TMAO promotes NLRP3 inflammasome activation via the ERS/NF-kb pathway in ox-LDL-induced THP-1 macrophages and accelerates the secretion of IL-1β、IL-18

Haiyan Zhao
The First Affiliated Hospital of Harbin Medical University
Xiaochen Yu
The First Affiliated Hospital of Harbin Medical University
Zhisheng Luo
The First Affiliated Hospital of Harbin Medical University
Mingxue Wang
The First Affiliated Hospital of Harbin Medical University
Wenxiao Wang
The First Affiliated Hospital of Harbin Medical University
Xiuru Guan (gxr0451@sina.com)
The First Affiliated Hospital of Harbin Medical University

Research Article

Keywords: TMAO, AS, NLRP3 inflammasome, NF-KB signaling pathway, Endoplasmic Reticulum stress

Posted Date: August 9th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1924809/v1

Abstract

Macrophages inflammation from variety of risk factors is critical in the rupture of atherosclerotic(AS) plaques. Trimethylamine oxide (TMAO), a dietary metabolite that depends on Gut microbiota, exerts strongly pro-inflammatory effects on Atherosclerosis. Nowadays, mounting research showed that NLRP3 inflammasome activation is essential for the pathogenesis of AS. The present study was to investigate the effect of TMAO on ox-LDL-induced NLRP3 inflammasomes activation of THP-1 cells, and to explore the potential mechanism. Used Cell Counting Kit-8 assay, LDH assay to evaluate the changes of macrophage activity under TMAO and ox-LDL, respectively, to clarify the appropriate dosage. Proteins related to NLRP3 inflammasomes, NF-kb and ERS were determined via Western Blot. Inflammatory cytokine secretion was then examined via ELISA. PCR detected gene levels of inflammatory markers, and caspase-1 activity assay was utilized to detect intracellular caspase-1 activity. The results showed that TMAO could activate NLRP3 inflammasome in ox-LDL-induced THP-1 macrophages and accelerate inflammatory factor release. In addition, TMAO can further activate the expression of ERS-related proteins(including BiP□p-PERK) and NF-KB pathway. NLRP3 inhibitor MCC950, NF-kb inhibitor JSH-23, and ER stress inhibitor 4-PBA reversed TMAO's promoting effect in ox-LDL-induced macrophage. Given these data, we conclude that TMAO promotes NLRP3 inflammasomes activation via the ERS/NF-kb pathway in ox-LDL induced THP-1 macrophages. Reducing the TMAO levels may be a viable approach to prevent atherosclerosis plaque development.

Introduction

With its intricate pathogenesis and high mortality rate, Atherosclerosis, one of the leading causes of cardiovascular and cerebrovascular diseases (CCVDs), seriously endanger the health of humans[1, 2]. Plaque deposition caused by excessive intake of ox-LDL is the basic pathological feature of atherosclerotic diseases and understanding how it develops is crucial to preventing atherosclerosis and improving the health of the whole people. Of which the macrophage inflammatory response, a crucial factor in accelerating plaque formation and plaque instability, could expand the lipid core□accelerates plaque rupture and promotes cardiovascular and cerebral vascular accidents development [3].

Nod-like receptor protein 3(NLRP3)inflammasome, a multi-protein complex composed of NLRP3, apoptosis-associated speck-like protein containing a CARD(caspase-1 recruitment domain)□ASC□, and pro caspase-1, play a major role in inflammation, innate immunological responses, and cardiovascular disease (CVD) progression [4-6]. Hyperlipidemia has been regarded as an important risk factor for atherosclerotic plaque formation. Growing research show that with stimulated by excessive ox-LDL, NLRP3 is initiated through some pathways such as the NF-kb signaling pathway, then the N-terminal pyrin domain (PYD) of NLRP3 assembles with the N-terminal PYD of ASC; the C-terminal CARD of ASC is mainly responsible for raising pro caspase-1, which compose the NLRP3 inflammasome and turn on the activation process. Locally high concentrations of pro-Caspase-1 in cells undergo auto-cleavage and become caspase-1 with activity. The latter can catalyze the cleavage of interleukin (IL)-1β and IL-18 precursors to IL-1b, IL-18 mature, and achieve the process of cellular inflammation. This sterile

inflammation, mediated by the NLRP3 inflammasome and inflammatory cytokine release, permeates the entire process of atherosclerosis development, promotes the progression of atherosclerotic plaques, and raises the danger of atherosclerotic plaque rupture[7].

Recently, accumulating studies have found that the occurrence of CCVDs is commonly accompanied by the imbalance of intestinal flora and changes in the levels of metabolites [8]. TMAO, one of the most extensively researched metabolites, has been shown closely linked to the increased risk of Atherosclerosis[9]. Mechanistic investigations have demonstrated that TMAO's pro-atherosclerotic effect is partly due to changing lipid metabolism, activating platelets, and causing vascular inflammation[10].Among them, vascular inflammation induced by NLRP3 inflammasome activation, as the key mechanism of plaque, has become a hot spot in the field of the relationship between TMAO and atherosclerosis. However, most of the research mainly focused on endothelial cells (EC)and smooth muscle cells (SMC) fields, with less attention paid to macrophages. This study chiefly focused on the connection and mechanism between TMAO and NLRP3 inflammasome activation in macrophage induced by ox-LDL, which provided a novel viewpoint for illuminating the molecular pathways by which TMAO fosters the development of atherosclerotic disease.

Materials And Methods

Materials

TMAO was purchased from Sigma-Aldrich (#317594, USA), and oxidized low-density lipoprotein (ox-LDL) was purchased from Yi yuan biotechnology (YB-002, Guangzhou, China). Phorbol-12-myristate-13-acetate (PMA) was bought from Sigma (USA). Primary antibodies: pro-caspase-1(1:1000, ab207802) IL-18 1:500, ab207324NF-kb(1:5000, ab76311)pro-IL-1b(1:1000,ab216995)β-actin(ab8226,1:10000) and GAPDH(1:5000, ab181602) were bought from Abcam(USA) ; NLRP3(1:700, D4D8T) was purchased from CST(USA); ASC(1:1000, WL02462)IL-1b mature(1:500, WL00891)caspase-1p20(1:1000, WL02996a)p-NF-KB(1:1000, WL02169)GRP78/Bip(1:1000, WL03157)p-PERK(1:1000, WL05295) were purchased from Wan lei Bio (Shenyang, China). While secondary antibodies: Goat Anti-Rabbit IgG HRP conjugate 1:10000, S0001, AffinityJiangsu)Goat Anti-Mouse IgG HRP conjugate (1:10000, Abcam ab205719,USA). NLRP3 inhibitor MCC950HY-12815A,25uM,4hand NF-KB inhibitor JSH-23HY-13982,30uM, 1hwere purchased from MCE (Shanghai, China), ER stress inhibitor 4-PBA (SML0309, 5mM30min) was obtained from Sigma (USA).

Cell culture and treat

THP-1 cells were grown in RPMI1640 with 10% fetal bovine serum (FBS) and then incubated at 37°C in a CO2 incubator. Cells with suspension state in the logarithmic growth phase were transferred to 6-well or 96-well plates and then were induced to differentiate into adherent M0 macrophages by stimulation with 100 ng/mL PMA for 48 h. Cells were separated into several groups at random, including the TMAO-induced group, ox-LDL-induced group, ox-LDL added TMAO group, MCC950 group, JSH-23 group, and 4-

PBA group. The TMAO-induced group and the ox-LDL-induced group were used to determine the dosage of TMAO and ox-LDL.

