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Evaluating ITPR-dependence of Apoptotic Signaling from the Endoplasmic Reticulum

Agustin Vargas
Pepperdine University

Jay L. Brewster
Pepperdine University

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Agustin Vargas and Jay L Brewster
Natural Science Division, Pepperdine University

Summer Undergraduate Research in Biology
Pepperdine University

Abstract

Stress within the endoplasmic reticulum (ER) can be induced by misfolded proteins accumulating in the lumen of this organelle. Signaling of ER stress to other parts of the cell results in altered gene expression, physiological adaptation, and with sustained stress, apoptosis (cell suicide). ER stress is often studied with highly toxic compounds that create severe ER stress rapidly, and a condition that is likely not physiologically relevant within an organism. In this study, we examine the apoptotic signaling induced by moderate ER stress, and in particular the inositol 1,4,5-trisphosphate receptor (ITPR). The ITPR regulates Ca2+ release from the ER lumen, and can induce apoptosis. We hypothesize that moderate levels of ER stress activate apoptosis via an ITPR-dependent signal. To induce moderate ER stress, we expose cells to 20-30nM concentrations of tunicamycin, an inhibitor of N-linked glycosylation in the ER. In this study, inclusion of an ITPR inhibitor (2-aminoethoxyphenyl borate, 2APB) protected cells from moderate ER stress, but did not protect cells from severe ER stress. A second methodology of assessing ITPR regulation of apoptosis includes overexpression of an ER-localized form of Bcl-2. The B-cell lymphoma 2 protein (Bcl-2has the ability to block the activation of cell suicide (apoptosis) by binding and inhibiting pro-apoptotic proteins (Bax family members). Bcl-2 is a membrane localized protein, found primarily in the mitochondrial outer membrane, and the endoplasmic reticulum (ER) membrane. In recent years, ER localized Bcl-2 has been shown to interact with the ITPR and inhibit pro-apoptotic Ca2+ signaling from the ER. We transfected cells with plasmids bearing forms of a Bcl-2 fusion protein to assess the capability of ER-Bcl-2 to protect cells from moderate apoptosis. The results of initial experiments did not show protection to either moderate or severe ER stress though some replicates of the experiment seemed to indicate protection. As this result is inconsistent with other results in our lab, we propose additional replicates of the experiment and using a drug-based mimic of this interaction to assess moderate ER stress signaling (Akl *et al.*, 2013).

Introduction

Apoptosis is the key mechanism by which unwanted or damaged cells are eliminated within an organism. The major players in apoptosis are the endoplasmic reticulum (ER), and the mitochondria, along with native proteins playing a part. The ER, the site of protein synthesis, folding, and glycosylation plays a major role in the initiation of apoptosis. As the ER becomes stressed and is unable to fold proteins, it activates what is known as the Unfolded Protein Response (UPR), where ER signaling activates pathways that signal for adaptation or activation of apoptosis(Akl, 2013). In intrinsic apoptosis, executioner signals of the Bcl-2 family of proteins within the mitochondria are activated. Bax in particular, homodimerizes in the mitochondrial outer membrane and causes for the permeabilization of the mitochondria and the release of cytochrome C (CytC) into the cytosol (Chan, 2004). CytC is a molecule that activates caspase proteases and apoptosome assembly to begin the process of programed cell death (Rodriguez, 2010). The pro and anti-apoptotic members of this family of proteins contain different Bcl-2 homology (BH) domains. Pro-apoptotic members contain BH1-3 domains, and a subgroup of pro-apoptotic members contain BH3 only domain. The anti-apoptotic members contain all four, BH1-4 domains (Rodriguez, 2013).

The inositol 1,4,5-trisphosphate (ITPR) receptor is a receptor that binds to the ITPR ligand in the ER. This receptor acts as a channel by which calcium is released from the ER stores into the cytosol and signal of apoptosis in the mitochondria. It has been shown that Bcl-2 suppresses ITPR receptor activity through its BH4 domain, thus inhibiting calcium release from the ER to the mitochondria (Akl, 2013).

Previous studies performed in our lab have shown that there is difference in the cell response to low and high concentrations of stress when during the inhibition of the IP_3 receptor with 2-Aminoethoxydiphenyl borate (2-APB). Using 2-APB on temperature sensitive BN7 (tsBN7) Hamster fibroblasts, it has been shown that cell viability was increased (by decreasing apoptotic percentages) at low levels of ER stress.

