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Full Length Article

Trimethylamine N-oxide promotes tissue factor expression and activity in vascular endothelial cells: A new link between trimethylamine N-oxide and atherosclerotic thrombosis

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ABSTRACT

Trimethylamine-N-oxide (TMAO), one of the products in choline metabolite, is recently reported to be associated with cardiovascular diseases (CVD) that mainly attribute to atherothrombosis. However, the mechanisms how TMAO functions in the pathogenesis of CVD and atherothrombosis remain elusive. Tissue factor (TF) has been implicated in the thrombogenicity of atherosclerotic plaques. In the present study, we demonstrated that TMAO promoted TF (but not TF pathway inhibitor) expression via activation of NF-κB signaling pathway in primary human coronary artery endothelial cells (HCAECs). TMAO strongly increased TF activity and thrombin production. Further, a small dose of TMAO significantly increased TF expression and nuclear translocation of NF-κB with the synergistic action of low-dose of pro-atherosclerotic factors, such as TNF-α and HMGB1. Importantly, plasma TMAO level was positively correlated with TF activity in patients with ST-elevation myocardial infarction (STEMI). Altogether, our data revealed that TMAO promoted thrombosis through increasing TF expression and activity. The understanding of the new link between TMAO and atherothrombosis may facilitate therapeutic strategy in the prevention and treatment of atherothrombosis.

1. Introduction

Thrombosis after atherosclerotic plaque disruption is the leading cause of most acute coronary syndromes [1]. Tissue factor (TF), a transmembrane glycoprotein, initiates an extrinsic coagulation cascade when exposed to circulation in the case of atherosclerotic plaque rupture, and eventually results in complete or partial vessel occlusion [1,2]. TF-mediated coagulation is pivotal in acute atherosclerotic thrombosis, especially acute myocardial infarction (AMI) [2–4]. TF activation is dispensable in the TF-mediated coagulation [5,6]. On the other hand, tissue factor pathway inhibitor (TFPI) is the only endogenous protein that effectively inhibits extrinsic coagulation pathway by producing factor Xa-mediated feedback inhibition of the factor VIIa/TF catalytic complex [7]. TFPI protects from atherosclerosis and is a central regulator of the thrombosis that occurs in the setting of AMI [8–10].

Recently, trimethylamine-N-oxide (TMAO) was reported to be closely related with atherosclerosis development [11]. Consistently, a large number of multi-center and multi-population clinical researches have demonstrated a strong association between the plasma levels of TMAO and cardiovascular diseases or AMI mortality. TMAO remarkably enhanced the cardiovascular risk or atherosclerosis [12–15], and the inhibition of TMAO production in vivo reduced thrombosis risk [16,17]. However, it is still not clear how TMAO promotes atherothrombosis. Since there is a low plasma level of TMAO in the majority of patients with atherothrombotic diseases and the low level of TMAO is still significantly associated with undesirable outcomes [15], TMAO may have synergic role with other pro-atherosclerotic factors including TNF-α and HMGB1 in pathogenesis of atherothrombosis. In addition, the involvement of TMAO in the modulation of TF activity has not yet been reported. The aim of our study is to investigate the underlying mechanisms of TMAO in atherothrombosis, which may facilitate the understanding of the link between TMAO and atherothrombosis.

2. Materials and methods

2.1. Cell culture and stimulation

Primary human coronary artery endothelial cells (HCAECs), isolated from normal human coronary arteries, were obtained from Clonetics (San Diego, CA) and cultured in Endothelial Growth Medium-2

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(Clonetics, Inc.) with full supplements and 5% FBS (Gibco) as described previously [18]. Before experiments, HCAECs were plated in 12-well plates in complete EGM-2 medium with full supplements and 5% FBS overnight. The HCAECs were then washed three times and stimulated (80%–90% confluence) with 10–200 μM trimethylamine-N-oxide (TMAO) (Sigma, USA) alone or incubated with 10–40 μM BAY-117082 (Sigma, USA) or 5–20 μM JSH-23 (Selleck, USA) 30 min prior to TMAO stimulation in EGM-2 medium containing 2% FBS. In some experiments, 10 μM TMAO was added 1 h before an incubation with tumor necrosis factor-alpha (TNF-α , 0.01 ng/mL, 4 h) (R&D Systems, USA) or high mobility group box 1 (HMGB1, 1 ng/mL, 4 h). Recombinant HMGB1 protein was produced in *E. coli* and purified as described previously [19]. After the treatments for the indicated time, the cells and the supernatants were collected for subsequent experiments.

2.2. Patients samples collection

Patients diagnosed with ST-elevation acute myocardial infarction (STEMI) [20] were included in the study. Inclusion criteria were: (a) chest pain lasting > 30 min, (b) ST-segment elevation ≥2 mm in at least 2 contiguous precordial leads (V1 to V6) of the electrocardiogram, (c) occlusion of an infarct-related artery (TIMI flow < 3), (d) visualization of coronary thrombosis, and (e) clinical indications for percutaneous coronary intervention (PCI) and thromboaspiration. Exclusion criteria were: malignancy, inflammatory diseases or severe acute ischemia other than myocardial ischemia, surgery within 1 month, renal replacement therapy, previous myocardial infarction or history of heart failure. Fourty four patients fulfilled the above criteria and were included in the study at the Third Xiangya Hospital of Central South University from January 2017 to September 2018. Eleven healthy individuals, matched for age and body mass index (BMI) with patients, were recruited as control subjects. Clinical characteristics of these subjects were recorded during hospital admission (shown in Table 1). The study protocol was approved by the ethics committee of the Third Xiangya Hospital of Central South University. Informed consent was obtained from all subjects prior to the commencement of the experiments.

