*ROLE OF REACTIVE OXYGEN SPECIES IN INFLAMMASOME ACTIVATION IN MICROGLIA UNDER STRESS CONDITIONS*

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**Abstract**

**Introduction:** Microglial cells are the resident immune cells of the Central Nervous System (CNS) intervening in adaptive immune responses by activating the inflammasome, which converts pro-IL-1β to its active form in response to diverse stimuli such as endoplasmic reticulum stress (ER stress) that is induced by the overload of misfolded protein in the ER lumen. Both ER stress and activation of inflammasome have been implicated in the pathogenesis of neurodegenerative diseases, namely in Alzheimer's disease (AD). However, the relationship between changes in ER proteostasis and neuroinflammation is still unclear. In this work we studied the involvement of ER stress in activation of the NLRP3 inflammasome in microglia.

**Methods:** ER stress was induced in the microglia cell line BV2 with brefeldin A (BFA). Using Western blot, MTT, Amplex Red and ELISA assays, the levels of ER stress markers, cell viability, inflammasome activation and generation of reactive oxygen species (ROS) were evaluated.

**Results:** We demonstrated that ER stress activates the NLRP3 inflammasome in microglial cells leading to the release of IL-1β. Furthermore, we showed that ER stress increases the production of ROS, which might act as an intermediate step between loss of ER proteostasis and inflammasome activation. Finally, we provided evidence that microglia cell survival is significantly compromised in response to ER stress.

**Discussion:** These data support that chronic induction of an ER stress response in microglia due to the accumulation of misfolded proteins in the ER lumen, which is characteristic of several neurodegenerative disorders, is followed by ROS generation and activation of the NLRP3 inflammasome. Prolonged ER stress, through several signaling pathways that trigger cell death mechanisms, decreases the viability of microglia cells. However, it remains to be determined whether NLRP3 activation under stress conditions is dependent from ROS accumulation in this cell type.

**Conclusion:** We provide evidence for a causal link between loss of proteostasis due to ER stress and inflammasome activation in microglia, a mechanism that might be implicated in neuroinflammation in protein misfolding neurodegenerative diseases.

**Keywords:** NLRP3 inflammasome; ER stress; microglia

**Resumo**

**Introdução:** As células da microglia são células imunitárias residentes no Sistema Nervoso Central (SNC) que intervêm na resposta imunitária adaptativa, ativando o inflamassoma que por sua vez converte a pró-interleucina 1β na sua forma ativa, em resposta a diversos estímulos como o stresse do retículo endoplasmático (RE) que é induzido pela acumulação de proteínas desenroladas no lúmen do RE. Tanto o stresse do RE como a ativação do inflamassoma estão envolvidos na patogénese de diversas patologias neurodegenerativas como a doença de Alzheimer (DA). No entanto, a relação entre a alteração da proteostase do RE e a neuroinflammação ainda não é clara. Neste projeto estudámos o envolvimento do stresse do RE na ativação do inflamassoma NLRP3 em células da microglia.

**Métodos:** O stresse do RE foi induzido na linha celular de microglia BV2 utilizando brefeldina A (BFA) e os níveis de marcadores de stresse do RE, viabilidade celular, ativação do inflamassoma e produção de espécies reativas de oxigénio (ROS) foram avaliados por Western blott, MTT, Amplex Red e ELISA, respetivamente.

**Resultados:** Demonstrámos que o stresse do RE induz a ativação do Inflamassoma NLRP3 em células de microglia levando à libertação de IL-1β. Além disso, os nossos resultados mostram que o stresse do RE estimula a produção de ROS, o que pode atuar como um passo intermédio entre a perda de proteostase no RE e a ativação do inflamassoma. Demonstrámos ainda que a sobrevivência de células de microglia está significativamente comprometida em resposta ao stresse do RE.

**Discussão:** Os nossos resultados suportam a hipótese de que a indução crónica de uma resposta ao stresse do RE nas células da microglia devido à acumulação de proteínas mal enroladas no lúmen do RE, característica de diversas doenças neurodegenerativas, aumenta a produção de ROS e induz ativação do inflamassoma NLRP3. O stresse prolongado do RE através da ativação de várias vias de sinalização que desencadeiam mecanismos de morte celular, reduz a viabilidade das células da microglia. No entanto, fica por esclarecer se a ativação do inflamassoma NLRP3 em condições de stresse é dependente da acumulação de ROS nestas células.

**Conclusão:** Este trabalho evidencia a existência de uma ligação causal entre a perda de proteostase devido a stresse do RE e a ativação de inflamassoma na microglia, que poderá estar envolvida na neuroinflamação que ocorre em doenças neurodegenerativas em que há acumulação de proteínas mal enroladas.

