Trimethylamine-N-Oxide Aggravates Kidney Injury via Activation of p38/MAPK Signaling and Upregulation of HuR

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Keywords
Trimethylamine-N-oxide · Inflammatory injury · p38/MAPK pathway · Human antigen R · Chronic kidney disease

Abstract
Background: Trimethylamine-N-oxide (TMAO) is an intestinal metabolic toxin, which is produced by gut flora via metabolizing high-choline foods. TMAO is known to increase the risk of atherosclerosis and cardiovascular events in chronic kidney disease (CKD) patients. Objectives: The objective of this study was to explore the role and mechanism of TMAO aggravating kidney injury. Method: We used the five-sixths nephrectomy (5/6 Nx)-induced CKD rats to investigate whether TMAO could aggravate kidney damage and its possible mechanisms. Six weeks after the operation, the two groups of 5/6 Nx rats were subjected to intraperitoneal injection with 2.5% glucose peritoneal dialysis fluid (2.5% PDF) and 2.5% PDF plus TMAO 20 mg/kg/day. Results: In this study, we provided evidence showing TMAO significantly aggravated renal failure as well as inflammatory cell infiltration and in five-sixths nephrectomy-induced CKD rats. We found that TMAO could upregulate inflammatory factors including MCP-1, TNF-α, IL-6, IL-1β, and IL-18 by activating p38 phosphorylation and upregulation of human antigen R. TMAO could aggravate oxidative stress by upregulating NOX4 and downregulating SOD. The result also confirmed that TMAO promoted NLRP3 inflammasome formation as well as cleaved caspase-1 and IL-1β activation in the kidney tissue. Conclusions: Taken together, the present study validates TMAO as a pro-inflammatory factor that causes renal inflammatory injury and renal function impairment. Inhibition of TMAO synthesis or promoting its clearance may be a potential therapeutic approach of CKD in the future.

Introduction
Chronic kidney disease (CKD) is a worldwide public health problem leading to end-stage renal disease (ESRD) [1]. Chronic inflammation is considered as a major driving force of CKD progression. The causes of systemic inflammation in CKD patients include direct induction by uremic toxins, oxidative stress, and microbial infections.

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Inflammation may interfere with intrarenal microcirculation regulation and recruit inflammatory cells to further aggravate renal injury. Several cytokines such as IL-1, IL-6, and TNF-α have been demonstrated to increase significantly in CKD patients. Numerous clinical studies have demonstrated that high TNF-α and IL-6 levels are closely associated with poor prognosis of CKD, such as large amount of albuminuria and progressive loss of renal function [2, 3].

The gut microbiota imposes a crucial effect on regulating mucosal immunity. CKD causes an imbalance of the intestinal flora and leads to immune disorders [4]. Previous studies have shown that the intestinal flora of CKD patients is significantly decreased in probiotics, while increased in toxigenic flora [5]. Imbalance of the gut flora and dysfunction of the intestinal barrier lead to translocation of intestinal flora. Their toxic products also transfer into the host circulatory system and lead to persistent systemic inflammation. Intestinal toxins inductoxyl sulfate and p-Cresyl sulfate have been demonstrated to promote inflammatory macrophage activation [6] and increase production of pro-inflammatory cytokines [7].

Uremic toxins may also promote the imbalance between formation of reactive oxygen species (ROS) and antioxidant capacity, which leads to excessive oxidative stress. Increased ROS can induce inflammation, endothelial dysfunction, atherosclerosis and fibrosis, and is considered to be a powerful promoter of CKD [8]. Active NADPH oxidases (NOX) are dominant sources of ROS, and NOX4 is the major type of NOX in the kidney. Previous studies have demonstrated that intrarenal NOX4 contributes to immune-cell activation in the progression of CKD [9].

