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Prototypic $P2X_7$ Receptor Agonist, BzATP, Induced the Expression of Unfolded Protein Response Genes in Human M1 Macrophages

Maryam Akhtari[1,2,3], Seyed Jalal Zargar[1], Ali Javinani[2], Amir Ashraf-Ganjouei[2], Mahdi Vojdanian[2], Ahmadreza Jamshidi[2], and Mahdi Mahmoudi[2,3]

[1] Department of Cell and Molecular Biology, School of Biology, College of Science, University of Tehran, Tehran, Iran
[2] Rheumatology Research Center, Tehran University of Medical Sciences, Tehran, Iran
[3] Inflammation Research Center, Tehran University of Medical Sciences, Tehran, Iran

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ABSTRACT

Purinergic receptors stimulation by adenosine triphosphate (ATP) contributes significantly to macrophage activation, and also macrophage cell death. Upon the macrophage activation, the protein load of the endoplasmic reticulum is increased which is resulted in the activation of unfolded protein response (UPR). In the current study, we aimed to evaluate the connection between prototypic $P2X_7$ receptor agonist, extracellular 2′(3′)-O-(4-Benzoylbenzoyl)-ATP (BzATP), and the UPR pathway in macrophages.

The monocyte-derived macrophages from blood samples of 14 healthy volunteers were skewed toward M1 macrophages after incubation with LPS and IFN-γ. M1 macrophages were treated with 200 μM BzATP. The expression levels of UPR genes, including *CHOP, HERP, GADD34, XBP1,* and *ATF6* in macrophages before and after treatment were measured using real-time polymerase chain reaction.

The results demonstrated that the expression of *CHOP, HERP,* and *ATF6* is significantly decreased and the expression level of *GADD34* and *XBP1* is significantly increased after M1 polarization. BzATP not only significantly increased the expression levels of *CHOP, GADD34*, *ATF6,* and *HERP* but also significantly decreases the *XBP1* expression level in M1 macrophages.

The present study showed that BzATP induces cellular stress in M1 macrophages by elevating the expression levels of UPR genes including *CHOP, GADD34*, *ATF6*, and reducing cell viability.

Keywords: 3'-O-(4-benzoyl) benzoyladenosine 5'-triphosphate; Cell death; Purinergic receptors; Unfolded protein response

Corresponding Authors: Seyed Jalal Zargar, PhD;
Department of Cell and Molecular Biology, School of Biology, College of Science, University of Tehran, Tehran, P.O.Box: 141556455, Iran. Tel: (+98 21) 6111 3646, Fax: (+98 21) 6649 2992, E-mail: zargar@ut.ac.ir.

Mahdi Mahmoudi, PhD;
Rheumatology Research Center, Tehran University of Medical Sciences, Tehran, P.O.Box: 1411713137, Iran. Tele/fax: (+98 21) 8822 0064, E-mail: mahmoudim@tums.ac.ir.

INTRODUCTION

Adenosine triphosphate (ATP) is the primary fuel of the cellular metabolism that is chiefly located in the intracellular compartment. However, the ATP in the abnormal milieu, including the extracellular environment could act as a danger signal and subsequently activates the danger sensing receptors.[1] Purinergic receptors are sensors of an evolutionary ancient signaling cascade that is mainly triggered by ATP and its metabolites. It consists of a diverse range of receptors in nearly every cell that mediates specific functions depending on the cellular context.[2]

P2X receptors are membrane-bound ion channels and P2Y ones are G-protein coupled receptors that are activated by extracellular nucleotides such as ATP.[2] These receptors play a significant role in controlling the immune system function and responses. During an inflammatory process, the inflammatory, necrotic and apoptotic cells release the ATP to the extracellular space. ATP makes the immune cells like neutrophils and macrophages secrete the pro-inflammatory mediators, including prostaglandins and cytokines that further aggravate the inflammatory process.[3]

