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TMAO, a carnitine-derived metabolite, prolongs the hypertensive effect of Ang II in rats

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Brief summary.

Clinical studies suggest that an elevated plasma TMAO level is associated with an increased risk of adverse cardiovascular events. Our study shows that TMAO prolongs the hypertensive effect of Ang II in rats. This implies that TMAO is not only a marker of an increased cardiovascular risk but may also be involved in the etiology of cardiovascular diseases.

Abstract.

Background. Recent evidence suggests that an elevated plasma trimethylamine N-oxide (TMAO) level is associated with an increased risk of adverse cardiovascular events in humans; however, the mechanism is not clear. The aims of this study were to establish plasma TMAO level in rats as well as to evaluate the effect of TMAO on arterial blood pressure (BP) and hemodynamic effects of angiotensin II (Ang II).

Methods. 12-week-old, Sprague-Dawley rats were implanted with telemetry transmitters and continuous recordings of heart rate, systolic (SBP) and diastolic (DBP) arterial blood pressures were made for 7 days before and 14 days during osmotic minipump-driven subcutaneous infusion of either: saline (controls), TMAO, low-dose Ang II or Ang II+TMAO. Plasma TMAO concentration was evaluated using liquid chromatography coupled with triple-quadrupole mass spectrometry.

Results. Plasma TMAO concentration in controls was 0.57 µmol/L, while in TMAO infused rats it was 58 µmol/L. Neither saline nor TMAO infusion affected SBP and DBP. Infusion of Ang II significantly increased SBP and DBP for the first 5 days of infusion only. In contrast, infusion of Ang II+TMAO produced hypertensive response which lasted until the end of the experiment. TMAO infusions did not affect body weight and motor activity.

Conclusions. We showed that physiological plasma TMAO concentration in rats was approximately ten times lower than that reported in humans. Furthermore, the new finding of the study is that TMAO does not affect BP in normotensive animals. However, it prolongs the hypertensive effect of Ang II.

INTRODUCTION.

Trimethylamine N-oxide (TMAO) is an organic compound whose concentration in blood increases after ingesting dietary l-carnitine and phosphatidylcholine, which are abundant in red meat [1-3]. Ingested phosphatidylcholine is broken down by intestinal lipases into choline and multiple compounds containing choline. These, in turn, are metabolized in the large bowel by intestinal bacteria producing trimethylamine (TMA). TMA is rapidly oxidized to TMAO by flavin-containing monooxygenase, a hepatic enzyme [4]. A deficiency of the enzyme results in trimethylaminuria or a "fish odor syndrome" which is a rare, autosomal recessive disease characterized by the accumulation of TMA in the body [5].

Recent evidence suggests that an elevated fasting plasma TMAO level is associated with an increased risk of major adverse cardiovascular events in humans [2]. It has been suggested than TMAO may constitute a link between high red meat consumption and cardiovascular diseases [1, 3]. It is worth noting, however, that high concentrations of TMAO are also found in saltwater fish [6], the consumption of which has been considered to have a beneficial effect on the circulatory system [7].

The effects of TMAO on arterial blood pressure (BP) have not yet been studied. Moreover, plasma level of TMAO in rats has not been established, whereas rat models have dominated research on cardiovascular diseases. In this study we performed direct quantitative analysis of plasma TMAO in rats. We also evaluated the effect of TMAO on BP. As the angiotensin II (Ang II) - dependent mechanisms play a key role in the pathophysiology and pharmacotherapy of cardiovascular diseases such as hypertension [8] we decided to explore possible interactions between hemodynamic effects of Ang II and TMAO.

MATERIALS AND METHODS.

The experiments were made according to Directive 2010/63/EU and approved by the Ethical Committee of the Medical University of Warsaw. We did the study on four groups (n=6) of male, 12-week-old, Sprague Dawley rats, fed standard laboratory chow with sodium content of 0.22%, (Labofeed H, Morawski, Poland). Rats were given ad libitum access to food and water, and were housed in 12/12-hour light-dark cycle.

