Experimental Neurology 322 (2019) 113059

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Review Article

Activation of endothelial ras-related C3 botulinum toxin substrate 1 (Rac1) improves post-stroke recovery and angiogenesis via activating Pak1 in mice

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ARTICLE INFO

Keywords:
Angiogenesis
Functional recovery
Ischemic stroke
Pak1
Rac1

ABSTRACT

Background and purpose: Long-term disability after stroke is common yet the mechanisms of post-stroke recovery are far from clear. It has been suggested that Ras-related C3 botulinum toxin substrate 1 (Rac1) contributes to functional recovery after ischemic stroke in mice. As Rac1 activation plays diverse roles in multiple cell types after central nervous system (CNS) injury, we herein examined the functional role of endothelial Rac1 in post-stroke recovery and angiogenesis.

Methods: Transient middle cerebral artery occlusion (MCAO) in mice and oxygen-glucose deprivation (OGD) in human brain endothelial cell line-5i (HEBC 5i) were performed to mimic ischemic stroke. Lentivirus vectors encoding Rac1 with GFP and endothelial promotor ENG were injected into the animal's brain after stroke to overexpress Rac1. After injection, stroke recovery was tested by multiple behavioral tests including novel object recognition, adhesive removal and single pellet reaching tests. Endothelial regeneration in the peri-infarct zone was detected by immunohistochemistry (IHC). In the vitro model, the effect of Rac1 and Pak1 inhibitors to cell proliferation and migration was examined by CCK-8 and wound healing assays after OGD. The cellular protein level of brain-derived neurotrophic factor (BDNF), phosphorylated cAMP response element-binding protein (CREB), extracellular signal-regulated kinase (ERK) 1/2 and mitogen-activated protein kinase kinase (MEK) 1/2 were detected by western blots.

Results: Delayed overexpression of endothelial Rac1 after MCAO improved cognitive and sensorimotor recovery from day 14 to 21 after stroke, increased vascular density and the protein level of pericytes in the peri-infarct zone without altering tissue loss in mice. Consistently, inhibition of Rac1 prevented endothelial proliferation and migration after OGD. Pak1 inhibition exerted a similar effect on endothelial cells. However, co-incubation of Rac1 and Pak1 inhibitors with cells did not lead to additive effects when compared with either inhibitor alone. Moreover, individual inhibition of Rac1 or Pak1 suppressed OGD-induced activation of pro-regenerative molecules, including CREB, MEK1/2 and ERK1/2, as well as the production of BDNF in vitro. The level of these proteins did not further decrease if both Rac1 and Pak1 were simultaneously inhibited.

Conclusions: We conclude that activation of endothelial Rac1 improves functional recovery and angiogenesis after stroke, and this process is mediated by Pak1 signaling. This study provides novel insight for Rac1 in the mechanism of long-term stroke recovery.

1. Introduction

Stroke is one of the leading causes of death and disability worldwide. Eighty-seven percent of strokes are characterized as an ischemic stroke (Liu et al., 2014a; Liu et al., 2018; Writing Group M et al., 2016). Clinically, the only pharmacological treatment for ischemic stroke is tissue plasminogen activator (tPA), which is limited by a narrow therapeutic window. However, there is no effective treatment for long-term recovery (Benowitz and Carmichael, 2010). It has been well recognized that angiogenesis is an essential tissue response in ischemia. This process is dependent on endothelial cell (EC) proliferation, migration and capillary tube formation, which could improve recovery and reduce long-term disability after stroke (Sawada et al., 2010; Beck and Plate, 2009).

Ras-related C3 botulinum toxin substrate 1 (Rac1) is a Rho-related small GTPase, which is ubiquitously expressed throughout the brain

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https://doi.org/10.1016/j.expneurol.2019.113059
Received 15 July 2019; Received in revised form 4 September 2019; Accepted 5 September 2019
Available online 06 September 2019

(Stankiewicz and Linseman, 2014). Specifically, the activity of Rac1 in ECs is essential for vessel development and remodeling (Sawada et al., 2010; Tan et al., 2008). Our previous work suggests that Rac1 contributes to stroke recovery, as pharmacological inhibition of cerebral Rac1 exacerbated functional recovery after ischemic stroke in mice (Liu et al., 2018). Similarly, overexpression of cerebral Rac1 improved behavioral recovery. This action of Rac1 may be partially attributed to its neuronal mechanism, as inhibition of Rac1 prevented axonal outgrowth in isolated cortical neurons from mice (Liu et al., 2018). However, whether endothelial Rac1 plays a role in recovery after brain ischemia remains unclear. In hind limb ischemic injury model of mice, endothelial Rac1 haploinsufficiency impaired neovascularization (Sawada et al., 2008), suggesting that endothelial Rac1 may play a role in tissue repair after ischemia. In this study, we investigated whether endothelial Rac1 mediates cerebral ischemia-induced angiogenesis in the brain and benefits post stroke recovery.

Rac1 interacts with specific downstream substrates that coordinate activation of a multitude of signaling cascades, influencing diverse physiological and pathological outcomes (Bustelo et al., 2007). Among the first described Rac1 effector proteins is the family of p21 activating kinases1 (PAK1) (Etienne-Manneville and Hall, 2002). Rac1 binds PAK1 in a GTP-dependent manner. This results in the transportation of signals through several downstream kinases, including the mitogen-activated protein kinase kinase (MEK) 1/2 and extracellular signal-regulated kinase (ERK) 1/2 (Frost et al., 1996; Fujita and Yamashita, 2014; Del Pozo et al., 2000). ERK1/2 can epigenetically modify the production of brain-derived neurotrophic factor (BDNF), via activating cAMP response element-binding protein (CREB) (Ciccarelli and Giustetto, 2014; Moosavi et al., 2016). BDNF can be produced by multiple cell types, including ECs, and is beneficial to stroke recovery (Beck and Plate, 2009). In this study, we explored the function of endothelial-specific Rac1 in post stroke recovery from broad behavior outcomes to its role in angiogenesis, as well as pathological consequences involving PAK1, MEK1/2, ERK1/2, CREB and BDNF.

2. Methods

2.1. Animals

All animal protocols were approved by the Center for Laboratory Animal Medicine and Care at the Medical School of University of Texas Health Science Center in Houston and were performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals. All experiments were carried out in compliance with ARRIVE guidelines. We used young male mice at 7–8 weeks old. A total of 14 C57BL/6 J wild-type (WT) mice were counted in analysis and purchased from Jackson Laboratory.

2.2. Middle cerebral artery occlusion (MCAO)

Transient MCAO procedure has been described in details previously (Pu et al., 2016). Briefly, after a skin incision along the midline of the mouse neck, 60 min MCAO was induced in mice using an intraluminal filament (size 6–0, coating diameter 0.21 ± 0.02 mm, coating length 5–6 mm, Doccol Corporation). After occlusion, mice were reperfused by suture withdrawal and sacrificed 21 days later for mechanistic study.