$P2X_7$ receptor ($P2X_7R$) is a member of P2X receptors with the most identified function in immune responses. The activation of $P2X_7R$ by ATP and lipopolysaccharide in macrophages leads to $P2X_7R$ opening and potassium ions efflux and inflammasome activation.[4] It is dominantly demonstrated in the context of malignant and infectious disorders that the extracellular ATP via $P2X_7R$ induces macrophage's cellular death and suppresses the normal immune defense against the cancerous and infected cells.[5,6]

Unfolded protein response (UPR) is a cellular pathway that mediates cellular responses to endoplasmic reticulum (ER) stress.[7] When the cellular demand of protein production and secretion overcomes the ER capacity, the UPR pathway is activated. Besides, during inflammation and immune cell activation, the protein load of the endoplasmic reticulum may be increased, which could activate the UPR.[8] Firstly, the UPR leads to the expression of proteins that expand the ER activity and also help the maturation of unfolded proteins. If this strategy cannot resolve the ER stress, autophagy, and cell death will occur to eliminate the cells with abnormal function. UPR pathway includes three signaling arms which are orchestrated with three ER transmembrane sensors: 1. Inositol-requiring kinase/endoribonuclease 1 (IRE-1), 2. Activating transcription factor 6 (ATF6), and 3. Protein kinase RNA-activated (PKR)-like ER kinase (PERK). These transmembrane sensors are bound to chaperon proteins like glucose-regulated protein (GRP78, Bip) under physiological conditions. However, during the ER stress, the chaperons are released and they become activated. After activation, IRE-1 with its RNase activity processes and splices XBP1 mRNA to generate a functional transcription factor. Activation of the IRE-1 arm leads to enhancement in protein folding, ER-associated degradation (ERAD), and phospholipid synthesis. PERK activation leads to synthesis attenuation of general proteins and activation of the transcription of genes involved in oxidant responses, apoptosis, and autophagy. ATF6 activation leads to increased expression of X-box binding protein 1 (XBP1) and genes involved in ERAD.[7,9]

Interestingly, the $P2X_7R$ activation via extracellular ATP could mediate the ER stress and cellular death in a neuronal cell.[10] Although it is found the eATP can induce cellular inflammation or cellular death in immune cells, data regarding the connection of the extracellular ATP and UPR pathway in immune cells is lacking. Due to the role of $P2X_7R$ activation on macrophages and the role of ER stress in macrophages function, we aimed to investigate the probable link between 2′(3′)-O-(4-Benzoylbenzoyl) adenosine-5′-triphosphate tri (triethylammonium) salt (BzATP) as a prototypic $P2X_7$ receptor agonist with the UPR genes expression in M1 macrophages.

MATERIALS AND METHODS

Participants

In the present study, 20 milliliters of blood samples were taken from 14 healthy individuals (11 men, 3 women, with a mean age of 32 years). The research was conducted with the human subjects' understanding and all contributors provided signed written informed consent before they participated in the study. This study was performed based on the Declaration of Helsinki guidelines and was approved by the Ethics Committee of Tehran University of Medical Sciences (Approval No: IR.TUMS.REC.1394.1504).

Monocyte Isolation and Macrophage Differentiation

Samples were drawn into an Ethylene diaminetetraacetic acid (EDTA) containing tubes and were processed in less than 4 hours. Blood samples were diluted by phosphate-buffered saline (PBS, ratio of 1:2 in the pH of 7.2). The peripheral blood mononuclear cells (PBMCs) were isolated from the samples using Ficoll (Innotrain, Germany) and by density gradient centrifugation. Extracted PBMCs were washed with PBS solution (Gibco Invitrogen, USA). Cells were primarily incubated with MACS CD14 microbeads for the isolation of monocytes from the PBMC samples. Applying magnetic-activated cell sorter columns, attached cells to CD14 microbeads were separated (MACS, all from MiltenyiBiotec, Germany). The flow cytometry analysis by anti-CD14 antibody (BD bioscience, USA) showed the purity of 92-95% of separated cells.[11] The isolated monocytes were cultured in Roswell Park Memorial Institute (RPMI, 500,000 cells per well, Gibco, Thermo Fisher Scientific, USA) in the presence of recombinant human macrophage-colony stimulating factor (0.05 µg/mL M-CSF; 7 days), for shifting them toward macrophage. L-glutamine (0.002 M, Biosera, France), streptomycin, penicillin (0.1 mg/mL and 100 U/mL respectively, Sigma-Aldrich, USA), and fetal bovine serum (10% FBS; Gibco, BRL, USA) were also contained in culture media. Macrophages surface proteins (CD163 and CD206) were analyzed by flow cytometry.[11]

