Advances in Medical Sciences 63 (2018) 57–63

Contents lists available at ScienceDirect

Advances in Medical Sciences

journal homepage: www.elsevier.com/locate/advms

Original research article

Trimethylamine-N-oxide, as a risk factor for atherosclerosis, induces stress in J774A.1 murine macrophages

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ARTICLE INFO

Article history:
Received 2 February 2017
Accepted 28 June 2017
Available online 16 August 2017

Keywords:
Heat-shock proteins
Endoplasmic reticulum stress
Trimethylamine-N-oxide
Tunicamycin
Macrophage

ABSTRACT

Purpose: Trimethylamine N-oxide (TMAO) is a biomarker for kidney problems. It has also been introduced as a risk factor for atherosclerosis. The classic risk factors for atherosclerosis trigger cellular and humeral immunoreaction in macrophages through induction of heat shock protein expressions and increased levels of GRP94 and HSP70 are associated with increased atherosclerosis risk. The present study evaluated the possible effect(s) of TMAO on the expression of GRP94 and HSP70 at protein levels.
Methods: J774A.1 murine macrophages were treated with different micromolar concentrations of TMAO and 4-phenylbutyric acid (PBA), a chemical chaperone, for 8, 18, 24, and 48 h intervals. Tunicamycin was also used as a control for induction of endoplasmic reticulum stress. Western blotting was used to evaluate the expression of GRP94 and HSP70 in macrophages at protein levels.
Result: Tunicamycin greatly increased protein levels of GRP94. Similarly, but to a lesser extent compared to tunicamycin, TMAO also increased GRP94. In 24 h treated cells, only 300 μM of TMAO, and in cells treated for 48 h, all doses of TMAO produced a significant increase in relative HSP70 protein levels compared to the control. PBA failed to induce any changes in GRP94 or HSP70 protein levels.
Conclusion: GRP94 and HSP70 are stress-inducible heat shock protein, so the elevation in J774A.1 murine macrophages can clearly define cells under stress and elucidate the contribution of stress induced by TMAO that may have a part in the abnormal activation of macrophages involved in foam cell formation.

1. Introduction

Atherosclerotic vascular disease, a major cause of death worldwide, is governed by many complex etiological factors that can be classified into three major categories: genetic factors, environmental factors, and another determinant called the “microbiome” [1,2]. The relationship between genetic and environmental determinants and cardiovascular risk have been previously established. However, the study of the latter category of CVD determinants is increasing in magnitude across the globe and has attracted the attention of health care systems of worldwide for the use of new preventive therapeutic strategies [2]. Recently, the research has been focused on its role as commensal intestinal microorganisms (gut flora) in the metabolism of food containing precursors of trimethylamine N-oxide (TMAO) to produce proatherogenic metabolites and the potential consequences for the pathogenesis of atherosclerosis.

In a metaorganismal pathway, TMAO is produced from trimethylamine during liver flavin-containing monooxygenase reactions in many species, including fish, animals, and humans. In this pathway, trimethylamine is derived from dietary choline, phosphatidylcholines (lecithin), or carnitine by gut microbial flora [3,4]. In Wang et al, for the first time, TMAO was introduced as an independent risk factor for CVD [3]. Subsequent studies have also confirmed the role of this metabolite as a risk factor for atherosclerosis and clinical prognostic significance of its circulating level for cardiovascular diseases and any other complications [5–7]. Other studies have also demonstrated that TMAO appears to alter the reverse cholesterol transport (RCT) system and/or other aspects of the cellular metabolism of cholesterol to help with foam cell formation and the promotion of atherosclerosis and other vascular events [6]. Warrier et al have suggested that the gut microbiota-driven TMA/FMO3/TMAO pathway plays a key role in regulating lipid metabolism and inflammation [8]. TMAO circulating levels can also be used as a biomarker for kidney problems as its levels become elevated in the plasma of subjects with end-stage

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http://dx.doi.org/10.1016/j.advms.2017.06.006
1896-1126/

renal disease [9]. Moreover, TMAO is known as an important natural osmolyte to upregulate and/or induce a conformational change in heat shock proteins 70 and 90 [10,11].

