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Drop in endo/sarcoplasmic calcium precedes the unfolded protein response in Brefeldin A-treated vascular smooth muscle cells

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ABSTRACT

The present study addresses the causal relationship between induction of endo/sarcoplasmic reticulum stress and dysregulation of calcium transport, while examining whether the most widely-used experimental endo/sarcoplasmic reticulum stressors can be considered appropriate for elucidating underlying cellular mechanisms involved during the progression of the unfolded protein response in vascular smooth muscle cells. Brefeldin A is most commonly cited as inducing the stress response through an accumulation of unfolded proteins in the lumen as a result of a blockage of protein transport from the endo/sarcoplasmic reticulum to the Golgi apparatus. We investigated the effects of Brefeldin A on cellular calcium regulation during the the unfolded protein response in cultured rat vascular smooth muscle cells. Acute exposure of cells to Brefeldin A caused a small transient increase in cytoplasmic calcium, which did not cause a significant decrease in endo/sarcoplasmic reticulum calcium content. However, over the time course of 0–12 h post-treatment with Brefeldin A, we observed that the endo/sarcoplasmic reticulum of vascular smooth muscle cells exhibited an approximate fifty percent decrease in calcium concentration after the first hour of exposure, which is maintained over the next eleven hours, whereas concentrations of unfolded protein response markers only began to increase significantly around nine to twelve hours post-treatment. We have concluded that the endo/sarcoplasmic reticulum calcium drop, which up to now, has been considered as a characteristic of the late onset of cellular stress response, occurs prior to the initiation of the unfolded protein response, rather than as a result of its many corrective pathways.

1. Introduction

Endo/sarcoplasmic reticulum (ER/SR) stress is a phenomenon resulting from the accumulation of unfolded proteins in the organelle's lumen (Xu et al., 2005). The affected cell immediately attempts to alleviate the disruptive symptoms of stress by initiating the unfolded protein response (UPR) (Cao and Kaufman, 2012). The UPR initiates a variety of processes, including increasing the concentration of chaperone proteins inside the ER/SR, lowering transcription and translation of new polypeptides, and increasing the destruction of improperly folded proteins (Schroder and Kaufman, 2005). The effectiveness of these damage control mechanisms ultimately determines the cell's fate, whether that be recovery or initiation of apoptotic cell death (Yorimitsu and Klionsky, 2007). Furthermore, the mitochondria that are found to be in close association with the ER/SR in smooth muscle cells also play an important role in the later stages of the ER stress response. When the UPR has clearly been initiated, as made obvious by the increased expression of many marker proteins, but its restorative measures are insufficient to save the cell, then Ca^{2+} dynamics between the ER/SR and mitochondria come into play. In this scenario, the ER/SR releases a significant amount of Ca^{2+} that is then taken up by the mitochondria (Deniaud et al., 2008; Ferreiro et al., 2008; Szegezdi et al., 2006). This process leads to the initiation of apoptosis in cells that cannot recover from the degree of stress to which they have been exposed. It is vital to address that this most commonly discussed mitochondria–ER crosstalk is a late-stage event, as the present study provides evidence for a completely separate SR Ca^{2+} drop that occurs hours before the pre-apoptotic Ca^{2+} movement. The important questions arising from current studies on stress response involve the timing of the changes in luminal calcium concentration ($[Ca^{2+}]_{SR}$) that occur during the ER/SR stress response. Many publications have so far indicated that cellular stress conditions lead to a significant drop in $[Ca^{2+}]_{ER}$ (Ma

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and Hendershot, 2004; Pal et al., 2012), but whether this decrease in luminal Ca^{2+} results directly from the onset of stress or whether it is the consequence of the UPR has yet to be determined.

In order to effectively study the changes in Ca^{2+} levels that characterize or define ER/SR stress, this cellular phenomenon needs to be induced using a pharmacological agent that has no immediate, direct effects on luminal Ca^{2+} content. In this context, we previously reported (Ziomek et al., 2014) that by this criterion, the most frequently used ER/SR stressor agent tunicamycin was not suitable for such research due to its Ca^{2+} ionophore-like properties.

Brefeldin A is another ER/SR stressor, commonly cited in the literature for its action of disrupting Golgi apparatus protein transport (Fujiwara et al., 1988; Klausner et al., 1992; Lippincott-Schwartz et al., 1989). The unhindered protein production process involves ribosomes situated on the rough ER/SR membrane, which ultimately translate mRNA strands into nascent polypeptides. These polypeptides are subsequently recognized and transported into the ER/SR lumen. From this point, characteristics determined by the specific sequence of individual amino acids within the polypeptide determine the final destination for the mature protein. The majority of synthesized proteins are first transported to the Golgi apparatus for further modification before ultimately being sent forward via vesicular transport to the cytoplasm or other organelles as their final destination. It is reported that Brefeldin A induces ER/SR stress by blocking nascent protein transport from the ER/SR lumen to the Golgi structure, thus resulting in an accumulation of the polypeptides inside the lumen (Fujiwara et al., 1988; Klausner et al., 1992; Misumi et al., 1986).

