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ORIGINAL ARTICLE

The Presence of High Levels of Circulating Trimethylamine *N*-Oxide Exacerbates Central and Peripheral Inflammation and Inflammatory Hyperalgesia in Rats Following Carrageenan Injection

Yanan Zhang,[1] Chunlian Zhang,[1] Haiou Li,[1] and Jingdong Hou[1,2]

Abstract— Gut microbiota-derived metabolite trimethylamine *N*-oxide (TMAO) has recently been shown to promote inflammation in peripheral tissues and the central nervous system (CNS), contributing to the pathogenesis of various human diseases. Here, we examined whether the presence of high levels of circulating TMAO would influence central and peripheral inflammation and inflammatory hyperalgesia in a carrageenan (CG)-induced rat model of inflammation. Rats were treated with vehicle or TMAO in drinking water. After 2 weeks of treatment, rats received intraplantar injection of saline or CG into the hind paw. Acute nociception was unaltered in TMAO-treated rats that had elevated plasma TMAO. Following CG injection, TMAO-treated rats were significantly more sensitive to thermal and mechanical stimulation of the inflamed paw and displayed greater paw edema. Molecular studies revealed that CG injection induced increases in recruitment of neutrophils/macrophages in the paw and activation of microglia in the spinal cord, along with increased activation of nuclear factor (NF)-kB and production of proinflammatory mediators in both vehicle-treated rats and TMAO-treated rats. However, the increases in the above parameters were more pronounced in TMAO-treated rats. Moreover, TMAO treatment decreased protein levels of anti-inflammatory mediator regulator of G protein signaling (RGS)-10 in both saline-injected rats and CG-injected rats. These findings suggest that the presence of high levels of circulating TMAO downregulates anti-inflammatory mediator RGS10 in both peripheral tissues and the CNS, which may increase the susceptibility to inflammatory challenge-induced NF-kB activity, leading to greater increase in production of inflammatory mediators and consequent exacerbation of peripheral inflammation and inflammatory hyperalgesia.

KEY WORDS: inflammatory hyperalgesia; trimethylamine *N*-oxide; NF-kB activity; inflammation; regulator of G protein signaling-10.

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INTRODUCTION

Inflammation is often associated with ongoing pain and increased sensitivity to stimuli, called hyperalgesia [1–3]. Following tissue injury or infection, circulating and resident immune cells, such as neutrophils and macrophages, are recruited rapidly into the inflamed sites where they release various inflammatory mediators including interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, and cyclooxygenase (COX)-2-derived prostaglandin E (PGE) 2 [1–3]. These inflammatory mediators can stimulate and sensitize nociceptors and increase the excitability of nociceptive primary afferent neurons (peripheral sensitization) [1–3]. In addition, the hyperexcitability of peripheral nociceptive neurons can induce activation of spinal glial cells to produce inflammatory mediators in the spinal cord, leading to the hyperactivity and hyperexcitability of spinal neurons (central sensitization) that are required for the maintenance of hyperalgesia [1–3]. Of note, the presence of some risk factors may enhance central and peripheral inflammation and inflammatory hyperalgesia [4, 5].

Recent evidence reveals that gut microbiota can influence pain *via* modulating inflammatory response in the peripheral and central nervous system [6, 7]. Trimethylamine *N*-oxide (TMAO), a gut microbiota-dependent metabolite of specific dietary nutrients, has been implicated in the pathogenesis of various human diseases, including metabolic, cardiovascular, and neurological disorders [8–10]. Elevated circulating TMAO causes inflammatory response in many peripheral tissues including the heart, aorta, and kidney [11–14]. Moreover, experimental and clinical studies have demonstrated elevated circulating TMAO levels in multiple diseases that are associated with augmented inflammation and inflammatory hyperalgesia in inflammatory state, such as obesity and diabetes [4, 5, 11, 15]. TMAO can rapidly cross the blood–brain barrier [16], and a recent study reported that the presence of high levels of circulating TMAO exacerbates microglia-mediated neuroinflammation in the brain in rats following surgical trauma, resulting in exaggeration of cognitive dysfunction [17]. Here, we examined whether the presence of high levels of circulating TMAO levels would influence peripheral inflammation and inflammatory hyperalgesia in a carrageenan (CG)-induced rat model of inflammation.

METHODS

Animals

All experiments were approved by the Institutional Animal Care and Use Committee of Jining No. 1 People's Hospital (No:20170038). Male Wistar rats (200–250 g) were purchased from Beijing Laboratory Animal Research Center (Beijing, China). Animals were individually housed in a climate-controlled room, with a 12-h light–dark cycle. Standard rodent chow pellets and water were provided *ad libitum*.