Rats were implanted with telemetry transmitters (Data Sciences International, St. Paul, U.S.A.) and continuous recordings of heart rate (HR), systolic blood pressure (SBP), diastolic blood pressure (DBP) and animal activity were made for 7 days before and 14 days during osmotic minipump (ALZET 2ML, Durect, Cupertino, U.S.A.) driven subcutaneous infusion of either: saline, TMAO [3.86 nmol × s^{-1} (0.29 µg × s^{-1}), Ang II, [0.76 pmol × s^{-1} (0.8 ng × s^{-1})] or Ang II + TMAO. All surgical procedures were performed under general anaesthesia with ketamine 100 mg/kg bw IP (Bioketan, Vetoquinol Biowet, Poland) and xylazine 10 mg/kg bw IP (Xylapan, Vetoquinol Biowet, Poland).

After the hemodynamic experiments the rats were anesthetized as described above, and venous blood was collected for evaluation of plasma TMAO concentration using liquid chromatography coupled with triple-quadrupole mass spectrometry, Waters Acquity Ultra Performance Liquid Chromatograph coupled with Waters TQ-S triple-quadrupole mass spectrometer (for detailed description of the method see online supporting materials). Afterwards, the anesthetized animals were killed by cervical dislocation and the minipumps were removed to check residual volumes of investigated compounds.

Compounds.

Following compounds were used: LC-MS grade - acetonitrile, 25% ammonium hydroxide and formic acid (J.T. Baker, Austin, USA), HPLC gradient grade Acetone (POCh, Gliwice, Poland). Ultra-pure water was obtained from water purification system (Mili-Q, Millipore, Milford, MA, USA). Angiotensin II, trimethylamine N-oxide dehydrate and trimethylamine - 13C3,15N Hydrochloride (TMAx – internal standard) were purchased from Sigma-Aldrich. Stock solutions of TMAO and TMAx were prepared in methanol and stored at -20^{o} C.

Statistics.

The results are expressed as means ± SE. To check the effects of SC infusions within series, we compared the average of over 12 hr dark cycle before the onset of infusion with the averages of the following 14 dark cycles during continuous SC infusions, with one-way analysis of variance (ANOVA) for repeated measures, followed by Tukey's test. Comparisons between series were evaluated by one-way ANOVA or multivariate ANOVA for repeated measures when appropriate, followed by Tukey's test. Two-sided $P < 0.05$ was considered significant. All analyses were conducted using STATISTICA 10.0 (StatSoft, Krakow, Poland).

RESULTS.

Plasma levels of TMAO in rats.

There were significant differences in plasma TMAO between the groups [$F(3,20) = 77.4$, $P< 0.05$], (Table 1). Plasma concentration of TMAO in rats infused with saline was 0.57 µmol/L ± 0.09 while in rats infused with TMAO it was 58 µmol/L ± 5.2.

Effects of chronic infusion of TMAO and Ang II on BP in rats.

Baseline DBP and SBP were comparable between the groups (Table 1). Neither infusion of saline nor TMAO affected DBP or SBP (Fig. 1A, 1B).

Infusion of Ang II significantly increased DBP [$F(1,14) = 5.1$ $P < 0.05$] and SBP [$F(1,14) = 3.4$ $P < 0.05$], however the increase was present for the first five days of infusion only (Fig. 1A, 1B). In contrast, combined infusion of Ang II and TMAO increased DBP [$F(1,14) = 2.0$ $P < 0.05$] and SBP [$F(1,14) = 2.51$ $P < 0.05$], which lasted until the end of experiments (Fig. 1A, 1B). HR was not affected significantly by infusions; however, ANG II + TMAO group showed small increase in HR during the experiment, while saline, TMAO, and Ang II groups showed bradycardic tendency during the last 4 days of the experiment (Fig. 1C).