**Abbreviations and symbols**

**AD** Alzheimer's Disease

**ASC** Apoptosis-Associated Speck-like protein containing a CARD

**ATF6** Activating Transcription Factor 6

**BFA** Brefeldin A

**BSA** Albumin Fraction V from Bovine Serum

**CARD** Caspase Recruitment Domain

**CNS** Central Nervous System

**COX2** Cyclooxygenase 2

**DOC** Sodium Deoxycholate

**DTT** 1,4-Dithiotreitol

**ECF** Enhanced Chemifluorescence

| | |
|---|---|
| **ER** | Endoplasmic Reticulum |
| **ERO1-α** | Endoplasmic Reticulum Oxidase 1-α |
| **FBS** | Fetal Bovine Serum |
| **GRP78** | Glucose-Regulated Protein 78 |
| **GSH** | Reduced Glutathione |
| **IFNɣ** | Interferon-ɣ |
| **IL-1β** | Interleukin-1β |
| **iNOS** | Inducible Nitric Oxide Synthase |
| **IRE1α** | Inositol-Requiring Enzyme 1$\alpha$ |
| **LPS** | Lipopolysaccharide |
| **NLRP3** | NOD-Like Receptor family, Pyrin domain containing 3 |
| **PDI** | Protein Disulfide Isomerase |
| **PERK** | Protein Kinase R-like Endoplasmic Reticulum Kinase |
| **PMSF** | Phenylmethylsulphonyl Fluoride |
| **PVDF** | Polyvinylidenedifluoride |
| **RIPA** | Radioimmunoprecipitation assay |
| **ROS** | Reactive Oxygen Species |
| **RPMI** | Roswell Park Memorial Institute Medium |
| **RT** | Room Temperature |
| **TBS-T** | Tris Buffered Saline with Tween |
| **UPR$^{ER}$** | Unfolded Protein Response of the Endoplasmic Reticulum |

## Introduction

Microglial cells are the resident immune cells of the Central Nervous System (CNS) that control the innate immunity and intervene in adaptive immune responses (1). When the CNS becomes threatened, microglia enter an activated state termed M1 releasing pro-inflammatory molecules such as IL-1β, IFNɣ, iNOS or cyclooxygenase 2 (COX2) (2). Depending on the type and duration of the insult microglia can also enter an alternative M2 activated state characterized by the expression of anti-inflammatory factors (2). In response to stimulus such as lipopolysaccharide (LPS), microglia upregulate the IL-1β pro-form, which is cleaved to active IL-1β by caspase-1 (3,4).

The release of pro-inflammatory IL-1β regulates the innate immune response in the CNS through a multiprotein complex denominated inflammasome (5). Although several types of inflammasomes have been described, the NLRP3 inflammasome is the most well described in microglia and has been implicated in several pathological conditions (5,6). Three major mechanisms have been proposed to explain NLRP3 activation, including changes in ion fluxes, overproduction of reactive oxygen species (ROS) or release of lysosomal cathepsins (7). Regarding NLRP3, it is described that two signals are required for its activation: a priming step that produces pro-IL-1β and a second step responsible for caspase-1 activation and thus cleavage of pro-IL-1β in its active form (6). Upon activation, NLRP3 associates with the adaptor protein ASC, which comprises a caspase recruitment domain (CARD) and a pyrin domain. The NLRP3:ASC complex oligomerizes and binds to caspase-1, forming active inflammasome complexes (NLRP3, ASC, caspase-1) that release active IL-1β (5).

The production of pro-inflammatory molecules in response to the M1 phenotype gives rise to a chronic neuroinflammatory state that has been implicated in the progression of several neurodegenerative disorders, including Alzheimer's disease (AD) (8,9). Recent studies have also demonstrated the activation of the NLRP3 inflammasome in response to the AD-associated β-amyloid peptide (10,11).

A neuropathological hallmark of AD and other neurodegenerative diseases is the selective accumulation of unfolded proteins in different brain areas (12). The organelle responsible for the synthesis and maturation of proteins in the secretory pathway is the Endoplasmic Reticulum (ER), which is mediated by ER-resident chaperones such as GRP78 (13). Disturbances in the ER function compromises its ability to properly fold proteins leading to the accumulation of unfolded/misfolded proteins in the lumen causing a state referred to as ER stress. Under these conditions, several signaling pathways downstream of the ER stress sensors IRE1α, PERK and ATF6, identified as the "Unfolded Protein Response" of the ER ($UPR^{ER}$), are activated to re-establish homeostasis (13). During $UPR^{ER}$ activation gene expression is affected through arresting of general translational to reduce the influx of newly synthesized proteins into the ER lumen, upregulating genes that enhance the ER protein folding capacity and quality control or those associated with redox homeostasis and energy metabolism, or promoting proteasome-mediated degradation of abnormal proteins (ERAD) and lysosome-mediated autophagy (14). Under prolonged ER stress, $UPR^{ER}$ activation triggers apoptosis to eliminate damaged cells (15). Increased ROS production occurs in response to ER stress as a byproduct of disulfide bond formation, during the transfer of electrons from protein thiol to molecular oxygen by ERO1 and PDI or, alternatively, upon GSH depletion. The over-production of ROS during ER stress can also arise from enhanced ER-to-mitochondria $Ca^{2+}$ transfer (16).

