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# ER-mediated stress induces mitochondrial-dependent caspases activation in NT2 neuron-like cells

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**Recent studies have revealed that endoplasmic reticulum (ER) disturbance is involved in the pathophysiology of neurodegenerative disorders, contributing to the activation of the ER stress-mediated apoptotic pathway. Therefore, we investigated here the molecular mechanisms underlying the ER-mitochondria axis, focusing on calcium as a potential mediator of cell death signals. Using NT2 cells treated with brefeldin A or tunicamycin, we observed that ER stress induces changes in the mitochondrial function, impairing mitochondrial membrane potential and distressing mitochondrial respiratory chain complex Moreover, stress stimuli at ER level evoked calcium fluxes between ER and mitochondria. Under these conditions, ER stress activated the unfolded protein response by an overexpression of GRP78, and also caspase-4 and-2, both involved upstream of caspase-9. Our findings show that ER and mitochondria interconnection plays a prominent role in the induction of neuronal cell death under particular stress circumstances. [BMB reports 2009; 42(11): 719-724]**

## INTRODUCTION

The endoplasmic reticulum (ER) has several important functions including the regulation of intracellular calcium homeostasis, protein glycosylation, formation of disulfide bounds and folding and assembly of newly synthesized secretory proteins.

Under various conditions, ER function is disturbed leading to the accumulation of unfolded proteins and activation of a sporadic ER stress response, also known as the unfolded protein response (UPR). In an attempt to survive, cells could develop self-protective mechanisms whereby conditions within the ER are communicated to the protein translation machinery and to the nucleus in order to restore cellular homeostasis. When cells are subjected to severe or prolonged ER stress, the transcriptional factor CHOP/Gadd153 is activated and apoptotic cell death may occur (1-3). ER stress has been implicated in many important diseases, including neurodegenerative disorders. We have recently demonstrated that the Alzheimer's disease-associated Aβ peptide induces ER stress contributing to the calcium release through ryanodine receptors (RyR) and inositol 1,4,5-triphosphate receptor ($IP_3R$) with subsequent increase in intracellular calcium levels and activation of apoptosis (4). Moreover, it has been demonstrated the up regulation of ER chaperones, such as protein disulfide isomerase (PDI) in postmortem brain tissues and cell culture models of Parkinson's disease (PD) that accumulates in Lewy bodies (5). Recent studies in cultured cells, including dopaminergic neurons, demonstrated that PD neurotoxins trigger ER stress and are also involved in the up-regulation of diverse UPR components (6, 7).

Mitochondria are often closely associated with the ER providing the conditions for a local and privileged communication between the two organelles (8).

Therefore, it seems worthwhile to unveil the molecular mechanisms that coordinate the interplay between ER stress and mitochondrial function and its relevance on the control of cell death.

In the present work, we report that mitochondria function is sensitive to ER stress stimuli and identify calcium fluxes in the ER and mitochondria axis as key factors of cell death signals triggered by ER and mitochondria stress.

## RESULTS AND DISCUSSION

### BFA and TUN activated UPR

To induce ER stress, we used two pharmacological agents employed as ER stress inducers that block ER to Golgi protein trafficking (brefeldin A, BFA) or that inhibit *N*-linked glycosylation reactions (tunicamycin, TUN).

Under stress conditions, ER activates the UPR by the induction of the ER resident stress proteins referred to as the glucose-regulated proteins (GRPs) (9). The GRPs are calcium binding chaperone proteins with protective properties. The best characterized GRP is GRP78, a 78-kDa protein also referred to as BiP. GRP78 is an essential regulator of ER function due to its

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role in protein folding and assembly, ER calcium binding and controlling the activation of transmembrane ER stress inducers (10, 11). The cytoprotective function of GRP78 has been well recognized in several experimental systems (12-14).

Fig. 1 shows a representative BiP/GRP78 immunoblot (Fig. 1A) and the corresponding protein expression levels (Fig. 1B) in cells incubated with ER stressors. Alpha-tubulin was used as a loading control. Results show that BIP/GRP78 expression levels were up-regulated by BFA or TUN treatment, indicating that BFA and TUN elicit ER stress.

