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# Contribution of toll-like receptor 2 and nicotinamide adenine dinucleotide phosphate oxidase to the trimethylamine N-oxide-induced inflammatory reactions in U937-derived macrophages

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**Original Article**

## Abstract

**BACKGROUND:** Trimethylamine N-oxide (TMAO) is emerging as a new generation of metabolites related to the activation of inflammatory reactions in the macrophages during atherosclerosis. Stress-activation of cell surface toll-like receptors (TLRs) as well as nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) is also assumed to be involved in TMAO-induced inflammatory reaction in the macrophages. To elucidate the possible contribution of TLRs and NOX to the mentioned signaling pathway, we aimed to simultaneously evaluate the expression level of TLR2, TLR6, and NOX2 in TMAO-treated macrophages.

**METHODS:** 2.5 × $10^6$ cells of U937-derived macrophages were treated in triplicates with different concentrations (37.5, 75, 150, and 300 µM) of TMAO for 24 hours. The cells were also treated with tunicamycin (TUN), as a positive control of stress. Normal control group (CTR) cells received no treatment. The viability of treated cells was checked by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT) assay. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was also used to evaluate the relative expression (fold change) of TLR2, TLR6, and NOX2 at messenger ribonucleic acid (mRNA) levels. One-way analysis of variance (ANOVA) with post-hoc Dunnett's test was performed to compare every mean with that of the control.

**RESULTS:** No cell death occurred because of treatments. Dose of 300 µM of TMAO significantly increased the relative expression of both TLR2 and NOX2 compared to the CTR cells ($P < 0.001$ for both). The elevation of TLR6 was not statistically significant in all groups of TMAO-treated cells ($P > 0.050$).

**CONCLUSION:** Our results provide documentation supporting contribution of TLR2 and NOX2 to previously described inflammatory reactions induced by TMAO in macrophages. In addition, they may clarify the proatherogenic role of TMAO in foam cell formation as well as abnormal activation of macrophages during atherosclerosis.

**Keywords:** Toll-Like Receptors; Atherosclerosis; Trimethylamine N-Oxide; Macrophages

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## Introduction

Trimethylamine N-oxide (TMAO) is an endogenous metabolite which is generally produced in the liver of human and other species.[1,2] It is produced from trimethylamine (TMA) by hepatic flavin-containing monooxygenase 3 (FMO3).[3]

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TMA itself is formed from dietary choline and other choline-containing precursors by intestinal gut microbial communities. The detailed information about this metaorganismal pathway was first provided by Wang et al. during a series of comprehensive metabolomics and functional studies to define the novel risk factors for atherosclerosis. The mentioned study, for the first time, introduced TMAO as a risk factor for atherosclerosis. Moreover, other investigation of this group showed that TMAO had a potential to induce the foam cell formation as well as inflammatory reactions in the macrophages and other vessel cells during atherosclerosis.[3] Atherosclerosis had been known as an inflammatory disease for a long time. Furthermore, macrophages are considered as a major component of inflammatory reactions.[4] Since then, TMAO is extensively studied for its contribution to the activation of inflammatory reactions in the various diseases such as atherosclerosis, non-alcoholic fatty liver,[5] Alzheimer's disease (AD),[6] type 2 diabetes mellitus (DM),[7] chronic kidney disease (CKD),[8] insulin resistance,[9] and gastrointestinal (GI) cancers.[10,11] The other studies also showed that gut flora-derived TMA/FMO3/TMAO pathway is correlated with induced inflammatory reactions in the endothelial cells of the vessel walls as well as adipose tissue.[12-14] Although the studies in this field are growing rapidly, the exact mechanism by which TMAO may contribute or initiate the inflammatory reactions in the vessel cells is not completely understood. It is also unclear whether TMAO has a potential to reacts with specific receptors on the surface of cells involved in the inflammatory reactions or not.[15]