2.3. Lentivirus and chemical administration in vivo

For overexpression of endothelial Rac1 in mice, lentiviral vectors, carrying Rac1 with GFP and endothelial promotor ENG, and control vectors without Rac1 were customized from Applied Biological Materals (Abm) Inc. The concentrations of the lentivirus were 1×10^9 transducing units/mL, which was titered by Abm prior to delivery. ECs proliferation begins as early as 12–24 h after MCAO and persists for up to several weeks thereafter in mice (Hayashi et al., 2003; Marti et al., 2000). Therefore, following established methods, lentiviral vectors were injected into the brain the following day after stroke (Liu et al., 2018). Briefly, a four-point injection was carried out at the following coordinates: 0.5 mm anterior to the bregma, 2.0 or 3.0 mm lateral to the sagittal suture at the ipsilateral side of MCAO (right), and 1.0 or 2.8 mm from the surface of the skull. A total of 1 μl of lentivirus was injected into each position at a rate of 0.5 μl/min with a 30-gauge needle on a 10 μl syringe (1701 RN, Hamilton). The needle remained in position for 5 min before it was withdrawn. For the proliferation study, bromodeoxyuridine (BrdU) was injected daily into the intraperitoneal space 3 days after stroke (dose: 50 mg/kg/day) for 14 days.

2.4. Behavior measurements

Given the majority of clinical survivors suffer sensorimotor and cognitive disability after stroke (Patel et al., 2002; Smania et al., 2008), evaluation of functional outcome in animal models is a key component in improving the clinical relevance of experimental studies. In this study, the novel object recognition test was used to evaluate cognition, particularly recognition memory (Bevins and Besheer, 2006). The adhesive removal (Bouet et al., 2009) and single pellet reaching tests (Liu et al., 2014b) were used to evaluate sensorimotor disability. Mice were placed in the behavioral room an hour prior to test for habituation, as well as pre-tested to control for the individual differences for each experiment. After MCAO, the adhesive removal and pellet reaching tests were performed at day 7, 14 and 21. The novel object recognition test was performed at day 21 after stroke. All behavioral tests were carried out by an examiner blinded to the treatment groups.

2.5. Novel object recognition test

This test is based on the tendency of mice to interact more with a novel object than with a familiar object, which allows us to investigate memory deficits after stroke (Harris et al., 2016). Briefly, animals were allowed to explore the arena with 2 identical objects for 5 min (trial 1). After a five minutes interval in a feeding cage, mice were placed into the same arena where one of the objects was replaced with a novel object, for another 5 min exploration (trial 2). The time spent at both the novel and the original object was recorded in seconds. It was reflected by a discrimination index (DI), calculated using the formula $(TN_{trial2}/TF_{trial2})/(TF_{trial1}/TF_{trial1})$, in which time spent on the novel object is TN and time spent on the familiar objects is TF. The arena was cleaned between tests to remove olfactory cues.

2.6. Adhesive removal test

Adhesive removal test is a sensitive method to assess sensorimotor deficits in mice (Chen et al., 2001). As previously described (Venna et al., 2014), adhesive-backed tape (25 mm^2) was used as tactile stimuli, placed on the distal–radial region of the left wrist. Elapsed time, in seconds, from tape adhesion to tape removal of 3 trials was averaged.

2.7. Single pellet reaching test

Pellet reaching is a well-organized movement involving complex neural control systems. This test has the capability to measure both the motor and sensory functions of the impaired limb and is increasingly used in long-term survival studies for stroke revoery (Alaverdashvili and Whishaw, 2013). As previously described (Liu et al., 2018), the mice were placed in a Plexiglas box with a vertical slot on the front wall. Animals received pre-training to use their left forepaw to extract 14 mg food pellets (F05684, Bio-Serv) through the slot. When the animal was able to bring food pellet to the mouth, a score of “success” was given. A “failure” was given if the animal dropped the pellet before eating. After 10 attempts by each mice, the success rate of pellet reaching was calculated according to the following equation (number of

successful reaches/total number of reaches × 100).

2.8. Immunohistochemistry on mice tissue

21 days after MCAO, mice were deeply anesthetized with 5% isoflurane and transcardially perfused by sodium phosphate buffer (PBS) followed by 4% Paraformaldehyde (PFA). Brain was post-fixed in 4% PFA overnight and cryoprotected in 30% sucrose solution for 48 h. 30 μm thick coronal sections were cut in cryostat and stored in −20 °C. Fluorescently-labeled immunohistochemistry was performed similarly as previously described (Liu et al., 2018; Jiang et al., 2019). Briefly, the sections were placed into sodium citrate buffer (pH 6.0) and heated in a high-temperature steamer for 10 min. After cooling, slices were incubated in 0.1% Trition × 100 in PBS for 15 min permeabilization, then blocked with 1% bovine serum albumin (BSA) for 15 min at room temperature (RT), to reduce non-specific binding. The slices were then incubated for 1 h with the primary antibodies: goat anti-CD105 (AF1320, R&D) at 1:100, rabbit anti-Rac1 (PA1-091, Thermo Fisher Scientific) at 1:100, rat anti-BrdU antibody (NB500-169, NOVUS Biologicals) at 1:300 and rabbit anti-NG2 (A11012, Thermo Fisher Scientific) at 1:300 at RT. Next, sections were washed and incubated for 30 min with Alexa Flour secondary antibodies: donkey anti-goat 647 (A32849, Thermo Fisher Scientific) at 1:1000, donkey anti-rabbit 594 (A21207, Thermo Fisher Scientific) at 1:1000, donkey anti-rat 594 (A21209, Thermo Fisher Scientific) at 1:1000 at RT. The nuclei were stained using DAPI (H-1500, Vector Lab). After staining, the images were recorded using a Zeiss Axiovert 200 M microscope (Carl Zeiss, Germany) with an X-Cite 120Q fluorescence illumination system (Lumen Dynamics Group Inc., Canada) and Zeiss image acquisition software (LSM 510, Zeiss). Identical digital imaging acquisition parameters were acquired from 3 random fields in the penumbra of the ischemic hemisphere at 40× objective magnification and analyzed using ImageJ (V1.52o). Rac1 and CD105 were semi-quantified as the integrated optical density (IOD), in which results were normalized by dividing each value by the mean IOD of the control groups. Merged staining was quantified as mean number of cells with DAPI positive signal per square millimeter.

Brain atrophy measurement was analyzed as previously described (Liu et al., 2018). Generally, the fixed brains were sliced for cresyl violet staining in accordance with previously described procedures. The brain tissue loss was determined following this calculation: %brain tissue lost = 100% × [(contralateral hemisphere area–contralateral ventricular area) − (ipsilateral hemisphere area–ipsilateral ventricular area)]/(contralateral hemisphere area–contralateral ventricular area).

