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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.

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

subjected to immunohistochemistry with antibodies against NeuN and CD68 (Serotec, Raleigh, NC). The cell nuclei were stained with hematoxylin. The immunoreactive signals were detected by incubation with biotinylated secondary antibody followed by avidin-biotin-peroxidase complex. Diaminobenzidine was used as a color substrate. For quantification, the number of immunoreactive signals was counted in 2 randomly selected fields per section in 6 continuous sections at a magnification of x 200.

2.7. Statistical analysis

The data are expressed as mean values ± standard deviation. Statistical analysis was carried out using one-way analysis of variance (ANOVA), followed by Dunnett's test to assess the statistical significance between treated and untreated groups. A level of $p < 0.05$ was considered statistically significant.

3. Results

3.1. TMP reduces ischemia-induced neuronal loss and inflammatory cell intracerebral infiltration

In the present study we used the same TMP treatment protocols and rat model of permanent cerebral ischemia as in our previous study [19]. There were no significant differences in physiological values for pH, pO_2, pCO_2, and hematocrit (%) among all groups (data not shown). The number of NeuN-positive cells, a specific neuronal nuclear protein, was maintained at a range of 64–83 positivity per field without statistical difference in all operated contralateral and sham-operated ipsilateral corresponding cortical areas. Loss of NeuN immunoreactivity was found in ischemic ipsilateral cortical areas, as evidenced by the results of immunohistochemistry (32 ± 17/field, $p < 0.01$, Fig. 1A). The loss of NeuN immunoreactivity in ipsilateral hemispheres was attenuated by TMP treatment (48 ± 12, $p < 0.05$, Fig. 1A). There was minimal detection of CD68-positive activated macrophages/microglia in all cortical areas except ischemic ipsilateral hemispheres. After the analysis of continuous sections obtained from animals in different ischemic groups, the corresponding slices of brain tissue treated with saline vehicle (33 ± 12/field) and TMP (18 ± 10/field, $p < 0.05$) showed reduction of CD68 immunoreactivity after TMP treatment (Fig. 1A). To elicit the effect of TMP on intracerebral neutrophil infiltration, neutrophil-associated MPO activity and $Ly6G^+/CD45^+$ neutrophil population were compared by enzymatic assay and fluorescence-activated cell sorting (FACS) analysis, respectively. The results showed that MPO activity (Fig. 1B) and the percentage of $CD45^+/Ly6G^+$-positive leukocytes (Fig. 1C) were significantly elevated in the ipsilateral hemispheres after ischemia and the increases were alleviated by TMP treatment.

3.2. TMP reduces ischemia-induced activation of circulating neutrophils

Since intracerebral neutrophils originate from hematogenous leukocytes, our next experiments were designed to analyze leukocyte activity in the bloodstream. Analysis of blood smear followed by Liu's stain (Table 1) revealed that the number of circulating leukocytes and the percentage of neutrophils increased but the percentage of lymphocytes decreased in ischemic groups when compared with sham groups. There was little effect on the percentage of monocytes after ischemia. However, an improvement of these changes was found in the TMP-treated ischemic group. Circulating neutrophils isolated from ischemic groups showed a greater preference for endothelium adhesion (Fig. 2A), fMLP chemotaxis (Fig. 2B), spontaneous NO production (Fig. 2C), and basal iNOS protein expression ($p < 0.01$, Fig. 2D) than circulating neutrophils isolated from sham groups. TMP had an inhibitory effect on neutrophils in ischemia-activated but not sham-operated animals (Fig. 2A–D). These changes of neutrophil activities were paralleled by upregulation of several intracellular regulatory molecules [4–6,19,22], including Akt phosphorylation ($p < 0.01$, Fig. 2D), ERK phosphorylation ($p < 0.05$, Fig. 2D), Nrf2 expression ($p < 0.05$, Fig. 2D), and HO-1 expression ($p < 0.05$, Fig. 2D) in ischemic groups. TMP attenuated ischemia-upregulated Akt phosphorylation ($p < 0.01$, Fig. 2D) and ERK phosphorylation ($p < 0.05$, Fig. 2D) but caused further elevation of Nrf2 protein expression ($p < 0.05$, Fig. 2D) and HO-1 protein ($p < 0.05$, Fig. 2D).

