

Investigation of Some Inflammation Related Gene Expressions Under Lipotoxic Endoplasmic Reticulum Stress in Mouse Macrophage Cell Line

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ABSTRACT

Aim: The accumulation of free fatty acids in non-adipose tissues lead to cellular lipotoxicity and induce endoplasmic reticulum (ER) stress. To restore ER homeostasis, cells evoke an adaptive mechanism that is known as the unfolded protein response (UPR). The aim of this study was to investigate the potential relationship between ER stress and some inflammasome complexes under lipotoxic ER stress conditions in the mouse macrophage cell line (RAW 264.7).

Materials and Methods: The mouse macrophage cells (RAW 264.7) were treated with Ethanol-BSA (control) or Palmitate-BSA (500µM). The expression of inflammation-related target genes was investigated using the qRT-PCR method.

Results: A significant induction at the mRNA levels of mTNFα, mNFκB1B, mIRF7, mCCL5, and reduction at the mRNA level of mIRF1 after lipid-induced metabolic ER stress were observed in RAW 264.7 cells.

Conclusion: Together, these findings confirm that UPR regulates the expression of key inflammation-related genes under saturated lipid-induced stress conditions.

Keywords: Endoplasmic reticulum stress, unfolded protein response, inflammation, lipotoxicity

LİPOTOKSİK ENDOPLAZMİK STRES SONUCU İNFLAMASYON İLE İLİŞKİLİ BAZI GEN İFADELERİNİN FARE MAKROFAJ HÜCRE HATTINDA ARAŞTIRILMASI

ÖZET

Amaç: Adipoz olmayan dokularda serbest yağ asitlerinin birikmesi, hücreli lipotoksisteye ve endoplazmik retikulum (ER) stresine neden olur. ER homeostazını geri kazanmak için hücreler, katlanmamış protein tepkisi (UPR) olarak bilinen bir uyarlamalı mekanizma uyandırır. Bu çalışmada, lipotoksik ER stres koşulları altında ER stres ve bazı inflamasyon kompleksleri arasındaki moleküler ilişki fare makrofaj hücre hattında (RAW 264.7) araştırılmıştır.

Gereç ve Yöntem: Fare makrofaj hücreleri (RAW 264.7), Ethanol-BSA (kontrol) veya Palmitate-BSA (500 µM) ile muamele edildi. İnflamasyonla ilişkili hedef genlerin ekspresyon değişimleri qRT-PCR yöntemi kullanılarak araştırıldı.

Bulgular: RAW 264.7 hücrelerinde lipit kaynaklı metabolik ER stres sonrasında; mTNFα, mNFκB1B, mIRF7, mCCL5 mRNA seviyelerinde artış ve mIRF1 mRNA seviyesinde azalma olduğunu gözlemledi.

Sonuç: Bu çalışmanın sonuçları UPR' nin, doymuş lipit kaynaklı stres koşulları altında temel inflamasyonla ilişkili genlerin ekspresyonunu düzenlediğini doğrulamaktadır.

Anahtar sözcükler: Endoplazmik retikulum gerilimi, katlanmamış protein cevabı, inflamasyon, lipotoksisteye

