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

by

Rachelle M. C. Mariani

A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Master of Applied Science
In
Kinesiology

Waterloo, Ontario, Canada 2015

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.

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**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 Unpublished work from our lab has specifically shown SERCA2 protection in whole left ventricle (LV) when co-expressed with its regulatory modulatory protein, PLN (Gamu & Tupling, unpublished).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 phopsholamban null ($PLN^{-/-}$) mice were exposed to varying concentrations of $ONOO^-$ (80 μM, 150 μM and 300 μM). 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 μM) 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 μM $ONOO^-$ 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 300μM $ONOO^-$ 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**

Thank you to my supervisors, Dr. Jim Rush and Dr. Russ Tupling, for providing me with this fantastic opportunity. It has opened my eyes to a whole new world and has left me a much different person than I was previously.

Thank you to Brendan for standing by me through everything the last two years has thrown my way. Without your love and support, I would not have seen my own potential.

To my family, I am so thankful for your unconditional love. No matter which path I choose in life, I know that I have you to look to for guidance.

I'd also like to appreciate all the people that I have met along this journey. The labmates, or better yet, friends who have made this adventure much more interesting than I could have ever thought possible.

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| | |
|---|---|
| 3-NY | 3-Nitrotyrosine |
| ACh | Acetylcholine |
| ADP | Adenosine diphosphate |
| Akt | Protein kinase B |
| ATP | Adenosine triphosphate |
| BSA | Bovine Serum Albumin |
| $Ca^{2+}$ | Calcium |
| cAMP | Cyclic adenosine monophosphate |
| cGMP | Cyclic guanosine monophosphate |
| CICR | Calcium Induced Calcium Release |
| cAMKII | Calmodulin-dependent protein kinase |
| CPA | Cyclopiazonic acid |
| Cys | Cyestine |
| DPMK | Dystrophia myotonica protein kinase |
| ER | Endoplasmic reticulum |
| $H_2O_2$ | Hydrogen Peroxide |
| His | Histadine |
| HSP70 | Heat Shock Protein 70 |
| $IP_3R$ | Inositol 1,4,5-triphosphate receptors |
| KCl | Potassium Chloride |
| kDa | Kilo daltons |
| LDH | Lactate Dehydrogenase |
| LE | Luminal extension |
| LV | Left Ventricle |
| NADH | Nicotinamide adenine dinucleotide |
| $NO^-$ | Nitric Oxide |
| NOS | Nitric Oxide Synthase |

| | |
|---|---|
| NOX | NADH Oxidase |
| $O^{-}_{2}$ | Superoxide |
| -OH | Hydroxyl Radical |
| $ONOO^{-}$ | Peroxynitrite |
| PARS | poly (adenosine 5'-diphosphoribose) synthase |
| PE | Phenylephrine |
| PK | Pyruvate Kinase |
| PKA | Protein Kinase A |
| PKG | Protein Kinase G |
| PLN | Phospholamban |
| $PLN^{-/-}$ | Phospholamban knockout |
| PMSF | Phenylmethylsulphonyl fluoride |
| PP | Protein Phosphatase |
| PVDF | Polyvinyldiene difluoride |
| RNS | Reactive Nitrogen Species |
| ROS | Reactive Oxygen Species |
| RyR | Ryanodine Receptor |
| sGC | Soluble Guanylyl Cyclase |
| SR | Sarcoplasmic reticulum |
| SERCA | Sarco(endo)plasmic reticulum $Ca^{2+}$-ATPase |
| SLN | Sarcolipin |
| SNP | Sodium Nitroprusside |
| TA | Thoracic Aorta |
| TBS-T | Tris buffered saline plus Tween-20 |
| Thr | Threonine |
| TM | Transmembrane Helice s |
| Tyr | Tyrosine |
| $V_{max}$ | Maximal SERCA activity |

| | |
|---|---|
| VSM | Vascular smooth muscle |
| WT | Wild Type |

## 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 $Ca^{2+}$ ($[Ca^{2+}]_f$) 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 ($IP_3Rs$) and ryanodine receptors (RyRs). Increases in $[Ca^{2+}]_f$ 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 $[Ca^{2+}]_f$ 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 $[Ca^{2+}]_f$ 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-transports $H^+$ 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.,

2005). 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 (E1-2$Ca^{2+}$ 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 (*ATP2A1–3*) generate multiple SERCA isoforms (SERCAla-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 E1-2$Ca^{2+}$ toward the cytosol, ii) slowed E1-2$Ca^{2+}$ →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 $Ca^{2+}$ 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 $Ca^{2+}$, exclusive of affecting maximal pump activity ($V_{max}$) (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 $Ca^{2+}$ (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 $Ca^{2+}$; 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, Hoying, 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 $[Ca^{2+}]_f$ 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).

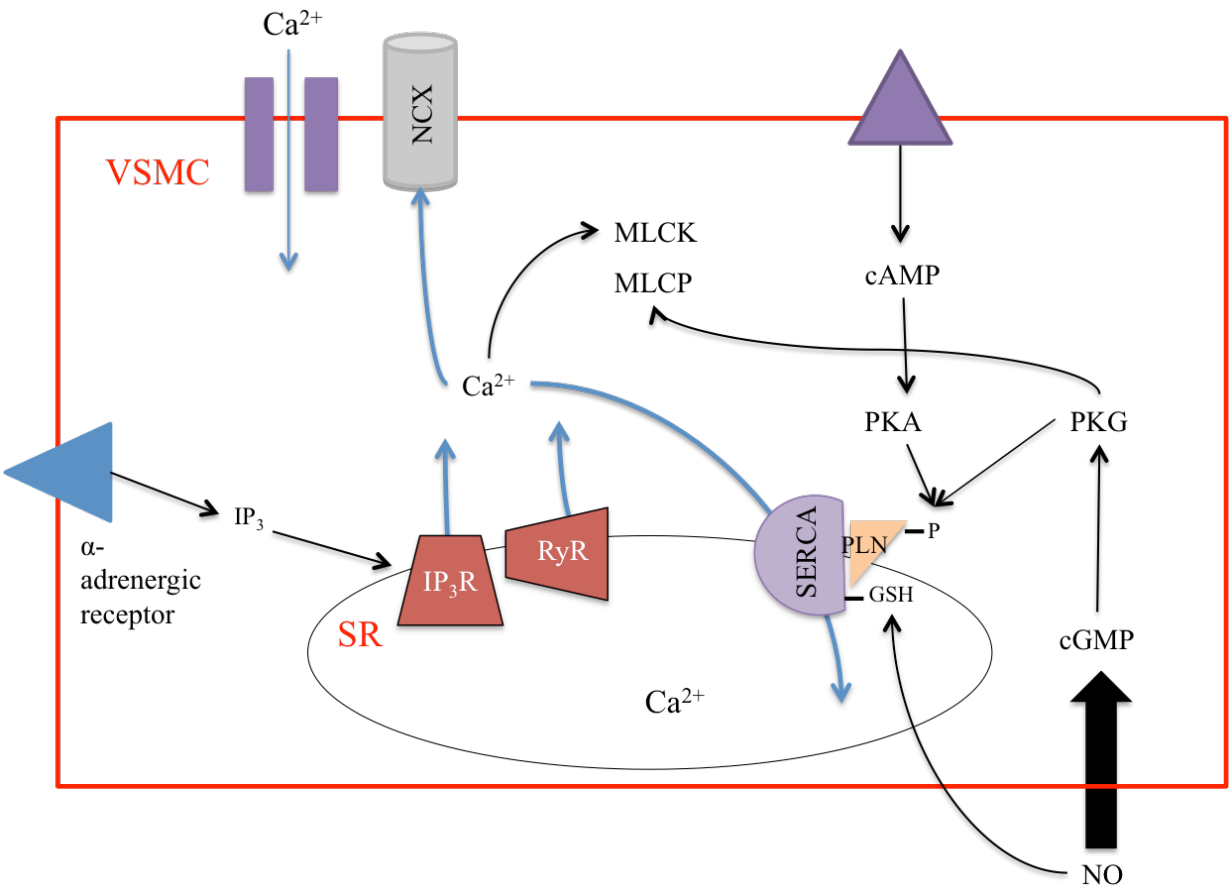


**Figure 1: Basic schematic of SERCA/PLN involvement in VSM contraction and relaxation.** Vasoconstrictors will bind to their receptors and elicit $Ca^{2+}$ release through $IP_3R$, or through CICR activation of the RyR. For example, PE will bind to its α-adrenergic receptor, which, through G-couple protein activation increases $IP_3$. $IP_3$ then binds to $IP_3R$ on the SR to activate $Ca^{2+}$ 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 $Ca^{2+}$ into the cytosol from the extracellular fluid. The increased cytosolic $Ca^{2+}$ triggers further $Ca^{2+}$ 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 $Ca^{2+}$ can then be reduced through the actions of SERCA, leading to relaxation and aiding in the refilling of the SR for $Ca^{2+}$ release upon subsequent stimulus. A small amount of $Ca^{2+}$ is returned to the extracellular fluid through the action of the $Na^{+}$-$Ca^{2+}$ 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 ($PLN^{-/-}$) 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 $PLN^{-/-}$ 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 $Ca^{2+}$through depolarization, while PE acts through receptor mediated $Ca^{2+}$ release from the SR. Due agonist induced contraction differences, Lalli et al. (1997) aimed to investigate if the $Ca^{2+}$stores within $PLN^{-/-}$ vessels are altered due to increased SERCA activity. Rings of VSM were held in a $Ca^{2+}$-free solution and stimulated with PE (10uM), and the $Ca^{2+}$-free scenario resulted in greater force production within the $PLN^{-/-}$ vessels compared to WT, suggesting that PLN ablation results in increased SR $Ca^{2+}$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 $Ca^{2+}$ 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 $PLN^{-/-}$ myocardium was observed. Due to the uninhibited SERCA pump and increased $Ca^{2+}$uptake, it is proposed that the decreased RyR aids to maintain appropriate cytosolic $Ca^{2+}$concentration (Chu et al., 1998). Rates of contractility and relaxation are increased in $PLN^{-/-}$ 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 ($O_2^{\cdot-}$), hydrogen peroxide ($H_2O_2$), nitric oxide (NO), hydroxyl radical (-OH), and peroxynitrite ($ONOO^-$). Typically, their concentration is balanced by endogenous scanvengers, 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, $H_2O$ and $O_2$ (Lounsbury, Hu, & Ziegelstein, 2000). As a stimulant and re-dox 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 $[Ca^{2+}]_f$ 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, $H_2O_2$, $O_2$ and $ONOO^-$ 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 $H_2O_2$-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, $Ca^{2+}$-$Mg^{2+}$-ATPase activity, and $Ca^{2+}$-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 $ONOO^-$ *in vivo* (Viner, Williams, & Schoneich, 1999). These studies highlight how the subtle differences in isoform amino acid confirmation affect susceptibility of the pump to ROS/RNS damage.

