The role of phospholamban (PLN) in potential protection of sarc(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) against oxidative stress

by

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AUTHOR’S DECLARATION
I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.
ABSTRACT

Sarco(endo)plasmic reticulum Ca\(^{2+}\) ATPase (SERCA) sequesters Ca\(^{2+}\) from the cytosol to: i) aid in muscular relaxation and ii) refill SR Ca\(^{2+}\) stores for release upon subsequent stimulus for contraction. SERCA function is modulated by physical interaction with phospholamban (PLN), where phosphorylation of PLN relieves its inhibitory action and increases SERCA activity. Protein-protein interactions have been shown to be protective against heat-induced SERCA2 modifications within HEK-293 cells (Fu & Tupling, 2009). Unpublished work from our lab has specifically shown SERCA2 protection in whole left ventricle (LV) when co-expressed with its regulatory modulatory protein, PLN (Gamu & Tupling, unpublished). The current thesis builds on this work by pairing vasomotor functional data from an isolated artery segment preparation with biochemical data to further explore the protective interaction between PLN and SERCA2. Peroxynitrite (ONOO\(^-\)) is an oxidant known to decrease SERCA2 function through irreversible damage (Viner, Williams, & Schöneich, 1999). Thoracic aorta from wild type (WT) and phospholamban null (PLN\(^{-/-}\)) mice were exposed to varying concentrations of ONOO\(^-\) (80 \(\mu\)M, 150 \(\mu\)M and 300 \(\mu\)M). Following this oxidative incubation, vasoactivity was assessed and compared. Furthermore, specific SERCA2 activity and structural adaptations through 3-NY formation were investigated. Interestingly, ONOO\(^-\) incubation (150 \(\mu\)M) elicited genotype differences throughout the incubation period, where PLN\(^{-/-}\) vessels had an increased basal tension response compared to WT vessels (p<0.05). Also, following 150 \(\mu\)M ONOO\(^-\) incubation, EC\(_{50}\) to SNP-induced relaxation was significantly increased in PLN\(^{-/-}\) vessels compared to WT (p<0.05). Both genotypes experienced reductions in PE-induced contractility, highlighting the ability for ONOO\(^-\) incubation to decrease contractility (p<0.05) though total SERCA2 activity was significantly reduced only following the 300\(\mu\)M ONOO\(^-\) incubation (p<0.05). No significant differences in SERCA2 activity were found between genotypes, indicating SERCA was not protected through its interaction with PLN from exposure to high ONOO\(^-\). This data shows high concentrations of ONOO\(^-\) incubation is able to significantly reduce SERCA2 activity, however we only have some evidence to support that PLN may be protective. This stands as a basis for future work to be completed regarding the protective nature of PLN and SERCA2 interaction.
ACKNOWLEDGEMENT

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<tr>
<td>3-NY</td>
<td>3-Nitrotyrosine</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>Calcium</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CICR</td>
<td>Calcium Induced Calcium Release</td>
</tr>
<tr>
<td>cAMKII</td>
<td>Calmodulin-dependent protein kinase</td>
</tr>
<tr>
<td>CPA</td>
<td>Cyclopiazonic acid</td>
</tr>
<tr>
<td>Cys</td>
<td>Cyestine</td>
</tr>
<tr>
<td>DPMK</td>
<td>Dystrophia myotonica protein kinase</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen Peroxide</td>
</tr>
<tr>
<td>His</td>
<td>Histadine</td>
</tr>
<tr>
<td>HSP70</td>
<td>Heat Shock Protein 70</td>
</tr>
<tr>
<td>IP$_3$R</td>
<td>Inositol 1,4,5-triphosphate receptors</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium Chloride</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo daltons</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
</tr>
<tr>
<td>LE</td>
<td>Luminal extension</td>
</tr>
<tr>
<td>LV</td>
<td>Left Ventricle</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NO$^-$</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric Oxide Synthase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NOX</td>
<td>NADH Oxidase</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>Superoxide</td>
</tr>
<tr>
<td>-OH</td>
<td>Hydroxyl Radical</td>
</tr>
<tr>
<td>ONOO$^-$</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>PARS</td>
<td>poly (adenosine 5'-diphosphoribose) synthase</td>
</tr>
<tr>
<td>PE</td>
<td>Phenylephrine</td>
</tr>
<tr>
<td>PK</td>
<td>Pyruvate Kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>PKG</td>
<td>Protein Kinase G</td>
</tr>
<tr>
<td>PLN</td>
<td>Phospholamban</td>
</tr>
<tr>
<td>PLN$^{+/−}$</td>
<td>Phospholamban knockout</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonyl fluoride</td>
</tr>
<tr>
<td>PP</td>
<td>Protein Phosphatase</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidiene difluoride</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive Nitrogen Species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine Receptor</td>
</tr>
<tr>
<td>sGC</td>
<td>Soluble Guanylyl Cyclase</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarco(endo)plasmic reticulum Ca$^{2+}$-ATPase</td>
</tr>
<tr>
<td>SLN</td>
<td>Sarcolipin</td>
</tr>
<tr>
<td>SNP</td>
<td>Sodium Nitroprusside</td>
</tr>
<tr>
<td>TA</td>
<td>Thoracic Aorta</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris buffered saline plus Tween-20</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane Helice s</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>Maximal SERCA activity</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>VSM</td>
<td>Vascular smooth muscle</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
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</table>
1.0 Introduction

1.1 Sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase

Muscular contraction and relaxation are regulated via Ca\(^{2+}\) movement throughout a myocyte (Ebashi, Endo, & Otsuki, 1969). Though precise mechanisms vary between muscle types, generally, an increase in intracellular free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{i}\)) will result in contraction, while a decrease will lead to relaxation (Devine, Somlyo, & Somlyo, 1972). Ca\(^{2+}\) levels within the cell are altered via an influx from the extracellular space through permeable ion channels or as a result of release from the cell’s internal Ca\(^{2+}\) stores (Sag, Wagner, & Maier, 2013). The sarcoplasmic reticulum (SR) makes up the primary storage compartment within the myocyte and is involved in rapid release of Ca\(^{2+}\) into the intracellular space. Upon stimulation Ca\(^{2+}\) is released from the SR through specific Ca\(^{2+}\) release channels, namely inositol 1,4,5-triphosphate receptors (IP\(_{3}\)Rs) and ryanodine receptors (RyRs).

Increases in [Ca\(^{2+}\)]\(_{i}\) results in Ca\(^{2+}\) binding or interaction with contractile proteins resulting in increased muscle tension (Ebashi et al., 1969). The SR also plays a key role in decreasing [Ca\(^{2+}\)]\(_{i}\) through the sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) pump which, when activated, catalyzes the ATP-dependent transport of Ca\(^{2+}\) against its concentration gradient into the lumen of the SR allowing relaxation to occur (MacLennan, 1990). Therefore SERCA serves two main functions; i) to induce relaxation by decreasing cytosolic [Ca\(^{2+}\)]\(_{i}\) and ii) to restore luminal SR Ca\(^{2+}\) stores for release upon subsequent muscular contractions (Periasamy & Kalyanasundaram, 2007).

SERCA localizes to the membrane of the SR in muscle cells and belongs to a family of P-type ATPases, which bind specific ions to be actively transported through an alternating access model (E1/E2 theory) (MacLennan, Rice, & Green, 1997). This implies a process by which ion-binding sites are accessible from either the cytosolic or extracytosolic side, interspersed with occlusion states where the pump is inaccessible from either side (Toyoshima, 2009). Large conformational changes powered by ATP hydrolysis allow the pump to operate appropriately. Standard SERCA function is able to transport two Ca\(^{2+}\) ions against the concentration gradient for every one ATP hydrolyzed (Periasamy & Kalyanasundaram, 2007). To work through the steps of the alternating access model, SERCA begins in the E2 state, and is said to be in its ground state having just released Ca\(^{2+}\) into the luminal SR. While SERCA primarily moves Ca\(^{2+}\) into the SR, it also counter-transport H\(^{+}\) as a measure to maintain structural integrity of the pump and to offset the charge imbalance in the absence of Ca\(^{2+}\) (Obara et al.,...
The conformational change following the release of Ca\(^{2+}\) causes the Ca\(^{2+}\) binding sites (site I and site II), now free of Ca\(^{2+}\), to increase their affinity to H\(^+\) and bind within the two known Ca\(^{2+}\) binding locations. The protonation causes a conformational change, causing SERCA to open to the cytosol subsequently releasing the bound H\(^+\) (E1 state). There is an increased affinity for Ca\(^{2+}\) once the binding sites are no longer protonated and both sites are available to accept Ca\(^{2+}\). The initial Ca\(^{2+}\) ion gains access to site I through site II, following which there is a conformational change at the Glu\(^{309}\) gating residue, stimulating a further increase in binding affinity at site II (E1-2Ca\(^{2+}\) state) (Toyoshima, 2009). ATP binding triggers phosphorylation-induced domain movements resulting in the closing of cytosolic access thus forming the occluded state (E1P state), and further acylphosphoryl transfer induces a conformational change resulting in the destruction of Ca\(^{2+}\) sites and release into the luminal SR (E2P state) (Kimura, Kurzydlowski, Tada, & Maclennan, 1997). One final transition releases the phosphate, closing luminal pump access and returns the pump to the energetically favourable ground state E2 (Ma, Lewis, Xu, Inesi, & Toyoshima, 2005).

In humans, three genes (\textit{ATP2A1–3}) generate multiple SERCA isoforms (SERCA1a-b, SERCA2a–c, SERCA3) through developmental or tissue-specific alternative splicing (Hovnanian, 2007). SERCA1 is expressed in fast twitch (Type 2) muscle, while SERCA2 is found in slow twitch (Type 1) muscle, and SERCA3 is found in non-muscle tissue such as endothelial lining within the vasculature or respiratory tract. The pumps differ in their regulatory and kinetic properties, with each being specifically tailored to their individual tissue expression (Brini & Carafoli, 2009; Liu et al., 1997; Wu, Bungard, Lytton, & Lyt-., 2001). Differences exist even within isoforms, for example SERCA2 has numerous isoforms, with SERCA2a and SERCA2b being the two most studied within this group. SERCA2a is found in slow twitch skeletal muscle and heart, while SERCA2b is more widely expressed and is the main isoform found in smooth muscle (Periasamy & Kalyanasundaram, 2007). Structurally, SERCA isoforms maintain a high level of homogeneity with the basic structure containing three domains projecting into the cytoplasm (A- actuator, N- nucleotide-binding, P- phosphorylation) and ten transmembrane (TM) helices (M1-M10). SERCA2b, unlike SERCA2a, is comprised of 11 TM helices in addition to a luminal extension (LE) made up from an extended C terminus of 49 residues also referred to as the 2b tail. This tail affects kinetic properties of the pump resulting in i) slowed dissociation from the high affinity binding sites in E1-2Ca\(^{2+}\) toward the cytosol, ii) slowed E1-2Ca\(^{2+}\) → E2P transition, iii) slowed E2P dephosphorylation, and iv) reduced rate of E2 → E1 (Clausen, Vandecaetsbeek, Wuytack, Vangheluwe, & Andersen, 2012). These changes to pump kinetics are in comparison to
the SERCA2a isoform, and overall illustrate that SERCA2b has a higher apparent affinity for Ca\textsuperscript{2+} coupled with a two-fold lower turnover rate (Vandecaetsbeek et al., 2009; H Verboomen, Wuytack, Van den Bosch, Mertens, & Casteels, 1994; Hilde Verboomen, Wuytack, De Smedt, Himpens, & Casteels, 1992). These characteristics have proven that even small changes to pump structure can have a large effect on function and activity. Even though variation exists between pump isoforms, the same small interactive proteins are responsible for modification of SERCA function.

1.2 Phospholamban

Phospholamban (PLN) is a small molecular weight protein (52 amino acids, 6 kDa) which regulates SERCA function whereby physical interaction reversibly inhibits pump activity by reducing its apparent affinity for Ca\textsuperscript{2+}, exclusive of affecting maximal pump activity (V\textsubscript{max}) (Bhupathy, Babu, & Periasamy, 2007a; MacLennan & Kranias, 2003). PLN is able to bind to all SERCA isoforms to affect affinity (Fujii et al., 1987; Fujii, Lytton, Tada, & MacLennan, 1988) and exists in the SR membrane of cardiac muscle, smooth muscle and slow skeletal muscle as both a pentamer and monomer; however, it is only the monomeric form that interacts with SERCA to decrease its apparent affinity for Ca\textsuperscript{2+} (MacLennan and Kranias, 2003). It has been found that the pentamer functions as an inactive/reservoir state and does not contribute to SERCA modulation (De Simone et al., 2013; Aschar-Sobbi, Emmett, Kargacin, & Kargacin, 2012). Sarcolipin (SLN) is a homologous protein to PLN and also acts to decrease SERCA’s apparent affinity for Ca\textsuperscript{2+}; however, it is found mainly in fast skeletal muscle and atria (Bhupathy et al., 2007). Both PLN and SLN directly interact with SERCA to affect its function and it is thought that the areas in which they bind are similar or even shared, with potential for both modulators to be present in one muscle fibre (Fajardo et al., 2013).

