Coupling of calcium (Ca\(^{2+}\)) release by ryanodine receptor (RyR) 3 to its extrusion by sodium/calcium exchanger (NCX) mediates endoplasmic reticulum (ER) Ca\(^{2+}\) discharge from vascular endothelial cells

by

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B.Sc., The University of British Columbia, 2015

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE in

The Faculty of Graduate and Postdoctoral Studies
(Pharmacology and Therapeutics)

THE UNIVERSITY OF BRITISH COLUMBIA (Vancouver)
January 2018

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Abstract

The endoplasmic reticulum (ER) is an organelle with multiple functions serving to maintain cellular health, the most important of which include its role as the cell’s largest store of the signaling ion calcium (Ca\textsuperscript{2+}). The regulation of this ion’s movement into and out of the ER, as well as the cell itself, is therefore of the utmost importance when considering the overarching goal of each cellular body to remain functional. The study outlined in the current thesis discusses hypotheses for the manner in which Ca\textsuperscript{2+} is released in a dramatic dysregulation event during ER stress, a cellular condition that often results in cell death and which has been implicated in multiple severe health conditions. This pathway was chosen to be studied in endothelial cells (ECs), those comprising the intimal (inner-most) layer of blood vessels. These cells are in direct contact with blood flowing through these tributaries of the vasculature, and their vast array of products controls constriction and many other properties of the blood vessel itself. Dysfunction of these cells results in the loss of their ability to exert protective effects on the affected blood vessel. Importantly, this type of damage to ECs can occur as a result of Ca\textsuperscript{2+} dysregulation at the level of the ER such as the dramatic ER Ca\textsuperscript{2+} drop being studied here. It is therefore the aim of the current thesis to present evidence for the mechanism whereby dramatic changes in Ca\textsuperscript{2+} levels within the ER lead to problems cellular function, with the ultimate goal of therapy development to prevent affected cells from becoming dysfunctional as a result of such ion dysregulation. Investigation into the way that cytoplasmic and luminal ER Ca\textsuperscript{2+} levels are affected by a range of agonists as well as relevant channel and pump blockers has culminated in a clearer understanding of the likely pathway for this Ca\textsuperscript{2+} movement early in the ER stress response, which may in future be manipulated at the point of ER stress induction to potentially deter subsequent cell failure, and ultimately, vascular disease.
Lay summary

The endoplasmic reticulum (ER) is the cell’s largest substructure, which makes molecules like proteins and stores calcium (Ca\(^{2+}\)). ER stress occurs when proteins accumulate inside the ER, often resulting in cell death if the stress is prolonged or particularly severe. The bulk of the ER stress response is preceded by a large drop in ER Ca\(^{2+}\), for which the release mechanism is unknown. In this project, we are monitoring changes to cellular, and specifically, ER Ca\(^{2+}\), concentrations while modifying the function of various Ca\(^{2+}\) pumps and channels in healthy endothelial cells. We hope to determine the physiological mechanism mirroring the ER stress Ca\(^{2+}\) drop, to be able to work towards preventing this dysregulation event and potentially avoiding the death of stressed cells in ER stress conditions.
Preface

All of the data presented in the current thesis was obtained from work done at the BC Children’s Hospital Research Institute (BCCHRI) and the Life Sciences Institute (LSI) at the University of British Columbia. The cell line used in the study is a generous gift from Dr. Pascal Bernatchez’s laboratory at the Center for Heart Lung Innovation at St. Paul’s Hospital in Vancouver.

Chapters 2 and 3 outline details pertaining to experiments designed and conducted in the van Breemen lab at the BCCHRI facilities and the LSI by Dr. Cornelis van Breemen and Gabriela Ziomek. I was responsible for executing all experimental protocols, collecting relevant data, and performing appropriate data analysis, with significant contribution from Dr. van Breemen with regard to experimental design.

Figures 1.4, 1.5 (Ziomek et al., 2014), and 1.8 (Ziomek, van Breemen and Esfandiarei, 2015) have been re-used within this thesis following their previous publication in the European Journal of Pharmacology. Following a query to Elsevier concerning reusing Elsevier content, I was granted permission from Elsevier’s editorial offices to use figures from my previous publications as long as the proper measures were taken to reference the journal in which they were published as the original source. Reference to the journal and specific articles from which the figures were repurposed was provided for each of the listed figures within the relevant figure legends.
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List of Abbreviations

µg Microgram
µM Micromolar
ATF6 Activating transcription factor 6
ATP Adenosine triphosphate
BAEC Bovine aortic endothelial cell
BFA Brefeldin A
BiP Binding immunoglobulin protein
BSA Bovine serum albumin
Ca\(^{2+}\) Calcium
[Ca\(^{2+}\)] Calcium concentration
[Ca\(^{2+}\)]\(_{\text{cyt}}\) Cytoplasmic calcium concentration
[Ca\(^{2+}\)]\(_{\text{ER}}\) Luminal endoplasmic reticulum calcium concentration
[Ca\(^{2+}\)]\(_{i}\) Intracellular calcium concentration
[Ca\(^{2+}\)]\(_{o}\) Extracellular calcium concentration
CALR Calreticulin
Cav1 Caveolin-1
CHOP C/EBP homologous protein
CICR Calcium-induced calcium release
CNX Calnexin
CVD Cardiovascular disease
DMEM Dulbecco’s Modified Eagle Medium
DMSO Dimethyl sulfoxide
DTT Dithiothreitol
EC Endothelial cell
E\(_{\text{Ca}}\) Equilibrium potential for Ca\(^{2+}\)
ED Endothelial dysfunction
EM Electron microscopy
E\(_{\text{Na}}\) Equilibrium potential for Na\(^{+}\)
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>E&lt;sub&gt;NCX&lt;/sub&gt;</td>
<td>Equilibrium potential for NCX</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial NOS</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>Endoplasmic reticulum-associated degradation</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin</td>
</tr>
<tr>
<td>F</td>
<td>Faraday’s number</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>GrpE</td>
<td>GroP-like gene E</td>
</tr>
<tr>
<td>HEPES</td>
<td>2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible NOS</td>
</tr>
<tr>
<td>IP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Inositol-1, 4, 5-triphosphate</td>
</tr>
<tr>
<td>IP&lt;sub&gt;3&lt;/sub&gt;R</td>
<td>Inositol-1, 4, 5-triphosphate receptor</td>
</tr>
<tr>
<td>IRE1</td>
<td>Inositol-requiring kinase 1</td>
</tr>
<tr>
<td>KBR</td>
<td>KB-R9743</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>Na&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Sodium</td>
</tr>
<tr>
<td>NCX</td>
<td>Sodium/calcium exchanger</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PERK</td>
<td>Pancreatic ER eukaryotic initiation factor 2α (eIF2α) kinase</td>
</tr>
<tr>
<td>PGI&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Prostacyclin</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>PMCA</td>
<td>Plasma membrane calcium ATPase</td>
</tr>
<tr>
<td>R</td>
<td>Gas constant</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine receptor</td>
</tr>
<tr>
<td>S1P</td>
<td>Site-1 protease</td>
</tr>
<tr>
<td>S2P</td>
<td>Site-2 protease</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarco/endoplasmic reticulum calcium ATPase</td>
</tr>
<tr>
<td>SOC</td>
<td>Store-operated channel</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>T</td>
<td>Temperature</td>
</tr>
<tr>
<td>Tg</td>
<td>Thapsigargin</td>
</tr>
<tr>
<td>TRP</td>
<td>Transient receptor potential channel</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>UTP</td>
<td>Uridine-5’-triphosphate</td>
</tr>
<tr>
<td>UUGT</td>
<td>Uridine 5’-diphosphate-glucose: glycoprotein glucosyltransferase</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
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Acknowledgements

I would like to take the opportunity to extend my grateful acknowledgement of the help I’ve received along the way to submitting this thesis. I hold myself in debt to the faculty of UBC who have invested so much of their time and effort in getting me to this point.

To Drs. Cornelis van Breemen and Mitra Esfandiarei, especially, I owe volumes of thanks. I thank Dr. van Breemen for teaching me how to navigate the problems a researcher consistently faces in constructing an interesting scientific story to tell the world. I thank Dr. Esfandiarei, in turn, for passing along to me the practical skills necessary to be able to tell that story in the first place. Both of these inspiring researchers mentored me and ignited an interest to pursue this project through their passion in their own work.

I would like to extend my thanks and gratitude to my committee members, Dr. Pascal Bernatchez and Dr. Ed Moore, for their questions, advice, and the encouragement, all of which were always forthcoming after every meeting.

I am also thankful to Mr. Jason Cui for his assistance with multiple endeavours throughout my time with the van Breemen lab. Similarly, I thank Mr. Arash Tehrani for his availability and help throughout completion of this project.

Finally, I would like to especially thank my parents and brother for their continuous support of my extended tenure in education. Without their backing, I would not have pursued my curiosity in the life of an academic for as long as I have. I also thank my partner James for his similarly unwavering support, made all the more meaningful as he progressed through his own degree at the same time as I did.
Chapter 1 - Introduction

1.1 Introduction

Calcium (Ca\(^{2+}\)) is the principal intracellular messenger controlling most bodily functions, including muscle contraction (Ebashi and Endo, 1968), neurotransmitter release (Neher and Sakaba, 2008), and cell proliferation (Balk et al., 1973).

Although it is involved in countless processes, the main focus here is intracellular signalling and protein folding. Ca\(^{2+}\) is a ubiquitous cation, found in every cell of the body, notably at varied concentrations within smaller domains of individual cells. Ca\(^{2+}\) is effectively compartmentalized between the cytoplasm and different cellular organelles, each having a different, highly-regulated concentration. For example, under steady-state conditions, extracellular free Ca\(^{2+}\) concentration as measured by equilibrium dialysis is approximately 1.5 millimolar (mM) (van Breemen, Daniel and van Breemen, 1966), significantly higher than the concentration maintained in the cell’s cytoplasm, which is about 100 nanomolar (nM) (Williams et al., 1985; Clapham, 1995). At rest, this low value is maintained by active pumping of Ca\(^{2+}\) out of the cytoplasm and into the extracellular space or uptake into cellular organelles such as the mitochondria, lysosomes, nucleus, and the endoplasmic reticulum (ER) (Clapham, 1995). The ER typically has a free Ca\(^{2+}\) concentration in the 100-200 micromolar (µM) range, a value also much higher than that of the cytoplasm (Ashby and Tepikin, 2001). In fact, the ER is the single largest store of Ca\(^{2+}\) inside the cell (Ashby and Tepikin, 2001) and movement of this ion into and out of the organelle forms the backbone of many different cellular signals. For example, changes to ER Ca\(^{2+}\) related to cellular stress responses are known to affect gene transcription within the nucleus, leading to significant changes in cellular metabolism (Hetz, 2012).
Figure 1.1. Schematic showing general differences in Ca\textsuperscript{2+} concentration between the extracellular space, cytosol, and ER lumen. mM: millimolar; µM: micromolar; nM: nanomolar.

ER Ca\textsuperscript{2+} is vital for many processes that maintain overall cellular health. For example, a significant portion of luminal ER Ca\textsuperscript{2+} is actually bound to proteins that aid in protein folding (Clapham, 1995; Michalak, Parker and Opas, 2002). Within the ER, many proteins known as chaperones are required for polypeptides to successfully reach their final conformations (Clapham, 1995; Michalak, Parker and Opas, 2002; Coe and Michalak, 2009). Importantly, these chaperones are commonly Ca\textsuperscript{2+}-dependent; they must be bound to Ca\textsuperscript{2+} ions in order to function properly. It is the interaction between functional chaperones and nascent polypeptides within the ER that allows for the final products of properly folded proteins to be successfully sent along their way (Coe and Michalak, 2009). Fluctuations in steady state Ca\textsuperscript{2+} levels therefore have the capacity to affect the functionality of this step along the polypeptides’ progression to their final and functional form. If the ER Ca\textsuperscript{2+} store is diminished as is the case for ER stress, the chaperone-bound Ca\textsuperscript{2+} would dissociate, rendering these molecules unable to perform their folding function. This has the potential to lead to negative outcomes for the cell’s health, such as induction of the ER stress response.
1.2 Calcium signalling

The ER is a tubular organelle that is distributed throughout the cell and actively stores and sequesters Ca$^{2+}$ inside its lumen via sarco/endoplasmic reticulum ATPase (SERCA) pump activity. Once Ca$^{2+}$ enters the organelle, it is buffered by binding proteins in the ER’s lumen. It is from these stores that localized Ca$^{2+}$ release can be activated in response to various physiological or pharmacological stimuli.

There are two types of channels mediating ER Ca$^{2+}$ release. The inositol triphosphate receptor (IP$_3$R) is a tetrameric protein containing a non-selective cation-permeable pore (Mikoshiba, 1993). This receptor is activated by binding inositol tri-phosphate (IP$_3$), with variable sensitivity depending on cytoplasmic and luminal ER Ca$^{2+}$ concentration ([Ca$^{2+}$]). For example, IP$_3$R is insensitive to IP$_3$ when Ca$^{2+}$ is at either very low or high concentration (Stehno-Bittel, Lückhoff and Clapham, 1995). The ryanodine receptor (RyR) is another tetrameric protein whose Ca$^{2+}$ release activity is also affected by both cytoplasmic and luminal [Ca$^{2+}$]. This receptor has multiple isotypes, RyR1, RyR2, and RyR3, with each having its own specific cellular distribution.