Toll-like receptors (TLRs), the homologues of the Drosophila toll protein, are transmembrane proteins and cell surface receptors which are presented on the macrophages, lymphocytes, fibroblasts, and epithelial and endothelial cells to sense pathogen-associated molecular patterns (PAMPs) expressed by microbial pathogens, and/or danger-associated molecular patterns (DAMPs) released from dead cells.[16-18] TLRs are well recognized as part of our innate immune system and their activation will trigger the expression of proinflammatory cytokines and other metabolic regulation. A similar contribution mechanism which is mediated by TLRs has also been proposed for the metabolic effects of intestinal flora.[13,19] TLR2 and TLR6, two members of this family, are also present on the surface of tissue macrophages.[16]

The nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) family include NOX1 to NOX5 and dual oxidase 1 (DUOX1) and DUOX2. They are involved in the production of cellular reactive oxygen species (ROS).[20] TMAO has also been shown to promote the production of ROS as a part of its proatherogenic mechanism.[14,21] Furthermore, TMAO increases the expression level of cluster of differentiation 36 (CD36) on the cell surface of macrophages. Moreover, TMAO activates nuclear factor kappa B (NF-κB) signaling pathways to promote vascular inflammation. It is supposed that TMAO may interact with CD36 to recruit TLRs/NF-κB pathway and to consequently activate the NOXs for producing ROS.[22] This hypothesis has been previously documented for oxidized low-density lipoprotein (ox-LDL) as another proatherogenic factor.[22] To complete our previous studies in this field, as well as to elucidate the possible contribution of TLRs and NOX to the inflammatory reaction induced by TMAO, we aimed to simultaneously evaluate the expression levels of TLR2, TLR6, and NOX2 in TMAO-treated macrophages.

## Materials and Methods

*Cell culture and treatments:* U937 which is a human monocyte cell line (obtained from Pasteur Institute, Tehran, Iran) was cultured in Roswell Park Memorial Institute (RPMI) 1640 (Gibco Co., USA) containing 10% fetal bovine serum (FBS) (Gibco Co., USA), 1% penicillin–streptomycin (Sigma-Aldrich Co., St. Louis, MO, USA), and 2 mM L-glutamine. Cells were maintained in a 5% carbon dioxide (CO2) incubator under a humidified atmosphere at 37 °C. About $2.5 \times 10^6$ cells of U937 were differentiated to macrophages by exposure to phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich Co., St. Louis, MO, USA) at a final concentration of 20 nM for 48 hours. After differentiation, the cells were treated in three separate replicates with different concentration of TMAO including 37.5, 75, 150, and 300 μM for 24 hours. These concentrations were selected based on previous studies to find any possible dose-dependent responses. Tunicamycin (TUN) was also used as a positive control for induction of endoplasmic reticulum stress (ER stress) at 2 μg/ml for 18 hours. A normal control group (CTR) with cells which were differentiated but received no TMAO or TUN treatment was also applied for final comparison. The viability of treated or untreated cells was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT) assays to determine any cell death because of treatments.

*MTT assay:* MTT assay was performed with a

few minor changes according to previously described protocol to check or calculate the viability of treated or untreated cells.[23] Briefly, 100 µl of a homogeneous cell suspension (100000 cells/ml) was added per well to a 96-well plate. Then the cells were treated in six replicates with different concentration of TMAO (37.5, 75, 150, or 300 µM) for 24 hours and TUN (2 µg/ml) for 18 hours. After that, 20 µl of 5 mg/ml MTT solution was added to each well aseptically and incubated for 3.5 hours. Afterward, 100 µl of MTT solvent [27 ml isopropanol, 3 ml triton X-100, 2.5 µl concentrated hydrochloric acid (HCl) for 30 ml] was added to each well and incubated for 4 hours at room temperature in the dark. Absorbance was then measured at 590 nm with a reference filter of 620 nm using a microplate reader (Synergy HTX, BioTek, USA). The percentage of cell viability was calculated with the following equation, using corrected absorbance: % cell viability = (mean of sample absorbance/mean of control absorbance) ×100