Despite the documentation of the drug's effectiveness in inducing ER/SR stress, obtained mainly by mapping the late events preceding the UPR, the short- and long-term effects of Brefeldin A on ER/SR Ca^{2+} levels have not been explored. The main objective of this study was to determine the chronological order of the UPR and changes in ER/SR Ca^{2+} content that lead to the development of ER/SR stress in response to Brefeldin A in vascular smooth muscle cells (VSMCs), with emphasis on documenting changes in SR luminal Ca^{2+} levels.

2. Materials and methods

2.1. Buffers and reagents

The 1.5 mM Ca^{2+} HEPES buffer was composed of 140 mM NaCl, 10 mM glucose, 5 mM KCl, 5 mM HEPES, 1.5 mM $CaCl_2$, and 1 mM $MgCl_2$. The nominally Ca^{2+} free solution was composed similarly excluding the 1.5 mM $CaCl_2$ component. Thapsigargin (an inhibitor of the endo/sarcoplasmic reticulum Ca^{2+}–ATPase), ionomycin (a Ca^{2+} ionophore), Brefeldin A (a protein transport inhibitor), 2-APB (an IP_3R blocker), FCCP (an uncoupler of mitochondrial oxidative phosphorylation), and tetracaine (a blocker of ryanodine receptors) were acquired from Sigma-Aldrich (Toronto, ON, Canada). Oligomycin was acquired from New England Biolabs, Ltd. (Whitby, ON, Canada). Stock solutions of thapsigargin, ionomycin, oligomycin, 2-APB, and Brefeldin A were prepared in dimethyl sulfoxide (DMSO) and stored in −20 °C conditions. Stock solutions of tetracaine and FCCP were prepared in water and 95% ethanol, respectively. Any further dilutions of these drugs were made using nominally Ca^{2+} free HEPES–PSS buffer. A 0.01% DMSO solution prepared in nominally Ca^{2+} free buffer was used as a control vehicle solution for experiments performed over the course of this study (designated as control groups). The Ca^{2+} indicator Fluo-4AM was procured from Invitrogen (Burlington, ON, Canada). All results obtained from drug-treated groups were subsequently compared to those derived from control vehicle treated groups.

2.2. Cell culture

Rat aortic smooth muscle cells (SMCs) were originally from the laboratories of Drs. Nicolas Demaurex and Urs Ruegg (University of Geneva, Geneva, Switzerland). These cells were prepared from the aorta of male Wistar Kyoto rats weighing 200–300 g, as previously described (Lo Russo et al., 1996). In preparation for experiments, cells (passages 22–26) were grown in Dulbecco's modified Eagle's medium with 10% heat-inactivated newborn calf serum, streptomycin (100 μg/ml), and penicillin G (100 μg/ml), all acquired from Invitrogen (Toronto, ON, Canada). Cells were grown 48 h prior to experiments, under consistent conditions of 37 °C and 5% CO_2 within a humidified incubator. This period of time allowed cells to grow sufficiently to reach approximately 75% confluency by the time of live imaging.

2.3. Cytoplasmic Ca^{2+} measurement

The Ca^{2+} indicator Fluo-4AM was used to load SMCs prior to each experiment. Cells were loaded for 1 h at room temperature (approximately 24 °C) and subsequently washed in HEPES–PSS buffer for a period of 5–10 min prior to imaging. Live imaging was done using a Leica TCS SP5 Confocal Microscopy System with an Argon–Krypton laser (excitation 488 nm/emission 555 nm). Image resolution was 512 × 512 pixels. All images were captured with the scanning speed of 700 Hz, and signal intensity was analyzed using Leica Application Suite 2.6.3 (Leica Microsystem Inc., Concord, ON, Canada). Representative traces denote the average fluorescent signals (F/F_0) from a small cluster of cells (5–10 cells) in each region of interest (ROI) from multiple independent experiments. The measured and reported changes in Fluo-4AM fluorescence intensity were proportional to the changes in cytoplasmic Ca^{2+} levels observed within the ROIs throughout the experiment. Formatting and analysis of the data from these imaging traces were performed using GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA). To observe the ER/SR luminal network, SMCs were transfected with CellLight® ER–RFP fusion construct (Life Technologies, NY, USA) approximately 15 min prior to imaging. Cells also underwent treatment with Brefeldin A for varied durations of time prior to imaging to allow the capture of any changes to the ER/SR structure with increased exposure time of cells. Images were captured on a DeltaVision Live Cell microscope at 63 × magnification. Cells were incubated at 37 °C with a 5% CO_2 supply for the duration of imaging.

2.4. Western blot analysis

The ER/SR stress antibody kit and Beta-actin antibody were purchased from New England Biolabs, Ltd. (Whitby, ON, Canada). Cultured cells were treated with control vehicle or Brefeldin A for the specified period of time. Cells were subsequently washed with ice-cold PBS buffer twice, before being placed on ice for 15 min in lysis buffer composed of 50 mM pyrophosphate, 50 mM NaCl, 50 mM NaF, 10 mM HEPES, 5 mM EDTA, 5 mM EGTA, 100 μM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 0.1% Triton X-100, and 10 μg/ml leupeptin. Cell lysates were collected and protein concentration of each sample was determined using Quick Start™ Bradford Protein Assay (Bio-Rad, Mississauga, ON, Canada). Approximately 20 μg of extracted protein samples were separated through electrophoresis using 7% SDS–polyacrylamide gels. Following separation, protein was transferred to nitrocellulose