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Trimethylamine-N-Oxide Instigates NLRP3 Inflammasome Activation and Endothelial Dysfunction

Krishna M. Boini¹, Tahir Hussain¹, Pin-Lan Li², and Saisudha Koka¹

¹Department of Pharmacological and Pharmaceutical Sciences, College of Pharmacy, University of Houston, Houston, TX, 77204, USA

²Department of Pharmacology and Toxicology, School of Medicine, Virginia Commonwealth University, Richmond, VA 23298, USA

Abstract

Background/Aim—Plasma trimethylamine-N-oxide (TMAO), a product of intestinal microbial metabolism of dietary phosphatidylcholine has been recently associated with atherosclerosis and increased risk of cardiovascular diseases (CVD) in rodents and humans. However, the molecular mechanisms of how TMAO induces atherosclerosis and CVD progression are still unclear. The present study tested whether TMAO induces NLRP3 inflammasome formation and activation and thereby contributes to endothelial injury initiating atherogenesis.

Methods—Inflammasome formation and activation was determined by confocal microscopy, caspase-1 activity was measured by colorimetric assay, IL-1 β production was measured using ELISA, cell permeability was determined by microplate reader and ZO-1 expression was determined by western blot analysis and confocal microscopy. In *in vivo* experiments, TMAO was infused by osmotic pump implantation.

Results—TMAO treatment significantly increased the colocalization of NLRP3 with Asc or NLRP3 with caspase-1, caspase-1 activity, IL-1 β production, cell permeability in carotid artery endothelial cells (CAECs) compared to control cells. Pretreatment with caspase-1 inhibitor, WEHD or Nlrp3 siRNA abolished the TMAO-induced inflammasome formation, activation and cell permeability in these cells. In addition, we explored the mechanisms by which TMAO activates NLRP3 inflammasomes. TMAO-induced the activation of NLRP3 inflammasomes was associated with both redox regulation and lysosomal dysfunction. In animal experiments, direct infusion of TMAO in mice with partially ligated carotid artery were found to have increased NLRP3 inflammasome formation and IL-1 β production in the intima of wild type mice.

Conclusion—The formation and activation of NLRP3 inflammasomes by TMAO may be an important initiating mechanism to turn on the endothelial inflammatory response leading to endothelial dysfunction.

Address for correspondence: Sai S. Koka, PhD, Department of Pharmacological and Pharmaceutical Sciences, College of Pharmacy, University of Houston, 3455 Cullen Blvd, Room 463, Houston, TX, 77204, Phone: 713 743 1086, Fax: 713 743 1232, sskoka@uh.edu.

CONFLICTS OF INTERESTS

The authors of this manuscript declare that they have no conflicts of interests.

Keywords

TMAO; inflammasome; tight junction protein; endothelial cell permeability

INTRODUCTION

Trimethylamine-N-oxide (TMAO) has been recently highlighted as a potential diagnostic marker for cardiovascular diseases (CVD). Recent studies demonstrated that elevated TMAO levels in plasma are associated with an increased risk of CVD [1–8]. Circulating TMAO levels were found to be elevated in distinct cohorts of cardiac patients with stable heart failure and were associated with increased risk for myocardial infarction, stroke and mortality [1–5, 9–15]. Plasma TMAO concentrations were found to independently predict coronary atherosclerosis and mortality in patients with chronic kidney disease [12]. Increased TMAO concentrations have also been associated with impaired glucose tolerance [16], diabetes [17]. Recently, TMAO have been associated with greater risk of colorectal and prostate cancer. Despite the clear association of TMAO with various chronic diseases, the exact mechanism through which TMAO leads to development and progression of various diseases is still unclear.

In this regard, some mechanisms postulated to date are alteration in host sterol/lipid metabolic pathway leading to changes in cholesterol transport and excretion [2, 7, 9, 15], modulation of platelet responsiveness [8] and activation of profibrotic pathways [3]. Recent reports indicated activation of mitogen-activated protein kinase, nuclear factor-kappa B signaling cascade and promotion of leukocyte adhesion *in vivo* [3]. Together, these studies suggests that TMAO may trigger endothelial and vascular inflammation, injury and fibrotic processes that may contribute to atherogenesis. However, the exact mechanism through which TMAO leads to development and progression of atherosclerotic vascular diseases is currently unclear.