$UPR^{ER}$ is chronically induced in the brain of patients and of animal models of neurodegenerative diseases and is implicated in neuronal cell demise, which is known to be exacerbated by the deleterious activation of microglia (12). However, the relationship between the ER stress-induced $UPR^{ER}$ and neuroinflammation is still unclear. To fill this gap we investigated whether microglia-associated inflammation is caused by the NLRP3 inflammasome activation as a response to oxidative stress occurring downstream of $UPR^{ER}$ induction, using the BV2 microglia cell line treated with the ER stress inducer brefeldin A (BFA).

## Materials

**Table 1 – Antibodies used.** Description of the antibodies used during the study and respective companies they were obtained from.

| **Antibodies** | **Obtained from** | **Ref.** |
|---|---|---|
| Mouse anti-BIP/GRP78 | BD Transduction Laboratories™, USA | 610978 |
| Mouse anti- ERO1-α/ERO1L | LifeSpan BioSciences, Inc | LS-C133740/49655 |
| Goat anti-IL-1β/IL-1F2 | R&D Systems®, USA | AF-501-NA |
| Rat Monoclonal anti h/m NLRP3/NALP3 | R&D Systems®, USA | MAB7578 |
| Mouse anti-Actin | Sigma-Aldrich®, USA | A5441 |
| Rat polyclonal anti-caspase -1 | abcam®, UK | Ab17820 |
| Goat Anti-Mouse IgG+IgM-AP conjugated | GE Healthcare UK Limited | NIF1317 |
| Chicken Anti-Rat IgG-AP conjugated | Santa Cruz Biotechnology, | SC-2960 |
| Rabbit Anti-Goat IgG-AP conjugated | Santa Cruz Biotechnology, | SC-2771 |

## Reagents

Lipopolysaccharide (LPS), Brefeldin A (BFA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Phenylmethylsulphonyl Fluoride (PMSF), Leupeptin, Pepstatin A, Chymostatin, Antipain, Penicilin, Streptomycin, Roswell Park Memorial Institute medium (RPMI medium) and Tween® 20 were purchased from SIGMA-ALDRICH®. 1,4-dithiotreitol (DTT) was purchased from AMRESCO®. Fetal Bovine Serum (FBS) was purchased from Gibco Invitrogen (Carlsbad, CA, USA). The Precision Plus Protein All Blue Standards and the Acrylamide were purchased from BIORAD and the Enhanced Chemifluorescnce (ECF) reagent was purchased from GE Healthcare. Albumin Fraction V from Bovine Serum (BSA) was purchased from Merck®. The AMPLEX RED Hydrogen peroxide/Peroxidase assay kit and the IL-1β ELISA Kit were obtained from Molecular Probes® (Thermo Fisher Scientific, Inc., MA USA) and BD Biosciences (NJ, USA), respectively.

## Methods

### Cell culture

The murine BV2 microglial cell line (Biological and Cell Banking Factory, Centro di Risorse Biologiche, Genova) was grown in RPMI medium supplemented with 23.8 mM sodium bicarbonate, 100 U/ml Penicilin, 100 µg/ml streptomycin and 10% heat inactivated fetal bovine serum (FBS). Cells were kept at 37 ºC in a 95% atmospheric air and 5% $CO_2$ humidified atmosphere. Numbers of viable cells were evaluated by counting trypan blue-excluding cells that were then plated at a density of $1x10^5$ cells/$cm^2$ for Western blot analysis, $0{,}50x10^5$ cells/$cm^2$ for the MTT assay or $1x10^5$ cells for Amplex Red assay. BV2 cells were treated with BFA (2-20 µM) for 3-6 h to induce ER stress or primed with LPS (300 ng/ml) for 3 h and then treated with BFA (2-20 µM) for 6 h to evaluate inflammasome activation.

### Cell viability - MTT reduction assay

To assess microglial injury caused by exposure to BFA, cell viability was evaluated through the MTT assay (17). After treatments, cell culture were washed twice in Krebs sodium medium at 37 ºC (in mM: 132 NaCl, 4 KCl, 1.2 $Na_2HPO_4$, 1.4 $MgCl_2$, 6 glucose, 10 Hepes, 1 $CaCl_2$, pH 7.4), and incubated with MTT (0.5 mg/ml) for 45 min at 37 ºC. The formazan crystals formed after the MTT sodium reduction were dissolved with equal volume of 0.04 M HCl in isopropanol and quantified spectrophotometrically by measuring the absorbance at 570 nm using a microplate reader (Spectramax Plus 384; Molecular Devices, California, USA). The results were expressed as a percentage of control values determined in untreated cells.