Two ATPase type pumps are vital to maintaining the steady-state low cytosolic [Ca$^{2+}$]: SERCA pumps two Ca$^{2+}$ per adenosine triphosphate (ATP) into the ER lumen, whereas the plasma membrane Ca$^{2+}$ ATPase (PMCA) moves one Ca$^{2+}$ from the cytosol to the extracellular space for each ATP hydrolysed. Also playing roles in maintaining cytosolic [Ca$^{2+}$] equilibrium are the store-operated channels (SOCs) and PM transient receptor potential channels (TRPs), each of which move Ca$^{2+}$ into the cytoplasm from the extracellular space.
The sodium-calcium exchanger (NCX) plays a similar role, as it removes Ca\(^{2+}\) from the cytosol in exchange for three sodium (Na\(^{+}\)) ions from the extracellular space. NCX is also able to reverse its activity, moving Ca\(^{2+}\) back into the cell while transporting Na\(^{+}\) out into the extracellular space. The direction of NCX activity is determined by both Na\(^{+}\) and Ca\(^{2+}\) gradients (Blaustein, 1977). The point of NCX activity’s reversal can be specified using the calculations for the equilibrium potentials of these two ions, \(E_{\text{Ca}}\) and \(E_{\text{Na}}\), where \(E_{\text{Ca}} = \frac{RT}{2F} \ln \left(\frac{[\text{Ca}^{2+}]_o}{[\text{Ca}^{2+}]_i}\right)\) and \(E_{\text{Na}} = \frac{RT}{F} \ln \left(\frac{[\text{Na}^{+}]_o}{[\text{Na}^{+}]_i}\right)\), where \(R\), \(T\), and \(F\) refer to the gas constant, temperature in Kelvin, and Faraday’s number, respectively (Blaustein and Lederer, 1999). The reversal potential for NCX overall can subsequently be calculated as follows: \(E_{\text{NCX}} = 3E_{\text{Na}} - 2E_{\text{Ca}}\) (Blaustein and Lederer, 1999).

It is important to note that the two main Ca\(^{2+}\)-extruding proteins, PMCA and NCX, generally serve the same function, though their capacities and affinities for Ca\(^{2+}\) differ significantly. PMCA, for example, binds tightly to Ca\(^{2+}\) (has a high affinity), but transports the ion relatively slowly (has a low capacity). These characteristics make it possible for PMCA to bind Ca\(^{2+}\) even when its intracellular concentration is low. NCX, on the other hand, has a low affinity for Ca\(^{2+}\) but a high capacity for its transport. NCX therefore only functions optimally where intracellular Ca\(^{2+}\) enters the micromolar range. Since endothelial cells lack voltage-gated Ca\(^{2+}\) channels, Ca\(^{2+}\) entry is mediated by a non-specific leak and receptor-activated channels (Adams et al., 1989).
Figure 1.2. Summary of main proteins involved in maintenance of Ca\(^{2+}\) homeostasis at the level of the ER and PM. PMCA: plasma membrane Ca\(^{2+}\) ATPase; SOC: store-operated channel; TRP: transient receptor potential channel; RyR: ryanodine receptor; IP\(_3\)R: inositol triphosphate receptor; SERCA: sarco/endoplasmic reticulum Ca\(^{2+}\) ATPase; NCX: sodium-calcium exchanger; CALR: calreticulin; CNX: calnexin

1.3 Protein folding

A single protein has multiple possible configurations, each with a specific free energy. The protein therefore begins folding with multiple pathways competing to reach different final shapes. It will, however, remain on the folding pathway that finally leads to folds embodying decreasing free energies, and will finally stop folding altogether when the final configuration with the lowest free energy has been acquired (Dobson, Šali and Karplus, 1998). Importantly, despite the potential for multiple different conformations, this final folding is largely determined by the protein’s original amino acid sequence.

The ER exhibits a set of particular characteristics, all of which alter how protein folding occurs in this organelle as compared to other cellular compartments. Most of the ER protein folding chaperones are Ca\(^{2+}\)-dependent, and their activity is altered by continuous changes in
luminal ER Ca\(^{2+}\) concentration. The effect that this ultimately has on normal protein folding within the ER depends on the protein of interest, as nascent polypeptides are dealt with by specific chaperones whose function may be affected differently in the face of ER Ca\(^{2+}\) fluctuation. Also important are the changes being made to the freshly-produced proteins in the ER, including N-linked glycosylation and formation of disulfide bonds between polypeptide residues (Schröder and Kaufman, 2005).

Another important consideration is that folding and degradation of a nascent polypeptide are closely associated, and may in fact be in direct competition of occurring at any given time (Hebert and Molinari, 2007). For the cell to optimally complete protein folding the cellular machinery necessary to accomplish degradation must also be present (Eriksson et al., 2004). Even with all such machinery present and operational, misfolding can still occur. For example, the products of mutated genes may affect polypeptide sequences in a way that mars the nascent protein’s folding pathway to its wild-type final conformation. Ideally, these faulty proteins are rapidly degraded, but that outcome is not guaranteed. Another possibility is their retention in the ER lumen and subsequent accumulation (Hebert and Molinari, 2007).

The ER has a large number of molecular chaperone molecules present in its lumen. While their most cited function is to help nascent single and oligomeric polypeptides reach their final conformation (Ellis, 2006), this action in of itself also helps prevent accumulation of unfolded proteins. Chaperones also serve to keep those proteins that remain to be folded in specific intra-organellar compartments, or micro-domains, where the largest concentration of folding enzymes can be found (Hebert and Molinari, 2007). The key molecular chaperones of the ER include calreticulin, calnexin, members of several heat shock protein (HSP) sub-families, as well as GroP-like gene E (GrpE)-like families (Ma and Hendershot, 2004).
Those proteins that do not end up properly folded are most commonly degraded through the work of the ER-associated degradation (ERAD) system, preventing any potential build-up of such proteins inside the ER (Ellgaard and Helenius, 2003). These failed proteins are ultimately fragmented sufficiently for their amino acid components to be re-used for synthesis of new polypeptides (Arias, Doyle and Schimke, 1969; Klausner and Sitia, 1990; Schubert et al., 2000). Literature pertaining to the topic differs in the estimates made as to the quantity of nascent polypeptides that undergo this degradation process, with data suggesting anywhere from approximately one third of all newly formed polypeptides (Schubert et al., 2000) to more conservative estimates stating that approximately one fifth of proteins are dealt with in this manner (Vabulas and Hartl, 2005). Importantly, work of this nature approximates the total amount of immature proteins that are degraded, including protein breakdown resulting from necessary ERAD activity for the creation of a supply of amino acids for biosynthesis. Studies of the kind referred to here are not estimating the amount of proteins that are improperly folded and degraded only as a result of mistakes made by cellular machinery which render them unfit to reach their final configuration, but rather the total proportion of proteins that are broken down for numerous reasons.
Figure 1.3. General possible outcomes for nascent polypeptides. Newly formed proteins enter the ER and are folded into their final configuration. Those that are folded properly are transported to the Golgi apparatus via vesicle and move further along the secretory pathway from there. Proteins that are folded improperly are detected by a cellular quality control system and redirected for eventual destruction by the ER-associated degradation system (a proteasome).

Within the many steps involved in proper protein folding, a few stand out with regard to their importance. For example, N-linked glycosylation is a relatively simple process with significant consequences for protein folding. The step involves the attachment of a sugar molecule (glycan) to a specific nitrogen atom on a protein residue. This process is generally accepted as being necessary for the majority of successful protein folding, as well as oligomerization when relevant. This notion is corroborated by the fact that blockage of N-linked glycosylation in protein folding by the antibiotic tunicamycin results in a failure of many proteins to reach their final conformations (Kornfeld, 1978; Helenius, 1994). Despite this experimental result, it is at the same time worth noting that some proteins missing their attached sugars are not affected at all, or are only moderately affected as far as reaching their final folded shape goes, as this process relies on multiple factors and is not always crippled by the lack of any one (Kornfeld,
1978). Also important is that as the protein progresses along its folding pathway, its advancement towards its final conformation is tracked by the trimming of sugar residues; this serves to provide a method whereby associated cellular machinery can determine the progression of the protein’s folding at any given time (Ellgaard, Molinari and Helenius, 1999).

1.4 Quality control

The cell follows the general rule that only those proteins that pass a strict quality assurance check are actually approved for progression down the secretory pathway. Quality control in this context encompasses the ability of the cell to sort proteins ready to be released from the ER from both similar but not correctly folded variants of themselves, as well as specifically-labeled ER resident proteins (Ellgaard, Molinari and Helenius, 1999). For proteins composed of multiple polypeptide chains, achievement of proper quaternary structure must also be confirmed.

Improperly folded proteins are largely caught at the level of the ER and either immediately retained in the organelle’s lumen or subjected to degradation by the ERAD system. Those that undergo further modification when released to the Golgi apparatus are still subject to the rigorous examination of the quality assurance system beyond the ER, in the sense that proteins can be brought back to the ER from organelles further along the secretory pathway. Proteins that fail to remain rigidly folded in their approved final configuration can have their final cellular destination altered. This commonly occurs by having proteins that have reached as far as the Golgi, for example, be redirected to lysosomes for destruction (Ellgaard, Molinari and Helenius, 1999).

One mechanism involved in keeping nascent polypeptides from moving beyond the ER lumen is their association with resident ER chaperones. While new peptide chains will certainly bind
these proteins during the folding process, chaperone molecules can also serve the purpose of anchoring as yet improperly folded, or immature, proteins to the confines of the ER. It appears that even inconsistent binding of a misfolded protein to a chaperone keeps the polypeptide in question from being transported further along the secretory pathway (Ellgaard, Molinari and Helenius, 1999). Certain chaperones are also capable of recognizing glycoproteins that have undergone N-linked glycosylation, an important step in the folding pathway of these types of protein. For example, the lectins (which recognize and bind to specific sugars) calreticulin and calnexin, bind indiscriminately to almost all glycoproteins formed in the ER, and proceed to ensure retention as well as proper folding of these bound proteins. These lectins also associate with a handful of other enzymes, such as the uridine 5’-diphosphate-glucose: glycoprotein glucosyltransferase (UUGT), as well as glucosidase II. Between these two enzymes’ activity, one promotes association between the proteins and sugar residues (UUGT), with the other induces the release of such associations (glucosidase II). Ultimately, the activity of these two enzymes in attaching or removing sugar residues to N-linked oligosaccharides affects glycoprotein binding and release from the lectins. This cycling through binding to calnexin and calreticulin is vital for proper folding in the case of many glycoproteins. UUGT senses which proteins have yet to reach their mature confirmation and acts on these to add further glucose residues to them. It therefore becomes clear how the decision of UUGT, whether to add more sugar residues or not to a nascent protein, will in turn determine whether it goes through another round of binding to the lectins for further folding (Parodi, 1999). Only proteins that are examined by UUGT and receive no further additions of glucose residues are released from the lectin-binding cycle and are able to progress to the next stage of the secretory pathway.
Another mechanism of retaining proteins in the lumen of the ER until they are deemed mature enough to undergo Golgi apparatus modifications, is simple aggregation. This applies in particular to oligomeric proteins that must have their individual proteins combine to be deemed travel-competent (Hurtley and Helenius, 1989). Improperly folded proteins are not the only polypeptides that are retained in the ER lumen. The multiple subunits of oligomeric proteins, as well as oligomeric proteins that have only been partially assembled, are also held back. Importantly, these proteins that are still in the process of being folded are recognized for what they are, and they are not retained in the ER through the same mechanism employed for misfolded monomeric polypeptides (simply being bound by one of the many ER resident molecular chaperones). Aggregation of proteins that are incompletely folded or assembled into their oligomeric configuration has been documented (Hurtley and Helenius, 1989), and it appears that the large size of the resulting protein aggregate mass prevents free diffusion or transport through the ER membrane (Ellgaard, Molinari and Helenius, 1999).

Once a protein has finally been properly folded, it will no longer be retained in the ER by components of the quality assurance system (such as UUGT and the lectin chaperones) and can access ER exit sites and make its way to the Golgi.

The story differs, however, for proteins that are improperly folded. Briefly, proteins that have been retained in the ER lumen for an extended period of time ultimately become destined for degradation (Klausner and Sitia, 1990), which is mainly performed by the 26S proteasome found in the cytosol in close proximity to the ER membrane. Once misfolded or unassembled oligomeric proteins have been recognized by any of a host of molecular chaperones, they are removed from the ER via translocon (Plemper et al., 1997). Multiple subsequent modifications are made, including the de-glycosylation of glycoproteins, and the ubiquitination of all proteins
passing through this process to mark them for degradation by the proteasome. Proteins tagged by poly-ubiquitination are then destroyed by the activity of the ERAD system.

Evident in this summary is that this system functions by monitoring structural aspects of the protein being folded, rather than any characteristics relating to their eventual function. This is a key point about how the quality control process works; indeed, a design in this manner is necessary because a significant portion of the nascent polypeptides synthesized at the ER leave this organelle in an inactive state. Combined quality control and degradation protocols clearly demonstrate that there is a complex system in place in the ER to ensure that very few, if any, improperly folded or immature proteins will be able to make it out of this organelle, and potentially induce harmful effects elsewhere in the cell.

1.5 Endoplasmic reticulum (ER) stress

What happens, however, when improperly-folded proteins are not dispatched to this fate?