*Reverse transcription-quantitative polymerase chain reaction (RT-qPCR):* Total ribonucleic acid (RNA) was extracted from 2.5 × $10^6$ cells of each (three) replicate of all groups using RNX-Plus (SinaClon Co., Iran). Purity and concentration of extracted RNA was assessed using a Picodrop-Take3 instrument (Synergy HTX, BioTek, USA). To remove the genomic deoxyribonucleic acid (DNA), about 1000 ng of RNA was treated with DNAse I (Scientific Inc., USA). Using PrimeScript™ RT reagent Kit (RR037A, Takara, Japan), reverse transcription (RT) reaction was performed in an Eppendorf Thermal Cycler (Germany) in one cycle including three steps as following: step one: 15 minutes at 37 °C for complementary DNA (cDNA) synthesis, step two: 5 seconds at 85 °C for heat inactivation of the RT enzyme followed by a final 5 minutes at 4 °C for final step. Samples were stored at –70 °C for future analysis. For analysis of gene expression, quantitative polymerase chain reaction (qPCR) assay was performed for actin beta (ACTB) (NM_001101.4), TLR2 (NM_001318796.1), TLR6 (NM_006068.4), and NOX2 (NM_000397.3) on a Rotor-Gene 6000 real-time polymerase chain reaction (PCR) machine (Corbett RG-6000, Australia) using SYBR Premix Ex Taq II (Tli Plus, Takara, Japan). About 50 ng of cDNA and 0.5 µl of specific primers (10 pmol/µl) were used in the following protocol: initial heat activation and denaturation for 5 seconds at 95 °C, 40 cycles of annealing and elongation for 30 seconds at 62 °C (primer-specific temperature). Primers were purchased from Bioneer Corporation (Korea). The following oligonucleotide primer pairs were used: ACTB (PCR product length: 234 bp), forward: 5'-GGACTTCGAGCAAGAGATGG-3', reverse: 5'-AGCACTGTGTTGGCGTACAG-3'; TLR2 (PCR product length: 162 bp), forward: 5'-ATC CTC CAATCAGGCTTCTCT-3', reverse: 5'-ACACCTCTGTAGGTCACTGTTG-3'; TLR6 (PCR product length: 108 bp), forward: 5'-GTGCCATTACGAACTCTA-3', reverse: 5'-TTGTTG GGA ATGCTGTT-3'; NOX (PCR product length: 111 bp), forward: 5'-TGTTCAGCTATGAGGTGGTGA-3', reverse: 5'-TCAGATTGGTGGCGTTATTG-3'. Specificity of the amplified PCR products was verified by melting point analyses and agarose gel electrophoresis. For normalization of the transcript levels, ACTB gene was used as the reference gene and quantified in parallel with the samples. The messenger RNA (mRNA) copy number (starting concentrations) of the targets and the reference genes were calculated by LinRegPCR software (version 2013.x).[24] After normalizing against ACTB, the relative amounts (fold change) of TLR2, TLR6, and NOX transcripts were calculated in treated cells with respect to controls. For example, the fold change values or relative amount of TLR2 mRNA level were calculated as: [(TLR2/ACTB) mRNA for each test]/[(TLR2/ACTB) mRNA for control cells]. Final values were expressed as mean ± standard error (SE) of three separate (replicate) measurements for each group.

*Statistical analysis:* The results of cell viability were presented as the mean ± SE of six replications of MTT assay (n = 6). The gene expression levels (or fold changes) were calculated and presented as mean ± SE of three separate measurements from RT-qPCR in three different population of cells (n = 3) for each group. Statistical analysis was performed using the SPSS software (version 16.0, SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) with post-hoc Dunnett's test was performed to compare every mean with that of the controls. P-values less than 0.05 were considered statistically significant.

## Results

The viability of treated or untreated cells was calculated from six replications of MTT assays for each group. The viability of treated U937 cells was higher than 96% in all groups. Therefore, no cell death occurred because of all treatments. In statistical analysis using one-way ANOVA with post-hoc Dunnett's test, no significant difference

was observed in the viability of treated groups when compared with CTR (P > 0.050, Figure 1).