3.3. TMP impairs ischemia-activated neutrophil stimulation

The above mentioned findings seemed to imply that TMP had a minimal effect on naïve neutrophils but tended to decrease neutrophil susceptibility to insult stimulation. Supporting evidence showed that *in vitro* stimulation with LPS/IFN-γ only caused further NO production in neutrophils isolated from ischemic groups and the increase was attenuated by TMP treatment (Fig. 3A). In parallel with up-regulation of intracellular molecules (Fig. 2D), Nrf2/HO-1 axis activator sulforaphane, MEK/ERK inhibitor U0126, and PI3K/Akt inhibitor LY294002 attenuated LPS/IFN-γ-stimulated NO production in neutrophils isolated from the ischemic vehicle group (Fig. 3B). Evidence suggests a role for HMGB1 and TLR4 signaling in cerebral ischemia-induced injury and neuroinflammation [6,23]. Results of Western blot revealed elevated expression of neutrophil TLR4 ($p < 0.05$, Fig. 3C) and plasma HMGB1 ($p < 0.05$, Fig. 3D) in ischemic groups. The up-regulation of neutrophil TLR4 ($p < 0.05$, Fig. 3C) and plasma HMGB1 ($p < 0.05$, Fig. 3D) was alleviated by TMP treatment.

4. Discussion

Experimental studies have demonstrated the beneficial effects of TMP against ischemic brain injury in rodent models of stroke, suggesting a pivotal role of TMP in anti-inflammatory activity [12–17]. Through systemic administration, our previous studies showed the resolution of neuronal apoptosis, oxidative stress, and cellular inflammatory responses in TMP-treated ischemic rats [18–20]. Despite the intensive investigation of inflammatory cell activation, pro-inflammatory cytokine overproduction, and accompanying intracellular regulatory molecules in brain parenchyma, the effects of TMP on post-stroke circulating neutrophils have been largely unexplored. To gain insights into the cellular inflammatory responses involved, we found that peripheral action of TMP that desensitized circulating neutrophils refractory to cerebral ischemic insult was accompanied by decreased intracerebral neutrophil infiltration, neuroinflammation, and neuronal loss. The immunosuppression or immune desensitization activated by TMP in ischemic rats was paralleled by a down-regulation of HMGB1, TLR4, and iNOS/NO expression, Akt and ERK phosphorylation as well as further up-regulation of Nrf2 and HO-1 expression. Current findings suggest that an alternative mechanism of the anti-inflammatory effect of TMP against cerebral ischemia might be activated by targeting circulating neutrophils. Despite the documentation of TMP's characteristic actions, the precise anti-inflammatory mechanisms of TMP against cerebral ischemia require further investigation.

Among the leukocytes, neutrophils play an essential role in building up a rapid innate immune response to combat invading pathogens through phagocytosis, release of proteolytic enzymes, and generation of reactive oxygen species. Under normal physiological conditions, the number and activation of neutrophils are