**Western blot analysis**

Cells were washed with PBS, scrapped and lysed on ice in with an ice-cold lysis RIPA buffer (150 mM NaCl, 1% (w/v) NP40, 0.1% (w/v) SDS, 0.5% (w/v) DOC, 50 mM Tris-HCl, pH 7.4), supplemented with 100 μM PMSF, 2 mM DTT and 1:1000 of a protease inhibitor cocktail (1 μg/ml leupeptin, pepstatin A, chymostatin, and antipain). Cell lysates were incubated on ice for 15 min and centrifuged at 20 000 x g for 15 min at 4 ºC and the supernatant collected. The total amount of protein was quantified using the Pierce™ BCA Protein Assay Kit. For each sample, 30-40 μg of protein present in total cellular extracts were separated by electrophoresis on 10-15% (w/v) SDS-polyacrylamide gel (SDS-PAGE) after denaturation at 95 ºC for 5 min in sample buffer (in mM): 100 Tris, 100 DTT, 4% (v/v) SDS, 0.2% (w/v) bromophenol blue and 20% (v/v) glycerol. To facilitate the identification of proteins of interest, the prestained Precision Plus Protein All Blue Standard (Bio-Rad, Hercules, CA, USA) was used. Proteins were then transferred to PVDF membranes (Millipore, USA), which were further blocked for 1 h at RT with 5% (w/v) BSA in Tris-buffered saline (150 mM NaCl, 50 mM Tris, pH 7.6) with 0.1% (w/v) Tween 20 (TBS-T). The membranes were next incubated overnight at 4 ºC with a primary mouse monoclonal antibody against GRP78 (1:1000 dilution in 5% BSA in TBS-T) or ERO1-α (1:1000 dilution in 5% BSA in TBS-T), a primary goat antibody against IL-1β (1:1000 dilution), a primary rat antibody against NLRP3 (1:250 dilution in 5% BSA in TBS-T) or with a primary rabbit antibody against caspase-1 (1:1000 dilution in 5% BSA in TBS-T). Control of protein loading was performed using a primary mouse antibody reactive against β actin (1:5,000 dilution in 5% BSA in TBS-T). After washing, membranes were incubated for 1 h at RT with an alkaline phosphatase conjugated secondary anti-mouse (1:20,000 dilution in TBS-T), anti-rabbit (1:20,000 dilution in TBS-T), anti-goat (1:2,500 dilution in TBS-T) or anti-rat antibody (1:2,500 dilution in TBS-T). Bands of immunoreactive proteins were visualized after membrane incubation with ECF reagent, on a Versa Doc 3000 Imaging System (Bio-Rad, Hercules, CA, USA).

**ELISA assay**

For the ELISA assay to quantify IL-1β levels, the culture supernatants of BV2 cells were collected after treatment and processed using the IL-1β ELISA Kit (BD Biosciences) according the manufacturer's instructions.

**Amplex Red**

The production of $H_2O_2$ in BV-2 cells was quantified by a fluorimetric assay with AMPLEX RED Hydrogen peroxide/Peroxidase assay kit (Molecular Probes® Thermo Fisher Scientific, Inc., MA USA) according to the manufacturer's instructions.

**Data Analysis**

Statistical significance was considered relevant for p values $<0.05$ using one-way ANOVA analysis of variance followed by Bonferroni's post hoc test for multiple comparisons. Data were presented as means ± standard error of mean (SEM). Every experimental condition was tested in at least three sets of independent experiments, and performed in duplicates.

## Results

### ER stress in BV2 microglial cells

When BV2 microglial cells were treated for 3 h with 2 or 10 μM brefeldin A (BFA), the protein levels of GRP78 were not affected (Fig. 1A). We found that prolonged incubation (6 h) with these concentrations of BFA induced a dose-dependent ER stress response in microglia detected by increased levels of GRP78 and ERO1-α, two ER stress markers (Fig. 1B,C). The increase in the levels of GRP78 and ERO1-α was statistically significant after treatment with 10 μM BFA ($p<0.01$ and $p<0.05$, respectively).

### NLRP3 levels and caspase-1 activation upon ER stress in microglia cells

Priming of BV2 cells with lipopolysaccharide (LPS) before treatment with increased concentrations of BFA triggered a significant increase in the protein levels of NLRP3 ($p<0.001$ and 0.05 for treatment with 2 and 10 μM BFA, respectively) and also of cleaved (active) caspase-1 (Fig. 2A,B).

