Trimethylamine-N-oxide (TMAO) Selectively Disrupts Endothelium-Dependent Hyperpolarization-Type Relaxations in a Time-Dependent Manner in Rat Superior Mesenteric Artery

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The vascular action of trimethylamine-N-oxide (TMAO)—the gut microbiota-derived metabolite—in contributing cardiovascular disease is a controversial topic. A recent study has shown that acute exposure of TMAO at moderate concentrations inhibits endothelium-dependent hyperpolarization (EDH)-type relaxations selectively in rat isolated femoral arteries, but not in mesenteric arteries. Here we determined the efficacy of higher TMAO concentrations with longer exposure times on vascular reactivity in rat isolated superior mesenteric arteries. Acetylcholine-induced EDH-type relaxations were examined before and after incubation with TMAO (0.1–10 mM) at increasing exposure times (1–24 h). One- and 4-h-incubations with TMAO at 0.1–3 mM did not cause any change in EDH-type relaxations. However, when the incubation time was increased to 24 h, responses to acetylcholine were reduced in arteries incubated with 1–3 mM TMAO. In addition, at higher TMAO concentration (10 mM) the decrease in EDH relaxations could be detected both in 4-h- and 24-h-incubations. The EDH-relaxations were preserved in rings incubated with 10 mM TMAO for 24 h in the presence of SKA-31 (10 µM), the small (SKCa)- and intermediate (IKCa)-conductance calcium-activated potassium channel activator. Contractile responses to phenylephrine increased in arteries exposed to 10 mM TMAO for 24 h. Interestingly, nitric oxide (NO)-mediated relaxations remained unchanged in arteries treated for 24 h at any TMAO concentration. Our study revealed that TMAO selectively disrupted EDH-type relaxations time-dependently without interfering with NO-induced vasodilation in rat isolated mesenteric arteries. Disruption of these relaxations may help explain the causal role of elevated TMAO levels in certain vascular diseases.

Key words relaxation; trimethylamine-N-oxide; endothelium-dependent hyperpolarization; rat superior mesenteric artery

INTRODUCTION

Trimethylamine N-oxide (TMAO) is a liver enzymatic oxidation product of trimethylamine (TMA), which is produced by gut microbiota as a waste product of carnitine, betaine and choline in nutrients. Plasma TMAO levels are relatively low in healthy people, but in case of kidney failure, old age, intestinal dysbiosis or western-type eating habits, its plasma level may exceed 50 mmol/L. Multiple clinical studies imply that enhanced plasma TMAO level is correlated with increased risk of cardiovascular disease such as heart failure, coronary artery disease, atherosclerosis and hypertension. Moreover, TMAO has the ability to heighten platelet reactivity that leads to enhanced potential for thrombotic events and stroke. People with high plasma TMAO levels are more prone to type-2 diabetes mellitus than the normal population. Although TMAO is considered to be a plasma biomarker that determines predisposition to cardiovascular disease, its direct association with vasculature has not been resolved yet. Limited information about the direct vascular effects of TMAO is based on a number of cell culture studies and in vivo disease models. In human umbilical vein endothelial cells TMAO increases oxidative stress and inflammation which are the main triggers of endothelial dysfunction. These factors have been claimed to be responsible for the reduction of endothelium-dependent nitric oxide (NO)-mediated relaxations in the aorta isolated from elderly rats with high plasma TMAO level. In rats with chronic renal failure, the decrease of endothelium-dependent relaxations has also been found closely associated with high plasma TMAO levels. In rat isolated aorta, direct contractile effects of TMAO at moderately high concentrations are seen when the endothelium is removed which implies that TMAO also exerts some detrimental effects on the vascular wall modulated by endothelium. However, the results of a recent study showing that chronic and low-dose TMAO administration in spontaneous hypertensive rats does not exacerbate high blood pressure further, indicates a controversy about the vascular effect of TMAO in the literature.