2.9. Oxygen-glucose deprivation (OGD) model

Human brain endothelial cell line-5i (HEBC 5i) of male donors were purchased from American Type Culture Collection (ATCC) and cultured as described previously (Sun et al., 2019). Briefly, cells were seeded at 50,000/ml and grown in DMEM/F12 (Thermo-Fischer Scientific) supplemented with 10% fetal bovine serum (FBS, Life Technologies) and 40 μg/ml endothelial growth supplement (ECGS, Thermo-Fisher Scientific). All cells were maintained at 37 °C in an incubator perfusing humidified atmosphere containing 5% CO_2. To mimic ischemic conditions that occur during stroke, OGD was performed as an “in vitro” experimental approach to ischemic stroke, as previously described (Sun et al., 2019) with modifications. Briefly, ECs were incubated in glucose-free DMEM (Thermo-Fisher Scientific), and placed in a hypoxic chamber (model MIC-101, Billups-Rothenberg) at a concentration of 5% CO_2-balanced N_2 at 37 °C for 16 h. Cells were then re-oxygenated and received DMEM. Control cells were maintained in DMEM without oxygen deprivation.

2.10. Cell proliferation and migration assay

To assess the roles of Rac1 and Pak1 in the capacity of ECs proliferation and migration after stroke, the cells were co-cultured with selective Rac1 inhibitor NSC2376630 at 30 μM (Liu et al., 2018) or Pak1 inhibitor IPA3 at 10 μM (Deacon et al., 2008) in DMEM/F12 medium starting from the 24th hour after OGD. Culture medium with 0.1% DMSO was used as the vehicle control for IPA3.

Endothelial cell proliferation was assessed by cell counting kit-8 (CCK-8, B34304, Bimake) according to the manufacturer's protocol. After 24 h co-incubation of cell with chemicals, CCK-8 reagent was added to the culture medium with the ratio 1:10 and incubated for an additional 1 h. The absorbance value of each well was determined at 450 nm using an automatic microplate reader (Bio-Rad, Hercules, CA).

Endothelial cell migration was assessed by the wound healing assay as previously described (Kochuparambil et al., 2011). The scratch was created on the monolayer endothelium 24 h after OGD, followed by immediate administration of chemicals. Next, cell plates were moved to time lapse phasecontrast microscopy and maintained at 37 °C with humidified atmosphere containing 5% CO_2 for 48 h. Images were taken at 0 and 48 h using at a 10× objective lens magnification. Cell migration was measured by the percentage of remaining wounding area versus normoxic control at 0 h after scratch.

2.11. Western blot analysis

After CCK-8 assay, cells were washed 3 times by PBS, collected in 1× NP-40 buffer (FNN0021, Thermo Fisher) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), protease inhibitor (04693116001, Roche) and phosphatase inhibitor (5870, CST) and sonicated for 10 s. Total protein was harvested from supernatant after cell lysate was centrifuged at 15,000 rpm for 20 min at 4 °C. Protein concentrations were measured using the bicinchoninic acid (BCA) assay (23,225, Pierce). Equal amounts of protein (20 μg/lane) were loaded onto 4%–15% precast protein gels (Bio-Rad) and transferred to polyvinylidene difluoride (PVDF, Bio-Rad) membranes.

In order to detect the activity and total expression of critical downstream signaling of Rac1, western blots were performed as previously described (Liu et al., 2018; Sun et al., 2019; Bu et al., 2018). Briefly, non-specific binding was blocked with 5% milk in tris-buffered saline with Tween-20 (TBST) for 1 h at RT. The membranes were then incubated with primary antibodies including rabbit anti-BDNF (ab108319, Abcam) at 1:1000, rabbit anti-phospho S133 CREB (9198, CST) at 1:1000, rabbit anti-CREB (ab32515, Abcam) at 1:1000, rabbit anti-phospho T202/Y204 ERK1/2 (4370,CST) at 1:1000, rabbit anti-ERK1/2 (9102,CST) at 1:1000, rabbit anti-phospho S217/221 MEK1/2 (9154,CST) at 1:1000 or rabbit anti-MEK1/2 (8727,CST) at 1:1000 overnight at 4 °C. Once excess primary antibody was washed off by TBST, membranes were incubated with horseradish peroxidase-linked anti-rabbit secondary antibody (7074, CST) at 1:1000 for 1 h at RT. Rabbit anti-β-actin antibody (3700, CST) at 1:1000 was used as a reference (Bu et al., 2018). The IOD results were normalized by dividing each value by the IOD of their reference.

2.12. Statistical analyses

Data from individual experiments was presented as mean ± SEM for analysis with GraphPad Prism 7.01 (GraphPad Software). Statistical significance was determined by Mann-Whitney test in immunohistological assessment, two-way ANOVA with subsequent Bonferroni correction for multiple comparison in behavioral, CCK-8 and wounding healing assessment, and Kruskal–Wallis test with subsequent Dunn's correction for multiple comparison in western blot assessment. Significant differences were detected when p value is < 0.05.

