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Expression levels of heat shock protein 60 and glucose-regulated protein 78 in response to trimethylamine-N-oxide treatment in murine macrophage J774A.1 cell line

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Abstract

Trimethylamine N-oxide (TMAO), a common metabolite in animals and humans, can induce changes in the expression or conformation of heat shock proteins. It has also been introduced as a risk factor for atherosclerosis and a biomarker for kidney problems. On the other hand, increased levels of heat shock proteins 60 and 70 KDa are associated with increased atherosclerosis risk. This study was therefore designed to evaluate the possible effect(s) of TMAO on the expression of HSP60 and GRP78 at the mRNA and protein levels. Murine macrophage J774A.1 cells were treated with micromolar concentrations of TMAO and 4-phenylbutyric acid (4-PBA), a chemical chaperon, for different time intervals. Tunicamycin was also used as a control for induction of endoplasmic reticulum stress. Tunicamycin greatly increased both mRNA and protein levels of GRP78. Similarly but to a lesser extent compared to tunicamycin, TMAO also increased mRNA and protein levels of GRP78 in a dose and time-dependent manner. In contrast, 4-PBA failed to induce any changes. Similar to GRP78, HSP60 was also increased only at mRNA level in TMAO treated cells. 4-PBA also increased HSP60 mRNA levels, whereas, tunicamycin did not show any effect on either protein or mRNA levels of HSP60. Since both heat shock proteins are stress inducible and the elevation of GRP78 is a hallmark for endoplasmic reticulum stress induction, it can be concluded that TMAO may induce endoplasmic reticulum stress or may act through elevation of these heat shock proteins.

Key words: Heat-Shock Proteins, Trimethylamine-N-Oxide, 4-Phenylbuteric acid, Tunicamycin, Endoplasmic Reticulum Stress, Macrophage.

Introduction

Osmolytes are small, and water soluble organic molecules produced by organisms to maintain their volume. They have powerful effects on protein stabilization (1-4). Trimethylamine N-oxide (TMAO), as a well-known natural osmolyte, has been found in large amounts in muscle tissues of fish and marine invertebrates (5, 6). It also acts in freeze-avoidance response in some species (7). In the in-vitro, as a nanocrowding particle, thermodynamically stabilize the protein folding against the pressure, temperature and chemical denaturants, such as urea (increased in kidney failure) or guanidine hydrochloride (5, 8-10).

The molecular mechanisms by which TMAO changes protein folding and stability (8, 9, 11, 12) or acts as a natural osmolyte in marine organisms (6, 13-15), as well as its contribution in the electron transfer chain in bacteria (16-21) has been extensively studied before. However, little is known about the other biological functions of TMAO in mammals, especially in humans.

TMAO is an oxidative product of trimethylamine by liver flavin-containing monooxygenase in many species, including fish, animals and humans. Trimethylamine is derived from choline, dietary phosphatidylcholines (lecithin) or carnitine by gut microbial flora (22-27). TMAO levels are elevated in plasma of subjects with end-stage renal disease and can be used as a biomarker for kidney problems (27).

Heat shock proteins (HSPs) which were reported for the first time in 1962, are produced as a cell response to elevation of temperature, and stress. They belong to a group of highly conserved proteins which are present in both prokaryotes and eukaryotes and based on their molecular weights are termed as; HSP10, HSP40, HSP60, HSP70, HSP90 and HSP110 (28-30).

The 70 kilodalton heat shock proteins (HSP70s) family which exist in all living organisms are close members with a unique pattern of expression and localization. They are an important part of the cell machinery for protein folding, and help to protect cells from stress (30-34). GRP78 also known as binding immunoglobulin protein (BiP) or heat shock 70 kDa protein 5 (HSPA5) is a HSP70 that is located in the lumen of the endoplasmic reticulum (ER) and by binding to the newly-synthesized proteins, holds them for subsequent proper folding. GRP78 is abundant in all stages of cell growth, however, its synthesis is markedly increased under conditions that lead to the accumulation of unfolded polypeptides in the ER. Its elevation is used as a hallmark for induction of ER stress and unfolded protein responses (UPR) activation (35-37).

