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RESEARCH ARTICLE

The Inhibitory Effect of Trimethylamine (TMA), an Intestinal Bacterial Metabolite, on Endothelial Vasorelaxation in Rat Mesenteric Artery

Melike Hacer ÖZKAN[*°]

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SUMMARY

The effect of the gut microbiota metabolite trimethylamine-(TMA) in isolated vessels is unknown. Previously TMAO, the hepatic oxidation product of TMA, at 3 mM has been shown to inhibit endothelium-dependent vasorelaxations of isolated arteries only after 24-hour interaction. In this study, the effects of TMA (at 1 mM) on endothelium-dependent relaxations with acute (1 or 4 hours) and longer (24 hours) incubation periods were evaluated in superior mesenteric arteries of rats. Acute exposure to TMA of 1 hour significantly inhibited acetylcholine-stimulated endothelium-derived hyperpolarizing (EDH) type relaxations, and this inhibition gradually intensified as the incubation period was prolonged to 4 and 24 hours. The area under the curves (AUCs) of the relaxation-response curves after 1 and 24 hours after TMA incubation were found to be significantly different from each other. In contrast, similar AUC values were obtained after 4 and 24 hours of incubation. Contractile responses to phenylephrine, and nitric oxide (NO)-mediated relaxations of acetylcholine were similar in arteries before and after pretreatment with TMA for 24 hours. These data indicate that TMA selectively inhibits EDH-type relaxations in rat isolated mesenteric arteries. Although the inhibitory effect of TMA intensifies over time, it appears to be more pronounced during acute incubation periods. The findings strengthen the evidence that TMA is a more toxic metabolite on vascular tone than TMAO.

Key Words: *Endothelial dysfunction, TMA, vascular, metabolite, mesenteric artery, rat.*

Bağırsak Bakteriyel Metaboliti Trimetilaminin (TMA) Sıçan Mezenter Arterinde Endotel-aracılı Gevşeme Üzerindeki İnhibitör Etkisi

ÖZ

Bağırsak mikrobiyota metaboliti trimetilamin'in (TMA) izole damarlardaki etkisi henüz bilinmemektedir. Daha önce, TMA'nın hepatik oksidasyon ürünü olan TMAO'nun izole arterlerde 3 mM konsantrasyonda 24 saatlik inkübasyon sonrası endotel-bağımlı gevşemeleri seçici olarak inhibe ettiği gösterilmiştir. Buradaki çalışmada sıçan izole superior mezenter arterlerinde TMA'nın (1 mM) akut (1 ve 4 saat) ve daha uzun (24 saat) süreli inkübasyonu sonrası endotel- bağımlı gevşemeler üzerindeki direkt etkileri incelendi. TMA'ya 1 saatlik akut maruz kalma, asetilkolin ile stimüle edilen endotel-kaynaklı hiperpolarizan (EDH)-tip gevşemeleri anlamlı olarak inhibe etti ve bu inhibisyon inkübasyon süresi 4 ve 24 saate uzatıldıkça şiddetlendi. Ne var ki, 1 ve 24 saatlik TMA inkübasyonu sonrasında gevşeme-yanıt eğrilerinin eğri altında kalan alanları (AUC) birbirine göre anlamlı derecede farklı iken, 4 ve 24 saatlik inkübasyon sonrasında benzerdi. Fenilefrin ile elde edilen kasılmalar ve asetilkolin ile stimüle edilen nitrik oksit (NO)-aracılı gevşemeler, 24 saatlik TMA inkübasyonu sonrası değişmedi. Bu veriler TMA'nın EDH-tipi gevşemeleri selektif olarak inhibe ettiğini göstermektedir. Her ne kadar TMA'nın inhibitör etkisi zamana bağlı şiddetlense de, bunun akut inkübasyon dönemlerinde daha belirgin olduğu görülmektedir. Bulgular, TMA'nın vasküler tonus üzerinde TMAO'dan daha toksik bir metabolit olduğuna dair kanıtları güçlendirmektedir.

Anahtar Kelimeler: *Endotel disfonksiyonu, TMA, vasküler, metabolit, mezenter arter, sıçan.*

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[*] ORCID: 0000-0003-1339-2616, Hacettepe Üniversitesi, Eczacılık Fakültesi, Eczacılık Meslek Bilimleri Bölümü, Farmakoloji A.B.D.

