13-Methyltetradecanoic acid mitigates cerebral ischemia/reperfusion injury

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Graphical Abstract

Abstract
13-Methyltetradecanoic acid can stabilize cell membrane and have anti-inflammatory, antioxidant and anti-apoptotic effects. Previous studies mainly focused on peripheral nerve injury, but seldom on the central nervous system. We investigated whether these properties of 13-methyltetradecanoic acid have a neuroprotective effect on focal cerebral ischemia/reperfusion injury, and detected the expression of basic fibroblast growth factor and vascular endothelial growth factor. This study established rat models of middle cerebral artery occlusion/reperfusion injury by ischemia for 2 hours and reperfusion for 24 hours. At the beginning of reperfusion, 13-methyltetradecanoic acid 10, 40 or 80 mg/kg was injected into the tail vein. Results found that various doses of 13-methyltetradecanoic acid effectively reduced infarct volume, mitigate cerebral edema, and increased the mRNA and protein expression of basic fibroblast growth factor and vascular endothelial growth factor at 24 hours of reperfusion. The effect was most significant in the 13-methyltetradecanoic acid 40 and 80 mg/kg groups. The findings suggest that 13-methyltetradecanoic acid can relieve focal ischemia/reperfusion injury immediately after reperfusion, stimulate the upregulation of basic fibroblast growth factor and vascular endothelial growth factor to exert neuroprotective effects.

Key Words: nerve regeneration; brain injury; 13-methyltetradecanoic acid; cerebral ischemia/reperfusion injury; basic fibroblast growth factor; vascular endothelial growth factor; cerebral infarction; cerebral edema; neural regeneration

Introduction
13-Methyltetradecanoic acid (13-MTD) is a saturated branched-chain fatty acid. Chemical formula: C_{15}H_{30}O_2, MW242.4. 13-MTD exists in the cell membrane of bacteria and some fungi, as an important ingredient of the bacterial cell membrane. 13-MTD has been shown to stabilize the mammalian cell membrane (Lindström et al., 2006), and promote cell proliferation (Yang et al., 2003). 13-MTD can be embedded into the lipid structure of the membrane, and affect cell function. When the fatty acid content in the lipid bilayer is above normal, 13-MTD can reduce the transmembrane diffusion. Studies have found that 12-MTD has anti-inflammatory and antioxidant effects (Kadri-Hassani et al., 1995; Yang et al., 2003; Krishnan et al., 2004). As congeners, 12-MTD and 13-MTD presumably have similar effects. Various reports showed that 13-MTD can stabilize mem-
branes and has anti-inflammatory, antioxidant, antithrombolytic and apoptosis-regulating effects in non-neuronal models (Faung et al., 1996; Lindstrom et al., 2006; Cai et al., 2009). Our research group has confirmed that different doses of 13-MTD have protective effect on oxygen paradox (such as oxygen-glucose deprivation/reperfusion) and H2O2-induced injury to SH-SY5Y neuroblastoma cells in vitro (Yu et al., 2010a, b, c). Oxygen paradox-induced apoptosis in rat embryonic cortical neurons and morphological injury also play a protective role (Yu et al., 2015). 13-MTD can also protect the primary cultured neonatal rat brain microvascular endothelial cells against oxygen paradox injury (Yu et al., 2010 a, b, c). The advantages of 13-MTD are that it has a low molecular weight, is very lipophilic, has low toxicity, few adverse reactions, and can be administered intravenously.

Both basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) are neuroprotective and angiogenic factors. The bFGF and VEGF expression promoted endogenous angiogenesis, increased blood flow in the infarct region, and increased the number of neuroblastoma cells that migrate towards the ischemic striatum (Burgess et al., 1989). bFGF, induced by ischemia, could regulate the expression of VEGF and act synergistically with VEGF (Stavri et al., 1995). It is beneficial for the processes of endothelial cells to penetrate the matrix and produce proteolytic enzymes, finally promoting vascular proliferation (Zhang et al., 1997).

