Trimethylamine-N-oxide Specifically Impairs Endothelium-Derived Hyperpolarizing Factor-Type Relaxation in Rat Femoral Artery

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Although substantial evidence suggests that an increase in the level of trimethylamine-N-oxide (TMAO) is associated with the risk of cardiovascular diseases, including atherosclerosis, chronic kidney diseases, and hypertension, the direct effect of TMAO on vascular endothelial function remains unclear. Therefore, we investigated the acute effects of TMAO on endothelium-dependent relaxation induced by acetylcholine (ACh) in the superior mesenteric arteries and femoral arteries of rat. In endothelium-intact preparations, it was observed that TMAO (300 µmol/L for 60 min) did not affect ACh-induced relaxation in either of the two arteries. In endothelium-derived hyperpolarizing factor (EDHF)-mediated relaxation under nitric oxide synthase (NOS) and cyclooxygenase (COX) inhibitions by Nω-nitro-L-arginine (L-NNa) and indomethacin, respectively, TMAO specifically impairs the relaxation in femoral arteries but not in the superior mesenteric arteries under the inhibitory actions of NOS and as well as blockade of intermediate-conductance calcium-activated potassium channel (IKCa) (by TRAM-34) and small-conductance calcium-activated potassium channel (SKCa) (by apamin), which are putative sources of EDHF, ACh-induced relaxation was low, and there were no differences between the control and TMAO-treated groups with respect to both arteries. In femoral arteries, TMAO slightly reduces ACh-induced relaxation in the presence of indomethacin (preserved NO and EDHF signals) but does not affect ACh-induced NO-mediated relaxation under the combined presence of indomethacin, TRAM-34, and apamin. These results suggest that acute treatment with TMAO specifically impairs EDHF-mediated relaxation in the femoral arteries but not in the superior mesenteric arteries. These novel observations show that TMAO is a causative factor in the development of peripheral arterial disease.

Key words  endothelium-derived hyperpolarizing factor; femoral artery; superior mesenteric artery; trimethylamine-N-oxide; endothelium-derived relaxing factor

INTRODUCTION

An important contribution of the gut microbiota to pathological processes, including immuneologic, metabolic, and cardiovascular diseases, has been recently suggested.1–4 An example is trimethylamine-N-oxide (TMAO), a compound generated by the liver via a flavin monoxygenase 3 oxidation of the gut microbiota-derived trimethylamine (TMA).4 Results of several recent studies have indicated that elevated circulating levels of TMAO are associated with an increased risk of cardiovascular diseases.5–7 At cellular levels of endothelial cells, there is a growing body of evidence suggesting that TMAO has various adverse effects including pro-inflammatory, increased oxidative stress, decreasing endothelial self-repair, and increased monocyte adhesion to endothelial cells.5–10 However, there is no direct evidence investigating the relationship between TMAO and vascular function.

Endothelial cells play a pivotal role in vascular tone regulation by producing of endothelial-derived relaxing factors (EDRFs), nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF), and prostacyclin (PGL), and contracting factors (EDCFs).11,12 Imbalance between EDCFs and EDRFs levels results in an endothelial dysfunction, which has been observed in several diseases, including hypertension, diabetes, and atherosclerosis.11,12 Although there are several reports suggesting the effect of gut-derived substances and vascular functions on endothelium-dependent relaxation,13,14 to the best of our knowledge, no study has investigated the direct effects of TMAO on endothelium-dependent relaxation.

In the present study, we hypothesized that acute exposure to TMAO affects endothelium-dependent relaxation. On this note, we aimed to evaluate the acute effects of TMAO on acetylcholine (ACh)-induced relaxation and related components in the superior mesenteric and femoral arteries in rats. These are both conduit arteries, but at the downstream region, they are different as the mesenteric arterial bed and lower limbs. The arteries also often exhibited endothelial dysfunction under pathophysiological states, including hypertension.15,16

MATERIALS AND METHODS

Animals and Measurement of Vascular Isometric Force Male Wistar rats (age: 8–10 weeks) were used in the study. All experiments were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals from the Committee for the Care and Use of Laboratory Animals of Hoshi University (Tokyo, Japan).