TMAO is produced by gut flora via metabolizing food containing choline, lecithin, betaine, and carnitine [10]. TMAO is predominantly excreted by the kidney, so its serum level is significantly increased in ESRD patients. It is in the spotlight recently because of its tight association with risk of cardiovascular events in both CKD and non-CKD patients [11, 12]. Many studies have demonstrated that dietary choline or TMAO can promote inflammatory activation of vascular endothelial cells and platelets aggregation, leading to atherosclerosis and CVD events. It has been demonstrated that choline TMA-lyase inhibitor, IMC, can suppress renal tubulointerstitial fibrosis caused by high-choline diet [13]. However, whether TMAO contributes to renal interstitial inflammation remains unknown. In the present study, we explored the role of TMAO in inflammatory injury and the underlying mechanism in the CKD rat model.

Method

Animal Experiments

Male Sprague-Dawley rats (at the age of 5–6 weeks, initial weight 180–200 g; Southern Medical University Animal Experiment Center) were subdivided into 3 groups randomly, and 5 in each group. The first group received sham operation, while the other 2 groups received five-sixths nephrectomy (5/6 Nx) as described previously [14]. Six weeks after the operation, the two groups of 5/6 Nx rats were subjected to intraperitoneal injection with 2.5% glucose peritoneal dialysis fluid (PDF; Baxter Health Care, Deerfield, IL, USA) and 2.5% glucose PDF plus TMAO 20 mg/kg/day (Sigma-Aldrich, St. Louis, MO, USA), respectively. The sham group was injected with PBS every day. Six weeks after injection, all the rats were sacrificed, and blood samples and kidney tissues were collected. Serum TMAO was detected by stable isotope dilution liquid chromatography tandem mass spectrometry (6460 Series Triple Quadrupole LC/MS; Agilent, Santa Clara, CA, USA).

Histological and Immunohistochemistry Staining

Paraffin-embedded kidney sections (2 μm) were subjected to hematoxylin and eosin (H&E) and Masson trichrome staining according to standard protocols. Tubular injury was graded with H&E-stained sections ranging from 0 to 4 according to the degree of tubular necrosis, dilatation, or cell swelling: 0, less than 5%; 1, 5–25%; 2, 25–50%; 3, 50–75%; and 4, over 75% [15]. At least 10 randomly chosen fields in the cortex region under the microscope (×400) were evaluated for each animal in a blinded manner, and an average score was calculated.

Interstitial fibrosis was assessed using Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA) on Masson trichrome stained sections. Ten visual fields (×400) were randomly selected for each animal and evaluated by a background subtraction method. Quantification is presented as the ratio of optical density of positive staining compared to the entire spectrum.

Immunohistochemistry staining was performed on 4-μm kidney sections. After antigen retrieval, sections were incubated with the primary antibodies against CD68 (Abcam, Cambridge, UK) or CD3 (Abcam). Images were taken by an Olympus BX51 micro-

Fig. 1. TMAO injection promotes renal functional decline in 5/6-nephrectomized rats and aggravates renal tubular injury. Six weeks after five-sixths nephrectomy, the CKD rats were randomly divided into 2 groups and received daily intraperitoneal injection of either PDF containing 2.5% glucose (2.5% PDF) or 2.5% PDF plus TMAO (20 mg/kg/day), respectively. The sham group was injected with PBS every day. Blood samples and kidney tissues were collected 24 h after 5 weeks of injection. A–C Serum levels of TMAO, Scr, and BUN. D Representative images of H&E and Masson’s trichrome staining of the kidney tissue. Original magnification, ×400; scale bar, 50 μm. Tubular injury score (E) and quantification assessment of kidney fibrosis (F). Bars depict mean values ± SD (n = 6 per group). Results were analyzed for statistical variance using one-way ANOVA analysis. *p < 0.05 versus sham group; **p < 0.05 versus CKD injected 2.5% PDF group. PDF, peritoneal dialysis fluid; TMAO, trimethylamine-N-oxide; Scr, serum creatinine; BUN, blood urea nitrogen; CKD, chronic kidney disease; H&E, hematoxylin and eosin.