Heat shock proteins (HSPs) belong to a group of highly conserved proteins in prokaryotes and eukaryotes. They are based on molecular weights as follows: HSP10, HSP40, HSP60, HSP70, HSP90, and HSP110 [12–14]. These proteins play a protective role under physiological conditions, but extracellular HSPs act as a "danger signal" for atherosclerosis and through some cell surface receptors that activate immunoinflammatory reactions [12,13,15,16]. Stress-inducible HSP70 is produced by all the cell types susceptible to atherosclerotic lesions including, aortic endothelial cells, macrophages, and smooth muscle cells [17]. GRP94 or Endoplasmin also known as gp96, ERp99, and TRA-1 are endoplasmic reticulum (ER) HSPs that have been implicated in the folding of proteins in the secretory pathway such as Toll-like receptors, integrins, and facilitates ER-associated degradation for misfolded proteins [18]. Its elevation is used as a marker for induction of ER stress and unfolded protein response activation [19,20].

Induced ER stress due to different risk factors is believed to correlate with all stages of atherosclerosis and is characteristic of lipid-rich macrophages in the atherosclerotic lesions [21–24]. Moreover, ER stress induction has been identified as an important inducer of abnormal macrophage activation, pathological inflammatory response, and, eventually, macrophage apoptosis, which all play important roles in atherosclerosis [25]. Therefore, considering the proatherogenic potential of TMAO as a risk factor, in this study, we measured GRP94 and HSP70 at protein level in response to trimethylamine N-oxide treatment in J774A.1 murine macrophage cell line to evaluate possible TMAO-induced stress.

2. Methods

2.1. Cell culture and treatments

Cell culture and treatments were performed as previously described [2]. J774A.1, a tumoral murine macrophage cell line (Pasture Institute, Tehran, Iran) were cultured in DMEM (Sigma) containing 10% fetal bovine serum (FBS, Sigma), 1% penicillin–