For the purposes of the current project, the binding position of PLN will be covered in more detail. Within the SERCA TM helices, a groove is formed between M2, M4, M6 and M9 in which PLN is able to bind and extend into the cytosol toward its second binding site within the N domain (Toyoshima et al., 2003). This binding position of PLN and SERCA is most accurate when SERCA is in its ground, E2, state. Phosphorylation of PLN at Ser16 by cAMP-dependent protein kinase A (PKA) or dystrophia myotonica protein kinase (DMPK) and/or at Thr17 by calcium calmodulin-dependent protein kinase (CaMKII) and Akt reverses the inhibitory protein-protein interaction between PLN and SERCA. This phosphorylation results in increasing SERCA’s
apparent affinity for Ca\(^{2+}\), allowing for an increased pump activity, thus increasing Ca\(^{2+}\) movement across the SR membrane (Asahi, Kurzydlowski, Tada, & MacLennan, 2002; Catalucci et al., 2009; Chen, Lah, Robinson, & Kemp, 1994; Gao et al., 2008).

Tissue-specific expression of PLN in various species has been highlighted through Western blot analyses, which have shown PLN to be expressed in left ventricle homogenate, portal vein homogenate and endothelial cells (Lalli, Shimizu, Sutliff, Kranias, & Paul, 1999; Sutliff, Hoyoing, Kadambi, Kranias, & Paul, 1999). This highlights the role of PLN as a functional modulator of many SERCA2 isoforms. The presence of PLN within endothelial cells has led to the suggestion that PLN also modulates SERCA3 function (Sutliff et al., 1999).

1.3 SERCA and PLN in Vascular Smooth Muscle and Cardiac Muscle

Vascular smooth muscle (VSM) comprises the medial layer of the vasculature throughout the body. In response to vasoconstrictors [e.g.: phenylephrine (PE), and angiotensin II] or vasodilators [e.g.: acetylcholine (Ach), and nitric oxide (NO)], VSM in arterioles and small arteries contracts or relaxes; in vivo these responses are important to maintain blood flow to all organs (Clark & Pyne-Geithman, 2005; Hai & Murphy, 1989; Horowitz, Menice, Laporte, & Morgan, 1996). VSM, like other muscle found in the body, relies heavily on changing [Ca\(^{2+}\)]\(_{i}\) for appropriate contraction-relaxation (Webb, 2003; Wray & Burdyga, 2010; Wu et al., 2001). Vasoconstrictors bind to their receptors on the VSM membrane and stimulate Ca\(^{2+}\) release from the SR through IP\(_3\)Rs and RyRs. Ca\(^{2+}\) can then bind to calmodulin forming a compound which is able to phosphorylate myosin light chain kinase, resulting in VSM contraction (Figure 1; Webb, 2003). On the other hand, vasodilators can indirectly phosphorylate PLN through receptor-mediated increases in cAMP or cGMP. PLN phosphorylation then decreases SERCA’s apparent affinity for Ca\(^{2+}\), causing increased activity and Ca\(^{2+}\) uptake into the SR, promoting relaxation (Figure 1; Karczewski et al., 1998). There are other means by which vasoactive molecules act upon VSM to elicit a response, for example through acting directly activating myosin light kinase or through the Rho-Kinase pathway to inhibit myosin light chain phosphatase causing contraction. However the brief description of pathways previously mentioned in detail involve SERCA function and will be the main focus for this document. Therefore proper SERCA2 function is required to decrease intracellular Ca\(^{2+}\) to induce VSM relaxation and to maintain intracellular Ca\(^{2+}\) stores needed for VSM contraction (Ashida, Schaeffer, Goldman, Wade, & Blaustein, 1988; Oloizia & Paul, 2008).
Figure 1: Basic schematic of SERCA/PLN involvement in VSM contraction and relaxation. Vasoconstrictors will bind to their receptors and elicit Ca\textsuperscript{2+} release through IP\textsubscript{3}R, or through CICR activation of the RyR. For example, PE will bind to its \(\alpha\)-adrenergic receptor, which, through G-couple protein activation increases IP\textsubscript{3}. IP\textsubscript{3} then binds to IP\textsubscript{3}R on the SR to activate Ca\textsuperscript{2+} release, which can then bind to calmodulin and activate myosin light chain kinase (MLCK) to initiate contraction. KCl induced contraction through mass depolarization of the cell, which opens Voltage Dependent Calcium Channels to allow Ca\textsuperscript{2+} into the cytosol from the extracellular fluid. The increased cytosolic Ca\textsuperscript{2+} triggers further Ca\textsuperscript{2+} release from the RyR. Vasodilators will then elicit relaxation through indirect activation of PKA and/or PKG to phosphorylate PLN and increase SERCA activity. Endothelial NO is also able to directly activate SERCA activity through s-glutathiolation, indicating PLN is not required to elicit relaxation. PKG is also able to activate myosin light chain phosphatase (MLCP) to increase smooth muscle relaxation. Cytosolic Ca\textsuperscript{2+} can then be reduced through the actions of SERCA, leading to relaxation and aiding in the refilling of the SR for Ca\textsuperscript{2+} release upon subsequent stimulus. A small amount of Ca\textsuperscript{2+} is returned to the extracellular fluid through the action of the Na\textsuperscript{+}-Ca\textsuperscript{2+} Exchanger (NCX). Figure adapted from (Kim, Cho, Han, Koh, & Perrino, 2005).
Numerous groups have shown, within VSM, regulation of SERCA2 function is primarily through PLN (MacLennan & Kranias, 2003; Nobe, Sutliff, Kranias, & Paul, 2001; Oloizia & Paul, 2008; Sutliff, Conforti, Weber, Kranias, & Paul, 2004). This has been observed by the global knockout of PLN resulting in altered smooth muscle function (Oloizia & Paul, 2008). The characteristics of smooth muscle as a result of the global knockout of PLN (PLN<sup>−/−</sup>) have shown that animals exhibited no physical changes (body weight) or changes in heart rate or blood pressure compared to their wild type (WT) littermates (Lalli, Harrer, Luo, Kranias, & Paul, 1997). When investigating vasoactive function in these two genotypes, no differences were observed in maximal isometric force production between the genotypes when stimulated with potassium chloride (KCl). However, when exposed to PE it was noted that the PLN<sup>−/−</sup> vessels were able to reach maximal tension faster when compared to WT, though no differences seen in total developed tension (Lalli et al., 1997). The difference between the two stimulants is the mechanism by which they activate vasoconstriction. KCl stimulates a large influx of extracellular Ca<sup>2+</sup> through depolarization, while PE acts through receptor mediated Ca<sup>2+</sup> release from the SR. Due agonist induced contraction differences, Lalli et al. (1997) aimed to investigate if the Ca<sup>2+</sup> stores within PLN<sup>−/−</sup> vessels are altered due to increased SERCA activity. Rings of VSM were held in a Ca<sup>2+</sup>-free solution and stimulated with PE (10uM), and the Ca<sup>2+</sup>-free scenario resulted in greater force production within the PLN<sup>−/−</sup> vessels compared to WT, suggesting that PLN ablation results in increased SR Ca<sup>2+</sup> stores (Lalli et al., 1997). The importance of PLN and proper SERCA function has also been established within the heart.

SERCA function is highly regulated by PLN within cardiomyocytes, and PLN expression within human cardiac muscle has been well established (Cerra & Imbrogno, 2012). Beta adrenergic signaling increases PLN phosphorylation at Ser16 and/or Thr17 relieving SERCA inhibition, though decreases in SR luminal Ca<sup>2+</sup> can also trigger a protein kinase dependent PLN phosphorylation to increase SERCA function (Bhogal & Colyer, 1998). Murine PLN ablation has been studied and its effects on the myocardium have previously been characterized (Luo et al., 1994; Tsuji et al., 2009; Watanabe et al., 2004). No changes to SERCA content was identified, though a reduction in RyR content within PLN<sup>−/−</sup> myocardium was observed. Due to the uninhibited SERCA pump and increased Ca<sup>2+</sup> uptake, it is proposed that the decreased RyR aids to maintain appropriate cytosolic Ca<sup>2+</sup> concentration (Chu et al., 1998). Rates of contractility and relaxation are increased in PLN<sup>−/−</sup> hearts compared to WT littermates, and there is an attenuation of beta-adrenergic stimulation seen compared to WT heart (Lalli et al., 1997).
1.4 SERCA Function and Oxidative Stress

Healthy muscle relies on reactive oxygen species (ROS) and reactive nitrogen species (RNS) for redox signaling within the cell (Adachi et al., 2004; Cohen et al., 1999; Rhodes, Packer, & Meiss, 1988). The primary production sites of RNS and ROS include the mitochondria, nitric oxide synthase (NOS) and NADPH oxidase (NOX) as well as storage pools of nitric oxide (Iovine et al., 2008; Trebak, Ginnan, Singer, & Jourd'heuil, 2010). Common ROS/RNS include superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), nitric oxide (NO), hydroxyl radical (·OH), and peroxynitrite (ONOO$^-$). Typically, their concentration is balanced by endogenous scavengers, including but not limited to; catalase, superoxide dismutase, glutathione (GSH), and glutathione peroxidase (GPx) (Pacher, Beckman, & Liaudet, 2007). These scavengers act on RNS/ROS, converting the potentially harmful free radicals into their molecular backbones, H$_2$O and O$_2$ (Lounsbury, Hu, & Ziegelstein, 2000). As a stimulant and redox signal, ROS and RNS play an important role, specifically within the VSM. NO created within the endothelium activates cGMP, which then activates protein kinase G (PK-G). PK-G is able to phosphorylate PLN at Ser16, thus relieving inhibition on SERCA allowing Ca$^{2+}$ to be moved into the SR, decreasing [Ca$^{2+}$], and inducing vasorelaxation (Bhogal & Colyer, 1998). There is also evidence for direct activation of SERCA2 whereby production of peroxynitrite (ONOO$^-$), from NO and O$_2^-$, increases activity through reversible s-glutathiolation at Cys-674 to increase pump activity and increase relaxation (Adachi et al., 2004). Pathological increases in ROS/RNS production without adequate removal or breakdown within muscle can result in cellular damage through oxidation or nitration of amino acids (Miller, Gutterman, Rios, Heistad, & Davidson, 1998; Touyz & Schiffrin, 2004; Zalba, Beaumont, & José, 2000). This is termed “high oxidative stress” and describes a situation in which cells are exposed to excessive levels of molecular oxygen or its’ chemical derivatives (Matsunaga et al., 2003; Zalba et al., 2000). Numerous situations or disease states can result in increased oxidative stress at the cellular level such as ageing, myocardial or cerebral ischemia-reperfusion, diabetes and atherosclerosis (Trebak et al., 2010).

All amino acids can be oxidized by high levels of circulating ROS and RNS, although certain amino acids are particularly sensitive to attack such as Trp, Tyr, His and Cys (Davies, Delsignore, & Lin, 1987; Stadtman, 1993). SERCA pumps contain numerous Cys and Tyr residues, making them highly susceptible to oxidative damage resulting in decreased function when exposed to high oxidative stress (Viner, Williams, et al., 1999; Ying, Sharov, Xu, & Jiang, 2008). Numerous studies have shown that SERCA damage can be caused in vitro through
heat shock, \( \text{H}_2\text{O}_2 \), \( \text{O}_2 \) and \( \text{ONO}^2- \) treatments (Adachi, Matsui, Weisbrod, Najibi, & Cohen, 2001; Adachi et al., 2004; Fu, MacLennan & Tupling, unpublished; Grover, Samson, & Misquitta, 1997; Viner, Hühmer, Bigelow, & Schöneich, 1996; Walia, Samson, Schmidt, Best, Whittington, et al., 2003). There is evidence to suggest that the SERCA isoforms vary in their susceptibility to oxidative damage and although SERCA isoforms have a high degree of homology, studies suggest that slight variations in amino acid sequence (i.e. number of Cys residues) and/or 3-D conformation may present different levels of susceptibility to oxidative stress (Grover, Samson, & Misquitta, 1997; Grover, Samson, Robinson, & Kwan, 2003). For example, microsomes from HEK-293 cells overexpressing SERCA3 proved to be more \( \text{H}_2\text{O}_2 \)-resistant compared to the same preparation of cells overexpressing SERCA2b (Grover et al., 1997). The significant resistance against inactivation in SERCA3 compared to SERCA2b was seen following three independent assays: ATP-dependent oxalate-stimulated azide-insensitive 45Ca\(^{2+}\) uptake by the microsomal vesicles, \( \text{Ca}^{2+}-\text{Mg}^{2+} \)-ATPase activity, and \( \text{Ca}^{2+} \)-dependent acylphosphate formation (Grover et al., 1997). This is proposed to be an important aspect of the SERCA3 isoform as it comes into contact with higher concentrations of ROS/RNS within the bloodstream therefore an increased resistance allows it to maintain function through increased exposure/contact. Research also shows that SERCA2a more readily undergoes nitration of Tyr residues compared to SERCA1 when exposed to \( \text{ONO}^2- \) \( \text{in vivo} \) (Viner, Williams, & Schöneich, 1999). These studies highlight how the subtle differences in isoform amino acid confirmation affect susceptibility of the pump to ROS/RNS damage.