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Kidney Blood Press Res 2022;47:61–71
DOI: 10.1159/000519603
then incubated with 25 mM glucose medium in the presence or absence of indicated concentration of TMAO for 24h. After 24h, all the cells were incubated with 3 μM dihydroethidium (Beyotime, MA, USA), anti-phosphorylated-p38 (Cell Signaling Technology), anti-p38 (Cell Signaling Technology), and anti-caspase-1 (Santa Cruz).

mRNA Analysis

RNA isolation and real-time quantitative polymerase chain reaction were conducted as recommended by MIQE guidelines. Total RNA was extracted from homogenized kidney cortex tissue. TNF-α, IL-6, IL-1β, IL-18, and MCP-1 mRNA levels were quantified by real-time quantitative polymerase chain reaction with the Applied Biosystems 7500 Fast Real-Time PCR System (Foster City, CA, USA). Gene expression was normalized to the housekeeping gene, β-actin. Primers used in this study were listed in online supplementary Table 1 (for all online suppl. material, see www.karger.com doi/10.1159/000519603).

Western Blot Analysis

Kidney tissues were lysed in lysis buffer for 30 min on ice. Western blot analysis was performed following procedure as described previously. The following primary antibodies were used: anti-IL-6 (Boster Biological Technology, Pleasanton, CA, USA), anti-IL-1β (Abcam), anti-β-actin (Boster Biological Technology), anti-human antigen R (HuR) (Santa Cruz, Santa Cruz, CA, USA), anti-TTP (Abcam), anti-NLRP3 (Cell Signaling Technology, Beverly, MA, USA), anti-phosphorylated-p38 (Cell Signaling Technology), anti-p38 (Cell Signaling Technology), and anti-caspase-1 (Santa Cruz). The following primary antibodies were used: anti-IL-6 (Abcam), anti-β-actin (Boster Biological Technology), anti-hu-

Primary renal tubular epithelial cells from C57BL/6 mice were cultured in DMEM/F12 (Gibco) medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 g/mL streptomycin (Hyclone) at 37°C in a 5% CO2 incubator. Fluorescence intensity was measured using flow cytometry (BD FACS; Calibur, San Francisco, CA, USA) at excitation/emission filters of 488/575 nm [17].

Dihydroethidium Fluorescence Analysis

Primary renal tubular epithelial cells from C57BL/6 mice were cultured on 12-well plates until they attained 80% confluency and then incubated with 25 mM glucose medium in the presence or absence of indicated concentration of TMAO for 24h. After 24h, all the cells were incubated with 3 μM dihydroethidium (Beyotime Biotechnology, Shanghai, China) for 30 min at 37°C in a 5% CO2 incubator. Fluorescence intensity was measured using flow cytometry (BD FACS; Calibur, San Francisco, CA, USA) at excitation/emission filters of 488/575 nm [17].

Statistical Analysis

For in vivo study and in vitro experiments, data were expressed as means ± SD. Results were analyzed for statistical variance using unpaired Student’s t test or one-way ANOVA analysis where appropriate. A two-sided p value <0.05 was considered to be statistically significant (SPSS software, version 20.0; SPSS, Inc., Chicago, IL, USA).

Result

TMAO Aggravates Renal Tubule Injury and Inflammatory Cells Infiltration

Previous studies have shown that TMAO is excreted through the kidneys and that TMAO levels gradually increase with renal function decline. As expected, serum levels of TMAO were significantly increased in CKD rats compared with those of the sham group (4.22 ± 1.98 μM vs. 2.14 ± 0.34 μM), while those of TMAO group were prominently higher than those of the CKD group (39.72 ± 11.97 μM vs. 4.22 ± 1.98 μM) (Fig. 1A). The data also showed that serum creatinine and blood urea nitrogen were both significantly elevated in the TMAO group compared with the CKD group (Fig. 1B, C), indicating that TMAO could further accelerate CKD progression.

Then, we performed histopathological examination to assess renal pathological injury. H&E staining revealed that in the TMAO group, the vacuolar and necrotic degeneration of renal tubular epithelial cells as well as inflammatory cells infiltration were much more severe than those in the CKD group. Masson trichrome staining also showed that TMAO treatment significantly exacerbated renal interstitial fibrosis in CKD rats (Fig. 1D, F). When evaluated by tubular injury score, the TMAO group had a significantly higher score than those of the CKD and sham groups (Fig. 1E).