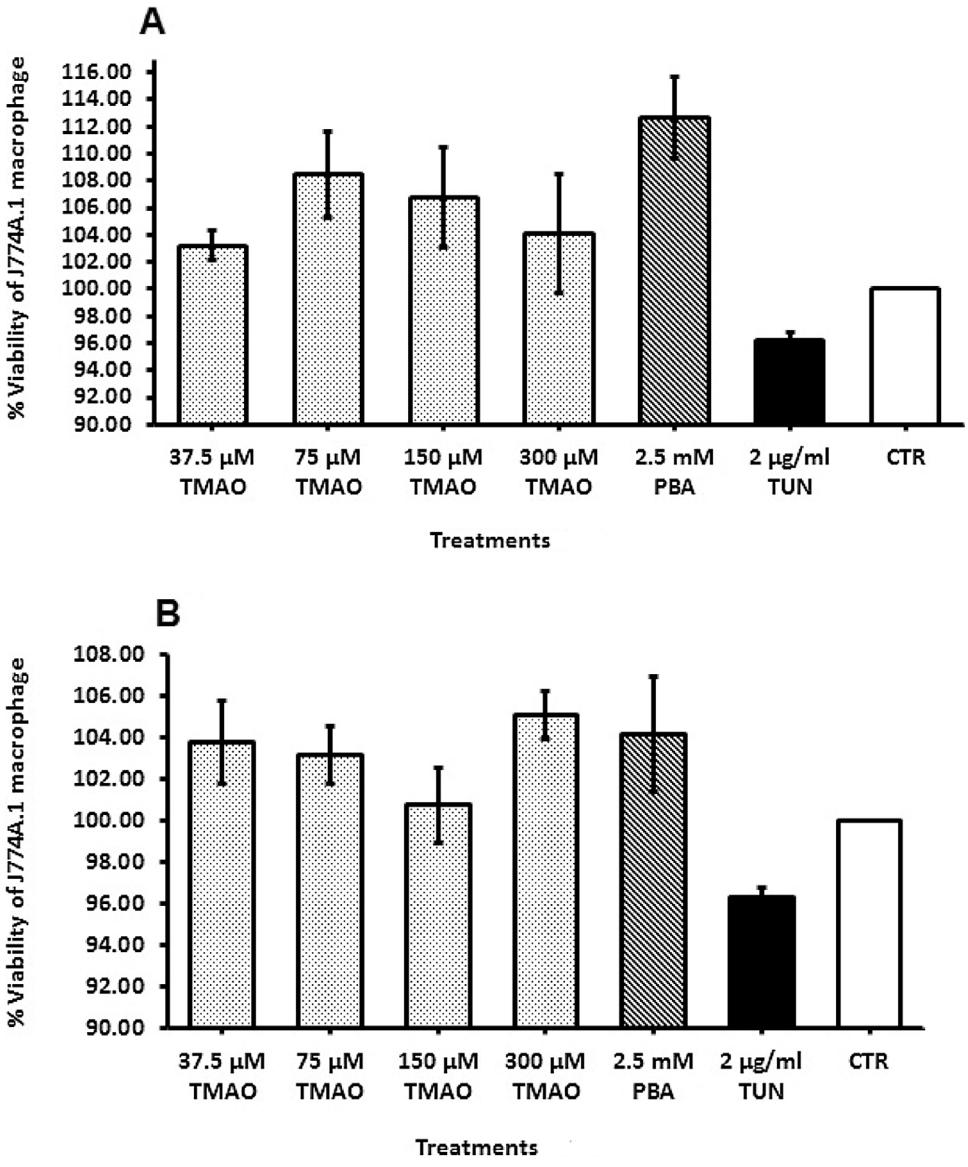

Fig. 1. The result of the MTT test for cell viability of J774A.1 murine macrophage cell line in different treatment and time intervals. A: Treatment 8 h. B: Treatment 18 h C: Treatment 24 h. D: Treatment 48 h. Values are mean ± standard error of six separate measurements.