In fact, $ONOO^-$ has been claimed to be the most effective inhibitor of $Ca^{2+}$ 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 $ONOO^-$ can result in irreversible damage (Viner et al., 1996; Viner, Williams, & Schöneich, 1999). Through the highly favourable reaction between NO and $O^-_2$, $ONOO^-$ 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 $ONOO^-$ 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 $ONOO^-$ production can occur in numerous disease states, including cerebral ischemia reperfusion (Dhar, Kaundal, & Sharma, 2006; Thiyagarajan, 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, Szabó, & 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 $Ca^{2+}$ 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 $Ca^{2+}$ 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 $Ca^{2+}$ into the SR lumen thereby affecting $Ca^{2+}$ storage and decreasing the $Ca^{2+}$ pool available for muscular contractility. This effect has been revealed by decreases in SERCA $Ca^{2+}$ 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 $Ca^{2+}$ (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 $PLN^{-/-}$ 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 *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 $ONOO^-$ would cause impairments in VSM function (i.e. impaired contraction and relaxation) and that the impairments would be greater in vessels from $PLN^{-/-}$ mice compared with WT mice. 2) It was hypothesized that the absence of PLN would expose SERCA2 to $ONOO^-$ 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 $ONOO^-$ incubation leading to increased nitrotyrosine formation, while PLN within the WT samples will prevent $ONOO^-$ induced nitrotyrosine content on SERCA2.

## 2.0 Methods

### *2.1 Animals*

The generation of $PLN^{-/-}$ 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 $PLN^{-/-}$) (>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 ($PLN^{+/-}$) mice, yielding 50% heterozygous pups, 25% WT ($^{+/+}$), and 25% $PLN^{-/-}$. 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 $MgCl_2$, 200 µM dNTP, 10x Taq buffer (containing 650 nM Tris-HCL, 200 mM $(NH_4)_2SO_4$, 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', $PLN^{-/-}$ forward: 5'-TCC TCG TTT ACG GTA TC-3', $PLN^{-/-}$ 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 intraparitoneal 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% $O_2$, 5% $CO_2$) 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) ($10^{-6}$M) was introduced to induce contraction. PE activates the α-adrenergic receptors present on vascular smooth muscle to increase cytosolic $Ca^{2+}$, 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 $H_2O$ 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 $H_2O$. 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.

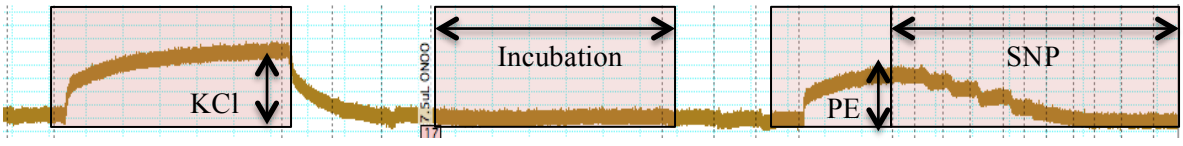


**Figure 2: Representative force tracing.** Following the myography protocol, as described above, each 2mm ring would have an associated tracing of force developed throughout the experiment. Figure 2 highlights the areas from the force tracing which were used in further data analysis.

*2.3 Calcium-dependent SERCA Activity*

Whole TA rings from WT and $PLN^{-/-}$ mice used in contractility experiments were powdered and homogenized to determine $Ca^{2+}$ dependent $Ca^{2+}$-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 $MgCl_2$, 1 mM EGTA, 10 mM $NaN_3$, 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 $Ca^{2+}$, 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 $Ca^{2+}$ in the wells was required to determine the maximal enzyme activity (Vmax) of SERCA2 and $pCa_{50}$, which is defined as the $[Ca^{2+}]_f$ 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 $Ca^{2+}$-ATPase activity measured in VSM homogenate.

All data was plotted against the negative logarithm of $[Ca^{2+}]_f$ (pCa) using basic statistatical software (GraphPad Prism™ version 6) to determine Vmax and $pCa_{50}$. More specifically, $pCa_{50}$ was determined by non-linear regression curve fitting using the sigmoidal dose response equation,

$$Y = Y_{bot} + (Y_{top} - Y_{bot}) / (1 + {}^{10(LogCa_{50} - x)} * n_H)$$

where $Y_{bot}$ is the bottom of the plateau, $Y_{top}$ is the top of the plateau, Log $Ca_{50}$ is the logarithm of $pCa_{50}$ and $n_H$ is the hill coefficient.

The accurate measurement of $[Ca^{2+}]_f$ used for the ATPase activity assay was measured using the $Ca^{2+}$ fluorophore, indo-1 on a spectrofluorometric plate reader (SPECTRAmax Gemini XS; Molecular Devices, Toronto, ON) as previously described (Duhamel et al., 2007). The 15 $[Ca^{2+}]_f$ 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 $Ca^{2+}$ concentrations were required for $Ca^{2+}$ measurement, a zero $Ca^{2+}$ and a max $Ca^{2+}$ 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 $[Ca^{2+}]_f$ with the following equation (Grynkiewicz et al., 1985);

$$[Ca^{2+}]_f == K_d * (G_{max}G_{min}) * (R-R_{min})/(R_{max}-R) \qquad \text{Equation 2.2}$$

where $K_d$ is the equilibrium constant for the interaction between $Ca^{2+}$ and indo-1, $R_{min}$ is the minimum value of R with zero $Ca^{2+}$, $G_{max}$ is the maximum value of G with zero $Ca^{2+}$, $R_{max}$ is the maximum value of R at max $Ca^{2+}$ (1mM) and $G_{min}$ 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 (Grynkiewicz 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 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 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 indo-1 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 $LN_2$, 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 $Ca^{2+}$-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, $H_2O$ 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$^o$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$^{o}$C after which 30μL of protein-A sepharose was added and the samples were rotated for another hour at 4$^{o}$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 $PLN^{-/-}$ and WT mice in control and treatment conditions for myography, $Ca^{2+}$-dependent $Ca^{2+}$ ATPase activity, and Western blotting data. The significance level was set at $P \leq 0.05$, 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).

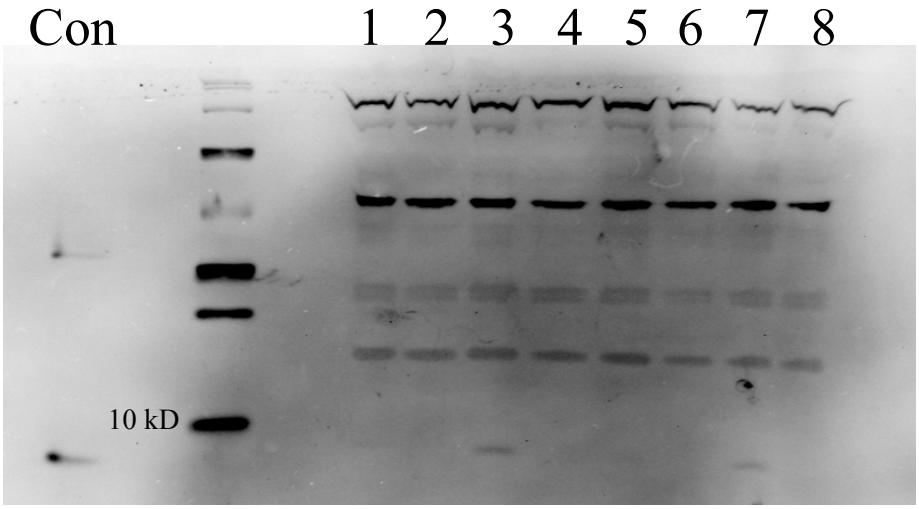


**Figure 3: Presence of PLN in VSM.** Whole TA homogenate was electrophoresed and blocked against PLN specific antibody. Lane "Con" contains human vastus lateralis homogenate as a positive control for PLN. Lanes 1,3,5, and 7 contain WT samples while lanes 2,4,6, and 8 contain $PLN^{-/-}$ samples. This 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 $PLN^{-/-}$ group consistently exhibited lower maximal relaxation compared with WT group ($p<0.05$).

| | Max KCl (g) | Max PE (Con, g) | Max PE (80μM $ONOO^-$, g) | Max PE (150μM $ONOO^-$, g) | Max PE (300μM $ONOO^-$, g) |
|---|---|---|---|---|---|
| WT | 0.580 ± 0.21 | 0.611 ± 0.064 | 0.323 ± 0.043 * | 0.408 ± 0.102 | 0.481 ± 0.046 |
| $PLN^{-/-}$ | 0.644 ± 0.21 | 0.597 ± 0.046 | 0.334 ± 0.065 * | 0.259 ± 0.087 * | 0.266 ± 0.087 * |

**Table 1: Developed KCl and PE induced force production.** WT and $PLN^{-/-}$ vessels were exposed to 60 mM KCl prior to $ONOO^-$ incubation and $10^{-6}$ M PE following $ONOO^-$ 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 $PLN^{-/-}$ vessels following any concentration of $ONOO^-$ incubation. Student's t-test reveal that Max PE tension is significantly lower than control following all concentrations of $ONOO^-$ within $PLN^{-/-}$ vessels (*, $p<0.05$). While only incubation with 80 μM $ONOO^-$ 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 $PLN^{-/-}$ 300 μM group)

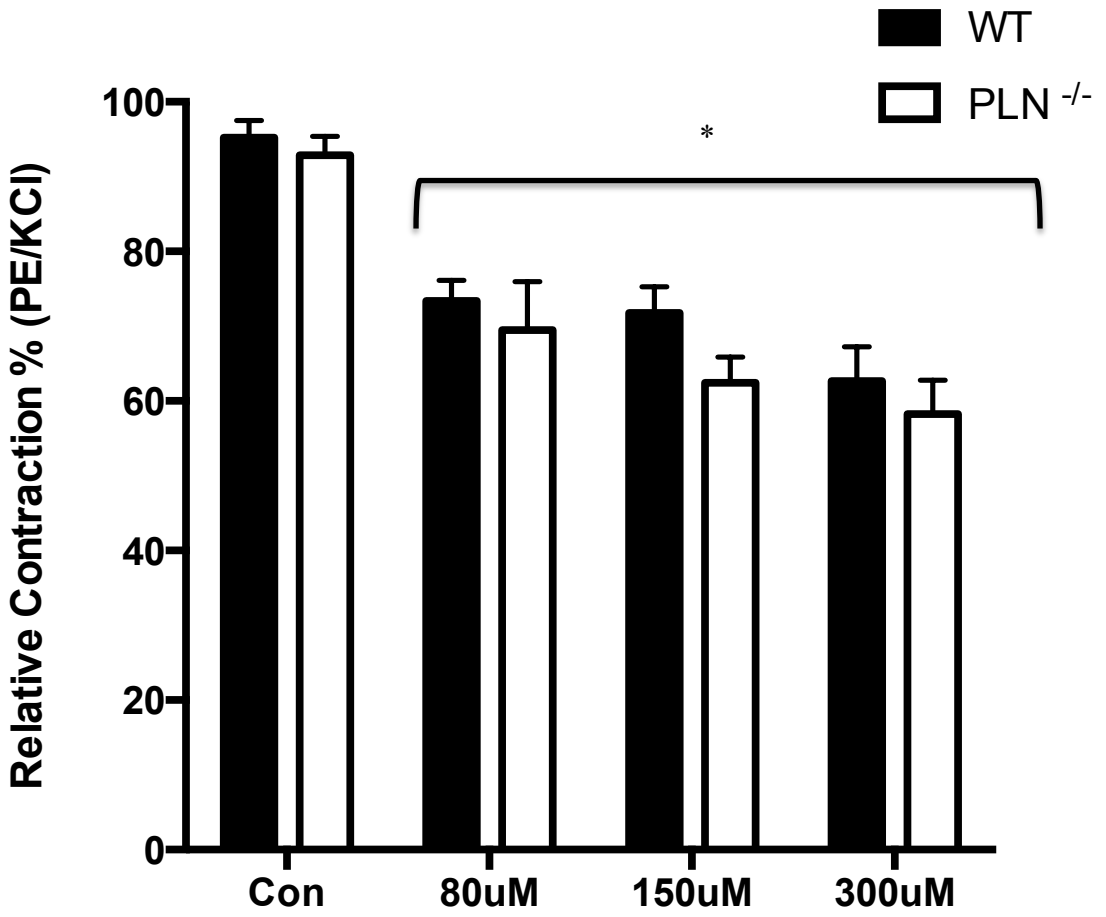


**Figure 4: Relative contraction of PE/KCl in vessels following $ONOO^-$ exposure.** Following exposure to varying $ONOO^-$ concentrations, TA rings from WT and $PLN^{-/-}$ animals were constricted by the addition of PE as described in "Methods". PE/KCL ratio was used to normalize PE induced force data, 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)