Experimental Protocol

Rats were treated with vehicle (VEH, tap water) or TMAO (120 mg/kg in tap water). After 2 weeks of treatment, tail vein blood samples were collected to confirm elevated TMAO levels in plasma. Rats then received intraplantar injection of saline (control) or CG (100 μl of 2% in saline) into the mid-plantar region of the right hind paw as described previously [18], leading to four experimental groups ($n = 10$ per group): (1) VEH-treated rats injected with saline (VEH + saline), (2) VEH-treated rats injected with CG (VEH + CG), (3) TMAO-treated rats injected with saline (TMAO + saline), and (4) TMAO-treated rats injected with CG (TMAO + CG). The dose of TMAO used in this study was based on previous studies [19]. The thermal and mechanical responses and paw volume on ipsilateral hind paw that received CG injection were measured before (baseline, time 0) and 2, 5, and 24 h after saline or CG injection. In addition, the rotarod test was performed before and 24 h after CG injection. At the termination of the study protocol, rats were euthanized by intraperitoneal injection of 200 mg/kg sodium pentobarbital to collect blood, paw, and spinal cord tissues for biochemical and molecular studies.

Behavioral Tests

Behavioral tests, including responses to thermal and mechanical stimulation, were performed as described previously [4, 5, 20]. Briefly, 5 days prior to the beginning of the tests, animals were placed into the behavioral testing boxes for 30 min twice daily for acclimation to the testing environment. The response thresholds to thermal and mechanical stimulation of ipsilateral hind paw were measured immediately before (time 0) and 2, 5, and 24 h after saline or CG injection. A plantar test instrument (Ugo Basile, Comerio

VA, Italy) was applied to measure the response to thermal stimulation. The thermal stimulation was carried out by an infrared beam (IR intensity 50) which is directed onto the plantar surface of the hind paw, and the latency to paw withdrawal was recorded. A cut off of 25 s was applied to prevent tissue damage. Mechanical response thresholds were performed by a dynamic plantar esthesiometer (Ugo Basile, Comerio VA, Italy). The esthesiometer consists of a unit that raises a 0.5-mm diameter metal filament until it touches the plantar surface of the hind paw and begins to exert an upwards force until the paw is withdrawn. A cut off of 50 g was applied to prevent tissue damage. For both behavioral tests, at least three readings were taken on each paw at each time point and averaged for analysis. All behavioral tests were performed by a single experimenter who was unaware of the group allocations (under blind conditions) to avoid unconscious bias.

Paw Edema

A plethysmometer (Ugo Basile, Comerio VA, Italy) was used to assess paw edema as described previously [21]. The paw volume was calculated by measuring water displacement when the paw was submerged into a water cell. The volume of ipsilateral paw was measured before (baseline, time 0) and 2, 5, and 24 h after saline or CG injection. Edema was expressed as the increase in paw volume (mL) after CG injection relative to the baseline value.

Rotarod Test

The integrity of motor function was evaluated before (time 0) and 24 h after saline or CG injection, as described previously [5, 20]. The animals were placed on a rotating rod turning at 10 rpm, and the time (s) that the animals stayed on a rotating rod was measured automatically in each case for periods of approximately 180 s. The trial was performed five times for each animal and the average performance time was used for data analysis.

ELISA Assay

Rats were decapitated under deep anesthesia and the paw and spinal cord tissues were quickly removed. Tissue samples were homogenized in a mammalian tissue lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) including protease inhibitors. After centrifugation at 12,000×*g* for 15 min at 4 °C, supernatants were collected and protein concentrations were measured with the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). The protein levels of inflammatory mediators IL-1β, TNF-α, IL-6 and COX-2, neutrophil marker myeloperoxidase (MPO) and macrophage-specific marker CD 68, and anti-inflammatory mediator regulator of G protein signaling (RGS)-10 in the paw and spinal cord tissues were measured with ELISA kits (kits for IL-1β, TNF-α and IL-6: R&D systems, Inc., Minneapolis, MN, USA; Kits for COX-2 and MPO: Biocompare, South San Francisco, CA, USA; Kit for CD68: LSBio, Seattle, WA, USA; Kit for RGS10: Aviva Systems Biology, Beijing, China) according to the manufacturer's instructions.

Western Blot Analysis

Proteins extracted from the paw and spinal cord tissues were separated by 10% polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore Corporation, Bedford, MA, USA). The membranes were immunoblotted with primary antibodies to microglia marker ionized calcium-binding adaptor molecule 1 (IBA-1), phospho-NF-kappaB p65 (P-NF-kB p65), and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C followed by HRP-conjugated second antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. The immunoreactive bands were analyzed with ImageJ software (NIH, Bethesda, Maryland, USA), and all data were normalized to β-actin.