Nlrp3 inflammasome act as a sensor to detrimental exogenous and endogenous substances and switch on both inflammatory and non-inflammatory responses which play a vital role in the development of atherosclerosis. Recent studies have indicated that Nlrp3 inflammasome activation is critical for the development of atherosclerosis upon atherogenic stimuli such as cholesterol crystals [18, 19]. However, whether Nlrp3 acts as a sensor to the recently recognized proatherogenic metabolite, TMAO, is unknown and the role of inflammasome signaling in TMAO-induced atherogenesis has not been explored. Since TMAO is a biologically active atherogenic molecule it is important to understand the role of TMAO in eliciting inflammatory and non-inflammatory responses via inflammasome activation during atherosclerotic vascular disease. The earliest event in the development of atherosclerosis is endothelium dysfunction, which can be triggered by several insults. Hence, it is plausible to determine whether TMAO induces endothelial inflammasome activation and contribute to the endothelial dysfunction in the very early stages of atherosclerosis.

MATERIAL AND METHODS

Cell culture and treatments

The mouse carotid arterial endothelial cells were isolated and characterized as described earlier [20, 21]. For the TMAO stimulation, cells were treated with TMAO (30 μ M) and then incubated for overnight. In case of inhibitors used, the cells were pretreated with 1 mmol/L Z-WEHD-FMK (WEHD; R&D Systems, Minneapolis, MN), cathepsin B inhibitor Ca-074Me (5 μ M, Sigma), potassium channel blocker glibenclamide (Glib, 10 μ M, Sigma) or ROS scavenger N-acetyl-L-cysteine (NAC, 10 μ M, Sigma) for 30 min.

Immunofluorescence microscopic analysis

Cells were grown on eight-well chamber slides and then treated as indicated. After the treatment, cells were fixed with 4% paraformaldehyde for 15 minutes. The cells were then washed in phosphate-buffer saline (PBS) and were incubated for 2 hours at 4°C with rabbit and/or mouse anti-Nlrp3 (1:500, Abcam), anti-ASC (1:500, Invitrogen, Abcam), anti-caspase 1 (1:1000; Abcam) and anti-ZO-1 (1:1000; Invitrogen). Double immunofluorescent staining was performed by incubating slides with Alexa Fluor 488 or Alexa Fluor 555-labeled secondary antibody (1:100, Invitrogen) for 1 hour at room temperature. The slides were visualized through sequentially scanning on an Olympus laser scanning confocal microscope (Fluoview FV1000, Olympus, Japan). Colocalization was analyzed by Image Pro Plus software, and the co-localization coefficient was represented by Pearson's correlation coefficient [22–25].

Caspase-1 Activity and IL-1 β production Assay

Cells were harvested and homogenized to extract proteins for caspase-1 activity assay using a commercially available kit (Biovision, CA). The data was expressed as the fold change compared with control cells. In addition, the cell supernatant was collected and IL-1 β production was measured by a commercially available ELISA Kit (R&D System, Minneapolis, MN) according to the protocol described by the manufacturer.

Immunoblotting

Cells were washed twice with ice-cold PBS and homogenized in ice-cold HEPES buffer containing 25 mM Na-HEPES, 255 mM sucrose, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride (pH 7.4). After centrifugation at 1000 \times g for 10 min at 4°C, the supernatants containing the membrane protein and cytosolic components, termed homogenates, were frozen in liquid N₂, and stored at –80°C until use. Cell homogenates were denatured with reducing Laemmli SDS-sample buffer and boiled for 5 min. Samples were run on SDS-PAGE gel, transferred into PVDF membrane and blocked. The membranes were probed with ZO-1 antibody (Life Technology, 1:1000) or β -actin overnight at 4°C followed by incubation with secondary antibody, and then conjugated to horseradish peroxidase-labeled immunoglobulin G. The immunoreactive bands were enhanced by chemiluminescence methods and imaged on Kodak Omat film. β -actin served as a loading control.