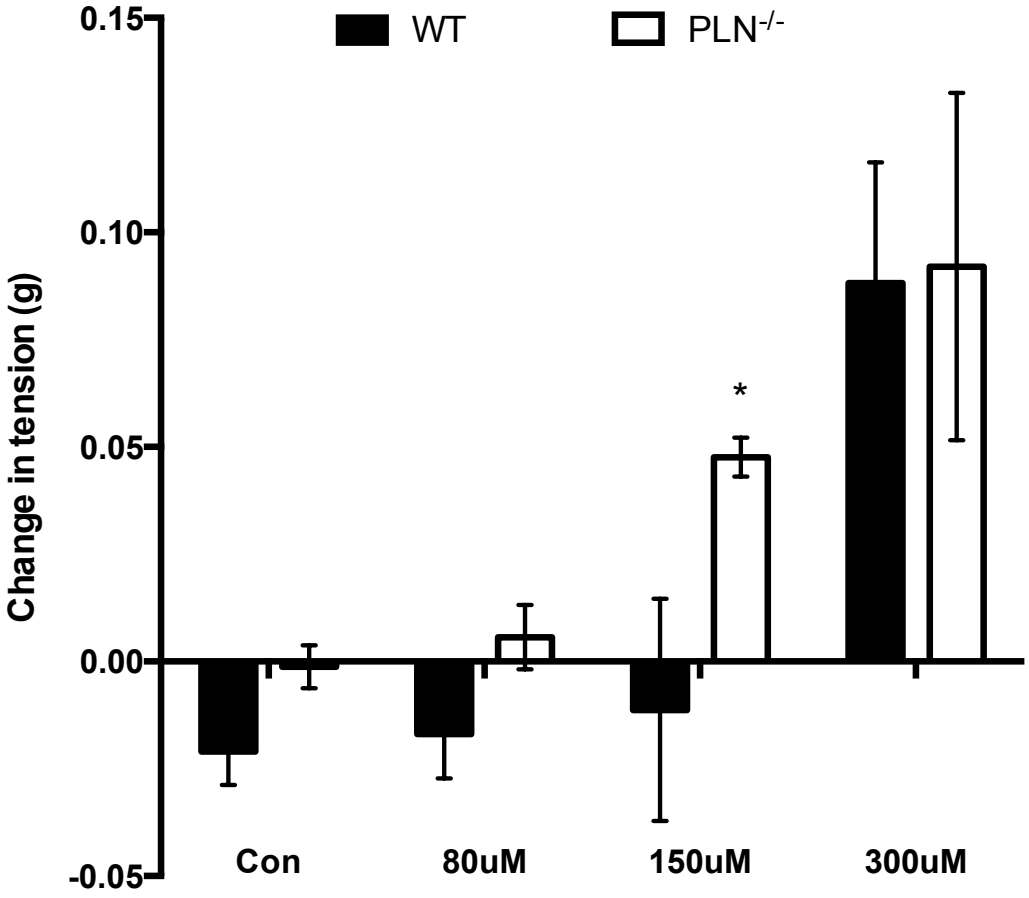


**Figure 5: Change in basal tension throughout $ONOO^-$ incubation.** TA segments from WT and $PLN^{-/-}$ animals were exposed to varying concentrations of $ONOO^-$ for 30 min as described in "Methods". Change in total developed tension throughout incubation is expressed in grams, mean ± SE. A significantly 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 $ONOO^-$ ($p<0.05$), indicated as *. (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)

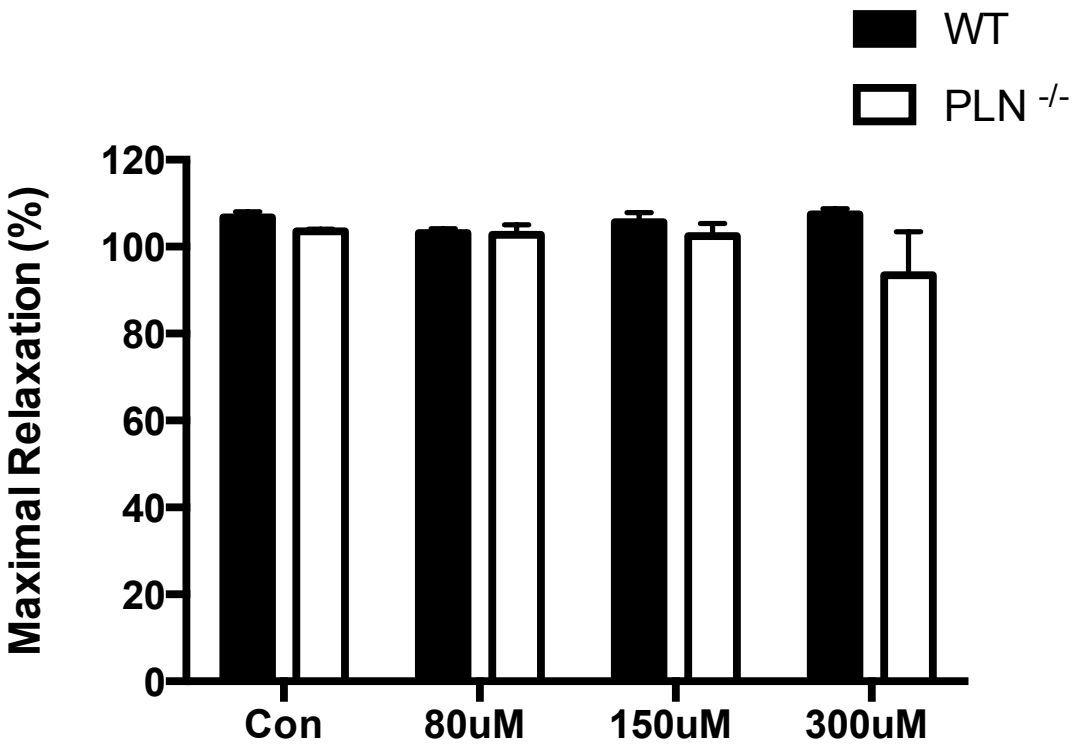


**Figure 6: Maximum relaxation to SNP following $ONOO^-$ exposure.** Vessels from WT and $PLN^{-/-}$ were exposed to varying concentrations of $ONOO^-$. Following exposure, the functionality of the vessels was measured as described in "Methods". The maximal relaxation following consecutive additions 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.

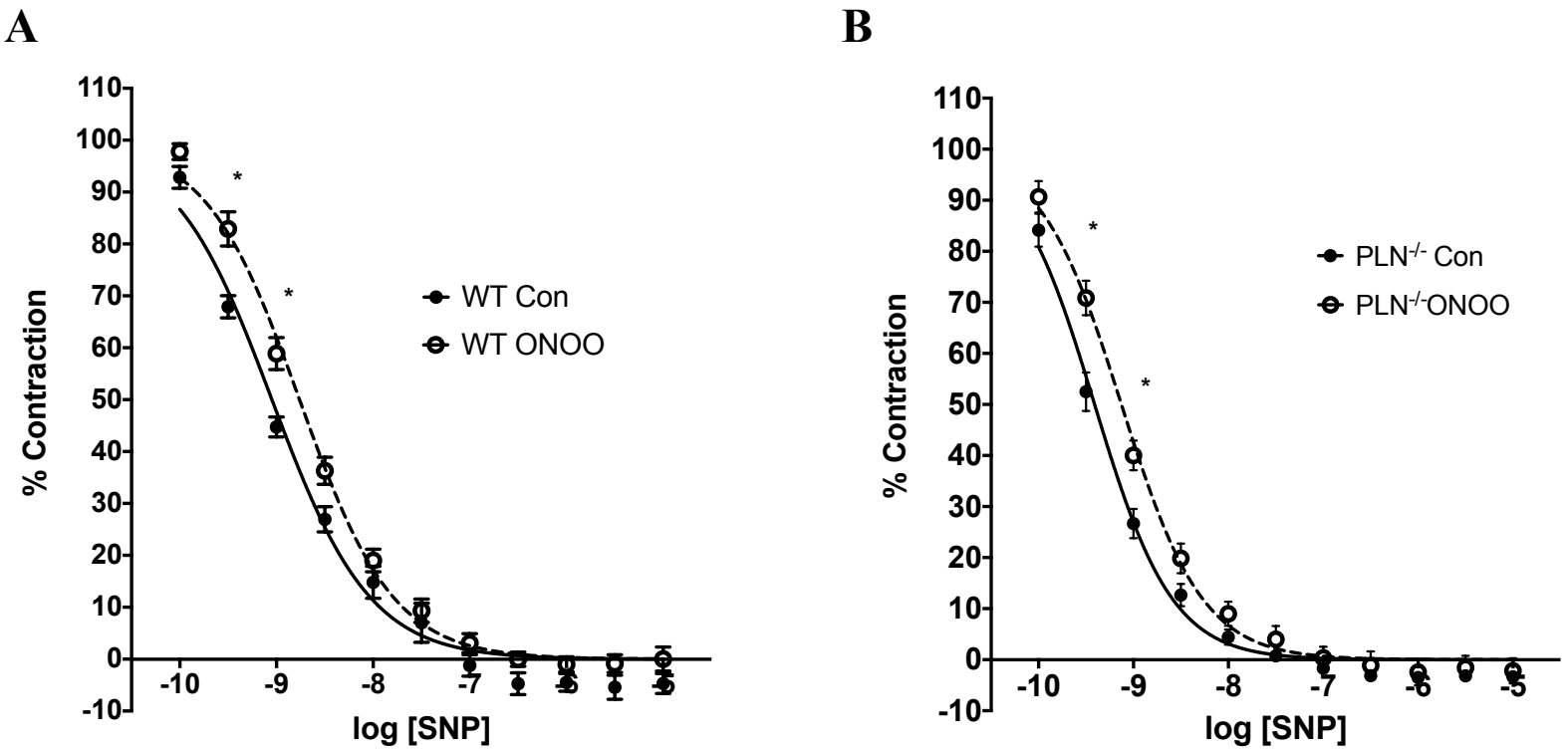


**Figure 7: SNP induced relaxation in thoracic aorta following $ONOO^-$ incubation (80 μM).** Thoracic aorta vessels were exposed to 30 mins of 80 μM $ONOO^-$. Following the incubation, vessels were pre-constricted with PE ($10^{-6}$M) and then relaxed with cumulative additions of SNP ($10^{-10}$-$10^{-5}$M). $ONOO^-$ 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).

