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Telmisartan regulates the development of cerebral ischemia by alleviating endoplasmic reticulum stress

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Recent studies have shown that telmisartan (TMS) is effective for the protection against ischemia/brain damage in rat models. However, the specific underlying mechanism is poorly understood. In line with previous results, our data showed that TMS improves CBF and physiological variables, including pH, pCO2, pO2. Through CD31 immunofluorescence staining, reduction of blood vessel density was found in MCAO group, but TMS treatment could enhance the cerebral vascular density in the ischemic area. Meanwhile, TMS treatment could enhance the number of BrdU/lectin double-positive cells. Furthermore, the reduction of nestin-positive cells was identified in the brain of MCAO rats, while the number of nestin-positive cells was significantly increased after TMS administration. Furthermore, the expression of ERS-related proteins, including GRP78, CHOP/GADD153, Caspase12 was increased after MCAO, but was decreased after administration of TMS, thereby enhancing angiogenesis and neuron regeneration.

1. Introduction

Stroke is characterized by high incidence, high morbidity and high mortality (Friedrich et al. 2016; Basu et al. 2017). At present, the drug therapy for ischemic stroke is limited (De Schryver and Halkes 2008; Bujak et al. 2011; Liu et al. 2014). For instance, recombinant tissue plasminogen activator (rtPA), which is approved by the Food and Drugs Administration (FDA), is found to be effective only if the administration is performed within 4.5 h after the stroke. Due to the narrow therapeutic window, few patients benefit from therapy (Kleindorfer et al. 2008). Hence, it is important to tap novel drugs for the therapy of ischemic stroke.

Apoptosis is a programmed cell death under pathological conditions (He et al. 2017). Studies have found that apoptosis can affect nerve function after spinal cord injury SCI (Lee et al. 2014) and have suggested that mitochondrial death receptor pathways were the two main ways that lead to apoptosis (Ohri et al. 2012; Matsuyama et al. 2014; Liu et al. 2015). Currently, endoplasmic reticulum stress (ERS) is also found to induce apoptosis (Ohri et al. 2012). The endoplasmic reticulum (ER) is an organelle in eukaryotic cells, important for the regulation of calcium ions, processing and synthesis of proteins (Ohri, Maddie et al. 2011). When multiple physiological and pathological causes changes in ER function, ER will respond or react (Penas et al. 2007; Ohri et al. 2014). The main sign of ERS is the expression of ERS proteins, including GRP78, GRP94 and GADD153 (Wu et al. 2016). If there is sustained severe ERS, the body can activate some pro apoptotic factors such as caspase-12, CHOP / GADD153, resulting in cell apoptosis and tissue damage (Zhang et al. 2014).

Telmisartan (TMS), an angiotensin receptor blocker with high lipid solubility, is also known as metabosartan. TMS is found to not only decrease blood pressure (BP), but also alleviate inflammation in the cerebral cortex and in adipose tissue (Sato et al. 2014). In the current study, we tried to explore the effect of TMS on ischemia stroke in rats. We first demonstrated that TMS enhanced the activation of AMPK and eNOS, thereby improving recovery from cerebral infarction.

2. Investigations and results

2.1. Improvement of blood parameters and CBF by TMS in MCAO rats

Blood parameters, including pO_2, pH and pCO_2, were measured in rats. Our data showed that pO_2 values were stable after MCAO, but pH values were decreased after MCAO and pCO_2 was enhanced after 24 h MCAO, indicating MCAO resulted in respiratory depression (Fig. 1A). Moreover, TMS therapy enhanced pO_2, reduced pCO_2 and increased pH to the sham group levels 5 weeks later (Fig. 1A). In addition, CBF in rats of MCAO group was significantly decreased. However, after TMS treatment for 5 weeks, CBF was significantly increased, indicating MCAO-induced shortage of brain blood supply could be partially restored to the normal level by TMS treatment (Fig. 1B).