Further analysis by immunohistochemistry staining showed the infiltration of macrophages (CD68+ cells) and lymphocytes (CD3+ cells) in the tubulointerstitial area. The amounts of both types of cells were significantly increased in the CKD group compared with the sham group, while the TMAO treatment drove the trend even further (Fig. 2). Our observations revealed that TMAO could promote inflammatory cells infiltration and renal tubular injury, which lead to the decline of kidney function.

TMAO Promotes Expression of Inflammatory Cytokines

As chemotaxis of inflammatory cells plays a crucial role in inflammation, we detected MCP-1, a key chemotactic cytokine involved in the migration of circulating monocytes. The result showed that MCP-1 was significantly increased in renal tissue by TMAO treatment (Fig. 3A, B). Next, we compared levels of inflammatory cytokines including TNF-α, IL-6, IL-1β, and IL-18 in kidney tissue among the groups. It was found that these inflammatory cytokines except IL-18 were significantly raised in the kidney tissues of the TMAO treatment group compared with those of the CKD group (Fig. 3C). Col-
lectively, these data demonstrated that TMAO could promote inflammatory cell chemotaxis and increase the expression of inflammatory cytokines.

**TMAO Exacerbates the Activation of the p38/MAPK Pathway Induced by 5/6 Nx**

Then, we tried to analyze the signaling pathway through which TMAO promotes kidney inflammation. First, we detected the NF-κB/p65 pathway, but the result did not support that TMAO increased the phosphorylation level of p65 in renal tissue (seen in online suppl. Fig. 1). Since the p38/MAPK pathway also plays a central role in the fibrosis process after inflammatory injury, we examined the level of phosphorylation of p38 as well as its downstream molecules. As is shown in (Fig. 4A, B), the ratio of phosphorylated p38/p38 was elevated in CKD rats...
compared with that of the sham group, and TMAO injection promoted the trend even further. Phosphorylated p38 is known to activate MK2/3 phosphorylation, which can upregulate HuR, while downregulate tristetraprolin (TTP) [18].

TMAO Upregulates HuR while Downregulating TTP
HuR is a type of RNA-binding protein that can combine with the AU-rich element (ARE) in the 3' untranslated region (3' UTR) of mRNA. It has been reported to maintain the mRNA stability of inflammatory cytokines such as TNF-α, IL-6, and IL-18 [19, 20]. TTP is another type of RNA-binding protein. But, TTP can promote the degradation of these cytokines, and HuR can competitively inhibit TTP. Consistently, we observed that TMAO treatment could significantly upregulate HuR and downregulate TTP (p < 0.05) (Fig. 4C, D). Our findings may partially explain why TMAO can promote the mRNA and protein levels of the inflammatory cytokines.

TMAO Increases NOX4 Expression and Aggravates Oxidative Stress
Upregulation of NOX4 is crucial in renal oxidative stress and kidney injury. As NOX4 mRNA also has numerous AREs in its 3'-UTR that can be bounded by HuR, we detected the expression of NOX4 in kidney tissue. The level of superoxide dismutase 2 (SOD2) in kidney tissue was also examined, because it is the major antioxidant enzyme catalyzing the dismutation of superoxide anions into oxygen and hydrogen peroxide [21]. The results showed that compared with the CKD group, TMAO treatment significantly raised the levels of NOX4 in kidney tissue, while pulled down the levels of SOD2 (Fig. 4E, F).

Fig. 4. TMAO activates the 5/6 Nx-induced P38/MAPK pathway and aggravates oxidative stress. A, B Protein levels of p-p38/p38 in the renal cortex were determined by Western blotting. The relative protein levels were expressed as the fold increase compared to that of the sham group in 3 independent experiments. C, D Representative Western blot images and quantification of protein levels of TTP and HuR in the renal cortex were presented. E, F Representative Western blot images and quantification of protein levels of NOX4 and SOD2 in the renal cortex. β-actin served as a loading control. Data are expressed as the mean ± SD (n = 5 per group). *p < 0.05 versus sham group; #p < 0.05 versus CKD injected 2.5% PDF.