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Endoplasmic Reticulum (ER) is an important metabolic organelle that plays critical roles in the cell, such as, protein synthesis and folding, degradation of misfolded proteins, lipid biogenesis and regulation of Ca^{2+} metabolism (1,2). The synthesis and folding of secreted transmembrane proteins, which comprise nearly one-third of all proteins produced in the cell, also take place in the ER (3,4). Many environmental conditions, both endogenous and exogenous, can disturb the protein-folding capacity of the ER and lead to accumulation of misfolded or unfolded proteins. Therefore, in such cases, ER homeostasis imbalance can lead to a condition known as ER stress. To maintain ER homeostasis, cells develop an adaptive mechanism, known as unfolded protein response (UPR) (5). The UPR transmits information about the protein folding status from the ER lumen to the nucleus and cytosol. The UPR is controlled by three major ER-transmembrane stress sensor proteins; Protein kinase RNA-like ER kinase (PERK); Inositol requiring protein-1 (IRE1); and Activating transcription factor-6 (ATF6) (6). Depending on the duration or dose of the stressors, firstly, the UPR promotes cell survival by activating transcription and translation of target genes or activating apoptotic gene expression in the cell to reestablish ER homeostasis (2,7). PERK responds to ER stress by phosphorylation of eukaryotic translation initiation factor 2 α (eIF2 α). This leads to a general inhibition of protein translation and reduces the accumulation of unfolded proteins in the ER. If ER stress is severe, PERK dependent ER signaling pathway can induce the transcription of C/EBP-homologous protein (CHOP) that drives the stressed cells to apoptosis (7). IRE1 is the other UPR sensor protein and it has RNase and kinase domains, which contribute to the re-establishment of ER homeostasis. IRE1 catalyzes the splicing of X-box binding protein 1 (XBP1) mRNA and spliced XBP1 (sXBP1) functions as a transcription factor that up-regulates the expression of ER chaperones and other ER stress response target genes. Furthermore, through its kinase domain, IRE1 can associate with some adaptor proteins to regulate inflammatory and apoptotic pathways. The final arm of the UPR is ATF6, which is an ER transmembrane protein with a transcription factor function. ATF6 has multiple domains with different functions, luminal domain (associates with BIP), cytosolic domain (contains bZIP motif), and transmembrane domain (contains Golgi target sequence) (8,9).

Studies reveal multiple levels of linkage between UPR and inflammation (10,11,12,13). Previous studies have shown that tauroursodeoxycholic acid (TUDCA)-treated, an effective inhibitor of ER stress, can reduce ER stress and inflammasome activation in tunicamycin-incubated

genetically obese mice ob/ob models (14). It was recently suggested that IRE1 could regulate inflammasome activation through TXNIP upregulation (12). UPR signaling has been shown to have crucial functions in innate and adaptive immune responses. For example, XBP1 has an important role in plasma and dendritic cell differentiation (15). In addition, XBP-1 contributes to CD8+ T cell differentiation during acute infection (16). NF- κ B is an important transcription factor in inflammation and studies have shown that ER stress conditions can lead to NF- κ B activation (17). NF- κ B inhibitor beta (NF κ BIB) inhibits NF- κ B in the cytoplasm of the cell (18). Although three UPR sensor proteins have also been shown to play a role in the regulation of NF- κ B protein, the association of NF κ BIB with the ER stress response under lipotoxic stress conditions has not yet been demonstrated. In addition to their role in NF- κ B activation, IRE1 and PERK can regulate inflammatory responses in many other ways. Studies have shown that the expression of tumor-necrosis factor- α (TNF α), one of the pro-inflammatory cytokines, can be regulated by IRE1- XBP1 branch (19). Moreover, it was shown that saturated fatty acids (SFA) activate UPR sensor proteins, especially IRE1, and lead to an accumulation of immune cells. SFAs also support the metabolic syndrome by activating Toll-like receptors (TLR) (20). Interferon regulatory factor 7 (IRF7), a transcription activator protein, has been shown to reduce the inflammation response through TLR. IRF7 and Interferon regulatory factor 1 (IRF1) are the transcriptional regulator of type I interferon (IFN) and type I IFN responsive genes and are downstream factors of TLRs. Hence, in this study, the effect of saturated fatty acid on IRF7 and IRF1 mRNA levels was investigated in RAW 264.7 cells.

Chemokine (C-C motif) ligand 5 (CCL5), also known as RANTES, is a highly critical protein for immune surveillance and inflammation. Increased expression of CCL5 was observed in tunicamycin induced ER stress conditions (21), but it has not been investigated under lipotoxic ER stress in mouse macrophage cells yet.

