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Gut microbe-derived metabolite trimethylamine N-oxide induces cardiac hypertrophy and fibrosis

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Abstract

Trimethylamine N-oxide (TMAO), a gut microbe-derived metabolite of dietary choline and other trimethylamine-containing nutrients, has been linked to increased cardiovascular disease risk. It is unknown whether TMAO plays a role in the development of cardiac hypertrophy. Transverse aortic constriction (TAC) was performed to induce cardiac hypertrophy in Sprague-Dawley (SD) rats. We observed that TMAO levels were significantly elevated in SD rats after 6 weeks of TAC, suggesting the potential role of TMAO in regulating cardiac hypertrophy. In cultured cardiomyocytes, TMAO treatment stimulated cardiac hypertrophy, as indicated by increased cell area of cardiomyocytes and expression of hypertrophic markers including atrial natriuretic peptide (ANP) and beta-myosin heavy chain (β -MHC). Additionally, TMAO treatment induced cardiac hypertrophy and cardiac fibrosis in SD rats. Reducing TMAO synthesis by antibiotics (Abs) attenuated TAC-induced cardiac hypertrophy and fibrosis. Furthermore, pharmacological inhibition of Smad3 by SIS3 significantly reduced the expression of ANP and β -MHC, and cardiomyocyte cell size in TMAO-treated group. These data for the first time demonstrate that gut microbe-derived metabolite TMAO induces cardiac hypertrophy and fibrosis involving Smad3 signaling, suggesting that inhibition of gut microbes or generation of TMAO may become a potential target for the prevention and treatment of cardiac hypertrophy.

Heart failure (HF) is a common, costly, and potentially fatal condition, which affected about 40 million people globally in 2015 [1]. Cardiac hypertrophy is a very common pathological process of cardiovascular diseases, such as

hypertension, valvular heart disease, cardiomyopathy, and HF. During the compensatory stage of hypertrophy, the heart initially increases in cardiomyocyte size and mass to maintain and even enhance cardiac function in response to hemodynamic overload. However, the sustained excessive cardiac overload can eventually compromise cardiac output, resulting in cardiac remodeling and HF. Cardiac hypertrophy is both an intermediate step and a determinant of HF [2]. Since the underlying mechanism of cardiac hypertrophy is not fully understood, identification and characterization of key molecules regulating cardiac hypertrophy will develop novel targeting therapies.

It has been demonstrated that gut microbiota play important roles in the pathogenesis of cardiovascular disease [3]. The role of gut microbiota on cardiac fibrosis has been suggested by previous studies [4, 5]. Trimethylamine N-oxide (TMAO), a gut microbe-derived metabolite of dietary choline and other trimethylamine-containing nutrients, is highly related to the risk of cardiovascular disease [6]. Dietary choline and carnitine can be metabolized to trimethylamine (TMA) by the gut microbiota. Subsequently, TMA is absorbed via the portal circulation

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and rapidly converted to TMAO by enzymes of the flavin monooxygenase (FMO) family, especially FMO3 in the liver [7]. In the past several years, TMAO has been demonstrated as a novel independent risk factor for major adverse cardiovascular events [8]. For example, TMAO promotes vascular inflammation, induces atherosclerosis, and enhances platelet hyperreactivity and thrombosis risk [9–11]. Importantly, several studies have indicated that TMAO is a very important factor involved in the development of HF [12, 13]. To date, it is largely unclear whether TMAO directly promotes the progression of cardiac hypertrophy. In the present study, we investigated the effect of TMAO on cardiac hypertrophy and fibrosis *in vitro* and *in vivo*. This may provide a new insight into understanding the pathogenesis of cardiac hypertrophy.