TMAO Measurement

Plasma levels of TMAO were determined by liquid chromatography coupled with triple-quadrupole mass spectrometry as described previously [22].

Statistical Analysis

All data were expressed as means ± SEM. Statistical analysis was made with GraphPad Prism 6.0 (GraphPad software for Science, San Diego, CA). A two-way ANOVA followed by a Bonferroni *post hoc* test was applied for statistical analysis. P values < 0.05 were considered statistically significant.

RESULTS

Plasma TMAO Levels Are Elevated in TMAO-Treated Rats Before and After CG Injection

After 2 weeks of TMAO treatment, plasma TMAO levels were similarly elevated in the two TMAO-treated groups compared with their respective VEH-treated groups before and after CG injection (Fig. 1). There were no

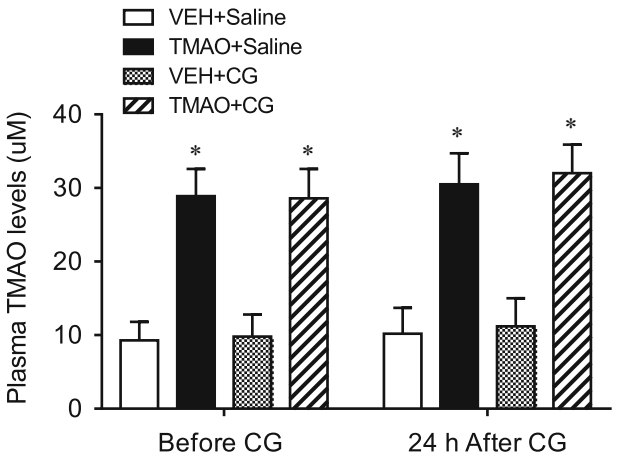

Fig. 1. Effects of TMAO treatment on plasma levels of TMAO before and 24 h after carrageenan (CG) injection. Values represent mean ± SEM (n = 10 for each group). *P < 0.05 *vs* VEH + saline or VEH + CG at each time point.

differences in plasma TMAO levels between the two TMAO-treated groups or between the two VEH-treated groups at both time points.

Inflammatory Hyperalgesia and Paw Edema Are Exacerbated in TMAO-Treated Rats Following CG Injection

As shown in Fig. 2, thermal hyperalgesia (Fig. 2a), mechanical allodynia (Fig. 2b) and paw edema (Fig. 2c) in the ipsilateral hind paw were comparable among the four experimental groups before CG injection (time 0). Intraplantar injection of CG but not saline induced significant thermal hyperalgesia, mechanical allodynia, and paw edema in the injected hind paw for at least 24 h in both VEH-treated rats and TMAO-treated rats.

In VEH-treated rats, paw withdrawal latency was significantly decreased from a baseline value (time 0) of 10.4 ± 0.7 to 7.3 ± 0.6 s at 2 h (P < 0.05 *versus* baseline) and 5.9 ± 0.5 s at 5 h (P < 0.01 *versus* baseline) following CG injection (Fig. 2a). Thermal hyperalgesia in TMAO-