### Calcium mediated ER to mitochondria communication

ER, besides playing a major role in regulating synthesis, folding, and transport of proteins, functions also as a dynamic calcium store. The dynamic changes in free/cytosolic and in ER calcium are regulated by a number of factors including intracellular calcium buffers and calcium transporting systems (channels and ATPases). Moreover, mitochondria have been suggested to play an important role in calcium buffering participating in the intracellular calcium handling machinery in stimulated cells (15, 16). More recently, mitochondria have been considered not only as high-capacity calcium buffers but also as active elements of the intracellular signaling system (17, 18).

Therefore, we appointed calcium as a potential mediator in the communication between these two organelles. To investigate this point, we determined ER calcium content after cell treatment with ER stressors. ER calcium content was assessed by changes in cytosolic calcium, monitored using Fura-2AM. Cytosolic calcium was measured before and after the addition of TPG to evoke ER calcium release. The difference in cytosolic calcium before and after TPG addition was taken as a measure of ER calcium store. For both stressors ER calcium achieved very low levels (Fig. 2A).

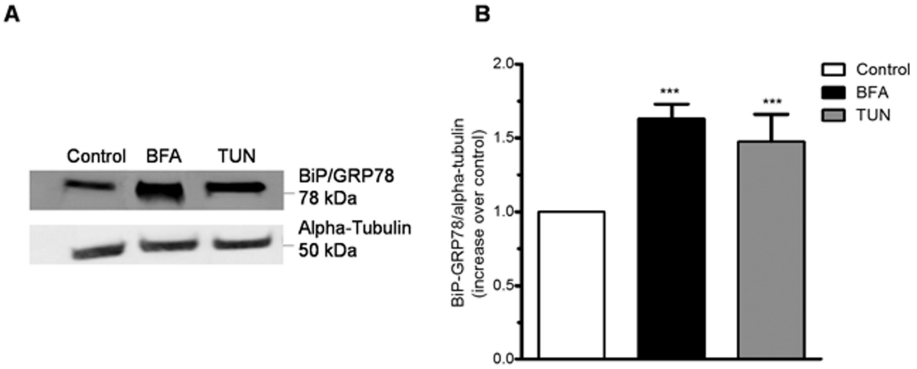


**Fig. 1.** Induction of UPR after NT2 cells exposure to ER stress. (A) Expression of BiP/GRP78 was determined by immunoblot analysis from untreated and ER (BFA, TUN) stressors treated cells. The blots were reprobed for alpha-tubulin to confirm equal protein loading. The data are representative blots. (B) Quantification of BiP/GRP78 expression levels by densitometric analysis of corresponding blots. Data are reported as the fold increase over the untreated cells values (control) and are represented as the mean ± SEM (n = 8, ***P < 0.001 compared to control).

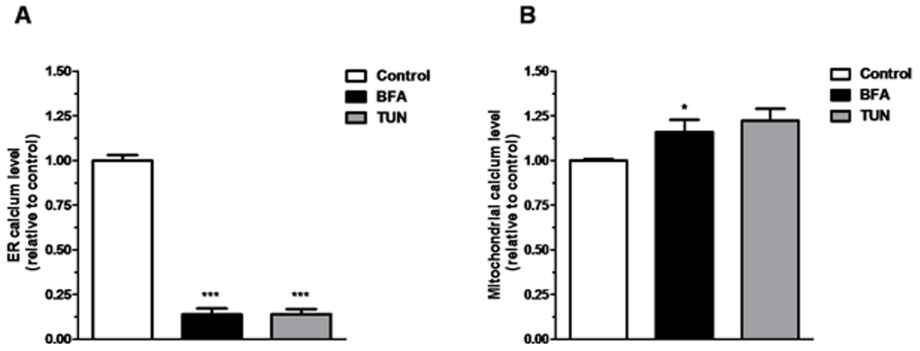


**Fig. 2.** Cellular calcium fluxes upon ER stress in NT2 cells. (A) Measurement of ER calcium released after TPG stimulation. Untreated (control), BFA or TUN treated cells were loaded with 5 μM Fura-2AM for the fluorescence measurement of ER calcium content. Data are reported in relation to untreated cells values (control) and are represented as the mean ± SEM (n = 4, ***P < 0.001 compared to control). (B) Measurement of $Ca^{2+}$ uptake by mitochondria. Untreated (control), BFA or TUN treated cells were loaded with 10 μM Rhod-2AM for the fluorescence measurement of mitochondrial calcium content. Data are reported in relation to untreated cells values (control) and are represented as the mean ± SEM (n = 4, *P < 0.05 compared to the corresponding control).