The focus of this study was to examine whether there is a difference in between moderate and sever levels of ER stress. Using ER localized Bcl-2 fused with green fluorescent protein (Bcl-2(ER)-GFP), wild-type (WT) Bcl-2-GFP, and green fluorescent protein (GFP), BHK21 cells were transfected to assess whether there would be a difference in apoptotic nuclei at low and high levels of ER stress. Considering that the Bcl-2 binds to the ITPR to inhibit calcium release into the cytosol, and thus arresting apoptosis at low level of ER stress; we proposed that there will be a difference in apoptotic nuclei between low and high levels of ER stress.

Results

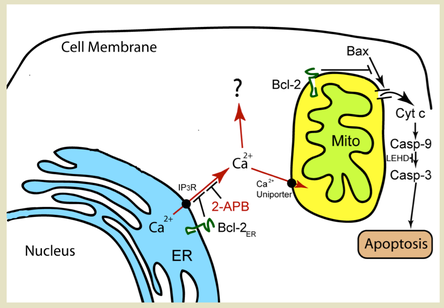
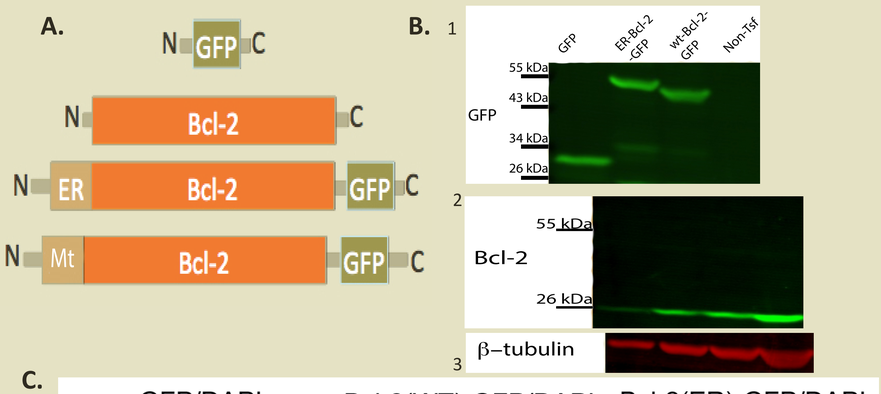


Figure 1: A working model apoptotic signaling induced by moderate ER stress.
Moderate ER stress activates mitochorial apoptotic signals via the ITPR-mediated release of Ca2+ from the ER lumen.
In recent studies, 2APB has been shown to be sufficient to inhibit apoptosis in cells exposed to moderate ER stress.



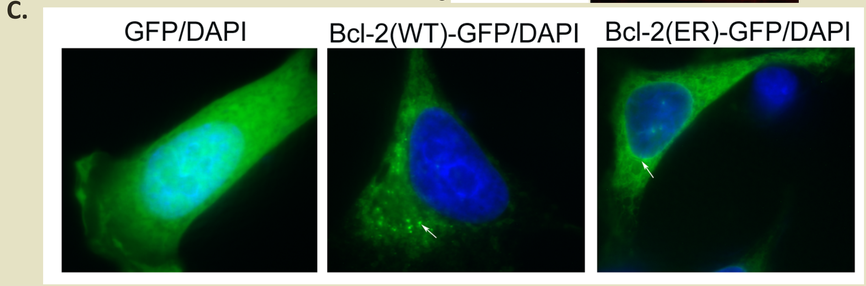


Figure2: Expression of Bcl-2 and GFP in BHK-21 36 hours after transfection
A) BHK21 cells were transfected using the plasmid constructs depicted.
B) After the 36 hours, cell lysates were obtained. 1) GFP transfected lysates show expression of GFP at the 30 kDa marker (GFP is 27 kDa), expression of ER-Bcl-2-GFP at the 55 kDa marker(Bcl-2 being 28 kDa; GFP 27 kDa) and wt-Bcl-2-GFP at the 45 kDa marker. There were no proteins present for the non-transfected BHK21 lysates. 2) Only native Bcl-2 seems to have been tagged by the antibody, appearing at approximately the 26 kDa marker. Immunoblot 3 shows the tubulin control.
C) After transfection and 36 hour exposure to low and high levels of ER stress, cells were fixed using 4% paraformaldehyde (PFA) and nuclei were DAPI stained.

