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Short communication

A likely protective effect of dimethyl itaconate on cerebral ischemia/reperfusion injury

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ABSTRACT

As a membrane-permeable derivative of itaconate, dimethyl itaconate (DMI) was recently showed to limit inflammatory response of activated macrophages, and to decrease the generation of reactive oxygen species and reduce cardiac ischemia/reperfusion injury. However, the effect of DMI in the context of cerebral ischemia/reperfusion injury remains unclear. Here, we treated the transient middle cerebral artery occlusion (tMCAO) mice with DMI or saline at the beginning of occlusion, and allowed them to recover for 3 days. We found that DMI obviously decreased the neurologic deficit score. Further, DMI significantly inhibited the toxic conversion of the *peri*-infarct microglia, and decreased the protein level of interleukin 1β. The present findings suggest that DMI might be recognized as a promising candidate for the treatment of ischemic stroke.

1. Introduction

Although preclinical studies on neuroprotective agents have been moving on, timely restoration of the interrupted cerebral blood flew after brain ischemia is still the most widely accepted and effective strategy. Following the made progress of thrombolytic therapy and endovascular treatment, ischemia/reperfusion brain injury has attracted close attention. Reperfusion induces the excessive reactive oxygen species (ROS) and subsequent oxidative stress which aggravates ischemic brain injury [1]. ROS also exacerbates neurotoxic inflammatory response [2]. Recent study highlighted the causal role of ischemic succinate accumulation in multiple ischemia/reperfusion injuries, and showed that dimethyl malonate, a competitive inhibitor of succinate dehydrogenase (Sdh), could reduce ischemic succinate accumulation and reperfusion damage [3], uncovering a possible target of treating ischemia/reperfusion diseases.

Nuclear factor erythroid 2-related factor (Nrf2) is well known for the endogenous defense against oxidative stress and inflammation. Nrf2 maintains physiological low expression through being associated with Kelch-like ECH-associated protein 1 (KEAP 1) [4]. The expression of Nrf2 and its target proteins like heme oxygenase (HO)-1 in brain are elevated after ischemic stroke, indicating an adaptively defensive mechanism [5]. It's also showed that the downstream metabolites of heme produced by HO-1 catalysis, such as carbon monoxide and bilirubin, could offer potent anti-inflammatory and antioxidant effects on ischemic brain injury [6,7]. Nrf2 pathway has been generally considered as a protective target of ischemic stroke.

Lately, dimethyl itaconate (DMI), which is a cell-permeable derivative of itaconate, was identified to suppress inflammatory response of activated bone marrow-derived macrophages (BMDMs) via inhibiting Sdh [8]. In a myocardial ischemia/reperfusion mouse model, DMI reduced infarct size through limiting Sdh and decreasing ROS levels [8], suggesting that DMI could alleviate Sdh-mediated oxidative stress. In addition, DMI could increase LPS-stimulated Nrf2 expression and decrease protein levels of inflammatory cytokines, for example interleukin 1β (IL1β), in vitro assay [9], indicating that DMI may function as the Nrf2 activator. However, whether DMI affects the outcomes of cerebral ischemia/reperfusion injury remains unknown. In this study, we investigated the role of DMI in the mouse model of transient middle cerebral artery occlusion (tMCAO) to test our hypothesis that DMI might protect against ischemia/reperfusion-induced brain injury.

2. Materials and methods

2.1. Animals

Adult male C57BL/6 mice (26–29 g) were obtained from the Animal Experimental Center of Zhengzhou University. Animal use and

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procedures were in accordance with the regulations for the management of experimental animals issued by the Ministry of Science and Technology of the People's Republic of China, and approved by the Animal Care and Use Committee of the Fifth Affiliated Hospital of Zhengzhou University. Every effort was made to minimize the number of mice used and their suffering.