ER stress is characterized most commonly by an accumulation of unfolded or misfolded proteins inside this organelle (Rutkowski and Kaufman, 2004; Xu, Bailly-Maitre and Reed, 2005; Chao et al., 2012). The cause for this phenomenon varies, and can involve anything from a disruption in red-ox regulation to a viral infection. The result of the induction of stress, however, follows the same steps regardless of the initiating factor. As the internal chemistry of the ER is affected by the initiating event, the protein-folding machinery of the organelle begins to fail, to the point that significantly larger quantities of proteins remain unfolded or are folded incorrectly than under healthy steady-state conditions. The protein accumulation is detectable after the fact, through the use of Western blot tests showing the temporal increase in concentration of relevant marker proteins (as seen in (Marciniak et al., 2004; Özcan et al., 2004; Lee, 2005). These
misfolded polypeptides do not pass the quality assurance check that allows them to be sent forth along the secretory pathway (Ellgaard and Helenius, 2003), and more proteins are ultimately retained inside the lumen of the ER than are sent onwards to other organelles. In the meantime, the ER continues to produce new polypeptides, therefore compounding the problem (Hampton, 2000). As the number of retained proteins is drastically increased, the ERAD system is unable to destroy proteins at a pace sufficient to clear this luminal accumulation. The result, then, is a clogged ER lumen, which the cell must attempt to clear if it is to recover from this stress.

While the term “ER stress” is not one widely known by the general population along the lines of for example, “cancer”, it is a phenomenon that plays a critical role in a variety of different disease conditions. ER stress-induced cell death has been implicated in many disease states, including cardiovascular complications (Minamino and Kitakaze, 2012), neurodegenerative disease (Lindholm, Wootz and Korhonen, 2006), and diabetes (Özcan et al., 2004). While not typically manifested in an obvious clinical symptom, at the cellular level there is consistent evidence in these conditions that cells experience ER stress and attempt to fight it off with the unfolded protein response (UPR) before being overwhelmed and sacrificing themselves through apoptosis. This is particularly important in cardiovascular pathophysiology. ER stress has previously been identified in a range of serious cardiac afflictions, such as atherosclerosis (Scull and Tabas, 2011), ischemic heart disease (Azfer et al., 2006), and heart failure (Okada et al., 2004; Song et al., 2011). ER stress tends to induce a vicious cycle, for example in a compromised vascular endothelial layer it will lead to numerous cells dying off within the blood vessel wall and thus further aggravation of vascular dysfunction. From such disruption a clear path to disease is evident; as soon as the integrity of the endothelium is compromised, so too is its overall protective effect on the vasculature. A stressed endothelial cell layer could ultimately
lead to plaque accumulation, subsequent atherosclerosis, and heart disease. This is merely one example of many that highlight the relevance of ER stress to the bigger picture of a wide range of disease states.

1.6 Natural and experimental induction of ER stress

Complicating any investigation into the ER stress phenomenon is that it can be induced by numerous causes, both endogenous and exogenous. Having run through the basic gamut of how a newly formed protein enters and then moves beyond the ER, it is clear that there are multiple steps that one such polypeptide must go through in its transformation. There are therefore many points at which the progression of a nascent polypeptide towards maturity can be prematurely halted. The potential for one such error to cause others ultimately leads to the possibility of an unwelcome accumulation of misfolded proteins in the ER’s lumen.

The concept of ER stress has developed over the last couple of decades into a relatively well-understood phenomenon, with many groups dedicated to investigating this cellular condition. Due to the many links that have been established between ER stress and a host of life-threatening diseases, further research into its mechanisms is required. While it is of course possible to prove the presence of ER stress in the cells of patients suffering from the relevant conditions such as diabetes, Parkinson’s, and a long list of others, the enormous magnitude of variables involved tend to make studying the mechanics of ER stress in this context too complex. Consequently, most research in this area has invoked the use of animal models for both whole tissue and cell culture work. In this scenario, cells will often be grown under optimal conditions, while ER stress is induced by applying one or more ER stressor agents.
The most common of the pharmacological agents for which the mechanism of ER stress induction is the best documented and understood are tunicamycin, thapsigargin, brefeldin A, and dithiothreitol. A search on PubMed indicates that tunicamycin, an antibiotic (Takatsuki, Arima and Tamura, 1971), is the most widely used agent for this purpose. It is employed mainly because of its presumed well-characterized manner of inducing stress, which is blockade of the N-glycosylation step of nascent polypeptide modification inside the ER lumen (Kuo and Lampen, 1974). This reaction is often important for the folding of glycoproteins into their final conformation; blocking it results in proteins unfit for passage onwards down the secretory pathway. However, our laboratory recently discovered a second mechanism through which this agent can induce ER stress (Ziomek et al., 2014).

![Tunicamycin exposure of cultured vascular smooth muscle cells induces immediate cytoplasmic Ca\(^{2+}\) transients in both the presence and absence of Ca\(^ {2+}\) due to permeabilization of ER and plasma membranes.](image)

**Figure 1.4.** Tunicamycin exposure of cultured vascular smooth muscle cells induces immediate cytoplasmic Ca\(^ {2+}\) transients in both the presence and absence of Ca\(^ {2+}\) due to permeabilization of ER and plasma membranes. (Ziomek et al., 2014)

In our recent report, we were able to demonstrate that tunicamycin has ionophoretic properties that severely affect cellular Ca\(^ {2+}\) levels when it is applied to cultured cells (Ziomek et
As shown in Figure 1.4, acute exposure of healthy vascular smooth muscle cells (VSMCs) to a dose within the range observed in the ER stress literature induces an immediate and significant Ca\textsuperscript{2+} transient, of similar magnitude to that caused by exposure of cells to the well-known Ca\textsuperscript{2+} ionophore ionomycin. As most ER stress research involves incubation of cells with the ER stressor of choice for a matter of hours, and this increase in Ca\textsuperscript{2+} permeability occurs within seconds of addition of tunicamycin to healthy cultured plates, it cannot be concluded that this drug induces ER stress only by its negative effects on protein N-glycosylation. While not the focus of our current work, this discovery has affected our subsequent ER stress study designs, wherein we traded tunicamycin in for another ER stressor (brefeldin A) with a confirmed insignificant effect on cellular and ER Ca\textsuperscript{2+} (Ziomek et al., 2014).

Thapsigargin is another stressor which affects cellular and ER Ca\textsuperscript{2+}, doing so by effectively blocking the SERCA pump (Thastrup et al., 1990). With SERCA blocked, Ca\textsuperscript{2+} continues to escape the ER without being replenished by the pump’s activity, ultimately leading to the organelle’s depletion. This lack of free luminal Ca\textsuperscript{2+} results in Ca\textsuperscript{2+} that was buffered in the ER lumen being released from Ca\textsuperscript{2+}-dependent chaperones. These unbound chaperones are then unable to perform their protein-folding function, ultimately leading to an excess of unfolded proteins forming in the ER lumen.

After discovering that tunicamycin is unsuitable for addressing Ca\textsuperscript{2+}-related ER stress questions, we shifted our focus to other ER stressors, such as brefeldin A (BFA) and dithiothreitol (DTT). BFA is a compound that prevents the transport of newly formed and modified proteins from the ER lumen to the Golgi apparatus (Misumi et al., 1986). It does this specifically by preventing the protein complex coating the transport vesicles from binding to the membrane of the Golgi. The failure in protein delivery results in retention inside of the lumen of
the ER of proteins that would otherwise move further down the secretory pathway, as the Golgi complex fuses with the ER as a result of the lack of transport vesicle formation (Fujiwara et al., 1988). DTT induces the ER stress response in an arguably simpler manner; this compound is a redox reagent (Cleland, 1964), and ultimately exerts its effect directly on nascent polypeptides in the lumen of the ER to reduce protein disulfide bonds. The effect of DTT’s reducing activity is essentially prevention of disulfide bonds forming between cysteine residues of individual proteins, or between those of multiple polypeptides that must associate to form oligomeric final products. This paves the way to ER stress by simply affecting the protein folding process itself, resulting in high numbers of polypeptides that do not reach their final conformation due to the lack of disulfide bond formation, which normally contributes to folding polypeptides’ requisite shape. Important to note is the fact that neither BFA nor DTT has any significant acute effect on ER or overall cellular Ca\textsuperscript{2+} movement, as was reported in our previous published work (Ziomek et al., 2014). The observed blip in cytoplasmic Ca\textsuperscript{2+} levels following addition of BFA to cultured cells maintained in physiological buffer (Fig. 1.5) was considered insignificant due to the negligible size of the transient, particularly when compared to the effect on cellular Ca\textsuperscript{2+} of adding a drug with ionophoric properties like tunicamycin (Fig 1.4), which induces an approximate 4-fold increase in cytoplasmic Ca\textsuperscript{2+} levels. The blip observed here is therefore likely an artefact of the acute addition of BFA to the plate of cultured cells, rather than any specific effect exerted by this drug’s actions on the cell.
Figure 1.5. Brefeldin A and dithiothreitol have no significant acute effect on steady-state cytoplasmic Ca\(^{2+}\) levels. Acute exposure of cultured VSMCs to these ER stressor agents showed no significant increases in cytoplasmic Ca\(^{2+}\) levels, indicating little effect of the agents on overall cytoplasmic Ca\(^{2+}\) movement, as well as that of ER Ca\(^{2+}\) stores. (Ziomek et al., 2014)

It is clear from this brief survey of a handful of ER stressor agents that there are various methods of causing protein accumulation inside the ER, and therefore, of inducing the stress response in an experimental setting. Also important to consider, however, is the way that this cellular phenomenon occurs naturally in the human body. These endogenous causes of ER stress can be manifested in multiple ways, and include, but are not limited to, genetic mutation, viral infection, chemical insult, nutrient deprivation, disruption to redox regulation, and Ca\(^{2+}\) dysregulation events (Rutkowski and Kaufman, 2004).

ER stress research tends to focus on circumstances stemming from direct, or near-direct disruption of protein folding, with little attention being paid to some of the less common avenues of induction as listed above (Schröder and Kaufman, 2005). The work presented here explores cellular events preceding the ER stress response. This is done so as to pinpoint the changes to cellular metabolism that occur in a cell experiencing the onset of the stress response for eventual application to an ER stress model.
1.7 The Unfolded Protein Response

Cells undergoing ER stress have the means to correct the problem in the form of the unfolded protein response, designed to decrease the number of improperly or misfolded proteins inside the ER lumen through multiple branches of action that affect cellular metabolism (Hetz, 2012). The three main changes observed following activation of the UPR are 1) increasing translation of chaperone proteins in an attempt to increase the rate of proper folding still occurring inside the ER, 2) decreasing translation of nascent polypeptides so as to prevent further addition to the accumulated proteins already present, and 3) upregulating the production of proteins involved in the degradation pathway to help mitigate the issue of protein build-up.

The UPR therefore clearly involves a complex series of changes being made to the normal functioning of a stressed cell. The main actions taken, as outlined above, are each initiated by a different branch of the UPR working in parallel. These three individual branches are headed by transmembrane proteins situated on the ER membrane (Cao and Kaufman, 2012), namely, by activating transcription factor 6 (ATF6), inositol-requiring kinase 1 (IRE1), and pancreatic ER eukaryotic initiation factor 2α (eIF2α) kinase (PERK). Each of these proteins act as sensors, with their luminal portions being able to detect levels of either unfolded or misfolded proteins present at a given time inside the ER (Cao and Kaufman, 2012). Following activation, each of these proteins activates its own signalling pathway, resulting in three significant changes to cellular metabolism. ATF6 activation results in the increased production of ER chaperone proteins, IRE1’s activation serves to increase assembly of ERAD components, and finally, PERK signaling ultimately results in decreased global messenger RNA (mRNA) translation within the ER.
Figure 1.6. Summary of the changes to cellular metabolism involved in the unfolded protein response (UPR). Disruption of steady-state misfolded protein levels in the ER results in the initiation of multiple signaling pathways headed by PERK, IRE1α, and ATF6. Activation of PERK results in reduction of protein synthesis to lighten the burden of accumulation in the ER lumen. IRE1α activity culminates in increased production of protein-folding chaperones, as well as upregulation of ERAD genes. Activation of ATF6 also serves to increase numbers of ER resident protein chaperones, as well as to increase gene expression of multiple components of the UPR.

All proteins entering the ER are, of course, initially found in their unfolded state, therefore it is clear that the thresholds for activation of each of ATF6, IRE1, and PERK must be regulated via feedback mechanisms (Walter and Ron, 2011). There are multiple theories on activation circulating in the literature, particularly with reference to the IRE1 branch, though the majority appears to believe that this process relies on competition for binding to the chaperone BiP (binding immunoglobulin protein), a member of the heat shock protein HSP70 family, between unfolded proteins and IRE1. ATF6 activation differs significantly from that of the other two transmembrane proteins, whereby ATF6, normally bound to BiP, is released following a detected
accumulation of mis/unfolded proteins. ATF6 is subsequently cleaved inside the Golgi apparatus by site-1 and site-2 proteases (S1P and S2P), resulting in the ATF6 p50 fragment. This fragment, a transcription factor, moves to the cell’s nucleus to ultimately activate gene expression, thereby serving to increase synthesis of chaperone proteins of different families, such as BiP (of the HSP70 group) and glucose-regulated protein 94 (of the HSP90 group) (Walter and Ron, 2011; Cao and Kaufman, 2012). The luminal domains of IRE1 and PERK are expected to exhibit similar structure, and therefore, the structures involved in their activation are also likely reminiscent of each other. In the case of the PERK branch of the UPR, following induction of ER stress, BiP is again released from the luminal domain of this transmembrane protein. PERK then oligomerizes and undergoes auto-phosphorylation for activation (Cao and Kaufman, 2012). Once activated, PERK goes on to phosphorylate a site on the α subunit of eIF2α. Phosphorylated eIF2α ultimately decreases ER protein translation with the effect of reducing incoming nascent polypeptides that need to be folded. A recent review indicates that there are multiple possibilities for the mode of IRE1 activation (Cao and Kaufman, 2012). This UPR branch is the most conserved across species, and functions as the only component of this rescue response in lower eukaryotes (Walter and Ron, 2011; Cao and Kaufman, 2012). First, it is possible that the unfolded proteins accumulating inside the ER lumen compete with the transmembrane protein for binding to BiP. When IRE1 is bound to this chaperone, signaling is inhibited; however, when IRE1 is released from BiP, it dimerizes, undergoes auto-phosphorylation, and ultimately activates ribonuclease (RNase) activation. This enzyme serves to degrade ribonucleic acid (RNA) into small segments, rendering the original strand useless for translation into nascent polypeptides. The second proposed model for IRE1 activation involves direct binding of un/misfolded proteins to its luminal domain, causing the signal protein to dimerize. Despite the
uncertainty surrounding IRE1’s activation, the end result of its signaling activity remains the same: a reduction in ER protein synthesis to lighten the luminal burden as the cell attempts to deal with the overload of misfolded protein.