In admission, all patients underwent urgent coronary angiography for PCI, and blood samples were collected into tubes containing 3.8% sodium citrate. Plasma was immediately obtained after centrifuges at 3000 ×*g* for 10 min. Plasma was then aliquoted and stored at −80 °C until the analysis for the level of TMAO, TF activity, HMGB1 and TNF-α. All enrolled patients were contacted for follow-up with a mean of two months after the PCI. Patients died of non-cardiovascular causes were excluded from analysis.

2.3. Western blot analysis

The cells were lysed in sodium dodecyl sulfate (SDS) buffer. Nuclear and cytoplasmic proteins were isolated using the NE-PER Kit as described by the manufacturer (Cat 78,833, Thermo Fisher Scientific, USA). Proteins were separated by 10% SDS-PAGE and transferred onto PVDF membranes (Millipore, Bedford, MA). The membranes were incubated with primary antibodies, including anti-human TF (Cat ab104513, Abcam, UK), TFPI (Cat ab66544, Abcam, UK), c-Rel (Cat 4727, Cell Signaling Technology, USA), p65 (Cat 8242, Cell Signaling Technology, USA), p-IκBα (Cat 2859, Cell Signaling Technology, USA) , and PCNA (Cat 13,110, Cell Signaling Technology, USA) at a dilution ratio of 1:1000 at 4 °C overnight. After incubation with secondary antibodies, the blots were visualized using ECL assay (Cat 32,106, Thermo Fisher Scientific, USA) and imaged using Gel Doc XR + (Bio-Rad, USA). Blots were normalized to the expression of β-actin (Cat 3700, Cell Signaling Technology, USA) or PCNA. Data were analyzed using Image J software.

2.4. TF protein assays

The cells were lysed in Cell Lysis Buffer 1 (5 mM HEPES, pH 8.0, 85 mM KCL, 0.5% Triton). Tissue factor (TF) protein levels were determined using a commercial kit: Human Coagulation Factor III/Tissue

Table 1
Patients' clinical characteristics (n = 44).

Subjects	STEMI (n = 44)		Health (n = 11)	P (Alive vs dead)
	Alive (32)	Dead (12)		
Males (%)	21 (65.6)	7 (58.3)	4 (36.4)	0.654
Age	64.28 ± 9.82	67.88 ± 10.97	50.46 ± 11.42	0.4099
BMI (kg/m^2)	23.92 ± 0.69	22.5 ± 0.59	22.45 ± 1.02	0.2460
Smokers (%)	14 (43.8)	9 (75)	3 (27.3)	0.065
Family history of CAD, n (%)	9 (28.1)	2 (16.7)	NA	0.267
Hypertension, n (%)	30 (93.8)	11 (91.7)	NA	0.807
Diabetes, n (%)	7 (21.9)	4 (33.3)	NA	0.434
Previous peripheral artery disease, n (%)	9 (28.1)	3 (25.0)	NA	0.957
Patients treated with				
ACE inhibitor/ARB, n (%)	19 (59.4)	7 (58.3)	NA	NA
β-Blocker, n (%)	2 (6.3)	2 (16.7)	NA	NA
Acetylsalicylic acid, n (%)	13 (40.6)	4 (33.3)	NA	NA
Calcium flow inhibitor, n (%)	11 (34.4)	5 (41.7)	NA	NA
Anticoagulants, n (%)	5 (15.7)	1 (8.3)	NA	NA
Total cholesterol (mmol/L)	4.21 ± 0.26	4.43 ± 0.40	1.39 ± 0.07	0.6509
LDL-cholesterol (mmol/L)	2.8 ± 0.14	2.58 ± 0.15	2.01 ± 0.13	0.3595
HDL-cholesterol (mmol/L)	1.11 ± 0.06	0.90 ± 0.06	1.21 ± 0.06	0.0503
Triacylglycerol (mmol/L)	1.57 ± 0.08	1.93 ± 0.23	1.33 ± 0.09	0.0761
TNF-α (pg/mL)	3.95 ± 0.48	5.90 ± 0.72*	1.55 ± 0.31	0.0291
HMGB1 (μg/L)	3.04 ± 0.20	4.58 ± 0.54**	1.17 ± 0.19	0.0024
TMAO (μmol/L)	3.53 ± 0.29	5.65 ± 0.54***	1.68 ± 0.21	0.0007
TF activity (pmol)	10.75 ± 0.54	15.32 ± 1.03***	3.64 ± 0.19	0.0001

STEMI: ST-elevation acute myocardial infarction; BMI: Body Mass Index; LDL: low-density lipoprotein; HDL: high-density lipoprotein; TNF-α: tumor necrosis factor α; HMGB1: high mobility group box-1 protein; TMAO: trimethylamine N-oxide; ACE: angiotensin converting enzyme; ARB: angiotensin II receptor blockers; NA: not available.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

Factor Immunoassay Kit (Cat DCF300, R&D Systems) according to the manufacturer's protocol.

2.5. RNA extraction and quantitative real-time (RT)-PCR

Total RNA was extracted using RNeasy Mini kit (Ambion, USA) according to the manufacturer's instructions. Afterward, 1 μg of total RNA was reversely transcribed to cDNA using the Reverse Transcription Kit (Cat #k1622, Thermo Fisher Scientific) and quantitative real-time PCR was performed using the following primers (synthesized by Sangon Biotech (Shanghai) Co., Ltd.): *tf*-F: TGATGTGGATAAAGGAGAAAACT ACTGT; *tf*-R: CTACCGGGCTGTCTGTACTCTTC [21]; *tfpi*-F: GATGGTC CGAATGGTTTCCAG; *tfpi*-R: GATTGCGGAGTCAGGGAGTTA (Primer Bank); *gapdh*-F: ACAACTTTGGTATCGTGGAAGG; *gapdh*-R: GCCATCAC GCCACAGTTTC (Primer Bank). The results were normalized to gapdh mRNA levels.