Intracellular heat shock proteins play a protective role under both physiological condition and stress. In pathologic events, such as in atherosclerosis, extra-

cellular forms of heat shock proteins act as a "danger signal" and through some cell surface receptors activate cellular and humoral immune reactions and immunoinflammation process (28, 29, 38-40). Both HSP60 (a common candidate autoantigen for atherosclerosis) and HSP70s (stress inducible heat shock protein and ligand for scavenger receptors) are produced by all cell types susceptible to atherosclerotic lesions including; aortic endothelial cells, macrophages and smooth muscle cells and $CD3^+$ lymphocyte T (34, 40-43).

Previous studies for the in-vitro beneficial effects of TMAO have mainly been carried out using millimolar or molar concentration of TMAO (44, 45). It has been shown that TMAO upregulates some heat shock proteins (45) or induces conformational changes to activate them (1). In addition, recently, the denaturant effects (3) and proatherogenic potential have been introduced for TMAO (22-26, 46). Moreover, it has been shown that the ER-stress induction and UPR activation of macrophages are associate with the reduction of ATP-binding cassette transporter-1 (ABCA1) in macrophages, hence contribute to the formation of foam cells and worsening atherosclerosis complications (47, 48). Considering the new role of TMAO in the foam cell formation (26) and the contribution of HSP60 and HSP70s overexpression of murine macrophages to atherosclerotic lesions (34), murine macrophages were selected to assess their possible stress conditions. Therefore, this study was performed to evaluate possible effects of micromolar doses of trimethylamine N-oxide on the expression of HSP60 and GRP78 at both protein and mRNA levels in J774A.1 murine macrophage cell line.

Materials and methods

Cell Culture and Treatments

J774A.1 (Pasture Institute, Tehran, Iran), a tumoral murine macrophage cell line, were cultured in DMEM (Sigma) containing 10% heat-inactivated fetal bovine serum (FBS, Sigma), 1% penicillin–streptomycin and 4 mM L-glutamine. Cells were maintained in a 5% CO2 incubator under humidified atmosphere at 37 ◦C. After reaching 90% confluency (approximately 7 days), cells were treated with 37.5, 75, 150 and 300 µM of TMAO for 8, 18 and 24 hour intervals. Three groups of cells, termed as normal control, chaperon control, and stress control were used as controls. For chaperon control cells, a well-known chemical chaperon, 4-PBA, was used at 2.5 or 5.0 mM concentrations. Stress control cells were treated with tunicamycin, 2 µg/mL, for 18 hours. Normal control cells received no treatment. Cell viability was examined by exclusion of trypan blue (higher than 96%) and MTT assays.

MTT Assay

First, a homogenous cell suspension with 100,000 cells per ml was prepared using complete medium (DMEM). Then 100 µl of this suspension (about 10000 cells) was added in triplicates to the wells and incubated overnight. On the next day the culture media was replaced with 100 µl of complete culture medium containing different doses of TMAO, 4-PBA and tunicamycin that were described in Section 2.1. Cells were then incubated for different time intervals as described earlier. At the appropriate time, 20 µl of 5 mg/ml MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) solution was added to each well aseptically and incubated for 3.5 hours. After this time, media was carefully removed and 100 µl of MTT solvent (27mL isopropanol, 3mL tritonX-100, 2.5uL concentrated HCl for 30 mL) was added to each well, covered and incubated for 4 h at room temperature in the dark. Absorbance was then measured at 590 nm with a reference filter of 620 nm in microplate reader (Synergy HT, BioTek, USA).