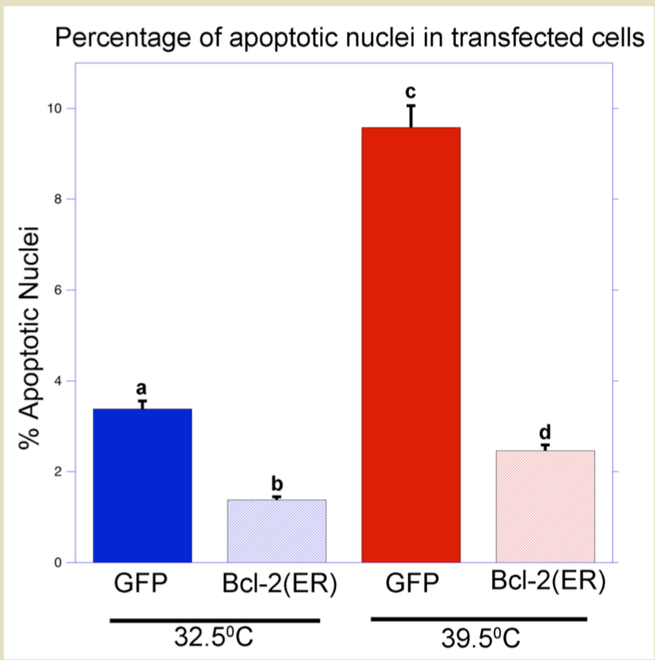
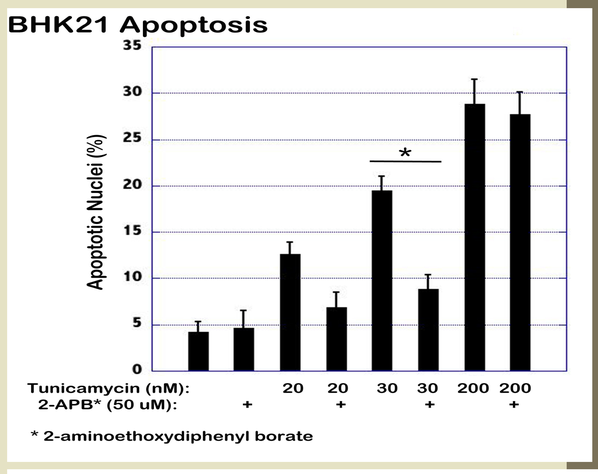


Figure 3: Over expression of Bcl-2(ER) protects in temperature-shifted tsBN7 cells from apoptosis
GFP-only or Bcl-2(ER)-GFP was transfected into tsBN7 cells seeded onto glass coverslips. Cells were incubated from 24 hours, fixed, and stained with DAPI. GFP-positive cells were scored for apoptosis. Results shown are the mean +/- SEM for three independent experiments. GFP-expressing cells displayed significantly higher apoptosis than Bcl-2(ER)-GFP-expressing cells at the restrictive temperature ($p<0.0002$). Statistical analysis was performed using one-way ANOVA analysis. (Data provided by Lindsey Long, SURB)

A



B

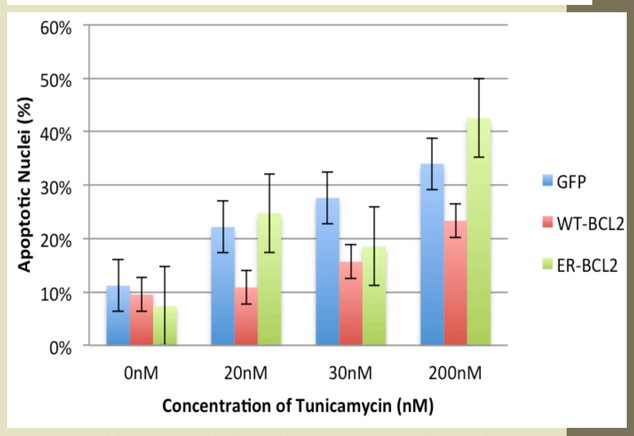


Figure 4: Rescue of BHK21s with ITPR inhibitor 2-APB, and ER-Bcl-2
A) Dependent experiment was performed on BHK21 cells using tunicamycin. 50 µM of 2-APB, a known ITPR receptor inhibitor, was used to inhibit calcium release. Cells were significantly rescued 30 nM concentration of Tm + 2-APB, compared to the non-treated 2-APB.
B) BHK21 cells were transfected to over express ER-Bcl-2-GFP, wt-Bcl-2-GFP, and GFP-only proteins then exposed to Tm for a 36 hour time period. Apoptotic percentages of transfected cells were obtained. A trend was observed, but there was no significance.

Conclusions

- tsBN7 cells transfected with the ER-Bcl-2-GFP plasmids were significantly rescued from undergoing apoptosis caused by temperature shifts, which would equate moderate levels of ER stress
- BHK21 cells exposed to moderate ER stress induced by tunicamycin (30nM) were significantly rescued by 2-APB, an ITPR inhibitor
- Rescue of BHK21 cells transfected with ER-Bcl-2-GFP and exposed to low and high levels of ER stress, was not significant when compared to the control
- There was a trend in apoptotic percentages in some of the experiments performed using ER-Bcl-2 transfected BHK21 cells but was not replicated; may be the focus of future studies

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