We further investigated whether the observed ER calcium changes are accompanied by changes in mitochondrial calcium content. The mitochondria, by virtue of their sustained membrane potential provide a sink for the buffering of cytoplasmic calcium after the ionophore A23187 treatment. Hence, we evaluated calcium clearance by mitochondrial uptake and subsequent calcium accumulation in the mitochondrial compartment following the treatments indicated before. After establishment of a stable baseline, as a measure of basal mitochondrial calcium stores, cells were challenged with the calcium ionophore A23, which rapidly increased the Rhod-2AM fluorescence, demonstrating a mitochondrial matrix calcium accumulation (data not shown). The ratio between mitochondrial matrix calcium before and after challenging with A23 was taken as an indirect measure of mitochondrial calcium content. We detected a significant increase in mitochondrial calcium content for both stressors (Fig. 2B). These results lead us to presume that mitochondria and ER are in a close communication, establishing a dynamic signalling pathway.

**BFA and TUN affected mitochondrial function**

Although we have shown that ER damage potentiates mitochondrial calcium uptake, we hypothesize that ER and mitochondria communication via calcium exchanges can induce mitochondria functional alterations.

Therefore, we investigated the functional significance of the ER stress induction on the mitochondrial function and its contribution to the initiation of cell death. We evaluated changes on the mitochondrial membrane potential (MMP). As shown in Fig. 3A, a slight decrease in the Rh123 retention induced by ER stress agents was observed, suggesting that these compounds did not incite a substantial mitochondrial depolarization. Meanwhile, we also evaluated the mitochondria respiratory chain complex I activity. Results in Fig. 3B demonstrated that both BFA and TUN provoked a statistically significant decrease in complex I activity. According to Sadek and colleagues (19), these findings could be explained regarding that complex I could be reversibly inhibited by calcium ions at low micromolar concentrations by a mechanism involving NADH-dependent oxidative modifications. Overall, these results indicate that mitochondrial respiratory chain activity is sensitive to the effects induced by BFA and TUN at ER level.

**BFA and TUN- induced stress activates ER- and mitochondria-dependent caspases**

Calcium release from the ER has been implicated as a key signaling event in many apoptotic models and it may influence the sensitivity of mitochondria to apoptotic alterations. Moreover, a recent discovery that a subpopulation of GRP78 can exist as an ER transmembrane protein implies that it can potentially interact directly with the cytosolic components of the apoptotic pathway and regulate their activity (13, 14). For instance, GRP78 has been reported to form complexes with procaspases, such as mouse caspase-12, which associates with the outer ER membrane (14).

So, we decided to examine the mechanisms through which these organelles initiate and propagate cell death signals and its involvement in the control of cell death.

Firstly, following the cascade of death signalling downstream of ER, we analysed the involvement of caspase-4. As shown in Fig. 4A, a significant increase in Ac-LEVD-*p*NA cleavage by activated caspase-4 was observed only upon BFA treatment.

Previously, it has been demonstrated that BFA and TUN promote the translocation of cytochrome c from the mitochondrial matrix to the cytosol (20, 21). In light of this, we further investigated the caspase-2 activity, which promotes cytosolic cleavage of Bid to tBid, acting upstream of cytochrome c release and being involved in ER stress-mediated death (22-24), and caspase-9 activity, which is involved in the cell death signals propagation triggered by mitochondria (25). As shown in Fig. 4B, only TUN activated caspase-2 (Ac-VDVAD- *p*NA cleavage). Both ER stressors activated caspase-9 (Ac-LEDH- *p*NA cleavage) (Fig. 4C). Interestingly, BFA and TUN differentially regulated the activation of caspase-4 and -2. BFA, by preventing anterograde transport and leading to the backflow of calcium into the ER lumen, can activate caspase-4, but TUN, as an inhibitor of the *N*-glycosylation reactions of proteins in ER membrane, cannot. In supporting this hypothesis, caspase 4 was shown to be activated by ER stress in a specific-manner and not by membrane- or mitochondria targeted signals (26).