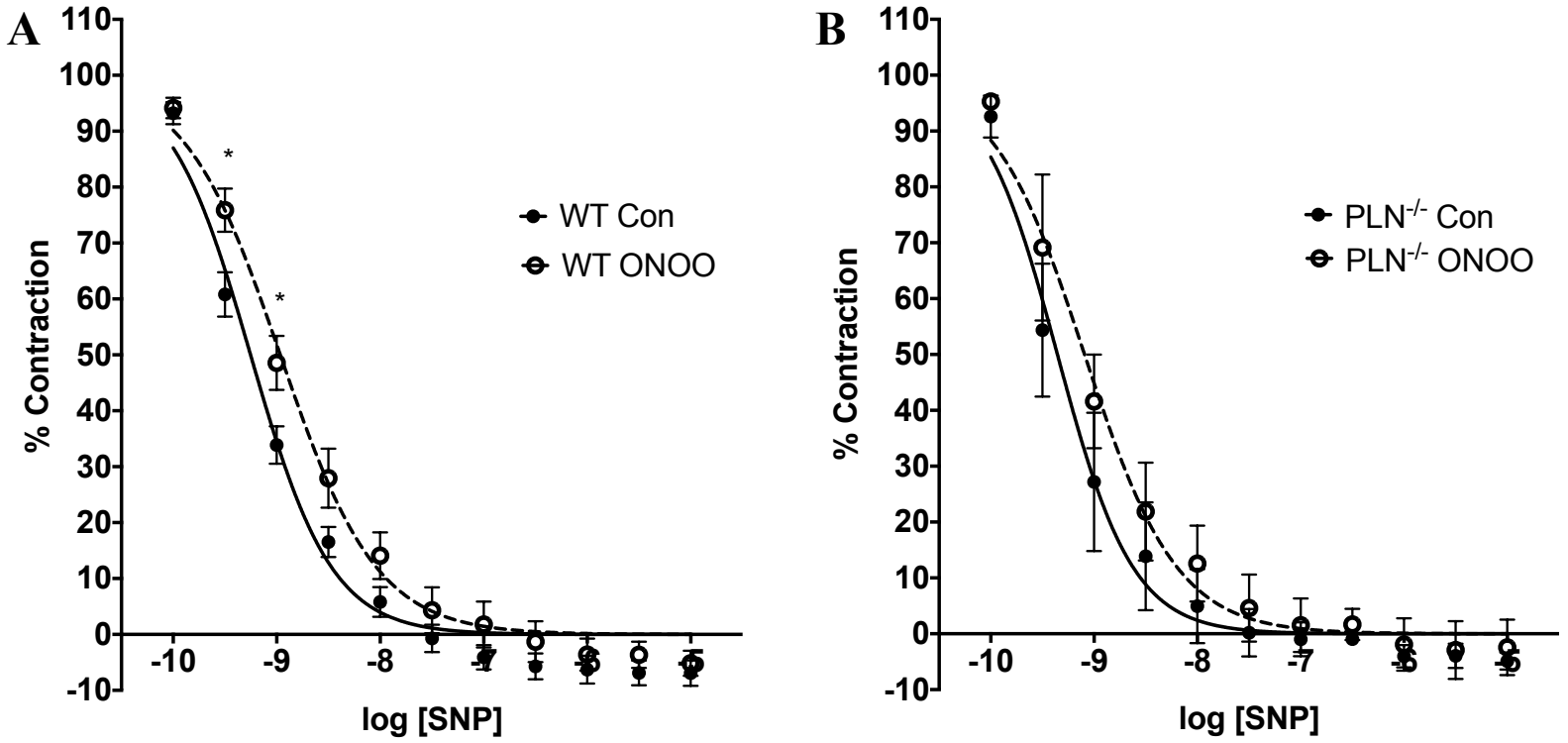


**Figure 8: SNP induced relaxation in thoracic aorta following $ONOO^-$ incubation (150 µM).** Thoracic aorta vessels were exposed to 30 mins of 150 µM $ONOO^-$. Following the incubation, vessels were pre-constricted with PE ($10^{-6}$M) and then relaxed with cumulative additions of SNP ($10^{-10}$-$10^{-5}$M). $ONOO^-$ 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).

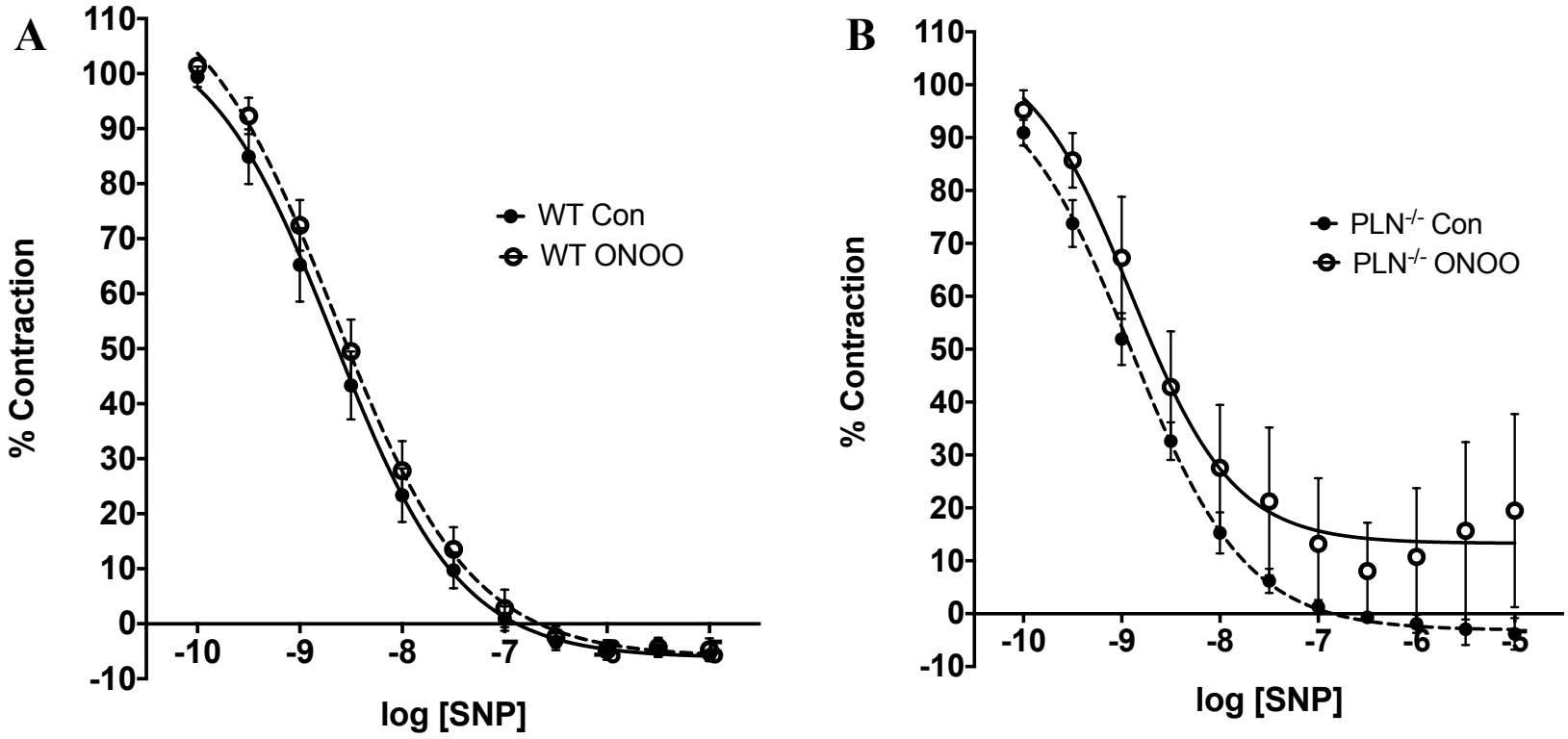


**Figure 9: SNP induced relaxation in thoracic aorta following $ONOO^-$ incubation (300 µM).** Thoracic aorta vessels were exposed to 30 mins of 300 µM $ONOO^-$. Following the incubation, vessels were pre-constricted with PE ($10^{-6}$M) and then relaxed with cumulative additions of SNP ($10^{-10}$-$10^{-5}$M). $ONOO^-$ treatment showed no significant effect in WT or $PLN^{-/-}$ vessels ($p>0.05$, n=4).