Blood pressure is mainly regulated by small-diameter arteries and arterioles of the cardiovascular system where endothelium-dependent vasorelaxation is substantially mediated by endothelium-dependent hyperpolarization (EDH), which is triggered by the activation of small (SKCa)- and/or intermediate (IKCa)-conductance calcium-activated potassium channels located in endothelium. As vessel diameter decreases, the role of EDH-type relaxations in the control of vascular tone increases. Superior mesenteric artery is the primary source of blood from the heart to the organs of the midgut. Mesenteric arterial network which receives 25% of cardiac output, contributes substantially to the total peripheral resistance, and therefore to arterial blood pressure. The main branch of the superior mesenteric artery is a convenient artery for studying EDH-mediated responses, since relaxations are largely preserved when NO and prostacyclin (PGI2) productions are

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inhibited. Moreover, the endothelium of this vessel has been shown to be structurally and functionally altered in hypertension.\textsuperscript{19,20} Impaired EDH-mediated hyperpolarization and relaxation contribute to the endothelial dysfunction in mesenteric arteries of spontaneously hypertensive rats (SHR).\textsuperscript{21,22} Therefore, impairment of NO and/or EDH-mediated relaxations in conduit arteries like superior mesenteric artery could be a causative factor initiating and/or accelerating the TMAO-associated endothelial dysfunction and related cardiovascular diseases such as hypertension.

In a recently published study, acutely exposure to TMAO has been shown to inhibit EDH-type relaxations selectively in rat isolated femoral arteries, but not in superior mesenteric arteries, which was attributed to the difference in the type of EDH-signaling between the two vessels.\textsuperscript{23} Since under pathological conditions such as hypertension and chronic renal failure, the tissues are exposed to high TMAO levels for an extended period of time, it is plausible to conduct in vitro experiments for prolonged incubation durations. Thus, the efficacy of higher TMAO concentrations with longer exposure times on endothelial vascular relaxations are not known and remains to be elucidated.

In the present study, we hypothesized that in vitro prolonged exposure to TMAO induces deterioration in vascular reactivity. Therefore, we aimed to examine how endothelium-dependent relaxations respond to incubation with TMAO at increasing exposure times (1, 4, and 24 h) in rat isolated superior mesenteric arteries. To verify this, short-term incubations with TMAO were done in organ bath (1 and 4 h), whereas the chronic exposure of TMAO (24 h) was carried out in a tissue culture system so as to maintain long-term tissue viability.

MATERIALS AND METHODS

Male Sprague Dawley rats 10–12 weeks old, weighing 250–350 g were obtained from Kocab Yatağan Experimental Animals Laboratory, Ankara, Turkey. Animal procedures and protocols were approved by the Animal Ethics Committee of University of Hacettepe (Ethics No: 2018-45). All animals were maintained on a 12 h light:12 h dark cycle with food and water available ad libitum.

Rats were euthanized with decapitation under CO\textsubscript{2} anesthesia. Main branch of the superior mesenteric artery was rapidly removed and placed into a petri dish containing Krebs-Henseleit solution (KHS) at 4 °C. The vessels were cleaned of fat and connective tissues and cut into rings of 2 mm in length. The rings were suspended in 5 mL organ baths containing KHS gassed with 95% O\textsubscript{2}–5% CO\textsubscript{2} at 37 °C and pH 7.4. KHS had the following composition (mM): NaCl 118.0, KCl 4.7, MgSO\textsubscript{4} 1.2, CaCl\textsubscript{2} 2.5, KH\textsubscript{2}PO\textsubscript{4} 1.2, NaHCO\textsubscript{3} 25.0 and glucose 11.1. Changes in tension were measured by iso- metric force transducers connected to a computer-based data acquisition system. Tissues were equilibrated at an optimal resting tension of 1 g for 1 h and washed out in every 10 min with freshly warmed KHS. Before starting the experimental protocol, tissues were treated with 80 mM KCl until reproducible contractions were obtained.