The concentration–relaxation responses of the vascular function in the superior mesenteric arteries and femoral arteries were determined by measuring the isometric force as reported previously.14,16 The superior mesenteric artery and femoral arteries were rapidly isolated from the rats under isoflurane-induced anesthesia and immersed in oxygenated, modified Krebs–Henseleit solution (KHS; composition

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in mmol/L: NaCl 118.0, KCl 4.7, NaHCO₃ 25.0, CaCl₂ 1.8, NaH₂PO₄ 1.2, MgSO₄ 1.2, and glucose 11.0). Subsequently, the artery was cleaned and cut into rings (length, 2 mm), suspended in a well-oxygenated (95% O₂, 5% CO₂) bath containing KHS at 37°C, and maintained at a resting tension of 1.0 g (superior mesenteric artery) or 0.5 g (femoral artery). Isometric tension was recorded using an isometric force-displacement transducer connected to an acquisition system.14–16)

To assess the acute effects of TMAO on the relaxation responses, we incubated arterial rings with or without 300 µmol/L TMAO for 60 min before applying phenylephrine (PE) or serotonin (5-HT) to the superior mesenteric artery or femoral artery, respectively. We used TMAO at 300 µmol/L because several reports have demonstrated that this concentration of TMAO deteriorates the functions. 8,17) Subsequently, ACh (10⁻⁹–10⁻⁵ mol/L) was added, and the concentration–response curves were observed. Next, we evaluated ACh-induced relaxation in the combined presence of the following drugs with or without TMAO (300 µmol/L for 60 min): 100 µmol/L Nω-nitro-L-arginine [L-NNA; NO synthase (NOS) inhibitor], 10 µmol/L indomethacin [cyclooxygenase (COX) inhibitor], 10 µmol/L TRAM-34 [specific intermediate-conductance calcium-activated potassium channel (IKCa) inhibitor], or 100 µmol/L apamin [specific small-conductance calcium-activated potassium channel (SKCa) inhibitor]. These inhibitors were applied 30 min before the application of PE or 5-HT.

**Statistical Analysis** Data were expressed as mean ± standard error of the mean (S.E.M.), with n representing the number of animals used in the experiments. The concentration–response curves were fitted using a non-linear regression-fitting program with a standard slope for each relaxation (GraphPad Prism ver. 8.0; GraphPad Software Inc., San Diego, CA, U.S.A.). We also analyzed the E₉₅ (the maximal response generated by ACh) and pD₂ (a negative logarithm of EC₅₀, which is the concentration of ACh producing 50% E₉₅) using the GraphPad Prism software. Significant differences were calculated using Student’s t-test. Statistical significance was set at α = 0.05, and p-values < 0.05 were considered significant.

**RESULTS**

To investigate the acute direct effect of TMAO on the endothelium-dependent relaxation in the rat superior mesenteric artery and femoral arteries, we performed a series of experiments in which ACh (10⁻⁹–10⁻⁵ mol/L) was cumulatively added to rings precontracted by PE and 5-HT, respectively. As shown in Figs. 1A and D, the relaxation produced with and without TMAO exposure was similar in the superior mesenteric artery. In femoral artery, TMAO specifically impairs ACh-induced EDHF-mediated relaxation.

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Fig. 1. The Concentration–Response Curves for ACh-Induced Relaxations of Isolated Rings of the Superior Mesenteric Arteries (A–C) and Femoral Arteries (D–H) from Rats Treated with (TMAO) and without (Control) TMAO (300 µmol/L).

Relaxations were evaluated as % of precontraction induced by PE (A–C) or 5-HT (D–H). n = 8–10. *p < 0.05, E₉₅, Control vs. TMAO. (I) Schematic summary in the present study. TMAO does not affect ACh-induced relaxation in superior mesenteric artery. In femoral artery, TMAO specifically impairs ACh-induced EDHF-mediated relaxation.
mesenteric artery \(E_{\text{max}}\) (% relaxation to PE): Control \((n = 10)\) 99.6 ± 0.3 \(\mu\)mol/L vs. TMAO \((n = 10)\) 99.1 ± 0.4; \(p > 0.05\), \(pD_2\); Control \((n = 10)\) 8.21 ± 0.07 vs. TMAO \((n = 10)\) 8.19 ± 0.07; \(p > 0.05\) (Fig. 1A) and femoral artery \(E_{\text{max}}\) (% relaxation to 5-HT): Control \((n = 9)\) 86.2 ± 3.7 vs. TMAO \((n = 8)\) 89.0 ± 2.5; \(p > 0.05\), \(pD_2\); Control \((n = 9)\) 7.33 ± 0.05 vs. TMAO \((n = 8)\) 7.37 ± 0.10; \(p > 0.05\) (Fig. 1D).