Western blotting

Cells were scraped and washed twice with ice cold PBS and homogenized in the protein lysate buffer [Radio-Immunoprecipitation Assay buffer (RIPA, Sigma); 50 mM Tris-HCl, pH 8.0, 150 mM sodium chloride, 1.0% legpal CA-630 (NP40), 0.5% sodium deoxycholate, 1.0% sodium dodecyle sulfate, 1µg/ml complete protease inhibitor cocktail, and 1 mM phenyl methyl sulfonyl fluoride] at 4 °C using an ultrasonic homogenizer (Hielscher, Germany). Cell debris were removed by centrifugation at 12,000xg for 15 min at 4°C. Protein concentration was measured using Bradford assay (49) and 100 µg protein was loaded per well on a 12% polyacrylamide gel for SDS-PAGE. Proteins were then transferred to a PVDF membrane (0.2 µm, BIO-RAD, USA) for 2.5 hrs at 250 mA in an ice bath. Transferred proteins were stained with ponceau S to ensure transfer of proteins. Membranes were then washed to remove the dye and then blocked with 5% skim milk in TBS-T buffer (20 mmol/L Tris, 137 mmol/L NaCl pH 7.6 containing 0.5%Tween-20) for 2 hours at room temperature. The membranes were then incubated with the primary monoclonal antibodies specific for HSP60 (ADI-SPA-806, Enzo life sciences) and GRP78 (ADI-SPA-827, Enzo life sciences) for 60 minutes at room temperature. The membranes were washed three times (20 min each) with TBS-T, and incubated with the second antibody (HAF007, R&D System) for 1 hour at room temperature. Immunoreactivity was detected by enhanced chemiluminescence (Perkin Elmer, Netherlands) using Molecular Imager® ChemiDOC™ XRS+ Imaging System (BIO-RAD, USA). Band densities and differences between the bands were analyzed and quantified using an Image Lab3 software (Bio-Rad, USA). After normalizing with GAPDH (as a reference), the relative amounts of HSP60, and GRP78 proteins in treated cells with respect to controls were expressed as mean ± standard error for four separate measurements.

Real-Time RT-PCR

Total RNA was extracted using Total RNA Purification kit (Jena Bioscience, Germany). RNA concentration and purity was then assessed using a Nanodrop instrument (ND-1000, Thermo Scientific Inc, USA). To remove the genomic DNA, about 1000 ng of RNA was treated with DNAse I (Thermo Scientific Inc, USA) for 30 min at 37 ◦C. To stop the reaction, 1µl of 50 mM NaEDTA was added to the tube and incubated for 10 min at 65 °C. Using AccuPower® CycleScript RT PreMix (dN_6) kit (Bioneer, Korea), reverse transcription reaction was performed in a Corbett Thermal Cycler (Qiagen, Germany) in 12 cycles (step1: 1 min at 25 ◦C for primer

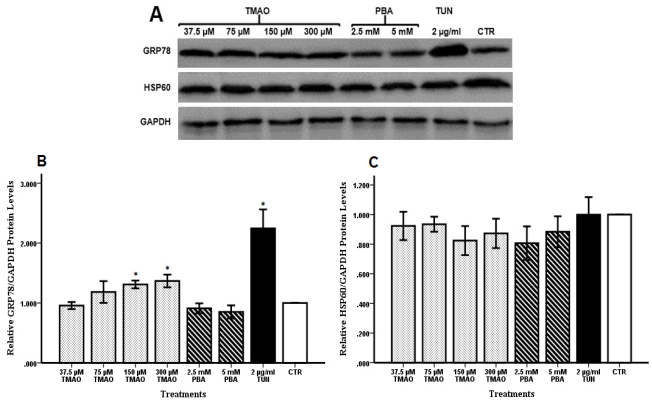

Figure 1. Dose dependent changes in GRP78 and HSP60 protein in murine macrophage J774A.1 cell line after 8h of treatment with TMAO and PBA, and 18h of treatment with tunicamycin. **A:** western blotting bands. **B:** relative GRP78/GAPDH protein levels. Relative GRP78/GAPDH protein level values were calculated as [(GRP78/GAPDH) protein for each test] / [(GRP78/GAPDH) protein for control cells]. **C:** relative HSP60/GAPDH protein levels. Relative HSP60/GAPDH protein level values were calculated as [(HSP60/GAPDH) protein for each test]/[(HSP60/GAPDH) protein for control cells]. TMAO: Therimthylamine-N-Oxide; PBA: 4-Phenylbutyric acid; TUN: Tunicamycin; CTR: Control. Values are mean ± standard error from four separate measurements. *P*-values less than 0.05 were considered significant. * shows $P<0.05$