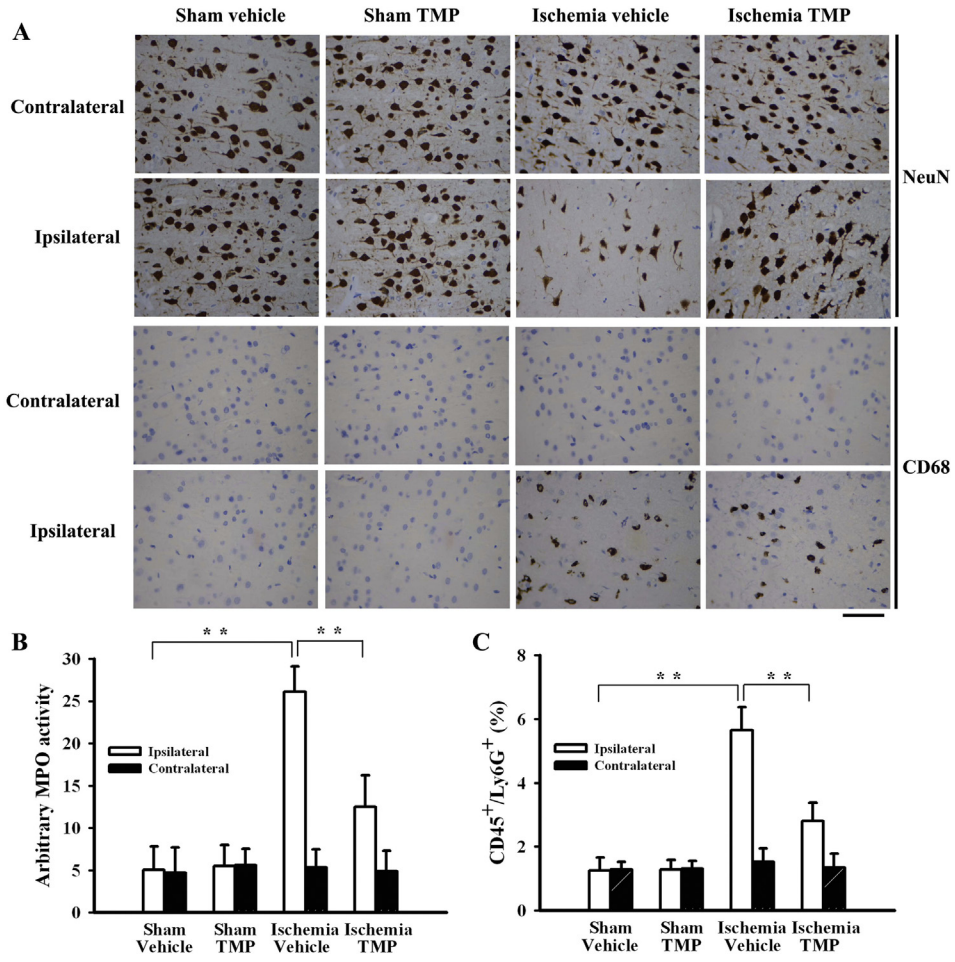

Fig. 1. TMP reduces ischemia-induced neuronal loss and inflammatory cell activation. (A) The paraffin sections of brain tissues obtained from sham and ischemic animals were subjected to immunohistochemistry with antibodies against NeuN and CD68 and counterstained with hematoxylin. Representative images show the signals in the core area of the ipsilateral cortex and the corresponding area of the contralateral cortex. Scale bar: 60 μm. (B) Cortical homogenates (ipsilateral and contralateral) from sham and ischemic animals were subjected to enzymatic assay for the measurement of MPO activity. (C) Leukocytes were isolated from sham and ischemic animals (ipsilateral and contralateral cortexes) and subjected to flow cytometric analysis. The average percentage of $CD45^+/Ly6G^+$ cells is depicted. $^{**}p < 0.01$ (n = 6/each group).

tightly controlled to avoid unexpected inflammatory damage [25]. In response to local tissue infection, inflammation, or damage, neutrophils undergo proliferation and activation and are rapidly mobilized. Although the onset varies, the intracerebral neutrophil infiltration is found in all types of cerebral ischemic injury [8]. Accumulating evidence suggests this intracerebral neutrophil infiltration has potentially damaging pro-inflammatory consequences in cerebral ischemia and highlights a crucial role of neutrophils in the prevention and treatment of stroke [5,7,9,10]. A recent study also showed that neutrophils and neutrophil-derived iNOS contribute to ischemic brain injury [26]. In accordance with those findings, our results showed that permanent cerebral ischemia increased the number of circulating neutrophils, chemotaxis, and endothelium adhesion, up-regulated neutrophil iNOS/NO expression, and promoted neutrophil intracerebral infiltration. TMP had an inhibitory effect on all levels of neutrophil activation after cerebral ischemia. Therefore, neutrophils might be an action target of TMP for suppression of neuroinflammation after cerebral ischemia.