Polarization Toward M1 Macrophages

To polarized monocyte-generated macrophages (M0 macrophages) toward M1 macrophages, lipopolysaccharide (0.1 µg/mL LPS; Sigma-Aldrich, USA), and interferon-gamma (1000 U/mL IFN-γ; R&D systems, USA) were added to culture media, and cells were incubated for 24 hours. After incubation, the transcriptional phenotype of M1 macrophage was analyzed by SYBR green real-time polymerase chain reaction (PCR). The expression of M1 macrophage-definite genes (CXCL11, IDO, and CCR7) was elevated and the expression of M0 macrophage-specific genes (CD36 and MRC1) was diminished in the macrophages after 1-day exposure to LPS/IFN-γ.[12]

Assessment of the UPR Genes Expression after BzATP Treatment

M1 macrophages were stimulated with BzATP (200 µM, Sigma-Aldrich, USA). Samples were incubated with or without BzATP for 24 hours. The total RNA was extracted from M0, BzATP treated, and untreated M1 macrophages by the High Pure RNA Isolation Kit (Roche, Germany). The complementary DNA (cDNA) was synthesized from an equal amount of total RNA by CellAmpdirect RNA prep kit for real-time PCR (Takara bio, Japan). The relative expression level of *C/EBP homologous protein (CHOP), Homocysteine-induced endoplasmic reticulum protein (HERP), Growth arrest and DNA damage-inducible protein (GADD34, PPP1R15A), X-Box binding protein 1 (XBP1),* and *Activating transcription factor 6 (ATF6)* genes was measured by SYBR green master mix (Ampliqon, Denmark) using StepOnePlus™ real-time polymerase chain reaction (PCR) system (Applied Biosystems, Foster City, USA). The housekeeping gene was *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) (Table 1). The comparative CT method ($2^{-\Delta CT}$) was performed[13] to compare the expression level of UPR pathway genes between the BzATP-incubated and the control group.

Statistical Analysis

All of the variables were scanned for normal distribution using the Shapiro-Wilk test. To compare the expression level of UPR genes between the BzATP-treated and control groups, the independent t-test was performed. *p-values* less than 0.05 were considered statistically significant. Graphs were designed by GraphPad Prism 6 and statistical analysis was performed using SPSS version 22.

RESULTS

The Expression Level of UPR Genes in M1 Macrophages

The basal gene expression level of UPR components was evaluated after M1 macrophages polarization (Figure 1). The expression of *CHOP* ($p \leq 0.01$, -3.4-fold), *HERP* ($p \leq 0.05$, -3.4-fold), and *ATF6* ($p \leq 0.001$, -1.4-fold) was significantly decreased after M1 polarization. However, the expression level of *GADD34* ($p \leq 0.01$, 1.3-fold) and *XBP1 (*$p \leq 0.01$, 1.6-fold) was significantly increased after the polarization toward the M1 type.

The Expression Level of UPR Genes in M1 Macrophages after BzATP Treatment

In the present study, the influence of BzATP (200 µM) as a $P2X_7R$ agonist on the UPR pathway in

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M1 macrophages was evaluated. The influence of BzATP treatment on gene expression is shown in Figure 2. BzATP significantly increased the expression level of *GADD34* ($p \leq 0.05$, 1.3-fold), *HERP* ($p \leq 0.01$, 1.6-fold), *CHOP* ($p \leq 0.05$, 1.4-fold), and ATF6 ($p \leq 0.01$, 1.4-fold) in M1 macrophages. The expression level of *XBP1* was decreased by -1.4-fold ($p \leq 0.01$) in M1 macrophages after BzATP treatment.