During the first 6 days of infusions we found significant differences between the groups in the changes of DBP [$F(3,20) = 12.8$, $P < 0.05$] and SBP [$F(3,20) = 15.2$, $P< 0.05$]. Ang II and Ang II + TMAO groups responded with significantly higher increase in DBP and SBP than TMAO and saline groups (Fig. 1A, 1B). The analysis of the following 8 days of infusions (day 7-14) also revealed significant differences between the groups in changes of DBP [$F(3,20) = 6.7$, $P< 0.05$] and SBP [$F(3,20) = 7.1$, $P< 0.05$]. Rats infused with Ang II + TMAO exhibited significantly higher increase in DBP and SBP than rats infused with saline, TMAO or Ang II alone (Fig 1A.1B).

Effects of chronic infusion of TMAO and Ang II on motor activity and body weight in rats.

There were no significant differences between the groups in body weight (Table 1) and motor activity (Fig. 1D).

DISCUSSION.

We have shown that fasting plasma TMAO concentration in young, healthy rats averages 0.57 µmol/L, which is approximately ten times lower than that reported in humans. The study reveals that a two week 100-fold increase in blood TMAO level does not affect BP in normotensive animals. However, it prolongs the hypertensive effect of chronic low-dose Ang II infusion.

Elevated concentration of plasma TMAO has been suggested to be a new marker of an increased risk of adverse cardiovascular events in humans, defined as death, myocardial infarction, or stroke [2]. Since TMAO is a metabolite of phosphatidylcholine and l-carnitine, both abundant in red meat, TMAO has been proposed to constitute a link between high consumption of red meat and cardiovascular diseases; [3] however, the mechanisms are not clear. Experimental studies show that TMAO may modulate cholesterol and sterol metabolism resulting in the development of atherosclerosis [3] . On the other hand, it is possible that an increase in blood TMAO may reflect the kidneys malfunction [9] and low glomerular filtration rate which is an independent cardiovascular risk factor [10]. In this context, low glomerular filtration rate along with other risk factors such as hypertension and diabetes mellitus were common in the top quartile of TMAO blood levels in patients with increased cardiovascular risk in the study by Tang and collaborators [2].

The concentration of TMAO in blood plasma in mammals has been evaluated in few studies and was reported to be in the range of 3 and 40 µmol/L in humans [1, 2, 9] and less than 5 µmol/L in mice [1, 3]. The blood TMAO level depends on several factors, such as the amount of dietary choline, the action of the gut microbiota and the activity of flavin-containing monooxygenase 3. In this context, it has been found that dietary choline challenge increases the concentration of TMAO, while treatment with broad-spectrum antibiotics reduces TMAO

concentration in the blood [1, 11, 12]. To our knowledge, a direct quantitative analysis of plasma TMAO in rats has not yet been done. Our study shows that concentration of TMAO in young, healthy rats is approximately 0.6 µmol/L which is at least 10 times lower than in humans. This may result from differences between humans and rats in guts' microbiota, a lower content of dietary choline in rats' chow, or both.

Hypertension is an important independent risk factor for adverse cardiovascular outcomes. Ang II plays a key role in the regulation of BP via its hemodynamic effects. Autocrine and paracrine effects of Ang II are recognized in cardiovascular remodelling [8]. Here, we have found that TMAO does not change resting BP in normotensive rats. However, it affects hemodynamic response to Ang II.

To explore a possible interaction between hemodynamic effects of Ang II and TMAO we treated rats with concomitant infusion of TMAO and low-dose Ang II. We decided to use a low-dose Ang II infusion protocol because rats infused with a high-dose Ang II show strong hypertensive response [13,14], and it has been shown that animals with high blood pressure fail to respond to pressor compounds [15,16]. We found that infusion of Ang II alone significantly increased BP for the first 5 days of infusion, while the concomitant infusion of Ang II and TMAO produced sustained increase in BP, which suggests that TMAO prolonged the hypertensive effect of Ang II.

There are several well-described rat models of hypertension induced by chronic subcutaneous infusion of Ang II at a dose of over 50 ng × min $^{-1}$ [13]. In contrast, chronic infusion of Ang II at a dose of 50 ng × min $^{-1}$ have been shown to produce sustained borderline hypertension [13,17] or the lack of significant hemodynamic effects [18]. In the present study rats infused with Ang II at the lower dose showed a significantly increase in SBP and DBP for the first 5 days of infusion, followed by return of their BP to baseline level. Inconsistent results of studies on

hemodynamic effects of a low-dose Ang II may be caused by differences in sodium content in rat's chow [17], different stress levels due to animal handling [19] or different recovery period after implantation of telemetry transmitters.