In preliminary experiments, vascular smooth muscle contractility was tested by obtaining cumulative contractions to phenylephrine (10 nM–100 µM) in each artery. Thereafter, the rings were precontracted with 1 or 3 µM phenylephrine submaximally, which caused a response equivalent to 60–80% of the maximum phenylephrine contraction, and then the endothelial function was determined by cumulative addition of acetylcholine (0.1 nM–10 µM). The tissues responding with relaxation more than 90% to acetylcholine were accepted as endothelium-intact and were used for further experiments. To determine the EDH- and NO-mediated components of acetylcholine-induced relaxations, responses were obtained in the presence of N\textsubscript{ω}-nitro-l-arginine methyl ester (l-NAME) (100 µM) + indomethacin (10 µM), and TRAM-34 (1 µM) + apamin (50 nM) + indomethacin (10 µM), respectively. All concentrations of the inhibitors were chosen based on previous studies.\textsuperscript{24–26} After obtaining control responses to acetylcholine, the experiments were repeated in the presence of TMAO (0.1–10 mM) at increasing exposure times (1, 4, and 24 h). For 1- and 4-h experiments, the arterial rings were exposed to TMAO directly in the organ baths. For 24-h incubations, arterial rings were immediately transferred into 0.5-mL cell-culture plates containing Dulbeco’s modified Eagle’s medium–high glucose (DMEM) (500 µL) and 1% (v:v) penicillin/streptomycin, and then exposed to TMAO for 24 h in a 5% CO\textsubscript{2} incubator at 37 °C. Tissues in the control group were only treated with 0.5 µL phosphate buffered solution (PBS), the solvent of TMAO. After 24 h, tissues were transferred to the organ baths and the rest of the experiments were conducted as described above. Phenylephrine-induced cumulative concentration-dependent contractions were also examined in the presence of TMAO (1, 3, and 10 mM) for 24 h. In another set of 24-h experiments the tissues were treated with TMAO (10 mM) in the absence and presence of SKA-31 (10 µM), the IK\textsubscript{Ca}/SK\textsubscript{Ca} activator. The control arteries were incubated with PBS (5 µL) together with dimethyl sulfoxide (DMSO) (0.5 µL), the solvents of TMAO and SKA-31, respectively.

Relaxations to sodium nitroprusside (SNP) (1 nM–10 µM), the NO donor, were also tested in the presence and absence of 1–10 mM TMAO for 24 h.

Chemicals and Solutions Acetylcholine chloride (Sigma-Aldrich, Germany, A6625), apamin (Sigma-Aldrich, A1289), SNP (Sigma, S-0501), l-NAME (Sigma, N-5751), l-phenylephrine hydrochloride (Sigma, P6126), were dissolved in distilled water. Indomethacin (Sigma, I-7378) was dissolved in distilled water containing 0.7% Na\textsubscript{2}CO\textsubscript{3} (wt/vol), SKA-31 (Sigma-Aldrich, S5576), and TRAM-34 was dissolved in DMSO.

DMEM (D5796) and penicillin-streptomycin (P4333) combination were purchased from Sigma. TMAO (Aldrich, 317594) was dissolved in PBS, which was prepared from PBS tablets pH 7.4 (PanReac AppliChem, A9162,0100). Eighty millimolar KC\textsubscript{I} solution was prepared by substitution of KC\textsubscript{I} with equimolar NaCl.

Statistics Data are presented as mean ± standard error of mean (S.E.M.). Contractions were expressed as a percentage of contractions induced by KC\textsubscript{I} (80 mM), and relaxations were expressed as a percentage of precontraction induced by phenylephrine. p\textsubscript{D2} (−logEC\textsubscript{50}, drug concentration which constitutes half-maximum response) values of concentration–response curves were calculated with Prism’s analysis program (GraphPad Prism 8.4.2). Values representing the area under the curve (AUC) were calculated with Prism’s AUC analysis program and expressed as arbitrary units (a.u). Comparison of concentration–response curves was done by using two-way ANOVA and Tukey’s post-hoc test. Differences be-
between $\text{AUC}$, $E_{\text{max}}$, and $pD_2$ values were analyzed with unpaired $t$ test or one-way ANOVA. $p < 0.05$ was considered significant.