Table 1. Primer sequences and product size of the studied genes

Gene name	Sequence	Size (bp)
GAPDH	F: 5' GAGTCAACGGATTTGGTCGT 3' R: 5' GACAAGCTTCCCGTTCTCAG 3'	185
CHOP	F: 5' TTGCCTTTCTCCTTCGGGAC 3' R: 5' TGATTCTTCCTCTTCATTTCCAGG 3'	132
GADD34	F: 5' TAAAGGCCAGAAAGGTGCGCT 3' R: 5' GGCTAAAGGTGGGTTCCTGAG 3'	167
XBP1	F: 5' GGCCATGAGTTTTCTCTCGT 3' R: 5' CGAATGAGTGAGCTGGAACA 3'	97
ATF6	F: 5' GCACCCACTAAAGGCCAGAC 3' R: 5' ACTGGGCTATTCGCTGAAGG 3'	146
HERP	F: 5' GAGCCTGCTGGTTCTAATCG 3' R: 5' GAAAGCTGAAGCCACCCATA 3'	168

GAPDH: glyceraldehyde-3-phosphate dehydrogenase, *CHOP*: C/EBP homologous protein, *GADD34*: growth arrest and DNA damage-inducible protein, *XBP1*: X-Box binding protein 1, *ATF6*: Activating transcription factor 6, *HERP*: Homocysteine-induced endoplasmic reticulum protein, bp: Base pair.

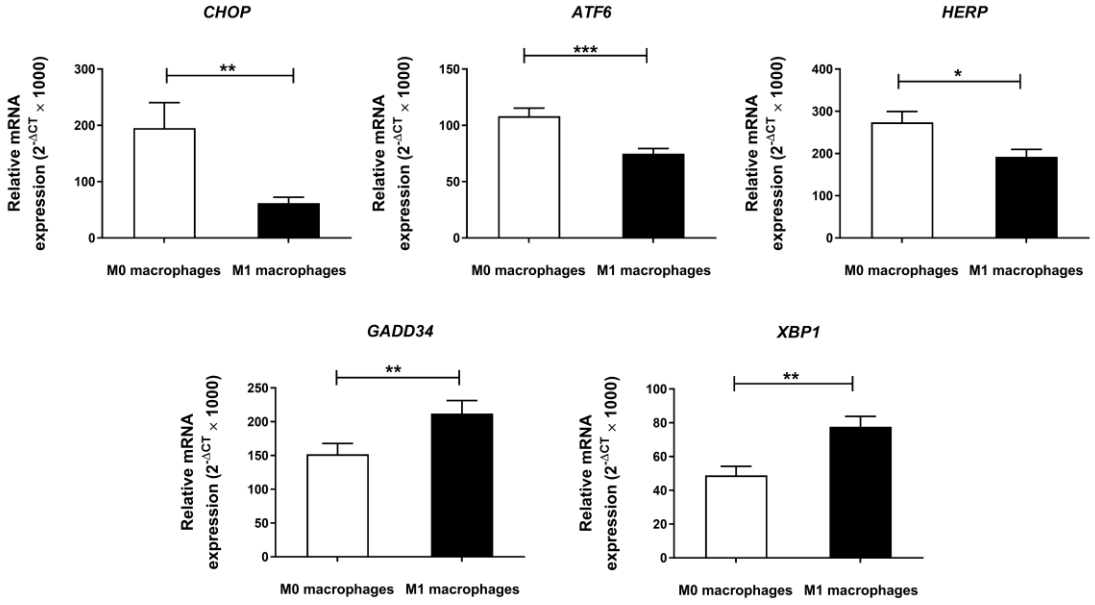

Figure 1. The relative mRNA expression level of *UPR* genes in M0 and M1 macrophages. The expression of *CHOP*, *HERP*, and *ATF6* genes was significantly decreased after M1 polarization. However, the expression level of *GADD34* and *XBP1* was significantly increased after proliferation toward the M1 type. *CHOP*: C/EBP homologous protein, *GADD34*: growth arrest and DNA damage-inducible protein, *XBP1*: X-Box binding protein 1, *ATF6*: Activating transcription factor 6, *HERP*: Homocysteine-induced endoplasmic reticulum protein. The data is presented as the mean±SEM (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