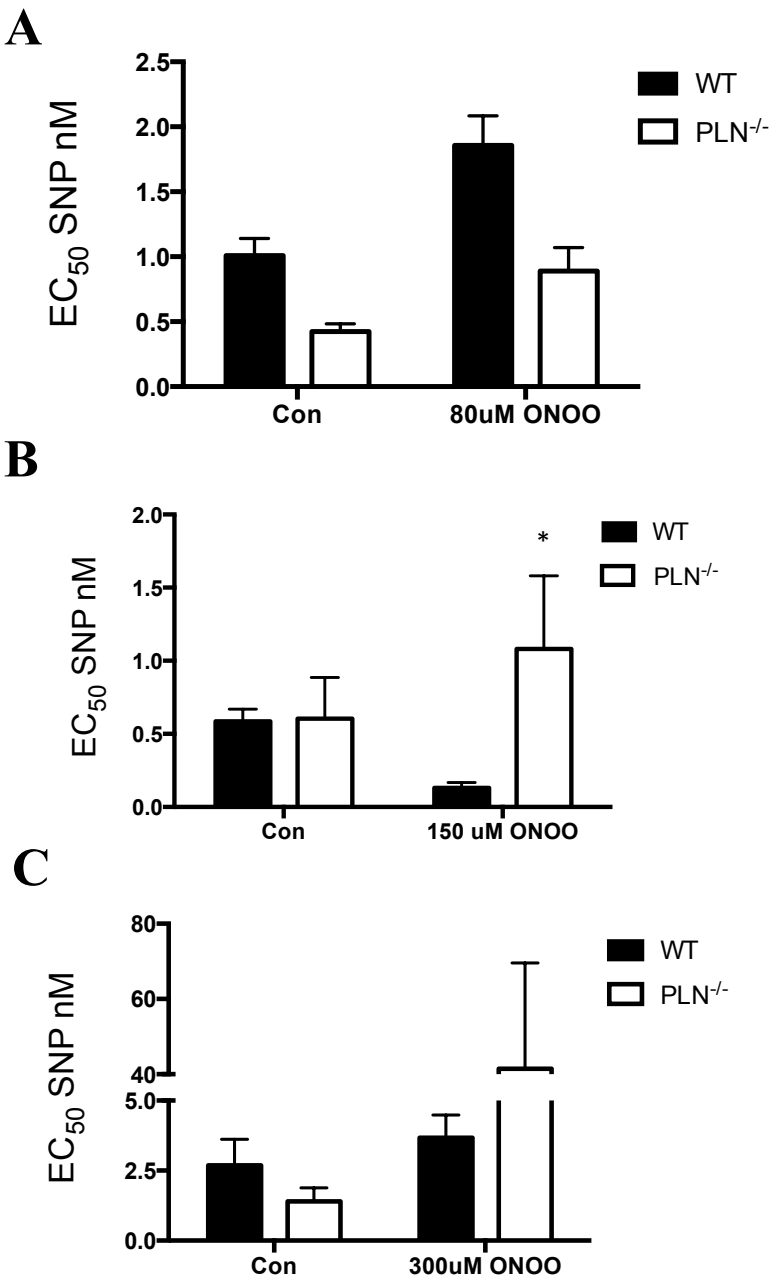


**Figure 10: $EC_{50}$ of ONOO treated vessels.** Thoracic aorta rings were exposed to varying concentrations of $ONOO^-$. Following which, the rings were pre-constricted with PE ($10^{-6}$ M) and a dose response to SNP ($10^{-10}$-$10^{-5}$ M) was performed as described in "Methods". $EC_{50}$ values 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 $Ca^{2+}$-dependent SERCA activity*

$Ca^{2+}$dependent $Ca^{2+}$-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, μmol/g protein/min, for all groups prior to normalization. Data were normalized to the WT control group within each treatment condition, either 80 μM or 300 μM, and analysis was completed comparing control to treatment groups within each concentration. The data presented in Figure 9 indicates control groups for both 80 μM and 300 μM $ONOO^-$ normalized to WT Con from the 80 μM 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 μM $ONOO^-$ treatment however; it was seen that 300 μM $ONOO^-$ incubation reduced SERCA activity in both the WT and $PLN^{-/-}$ groups ($p<0.05$).

| | | Max ATPase (µmol/g pro/min) | |
|---|---|---|---|
| | | **80 µM** | **300 µM** |
| **WT** | **Con** | 193.15 ± 74.9 | 219.31 ±20.2 |
| | **$ONOO^-$** | 176.40 ± 45.1 | 151.33 ± 43.7 |
| **$PLN^{-/-}$** | **Con** | 222.92 ± 46.9 | 290.86 ± 82.6 |
| | **$ONOO^-$** | 243.63 ±103.4 | 193.97 ± 68.8 |

**Table 2: Maximal $Ca^{2+}$ dependent $Ca^{2+}$-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).

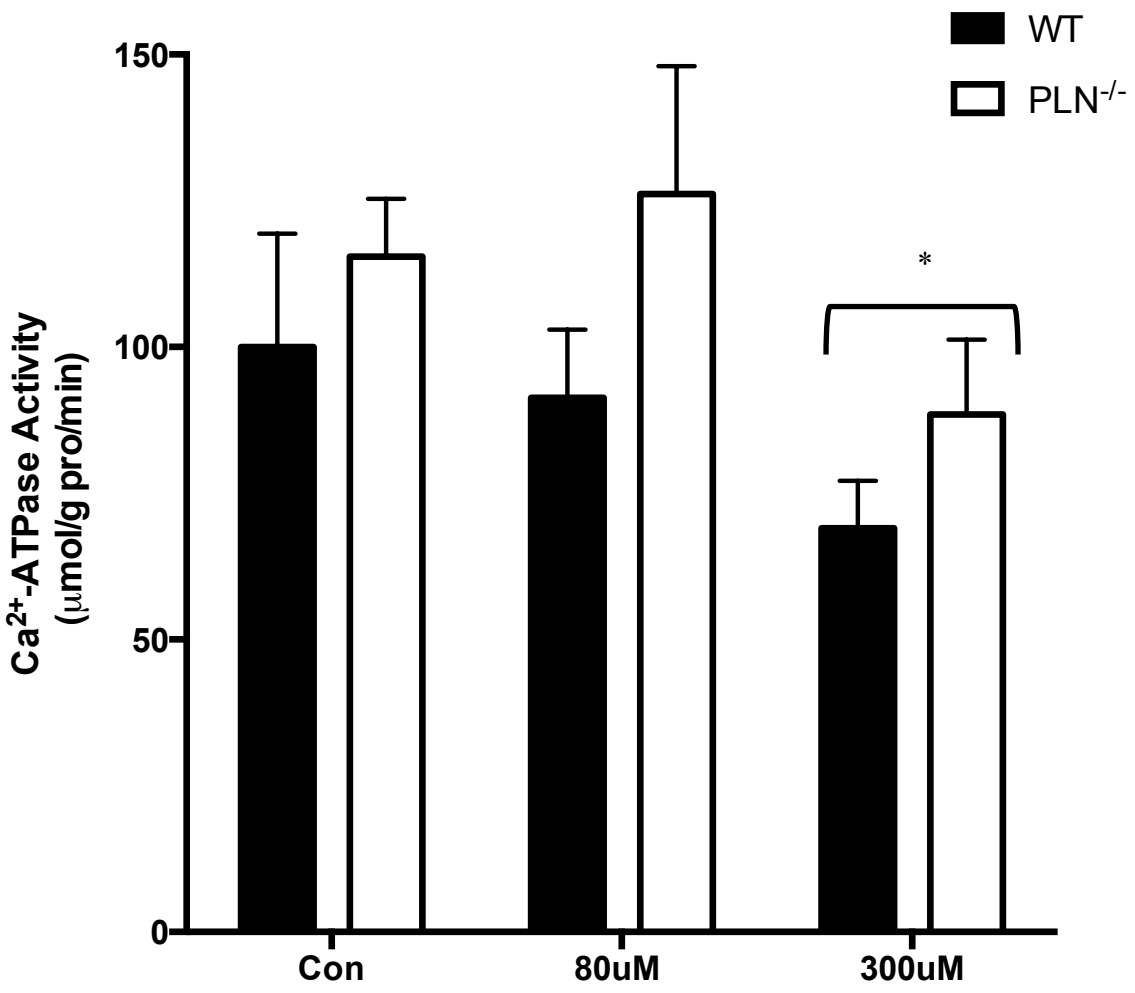


**Figure 11: $Ca^{2+}$ dependent $Ca^{2+}$-ATPase activity of SERCA2.** LV homogenates from WT or $PLN^{-/-}$ animals were exposed to $ONOO^-$ for a total of 30 mins (37°C). Control samples were exposed to heat only. This graph shows maximal ATPase values normalized to WT control. For analysis, each 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 $Na^+/K^+$-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 ($p<0.05$).

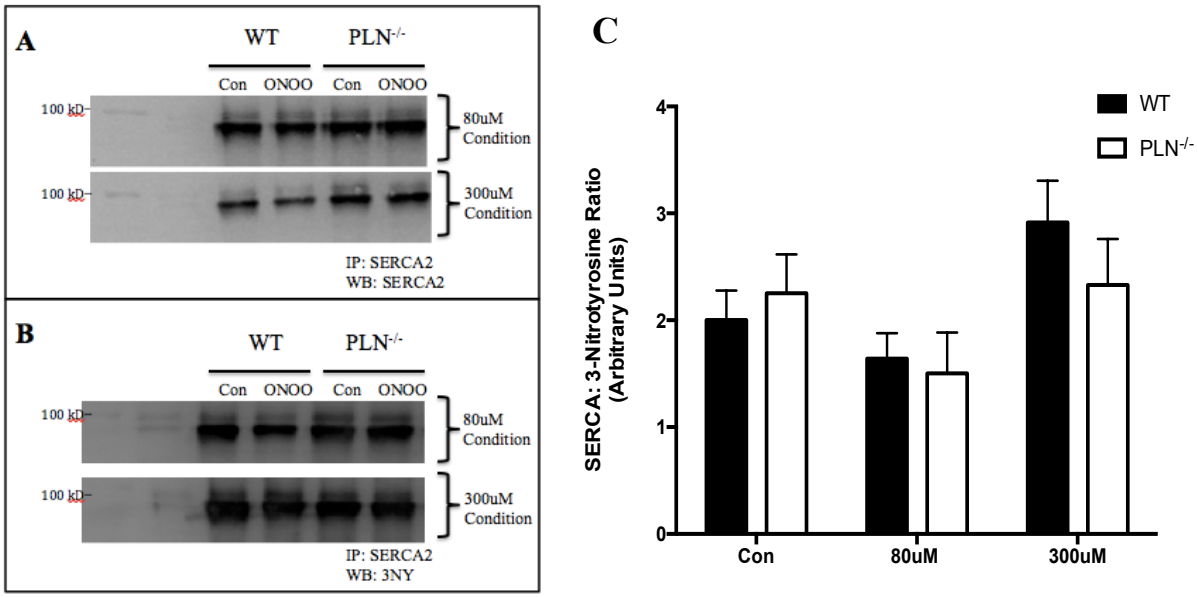


**Figure 12: SERCA2 3-NY content following $ONOO^-$ exposure.** SERCA2 was immunoprecipitated from control and $ONOO^-$ treated LV homogenates from WT and $PLN^{-/-}$ animals. Panel A shows presence of SERCA2 within the LV for all conditions, while Panel B depicts the 3-NY formation on SERCA2. Graph 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