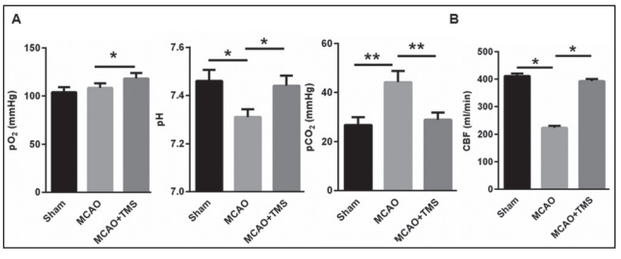

Fig. 1: The improvement of blood parameters and CBF was identified by TMS in MCAO Rats. (A) TMS therapy enhanced pO_2, reduced pCO_2 and increased pH to the sham group levels 5 weeks later. (B) After TMS treatment for 5 weeks, CBF was significantly increased. *p<0.05, **p<0.01 *vs*. control.

2.2. Cerebral infarction was relieved in rats after TMS treatment

Furthermore, TTC and H&E staining were carried out to evaluate the effects of TMS on cerebral infarction. Not surprisingly, cerebral infarction was obvious in the MCAO group but the infarction

volume was decreased after TMS treatment (Fig. 2A). Meanwhile, decreased neurological deficits, cerebral infarct volume, and brain edema rate were found in MCAO group, but TMS treatment could obviously improve these parameters (Figs. 2B, 2C and 2D). Besides, reduced neuron counts were identified in the MCAO group, but TMS treatment could significantly enhance the number of neuron counts (Fig. 2E), suggesting a protective role of TMS in cerebral infarction.

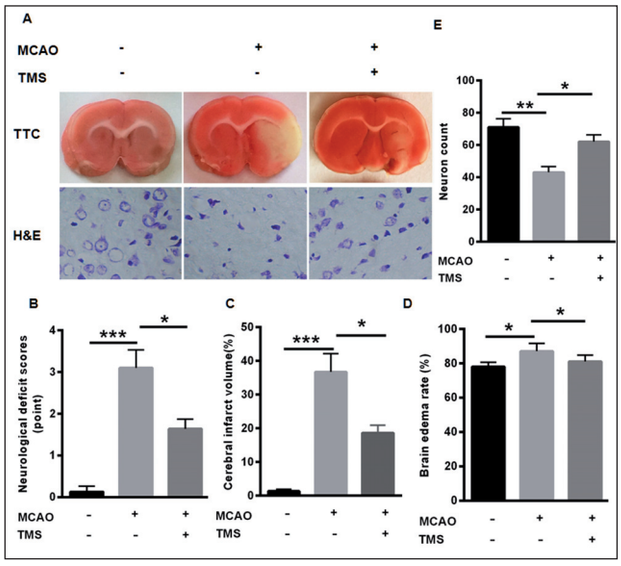

Fig. 2: Cerebral infarction was relieved in rats after TMS treatment. (A) cerebral infarction was obvious in MCAO group but the infarction volume was decreased after TMS treatment. Decreased neurological deficits (B), cerebral infarct volume (C), and brain edema rate (D) were found in MCAO group, but TMS treatment could obviously improve these parameters. (E) Reduced neuron counts were identified in MCAO group, but TMS treatment could significantly enhance the number of neuron counts. *p<0.05, **p<0.01 *vs*. control.

2.3. Angiogenesis was enhanced after TMS treatment

Vascular regeneration after ischemia can increase the perfusion of brain tissue and improve the repair of ischemic tissue. Here, we evaluated the density of blood vessels in the ischemic area through CD31 immunofluorescence staining. Decreased blood vessel density was found in MCAO group, but TMS treatment could enhance the cerebral vascular density in the ischemic area (Fig. 3A). The BrdU/lectin staining was also carried out to evaluate the effect of TMS on angiogenesis. As shown in Fig. 3B, MCAO surgery reduced the number of functional vessels (Fig. 3B). However, TMS treatment could enhance the number of BrdU/lectin double-positive cells (Fig. 3B), suggesting the promotion of angiogenesis by TMS.