Cell viability assay

THP-1 cells in the logarithmic growth phase were sown in 96-well plates, and 100μl cell suspension with 2% serum and PMA was added to per well.10μL CCK-8(C0005, Target MOI) solution was subsequently added per well in the dark and measured the absorbance at 450nm wavelength after being cultured for 1-2 h.

Detection of LDH release

After the respective treatment of cells, the supernatant was collected, the appropriate LDH reagent was applied to each well in accordance with the manufacturer's instructions (A020-2-2, Jiancheng Biology, Nanjing), and absorbance was measured at 450 nm wavelength.

Western blot (WB)analysis

Cells were lysed using RIPA lysis buffer (Beyotime, P0013B, China) containing protease inhibitor PMSF (Solarbio, P0100, China) and phosphatase inhibitor (BL615A, Bio sharp, China) after treatment, BCA protein detection kit (Solarbio, China) was used to measure the protein concentration per samples after collecting. Polyvinylidene fluoride (PVDF) membranes were used to transfer the proteins after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separated them (ISEQ00010, Merck). After being blocked with 5% nonfat dry milk, the PVDF membranes was incubated in the appropriate primary antibody solution at 4° for an overnight period, rinsed with PBST, and then incubated with the corresponding secondary antibody for an hour at room temperature. After adding the ECL luminescent agent（P10100,NCM）, exposure and development were conducted, and the quantitative analysis of gray value was performed by using ImageJ software.

Enzyme-linked immunosorbent assay （ELISA）

The levels of IL-1β and IL-18 were measured according to the manufacturer's instructions of ELISA kit (JLC6380, JLC6382, Jingkang Biological, Shanghai) after being handled appropriately and harvested supernatants.

Caspase-1 activity detection

Caspase-1 activity assay kit (C1102，Beyotime, Shanghai) was used to measure the activity levels of caspase-1 This assay was based on the ability of caspase-1 to catalytic acetyl-Tyr-Val-Ala-Asp p-nitroaniline (Ac-YVAD-pNA) to the yellow formazan product p-nitroaniline (pNA) .A standard curve for pNA was drawn by measuring the OD values in 405nm. The production of pNA in the test sample indicates the activation level of caspase 1.

Reverse transcription-quantitative PCR (qRT-PCR)

Use of the Trizol reagent extracted total RNA from the THP-1 cells. The reverse transcription kit (RR036A,Takara, Harbin, China) was used to synthesize cDNA, and the target gene was amplified by the SYBER Green method（RR820A,Takara, Harbin, China）.NLRP3-related primers came from the remaining in our research group, Bip and PERK primers sequences were designed from Rui Biotech (Beijing, China). The relative expression of the target gene was shown as CT value, the targeting gene's mRNA level was standardized to GAPDH, and calculated by the formula x=2-ΔΔCT (Table 1).

Table 1: primer sequences of gene

Target gene	primer sequences	
GAPDH	F	TCGGAGTCAACGGATTTGGT
	R	TCGGAGTCAACGGATTTGGT
IL-18	F	ACTGGTTCAGCAGCCATCTT
	R	TGCAGTCTACACAGCTTCGG
IL-1b	F	CCTGCAGCTGGAGAGTGTG
	R	TGTGCTCTGCTTGTGAGGTGC
NLRP3	F	CTTCCTTTCCAGTTTGCTGC
	R	TCTCGCAGTCCACTTCCTTT
Caspase-1	F	GCCCAAGTTTGAAGGACAAA
	R	GGTGTGGAAGAGCAGAAAGC
GRP78/Bip	F	GAACGTCTGATTGGCGATGC
	R	TCTTTGGTTGCTTGGCGTTG
PERK	F	CCAGTTTTGTACTCCAATTGCA
	R	CAGATACAGCTGGCCTCTATAC

Statistical analysis

GraphPad 9.0 software was used for statistical analysis, and all data were reported as mean ± SD. A t-test or one-way analysis was utilized to analysis differences, which were deemed statistically significant when $p < 0.05$.

Results

1 Effects of TMAO on cell survival and NLRP3 inflammasome expression inTHP-1 cells

To investigate the cytotoxicity of TMAO towards THP-1cells, we first measured the cell viability after being exposed to TMAO at different concentrations (0/50/100/200/400/800uM TMAO) and durations (400uM for 0h/4h/8h/12h/24h/48h). CCK-8 results showed that the concentration and duration of TMAO had no discernible impact on the cell survival (figure1 A, B). We further explored the effect of TMAO on macrophage inflammation, results of Western Blot showed that pro IL-1b, IL-18, ASC, and NLRP3 protein expression was increased by TMAO in THP-1 macrophages in a dose- and time-dependent manner, while pro-caspase-1 expression was decreased—most significantly at 400uM TMAO over 24 hours (figure1C, D).We concluded that TMAO had no significant effect on macrophage activity, possibly affected cellular function by affecting cellular inflammation .Combined with CCK-8, we determine 400uMTMAO treatment for 24h as the subsequent experimental condition.

2 TMAO accelerates IL-1b、IL-18 secretion by activating the NLRP3 inflammasome in ox-LDL-induced THP-1 cells.

Inflammation induced by excessive phagocytosis of ox-LDL in macrophages is critical for atherosclerotic plaques development. To explore the relationship between TMAO and ox-LDL induced inflammation in THP-1 cells, we firstly treated THP-1 macrophages with different concentrations of ox-LDL to determine the concentration of ox-LDL in following experiment. LDH results showed that ox-LDL promoted the release of LDH in THP-1 cells, decreased cell viability in a concentration-dependent manner (Fig2A).Western Blot also showed that gradually increased expression of NLRP3 indicators and IL-18、IL-1b(Fig2B), which was consistent with the earlier results[11]. Considering the strong cytotoxicity of 75-100ug/ml ox-LDL to macrophages, we decided to use 50ug/ml ox-LDL in the following studies. Subsequently, we examined the inflammation and viability effect of TMAO on THP-1 cells under 50ug/ml ox-LDL. After treating with 50ug/ml ox-LDL for 24h, added TMAO to continue treatment for 24h, according to the results of CCK-8, none concentration of TMAO had significant effect on the survival of ox-LDL-induced macrophages(Fig2C), while the results of WB and PCR indicated that TMAO can significantly promote the expression and mRNA levels of IL-1b、IL-18 in the ox-LDL-induced THP1 cells(Fig2 D, E). Additionally, ELISA results showed that TMAO also promoted the IL-1b、IL-18 secretion (Fig.2F).

