VEGF receptor 2(FIK-1)/CD34, and Brdu/nestin staining. Small interfering RNA targeting bFGF blocked the protective effects of bFGF. In addition, 4 weeks of post-stroke recovery still ameliorated ischemia-induced damage without bFGF shRNA. These findings suggest a novel mechanism underlying the beneficial effects of bFGF following stroke, and indicate that treadmill exercise may aid stroke recovery by regulating the caveolin-1/VEGF pathway in the ischemic zone.

1. Introduction

Strokes account for about 55% of neurological diseases and are considered to be the leading cause of permanent physical and mental disability (Posada-Duque et al., 2014). However, therapeutic measures for ischemic stroke remain limited, and the only drug currently approved for stroke, recombinant tissue plasminogen activator, has side effects including increased bleeding risk and limited 'golden time' (Hosseini et al., 2015). In addition, blood reperfusion after thrombolysis may trigger oxidative and inflammatory events, resulting in ischemia-reperfusion injury to the brain (Rhim et al., 2013). Although our understanding of the molecular basis of the cerebral ischemia cascade has improved, the findings have not been successfully translated into clinical neuroprotective strategies, at least partly because of inappropriate target selection.

Shen et al. suggested that growth factors may be an appropriate therapeutic target for ischemic brain disease (Shen et al., 2013). Fibroblast growth factors (FGFs) are involved in many biological processes, including angiogenesis, embryogenesis, differentiation,

Abbreviations: FGF, fibroblast growth factor; bFGF, basic FGF; MCAO, middle cerebral artery occlusion; VEGF, vascular endothelial growth factor; NVU, neurovascular unit; shRNA, small hairpin RNA; RNAi, RNA interference; S, sham-operation rats; M7 and M28, focal cerebral ischemia rats; EM7 and EM28, treadmill training after focal cerebral ischemia; siM7 and siM28, lateral ventricle injection of lentivirus-mediated bFGF shRNA before focal cerebral ischemia; siEM7 and siEM28, lateral ventricle injection of lentivirus-mediated bFGF shRNA and treadmill training after focal cerebral ischemia; NCEM7 and NCEM28, lateral ventricle injection of lentivirus-mediated negative control shRNA and treadmill training after focal cerebral ischemia; ECA, external carotid artery; Brdu, 5-bromo-2′-deoxyuridine.

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and proliferation (Galzie et al., 1997), and have demonstrated survival-promoting and protective effects on brain neurons, and promotion of neural outgrowth, synapse formation, and neurogenesis in the brain (Rai et al., 2007). Basic FGF (bFGF) has been shown to contribute to functional neurologic recovery not only by increasing dendritic length and spine density (Nemati and Kolb, 2011), but also by driving the proliferation of neural stem cells to differentiate into neurons and astrocytes after ischemic brain injury in rats (Jinqiao et al., 2009). Recent research into cerebrovascular disease has focused on interactions among neurons, pericytes, astrocytes, and the extracellular matrix, leading to adoption of the term 'neurovascular unit' (NVU). However, the role of bFGF in the NVU remains unknown.

Vascular endothelial growth factor (VEGF) is considered to have protective effects against stroke. VEGF was shown to participate in neuronal survival, increase microvascular density, and promote glial proliferation and migration after cerebral ischemia (Sanchez et al., 2010). Intimate cross-talk exists between bFGF and VEGF family members during angiogenesis, and vasculogenesis (Ito et al., 2013) (Yu et al., 2016). Previous studies demonstrated elevated expression levels of bFGF and VEGF during cerebral ischemia and reperfusion in rats (Wang et al., 2008), and several experiments have shown that both endothelial and exogenous bFGF modulate VEGF expression in endothelial cells (Seghezzi et al., 1998). Interestingly, recent reports also demonstrated that expression of FGF receptor (FGFR) 1 or FGFR2 on glioma cells decreased tumor vascularization in parallel with VEGF down-regulation (Auguste et al., 2001). Overall, this evidence suggests that bFGF may induce neovascularization indirectly via activation of VEGF.

Caveolins and caveolae are novel pathologically activated factors that assist thrombolysis and neurorestoration in ischemic stroke (Xu et al., 2015). Caveolin-1, a major structural protein of caveolae, is known to be involved in vesicular trafficking, endocytosis, and signal transduction (Lisanti et al., 1994), and is present in different cell types in the central nervous system, especially in the NVU. Treatment of spinal cord injury model rats with bFGF maintained the blood–spinal cord barrier integrity associated with expression of caveolin-1, while introduction of caveolin-1 small hairpin RNA (shRNA) into brain microvascular endothelial cells eliminated the protective effect of bFGF under anaerobic conditions (Ye et al., 2016). We previously demonstrated that treadmill exercise activated the caveolin-1/VEGF signaling pathway to enhance angiogenesis in MCAO rats (Gao et al., 2014). These results suggest that bFGF may be a critical mediator of the caveolin-1/VEGF pathway after MCAO.