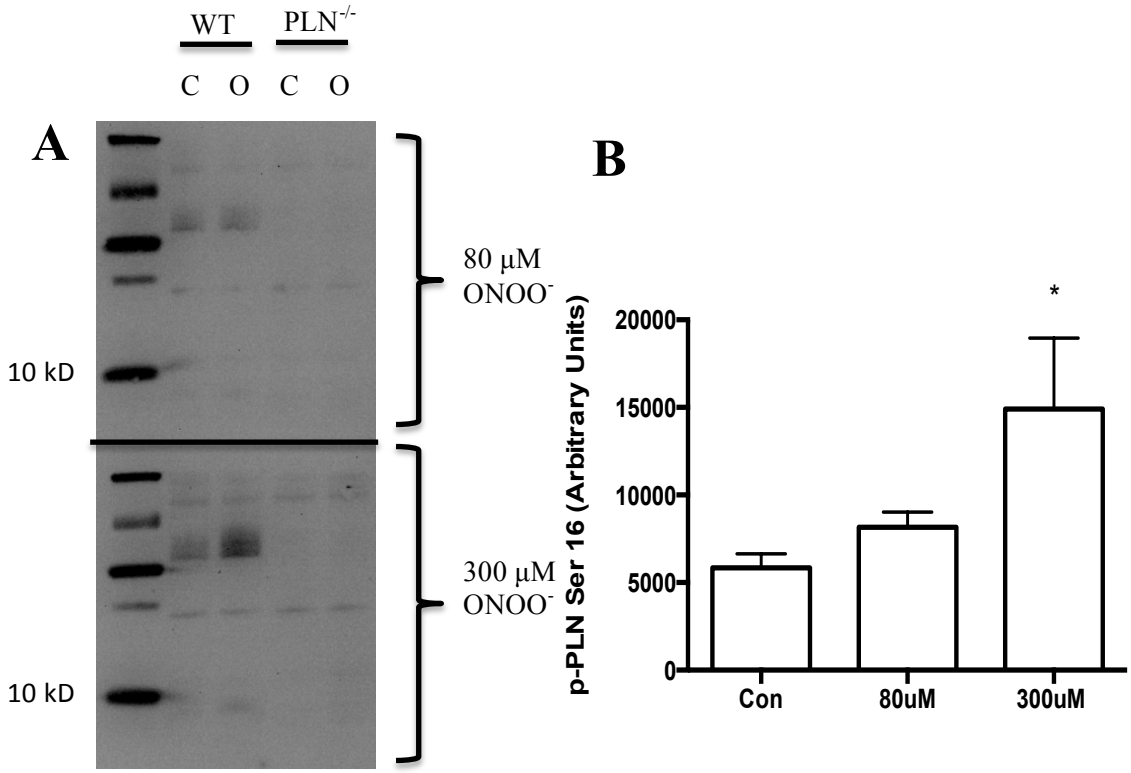


**Figure 13: p-PLN at Ser 16 in LV following $ONOO^-$ exposure.** LV homogenates were exposed to varying concentrations of $ONOO^-$ for 30 mins at 37°C as described in "Methods". Samples were then run against a p-PLN Ser16 specific antibody to detect any changes in phosphorylation level. Panel A depicts 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 μM, 150 μM or 300 μM) 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 μM $ONOO^-$. It was also found that SERCA2 activity was significantly reduced in both the WT and $PLN^{-/-}$ following incubation with 300 μM $ONOO^-$. 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 μM, 150 μM or 300 μM) or control (0 μM $ONOO^-$). 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 μM treatment and an increased basal tension in both WT and $PLN^{-/-}$ following 300 μM 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 μM $ONOO^-$ incubation, consistent with research previously reported by Li et al., who found incubation of rat aortic rings with $10^{-8}$-$10^{-4}$ 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 base line 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 base line 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 $ONOO^-$. 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 $Ca^{2+}$. As the $ONOO^-$ concentration increases, differences in tension production occur which can be potentially explained by an increase in PKA. For example, with 150μM $ONOO^-$, 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 $Ca^{2+}$ will decrease resulting in increased cytosolic $Ca^{2+}$ 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 nitrosylaton of SERCA occurs with increased amounts of oxidants, and though not detected in this data could explain the increased base line 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 $Ca^{2+}$pumping efficiency, resulting in lower overall contraction due to lower $Ca^{2+}$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 $Ca^{2+}$ release channel on the SR, PP has been shown to increase the open probability of RyR channels resulting in $Ca^{2+}$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 probably 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 $EC_{50}$. The lack of effect of high concentrations of $ONOO^-$ on $EC_{50}$ may be due to the significant reduction in overall force production following exposure to 300 μM $ONOO^-$. Due to the ~60% reduction in PE/KCl following 300 μM $ONOO^-$ exposure, the slight changes in relaxation needed to determine the $EC_{50}$ through a sigmoidal dose-response analysis were difficult to detect. In addition, it is possible that $ONOO^-$ 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 $Ca^{2+}$, pumping it against its concentration gradient into the SR. As high concentrations of $ONOO^-$ have been shown to decrease $Ca^{2+}$-dependent SERCA activity, it was hypothesized that this would lead to impairments in whole muscle relaxation. It was observed that $PLN^{-/-}$ vessels had a significantly lower maximal relaxation across all treatment conditions. $ONOO^-$ incubation did not have a greater effect on the $PLN^{-/-}$ 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 $PLN^{-/-}$ group following exposure to 300 μM $ONOO^-$, 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 $ONOO^-$ 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 $PLN^{-/-}$ 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). $ONOO^-$ 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 $ONOO^-$ 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 $ONOO^-$, 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 $ONOO^-$, both WT and $PLN^{-/-}$ groups demonstrated less relaxation at SNP $10^{-9.5}$ and $10^{-9}$ M. Incubation with 150 μM $ONOO^-$ 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 $Ca^{2+}$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 $Ca^{2+}$ 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 $Ca^{2+}$, 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-Martin et al (2004) showed that a single bolus of $ONOO^-$ (200-300 μM, 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 μM $ONOO^-$. 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 μM and a significant increase following 300 μM $ONOO^-$. In a whole heart model, this would translate to reduced contractility following incubation with 80 μM $ONOO^-$, while an increased contractility following 300 μM $ONOO^-$ 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 μM $ONOO^-$. 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 uM 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 $ONOO^-$ exposure with other cellular functions.

*4.3 $ONOO^-$ 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 $ONOO^-$ 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 $Na^+/K^+$-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 $ONOO^-$ would increase 3-NY content within LV homogenate. However, treatment of WT LV sample with $ONOO^-$ did not increase 3-NY content on SERCA compared to control. Additionally, the presence of PLN under control and $ONOO^-$ 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 $ONOO^-$ exposure experiments (Viner et al., 1996). Other states of high oxidative stress, such as aging, have been shown to increase SERCA2 3-NYcontent 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 $ONOO^-$ incubation.

**Summary**

In summary, these data does not show conclusive evidence that PLN ablation increases $ONOO^-$ 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 $ONOO^-$. It was seen that $ONOO^-$ was able to cause a reduction in SERCA2 $Ca^{2+}$-ATPase activity in both the WT and $PLN^{-/-}$ LV as well as a reduction in maximal tension, maximal relaxation and increase in $EC_{50}$ within aortic rings for both genotypes. Few pieces of data from this thesis stand to serve as clues that PLN ablation does increase $ONOO^-$ induced SERCA2 damage, one of which is the reduced $EC_{50}$ in WT vessels following 150μM $ONOO^-$ treatment. This could represent a protective mechanism of PLN within the WT vessels, as the $PLN^{-/-}$ vessels showed a significantly increased $EC_{50}$ possibly highlighting increased SERCA2 damage. Structural SERCA2 damage was not shown to be higher in the $PLN^{-/-}$ group following $ONOO^-$ 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 $ONOO^-$.

**Conclusion**

The results of this thesis indicate the ability for $ONOO^-$ incubation to greatly reduce PE mediated vascular contraction, while having little effect on SNP induced vascular relaxation. It also highlights the ability for $ONOO^-$ incubation to decrease overall maximal SERCA activity through mechanisms other than 3-NY formation. Since differences between WT and $PLN^{-/-}$ 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 $ONOO^-$ 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 $11^{th}$ 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 $EC_{50}$ 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**

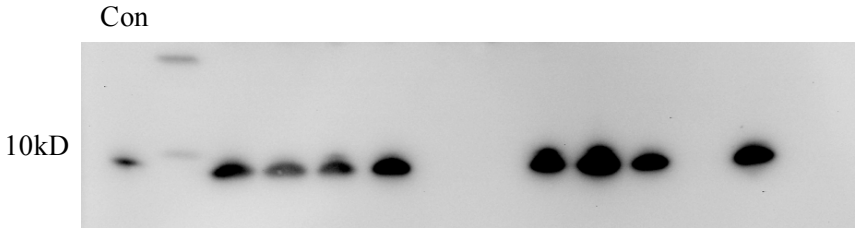


**Figure 14: PLN in LV**. Whole LV homogenate was electrophoresed and blotted against PLN antibody as a genotype check. This figure represents the blot completed and shows the presence or absence of PLN in LV just below the 10kD ladder marker. Control lane contains human vastus lateralis as a positive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

**References**


Adachi, T., Matsui, R., Weisbrod, R. M., Najibi, S., & Cohen, R. a. (2001). Reduced sarco/endoplasmic reticulum Ca(2+) uptake activity can account for the reduced response to NO, but not sodium nitroprusside, in hypercholesterolemic rabbit aorta. *Circulation*, *104*(9), 1040–1045. doi:10.1161/hc3501.093798

Adachi, T., Weisbrod, R. M., Pimentel, D. R., Ying, J., Sharov, V. S., Schöneich, C., & Cohen, R. A. (2004). S-Glutathiolation by peroxynitrite activates SERCA during arterial relaxation by nitric oxide. *Nature Medicine*, *10*(11), 1200–7. doi:10.1038/nm1119

Asahi, M., Kurzydlowski, K., Tada, M., & MacLennan, D. H. (2002). Sarcolipin inhibits polymerization of phospholamban to induce superinhibition of sarco(endo)plasmic reticulum Ca2+-ATPases (SERCAs). *The Journal of Biological Chemistry*, *277*(30), 26725–8. doi:10.1074/jbc.C200269200

Asahi, M., McKenna, E., Kurzydlowski, K., Tada, M., & MacLennan, D. H. (2000). Physical interactions between phospholamban and sarco(endo)plasmic reticulum Ca2+-ATPases are dissociated by elevated Ca2+, but not by phospholamban phosphorylation, vanadate, or thapsigargin, and are enhanced by ATP. *The Journal of Biological Chemistry*, *275*(20), 15034–8.

Aschar-Sobbi, R., Emmett, T. L., Kargacin, G. J., & Kargacin, M. E. (2012). Phospholamban phosphorylation increases the passive calcium leak from cardiac sarcoplasmic reticulum. *Pflügers Archiv : European Journal of Physiology*, *464*(3), 295–305. doi:10.1007/s00424-012-1124-9

Ashida, T., Schaeffer, J., Goldman, W. F., Wade, J. B., & Blaustein, M. P. (1988). Role of sarcoplasmic reticulum in arterial contraction: comparison of ryanodine's effect in a conduit and a muscular artery. *Circulation Research*, *62*(4), 854–864.

Belevych, A., Kubalova, Z., Terentyev, D., Hamlin, R. L., Carnes, C. A., & Györke, S. (2007). Enhanced ryanodine receptor-mediated calcium leak determines reduced sarcoplasmic reticulum calcium content in chronic canine heart failure. *Biophysical Journal*, *93*(11), 4083–92. doi:10.1529/biophysj.107.114546

Belik, J., Jankov, R. P., Pan, J., & Tanswell, A. K. (2004). Peroxynitrite inhibits relaxation and induces pulmonary artery muscle contraction in the newborn rat. *Free Radical Biology & Medicine*, *37*(9), 1384–92. doi:10.1016/j.freeradbiomed.2004.07.029

Bhattacharyya, J., Biswas, S., & Datta, A. G. (2004). Mode of action of endotoxin: role of free radicals and antioxidants. *Current Medicinal Chemistry*, *11*(3), 359–68.

Bhogal, M. S., & Colyer, J. (1998). Depletion of Ca2+ from the sarcoplasmic reticulum of cardiac muscle prompts phosphorylation of phospholamban to stimulate store refilling. *Proceedings of the National Academy of Sciences of the United States of America*, *95*, 1484–1489.