Endothelial permeability

CAECs were cultured in 24-well transwell plates and treated as indicated for 24 hr. The transwell inserts were moved into non-used wells with 200 μ l fresh media. 100 μ l Fluorescein isothiocyanate (FITC)–dextran (10 KDa, Invitrogen) solution was added into each insert and the plate was incubated at 37°C for 2 hours to allow fluorescein molecules flow through the endothelial cell monolayer. The inserts were then removed and fluorescent intensity in each well was determined at excitation/emission of 485/530 nm using a fluorescent microplate reader (FL \times 800, BIO-TEK Instruments). The arbitrary fluorescence intensity was used to calculate the relative permeability.

Partial Carotid Ligation and Osmotic Pump Implantation

Eight-week-old male C57BL/6J wild-type mice were used. All protocols were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. Partial carotid ligation surgery was performed as previously reported [26]. In brief, animals were sedated with 2% isoflurane that was provided through a nose cone. Next, a ventral midline incision of 4 to 5 mm was made in the neck. With the use of blunt dissection, muscle layers were separated with curved forceps to expose the left carotid artery (LCA). Three of four branches of the LCA (left external carotid, internal carotid, and occipital arteries) were ligated by using a 6-0 silk suture. The superior thyroid artery was left intact, providing the sole source for blood circulation. The incision was then closed, and the animals were kept on a heating pad until they gained consciousness. In the TMAO infusion group, the osmotic pump (model 2002; Alzet, Cupertino, CA) filled with TMAO was implanted subcutaneously, and the catheter was inserted into the external jugular vein. In another group, mice were injected intraperitoneally with WEHD, a caspase-1 inhibitor, at a dosage of 1 mg/kg per day before implantation of the TMAO pump. Fourteen days after partial ligation, animals were sacrificed by cervical dislocation after the administration of anesthesia. Blood samples were collected, LCAs and right carotid arteries were then harvested for immunohistochemistry, dual fluorescence staining, and confocal analysis.

Immunohistochemistry

Formalin-fixed, paraffin-embedded carotid arterial tissue sections (4 μ m) were stained with primary antibodies (1:50 dilution) overnight at 4 °C after a 20 min wash with 3% H₂O₂ and 30 min blocking with serum. The slides were sequentially treated with CHEMICON IHC Select HRP/DAB Kit (EMD Millipore, MA) according to the protocol described by the manufacturer. Finally, the slides were counterstained with hematoxylin. Negative controls were prepared by leaving out the primary antibodies.

Statistics

Data are presented as means \pm SEM. Significant differences between and within multiple groups were examined using ANOVA for repeated measures, followed by Duncan's multiple-range test. $P < 0.05$ was considered statistically significant

RESULTS

TMAO induces formation and activation of NLRP3 inflammasome in mouse carotid artery endothelial cells (CAECs)

We tested the hypothesis that TMAO induces inflammasome formation and activation and thereby contributes to endothelial injury. In cultured CAECs, we examined whether TMAO could trigger the formation and activation of Nlrp3 inflammasome complexes by analyzing the co-localization of Nlrp3 inflammasome components, the cleavage of pro-caspase-1 to activate caspase-1, and the production of IL-1 β . Our confocal microscopic images showed that TMAO-induced co-localization of inflammasome molecules between Nlrp3 (green) with ASC (red) or Nlrp3 (green) with Caspase-1 (red) as shown by increased yellow staining (yellow spots) in CAECs, which were blocked by caspase-1 inhibitor (WEHD) or silencing Nlrp3 gene by Nlrp3 siRNA (Nlrp3si) transfection (Fig. 1). Nlrp3 inflammasome complex formation results in cleavage of pro-caspase-1 protein to their bioactive form, which in turn binds to and cleaves its substrates such as pro-interleukin 1 β (IL-1 β). In line with the confocal findings of inflammasome complex formation, we have shown that TMAO increased caspase 1 activity (Fig. 2A) and also enhanced IL-1 β production (Fig. 2B). Caspase-1 activity and IL-1 β production were abolished in CAECs with prior treatment of Nlrp3 gene silencing.