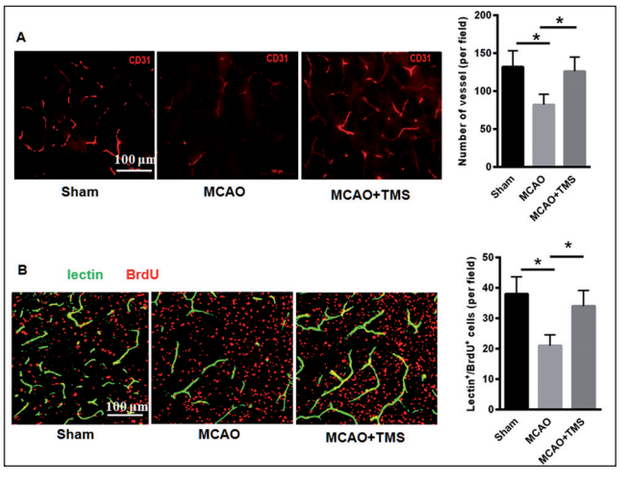

Fig. 3: TMS treatment increased angiogenesis. (A) CD31 immunofluorescence staining indicated that TMS treatment could enhance the cerebral vascular density in the ischemic area. (B) TMS treatment could enhance the number of BrdU/lectin double-positive cells. *p<0.05, **p<0.01 *vs*. control.

2.4. Enhanced nestin-positive cells in the subventricular zone (SVZ) after TMS therapy

Nestin, the marker of neural stem cells, was examined in the SVZ after ischemia. In the sham group, nestin-positive cells were sparse in the SVZ region. However, they were more obvious seven days after MCAO in the ischemic hemisphere (Fig. 4), indicating a spontaneous recovery. Interestingly, more nestin-positive cells were identified in the SVZ region than MCAO group (Fig. 4), suggesting the proliferation of neural stem cells after TMS treatment.

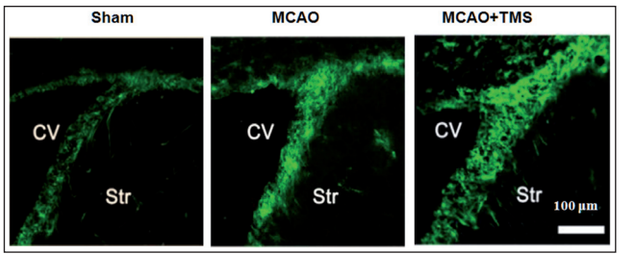

Fig. 4: TMS treatment increased nestin-positive cells in the subventricular zone. *p<0.05, **p<0.01 vs. control. *p<0.05, **p<0.01 *vs*. control.

2.5. TMS activated ERS signaling in ischemia brain

In order to detect whether MCAO can induce ERS, we examined the expression of ERS-related protein GRP78 using Western blot. Under normal conditions, the expression of GRP78, CHOP and caspase12 is relatively low (Fig. 5A), but the expression of GRP78, CHOP and caspase12 were increased after MCAO (Fig. 5A). Thus, MCAO may provoke ERS. But after TMS treatment for 7 d, the expression of GRP78, CHOP and caspase12 was markedly decreased (Fig. 5B). These data indicated the protective role of TMS in ERS.

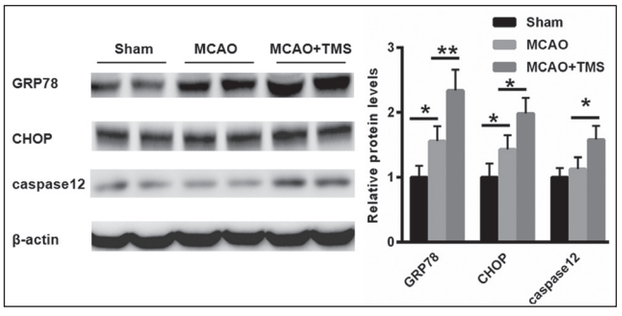

Fig. 5: Western blot assay showed that TMS treatment further resulted in the suppression of ERS signaling. *p<0.05, **p<0.01 *vs*. control.

3. Discussion

Telmisartan is reported to exert a special protective effect on both acute brain damage and chronic neurodegeneration (Sato et al. 2014). In line with previous study, our data showed that TMS improves CBF and physiological variables, including pH, pCO2, pO2. Moreover, TTC and H&E staining indicated that cerebral infarction and neuronal death was improved in the SVZ region after TMS treatment. These findings suggest that TMS directly protects rats from cerebral ischemia.