Materials and methods

Animal models

All study protocols were approved by the Animal Care and Use Committee of the Southern Medical University, and strictly complied with the National Institutes of Health Guidelines on the Use of Laboratory Animals (NIH publication No. 85–23, revised 1996). Adult male Sprague-Dawley (SD) rats aged 4–5 weeks (weighing 80–100 g) were obtained from Guangdong Medical Lab Animal Center ($n = 10$ for each Group). All animals were housed and maintained in a temperature-controlled ($20 \pm 5^\circ\text{C}$), and a humidity-controlled (40–70%) animal facility adhering to a 12-h light/dark cycle, in non-directional airflow cages. Rats were provided with water and rodent chow *ad libitum* (supplied by the Southern Medical University Experimental Animal Center, China). For the transverse aortic constriction (TAC) surgery duration, rats were fasted for 8 h and then were anesthetized using 5% chloral hydrate (6 ml/kg). Briefly, a midline laparotomy was performed and the suprarenal abdominal aorta was exposed by using blunt dissection. A ligation was made along the abdominal aorta 3 mm above the branch point of the renal artery, which was achieved by ligating a 24-gauge blunt needle with 4–0 nylon sutures. Subsequently, the needle was removed promptly to create an abdominal aortic constriction [14]. Sham-operated rats subjected to the same surgery, except for the ligation of the aortic band, and served as controls. Thereafter, the abdomen was closed in layers. For a 6-week period, the postoperative rats were administered a chow diet in the absence or presence of antibiotics (Abs) (Vancomycin 100 mg/kg/d of body weight, Neomycin 200 mg/kg/d, Metronidazole 200 mg/kg/d, Ampicillin

200 mg/kg/d) and 0.9% saline was administered to Sham-operated rats. For the TMAO treatment studies, rats were administered TMAO (15 mg/100 g body weight/day, Sigma-Aldrich) by intraperitoneal injection for 1 or 2 weeks respectively and 0.9% saline was administered to control rats. At the end of the treatment period, systemic blood pressure (BP-2010A, Softron, Tokyo) and body weight were measured and animals were fasted for 8 h before blood and tissues were collected for further analysis.

Cell culture

Cardiomyocytes were isolated and cultured from the ventricles of 1–3-day-old neonatal SD rats. Rat hearts were removed and large vessels and atria discarded aseptically. The ventricles were then washed and cut into small pieces in Balanced Salt Solution (D-Hank's), followed by digestion in 0.25% trypsin (Gibco, USA) at 4°C for 12–15 h. The cells were centrifuged and cultured in Dulbecco's Modified Eagle's Medium (DMEM) for 2 h. This purification process was repeated twice in order to enrich for cardiomyocytes and to deplete non-myocytes. Afterwards, cardiomyocytes were resuspended and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) in a CO_2 incubator at 37°C and were further cultured for 24–48 h. Next, the culture medium was changed to serum-free DMEM for 24 h, and the cardiomyocytes were treated with $5\ \mu\text{M}$ TMAO (Sigma-Aldrich) for 18 h. SIS3 ($3\ \mu\text{M}$, Selleck), a novel and specific smad3 inhibitor [15], was used to treat cardiomyocytes for 72 h in some experiments. Cells analyzed for cardiomyocyte size in Fig. 2a were randomly selected and blindly analyzed.

Real-time quantitative PCR

Quantitative analysis of atrial natriuretic peptide (ANP) and β -myosin heavy chain (β -MHC) mRNA relative expression in cardiomyocytes and cardiac tissues was evaluated by quantitative PCR (q-PCR). Total RNA extraction of cardiomyocytes and cardiac tissues was extracted using Trizol reagent (TaKaRa, Japan), according to the manufacturer's instructions. To obtain cDNA, RNA samples were reverse transcribed with random primers using a High-Capacity cDNA Reverse Transcription Kit (TaKaRa, Japan). Cardiomyocyte ANP and β -MHC levels were quantified by q-PCR using a TaKaRa SYBR Fast q-PCR kit. The q-PCR was performed on a 7500 FAST Real-Time PCR System (Applied Biosystems, USA). The sequences of primers used for amplification were as follows: ANP, 5'-GGGCTTCTTCCTCTTCCTGG-3' and 5'-TCTGAGACGGGTTGACTTCC-3'; β -MHC, 5'-AGGGCAAAGGCAAAGCAAAGA-3'

and 5'-TACAAAGTGAGGGTGCCTGGA-3'; GAPDH, 5'-GGCAAGGTCATCCCAGAGCT-3' and 5'-CCCAGGATGCCCTTTAGTGG-3'. Data were analyzed using the comparative Ct ($\Delta\Delta C_t$) method for relative quantification. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.