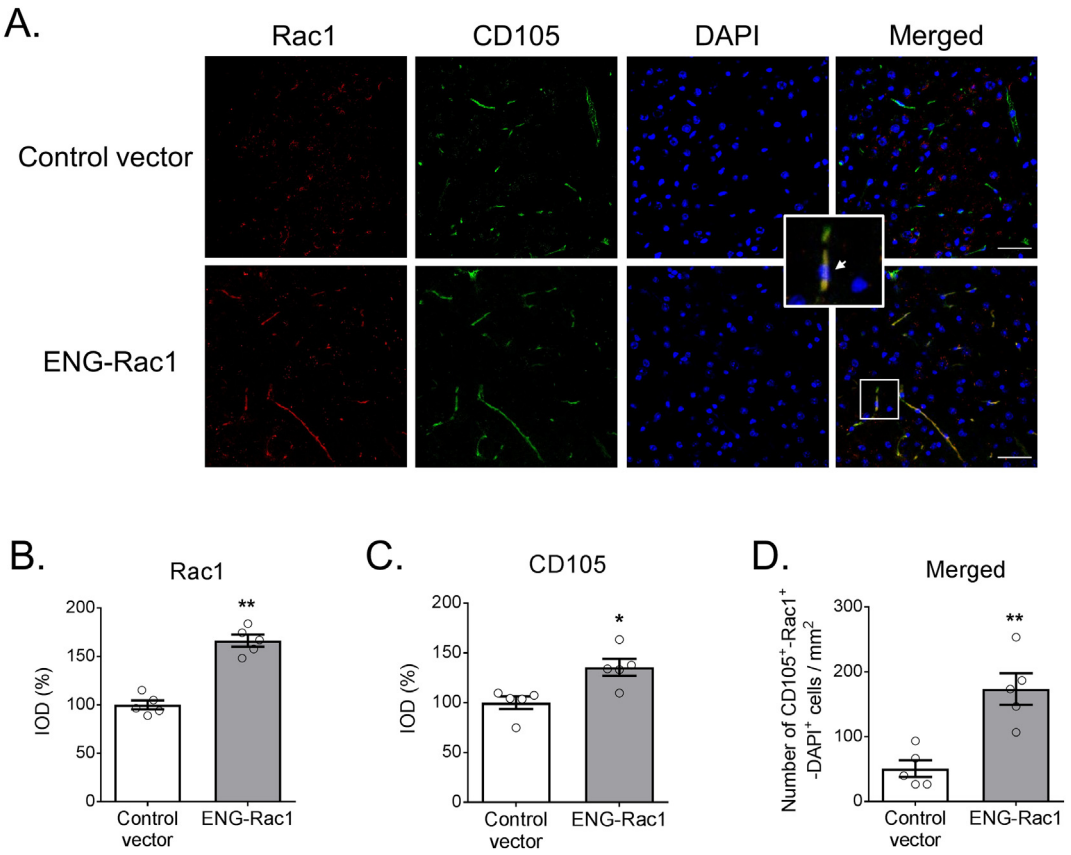

Fig. 1. Delayed overexpression of endothelial Rac1 promoted the formation of endothelial cells in penumbra after ischemic stroke in mice. Lentivirus vectors encoding Rac1 with GFP and endothelial promotor ENG were injected into ipsilateral cortex and striatum of WT mice 1 day after MCAO. Same process was performed using lentivirus without Rac1 sequence as controls. Mice was sacrificed for IHC 20 days after lentivirus injection. (A) Representative images taken from the ipsilateral peri-infarct areas, showing immunofluorescence of Rac1 (red), endothelial cells marker CD105 (green) and nuclear marker DAPI (blue). Rectangle: the region enlarged in high-power images. Arrow showed one endothelial cells expressing Rac1 (yellow). IOD: integrated optical density. Scale bar: 50 μm. (B-D) Relatively quantitative assessment of Rac1, CD105 and their colocalization. Dots represent individual values. N = 5 for each group. Data were presented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Validation of endothelium-specific Rac1 overexpression

To elucidate the specific role of Rac1 in ECs, lentiviral vector encoding Rac1 with GFP and endothelial promotor ENG was injected into the brain of mice 1 day after stroke. We found 20 days after injection, the expression of endothelial Rac1 was significantly enhanced (Fig. 1A and B). The increase was manifested by an increase in the ratio of Rac1 signal intensity in ENG-Rac1 group to that in control group (100 ± 4.62 vs. 166.3 ± 6.23, $^{**}p < 0.01$, u = 0). Similarly, endothelial marker CD105 also was increased in ENG-Rac1 group (100 ± 6.37 vs. 136.6 ± 8.53, $^{*}p < 0.05$, u = 0.5, Fig. 1A and C), compared to that of the control. If counting the co-localization of $Rac1^{+}$, $CD105^{+}$ and $DAPI^{+}$ signals, a significant increase was observed in ENG-Rac1 group compared to control (50.67 ± 12.93 cells/mm^2 vs. 173.3 ± 24.22 cells/mm^2, $^{**}p < 0.01$, u = 0, Fig. 1A and D), confirming endothelium-specific overexpression of Rac1, and also suggesting an improvement of endothelial regeneration by Rac1.

3.2. Delayed overexpression of endothelial Rac1 promoted functional recovery after stroke in vivo

We next investigated whether overexpression of endothelial Rac1 could lead to long-term functional recovery after stroke in mice. To comprehensively evaluate sensorimotor and cognitive functions after stroke, three different behavioral tests were performed before and up to 21 days after stroke (Fig. 2). In the control vector group, MCAO-induced sensorimotor deficiencies (single pellet reaching and adhesive removal tests) were the most prominent at day 7 after MCAO, followed by gradual recovery over 3 weeks (Fig. 2A and B). In the single pellet reaching test, ENG-Rac1 mice performed significantly better than control mice at day 7 (20 ± 3.16% vs. 35 ± 94.28%, $^{*}p < 0.05$, $t = 2.69$) and day 14 (26.67 ± 3.33% vs. 43.33 ± 94.22%, $^{*}p < 0.05$, $t = 3.14$) (Fig. 2A). In the adhesive removal test, ENG-Rac1 mice performed better at day 7 (69.7 ± 9.74 s vs. 32.03 ± 7.43 s, $^{*}p < 0.05$, $t = 3.11$) (Fig. 2B). Both groups recovered to comparable levels in both behavioral tests within 21 days after MCAO. Overexpression of endothelial Rac1 by ENG-Rac1 lentivirus promoted the improvement of memory/cognitive function (0.96 ± 0.1 vs.1.42 ± 0.06) up to 21 days after MCAO compared to control vector group (Fig. 2C, $^{*}p < 0.05$, $t = 2.44$). These data demonstrated that endothelial Rac1 accelerates the recovery of sensorimotor function at earlier time points, as well as improves memory/cognitive function at later time points after stroke. Our previous studies have shown that overexpression of Rac1 using lentivirus did not alter sensorimotor function assessed by single pellet reaching test in the sham operated groups (Liu et al., 2018). Therefore similar experiments in sham mice were not performed in the current study.

3.3. Delayed overexpression of endothelial Rac1 promoted endothelial proliferation without altering tissue loss after stroke in vivo