The SVZ is an important region for nerve regeneration in the adult mammalian brain (Chen et al. 2016; Liu et al. 2016). Under normal status, the neurons migrate to the olfactory bulb and hippocampus

from SVZ. Research has shown that increased nerve regeneration is identified in the circumstances of cerebral ischemia and hemorrhage (Liu et al. 2016). In the recovery process of stroke, post-ischemic neurogenesis plays an important role (Bellenchi et al. 2013). Hence, the proliferation, migration, and differentiation of neural stem cells are key therapeutic targets for the functional improvement of stroke (Christie and Turnley 2012). For the first time, we showed that TMS enhanced nestin positive cells in the SVZ area, indicating enhanced neuron repair after ischemia. In line with nerve regeneration and neuronal plasticity, there is complex vascular remodeling during the recovery phase of ischemia. Angiogenesis is found in brain tissue after ischemia in both humans and rats (Yao et al. 2016). CD31 staining showed that TMS treatment increased the density of blood vessels in the ischemic area. Hence, TMS improves nerve regeneration and angiogenesis in the ischemic rat brains.

ERS regulates the homeostasis of the organism by modulating protein degradation and protein folding (Wang et al. 2016). Under non stress conditions, ER chaperone glucose regulated protein 78 (GRP78) combines with the three molecules of PERK, ATF6 and IRE1 to maintain its non-activation state (Saraswat Ohri et al. 2018). In the circumstance of unfolded protein response (UPR), unfolded proteins are accumulated in the ER, which then triggers ERS-induced cell apoptosis (Zhu et al. 2016). GRP78, CHOP / GADDl53, and Caspase12 are ERS specific transcription factors that are not activated in the death receptor and mitochondrial signaling pathway (Liu et al. 2015). ER stress signaling pathway is suggested to play a key role in nerve cell damage diseases (Kuroiwa et al. 2014; Son et al. 2014; Rozpedek et al. 2015). For instance, ESR–induced cell death is thought to play a central role in Alzheimer's disease (Rozpedek et al. 2015). In the Parkinson neurotoxin model, ER calcium levels were disordered and the activation of CHOP was found in apoptotic neurons (Son et al. 2014). Meanwhile, studies have shown that neuronal and glial cell apoptosis play an important role in spinal cord injury, and inhibition of neuronal and glial cell apoptosis may be a strategy for the treatment of SCI (Zhang et al. 2015; Xu et al. 2017). In line with previous studies, our data showed that ERS was activated in the brains of MCAO rats. Our data indicated that the activation of ERS could be alleviated by TMS, which is accompanied by improved nerve vascular regeneration. Hence, we propose that suppression of ERS by TMS could improve nerve regeneration, angiogenesis and brain function in the chronic recovery phase of cerebral infarction.

In conclusion, for the first time, our data showed that TMS improves the recovery of nerve function and angiogenesis by suppressing ERS signaling during the recovery period of ischemia.

4. Experimental

4.1. Drug administration in middle cerebral artery occlusion (MCAO) in rats

A total of 60 male Sprague Dawley (SD) weighing 200–240 g were used in this study. Rats were housed in the same animal care facility during a 12 h light/dark cycle throughout the protocol with free access to food and water. Briefly, the rats were anesthetized with intraperitoneal injection of 4 % chloral hydrate (1 mL/100g) of rats and arterial blood samples obtained *via* a femoral catheter were collected to measure pO_2, pCO_2 and pH with an AVL 998 Blood Gas Analyzer (Roche Co., Basel, Switzerland). The rectal temperature was maintained at 37±0.5 °C during MCAO *via* a temperature-regulated heating lamp. A fiber-optic probe was attached to the parietal bone overlying the middle cerebral artery territory 5 mm posterior and 5 mm lateral to the bregma, and it was connected to a laser Doppler flowmeter (PeriFlux System 5000, Stockholm, Sweden) for continuous monitoring of the cerebral blood flow (CBF). A 4-0 nylon monofilament suture with a heat-blunted tip was introduced into the internal carotid artery through the stump of the external carotid artery. It was gently advanced for a distance of 18 mm from the common carotid artery bifurcation to block the origin of the middle cerebral artery for 90 min and then withdrawn to allow reperfusion. After the wound had been closed, the animals were allowed to recover from anesthesia before they were returned to their home cages. Sixty adult male SD rats were randomly divided into sham operation group, ischemia reperfusion group (MCAO) and MCAO+TMS treated group. The rats were intraperitoneally treated with either 50 µl of saline or TMS (1 mg/kg; Lipomed AG, Arlesheim, Switzerland) for five weeks.