Cerebral ischemia is a complicated disorder with a sterile inflammatory reaction [7]. Thus, damage-associated molecular patterns derived from the course of permanent cerebral ischemia are

Table 1
Effects of TMP on circulating white blood cells (WBCs).

	Sham		Ischemia	
	Vehicle	TMP	Vehicle	TMP
Blood WBCs (x 10^6/ml)	4.0 ± 0.6	4.3 ± 1.1	7.2 ± 1.1**	5.0 ± 1.1$^{\#\#}$
Differential WBCs (%)Monocytes	1.0 ± 0.9	0.9 ± 0.7	0.4 ± 0.3	0.6 ± 0.6
Lymphocytes	80.5 ± 3.7	81.9 ± 2.2	56.5 ± 6.8**	76.2 ± 4.8$^{\#\#}$
Neutrophils	14.6 ± 2.9	14.8 ± 3.1	41.3 ± 8.0**	21.0 ± 3.7$^{\#\#}$

Rats were subjected to sham operation or permanent ischemia receiving saline vehicle or TMP administration (30 min before occlusion and 60 min after occlusion, 20 mg/kg). The blood samples were collected and subjected to WBC counting and typing. Data are expressed as means ± SD, n = 6. $^{**}p < 0.01$ vs. sham vehicle and ##p < 0.01 vs. ischemia vehicle.