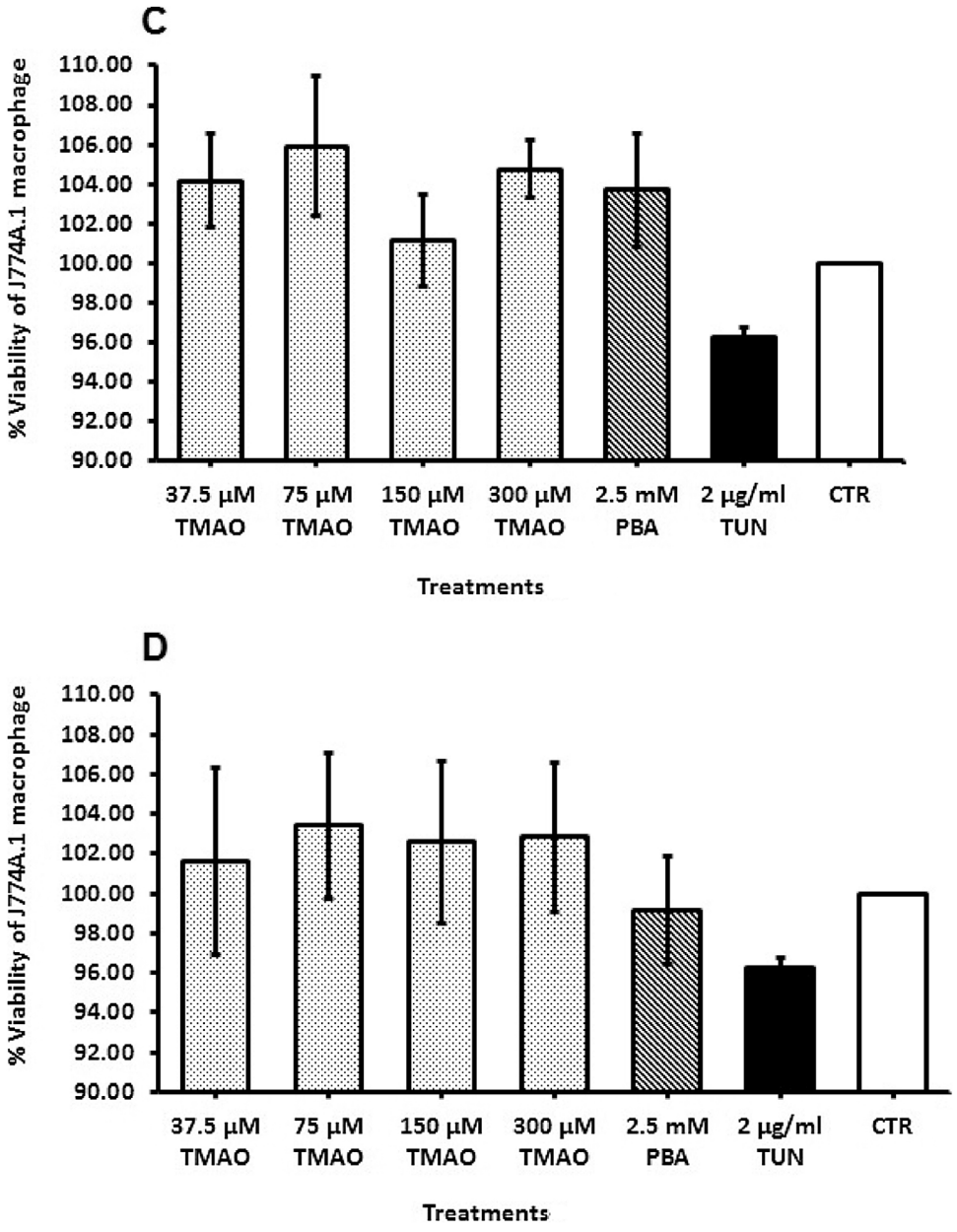

Fig. 1. (Continued)

streptomycin (Sigma), and 4 mM L-glutamine (Sigma). Cells were maintained in a 5% CO_2 incubator under humidified atmosphere at 37 °C. After reaching 90% confluency, cells were treated with 37.5, 75, 150, and 300 μM of TMAO for 8, 18, 24, and 48 h 4-phenylbutyric acid (4-PBA), as a well-known chemical chaperon, was used at 2.5 mM concentration for 8, 18, 24, and 48 h intervals. Tunicamycin was also used as a control for induction of endoplasmic reticulum stress at 2 μg/ml for 18 h. Normal control cells received no treatment. Cell viability was examined by the exclusion of trypan blue and MTT assays.

2.2. MTT assay

MTT Assay was performed as previously described [26]. Briefly, 100 μl of a homogenous cell suspension (100,000 cells per ml in a complete medium) was added in triplicate to the wells in 96-well plates 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 as described in the previous section. After appropriate time intervals for cell incubation, 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 h. After this time, media was carefully removed and 100 μl of MTT solvent (27 ml isopropanol, 3 ml tritonX-100, 2.5 μl concentrated HCl for 30 ml) was added to each well 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 using a microplate reader (Synergy HT, BioTek, USA).

2.3. Western blotting

Cells were scraped and washed twice with ice-cold PBS and homogenized in the protein lysate buffer (Radio-

Immunoprecipitation Assay buffer or RIPA, Sigma) containing complete protease inhibitor cocktail 1 μg/ml, and 1 mM phenyl methyl sulfonyl fluoride at 4 °C using an ultrasonic homogenizer (Hielscher, Germany). Cell debris was removed by centrifugation at 12,000 × *g* for 15 min at 4 °C. Then 100 μg of 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 h at 250 mA in an ice bath. The membranes 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 for 2 h at room temperature. The membranes were then incubated with the primary monoclonal antibodies specific for stress-inducible HSP70 (ADI-SPA-810, Enzo life sciences) and GRP94 (ADISPA-827, Enzo life sciences) for 60 min at room temperature. The membranes were washed three times (20 min each) with TBS-T and incubated with the second antibody (HAF007, R&D) for 1 h 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 HSP70 and GRP94 proteins in treated cells with respect to controls were expressed as a mean ± standard error of three separate measurements.