**RESULTS**

One and 4-h incubations with TMAO at concentrations between 0.1–1 mM did not cause any change in acetylcholine-induced EDH-type relaxations in rat superior mesenteric arteries (data not shown). However, in arterial rings incubated for 24 h with TMAO at 1 mM, responses to acetylcholine were significantly reduced (Fig. 1), but at smaller concentrations (0.1 and 0.3 mM) no inhibition was observed (data not shown). The $pD_2$ for 1 mM-TMAO group was not significantly different from control, while the $AUC$ value significantly reduced in the presence of 1 mM TMAO ($n = 5$) ($p < 0.05$) (Table 1, Fig. 1).

We next examined higher concentrations of TMAO on EDH-mediated relaxations. At 3 mM, relaxant responses to TMAO incubated for 1 and 4 h still did not differ from control, while they significantly shifted to right when the exposure...
duration was extended to 24 h (n = 4–6) (Fig. 2). The pD₂ and AUC values significantly reduced at 24-h group compared to 1- and 4-h group (n = 4–6) (p < 0.05) (Table 1, Fig. 2).

Increasing TMAO concentration to 10 mM caused a significant decrease in EDH relaxations both in arterial rings exposed to TMAO for 4 h (pD₂ value was 7.46 ± 0.14, and 6.81 ± 0.13 in the absence and presence of 10 mM TMAO, respectively) (n = 5) (p < 0.05) and for 24 h (p < 0.05) (Table 1, Fig. 2).
The extent of rightward shift in 24-h group tended to be much more than the 4-h group, but the difference did not reach to statistical significance ($n = 5$) (Fig. 3).

Phenylephrine-induced contractions were also examined in the tissues incubated with 1, 3, and 10 mM TMAO for 24 h. In the presence of 10 mM TMAO contraction-response curves to phenylephrine shifted significantly to the left ($n = 6$) ($p < 0.05$) (Fig. 4), while lower concentrations of TMAO were ineffective (data not shown).

In order to investigate the mechanisms underlying impaired EDH-mediated responses, we tested the role of SKA-31 (10 $\mu$M) on impaired EDH-type responses by TMAO. The results showed that SKA-31 (10 $\mu$M) ameliorated the reduced EDH-type vasorelaxations by TMAO (10 mM) ($n = 6$) ($p < 0.05$) (Table 1, Fig. 3). The extent of rightward shift in 24-h group tended to be much more than the 4-h group, but the difference did not reach to statistical significance ($n = 5$) (Fig. 3).

The AUC values show the area under the curves of acetylcholine-induced EDH-type relaxations ($n = 5–6$) (two-way ANOVA *$p < 0.05$ values significantly different vs. control; unpaired $t$ test *$p < 0.05$ values significantly different vs. other group; ns $p > 0.05$).
2, Fig. 5). The extent of leftward shift of the concentration–response curves by SKA-31 in the presence of TMAO was almost the same as that observed in control arteries incubated with SKA-31 alone (Table 2) (Figs. 5B, C). The increased EDH-mediated relaxations were inhibited in the presence of TRAM-34 (1 µM) and apamin (50 nM) (n = 6) (p < 0.05) (Fig. 5C).

In order to reveal whether the efficacy of TMAO was restricted to EDH-type relaxations or not, another set of experiments were conducted to examine its effect on NO-mediated relaxations. Interestingly, these responses failed to change in superior mesenteric arteries treated for 24 h with 3 and 10 mM TMAO (AUC values were 324.2 ± 13.06 for control, 284.5 ± 15.03 for 3 mM TMAO, and 301.8 ± 17.03 for 10 mM TMAO) (Fig. 6). Pretreatment with 1, 3, and 10 mM TMAO for 24 h did not also impair relaxation induced by SNP (0.1 nM–1 µM) either (Fig. 7).