[°] Corresponding Author; Melike Hacer ÖZKAN
E-mail: melikeo@hacettepe.edu.tr

INTRODUCTION

Trimethylamine (TMA) is produced as an intermediate digestion product of animal foods through the metabolism of the intestinal microbiota. After passing the systemic circulation, TMA is immediately metabolized to trimethylamine N-oxide (TMAO) via flavin-monooxygenase-3 (FMO-3)-mediated oxidation in hepatic tissue (al-Waiz, Mikov, Mitchell, & Smith, 1992). Clinical studies show that TMAO is independently associated with cardiovascular disease (CVD) and that the risk of myocardial damage can be predicted by the plasma level of TMAO (Roncal, Martinez-Aquilar, Orbe, Ravassa, Fernandez-Montero,… Paramo, 2019). Essentially level of both metabolites in the circulatory system increases in patients having renal dysfunction or trimethylaminuria (Treacy, Akerman, Chow, Youil, Bibeau, Lin,... Forrest, 1998; Bain, Faull, Fornasini, Milne, & Evans, 2006). However, the direct vascular effects of these metabolites remain to be solved. Although TMA is known to be a more toxic metabolite on cells than TMAO, a limited number of *in vitro* studies have comparatively examined the vascular effects of TMA together with TMAO in the same experimental design. Both TMA and TMAO might change vascular tone. In human umbilical arteries, the potency and efficacy of TMA-induced contractile responses have been reported to be significantly higher than TMAO (Ongun, Orgul, Celik, & Bariskaner, 2023). We have also reported at pharmacological concentrations that TMAO impairs endothelium-derived and hyperpolarizing vasodilation in the rat isolated mesenteric artery, which implies the contribution of TMAO to endothelial disruption (Hamad, Ozkan, & Uma, 2021; Ongun, Orgul, Celik, & Bariskaner, 2023). However, no studies have investigated whether TMA could influence the function of endothelium in rat isolated mesenteric arteries. In the current study, the experimental protocol was applied with TMA at 1 mM concentration in both acute (1 hour and 4 hours) and long-term exposure times (24 hours) in rat small-sized arteries by measuring *ex vivo* isolated vessel function.

MATERIAL AND METHODS

Twenty-eight male Sprague Dawley rats 200-250 g in weight were purchased from Kobay Company (Ankara, Turkiye). The ethical approval was obtained by the local ethics committee of Hacettepe University (No: 2018-45). The animals were anesthetized by CO_2 and euthanized by decapitation. The feeding branch of superior mesenteric artery was carefully dissected and cleaned from adipose and connective tissues. The four mesenteric arterial rings were obtained from one rat mesenteric artery, and the rings were mounted into the organ baths of 5 ml. Isometric tensions were measured using a transducer and recorded on a computer. The rings were precontracted with phenylephrine submaximally, and then endothelium-dependent relaxations were obtained by cumulative acetylcholine concentrations (0.1 nM - 10 μM). After the first relaxation responses to acetylcholine, N(gamma)-nitro-L-arginine methyl ester (L-NAME) (100 μM)+indomethacin (INDO; 10 μM) were applied into the baths for 45 min to obtain endothelium-derived hyperpolarizing (EDH)-type vasorelaxations. Thereafter, the rings were incubated with TMA (1 mM) at increasing exposure times. For 1- and 4-hour incubations, the rings were exposed to TMA in Krebs-Henseleit Solution (KHS) of organ baths. Then acetylcholine responses were repeated in the presence of the inhibitors, as mentioned above. 24-hour-incubations (unpaired data) were carried out with tissue culture studies, in which arterial rings were pretreated with TMA in a cell culture medium containing DMEM (0.5 mL) and penicillin/streptomycin (1%) in an incubator at 37 °C for 24 hours. PBS was applied to the rings as a solvent control. At the end of the incubation period, TMA- and PBS-treated rings were mounted to organ baths, and the remaining experimental protocol were conducted, as mentioned over. In addition to EDH-type relaxations, NO-mediated relaxations of acetylcholine were also obtained in TMA-treated rings in the presence of apamin (APA; 50 nM)+INDO (10 μM) and TRAM-34

(1 µM) for 45 min. To examine vascular contractility, cumulative contractions to phenylephrine (10 µM - 100 µM) were obtained in TMA-treated rings for 4 hours and 24 hours.