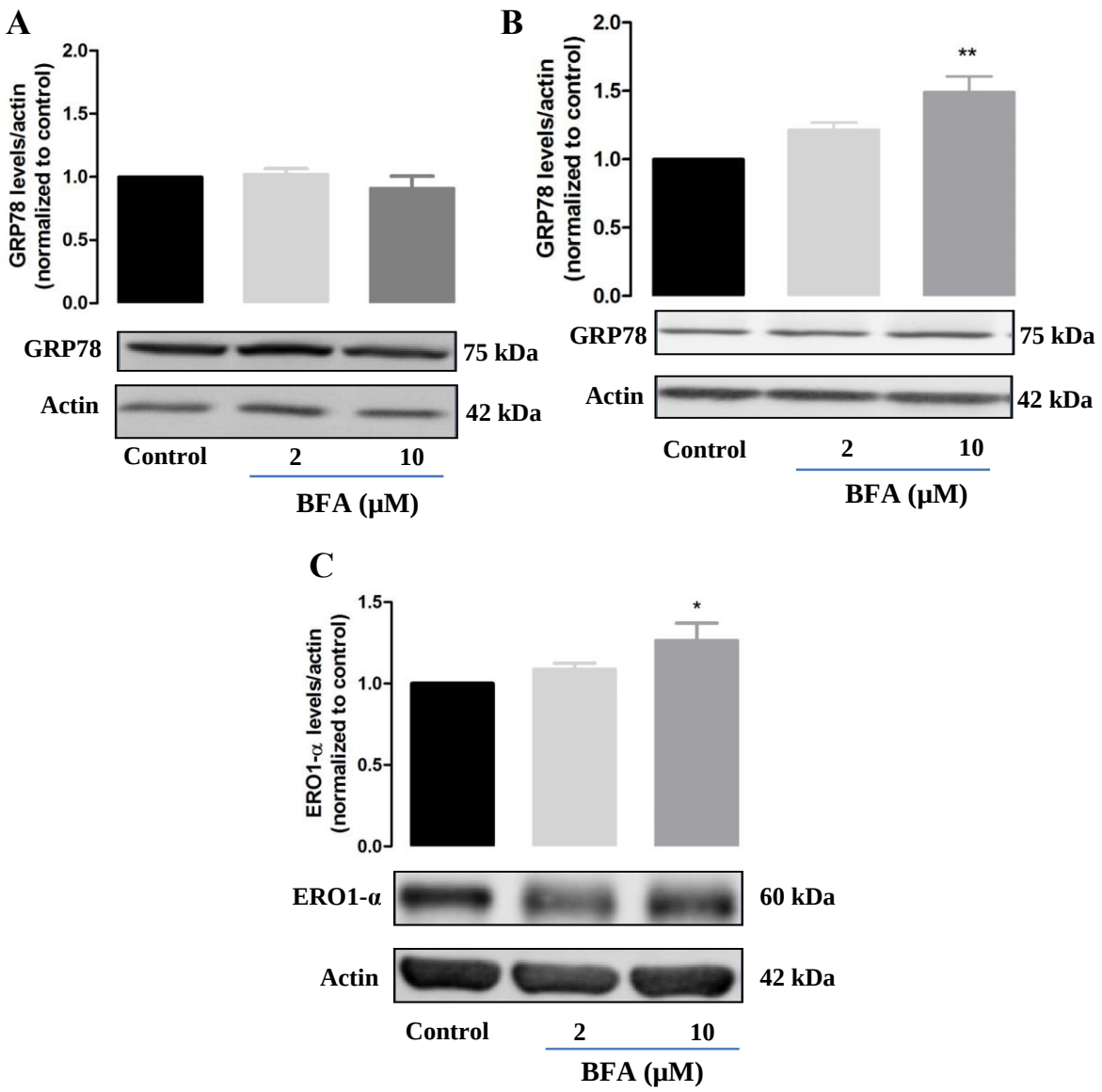


**Fig. 1 – *ER stress in BV2 microglia cells***. BV2 cells were incubated with increased concentrations of BFA for 3 h (A) or 6 h (B, C) and the levels of the ER stress markers GRP78 and ERO1-α were analyzed by Western blot. *p<0.05 and **p<0.01 significantly different from control, in the absence of the ER stress inducer.