Echocardiography assessment

Rats from all groups were weighed and anesthetized with chloral hydrate. Transthoracic two-dimensionally guided M-mode echocardiography was performed on an IE33 echocardiographic system (Philips Medical Systems, the Netherlands) during those animals received light anesthesia with isoflurane (0.25–0.50%) supplemented with 100% O₂. The following cardiac function parameters were measured: left ventricular (LV) ejection fraction (EF), LV internal dimensions at both diastole and systole (LVIDd and LVIDs, respectively), LV anterior wall dimensions at both diastole and systole (LVAWd and LVAWs, respectively), and LV posterior wall dimensions at both diastole and systole (LVPWd and LVPWs, respectively). Each echocardiographic value was taken from the mean of three consecutive cardiac cycles.

Histological analysis

Rat hearts were harvested at indicated time points. LV mass-to-body weight ratio (LVm/BW) was then calculated. Rat hearts and small intestine were fixed in 4% paraformaldehyde, embedded in paraffin, cut into 6 μ m sections and stained with hematoxylin and eosin (H&E) to analyze the organizational morphology. Heart samples were stained with either Masson's Trichrome or Picrosirius Red to analyze the myocardial fibrosis. To measure myocyte cross-sectional area, cardiac sections were stained with wheat germ agglutinin (WGA, Thermo Fisher Scientific Inc., USA) solution for 10 min, and washed with PBS three times. After that, sections were imaged using a Leica Microsystems microscope and quantified using Image Pro Plus 6.0.

Quantification of TMAO

Levels of TMAO in plasma samples were quantified using stable isotope dilution liquid chromatography-tandem mass spectrometry (LC-MS). Briefly, the internal standard, trimethylamine-d₉ N-Oxide TMAO (d₉-TMAO) was added into serum samples before protein

precipitation and monitored in positive MRM mode at m/z 85 $\rightarrow$ 66 [16]. Concentrations of TMAO standards and a fixed amount of internal standard were spiked into control serum to prepare the calibration curves for quantification of serum analytes, provided by the Agilent chemical workstation.

Measurement of the cell surface area

Cultured cardiomyocytes were plated onto coverglass Bottom Dish, fixed with 4% paraformaldehyde for 30 min, and permeabilized with 0.5% Triton X-100 in PBS for 5 min, followed by being incubated with TRITC Phalloidin (200 nM, Yeasen, China) for 30 min. Nuclear staining was performed by incubating with 4', 6-diamidino-2-phenylindole (DAPI, Beyotime, China) for 1 min both at room temperature. Cardiomyocytes were imaged using a microscope (Leica Microsystem, Germany) and cardiomyocyte surface area was determined using Image Pro Plus software (version 6.0, Media Cybernetics).

Western blot analysis

Total protein was extracted from cultured neonatal cardiomyocytes and protein concentration was measured using a BCA protein assay kit (Thermo Scientific, USA). Protein samples were separated by 8% or 12% acrylamide denaturing gels (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes. Following transfer, membranes were washed with tris-buffered saline and polysorbate 20 (TBST), and then blocked in 5% skim milk (Gibco, USA) in TBST for 2 h at room temperature, followed by incubation with anti-ANP (1:1000 dilution, Santa Cruz Biotechnology), anti- β -MHC (1:1000 dilution, Santa Cruz Biotechnology), anti-phospho-Smad3 antibody (p-Smad3, 1:1000 dilution, CST), anti-Smad3 antibody (1:1000 dilution, CST) and anti-GADPH (1:10,000 dilution, BOSTER) antibodies on a shaker overnight at 4 °C. The following day, membranes were washed in TBST and then incubated with secondary antibodies (1:8000 dilution, BOSTER) for 2 h at room temperature. Antibody-antigen complexes in all membranes were detected using the Imaging System (GE, Amersham Image 600) and protein bands were quantified by Image Pro Plus 6.0.