In fact, \( \text{ONO}^2- \) has been claimed to be the most effective inhibitor of \( \text{Ca}^{2+} \) uptake through SERCA damage (Walia, Samson, Schmidt, Best, Kwan, et al., 2003; Walia, Samson, Schmidt, Best, Whittington, et al., 2003) and studies have shown that high concentrations of \( \text{ONO}^2- \) can result in irreversible damage (Viner et al., 1996; Viner, Williams, & Schöneich, 1999). Through the highly favourable reaction between NO and \( \text{O}_2 \), \( \text{ONO}^2- \) is formed (Groves, 1999; Pryor & Squadrito, 1995), and under physiological conditions remains at low levels through the actions of endogenous superoxide dismutase (Radi, Cassina, Hodara, Quijano, & Castro, 2002; Radi, Cassina, & Hodara, 2002). This is important as \( \text{ONO}^2- \) plays a critical role in healthy cell function, specifically aiding in microbial defense within neutrophils and macrophages (MacMicking et al., 1997; Nathan & Shiloh, 2000). However, a pathological rise in \( \text{ONO}^2- \) production can occur in numerous disease states, including cerebral ischemia reperfusion (Dhar, Kaundal, & Sharma, 2006; Thiagarajan, Kaul, & Sharma, 2004), myocardial ischemia-reperfusion (Cheung, Wang, & Schulz, 2000; Wang & Zweier, 1996), heart failure (Ferdinandy, Danial,
Ambrus, Rothery, & Schulz, 2000; P. Zhang et al., 2007), atherosclerosis (Buttery et al., 1996; Luoma et al., 1998), diabetes (Suarez-Pinzon et al., 2001; Suarez-Pinzon, Szabol, & Rabinovitch, 1997), and septicemia (Bhattacharyya, Biswas, & Datta, 2004) reaching pathophysiological levels, resulting in detrimental cell function.

ONOO⁻ has been shown to elicit a biphasic effect on cardiac myocytes, whereby low concentrations (< 30 μM) have a positive inotropic effect and high concentrations (>100 μM) elicit a negative inotropic effect (Kohr et al., 2008a). At low concentrations ONOO⁻ has been shown to activate PKA, which acts to phosphorylate RyR and PLN, resulting in increased Ca²⁺ release from the RyR as well as increased SERCA activity. In addition to phosphorylation through PKA, ONOO⁻ is able to elicit a reversible s-nitrosylation of the SERCA pump increasing its affinity for Ca²⁺ and its overall activity independent of PLN (Cohen & Adachi, 2006). However, concentrations >100 μM result in the ONOO⁻-dependent selective nitration of SERCA2 at Tyr294-Tyr295 which are located in the M4 transmembrane domain, and at Tyr753 located in the stalk region of the enzyme (Squier & Bigelow, 2000; Sharov et al, 2006). These site-specific modifications result in SERCA’s severely reduced ability to move Ca²⁺ into the SR lumen thereby affecting Ca²⁺ storage and decreasing the Ca²⁺ pool available for muscular contractility. This effect has been revealed by decreases in SERCA Ca²⁺ uptake as well as decreased β-adrenergic stimulation (Davidson, Kaminski, & Wolin, 1997; Dremina, Sharov, Davies, & Schöneich, 2007). Additionally, high levels of ONOO⁻ have been shown to activate Protein Phosphatase (PP), which has the potential to dephosphorylate PLN resulting the re-integration of PLN and SERCA, increasing SERCA’s apparent affinity for Ca²⁺ (Kohr, Davis, & Ziolo, 2009).

1.5 SERCA protection through protein-protein interaction

Various protein-protein interactions are known to be beneficial for stability against degradation. Heat shock protein 70 (HSP70) has been shown to bind with SERCA under heat stress conditions and protect it against damage in HEK-293 cells (Fu & Tupling, 2009). This binding prevented the reduction in SERCA2 activity seen in cells only expressing SERCA2 compared to those expressing both SERCA2 and HSP70. Similarly, preliminary data from our lab has shown that cultured cells transfected with SERCA2a and PLN present with fewer signs of SERCA damage following heat stress compared to cells containing only SERCA2a (Fu et al., unpublished). Specifically, after one hour of incubation at 39.2°C, maximal SERCA2a activity was significantly decreased in cells expressing SERCA2a only, however SERCA2a activity was unchanged when expressed with PLN (Fu et al.,
unpublished). Furthermore, consistent with cell culture experiments, whole heart homogenate from PLN<sup>−/−</sup> animals has shown increased SERCA impairment following a similar heat stress protocol described previously compared to homogenate collected from a wild type animal (Gamu et al, unpublished). Taken together, these results support the notion that PLN protects SERCA from loss of activity due to heat stress. Due to the location of PLN binding and the specific sites of damage caused by heat stress, it is thought that PLN acts to prevent the damage to key amino acids on SERCA. PLN interacts with SERCA at the M2, M4, M6 and M9 transmembrane helices (M Asahi, McKenna, Kurzydlowski, Tada, & MacLennan, 2000; Toyoshima, Nakasako, Nomura, & Ogawa, 2000). Since Tyr294-295 at the M4 transmembrane domain renders SERCA2 susceptible to nitrosylation, it is plausible that the protein-protein binding of PLN and SERCA could stabilize the SERCA pump by preventing nitrosylation and thereby preserving its function and structure during oxidative stress.
Statement of the problem

SERCA2 functions to regulate intracellular Ca\(^{2+}\) and is known to be highly susceptible to oxidative damage. PLN physically interacts with SERCA2 and may act to protect against oxidative damage by binding with residues highly susceptible to nitrosylation. Work from our laboratory has shown that PLN protects SERCA2 from heat stress induced damage. However, whether ablation of PLN can further decrease SERCA2 function \textit{in vivo} remains unknown. Furthermore, if PLN ablation can further decrease whole cellular function (i.e. contraction and relaxation) following incubation with ONOO` will be investigated. These will be examined using the PLN\(^+-\) murine model (Luo et al, 1994).

Objectives

The purpose of this study is threefold; 1) to determine if physiological levels of PLN aids in the maintenance of vasomotor function following oxidative stress induced by ONOO` exposure; 2) to determine if PLN protects SERCA2 activity from exposure to ONOO` and; 3) to determine if PLN prevents ONOO` induced irreversible structural modifications to SERCA.
Hypotheses

1) It was hypothesized that exposure of thoracic aorta to ONOO\textsuperscript{−} would cause impairments in VSM function (i.e. impaired contraction and relaxation) and that the impairments would be greater in vessels from PLN\textsuperscript{−/−} mice compared with WT mice. 2) It was hypothesized that the absence of PLN would expose SERCA2 to ONOO\textsuperscript{−} induced reduction in activity and that the WT samples would show unchanged SERCA activity following exposure. 3) It was also hypothesized that the absence of PLN will de-stabilize the SERCA2 structure following ONOO\textsuperscript{−} incubation leading to increased nitrotyrosine formation, while PLN within the WT samples will prevent ONOO\textsuperscript{−} induced nitrotyrosine content on SERCA2.
2.0 Methods

2.1 Animals

The generation of PLN\textsuperscript{-/-} mice has been previously described (Luo et al., 1994). Animals were housed 2-4 per cage in an environmentally controlled room with 12:12- h light dark cycle with free access to food and water. A total of 80 mice (40 WT, 40 PLN\textsuperscript{-/-}) (>4 months of age) were used for this study. Genotype was confirmed through PCR and Western blotting. All work was approved by the University of Waterloo Animal Care Committee and adhered to the guidelines of the Canadian Council of Animal Care.

2.1.1 Genotyping

Animals were generated through the breeding of heterozygous (PLN\textsuperscript{+/+}) mice, yielding 50% heterozygous pups, 25% WT (\textsuperscript{++}), and 25% PLN\textsuperscript{-/-}. For labeling and determining genotype of each pup, ear clippings were taken at four weeks of age. These clippings were digested, and DNA extracted using a commercially available kit (Purelink DNA Mini Kit; Invitrogen). Extracted DNA was subject to PCR for amplification of target DNA. Briefly, ~40 ng of DNA were added to a Taq DNA polymerase mixture (Fermentas) containing 3mM MgCl\textsubscript{2}, 200 µM dNTP, 10x Taq buffer (containing 650 nM Tris-HCL, 200 mM (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, and 0.1% (v/v) Tween 20), 1.5 µL Taq DNA polymerase, and 0.4 µM of forward and reverse primers (PLN-WT forward: 5’CAC GTC AGA ATC TCC AGA ACC-3’, PLN-WT reverse: 5’-TCC CCC TTT AAC TCT CATAAG C-3’, PLN\textsuperscript{-/-} forward: 5’-TCC TCG TTT ACG GTA TC-3’, PLN\textsuperscript{-/-} reverse: 5’-ACA ACC ACT TCC TCT CTG CGA GAT CA-3’). The DNA containing mixture was prepared in 200 µL thin walled PCR tubes and run in a thermal cycler (S1000 Thermal Cycler, Bio-Rad). The DNA denatured at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 59°C for 30 sec, extension at 72°C for 60 sec, with a final extension at 72°C for 10 min. Amplified target DNA was then resolved by electrophoresis on a 3% agarose gel containing 0.013% ethidium bromide (BioShop) and identified under UV light using a bio-imaging system and GeneSnap softwater (Syngene).

2.2 Vessel Contractility

In order to understand the protective role PLN may have in stabilizing the structure and function of SERCA2 during oxidative stress, we used standard vessel myography to identify changes in contractile responses
following ONOO⁻ exposure. PLN⁻/⁻ mice and their WT counterparts were sacrificed via intraperitoneal injection of 1mg sodium pentobarbital per gram body weight. The thoracic aorta (TA) from each animal was excised immediately and excess fat mechanically removed under a microscope. Any blood that remained in the aorta was removed and the whole vessel was cut into 2mm rings (6 rings per TA). To rule out any interplay with SERCA 3 and PLN present in the endothelial cells (Sutliff et al, 1999), the intimal surface of each ring was gently rubbed with a wire to remove the endothelial layer (Esfandiarei et al., 2011). Rings were then mounted between two wires, one fastened to a fixed foot and the other to a force transducer that recorded tension throughout the protocol.

The vessels were suspended in gassed Krebs Bicarbonate Buffer (95% O₂, 5% CO₂) under a tension of 0.65 g for 1-hour equilibration. Throughout this time the incubating buffer was changed every 20 minutes. Following the equilibration period, the rings were exposed to 60 mM KCl to elicit contractility for 30 minutes. This was used as a test for viability, thus any rings that did not contract from this depolarization were removed from the experiment.

Following the removal of KCl, ONOO⁻ (80 µM, 150 µM or 300 µM Calbiochem) was added to three of the six baths to induce oxidative stress; the remaining 3 baths were used as internal controls. Following a 30-minute incubation protocol, the buffer was changed to remove any remaining ONOO⁻, and phenylephrine (PE) (10⁻⁶M) was introduced to induce contraction. PE activates the α-adrenergic receptors present on vascular smooth muscle to increase cytosolic Ca²⁺, thereby causing vasoconstriction. This contraction was given 15 minutes to stabilize, followed by exposure to increasing concentrations of sodium-nitroprusside (SNP) (10⁻¹⁰ - 10⁻⁵ M). More specifically, SNP was weighed and dissolved with ultrapure H₂O for a 10⁻⁴ stock solution. From this solution, a 10⁻⁴.5 solution was made by adding 32 µL of 10⁻⁴ SNP to 68 µL ultrapure H₂O. Both of these solutions (10⁻⁴ and 10⁻⁴.5) were serial diluted by 10x repeatedly to create solutions ranging from 10⁻⁵—10⁻¹⁰ for a total of 11 concentrations.

Once all additions of SNP were complete, rings were removed, snap frozen and held at -80°C until use in further analyses.