In certain circumstances, together, these dramatic changes to cellular metabolism manage to clear the accumulation of misfolded proteins and the cell recovers from the stress experienced. However, in other cases, the UPR will ultimately fail to correct the problem and will veer from attempting to orchestrate cellular recovery to instead resigning the cell to apoptosis (also known as programmed cell death) (Wu and Kaufman, 2006).

1.8 Apoptosis

A cell is likely to activate an apoptotic pathway if it has battled an accumulation of unfolded proteins for a prolonged period. In some cases, the initial insult is so severe that it induces a rapid and overwhelming accumulation of unfolded protein, leading to the activation of pro-apoptotic pathways even as the UPR is at its initial stage (Rutkowski and Kaufman, 2004). Important to note is that there are both intrinsic and extrinsic apoptotic pathways. Briefly, intrinsic stimulation of apoptosis results in activating pro-apoptotic proteins of the Bcl-2 protein group, while downregulating those with anti-apoptotic tendencies. Important to the intrinsic apoptotic pathway is the release of cytochrome-c, which initiates a series of protein interactions and signals being sent to activate multiple caspases, among them caspase-3, also known as the ‘executioner’ (Rutkowski and Kaufman, 2004). The extrinsic pathway also involves the caspase family. In this case, it is the activation of members of the death receptor family that leads to the recruitment of caspase-8, also known as the “initiator”, and the subsequent activation of caspase-3 that results in apoptotic cell death. Despite the relatively strong understanding of these different apoptotic pathways, their relation to the ER stress response remains unclear. Some
studies have reported that ER stress-related apoptosis can involve components of both intrinsic and extrinsic pathways (Szegezdi, Fitzgerald and Samali, 2003).

Not unexpectedly, changes to intracellular Ca\(^{2+}\) concentrations are also tied to the apoptotic process. It has been suggested that changing concentrations of this ion within the cytoplasm, ER, and other cellular compartments like the mitochondria can also embody a cause for apoptosis (Rutkowski and Kaufman, 2004). A potential pathway appears to involve the pro-apoptotic members of the Bcl-2 family, Bad, Bax, and Bak, wherein following ER stress induction, both Bak and Bax change their conformation at the ER membrane. Bak, now located at the ER, affects Ca\(^{2+}\) signaling to result in ER Ca\(^{2+}\) release and the ultimate luminal activation of caspase-12 in the ER (Zong et al., 2003). The release of ER Ca\(^{2+}\) brings forward an important interaction with nearby mitochondria, whereby these organelles take up the freed Ca\(^{2+}\) at areas of close approximation of ER and mitochondrial membranes.

As previously mentioned, early changes to cellular metabolism as initiated by the UPR affect the Bcl-2 family. The activity of these proteins in an ER stress scenario impede the effects of Bax and Bad of the BH3-only group. For example, Bcl-2’s localization is affected in response to the induction of ER stress, and if it remains situated at the ER membrane, has even been shown to block apoptosis from occurring (Häcki et al., 2000). On the other hand, the transcription factor C/EBP homologous protein (CHOP), is a pro-apoptotic molecule. During ER stress and the decision to kill the cell, CHOP decreases Bcl-2 transcription, therefore effectively swinging the cell’s activity from attempting to pave a road to survival to being pro-apoptotic (Rutkowski and Kaufman, 2004).
Figure 1.7. Schematic showing the main avenue for apoptotic signaling in cells experiencing severe ER stress. Prolonged CHOP activation is known to promote cellular apoptosis, with this protein exerting multiple actions promoting cell death.

The fact that prolonged UPR activity is required for attempts to save the cell serves as an indication that the ER stress experienced cannot be easily halted and reversed. Considering this cellular decision in a wider framework, this initiation of apoptosis has been suggested to serve as a manner of protecting the affected individual as a whole from the small subset of cells that may be considered to have gone “rogue” and are unable to rectify the problem in an efficient way (Walter and Ron, 2011). In this context, then, this group argues that this decision to pursue cellular death over recovery makes the UPR’s role in various diseases more evident (Walter and Ron, 2011). Namely, when the normal use of the UPR fails, its cell-killing function may result in the death of healthy, beneficial cells in proximity to those that are excessively stressed.

1.9 Cardiovascular disease

The cardiovascular system involves the heart, blood vessels, and blood. It functions to deliver oxygen and nutrients to the body’s tissues while removing waste products for delivery to the relevant organs involved in their metabolism. The cardiovascular system thus has a main role in maintaining control of the body’s overall homeostasis. This includes control over the body’s
internal temperature, blood pH level, and cellular osmotic balance. Simply by noting the widespread reach of this system in keeping the body functional, it becomes equally apparent how easily it could be compromised.

Cardiovascular disease (CVD) includes a wide range of afflictions with serious health implications for those affected. It covers all conditions relating to the heart and associated blood vessels, including hypertension, stroke, arrhythmias, myocardial infarction, angina (both also referred to as coronary artery diseases), heart failure, congenital heart disease (heart defects present at birth), aortic aneurisms (significant dilation of the aorta), cardiomyopathies (a subgroup of conditions that affect heart muscle), myocarditis (inflammation of the heart or surrounding layers/tissue), and many more (Mendis, Puska and Norrving, 2011). The underlying causes for these conditions as a group can vary substantially, though there are known key risk factors which put any individual at greater risk of suffering from some form of CVD. By now this information has been widely documented and it is virtually common knowledge that factors such as smoking, obesity, high blood cholesterol, alcohol consumption, and high blood pressure, among others, contribute to a high risk profile (Kannel and McGee, 1979; Hubert et al., 1983; Wilson et al., 1998; Mendis, Puska and Norrving, 2011). While the majority of CVD cases are thought to be preventable (McGill, McMahan and Gidding, 2008), it is the unfortunate truth that this group of conditions together forms the leading cause of death worldwide (Mendis, Puska and Norrving, 2011).

As CVD encompasses not only problems with the heart, but also the associated vasculature (Mendis, Puska and Norrving, 2011), there are innumerable issues that may arise across the system as a whole to compromise an individual’s health. Of particular interest to the current project is the endothelium, the innermost layer of cells lining the body’s blood vessels, which
performs multiple functions vital to cardiovascular health. When the endothelial layer is compromised, a host of problems will likely follow for the affected individual.

A brief outline of one of the main instigators of CVD, atherosclerosis, reveals how endothelial layer health is involved in this condition, as well as in other, similar disease states. Atherosclerosis involves the thickening of an artery wall following an accumulation of cells on the inner lining of the vessel, contributing to plaque formation. It can take decades for this plaque to reach significant size and become symptomatic (Ross, 1999). Nevertheless, with time, these accumulations can grow in size to interfere with blood flow through the affected vessel, leading to restriction of blood flow to tissues (ischemia). Atherosclerosis in coronary arteries is a major cause of myocardial infarction (Ross, 1999).

Investigation pertaining to the development of atherosclerosis revolves around endothelial dysfunction (ED), wherein the normal protective function of the endothelial cell (EC) layer lining blood vessels is disrupted as a result of problems with creation of this cell type’s many products (Deanfield et al., 2005). Previous work had outlined a similar theory for the cause of this complication based on endothelial denudation (Ross and Glomset, 1973). This hypothesis, however, later gave way to the current notion of ED. While there are a host of causes for ED, once it has been induced, it appears that atherosclerosis is a common result. When this cell layer has been compromised, likely by injury to the area of concern, its ability to prevent adhesion of platelets and leukocytes, as well as maintenance of its usual low permeability, is lost (Ross, 1999). Due to their importance to vascular health, the current study uses endothelial cells to investigate ER stress and its implication in CVD.
1.10 Endothelial dysfunction

Blood vessels are composed of three layers, the adventitia (outermost), the media (middle), and the intima (innermost layer). The tunica intima is composed of a thin layer of ECs, whereas the tunica media and adventitia are thicker as a result of their composition of smooth muscle, elastin and collagen. The ECs of the tunica intima form a thin layer in between blood flowing through a vessel and the vessel wall itself, and are therefore in direct contact with blood flowing through tributaries of the vasculature.

Endothelial cells make an important contribution to vascular health, through their role in creating and releasing a wide range of products affecting the function of smooth muscle and blood cells. For example, endothelial cells can release coagulants and anticoagulants, vasodilators and vasoconstrictors, and a host of other similar chemical pairs capable of exerting opposite effects on the blood vessel in question (Stern et al., 1985; Ignarro et al., 1987). A healthy EC layer most commonly functions to serve a protective function by controlling blood pressure, preventing inflammation, preventing adherence of circulating cells and molecules to the walls of the vessel, and serving as a semi-permeable barrier between blood and extracellular fluid. The ability of the endothelium to exert its protective roles is based on substances found in the blood, as well as factors produced by ECs themselves. Often, it is substances within the blood, such as insulin, glucose, thrombin, and more, that induce ECs to release specific compounds in response. The main endothelial factors secreted are nitric oxide (NO), endothelin (ET-1), and prostacyclin (PGI₂), each of which functions as either a vasodilator (NO and PGI₂) or a vasoconstrictor (ET-1) (Lüscher, 1990).
NO is produced by many different cell types, but has particular importance in the vascular endothelium. This is due to the product’s role in regulating blood flow, as well as its anti-inflammatory, anti-proliferative, and anti-thrombotic effects (Rees, Palmer and Moncada, 1989).

NO is produced as a result of the activity of the nitric oxide synthase (NOS) enzyme. NOS can be found in multiple forms in the endothelium, either as inducible NOS (iNOS) or endothelial NOS (eNOS), also known as constitutive NOS (Aktan, 2004). Under physiological conditions, the bulk of NO is produced by eNOS in a Ca\(^{2+}\)-dependent manner. iNOS is usually not present in healthy, resting cells, but its expression can be induced following disturbances in multiple cell lines, including ECs (Aktan, 2004). For normal NO production to occur, the ECs of a blood vessel are acted on by the shear force resulting from blood flow across their surfaces. These shear forces induce Ca\(^{2+}\) release, which in turn activates eNOS, which catalyzes the reaction turning the amino acid L-arginine into NO (Alderton, Cooper and Knowles, 2001). This process is better known as flow-dependent NO formation, due to the requirement of blood flow-generated shear force for NOS activation. In addition, activation of a variety of endothelial receptors by autocoids such as acetylcholine, adenosine, bradykinin, stimulates Ca\(^{2+}\) signals, which dissociate calmodulin from eNOS to cause its activation (Lüscher, 1990).

iNOS activation differs from that of eNOS in that it is a Ca\(^{2+}\)-independent process (Alderton, Cooper and Knowles, 2001). Its activity is also very low when the endothelium is healthy. When problems arise, however, such as inflammation, iNOS is activated (Cooke and Davidge, 2002; Aktan, 2004). Under these non-physiological conditions, the roles are reversed, and iNOS can produce significantly greater amounts of NO than its constitutive counterpart. Unfortunately, NO generated by iNOS leads to accumulation of harmful reactive oxygen species (ROS).
Any disturbance to NO’s formation and release could have devastating effects on blood flow and the vessel wall. Significant loss of NO at the endothelial layer will ultimately result in vasoconstriction of the affected blood vessel, adhesion of platelets and other substances to the surface of the vascular endothelium, and inflammation following upregulation of endothelial adhesion molecules that allow substances to bind the cells’ surfaces (Cai and Harrison, 2000). As a result of these outcomes, ED often plays a role in the development of a host of vascular diseases, including ischemic heart disease, atherosclerosis, and heart failure, among others (Flammer et al., 2012; Eren, Yilmaz and Aydin, 2013). ED has also been implicated in a range of other diseases. In diabetes, for example, it is relatively well known that there is a connection between changes to steady state Ca$$^{2+}$$ signaling and the development of ED (Fernández-Velasco et al., 2014), though the exact processes involved have not yet been comprehensively studied or understood. However, it is known that the diabetes-plagued endothelium suffers from many disturbances to its normal surroundings, including, but not limited to, hyperglycemia, reduced production of NO, as well as the consequences of ER stress and apoptosis (Ding and Triggle, 2010; Basha et al., 2012). One general conclusion from this field of research is that the majority of reported changes to cellular environment have an effect on Ca$$^{2+}$$ homeostasis (Fernández-Velasco et al., 2014).