DISCUSSION

The relationship between plasma TMAO elevation and vascular diseases such as atherosclerosis, thrombosis, coronary artery disease and hypertension has been demonstrated by several animal experiments and human studies.\(^4,7,8,27,28\) In a recent meta-analysis, it has been suggested that prevalence of hypertension increases by 20% per 10 µM increment in circulating TMAO concentration.\(^27\) However, the causal relationship between hypertension and TMAO has not been resolved yet. In the current study we conducted in vitro experiments to investigate the potential direct effects of TMAO on endothelium-dependent relaxations that largely diminish in aforementioned vascular disorders. TMAO at relatively low concentrations (0.1 and 0.3 mM) did not exert a direct action on vasodilation. However, at 1 mM and higher concentrations, 24-h incubation with TMAO selectively impaired EDH-type relaxations of acetylcholine, without affecting NO-mediated responses. In support of our results, a recent study reported that acute tissue incubation (60 min) with 0.3 mM TMAO failed to affect the endothelium-mediated vasodilation in rat superior mesenteric artery, while same concentration and exposure time selectively disrupted EDH-type responses in rat femoral artery.\(^23\) It is likely that the lack of effect of TMAO in superior mesenteric arteries is presumably the result of low concentration they have used, as well as the short exposure duration. Our finding that TMAO failed to alter NO-mediated relaxations was further confirmed with experiments conducted in rat isolated aorta, where acetylcholine-induced NO-dependent relaxations were found totally resistant to high concentrations of TMAO incubated for 24 h (data not shown). However, in aged rats and in rats with chronic kidney damage, NO-mediated relaxations have been reported to decrease in isolated aortic rings due to increased TMAO in plasma.\(^13,15\) This discrepancy may be the result of several factors. In rats with kidney damage or in aged rats, additional pathophysiological changes such as inflammation and oxidative stress are likely to contribute to the inhibitory effects of TMAO on vasculature. Indeed, TMAO has been shown to exaggerate cardiac fibrosis in doxycycline-treated mice, whereas it was observed in very low levels in healthy mice.\(^29\) Similarly, the potentiating effect of TMAO on CD36 expression in macrophages could be seen only in the presence of oxidized low-density lipoproteins (LDL).\(^30\) Therefore, the vascular changes attributed to TMAO in animal disease models may be a result of the sum of pathophysiological changes seen in the vascular microenvironment. In this respect, usage of in vitro methods may help understand the underlying mechanism of action in an uncomplicated manner.

In our study the inhibitory effect of TMAO on endothelial responses appeared at relatively high concentrations (>1 mM) when the exposure time was prolonged to 24 h. There is evidence in the literature implicating that biological effects of TMAO on different cells are likely to be dependent on its concentration. For example, the enhancing effect of TMAO on platelet activation induced by ADP is seen at concentrations as low as 100 µM,\(^8\) whereas in human vascular endothelial cell cultures, production of reactive oxygen species and proinflammatory cytokines is triggered after incubation for at least 8 h with 600 µM TMAO.\(^31\) There are also some other in vitro

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**Fig. 4. The Concentration–Response Curves for Phenylephrine-Induced Contractions in the Absence (Control) and Presence of 10 mM TMAO Incubated for 24 h in Rat Superior Mesenteric Arterial Rings (n = 6)**

Two-way ANOVA; *p < 0.05 values significantly different vs. control.