To verify whether the increased secretion of IL-1β and IL-18 under TMAO stimulation attribute to activation of the NLRP3 inflammasome, we further examined the effect of TMAO on NLRP3 inflammasome activation under ox-LDL. Results of Western Blot showed that TMAO can promote the protein expression of NLRP3, ASC, and caspase-1 p20 in ox-LDL-induced THP-1 cells and reduce the protein expression of procaspase-1 (figure2G). The PCR results were consistent with it (figure2H). Similarly, the caspase-1 activity assay demonstrated that TMAO can promote the enzymatic activity of caspase-1 in ox-LDL-induced THP-1 cells（figure2I）, which revealed the possible role of NLRP3 inflammasome activation in TMAO pro- inflammation. To further clear the role of NLRP3 inflammasome in the secretion of IL-1b and IL-18 induced by TMAO, We pretreated with the NLRP3 inhibitor MCC950 after 24 hours of treatment with 50ug/ml ox-LDL, followed by TMAO treatment and found that MCC950 significantly reversed the synergistic promotion of TMAO on ox-LDL-induced expression of NLRP3, ASC,

and caspase-1 p20, which means the MCC950 is effective, subsequently, in ox-LDL-induced macrophages ,the proIL-1b, IL-18 expression upregulated by TMAO was also inhibited by MCC950(figure2J). ELISA and PCR results indicated that MCC950 could decrease the mRNA level and release of IL-1b and IL-18 (figure2K, L), which all suggest that TMAO can promote the activation of NLRP3 inflammasome in ox-LDL-induced macrophages, and then accelerate the release of IL-1b and IL-18.

3 TMAO promotes ox-LDL-induced NLRP3 inflammasome initiation and activation via the NF-KB pathway in THP-1 cells

Next, we aimed to explore the molecular mechanism by which TMAO regulated NLRP3 inflammasome activation during ox-LDL-induced THP-1 macrophage. WB showed that phosphor-NF-κB p65 protein levels and the ratio of p-NF-KB/T-NF-KB were increased in ox-LDL-induced THP-1 in response to TMAO （figure3A）, which indicates that NF-κB pathway maybe one of the mechanism by which TMAO activates the NLRP3 inflammasome in ox-LDL-induced macrophages.

To further verify the above speculation, the NF-κB p65 nuclear translocation inhibitor, JSH-23, was added before TMAO. Our results indicated that JSH-23 treatment prevented TMAO-induced increases in NF-κB p65 phosphorylation in ox-LDL-treated THP-1macrophage, moreover, increased NLRP3、ASC and IL-1β、IL-18 production was also subsequently reversed by JSH-23 (figure 3B).In addition, increased IL-1β, IL-18, NLRP3, and caspase-1 mRNA levels induced by TMAO were prevented by JSH-23 treatment in ox-LDL-treated macrophage, and ELISA results were consistent with it. (Figure3 C, D). Caspase-1 activity assay also indicated that JSH-23 significantly reduced the activity of caspase-1 I in foam cells induced by TMAO. (Figure3 E). All these results demonstrate that TMAO promoted the initiation of NLRP3 via the NF-κB pathway in ox-LDL-induced macrophages and accelerated the activation of the NLRP3 inflammasomes.

4 TMAO promotes ox-LDL-induced NLRP3 inflammasome activation by ERS/NF-kb pathway in THP-1cells

We next investigated whether ERS regulated the activation of NF-KB and NLRP3 inflammasomes promoted by TMAO in ox-LDL-treated macrophage cells. Western blot results showed that TMAO further promoted the upregulation of Bip and p-PERK expression mediated by ox-LDL treatment in THP-1 cells (Figure 4A). Furthermore, we found that treatment of ox-LDL-induced macrophages with an ERS inhibitor (4-PBA) prior to TMAO significantly suppressed p-PERK and Bip expression compared with ox-LDL mixed with TMAO treatment. We also verified that the increases in p-NF-KB, NLRP3, caspase-1 p20, IL-1β, and IL-18 expression mediated by TMAO in ox-LDL-induced macrophages were also reversed by 4-PBA(Figure 4B). PCR results showed that compared with the TMAO added ox-LDL group, 4-PBA group exerted the decreasing mRNA levels of PERK, Bip/GRP78、NLRP3, IL-1b,and IL-18. (Figure 4C). In addition, evidence from ELISA and caspase-1 activity assay also showed that inhibition by 4-PBA decreases the activity of Caspase-1 and the release of IL-18, and IL-1b (Figure4D, E), which all indicate that TMAO promoted the release of IL-18 and IL-1b in ox-LDL-induced THP-1 cells by activating the ER stress/NF-KB/NLRP3 pathway.

Discussion

Data obtained in this study demonstrated that although there was no effect on macrophage viability, TMAO remain activated the macrophage NLRP3 inflammasome in a dose-dependent manner, which mainly provided a dose reference for the study of TMAO in the foam cell model. Moreover, ox-LDL dose-dependently triggered NLRP3 inflammasome activation, and secretion of IL-18、IL-1β in macrophages, and we regard 50 μg/mL ox-LDL for 48h as the optimum concentration. On this basis, we clarified the effect of TMAO on ox-LDL-induced activation of the NLRP3 inflammasome in macrophages. Notably, this study identified a pro-inflammatory process of TMAO associated with ERS and the NF-kb pathways. Therefore, these results may support a novel role of TMAO in ox-LDL-induced NLRP3 inflammasome activation in macrophages by activating the ERS/NF-kb axis, which provides a new perspective for elucidating the mechanism by which TMAO promotes atherosclerosis.

It has been established that the risk factors for enlarging the necrotic core and quickening the process of plaque rupture are the activation of the macrophage inflammasome and the cell death that follows it in vulnerable plaques during the development of atheromatous plaques[3]. NLRP3 inflammasome, the most widely studied inflammasome, has been identified as a multi-protein complex composed of NLRP3, ASC, and procaspase 1. NLRP3 binds and activates pro caspase into active caspase-1 through ASC when cells are activated, which expedites the release of IL-18 and Il-1β[12]. Accumulating studies have reported the relationship between excessive ox-LDL and the activation of NLRP3 inflammasome in macrophages, those shown that ox-LDL activates the NLRP3 inflammasome by enhancing oxidative stress and other pathways, and then activates caspase-1, on the one hand, which cleaves the formation of GSDMD into pyroptotic marker GSDMD-N triggers pyroptosis, and on the other hand, induces pro-IL-18 and Pro-IL-1β to become IL-18 and IL-1β mature bodies, leading to the inflammatory response [13, 14]. Similarly, our study also proved that 50ug/ml ox-LDL treatment for 48h can promote the expression of NLRP3, ASC, IL-18, and IL-1β and reduce cell viability in THP-1 macrophages. Zeng et al demonstrated that treatment with the NLRP3 inhibitor MCC950 significantly reduced plaque burden and intraplaque macrophage content, which was associated with reduced IL-1β and IL-18 lysates production and pyroptosis in macrophage as well as aortic researched by vitro experiments[15]. This undoubtedly affirms the critical role of macrophage NLRP3 inflammasome activation in the progression of atherosclerosis.