1.11 Ca$$^{2+}$$ dysregulation in ER stress

ER stress literature commonly makes reference to a drop in ER Ca$$^{2+}$$ content, considered characteristic of the stress response. While much of the work pertaining to ER stress makes mention of this phenomenon, until recently there was little to no information on its magnitude or timing. Our laboratory has attempted to generate answers to both of these questions in a recent publication (Ziomek, van Breemen and Esfandiarei, 2015). In a brefeldin-A induced ER stress
model using VSMCs, it was observed that approximately 50% of the ER’s Ca\textsuperscript{2+} store was depleted within an hour of the cells’ exposure to the stressor. This drop is clearly evident at the 1-hour mark and appears to partially recover over the following 12 hours of exposure. This Ca\textsuperscript{2+} loss precedes any sign of the UPR in affected cells, which only becomes evident at approximately 6-9 hours in as indicated in the Western blot sample exemplified in Figure 1.8.

Figure 1.8. Ca\textsuperscript{2+} dysregulation event in the ER stress response precedes evidence of initiated UPR in vascular smooth muscle cells. Left: Western blot results showing the concentration of UPR protein IRE1\textalpha over time-course experiment with cells exposed to the ER stressor brefeldin A for varied amounts of time. Significant increase in IRE1\textalpha from control is only visibly noticeable following 6-9 hours of exposure. Right: Bar graph depicting the amount of Ca\textsuperscript{2+} released from the ER stores following stimulation of cells with agonist in 0 Ca\textsuperscript{2+} conditions. A significant drop in the amount of ER Ca\textsuperscript{2+} present in the organelle’s lumen is evident within 1 hour of cellular exposure to BFA. (Ziomek, van Breemen and Esfandiarei, 2015)

While stress is generally indicated in cultured cells or other models by the presence of UPR marker proteins measured by Western blot analysis, our data indicates that the stress response is induced at a much earlier time point. The importance of this lies in the potential relevance of the documented ER Ca\textsuperscript{2+} dysregulation event. Namely, could this phenomenon serve as a trigger for the remainder of the stress response? Is a stress-inducing event normally closely followed by this significant drop in ER Ca\textsuperscript{2+}? Such a trigger would inactivate chaperones in the lumen of the ER to inhibit steady state protein folding. An approximate 50% decrease in free ER Ca\textsuperscript{2+} would
result in some buffered Ca\textsuperscript{2+} being released from these chaperones as the organelle attempts to re-establish equilibrium with the rest of the cell, leaving these molecules unable to perform their protein-folding function and ultimately increasing the rate at which unfolded proteins accumulate in the organelle’s lumen.

1.12 Mechanisms for Ca\textsuperscript{2+} egress from the ER

The ER is a pump/leak system with respect to its luminal Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{ER}). It takes up Ca\textsuperscript{2+} from the cytoplasm via SERCA and releases Ca\textsuperscript{2+} via IP\textsubscript{3}R and RyR. At rest, influx and efflux are balanced to create a steady state, but during activity periodic release events are restored by subsequent SERCA-mediated refilling. In order to disrupt the above balance to achieve a net loss of [Ca\textsuperscript{2+}]\textsubscript{ER}, the released Ca\textsuperscript{2+} must be extruded from the cytoplasm via PMCA and NCX. The role of the latter has not yet been properly defined, as the low Ca\textsuperscript{2+} affinity of NCX only allows it to function when [Ca\textsuperscript{2+}]\textsubscript{cyt} is in the µM range, which is above expected values within the bulk cytoplasm.

The conventional view holds that IP\textsubscript{3}R is the main avenue through which Ca\textsuperscript{2+} release occurs, as it is known that release from these receptors plays roles throughout the cell, including initiating metabolic pathways through providing Ca\textsuperscript{2+} to the mitochondria, and increasing signals from cellular lysosomes (Kania \textit{et al.}, 2017). In the current thesis, we are proposing a pathway for Ca\textsuperscript{2+} release focused instead on RyR as the main avenue for this ion’s movement out into the cytoplasm. It is possible that an ER Ca\textsuperscript{2+} drop through RyR in ER stress mirrors a similar process documented in an Alzheimer’s disease model, wherein a dysfunctional RyR2-instigated leak activates signaling pathways further downstream that contribute to the condition’s pathogenicity (Lacampagne \textit{et al.}, 2017). A similar story has previously been outlined with regard to the development of heart failure, where again RyR2 activity is affected by extracellular signals,
resulting in above-normal levels of ER Ca\textsuperscript{2+} release (Wehrens, Lehnart and Marks, 2005). Indeed, the theme of a wayward protein affecting normal Ca\textsuperscript{2+} signaling to wreak havoc is a relatively widespread notion, such as in the recent discussion of transmembrane Bax Inhibitor-1 Motif-containing proteins, which can play a hand in both cellular Ca\textsuperscript{2+} homeostasis and in mediating cell death (Liu, 2017).

1.13 RyR and NCX in the vascular endothelium

As previously mentioned, the IP\textsubscript{3}R is one of the two main ER Ca\textsuperscript{2+} release channels present in the cell, and its activity is regulated by the ambient Ca\textsuperscript{2+} concentration, on both sides of the ER membrane. For example, this receptor will show insensitivity to its ligand IP\textsubscript{3} when the Ca\textsuperscript{2+} levels in its vicinity are found to be either low or high. Ca\textsuperscript{2+}-mediated activation of RyR, the other important Ca\textsuperscript{2+} release receptor, behaves similarly, in that its capacity for release of ER Ca\textsuperscript{2+} is affected by the cell’s cytoplasmic and luminal [Ca\textsuperscript{2+}]. The importance of RyR to cellular Ca\textsuperscript{2+} movement has been well established, though its localization remains unclear across many cell types. The receptor’s specific positioning has been visually and statistically confirmed in the cardiac myocyte (Asghari et al., 2014). Electron tomography was recently used to demonstrate how individual RyR units are spaced in relation to each other within the small space of a cardiac dyad (Asghari et al., 2014). The distribution of cardiac RyR and NCX have also been examined using immunofluorescence (IF) in early development, and it was found that in newborn rabbits, there was a clear pattern of NCX and RyR clusters at the cell surface, with clusters of the two exhibiting similar periodicity. In adults, however, RyR’s periodicity was longer while NCX’s remained the same, showing a decreased overlap in localization of these two proteins (Dan et al., 2007). Despite these very specific discoveries in the cardiac cell model, a specific location of RyR in ECs has so far not been established. Early work on RyR localization in the EC indicated
its presence throughout the cytoplasm in an unclear pattern (Lesh *et al.*., 1993), likely outlining the receptor’s widespread distribution across the membrane of the sprawling ER. It was later published that only approximately 5% of EA.hy926 endothelial cells (a commonly used cell line for cell culture technique) showed any presence of the RyR3 isotype of this receptor, and the absence of RyR1 and 2 (Köhler *et al.*, 2001). More recently, the localization of RyR in the sinus endothelial cells of the rat spleen was investigated, and it was shown that the RyR3 isoform was prevalent at organelle membranes close to the PM (Uehara *et al.*, 2004). Although our knowledge is incomplete, the data obtained thus far suggests that in ECs RyR is found throughout the cell and may concentrate in regions where the peripheral ER membrane comes in close proximity to the PM and membranes of other organelles.

Although several studies have attempted to investigate the question, the exact role of NCX in ECs remains unknown. It is known that NCX can function to move Ca\(^{2+}\) both in and out of a cell by shifts between forward and reverse modes of operation, for example due to binding activity of an extracellular ligand like acetylcholine which affects the Ca\(^{2+}\) gradients, as demonstrated recently (Berra-Romani *et al.*, 2010). The activity of this ion exchange carrier has previously been suggested to be influenced by, as well as able to influence, ER Ca\(^{2+}\) channels. The same group suggested that release of this ion through the IP\(_3\)R could potentially stimulate NCX to extrude Ca\(^{2+}\) from the cytoplasm (Berra-Romani *et al.*, 2010), similar to our proposed interaction between the NCX in the PM and RyR on the ER membrane. Such potential concerted activity between PM and ER channels in endothelial cells was also shown by earlier studies (Wang *et al.*, 1995). It was suggested that NCX has the ability to refill or empty the ER, based on cellular stimulation with a series of agonists (Chan, 2004). This idea has received support from modelling of Ca\(^{2+}\) movement and gradients between these transporters (Fameli, van Breemen...
and Kuo, 2007). After examining channel distribution during development in newborns, it was postulated that influx of Ca\(^{2+}\) through reverse NCX activity could couple with nearby, peripheral Ca\(^{2+}\) stores to cause calcium-induced-calcium release (CICR) (Dan et al., 2007). Research designed to investigate the function of nano-junctions has revealed that close approximation of sarcoplasmic reticulum (SR) and lysosomal membrane exhibits specific localization of SR RyR3 near the lysosomal membrane, showing a 41% overlap (Kinnear et al., 2008). Again, this discovery puts forth the notion that specially localized E/SR channels may interact specifically with complementary transporters on other, close by membranes. Previous investigations have therefore laid the groundwork for understanding how NCX may be involved in modulating ER Ca\(^{2+}\) levels and ultimately the signals processed by the ER.

**1.14 Nano-junctions**

A cytoplasmic nano-space (junction) can be defined as a region partially limited by cell and organellar membranes in close proximity (<50 nanometers - nm) to each other, which restricts diffusion to and from the bulk cytoplasm (Evans, 2017). It has been shown that such a nano-space populated with Ca\(^{2+}\) channels, pumps or exchangers exhibits fluctuations in [Ca\(^{2+}\)] far greater than in the bulk cytoplasm. The goal of the current study is to measure Ca\(^{2+}\) dynamics related to endothelial ER, in the context of the nano-junction hypothesis presented. The model prescribed here focuses on the potential coupling of the NCX in the PM and RyR in the ER to explain how NCX assists in unloading ER Ca\(^{2+}\).
Localized interactions between ion transporters have been documented for decades, with the first well-described instance being the neuromuscular junction (Fatt and Katz, 1950). Electron micrographs from studies published in the 1970s demonstrated similarly narrow spaces between the PM and SR inside smooth muscle cells (Devine, Somlyo and Somlyo, 1972; Gabella, 1972). However, functional importance with respect to intracellular Ca$^{2+}$ signalling in smooth muscle was first demonstrated by measurements of unidirectional and net Ca$^{2+}$ fluxes (van Breemen, 1977). This early paper provided a focus for many studies with a number of coworkers, which led us to define the nano-junction as being composed of two biological membranes that are separated by no more than 50 nanometers and typically exhibiting a length of multiple hundreds of nm, with both membranes housing ion transporters that could work in tandem to produce or receive Ca$^{2+}$ signals (Evans, 2017).
Chapter 2 – Experimental design

2.1 Research focus and rationale

The significant drop in ER Ca\(^{2+}\) that we were previously able to pinpoint as occurring prior to any measurable increase in ER stress markers (Ziomek, van Breemen and Esfandiarei, 2015) is key to understanding how Ca\(^{2+}\) dysregulation can have far-reaching effects on the rest of the body’s health, from cells to endothelial layers to the affected vasculature as a whole. Our aim is to better understand the mechanisms that maintain Ca\(^{2+}\) homeostasis in the endothelial ER, taking the approach that in order to understand what goes wrong with Ca\(^{2+}\) extrusion from the ER, we must first know what mechanisms are in place physiologically for ER Ca\(^{2+}\) release.

The intricacies of Ca\(^{2+}\) movement make studying a specific pathway difficult, and this becomes particularly evident in a non-physiological situation such as a model system wherein ER stress has already been induced. The current study therefore explores the ER Ca\(^{2+}\) release event under physiological conditions to first determine a likely pathway for this ion’s movement. In future, information gleaned from this work will ultimately be applied to an ER stress model. Whether or not this ER Ca\(^{2+}\) drop serves as a trigger for the remainder of the stress response could ultimately be determined by manipulating the pathway uncovered in the current study and observing the effects, if any, this may have on the unfolding of ER stress in affected cells.

The aim of this thesis is therefore to provide new evidence on a novel physiological pathway, which unloads Ca\(^{2+}\) from the ER lumen to the extracellular space, while bypassing the bulk cytoplasm. Following our investigation, we suspect that the ER stress Ca\(^{2+}\) drop occurs through Ca\(^{2+}\) leaving the ER mainly through RyR, with IP\(_3\)R playing a minimal role, if it participates at
all. We further suspect that RyR is coupled to PM NCX, which functions to remove the released Ca\(^{2+}\) from the cell altogether.

Ultimately, investigation in the current work has culminated in a clearer understanding of the complex Ca\(^{2+}\) movements constantly occurring intracellularly, and how they may in future be manipulated to prevent dramatic changes commonly implicated in ER stress, subsequent cell failure, and ultimately vascular disease. The experiments outlined below therefore do not directly relate to ER stress; they are instead designed to obtain a more complete understanding of ER Ca\(^{2+}\) homeostasis in ECs.

The majority of experiments performed involved cultured ECs being used for live cell imaging. These cells were incubated with different Ca\(^{2+}\) indicators as called for by specific experiments and to allow real-time Ca\(^{2+}\) measurement to be recorded in both the ER and cytoplasm. Fluctuations in cellular and ER Ca\(^{2+}\) levels were induced during experiments through exposure of cultured cells to different combinations of pharmacological agonists and blockers to affect the activity of specific ion channels, pumps, and receptors. Cellular conditions were also altered by solution changes, with cells exposed to physiological (1.5 mM Ca\(^{2+}\)), nominally-Ca\(^{2+}\)-free, or high Ca\(^{2+}\) (5 mM) buffers as called for by the distinct goal of each experiment.