Effect of TMAO on tight junction proteins and endothelial cell permeability in CAECs

Endothelial cells are connected by tight junction proteins which maintain the integrity of the endothelium. Tight junctions function as a barrier in regulating paracellular permeability and maintaining cell polarity. ZO-1 is an essential tight junction protein which is associated with junction integrity and its down regulation leads to junctional disruption and enhanced cellular permeability. Hence, we investigated whether TMAO-induced Nlrp3 inflammasome activation could cause disassembly of tight junction protein ZO-1. Our confocal analysis showed that TMAO markedly decreased the expression of tight junction protein ZO-1 on endothelial cell monolayers (Fig. 3A). TMAO induced downregulation of ZO-1 which was prevented by silencing Nlrp3 in the CAECs. Down-regulation of ZO-1 by TMAO was further confirmed by western blot analyses which indicate that TMAO decreased ZO-1 protein expression (Fig. 3B and 3C). To further determine the functional significance of NLRP3 inflammasome activation, we examined its influence on TMAO-induced changes in barrier function of endothelial monolayers. As shown in Fig. 4, dextran flux significantly increased in ECs treated with TMAO compared to vehicle treated ECs. This TMAO-induced increase in EC permeability was markedly reduced in the presence of Nlrp3 siRNA transfection (Fig. 4). These results indicate that activation of Nlrp3 inflammasome by TMAO causes disruption of tight-junction proteins and alters EC permeability.

TMAO-induced inflammasome signaling pathways

Endothelial inflammasomes are known to be activated by three major signaling pathways which include reactive oxygen species (ROS) activation, lysosome rupture and ion channel gating (K⁺ efflux). We examined TMAO-induced inflammasome signaling mechanisms by using inhibitors of the above mentioned pathways which lead to activation of Nlrp3 inflammasomes. It was found that inhibition of both cathepsin B activity (Ca-074Me) and

ROS release (N-acetyl-L-Cysteine or Nac) in ECs markedly attenuated TMAO-induced caspase-1 activity in ECs (Fig. 5). In contrast, K Channel blocker (Glibenclamide) had a no significant effect on TMAO-induced Nlrp3 inflammasome activation. Therefore our results indicate that TMAO could act via lysosomal destabilization and blockade of ROS.

TMAO-induced endothelial inflammasome formation and activation in the carotid arteries of mice

Confocal microscopic analysis demonstrated that TMAO treatment increased the co-localization of NLRP3 with ASC in carotid arteries of wild type mice (Fig. 6A). In addition, the TMAO-induced IL-1 β production in the intima in wildtype mice (Fig. 6B). This data suggests the formation and activation of NLRP3 inflammasomes in the endothelium of these arteries.

DISCUSSION

The primary goal of the present study was to reveal whether TMAO induced NLRP3 inflammasome activation and leads to the development of endothelial dysfunction. We first confirmed that TMAO stimulation induced the formation and activation of the NLRP3 inflammasome complex in CAECs. However, such inflammasome formation and activation were abolished in ECs with prior treatment with *Nlrp3* siRNA or Caspase-1 inhibitor, WEHD. Thus TMAO lead to the formation and activation of NLRP3 inflammasomes in ECs. The findings demonstrate the critical role of TMAO in the activation of NLRP3 inflammasomes which could be associated with subsequent endothelial dysfunction and atherogenesis.

Recently, NLRP3 inflammasome has been implicated in different auto inflammatory diseases such as gout, myocardial infarction, and type II diabetes, obesity, glomerular injury [18, 24, 27–36] and also to a number of other diseases including silicosis, liver toxicity, Alzheimer's disease, cystic fibrosis and acute lung injury [23, 24, 37–42]. However, little is known about inflammasome contribution to the initiation or development of atherosclerosis. Among different types of inflammasomes, the NLRP3 inflammasome has been well characterized, which consists of a proteolytic complex formed by Nlrp3, the adaptor protein ASC, and caspase-1. Caspase-1 is activated when the inflammasome complex is formed to produce active IL-1 β and IL-18 by cleavage of their precursors [22, 29, 43]. NLRP3 acts as the sensory component to recognize both endogenous and exogenous danger signals [44–46], when ASC and caspase-1 are recruited to form a protein complex, where caspase-1 is activated [47–49]. The active caspase-1 not only proteolytically cleaves IL-1 β and/or IL-18 into their biologically active form [22, 29, 43]. In macrophages, NLRP3 inflammasome activation is critical for the foam cell formation and other atherosclerotic lesions upon proatherogenic stimuli such as cholesterol crystals (ChC) [18, 19]. More interestingly, some non-atherogenic endanger factors also activate NLRP3 inflammasomes including adenosine triphosphate (ATP), uric acid, visfatin and DAMPs [26, 28, 30, 34, 50–53], which may enhance the susceptibility to atherosclerosis or other vascular diseases, cell pyroptosis and alterations of cell membrane permeability, turning on the inflammatory response and directly inducing cell dysfunction or injury. Moreover, recent study shows that TMAO

via activation of Nlrp3 inflammasomes (Fig. 4) which is prevented by inhibition of Nlrp3 by Nlrp3 siRNA.