**Table 2. The Precontractile Tone, pD2, Emax, and AUC Values of Acetylcholine-Induced EDH-Type Relaxations in Rat Isolated Mesenteric Arteries Incubated with (PBS + DMSO) or TMAO (10 mM) in the Presence and Absence of SKA-31 (10 µM) for 24 h**

| Tissue incubation 24-h | Acetylcholine-induced EDH-type relaxations (0.1 nM–10 µM) | pD2 | Emax (%) | AUC (a.u) |
|-----------------------|--------------------------------------------------------|-----|----------|----------|
| Control (PBS + DMSO)  | 0.696 ± 0.05                                          | 249.8 ± 6.99 | 91.68 ± 2.39 | 7.77 ± 0.16 |
| SKA-31 10 µM          | 0.666 ± 0.05                                          | 342.1 ± 8.59* | 91.47 ± 2.28 | 9.10 ± 0.12* |
| TMAO 10 mM           | 0.755 ± 0.05                                          | 151.8 ± 9.94* | 84.72 ± 5.59 | 6.34 ± 0.24* |
| TMAO 10 mM + SKA-31 10 µM | 0.815 ± 0.08                                         | 337.4 ± 14.33** | 91.52 ± 2.6 | 8.8 ± 0.29** |

One-way ANOVA *p < 0.05 vs. control; **p < 0.05 vs. TMAO 10 mM; # p < 0.05 vs. control. Data are expressed as mean ± S.E.M. of 4–6 animal. pD2: negative logarithm of the concentration that produces a 50% of maximal response; Emax: Maximal response. AUC: Area under the curve.
studies that have used TMAO in a concentration range of 0.1 to 500 mM showing that it does not give damage to cell viability even at concentrations exceeding 1000 times the physiological level.\(^{32,33}\) Nevertheless, because the effective concentrations used in the present work are far above the circulating levels of TMAO observed under in vivo conditions, we have to admit that our results do not provide convincing evidence for the involvement of a diminished EDH-type vasodilation as a causative factor in TMAO-evoked hypertension and/or other cardiovascular diseases.