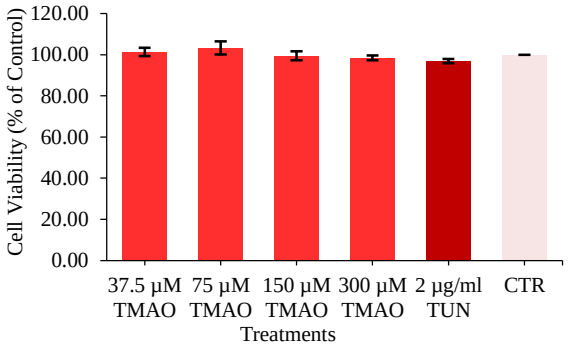


**Figure 1.** Cell viability of U937 cell line after 24 hours of treatment with TMAO and 18 hours of treatment with TUN; values are mean ± standard error (SE) of six separate measurements (n = 6) and P-values less than 0.05 were considered significant. TAMO: Trimethylamine N-oxide; TUN: Tunicamycin; CTR: Control group

The results of gene expression level (fold changes) were calculated from three separate measurements of RT-qPCR in three different population of cells for each group. The fold change of expression or relative amount of TLR2 mRNA level was calculated as: [(TLR2/ACTB) mRNA for each test]/[(TLR2/ACTB) mRNA for control cells]. Figure 2 shows relative expression change of TLR2 at mRNA levels in macrophages treated with different doses of TMAO for 24 hours and TUN for 18 hours.

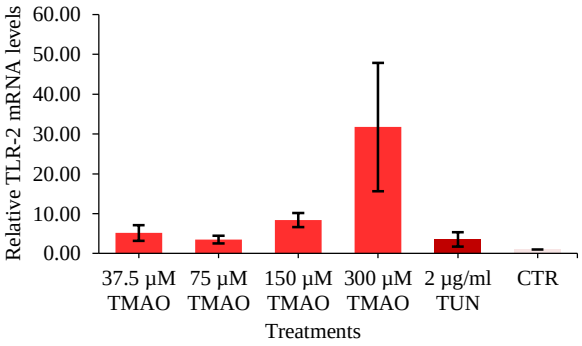


**Figure 2.** Relative toll-like receptor 2 messenger ribonucleic acid (TLR2 mRNA) level of U937 cell line after 24 hours of treatment with TMAO and 18 hours of treatment with TUN; relative TLR2 [TLR2/actin beta (ACTB)] mRNA level values were calculated as [(TLR2/ACTB) mRNA for each test]/[(TLR2/ACTB) mRNA for control cells]. Values are mean ± standard error (SE) of three separate measurements (n = 3) and P-values less than 0.05 were considered significant. TMAO: Trimethylamine N-oxide; TUN: Tunicamycin; CTR: Control group; *** P < 0.001 in comparison with CTR group

One-way ANOVA showed a statistically significant difference in relative expression levels of TLR2 between groups (P = 0.001). Post-hoc Dunnett's test showed that although all concentrations of TMAO increased the expression level of TLR2, the effect of 300 µM was only significant in comparison with CTR (P < 0.001).

The fold change of expression or relative amount of TLR6 mRNA level was calculated as: [(TLR6/ACTB) mRNA for each test]/[(TLR6/ACTB) mRNA for control cells]. Figure 3 shows the fold changes of TLR6 mRNA levels in macrophages treated with different concentrations of TMAO for 24 hours and TUN for 18 hours. One-way ANOVA showed a statistically significant difference in relative expression levels of TLR6 between groups (P = 0.025). Post-hoc Dunnett's test showed that only TUN significantly increased the relative TLR6 mRNA levels (P = 0.010). In all groups of TMAO-treated cells, no significant elevation was observed in relative TLR6 mRNA levels in comparison with CTR cells (P > 0.050).