![Figure 2: Representative force tracing](image)

**Figure 2: Representative force tracing.** Following the myography protocol, as described above, each 2mm ring would have an associated tracing of force developed throughout the experiment. Figure 2 highlights the areas from the force tracing which were used in further data analysis.
2.3 Calcium-dependent SERCA Activity

Whole TA rings from WT and PLN<sup>-/-</sup> mice used in contractility experiments were powdered and homogenized to determine Ca<sup>2+</sup>-dependent Ca<sup>2+</sup>-ATPase activity using a spectrophotometric assay developed by Simonides & Van Hardeveld (1990) and modified by our laboratory to accommodate a 96-well plate reader (SPECTRAMax Plus; Molecular Devices, Toronto, ON; Duhamel et al., 2007). Briefly, reaction buffer (200 mM KCl, 20 mM HEPES (pH 7.0), 15 mM MgCl<sub>2</sub>, 1 mM EGTA, 10 mM NaN<sub>3</sub>, 5 mM ATP and 10 mM PEP) containing 18 U/mL of both LDH and PK, as well as homogenate were added to test tubes containing 15 different concentrations of Ca<sup>2+</sup>, ranging between 6.5 and 4.7 pCa units in the presence of ionophore A23187 (4.2 µM). Aliquots (60 µl) were then transferred in triplicate to a clear bottom 96-well plate (Costar, Corning Incorporated, NY), where 0.3 mM NADH was added to start the reaction. The plate was read at a wavelength of 340 nm for 30 min at 37°C. The different concentrations of Ca<sup>2+</sup> in the wells was required to determine the maximal enzyme activity (Vmax) of SERCA2 and pCa<sub>50</sub>, which is defined as the [Ca<sup>2+</sup>]<sub>r</sub> required to achieve 50% of Vmax. Lastly, cyclopiazonic acid (CPA; 40 µM), a highly specific SERCA inhibitor (Seidler et al., 1989), was utilized to determine background activity and subtracted from the total Ca<sup>2+</sup>-ATPase activity measured in VSM homogenate.

All data was plotted against the negative logarithm of [Ca<sup>2+</sup>]<sub>r</sub> (pCa) using basic statistical software (GraphPad Prism<sup>TM</sup> version 6) to determine Vmax and pCa<sub>50</sub>. More specifically, pCa<sub>50</sub> was determined by non-linear regression curve fitting using the sigmoidal dose response equation,

\[
Y = Y_{bot} + (Y_{top} - Y_{bot}) \cdot \left(1 + 10^{(\log \text{Ca}_{50} - x)} \right) \cdot n_H
\]

where \(Y_{bot}\) is the bottom of the plateau, \(Y_{top}\) is the top of the plateau, \(\log \text{Ca}_{50}\) is the logarithm of pCa<sub>50</sub> and \(n_H\) is the hill coefficient.

The accurate measurement of [Ca<sup>2+</sup>]<sub>r</sub> used for the ATPase activity assay was measured using the Ca<sup>2+</sup> fluorophore, indo-1 on a spectrofluorometric plate reader (SPECTRAMax Gemini XS; Molecular Devices, Toronto, ON) as previously described (Duhamel et al., 2007). The 15 [Ca<sup>2+</sup>]<sub>r</sub> concentrations ranging from 6.5 to 4.7 pCa units and all other components found in the above assay cocktail were added with the addition of indo-1 (1.5 µM); note however that NADH was not added, as it has extremely fluorescent properties which can interfere with the Indo-1 signal. In addition, two other Ca<sup>2+</sup> concentrations were required for Ca<sup>2+</sup> measurement, a zero Ca<sup>2+</sup> and a max Ca<sup>2+</sup> concentration (1mM). These were then added into a black 96 well plate (Costar, Corning Incorporated, NY) in triplicate and read on a spectrofluorometric plate reader (SPECTRAMax Gemini XS;
Molecular Devices, Toronto, ON) after a 15min incubation period at 37 °C. This assay is based on the difference in the maximal emission wavelength between Ca$^{2+}$ bound indo-1 (F) and Ca$^{2+}$ free indo-1 (G) complexes which have emission wavelengths of 405 and 485 nm upon excitation with a 355 nm wavelength, respectively. The ratio (R) of bound (F) to free (G) indo-1 complexes were used to calculate $[\text{Ca}^{2+}]_i$ with the following equation (Gryniewicz et al., 1985);

$$[\text{Ca}^{2+}]_i = K_d * (G_{\text{max}}G_{\text{min}}) * (R_{\text{min}})/(R_{\text{max}}-R)$$  

Equation 2.2

where $K_d$ is the equilibrium constant for the interaction between Ca$^{2+}$ and indo-1, $R_{\text{min}}$ is the minimum value of R with zero Ca$^{2+}$, $G_{\text{max}}$ is the maximum value of G with zero Ca$^{2+}$, $R_{\text{max}}$ is the maximum value of R at max Ca$^{2+}$ (1mM) and $G_{\text{min}}$ is the minimum value of G at max Ca$^{2+}$ (1mM). The $K_d$ value of the indo-1 Ca$^{2+}$ dye complex is 250 for muscle homogenates (Gryniewicz et al., 1985).

2.3.1 Steps to optimize Ca$^{2+}$-dependent Ca$^{2+}$-ATPase for VSM

Results from this assay yielded questionable and highly variable SERCA2 activity, which was incongruent with the literature. Therefore, a lot of time was spent trouble shooting the assay in an attempt to optimize for use with VSM. Below I have documented the various attempted experimental designs (approximately 14 iterations). In the end, none of these attempts yielded data consistent with the literature. As the results from these individual changes were limited, they were not analyzed for statistical significance and all the data only serves to act as a rationale for the discontinuation of the SERCA activity assay on VSM.

Initially, the inability for the assay to work was thought to be a result of experimenter error, however following successful repetitions on samples of white quadriceps, known to be high in SERCA content and pump activity, this was ruled out. It was thought that the low (barely detectable) activity was a result of low protein concentration present in VSM as a consequence of minute amount of tissue being homogenized (1-2 µg/µL per 2 mm ring). To increase total protein content two whole thoracic samples were pooled to increase total protein content (~7 µg/µL). This attempt resulted in sporadic SERCA activity and inconsistency within samples, which led us to believe the preparation of homogenate was not effectively breaking up connective tissue. Thus, leaving large particles in the assay preparation interfering with the spectrophotometric reading. To account for this, a polytron was utilized to replace hand held glass-on-glass homogenization to reduce variability between samples.
However, sporadic activity was seen in both preparations. We returned to hand homogenization and ensured full powdering of sample under LN$_2$ before re-suspending for glass-on-glass homogenization.

The next attempt of detecting Ca$$^{2+}$$-ATPase in VSM used a whole thoracic aorta (~6 µg/µL), resulting in saturated SERCA activity. In order to determine a full SERCA activity curve, it was noted that a lower Ca$$^{2+}$$ range be used and increasing the homogenate volume in the preparation would improve the results. Therefore, the CaCl$_2$ additions were diluted by half the original concentration, and homogenate used for sample prep was increased to 100 µL from 60 µL used in all previous attempts. This attempt left us with another saturated signal at low Ca$$^{2+}$$. The CaCl$_2$ additions were diluted further, for a solution one quarter its original potency, and again SERCA activity peaked with low Ca$$^{2+}$$.

To continue optimization, the next step was to attempt to quench already existing Ca$$^{2+}$$ within the sample preparation, as an increased Ca$$^{2+}$$ within the homogenate could result in premature SERCA activity saturation. Additional EGTA was added to the ATPase buffer, typically made up with 1mM EGTA. Adding .2 mM and .5 mM EGTA to separate cocktail preparations was seen to again result in low SERCA activity values. In an attempt to understand Ca$$^{2+}$$ presence in VSM homogenate and determine the optimal EGTA to quench free Ca$$^{2+}$$, an indol assay was completed. This assay is described in “Methods”, however this procedure did not include Ca$$^{2+}$$, and EGTA was added in increasing concentrations. It was found that the initial concentration was adequately binding free Ca$$^{2+}$$ within the VSM sample prep (1mM). With this information, additional assays were completed with the original EGTA (1mM), decreased Ca$$^{2+}$$ (25% of original concentration), and increased sample volume (100 µL). These attempts were inconsistent and variable leading to further assay adjustments.

From the literature, it was found that groups who have assessed SERCA activity through NADH oxidation in VSM typically use large samples of VSM, for example the porcine coronary artery, and can therefore complete the assay using a fractioned sample of VSM homogenate (Grover & Samson, 1989; Suzuki & Ford, 1991). Due to the low sample volume following homogenization of murine TA (~125 µL), this was not possible. However, following fractionation the literature describes a microsomal prep buffer used to complete SERCA activity assays. We aimed to replicate this buffer by decreasing KCl, known to inhibit myosin ATPase, along with lower concentrations of NaN$_3$, responsible for decreasing mitochondrial function. Altering these components showed no positive affect on SERCA activity, and it remained sporadic, low, and inconsistent.
Following this attempt, we aimed to determine if the powdering of sample under LN2 was affecting SERCA activity. Typically, sample used in this assay is exposed to one freeze-thaw cycle. The tissue is collected, immediately homogenized and held at -80°C until further use. The method by which VSM homogenization occurs includes powdering the sample under LN2, re-suspending the powder in homogenization buffer (PMSF) followed by glass-on-glass homogenization. Following which, homogenate was then transferred into a clean 1.5 mL Eppendorf tube and held at -80°C until the Ca\(^{2+}\)-ATPase assay was completed. This introduces an additional freeze-thaw cycle as the sample must be frozen prior to powdering, and this was thought to cause reduced SERCA activity. Fresh homogenization, completed just prior to assay, was performed to ensure only one freeze-thaw cycle. This method seemed to result in higher activity compared to previously frozen samples, however results were still inconsistent.

Due to the low volume of homogenate from each sample (∼125 µL), running samples in duplicate was not possible as each preparation utilized 100 µL of sample. Whole TA homogenate was once again pooled from two animals to increase overall sample volume. Two whole thoracic aortas were pooled and homogenized to gather a total of ∼250 µL sample. This allowed duplicate runs to be performed on fresh homogenate with half concentration Ca\(^{2+}\) and previously outlined ATPase buffer. As this was the most promising lead for detecting SERCA activity within VSM, this set up was attempted on seven samples run in duplicate. One of seven duplicate runs was consistent, while the remaining six showed a high degree of variance between samples.

We concluded that our efforts to quantify VSM SERCA activity did not produce the anticipated results and a surrogate marker was required to determine if PLN could protect SERCA function following ONOO⁻ exposure. Within the VSM, SERCA2b is the main isoform with PLN binding occurring in the groove formed between M2, M4, M6 and M9. This is the same binding domain within the SERCA2a isoform found mainly in the left ventricle. We used left ventricle from WT and PLN\(^{-/-}\) mice as an alternative model to elicit the potential protective effects of PLN. LV was collected, homogenized, divided into aliquots and kept at -80° until further use. ONOO⁻ treatment was administered by adding ONOO⁻ directly (80 µM or 300 µM) to homogenate and allowed incubation for 30 min at 37°C. Our lab has shown SERCA activity reduction following heat stress, therefore the control homogenate was also held in the water bath for 30 min to ensure any changes in activity were a direct result of ONOO⁻ exposure. Following the incubation period, the assay was completed as originally described above in Section 2.3.
2.4 PLN Protein Expression in LV and VSM

The left ventricle and thoracic aorta from each animal were collected and homogenized analyzed via Western blot to ensure genotype. Protein concentration was determined through a Bicinchoninic Acid protein assay utilizing BSA as a protein standard. Specifically, collected tissue was immediately homogenized in cold PMSF buffer, solubilized in 5X Lamelli sample buffer (60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) and boiled at 95°C for 5 min. To analyze VSM, reduced sample, 45 ug, was electrophoresed through 13% acrylamide gels using a tricine-based system, while LV, 2 ug, was electrophoresed through 7.5% acrylamide gels using a glycine-based system. Following this, both LV and VSM were transferred to a polyvinylidene fluoride (PVDF) membrane and blocked in 5% milk-TBST. The membranes were then incubated with an antibody directed against PLN (Pierce Antibodies, monoclonal, clone 2D12, mouse, 1:2000, 5% milk-TBST), washed and incubated with horseradish peroxidase-conjugated goat anti-mouse in 5% milk-TBST (SantaCruz, 1:2000) for one hour. The presence of PLN was detected using enhanced chemiluminescence. A 10-250 kDa wide range protein molecular weight marker was used to determine the proteins of interest through known molecular weights following imaging on a Chemigenius 2 Bio-imaging System (Syngene).

2.5 Isolation of SERCA2 by immunoprecipitation

It was necessary to immunoprecipitate SERCA2 from the LV homogenate in order to measure nitrotyrosine (3-NY) content of SERCA2. This is due to an abundance of the Na⁺/K⁺-ATPase in addition to SERCA2 present in LV. Both of these pumps are members of the P-type transporter family and are of similar size (~100 kDa) and 3-D structure (Toyoshima et al., 2000). Therefore, it would be impossible to discern the Ca²⁺-ATPase band from the Na⁺/K⁺-ATPase band when blotting for 3-NY content. In addition, by immunoprecipitating SERCA2 the sample becomes concentrated with SERCA2, which improves the ability to a) detect 3-NY and to b) detect differences between groups. Therefore, SERCA2 was first immunoprecipitated from the whole heart homogenate. Briefly, homogenates (1 mg/mL) were diluted with homogenizing buffer, H₂O and 2X IP buffer (20 mM Tris, pH 7.4, 600 mM Sucrose, 1% CHAPS & 1 protease inhibitor tablet; Complete© Roche) and mixed gently by inversion. The samples were then centrifuged for 10 min at 6000 g at 4°C. The supernatant of each sample was transferred to new tubes and 8 µL of 25% BSA and 1 µg of anti-SERCA2 antibody added to each tube.
The samples mixed via rotation for 1 hour at 4°C after which 30µL of protein-A sepharose was added and the samples were rotated for another hour at 4°C. The protein-A sepharose was then centrifuged at 8000g for 5 minutes and the supernatant discarded. The sepharose beads were washed 3 times with 1X RIPA buffer. After washing, 10 µL of 2X sample buffer was then added to each tube and directly used in a Western blot protocol to determine nitrotyrosine content.