To evaluate the non-NO, non-prostaglandin-mediated relaxation (EDHF-type relaxation evoked by ACh in both arteries), we derived the concentration–response curve for ACh in the presence of 100 \(\mu\)mol/L l-NAME plus 10 \(\mu\)mol/L indomethacin. Surprisingly, the EDHF-type relaxation was similar between the control and TMAO-treated groups in the superior mesenteric arteries \(E_{\text{max}}\) (% relaxation to PE): Control \((n = 10)\) 66.6 ± 3.7 vs. TMAO \((n = 10)\) 69.2 ± 5.3; \(p > 0.05\), \(pD_2\); Control \((n = 10)\) 7.31 ± 0.05 vs. TMAO \((n = 10)\) 7.20 ± 0.10; \(p > 0.05\) (Fig. 1B), whereas the relaxation was significantly weaker in the TMAO-treated femoral artery \((\text{vs. control})\) \(E_{\text{max}}\) (% relaxation to 5-HT): Control \((n = 9)\) 51.3 ± 6.1 vs. TMAO \((n = 10)\) 29.4 ± 6.5; \(p < 0.05\), \(pD_2\); Control \((n = 9)\) 6.73 ± 0.15 vs. TMAO \((n = 10)\) 6.73 ± 0.10; \(p > 0.05\) (Fig. 1E). The ACh-induced EDHF-type relaxation was largely inhibited by apamin \(100 \mu\)mol/L, SKCa inhibitor) plus TRAM-34 \((10 \mu\)mol/L, IKCa inhibitor) in the superior mesenteric \(E_{\text{max}}\) (% relaxation to PE): Control \((n = 9)\) 7.2 ± 2.7 vs. TMAO \((n = 8)\) 7.2 ± 3.4; \(p > 0.05\), \(pD_2\) values were not shown because the values were not calculated in some curves (Fig. 1C) and femoral arteries \(E_{\text{max}}\) (% relaxation to 5-HT): Control \((n = 8)\) 26.4 ± 5.0 vs. TMAO \((n = 9)\) 26.4 ± 4.3; \(p > 0.05\), \(pD_2\); Control \((n = 8)\) 6.28 ± 0.07 vs. TMAO \((n = 9)\) 6.18 ± 0.13; \(p > 0.05\) (Fig. 1F) and such relaxations were similar between control and TMAO-treated groups.

Next, we investigated the effect of TMAO on EDRFs-mediated relaxation evoked by ACh following two conditions: the preserved NO and EDHF components by indomethacin \((10 \mu\)mol/L, COX inhibitor) (Fig. 1G) and the preserved NO component by indomethacin \((10 \mu\)mol/L) plus apamin \((100 \mu\)mol/L) and TRAM-34 \((10 \mu\)mol/L). As shown in Fig. 1G, ACh-induced relaxation was slightly decreased in the TMAO-treated group compared with that in the control group under COX inhibition, but this was not significant \(E_{\text{max}}\) (% relaxation to 5-HT): Control \((n = 10)\) 93.7 ± 2.1 vs. TMAO \((n = 10)\) 87.7 ± 2.9; \(p > 0.05\), \(pD_2\); Control \((n = 10)\) 7.42 ± 0.10 vs. TMAO \((n = 10)\) 7.11 ± 0.12; \(p = 0.057\). As shown in Fig. 1H, ACh-induced NO-mediated relaxation was similar between the control and TMAO-treated groups under COX and SKCa/IKCa inhibitions \(E_{\text{max}}\) (%relaxation to 5-HT): Control \((n = 10)\) 87.7 ± 3.6 vs. TMAO \((n = 10)\) 88.7 ± 3.3; \(p > 0.05\), \(pD_2\); Control \((n = 10)\) 7.23 ± 0.11 vs. TMAO \((n = 10)\) 7.30 ± 0.10; \(p > 0.05\).

**DISCUSSION**

In this study, we report that TMAO specifically impairs EDHF-type relaxation in the rat femoral artery but not in the superior mesenteric artery as summarizing Fig. 1H.

Although several reports suggest that TMAO causes cellular abnormalities such as inflammation and increased oxidative stress in endothelial cells, its relationship with endothelium-dependent vascular reactivity has not been elucidated. Recently, Li et al. found an association between elevated circulating TMAO levels and endothelial dysfunctions including decreased endothelial NOS (eNOS)-derived NO bioavailability in Fisher-344 rat aorta with aging, and suggested that TMAO is a causative factor in the development of endothelial dysfunction. In the current study, we investigated the direct effects of TMAO on endothelium-dependent relaxation in two arteries, specifically, the superior mesenteric artery and femoral arteries. Our results indicate that ACh-induced endothelium-dependent relaxations were similar between the control and TMAO-treated groups in rat superior mesenteric and femoral arteries. Because ACh can generate and release three EDRFs (viz. NO, PGI, and EDHF), there may be substantial compensatory interactions among these within a given blood vessel. Thus, we further explored the possible role of EDHF-mediated relaxation induced by ACh in both arteries because this has been reported to partly contribute to ACh-induced relaxation in arteries.