annealing, step2: 4 min at 43 ◦C for cDNA synthesis, and step3: 30s at 55 ◦C for melting secondary structure and cDNA synthesis), followed by a final 5 min at 95 ◦C for heat inactivation of the enzyme. Samples were stored at –70 ◦C for further analysis. To verify successful cDNA synthesis and ensure that the genomic DNA has been removed, selected samples were subjected to standard PCR (30 cycles, 30 s at 95 ◦C, 30 s at 56 ◦C, 30s at 72 ◦C). Subsequently, PCR products were analyzed on a 2 % agarose gel stained with Ethidium bromide. A VC100-base pair (bp) DNA was used as the ladder (Vivantis). For analysis of gene expression, quantitative PCR assay was performed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, NM_008084.2), HSP60 (NM_010477.4), and GRP78 (NM_001163434.1) on a Rotor-Gene™ 6000 real-time PCR machine (Qiagen, Germany) using Quantifast SYBR Green PCR Kit (Qiagen, Germany). Approximately 50 ng of cDNA and 1µL of specific primers (10 pmol/µl) were used in the following protocol: initial heat activation for 300 s at 95 ◦C, 40 cycles of, denaturation: 10 s at 95 ◦C, annealing: 15 s at 56 ◦C (primer-specific temperature), and elongation for 20 s at 72 ◦C. Primers were purchased from BIONEER (Korea). The following oligonucleotide primer pairs were used: GAPDH (PCR product length 198 bp), forward: 5'- CCATCCGGGTTCCTATAAAT -3', reverse: 5'- AATCTCCACTTTGCCACTG -3'; HSP60 (PCR product length 112 bp), forward: 5'-GGAAGCCATTGGTCATAATC-3', reverse: 5'-AATCCTGGAGCTTTGACTG-3'; GRP78 (PCR product length 182 bp), forward: 5'-ACGCACTTGGAATGACCCT-3', reverse: 5'-AATACGCCTCAGCAGTCTC-3'. Specificity of the amplified PCR products was verified by melting point analyses and agarose gel electrophoresis. For normalization of the transcript levels, GAPDH gene was used as the reference and quantified in parallel with the samples. The starting concentrations (mRNA copy number) of the targets and the reference genes were calculated by version 2013.x of LinRegPCR software (50). After normalizing against GAPDH, the relative amount of HSP60, and GRP78 transcripts in treated cells with respect to controls were expressed as mean ± standard error for four separate measurements.

Statistical Analysis

Data are presented as mean ± standard error of the mean (S.E.M.). Statistical analysis were performed using the SPSS software package version 20. A two-tailed student's t test was performed to compare every mean with that of control. The *P*-values of less than 0.05 were considered statistically significant.

Results

Cell viability of macrophages in different groups was higher than 96% and no cell death because of treatments occurred.

Protein Levels of GRP78 and HSP60

Figures 1B to 3B and 1C to 3C, respectively, show relative changes in GRP78/GAPDH and HSP60/GAPDH protein levels in macrophages treated with different doses of TMAO and PBA for 8, 18 and 24 hours and tunicamycin for 18 hours. Cells treated with 150 µM and 300 µM of TMAO for 8 hours (Figure 1B) produced a significant increase in relative GRP78/GAPDH protein levels compared to the control (P=0.018, and 0.040 respectively) (Figure 1). After 18 hours of treatment (Figure 2B), except for the 75 µM, other doses of TMAO, and tunicamycin produced a significant increase in relative GRP78/GAPDH protein levels compared to the control ($P<0.05$) (Figure 2). In cells treated for 24 hours (Figure 3B), treatment with 150 µM and