Although part of studies has identified elevated TMAO levels in various inflammatory diseases including diabetes and chronic kidney disease, studies still mainly focus on exploring the relationship between TMAO and atherosclerotic disease currently[9, 16]. Many previous studies have found that most patients with atherosclerotic disease are often accompanied by elevated plasma TMAO levels [17]. Some studies have also affirmed the role of prebiotics, probiotics, and other substances in promoting the growth of beneficial gut microbiota, reducing TMAO levels and cardiovascular disease risk [18]. In addition, some evidence has demonstrated that resveratrol [19]、DMB[20] reduces the level of TMAO commonly accompanied by obvious anti-inflammatory and interventional effects on the progression of atherosclerosis. Recently, part of the research has shown that high levels of TMAO can activate NLRP3 inflammasome by ROS-TXNIP, NF-kb, and MAPK in endothelial cells (EC) and smooth muscle cells (SMC)

to participate in vascular inflammation and vascular calcification and accelerate plaque formation and rupture. [21, 22], which not only confirms the close link between TMAO and atherosclerosis but also emphasizes the relationship between this process and the NLRP3 inflammasome activation in cells. However, at present, most studies mainly focus on EC and SMC fields. This study highlighted the relationship between the NLRP3 inflammasome activated by TMAO in macrophages and atherosclerotic plaque progression, which not only activated the NLRP3 inflammasome in macrophages in a time- and concentration-dependent manner, TMAO but also promoted the role of ox-LDL synergistically. In the foam cell, TMAO treatment can promote the protein expression and gene expression of NLRP3 indicator in ox-LDL-induced macrophages, increase the activity of caspase-1, and release inflammation factors. In summary, data was gathered to demonstrate that not only in macrophages but more importantly in a foam cell model, TMAO had a synergistic effect on ox-LDL in activating the NLRP3 inflammasome, which filled the gap between TMAO and foam cell NLRP3 inflammation and enriched the network of mechanisms by which TMAO promoted vascular inflammation and accelerated atherosclerotic plaque formation.

Studies has reported that NLRP3 can be activated through multiple pathways including NF-kb[23]、ERS[24]、ROS[25]and MAPK[26]. Among them, NF-KB has been widely recognized as the canonical pathway of NLRP3 activation. Consistent with most studies, our experiment found that JSH-23, NF-KB pathway inhibitor, inhibited the phosphorylation of NF-kb and following reverse the effect of TMAO on activating NLRP3 inflammasome, which demonstrated the regulatory role of NF-kb in this process. Notably, this study was the first to identify the role of ER stress in the above process. While ERS was an adaptive mechanism, sustained or excessive ERS induced inflammation, apoptosis, and tissue damage. Studies had shown that ERS a common mechanism by which various risk factors participated in the development of atherosclerosis, and the use of the inhibitor 4-PBA ER stress process and then reversed atherosclerotic vascular calcification and foam cell formation aggravated by ox-LDL and Hcy[27], which had also already been confirmed by vivo experiments in ApoE-/- Mice [28]. Recently ,a study demonstrated that TMAO can directly bind and activate PERK in hepatocytes to induce endoplasmic reticulum stress and lead to metabolic disorders, Which making it clear that PERK can act as a TMAO binding target and involve in the metabolic disorders [29] .In addition, a study by feeding hyper choline mice with TMA inhibitor DMB found that DMB treatment can significantly reverse TMAO-induced inflammasome expression thereby reducing vascular remodeling, and treating atherosclerosis, mechanistic studies suggest that this may be related to its regulation of endoplasmic reticulum stress in smooth muscle cells[20]. Based on this, we speculated whether the pro-inflammatory effect of TMAO in ox-LDL-induced macrophages also involves the ERS pathway. In the following experiments, we did observe that TMAO promoted the expression of GRP78, p-PERK induced by ox-LDL in macrophages, adding 4-PBA reversed TMAO-induced phosphorylation of NF-kb and subsequent activation of NLRP3 in foam cells, suggesting that ERS and NF-kb signaling pathway are linked in macrophage NLRP3 inflammasome activation.

In conclusion, as shown in Fig. 5, TMAO promoted ox-LDL-induced activation of the NLRP3 inflammasome and release of IL-1β and IL-18 in THP-1 macrophages via ER stress and the NF-kb

pathway. It is undeniable that our conclusions will be completer and more reliable if this study can fill the gap about lacking vivo study, which is what we seek in our future studies. Our findings suggest that the ERS/NF-kb axis plays an important role in the NLRP3 inflammasome activation and IL-18 IL-1β release in ox-LDL-treated macrophages promoted by TMAO, which may be useful for targeting gut microbiota points to a new direction for the clinical prevent of atherosclerosis.

Declarations

Funding:

This research was supported by the National Natural Science Foundation of China (No. 81672084)

References

1. Spence J. D. Diet for stroke prevention. Stroke and vascular neurology,2018,3,44-50.
2. Torres N, Guevara-Cruz, M, Velázquez-Villegas, L. A., Tovar, A. R. Nutrition and Atherosclerosis. Arch Med Res. 2015,46,408-26
3. Zhu, Y., Xian, X., Wang, Z., Bi, Y., Chen, Q., Han, X., Tang, D., & Chen, R. Research Progress on the Relationship between Atherosclerosis, and Inflammation. Biomolecules, 2018 ,8, 80..
4. Pavillard, L. E., Marín-Aguilar, F., Bullon, P., Cordero, M. D. Cardiovascular diseases, NLRP3 inflammasome, and western dietary patterns. Pharmacological research, 2018, 131, 44–50.
5. Zhang, Y., Yang, W., Li, W., & Zhao, Y. NLRP3 Inflammasome: Checkpoint Connecting Innate and Adaptive Immunity in Autoimmune Diseases. Frontiers in immunology, 2021,12, 732933
6. Zeng, Q. Z., Yang, F., Li, C. G., Xu, L. H., He, X. H., Mai, F. Y., Zeng, C. Y., Zhang, C. C., Zha, Q. B., & Ouyang, D. Y. Paclitaxel Enhances the Innate Immunity by Promoting NLRP3 Inflammasome Activation in Macrophages. Frontiers in immunology, 2019, 10, 72.
7. Yang, Y., Wang, H., Kouadir, M., Song, H., & Shi, F. Recent advances in the mechanisms of NLRP3 inflammasome activation and its inhibitors. Cell death & disease, 2019,10(2), 128.
8. Jin, M., Qian, Z., Yin, J., Xu, W., & Zhou, X. The role of intestinal microbiota in cardiovascular disease. Journal of cellular and molecular medicine,2019.23, 2343–2350.
9. Croyal, M., Saulnier, P. J., Aguesse, A., Gand, E., Ragot, S., Roussel, R., Halimi, J. M., Ducrocq, et al,Plasma Trimethylamine N-Oxide and Risk of Cardiovascular Events in Patients With Type 2 Diabetes. The Journal of clinical endocrinology and metabolism, 2020.105, dgaa188
10. Zhu, Y., Li, Q., Jiang, H. Gut microbiota in atherosclerosis: focus on trimethylamine N-oxide. APMIS : acta pathologica, microbiologica, et immunologica Scandinavica, 2020.128(5), 353–366.
11. Liu, J., Wang, C., Li, J., Yu, Y., Liu, Y., Liu, H., Peng, Q.et al. Autophagy blockage promotes the pyroptosis of ox-LDL-treated macrophages by modulating the p62/Nrf2/ARE axis. Journal of physiology and biochemistry, 2021,77, 419–429.

