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Tetramethylpyrazine inhibits neutrophil activation following permanent cerebral ischemia in rats

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ABSTRACT

Experimental studies have demonstrated the beneficial effects of tetramethylpyrazine (TMP) against ischemic stroke and highlighted its crucial role in anti-inflammatory activity. This study provides evidence of an alternative target for TMP and sheds light on the mechanism of its anti-inflammatory action against ischemic brain injury. We report a global inhibitory effect of TMP on inflammatory cell intracerebral activation and infiltration in a rat model of permanent cerebral ischemia. The results of immunohistochemistry, enzymatic assay, flow cytometric analysis, and cytological analysis revealed that intraperitoneal TMP administration reduced neuronal loss, macrophage/microglia activation, brain parenchyma infiltrative neutrophils, and circulating neutrophils after cerebral ischemia. Biochemical studies of cultured neutrophils further demonstrated that TMP attenuated neutrophil migration, endothelium adhesion, spontaneous nitric oxide (NO) production, and stimuli-activated NO production after cerebral ischemia. In parallel with these anti-neutrophil phenomena, TMP also attenuated the activities of ischemia-induced inflammation-associated signaling molecules, including plasma high-mobility group box-1 protein (HMGB1) and neutrophil toll-like receptor-4 (TLR4), Akt, extracellular signal-regulated kinase (ERK), and inducible nitric oxide synthase. Another finding in this study was that the anti-neutrophil effect of TMP was accompanied by a further elevated expression of NF-E2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) in neutrophils after cerebral ischemia. Taken together, our results suggest that both the promotion of endogenous anti-inflammatory defense capacity and the attenuation of pro-inflammatory responses via targeting of circulating neutrophils by elevating Nrf2/HO-1 expression and inhibiting HMGB1/TLR4, Akt, and ERK signaling might actively contribute to TMP-mediated neuroprotection against cerebral ischemia.

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1. Introduction

Stroke resulting from the interruption of cerebral blood circulation is a devastating and disabling neurological disorder worldwide. The cessation of cerebral blood circulation causes an acute ischemic brain injury for which treatment options are limited. Although early perfusion strategies remain the best treatment option, they can only provide limited clinical benefit because of the narrow therapeutic window and potential reperfusion-accompanied inflammatory responses [1]. Despite the complicated pathogenesis of ischemic stroke, neuroinflammation is assumed to be essential for the primary and secondary progression of brain injury [2,3]. Supporting evidence further shows that anti-inflammatory treatments and immune deficits lead to better outcomes in ischemic stroke [4–7]. Therefore, inflammatory mechanisms represent a key target of current translational cardiovascular

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researches and these phenomena highlight the importance of better understanding of post-stroke neuroinflammation in the development of therapeutic strategies.

In addition to brain resident macrophages/microglia, hematogenous leukocytes have been shown to play a pivotal role in post-stroke neuroinflammation. Among white blood cells, neutrophils have attracted much interest recently and have been intensively studied. The recruitment of neutrophils to brain parenchyma is found in ischemic brain injury [8]. The neutrophil-to-lymphocyte ratio is a prognostic marker in ischemic cerebrovascular diseases and the depletion or functional inhibition of neutrophils leads to decreased neuroinflammation and neuronal apoptosis in stroke subjects [5,7,9,10]. Despite the potential compromise of the blood-brain barrier (BBB) in ischemic brain injury, this structure still remains a determinant barrier of therapeutic drug feasibility. Therefore, peripheral circulating neutrophils might be a practical target for intervention.

Ligusticum wallichii Franchat (Chuan Xiong) has traditionally been used to treat neurovascular and cardiovascular diseases. One of its active ingredients, 2,3,5,6-tetramethylpyrazine (TMP), is widely used in the treatment of ischemic stroke [11]. The beneficial effects of TMP and its analogue against ischemic brain injury have been demonstrated in diseased animals [12–14]. Apart from vascular, anti-apoptotic, and antioxidant effects, anti-inflammatory effects are assumed to be crucial for the neuroprotective actions of TMP [15–17]. The above mentioned animal studies showed an association between decreased neutrophil infiltration and TMP neuroprotection; however, the impact and potential role of TMP in the regulation of neutrophil activity remain to be elucidated. Our previous reports demonstrated that systemic TMP administration had neuroprotective, anti-apoptotic, antioxidant, and anti-inflammatory activities in both cerebral ischemia/reperfusion and permanent cerebral ischemia rodent models [18–20]. To extend the scope of relevant studies, we therefore wanted to examine whether systemic TMP treatment would alleviate post-stroke neuroinflammation by reducing circulating neutrophil activation after permanent cerebral ischemia, and if it did, to determine the intracellular characteristics of the beneficial anti-neutrophil response.