We propose that 13-MTD intervention upregulated bFGF and VEGF expression after focal cerebral ischemia/reperfusion injury, and thus plays a neuroprotective role. This study observed the influence of 13-MTD intervention on bFGF and VEGF expression after focal cerebral ischemia and reperfusion injury.

Materials and Methods

Animals
Thirty adult male Sprague-Dawley rats weighing 255 ± 20 g were provided by the Shanghai SLAC Experimental Animal Technical Co., Ltd., China (SCXK [Hu] 2010-0002). All rats were maintained and housed at 22°C in 12-hour reversed light/dark cycle, and allowed free access to food and water. All surgery was performed under anesthesia (intraperitoneal injection of 10% chloral hydrate 0.3 mL/kg). All procedures were performed to minimize the pain and distress of the experiment animals. All animal experiments were carried out in accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animal (NIH Publication No. 85-23, revised 1986).

Middle cerebral artery occlusion/reperfusion (MCAO/R) modeling
Sprague-Dawley rats were equally and randomly assigned to the following five groups: sham group, vehicle + MCAO group (vehicle group), 13-MTD 10, 40, 80 mg/kg + MCAO groups (M10, M40, M80 groups). Sham group received the same intervention without inserting a thread. Rats in the M10, M40, and M80 groups were intraperitoneally injected with 13-MTD 10, 40, 80 mg/kg, respectively at the initiation of reperfusion. The vehicle group received an equal volume of solvent.

MCAO 2 hours/reperfusion 24 hours (MCAO 2 h/R 24 h) rat models were established according to Longa's method (Longa et al. 1989). Rats were anesthetized with 10% chloral hydrate. After a median neck incision and blunt dissection, the common carotid artery, internal carotid and external carotid arteries were exposed. The external carotid artery was transected by electrocoagulation. A nylon filament (diameter 0.205 mm) was then inserted from external carotid artery and gently advanced into internal carotid artery, approximately 18.5 ± 0.5 mm from the carotid bifurcation to the origin of the right middle cerebral artery in the circle of Willis. Rats walking in circles to the hemiplegic (predominantly left) side after regaining consciousness were the sign of success. To allow reperfusion, the nylon filament was withdrawn 2 hours after MCAO.

Administration of drugs
The sham group received the same intervention without inserting a thread. After fasting for 12 hours and free drinking water before surgery, rats were intraperitoneally anesthetized with 10% chloral hydrate 0.3 mL/kg and fixed in the supine position. After disinfection with tincture of iodine, a 3-cm median incision was made on the neck. The carotid artery, external carotid artery and internal carotid artery were bluntly dissected. MCAO 2 h/R 24 h rat models were induced on the right side. Rats in the M10, M40, and M80 groups were intraperitoneally injected with 13-MTD 10, 40, 80 mg/kg, respectively at the initiation of reperfusion (13-MTD, synthesized in the Pharmaceutical College of Fujian Medical University, China; the purity was 98.5% tested by gas chromatograph). Rats in the vehicle group were injected with an equal volume of solvent. All rats were decapitated 24 hours after reperfusion.

Measurement of infarct volume and cerebral edema analysis
The brains were coronally cut into five 2-mm-thick slices. These slices were dyed immediately and incubated in 2% 2,3,5-triphenyltetrazolium chloride (TTC) phosphate buffer solution (Shanghai Medical Group Shanghai Chemical Reagent Company, Shanghai, China) for 30 minutes at 37°C in the dark. The percentage of infarct volume within the total cerebral volume and cerebral edema was measured using AutoCAD image processing software. The percentage of infarct volume to total brain volume was considered as a statistical parameter. Cerebral edema (%) was equal to (ischemic cerebral hemisphere volume – non-ischemic cerebral hemisphere volume)/non-ischemic hemisphere volume × 100%.