12. Wang, R., Wang, Y., Mu, N., Lou, X., Li, W., Chen, Y., Fan, D,et al. Activation of NLRP3 inflammasomes contributes to hyperhomocysteinemia-aggravated inflammation and atherosclerosis in apoE-deficient mice. Laboratory investigation; a journal of technical methods and pathology, 2017,97, 922–934.
13. Jiang, Y., Wang, M., Huang, K., Zhang, Z., Shao, N., Zhang, Y., Wang, W., & Wang, S. Oxidized low-density lipoprotein induces secretion of interleukin-1β by macrophages via reactive oxygen species-dependent NLRP3 inflammasome activation. Biochemical and biophysical research communications, 2012,425, 121–126..
14. . Li, W. L., Hua, L. G., Qu, P., Yan, W. H., Ming, C., Jun, Y. D., Yuan, L. D., & Nan, N. NLRP3 inflammasome: a novel link between lipoproteins and atherosclerosis. Archives of medical science : AMS, 2016, 12, 950–958
15. Zeng, W., Wu, D., Sun, Y., Suo, Y., Yu, Q., Zeng, M., Gao, Q., Yu, B., Jiang, X., & Wang, Y.. The selective NLRP3 inhibitor MCC950 hinders atherosclerosis development by attenuating inflammation and pyroptosis in macrophages. Scientific reports, 2021.11 19305.
16. Lai, Y., Tang, H., Zhang, X., Zhou, Z., Zhou, M., Hu, Z., Zhu, F., Zhang, L., & Nie, J.. Trimethylamine-N-Oxide Aggravates Kidney Injury via Activation of p38/MAPK Signaling and Upregulation of HuR. Kidney, blood pressure research, 2022, 47, 61–71.
17. Tang, W. H., Wang, Z., Levison, B. S., Koeth, R. A., Britt, E. B., Fu, X., Wu, Y., & Hazen, S. L. Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. The New England journal of medicine, 2013. 368, 1575–1584.
18. O'Morain, V. L., Chan, Y. H., Williams, J. O., Alotibi, R., Alahmadi, A., Rodrigues, N. P., Plummer, S. F., Hughes, T. R., Michael, D. R., & Ramji, D. P. The Lab4P Consortium of Probiotics Attenuates Atherosclerosis in LDL Receptor Deficient Mice Fed a High Fat Diet and Causes Plaque Stabilization by Inhibiting Inflammation and Several Pro-Atherogenic Processes. Molecular nutrition & food research, 2021, 65, e2100214.
19. Chen, M. L., Yi, L., Zhang, Y., Zhou, X., Ran, L., Yang, J., Zhu, J. D., Zhang, Q. Y., & Mi, M. T. Resveratrol Attenuates Trimethylamine-N-Oxide (TMAO)-Induced Atherosclerosis by Regulating TMAO Synthesis and Bile Acid Metabolism via Remodeling of the Gut Microbiota. mBio, 2016. 7, e02210–e2215.
20. Chen, C. Y., Leu, H. B., Wang, S. C., Tsai, S. H., Chou, R. H., Lu, Y. W., Tsai, Y. L., Kuo, C. S., Huang, P. H., Chen, J. W., & Lin, S. J. Inhibition of Trimethylamine N-oxide attenuates neointimal formation through reduction of inflammasome and oxidative stress in a mouse model of carotid artery ligation. Antioxidants redox signaling, 2022,10.1089
21. Zhang, X., Li, Y., Yang, P., Liu, X., Lu, L., Chen, Y., Zhong, X., Li, Z., Liu, H., Ou, C., Yan, J., & Chen, M. Trimethylamine-N-Oxide Promotes Vascular Calcification Through Activation of NLRP3 (Nucleotide-Binding Domain, Leucine-Rich-Containing Family, Pyrin Domain-Containing-3) Inflammasome and NF-κB (Nuclear Factor κB) Signals. Arteriosclerosis, thrombosis, and vascular biology, 2020,40, 751–765.

22. Sun, X., Jiao, X., Ma, Y., Liu, Y., Zhang, L., He, Y., & Chen, Y. Trimethylamine N-oxide induces inflammation and endothelial dysfunction in human umbilical vein endothelial cells via activating ROS-TXNIP-NLRP3 inflammasome. Biochemical and biophysical research communications, 2016 481, 63–70
23. Xu, S., Chen, H., Ni, H., & Dai, Q. Targeting HDAC6 attenuates nicotine-induced macrophage pyroptosis via NF-κB/NLRP3 pathway. Atherosclerosis, 2021 317, 1–9.
24. Ji, C., Yi, H., Huang, J., Zhang, W., & Zheng, M. Propofol alleviates inflammation and apoptosis in HCY-induced HUVECs by inhibiting endoplasmic reticulum stress. Molecular medicine reports, 2021 23, 333.
25. Akhter, N., Wilson, A., Thomas, R., Al-Rashed, F., Kochumon, S., Al-Roub, A., Arefanian, H., Al-Madhoun, A., Al-Mulla, F., Ahmad, R., & Sindhu, S. ROS/TNF-α Crosstalk Triggers the Expression of IL-8 and MCP-1 in Human Monocytic THP-1 Cells via the NF-κB and ERK1/2 Mediated Signaling. International journal of molecular sciences, 2021,22, 10519.
26. Yu, G., Yu, H., Yang, Q., Wang, J., Fan, H., Liu, G., Wang, L., Bello, B. K., Zhao, P., Zhang, H., & Dong, J. Vibrio harveyi infections induce production of proinflammatory cytokines in murine peritoneal macrophages via activation of p38 MAPK and NF-κB pathways but reversed by PI3K/AKT pathways. Developmental and comparative immunology, 2022 127, 104292..
27. Wu, H., Chen, Z., Chen, J. Z., Pei, L. G., Xie, J., Wei, Z. H., Kang, L. N., Wang, L., & Xu, B. High Mobility Group B-1 (HMGB-1) Promotes Apoptosis of Macrophage-Derived Foam Cells by Inducing Endoplasmic Reticulum Stress. Cellular physiology and biochemistry: international journal of experimental cellular physiology, biochemistry, and pharmacology, 2018,48, 1019–1029.
28. Lynn, E. G., Lhoták, Š., Lebeau, P., Byun, J. H., Chen, J., Platko, K., Shi, C., O'Brien, E. R., & Austin, R. C. 4-Phenylbutyrate protects against atherosclerotic lesion growth by increasing the expression of HSP25 in macrophages and in the circulation of Apoe-/- mice. FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 2019,33, 8406–8422
29. Chen, S., Henderson, A., Petriello, M. C., et al. Trimethylamine N-Oxide Binds and Activates PERK to Promote Metabolic Dysfunction. *Cell metabolism*, *30*(6), 1141–1151.e5.