Statistical analysis

All statistical analyses were performed using SPSS Statistical software (version 20.0). Data are presented as the mean $\pm$ standard error of mean (SEM). Independent Student's *t*-tests and one-way ANOVA were used for comparisons. All statistical significance was two-tailed and a value of $p < 0.05$ was considered significant.

Fig. 1 Plasma levels of TMAO were increased in TAC-induced cardiac hypertrophy model in rats ($n = 10$). **a** Left ventricular (LV) anterior wall dimensions at diastole (LVAWd; in mm); **b** LV anterior wall dimensions at systole (LVAWs; in mm); **c** LV internal dimensions at diastole (LVIDd; in mm); **d** LV dimensions at systole (LVIDs; in mm); **e** LV posterior wall dimensions at diastole (LVPWd; in mm);

f LV posterior wall dimensions at systole (LVPWs; in mm); **g** plasma levels of TMAO in rat model of TAC-induced cardiac hypertrophy were measured; **h** cross sections of the small intestine of sham and TAC were stained with H&E to examine the villi pathology (Scale bar: 200 μ m). * $p < 0.05$ and ** $p < 0.01$ vs. sham

Results

TMAO levels are increased in rats in the TAC model

Cardiac structure and function in rats were assessed by echocardiography at 6 weeks post surgery. Serial echocardiography analyses revealed that the rat model of compensatory cardiac hypertrophy induced by TAC was successfully established. The parameters of LV wall thickness including LVAWd and LVAWs (Fig. 1a, b), LVPWd and LVPWs (Fig. 1e, f) were significantly increased in TAC rats compared with sham rats, whereas no change in LVIDd and LVIDs were detected between the two groups (Fig. 1c, d). These data indicated that TAC surgery induced compensatory cardiac hypertrophy in rats. Of note, the levels of circulating TMAO were significantly increased in TAC rats compared with sham-operated animals (Fig. 1g). Next, we

investigated whether the increased levels of circulating TMAO are associated with alterations in gut pathology. As shown in Fig. 1h, intestinal villi in TAC-treated rats were much shorter and appeared stunted, compared with the sham control, suggesting that the increased permeability of small intestine could be induced by TAC. In addition, we also examined the protein expression of FMO3, a key enzyme responsible for converting TMA to TMAO in liver and TAC treatment has no effect on FMO3 protein expression (Supplementary Fig. 1). Taken together, TAC-induced increased levels of plasma TMAO could be due to increased intestinal permeability in TAC-treated animals.

TMAO induces cardiac hypertrophy in vitro

To assess the effect of TMAO on cardiac hypertrophy, neonatal rat cardiomyocytes were treated with 5 μ M

Fig. 2 Effect of TMAO on cardiac hypertrophy in vitro. Neonatal mouse cardiomyocytes were treated with TMAO (5 μ M) for 18 h. **a** Representative microscopic images of cardiomyocytes with rhodamine-phalloidin staining of cytoskeleton and DAPI staining of the nucleus (Scale bar: 25 μ m) and cell surface area analysis. **b** The mRNA expression levels of ANP and β -MHC were analyzed by q-PCR. **c** Western blot analysis of hypertrophic genes expression. ** p <0.01 vs. control

TMAO. We found that TMAO treatment significantly increased the cell area of cardiomyocytes as assessed by cardiomyocyte immunofluorescence and cell surface area analysis (Fig. 2a). Furthermore, q-PCR and western blot analysis showed that TMAO significantly increased the mRNA and protein levels of hypertrophic markers including ANP and β -MHC (Fig. 2b, c). These results indicated that TMAO positively regulates cardiac hypertrophy in vitro.