2.6. Flow cytometry assays

The HCAECs were gently detached with 0.25% trypsin (Gibco), washed and incubated in 100 μL PBS containing 5 μL PE anti-human CD142 antibody (Cat 365,204, Biolegend, USA) and 1% BSA for 30 min on ice and in dark. The cells were washed and resuspended in 300 μL PBS supplemented with 1% BSA and analyzed on the CantoII-Flow Cytometer (BD Biosciences, USA). Data were analyzed using FlowJo software.

2.7. TF activity analysis and thrombin activity assay

TF activity of samples was determined using the TF activity assay kit (Cat CT10002B, AssayPro, USA) according to the manufacturer's instructions. Briefly, cell lysates (10 μg) were added to the assay buffer containing FVII and FX, and incubated at 37 °C 30 min, followed by adding 20 μL FXa substrate. The absorbance at 405 nm was recorded and the TF activity in samples was calculated from the Standard Curve. The cellular TF pro-coagulant activity was assessed using SensoLyte AFC Thrombin Activity Assay Kit (Cat AS-72129, AnaSpec, USA). Specifically, the protein samples (10 μg) were mixed with 20% human platelet-poor plasma, and then 50 μL of thrombin substrate was added into 96-well black microplate. The reaction mixture was incubated for 40 min and was protected from light. Finally, the fluorescence intensity was measured at Ex/Em = 380 nm/500 nm.

2.8. Plasmids and transient transfection

Wild-type human TF promoter (−227) with or without a mutation in the NF-κB binding sites and luciferase were constructed in a pGL3 plasmid as described elsewhere [22]. The HCAECs were plated in 12-well culture plate overnight (3×10^5/well). Afterward, the cells were transfected with 800 ng DNA/well for 6 h using the Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. All groups were co-transfected with equal amounts of a pRL-TK plasmid encoding Renilla luciferase (Renilla Luciferase, Promega) for compensating variations in transfection efficiencies. Twenty-four hours post the transfection, the cells were incubated with 100 μM TMAO for 12 h. Then the cells were collected for luciferase assays using the dual luciferase reporter system (Promega, USA) according to the manufacturer's protocol. Briefly, the firefly luciferase reporter was measured with a luminometer first by adding 100 μL Luciferase Assay Reagent II (LAR II) into 20 μL cell lysate. Next, the Renilla luciferase reaction was simultaneously initiated by adding 100 μL Stop & GloR Reagent (stopping firefly luciferase reaction) to the same tube. The firefly luciferase signal was normalized to the Renilla luciferase signal.

2.9. Measurement of plasma TMAO

Ultra-high performance liquid chromatography (UHPLC)-MS/MS with electrospray for the analysis of TMAO in blood was used in the present study [14]. Briefly, 100 μL of plasma was transferred to a tube followed by addition of d9-TMAO (5 μM), with vortex for 1 min and incubation at 37 °C for 30 min. Afterward, samples were centrifuged at 20,000 × *g* and 4 °C for 60 min and the supernatants were analyzed using LC-MS/MS. As internal standards, various concentrations of TMAO standards were added to control healthy plasma to generate calibration curves that allowed us to quantify plasma TMAO levels. For LC-MS-MS, The Agilent (Agilent Technologies) 1260 HPLC system was equipped with a G1322A vacuum degasser, a G1312B binary pump, a G1316B column oven and a G1367D autosampler. The detection was performed on an Agilent 6410 Series Triple Quadrupole mass spectrometer (Agilent Technologies, Wilmington, DE, USA) with an ESI source. The mobile phase contained methanol with 0.1% formic acid (phase A) and water with 0.1% formic acid (phase B). The ratio of phase A to B was 40:80 with a flow rate of 0.5 mL/min. TMAO were monitored in Multiple Reaction-Monitoring (MRM) modes using characteristic precursor-product ion transitions *m/z* 598.9/99.5 and m/z 544.9/442.7. Data collection and analysis was performed using Agilent MS workstation Masshunter 1.0 software.

2.10. Measurement of plasma HMGB1 and TNF-α

Plasma levels of HMGB1 in subjects were measured using HMGB1 EILSA KIT (Shino-Test Corporation, Kanagawa, Japan). Plasma TNF-α levels were measured using ELISA KIT (Invitrogen) according to the manufacturers' instructions. The overall intra-assay and inter-assay coefficients of variance were both < 10%.

2.11. Statistical analysis

Statistical analyses were performed with GraphPad Prism7.0, and all data were presented as mean ± SD. Statistical significance was determined with the 2-tailed Student's *t*-test. For the comparison of > 2 groups, one-way ANOVA with Tukey's post-hoc test was used. Pearson's correlation coefficient was calculated to analyze relevance. Values of $P \leq 0.05$ were considered statistically significant.

3. Results

3.1. TMAO up-regulated TF expression but did not influence TFPI expression in HCAECs

To investigate the effect of TMAO on TF expression in HCAECs, TF expression levels were determined after exposure to TMAO at various concentrations and different time points. Western blot and ELISA analysis revealed that stimulation of HCAECs with TMAO (10 to 200 μM) led to a concentration- and time-dependent induction of TF expression. The maximal effect was observed at 8 h following stimulation with TMAO (200 μM) (Fig. 1A to D). Furthermore, TF mRNA expression was also boosted by TMAO in a concentration- and time-dependent manner (Fig. 1E and F). A maximum increase of TF mRNA expression, up to 65 folds, was observed in HCAECs after treatment with TMAO (Fig. 1E), indicating that TMAO induced TF expression at the transcriptional level. As intracellular TF are functionally inactive [23], we measured TF expression on HCAECs surface by flow cytometry. TF expression on HCAECs surface was significantly higher in the TMAO-stimulated HCAECs, with a maximum effect at 8 h (Fig. 1G). Tissue factor pathway inhibitor (TFPI) is the direct physiological inhibitor of the TF/FVIIa complex. Interestingly, TFPI mRNA and protein were not influenced by the treatment of TMAO in both dose-dependent and time-dependent manner (Fig.1H to K). Taken together, our results indicated that TMAO enhanced TF expression but did not influence TFPI expression.