2.2. tMCAO model

We carried out the tMCAO procedure as previously published [6,10,11]. In brief, after anesthetizing mouse with an appropriate dose of 5% chloral hydrate via the intraperitoneal route, we carefully advanced a 6.0 monofilament nylon suture with silicone-coated tip through the common carotid artery to block the origin of the right middle cerebral artery, and withdrew it 60 min later. Sham operation was defined as immediately withdrawing the filament post advancing it to the origin of the right middle cerebral artery.

tMCAO mice (16 mice/group) and sham mice (8 mice/group) were randomly [6,11] assigned to either DMI-treated or saline-treated group.

We delivered the DMI (Sigma-Aldrich, St. Louis, MO, USA; 20 mg in 0.5 ml saline per mouse) or the saline through the intraperitoneal route at the beginning of occlusion [12]. All the treated mice were examined daily for infection or illness, and were allowed to recover for 72 h.

2.3. Measurement of neurologic deficits

In a blinded manner, an investigator measured the neurologic deficits according to a five-point scale [6,11]. Briefly, zero-point indicated that mouse did not have neurologic deficit, one-point indicated that mouse failed to fully extend contralateral forepaw, two-point indicated that mouse circled to the contralateral side, three-point indicated that mouse fell to the left, four-point indicated that mouse did not have spontaneous walking, five-point indicated that mouse had depressed level of consciousness.

2.4. Immunofluorescence

We obtained coronal brain sections and performed immunofluorescence staining as stated previously [6,10,11]. After incubating the sections with 0.3% Triton X-100 in phosphate-buffered saline (PBST) and blocking them with 1% bovine serum albumin (BSA) dissolved in PBST, we stained them overnight at 4 °C with antibodies against ionized calcium-binding adapter molecule 1 (Iba-1; 1:400, Sigma-Aldrich) and CD16/CD32 (1:100, Novus Biologicals, Littleton, CO, USA), incubated them with appropriate secondary antibodies, and then mounted the stained sections with the mounting medium containing 4′,6′-diamidino-2-phenylindole (DAPI; Santa Cruz Biotech, Dallas, TX, USA). The investigator blinded to the treatment groups randomly chose three separate sections of each mouse and three non-overlapping 20× fields in *peri*-infarct regions for observation under a fluorescence microscope (ZEISS Scope A1, ZEISS, Germany).

2.5. Western blot analysis

We measured the protein level of IL1β following the previous protocol [6,10,11]. Briefly, we transferred the protein extracted from the right hemisphere to polyvinylidene difluoride membranes (Millipore, Bedford, MA), and incubated the membranes overnight at 4 °C with antibody against IL1β (1:2000, Abcam, Cambridge, MA, USA) and β-actin (1:5000, Affinity Biosciences, OH, USA) which served as the loading control. Protein bands were visualized by enhanced chemiluminescence kit (Affinity Biosciences). The optical density of the band was quantified by an investigator blinded to the experimental groups using Gel Analysis V 2.02 software (ClinX Science Instruments, Shanghai, China).

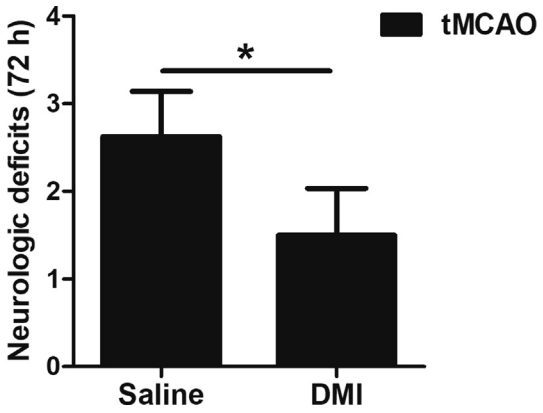

Fig. 1. DMI promotes recovery 3 days post tMCAO. The measurement of neurologic deficits indicated that DMI contributed to better neurologic function. (*$p < 0.05$, $n = 8$ per group). Data are shown as mean ± SD.

2.6. Statistical analysis

All data were analyzed using SPSS version 13.0. Results are expressed as mean ± SD. We used student's *t* test or one-way ANOVA followed by LSD test to assess differences in neurologic deficit score, immunofluorescence and Western blot analysis. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. DMI alleviates neurologic deficits after tMCAO

We assessed the neurologic deficit score on day 3 post stroke, and found that the DMI-treated mice had significantly lower score than did those treated with saline (Fig. 1).