TMAO induces cardiac hypertrophy in rats

To further verify the effects of TMAO in cardiac hypertrophy in vivo, 200 μ M of TMAO was used to treat rats by intraperitoneal injection for 1 week or 2 weeks. Plasma TMAO levels were assessed in animals treated with TMAO. Of note, TMAO-treated rats displayed higher levels of TMAO than control rats, especially in

the 2-week TMAO-injected rats (Fig. 3c). As shown in Fig. 3a, echocardiography analysis revealed that TMAO exerted adverse effects on cardiac remodeling, as indicated by increased LVAWd, LVAWs and LVPWd in rats injected with TMAO for 2 weeks compared with the controls. HE staining showed that LV chamber thickness was increased in TMAO-treated hearts compared with the controls (Fig. 3b). As demonstrated in Fig. 3d, LVm/BW was significantly increased in rats injected with TMAO for 2 weeks compared with the controls. Moreover, 2 weeks of TMAO injection significantly increased ANP and β -MHC mRNA levels (Fig. 3e). Furthermore, WGA staining of gross heart tissue confirmed that TMAO treatment increased cardiomyocyte size, suggesting the positively regulatory effect of TMAO on cardiac hypertrophy (Fig. 3f, g). Collectively, these data demonstrated a detrimental effect of TMAO on cardiac hypertrophy.

Fig. 3 Effect of TMAO on cardiac hypertrophy in rats. Rats were injected intraperitoneally with TMAO (200 μ M) for 1 week or 2 weeks ($n = 10$). **a** Echocardiography parameters: left ventricular (LV) anterior wall dimensions at diastole (LVAWd; in mm), LV anterior wall dimensions at systole (LVAWs; in mm), LV posterior wall dimensions at diastole (LVPWd; in mm), LV posterior wall dimensions at systole (LVPWs; in mm), LV internal dimensions at diastole (LVIDd; in mm), LV internal dimensions at systole (LVIDs; in mm). **b** Photomicrographs of left ventricular tissue sections stained with

hematoxylin and eosin (H&E) and chamber thickness of H&E (mm). **c** Plasma levels of TMAO were increased in TMAO-treated rats. **d** Ratio of heart weight to body weight (LVm/BW, mg/g). **e** The mRNA expression levels of ANP and β -MHC were analyzed by q-PCR. **f** Photomicrographs of left ventricular tissue sections stained with wheat germ agglutinin (WGA, Scale bar: 25 μ m). **g** Quantification of cell surface areas (relative to control). * $p < 0.05$ and ** $p < 0.01$ vs. control

Fig. 4 Effect of TMAO on cardiac fibrosis in rats ($n = 10$). **a** Representative photomicrographs of Masson Trichrome-stained heart sections in control and TMAO groups (Magnification, $\times 400$). **b** Cardiac fibrosis as a percent (%) of left ventricular area was calculated from Masson Trichrome-stained hearts. **c** Picosirius Red staining for perivascular fibrosis (Scale bar: 50 μm) using Polarizing Microscopy. ** $p < 0.01$ vs. control

Recent studies indicated that the metabolites/products of gut microbe are involved in the regulation of hypertension [17–19]. Since TMAO is one of the key metabolites of the gut microbe, we measured the blood pressure of the TMAO-treated rats. Both systolic blood pressure (SBP) and diastolic blood pressure (DBP) of rats were significantly increased especially after TMAO treatment for 2 weeks (Supplementary Table 1). Our findings imply that TMAO may induce myocardial hypertrophy partly through increasing blood pressure.

TMAO induces cardiac fibrosis in rats

Cardiac hypertrophy usually accompanies fibrosis. Next we performed Masson Trichrome and Picosirius Red staining on cardiac tissues to examine the effect of TMAO on cardiac fibrosis in rats. Masson staining of heart tissue showed increased interstitial fibrosis in hearts from 2-week TMAO-treated animals compared with the controls (Fig. 4a). Quantification analysis of the intensity of Masson staining (Fig. 4b) confirmed significantly increased levels of

interstitial fibrosis in the TMAO group compared with the controls. Picosirius Red staining was used to detect degree of collagen fibrosis in the present study. As showed in Fig. 4c, Picosirius Red staining assay revealed that the perivascular fibrosis in TMAO-treated rats was increased when compared with the controls. Taken together, these findings indicate that TMAO acts as an inducer for cardiac fibrosis.