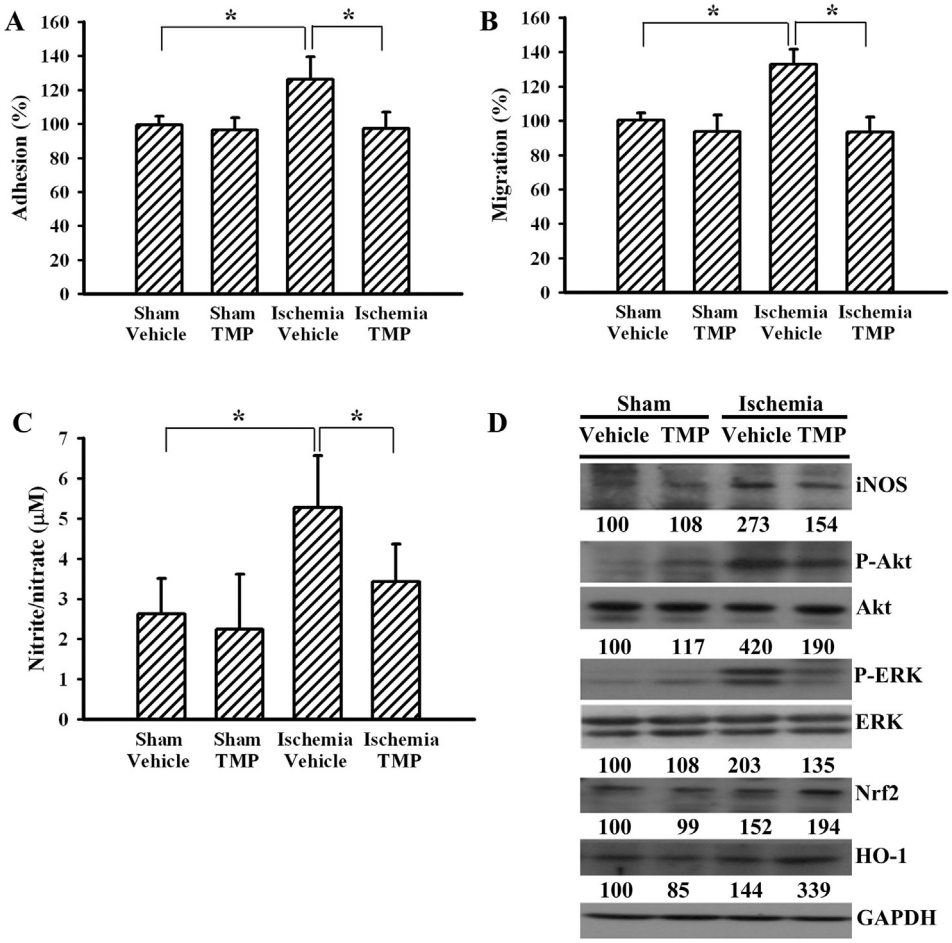

Fig. 2. TMP reduces ischemia-induced neutrophil activation. Circulating neutrophils were isolated from blood and subjected to cell adhesion (A) and migration (B) assay. (C) The obtained neutrophils were plated onto 96-well plates for 12 h and the supernatants were subjected to nitrite/nitrate measurement. (D) Proteins were extracted from the obtained neutrophils and subjected to Western blot analysis with the indicated antibodies. One representative blot is shown. The fold of relative protein content is depicted under the panel (the first lane was defined as 100%). *$p < 0.05$ ($n = 6$/each group).

of importance to initiate activation of cascades of circulating neutrophils. Harakawa et al. [23] reported that cerebral ischemia elevated HMGB1 expression and increased plasma HMGB1 levels leading to neutrophil activation. Permanent cerebral ischemia increased plasma levels of HMGB1 and elevated neutrophil TLR4 expression, an action receptor of HMGB1. Therefore, the anti-neutrophil effect of TMP could be a consequence of suppression of HMGB1/TLR4 signaling. TMP can traverse the BBB to the brain parenchyma [24]; thus, the inhibition of neutrophil HMGB1/TLR4 signaling could be secondary to TMP's neuroprotective effect. Specific blockade of intracellular signaling events critical to neutrophil activation might be another possibility.

The engagement of HMGB1 and its receptor TLR4 triggers an intracellular signaling cascade resulting in altered gene expression and cell activation. The activation of the HMGB1/TLR4 axis and its downstream effectors Akt and ERK has been implicated in the pathogenesis of cerebral ischemia, expression of pro-inflammatory cytokines, and activation of neutrophils [5,6,22,23]. Thus, our findings suggest that the attenuation of neutrophil activation and iNOS/NO expression by TMP could be a result of inhibition of Akt and ERK activities. It should be noted that Akt and ERK can cause convergence of multiple extracellular and intracellular signals to regulate gene expression and cell activity. That is, the interruption of Akt and ERK signaling by TMP might be independent of HMGB1/TLR4 inhibition. In addition to the studies of Akt and ERK signaling, other studies showed that Nrf2 and HO-1 are activated upon inflammatory stimulation and play a regulatory role in neuronal cell survival and inflammatory response in the brain. Silencing of Nrf2 or HO-1 sensitizes animals to, and their overexpression or activation reduces, cerebral ischemia-induced neuroinflammation and injury [27–29]. An inverse relationship has been demonstrated between Nrf2/HO-1 and signaling molecules and transcription factors [27,29]. Our previous findings showed that TMP further elevated expression of Nrf2/HO-1 in ipsilateral neurons and macrophages/microglia after permanent cerebral ischemia [19]. And, we also found that TMP caused further expression of Nrf2/HO-1 in circulating neutrophils after permanent cerebral ischemia. In consideration of the above mentioned Nrf2/HO-1 biological activities, Nrf2/HO-1 is assumed to be an action target of TMP by which it attenuates intracellular signaling crucial to neutrophil activation and/or enhances neutrophil resistance to activation. Our findings also showed that the regulation of Nrf2/HO-1 expression might be tightly controlled. TMP treatment was not sufficient to induce the expression of Nrf2/HO-1 in naïve neutrophils; however, it promoted the expression of Nrf2/HO-1 after ischemic insult. The regulatory signature of Nrf2/HO-1 expression by TMP requires further investigation.