Cerebral edema analysis
TTC phosphate buffer solution is a fat-soluble light-sensitive compound which produces a dark red fat-soluble substance
by reacting with the succinate dehydrogenase in normal mitochondria. After ischemic injury, the dehydrogenase was lost, due to cell membrane damage, so the ischemic tissue remains white. At 24 hours after reperfusion, rats were decapitated. The brain was dissected out and coronally cut into five 2-mm-thick slices. These slices were immediately placed in 2% TTC in phosphate buffer (pH 7.4) in the dark at 37°C for 30 minutes. The infarct area was white, and non-infarct area was red. After incubation, the brain slices were stored in 10% formalin in the dark for further use (Joshi et al., 2004). After TTC staining, cerebral edema (%) was calculated by (ischemic hemisphere volume–non-ischemic hemisphere volume)/non-ischemic hemisphere volume × 100%.

Figure 1 Effects of 13-MTD on infarct volume and cerebral edema in rats subjected to ischemia for 2 hours and reperfusion for 24 hours. (A) General observation of coronal sections with TTC staining; (B) percentage of infarct volume within the total cerebral volume and percentage of cerebral edema at 24 hours reperfusion in MCAO rats (###P < 0.01, vs. vehicle group; ††P < 0.01, vs. M10 group; mean ± SD, n = 7 rats, M10: n = 6 rats, M40: n = 6 rats, M80: n = 8 rats; one-way analysis of variance and Student-Newman-Keuls test). M10, M40, M80: 13-MTD 10, 40, 80 mg/kg groups, respectively. 13-MTD: 13-Methyltetradecanoic acid; MCAO: middle cerebral artery occlusion; TTC: 2,3,5-triphenyltetrazolium chloride.

Figure 2 Comparison of Evans blue content in rat brain tissues from different groups after 2 hours of ischemia and 24 hours of reperfusion. (A) General observation of coronal sections; (B) Evans blue content in each group. Key to significant differences between groups: *P < 0.05, **P < 0.01, vs. sham group; #P < 0.05, ###P < 0.001, vs. vehicle group; †P < 0.05, vs. vehicle group; ††P < 0.05, vs. M10 and M80 groups (mean ± SD, n = 7 rats, one-way analysis of variance and Student-Newman-Keuls test). M10, M40, M80: 13-MTD 10, 40, 80 mg/kg groups, respectively. 13-MTD: 13-Methyltetradecanoic acid.

Figure 3 13-MTD effects on mRNA expression of bFGF and VEGF in brain tissues of rats with ischemia/reperfusion injury. The mRNA expression of bFGF and VEGF was measured as the absorbance ratio of bFGF/β-actin mRNA and VEGF/β-actin. **P < 0.01, vs. sham group (1); ###P < 0.01, vs. vehicle group (2); ††P < 0.01, vs. M10 group (3); §§P < 0.01, vs. M40 group (4) (mean ± SD, n = 6 rats, one-way analysis of variance and Student-Newman-Keuls test). M10, M40, M80 (3–5): 13-MTD 10, 40, 80 mg/kg groups, respectively. 13-MTD: 13-Methyltetradecanoic acid; bFGF: basic fibroblast growth factor; VEGF: vascular endothelial growth factor.
Blood-brain barrier (BBB) permeability detection

Evans blue (Xiamen Krunghai Biotechnology Co., Ltd., Xiamen, Fujian Province, China) was used as a tracer to detect the degree of BBB damage (Belayev et al., 1996). Evans blue (4 mg) was dissolved in 25 mL normal saline as the original solution. The original solution was diluted with formamide to the concentration of 8, 4, 2, 1, 0.5, 0.25, 0.125 μg/mL. Optical density values were measured colorimetrically ($\lambda = 570$ nm) after incubation at 54°C for 24 hours.