TMAO synthesis inhibition by Abs attenuates cardiac hypertrophy and cardiac fibrosis

Since previous studies have demonstrated that Abs can reduce TMAO levels by inhibiting the gut microbiota [7, 20], Abs were used to treat TAC-operated rats in this study. Similarly, we also found that Abs-treated rats displayed reduced levels of TMAO (Fig. 5a). Given that Abs might affect rat body weight and growth, we examined the body weights of the TAC model rats. We found that Abs treatment has no effect on the body weights of the TAC model rats (Supplementary Fig. 2). In Abs-treated rats there was a

significant decrease in cardiac hypertrophy compared with TAC-treated rats, but without damage on cardiac systolic function (Fig. 5b). Of note, LVm/BW was significantly

decreased in rats supplemented with Abs compared with TAC rats as shown in Fig. 5c. Consistently, whole mount and HE staining of gross heart tissue showed that chamber

◀ **Fig. 5** TMAO synthesis inhibition by Abs resulted in a significant decrease index of myocardial hypertrophy and cardiac fibrosis after TAC in rats ($n = 10$). **a** Plasma levels of TMAO in rats; **b** LV ejection fraction (EF, %); **c** ratio of heart weight to body weight (LVm/BW, mg/g); **d** representative images of the whole heart; **e** photomicrographs of left ventricular tissue sections stained with hematoxylin and eosin (H&E); **f** chamber thickness of H&E staining of gross heart tissue; **g** the mRNA expression levels of ANP and β -MHC in hearts were analyzed by q-PCR; **h** quantification of cell surface areas (relative to controls); **i** cardiac fibrosis as a percent (%) of left ventricular area was calculated from Masson Trichrome-stained hearts; **j** photomicrographs of left ventricular tissue sections stained with wheat germ agglutinin (WGA, Scale bar: 25 μ m); **k** photomicrographs of Masson Trichrome staining for interstitial fibrosis showing myocardium in red and fibrosis in blue (Magnification, $\times 400$); **l** Picrosirius Red staining for perivascular fibrosis (Scale bar: 50 μ m) using Polarizing Microscopy; * $p < 0.05$, ** $p < 0.01$ vs. sham; # $p < 0.05$, ## $p < 0.01$ vs. TAC

thickness was remarkably increased in TAC rats at 6 weeks post surgery, while no change in the Abs-treated group (Fig. 5d–f). In addition, q-PCR analysis of expression of hypertrophic genes showed that ANP and β -MHC mRNA levels were enhanced in the TAC group when compared with the sham-operated group. Of note, ANP and β -MHC mRNA levels in Abs-treated rats were remarkably lower than TAC rats without Abs (Fig. 5g). In addition, WGA staining of gross heart tissue also showed that surface areas of cardiomyocytes were significantly decreased in Abs-treated group compared with TAC group (Fig. 5h–j). These data suggest that TAC-induced cardiac hypertrophy could be partly alleviated by inhibition of TMAO synthesis by Abs. To further confirm the effect of TMAO on cardiac fibrosis, we used Abs to reduce TMAO synthesis in TAC rats. Masson Trichrome staining showed a significantly increased heart fibrosis in the TAC rats compared with sham-operated rats, which can be prevented by Abs treatment (Fig. 5i, k). Additionally, similar results were observed in the perivascular sections of heart tissue, as indicated by Picrosirius Red staining (Fig. 5l). Taken together, these findings indicate that TMAO synthesis inhibition by Abs alleviates cardiac fibrosis.

TGF- β 1/Smad3 signaling is required for TMAO-induced cardiac hypertrophy

Western blot analysis showed that TGF- β 1 expression was up-regulated in TMAO-treated cardiomyocytes (Fig. 6a). Consistently, an increased protein expression of TGF- β 1 was also observed in TMAO-treated rats compared with sham control (Fig. 6b). Previous studies have shown that Smad3 signaling is involved in the development of cardiac hypertrophy [21]. In addition, Smad3 is a well-known signaling pathway in the process of fibrosis [22]. Therefore, we decided to test whether Smad3 signaling pathway participates in the progression of TMAO-induced cardiac hypertrophy. SIS3, a pharmacological inhibitor of Smad3 was