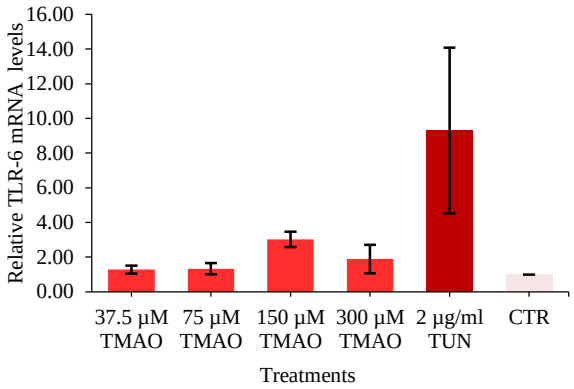


**Figure 3.** Relative toll-like receptor 6 messenger ribonucleic acid (TLR6 mRNA) level of U937 cell line after 24 hours of treatment with TMAO and 18 hours of treatment with TUN; relative TLR6 [TLR6/actin beta (ACTB)] mRNA level values were calculated as [(TLR6/ACTB) mRNA for each test]/[(TLR6/ACTB) mRNA for control cells]. Values are mean ± standard error (SE) ofthree separate measurements (n = 3) and P-values less than 0.05 were considered significant. TMAO: Trimethylamine N-oxide; TUN: Tunicamycin; CTR: Control group; * P < 0.050 in comparison with CTR group

Figure 4 shows the relative amounts or fold change of NOX2 after 24 hours of treatments. The fold change of expression or relative amount of NOX2 mRNA levels was calculated as: [(NOX2/ACTB) mRNA for each test]/[(NOX2/ACTB) mRNA for CTR cells].

One-way ANOVA showed a statistically significant difference in relative expression levels of TLR6 between groups (P = 0.001). Post-hoc Dunnett's test showed that after 24 hours of treatment, only 300 µM of TMAO significantly increased the relative amount of NOX2 mRNA when compared to the CTR (P < 0.001, Figure 4).

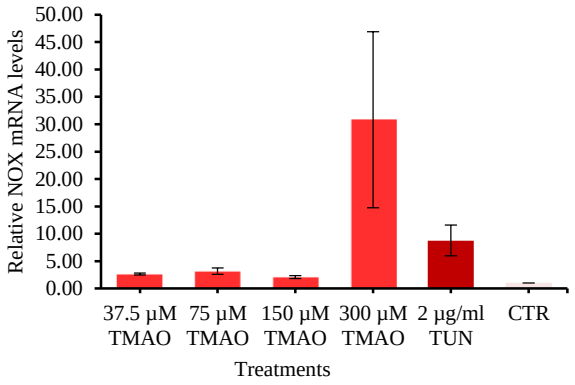


**Figure 4.** Relative NADPH oxidase 2 messenger ribonucleic acid (NOX2 mRNA) level of U937 cell line after 24 hours of treatment with TMAO and 18 hours of treatment with TUN; relative NOX2 [NOX2/actin beta (ACTB)] mRNA level values were calculated as [(NOX2/ACTB) mRNA for each test]/[(NOX2/ACTB) mRNA for control cells]. Values are mean ± standard error (SE) of three separate measurements (n = 3) and P-values less than 0.05 were considered significant. NADPH: nicotinamide adenine dinucleotide phosphate; TMAO: Trimethylamine N-oxide; TUN: Tunicamycin; CTR: Control group; $^{***}$P < 0.001 in comparison with CTR group

## Discussion

Our results showed that TMAO had a potential to induce the expression level of TLR2 in macrophages. To support this finding, we should refer to the previous studies of TMAO. Under aerobic conditions, TMAO as uncoupling agent inhibits the electron transport system which in turn, leads to production of ROS.[25] This effect leads to oxidative stress in the vascular cells.[26] Therefore, TMAO can induce the ER stress and consequently, activates the unfold protein response pathway.[23] The elevation of 78 kDa glucose-regulated protein (GRP78) in response to treatments of macrophages with TMAO is a hallmark for unfolds protein response activation.[27-29] It has been shown that TMAO increases the expression levels of GRP78[23] as well as stress-inducible (cytoplasmic) isoform of heat shock protein 70 (Hsp70)[30] in the macrophages. Hsp70 interacts with TLR2 to induce inflammation.[17,31] Therefore, Hsp70 may provide a link between TMAO and its potential to induce proinflammatory cytokine production.