After identification of a RyR-NCX linked Ca\(^{2+}\) transport pathway that could be involved in the ER stress Ca\(^{2+}\) drop, different methods were required to pursue the question of which isotype of RyR were present in our BAECs, with future goals to establish any co-localization of PM NCX and RyR at the ER membrane. Answering this question required the use of IF technique. Working through a significant number of protocol changes to optimize IF for the BAECs being used, we were ultimately able to draw conclusions on the RyR isotype present in these cells. The
question of whether NCX and RyR co-localize in our cells could not be tackled in the current thesis.

2.2 Hypothesis

Previous investigation into the ER stress model has demonstrated that an approximate 50% drop in ER Ca\(^{2+}\) stores is evident early on in the stress response (Ziomek, van Breemen and Esfandiarei, 2015). There are multiple avenues through which ER Ca\(^{2+}\) levels may be depleted, and which may need to be investigated to better understand this particular ER stress Ca\(^{2+}\) event. These include release from RyR, release from IP\(_3\)R, lack of Ca\(^{2+}\) re-uptake by SERCA, as well as general, as yet undefined, membrane leakage. Insofar, as the current study is concerned, the most likely avenue of release for the event being characterized was limited to the first three of the listed options. One clear method of release could be via the IP\(_3\)R, which release intracellular Ca\(^{2+}\) stores following activation by an agonist. Another candidate for ER Ca\(^{2+}\) release of the magnitude observed in the stress response would be RyR. It is also expected that the Ca\(^{2+}\) dispelled from the ER in the studied pathway is ultimately extruded from the cell, rather than being released into the bulk cytoplasm. A stressed cell normally exhibits the opposite in terms of Ca\(^{2+}\) signaling, wherein the release of ER Ca\(^{2+}\) can either activate pro-survival or apoptotic pathways, depending on the severity of the stress experienced, through the ion’s release into the cytoplasm. The main hypothesis of the work done for this thesis is that the ER Ca\(^{2+}\) released leaves the organelle through RyR and is subsequently removed from the cell through NCX activity at the PM. The aim of the experiments described below is to provide evidence for such a novel pathway, which mediates a net transfer of Ca\(^{2+}\) present in endothelial ER to the extracellular space, while bypassing the bulk cytoplasm.
2.3 Materials and methods

2.3.1 Buffers and reagents

HEPES-PSS buffer of pH 7.4 was used for cytoplasmic Ca\(^{2+}\) measurement experiments. The 1.5 mM Ca\(^{2+}\) solution was composed of 140 mM NaCl, 10 mM glucose, 5 mM KCl, 5 mM HEPES, 1.5 mM CaCl\(_2\), as well as 1 mM MgCl\(_2\). The nominally Ca\(^{2+}\)-free HEPES buffer was prepared using the same components with the exception of the 1.5 mM CaCl\(_2\). D1ER buffer of pH 7.6 was employed for luminal ER Ca\(^{2+}\) tests. The 1.5mM Ca\(^{2+}\) D1ER buffer was composed of 145 mM NaCl, 5 mM KCl, 1 mM MgCl\(_2\), 10 mM D-(+)-glucose, 5 mM HEPES (sodium salt), and 1.2 mM CaCl\(_2\) dihydrate. The nominally Ca\(^{2+}\)-free version contained the same components except for the CaCl\(_2\). Thapsigargin (a SERCA inhibitor), uridine-5'-triphosphate (a purinergic receptor agonist), tetracaine (a RyR inhibitor), caffeine (used as a RyR stimulator), and KB-R7943 (KBR - a selective inhibitor of NCX) were obtained from Sigma-Aldrich (Toronto, ON, Canada). Stock solution of thapsigargin was prepared in dimethyl sulfoxide (DMSO) and stored in -20\(^\circ\)C conditions. Stock solutions of tetracaine and caffeine were prepared in water and stored in -20\(^\circ\)C conditions and prepared as needed. Stock solutions of uridine-5’-triphosphate (UTP) and KBR were prepared in distilled water and DMSO, respectively, and were stored in -20\(^\circ\)C conditions for use as needed. Any further dilutions of these drugs were made using nominally Ca\(^{2+}\)-free HEPES-PSS or D1ER buffer, as needed. 0.01% DMSO solution prepared in nominally Ca\(^{2+}\)-free buffer was employed as an experimental control vehicle over the duration of the current study. The ER-specific Ca\(^{2+}\) indicator D1ER was a kind gift from Dr. Wayne Chen (University of Alberta, Canada). The cytoplasmic Ca\(^{2+}\) indicator Fluo-4, AM was obtained from Invitrogen (Burlington, ON, Canada). For experiments involving treatment of cells with the drugs listed above, the agents were added to culture plates for 10-15 minutes prior to recording.
to ensure diffusion across the plate prior to further experimental manipulation. Results derived from drug-treated plate groups were subsequently compared to plates treated with control vehicle (DMSO dilution).

2.3.2 Cell culture

Bovine aortic endothelial cells (BAECS) were obtained from the laboratory of Dr. Pascal Bernatchez (University of British Columbia, Canada). Cells were prepared by Arash Tehrani from fresh bovine aortas, which were collected on the day of isolation from a slaughter house. The resulting isolated ECs were then grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 15% heat-inactivated fetal bovine serum, penicillin G (100 µg/ml) and the antibiotic streptomycin (100 µg/ml) (all acquired from Invitrogen, ON, Canada). Cells grown for experimental use were held at 37°C in 5% CO2 conditions inside a humidified incubator. Passages of BAECS between 2-12 were used for experiments throughout the course of this study. Cells were cultured onto glass-bottomed microscopy dishes 48 hours prior to experiments, thus allowing sufficient time for recently plated cells to reach approximately 80% confluency on the day of planned live imaging experiments.

2.3.3 Measurement of cytoplasmic Ca2+

Fluo-4, AM, an intracellular Ca2+ indicator, was used to load BAECs prior to relevant experiments. ECs were loaded at room temperature (around 24°C) for 1 hour, before being washed in 1.5 mM or nominally Ca2+-free HEPES-PSS buffer for approximately 5-10 minutes before initiating recording. Live imaging was accomplished using a Leica TCS SP5 Confocal Microscopy System with an Argon-Krypton laser (excitation 488 nm/emission 555 nm) for illumination of cells. Derived image resolution was 512 x 512 pixels. Images were obtained
using a 700 Hz scanning speed. Signal intensity was analyzed with the Leica Application Suite 2.6.3 (Leica Microsystems Inc., Concord, ON, Canada). Single representative Ca$^{2+}$ trace recordings depict the average fluorescent signals ($F/F_0$) collected from small groupings of cells (approximately 5-10 at a time) in the region of interest (ROI) for each of multiple independent experiments. Changes to the intensity of the Fluo-4, AM fluorescence as reported in the current study were in proportion to alternations to cytoplasmic Ca$^{2+}$ levels observed during individual experiments within specified ROIs. Data from experiments were analyzed and formatted using the GraphPad Prism 5.0 program (GraphPad Software, Inc., San Diego, CA, USA).

### 2.3.4 Measurement of luminal ER Ca$^{2+}$

The D1ER cameleon used in the current study was a kind gift from Dr. Wayne Chen at the University of Alberta (Canada). Briefly, important indicator characteristics include the fact that if luminal ER/SR Ca$^{2+}$ concentration drops, then a conformational change to the CaM-M13 core domain of the indicator results in a decrease in fluorescence resonance energy transfer (FRET) between the CFP and YFP regions that flank CaM-M13. This results in a corresponding drop in the signal intensity recorded for FRET. BAECs were transfected with the D1ER indicator adenoviral construct at a multiplicity of infection (MOI) of 100 to ensure a high transfection efficiency. Cells were then incubated overnight at 37°C with 5% CO$_2$, after which they were replenished with a fresh 1 ml of medium. Transfection efficiency was assessed using fluorescence microscopy 48 hours after initial transfection. Efficiency of approximately 80-90% was achieved for all relevant experiments. The FRET Wizard of Leica Application Suite 2.6.3 (Leica Microsystems Inc., Concord, ON, Canada) was used to perform FRET measurements. Briefly, cultured BAECs were excited in sequence at 440 nm (donor and FRET) and 515 nm (acceptor). Emission wavelengths were collected at 488 nm (donor) and 535 nm (FRET and
acceptor) using spectral photomultiplier tube detectors. Recorded traces were collected using a fast point scanner running at 8000 Hz. This resulted in an imaging speed of 30 ms/frame in the collection of 512 x 512 pixel images. The data obtained from the resulting traces were analyzed and formatted using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA).

2.3.5 Immunofluorescence

BAECs were cultured on glass coverslips and maintained in DMEM with 10% FBS for approximately 48 hours prior to fixation. Cultured BAECs were fixed with 4% buffered paraformaldehyde for a 10-minute period. Newly fixed cells were then treated with glycine buffer for another 10 minutes. BAECs were next permeabilized using Triton X-100 (0.05%) for 2 minutes. Cells were then washed for another block of 10 minutes with phosphate-buffered saline (PBS) prior to treatment with the primary antibody. Following this wash period, the cells were incubated with RyR3 (Alamone labs) and caveolin-1 (Cav-1) H-97 rabbit polyclonal IgG (Santa Cruz Biotechnology) antibodies on separate coverslips for eventual imaging. Primary antibody dilution was 1:100 for Cav-1-treated cells, and 1:20 for RyR3-treated cells, with antibody added to antibody buffer solution (2% goat serum, 0.05% Triton X-100, 1% bovine serum albumin (BSA), and 3mM sodium azide in saline-sodium citrate buffer). The relevance of including goat serum and BSA in this buffer was overall to block non-specific binding sites. Cells were incubated with the primary antibody overnight. Following this incubation, primary antibody was removed over a series of 3 PBS washes 10 minutes in duration. Cells were subsequently incubated with Alexa-conjugated Alexa Fluor 488 donkey anti-rabbit IgG (Life Technologies) diluted to 1:100 in antibody buffer, for 75 minutes at room temperature.

Following addition of secondary antibody to coverslips, the cell culture plate was wrapped in
paper towel and cling film to keep coverslips moist over the course of incubation. The plate was also wrapped in aluminum foil to protect the antibodies from exposure to light. Following the 75-minute incubation, the cells were washed 3 times with PBS for 10-minute periods. Individual coverslips were then mounted on to glass microscope slides using ProLong Gold Antifade Mountant (Thermo Fisher Scientific) and sealed in place using nail polish. Slides were transferred to 4°C storage to allow for initial curing; following this period they were ready for imaging. Control coverslips were prepared for each set of experiments performed. Single staining was executed in order to ensure that each primary antibody was functional. Additionally, to determine the extent of non-specific binding occurring, control coverslips with only secondary antibody were also prepared. Finally, to determine the level, if present, of any auto-fluorescence in the BAECs used, control experiments with cells’ only being incubated with antibody buffer were performed.

Following cell preparation, staining, and mounting onto glass slides, cells were imaged on a Zeiss LSM 700 confocal microscope with the 63x objective and a laser set to illuminate cells at a wavelength of 488 nm. Resulting images had a pixel size of 0.40 micrometers. Images were collected and deconvolved on the Zen Black software from Zeiss Microscopy and subsequently observed using the ImageJ image processing program (National Institutes of Health).

2.3.6 Statistical analysis

Data analysis was performed using GraphPad Prism 5.0 Software (GraphPad Software Inc., San Diego, CA, USA). The main form of analysis involved comparison of changes to Ca²⁺ signal intensity following changes to buffer solution or addition/wash out of pharmacological agents. Figures show the average obtained from multiple traces with mean and standard error of the mean (SEM) values pertaining to the relevant set of experiments. The unpaired Student’s t test
was used to confirm significance between sets of two experimental groups, where a resulting value of $P$ less than 0.05 was determined to indicate a statistically significant difference between compared groups. When mention was made to differing rates of Ca$^{2+}$ movement, this was confirmed by observing the slope of the relevant line by determining the difference between two points using linear regression analysis. The number of repetitions performed per round of experiments (n) varied across tests performed; number of repetitions per experimental group is indicated in corresponding figures and legends. Many experiments served to compare control plates (cells were treated with control vehicle of 0.01% DMSO solution) versus those where cells were treated prior to recording with specific pharmacological agents.
Chapter 3 - Results

3.1 UTP partially releases ER Ca\(^{2+}\):

Figure 3.1 A shows that UTP causes partial (approximately 50%) depletion of the contents of the ER in cells incubated in 1.5mM Ca\(^{2+}\) physiological solution, as confirmed by further subsequent release following a 2 \(\mu\)M dose of thapsigargin (Tg), a potent SERCA inhibitor. Figure 3.1 B demonstrates similar results for cells incubated in the absence of external Ca\(^{2+}\).

![Graph A](image1.png)

![Graph B](image2.png)

**Figure 3.1 (D1ER).** The purigenic receptor agonist UTP partially depletes \([\text{Ca}^{2+}]_{ER}\). A) Cultured BAECs were held in 1.5mM Ca\(^{2+}\) D1ER buffer prior to addition of 1mM UTP and a subsequent dose of 2mM Tg. B) Another group of cultured cells were incubated in nominally Ca\(^{2+}\)-free D1ER buffer prior to the same sequential addition of agonists. Combined effect of UTP and Tg empties the ER consistently under both conditions.