2. Materials and methods

2.1. Animals and cerebral ischemia

Seventy-two male Sprague–Dawley rats (250–300 g) were randomly allocated into sham and ischemia groups and then further divided into vehicle treatment and TMP treatment subgroups. Rats were anesthetized with 4% isofurane. Permanent cerebral ischemia was produced by occluding the common carotid arteries and the right middle cerebral artery, as described previously [19]. In sham operations, all surgical procedures except arterial occlusion were the same as those mentioned above. TMP (20 mg/kg) or saline vehicle was injected intraperitoneally twice, 30 min before and 60 min after the occlusion. All animals were sacrificed 3 days after surgery and subjected to further analyses. The protocol of this animal study was approved by the Ethics Committee of Taichung Veterans General Hospital.

2.2. Isolation and analysis of blood leukocytes

Rats (6 animals/group) were euthanized and blood was withdrawn from the left femoral artery via intra-arterial catheterization. The types of white blood cells were identified through blood smears followed by Liu's stain. Some of the plasma samples were kept at −70 °C until use. Neutrophils were purified by dextran sedimentation, then centrifuged through Ficoll-Hypaque according to our previously reported protocols [21]. Analysis of neutrophil migration was performed with a modified 24-well Transwell after the cells were labeled with calcein AM. The labeled neutrophils (1×10^6) were added to the upper well of the chamber, which was separated from the lower well by 3-μm pore polycarbonate filters. RPMI containing 0.1 μM of fMLP was added to the lower well and the chamber was incubated for 1 h at 37 °C. Migrating cells attached to the lower surfaces were evaluated by measuring the fluorescent signals (E_x 488 nm and E_m 538 nm). Murine SVEC endothelial cells (ATCC® CRL-2181™) were plated onto 24-well plates until they reached confluence. The labeled neutrophils (1×10^6) were added to the monolayers of SVEC cells for 30 min. After the unattached cells were washed, the level of neutrophil adhesion was evaluated by measuring the fluorescent signals. For nitric oxide (NO) analysis, the obtained neutrophils were incubated with RPMI alone or stimulated with lipopolysaccharide (LPS, 10 ng/ml)/interferon-gamma (IFN-γ, 10 U/ml) for 12 h. The supernatants were collected and subjected to measurement of nitrite/nitrate using Griess reagent.

2.3. Flow cytometry

Rats (6 animals/group) were euthanized and the ipsilateral and contralateral cortical tissues were collected. The dissected tissues were dissociated into single cells and the inflammatory cells were collected by overlaying the dissociated materials on a Percoll gradient in accordance with reported protocols [19]. For the detection of neutrophils, the isolated cells were washed in PBS and stained with monoclonal antibody against CD45 and Ly6G (BD Biosciences, San Diego, CA). Characterization of antibody-labeled cells was performed on a BD FACSCalibur flow cytometer using Cell Quest software.

2.4. Myeloperoxidase activity (MPO) assay

Rats (6 animals/group) were euthanized and the ipsilateral and contralateral cortical tissues were collected. A commercially available MPO colorimetric activity assay kit (BioVision, Milpitas, CA) was used to measure MPO activity. The extraction of proteins and the enzymatic assay were carried out according to the manufacturer's instructions.

2.5. Western blot

Rats (6 animals/group) were euthanized and the circulating neutrophils and plasma were isolated. Protein extracts of neutrophils and plasma samples were resolved by SDS-PAGE, and transferred onto a PVDF membrane. The membranes were incubated with antibodies against inducible nitric oxide synthase (iNOS), toll-like receptor-4 (TLR4), high-mobility group box-1 protein (HMGB1), NF-E2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), Akt, phosphorylated Akt, extracellular signal-regulated kinase (ERK), phosphorylated ERK, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, Santa Cruz, CA). The immunoreactive protein bands were recognized by a horseradish peroxidase-conjugated secondary antibody and visualized by enhanced chemiluminescence. The intensity of each band was quantified by densitometry.

2.6. Immunohistochemistry

Rats (6 animals/group) were euthanized and perfused with heparinized PBS, followed by perfusion with 10% formalin in PBS [18]. A series of 8 μm-thick paraffin sections of brain tissues were

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