As previously mentioned, TLRs are immune receptors presented on the macrophages and other vascular cells. When TLRs are activated, they promote the production of the proinflammatory cytokines[32] and consequently, initiate the inflammation.[33] CD36 recognizes the endogenous ligands in atherosclerosis. It facilitates the signaling of TLR2.[34] It is supposed that CD36 may provide another link between TMAO and TLR pathways. To support this hypothesis, there are studies which show that TMAO induces the expression level of CD36 to promote the inflammatory reaction in the macrophages.[3] Again, TMAO as an endogenous ligand may interact with CD36 which in turn, induces TLR2 to produce the proinflammatory cytokines.[35,36]

In the present study, we also found that TMAO increased the expression level of NOX. The previous works of Chen et al. confirm this finding. They showed that TMAO activated the nod-like receptor pyrin domain containing 3 (NLRP3) inflammasome through the SIRT3-SOD2-mtROS signaling pathway to induce vascular inflammation.[21] As previously noted, TMAO has also a potential to generate the ROS by itself.[25] Therefore, TMAO may induce the expression levels of NOX through a pathway mediated by TLR2 to promote ROS production in a similar mechanism which is previously mentioned for ox-LDL.[22]

As previously noted, intestinal bacteria produce TMA from dietary foods containing phosphatidylcholine (PC), choline, and L-carnitine. TMA is absorbed in the intestine and then converted to TMAO by hepatic FMO3 in the liver.[3,37] TMAO induces inflammation in the macrophages and promotes the foam cell formation.[3] It also reduces the reverse cholesterol transport pathway through down-regulation of cholesterol 7 alpha-hydroxylase (7α-hydroxylase).[12] This enzyme catalyzes the conversion of cholesterol into bile acids. The intracellular reduction of hepatic bile acids production through a feedback pathway suppresses the expression of liver x-receptors (LXR) which in turn, initiates the production of proinflammatory cytokines as another mechanism for activation of inflammation.[12] Previous studies have also shown that TMAO promotes the vascular inflammation through activation of mitogen-activated protein kinase (MAPK) and NF-κB signaling pathways.[14] TMAO specifically induces the expression of interleukin 1β (IL-1β) in the macrophages.[38]

## Conclusion

In the recent years, many valuable studies have been done to elucidate the pathologic mechanism by which TMAO activates the inflammatory reactions in the macrophage and other vascular cells. Our previous studies documented an elevation in the expression levels of proinflammatory cytokines in response to treatment of U937-derived macrophages with TMAO. Based on arguments that were provided in the discussion as well as previous studies which were presented earlier, the results of this study provide more findings to support the possible contribution of TLR2 and NOX2 as other components which may be involved in TMAO-induced inflammatory reactions in the macrophages. It also may in part elucidates the proatherogenic role of TMAO for the foam cell formation and abnormal activation of macrophages. To confirm this conclusion, further investigation is required to evaluate the direct interaction of TMAO with TLR2 and other cell surface receptors presented on the macrophages using targeted receptor assay. Laboratory studies of this section were not possible for us due to the limitations of the instrumentation and financial resources.

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## Conflict of Interests

Authors have no conflict of interests.

## Authors' Contribution

Abbas Ahmadi provided assistance in the design of the study, coordinated manuscript preparation. Zakaria Vahabzadeh carried out the design and supervised the study, and participated in manuscript preparation. Mohammadraman Moloudi assisted in the manuscript preparation and statistical analysis. Leila Farhadi provided assistance in the cell culture. Sara Shirahmadi provided assistance in the laboratory works. All authors have read and approved the content of the manuscript. The authors declare that they have no competing interests.

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