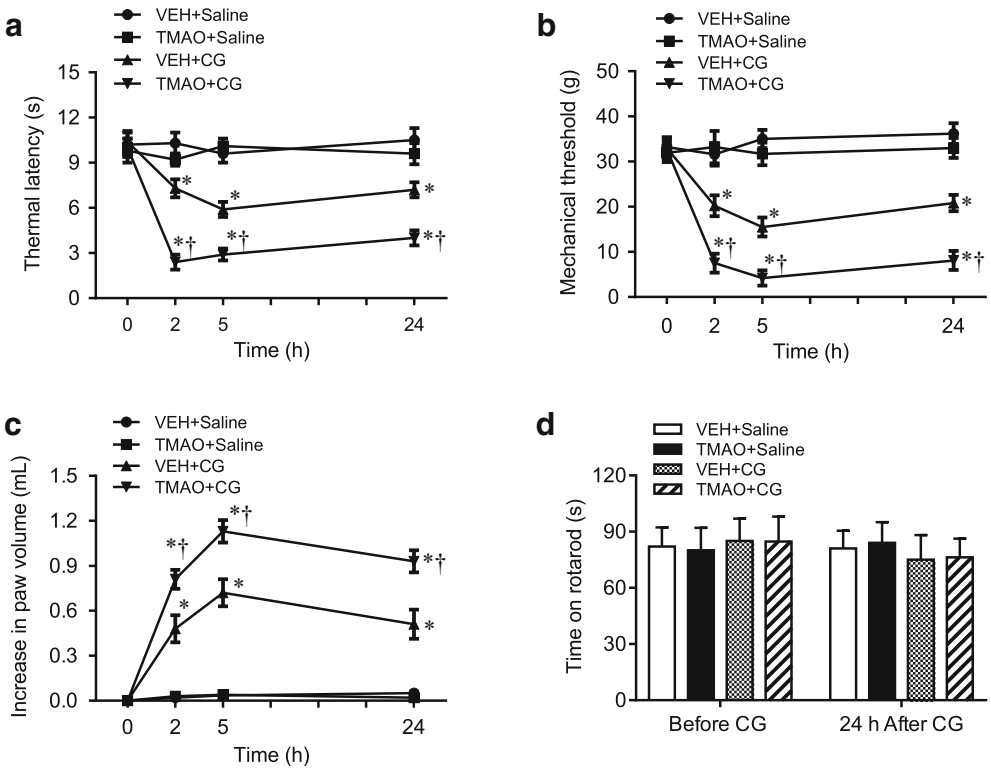

Fig. 2. Effects of TMAO treatment on paw withdrawal latency to thermal stimulation (**a**), paw withdrawal threshold to mechanical stimulation (**b**) and paw edema (**c**) before (baseline, time 0) and 2, 5, and 24 h after CG injection and effect of TMAO treatment on motor coordination before and 24 h after CG injection (**d**). Edema was expressed as the increase in paw volume (mL) after CG injection relative to the baseline value. Values represent mean ± SEM (n = 10 for each group). *P < 0.05 *vs* VEH + saline or TMAO + saline at each time point; †P < 0.05, TMAO + CG *vs* VEH + CG at each time point.

treated rats was significantly more pronounced than that in VEH-treated rats from 2 h following CG injection. Maximal thermal hyperalgesia in TMAO-treated rats was observed at 2 h, occurring earlier than in VEH-treated rats.

Mechanical withdrawal threshold in VEH-treated rats was markedly decreased from a baseline of 33.0 ± 2.4 to 20.2 ± 2.3 g at 2 h ($P < 0.05$ *versus* baseline) and 15.5 ± 2.1 g at 5 h ($P < 0.01$ *versus* baseline) following CG injection (Fig. 2b). Mechanical allodynia was significantly greater in TMAO-treated rats compared with VEH-treated rats at all time points after CG injection. The maximal decrease in mechanical withdrawal threshold was shown at 5 h following CG injection in both groups.

Compared with VEH-treated rats, TMAO-treated rats had greater paw edema from 2 h following CG injection. The increase in paw volume in VEH-treated rats reached a maximum of 0.72 ± 0.09 mL at 5 h following CG injection. In TMAO-treated rats, the increase in paw volume reached a maximum of 1.13 ± 0.08 mL at 5 h following CG injection.

The Presence of High Levels of Circulating TMAO Does Not Alter the Integrity of Motor Function

To exclude possible alteration in subtle systems that might confound the behavioral assessment, we evaluated the integrity of motor function using the rotarod test. As shown in Fig. 2d, there was no difference in time on the rotarod across the four groups at both baseline and 24 h following CG injection, indicating that elevated circulating TMAO did not affect motor coordination.

The Presence of High Levels of Circulating TMAO Enhances Expression of Inflammatory Mediators in the Paw and Spinal Cord in Rats Following CG Injection

Intraplantar injection of CG induced significant increases in protein levels of inflammatory mediators IL-1β (Fig. 3a), TNF-α (Fig. 3b), IL-6 (Fig. 3c), and COX-2 (Fig. 3d) in the paw and spinal cord in both VEH-treated rats and TMAO-treated rats, compared with intraplantar injection of saline at 24 h. Of note, the increases in protein levels of these inflammatory mediators in the paw and spinal cord were more pronounced in TMAO-treated rats as compared to VEH-treated rats following CG injection. There were no differences in protein levels of IL-1β, TNF-α, IL-6, and COX-2 in the paw and spinal cord between the two saline-injected groups.