used to inhibit the phosphorylation of Smad3 in this study. As shown in Fig. 6c, d, we found that TMAO treatment stimulated the phosphorylation of Smad3 and significantly increased protein and mRNA levels of hypertrophic markers including ANP and β -MHC in cardiomyocytes, while inhibition of Smad3 phosphorylation by SIS3 was sufficient to block TMAO-induced expression of ANP and β -MHC in cardiomyocytes. Furthermore, TMAO markedly increased the cell surface area of cardiomyocytes, which can be blocked by SIS3 (Fig. 6e, f). Taken together, these results suggest that TGF- β 1/Smad3 signaling activation mediates TMAO-induced cardiac hypertrophy.

Discussion

In this study, we demonstrate that TMAO directly induces cardiac hypertrophy and fibrosis. Firstly, a significant increased TMAO level in plasma was detected in rat model of cardiac hypertrophy induced by TAC. Secondly, TMAO treatment directly stimulated cardiac hypertrophy and fibrosis in vitro and in vivo. Thirdly, Ab treatment reduced plasma TMAO levels and attenuated cardiac hypertrophy in TAC-treated rats. Finally, Smad3 signaling was found activated in TMAO-induced cardiac hypertrophy and inhibition of Smad3 by SIS3 attenuated TMAO-induced cardiac hypertrophy. Taken together, these data indicate that TMAO stimulates cardiac hypertrophy involving the Smad3 signaling, thus providing a novel insight into TMAO signaling mechanism underlying cardiac hypertrophy.

Gut microbes play an important role in regulating human health and disease [23]. TMAO, the gut microbiota metabolite, has been demonstrated as a key mediator of the development of cardiovascular diseases as mentioned above. Previous studies have demonstrated that TMAO provokes fibrosis in heart [5, 24]. However, there are some other studies did not show an association between an elevated blood TMAO and an increased cardiovascular risk [25, 26]. This discrepancy can be explained by differences in the study design and clinical context, geographic and ethnic background, or dietary habits. In this study, TAC was used to induce rat model of compensatory cardiac hypertrophy. We found that the levels of circulating TMAO were significantly increased in TAC rats. The elevated circulatory TMAO levels are probably due to increased intestinal permeability and decreased renal clearance. There is a gut hypothesis about the relation between HF and gut microbiota indicating that substantial hemodynamic changes possibly affect the growth and composition of gut microbiota and ultimately exacerbate TMAO translocation into systemic circulation in HF [23, 27, 28]. To investigate whether increased intestinal permeability or upregulation of FMO3 in the liver could result in increased levels of plasma TMAO in

TAC-treated rats, we analyzed the permeability of small intestine and FMO3 protein expression in liver. H&E staining revealed that intestinal villi in TAC-treated rats were

much shorter and appeared stunted, compared with the sham control, suggesting that the increased permeability of small intestine could be induced by TAC. In addition, western blot

◀ **Fig. 6** Effect of SIS3, Smad3 inhibitor on TMAO-induced Cardiac Hypertrophy. SIS3 (3 μ M) was used to treat cardiomyocytes in the presence or absence of TMAO for 72 h. **a** Western blot analysis of TGF- β 1 protein expression in cardiomyocytes; **b** western blot analysis of TGF- β 1 protein expression in hearts from control or TMAO-treated rats; **c** western blot analysis of p-Smad3, ANP and β -MHC protein expression; **d** the mRNA expression levels of ANP and β -MHC were analyzed by q-PCR; **e** quantification of cardiomyocyte surface area (relative to control); **f** the cell areas were detected by an immunofluorescence assay in neonatal mouse cardiomyocytes (Scale bar: 25 μ m). ** p <0.01 vs. control; ## p <0.01 vs. TMAO

analysis showed no change of FMO3 protein expression between the TAC-treated rats and the sham control. Taken together, increased intestinal permeability in TAC-treated animals could explain why plasma TMAO significantly increased by transient aortic constriction.