2.4. Statistical analysis

Data are presented as mean ± standard error of the mean (S.E.M.). Statistical analysis was performed using the SPSS software package version 20. One-Way ANOVA with post-hoc Dunnett test was performed to compare every mean with that of the controls. *P*-values less than 0.05 were considered statistically significant.

3. Results

Based on the results of the MTT test (Fig. 1), cell viability of macrophages in all different treatments and time intervals was higher than 96% and no cell death from the treatments occurred. Figs. 2 B–5 B show relative changes in GRP94 protein levels in macrophages treated with different doses of TMAO and PBA for 8, 18, 24, and 48 h and tunicamycin for 18 h. Tunicamycin-treated cells showed a high significant increase in the relative GRP94 protein levels compared to the control ($P < 0.0001$). After 24 h of treatment (Fig. 4B), except for the 37.5 μM, other doses of TMAO produced a significant increase in relative GRP94 protein levels compared to the control ($P < 0.05$). No significant difference was observed in relative GRP94 protein levels between control and TMAO-treated cells for 8, 18, and 48 h ($P > 0.05$). In macrophage treated for different time intervals, PBA failed to induce any changes in relative HSP70 or GRP94 protein levels. HSP70 was not detected in the control cells and the cells treated with different concentration of TMAO for 8 and 18 h time periods. Figs. 4 C and 5 C show the relative changes in HSP70 protein levels of macrophages treated with different doses of TMAO and PBA for 24 and 48 h and tunicamycin for 18 h. In 24 h treated cells (Fig. 4C), 300 μM of TMAO greatly induced the expression of the HSP70 at protein levels, which was significantly greater than the control and the tunicamycin-treated cells ($P < 0.0001$). In cells treated for 48 h, all doses of TMAO produced a significant increase in relative HSP70 protein levels compared to the control (Fig. 5C).

4. Discussion

Although HSPs were found initially as a cell response to elevations in temperature [27,28], but they are also induced in response to exposure with a wide range of environmental stresses including oxidative stress, ultraviolet irradiation, chemical agents, viruses, and dietary deficiencies [29,30]. HSPs are known as important intracellular chaperones with cytoprotective functions to facilitate folding and stabilize proteins during new synthesis or under denaturing conditions [12]. However, other physiological roles such as intercellular signaling were also found. Furthermore, HSPs can be released into the extracellular space where then in turn they act as immunoregulatory agents, elicit cytokine

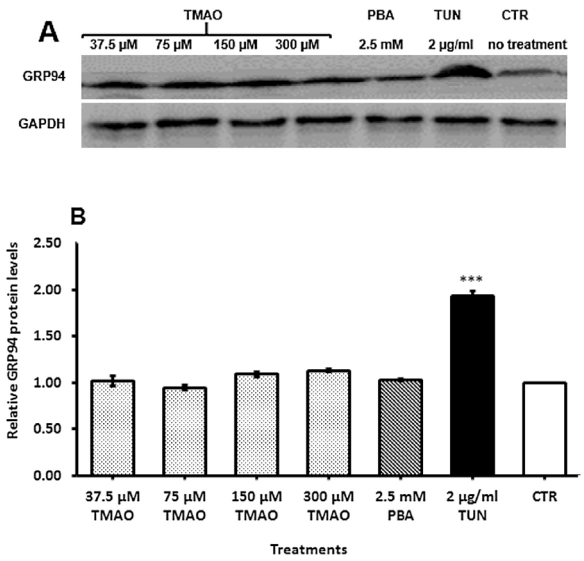

Fig. 2. Changes in GRP94 protein in the murine macrophage J774A.1 cell line after 8 h of treatment with TMAO and PBA, and 18 h of treatment with tunicamycin. **A:** western blotting bands. **B:** relative GRP94 protein levels. TMAO: Trimethylamine-N-Oxide; PBA: 4-Phenylbutyric acid; TUN: Tunicamycin; CTR: Control. Values are mean ± standard error of three separate measurements. P-values less than 0.05 were considered significant. *** shows $P < 0.001$.