The Presence of High Levels of Circulating TMAO Further Increases Recruitment of Neutrophils/Macrophages in the Paw and Enhances Activation of Microglia in the Spinal Cord in Rats Following CG Injection

The recruitment of neutrophils/macrophages to the inflamed site and activation of microglia in the spinal cord contribute to the production of various inflammatory mediators that elicit peripheral inflammation and inflammatory pain [2, 18]. We therefore measured protein levels of MPO (a marker of neutrophils) and CD68 (a marker of macrophage) in the paw and protein levels of IBA-1 (a marker of microglia) in the spinal cord. The two CG-injected groups, compared with their respective saline-injected groups, exhibited significantly increased protein levels of MPO (Fig. 4a) and CD68 (Fig. 4b) in the paw and protein levels of IBA-1 (Fig. 4c) in the spinal cord, but the levels for MPO, CD68, and IBA-1 were higher in the TMAO-treated group compared with VEH-treated group at 24 h after CG injection. Protein levels of MPO, CD68, and IBA-1 were not different between the two saline-injected groups.

The Presence of High Levels of Circulating TMAO Exaggerates Activation of NF-kB in the Paw and Spinal Cord in Rats Following CG Injection

NF-kB signaling pathway plays a pivotal role in regulating activity of neutrophils/macrophages and microglia as well as the subsequent production of inflammatory mediators [23–25]. We next evaluated the activity of NF-kB in the paw and spinal cord. Compared with their respective saline-injected groups, the two CG-injected groups had significantly increased protein levels of P-NF-kB p65 in the paw and spinal cord, with greater increases in TMAO-treated group at 24 h following CG injection (Fig. 4d). Protein levels of P-NF-kB p65 in the paw and spinal cord did not differ between the two saline-injected groups.

The Presence of High Levels of Circulating TMAO Downregulates RGS10 Expression in the Paw and Spinal Cord

RGS10 has recently been suggested to negatively modulate NF-kB activity and inflammatory response in macrophages and microglia [26, 27]. To further examine possible mechanisms by which TMAO exacerbates NF-kB-mediated inflammation after CG injection, we measured protein levels of RGS10 in the paw and spinal cord. As shown in Fig. 5, there were no differences in protein

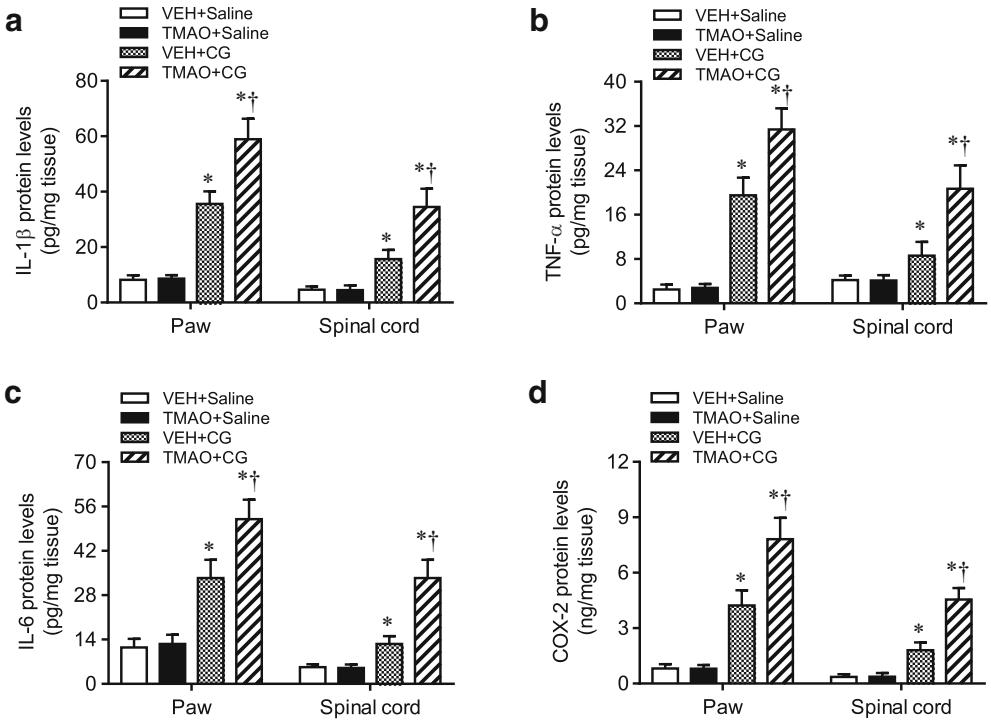

Fig. 3. Effects of TMAO treatment on protein levels of inflammatory mediator interleukin (IL)-1β (**a**), tumor necrosis factor (TNF)-α (**b**), IL-6 (**c**), and cyclooxygenase (COX)-2 (**d**) in the paw and spinal cord at 24 h after CG injection. Values represent mean ± SEM ($n = 10$ for each group). *$P < 0.05$ *vs* VEH + saline or TMAO + saline; †$P < 0.05$, TMAO + CG *vs* VEH + CG.