Several recent clinical studies have revealed a strong association between TMAO and HF [12, 23]. A recent animal study has shown that either supplemental choline or TMAO leads to adverse cardiac remodeling and LV dysfunction in HF after TAC [5]. Our study extends these findings by assessing the effect of TMAO on cardiac hypertrophy in vitro and in vivo. Our data have shown that TMAO directly induces the development of cardiac hypertrophy, as evidenced by increased cardiomyocyte size and high levels of hypertrophic markers including ANP and β -MHC in hearts after TMAO treatment. Additionally, cardiac hypertrophy can be markedly inhibited by reducing TMAO synthesis via Ab treatment. These data support the conclusion that TMAO plays a contributory role in the progression of cardiac hypertrophy, providing a new insight into the molecular mechanisms responsible for cardiac hypertrophy. Abundant evidence reveals that the metabolites/products of gut microbe are involved in the hypertension [17–19]. In addition, hypertension plays an important role in cardiac hypertrophy. We found that TMAO as a metabolite of the gut microbe, can increase the blood pressure of the rats. These finding might further support the hypothesis that TMAO is related to the cardiac hypertrophy. Recent studies indicated that high blood pressure increases the permeability and histological changes of the gut, and these changes are related to RAS [18, 19]. We did not rule out the possibility that RAS is involved in TAC-induced cardiac hypertrophy. A previous study has demonstrated that TMAO promotes TAC-induced cardiac hypertrophy [5]. In our study, we have shown that TMAO treatment directly induces cardiac hypertrophy both in vitro and in vivo, suggesting that TMAO could become an independent risk factor for cardiac hypertrophy. It is noteworthy that Abs are able to inhibit TMAO synthesis via remodeling gut microbiota, but Abs could affect other biological processes in the gut. For example, broad spectrum Abs would cause diarrhea and affect hemodynamic response. Therefore, we examined the body weights of the TAC model rats

and found that Abs treatment has no effect on the body weights of the TAC model rats. However, a specific inhibitor of TMAO was not used in this study, and we cannot completely rule out the possibility that Abs treatment may attenuate cardiac hypertrophy through affecting other biological processes in the gut.

Although the plasma TMAO concentration in TAC-treated rats is similar to rats treated with TMAO for 1 week, TAC-induced cardiac hypertrophy is much more severe compared with the latter, suggesting that TAC results in cardiac hypertrophy possibly through, other mechanisms, such as increased levels of cardiac overloading and neuro-humoral regulation, in addition to TMAO pathway [29, 30]. In this study, reduction of TMAO by Abs significantly attenuated cardiac hypertrophy induced by TAC. These findings support the gut hypothesis that effect of TAC on cardiac hypertrophy at least partly depends upon gut microbes and the production of the metabolite TMAO.

Transforming growth factor- β 1 (TGF- β 1) is a well-known signaling pathway involved in the process of cardiac hypertrophy and fibrosis [28]. Smad3 is a downstream mediator of TGF- β 1, which is phosphorylated by activated type I receptor and then complexed with Smad4, and translocated from cytoplasm to the nucleus, where activates the transcription of downstream targeting genes promoting cardiac hypertrophy and fibrosis [31]. Previous studies have shown that Smad3 signaling participates in the development of cardiac hypertrophy and fibrosis [32]. In the present study, we found that TMAO could activate Smad3 signaling, while inhibition of Smad3 by SIS3 could attenuate TMAO-induced cardiac hypertrophy. In addition, western blot analysis showed that TGF- β 1 expression was up-regulated in TMAO-treated group. Taken together, TMAO induces cardiac hypertrophy and fibrosis involving TGF- β 1/Smad3 signaling pathway.

In summary, our studies have demonstrated that supplement of TMAO is an effective approach to induce cardiac hypertrophy and fibrosis. Although angiotensin II and norepinephrine have been widely used for generation of models of cardiac hypertrophy [33, 34], several disadvantages including expensive cost and not being in agreement with disease model exist. TMAO-induced model of cardiac hypertrophy shows several advantages including stable performance, simple operation, convenience and low cost. Hence, TMAO could be used as an inducer for generation of a novel and effective model of cardiac hypertrophy and targeting TMAO generation may become a therapeutic approach for the treatment of cardiac hypertrophy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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