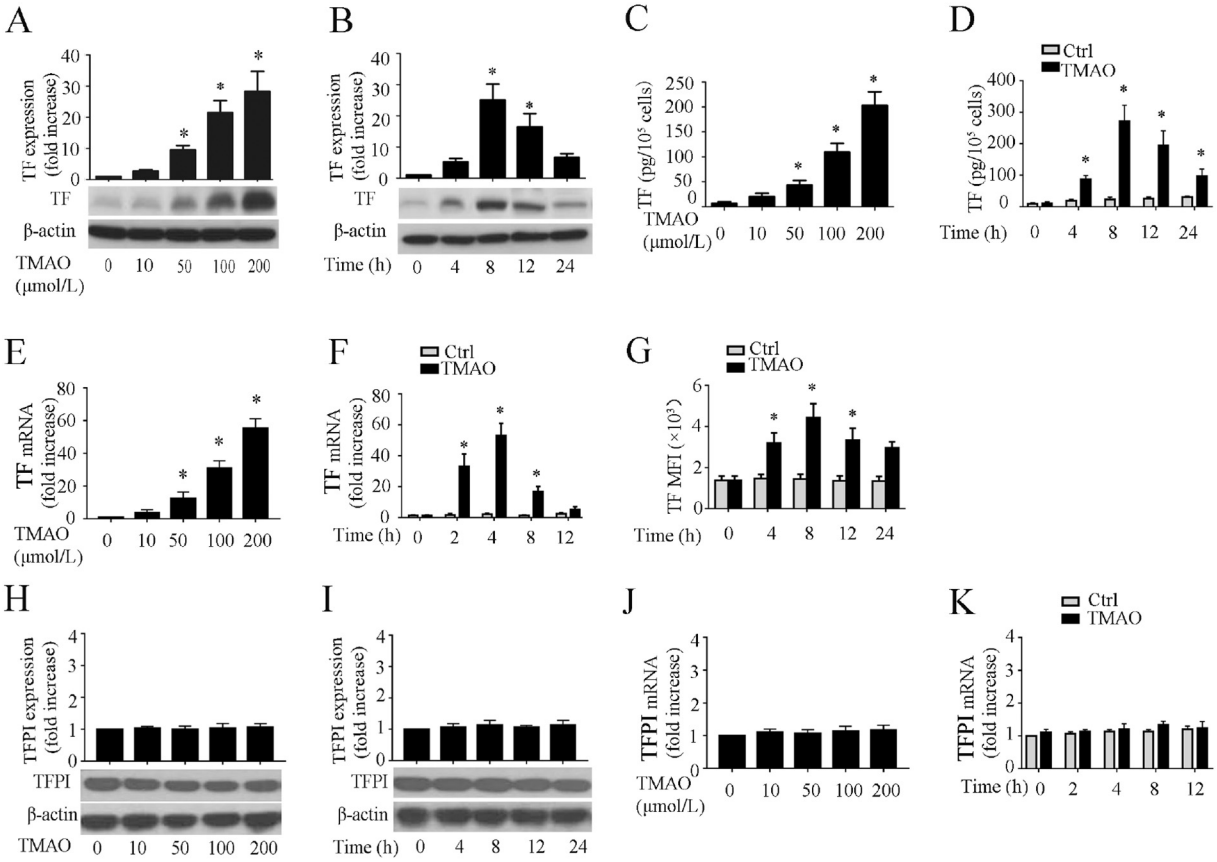

Fig. 1. TMAO up-regulated TF expression but did not influence TFPI expression in HCAECs.
(A and C) TF protein expression in HCAECs stimulated with various concentrations of TMAO (10 to 200 μM) for 8 h as detected by Western blot and ELISA; (B and D) TF protein expression in HCAECs stimulated with 100 μM TMAO for different time points (4 to 24 h) as detected by Western blot and ELISA; (E) The level of TF mRNA in HCAECs stimulated with various concentrations of TMAO (10 to 200 μM) for 4 h as detected by RT-PCR; (F) The level of TF mRNA in HCAECs stimulated with 100 μM TMAO for different time points (2 to 12 h) as detected by RT-PCR; (G) TF expression on the surface of HCAECs stimulated with 100 μM TMAO for different time points (4 to 24 h) as detected by flow cytometry. (H) TFPI protein expression in HCAECs stimulated with various concentrations of TMAO (10 to 200 μM) for 8 h as detected by Western blot. (I) TFPI protein expression in HCAECs stimulated with 100 μM TMAO for different time points (4 to 24 h) as detected by Western blot. (J and K) TFPI mRNA expression in HCAECs stimulated with various concentrations of TMAO (10 to 200 μM) for 4 h or 100 μM TMAO for different time points (2 to 12 h) as detected by RT-PCR; MFI: mean fluorescence intensities. Data are representative of five independent experiments; (*P < 0.05 vs Control group).

3.2. TMAO enhanced TF activity and thrombin production in HCAECs

TF activity in HCAECs was significantly increased at 4, 8, 12 and 24 h of treatment using 100 μM TMAO with a maximum effect at 8 h (Fig. 2A), which consisted with the results of TF protein expression. To further confirm the TF pro-coagulant activity (PCA), the Thrombin Activity Assay Kit was utilized. Similar results were observed (Fig. 2B). Thus, TMAO enhanced TF activity and thrombin production in HCAECs.