Next we examined how TMAO-induced NLRP3 inflammasome activation in endothelial cells. Several mechanisms underlying inflammasome activation have been reported, including lysosome rupture, K⁺ channel gating, and reactive oxygen species (ROS) activation [25]. We first tested which of these pathways are involved in TMAO-induced NLRP3 inflammasome activation. Using blockers or inhibitors of individual pathway, we found that TMAO-induced NLRP3 inflammasome formation and activation in endothelial cells were significantly attenuated or abolished by ROS scavenger, N-acetyl-L-cysteine (NAC) and cathepsin B inhibitor, Ca-074Me, but not by potassium channel blocker, glibenclamide (Glib). These results suggest that TMAO is able to activate NLRP3 inflammasomes in ECs at least *via* two reported pathways involving increased ROS and frustrated lysosomes and enhanced cathepsin B activity.

In summary, this work has studied the formation and activation of NLRP3 inflammasomes by TMAO which may be an important initiating mechanism to turn on the endothelial inflammatory response leading to endothelial dysfunction. Our data suggest that TMAO induces inflammasome-dependent endothelial hyperpermeability via activation of the Nlrp3 inflammasome in endothelial cells. Thus, our findings provide novel insights that TMAO-induced endothelial hyperpermeability via inflammasome activation may facilitate endothelial barrier dysfunction thereby contributing to endothelial dysfunction and atherogenesis.

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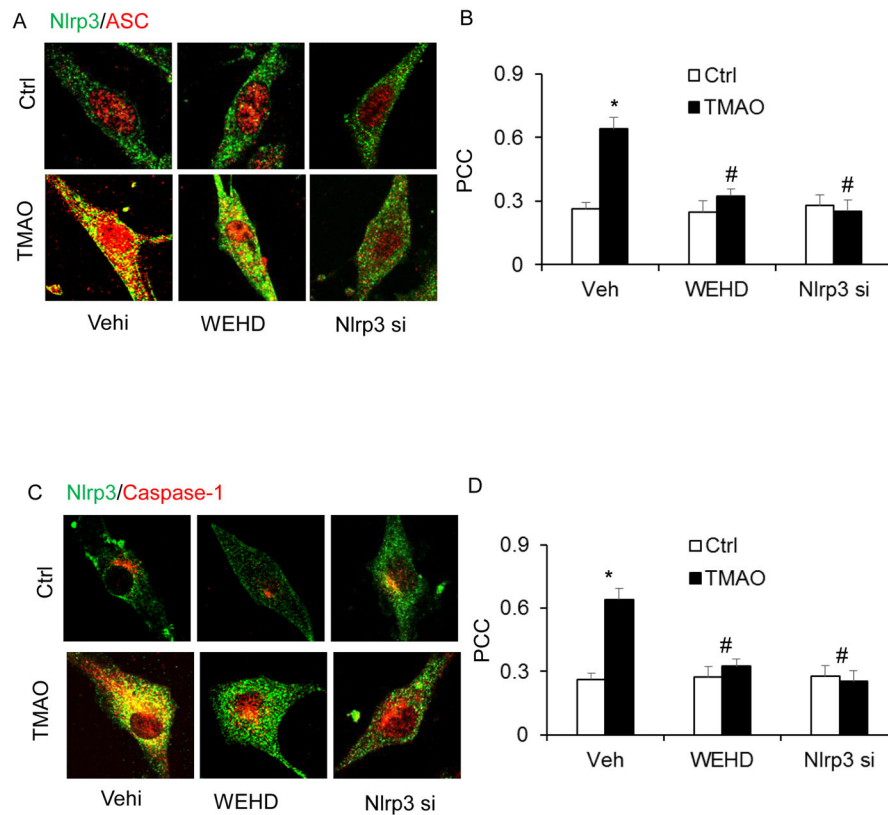