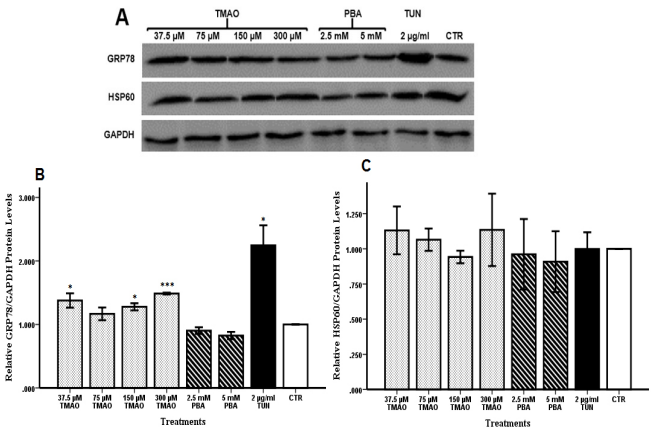

Figure 2. Dose dependent changes in GRP78 and HSP60 protein in murine macrophage J774A.1 cell line after 18h of treatment with TMAO, PBA and tunicamycin. **A:** western blotting bands. **B:** relative GRP78/GAPDH protein levels. Relative GRP78/GAPDH protein level values were calculated as [(GRP78/GAPDH) protein for each test]/[(GRP78/GAPDH) protein for control cells]. **C:** relative HSP60/GAPDH protein levels. Relative HSP60/GAPDH protein level values were calculated as [(HSP60/GAPDH) protein for each test]/ [(HSP60/GAPDH) protein for control cells]. TMAO: Therimthylamine-N-Oxide; PBA: 4-Phenylbutyric acid; TUN: Tunicamycin; CTR: Control. Values are mean ± standard error from four separate measurements. *P*-values less than 0.05 were considered significant. * shows $P<0.05$ and *** shows $P<0.001$

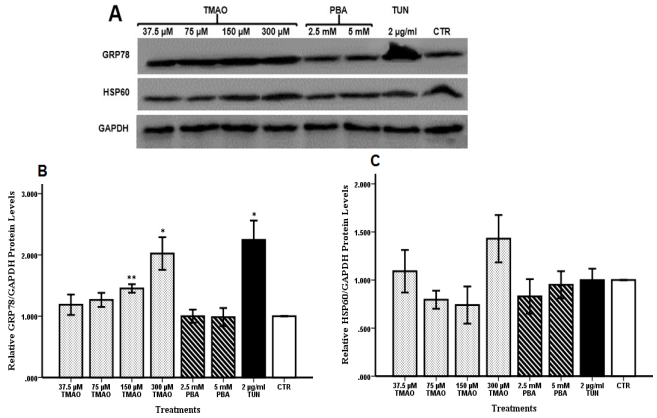

Figure 3. Dose dependent changes in GRP78 and HSP60 protein in murine macrophage J774A.1 cell line after 24h of treatment with TMAO and PBA, and 18h of treatment with tunicamycin. **A:** western blotting bands. **B:** relative GRP78/GAPDH protein levels. Relative GRP78/GAPDH protein level values were calculated as [(GRP78/GAPDH) protein for each test]/[(GRP78/GAPDH) protein for control cells]. **C:** relative HSP60/GAPDH protein levels. Relative HSP60/GAPDH protein level values were calculated as [(HSP60/GAPDH) protein for each test]/[(HSP60/GAPDH) protein for control cells]. TMAO: Therimthylamine-N-Oxide; PBA: 4-Phenylbutyric acid; TUN: Tunicamycin; CTR: Control. Values are mean ± standard error from four separate measurements. *P*-values less than 0.05 were considered significant. * shows $P<0.05$ and ** shows $P<0.01$

300 µM TMAO, produced significant increase in relative GRP78/GAPDH protein levels compared to control ($P=0.007$, and 0.031 respectively) (Figure 3). No significant difference was observed in HSP60/GAPDH protein levels between treated cells and control for all time periods (Figures 1C to 3C).