Bhupathy, P., Babu, G. J., & Periasamy, M. (2007). Sarcolipin and phospholamban as regulators of cardiac sarcoplasmic reticulum Ca 2 + ATPase. *Journal of Molecular and Cellular Cardiology*, *42*, 903–911. doi:10.1016/j.yjmcc.2007.03.738

Bonnevier, J., & Arner, A. (2004). Actions downstream of cyclic GMP/protein kinase G can reverse protein kinase C-mediated phosphorylation of CPI-17 and Ca(2+) sensitization in smooth muscle. *The Journal of Biological Chemistry*, *279*(28), 28998–9003. doi:10.1074/jbc.M404259200

Bonnevier, J., Fässler, R., Somlyo, A. P., Somlyo, A. V, & Arner, A. (2004). Modulation of Ca2+ sensitivity by cyclic nucleotides in smooth muscle from protein kinase G-deficient mice. *The Journal of Biological Chemistry*, *279*(7), 5146–51. doi:10.1074/jbc.M306532200

Brini, M., & Carafoli, E. (2009). Calcium pumps in health and disease. *Physiological Reviews*, *89*(4), 1341–78. doi:10.1152/physrev.00032.2008

Buttery, L. D., Springall, D. R., Chester, A. H., Evans, T. J., Standfield, E. N., Parums, D. V, … Polak, J. M. (1996). Inducible nitric oxide synthase is present within human atherosclerotic lesions and promotes the formation and activity of peroxynitrite. *Laboratory Investigation; a Journal of Technical Methods and Pathology*, *75*(1), 77–85.

Catalucci, D., Latronico, M. V. G., Ceci, M., Rusconi, F., Young, H. S., Gallo, P., … Condorelli, G. (2009). Akt Increases Sarcoplasmic Reticulum Ca2+ Cycling by Direct Phosphorylation of Phospholamban at Thr17. *Journal of Biological Chemistry*, *284*(41), 28180–28187. doi:10.1074/jbc.M109.036566

Cerra, M. C., & Imbrogno, S. (2012). Phospholamban and cardiac function: a comparative perspective in vertebrates. *Acta Physiologica (Oxford, England)*, *205*(1), 9–25. doi:10.1111/j.1748-1716.2012.02389.x

Chabot, F., Mitchell, J. A., Quinlan, G. J., & Evans, T. W. (1997). Characterization of the vasodilator properties of peroxynitrite on rat pulmonary artery: role of poly (adenosine 5'-diphosphoribose) synthase. *British Journal of Pharmacology*, *121*(3), 485–90. doi:10.1038/sj.bjp.0701162

Chen, W., Lah, M., Robinson, P. J., & Kemp, B. E. (1994). Phosphorylation of phospholamban in aortic smooth muscle cells and heart by calcium/calmodulin-dependent protein kinase II. *Cellular Signalling*, *6*(6), 617–30.

Chesnais, J.-M., Fischmeister, R., & Mery, P.-F. (1999). Peroxynitrite is a positive inotropic agent in atrial and ventricular fibres of the frog heart. *The Journal of Physiology*, *521*(2), 375–388. doi:10.1111/j.1469-7793.1999.00375.x

Cheung, P. Y., Wang, W., & Schulz, R. (2000). Glutathione protects against myocardial ischemia-reperfusion injury by detoxifying peroxynitrite. *Journal of Molecular and Cellular Cardiology*, *32*(9), 1669–78. doi:10.1006/jmcc.2000.1203

Chu, G., Ferguson, D. G., Edes, I., Kiss, E., Sato, Y., & Kranias, E. G. (1998). Phospholamban ablation and compensatory responses in the mammalian heart. *Annals of the New York Academy of Sciences*, *853*, 49–62.

Clark, J., & Pyne-Geithman, G. (2005). Vascular smooth muscle function: The physiology and pathology of vasoconstriction. *Pathophysiology*, *12*, 35–45. doi:10.1016/j.pathophys.2005.02.007

Clausen, J. D., Vandecaetsbeek, I., Wuytack, F., Vangheluwe, P., & Andersen, J. P. (2012). Distinct roles of the C-terminal 11th transmembrane helix and luminal extension in the partial reactions determining the high Ca2+ affinity of sarco(endo)plasmic reticulum Ca2+-ATPase isoform 2b (SERCA2b). *The Journal of Biological Chemistry*, *287*(47), 39460–9. doi:10.1074/jbc.M112.397331

Cohen, R. a, Weisbrod, R. M., Gericke, M., Yaghoubi, M., Bierl, C., & Bolotina, V. M. (1999). Mechanism of nitric oxide-induced vasodilatation: refilling of intracellular stores by sarcoplasmic reticulum Ca2+ ATPase and inhibition of store-operated Ca2+ influx. *Circulation Research*, *84*(2), 210–219. doi:10.1161/01.RES.84.2.210

Cohen, R., & Adachi, T. (2006). Nitric-Oxide-Induced Vasodilatation: Regulation by Physiologic S-Glutathiolation and Pathologic Oxidation of the Sarcoplasmic Endoplasmic Reticulum. *Trends in Cardiovascular Medicine*, *16*(4), 109–114.

Colyer, J. (1998). Phosphorylation states of phospholamban. *Annals of the New York Academy of Sciences*, *853*, 79–91.

Davidson, C. A., Kaminski, P. M., & Wolin, M. S. (1997). Endogenous peroxynitrite generation causes a subsequent suppression of coronary arterial contraction to serotonin. *Nitric Oxide : Biology and Chemistry / Official Journal of the Nitric Oxide Society*, *1*(3), 244–53. doi:10.1006/niox.1997.0128

Davies, K. J., Delsignore, M. E., & Lin, S. W. (1987). Protein damage and degradation by oxygen radicals. II. Modification of amino acids. *The Journal of Biological Chemistry*, *262*(20), 9902–7.

De Simone, A., Gustavsson, M., Montalvao, R. W., Shi, L., Veglia, G., & Vendruscolo, M. (2013). Structures of the excited states of phospholamban and shifts in their populations upon phosphorylation. *Biochemistry*, *52*(38), 6684–94. doi:10.1021/bi400517b

Devine, C., Somlyo, A., & Somlyo, A. (1972). Sarcoplasmic reticulum and excitation-contraction coupling in mammalian smooth muscles. *The Journal of Cell Biology*, *52*, 690–718.

Dhar, A., Kaundal, R. K., & Sharma, S. S. (2006). Neuroprotective effects of FeTMPyP: a peroxynitrite decomposition catalyst in global cerebral ischemia model in gerbils. *Pharmacological Research : The Official Journal of the Italian Pharmacological Society*, *54*(4), 311–6. doi:10.1016/j.phrs.2006.06.009

Dremina, E. S., Sharov, V. S., Davies, M. J., & Schöneich, C. (2007). Oxidation and inactivation of SERCA by selective reaction of cysteine residues with amino acid peroxides. *Chemical Research in Toxicology*, *20*(10), 1462–9. doi:10.1021/tx700108w

Duhamel, T. A., Green, H. J., Stewart, R. D., Foley, K. P., Smith, I. C., & Ouyang, J. (2007). Muscle metabolic , SR Ca 2 + -cycling responses to prolonged cycling , with and without glucose supplementation Muscle metabolic , SR Ca 2 ∂ -cycling responses to prolonged cycling , with and without glucose supplementation. *Journal of Applied Physiology*, *103*, 1986–1998. doi:10.1152/japplphysiol.01440.2006

Ebashi, S., Endo, M., & Otsuki, I. (1969). Control of muscle contraction. *Quarterly Reviews of Biophysics*, *2*(4), 351–84.

Esfandiarei, M., Lam, J. T. N., Yazdi, S. A., Kariminia, A., Dorado, J. N., Kuzeljevic, B., … van Breemen, C. (2011). Diosgenin modulates vascular smooth muscle cell function by regulating cell viability, migration, and calcium homeostasis. *The Journal of Pharmacology and Experimental Therapeutics*, *336*(3), 925–39. doi:10.1124/jpet.110.172684

Fajardo, V. A., Bombardier, E., Vigna, C., Devji, T., Bloemberg, D., Gamu, D., … Tupling, A. R. (2013). Co-expression of SERCA isoforms, phospholamban and sarcolipin in human skeletal muscle fibers. *PloS One*, *8*(12), e84304. doi:10.1371/journal.pone.0084304

Ferdinandy, P., Danial, H., Ambrus, I., Rothery, R. A., & Schulz, R. (2000). Peroxynitrite is a major contributor to cytokine-induced myocardial contractile failure. *Circulation Research*, *87*(3), 241–7.

Fu, M., MacLennan, D., & Tupling, A. R. (n.d.). PLN Prevents Thermal Inactivation of SERCA2a, *05*.

Fu, M., & Tupling, A. R. (2009). Protective effects of Hsp70 on the structure and function of SERCA2a expressed in HEK-293 cells during heat stress. *American Journal of Physiology- Heart and Circulatory Physiology*, *296*(4), H1175–83. doi:10.1152/ajpheart.01276.2008

Fujii, J., Lytton, J., Tada, M., & MacLennan, D. H. (1988). Rabbit cardiac and slow-twitch muscle express the same phospholamban gene. *FEBS Letters*, *227*(1), 51–5.

Fujii, J., Ueno, A., Kitano, K., Tanaka, S., Kadoma, M., & Tada, M. (1987). Complete complementary DNA-derived amino acid sequence of canine cardiac phospholamban. *The Journal of Clinical Investigation*, *79*(1), 301–4. doi:10.1172/JCI112799

Gao, M. H., Tang, T., Guo, T., Miyanohara, A., Yajima, T., Pestonjamasp, K., … Hammond, H. K. (2008). Adenylyl Cyclase Type VI Increases Akt Activity and Phospholamban Phosphorylation in Cardiac Myocytes *. *The Journal of Biological Chemistry*, *283*(48), 33527–33535. doi:10.1074/jbc.M805825200

Gonzalez, D. R., Treuer, A. V, Castellanos, J., Dulce, R. A., & Hare, J. M. (2010). Impaired S-nitrosylation of the ryanodine receptor caused by xanthine oxidase activity contributes to calcium leak in heart failure. *The Journal of Biological Chemistry*, *285*(37), 28938–45. doi:10.1074/jbc.M110.154948

Grover, A. K., Kwan, C.-Y., & Samson, S. E. (2003). Effects of peroxynitrite on sarco/endoplasmic reticulum Ca2+ pump isoforms SERCA2b and SERCA3a. *American Journal of Physiology. Cell Physiology*, *285*(6), C1537–43. doi:10.1152/ajpcell.00299.2003

Grover, A. K., & Samson, S. E. (1989). Protection of Ca pump of coronary artery against inactivation by superoxide radical. *The American Journal of Physiology*, *256*(3 Pt 1), C666–73.

Grover, A. K., Samson, S. E., & Misquitta, C. M. (1997). Sarco(endo)plasmic reticulum Ca2+ pump isoform SERCA3 is more resistant than SERCA2b to peroxide. *The American Journal of Physiology*, *273*(2 Pt 1), C420–5.