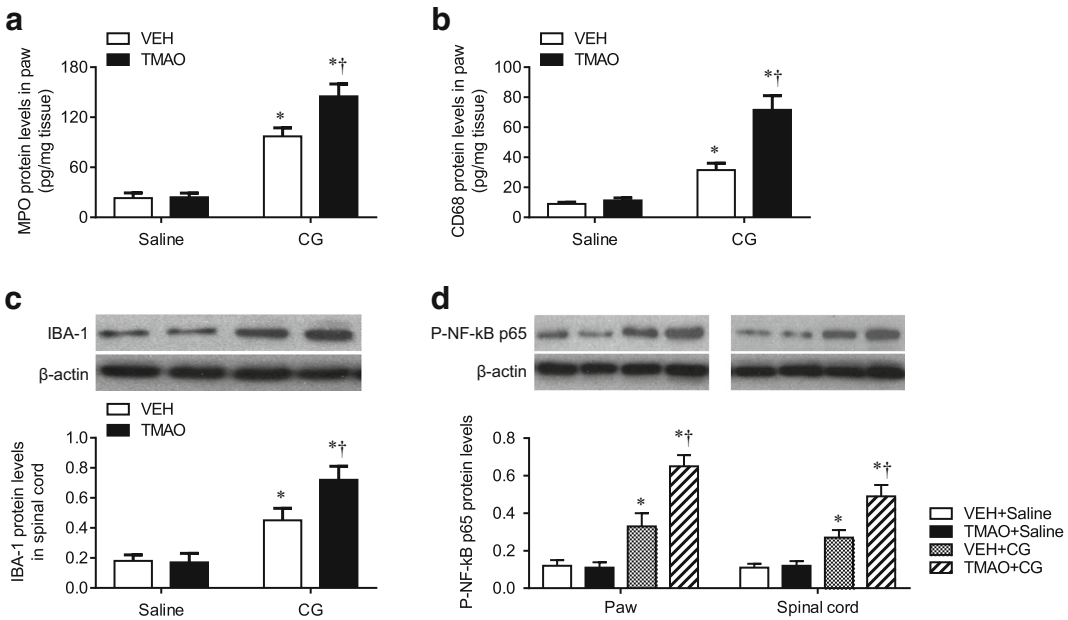

Fig. 4. Effects of TMAO treatment on myeloperoxidase (MPO, an indicator for recruitment of neutrophils, **a**) and CD68 (an indicator for recruitment of macrophages, **b**) in the paw and ionized calcium-binding adaptor molecule 1 (IBA-1, an indicator for activation of microglia, **c**) in the spinal cord, and expression of phospho (P)-NF-κB p65 (**d**) in the paw and spinal cord at 24 h after CG injection. Values represent mean ± SEM ($n = 10$ for each group). *$P < 0.05$ *vs* VEH + saline or TMAO + saline; †$P < 0.05$, TMAO + CG *vs* VEH + CG.

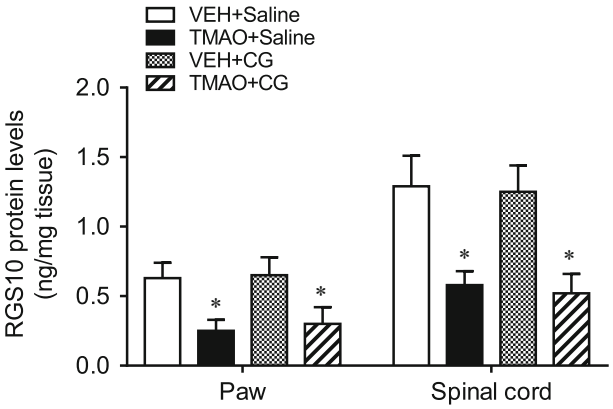

Fig. 5. Effects of TMAO treatment on anti-inflammatory mediator regulator of G protein signaling (RGS) 10 in the paw and spinal cord at 24 h after CG injection. Values represent mean ± SEM ($n = 10$ for each group). *$P < 0.05$ *vs* VEH + saline or VEH + CG.

levels of RGS10 in the paw and spinal cord between the two VEH-treated groups at 24 h after CG injection. However, TMAO treatment equally reduced protein levels of RGS10 in the paw and spinal cord in both saline-treated group and CG-treated group.