3.2. DMI attenuates the toxic conversion of the microglia 3 days after tMCAO

Immunofluorescence staining showed that the number of the *peri*-infarct toxic M1 microglia labeled by Iba-1 and CD16/CD32, was notably fewer in the DMI-treated group than that in the saline-treated group (Fig. 2A, B). Further, DMI significantly limited the tMCAO-induced elevation of IL1β protein level (Fig. 2C, D).

4. Discussion

We studied whether the itaconate derivative DMI could be beneficial for the cerebral ischemia/reperfusion injury. Using tMCAO mouse model, we showed that DMI decreased neurologic deficits, indicating a potentially protective effect of DMI on ischemic stroke. Further, DMI inhibited the toxic M1 microglia polarization and decreased the expression of IL1β. These findings preliminarily suggest that DMI may likely be an option for the treatment of the ischemia/reperfusion-induced brain injury.

Itaconate is generated by immune responsive gene 1 (Irg1)-encoded enzyme [13]. Recent study characterized that itaconate was highly induced in lipopolysaccharide (LPS)-stimulated BMDMs and had the anti-inflammatory action [8,12]. DMI is a cell-permeable derivative of itaconate [8]. Although previous report defined that exogenous DMI couldn't mimic endogenous itaconate because of its rapid degradation in cells [14], recent study showed the structural similarity between itaconate and the well-known Sdh inhibitor malonate, and further identified that DMI could competitively inhibit Sdh [8]. In addition, DMI was currently showed to boost the expression of Nrf2 and its downstream target genes, and to inhibit inflammation in activated macrophages, functioning like the Nrf2 activator dimethyl fumarate