Unlike macrophages/microglia, neutrophils are relatively refractory to LPS stimulation [22]. Another interesting finding in this study was that permanent cerebral ischemia turned LPS-resistant

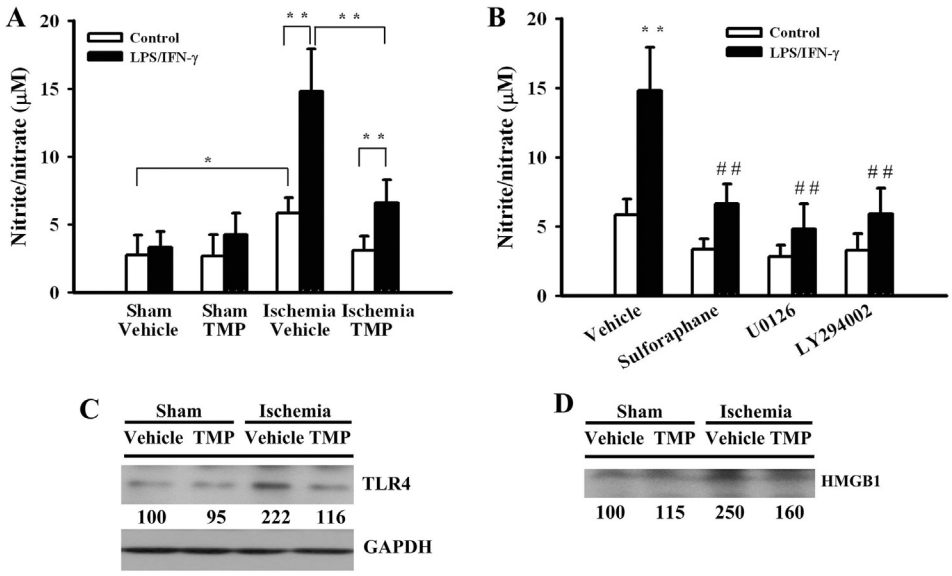

Fig. 3. TMP reduces ischemia-activated TLR4 signaling in neutrophils. Circulating neutrophils were isolated from blood. (A) The obtained neutrophils were plated onto 96-well plates for 12 h in the absence or presence of LPS (10 ng/ml)/IFN-γ (10 U/ml) and the supernatants were subjected to nitrite/nitrate measurement. $^{*}p < 0.05$ and $^{**}p < 0.01$ (n = 6/each group). (B) The neutrophils obtained from the ischemia/vehicle group were plated onto 96-well plates, pretreated with vehicle, sulforaphane (10 μM), U0126 (10 μM), or LY294002 (10 μM) for 1 h, and exposed to LPS (10 ng/ml)/IFN-γ (10 U/ml) for an additional 12 h. The supernatants were subjected to nitrite/nitrate measurement. $^{**}p < 0.01$ vs. vehicle control and ##p < 0.01 vs. vehicle LPS/IFN-γ, n = 6. (C) Proteins were extracted from the obtained neutrophils and subjected to Western blot analysis with the indicated antibodies. (D) Plasma samples were subjected to Western blot analysis with the indicated antibodies. One representative blot is shown (n = 6/each group). The fold of relative protein content is depicted under the panel (the first lane was defined as 100%).

neutrophils into LPS-sensitive neutrophils. Cerebral ischemia initiates multiple cellular and biochemical events and these alterations are supposedly able to form a microenvironment that favors cell activation or inactivation. Thus, we hypothesized that the up-regulation of TLR4 in neutrophils after ischemia might be a possibility. This hypothesis and other potential hypotheses should be fully investigated.

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. One limitation of this study was that our data on the mechanisms of the effects of TMP were based on associations and thus it was not possible to establish causal relationships. Therefore, further studies are required to determine the precise anti-neutrophil and action signaling mechanisms of TMP.

Conflict of interest

None.

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Transparency document

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