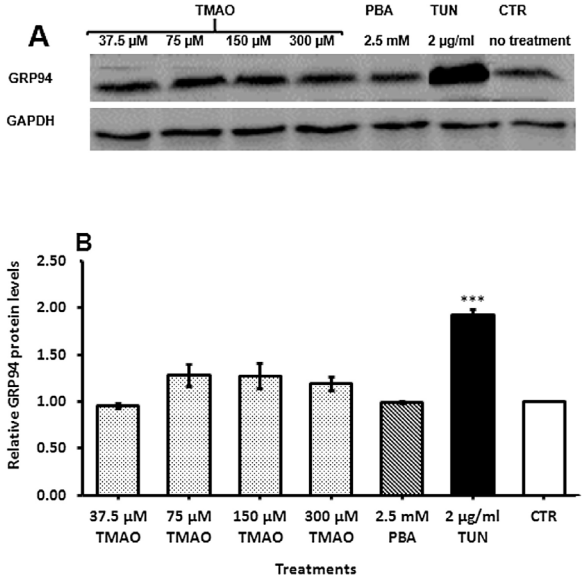

Fig. 3. Changes in GRP94 protein in the murine macrophage J774A.1 cell line after 18 h of treatment with TMAO, PBA, and tunicamycin. **A:** western blotting bands. **B:** relative GRP94 levels. TMAO: Trimethylamine-N-Oxide; PBA: 4-Phenylbutyric acid; TUN: Tunicamycin; CTR: Control. Values are mean ± standard error of three separate measurements. P-values less than 0.05 were considered significant. *** shows $P < 0.001$.

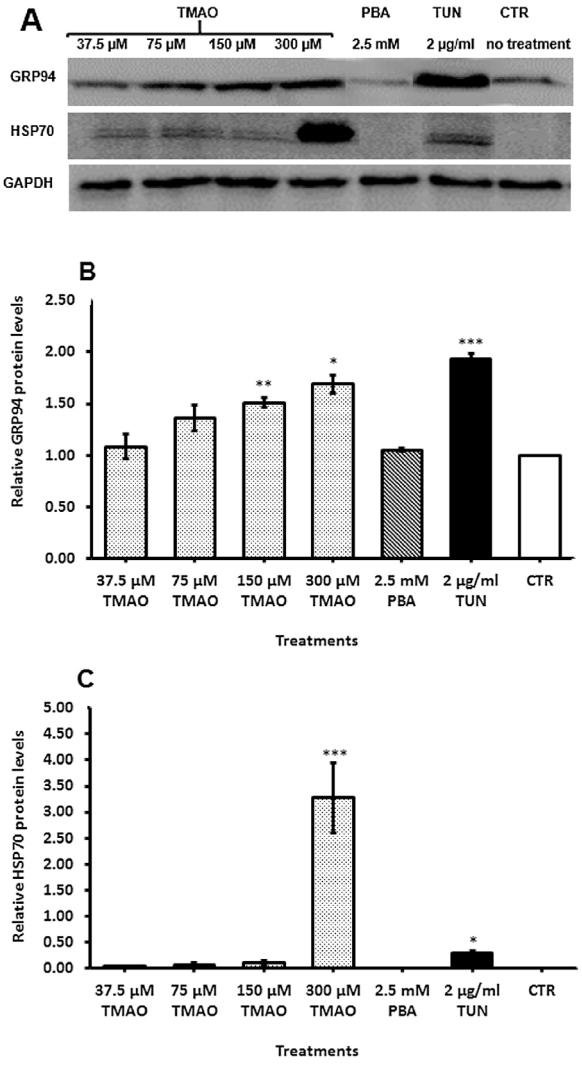

Fig. 4. Changes in GRP94 and HSP70 protein in the murine macrophage J774A.1 cell line after 24 h of treatment with TMAO and PBA, and 18 h of treatment with tunicamycin. **A:** western blotting bands. **B:** relative GRP94 protein levels. **C:** relative HSP70 protein levels. TMAO: Trimethylamine-N-Oxide; PBA: 4-Phenylbutyric acid; TUN: Tunicamycin; CTR: Control. Values are mean ± standard error of three separate measurements. P-values less than 0.05 were considered significant. * shows $P < 0.05$, ** shows $P < 0.01$ and *** shows $P < 0.001$.