Blood-borne Ang II is known to produce a long-term hypertensive response by both peripheral and brain mechanisms [20-21]. Since the hypertensive effect of combined treatment with Ang II and TMAO appeared after 5 days of infusions, it may be hypothesized that the effect was caused rather by long-term Ang II-dependent mechanisms such as an increase in blood volume or an increase in sympathetic drive due to the activation of central mechanisms involved in BP control [13,21] . This notion may also be supported by our preliminary experiments in which we found that short, 30-seconds-long IV infusions of TMAO at a dose of 1 to 12 mmol × s^{-1} did not affect resting BP and hypertensive effects of Ang II in rats (data not presented).

In this study, the effect of TMAO on the hemodynamic response to Ang II was present in animals whose blood TMAO level was 100 times higher than that under physiological conditions in young, healthy rats, and ten times higher than that found to be associated with increased cardiovascular risk in humans [2]. Interestingly, such high plasma TMAO level had no effect on rat's body weight and motor activity. The lack of any toxic effect of TMAO at a concentration of 50-60 µmol/L may be less surprising if compared to TMAO blood level in deep-sea animals whose blood TMAO concentration ranges from 100 to 300 µmol/L [6].

In mammals TMAO is considered as a waste product of choline metabolism; however, it plays an important role as an osmolyte. In general, osmolytes are small compounds used by cells to maintain cell volume when exposed to osmotic and hydrostatic pressure stresses. Deep-sea fishes utilize TMAO to counteract the protein-destabilizing effects of osmotic and

hydrostatic pressures [6, 22]. In humans, osmolytes are of particular importance in the renal medulla [22] in which osmolarity may exceed osmolarity of plasma four times.

It has been found that osmolytes such as TMAO can enhance protein folding, ligand binding and can counteract perturbations caused by urea or inorganic ions [22-24]. Therefore, it is possible that TMAO affects the structure of receptors and peptide hormones such as Ang II, and may be not only a marker of increased cardiovascular risk, but also a mediator in etiology of cardiovascular diseases.

A limitation of the study is that the hemodynamic measurement during sc infusions lasted 14 days only. Besides, to achieve subpressor effect of Ang II we infused rats with low dose Ang II, while the number of studies on the effects of chronic low dose Ang II on BP is limited and the results are inconsistent [13,18]. Further research including an evaluation of water-electrolyte balance and the activity of sympathetic nervous system and the renin-angiotensin-aldosterone system is needed to provide mechanistic explanation for our findings.

Conclusions.

This is the first study of hemodynamic effects of TMAO in rats. The results show that a two week 100-fold increase in blood TMAO does not affect BP in rats; however, it prolongs the hypertensive effect of chronic low-dose Ang II infusions. Whether TMAO plays a role of a mediator in the etiology of cardiovascular diseases or whether its high concentration only coexists with factors hindering homeostasis of the circulatory system remains to be elucidated.

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Conflict of Interest

None.

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Table 1. Hemodynamic parameters, trimethylamine N-oxide (TMAO) plasma concentration and body weight of rats. Rats infused with saline (saline group), trimethylamine N-oxide (TMAO group), angiotensin II (Ang II group) or Ang II + TMAO (Ang II + TMAO group). Means ± SE.

Group	**Saline**	**TMAO**	**Ang II**	**Ang II + TMAO**
Baseline SBP (mmHg)	135.5±1.7	135.0±1.6	133.4±2.9	134.2±2.5
Baseline DBP (mmHg)	95.3±1.3	95.5±2.3	95.5±3.0	93.8±2.7
Baseline HR (beats/min)	381±12	371±6	370±12	379±6
Plasma TMAO concentration (μmol/L)	0.57±0.09$^{*\#}$	58±5.2	0.69±0.10	65±5.0
Body mass (g)	317±6	321±7	320±5	315±5
the first and the last day of experiments	346±8	347±5	344±5	341±6

*-Saline *vs* TMAO group P<0.05, $^{\#}$-Saline *vs* Ang II+TMAO group P<0.05.