3.3. TMAO-induced TF expression depended on the activation of the NF-κB signaling pathway

The transcription factor, nuclear factor-κB (NF-κB), is responsible for regulating TF expression [22,23]. Since NF-κB activation requires IkBα phosphorylation and NF-κB nuclear translocation, where it functions as nuclear transcription factor [24], we detected the level of cytoplasmic p-IkBα and nuclear levels of NF-κB (p65/c-Rel) by Western blot. Following stimulation with TMAO, the level of p-IkBα and nuclear levels of p65/c-Rel were significantly increased in a concentration-

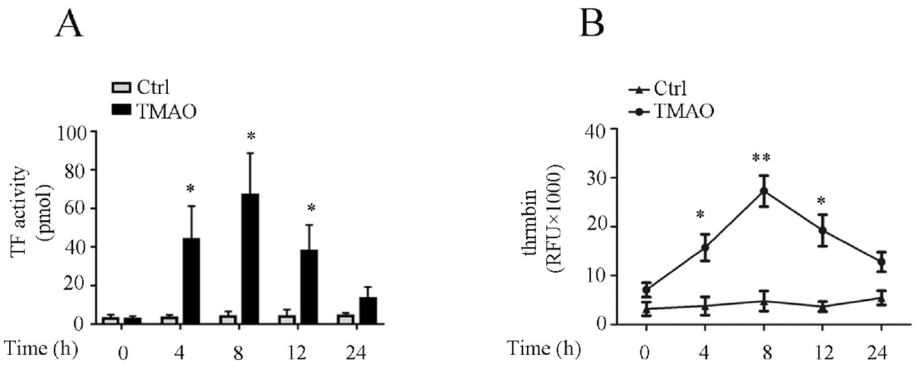

Fig. 2. TMAO enhanced TF activity and thrombin production in HCAECs.
HCAECs were stimulated with or without 100 μM for 4, 8, 12 or 24 h. (A) TF activity and (B) thrombin production were detected in HCAECs. All the fluorescence readings are expressed in relative fluorescence units (RFU). Data are representative of five independent experiments. (*P < 0.05, **P < 0.01 vs Control group).

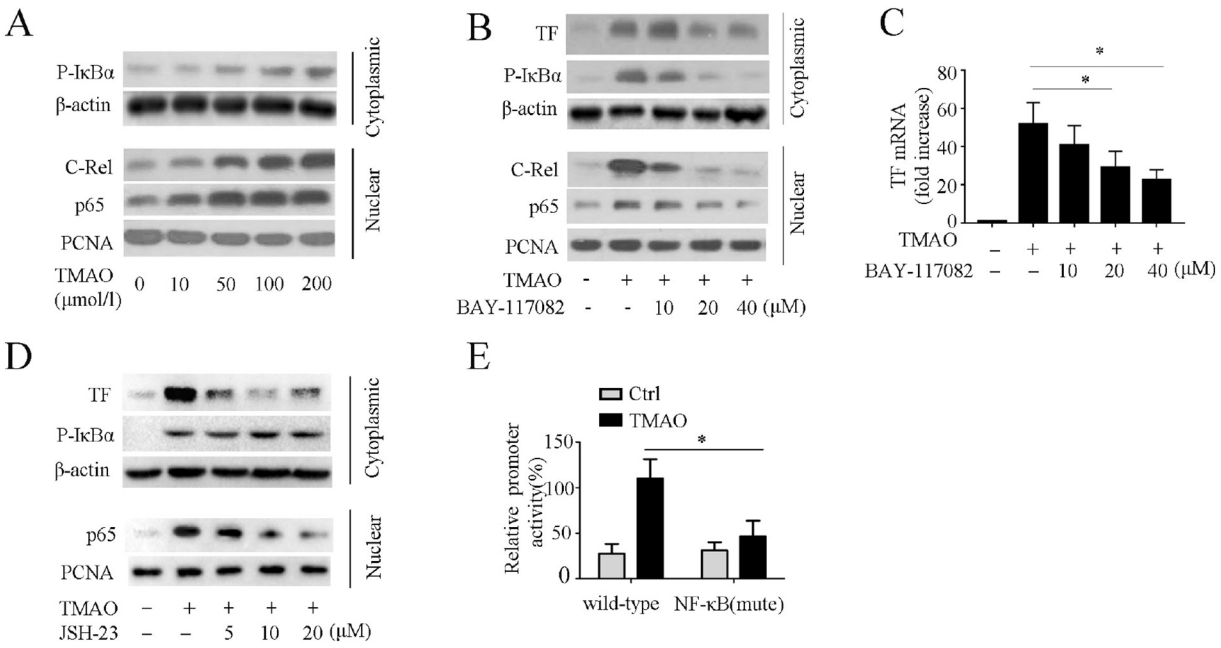

Fig. 3. TMAO-induced TF expression depended on the activation of the NF-κB signaling pathway.
(A) Western blot analysis of the level of cytoplasmic p-IκBα and NF-κB (c-Rel/p65) nuclear translocation in HCAECs following the stimulation with various concentrations of TMAO; (B to D) HCAECs were pretreated with or without NF-κB inhibitor (BAY-117082 or JSH-23) 30 min prior to stimulation with 100 μM TMAO. (B and D) The levels of cytoplasmic p-IκBα and TF, and NF-κB (c-Rel/p65) nuclear translocation were detected by Western blot; (C) The level of TF mRNA as reveled by RT-PCR; (E) The promoter activity of TF in HCAECs transfected with wild-type or mutated NF-κB binding sites plasmids in response to 100 μM TMAO stimulation. Values of C are presented as the fold increase over the un-stimulated control. Data are representative of three independent experiments. (*$P < 0.05$.)