Phenylephrine (Sigma, P6126), acetylcholine (Sigma-Aldrich, A6625), L-NAME (Sigma, N-5751) and apamin (Sigma-Aldrich, A-1289) were prepared in distilled water. Indomethacin (Sigma, I-7378) was prepared with sodium bicarbonate-water mixture. KHS (mM): NaCl 118.0, KCI 4.7, $MgSO_4$ 1.2, $CaCI_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 11.1. NaCl was replaced with equimolar KCI to create an 80 mM KCI solution.

DMEM (D-5796) and penicillin-streptomycin (P-4333) were from Sigma. TMA (Sigma-Aldrich, 243205) was dissolved in PBS at pH 7.4.

Results are shown as the Mean ± Standard Error of the Mean (SEM). Contractions are expressed % of contraction obtained with high KCI (80 mM), and relaxations % of precontraction. The area under the curves (*AUCs*) are expressed as arbitrary units (a.u). pD_2 values are represented as the -logEC_{50}, the half maximal effect occurred by sigmoid curve fitting (GraphPad, Prisim 8). Two response curves were analysed with two-way ANOVA. Multiple comparisons were done using one-way ANOVA and *Tukey's* or *Dunnett's post-hoc* tests. $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

In this *ex vivo* study, it was examined whether TMA showed similar disruptive effects as its hepatic metabolite TMAO on endothelium-dependent relaxation in the rat isolated mesenteric artery. The TMA concentration was chosen as 1 mM to avoid a possible cytotoxic effect that may occur above this concentration (Jaworska, Bielinska, Gawrys-Kopczynska, & Ufnal, 2019). In rat mesenteric arteries, endothelium-dependent relaxations are mainly mediated by EDH and NO. To examine EDH-type vasorelaxations, cumulative and endothelium-dependent relaxations of acetylcholine were first obtained in the presence of L-NAME (100 µM)+INDO (10 µM). The same preparations were then pretreated with 1 mM TMA in bath media for 1 and 4 hours of acute exposure. Acetylcholine-induced EDH-type relaxations were significantly reduced by 1- and 4-hour TMA incubation (Figure 1A and 1B). The acute effects of TMA on the EDH-type relaxations were detected as early as 1 hour and intensified within 4 hours. We have previously shown that acute treatment with 3 mM TMAO does not cause any changes in EDH-type in the same preparations, and the responses to acetylcholine are significantly reduced by increasing the incubation time to 24 hours (Hamad, Ozkan, & Uma, 2021). Similar to this data, extending the incubation period with 1 mM TMA up to 24 hours caused a more significant decrease in EDH-mediated relaxations compared to 1-hour incubation (Figure 1C). However, similar inhibitions were obtained with 4 and 24 hours of incubations. The AUC values of the relaxation-response curves obtained with acetylcholine after 1 and 24 hours of TMA incubation were found to be significantly different compared to each other ($p < 0.05$) (Figure 1D).