mRNA Levels of GRP78 and HSP60

Figures 4A through 6A show relative changes in GRP78/GAPDH mRNA levels for different doses of TMAO at different time periods. In cells treated for 8 hours (Figure 4A), except for the 150 µM, other doses of TMAO showed a significant increase compared to the control cells ($P<0.05$) (Figure 4). In cells treated for 18 (Figure 5A) all doses of TMAO produced a significant increase in relative GRP78/GAPDH mRNA levels compared to the control ($P<0.05$). Also, tunicamycin treated cells showed a significant increase in the mRNA levels compared to the control ($P=0.001$). (Figure 5). In cells treated for 24 hours (Figure 6A), except for the 37.5 µM, other doses of TMAO produced a significant increase in relative GRP78/GAPDH mRNA levels compared to control cells ($P<0.05$) (Figure 6).

Figures 4B to 6B show relative HSP60/GAPDH mRNA levels in macrophages treated with TMAO and PBA for 8, 18 and 24 hours and tunicamycin for 18 hours. Cells treated with 37 µM and 75 µM of TMAO for 8 hours (Figure 4B) showed a significant increase in relative HSP60/GAPDH mRNA levels compared to control cells ($P=0.046$, and 0.042 respectively). After 18 hours of treatment (Figure 5B), all doses of TMAO and PBA produced a significant increase in relative HSP60/GAPDH mRNA levels compared to the control cells ($P<0.05$). In 24 h treated cells (Figure 6B), only 2.5 and 5 mM of PBA produced a significant difference in relative HSP60/GAPDH mRNA levels compared to

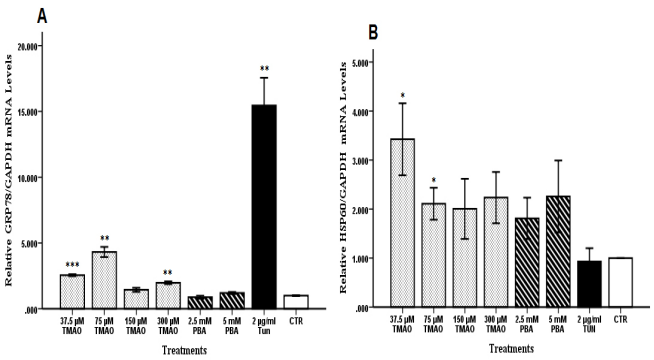

Figure 4. Dose dependent changes in GRP78 and HSP60 mRNA in murine macrophage J774A.1 cell line after 8h of treatment with TMAO and PBA, and 18h of treatment with tunicamycin. **A:** relative GRP78/GAPDH mRNA levels. Relative GRP78/GAPDH mRNA level values were calculated as [(GRP78/GAPDH) mRNA for each test]/[(GRP78/GAPDH) mRNA for control cells]. **B:** relative HSP60/GAPDH mRNA levels. Relative HSP60/GAPDH mRNA level values were calculated as [(HSP60/GAPDH) mRNA for each test]/[(HSP60/GAPDH) mRNA for control cells]. TMAO: Therimthylamine-N-Oxide; PBA: 4-Phenylbutyric acid; TUN: Tunicamycin; CTR: Control. Values are mean ± standard error from four separate measurements. *P*-values less than 0.05 were considered significant. * shows $P<0.05$, ** shows $P<0.01$ and *** shows $P<0.001$ shows