All experimental protocols described in this study were approved by the Ethics Review Committee for Huaihe Hospital of Henan University.

4.2. Assessment of neurological deficit score and analysis of survival rates

Rats were sacrificed 24 h after reperfusion and the neurological deficit score was evaluated. Two examiners were kept unaware of the identity of the rat and the treatment protocol. The following neurological deficit scoring (NDS) system was used: 0, no motor deficits (normal); 1, forelimb weakness and torso turning to the ipsilateral side when held by tail (mild); 2, circling to the contralateral side but normal posture at rest (moderate); 3, unable to bear weight on the affected side at rest (severe); and 4, no spontaneous locomotor activity or barrel rolling (critical). If no deficit was observed 2 h after recovering from anesthesia, the animal was removed from further study.

4.3. Edema measurement

The ipsilateral and contralateral hemispheres were dissected and the wet weight of the tissue was determined. The tissues were dried at 120 °C for 24 h. The percent cerebral water was determined as (wet weight – dry weight)/dry weight ×100.

4.4. Infarct analysis

At 72 h after stroke, the brain was removed and cut into 5 2-mm slices and stained with 1.5% 2, 3, 5 triphenyltetrazolium (TTC) for 30 min at 3 °C. In animals assessed chronically post-stroke, rats were sacrificed at 5 weeks by a pentobarbital overdose and perfused transcardially with cold PBS followed by 4% paraformaldehyde; the brain was postfixed for 18 h and placed in cyroprotectant (30% sucrose). The brain tissue was cut into 40-µm free-floating sections on a freezing microtome and every eighth slice was stained by cresyl violet staining for evaluation of ischemic cell damage. Infarct volume, expressed as a percentage of whole-brain volume, was measured by an Image-Processing and analysis system (1.25×objective, Q570IW; Leica, Wetzlar, Germany) and calculated by integration of the infarct area on each brain section along the rostralcaudal axis.

4.5. Western blot analysis

Western blots were performed as described previously (Vergoni et al. 2000). After the onset of cerebral ischemia, rats were sacrificed, brains were homogenized using lysis buffer and protein was loaded on a 4% to 15% gradient SDS–polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. GAPDH, cleaved-caspase-3, GRP78, CHOP / GADD153 and caspase12 (Cell Signaling), was used as the loading control. Blots were incubated overnight in primary antibody at 4 °C in TBS containing 4 % bovine serum albumin and 0.1 % Tween20. Secondary antibodies (goat anti-rabbit IgG 1:5,000, Zhongshanjinqiao, Beijing, China) were diluted and ECL (pico) detection kit (ThermoScientific) was used for signal detection.

4.6. Immunostaining

Briefly, brain sections were blocked with 10 % FBS for 1 h and then incubated with BrdU staining in 2 M HCl at 37 °C for 20 min and rinsed in 0.1 M borate buffer (pH 8.5) before blocking. For CD31, lectin and nestin (1:50 dilution; Cell Signaling Technology, Inc., Boston, MA, USA) staining, antigens were retrieved with citrate buffer (10 mM [pH 6.5]) for 20 min at 95 °C before blocking at 4 °C overnight. After washing, brain sections were incubated with the appropriate second antibodies for 1 h. Brain sections were examined using a confocal microscope (Leica, Solms, Germany) and photographs were taken for further analysis.

4.7. Statistical analysis

Data were expressed as mean±SD. Statistics were performed either with Student t-test, one-way analysis of variance with Tukey post hoc test for multiple comparisons. p value < 0.05 was considered to be statistically significant.

Conflicts of interest: None declared.

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