Evans blue (2%, 2 mL/kg) was injected into the rat tail vein after reperfusion for 2 hours. Rats were decapitated 24 hours after reperfusion and the whole brain was taken out and weighed immediately. The tissue on the injured side was homogenized with 3 mL formamide, incubated at 54°C for 24 hours, and centrifuged at 15,000 r/min for 20 minutes. The supernatants (200 μL each) were placed in 96-well plates. The optical density values of each group were determined by a microplate reader ($\lambda = 570$ nm; Thermo Labsystems Company, Finland). Evans blue content was calculated according to the standard curve. The permeability of the BBB to Evans blue was calculated in accordance with the measured concentration, indicated as the amount of the Evans blue (μg/g) per gram of wet brain tissue.

Reverse transcription-polymerase chain reaction (RT-PCR)

After model establishment, brain tissue was immediately taken from the injured side. Total RNA was extracted by TRIzol (Invitrogen Trading (Shanghai) Co., Ltd., Shanghai, China) according to the manufacturer's instructions. cDNA was synthesized from 2.0 μg of the total RNA with a reverse transcription kit by AMV reverse transcriptase (Promega Company, CA, USA), and expressed as the absorbance ratio analyzed by Gel imaging analysis system (Syngene Genius Company, CA, USA). The white infarcts in 13-MTD groups were significantly smaller than that in the vehicle group ($P < 0.01$), especially in the M40 and M80 groups ($P < 0.01$; Figure 1).

Immunohistochemistry of bFGF and VEGF

After ventricular perfusion with 1% heparin and 10% formalin, rats were rapidly decapitated. Brain tissue from the optic chiasma to the infundibular stem was coronally sliced into sections. These sections were fixed in 10% formalin, dehydrated, permeabilized, and embedded in paraffin. 5-μm-thick coronal slices were placed on polylysine-coated glass slides, which were baked in a 60°C oven for 6 hours. Paraffin-embedded sections were used to assess the immunoreactivities of bFGF and VEGF. Brain sections were incubated with rabbit anti-rat bFGF and VEGF polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted at 1:200 in phosphate buffered saline (PBS) overnight at 4°C and incubated at 37°C for 1 hour. Sections were incubated at room temperature for 20 minutes with polymer enhancer, 30 minutes with enzyme-marked secondary goat anti-rabbit antibody (1:200) and 45 minutes at 37°C and stained with 3,3′-diaminobenzidine (Fuzhou Maixin Biotech. Co., Ltd., Fuzhou, China). The slides were closed with neutral gum after staining with hematoxylin. Immunoreactivity was recorded with an optical microscope and the image pickup system (Olympus, Tokyo, Japan). Motic Images Advanced 3.0 image analysis system (Hong Kong MOTIC, Hong Kong, China) was used for automatic analysis. Results were expressed as mean gray value of immunoreactive cells, the number of immunoreactive cells and the amount of immunoreactive cells.

Statistical analysis

Data were expressed as the mean ± SD, and analyzed by SPSS 13.0 statistical software (SPSS, Chicago, IL, USA). Data between groups were compared by one-way analysis of variance and Student-Newman-Keuls test. A value of $P < 0.05$ was considered statistically significant.

Results

Changes in infarct volume and cerebral edema after ischemia/reperfusion injury

The whole brain sections in the sham group showed red after staining with TTC. In the vehicle group, white infarct was obviously seen in the area supplied by the middle cerebral artery. The white infarcts in 13-MTD groups were significantly smaller than that in the vehicle group ($P < 0.01$), especially in the M40 and M80 groups ($P < 0.05$). Cerebral edema was significantly reduced in the treatment groups ($P < 0.01$), especially in the M40 and M80 groups ($P < 0.05$; Figure 1).

Evans blue content in brain tissue of rats with ischemia/reperfusion injury

Significant blue-stained area was seen in the vehicle group. Evans blue content significantly increased in the vehicle group, but decreased in the M40 group ($P < 0.001$), and still significantly decreased in the M10 group ($P < 0.05$; Figure 2).