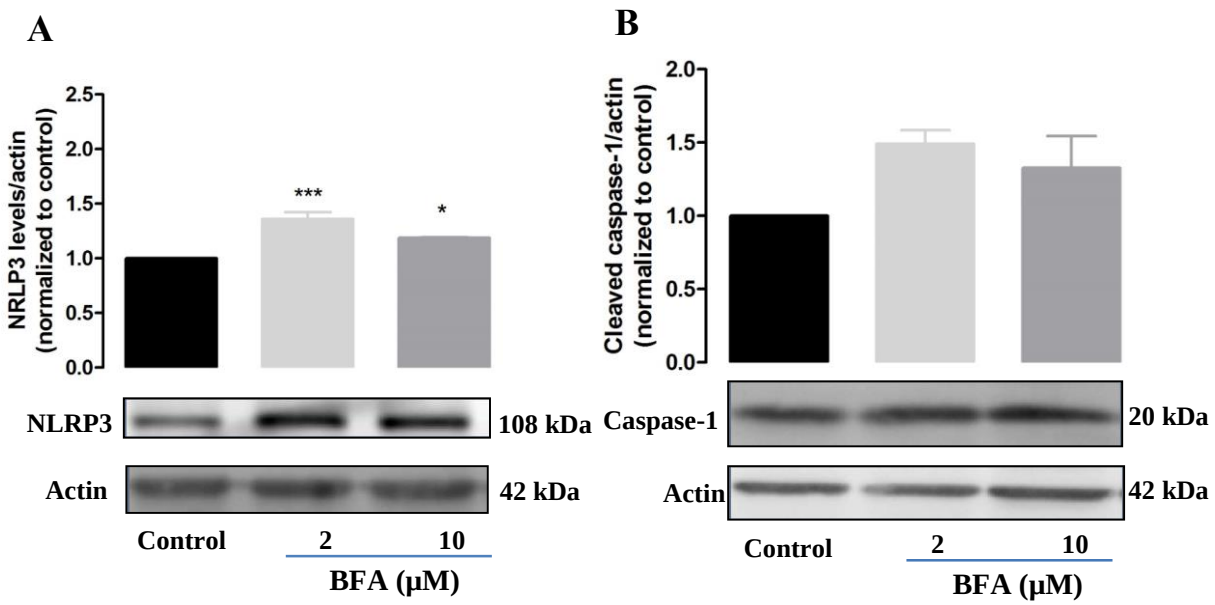


**Fig. 2 –** ***ER stress increases NLRP3 levels and induces caspase-1 activation in microglia***. BV2 cells were primed with 300 ng/ml LPS for 3 h and then treated with increased concentrations of BFA for 6 h. In these conditions, the levels of NLRP3 (A) and cleaved caspase-1 (B) were evaluated by Western blot *p<0.05 and ***p<0.001 significantly different from control, in the absence of the ER stress inducer.

**IL-1β release under ER stress conditions in microglia cells**

Increased levels of pro-IL1β were detected in BV2 cells primed with LPS and then exposed to 2 µM BFA (p<0.001) or 10 µM BFA (p<0.01) (Fig. 3A). The maximal response was observed upon incubation with the lower BFA concentration that was tested. The amount of IL-1β was analyzed using an ELISA assay and it was demonstrated that a dose-dependent increase in the release of IL-1β occurs in response to ER stress (Fig. 3B). IL-1β levels significantly increased in primed cells upon treatment with 2 µM BFA (p<0.05) or 10 µM BFA (p<0.001), compared with cells treated with LPS (priming stimulus) alone (p<0.01).