Figure 1. TMAO-induced NLRP3 inflammasome formation and activation in CAECs
 Representative confocal fluorescence images show the colocalization of NLRP3 with ASC (A) or NLRP3 with caspase-1 (C). Summarized data shows the fold changes of Pearson coefficient correlation (PCC) for the colocalization of NLRP3 with ASC (B) and NLRP3 with caspase-1 (D) in CAECs of *Nlrp3*^{+/+} mice. * Significant difference ($P < 0.05$) compared to the values from control cells, # Significant difference ($P < 0.05$) compared to the values from TMAO treated group. Nlrp3 si, Nlrp3 siRNA; cells were transfected with Nlrp3 siRNA or WEHD and then stimulated with TMAO. N=5–6.

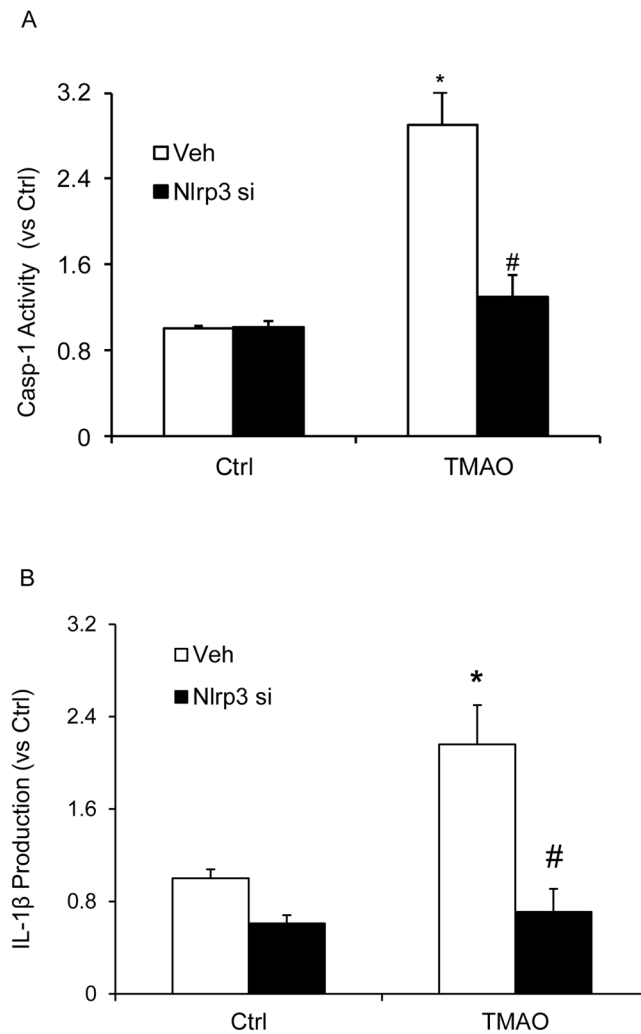


Figure 2. Effects of TMAO on caspase-1 activity and IL-1 β production in CAECs

Values are arithmetic means \pm SEM (n=6 each group) of caspase-1 activity (A), IL-1 β production (B) in CAECs of Nlrp3^{+/+} mice with or without stimulation of TMAO and/or Nlrp3 siRNA transfection. * Significant difference ($P < 0.05$) compared to the values from control cells, # Significant difference ($P < 0.05$) compared to the values from TMAO treated group. Nlrp3 si, Nlrp3 siRNA; cells were transfected with Nlrp3 siRNA or WEHD and then stimulated with TMAO. N=6.