Expression of bFGF and VEGF mRNA in brain tissue of rats with ischemia/reperfusion injury

After injury, bFGF mRNA expression was significantly higher in
the vehicle group than in the sham group ($P < 0.01$). Compared with the vehicle group, bFGF mRNA expression significantly increased in the M40 and M80 groups ($P < 0.01$). VEGF mRNA expression was significantly higher in the vehicle group than in the sham group ($P < 0.01$). VEGF mRNA expression was significantly higher in the M10, M40 and M80 groups than in the vehicle group ($P < 0.01$). VEGF mRNA expression was significantly higher in the M40 group than in the M10 and M80 groups ($P < 0.01$; Figure 3).

**Immunoreactivities of bFGF and VEGF in brain tissue of rats with ischemia/reperfusion injury**

The positive expression of bFGF protein was observed as brown particles in the cytoplasm or the nucleus of bFGF-immunoreactive cells. Compared with the sham group, bFGF immunoreactivity was stronger; the number of bFGF-immunoreactive cells was significantly higher ($P < 0.01$), especially in the ischemic penumbra area; mean gray value was significantly lower ($P < 0.01$) in the vehicle group. 13-MTD 10, 40, and 80 mg/kg significantly increased bFGF protein expression, increased the area of immunoreactivity ($P < 0.01$; Figure 4), significantly decreased mean gray value ($P < 0.01$), and increased the number of immunoreactive cells ($P < 0.01$; Figure 5), especially in the M80 group ($P < 0.01$; Figures 4, 5).

VEGF-immunoreactive cells were mainly distributed in the cortex, as the presence of yellow or brown cytoplasm, and mainly in the ischemic area. VEGF protein expression was weak in the sham group. In the vehicle group, the expression of VEGF protein was upregulated, its mean gray value decreased ($P < 0.01$) and the number of immunoreactive-cells increased ($P < 0.01$). In each 13-MTD group, the number of VEGF-immunoreactive cells increased ($P < 0.01$) and the mean gray value significantly decreased ($P < 0.01$), especially in the M80 group ($P < 0.01$) (Figures 4, 5).

**Discussion**

The lesions in the vehicle group could involve the striatum, frontal and parietal cortex, all supplied by the middle cerebral artery and sensitive to ischemic injury, especially irreversible damage. However, the frontal and parietal cortex is supplied by the anterior cerebral artery with penetrating branches beside the middle cerebral artery, so the damage is relatively mild and occurred later. The expression of bFGF and VEGF on the injured side increased; cerebral infarction and edema appeared; the permeability of BBB increased after MCAO 2 h/R 24 h. After treatment with 13-MTD, cerebral infarction and edema were mitigated; the permeability of BBB reduced; the expression of bFGF and VEGF further increased, especially in M40 and M80 groups. These results showed that 13-MTD could promote the proliferation of endothelial cells and angiogenesis in the ischemic penumbra through increasing the expression level of bFGF and VEGF. Together, they regulated an improvement in the blood supply to the injured region. Moreover, 13-MTD could effectively reduce the infarct volume, cerebral edema and the permeability of BBB. Thus, 13-MTD has a clear protective effect on cerebral ischemia/reperfusion injury.

bFGF and VEGF are neuroprotective and angiogenic factors. The up-regulated bFGF and VEGF expression also can promote endogenous angiogenesis and blood flow within the infarct area and increase the number of neuroblasts migrating toward the ischemic striatum (Oyamada et al., 2008). bFGF has been shown to play a direct role in neurotrophy, promote the multiplication of stem cells during development period of the central nervous system, induce axon to grow, promote new neuron survival and angiogenesis (Li et al., 2005). Li et al. (2002) found that neurological deficits were clearly improved after bFGF administration, and the infarct volume reduced by 32% after MCAO for 24 hours in rats. Sugimori et al. (2001) confirmed that bFGF reduced the infarct volume by 27% after MCAO for 3 months in rats. The intravenous administration of bFGF has been shown to be effective in reducing infarct volume and promoting the recovery of nervous functions in several species of animals (Liu et al., 2006). Ay et al. (2001) verified that the infarct volume was reduced when bFGF was used. bFGF can reduce DNA fracturing, increase the immune response of anti-apoptotic protein bcl-2, and thus reduce the apoptosis of ischemic brain tissues. In this study, bFGF expression increased after MCAO 2 h/R 24 h in rats and further increased in 13-MTD groups, suggesting that 13-MTD plays a neuroprotective effect through increasing bFGF expression.