Figure Legends.

Figure 1. Changes from baseline in hemodynamic parameters and motor activity in Sprague Dawley rats during chronic SC infusions of saline (saline), trimethylamine N-oxide (TMAO), angiotensin II (Ang II) or combined infusion of Ang II + TMAO. Means ± SE are shown.

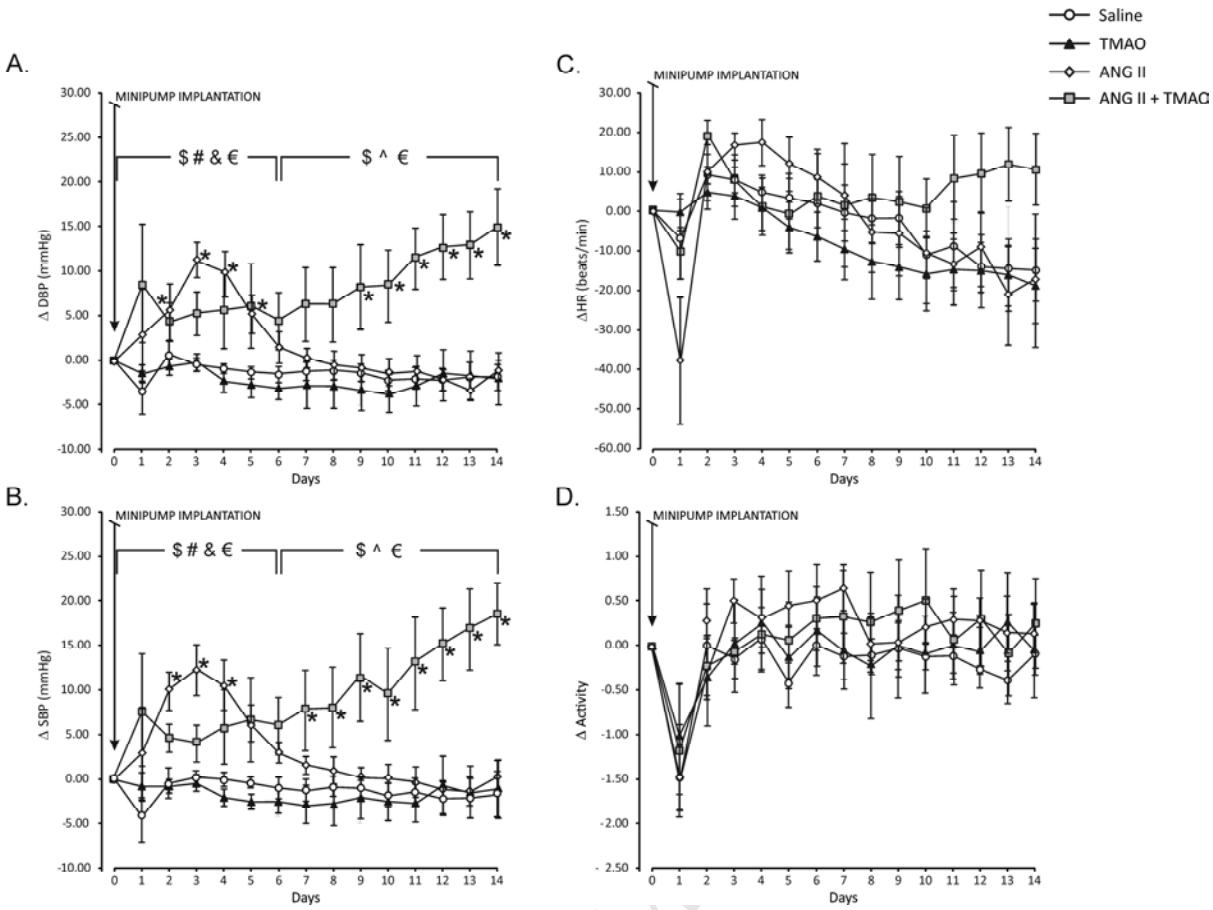
* - *vs* baseline, $ - Saline *vs* Ang II + TMAO group P<0.05, # - Saline *vs* Ang II group P<0.05, & - Ang II *vs* TMAO II group P<0.05, € - Ang II *vs* Ang II + TMAO group P<0.05, ^ - TMAO *vs* Ang II + TMAO group P<0.05.