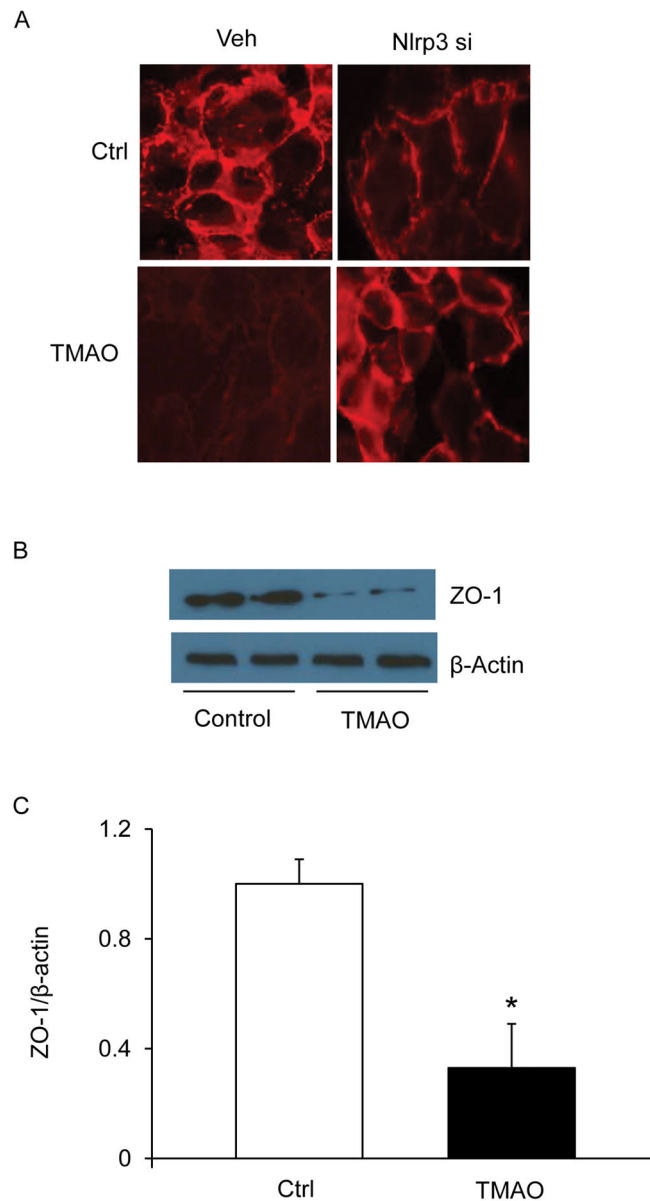


Figure 3. Effects of Nlrp3 gene silencing on TMAO-induced tight junction protein ZO-1 in CAECs

A: Representative fluorescence images shows the ZO-1 expression in CAECs with or without stimulation of TMAO and/or Nlrp3 siRNA transfection (n=5). B: Representative Western blot gel document showing the expression of ZO-1 (n=3–5). C: Summarized data showing the expression of ZO-1 (n=3–5). * Significant difference ($P<0.05$) compared to the values from control cells.

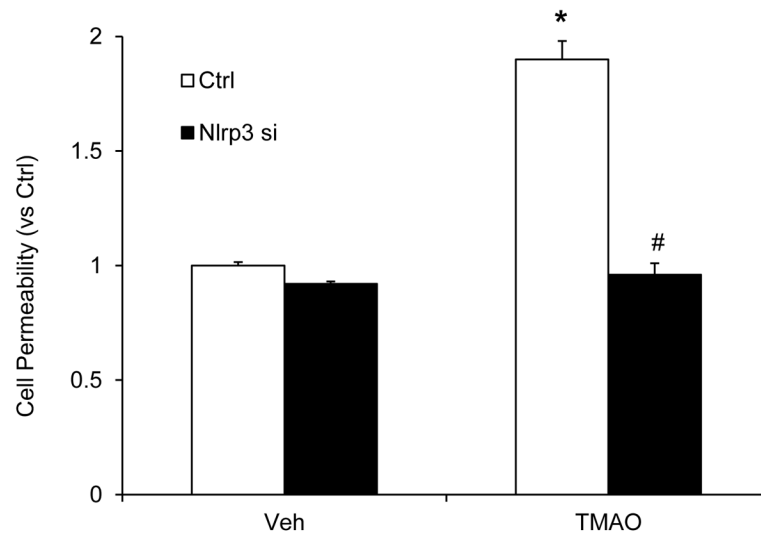


Figure 4. Inhibition of inflammasome abolishes TMAO-induced cell permeability in CAECs Values are arithmetic means \pm SEM (n=6 each group) of cell permeability in CAECs of Nlrp3^{+/+} mice with or without stimulation of TMAO and/or Nlrp3 siRNA transfection. * Significant difference ($P < 0.05$) compared to the values from control cells, # Significant difference ($P < 0.05$) compared to the values from TMAO treated group. Nlrp3 si: Nlrp3 siRNA; cells were transfected with Nlrp3 siRNA and then stimulated with TMAO.