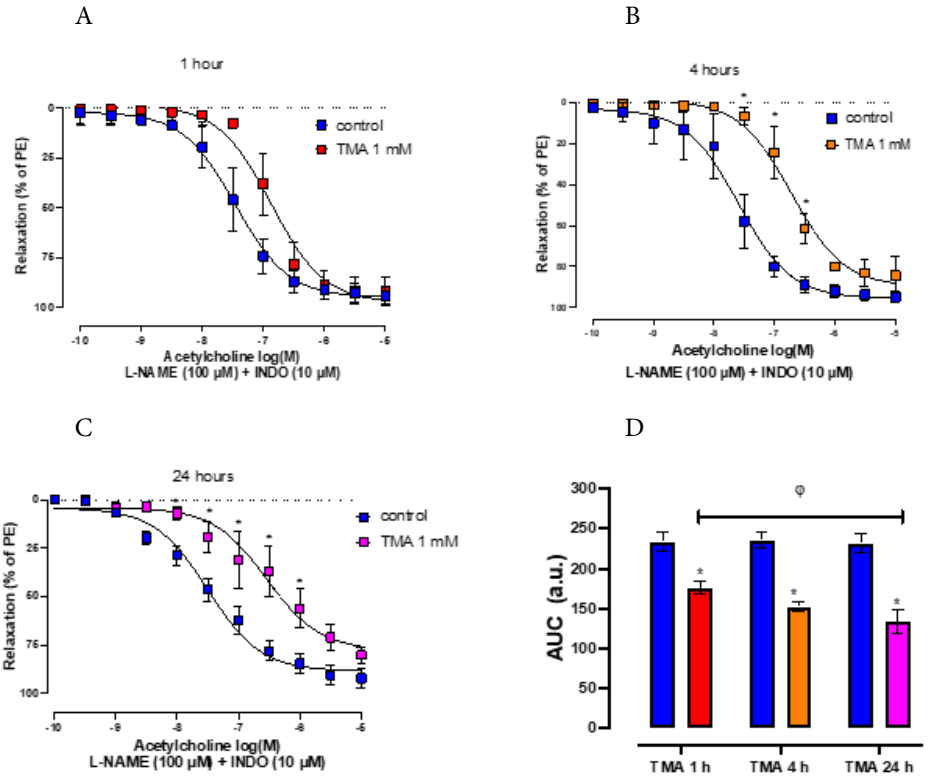

Figure 1. Acetylcholine-induced EDH-type vasorelaxations after 1-, 4- or 24-hour TMA exposure in mesenteric arteries after precontraction with phenylephrine (PE; 0.3 µM). Concentration-dependent relaxations obtained with acetylcholine in the presence of L-NAME (100 µM) and indomethacin (INDO: 10 µM) significantly decreased after 1 hour (A), 4 hours (B), and 24 hours (C) of incubation with TMA (1 mM). The AUC values (D) of the relaxation-response curves obtained with acetylcholine after 1 and 24 hours of TMA incubation were significantly different compared to each other (n=7) (*significantly different from its control; $^{\Phi}$significantly different from 1-hour incubation with TMA) ($p<0.05$).

In another series of experiments, long-term TMA incubations were done in organ culture media to evaluate its effect on NO relaxations. After 24 hours of exposure to TMA in cell-culture medium, the arterial rings were placed in organ baths, and then acetylcholine-stimulated endothelium-dependent and NO-mediated relaxations were obtained in the presence of TRAM-34+INDO+APA. Interestingly, these responses were unchanged in mesenteric arteries treated with 1 mM TMA or its solvent PBS for 24 hours (Figure 2).

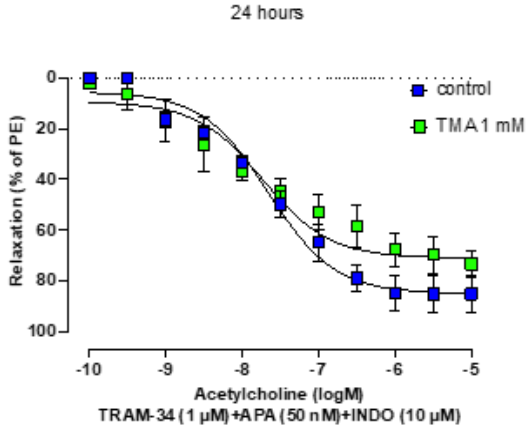

Figure 2. Acetylcholine-induced NO-mediated vasorelaxations after 24 hours of TMA (1 mM) or PBS (control) exposure in rat mesenteric arteries incubated with TRAM-34+INDO+APA. The long-term tissue presence of TMA did not lead to a change in the amplitude or sensitivity of the relaxations to acetylcholine (n=7).

Phenylephrine-induced cumulative contractions were also found to be TMA-resistant after 24-hour incubation (Figure 3).