3.2 Reverse NCX has no role in the ER refilling pathway.

Cells transfected with D1ER, an ER Ca\(^{2+}\) indicator, and maintained in nominally Ca\(^{2+}\)-free D1ER buffer were exposed to UTP with the goal of studying net Ca\(^{2+}\) release from the ER. When the decrease in \([\text{Ca}^{2+}]_{ER}\), stabilized, UTP was washed out and Ca\(^{2+}\) was replenished to refill the ER. As shown in figure 3.2 A, ER Ca\(^{2+}\) levels increased substantially following addition of Ca\(^{2+}\).
These preliminary experiments indicate that the best way to study net ER Ca\(^{2+}\) release is to preemptively overload the ER with Ca\(^{2+}\). This experiment was repeated with cells treated with 30 \(\mu\)M KBR to block NCX. As seen in figure 3.2 B, treated cells exhibited a similar response to the untreated controls, indicating that reverse NCX did not contribute to refilling the ER under the present conditions.

![Graph](image)

**Figure 3.2 (D1ER).** Reverse NCX does not contribute to the ER refilling pathway under control conditions in the absence of agonist. **A)** Cultured cells were held in nominally Ca\(^{2+}\)-free solution prior to acute treatment with UTP, wash with 0Ca\(^{2+}\) solution, and a subsequent increase to 1.5mM Ca\(^{2+}\). **B)** Cultured cells pretreated with 30 \(\mu\)M NCX blocker in nominally Ca\(^{2+}\)-free solution prior to same addition of agonist, wash period, and dose of Ca\(^{2+}\).

### 3.3 NCX contributes to ER Ca\(^{2+}\) extrusion after overload of the ER.

The potential involvement of NCX in the ER Ca\(^{2+}\) release pathway was investigated using D1ER-loaded BAECs to observe changes in luminal ER Ca\(^{2+}\) concentrations. The BAECs were first exposed to high-Ca\(^{2+}\) (5 mM) D1ER buffer to ensure overloading of the ER Ca\(^{2+}\) stores, followed by washing with nominally Ca\(^{2+}\)-free solution. Figure 3.3 A shows an immediate fall in
[Ca\textsuperscript{2+}]_\text{ER} following Ca\textsuperscript{2+} removal, which is almost completely blocked by the NCX inhibitor KBR (Fig. 3.3 B).

A) B)

**Figure 3.3 (D1ER).** NCX contributes to extrusion of ER Ca\textsuperscript{2+} after overload due to an increase in extracellular Ca\textsuperscript{2+}. A) Cultured ECs were exposed to high Ca\textsuperscript{2+} buffer to cause ER loading, and subsequently washed with nominally Ca\textsuperscript{2+}-free solution. A decline in ER Ca\textsuperscript{2+} is evident following the buffer change, indicating loss of ER Ca\textsuperscript{2+} from luminal stores. B) Cultured ECs were exposed to high Ca\textsuperscript{2+} buffer and subsequently washed with nominally Ca\textsuperscript{2+}-free solution in the presence of KBR. No significant decline in ER Ca\textsuperscript{2+} was observed following the change of solution; depletion of ER Ca\textsuperscript{2+} stores in response to removal of external Ca\textsuperscript{2+} was blocked by KBR, indicating that NCX is involved in the ER Ca\textsuperscript{2+} extrusion pathway.

### 3.4 RyR involvement in net ER Ca\textsuperscript{2+} extrusion.

Although NCX appears to mediate the observed net loss of [Ca\textsuperscript{2+}]_\text{ER}, its location in the PM suggests that it extrudes Ca\textsuperscript{2+} released by the ER into the cytoplasm. We examined the possibility that RyR mediated this Ca\textsuperscript{2+} release by its inhibition with 100 µM tetracaine. Ca\textsuperscript{2+} release from an overloaded ER was induced by a solution change to nominally Ca\textsuperscript{2+}-free D1ER buffer. Cells exposed to this sequence exhibited a clear decrease in [Ca\textsuperscript{2+}]_\text{ER} (Fig. 3.4 A). We observed that blocking RyR activity in cultured BAECs abolished the decline in ER Ca\textsuperscript{2+} levels recorded in the
control experiment (Fig. 3.4 B). The results presented here indicate that ER Ca\(^{2+}\) release is prevented when RyR activity is blocked.

A) Control

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{control.png}
\caption{Control experiment (Fig. 3.4 B).}
\end{figure}

B) Pretreat 15’ tetracaine

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{pretreat.png}
\caption{Pretreat 15’ tetracaine (Fig. 3.4 B).}
\end{figure}

**Figure 3.4 (D1ER).** RyR act as release valves on overloaded ER. A) Cultured ECs were exposed to a high concentration of Ca\(^{2+}\) to ensure ER loading, and were subsequently washed with nominally Ca\(^{2+}\)-free solution. A decline in ER Ca\(^{2+}\) is evident following Ca\(^{2+}\) removal, indicating loss of ER Ca\(^{2+}\) from luminal stores. B) Cultured ECs were pretreated with 100 µM of RyR blocker tetracaine and maintained in 1.5mM Ca\(^{2+}\) HEPES buffer. Steady recording was followed by addition of a high concentration dose of Ca\(^{2+}\) and a wash with 0 Ca\(^{2+}\) D1ER buffer following re-established baseline after transient. Ca\(^{2+}\) release from the ER is prevented when RyR is blocked by tetracaine. These results lead to the hypothesis that unloading of the ER proceeds via RyR-mediated Ca\(^{2+}\) release.

The significant findings illustrated in Figures 3 and 4 above is that either inhibition of NCX or inhibition of RyR will abolish the net release of Ca\(^{2+}\) from the ER. This strongly suggests that the two Ca\(^{2+}\) transporters are functioning in series rather than in parallel. Two main possibilities therefore logically move to the forefront; in this pathway, Ca\(^{2+}\) is either released by RyR into the bulk cytoplasm for eventual removal, or ER Ca\(^{2+}\) is released into a nano-junction between the superficial ER and PM, where it reaches a micromolar concentration to activate NCX-mediated extrusion.
3.5 Ca\textsuperscript{2+} released from ER is extruded at nano-junctions.

We examined the possibility that Ca\textsuperscript{2+} released by RyR in the studied pathway enters the bulk cytoplasm for eventual extrusion by NCX. If this were indeed the route of net extrusion of Ca\textsuperscript{2+} from the ER, and since Ca\textsuperscript{2+} is the physiological activator of RyR, we would expect to see a change in [Ca\textsuperscript{2+}]\textsubscript{cyt} on switching from high to zero [Ca\textsuperscript{2+}].

Cells were loaded with Ca\textsuperscript{2+} prior to induction of Ca\textsuperscript{2+} release from the overloaded ER through a change in solution from physiological buffer to nominally Ca\textsuperscript{2+}-free solution. Cells exposed to this protocol exhibited a strong cytoplasmic transient in response to the dose of high Ca\textsuperscript{2+}, followed by a steady decline in intracellular Ca\textsuperscript{2+} towards baseline levels (Fig. 3.5 A). However, there was no observable transient in [Ca\textsuperscript{2+}]\textsubscript{cyt} following removal of extracellular Ca\textsuperscript{2+}. The lack of a cytoplasmic transient following induction of ER Ca\textsuperscript{2+} store depletion in Fluo-4, AM-incubated cells suggests that Ca\textsuperscript{2+} release from the overloaded ER is directed towards the PM rather than the bulk cytoplasm (Fig. 3.5 A). In cells that were pretreated with RyR blocker tetracaine (Fig. 3.5 B), the resulting traces were virtually identical to those obtained for figure 3.5 A, lending further support to the notion that the bulk cytoplasm was not involved in the process. These results provided evidence that RyR releases Ca\textsuperscript{2+} into a junctional nano-space, which does not affect the bulk [Ca\textsuperscript{2+}]\textsubscript{cyt}, where it raises the [Ca\textsuperscript{2+}] sufficiently to activate NCX.
Figure 3.5 (Fluo-4, AM). A) Cultured BAECs were maintained in 1.5mM Ca$^{2+}$ HEPES buffer and subsequently treated with a high dose of Ca$^{2+}$ to induce a cytoplasmic Ca2+ transient. Cells were then washed with nominally Ca$^{2+}$-free HEPES buffer to observe effect, if any, on [Ca$^{2+}$]$_{cyt}$. The large transient following addition of CaCl$_2$ is followed by steady decline in cytoplasmic Ca$^{2+}$ levels, unaffected by removal of external Ca$^{2+}$. B) Cultured BAECs pretreated with a dose of RyR blocker tetracaine were maintained in 1.5mM Ca$^{2+}$ HEPES buffer and subjected to the same treatment as in A). The magnitude of the CaCl$_2$-induced transient was similar, indicating that the RyR blocker had no effect on Ca$^{2+}$ loading capabilities, and cytoplasmic Ca$^{2+}$ showed a steady decline following wash with 0 Ca$^{2+}$ solution. Lack of a noticeable cytoplasmic transient when ER Ca$^{2+}$ is forcibly emptied indicates that RyRs release Ca$^{2+}$ from the overloaded ER to supply ER Ca$^{2+}$ directed to the extracellular space, rather than to the cytoplasm. These results indicate that unloading of the ER proceeds via RyR mediated Ca$^{2+}$ release from the peripheral ER coupled to Ca$^{2+}$ extrusion by NCX.

3.6 Functional evidence for RyR in BAECs.

Previous experiments (Fig. 3.4) indicate that RyR is involved in the net Ca$^{2+}$ loss from the ER after Ca$^{2+}$ overload. Although direct stimulation of RyR with caffeine does not cause a significant cytoplasmic Ca$^{2+}$ signal, figure 3.6 shows that it does deplete the ER of agonist-releasable Ca$^{2+}$. Cells exposed to nominally Ca$^{2+}$-free buffer and then treated with a dose of UTP, exhibited a large cytoplasmic Ca$^{2+}$ transient. This is indicative of ER Ca$^{2+}$ release due to the 0 Ca$^{2+}$ conditions employed. Interestingly, when the protocol was performed with an additional step wherein a 2 mM dose of caffeine was added to the cells soon after stabilization of
the trace after the buffer switch, the UTP-induced Ca\textsuperscript{2+} peak was significantly diminished. A small peak following acute addition of caffeine indicates some stimulation of RyR channels, which if given enough time, will deplete the ER.

Figure 3.6 (Fluo-4, AM). Caffeine-induced RyR stimulation affects ER Ca\textsuperscript{2+} unloading. A) Cultured cells were incubated in 1.5mM Ca\textsuperscript{2+} HEPES buffer prior to being washed with nominally Ca\textsuperscript{2+}-free solution and subsequent addition of 1mM UTP. B) Cultured cells were incubated in 1.5mM Ca\textsuperscript{2+} HEPES buffer prior to being washed with nominally Ca\textsuperscript{2+}-free solution. Following a 3-minute recording after the solution change, 2 mM caffeine was added to the cells before UTP was added at the same time point as plates in A). C) Comparison of peak cytoplasmic Ca\textsuperscript{2+} transient values following addition of UTP, caffeine, and caffeine followed by
UTP to cultured BAECs as shown in pooled trace form in A) and B). Addition of the RyR activator caffeine had a significant inhibitory effect on the cytoplasmic Ca$^{2+}$ transient induced by subsequent addition of UTP, indicating that RyR is the key ER Ca$^{2+}$ channel involved in the Ca$^{2+}$ unloading pathway.

### 3.7 Determination of RyR3 isotype in cultured BAECs.

Following the clear indication from our dynamic [Ca$^{2+}$] measurements that RyR is involved in the ER Ca$^{2+}$ release pathway discussed, further investigation into this receptor was required. Namely, our next step was geared towards determination of the specific RyR isotype present in the BAECs used, a line of investigation that was addressed using IF technique to label these proteins. Following a series of initial experiments and protocol modifications to optimize the experimental parameters of fixative concentration, permeabilization time, and primary antibody dilution, we were able to test for all of RyR1, RyR2, and RyR3 in our current cell line. Our final results indicated that neither of the RyR1 or RyR2 isotypes are present in our ECs. However, clear labeling of RyR3 with some clustering near the plasma membrane was observed across multiple experiments.

A)  

B)
Figure 3.7. Presence of RyR3 in cultured BAECs. Cultured cells were fixed, permeabilized, and incubated with primary and secondary antibodies prior to mounting on glass slides for viewing under a confocal microscope. A) Primary control. Cells were incubated with caveolin-1 primary antibody (1:100) prior to treatment with the Alexa Fluor 488 donkey anti-rabbit IgG. Cells showed clear labeling characteristic of Cav-1 positioning; found prominently at the plasma membrane. B) BAECs were incubated with RyR3 primary antibody (1:20) prior to further incubation with the same secondary antibody as in A). Cells demonstrated strong labeling on portions of the plasma membrane. Positioning of RyR3 indicates labeling of ER network throughout the cell, including regions in close proximity to the plasma membrane. C) Secondary control. Cells were incubated with the Alexa Fluor 488 donkey anti-rabbit IgG. Subsequent imaging showed a lack of signal strength, as well as any obvious patterning reminiscent of what was observed in either Cav-1 or RyR3-labeled cells. N = 3 for all cell treatments.
Chapter 4 - Discussion

4.1 Discussion

The current study outlines a thorough investigation into the pathway for Ca\(^{2+}\) movement involved in the significant Ca\(^{2+}\) dysregulation event evident in the early stage of the ER stress response. Using healthy cells to investigate the likely mechanism for this movement, we were able to determine a possible pathway for ER Ca\(^{2+}\) release in a physiological setting, for later potential application to a cellular level ER stress model. The main conclusion drawn from collected data is that the characteristic drop in ER Ca\(^{2+}\) stores occurs through release from the ER via RyR, followed by subsequent extrusion of this ER Ca\(^{2+}\) from the cell through NCX activity, as outlined in Figure 4.1

Figure 4.1. Diagram depicting the proposed pathway for the ER stress Ca\(^{2+}\) drop, as deduced from a physiological endothelial cell model. Ca\(^{2+}\) appears to be released from the ER lumen via RyR and subsequently extruded from the cell via NCX activity at the plasma membrane. This coupling between ion transporters is expected to be facilitated by the proteins’ being found in close proximity to each other at PM-ER nano-junctions.