Figures

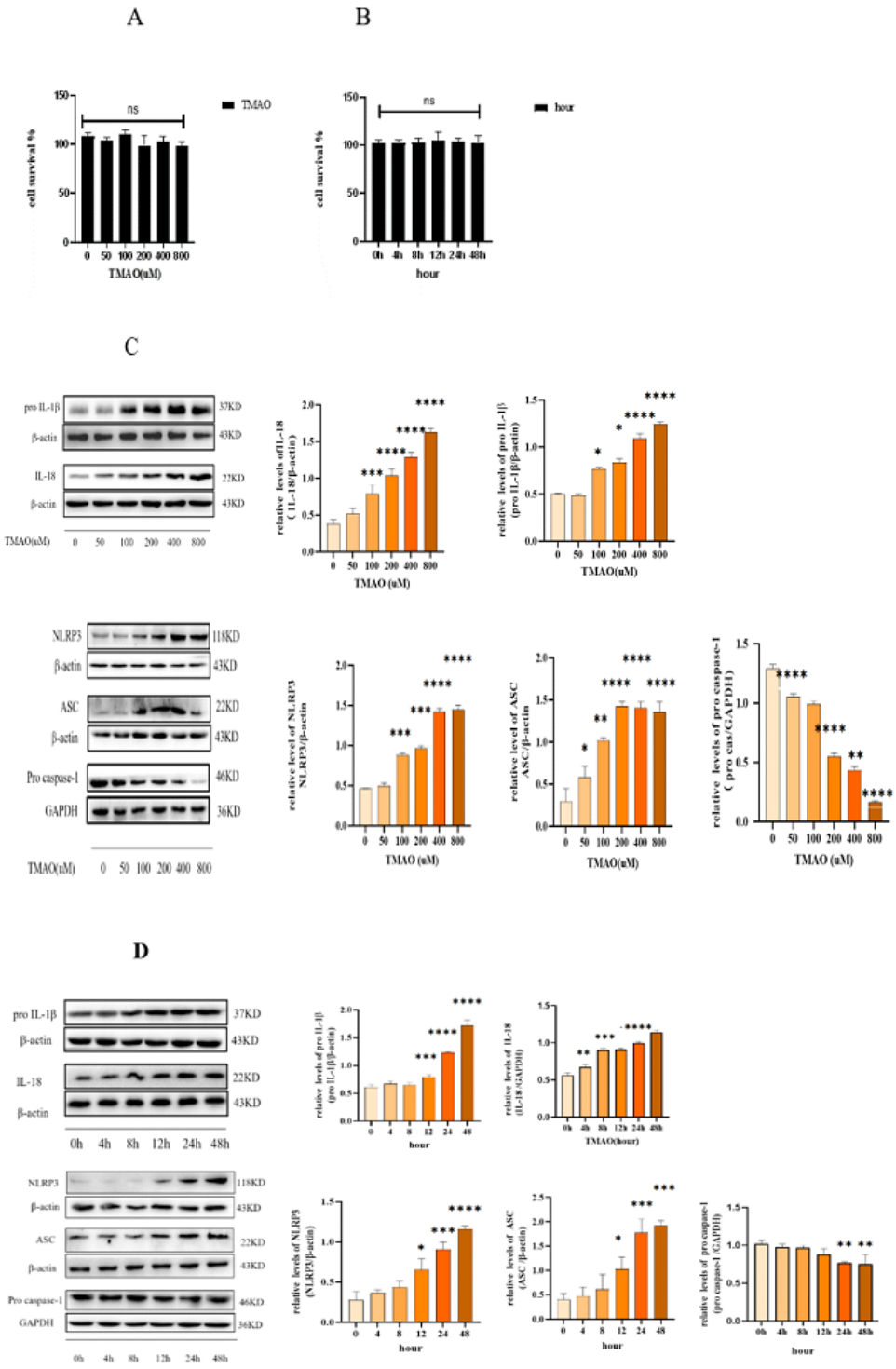

Figure 1

Effects of TMAO on cell survival and NLRP3 inflammasome expression in THP-1 cells. Cells were respectively treated with different concentration and duration of TMAO. (A, B) Cell viability was measured by using CCK-8 detection kit ; (C, D)Results of Western blot about the level of NLRP3 inflammasome related indicators treated with different TMAO dosages. Values are shown as mean ± SD.n=3. Statistical

significance compared to unstimulated groups is denoted by asterisks (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001)