Previous studies show that angiogenesis after stroke contributes to

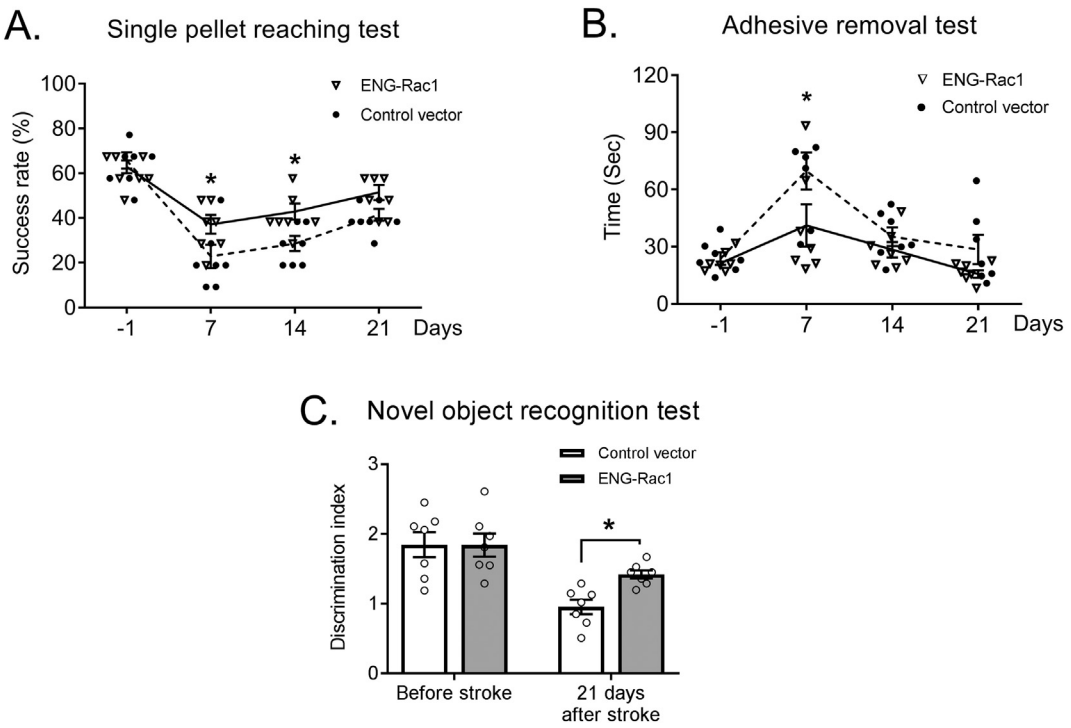

Fig. 2. Delayed overexpression of endothelial Rac1 promoted functional recovery after stroke in mice. Lentivirus vectors encoding Rac1 with GFP and endothelial promotor ENG were injected into ipsilateral cortex and striatum of WT mice 1 day after MCAO. Same process was performed using lentivirus without Rac1 sequence as controls. Mice was sacrificed for behavioral tests 20 days after injection. (A–C) A battery of behavioral tests was performed before and up to 21 days after MCAO to assay sensorimotor function recovery by (A) single pellet reaching and (B) adhesive removal tests, and to assay memory/cognitive function recovery by (C) novel object recognition test. Dots and triangle represent individual values. N = 7 for each group. Data were presented as mean ± SEM. *p < 0.05.

the functional recovery in rodents (Yu et al., 2014; Yang et al., 2010). Herein we investigated whether endothelial Rac1 could involve in angiogenesis besides improving functional recovery after MCAO. We found that delayed overexpression of endothelial Rac1 resulted in a significant increase in BrdU colocalized with endothelium marker CD105, compared to control group (42.67 ± 7.77/mm^2 vs. 104 ± 13.6/mm^2, *p < 0.05, u = 0.5, Fig. 3A). This suggests that Rac1 activation induces EC proliferation after stroke. Because pericytes, the mural cells of blood microvessel, is indispensable for stable vessel structure (Armulik et al., 2011), we next investigated whether pericytes increase concurrently with endothelial proliferation. As expected, NG2 positive cells were increased in the penumbra area in ENG-Rac1 mice after MCAO compared to control vector (122.7 ± 22.86/mm^2 vs. 664 ± 65.24/mm^2, **p < 0.01, u = 0, Fig. 3B). Additionally, the cavity sizes were not altered after EC Rac1 overexpression (Fig. 3C), suggesting that the effect of EC Rac1 overexpression in angiogenesis is not due to any effect in injury size following stroke.

3.4. Delayed inhibition of Rac1 activity reduced endothelial proliferation and migration via Pak1 in vitro

To investigate the mechanisms underlying endothelial Rac1 in mediating revascularization after stroke, we performed pharmacological experiments in ischemic model in vitro. The OGD model was first validated by CCK-8. Similar to the previous study (Sun et al., 2019; Huang and Sheibani, 2008), we found that cell viability (100 ± 4.7 vs. 77.4 ± 1.17, ***p < 0.001, t = 6.299, Fig. 4A) and migration (1.53 ± 0.35 vs. 12.63 ± 0.79, *p < 0.05, t = 2.934, Fig. 4B and C) were significantly stunted compared to the control. When Rac1 inhibitor NSC23766 at 30 μM was incubated with HBEC-5i cells under normoxic condition, cell viability was not altered compared with the vehicle control (Fig. 4A). This suggests that NSC23766 at 30 μM displayed no cytotoxicity under normoxic condition. Differently, inhibition of endothelial Rac1 using NSC23766 after OGD significantly reduced cell numbers 48 h after OGD (77.4 ± 1.17 vs. 64.8 ± 2.75, *p < 0.05, t = 3.51, Fig. 4A), and migration 72 h after OGD (12.63 ± 0.79 vs. 32.85 ± 2.69, ***p < 0.001, t = 4.78, Fig. 4B), compared to the vehicle control. Our data demonstrated that Rac1 plays critical roles in cell proliferation after ischemia.

It is known that one of the major downstream effectors of Rac1 is Pak1 (Kumar et al., 2006; Shutes et al., 2007), which contributes to the vessel cell migration and formation (Kiosses et al., 1999; Orr et al., 2007). To investigate whether Pak1 could also regulate endothelial proliferation and migration, a Pak1 inhibitor was applied on HBEC 5i cells. Similarly to Rac1, inhibition of Pak1 using IPA3 at 10 μM significantly reduced cell numbers (77.13 ± 3.19 vs. 57.16 ± 1.88, **p < 0.01, t = 5.56, Fig. 4A), and migration (12.47 ± 1.74 vs. 32.44 ± 3.46, **p < 0.01, t = 3.93, Fig. 4B) after OGD, compared to 0.1% DMSO vehicle control, whereas no inhibitory effect under normoxic condition. Interestingly, incubation of cells with Rac1 and Pak1 inhibitors resulted in a comparable inhibitory effect, as the Pak1 inhibitor alone in either viability assay (77.13 ± 3.19 in vehicle vs. 60.16 ± 2.11 in NSC23766 + IPA3, **p < 0.01, t = 4.73, Fig. 4A) or migration assay (12.47 ± 1.74 in vehicle control vs. 31.11 ± 1.45 in NSC23766 + IPA3, **p < 0.01, t = 3.67, Fig. 4B). This suggests that Pak1 involves in the Rac1 signaling in mediating revascularization after stroke.

3.5. Endothelial Rac1 inhibition reduced activation of BDNF signaling pathway in vitro

It is known that vascular recovery after ischemic stroke can be modulated through the release of trophic factors, such as BDNF (Liu et al., 2014c; Navaratna et al., 2009; Zhang et al., 2012). Our previous study showed that global inhibition of cerebral Rac1 suppressed MCAO-induced activation of MEK1/2 and ERK1/2[3] after stroke. Astrocytic