2.6 SERCA2 nitrotyrosine content

The degree of nitrosylation of SERCA2 was determined using the methods of Li et al. (2006) with slight modifications. After immunoprecipitation of SERCA2 from LV homogenate, the sample was divided with 12 µL of being loaded onto 2 7.5% acrylamide gels, separated by standard SDS-PAGE protocols and transferred to PVDF membranes. The membranes were probed with a monoclonal primary antibody specific for nitrotyrosine residues (1;1000, Cayman Chemicals) and SERCA2, respectively (1:2000, Pierce Thermo). After washing with TBS-T the membrane was incubated with goat anti-mouse secondary antibodies (1:2000, Santa Cruz) and visualized using an ECL kit (GE HealthCare) on a Chemigenius 2-Bio-imaging System (Sygnene). The duplicate sample probed for SERCA2 content was used to normalize the nitrotyrosine content.

2.7 Statistical Analysis

A two-way ANOVA was used to test for differences between PLN<sup>+/−</sup> and WT mice in control and treatment conditions for myography, Ca<sup>2+</sup>-dependent Ca<sup>2+</sup> ATPase activity, and Western blotting data. The significance level was set at P ≤ 0.05, and when appropriate, a Newman-Keuls post hoc test was used to compare specific means. To elucidate any differences within groups, pre-planned comparisons within genotype were performed using Student’s t-tests. Values are means ± SE. Sample sizes for all statistics will be described in the “Results” section. Variation in sample size between groups is present as a result of removing outliers (±2SD) for statistical analysis.
3.0 Results

3.1 Presence of Phospholamban

The genotype of all animals used was confirmed through a western blot run on whole LV homogenate blotted with a monoclonal antibody against PLN. This tissue is known to be highly abundant in PLN providing easy detection for genotype verification (Appendix). The presence or absence of PLN within the VSM was confirmed through western blot of whole thoracic aorta homogenates. The blot is the first documented in the literature indicating PLN protein in WT whole VSM tissue and also confirms the absence of PLN protein in PLN$^{-/-}$ TA (Figure 1).

![Figure 3: Presence of PLN in VSM](image)

**Figure 3: Presence of PLN in VSM.** Whole TA homogenate was electrophoresed and blocked against PLN specific antibody. Lane “Con” contains human vastus lateralis homogenate as a positive control for PLN. Lanes 1,3,5, and 7 contain WT samples while lanes 2,4,6, and 8 contain PLN$^{-/-}$ samples. This blot confirms the presence of PLN within the WT VSM, seen just below the 10kD marker.
3.2 Contractility

Standard vessel myography was used to determine if physiological levels of PLN could aid in maintaining vasomotor function following ONOO$^-$ induced damage. Prior to mounting vessels in the organ bath, rings were cut to 2 mm in width. Though this was completed microscopically, slight variance in ring size is possible resulting in increased force production within rings of larger size. To normalize total force production, as slight ring error is likely, we utilize the PE/KCl ratio to compare responses to PE across groups. The total force production with KCl represents the total force produced through depolarization of the vessel, while total force production through PE represents receptor mediated force production. Using a ratio between the two can allow normalization for overall size of the vessel and comparisons between rings. Table 1 indicates total tension developed in response to KCl (60 mM) and PE ($10^{-6}$ M), showing no difference in KCl force production between WT and PLN$^{-/-}$ control vessels. Therefore using the PE/KCl ratio is an appropriate indicator of PE induced force production in both genotypes while also normalizing for vessel dimensions. This finding is consistent with the literature as it has been shown that in response to KCl, WT and PLN$^{-/-}$ vessels produce equal force (Lalli et al., 1997). Our data indicate no difference between the genotypes following ONOO$^-$ treatment, although ONOO$^-$ causes decreased overall contraction across all treatment conditions (p<0.05; Figure 2). One-way ANOVAs within genotype reveal a significant decrease in force production in both genotypes with each concentration being significantly different from control (p<0.05) and no significant differences between concentrations (p>0.05). We can thus state that the effect of ONOO$^-$ incubation on contraction is equivalent in both genotypes.

Ideally, resting tension throughout incubation should remain unchanged to ensure the oxidant is not interfering with baseline measurements but a change in resting tension could indicate interaction of ONOO$^-$ incubation with contraction pathways. Incubation with 300 µM ONOO$^-$ significantly increased basal tension in both the WT and PLN$^{-/-}$ vessels (p<0.05) however basal tension during 150 µM incubation was significantly higher in PLN$^{-/-}$ compared to WT (p<0.05, Figure 3).

Total relaxation was also assessed to determine if ONOO$^-$ affected the ability of the vessels to achieve complete relaxation. Relaxation was elicited through cumulative additions of SNP ($10^{-10}$ to $10^{-5}$ M). During the cumulative dose-response experiment the tension response to each concentration was allowed to stabilize before the subsequent concentration was added. Figure 4 shows ONOO$^-$ does not affect the ability of the vessels to relax,
however it was seen that the PLN<sup>-/-</sup> group consistently exhibited lower maximal relaxation compared with WT group (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Max KCl (g)</th>
<th>Max PE (Con, g)</th>
<th>Max PE (80µM ONOO&lt;sup&gt;-&lt;/sup&gt;, g)</th>
<th>Max PE (150µM ONOO&lt;sup&gt;-&lt;/sup&gt;, g)</th>
<th>Max PE (300µM ONOO&lt;sup&gt;-&lt;/sup&gt;, g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.580 ± 0.21</td>
<td>0.611 ± 0.064</td>
<td>0.323 ± 0.043 *</td>
<td>0.408 ± 0.102</td>
<td>0.481 ± 0.046</td>
</tr>
<tr>
<td>PLN&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>0.644 ± 0.21</td>
<td>0.597 ± 0.046</td>
<td>0.334 ± 0.065 *</td>
<td>0.259 ± 0.087 *</td>
<td>0.266 ± 0.087 *</td>
</tr>
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</table>

**Table 1: Developed KCl and PE induced force production.** WT and PLN<sup>-/-</sup> vessels were exposed to 60 mM KCl prior to ONOO<sup>-</sup> incubation and 10<sup>-6</sup> M PE following ONOO<sup>-</sup> incubation. This table highlights the total force produced by each vasoconstrictor, indicating no difference in KCl force development between genotypes (p>0.05), and a significant effect of force reduction in both WT and PLN<sup>-/-</sup> vessels following any concentration of ONOO<sup>-</sup> incubation. Student’s t-test reveal that Max PE tension is significantly lower than control following all concentrations of ONOO<sup>-</sup> within PLN<sup>-/-</sup> vessels (*, p<0.05). While only incubation with 80 µM ONOO<sup>-</sup> results in a significantly reduced Max PE tension in WT vessels. (n= 21 control groups, n=12 80 µM groups, n=6 150 µM groups, n=4 WT 300 µM group, n=3 PLN<sup>-/-</sup> 300 µM group)
**Figure 4: Relative contraction of PE/KCl in vessels following ONOO⁻ exposure.** Following exposure to varying ONOO⁻ concentrations, TA rings from WT and PLN⁻/⁻ animals were constricted by the addition of PE as described in "Methods". PE/KCl ratio was used to normalize PE induced force data, and determine relative contraction; all values represent means ± SE. Incubation with any concentration of ONOO⁻ resulted in significant reduction in PE stimulated force production in both WT and PLN⁻/⁻ groups, indicated by * (p<0.05) (n= 21 control groups, n=12 80 µM groups, n=6 150 µM groups, n=4 WT 300 µM group, n=3 PLN⁻/⁻ 300 µM group)
Figure 5: Change in basal tension throughout ONOO⁻ incubation. TA segments from WT and PLN⁻/⁻ animals were exposed to varying concentrations of ONOO⁻ for 30 min as described in "Methods". Change in total developed tension throughout incubation is expressed in grams, mean ± SE. A significantly higher tension developed within the PLN⁻/⁻ group compared to WT regardless of treatment condition (p<0.05). There is also a significant increase in tension developed as a result of ONOO⁻ exposure (p<0.05). Planned comparisons also show a significant difference between the WT and PLN⁻/⁻ vessels incubated with 150 µM ONOO⁻ (p<0.05) (n=12 for 80 µM groups, n=5 for WT 150 µM groups, n=3 for PLN⁻/⁻ 150 µM groups, n=4 for 300 µM groups).
Figure 6: Maximum relaxation to SNP following ONOO⁻ exposure. Vessels from WT and PLN⁻/⁻ were exposed to varying concentrations of ONOO⁻. Following exposure, the functionality of the vessels was measured as described in "Methods". The maximal relaxation following consecutive additions of SNP is expressed in this graph and shown as a percentage of total constriction to PE, all values are mean ± SE. PLN⁻/⁻ vessels showed a significantly lower maximal relaxation compared to WT vessels across all treatment concentrations (p<0.05) (n= 21 for Con groups, n=12 for all 80 µM groups, n=5 for WT 150 µM, n=4 for WT 300 µM group, n=3 for PLN⁻/⁻ 150 µM and 300 µM groups).
Within each genotype, the SNP dose response was compared between treatment conditions. Following 80 µM ONOO\(^-\) incubation, the relaxation to SNP was affected at \(10^{-9.5}\) and \(10^{-9}\) M within both the WT and PLN\(^{-/-}\) groups (p<0.05, Figure 5). Similar changes were seen following 150 µM ONOO\(^-\) incubation, although the differences were only significant within the WT group (p<0.05, Figure 6). After 300 µM ONOO\(^-\) incubation, no statistical significant differences were observed between control and treatment groups at any concentration of SNP (p>0.05, Figure 7).

\(EC_{50}\) describes the concentration of drug required to elicit half relaxation: a lower \(EC_{50}\) indicating a greater sensitivity to the drug. Comparing \(EC_{50}\) within treatment condition and across genotypes shows that 80 µM ONOO\(^-\) significantly increased \(EC_{50}\) in both genotypes, suggesting a decreased sensitivity to SNP following ONOO\(^-\) treatment (p<0.05). Incubation with 150µM ONOO\(^-\) shows a differing response compared to 80 µM ONOO\(^-\). Within the WT vessels 150µM ONOO\(^-\) was able to significantly decrease the \(EC_{50}\), while the same concentration was able to significantly increase the \(EC_{50}\) within PLN\(^{-/-}\) vessels (p<0.05). Incubation with 300µM ONOO\(^-\) caused no significant changes in \(EC_{50}\) in either WT or PLN\(^{-/-}\) vessels (p>0.05, Figure 8). The control values for each level of drug concentration used in this experiment show large variation, therefore comparisons between groups would mask effects within each group. For this reason, comparisons were held to only within each concentration group.
Figure 7: SNP induced relaxation in thoracic aorta following ONOO⁻ incubation (80 µM). Thoracic aorta vessels were exposed to 30 mins of 80 µM ONOO⁻. Following the incubation, vessels were pre-constricted with PE (10⁻⁶ M) and then relaxed with cumulative additions of SNP (10⁻¹⁰ - 10⁻⁵ M). ONOO⁻ treatment showed the same effect in both WT (A) and PLN⁻/- (B) vessels, whereby at 10⁻⁹.5 and 10⁻⁹ M SNP, the ONOO⁻ treated vessels had a decreased relaxation. * (p<0.05, n=12).
Figure 8: SNP induced relaxation in thoracic aorta following ONOO⁻ incubation (150 µM). Thoracic aorta vessels were exposed to 30 mins of 150 µM ONOO⁻. Following the incubation, vessels were pre-constricted with PE (10⁻⁶M) and then relaxed with cumulative additions of SNP (10⁻¹⁰-10⁻⁵M). ONOO⁻ treatment showed an effect in WT vessels, whereby at 10⁻⁹.5 and 10⁻⁹ M SNP, the ONOO⁻ treated vessels had a decreased relaxation, * (p<0.05, n=5).
Figure 9: SNP induced relaxation in thoracic aorta following ONOO⁻ incubation (300 µM). Thoracic aorta vessels were exposed to 30 mins of 300 µM ONOO⁻. Following the incubation, vessels were pre-constricted with PE (10⁻⁶M) and then relaxed with cumulative additions of SNP (10⁻¹⁰-10⁻⁵M). ONOO⁻ treatment showed no significant effect in WT or PLN⁻/⁻ vessels (p>0.05, n=4).
Figure 10: EC$_{50}$ of ONOO treated vessels. Thoracic aorta rings were exposed to varying concentrations of ONOO$^-$. Following which, the rings were pre-constricted with PE (10$^{-6}$ M) and a dose response to SNP (10$^{-10}$-10$^{-5}$ M) was performed as described in “Methods”. EC$_{50}$ values are shown as mean ± SE. (A) In response to 80 µM ONOO$^-$ treatment, there was a significant increase in EC$_{50}$ in both the WT and PLN$^{-/-}$ vessels (p<0.05). WT vessels also showed to have a significantly higher EC$_{50}$ in both the control and treatment conditions (p<0.05, n=12). (B) ONOO$^-$ treatment of 150 µM elicited a significantly decreased EC$_{50}$ in WT vessels and a significantly increased EC$_{50}$ in PLN$^{-/-}$ vessels, indicated as * (p=0.05, n=5). (C) Following 300 µM ONOO$^-$ treatment, there were no significant differences seen in EC$_{50}$ data (p>0.05, n=4 for WT n=3 for PLN$^{-/-}$).
3.3 \( \mathrm{Ca}^{2+} \)-dependent SERCA activity

\( \mathrm{Ca}^{2+} \)-dependent \( \mathrm{Ca}^{2+} \)-ATPase activity was examined to determine if PLN could protect SERCA function following incubation with an oxidant. This assay was completed in LV homogenate of WT and PLN\(^{-/-}\) animals, as the method could not be adapted to vascular smooth muscle, described in “Methods”.