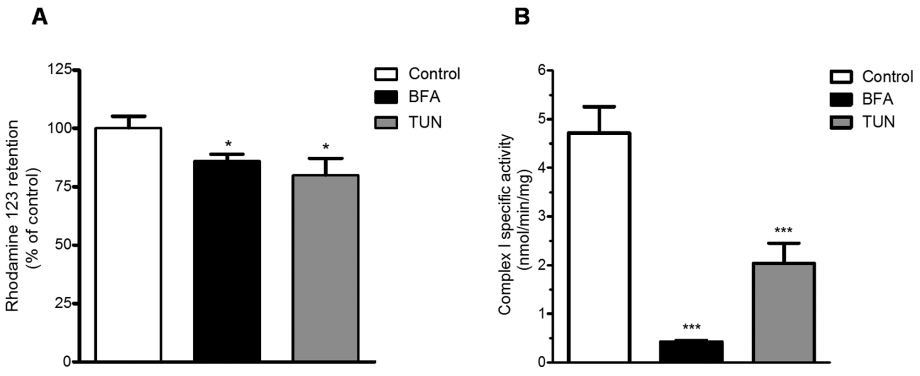


**Fig. 3.** Effect of BFA and TUN on mitochondrial function. (A) Changes in mitochondrial membrane potential induced by BFA and TUN stressors were estimated using the fluorescent cationic dye Rh123. Data are expressed as a percentage of the untreated cells values (control) and are represented as the mean ± SEM (n = 3, *P < 0.05 compared to control). (B) Mitochondrial respiratory chain complex I activity was determined as described in Materials and Methods. Data are reported in nmol/min/mg as the mean ± SEM (n = 4, ***P < 0.001 compared to control).

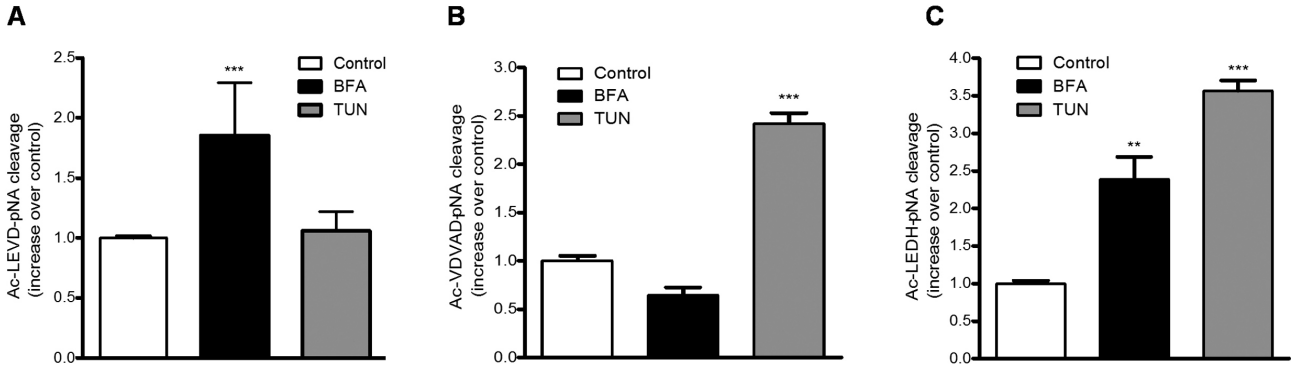


**Fig. 4.** ER stress-induced caspases activation in NT2 cells. Effect of BFA and TUN on caspase 4-like activity (Ac-LEVD-*p*NA cleavage) (A), caspase 2-like activity (Ac-VDVAD-*p*NA cleavage) (B) and caspase 9-like activity (Ac-LEDH-*p*NA cleavage) (C). Data are expressed relative to the basal activity observed in the untreated NT2 cells, with the mean ± SEM derived from 3-6 different experiments. (**P < 0.01, ***P < 0.001 compared to the corresponding control).