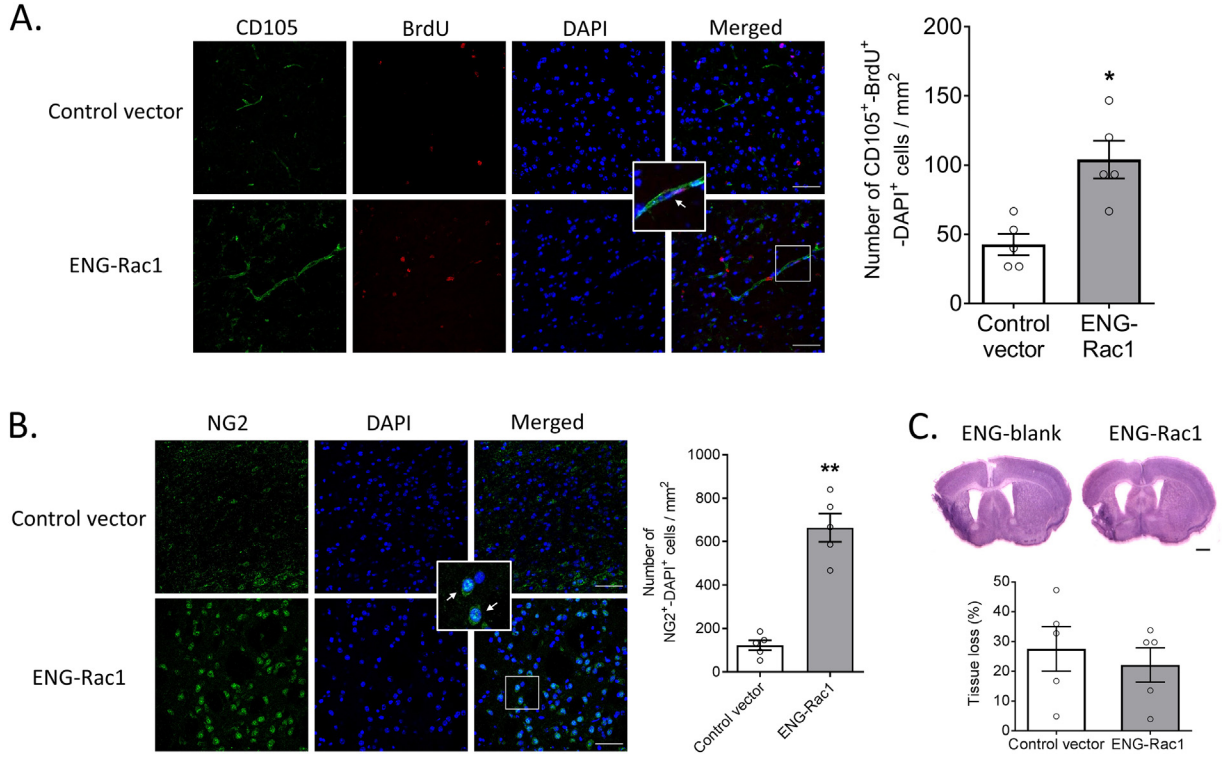

Fig. 3. Delayed overexpression of endothelial Rac1 promoted endothelial proliferation with no effect on tissue loss 21 days after stroke in mice. (A-left) Representative images taken from the ipsilateral peri-infarct areas, showing immunofluorescence of endothelial cells marker CD105 (green), BrdU (red), and nuclear marker DAPI (blue). Rectangle: the region enlarged in high-power images. Arrow showed proliferating endothelial cells. Scale bar: 50 μm. (A-right) Assessment of the colocalization of Rac1, CD105 and DAPI. (B-left) Representative images taken from the ipsilateral peri-infarct areas, showing immunofluorescence of pericytes marker NG2 (green) and nuclear marker DAPI (blue). Arrow showed pericytes. Scale bar: 50 μm. (B-right) Assessment of the colocalization of NG2 and DAPI. (C) Representative images and quantitative assay of brain tissue loss 21 days after MCAO using CV staining. Scale bar: 1 mm. Dots represent individual values. N = 5 for each group. Data were presented as mean ± SEM. *p < 0.05, **p < 0.01. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ERK1/2 can activate CREB, further enhance BDNF production, and induce neurogenesis in the hippocampus after brain ischemia (Ciccarelli and Giustetto, 2014). In this study, we investigated whether the activation of endothelial MEK1/2, ERK1/2 and CREB, and the production of BDNF, could be induced after stroke, and whether these could be mediated by Rac1 and Pak1 signaling. As expected, 48 but not 24 h after OGD, the production of BDNF (0.74 ± 0.03 vs. 0.98 ± 0.06, *p < 0.05), and the activity of CREB (1.05 ± 0.06 vs. 1.34 ± 0.03, *p < 0.05), ERK1/2 (1.05 ± 0.05 vs. 1.37 ± 0.11, *p < 0.05) and MEK1/2 (0.55 ± 0.06 vs. 0.97 ± 0.09, *p < 0.05) were increased compared to the normoxic control (Fig. 5A). In addition, delayed inhibition of either Rac1 or Pak1 significantly suppressed the production of BDNF (0.91 ± 0.04 in H_2O vs. 0.58 ± 0.03 in NSC23766, *p < 0.05; 0.75 ± 0.02 in 0.1% DMSO vs. 0.48 ± 0.03 in IPA3, *p < 0.05), and the activity of CREB (1.38 ± 0.05 in H_2O vs. 1.08 ± 0.04 in NSC23766, *p < 0.05; 1.42 ± 0.06 in 0.1% DMSO vs. 1.07 ± 0.06 in IPA3, *p < 0.05), ERK1/2 (1.54 ± 0.03 in H_2O vs. 1.11 ± 0.05 in NSC23766, *p < 0.05; 1.45 ± 0.03 in 0.1% DMSO vs. 0.96 ± 0.08 in IPA3, *p < 0.05) and MEK1/2 (0.73 ± 0.02 in H_2O vs. 0.4 ± 0.02 in NSC23766, *p < 0.05; 0.61 ± 0.03 in 0.1% DMSO vs. 0.32 ± 0.02 in IPA3, *p < 0.05) compared to the selected control group (Fig. 5B). Neither MCAO nor delayed treatment altered the protein level of total CREB, Erk1/2 or MEK1/2 (quantification not shown, Fig. 5). Furthermore, addition of Rac1 inhibitor to the Pak1 inhibitor after OGD had no further inhibitory effect to the content of BDNF, as well as the activated CREB, ERK1/2 and MEK1/2 compared to either inhibitor alone (Fig. 5B). These data suggest that endothelial Rac1 regulates BDNF production, as well as MEK1/2, ERK1/2 and CREB activity via recruiting Pak1 after stroke.

4. Discussion

In this study, we used multiple approaches to demonstrate the critical role of endothelial Rac1 in stroke recovery, in vivo and in vitro. We first used an in vivo approach to show that activation of endothelial Rac1 improves angiogenesis and functional recovery after stroke. The OGD model was used to further investigate the mechanisms of Rac1 underpinning angiogenesis. In the in vitro study, we first confirmed the involvement of Rac1 in angiogenesis in ECs. We then demonstrated that Pak1 signaling is the key downstream mediator of Rac1 in angiogenesis, as we showed the lack of additive effect when both inhibitors of Rac1 and Pak1 were used. This finding provides novel insight into Rac1/Pak1 signaling in the mechanisms underpinning angiogenesis, which may be a potential target for stroke recovery.