Therapeutic exercise is the most common mode of rehabilitation and can significantly reduce recurrence risk and minimize the severity of functional damage after stroke (Middleton et al., 2013). Physical exercise is associated with enhanced neurotropism and growth factor expression, synaptogenesis, neurogenesis and angiogenesis (Ward, 2004; van Praag et al., 2005). Niwa et al. (2016) found that voluntary wheel running enhanced the expression of bFGF, correlated with the proliferation and differentiation of hypothalamic neurons in stroke-prone spontaneously hypertensive rats.

RNA interference (RNAi) has recently become widely used as a gene-silencing tool with high specificity and efficiency , and has also emerged as an efficient approach for treating focal ischemic brain injury in rats (Hu et al., 2011). In the present study, we therefore transfected lentivirus-mediated shRNA against bFGF to elucidate the role of the caveolin-1/VEGF pathway in bFGF-enhanced angiogenesis and neurogenesis, and to determine the correlation between the beneficial effects of treadmill exercise and expression of bFGF.

Table 1
Results of the neurological scores in each group after MCAO (score).

Groups	1 day	7 days	28 days
S	0	0	0
M	2.5 ± 0.58	2.17 ± 0.41	1.67 ± 0.55
EM	2.25 ± 0.50	0.83 ± 0.75[a]	0.50 ± 0.55[a]
siM	2.75 ± 0.50	2.27 ± 0.47[a]	1.67 ± 0.75[a]
siEM	2.75 ± 0.50	1.31 ± 0.48[b]	0.67 ± 0.52[b]
NCEM	2.5 ± 0.58	1.25 ± 0.50	0.75 ± 0.50

Data were presented as mean ± SD, *T*-test, a: $p < 0.05$ as compared to the same period of M, b: $p < 0.05$ as compared to the same period of siM.

2. Results

2.1. Treadmill training attenuated the neurological deficit score and decreased infarct size, while bFGF shRNA increased infarct injury

There was a significant difference among the groups in terms of neurological function assessed by Zea Longa scores after ischemic injury ($p < 0.05$; Table 1 and Fig. 1e). Rats exposed to ischemia followed by exercise for 7 or 28 days after ischemic injury (EM group) had significantly better (lower) scores than rats without exercise (M group) ($p < 0.05$). Scores in rats injected in the lateral ventricle with lentivirus carrying shRNA against bFGF prior to ischemia (siM group) were higher at 7 or 28 days after exercise compared with the equivalent M groups. Staining with 2,3,5-triphenyltetrazolium chloride showed that the EM groups had smaller infarct volumes while the siM groups had larger infarct volumes compared with the M groups ($p < 0.05$; Table 2 and Fig. 1a, b).

Neuronal morphology of the rat brain was observed after MCAO by hematoxylin and eosin (HE) staining (Fig. 1c). No intact neurons were present in brain slices from the control groups, while the infarct site was observed in the ischemic area in the other groups. Brain tissues from rats in the EM groups showed a more regular arrangement than tissues from rats in the M groups. Changes in the siM groups were more severe compared with the M groups, with multiple vacuolated interspaces and dead neurons, correlated with significantly poorer motor function.

2.2. Treadmill training increased protein expression levels of bFGF, caveolin-1, and VEGF in the ischemic penumbra

We examined the effect of treadmill training on protein levels by subjecting MCAO rats to treadmill exercise for 20 m/min, 30 min/day, 5 days/week. Western blot analysis 7 or 28 days after ischemic injury showed that expression levels of bFGF, caveolin, and VEGF were significantly improved in the EM groups compared with the M groups. These results indicated that treadmill training increased the expression of ischemia-induced bFGF, caveolin, and VEGF in the ischemic penumbra ($p < 0.05$; Fig. 5).

2.3. bFGF shRNA blocked the caveolin-1/VEGF pathway in the ischemic penumbra

Although we previously demonstrated that the caveolin-1/VEGF pathway played a critical role in the ischemic zone, the relationship between bFGF and this pathway remains unclear. To investigate the hypothesis that the effect of bFGF is mediated via the caveolin-1/VEGF pathway, we transfected bFGF shRNA into rat brains using a lentivirus vector. After 3 days of virus injection rats were sacrificed. The GFP fluorescence showed the position of lateral ventricle was correct and the percentage of GFP fluorescence following virus injection of bFGF shRNA was about 37%. (Fig. 6) The level of bFGF knockdown was assessed by comparing bFGF mRNA expression levels following transfection with bFGF and neg-