G Primary renal tubular epithelial cells of rats were cultured with high-glucose medium (glucose 25 mmol/L) and then treated with gradient concentrations of TMAO (0–400 μM). The levels of superoxides in these cells were detected by DHE fluorescence. Data are expressed as the mean ± SD (n = 5 per group). *p < 0.05 versus control; #p < 0.05 versus cells cultured with high-glucose medium solely. HuR, human antigen R; TTP, tristetraprolin; DHE, dihydroethidium; TMAO, trimethylamine-N-oxide; CKD, chronic kidney disease; PDF, peritoneal dialysis fluid.

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DOI: 10.1159/000519603
In addition, we tested the effect of TMAO on ROS production in renal tubular epithelial cells. High glucose-cultured PTECs were stimulated with gradient concentrations of TMAO (0−400 μM) for 24h. Dihydroethidium fluorescence analysis showed that levels of superoxide in renal tubular cells were significantly elevated by TMAO treatment (Fig. 4G). These data demonstrated that TMAO could aggravate oxidative stress in renal tissue.

**TMAO Promotes NLRP3 Inflammasomes Activation**

Inflammasomes turn oxidative stress signal signals into the release of inflammatory cytokines which rapidly set off local and systemic inflammation. We also assessed the levels of major members involved in the NOD-like receptor protein 3 (NLRP3) inflammasome pathway by Western blot. The result revealed that TMAO significantly elevated the protein levels of NLRP3, cleaved caspase-1, and cleaved IL-1β compared with the nontreated CKD group (Fig. 5). The data above demonstrated that TMAO could aggravate tubular inflammation via NLRP3 inflammasome activation.

**Fig. 5.** TMAO activates 5/6 Nx-induced NLRP3 inflammasomes. A Representative Western blots showed the abundance of NLRP3, cleaved caspase-1, and cleaved IL-1β in the renal cortex in sham rats and CKD rats injected with 2.5% PDF or 2.5% PDF plus TMAO. B Relative protein levels of NLRP3, cleaved caspase-1, and cleaved IL-1β compared to β-actin. Data are expressed as the mean ± SD (n = 5 per group). Data were analyzed for statistical variance using one-way ANOVA analysis. *p < 0.05 versus sham group; † p < 0.05 versus CKD injected 2.5% PDF. TMAO, trimethylamine-N-oxide; CKD, chronic kidney disease; PDF, peritoneal dialysis fluid.
Discussion

TMAO, a metabolite of intestinal flora, has been recognized to promote atherosclerosis, but its role in CKD progression is still unknown. Based on previous studies, we explored the effects of TMAO on renal interstitial inflammation. There are several important findings in the present study. First, the study found that TMAO could significantly aggravate renal function decline and tubular interstitial injury. Second, TMAO could activate the inflammatory pathway through increasing p38 phosphorylation and HuR level. Last, TMAO upregulated NOX4 as well as promoted oxidative stress and NLRP3 activation.

Chemotaxis and infiltration of inflammatory cells is the key step of renal inflammatory injury [22]. We found that the infiltration of CD68+ and CD3+ cells were significantly increased in the TMAO group. TMAO treatment also stimulated the expression of MCP-1 as well as inflammatory factors including IL-6, IL-1β, IL-18, and TNF-α in the kidney tissue. Consistently, the renal tubular injury score in the TMAO group was significantly higher than that in the CKD group. It is still controversial whether mature IL-1β can be secreted from renal intrinsic cells. Some studies suggest that IL-1β comes from activated macrophages. From the results above, we speculate that TMAO can induce renal tubular epithelial cell injury and recruit macrophages, which in turn release more inflammatory cytokines.

Then, we sought to identify the pathway by which TMAO activated the inflammatory signaling in the kidney. As is well known, p38 is the core component of the MAPK pathway, and phosphorylated p38 can turn on the transcription of many target genes, such as TNF-α, MCP-1, and NLRP3 inflammasome [23, 24]. Our results revealed that TMAO had a promoting effect on p38 phosphorylation in the MAPK pathway but not on phosphorylation of either ERK or p65. Seldin et al. [25] has reported that TMAO could induce the MAPK signaling cascade in vascular endothelial cells in the model of aortic disease. p38/MAPK is also a major pro-inflammatory pathway for other intestinal flora toxins. For example, the uremia toxin p-cresyl sulfate is reported to activate JNK/p38 MAPKs and stimulate ROS production [26].