How does delayed activation of endothelial Rac1 improve functional recovery after stroke? It has been described that in ischemic stroke patients, a correlation was found between the number of new vessels in the ischemic penumbral regions with prolonged survival (Zhang et al., 2012), suggesting that activated angiogenesis could be beneficial for the ischemic brain. In the current study, we showed the increase of endothelial proliferation by activation of endothelial Rac1. This was accompanied with the improvement of behavioral recovery after ischemic-stroke insult, supporting the likelihood of endothelial Rac1 improving stroke recovery via remodeling brain vessels. Interestingly, it is reported that constitutive hemizygous deletion of Rac1 (Rac1 +/−) in mouse ECs attenuates brain injury and edema after focal cerebral ischemia (Sawada et al., 2009). This detrimental effect of ECs Rac1 seems to be contradictory with its regenerative effect to ECs in our current study. The discrepancies seen in two studies may be due to the

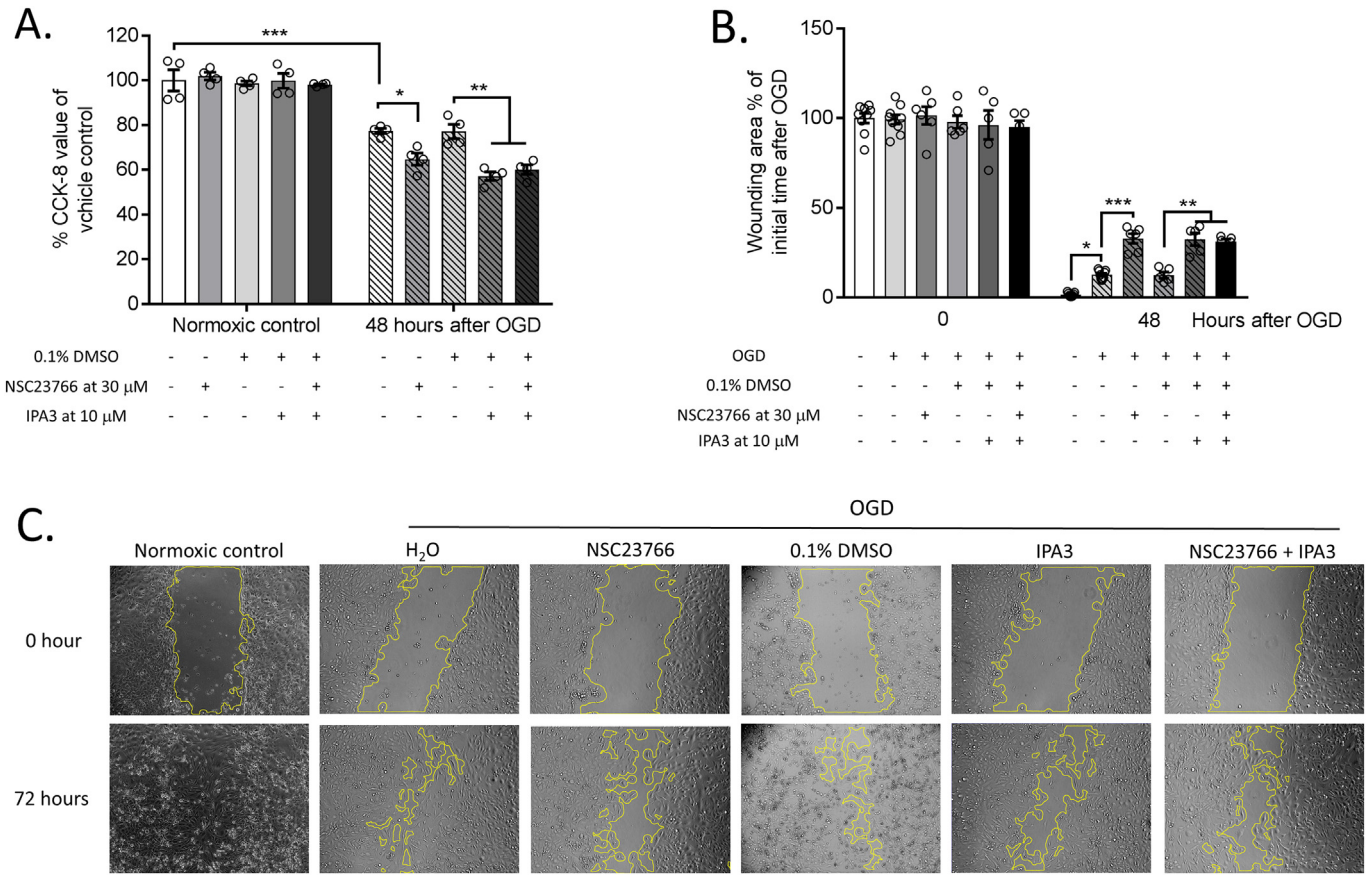

Fig. 4. Delayed inhibition of either Rac1 or Pak1 prevented endothelial proliferation and migration, while inhibiting both kinases concurrently produced no additive effect after OGD. (A) Assessment of endothelial proliferation by CCK-8 48 h after OGD. (B) Assessment of endothelial migration by wounding healing approach 72 h after OGD. (C) Representative images of wounding healing. N = 4–9 for each group. Data were presented as mean ± SEM. *p < 0.05. **p < 0.01. ***p < 0.001.

timing of Rac1 deletion/activation after stroke. In our study, Rac1 vector was injected 1 day after stroke, while Rac1 was inhibited pre-stroke in Sawada's study (Sawada et al., 2009). Rac1 in ECs may predominantly display pro-oxidative and anti-survival effects (Sawada et al., 2009) in the acute ischemia pathological context. While in the post-stroke recovery phase, its dominant effect is pro-regenerative: it activates neurotropic pathways as seen in our study and plays a less important effect in cellular survival. Indeed, we found the tissue loss caused by stroke was not altered by post-stroke ECs Rac1 over-expression, which also indicates that the pro-angiogenetic effect is not related with size of injury. Therefore, it is likely endothelial Rac1 possesses dual and phase dependent effects in stroke.