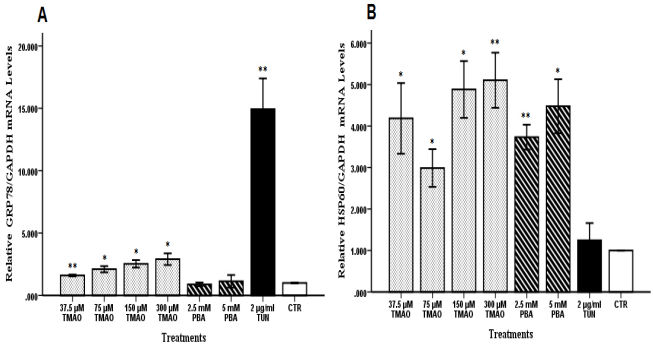

Figure 5. Dose dependent changes in GRP78 and HSP60 mRNA in murine macrophage J774A.1 cell line after 18h of treatment with TMAO, PBA and tunicamycin. **A:** relative GRP78/GAPDH mRNA levels. Relative GRP78/GAPDH mRNA level values were calculated as [(GRP78/GAPDH) mRNA for each test]/[(GRP78/GAPDH) mRNA for control cells]. **B:** relative HSP60/GAPDH mRNA levels. Relative HSP60/GAPDH mRNA level values were calculated as [(HSP60/GAPDH) mRNA for each test]/[(HSP60/GAPDH) mRNA for control cells]. TMAO: Therimthylamine-N-Oxide; PBA: 4-Phenylbutyric acid; TUN: Tunicamycin; CTR: Control. Values are mean ± standard error from four separate measurements. *P*-values less than 0.05 were considered significant. * shows $P<0.05$) and ** shows $P<0.01$

control ($P=0.001$, and 0.015 respectively).

Discussion

TMAO is known as an important osmolyte and a chaperone to stabilize proteins under denaturing conditions in fish and mammals (6, 51). Nevertheless, more recently, TMAO has been introduced as a risk factor for atherosclerosis in terms of helping foam cell formation and exacerbation of inflammation (26). It has been shown that plasma levels of TMAO is under a complex genetic, dietary and hormonal regulation (46). TMAO has also shown a dose dependent relationship with the

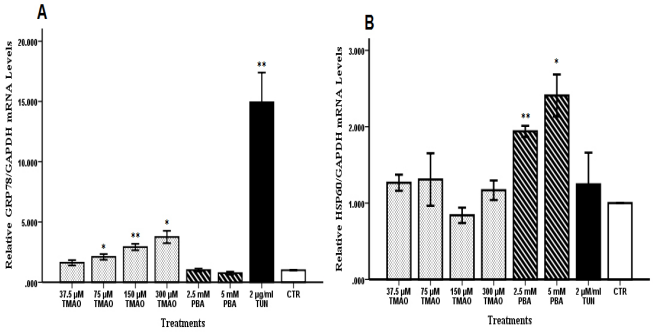

Figure 6. Dose dependent changes in GRP78 and HSP60 mRNA in murine macrophage J774A.1 cell line after 24h of treatment with TMAO and PBA, and 18h of treatment with tunicamycin. **A:** relative GRP78/GAPDH mRNA levels. Relative GRP78/GAPDH mRNA level values were calculated as [(GRP78/GAPDH) mRNA for each test]/[(GRP78/GAPDH) mRNA for control cells]. **B:** relative HSP60/GAPDH mRNA levels. Relative HSP60/GAPDH mRNA level values were calculated as [(HSP60/GAPDH) mRNA for each test]/[(HSP60/GAPDH) mRNA for control cells]. TMAO: Therimthylamine-N-Oxide; PBA: 4-Phenylbutyric acid; TUN: Tunicamycin; CTR: Control. Values are mean ± standard error from four separate measurements. *P*-values less than 0.05 were considered significant. * shows $P<0.05$) and ** shows $P<0.01$

incidence of cardiovascular disease, the extent of atherosclerotic plaques and various phenotypes of atherosclerosis (26).

Nowadays atherosclerosis is known as a chronic inflammatory disease, and autoimmune mechanisms are of the core processes involved in atherosclerotic inflammation. A number of evidences have been provided for the involvement of antibodies produced against autoantigens, including HSPs (28). In fact, in-vivo and in-vitro experiments have shown that the classic risk factors of atherosclerosis act as endothelial stressors. By stimulating the expression of HSP's (such as HSP60) and adhesion molecules in mitochondria and cytoplasm, as danger signals, they activate cellular and humoral immunity in atherosclerotic lesions (29, 38, 39, 42, 52).