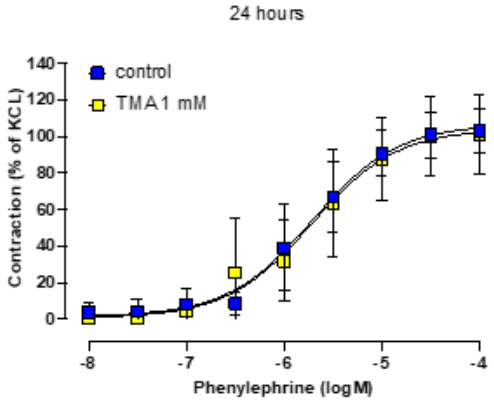

Figure 3. Phenylephrine-induced contractions after 24 hours of TMA (1 mM) exposure in rat isolated mesenteric arterial rings. The long-term presence of TMA in bath media did not affect the contractile responses in the mesenteric arterial rings (n=7).

In this study, we conducted *ex vivo* experiments to examine the possible effects of TMA, the precursor of TMAO, on endothelium-dependent vasorelaxations obtained with acetylcholine. TMA and TMAO levels in plasma increase in cardiovascular patients with decreased glomerular filtration rates, and in aged rats (Jaworska, Bielinska, Gawrys-Kopczynska, & Ufnal, 2019). Previously, we have shown no deterioration in endothelium-dependent relaxations obtained with acetylcholine after 1 or 4 hours of acute exposure to TMAO (1 or 3 mM) in rat isolated mesenteric arteries. However, TMAO at 3 mM concentration selectively inhibited EDH-type relaxations in response to acetylcholine when the incubation period was prolonged up to 24 hours (Hamad, Ozkan, & Uma, 2021). In the current study, a similar experimental protocol was applied to investigate whether TMA would worsen endothelium-dependent relaxations similarly to TMAO. Interestingly, TMA at 1 mM concentration inhibited EDH-type vasorelaxations to acetylcholine as early as acute incubations of 1 and 4 hours. However, extending the duration of treatment with TMA to 24 hours maintained a decrease in EDH-type responses, but those were similar to that obtained at 4-hour incubation group. Although previously we have shown that TMAO has a time-dependent progression in its destructive effects, leading to a decrease in EDH-type vasorelaxations (Hamad, Ozkan, & Uma, 2021), the inhibitory potency of TMA on the same type of relaxations might appear more pronounced and severe in the acute exposure periods. These data propose that TMA can worsen endothelial function in a much shorter time and more severely than TMAO when its level increases in acute cardiovascular events (Jaworska, Konop, Hutsch, Perlejewski, Radkowski, Grochowska, ... Ufnal, 2020; Hamad, Ozkan, & Uma, 2021). The signaling pathways and subsequent events triggered by TMA in vascular endothelium are precisely unknown. The inhibition of EDH-type relaxations with TMA may be due to TMA somehow inhibiting membrane hyperpolarization through post-receptor events, thus preventing calcium-activated potassium channels from opening and vasorelaxation. However, to support this, some specific experiments such as membrane potential or calcium measurements in the vascular endothelium need to be done. Previously some G-protein coupled receptors like Olr78 and Gpr41 expressed on endothelium, the target of short-chain fatty acids, which are gut microbiota metabolites, which implies such a target may also apply to TMA or TMAO (Natarajan, Hori, Flavahan, Steppan, Flavahan, Berkowitz, & Pluznick, 2016). Recently, TMA has been suggested to be an agonist for trace amine-associated receptor 5 (TAAR5) in human olfactory epithelial cells (Wallrabenstein, Kuklan, Weber, Zborala, Werner, Altmuller, ... Gisselmann, 2013). However, it remains unclear what the target molecules are that mediate the effect of TMA on vascular endothelium or smooth muscle layer. Interestingly, the FMO-3 enzyme subtype, which is responsible for the oxidation of TMA to TMAO, has also been detected in rat aortic perivascular adipose tissue (Restini, Fink, & Watts, 2021). Since perivascular adipose tissue functions with anticontractile effects in regulating vascular tone (Chang, Garcia-Barrio, & Chen, 2020), it may also play a modulatory role in TMA metabolism and trigger the conversion to TMAO, a less toxic molecule, against increasing plasma TMA levels. All these studies provide that TMA might play a potential role in vascular diseases and contribute to endothelial dysfunction through direct and, or indirect effects on the endothelium.

In conclusion, the findings of the current study confirm that TMA inhibits EDH-type vasorelaxations without causing disruption with NO-mediated relaxations like TMAO. Moreover, short exposure periods to TMA are sufficient to reduce EDH-type relaxations in rat isolated mesenteric arteries.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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