Previously, it was shown that lipids activate UPR in macrophages (22) and activated UPR increases reactive oxygen species (ROS), inflammation and disrupts cellular functions eventually leads to the cells death (23). Based on this information, it was aimed to investigate the effect of saturated fatty acid, palmitic acid, induced ER stress on TNF α , NF κ BIB, IRF7, CCL5 and IRF1 mRNA levels in RAW 264.7 mouse macrophage cell lines.

Methods

Preparation of palmitic acid-bovine serum albumin complex

Palmitic acid (PA) was prepared as 500 mM stock solution by dissolving it with absolute ethanol and stored at -80°C . To obtain a working concentration, palmitic acid was diluted in 1% fatty acid-free Bovine Serum Albumin (BSA) in RPMI-1640 medium (without serum) and warmed at 50°C for 30 minutes with occasional vortexing (22).

Cell Culture and treatments

The RAW 264.7 mouse leukemic monocyte-macrophage cell line was acquired from American Type Culture Collection (ATCC) and grown in 10% heat-inactivated Fetal Bovine Serum (FBS), FBS was incubated in 55°C for 1 hour for heat inactivation and supplied with 1% L-glutamine RPMI medium (24). The cells were treated with $500\mu\text{M}$ palmitic acid (Sigma, P0500) for 6 hours in order to generate lipotoxic ER stress on the cells.

RNA Isolation and Quantitative RT-PCR

Total RNAs were isolated from palmitic acid-treated and control RAW 264.7 cells using Trisure/TRIzure (Bioline) according to manufacturer's protocols. Revert Aid First-strand cDNA synthesis kit (Thermo Scientific; K1622) was used for reverse-transcription of RNA into cDNA according to the manufacturers' instructions. PCR amplification was performed in the Rotor-Gene (Qiagen) Realtime PCR instrument using specific primers (Table 1). $2\mu\text{l}$ of diluted cDNA was used in the reaction. Each PCR was carried out in a $10\mu\text{l}$ reaction mixture containing $5\mu\text{l}$ SYBR mix, $1\mu\text{l}$ forward and reverse primers. The thermal cycling conditions were as follows; initial hold 15 min at 95°C , followed by 45 cycles 10 sec at 95°C , 30 sec at 60°C and 30 sec at 72°C . GAPDH transcript levels were used for normalization.

Statistical Analysis

Fold change expression of interested genes were calculated by using $\Delta\Delta\text{CT}$ method; (Primer efficiency)- $\Delta\Delta\text{CT}$ where $\Delta\Delta\text{Ct}$ means ΔCt (target gene) - ΔCt (reference gene) and Ct means (threshold cycle) (25). Results were analyzed from three independent experiments using the Student's t-test.

Results

Regulation of some inflammation-associated gene expression levels under lipid-induced ER stress conditions in RAW 264.7 mouse macrophage cell lines.

Lipids are structural components of all cellular membranes and act as signaling molecules. Excess lipids in cells that could not be stored may promote the generation of lipotoxicity. So, it triggers ER stress and inflammation, which can lead to apoptotic cell death (23). Studies showed that immune cells such as macrophages treated with saturated fatty acid increases ER stress (26), and regulates IL- 1β mRNA production and mature IL- 1β secretion from the cell (22). In this study, the effect of palmitic acid-induced ER stress on gene expression associated with inflammation was investigated in RAW 264.7 cells, and the levels of some inflammation-related gene expression were significantly shown to change under lipotoxic ER stress (Figure 1A-E). Previously, the expression of TNF α was shown to be increased in macrophages under palmitic acid-induced lipotoxic conditions (27). As expected, the mRNA expression of TNF α was increased more than two-fold in palmitic acid-treated RAW 264.7 cells (Figure 1A). Additionally, NF- κB is an important transcription factor in inflammation and studies have shown that ER stress conditions can also lead to NF- κB activation (19). NF κB