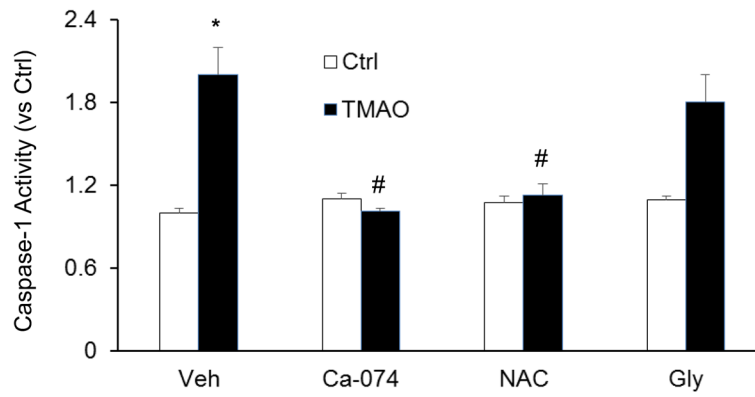


Figure 5. Effect of cathepsin B inhibition, potassium channel blockade or ROS scavenging on TMAO- induced NLRP3 inflammasomes activation in CAECs

Summarized data showing the, caspase-1 activity in CAECs with or without stimulation of TMAO. Ca-074: Ca-074Me, cathepsin B inhibitor, Gly: Glybeclamide, potassium channel blocker, NAC: N-acetyl-L-cysteine, ROS scavenger. * $P < 0.05$ vs. Ctrl group; # $P < 0.05$ vs. TMAO (n=6).

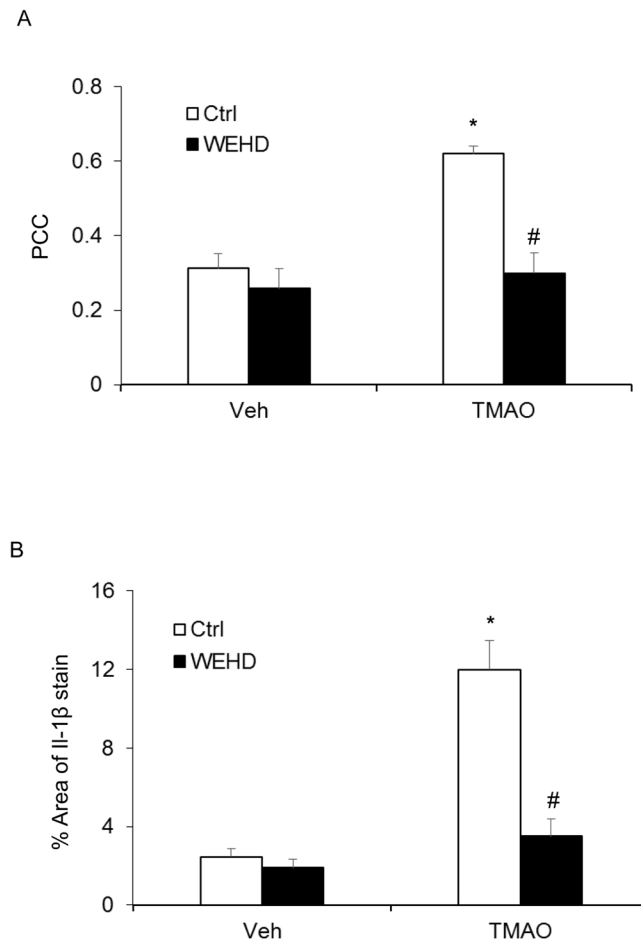


Figure 6. Nlrp3 inflammasome formation and activation in wild type mice with or without stimulation of TMAO and PLCA

A: Summarized data showing the co-localization coefficient (PCC) of Nlrp3 with Asc. B: IL-1 β production in the intima of vehicle or TMAO treated wild type mice. * Significant difference ($P < 0.05$) compared to the values from control mice. # Significant difference ($P < 0.05$) compared to the values from mice on the TMAO.