Our results revealed that stress in the ER and the subsequent ER calcium release triggers a caspase-4 dependent pathway and also a mitochondrial-dependent apoptotic pathway, involving caspase-2 as an initiator caspase. We presume that these two components of the ER and mitochondria stress can apparently work independently, converging on the activation of caspase-9. These results are consistent with a recent study which showed that BFA and TUN induce morphological apoptosis features, such as apoptotic bodies in HeLa cells (27).

First, our results demonstrated that ER stressors induce ER calcium release to the cytosol. Second, perturbations at ER level by BFA and TUN potentiated calcium uptake by mitochondria. Third, ER stress led to a low decrease of MMP, with a substantial decline in the rotenone sensitive NADH oxidation. Taken together, these results led us to infer the inevitable role of calcium fluxes between ER and mitochondria and subsequent caspases activation, representing a link between ER deregulation and mitochondria-dependent apoptotic pathways.

## MATERIALS AND METHODS

### Cell culture and treatments

Human teratocarcinoma NT2 cells, a neuronally committed human teratocarcinoma cell line (28) were purchased from Stratagene and were cultured as described previously (29). Briefly, cells were grown in Opti-MEM medium (Gibco) with 10% heat inactivated fetal calf serum (FBS) (Gibco), containing 10,000 U/ml penicillin and 10 µg/ml streptomycin (Gibco), under a humidified atmosphere of 95% air, 5% $CO_2$ at 37°C. Brefeldin A and Tunicamycin (Sigma) (2 µM) were added from 5 mM stock solutions prepared in DMSO. The final concentration of DMSO in culture media did not exceed 0.05% (v/v) and under these conditions, no alterations in cell's viability were observed. For all experimental procedures, controls were performed in the absence of the stress agents.

### Western blot analysis

$2.5 \times 10^5$ cells/well were seeded in 6-well plates and treated with the different agents for 24 h. Afterward, cells were washed in ice-cold phosphate-buffered saline (PBS) and lysed in 0.1% Triton X-100 containing hypotonic lysis buffer (25 mM HEPES, pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA and 1 mM EGTA supplemented with 2 mM DTT, 0.1 mM PMSF and a 1 : 1,000 dilution of a protease inhibitor cocktail). For each sample, 40 µg of protein was separated under reducing conditions on 12% SDS-polyacrylamide gels. After transfer to a Hybond-P PVDF membranes (GE Healthcare), the membranes were incubated for 1h in 5% non fat milk and 0.1% Tween 20 in Tris-buffered saline (TBS), followed by an overnight incubation with the anti-BiP/GRP78 (BD Biosciences) or anti-tubulin (Sigma) primary antibody, at 4°C.

Membranes were further washed three times with TBS, 0.1% Tween 20 and then incubated with the corresponding alkaline phosphatase - conjugated secondary antibody (1 : 20,000 dilution, GE Healthcare) for 2 h at room temperature. The membranes were washed again three times and bound antibodies detected using the enhanced chemifluorescence reagent ECF (Amersham Biosciences UK Limited) according to the manufacturer's instructions. Blots were visualized using a VersaDoc imaging system (Bio-Rad) and quantified using Quantity-One software (Bio-Rad).

### Fluorometric measurement of endoplasmic reticulum and mitochondrial calcium levels

Measurement of ER calcium content was assessed according to the method described by Nutt *et al*. (30), with some modifications. $1.0 \times 10^5$ cells/well were seeded in 12-well plates and treated with the ER stress agents for 24 h. Afterward, treated and control cells were washed twice in Krebs buffer (pH 7.4) composed of 132 mM NaCl, 4 mM KCl, 1.4 mM $MgCl_2$, 6 mM Glucose, 10 mM HEPES, 10 mM $NaHCO_3$, 1 mM $CaCl_2$ and loaded with Fura-2AM (5 µM) (Molecular Probes) supplemented with 0.01% Pluronic F-127 (Molecular Probes) and

1% BSA in Krebs buffer for 40 min, at 37°C. After loading, cells were washed 3 times in calcium-free Krebs buffer and were kept in calcium and dye free-medium at 37°C for 30 min, thereby permitting the cells to hydrolyze the acetoxymethyl ester completely. From the entire loading process onwards, the cells were shielded from ambient light. Then, the recordings were carried out. After fluorescence baseline stabilization, cells were stimulated with TPG (5 μM), in the absence of extracellular calcium, to empty calcium from ER. Fura-2AM fluorescence was recorded at 340/380 nm excitation and 512 nm emission.The peak amplitude of Fura-2AM fluorescence (ratio at 340/380 nm) was used to evaluate ER calcium levels.