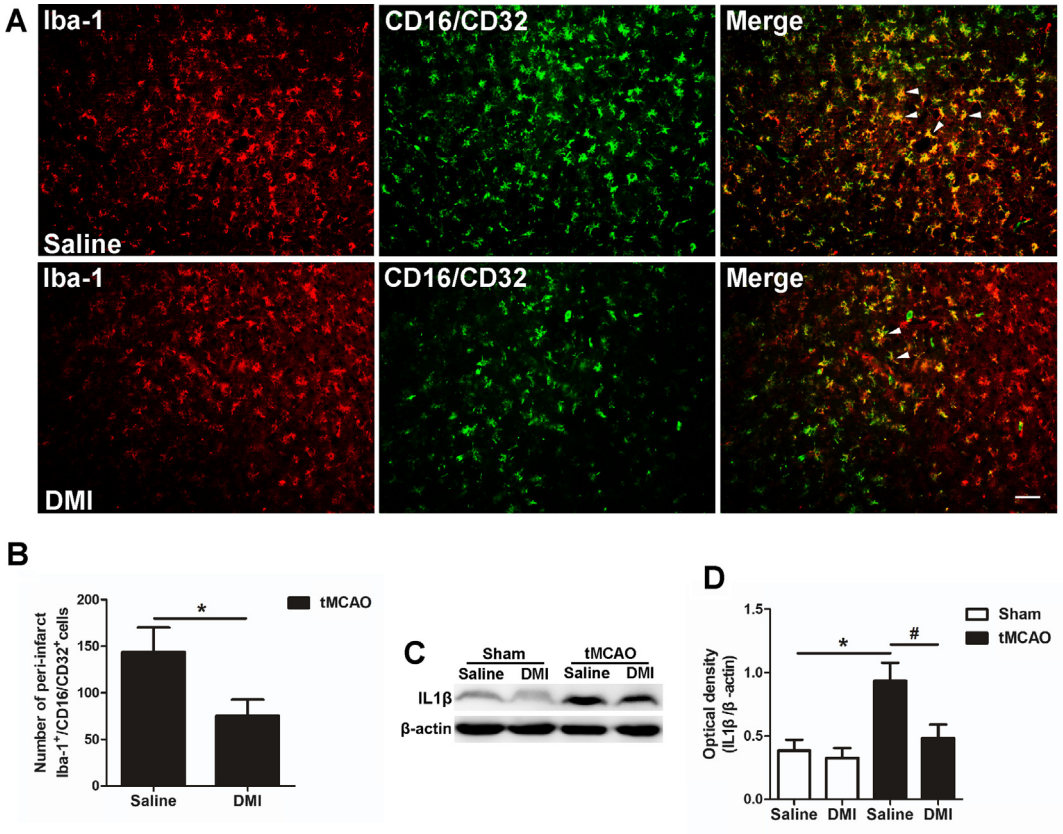

Fig. 2. Effect of DMI on the toxic M1 microglia. **(A)** Representative images of double immunostaining of the *peri*-infarct areas of the saline-treated and the DMI-treated tMCAO mice, depicting colocalization of Iba-1 (red) with CD16/CD32 (green). The white arrowheads show M1 microglia. Scale bar = 50 μm. **(B)** Quantification of the M1 microglial cell number per 20× field, showing that DMI inhibited the toxic polarization of the microglia 3 days after tMCAO. (*$p < 0.05$, $n = 8$ per group). **(C)** Western blot analysis of IL1β. **(D)** Quantification of IL1β expression in each group (*$p < 0.05$ vs. Saline-treated sham group, $^{\#}p < 0.05$ vs. DMI-treated tMCAO group, $n = 8$ per group). Data are shown as mean ± SD.

[9]. DMI was also identified to regulate IκBζ-ATF3 inflammatory axis [12]. Moreover, DMI was showed to lessen infarct area in the mouse model of myocardial ischemia/reperfusion injury [8]. In this study, we intraperitoneally administered DMI with a dose of 20 mg per mouse based on the published literature which proved a therapeutic effect of DMI in the psoriasis mouse model without toxic activity [12]. We demonstrated that the immediate DMI administration at the beginning of occlusion could obviously promote functional recovery 3 days post tMCAO, suggesting a potential therapeutic effect of DMI on ischemic stroke. Further investigations into the influence of DMI on brain infarct volume and the additional detailed behavior tests should be performed to better support the protective action of DMI.

Microglial cells respond rapidly to the interruption of cerebral blood flow and polarize into at least two subtypes termed M1 (pro-inflammatory) and M2 (anti-inflammatory) [15]. Previous study has revealed that the toxic M1 microglia, which could be recognized as Iba1/CD16/CD32-positive cells [16–20], play dominant role 3 days after brain ischemia [11,15]. We observed the role of DMI in the conversion of the toxic M1 microglia, and found that DMI apparently blocked this conversion, partly suggesting that DMI could suppress inflammation after stroke. Confocal images presented in a 3D format and flow cytometry showing the changes of microglial population's phenotype will be helpful to display the regulatory effect more clearly. It is well known that nucleotide-binding domain (NOD)-like receptor protein 3 (NLRP3) signaling contributes to the polarization of M1 microglia post ischemic stroke [15]. Recent study revealed that DMI inhibited NLRP3 activation in BMDMs [8], which suggests a possible mechanism underlying the effect of DMI on reactive microglia after stroke.

This study has some limitations. A major limitation is the lack of further investigation into the molecular mechanism(s) contributing to the negative role of DMI in the conversion of microglia after ischemic stroke. Although the finding of the assessment of neurologic deficits supported the DMI-mediated protection, more specific and long-time tests should be carried out. The optimized route, dose and time-window of DMI treatment needs further confirmation. Additionally, Study about the effect of DMI on the chronic pathophysiological processes of cerebral ischemia/reperfusion injury, such as angiogenesis and neurogenesis, and the mechanisms could provide more comprehension of itaconate and its derivatives.

5. Conclusion

In conclusion, we partly identified that DMI could promote functional recovery, and suppress the toxic polarization of the microglia in tMCAO mice. These findings might suggest DMI as a potential applicable agent for the treatment of cerebral ischemia/reperfusion injury.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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