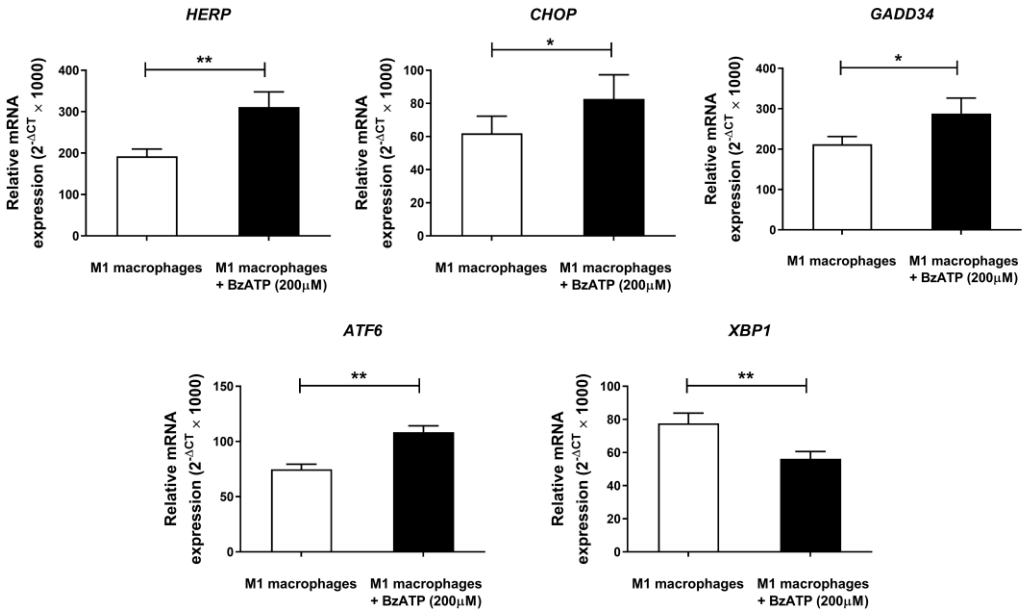

Figure 2. The relative mRNA expression level of *UPR* genes in 200 μM BzATP-treated and untreated M1 macrophages. BzATP significantly increased the expression level of *HERP*, *CHOP*, *GADD34*, and *ATF6* and significantly decreased *XBP1* expression in M1 macrophages. *CHOP*: C/EBP homologous protein, *GADD34*: growth arrest and DNA damage-inducible protein, *XBP1*: X-Box binding protein 1, *ATF6*: Activating transcription factor 6, *HERP*: Homocysteine-induced endoplasmic reticulum protein. The data is presented as the mean±SEM (p*≤0.05, ***p*≤0.01, ****p*≤0.001).**

DISCUSSION

In the present study, we have shown that the expression of UPR genes is changed by the polarization of primary human macrophages toward the M1 type. Our findings showed that the expression of *GADD34* and *XBP1* genes was increased in M1 macrophages in comparison to M0 macrophages. These changes are compatible with the secretory nature of M1 macrophages and it seems to be crucial for enhancing the macrophages' production capacity of pro-inflammatory cytokines. In line with our findings, previous studies have shown that IRE1 activation following induced ER-stress in macrophages, resulted in cytokine production by XBP1.[14] It was also demonstrated that over-expression of XBP1 by Toll-like receptor (TLR) is needed to overcome the increasing demand on macrophages for cytokine production[15]. Moreover, it was shown that the rising level of GADD34 could help protein synthesize by dephosphorylating the eukaryotic initiation factor (eIF)-2α.[16] In a previous report by Nakayama et al, it was demonstrated that apoptosis is hampered in LPS-treated macrophages. The authors believed that it was because of the overexpression of BIP chaperon and the protective mechanisms of ER function, which prevent CHOP expression.[17] In the current study, we also observed reduced *CHOP* and *ATF6* gene expression in M1 macrophages. Taken together, considering increased *GADD34* and *XBP1* expression and reduced *CHOP* and *ATF6* expression in M1 macrophages, our findings suggested that upon M1 macrophage polarization, the ER stress and UPR pathway is activated toward increasing the secretory capacity of M1 macrophages.