A novel, intriguing, and potentially important finding made in the present study is the presence of a specific impairment of EDHF-type relaxation in rat femoral arteries but not in rat superior mesenteric arteries. This idea is supported by several pieces of evidence. First, although the ACh-induced femoral arterial relaxation was similar between the control and TMAO-treated groups, the ACh-induced femoral arterial relaxation observed in the presence of l-NAME plus indomethacin was greatly impaired in the TMAO-treated group. Second, under indomethacin treatment (that is, the preserved NO and EDHF components), ACh-induced femoral arterial relaxation was slightly weaker in the TMAO-treated group than that in the control group. Third, ACh-induced NO-mediated femoral arterial relaxation under treatment with indomethacin and two KCa channel inhibitors related to the source of EDHF by TRAM-34 and apamin was similar between the control and TMAO-treated groups. Fourth, ACh-induced femoral arterial relaxation was very small and similar between the two groups under three EDRFs blockades by treatment with l-NAME, indomethacin, TRAM-34, and apamin. ACh-induced relaxation was similar between the control and TMAO-treated groups under intact, l-NAME plus indomethacin, and l-NAME, indomethacin, TRAM-34, plus apamin conditions in the rat superior mesenteric arteries. These results suggest that TMAO could specifically affect endothelial function, and its sensitivity varies between vessels.

There are certain limitations associated with the present study. First, molecular mechanisms underlying the specific impairment of the ACh-induced EDHF-mediated relaxation by TMAO exposure in the femoral arteries but not the superior mesenteric arteries were not investigated. Numerous efforts have been made to identify the specific factor responsible for endothelium-dependent hyperpolarization. The putative mediator of the EDHF response might be only one or a combination of various candidates and be provably existing in the heterogeneity among arteries, species, and pathophysiological states. A number of different factors including KCa, hydrogen peroxide \((H_2O_2)\), and epoxyeicosatrienoic acids (EETs) which are CYP epoxygenase-derived metabolites of arachidonic acid), and non-diffusible phenomena, such as cell–cell contact-mediated mechanisms through myoendothelial gap junctions. Although the extent of regulating the production/release of EDHF between mesenteric and femoral arteries remains unclear, several reports suggest that there is a difference in EDHF signaling between mesenteric and femoral arteries.
arteries. For example, Shi et al. found that Ach-induced EDHF-type relaxation was reduced by sulfaphenazole, a selective inhibitor of CYP2C9, in rat femoral arteries but not in mesenteric arteries. Sandow et al. found that myoendothelial gap junctions allow the transmission of electrical responses between endothelial cells and smooth muscle cells. Furthermore, they function as a pathway for the spread of endothelial cell hyperpolarization to smooth muscle cells in the rat mesenteric artery, whereas a lack of myoendothelial gap junctions in the rat femoral artery precludes this. Judging from these reports and our present data, TMAO may affect diffusible factors but not the myoendothelial gap junctions in femoral arteries. At present, moreover, it is known that the primary components interact with TMAO, and the effects of such interactions between the superior mesenteric and femoral arteries are unknown. Although a receptor for TMAO has not yet been identified to date, Seldin et al. suggested a G protein-coupled receptor-mediated mechanism for TMAO, specifically via the activity of the Gβγ subunit complex resulting in the promotion of endothelial cell inflammation. In our study, the arteries were exposed to TMAO for 60 min. Such a short duration of exposure may affect the putative EDHF components in the femoral arteries, including K+ channel, EETs, and H2O2 rather than the gene expression of the functional proteins. An investigation of the cross talk between Ach-induced EDHF-mediated signaling and molecular signaling upon TMAO exposure is needed in a future study. Second, previous studies have reported that the circulating levels of TMAO are approximately between 10−6 and 10−4 mol/L in vivo for animal models and humans. Although we used a higher concentration of TMAO compared with plasma levels, the local level of TMAO may transiently reach higher concentrations. Elevated circulating TMAO levels may be an important prognostic marker in patients with a peripheral arterial disease.

Endothelial dysfunction is a precursor for the development of atherosclerosis, and is seen in femoral arteries in diseases associated with peripheral arterial disease, including hypertension and diabetes. Therefore, our findings suggest that TMAO is a causative factor in the development of peripheral arterial disease. To get a comprehensive understanding of the effects of TMAO on vascular function, future studies are required on the relationship among exposed concentration, exposed time, vessel types including resistance-size arteries, vascular functions, and TMAO.

In conclusion, our results suggest that exposure to TMAO specifically impairs EDHF-mediated relaxation in the rat femoral arteries but not in the superior mesenteric arteries. A comprehensive understanding of the direct effects of TMAO on vascular function could provide a potentially novel therapeutic target as a causative factor in peripheral arterial diseases.

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Author's Contributions TM designed research. TM, MK, KT, TK, and KT conducted experiments and analyzed data. TM and TK wrote the manuscript. All authors have read and approved the manuscript.

Conflict of Interest The authors declared no conflict of interest.

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