In addition to promoting p38 phosphorylation, we found that TMAO could upregulate HuR and downregulate TTP in the kidney. Previous studies have shown that HuR could regulate several pro-inflammatory factors such as IL-6, TNF-α, and C-reactive protein via binding to AREs in their 3′-UTR. One study in glioblastoma cells showed that IL-1β could enhance IL-6 mRNA through the p38 MAPK-HuR pathway [27]. HuR increases in various kidney disease models and is involved in glomerulosclerosis. Another research revealed that inhibition of HuR could significantly ameliorate podocyte injury, macrophage cell infiltration as well as fibrogenic protein deposition [28]. As cytoplasmic localization of HuR is controlled by the p38/MAPK pathway, the results above support the speculation that TMAO indirectly promotes inflammation by regulating transcription factor HuR and TTP.

Our data also revealed that TMAO increased the NOX4 level, while decreased the SOD2 level in the kidney. It is known that NOX4 expression is increased in diabetic kidney disease, accompanied with ROS generation and mesangial-cell fibrotic injury [29]. NOX4 has numerous AREs in its 3′-UTR that can be bounded by HuR [30]. Shi et al. [30] observed that HuR could bind to the NOX4 promoter and increase the ROS level of mesangial cells in response to high glucose. Therefore, we hypothesize that TMAO could increase the NOX4 level by upregulating HuR, thus aggravating oxidative stress in CKD. Since direct inhibition of oxidative stress and NOX4 inhibitors have not been clinically beneficial, inhibition of TMAO synthesis may be a feasible treatment to reduce oxidative stress and ameliorate CKD.

Another novelty of this study is that TMAO could promote NLRP3 inflammasome formation as well as caspase-1 activation and IL-1β synthesis in the CKD model. It has been reported that TMAO promotes vascular calcification through activation of NLRP3 inflammasome and NF-κB signaling [31]. The mechanism by which TMAO activates NLRP3 remains unclear. In general, bacterial endotoxins stimulate inflammation via the toll-like receptor 4 and NLRP3 inflammasome pathways [32]. Oxidative stress is considered to be another inducer of NLRP3 inflammasome activation. It has also been demonstrated that NOX4 can downregulate the fatty-acid oxidation pathway by inhibiting carnitine palmitoyl transferase 1A and then stimulates NLRP3 inflammasome activation and IL-1β production [32, 33]. In summary, TMAO may activate the NLRP3-IL-1β axis, then promote inflammatory cell chemotaxis and inflammatory cytokine release, and finally lead to tubulointerstitial injury.

There are several limitations in the current study. Firstly, in order to directly test the effects of TMAO, rather than other intestinal metabolites from high-choline diet, intraperitoneal injection of TMAO was used in this study. Nevertheless, repeated injections may also increase the risk of peritoneal inflammation and intestinal damage.
Secondly, we did not collect the urine for urinary albumin measurement, which is also an important indicator for CKD model. Finally, in addition to the effect of TMAO on the protein levels of HuR and TTP, the impact of TMAO on their function and the combination with downstream genes should also be explored in future studies.

Conclusion

We provide evidence showing that in the CKD rat model, TMAO could regulate the expression of downstream inflammatory factors by activating p38 phosphorylation and upregulation of HuR. Activation of inflammatory pathways results in the upregulation of NOX4 and activation of NLRP3 inflammasome in kidney tissue. The increasing inflammation cytokines and oxidative stress aggravate inflammatory cell infiltration, and eventually lead to renal function deterioration. TMAO may become a therapeutic target to ameliorate renal tubular interstitial injury and delay the progression of CKD.

Acknowledgment

Dr. Boxin Zhao is acknowledged for performing the serum TMAO quantification.

Statement of Ethics

This study protocol was reviewed and approved by the Nanfang Hospital Animal Ethic Committee, approval number NFYY-2017-118.

Data Availability Statement

The data that support the findings of this study are openly available in figshare at 10.6084/m9.figshare.14703138.

Conflict of Interest Statement

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work; there is no professional or other personal interest of any nature or kind in any product, service, and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled “Trimethylamine-N-oxide aggravates kidney injury via activation of p38/MAPK signaling and upregulation of HuR.”

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