The determination that these specific ion channels are involved was supported by experiments monitoring changes to ER luminal and cytoplasmic Ca\(^{2+}\) levels following manipulations of
buffers and addition of specific agonists or blockers to cultured bovine ECs. The involvement of RyR was determined through a simple comparison between luminal ER Ca\textsuperscript{2+} movement when RyR was functioning normally, and when it was blocked. Blocking RyR completely abolished the net release of Ca\textsuperscript{2+}, indicating the receptor’s role as the main avenue of ER Ca\textsuperscript{2+} release in the pathway being investigated. Important to note is that without overloading of the ER Ca\textsuperscript{2+} stores, we don’t consistently see the drop in ER Ca\textsuperscript{2+} observed in Fig. 3.3 A following the solution change, thus exemplifying the usefulness of overloading the ER in being able to clearly observe net ER Ca\textsuperscript{2+} release.

The involvement of NCX was determined similarly. Namely, experiments showed that the overloaded ER can normally be induced to release its Ca\textsuperscript{2+} at a steady rate when cultured cells are exposed to 0Ca\textsuperscript{2+} extracellular conditions. When PM NCX is blocked, however, this release is abolished, despite no ER Ca\textsuperscript{2+} receptors or channels having been pharmaceutically manipulated. This lack of Ca\textsuperscript{2+} release from the ER itself when a PM exchanger is blocked indicates a clear link between RyR and NCX. As it is proposed in the current thesis, direct and close opposition of the ER and PM at nano-junctions is necessary in order for this rapid release and extrusion to occur without a noticeable cytoplasmic Ca\textsuperscript{2+} transient.

The experimental result that first led us to consider the possibility of PM NCX and ER RyR facing off within a nano-junction structure was that wherein fully operational RyR activity was completely abolished following the blockage of PM NCX. One possible conclusion indicated by this result, wherein RyR’s functionality appears to be inhibited in the presence of NCX blocker KBR, could be that preventing the removal of Ca\textsuperscript{2+} from the nano-junction through NCX activity causes this ion to increase in concentration to inhibitory levels in this region. RyR can be either activated or inhibited by Ca\textsuperscript{2+} when present in micromolar and millimolar concentrations,
respectively (Laver and Lamb, 1998). Therefore, when NCX at the PM is blocked and is no longer removing Ca^{2+} from the cell, it is possible that in the microenvironment of the nano-junction where RyR and NCX are found in close proximity, the [Ca^{2+}] increases to the point that RyR is inhibited.

Distinct pathways for localized Ca^{2+} movements are required to generate a multitude of Ca^{2+} signals vital for maintenance of cellular health and physiological functions. The ER is the main Ca^{2+} regulatory organelle in the cell, which, according to the pan-junctional ER/SR theory, executes this role by localized supply or removal of cytoplasmic Ca^{2+} at junctions with the PM and other organelles, which typically have a width of 20 nm and are characterized by restricted diffusion (van Breemen, Fameli and Evans, 2013). In this fashion targets which affect a specific function, such as membrane polarity, can be activated without triggering other targets in the cell (for example, those initiating contractile activity or nuclear transcription). The current study adds another dimension to this concept with evidence showing that linked Ca^{2+} transport between RyR localized in the peripheral ER and NCX clustered in the PM across PM-SR nano-junctions regulates luminal [Ca^{2+}] in the endothelial ER. This type of localized control over [Ca^{2+}]_{ER}, which bypasses the bulk cytoplasm, is critically important for an independent role for the ER in the generation of site- and time-specific cellular Ca^{2+} signalling.

The literature includes a large number of publications that make reference to ER Ca^{2+} depletion during the stress response (Benali-Furet et al., 2005; Cardozo et al., 2005; Yoshida et al., 2006). While these groups acknowledged that some form of Ca^{2+} dysregulation was involved in this cellular phenomenon, little to no other investigation could be found concerning the relevance of such an event to the progression of the stress response itself. What remains to be fully understood, then, is whether the Ca^{2+} drop serves as a trigger initiating the ER stress
response, or whether this depletion event is a result of stress already having been induced by some other cause. It is well known that a significant Ca\(^{2+}\) dysregulation event can induce stress by its own means, but unclear why a significant drop in ER stores is observed despite ER stress being caused by a different mechanism of action, whether that be by prevention of the N-glycosylation of nascent polypeptides (tunicamycin’s reported mechanism of action), blocking of their transport from the ER to Golgi apparatus (by brefeldin A), inhibition of disulfide bond formation between protein residues (by dithiothreitol), or any of a host of other means to do so.

From a translational point of view, the mechanism behind removal of ER Ca\(^{2+}\) under physiological conditions is vital to making progress towards understanding the significant Ca\(^{2+}\) drop observed in early ER stress. We have previously reported that this substantial decrease in ER Ca\(^{2+}\) stores occurs within 1 hour of exposure to a stressor agent (Ziomek, van Breemen and Esfandiar, 2015), indicating that this Ca\(^{2+}\) dysregulation event is a potential trigger for the remainder of the full-blown cellular stress response. As our current study was performed using endothelial cells, knowledge of this physiological pathway for Ca\(^{2+}\) release may prove important to future work on ER stress in the endothelial layer, in relation to the host of disease conditions invoked by excessive die-off of stressed ECs. A rash of work focusing on the endothelium’s link to cardiovascular disease, for example, indicates how dysfunction at this cellular level can have significant negative effects on an individual’s overall health (Cai and Harrison, 2000; Heitzer et al., 2001; Davignon and Ganz, 2004). A more complete understanding of the cellular and molecular mechanisms of endothelial Ca\(^{2+}\) homeostasis will ultimately reveal novel target sites for drug therapy.

In conclusion, this study demonstrates a link in activity between ER RyR and PM NCX in the context of a pathway for ER Ca\(^{2+}\) release and subsequent extrusion from the cell. Previous
studies have shown that Ca$^{2+}$ can pass via STIM-ORAI or TRP channels into PM-ER junctional domains for SERCA-mediated refilling of the ER in a manner, which bypasses the bulk cytoplasm. When this ER filling pathway works in some form of synchrony (oscillatory or steady state) with the pathway described herein, which “directly” promotes net Ca$^{2+}$ extrusion from the ER, then the organelle is capable of autonomous control. Such control over its Ca$^{2+}$ homeostasis is essential for its role in site and function specific cellular Ca$^{2+}$ signalling, where the ER actively creates localized cytoplasmic Ca$^{2+}$ signals, rather than passively responding to changes in [Ca$^{2+}$]$_{cyt}$. Investigation into this pathway allows for a more complete understanding of the physiological mechanisms for Ca$^{2+}$ release from the ER, and could ultimately reveal new target sites for preventing the Ca$^{2+}$ drop that occurs during ER stress in afflicted cells. Further studies using known ER stress-inducing agents in ECs will test whether the proposed pathway indeed does play a role in the stress response, and whether its manipulation could have any effect on the survival of stressed cells.

4.2 Limitations of methods employed

4.2.1. Cell culture

It must be acknowledged that when growing cells for experimental purposes, those being cultured do not necessarily represent the physiological phenotype. Culturing cells using sterile technique to ensure that they do not fall prey to contamination and death is necessary for their growth for experimental purposes. Nevertheless, it is clear that cells grown in petri dishes and supplied with DMEM, heat, and carbon dioxide amount to conditions relatively removed from what cells would experience in a purely physiological model. Therefore, a key limitation of using cell culture is the fact that the cells used for experimentation will not quite represent the native cell type as found in vivo in the model organism. For the current thesis, BAECs were cultured for
experimental purposes due to their accessibility and potential for replenishment, as they were kindly provided by Dr. Bernatchez’s research group upon multiple occasions over the course of the project. Cells were used consistently over passages 2-12 over the duration of the project.

Also important to note is the particular cell type employed. Bovine aortic ECs were used for all cell culture work and related dynamic Ca\textsuperscript{2+} experiments, as well as the attempts at immunofluorescence at the tail end of the overall project. Due to the fact that the IF work entailed characterization of the relevant RyR isoform evident in the BAECs, new antibodies were purchased and tried out on these cells in attempts to visualize their distribution of RyR. Briefly, it is vital to acknowledge simply that on the industry side of research, the majority of antibodies produced are not tested in bovine cells, with work instead focusing on showing their efficiency in rat, human, and rabbit cells above all others. This lack of information in relation to bovine cells of course lent a certain degree of uncertainty concerning whether or not any bought antibodies would work with our cultured cells.

4.4.2. *Fluo-4, AM indicator/cytoplasmic Ca\textsuperscript{2+} measurements*

One of the key limitations of using the Fluo-4, AM Ca\textsuperscript{2+} indicator is the fact that it measures cytoplasmic Ca\textsuperscript{2+} only. This means that despite efforts to manipulate experimental cellular conditions to investigate what is happening at the level of ER Ca\textsuperscript{2+}, conclusions drawn can only ever involve extrapolation based on what is considered likely to be occurring inside the organelle. This indirect investigation into ER Ca\textsuperscript{2+} is commononly a significant limitation of using a cytoplasmic indicator. However, in the current thesis this roadblock was largely sidestepped through the complementary use of the D1ER cameleon, which allowed for observation of luminal ER Ca\textsuperscript{2+} levels over the course of executed experiments.
4.3 Summary

Recent investigation into ER stress has revealed a significant Ca\textsuperscript{2+} dysregulation event that occurs early on in the cellular response, resulting in an approximate 50% drop in overall ER Ca\textsuperscript{2+} levels as compared to control. While the timing of this ER Ca\textsuperscript{2+} release has been narrowed down to within an hour of cellular exposure to the ER stressor, no work has previously outlined the likely pathway that the released Ca\textsuperscript{2+} takes from the ER itself and beyond.

The current study has revealed evidence indicating that following induction of ER stress, this substantial portion of luminal ER Ca\textsuperscript{2+} is released from the organelle via RyR, and subsequently removed from the cell through plasma membrane NCX activity. The rapid removal of ER Ca\textsuperscript{2+} from the cell, causing no significant perturbation in cytoplasmic Ca\textsuperscript{2+} levels at the time of release from the ER, indicates coupling between the ER RyR and PM NCX. Under physiological conditions, it was shown that agonist-induced Ca\textsuperscript{2+} movement indeed does occur largely through RyR, and that blocking NCX at the PM prevents this release altogether. These experimental results strongly support the model proposed here that close interaction between clusters of RyR and NCX on closely apposing membranes is necessary to explain the ER and cytoplasmic Ca\textsuperscript{2+} level fluctuations evident in the presented Ca\textsuperscript{2+} traces. The delineation of this pathway for intracellular Ca\textsuperscript{2+} movement has allowed for better understanding of the likely mechanics of the goings-on of the early ER stress response, as well as adding to the mass of information required to really comprehend the multitudes of Ca\textsuperscript{2+} signals that are occurring at any given time inside a cell.
4.4 Future directions

An important caveat about the conclusions drawn from the current study is that in our experimental set-up the ER stress Ca\(^{2+}\) drop was merely simulated using an appropriate agonist in an otherwise healthy cell. To truly ascertain whether the pathway for Ca\(^{2+}\) movement outlined here is evident in the stress response, it would be necessary to investigate the Ca\(^{2+}\) drop in cells actually experiencing ER stress and initiating the UPR. Therefore, additional studies are required, in which ER stress is induced in the BAECs via some method that does not overtly perturb steady-state cellular Ca\(^{2+}\) concentrations. This would likely mirror our earlier study wherein we employed BFA as an appropriate ER stressor in VSMCs (Ziomek et al., 2015) because of its specific mechanism of action and its lack of any significant acute effect on cellular Ca\(^{2+}\) levels. Overall, having outlined a likely mechanism for ER Ca\(^{2+}\) release from the organelle as well as the cell in a simulated-stress environment, the ultimate goal is to understand whether this pathway plays a similar role under pathophysiological circumstances.

Furthermore, while we performed work on characterizing the specific isoform of RyR present in the BAECs used, further investigation into the isoform of NCX present, as well as both of these proteins’ cell-wide distributions would also allow for significant insight into different methods of Ca\(^{2+}\) movement and signaling in this cell type, in both healthy and ER-stress compromised circumstances. Also vital is deeper study into the PM-ER nano-junctions evident in the cell line employed in the current study, as well as ultrastructural characterization of the many other nano-junctions likely found in BAECs. It would be of significant interest to map out the dimensions and numbers of such junctions and compare their size and prevalence to those that are relatively well documented in other cell types, such as vascular smooth muscle.
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Appendix A: Publications and abstracts

Publications


Abstracts

Ziomek G, Esfandiarei M, & van Breemen C. Ca\(^{2+}\) extrusion from the ER of vascular endothelial cells proceeds via PM-ER nano-junctions. (Anesthesiology, Pharmacology, and Therapeutics Research Day 2017 - Poster session)

Ziomek G, Horvath G, van Karnebeek C, van Breemen C, & Fameli N. Intracellular Calcium Signaling and Severe Epilepsy. (BC Children’s Hospital Research Institute MIND Theme Research Day 2016 - Poster session)