Table 2 highlights the maximal SERCA activity, \( \mu \text{mol/g protein/min} \), for all groups prior to normalization. Data were normalized to the WT control group within each treatment condition, either 80 \( \mu \text{M} \) or 300 \( \mu \text{M} \), and analysis was completed comparing control to treatment groups within each concentration. The data presented in Figure 9 indicates control groups for both 80 \( \mu \text{M} \) and 300 \( \mu \text{M} \) ONOO\(^{-} \) normalized to WT Con from the 80 \( \mu \text{M} \) group. However, statistical comparisons were made between each treatment group and their corresponding control group. No statistically significant differences were seen between control and 80 \( \mu \text{M} \) ONOO\(^{-} \) treatment however; it was seen that 300 \( \mu \text{M} \) ONOO\(^{-} \) incubation reduced SERCA activity in both the WT and PLN\(^{-/-}\) groups (\( p < 0.05 \)).
<table>
<thead>
<tr>
<th></th>
<th>Max ATPase (µmol/g pro/min)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>80 µM</td>
</tr>
<tr>
<td><strong>WT</strong></td>
<td></td>
</tr>
<tr>
<td>Con</td>
<td>193.15 ± 74.9</td>
</tr>
<tr>
<td>ONOO⁻</td>
<td>176.40 ± 45.1</td>
</tr>
<tr>
<td><strong>PLN⁻/⁻</strong></td>
<td></td>
</tr>
<tr>
<td>Con</td>
<td>222.92 ± 46.9</td>
</tr>
<tr>
<td>ONOO⁻</td>
<td>243.63 ± 103.4</td>
</tr>
</tbody>
</table>

**Table 2: Maximal Ca²⁺ dependent Ca²⁺-ATPase activity.** LV homogenates from WT or PLN⁻/⁻ animals were exposed to 80 µM or 300 µM ONOO⁻ for 30 mins. Table indicates maximal activity achieved in each condition. Two-way ANOVA of data shows that, PLN⁻/⁻ samples had a significantly higher maximal SERCA activity level (p<0.05) and ONOO⁻ treated samples had lower maximal SERCA activity than control (p=0.05, n=4 WT Con & WT 80 µM group, n=6 WT 300 µM, PLN⁻/⁻ Con, 80 µM & 300 µM groups).
Figure 11: Ca$^{2+}$ dependent Ca$^{2+}$-ATPase activity of SERCA2. LV homogenates from WT or PLN$^{-/-}$ animals were exposed to ONOO$^-$ for a total of 30 mins (37°C). Control samples were exposed to heat only. This graph shows maximal ATPase values normalized to WT control. For analysis, each treatment group was compared to their own corresponding control group however the graph depicts a lumped group of all control samples used throughout the experiment. A significant reduction in SERCA activity was seen following 300 µM ONOO$^-$ treatment with no differences seen between the WT and PLN$^{-/-}$ groups, * (p<0.05, n=4 WT Con & WT 80 µM group, n=6 WT 300 µM, PLN$^{-/-}$ Con, 80 µM & 300 µM groups)
3.4 Co-immunoprecipitation

LV homogenate from WT and PLN−/− animals were exposed to varying concentrations of ONOO− (80 µM & 300 µM) or Con (0 µM), while incubating in a water bath (37°C, 30 min). To determine if PLN could protect SERCA through physical interaction, IP for SERCA was performed on samples in order to isolate ONOO− induced damage to the SERCA pump specifically. This is important as the Na+\(\text{/K}^+\)-ATPase found in LV is of similar size to SERCA and when electrophoresed, migrates to a very similar band position as SERCA. If IP was not completed, both pumps could be contributing to the 3-NY content detected in immunoblotting experiments. Therefore, it was important to pull down SERCA2 from the homogenate to ensure all 3-NY detected was on SERCA2. Western blot analysis showed no differences in 3-NY content on SERCA2 between Con and ONOO− exposed groups (p<0.05, Figure 10).

3.5 p-PLN following ONOO− incubation in LV

Figure 11 shows the changes in p-PLN at Ser16 following 80 µM and 300 µM ONOO− incubation. As high concentrations of ONOO− are known to increase protein phosphatase activity, the phosphorylation status of PLN was detected to aid in determining functional implications of ONOO− incubation. It has also been shown that low concentrations (<30 µM) can activate SERCA activity through PKA dependent phosphorylation of PLN, therefore it was suspected that incubation with 80 µM ONOO− could show increases in p-PLN while incubation with 300 µM ONOO− would indicate decreased p-PLN. However, it was seen that high concentration ONOO− significantly increased PLN phosphorylation compared to control (p<0.05).
Figure 12: SERCA2 3-NY content following ONOO' exposure. SERCA2 was immunoprecipitated from control and ONOO' treated LV homogenates from WT and PLN⁻/⁻ animals. Panel A shows presence of SERCA2 within the LV for all conditions, while Panel B depicts the 3-NY formation on SERCA2. Graph C represents the optical density of Western blots for 3-NY content on SERCA2 (n=3). Values were normalized to SERCA2 content in each group.
Figure 13: p-PLN at Ser 16 in LV following ONOO⁻ exposure. LV homogenates were exposed to varying concentrations of ONOO⁻ for 30 mins at 37°C as described in "Methods". Samples were then run against a p-PLN Ser16 specific antibody to detect any changes in phosphorylation level. Panel A depicts two representative blots following incubation, were the effects control (C) and ONOO treatment (O) in LV on p-PLN can be seen just below the 10kD marker. Panel B indicates the optical density analysis of WT p-PLN from the blots. It was seen that incubation with 300 μM ONOO⁻ significantly increase p-PLN at the described site (p<0.05). All group values are means ± SE. (n=3)
4.0 Discussion

The objectives of this study were to test the hypothesis that PLN is able to protect SERCA2 structure and function from deleterious effects associated with exposure to ONOO\(^{-}\). More specifically, this study aimed to determine if physiological levels of PLN aid in the maintenance of vasomotor function following ONOO\(^{-}\) exposure. This study also aimed to determine if PLN protects SERCA2 activity following ONOO\(^{-}\) exposure, and to determine if PLN prevents ONOO\(^{-}\) induced 3-NY modifications to SERCA. It was found that endogenous levels of PLN were unable to protect vasomotor function following incubations with various concentrations of ONOO\(^{-}\) (80 \(\mu\)M, 150 \(\mu\)M or 300 \(\mu\)M) as both the WT and PLN\(^{-/}\) were affected equally. The only exception was that the EC\(_{50}\) for SNP-induced relaxation was significantly increased in the PLN\(^{-/}\) group while it was significantly decreased in the WT group following incubation with 150 \(\mu\)M ONOO\(^{-}\). It was also found that SERCA2 activity was significantly reduced in both the WT and PLN\(^{-/}\) following incubation with 300 \(\mu\)M ONOO\(^{-}\). SERCA2 3-NY content was unchanged with treatment in both genotypes. Overall, the results from this thesis do not support the notion that PLN can protect SERCA2 structure and function against ONOO\(^{-}\) induced damage.

4.1 Effects of ONOO\(^{-}\) incubation on vasomotor function in WT and PLN\(^{-/}\) vessels

Using standard vessel myography, rings from the thoracic aorta of WT and PLN\(^{-/}\) were exposed to 30 min of ONOO\(^{-}\) (80 \(\mu\)M, 150 \(\mu\)M or 300 \(\mu\)M) or control (0 \(\mu\)M ONOO\(^{-}\)). Previous studies have highlighted no morphological differences exist between WT and PLN\(^{-/}\) mice (Lalli et al., 1997). It has also been reported that aortic smooth muscle of PLN\(^{-/}\) mice is more sensitive to PE stimulation, indicating increased SR Ca\(^{2+}\) release (Lalli et al., 1997), though that was not replicated here. During incubation with ONOO\(^{-}\), it was observed that the PLN\(^{-/}\) vessels elicited an increased basal tension following the 150 \(\mu\)M treatment and an increased basal tension in both WT and PLN\(^{-/}\) following 300 \(\mu\)M treatments. These dissipated rapidly after oxidant removal, signifying contraction occurred as a result of ONOO\(^{-}\) presence and was reversible upon the removal of oxidant pressure. Work presented here shows no differences in baseline tension with 80 \(\mu\)M ONOO\(^{-}\) incubation, consistent with research previously reported by Li et al., who found incubation of rat aortic rings with \text{10}^{-8}\text{-10}^{-4} M ONOO\(^{-}\) had no effect on baseline tension (Li, Li, Altura, & Altura, 2005). However, when using a different model of smooth
muscle, canine cerebral arteries, Li et al found that $10^{-7}$-10$^{-6}$ M ONOO$^-$ was able to significantly reduce baseline tension (Li, Li, Altura, & Altura, 2004). Another group reported that incubation with ONOO$^-$ (100 µM) was able to cause constriction in rat pulmonary arteries, though the data did not reach significance (Chabot, Mitchell, Quinlan, & Evans, 1997). The differing ONOO incubation effects established in the literature could result from variances in experimental protocols including parameters such as length of incubation, origin or type of ONOO$^-$ (endogenous vs. exogenous), state of the vessel during exposure, and concentrations used. The most consistent finding in the literature is that ONOO$^-$ incubation causes smooth muscle relaxation, and numerous groups have proposed different mechanisms of action for this ONOO$^-$ induced response. Specifically, one group has suggested that relaxation is a result of the endogenous ONOO$^-$ formation consuming existing NO created within the endothelium. This increased NO consumption results in a subsequent release of NO to replace that used for ONOO$^-$ formation, causing an overall increase in vessel relaxation (Davidson et al., 1997). The vessels used in data presented here had the endothelial layer removed to ensure no interplay of SERCA3, with the results aiming to decipher the effects of ONOO$^-$ incubation on SERCA2. Therefore, the vessels used in this experiment were unable to produce endogenous endothelial-derived NO. Also, ONOO$^-$ used in this study was commercially purchased and directly added to the bath, where Davidson et al. describes a shift in NO production only through endogenous ONOO$^-$ formation (1997). To refute this mechanism further, Li et al (2005) have shown relaxation in denuded TA vessels implying that the endothelium is not required to elicit ONOO$^-$ induced relaxation. Instead, the relaxant effects may be mediated via the ONOO$^-$ induced activation of soluble guanylyl cyclase (sGC) which enhances cGMP levels resulting in vascular relaxation through PLN phosphorylation (Li et al., 2005). This method of relaxation would present differently within WT and PLN$^{-/-}$ vessels, however data presented here showed the opposite results with increased force production in only the PLN$^{-/-}$ group following 150 µM ONOO$^-$ incubation, and increased force production in both WT and PLN$^{-/-}$ groups following 300 µM ONOO$^-$ incubation. If PLN phosphorylation was the main mechanism affecting baseline tension, you would expect to see decreased force within the WT group compared to the PLN$^{-/-}$ group; however, the observed increase in tension within the PLN$^{-/-}$ group with 150 µM ONOO$^-$ exposure and both genotypes with 300 µM ONOO$^-$ is the result of more than PLN phosphorylation, as no change in p-PLN were seen following 150 µM ONOO$^-$ exposure. The literature covers much detail regarding the biphasic nature of ONOO$^-$, low concentrations can activate PKA/PKG to stimulate phosphorylation of PLN and reversible nitration of SERCA, along with many other targets, to increase muscular relaxation (Belik,
Jankov, Pan, & Tanswell, 2004; Chabot et al., 1997; Chesnais, Fischmeister, & Mery, 1999; Kamat, 2006; Kohr, Traynham, Roof, Davis, & Ziolo, 2010). High concentrations have been shown to increase protein phosphatase activity resulting in dephosphorylation of PLN resulting in movement back to its original inhibitory state (Kohr, Roof, Zweier, & Ziolo, 2012; Maneen, Hannah, Vitullo, DeLance, & Cipolla, 2006; Viner et al., 1996). The data presented here indicates this biphasic disposition of ONOO⁻. Incubation with low concentrations of ONOO⁻ can lead to PLN phosphorylation causing dissociation from SERCA in the WT vessels, resulting in the ability for SERCA to constantly clear excess cytosolic Ca²⁺. As the ONOO⁻ concentration increases, differences in tension production occur which can be potentially explained by an increase in PKA. For example, with 150 µM ONOO⁻, the WT vessels showed no change, which could possibly be a result of increased PKA, and the large increase in baseline tension within the PLN⁻/- vessels could possibly be an indication of SERCA damage. If SERCA is being oxidized, its ability to sequester Ca²⁺ will decrease resulting in increased cytosolic Ca²⁺ and contraction. We cannot exclude the possibility that the WT vessels experienced no change in tension throughout incubation with 150 µM ONOO⁻ as a result of PLN binding and protecting SERCA function. On the other hand, perhaps high oxidant levels (300 µM ONOO⁻), were enough to damage SERCA irrespective of PLN binding. Irreversible nitrosylation of SERCA occurs with increased amounts of oxidants, and though not detected in this data could explain the increased baseline tension in both the WT and PLN⁻/- vessels (Knyushko, Sharov, Williams, Schöneich, & Bigelow, 2005).