dependent manner (Fig. 3A). Furthermore, NF-κB signaling inhibitor (BAY-117082, the IkB kinases inhibitor) efficiently abrogated TF protein and mRNA expression in a concentration-dependently manner (Fig. 3B and C). To confirm this result, another specific inhibitor (JSH-23) for NF-kB (P65) nuclear translocation, without effecting p-IkBα degradation [25], was utilized. Similar results were observed (Fig. 3D). To further investigate the role of NF-κB in the TMAO-mediated TF expression, wild-type and mutated NF-κB binding sites plasmids containing luciferase reporter gene were transfected transiently into HCAECs. TMAO stimulation resulted in a 4.4-fold increase ($P < 0.05$) in luciferase reporter activity in HCAECs transfected with the plasmid containing wild-type NF-κB binding sites as compared to an insignificant increase ($P > 0.05$) in HCAECs transfected with the plasmid containing mutated NF-κB binding sites (Fig. 3E). Thus, our results supported an important role of NF-κB in the regulation of TMAO-mediated TF expression in HCAECs.

3.4. Low-dose TMAO significantly promoted low-dose TNF-α- or HMGB1-stimulated TF expression via activating NF-κB signaling in HCAECs

Tumor necrosis factor-alpha (TNF-α) and high mobility group box 1 (HMGB1) were demonstrated in the human atherosclerotic plaques and reported to be involved in the diverse pathological processes of atherosclerosis [26–29]. Whether TMAO promotes TNF-α and HMGB1-mediated atherothrombotic effect has attracted much attention. Here, we found that low dose of TMAO (10 μM), inactive by itself alone, strongly up-regulated the TF expression by co-stimulation with TNF-α (0.01 ng/mL) or HMGB1 (1 ng/mL) in vascular endothelial cells (Fig. 4A and B). Further, low dose of TMAO significantly increased TF activity and thrombin production through synergistic action of TNF-α and HMGB1 (Fig. 4C and D). The level of TF mRNA was also increased with the co-stimulation of TMAO and TNF-α or HMGB1 (Fig. 4E), indicating that synergistic effect induced TF expression at the transcriptional level. The level of cytoplasmic p-IkBα and nuclear levels of NF-κB (p65/c-Rel) were also elevated with the co-stimulation (Fig. 4F). Inhibiting NF-κB signaling with BAY-117082 or JSH-23 uniformly diminished the TF expression induced by TMAO and TNF-α or HMGB1 (Fig. 4G and H). Taken together, these findings established that TMAO, even in low dose, significantly promoted low-dose TNF-α or HMGB1-mediated TF expression via activating NF-κB signaling.

3.5. Plasma TMAO was positively correlated with TF activity in patients with STEMI

To better reveal the pro-coagulant potential of TMAO during atherosclerotic plaque rupture and thrombosis, we measured plasma levels of TMAO and TF activity in patients with STEMI. Plasma TMAO and TF activity as well as TNF-α and HMGB1 levels were elevated in STEMI patients compared with healthy controls (Table 1). Furthermore, after a follow-up period of two months, plasma TMAO and TF activity levels in STEMI non-survival patients were significantly higher than those in survivors (Table 1). Thus, plasma TMAO levels and TF activity are shown to be associated with increased mortality in STEMI patients. Finally, we evaluated potential associations of TMAO level with TF activity among STEMI patients, and observed that TMAO level was positively correlated with TF activity (Fig. 5). These results suggested that TMAO may promote atherosclerotic thrombosis by enhancing TF activity.

4. Discussion

Recently, a large number of studies involving different population subsets have demonstrated an association between the plasma levels of TMAO and CVD or mortality [12–14]. Increased TMAO levels showed an association with poor prognosis and a superiority over contemporary biomarkers [30]. However, how TMAO enhances the potential of thrombotic events is not clear. A previous study has reported that TMAO can directly induce platelet aggregation and promote the formation of atherosclerotic disease thrombosis [12]. Besides, TMAO could induce endothelial cells dysfunction [31]. Here, we firstly