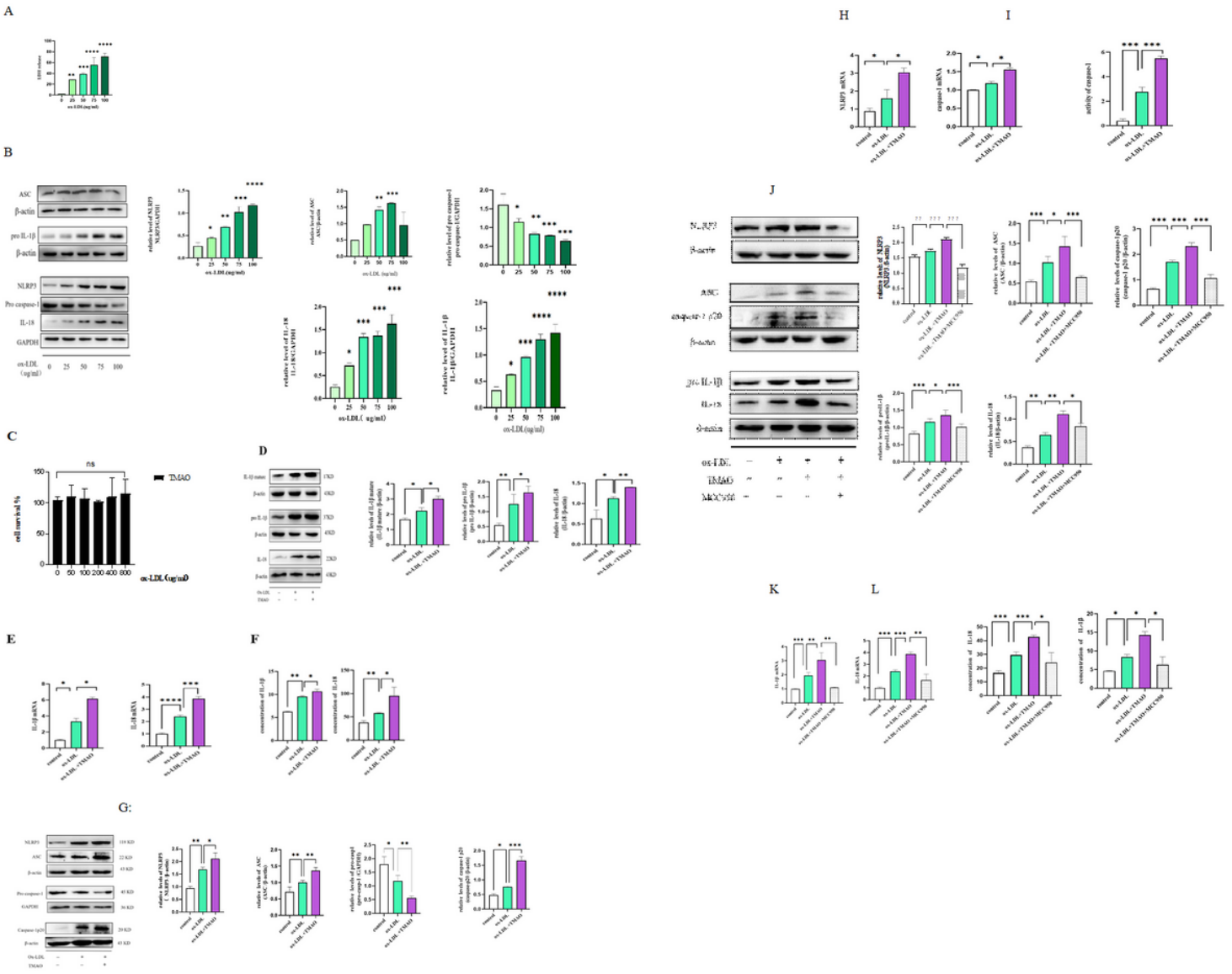

Figure 2

TMAO accelerates the release of IL-1β、IL-18 by activating the NLRP3 inflammasome in ox-LDL induced THP-1 cell. (A)Results of LDH release under different ox-LDL concentrations was performed by LDH detection kit ; (B) Western blot was performed to determine the level of NLRP3, ASC, pro-caspase-1, pro-IL-1b, and IL-18 induced by ox-LDL; (C) The effect of TMAO on the viability of 50 ug/ml ox-LDL cells detected by CCK-8 ;(D) The expressions of IL-1β、IL-18 in ox-LDL-induced macrophages by TMAO were detected by WB, TMAO:400uM treat 24h.ox-LDL:50ug/ml treat 48h; (E, F) ELISA and PCR were used to determine the secretion and mRNA level of IL-1β and IL-18; (G) Western blot results of NLRP3, ASC, caspase-1 in response to TMAO ;(H) PCR results of NLRP3 and caspase-1 mRNA level; (I) caspase-1 activity assay in response to TMAO in ox-LDL-induced THP-1cells. (J) Results of Western blot about IL-18,IL-1b and NLRP3 expression under MCC950. (K) PCR results of IL-18 and IL-1β in response to MCC950;

(L) ELISA results treated with MCC950. Values are presented as mean ± SD. Asterisks signify statistical significance (*:p < 0.05, **:p < 0.01, ***:p < 0.001, ****:p < 0.0001.)

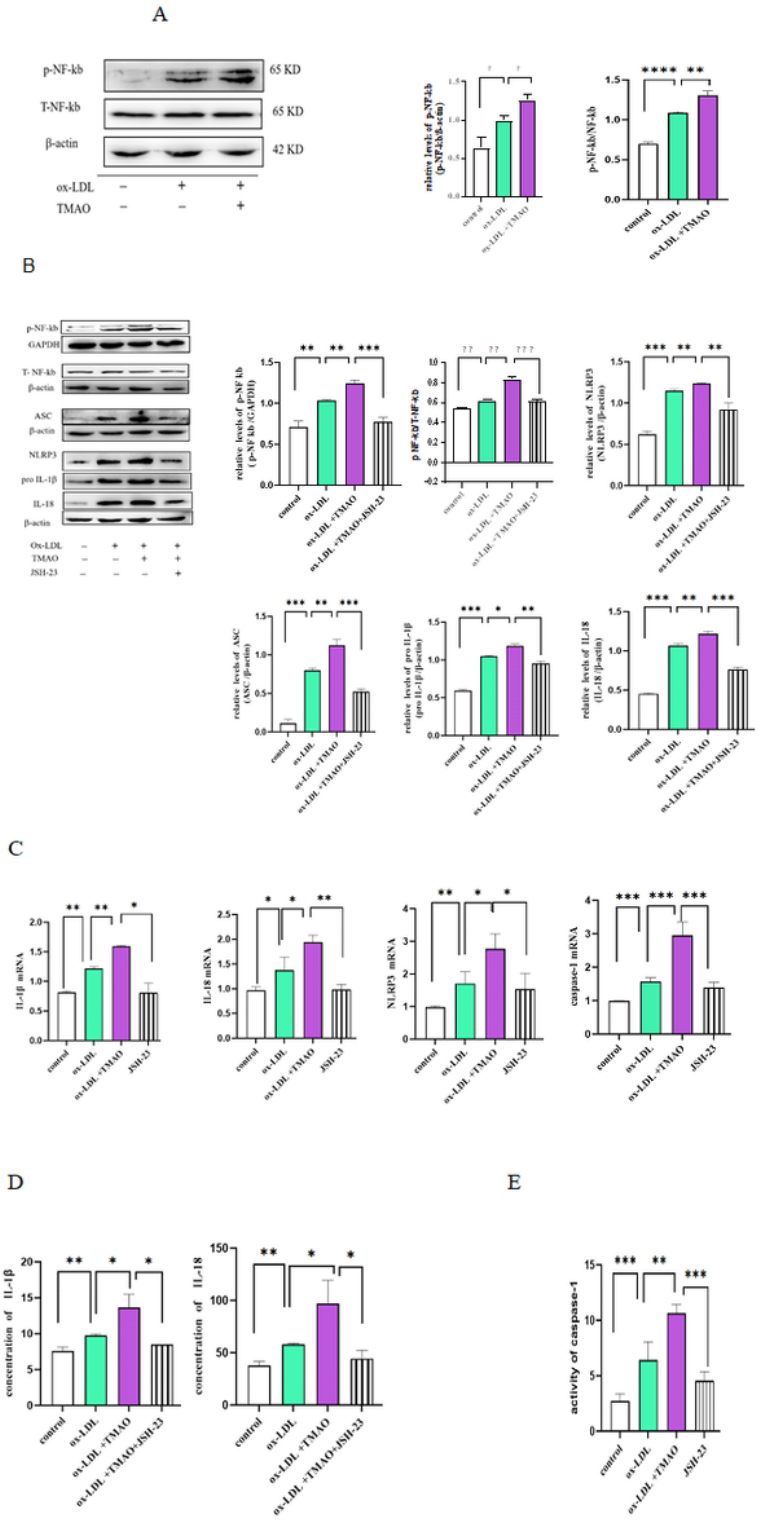

Figure 3

TMAO regulated the initiation and activation of NLRP3 via NF-κB pathway in ox-LDL-induced macrophage (A) P-NF-kb, T-NF-kb levels were measured by western blot; (B) WB result of NF-kb ,NLRP3

,ASC,caspase-1 p20 ,IL-1b,IL-18 treated with JSH-23(30uM,1h).(C) mRNA level of IL-1b,IL-18,NLRP3,and caspase-1 in response to JSH-23 were detected by qRT-qPCR. (D) The secretion of IL-1b and IL-18 was measured by ELISA. (E) caspase-1activity assay was utilized to calculate the caspase activity intracellular under JSH-23. Data are presented as mean ± SD. Asterisks denote statistical significance. N = 3 in each group. (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.)