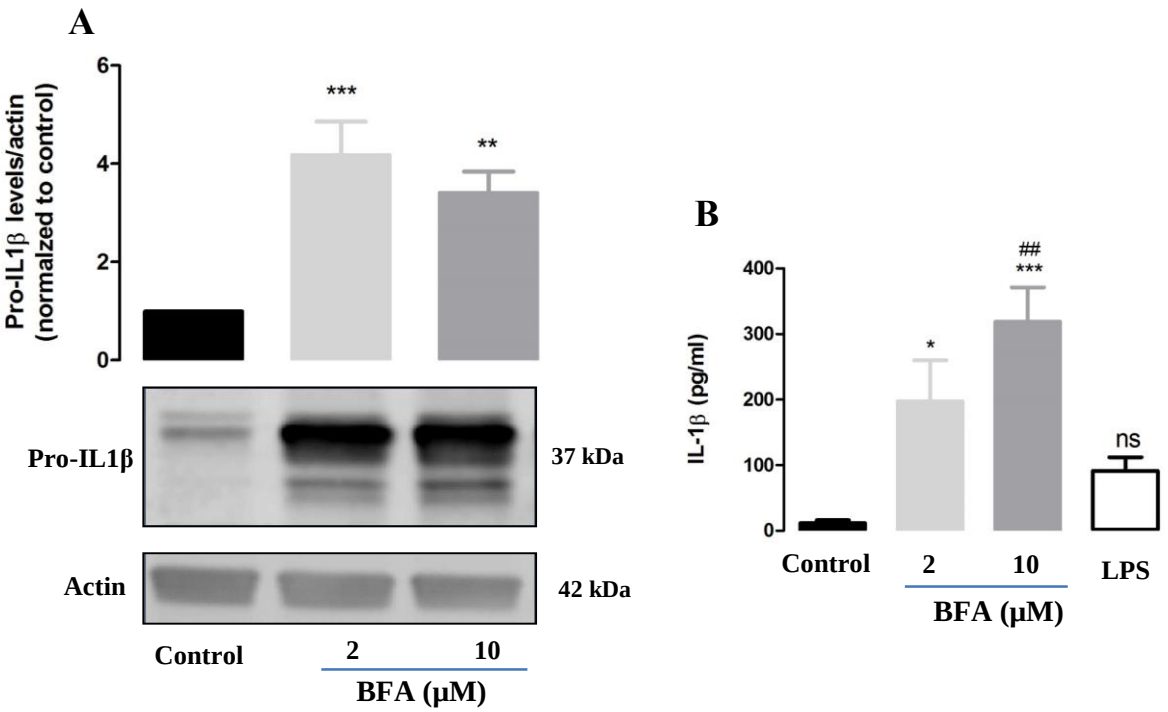


**Fig. 3 – *ER stress induces IL-1β release by microglia***. BV2 cells were primed with LPS and then exposed to increased concentrations of BFA for 6 h. In these conditions, the levels of Pro-IL1β in cell lysates were evaluated by Western blot (A) and the levels of released IL-1β to cell culture medium were measured by an ELISA assay (B). *p<0.05, **p<0.01 and ***p<0.001 significantly different from control, in the absence of ER stress inducer. ##p<0.01 significantly different from LPS-treated cells. ns- not significant.

**Microglia cell viability under ER stress**

BV2 cells treated with increased concentrations of BFA for 6 h showed decreased cell viability (p<0.001), as evaluated by the MTT assay.

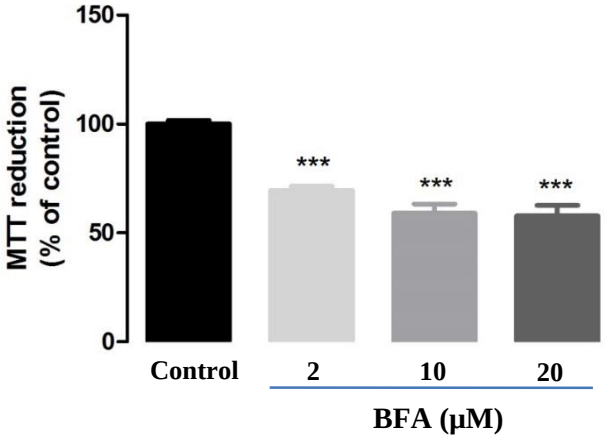


**Fig. 4 –** ***ER stress decreases cell viability in microglia***. BV2 cells were treated with increased concentrations of BFA (2, 10 and 20 µM) for 6 h and cell viability was then evaluated by the MTT assay. *p<0.05, **p<0.01 and ***p<0.001 significantly different from control, in the absence of ER stress inducer.

**Production of hydrogen peroxide ($H_2O_2$) by microglia upon ER stress induction**

BV2 cells were treated with increased concentrations of BFA and then the production of ROS, namely $H_2O_2$, was measured using the Amplex Red assay. A dose-dependent increase in $H_2O_2$ levels was observed in BFA-treated BV2 cells in comparison with control cells.

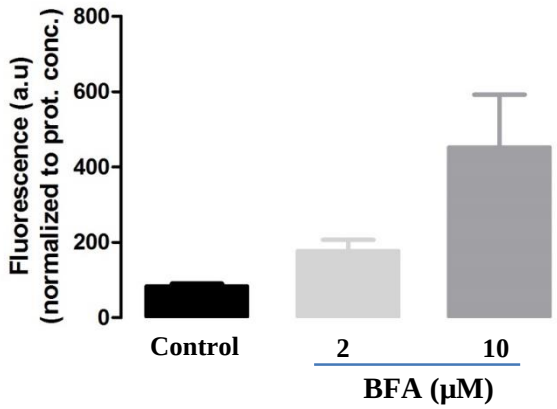


**Fig. 5 –** ***ER stress enhances the production of hydrogen peroxide ($H_2O_2$) in microglia***. BV2 cells were treated with increased concentrations of BFA for 6 h and the production of $H_2O_2$ was evaluated using a fluorimetric assay with Amplex Red reagent.

**Discussion and conclusion**

The present study addresses the casual relationship between ER stress and inflammasome activation in microglia. It has been previously demonstrated that toxic stimuli such as the AD-associated Aβ peptide induces ER stress in different cell types like neuronal and endothelial cells (18,19). Indeed, Aβ1-42 oligomers induce ER stress in neuronal cell cultures, detected by an increase in GRP78 levels and a decrease in the pro-caspase-12 levels (18). Moreover, brain endothelial cells treated with Aβ1-40 induced ER stress activating an apoptotic pathway leading to decreased cell survival (19). These data suggest that ER stress triggered by the accumulation of Aβ, which is associated with the neurodegenerative process in AD, affects not only neurons but also other types of cells in the brain. However, the role of ER stress in microglia activation is still unclear.