Fig. 1A. Changes in diastolic arterial blood pressure (DBP, mmHg).

Fig. 1B. Changes in systolic arterial blood pressure (SBP, mmHg).

Fig. 1C. Changes in heart rate (HR, beats/min).

Fig. 1D. Changes in motor activity (counts/min). Semiquantitative activity output, a non-linear index of a transmitter movement.



Quantitative analysis of TMAO in plasma.

Sample preparation. Sample preparation was as follows: 10 μL of plasma was transferred into 1.5 mL silanized conical test tube, then 1000 μL of Acetone with TMAx (300 ng/ml) was added for protein precipitation and analytes extraction. After the mixture was vortexed (1 min) and centrifuged (2 min at 18626 RCF), whole supernatant was transferred to the vial and analyzed by LC/MS/MS. Some samples exceeded concentration of the highest calibration point we prepared. Those were repeated using 2.5 μL of plasma, 7.5 μL of Mili-Q water and 1000 μL of Acetone with TMAx and the results were corrected accordingly.

Instrumentation. Instrumentation consisted of Waters Acquity Ultra Performance Liquid Chromatograph coupled with Waters TQ-S triple-quadrupole mass spectrometer. For the instrument control and data acquisition MassLynx software was used. LC/MS/MS analyses were performed in positive electrospray ionization mode (ESI). Mass spectrometer operated in multiple-reaction monitoring (MRM). The concentration of each analyte was calculated using TMAx as internal standard.

For chromatographic separation we applied UPLC BEH HILIC column (50 x 2,1 mm, 1,7 μm, Waters) thermostatted at 70 °C. Mobile phase A was Mili-Q water with addition of 1 mL of 25% NH4OH per 1000 mL of water, and mobile phase B was pure Acetonitrile. The flow rate of mobile phase was set at 0.5 mL/min and the injection volume was 3 μL. The gradient scheme was reversed due to using HILIC column: 70% B initially, decrease to 4% B at 0,8 min. At 1,9 min the mobile phase reverted to initial condition (70% B). The total analysis time was 2,5 min including re-equilibration time.

For all analyzed compounds mass spectrometer optimized settings were as follows: capillary voltage = 3.2 kV, desolvation temperature = 650 ºC, desolvation gas flow = 900 L/h, cone gas flow = 250 L/h, nebuliser gas pressure = 7.0 bar, source temperature = 150 °C. MRM transitions, cone voltages, collision energies and retention times used in described methods are presented in Table 1. The first MRM transition of each compound served as a quantitative transition, the second as a confirmation transition.

To define the relationship between the concentration and detector response for analytes 6 calibration points were prepared with concentration as follows: 9.79 μg/ml, 48.96 μg/ml, 97.92 μg/ml, 199.104 μg/ml, 398.208 μg/ml, 499.392 μg/ml. Due to unsuccessful attempt to obtain TMAO free plasma from rats fed with broad spectrum antibiotics, calibration curve was prepared using Mili-Q water as a matrix. We decided that it is acceptable solution because in preparation stage sample is diluted 1:100 and the matrix effect is negligible.

Table

Table 1. Monitored transitions, cone voltages, collision energies and retention times of analyzed compounds.

Analyte	**MRM transition**	**Cone voltage**	**Collision energy**	**Retention time [min]**
TMAO	76.076>57.97 (qt*)	15	50	0.65
	76.076>41.95	15	50	

TMAx (IS)	64.09>47.04 (qt)	20	18	1.3
	64.09>48.05	20	18	

- * qt – quantification transition