Table 1. Real Time PCR Primers

Gene	Forward	Reverse
Mouse	5'-3'	5'-3'
TNF α	AAGCCTGTAGCCACGTCGTA	GGCACCCTAGTTGGTTGTCTTTG
NF κB	GCGGATGCCGATGAATGGT	TGACGTAGCCAAAGACTAAGGG
IRF7	GAGACTGGCTATTGGGGAG	GACCGAAATGCTTCCAGGG
IRF1	ATGCCAATCACTCGAATGCG	TTGTATCGGCCTGTGTGAATG
CCL5	GCTGCTTTGCCTACCTCTCC	TCGAGTGACAAACACGACTGC
GAPDH	GTGAAGGTCGGTGTGAACG	GGTCGTTGATGGCAACAATCTC
sXBP1	TGAGAACCAGGAGTTAAGAACACGG	CCTGCACCTGCTGCGGAC
CHOP	AAGATGAGCGGGTGGCAGCG	GCACGTGGACCAGGTTCTGCT

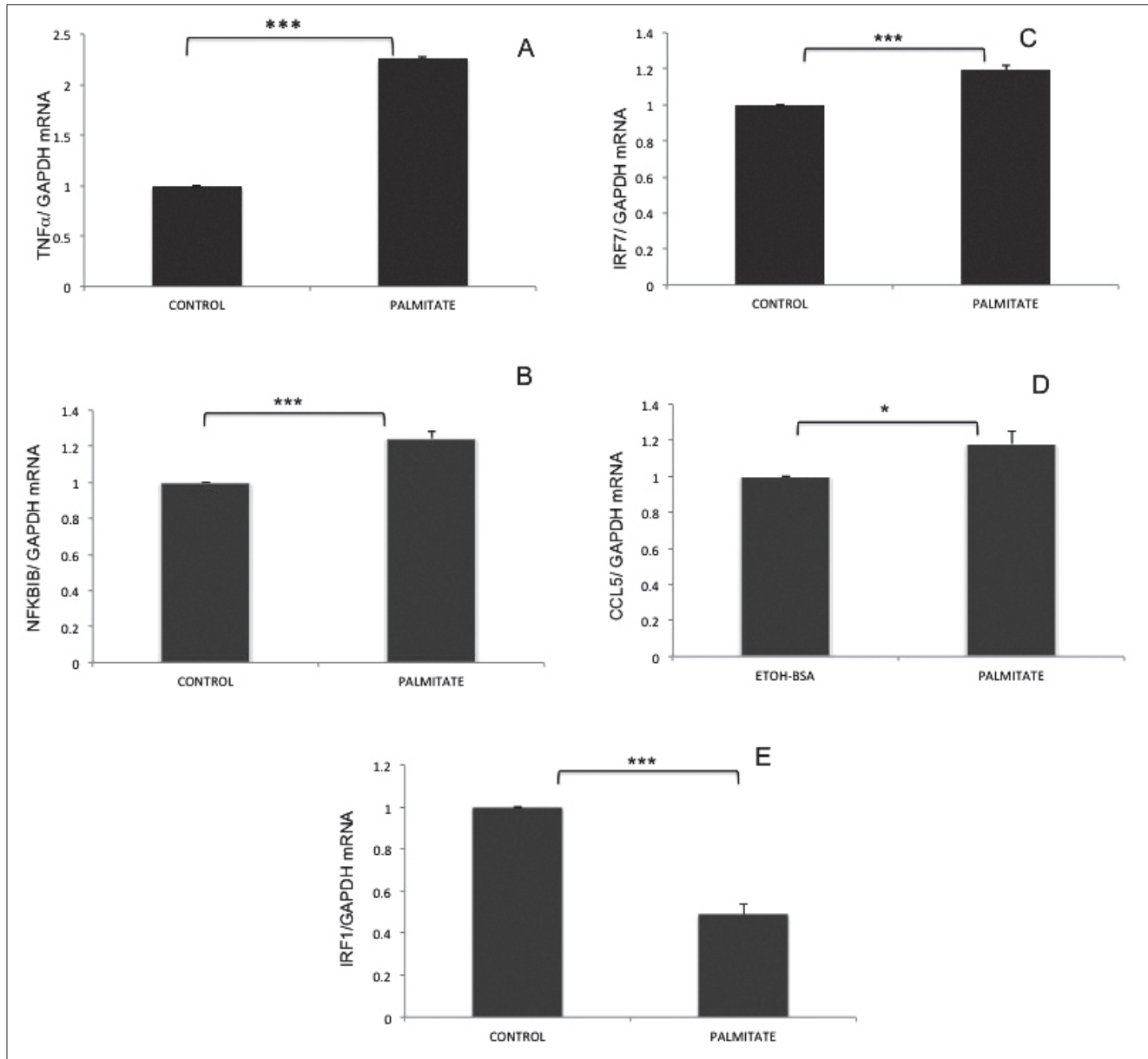


Figure 1. The effect of lipotoxic ER stress on some inflammation related gene expression.

(A-E) qRT-PCR analysis of some inflammation related genes, mTNF α , mNF κ B1B, mIRF7, mCCL5, mIRF1, in RAW 264.7 mouse cell lines treated with 500 μ M Palmitic acid or ETOH-BSA (control). Data: mean values \pm SEM; n = 3. Student's t test. *P \leq 0.05; **P \leq 0.01; ***P \leq 0.001.

inhibits NF- κ B activation under normal conditions; in this study, the mRNA expression level of NF κ B1B was increased 1.2 fold under lipotoxic stress conditions (Figure 1B). Similarly, the mRNA levels of a highly critical protein for immune surveillance and inflammation (CCL5) were increased in RAW 264.7 cells under the same conditions (Figure 1D). Studies have shown the activation of Toll-like receptors by SFAs (28, 29). So, the effect of palmitic acid on the expression levels of TLR related transcription factors IRF7 and IRF1 was also investigated. The mRNA level of IRE7 was increased by 1.2 fold (Figure 1C), while the

IRF1 was decreased by half in palmitic acid-treated mouse macrophage cells (Figure 1E).

The critical role of saturated fatty acids in ER stress response was shown in previous studies. So, the effect of lipotoxic ER stress on IRE1 related sXBP1 mRNA level and PERK related CHOP mRNA level in palmitic acid-treated RAW 264.7 mouse cells were analyzed in this study. It was observed that PERK-CHOP pathways did not show a significant change at the mRNA expression level of CHOP, whereas sXBP1 expression was increased more than five-fold due to lipotoxicity (P < 0.001) (Figure 2A and 2B).