To induce ER stress in microglial cells we have used BFA that is reported to block the secretory pathway, leading to protein accumulation within the ER lumen (20). BFA, as well as other ER stress inducers such as tunicamycin and thapsigargin, have been used to induce ER stress in different type of cells namely macrophages, hepatocytes, smooth muscle cells and neurons (21–24) but the effect of ER stress in microglia cells is still not well established. In this work we observed that BFA increased the levels of the ER stress markers GRP78 and ERO1-α in the microglia cell line BV2. The upregulation of GRP78 occurred in response to lower BFA concentrations compared to that of ERO1-α, which could be explained by the sequential activation of UPR mediators. When cells are submitted to mild ER stress due to the accumulation of unfolded proteins, the levels of the chaperone GRP78 are increased to control the overload of misfolded proteins (14). In the continuous presence of the stimulus the activation of the PERK branch of the UPR is sustained and up-regulates the pro-apoptotic transcription factor C/EBP homologous protein CHOP/GADD153, which promotes the expression of ERO1-α. The excessive activation of this oxidoreductase involved in protein folding leads to the generation of oxidant species and depletion of the antioxidant glutathione

(GSH) (15), which is concordant with our data showing that ERO1-α up-regulation occurs in response to the higher BFA concentration tested.

Upon inducing ER stress in LPS-primed microglial cells, we observed an increase in the levels of NLRP3 and cleaved (active) caspase-1, both constituents of the inflammasome complex (5), providing evidence that in response to a chronic danger signal such as ER stress, the NLRP3 inflammasome is assembled in microglia. Lower BFA doses were shown to be significantly effective in the assembly of NLRP3, contrary to the higher concentrations used to activate UPR. This phenomenon could mean that, in response to a danger signal microglia activate pathways that assemble and activate the NLRP3 inflammasome before the initiation of UPR. In macrophages, inflammasome was demonstrated to be activated through an UPR-independent mechanism (21). Indeed, Menu *et al.* established that THP-1 cells expressing shRNA against IREα and PERK showed no alteration in the secretion of mature IL-1β in response to BFA or tunicamycin. Furthermore, macrophages derived from ATF6 knockout mice did not differ from WT littermates in their response to tunicamycin and thapsigargin, thus concluding that a possible "fourth branch" of ER stress response regulates NLRP3 inflammasome activation (21).

Upon activation of the NLRP3 inflammasome, increased levels of pro-IL1-β were detected in response to ER stress indicating that not only the assembly of NLRP3 inflammasome but also its activation is regulated in response to a danger signal such as ER stress. Concentration of active IL-1β in medium significantly increased in response to BFA-induced ER stress. Similarly, it was previously described an increased secretion of active IL-1β in primary cell cultures of human macrophages treated with the widely used ER stress inducers BFA, tunicamycin and thapsigargin (21).

Our results demonstrated that microglial cells respond to a danger stimulus such as ER stress by inducing the signaling pathways to re-establish homeostasis and activate the NLRP3 inflammassome under conditions of sustained ER stress. Although ER stress can directly lead to inflammasome activation, a more likely scenario involves a more intricate pathway. One of the

mechanisms described to link ER stress and inflammasome activation is the over-production of ROS (21), which can occur in response to ER stress as a byproduct of disulfide bond formation, during the transfer of electrons from protein thiol to molecular oxygen by ERO1-α and PDI or, alternatively, upon GSH depletion. The over-production of ROS during ER stress can also arise from enhanced ER-to-mitochondria $Ca^{2+}$ transfer (16). An increase in $H_2O_2$ production was detected in microglia cells in response to ER stress. Although further testing is required, a BFA dose-response $H_2O_2$ production was observed that was similar to the changes in ERO1-α levels, suggesting that ROS arise from ERO1-α up-regulation and activation. From these data we can conclude that upon activation of ER stress-induced UPR, ERO1-α is activated leading to the overproduction of ROS that can then activate the NLRP3 inflammassome (25). To further implicate ROS in the NLRP3 inflammasome activation an antioxidant such as GSH (Esteves et al., 2009) should be tested in the future work (26).

The inflammasome activation and production of IL-1β initiate a cascade of events that induce pyroptotic cell death (7). Our data demonstrating a significant decrease in cell viability in BFA-treated microglia correlates with previous findings (19) showing that cell viability decreases significantly in a dose-dependent manner upon exposure to ER stress inducers. In order to access the influence of inflammasome activation in cell survival, the inhibition of inflammasome activation would be an appropriate approach. Cell survival could be determined after treating cells with BFA in the presence of Glyburide, described to inhibit NLRP3 inflammasome and subsequent caspase-1 activation and IL-1β secretion (27).

Taken together, we can conclude from results that ER stress triggers ROS generation and activates the NLRP3 inflammasome, decreasing cell viability in microglia. These findings suggest that the ER could be a suitable therapeutic target in diseases associated with excessive microglia activation.

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