Once the incubation period had elapsed, ONOO⁻ was removed from the bath to ensure no interference with subsequent testing. It was hypothesized that lasting effects of ONOO⁻ exposure would be maintained for the remainder of the experiment and that changes seen in contraction or relaxation were the result of PLN’s effect on SERCA. The long-term effects of ONOO⁻ incubation were evident through the reduction in contraction of both WT and PLN⁻/- vessels. ONOO⁻ has been reported to decrease peak tension to KCl by ~10% following 150 µM incubation (Schmidt, Zaib, Samson, Kwan, & Grover, 2004). One mechanism which has been observed in the literature is the ONOO⁻ induced SERCA oligomerization, thereby decreasing Ca²⁺ pumping efficiency, resulting in lower overall contraction due to lower Ca²⁺ release (Grover, Kwan, & Samson, 2003). The literature has also demonstrated that ONOO⁻ incubation increases the activity of protein phosphatase (PP), which has numerous downstream effects, one of particular interest is the RyR. As the primary Ca²⁺ release channel on the SR, PP has been shown to increase the open probability of RyR channels resulting in Ca²⁺ release (Terentyev, Viatchenko-
Karpinski, Gyorke, Terentyeva, & Gyorke, 2003). Low level oxidation has also been shown to increase redox-modifications on the RyR, independent of PP activity, leading to an increase in open probability to facilitate Ca$^{2+}$ release/leak from the SR (Aschar-Sobbi, Emmett, Kargacin, & Kargacin, 2012; Belevych et al., 2007; Gonzalez, Treuer, Castellanos, Dulce, & Hare, 2010). This leads to reduction or depletion of the SR Ca$^{2+}$ stores and upon subsequent stimulus for contraction less Ca$^{2+}$ is released leading to lower force production (Belevych et al., 2007). Another downstream target of PP is PLN with the overall effect of decreasing SERCA activity through PLN dephosphorylation (M Asahi et al., 2000). Once PLN is dephosphorylated, it is able to return to its resting inhibitory state, decreasing SERCA activity by decreasing its affinity for Ca$^{2+}$ (Colyer, 1998). This was not observed in the data presented, as a significant reduction in PE induced force following ONOO$^-$ incubation occurred, with no changes between WT and PLN$^{-/-}$ groups (p<0.05). If the main mechanism by which ONOO$^-$ can decrease force production is through PP phosphorylation of PLN, there would be a difference between the genotypes. Therefore, given the data presented, it is more likely that decreased peak PE-induced tension is a result of depleted SR Ca$^{2+}$ stores occurring through increased open probability of the RyR, though that action was not directly investigated in this body of work. Another possible mechanism that was not directly investigated, though would be consistent with our results in the aggregation of SERCA pumps to decrease their overall efficiency. Coupled with decreased SR Ca$^{2+}$ pools would result in reduced peak force in both the WT and PLN$^{-/-}$ groups.

In vivo, endogenous ONOO$^-$ formation is frequently used as a replication of that which occurs in diseased states to investigate its effects on whole cell function. Specifically, SIN-1 is used as a NO donor which results in the formation of ONOO$^-$ (Stojanovic, Ziolo, Wahler, & Wolska, 2001). It has been shown that incubation with SIN-1 can result in decreased β-adrenergic stimulated contraction (Stojanovic et al., 2001; Yin, Shan, Deng, & Bourreau, 2002). More specifically, SIN-1 exposure was able to decrease the positive inotropic effects of isoproterenol and was associated with increased cGMP, decreased cAMP, as well as reduced PLN and troponin-I phosphorylation (Stojanovic et al., 2001). Research shows that cGMP and cAMP, mediated through phosphodiesterases, are able to decrease myocyte shortening and decrease Ca$^{2+}$ transients following SIN-1 incubation (Stojanovic et al., 2001). Matched with the ability for ONOO$^-$ to increase the rate of relaxation through phosphorylation of regulatory cross-bridge formation proteins, and stimulation of SERCA through phosphorylation of PLN, SIN-1 has shown to decrease force production through numerous pathways (Zhang, Zhao, Mandveno, & Potter, 1995). These provide alternative pathways through which ONOO$^-$ incubation causes a
reduction in PE stimulated contraction. As no differences were observed between WT and PLN\(^{-/-}\) groups, decreased Ca\(^{2+}\) transients through ONOO\(^{-}\) cGMP pathways could be another mechanism of action.

EC\(_{50}\) is defined as the drug concentration necessary to elicit half relaxation of a measured response. In this case, a change in EC\(_{50}\) value can depict a smooth muscle change in drug sensitivity, where an increased EC\(_{50}\) is indicative of decreased sensitivity and a decreased EC\(_{50}\) implies an increased sensitivity. Looking closely at the EC\(_{50}\), we can determine if ONOO\(^{-}\) incubation affects the sensitivity of TA rings to SNP relaxation. SNP elicits relaxation through PKG-induced pathways, a major pathway of interest being phosphorylation of PLN (Kim, Han, Koh, & Perrino, 2006), though it has also been shown to indirectly activate MLCP and decrease Ca\(^{2+}\) sensitivity of contractile proteins (Bonnevier & Arner, 2004; Bonnevier, Fässler, Somlyo, Somlyo, & Arner, 2004).

Comparisons were made at each SNP concentration and it was seen that 80 µM ONOO\(^{-}\) treatment was able to significantly increase the EC\(_{50}\) in both the WT and PLN\(^{-/-}\) vessels (p<0.05). This was seen following pre-stimulation with PE, to which there was a large reduction in overall force production with 80 µM ONOO\(^{-}\). The ability for oxidants as well as oxidant-induced PP to increase open probability of RyRs has been discussed, which would result in an increased SR Ca\(^{2+}\) release/leak leading to reduced force upon subsequent contraction. In addition to this, ONOO\(^{-}\) induced elevations in cGMP could be acting to decrease contractile Ca\(^{2+}\) sensitivity, which would result in increased [Ca\(^{2+}\)]\(_{f}\) with no accompanied increase in force. This would then lead to an increased EC\(_{50}\) in both the WT and PLN\(^{-/-}\) groups as additional Ca\(^{2+}\) needs to be pumped, through SERCA, into the SR. A different response was seen following 150 µM ONOO\(^{-}\) incubation where a significant decrease was seen in the WT vessels while the PLN\(^{-/-}\) vessels had a significantly increased EC\(_{50}\) (p<0.05). With the ablation of PLN, this increased EC\(_{50}\) could be seen as damage to SERCA as the increase was not replicated within the WT group. SERCA damage could result in decreased pump activity or decreased coupling ratio, therefore taking longer to effectively move Ca\(^{2+}\) into the SR causing a large increase in EC\(_{50}\) of SNP-induced relaxation. The significant reduction in EC\(_{50}\) in the WT could be the result of ONOO\(^{-}\)-induced PKG mediated activation of MLCP and increased relaxation. Paired with maintained SERCA activity due to PLN presence, would then increase overall relaxation and decrease EC\(_{50}\) to SNP-induced relaxation. However, there were no changes seen in EC\(_{50}\) following incubation with 300 µM ONOO\(^{-}\) though large variances observed within the PLN\(^{-/-}\) group and low sample size could affect results. It was hypothesized that increasing concentrations of ONOO\(^{-}\) would have an increasing effect on altering EC\(_{50}\), suggesting higher concentrations would result in a more pronounced decrease to SNP sensitivity,
increased EC\textsubscript{50}. The lack of effect of high concentrations of ONOO\textsuperscript{−} on EC\textsubscript{50} may be due to the significant reduction in overall force production following exposure to 300 µM ONOO\textsuperscript{−}. Due to the ~60% reduction in PE/KCl following 300 µM ONOO\textsuperscript{−} exposure, the slight changes in relaxation needed to determine the EC\textsubscript{50} through a sigmoidal dose-response analysis were difficult to detect. In addition, it is possible that ONOO\textsuperscript{−} incubation is not affecting the response to SNP in a consistent way, and that effects could be more apparent if other vasodilator agents were used.

SERCA serves to aid in the relaxation of a muscle through decreasing cytosolic Ca\textsuperscript{2+}, pumping it against its concentration gradient into the SR. As high concentrations of ONOO\textsuperscript{−} have been shown to decrease Ca\textsuperscript{2+}-dependent SERCA activity, it was hypothesized that this would lead to impairments in whole muscle relaxation. It was observed that PLN\textsuperscript{−/−} vessels had a significantly lower maximal relaxation across all treatment conditions. ONOO\textsuperscript{−} incubation did not have a greater effect on the PLN\textsuperscript{−/−} vessels as the control group also expressed lower levels of relaxation leading to the conclusion that the presence of PLN was unable to exert a protective effect. There was large variability seen within the PLN\textsuperscript{−/−} group following exposure to 300 µM ONOO\textsuperscript{−}, possibly indicating damage to some of the vessels more so than others but this is speculative.

In the data presented here, relaxation was elicited through cumulative additions of SNP. It is possible that if a single dose was used, differences between genotypes could be more apparent. Then, a time dependent response could be investigated and the resulting effects of ONOO\textsuperscript{−} could be compared to the known differences between the groups for a more decisive understanding of the potential contribution of PLN. It is known that PLN\textsuperscript{−/−} vessels produce force at a faster rate and also relax at a faster rate than WT vessels in response to a single addition of PE (Lalli et al., 1997). ONOO\textsuperscript{−} may be able to affect the time course in which force is developed or relaxation is attained, therefore it could be beneficial to determine time course dependent alterations from ONOO\textsuperscript{−} incubation and then determine the role of PLN in protecting SERCA activity.

To further investigate the role of PLN in protecting SERCA function and thus VSM relaxation following incubation with ONOO\textsuperscript{−}, an SNP dose response curve was completed. SNP results in vasodilation by acting as an NO donor, thus increasing cGMP and phosphorylating PLN through PKG to decrease cytosolic calcium levels. Following low concentration (80 µM ONOO) incubation with ONOO\textsuperscript{−}, both WT and PLN\textsuperscript{−/−} groups demonstrated less relaxation at SNP $10^{-6.5}$ and $10^{-9}$ M. Incubation with 150 µM ONOO\textsuperscript{−} resulted in a similar change as that seen
with 80μM; however, only within the WT group. Finally, following incubation with 300 μM ONOO⁻ no changes were observed in response to SNP in either WT or PLN⁻/⁻ groups. Work by Grover et al. demonstrates the ability for ONOO⁻ incubation to decrease SERCA activity (Grover et al., 1997). Using a measure of SNP dose response does not appear to be indicating SERCA damage, as many pathways are involved in SNP induced relaxation. The differences seen in SNP relaxation as a result of low level ONOO⁻ incubation could be highlighting ONOO⁻ incubation acting on pathways not directly related to SERCA function. For instance, it has been suggested that ONOO⁻ incubation increases myosin light chain phosphatase activity and activates K⁺ channels resulting in an increased relaxation (Li et al., 2004; Maneen et al., 2006). However, seen in the data presented here is a decreased relaxation whereby following incubation of ONOO⁻ at 80 μM or 150 μM, the vessels of WT and PLN⁻/⁻ animals are less sensitive to low doses of SNP. This could indicate the ability for ONOO⁻ to affect numerous pathways within the smooth muscle cell, leading to differing results following incubation.