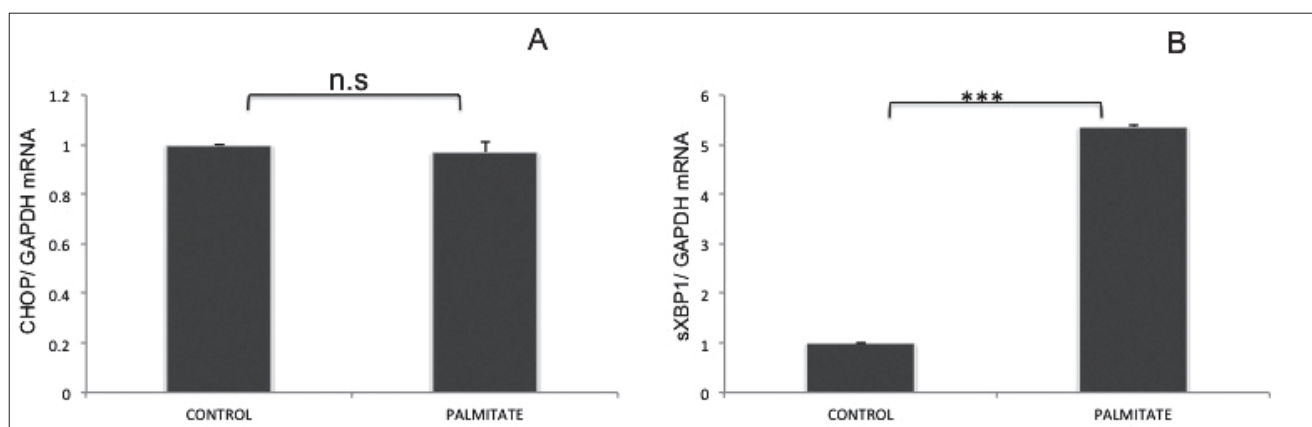


Figure 2. Effect of lipotoxic ER stress on sXBP1 and Chop mRNA levels in RAW 264.7 mouse cells
 A) q-RT-PCR analysis of PERK-dependent CHOP mRNA expression. B) q-RT-PCR analysis of IRE1-dependent sXBP1 mRNA expression in RAW 264.7 cells treated with 500 μ M Palmitic acid or with ETOH-BSA (control). Data: mean values \pm SEM; n = 3. Student's t test. ns, not significant. ***P \leq 0.001.

Discussion

Many metabolic diseases including obesity, type 2 diabetes and cardiovascular diseases are characterized by increased cytokine production and inflammatory gene expression (30). Inflammation is an innate immune response to repair tissue damage and occurs mainly through myeloid cells such as monocytes, macrophages, and neutrophils (31). Excessive intake of saturated fatty acids is an important factor that plays a crucial role in many metabolic disorders. Saturated fatty acid overload induces inflammatory signaling as well as stress signaling pathways such as endoplasmic reticulum stress, reactive oxygen species production and apoptosis (32). Studies show that one of the major causes of these diseases is the excess fatty acid accumulation in non-adipose tissues, which leads to lipotoxicity (33). Under cellular conditions, the ER regulates the composition of the intracellular fatty acid pool and plays a central role in managing lipotoxicity. Thus, lipid metabolism has an important role in maintaining the ER membrane function and its disturbance can cause major challenges that might disrupt the ER function and lead to the accumulation of unfolded protein in the ER. Prolonged ER stress is harmful to the cells and plays an important role in the development and progression of many metabolic diseases such as obesity, diabetes, atherosclerosis and cancer (34). Cells adapt to ER stress by activating an integrated signal transduction pathway called the unfolded protein response. UPR attempts to restore cellular homeostasis and maintain cell survival via reducing unfolded protein levels, however, chronic exposure to ER stress by excess lipids can activate many pro-apoptotic and inflammatory pathways. Saturated fatty acid uptake has been associated with inflammation, but it has not yet

been elucidated in the studies on which this effect is induced by which intracellular mechanisms?!?. Although the UPR is known to be involved in the pathogenesis of inflammation, the cellular mechanism of ER related inflammation has not been deeply investigated yet.

In this study, a significant induction at the mRNA levels of mTNF α , mNf κ B, mIRF7, mCCL5, and reduction at the mRNA level of mIRF1 were observed after lipid-induced metabolic ER stress in RAW 264.7 cells. In addition, the expression of sXBP1 was increased, which induces the unfolded protein response via the IRE1 signaling pathway activated in the cell as a result of the lipotoxic ER stress, but the mRNA expression level of CHOP did not show any significant change relative to IRE1.

These findings suggest that lipid stress causes increased expression of some of the important genes associated with inflammation through the IRE1-XBP1 pathway, and that in future studies, these genes can be targeted to reduce inflammation in many diseases. In addition, it was confirmed that UPR regulates the expression of key inflammation-related genes under saturated lipid-induced stress conditions. More detailed studies are needed to determine, which UPR arm is responsible for the changes in mRNA levels of inflammatory genes in mouse macrophage cells.

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