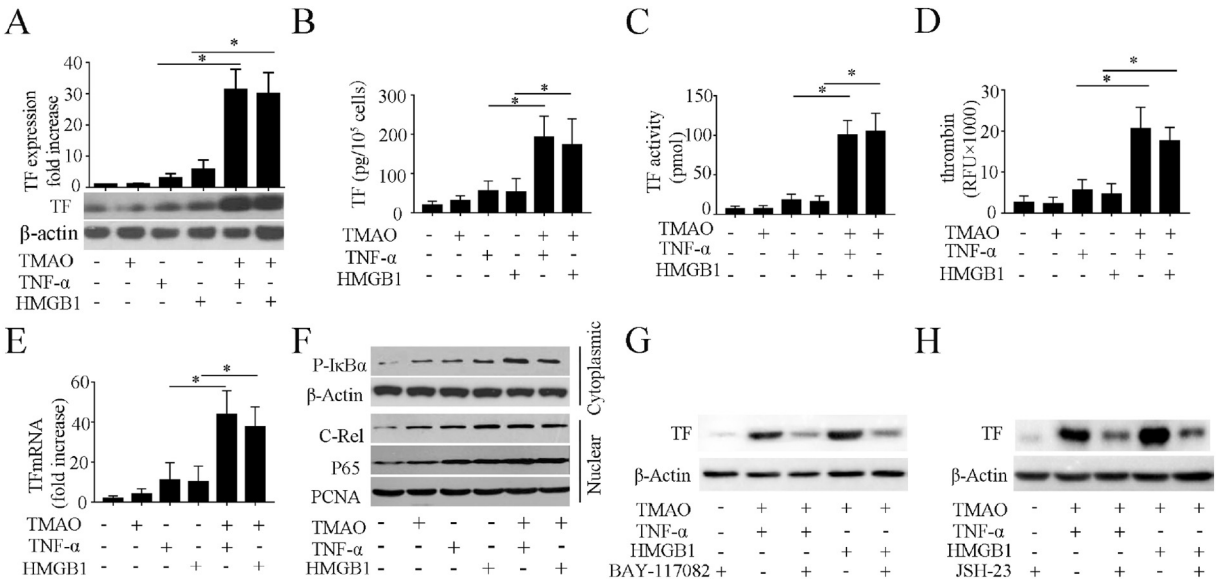

Fig. 4. Low-dose TMAO significantly promoted low-dose TNF-α- and HMGB1-induced TF expression via activating NF-κB signaling in HCAECs.
(A–F) The HCAECs were pre-incubated with TMAO (10 μM) 1 h before stimulation with TNF-α (0.01 ng/mL,) or HMGB1 (1 ng/mL) for 4 h. TF protein levels (A and B), TF activity (C), TF thrombin generation (D), and TF mRNA (E) were detected; The levels of cytoplasmic p-IκBα and nuclear NF-κB subunit c-Rel and p65 were shown by Western blotting (F); (G and H) The levels of TF protein in HCAECs pretreated with BAY-117082 (40 μM) or JSH-23 (20 μM) 30 min before stimulation of TMAO and TNF-α or HMGB1. Data are representative of three independent experiments. Blots are normalized to β-actin or PCNA expression. Values of A and E are given as the fold increase over the un-stimulated control (*$P < 0.05$ vs. TNF-α- or HMGB1-stimulated alone).

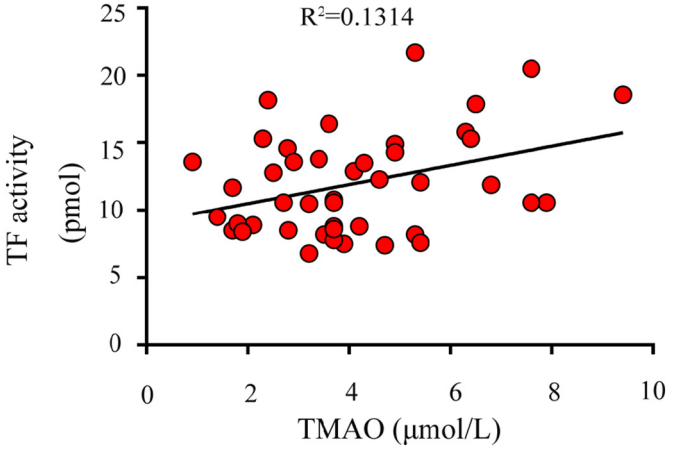

Fig. 5. Plasma TMAO level was positively correlated with TF activity in STEMI patients.
The association between levels of TMAO and TF activity in the plasma from STEMI patients ($P < 0.05$).

investigated the effect of TMAO on the TF and TFPI expression in HCAECs. We identified that TMAO up-regulated the TF expression and activity in HCAECs in both dose-dependent and time-dependent manner. However, TMAO treatment did not affect the TFPI expression in HCAECs. Therefore, the results suggested that TMAO promoted TF activity through increasing TF protein expression.

TF gene expression is complex and regulated by a number of transcription factors. NF-κB is one of the most important transcription factors. Our research showed that TMAO-mediated TF expression depended on the activation of NF-κB. TMAO-induced promoter activity and TF expression were significantly reduced by mutation of the NF-κB binding site in the TF gene promoter or using NF-κB inhibitors. Thus, our study indicated TMAO promoted TF protein expression via activating NF-κB signaling pathway.

An interesting finding in our study is that TMAO enhanced TNF-α- or HMGB1-mediated TF expression and TF activity. At present, clinical researches indicated that the plasma TMAO concentrations ranged from 0.06 μM to 312 μM, mostly about 10 μM, in the patients with atherosclerosis [13,15]. Atherosclerosis is a multi-factorial disease. We explored whether TMAO assisted other pro-atherosclerotic factors to promote TF expression in HCAECs. We selected TNF-α and HMGB1, both of which were recognized to play key roles in the pathogenesis of atherosclerosis and were predictive factors for incident coronary and cardiovascular events and total mortality [27–29]. Our results indicated that TMAO, in a low dose (10 μM) that is inactive by itself, significantly promoted TNF-α- or HMGB1-induced TF expression and activity in ECs. Therefore, TMAO itself or in combination with other pathogenic factors contribute to TF expression.

Finally, we confirmed our results in STEMI patients. We observed a positive correlation between TMAO with TF activity in patients with STEMI. The results are consistent with previous reports demonstrating an association of circulating TMAO levels or TF activity with the mortality of STEMI patients [4,30]. Thus, we consolidated our results that TMAO was closely related with TF activity, which may promote thrombosis in human cardiovascular diseases.

In conclusion, our study demonstrated the underlying mechanisms of TMAO–mediated thrombosis. TMAO induced TF expression and activity through NF-κB activation. Low-dose TMAO significantly promoted TNF-α- or HMGB1-induced TF expression. Increased plasma TMAO was related with increased TF activity in STEMI patients. The results of the present study will provide a profound understanding regarding TMAO-promoted thrombosis risk, which may facilitate the prevention and treatment of atherothrombosis.

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Author contributions

Xinyu Yang and Chuang Yuan conceived the project, designed the experiments, and wrote the manuscript; Xiaoye Cheng, Xianhui Qiu and Yukun Liu did the experiments; Xiaoye Cheng analyzed the data and made the figures.

Conflict of interest

The authors declare no conflict of interest.