4.2 Effect of PLN on SERCA2 activity following ONOO⁻ exposure

Due to the inability to adapt our Ca²⁺ ATPase assay to VSM, LV was used as a surrogate system for assessment of SERCA2 function. The main isoform of SERCA2 found in the LV is SERCA2a, whereas SERCA2b is primarily found in smooth muscle. PLN is able to interact with both isoforms in the same manner, in that PLN binding is able to reduce the apparent affinity for Ca²⁺ by a factor of two (Verboomen et al., 1992). The main difference between these two isoforms is the additional TM helix found in SERCA2b, which extends into a luminal tail acting as a stabilizing agent for the pump (Clausen et al., 2012). Compared to one another, SERCA2b has an increased apparent affinity for Ca²⁺, however a lower turnover rate compared to SERCA2a (Ver Heyen et al., 2001). Though functional differences exist between the isoforms, the binding of PLN is unchanged so it was determined that the use of LV in place of VSM was appropriate (Verboomen et al., 1992). Incubation of whole LV homogenate with ONOO⁻ (80 μM and 300 μM) resulted in no change and ~30% reduction in SERCA activity, respectively, with no differences between genotypes. These results are in agreement with the hypothesis that incubation with ONOO⁻ will reduce SERCA2 activity. Numerous studies have examined the effect of ROS/RNS on SERCA activity and most indicate decreased activity following exposure (Dremina et al., 2007; Squier & Bigelow, 2000; Viner, Ferrington, Williams, Bigelow, & Schöneich, 1999; Xu, Zweier, & Becker, 1997).
Gutierrez-Martín et al (2004) showed that a single bolus of ONOO\(^-\) (200-300 \(\mu M\), 10 min) reduced SERCA activity by \(~50\%) in both SR vesicles and fast-twitch skeletal muscle. This group also found that serial additions of ONOO\(^-\) (over 50 min) can further reduce SERCA activity and states the physiological importance of these serial pulses as being more representative of chronic ONOO\(^-\) exposure (Gutiérrez-Martín, Martín-Romero, Iñesta-Vaquera, Gutiérrez-Merino, & Henao, 2004). The work presented here saw similar decreases in SERCA function following incubations with 300 \(\mu M\) ONOO\(^-\). It has also been shown that perfusion of SIN-1, a ONOO\(^-\) donor, in whole heart reduces contractility through activation of protein phosphatase thereby reducing PLN phosphorylation at Ser16 (Kohr et al., 2008b, 2009). A change in protein phosphatase was not directly measured in this thesis, although a western blot for p-PLN at Ser16 shows a reduction following incubation with 80 \(\mu M\) and a significant increase following 300 \(\mu M\) ONOO\(^-\). In a whole heart model, this would translate to reduced contractility following incubation with 80 \(\mu M\) ONOO\(^-\), while an increased contractility following 300 \(\mu M\) ONOO\(^-\) incubation would occur. The reason for differences with the data presented here and the work completed by Kohr et al (2009) could be a result of the administration of ONOO\(^-\). Kohr et al. used a whole perfused heart model to study the effects of ONOO\(^-\) incubation, while the p-PLN data shown in this thesis was completed on LV homogenate where ONOO\(^-\) was added directly to the sample homogenate. This causes the ONOO\(^-\) to remain in the sample prep where potential interactions could continue to occur and cause a varied result compared to that reported by Kohr et al. (2009).

Many studies previously showing ONOO\(^-\) damage on SERCA2 have been completed in preparations of SR vesicles or SR enriched fractions. For example, Walia et al (2003) reported a significant reduction in SERCA uptake in primary cultured smooth muscle cells following 30 min incubation with 250 \(\mu M\) ONOO\(^-\). Grover et al also saw a similar reduction in SR fractions collected from pig coronary arteries (Grover, Kwan, et al., 2003). The robust changes to SERCA function shown in the literature may be a result of administration of the oxidant and its differing affect in vivo and in vitro. The whole muscle contraction and relaxation work presented here used intact vascular rings with an ex vivo model. This model relies on the assumption that drugs administered to the bath are evenly diffusing into and throughout the vessel ring suspended in buffer. Since reductions in force production and impaired SNP-induced relaxation, to low incubation with 80 and 150 \(\mu M\) ONOO, were evident, it is clear that ONOO\(^-\) was able to diffuse into the vessel. However, the ability for ONOO\(^-\) to react within the sites of interest
could be limiting. If exposure of the PLN-SERCA binding locations is limited through this preparation, than the results could be indicative of ONOO\textsuperscript{−} exposure with other cellular functions.

4.3 ONOO\textsuperscript{−} shows no effect of 3-NY formation within SERCA2

As a measure of SERCA2 damage, this study aimed to detect levels of 3-NY on SERCA2 as this parameter has been reported to increase with ONOO\textsuperscript{−} exposure and cause decreased SERCA2 activity (Dremina et al., 2007; Grover, Samson, et al., 2003). To accurately determine the amount of 3-NY on SERCA2, it was necessary to immunoprecipitate SERCA2 from whole LV homogenate. This was an important step because pumps of similar size exist within the LV such as the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase, which share functional and structural similarities to SERCA resulting in vulnerability to oxidative damage. Both pumps maintain a very similar 3D structure and when applied to SDS-page these two pumps migrate identically (~100 kDa). Due to the parallels between the two, it is highly likely that if whole homogenate were to be separated through SDS-page and probed for 3-NY, the differences seen could not be attributed solely to changes within the SERCA pump.

It was hypothesized that ONOO\textsuperscript{−} would increase 3-NY content within LV homogenate. However, treatment of WT LV sample with ONOO\textsuperscript{−} did not increase 3-NY content on SERCA compared to control. Additionally, the presence of PLN under control and ONOO\textsuperscript{−} conditions had no effect on 3-NY content as none was present following exposure. Previously, Viner et al. have observed decreased SERCA activity with no changes to SERCA 3-NY content in ONOO\textsuperscript{−} exposure experiments (Viner et al., 1996). Other states of high oxidative stress, such as aging, have been shown to increase SERCA2 3-NY content within highly purified rabbit fast-twitch muscle which was associated with decreased SERCA2 function (Viner et al., 1999). Oxidants are known to have vast effects on proteins and can alter structure, binding sites or substrate affinity. Literature suggests targeted oxidant damage to tyrosine residues is most affective in altering SERCA structure and activity thereby decreasing its overall function (Gutiérrez-Martín et al., 2004; Knyushko et al., 2005; R I Viner et al., 1999). However, cysteine residues within the SERCA structure have also been suggested as more important oxidant targets to maintain functional ability (Davies et al., 1987; Sharov, Dremina, Galeva, Williams, &
Schoneich, 2006). It is possible that western blotting for oxidant damage within the cysteine residues could identify further evidence to support SERCA damage following ONOO\textsuperscript{\textperiodcentered} incubation.

**Summary**

In summary, these data does not show conclusive evidence that PLN ablation increases ONOO\textsuperscript{\textperiodcentered} induced SERCA2 damage in VSM or LV. If PLN were able to protect SERCA2, we would have expected to detect genotype differences following exposure to ONOO\textsuperscript{\textperiodcentered}. It was seen that ONOO\textsuperscript{\textperiodcentered} was able to cause a reduction in SERCA2 Ca\textsuperscript{2+}\text-supplied ATPase activity in both the WT and PLN\textsuperscript{-/-} LV as well as a reduction in maximal tension, maximal relaxation and increase in EC\textsubscript{50} within aortic rings for both genotypes. Few pieces of data from this thesis stand to serve as clues that PLN ablation does increase ONOO\textsuperscript{\textperiodcentered} induced SERCA2 damage, one of which is the reduced EC\textsubscript{50} in WT vessels following 150µM ONOO\textsuperscript{\textperiodcentered} treatment. This could represent a protective mechanism of PLN within the WT vessels, as the PLN\textsuperscript{-/-} vessels showed a significantly increased EC\textsubscript{50} possibly highlighting increased SERCA2 damage. Structural SERCA2 damage was not shown to be higher in the PLN\textsuperscript{-/-} group following ONOO\textsuperscript{\textperiodcentered} incubation at any concentration indicating that there were no increased 3-NY induced structural modifications accompanying the decreased activity. Through the experiments conducted in the thesis presented here, the absence of PLN did not clearly indicate increased SERCA2 impairment following exposure to ONOO\textsuperscript{\textperiodcentered}.

**Conclusion**

The results of this thesis indicate the ability for ONOO\textsuperscript{\textperiodcentered} incubation to greatly reduce PE mediated vascular contraction, while having little effect on SNP induced vascular relaxation. It also highlights the ability for ONOO\textsuperscript{\textperiodcentered} incubation to decrease overall maximal SERCA activity through mechanisms other than 3-NY formation. Since differences between WT and PLN\textsuperscript{-/-} tissue were not seen across all experiments, we can conclude that, in respect to the specific outcomes assessed within the scope of this project, PLN ablation does not enhance ONOO\textsuperscript{\textperiodcentered} induced damage.
Limitations

Ideally, all works in this thesis were to be completed on a single type of muscle to better understand the potential ability for PLN to protect against SERCA2 damage. As described earlier, the assay for SERCA activity in VSM could not be optimized and LV was used as a surrogate marker to understand the effects of PLN on SERCA2 activity following ONOO⁻ exposure. Different SERCA isoforms exist in these two tissues, with SERCA2a being predominantly found in LV and SERCA2b found mainly in VSM. The binding of PLN to each of these isoforms is the same, however they differ in susceptibility to oxidative damage as SERCA2b comprises an 11\textsuperscript{th} TM helix with a luminal extension that acts to stabilize the pump compared to SERCA2a (Verboomen et al., 1992). Therefore, it is difficult to infer the effects on one isoform from information on the other. This stands as a major limitation of the thesis, as ONOO⁻ incubation affects each isoform differently.

Specific to myography data, limitations exist that could affect the results and interpretation of the data. We observed large variability between control conditions used for each concentration cohort which serves as a large limitation in comparing ONOO⁻ treatment across concentrations. When analyzing data as one combined dataset, statistical significance was likely masked due to the variability in control data. We were able to analyze each treatment condition with its corresponding control group, however a large limitation to the contraction data is that each animal was not exposed to all three-treatment conditions; rather different cohorts were used for the different treatment conditions. Originally, this project aimed to look at one level of ONOO⁻ incubation. As the project evolved, additional concentrations were added leading to each level of treatment being completed separately. Performing all levels of ONOO⁻ concentration (80µM, 150µM and 300µM) on rings collected from one animal would have allowed for a stronger assessment between the concentrations and their effects on vasoreactivity. Another limitation to the myography work is the level of contraction impairment following 300 µM ONOO⁻ incubation. The ~60% reduction in PE/KCl ratio made it very difficult to determine a full relaxation curve. This resulted in large variability when aiming to calculate EC\textsubscript{50} from the curve fit parameters.
Future Directions

Future studies should aim to detect the functional and structural changes within one type of muscle. More specifically, to use the PLN null mouse model and examine the effects of ONOO' incubation on whole muscle function and then decipher structural changes caused by the exposure. This could be completed through use of the Langendorff system to study a whole heart model. This would provide a more precise understanding of the ability for PLN to protect SERCA. Working with a complete model to understand protective mechanisms could also aid in providing further insight on specific pathways of ONOO' induced SERCA damage and inactivation. To continue work within VSM would possibly require the use of a larger animal model, such as that from a rat or rabbit. This would provide increased tissue size and following incubation to an oxidative stress, fractionation of the sample could provide the SR-enriched fraction. Ca\(^{2+}\)-uptake or SERCA activity assays on an SR-enriched sample would offer more insight to the direct actions of SERCA and PLN’s ability to protect its structure and function.

It has been identified that aortic vessels absent of PLN have a faster response to a single bolus of PE than those from WT animals (Lalli et al., 1997). This indicates a time course difference between the genotypes and could be an area of interest for future oxidative damage studies. Specifically identifying the ability for ONOO' to affect the time course of force production could help to further understand the ability for PLN to protect SERCA. Using force developed, or relaxation achieved, over time could provide more insight into the mechanisms by which ONOO' is able to decrease force production and potentially identify whole muscle relaxation deficits.

It will also be important to investigate numerous sites of SERCA damage following ONOO' incubation. The literature indicates that both cysteine oxidation and 3-NY formation can result in decreased SERCA activity. Therefore it is important for future studies to research the effects of ONOO within numerous key residues to further understand PLNs ability to protect SERCA.

As many of the differences in VSM function were seen following 150 µM ONOO' exposure, further work needs to be completed to better understand concentration dependent effects of ONOO' incubation. Biochemical data following 150 µM ONOO' exposure, could help to elucidate the function of PLN in potential protection of SERCA. Future works could include a wider variety of ONOO' concentrations to understand, in more detail, the biphasic effect of ONOO' and its effects on the PLN-SERCA interaction.
Appendix

Figure 14: PLN in LV. Whole LV homogenate was electrophoresed and blotted against PLN antibody as a genotype check. This figure represents the blot completed and shows the presence or absence of PLN in LV just below the 10kD ladder marker. Control lane contains human vastus lateralis as a positive control for PLN, and each remaining lane contains sample from a different animal.
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