Additionally, we have investigated the effect of extracellular BzATP on the UPR pathway. This study is the first that investigates the connection of the extracellular ATP and the UPR pathway in immune cells. BzATP is the prototypic $P2X_7R$ agonist and can also activate some P2Y receptors.[18] The UPR is detected in diverse cellular lineage, including the human immune system, and contributes to immune system development. It consists of three major arms that synergistically participate in managing the proteosynthesis pathway upon ER activation and skew the cell toward either ER survival or cell death.[19,20] Activation of ATF6 and IRE1α pathways causes *XBP*

expression that will further expand the ER function and augment the secretory capacity of the cells. Moreover, activation of these two arms increases the expression of ER chaperones, including BIP. Nonetheless, intense activation of UPR induces the expression of *CHOP* via the PERK/eIF-2α pathway and induces cell death. This pathway is a commonly shared signaling cascade between UPR and other cellular stress pathways. Whether which one of these three arms dominates upon the UPR activation is probably depends on the cellular micro-environment, co-activation of other cellular pathways, intensity, and duration of the ER stress.[21] The present study showed that the BzATP significantly increased the expression of *CHOP*, *GADD34*, *ATF6*, and *HERP* and diminished the expression of *XBP1*. Previous reports suggested that an increased rate of cell death was occurred by over-expression of *CHOP*, which codes proteins contributed to programmed cell death. CHOP is activated through the PERK/ATF4 pathway and induced ER-stress mediated death.[22] It is also found that ATF6 has an important role in CHOP production and cell death fate.[23] Also, *HERP* is one of the genes that its expression is regulated during ER stress through the PERK/ATF4 arm. During ER stress, eIF-2α phosphorylation induces ATF4 which then directly activates *HERP* and *CHOP* expression.[19] Previously Chao et al. revealed P2X7R-mediated ER stress in the differentiated NG108-15 neuronal cells with an increased level of CHOP and caspase-3 following BzATP treatment.[10]

Extracellular ATP can induce the NLRP3 inflammasome pathway through $P2X_7$ receptors and leads to caspase-1 activation. Caspase-1 contributes to the programmed cell death and inflammatory release of intracellular components.[24] It has been found that prolonged stimulation of LPS-primed macrophages with ATP leads to caspase-1 activation and cell death.[25] It has been also shown that extracellular ATP induces cell apoptosis via $P2X_7R$ and caspase-3/7 activation in mouse macrophages.[26] In addition, Wang and colleagues found that BzATP increases cell death in human cervical epithelial cells.[27] Besides, it is discovered that high concentrations of extracellular ATP can diminish cellular viability in macrophages and induce apoptosis by producing reactive oxygen species (ROS).[28] It is suggested that induced cell death of infected host macrophages via $P2X_7R$ activation is one of the recognized mechanisms for eradicating intracellular parasites.[29,30] In contrast, it is shown that several unknown mediators, including ATP, could elicit cell death in tumor-associated macrophages that subsequently favor tumor propagation and survival.[5] Taken together, these observations suggest that the extracellular ATP induces cell death in macrophages that could be beneficial or detrimental. In line with these results, we previously showed that LPS and INF-γ primed macrophages have decreased cell viability after BzATP treatment.[31]

Considering increased expression of *CHOP*, *ATF6*, *HERP*, and *GADD34* decreased *XBP1* expression, and increased cell death rate in BzATP treated macrophages[31] it is suggested that BzATP activated the ER stress in M1 macrophages toward PERK/eIF-2α arm and cellular death rather than IRE1/XBP1 pathway. However, further studies are required to identify the connection pathway between the extracellular ATP and the UPR pathway in immune cell regulation.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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