VEGF is a powerful angiogenic factor and has direct neurotrophic and neuroprotective effect. In this study, we found that VEGF expression increased after cerebral ischemia/reperfusion injury, and further increased after treatment with 13-MTD. Infarct volume and brain edema mitigated, and BBB permeability decreased. We infer that 13-MTD plays a protective role by up-regulating VEGF expression. Wang et al. (2009) injected human VEGF$\textsubscript{165}$—expressing plasmids into the lateral ventricle of rat brains after MCAO for 30 minutes, resulting in a significant reduction in the infarct volume and increase in the number of cortical newborn GABAergic neurons at 2, 4 and 8 weeks after MCAO. Yang et al. (2009) showed that perifocal administration of VEGF protected the brain from ischemia/reperfusion injury (MCAO 3 h/R 3 d) in rabbits. It reduced the infarct volume and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL)-positive cells, and effectively inhibited the expression of caspase-12 and caspase-3. The VEGF gene transplanted into adult rat hippocampus could increase neurogenesis and improve cognitive function, but neurogenesis would be blocked completely if VEGF expression was inhibited at RNA levels (During et al., 2006). Zhao et al. (2010) found that VEGF expression increased after MCAO 2 h/R 24 h in rats and that electroacupuncture treatment raised VEGF levels further. Electroacupuncture also decreased induced nitric oxide synthase activity, alleviated ischemic injury, and regulated the quantity of endothelial progenitor cells in blood. They suggested that high expression of VEGF could accelerate mobilization, chemotaxis and homing of endothelial progenitor cells, promote neovascularization and improve cerebral ischemia/reperfusion injury. Nie et al. (2010) used candesartan pretreatment in MCAO
Our preliminary studies verified that 13-MTD promoted the neurological function and reduced infarct volume. 2 h/R 24 h rats and found increased VEGF expression, improved cell activity of the brain tissue, reducing BBB permeability and mitigating cerebral ischemia/reperfusion injury effectively.

Our results not only verify the effect of 13-MTD, but also elucidate the variation of bFGF and VEGF in a focal cerebral ischemic injury, which is helpful to further understand the characteristic and mechanism of the recovery in cerebral ischemia/reperfusion injury. Studies are underway to further define the mechanism of 13-MTD action in cerebral ischemia/reperfusion injury.

2 h/R 24 h rats and found increased VEGF expression, improved neurological function and reduced infarct volume. Our preliminary studies verified that 13-MTD promoted the proliferation of SH-SY5Y cells in vitro (Yu et al., 2010a, b, c). Therefore, we infer that 13-MTD may promote proliferation through increasing VEGF expression, improving blood supply and cell activity of the brain tissue, reducing BBB permeability and mitigating cerebral ischemia/reperfusion injury effectively.

Our results not only verify the effect of 13-MTD, but also elucidate the variation of bFGF and VEGF in a focal cerebral ischemic injury, which is helpful to further understand the characteristic and mechanism of the recovery in cerebral ischemia/reperfusion injury. Studies are underway to further define the mechanism of 13-MTD action in cerebral ischemia/reperfusion injury.

**Author contributions:** JY and YYW conceived and designed the study, collected, analyzed and interpreted data, participated in statistical expertise, obtained funding, and provided administrative, technical or material support, and supervision. JY; LNY and BHL wrote the paper or provided critical revision of the paper for intellectual content. JY designed research. YYW performed research. LNY and BHL analyzed data. JY, LNY and BHL wrote the paper. SMW and CLH participated in this
This paper was double-blinded and stringently reviewed by international expert reviewers.

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