References

[1] C. Weber, H. Noels, Atherosclerosis: current pathogenesis and therapeutic options, Nat. Med. 17 (11) (2011) 1410–1422.
[2] S.P. Grover, N. Mackman, Tissue factor: an essential mediator of hemostasis and trigger of thrombosis, Arterioscler. Thromb. Vasc. Biol. 38 (4) (2018) 709–725.
[3] V. Toschi, et al., Tissue factor modulates the thrombogenicity of human atherosclerotic plaques, Circulation 95 (3) (1997) 594–599.
[4] B.A. Steppich, et al., Plasma TF activity predicts cardiovascular mortality in patients with acute myocardial infarction, Thromb. J. 7 (2009) 11.
[5] Y.Z. Ohkubo, J.H. Morrissey, E. Tajkhorshid, Dynamical view of membrane binding and complex formation of human factor VIIa and tissue factor, J. Thromb. Haemost. 8 (5) (2010) 1044–1053.
[6] N. Mackman, Role of tissue factor in hemostasis, thrombosis, and vascular development, Arterioscler. Thromb. Vasc. Biol. 24 (6) (2004) 1015–1022.
[7] A.E. Mast, Tissue factor pathway inhibitor: multiple anticoagulant activities for a single protein, Arterioscler. Thromb. Vasc. Biol. 36 (1) (2016) 9–14.
[8] R.J. Westrick, et al., Deficiency of tissue factor pathway inhibitor promotes atherosclerosis and thrombosis in mice, Circulation 103 (25) (2001) 3044–3046.
[9] B. Steppich, et al., Tissue factor pathway inhibitor on circulating microparticles in acute myocardial infarction, Thromb. Haemost. 93 (1) (2005) 35–39.
[10] E. de Jonge, et al., Tissue factor pathway inhibitor dose-dependently inhibits coagulation activation without influencing the fibrinolytic and cytokine response during human endotoxemia, Blood 95 (4) (2000) 1124–1129.
[11] Z. Wang, et al., Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease, Nature 472 (7341) (2011) 57–63.
[12] W. Zhu, et al., Gut microbial metabolite TMAO enhances platelet hyperreactivity and thrombosis risk, Cell 165 (1) (2016) 111–124.
[13] W.H. Tang, et al., Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk, N. Engl. J. Med. 368 (17) (2013) 1575–1584.
[14] R.A. Koeth, et al., Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis, Nat. Med. 19 (5) (2013) 576–585.
[15] G.G. Schiattarella, et al., Gut microbe-generated metabolite trimethylamine-N-oxide as cardiovascular risk biomarker: a systematic review and dose-response meta-analysis, Eur. Heart J. 38 (39) (2017) 2948–2956.
[16] A.B. Roberts, et al., Development of a gut microbe-targeted nonlethal therapeutic to inhibit thrombosis potential, Nat. Med. 24 (9) (2018) 1407–1417.
[17] Z. Wang, et al., Non-lethal inhibition of gut microbial trimethylamine production for the treatment of atherosclerosis, Cell 163 (7) (2015) 1585–1595.
[18] S. Demyanets, et al., Interleukin-33 induces expression of adhesion molecules and inflammatory activation in human endothelial cells and in human atherosclerotic plaques, Arterioscler. Thromb. Vasc. Biol. 31 (9) (2011) 2080–2089.
[19] H. Wang, et al., HMG-1 as a late mediator of endotoxin lethality in mice, Science 285 (5425) (1999) 248–251.
[20] K. Thygesen, et al., Fourth universal definition of myocardial infarction (2018), Eur. Heart J. 40 (3) (2019) 237–269.
[21] B. Szotowski, et al., Alterations in myocardial tissue factor expression and cellular localization in dilated cardiomyopathy, J. Am. Coll. Cardiol. 45 (7) (2005) 1081–1089.
[22] Bavendiek, U., et al., Induction of tissue factor expression in human endothelial cells by CD40 ligand is mediated via activator protein 1, nuclear factor kappa B, and Egr-1. J Biol Chem, 2002. 277(28): p. 25032–9.
[23] N. Mackman, Regulation of the tissue factor gene, FASEB J. 9 (10) (1995) 883–889.
[24] A. Hoffmann, et al., The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation, Science 298 (5596) (2002) 1241–1245.
[25] H.M. Shin, et al., Inhibitory action of novel aromatic diamine compound on lipopolysaccharide-induced nuclear translocation of NF-kappaB without affecting IkappaB degradation, FEBS Lett. 571 (1–3) (2004) 50–54.
[26] W. Koch, et al., Interleukin-10 and tumor necrosis factor gene polymorphisms and risk of coronary artery disease and myocardial infarction, Atherosclerosis 159 (1) (2001) 137–144.
[27] M. Andrassy, et al., HMGB1 as a predictor of infarct transmurality and functional recovery in patients with myocardial infarction, J. Intern. Med. 270 (3) (2011) 245–253.
[28] K. Tuomisto, et al., C-reactive protein, interleukin-6 and tumor necrosis factor alpha as predictors of incident coronary and cardiovascular events and total mortality. A population-based, prospective study, Thromb. Haemost. 95 (3) (2006) 511–518.
[29] M. Sack, Tumor necrosis factor-alpha in cardiovascular biology and the potential role for anti-tumor necrosis factor-alpha therapy in heart disease, Pharmacol. Ther. 94 (1–2) (2002) 123–135.
[30] T. Suzuki, et al., Trimethylamine N-oxide and risk stratification after acute myocardial infarction, Clin. Chem. 63 (1) (2017) 420–428.
[31] T. Li, et al., Increased circulating trimethylamine N-oxide contributes to endothelial dysfunction in a rat model of chronic kidney disease, Biochem. Biophys. Res. Commun. 495 (2) (2018) 2071–2077.