Superior mesenteric arteries are small vessels that contribute to local control of the vascular tone and blood flow according to perfusion need of the tissue. In these arteries, contrary to the aorta, the main part of endothelium-dependent relaxations is preserved after the inhibition of NO synthase and cyclooxygenase pathways. These relaxations, called EDH-type, occur as a result of the opening of endothelial IKCa and/or SKCa channels leading to hyperpolarization. It is subsequently spread to the smooth muscle cells underneath by means of one or several factors including K\(^+\),\(^{34}\) hydrogen peroxide,\(^{35}\) arachidonic acid metabolites,\(^{36}\) or myoendothelial gap junctions.\(^{37}\) Since the inhibition of EDH-type relaxations by TMAO did not appear upon acute exposure, but were apparent after 24-h incubation, we can speculate that the effect could be due to impairment of gene expression of the functional proteins rather than deactivation of the putative factors mentioned above. The preserved NO-mediated vasodilation in response to TMAO may indicate that the two endothelium- and calcium-dependent relaxing pathways are differently affected by TMAO. The inability of TMAO to affect SNP-induced relaxations further support the point of view that NO-cyclic guanosine monophosphate (cGMP) pathway is not involved in its inhibitory mechanism of action. Cytosolic calcium activating endothelial IKCa/SKCa channels and NO synthase may come from different sources. While NO synthase uses mainly intracellular calcium stores, calcium influx from plasma membrane via transient receptor potential (TRP) channels constitutes an additional driving force for the activation of IKCa/SKCa channels.\(^{38}\) At present, the primary target of TMAO in rat superior mesenteric arteries is not exactly known. Some gut-microbial metabolites such as short-chain fatty acids can act as ligands for G-protein coupled recep-
phenylephrine-induced contractions observed in the presence and thus EDH-type relaxations. In this regard, the enhanced have been affected by TMAO that reduces hyperpolarization muscle cells. It can be speculated that TRP channels might govern membrane polarization of endothelium and smooth cation channels, which not only do allow calcium influx into directly or indirectly alter ion channel functions like calcium channels or TRP channels. TRP channels are non-selective in vascular endothelium. 39) TMA, the precursor of TMAO, is also known to be a full agonist at human G-protein coupled trace amin-associated receptor 5 (TAAR5) that does not recognize TMAO.40) Recently, in rat isolated aortic rings TMAO has been shown to have a receptor-independent direct contractile effect that could be modulated by perivascular adipose tissue and endothelium, the mechanism of which remains to be solved.46) Similarly, TMAO increases cardiac contractility by modulating intracellular calcium levels in human and mice cardiac tissues \textit{ex vivo}.41) These data imply that TMAO may directly or indirectly alter ion channel functions like calcium channels or TRP channels. TRP channels are non-selective cation channels, which not only do allow calcium influx into endothelium, but also are permeable to sodium ions that determine the membrane potential. Therefore, these channels also govern membrane polarization of endothelium and smooth muscle cells. It can be speculated that TRP channels might have been affected by TMAO that reduces hyperpolarization and thus EDH-type relaxations. In this regard, the enhanced phenylephrine-induced contractions observed in the presence of 10mM TMAO for 24h supports our view because of the slow depolarization of the smooth muscle membrane seen when endothelial KCa channel function is decreased.30) We admit that lack of measurement of membrane potential and lack of examination of TRP channel modulators constitute the limitations of the present study. Of course, a possible blockade of IKCa/SKCa channel activity by TMAO cannot be ruled out. Our findings showed that the IKCa/SKCa channel opener SKA-31 (10μM) ameliorated the reduced EDH-type vasorelaxations induced by TMAO (10mM) after 24-h-incubation, implying that the effect of TMAO can be reversed by increasing KCa channel function. The gap junctional transmission between endothelium and smooth muscle cells might also have been affected by TMAO for it has been previously shown that myoendothelial gap junctions provide critical link for the action of IKCa/SKCa-mediated EDH response in rat isolated superior mesenteric artery.42) Although we did not examine the effect of gap junction inhibitors, our results showed that the extend of leftward shift of the concentration–response curves by SKA-31 in the presence of TMAO was almost the same as that observed in control arteries incubated with SKA-31 alone, implicating that endothelial hyperpolarization is likely to be transmitted to the smooth muscle without any interruption caused by TMAO. This leads us to speculate that gap junctional communication seems unlikely to be responsible for TMAO effect in our experiments. Indeed, experimental hypertension studies imply that the altered function of gap junctions should not be a major contributing factor to impaired EDH-mediated responses during hypertension.43,44)

In conclusion, the current study has examined the direct vascular action of TMAO as a potential substance affecting endothelial function in rat isolated superior mesenteric arteries through \textit{in vitro} organ bath method and our findings confirmed that TMAO at pharmacological concentrations selectively disrupts EDH-type vasorelaxations, without interfering with NO-induced vasodilatation. We revealed that incubation time of TMAO was critical for reduction in EDH-type relaxations. In conditions like hypertension, aging, diabetes, and other cardiovascular diseases, where NO-mediated relaxations are blunted, endothelial function is largely maintained by other cardiovascular diseases, where NO-mediated relaxations are blunted, endothelial function is largely maintained by perivascular adipose tissue. This leads us to speculate that gap junctional communication seems unlikely to be responsible for TMAO effect in our experiments. Indeed, experimental hypertension studies imply that the altered function of gap junctions should not be a major contributing factor to impaired EDH-mediated responses during hypertension.43,44)

**Conflict of Interest** The authors declare no conflict of interest.

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**Fig. 6. AUC Values for Acetylcholine-Induced NO-Mediated Relaxations in the Absence (Control) and Presence of TMAO (3 and 10mM) Incubated for 24h in Rat Isolated Superior Mesenteric Arterial Rings (n = 4)** ns p > 0.05.

**Fig. 7. The Concentration–Response Curves for SNP-Induced Relaxations in the Absence (Control) and Presence of TMAO (1, 3, and 10mM) Incubated for 24h in Rat Superior Mesenteric Arterial Rings (n = 7)**

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