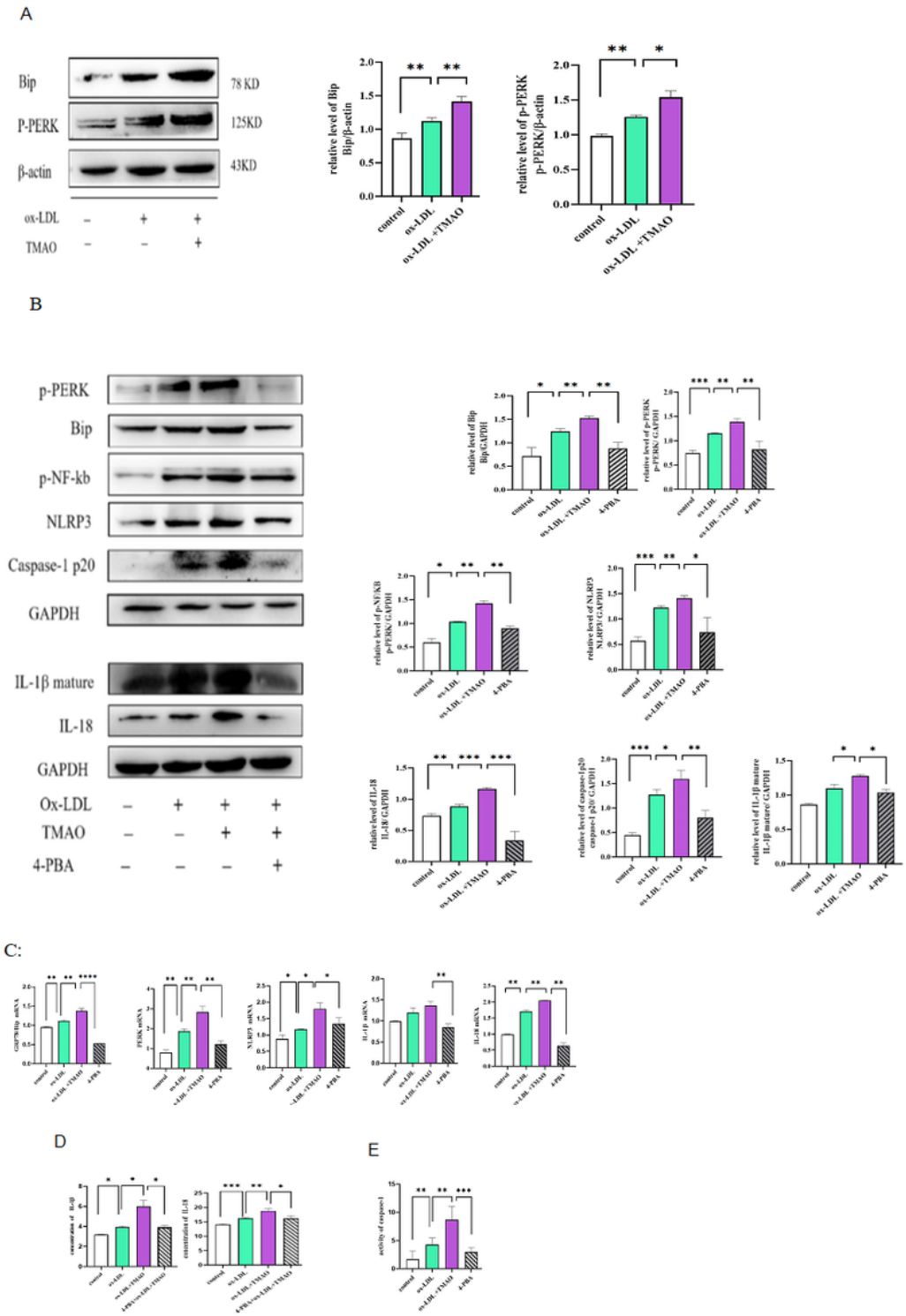

Figure 4

TMAO promotes ox-LDL-induced NLRP3 inflammasome activation by ERS/NF-kb pathway in THP-1 cells. Use of ER stress inhibitor 4-PBA inhibits ERS pathway and NF-kb phosphorylation and NLRP3 inflammasome activation. (A) Bip, p-PERK expression and analysis data were performed by WB and ImageJ;(B) IL-1β,IL-18, NLRP3, casp1 P20, p-NF-KB, Bip and p-PERK levels were detected by Western blotting;(C) IL-1β, IL-18, NLRP3, Bip, and PERK levels were detected by qRT-PCR;(D) ELISA detect the concentration of IL-18 and IL-1β in supernatant (E)Result of caspase-1 activity in response to 4-pBA. Values are expressed as the mean ± SD (n = 3). (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001)

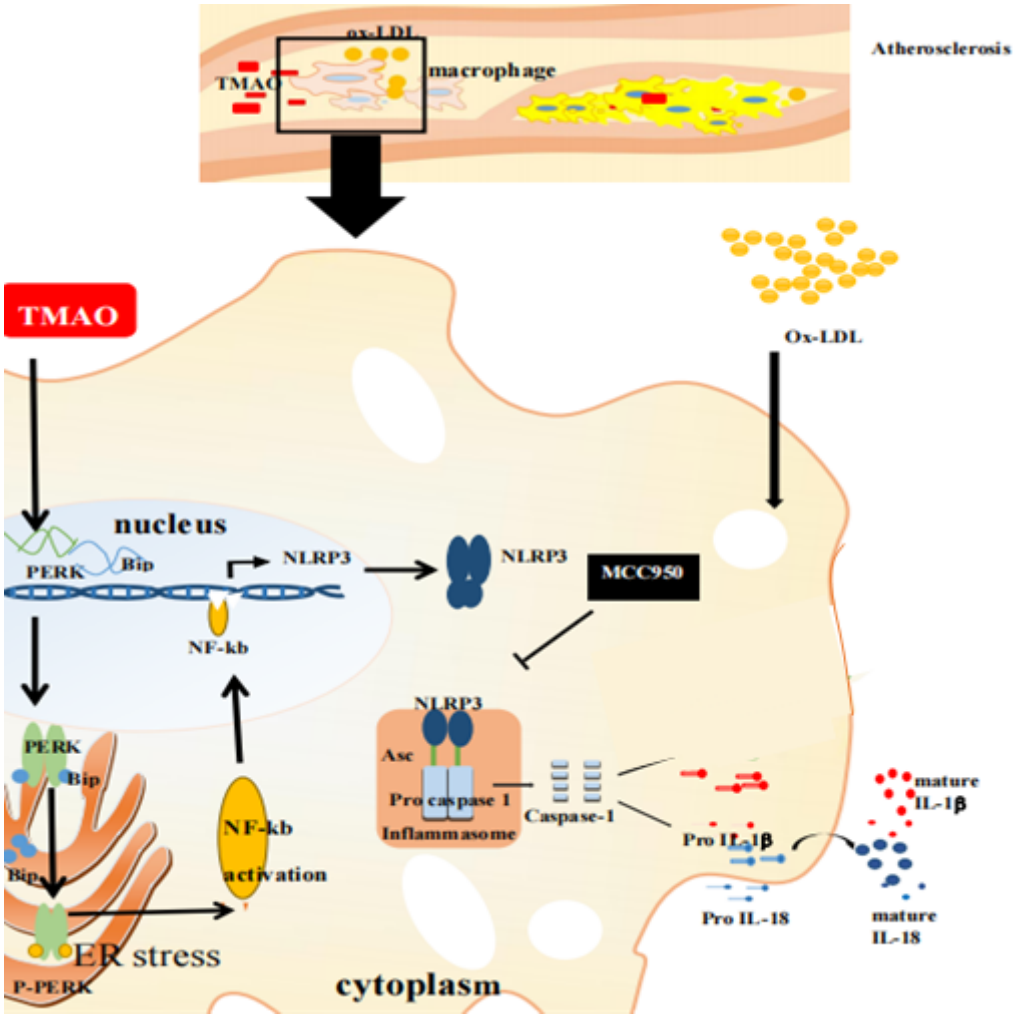

Figure 5

The proposed schematic: TMAO activated NLRP3 inflammasomes in ox-LDL-induced THP-1 cells by activating NF-kb and ERS, TMAO activates the NLRP3 inflammasome and accelerates the release of IL-1β□IL-18, endoplasmic reticulum stress and NF-kb pathway activated by TMAO regulated the degree of NLRP3 activation and IL-1β □IL-18 production induced by ox-LDL in THP-1 macrophages.