Since ER is the major site for protein synthesis, folding and trafficking, when the rate of de novo protein synthesis, and/or the extent of stress-induced conformational changes of proteins exceeds the capacity of ER to restore their proper folding, the accumulation of unfolded or misfolded proteins in the ER results in ER stress and initiates "unfolded protein response" (UPR). In eukaryotes, three ER resident proteins sense ER stress: ATF6 (activating transcription factor 6) (ATF6), PERK (protein kinase RNA-like ER kinase), and IRE1 (inositol requiring protein 1). In normal conditions, GRP78 binds to IRE1, ATF6, and PERK and repress the UPR. When ER stress initiates, GRP78 dissociates and activates UPR signaling. (35-37, 53, 54). This signaling cascade via transcriptional up-regulation of GRP78, transcription factors and so on, restores ER homeostasis or results in cell death (35, 36, 55).

In the present study we evaluated the effect of TMAO on the expression of HSP60 and GRP78 at mRNA and protein levels in the murine macrophage J774.A1 cell line. TMAO increased the GRP78 mRNA and protein levels in a dose and time-dependent manner. Recently, Macdonald and his coworkers showed that TMAO, because of its hydrophobic interactions, may acts as denaturant (3). It has also been shown that TMAO inhibits electron transport in staphylococcus aureus under aerobic conditions (56). If this occurs in the vascular cells, it can lead to oxidant stress via mitochondrial uncoupling and generation of the reactive oxygen species (24). Considering these findings, it can be concluded that TMAO may induce endothelial stress and UPR pathway, possibly by changing the conformation of proteins. The elevation of GRP78 as hallmarks of ER stress and UPR activation will support this conclusion.

Gong et.al also showed that TMAO cause moderate upregulation of HSP70 and probably by this way it affects proteins folding (45). Moreover, Timothy and coworkers showed that TMAO can induce specific conformational changes in heat shock proteins such as HSP90 to activate it (1). Therefore, without any conclusions for the role of TMAO (as an ER stressor or as an inducer of conformational change in proteins) our results are consistent with previous findings in terms of upregulation of HSP70s.

In our study, although TMAO caused significant dose and time-dependent increase in HSP60-mRNA, but failed to induce these changes at the protein levels. Perhaps part of the reason for this observation may be due to differences in the stability of mRNA and protein, possibly due to the role of TMAO in increasing the mRNA stability. It was shown that the protonated form of trimethylamine N-oxide (TMAOP) mimics behavior of cations and by forming electrostatic bond with phosphate groups of specific sites of RNA backbone, it stabilizes RNA's tertiary structure (57). In comparison, tunicamycin also failed to induce any changes in the protein or mRNA levels of HSP60. HSP60 or chaperonin 60 is a mitochondrial heat shock protein that facilitates the correct folding of imported protein to mitochondrial matrix, hence preventing the accumulation of misfolded or unfolded polypeptides generated under stress conditions in the mitochondria (33).

TMAO has been shown to regulate some steps of both reverse and forward cholesterol transport, in part by regulation of specific steps in cholesterol metabolism and macrophage foam cell formation through inducing scavenger receptors (23, 26). The mechanism by which TMAO levels promote atherosclerosis and affect cellular metabolism has not been revealed yet, and, it is not known how TMAO levels are sensed to elicit these pathological responses. The results of this study may elucidate a part of mechanism(s) involved in proatherogenic role of TMAO.

In conclusion, significant elevation of GRP78 in macrophages produced by TMAO is an indication of ER stress, suggesting that TMAO may act as an ER stressor. To confirm this conclusion, further investigation on the molecular mechanisms and other mediators of unfolded protein response are required. Taken together, TMAO may directly induce endoplasmic reticulum stress or may act through elevation of the heat shock proteins 60 and 70 kDa, and further studies are necessary to elucidate these effects of TMAO.

Authors' contributions

Abbas Mohammadi carried out the design and supervised the study, and participated in manuscript preparation. Ahmad Gholamhoseyniannajar provided assistance in the design of the study, coordinated in

manuscript preparation. Mohammad Mehdi Yaghoobi provided assistance in the design of primers and real time PCR experiments and participated in manuscript preparation. Yunes Jahani assisted in the statistical analysis of the study. Zakaria Vahabzadeh carried out all the experiments and prepared the manuscript. All authors have read and approved the content of the manuscript.

Acknowledgments

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