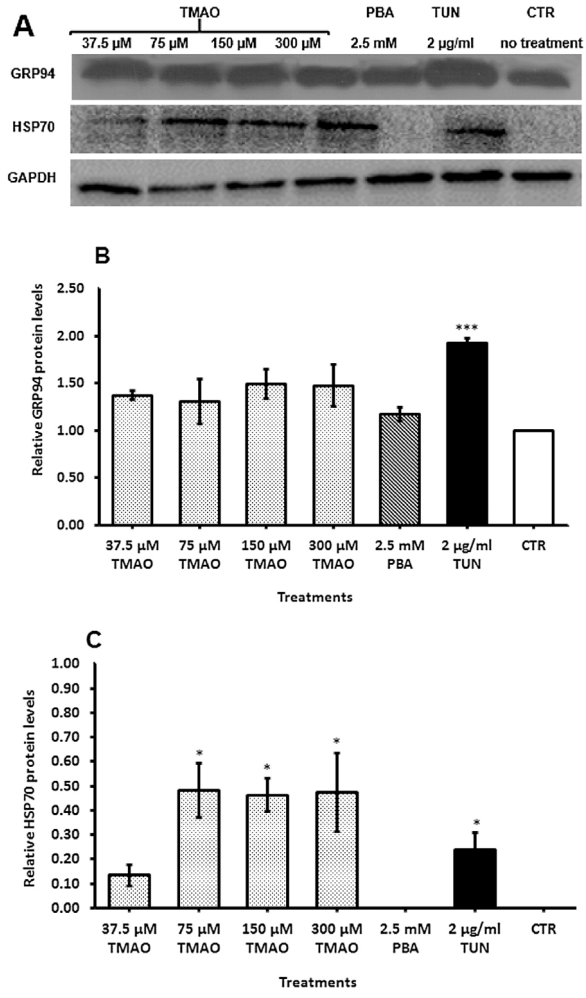

Fig. 5. Changes in GRP94 and HSP70 protein in the murine macrophage J774A.1 cell line after 48 h of treatment with TMAO and PBA, and 18 h of treatment with tunicamycin. **A:** western blotting bands. **B:** relative GRP94 protein levels. **C:** relative HSP70 protein levels. TMAO: Trimethylamine-N-Oxide; PBA: 4-Phenylbutyric acid; TUN: Tunicamycin; CTR: Control. Values are mean ± standard error of three separate measurements. P-values less than 0.05 were considered significant. * shows $P < 0.05$ and *** shows $P < 0.001$.

production, or deliver signals to other cells through receptor-mediated interactions [29–31]. Moreover, there are many pieces of evidence showing their role in pathologic situations such as in atherosclerotic vascular diseases [12,29].

In the present study, we evaluated the effect of TMAO on the expression of GRP94 at protein level in the J774.A1 macrophage cell line. TMAO increased the protein level of GRP94 in a dose/time-dependent manner, so that this elevation was only significant in macrophages treated with high concentration of TMAO for 24 h. GRP78 and GRP94 have been previously introduced as hall marks of ER stress [32,33]. Normally, HSPs bind to three biosensors proteins that are located at ER, including inositol requiring protein 1 (IRE1), activating transcription factor 6 (ATF6), and protein kinase RNA-like ER kinase (PERK). When ER stress is induced, the GRPs are released (and consequently elevated) from the sensor proteins which in turn activates the unfold protein response (UPR) [20,34]. Macdonald et al showed that TMAO through hydrophobic interactions may act as a denaturant agent [35]. Furthermore, it has been noted that TMAO may induce the generation of a reactive oxygen species in part by its potential to inhibit electron transport in some organisms under aerobic conditions [36]. Considering the above-mentioned effects for TMAO, we can argue that TMAO may induce ER stress and UPR pathway. In our study, tunicamycin, a well-established inducer of ER stress, significantly increased the protein level of GRP94. This finding confirms our argument. Although to validate this hypothesis, more research is needed to evaluate the biosensor proteins of ER stress as well as to perform a functional assay for atherosclerosis, like foam cell formation to establish the link between TMAO, ER stress, and atherosclerosis.