Likewise, to monitor mitochondrial calcium levels we used 10 μM of the fluorescent membrane permeable probe Rhod-2AM (Molecular Probes) ($\lambda_{ex}$ = 552 nm and $\lambda_{em}$ = 581 nm). In its AM form, Rhod-2 carries a delocalized positive charge that causes preferential accumulation of this probe in mitochondrial matrix driven by $\Delta\psi_m$ according to Nernst equation. After deesterification, this indicator remains trapped in the mitochondria and reports calcium levels variations in the mitochondrial lumen. To assure a selective loading of Rhod-2 into mitochondria, probe loading procedure was performed at low temperature followed with warm incubation at 37°C. Mitochondrial maximal calcium uptake ability was further assessed by challenging mitochondria with the subsequent addition of 5 μM calcium ionophore A23187 (A23) (Sigma) as described previously by (31). As indicated earlier, this stimulant was also added after establishment of a stable fluorescence baseline. In both cases, the variations of fluorescence were monitored using a Spectramax Plus 384 spectrofluoremeter (Molecular Devices).

### Analysis of mitochondrial membrane potential ($\Delta\psi_m$)

Changes in mitochondrial membrane potential were estimated using the fluorescent cationic dye rhodamine 123 (Rh123) (Sigma).

After 24 h treatment, cells were loaded with 0.5 μM Rh123 (in the dark, at 37°C) and the fluorescence ($\lambda_{exc}$ = 505 nm and $\lambda_{em}$ = 525 nm) was recorded during 45 min before, and also for 10 min after mitochondrial depolarization, using a Spectramax Plus 384 spectrofluoremeter (Molecular Devices). Maximal mitochondrial depolarization ($\Delta\psi_m$ collapse) was performed in every individual experiment by adding 1 μM FCCP (protoionophore), which was always preceded by oligomycin (2 μg/ml) to prevent ATP synthase reversal.

Rh123 retention was determined by the difference between total fluorescence (after depolarization) and the initial value of fluorescence. Because positively charged Rh123 is retained by functional mitochondria with a high $\Delta\psi_m$, a decrease of cellular retention of Rh123 has been associated with a decrease in $\Delta\psi_m$.

### Mitochondrial respiratory chain complex I (NADH-Ubiquinone Oxidoreductase) activity

Mitochondrial complex I activity was determined by using a modified version of the method of Ragan (32) which follows the decrease in NADH absorbance at 340 nm ($\varepsilon$ = 6.81 $mM^{-1}cm^{-1}$) that occurs when ubiquinone (CoQ1) is reduced to form ubiquinol The reaction was initiated by adding CoQ1 (50 μM) to the reaction mixture, at 30°C. After 5 min, rotenone (10 μM) was added and the reaction was monitored for a further 5 min. Complex I activities are expressed in nanomoles per minute per milligram of protein and represent the rotenone sensitive rates.

### Caspases activation assays

Caspase activation assays were performed by using the method described by (33) with minor modifications. Cell extracts containing 50 μg or 100 μg of protein were incubated at 37°C for 2 h in 25 mM HEPES, pH 7.5 containing 0.1% (w/v) CHAPS, 10% (w/v) sucrose, 2 mM DTT with 100 μM Ac-VDVAD-*p*NA (Sigma), 50 μM Ac-LEVD-*p*NA (MBL International Corporation) or 50 μM Ac-LEDH-*p*NA (Calbiochem), colorimetric substrates for caspase-2, -4 and -9, respectively. Substrate cleavage was detected at 405 nm using a Spectramax Plus 384 spectrophotometer (Molecular Devices).

### Data analysis

All data were expressed as mean ± SEM of at least three independent experiments performed in triplicate. Statistical analyses were performed using one-way ANOVA followed by Bonferroni Multiple-Comparisons Procedure as post-hoc test. A P value $< 0.05$ was considered statistically significant.

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