DISCUSSION

This study investigated the effect of elevated circulating TMAO on central and peripheral inflammation and inflammatory hyperalgesia in a CG-induced rat model of inflammation. The novel findings are that (1) the presence of high levels of circulating TMAO leads to more pronounced hyperalgesia and paw edema in rats following CG injection, (2) the presence of high levels of circulating TMAO causes greater increases in recruitment of neutrophils/macrophages in the paw and activation of microglia in the spinal cord, along with enhanced activation of NF-kB and production of proinflammatory mediators, and (3) the presence of high levels of circulating TMAO downregulates anti-inflammatory mediator RGS10 expression in the paw and spinal cord.

Recently, more attention has been paid to gut microbiota-dependent metabolite TMAO as it is closely associated with the pathogenesis of many human diseases, including obesity, type 2 diabetes mellitus, cardiovascular disease, renal dysfunction, and neurological disorders [8–15, 17]. TMAO is synthesized by the flavin monooxygenases 3 (FMO3) in the liver from trimethylamine (TMA), which is produced by the action of gut microbiota from dietary choline and phosphatidylcholine [28]. Under normal physiologic conditions, circulating TMAO is rapidly cleared by the kidney [29]. Alterations in gut microbiota composition (known as dysbiosis), FMO3 activity, and kidney function may lead to elevated circulating TMAO [30]. Previous studies have shown that average plasma TMAO concentrations are 5.8 μM in healthy control subjects [31] and are approximately 1.3 to 9.1 times higher in patients with diabetes mellitus (7.5 μM) [32], heart failure (17.3 μM) [33] and chronic kidney disease (53.4 μM) [31] than healthy control subjects. Numerous studies have shown that elevated circulating TMAO promotes inflammatory response in peripheral tissues including skin, joints, aorta, heart, and kidney, contributing to the development of autoimmune disease and multiple cardiovascular and renal diseases [12–14, 34]. Circulating TMAO is capable of crossing the blood–brain barrier and has been suggested to be relevant to neurological disorders [35]. A recent study reported that the presence of elevated circulating TMAO exacerbates activation of microglia and neuroinflammation in rats following surgical trauma, leading to exaggeration of cognitive dysfunction [17]. To date, however, no studies have examined the influences of elevated circulating TMAO on inflammatory hyperalgesia. In the present study, animals were pre-treated with VEH or TMAO at a dose of 120 mg/kg for 2 weeks and circulating TMAO levels were approximately three times higher in TMAO-treated rats than VEH-treated rats before CG injection. Such levels of plasma TMAO in TMAO-treated rats may well reflect high levels of plasma TMAO in patients under some pathological conditions. We found that intraplantar injection of CG in VEH-treated rats induced thermal hyperalgesia, mechanical allodynia, and paw edema as indicated by decreases in thermal latency and mechanical threshold and increase in paw volume. These results are consistent with previous studies [18, 36, 37]. More importantly, our results showed that intraplantar injection of CG in TMAO-treated rats resulted in exaggerated thermal hyperalgesia and mechanical allodynia and more pronounced paw edema. These results suggest that the presence of high levels of circulating TMAO increases susceptibility to peripheral inflammatory response and inflammatory hyperalgesia.

It is well known that inflammatory mediators in peripheral tissues and the central nervous system (CNS) play a critical role in mediating peripheral inflammatory response and inflammatory hyperalgesia [1, 2]. Tissue injury or infection leads to the release of pronociceptive mediators from damaged cells including bradykinin, proinflammatory cytokines, and chemokines [1, 2]. These pronociceptive mediators recruit immune cells (neutrophils and macrophages) to the inflamed site where they produce further mediators including proinflammatory cytokines IL-1β, TNF-α, and IL-6. These