To further understand the downstream mechanism of EC Rac1 in mediating revascularization, we used pharmacological approaches on pure ECs, to detect the content of promising effectors of Rac1 in OGD model. Pak1 are reported to be primarily expressed in brain, residing in the cytosol and functioning as a direct effector of Rac1 in proliferation and pathological vascular remodeling (Hinoki et al., 2010). In our in vitro stroke model, we found both delayed inhibition of Rac1 and Pak1 prevents the endothelial proliferation and migration. More interestingly, combined use of Pak1 inhibitor with Rac1 inhibitor did not result in any additive effects, suggesting that Rac1 mediates angiogenesis via recruiting Pak1. In addition, inhibition of the Rac1/Pak1 signaling suppressed the OGD-induced activation of MEK1/2, ERK1/2 and CREB, as well as suppressed the increase in protein level of BDNF in ECs. MEK1/2 and ERK1/2 are well-known pro-regenerative molecules (Ciccarelli and Giustetto, 2014; Moosavi et al., 2016), as they stimulate the activity of CREB and further modify the production of pro-regenerative genes, such as BDNF epigenetically (Ciccarelli and Giustetto, 2014, Moosavi et al., 2016). BDNF is produced and secreted by ECs and promotes angiogenesis and functional recovery in multiple stroke models (Beck and Plate, 2009; Clarkson et al., 2015). Furthermore, exogenous application of BDNF can reduce GFAP positive reactive astrocytes, which leads to growth cone collapse (Yiu and He, 2006), in the late phase after spinal cord injury (Jain et al., 2006); and also promotes neurogenesis and functional recovery after stroke (Beck et al., 1994; Bejot et al., 2011). Together with our observation, it is likely that endothelial Rac1 improves BDNF production and secretion. BDNF may further improves stroke recovery through broadly potentiating autocrine (acts on ECs) and paracrine (acts on astrocytes and neurons) activities.

One limitation of this study is that we only studied EC Rac1 in young male mice. Future studies of EC Rac1 in angiogenesis should be conducted using aged subjects and female subjects. For instance, the contribution of endothelial Rac1 to functional recovery may be dependent on age. It is well known that aged animals show significant decreases in capillary angiogenesis, which results in a poor prognosis, compared to young animals following stroke (Sohrabji et al., 2013). Enhancing angiogenesis in aged subjects with Rac1 manipulation may produce an effect after stroke different from what is seen in young animals.

In summary, this study utilized multiple approaches and stroke models to improve our understanding of endothelial Rac1 in functional recovery after stroke. Our experiments in mice shows that endothelial Rac1 improves angiogenesis and post-stroke outcome through Pak1 signaling pathways including the activation of ERK1/2, MEK1/2 and CREB, and the production of BDNF. Targeting endothelial Rac1 may offer a potential therapeutic target for promoting brain plasticity and functional recovery after stroke.

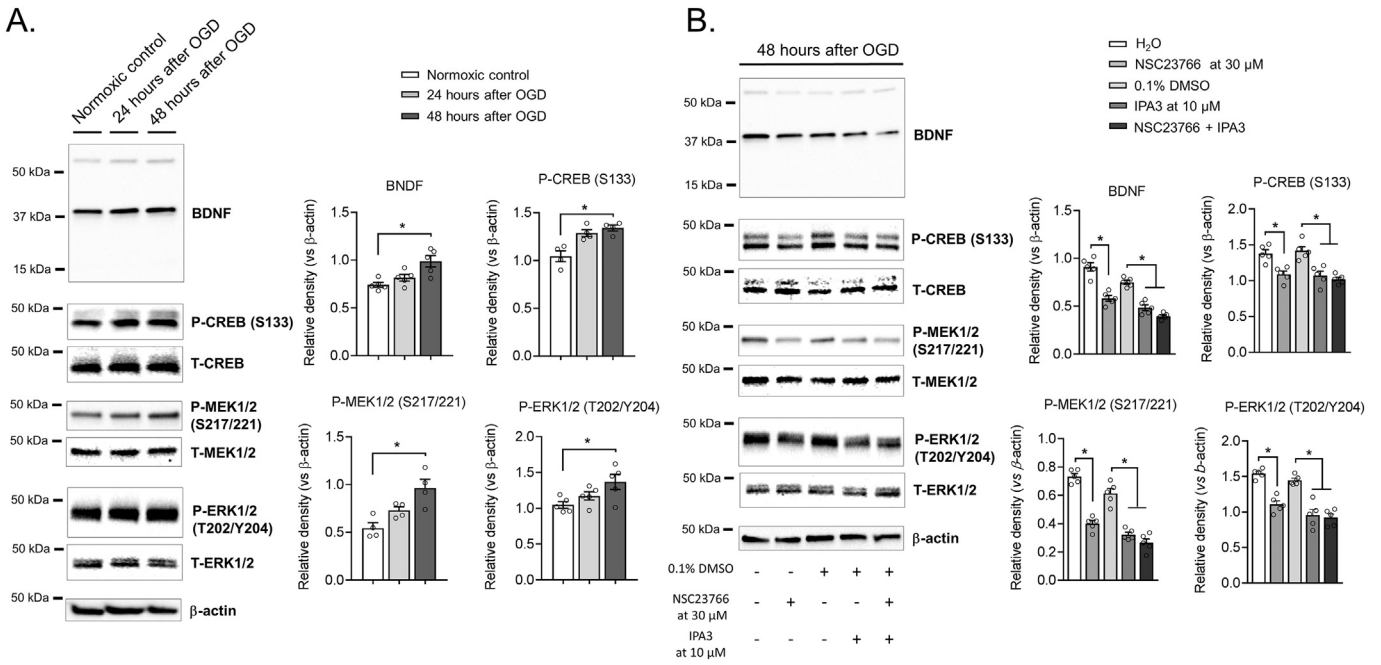

Fig. 5. Delayed inhibition of Rac1 in endothelial cells suppressed OGD-induced production of BDNF and activation of CREB, ERK1/2 and MEK1/2. (A) BDNF and p-CREB (S133), p-ERK1/2 (T202/Y204) and p-MEK1/2 (S217/221) increased 48 h after OGD. (B) Delayed application of either Rac1 inhibitor NSC23766 at 30 μM or Pak1 inhibitor IPA3 at 10 μM after OGD suppressed BDNF and p-CREB (S133), p-ERK1/2 (T202/Y204) and p-MEK1/2 (S217/221) compared to the controls. Addition of NSC23766 to IPA3 did not add additional inhibitory effect. Equal loading was indicated by β-actin. Quantitative analysis of relative intensity of interest proteins by normalizing them to β-actin. Neither MCAO nor delayed treatment altered the protein level of total CREB, Erk1/2 or MEK1/2 (quantification not shown). Our ERK1/2 and CREB antibodies recognize a doublet of bands on immunoblots, although, for p-ERK 1/2, some of the bands with higher intensity appear single due to signal overlapping of the doublet (A). We therefore measured the sum of both by densitometry as done previously by other studies (Sawada et al., 2010). The total signal of bands was assessed and used for quantitation. N = 4–5 for each group. Data were presented as mean ± SEM. *$p < 0.05$.

Author contribution statement

F B performed the majority of the experiments, analyzed data and drafted manuscript; J-W M and Y M performed experiments; Y-J L and L Q provided technical support; A U and L D M helped with data analysis and interpretation; J L conceived the research project, helped design the experiments and finalized the paper. All authors read and approved the final manuscript.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and publication of this article: This work was supported by National Institutes of Health grant R01 NS099628 (J.Li).

Declaration of competing interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Acknowledgements

The authors thank the University of Pittsburgh neurodegenerative brain bank for providing human samples.

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