It has been shown that extracellular HSPs activate the cellular and humoral immunoinflammatory processes which in turn correlates with the pathogenesis of atherosclerosis [13,16,31,37,38]. HSPs are commonly released during some physiologic or pathologic events and some cell types including neuronal cells, monocytes, macrophages, B cells, and tumoral cells have a specific capacity for this phenomenon [16,39,40]. Several *in-vitro* and *in-vivo* studies have shown that the classic and common risk factors for atherosclerosis such as oxidative stress, hypertension, infection, and other biochemical stressors through activation

of HSP transcription factor 1 to stimulate the HSP expressions in macrophages, smooth muscle, and endothelial cells. During atherosclerosis, circulating HSPs can interact with cell surface receptors such as Toll-like receptor 4/CD14 complex and activate the nuclear factor κb, which triggers innate immune response reactions including the production of inflammatory cytokines in macrophages as well as adhesion molecules in endothelial cells [41,42]. These proinflammatory responses, as well as autoimmunity against the released HSPs [13], correlate with the progression of atherosclerosis with their induction [12]. Therefore, HSPs have a potential to mediate some induced reactions in response to stress exerted on the arteries during atherosclerosis [41].

As previously mentioned, it is believed that atherosclerosis's risk factors induce ER stress as a characteristic feature at different stages of atherosclerosis which in turn triggers an abnormal activation of macrophages and plays an important role in the pathogenesis of atherosclerosis [21–24]. Furthermore, ER stress induction appears to correlate with the upregulation of scavenger receptor A1 and/or ATP-binding cassette transporter A1 reduction in macrophages that are involved in the pathogenesis of atherosclerosis [19]. Similar findings and evidence are also shown by the presence of high circulating levels of TMAO in mice [3] and human [6] or in TMAO treated macrophages [43]. In agreement with the above-mentioned findings, the result of this study and our previous work [26] showed an elevation of GRP94 and GRP78 is a hallmark for ER stress induction in response to the treatment of macrophages with TMAO.

In the present study, we also evaluated the effect of TMAO on the expression of stress-inducible and cytoplasmic isoform of HSP70 at protein levels in the J774.A1 macrophage cell line. HSP70 was not detected in the cells treated with different concentrations of TMAO for 8 and 18 h. TMAO differently induced the expression of HSP70 protein level in cells treated for 24 and 48 h. Usually, the expression of HSPs changes are dependent on the duration and concentration of stimulus (inducer) in a time and/or dose dependent manner. These responses are usually short term and differ in different HSPs [14]. Furthermore, the different stability of the protein and mRNA of HSPs as well as their different extracellular secretion should also be considered for the observed different time and dose dependent responses of HSP70 for cells treated with TMAO in the present study. HSP70 was found to be expressed by all major cell types of the atherosclerotic lesions prone area including macrophages, endothelial, and smooth muscle cells [17]. We evaluated stress-inducible isoform of HSP70 in this study and found that it is not constitutively present in mice [17], but under stress conditions, its expression is stimulated. For this reason, the expression of the stress-inducible isoform of HSP70 in TMAO treated cells can clearly indicate stressed cells.

5. Conclusion

Our results showed that TMAO, a well-established risk factor of atherosclerosis, significantly induced the elevation of GRP94 or the expression of HSP70, suggesting that TMAO may act as a stress inducer in the J774A.1 murine macrophage cell line. Consequently, the result of this study may elucidate a contribution with TMAO in the abnormal activation of macrophages and the possibility of immuno-inflammation reactions involved in the foam cell formation macrophages. To confirm this conclusion, further investigation is required to evaluate the other major ER biosensors, the expression of Toll-like receptor family, and to perform a functional assay for foam cell formation to establish a link between TMAO, ER stress, and atherosclerosis.

Conflicts of interest

The authors declare no conflict of interests.

Financial disclosure

The authors have no funding to disclose.

Acknowledgements

We thank heads of the Cellular & Molecular Research Center, Kurdistan University of Medical Sciences for their valuable assistance.

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