proinflammatory cytokines not only cause peripheral inflammatory response but also activate peripheral nociceptors directly to induce pain or enhance nociceptor sensitization indirectly by stimulating COX-2 and release of PGE2 [1, 2]. Additionally, the persistent nociceptive input in turn leads to activation of glial cells in the spinal cord [2] to produce various proinflammatory mediators including proinflammatory cytokines, which induce hyperactivity and hyperexcitability of spinal neurons (central sensitization), contributing to generation and maintenance of inflammatory hyperalgesia [1, 2]. Interventions that reduce proinflammatory mediators in either peripheral tissues or spinal cord have been shown to ameliorate peripheral inflammatory response as well as inflammatory hyperalgesia in different animal models of inflammation [20, 36, 38, 39]. In agreement with previous studies [18, 36, 37], we found that intraplantar injection of CG induced significant increases in protein levels of proinflammatory mediators IL-1β, TNF-α, IL-6, and COX-2 in the paw and spinal cord in both VEH-treated rats and TMAO-treated rats at 24 h following CG injection. In addition, intraplantar injection of CG also led to recruitment of neutrophils and macrophages to the paw as indicated by increased protein levels of MPO and CD68 and activation of microglia in the spinal cord as indicated by increased expression of IBA-1, along with augmented activation of transcription factor NF-kB p65 that is required for activation of immune cells and induction of proinflammatory mediators. However, CG-induced increases in all these measured parameters were more pronounced in TMAO-treated rats as compared to VEH-treated rats. These findings suggest that the presence of high levels of circulating TMAO may further enhance activation of immune cells (neutrophils, macrophages, and microglia) and NF-kB both peripherally in the inflamed site and centrally in the spinal cord, leading to greater production of proinflammatory mediators and consequently exaggeration of peripheral inflammatory response and inflammatory hyperalgesia. Indeed, a recent study has reported that the presence of high levels of circulating TMAO aggravates neuroinflammation and oxidative stress in the hippocampus, resulting in exaggeration of cognitive decline following surgical trauma [17]. Of note, the presence of high levels of circulating TMAO did not alter activation of immune cells and NF-kB, production of proinflammatory mediator in the paw and spinal cord, peripheral inflammatory response, and inflammatory hyperalgesia in rats following saline injection. These findings are consistent with previous study showing that elevated circulating TMAO had no effects on hippocampal proinflammatory mediator expression as well as cognitive function in sham rats [17], suggesting that elevated circulating TMAO alone in a short time period may be insufficient to induce production of proinflammatory mediator in peripheral tissues and the CNS under physiological conditions.

To further explore the possible mechanism by which elevated circulating TMAO leads to greater increases in production of proinflammatory mediator following CG injection, we measured protein levels of RGS10, a GTPase accelerating protein for G alpha subunits that has been shown to negatively regulate NF-kB activity and inflammatory mediators in peripheral tissues and the CNS [26, 27]. RGS10 is expressed in peripheral immune cells, brain, and spinal cord microglia [26, 27]. Recent studies show that peripheral macrophages and brain microglia from RGS10 knockout mice display hypersensitivity to bacteria stimuli (*i.e.*, lipopolysaccharide) by further enhancing NF-kB activity and producing greater amounts of proinflammatory mediators including IL-1β, TNF-α, IL-6, and COX-2 [26, 40, 41]. Whereas RGS10 overexpression suppresses microglial activation and attenuates activation of microglial NF-κB, proinflammatory cytokine release, and inflammatory neurotoxicity [41, 42]. In the present study, we observed significant reduction in RGS10 protein levels in the paw and spinal cord in both saline-injected rats and CG-injected rats after a 2-week TMAO treatment, although the underlying mechanisms were unclear. This observation suggests that the elevated circulating TMAO leads to reduced RGS10 protein levels in peripheral tissues and spinal cord, which might increase the susceptibility to CG-induced NF-kB activity in the peripheral immune cells and spinal cord microglia, resulting in greater increase in production of proinflammatory mediators and exaggeration of peripheral inflammatory response and inflammatory hyperalgesia following CG injection.

It should be noted that only one dose of CG was used to produce inflammatory pain in this study. This dose of CG induced inflammatory pain in both VEH-treated rats and TMAO-treated rats. It is unclear whether injection of a lower dose of CG (one that does not induce a prominent inflammatory response) can cause pain in the presence of high circulating TMAO levels or low levels of RGS10 in the paw and spinal cord. If injection of low-dose CG can induce pain in animals after TMAO treatment, that would suggest that the presence of high circulating TMAO levels may result in pain in those that would not normally have pain and would have important clinical implication.

One major limitation of the present study should be acknowledged. Although clinical studies have demonstrated that TMAO can cross the blood–brain barrier, we did not measure TMAO levels in the spinal cord and could not find the evidence showing that this exact experimental condition increases TMAO levels in the spinal cord. Further studies are necessary to examine whether TMAO

treatment increases TMAO levels in the spinal cord and whether direct intervention to reduce TMAO levels in the spinal cord can prevent or eliminate inflammatory pain in the presence of high circulating TMAO levels.

In conclusion, the present study demonstrates that the presence of high levels of circulating TMAO downregulates RGS10 in the paw and spinal cord, which may increase the susceptibility to CG-induced NF-kB activity, leading to greater increase in production of inflammatory mediators and consequent exacerbation of peripheral inflammatory response and inflammatory hyperalgesia following CG injection. Elevated circulating TMAO has been reported in animal models and patients with different diseases including Alzheimer's disease, obesity, type 2 diabetes mellitus, and cardiovascular and kidney disease. Interventions that reduce circulating TMAO may be a novel strategy to prevent the exacerbation of peripheral inflammatory response and inflammatory hyperalgesia in patients with high circulating TMAO.

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