Grover, A. K., Samson, S. E., Robinson, S., & Kwan, C. Y. (2003). Effects of peroxynitrite on sarcoplasmic reticulum Ca2+ pump in pig coronary artery smooth muscle. *American Journal of Physiology. Cell Physiology*, *284*, C294–C301. doi:10.1152/ajpcell.00297.2002

Groves, J. T. (1999). Peroxynitrite: reactive, invasive and enigmatic. *Current Opinion in Chemical Biology*, *3*(2), 226–35. doi:10.1016/S1367-5931(99)80036-2

Gutiérrez-Martín, Y., Martín-Romero, F. J., Iñesta-Vaquera, F. A., Gutiérrez-Merino, C., & Henao, F. (2004). Modulation of sarcoplasmic reticulum Ca(2+)-ATPase by chronic and acute exposure to peroxynitrite. *European Journal of Biochemistry / FEBS*, *271*(13), 2647–57. doi:10.1111/j.1432-1033.2004.04193.x

Hai, C. M., & Murphy, R. A. (1989). Ca2+, crossbridge phosphorylation, and contraction. *Annual Review of Physiology*, *51*, 285–98. doi:10.1146/annurev.ph.51.030189.001441

Horowitz, A., Menice, C., Laporte, R., & Morgan, K. G. (1996). Mechanisms of smooth muscle contraction. *Physiological Reviews*, *76*(4), 967–1001.

Hovnanian, A. (2007). SERCA pumps and human diseases. *Sub-Cellular Biochemistry*, *45*, 337–63.

Iovine, N. M., Pursnani, S., Voldman, A., Wasserman, G., Blaser, M. J., & Weinrauch, Y. (2008). Reactive nitrogen species contribute to innate host defense against Campylobacter jejuni. *Infection and Immunity*, *76*(3), 986–93. doi:10.1128/IAI.01063-07

Kamat, J. P. (2006). Peroxynitrite: a potent oxidizing and nitrating agent. *Indian Journal of Experimental Biology*, *44*(6), 436–47.

Karczewski, P., Hendrischke, T., Wolf, W. P., Morano, I., Bartel, S., & Schrader, J. (1998). Phosphorylation of phospholamban correlates with relaxation of coronary artery induced by nitric oxide, adenosine, and prostacyclin in the pig. *Journal of Cellular Biochemistry*, *70*(1), 49–59.

Kim, M., Cho, S. Y., Han, I. S., Koh, S. D., & Perrino, B. A. (2005). CaM kinase II and phospholamban contribute to caffeine-induced relaxation of murine gastric fundus smooth muscle CaM kinase II and phospholamban contribute to caffeine-induced relaxation of murine gastric fundus smooth muscle. *American Journal of Physiology. Cell Physiology*, *288*, C1202–C1210. doi:10.1152/ajpcell.00299.2004

Kim, M., Han, I. S., Koh, S. D., & Perrino, B. A. (2006). Roles of CaM kinase II and phospholamban in SNP-induced relaxation of murine gastric fundus smooth muscles. *American Journal of Physiology. Cell Physiology*, *291*(2), C337–47. doi:10.1152/ajpcell.00397.2005

Kimura, Y., Kurzydlowski, K., Tada, M., & Maclennan, D. H. (1997). Phospholamban inhibitory function is activated by depolymerization. *The Journal of Biological Chemistry*, *272*(24), 15061–15064.

Knyushko, T. V, Sharov, V. S., Williams, T. D., Schöneich, C., & Bigelow, D. J. (2005). 3-Nitrotyrosine modification of SERCA2a in the aging heart: a distinct signature of the cellular redox environment. *Biochemistry*, *44*(39), 13071–81. doi:10.1021/bi051226n

Kohr, M. J., Davis, J. P., & Ziolo, M. T. (2009). Peroxynitrite Increases Protein Phosphatase Activity and Promotes the Interaction of Phospholamban with Protein Phosphatase 2a in the Myocardium. *Nitric Oxide : Biology and Chemistry / Official Journal of the Nitric Oxide Society*, *20*(3), 217–221. doi:10.1016/j.niox.2009.01.003

Kohr, M. J., Roof, S. R., Zweier, J. L., & Ziolo, M. T. (2012). Modulation of myocardial contraction by peroxynitrite. *Frontiers in Physiology*, *3*, 468. doi:10.3389/fphys.2012.00468

Kohr, M. J., Traynham, C. J., Roof, S. R., Davis, J. P., & Ziolo, M. T. (2010). cAMP-independent activation of protein kinase A by the peroxynitrite generator SIN-1 elicits positive inotropic effects in cardiomyocytes. *Journal of Molecular and Cellular Cardiology*, *48*(4), 645–8. doi:10.1016/j.yjmcc.2010.01.007

Kohr, M. J., Wang, H., Wheeler, D. G., Velayutham, M., Zweier, J. L., & Ziolo, M. T. (2008a). Biphasic effect of SIN-1 is reliant upon cardiomyocyte contractile state. *Free Radical Biology & Medicine*, *45*(1), 73–80. doi:10.1016/j.freeradbiomed.2008.03.019

Kohr, M. J., Wang, H., Wheeler, D. G., Velayutham, M., Zweier, J. L., & Ziolo, M. T. (2008b). Targeting of phospholamban by peroxynitrite decreases beta-adrenergic stimulation in cardiomyocytes. *Cardiovascular Research*, *77*(2), 353–61. doi:10.1093/cvr/cvm018

Lalli, J., Harrer, J. M., Luo, W., Kranias, E. G., & Paul, R. J. (1997). Targeted ablation of the phospholamban gene is associated with a marked decrease in sensitivity in aortic smooth muscle. *Circulation Research*, *80*(4), 506–13.

Lalli, M. J., Shimizu, S., Sutliff, R. L., Kranias, E. G., & Paul, R. J. (1999). [Ca2+]i homeostasis and cyclic nucleotide relaxation in aorta of phospholamban-deficient mice. *American Journal of Physiology- Heart and Circulatory Physiology*, *277*, H963–J970.

Li, J., Li, W., Altura, B. T., & Altura, B. M. (2004). Peroxynitrite-induced relaxation in isolated canine cerebral arteries and mechanisms of action. *Toxicology and Applied Pharmacology*, *196*(1), 176–82. doi:10.1016/j.taap.2003.12.007

Li, J., Li, W., Altura, B. T., & Altura, B. M. (2005). Peroxynitrite-induced relaxation in isolated rat aortic rings and mechanisms of action. *Toxicology and Applied Pharmacology*, *209*(3), 269–76. doi:10.1016/j.taap.2005.04.016

Liu, L. H., Paul, R. J., Sutliff, R. L., Miller, M. L., Lorenz, J. N., Pun, R. Y., … Shull, G. E. (1997). Defective endothelium-dependent relaxation of vascular smooth muscle and endothelial cell Ca2+ signaling in mice lacking sarco(endo)plasmic reticulum Ca2+-ATPase isoform 3. *The Journal of Biological Chemistry*, *272*(48), 30538–45.

Lounsbury, K. M., Hu, Q., & Ziegelstein, R. C. (2000). Calcium signaling and oxidant stress in the vasculature. *Free Radical Biology & Medicine*, *28*(9), 1362–1369.

Luo, W., Grupp, I. L., Harrer, J., Ponniah, S., Grupp, G., Duffy, J. J., … Kranias, E. G. (1994). Targeted ablation of the phospholamban gene is associated with markedly enhanced myocardial contractility and loss of beta-agonist stimulation. *Circulation Research*, *75*(3), 401–409. doi:10.1161/01.RES.75.3.401

Luoma, J. S., Strålin, P., Marklund, S. L., Hiltunen, T. P., Särkioja, T., & Ylä-Herttuala, S. (1998). Expression of extracellular SOD and iNOS in macrophages and smooth muscle cells in human and rabbit atherosclerotic lesions: colocalization with epitopes characteristic of oxidized LDL and peroxynitrite-modified proteins. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *18*(2), 157–67.

Ma, H., Lewis, D., Xu, C., Inesi, G., & Toyoshima, C. (2005). Functional and Structural Roles of Critical Amino Acids within the "N", "P", and "A" Domains of the Ca2+ ATPase (SERCA) Headpeice. *Biochemistry*, *44*, 8090–8100.

MacLennan, D. H. (1990). Molecular tools to elucidate problems in excitation-contraction coupling. *Biophysical Journal*, *58*(6), 1355–65. doi:10.1016/S0006-3495(90)82482-6

Maclennan, D. H., Rice, W. J., & Green, N. M. (1997). The mechanism of Ca2+ transport by sarco(endo)plasmic reticulum Ca2+ ATPase. *The Journal of Biological Chemistry*, *272*(46), 28815–28818.

MacLennan, D., & Kranias, E. (2003). Phospholamban: a crucial regulator of cardiac contractility. *Nature Reviews Molecular Cell Biology*, *4*, 566–577. doi:10.1038/nrm1151

MacMicking, J. D., North, R. J., LaCourse, R., Mudgett, J. S., Shah, S. K., & Nathan, C. F. (1997). Identification of nitric oxide synthase as a protective locus against tuberculosis. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(10), 5243–8.

Maneen, M. J., Hannah, R., Vitullo, L., DeLance, N., & Cipolla, M. J. (2006). Peroxynitrite diminishes myogenic activity and is associated with decreased vascular smooth muscle F-actin in rat posterior cerebral arteries. *Stroke; a Journal of Cerebral Circulation*, *37*(3), 894–9. doi:10.1161/01.STR.0000204043.18592.0d

Matsunaga, S., Inashima, S., Yamada, T., Watanabe, H., Hazama, T., & Wada, M. (2003). Oxidation of sarcoplasmic reticulum Ca(2+)-ATPase induced by high-intensity exercise. *Pflügers Archiv : European Journal of Physiology*, *446*(3), 394–9. doi:10.1007/s00424-003-1040-0

Miller, F., Gutterman, D., Rios, C., Heistad, D. D., & Davidson, B. L. (1998). Superoxide production in vascular smooth muscle contributes to oxidative stress and impaired relaxation in atherosclerosis. *Circulation Research*, *82*, 1298–1305. doi:10.1161/01.RES.82.12.1298

Nathan, C., & Shiloh, M. U. (2000). Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(16), 8841–8.

Nobe, K., Sutliff, R. L., Kranias, E. G., & Paul, R. J. (2001). Phospholamban regulation of bladder contractility: evidence from gene-altered mouse models. *The Journal of Physiology*, *535*(3), 867–878. doi:10.1111/j.1469-7793.2001.00867.x

Obara, K., Miyashita, N., Xu, C., Toyoshima, I., Sugita, Y., Inesi, G., & Toyoshima, C. (2005). Structural role of countertransport revealed in Ca(2+) pump crystal structure in the absence of Ca(2+). *Proceedings of the National Academy of Sciences of the United States of America*, *102*(41), 14489–96. doi:10.1073/pnas.0506222102

Oloizia, B., & Paul, R. (2008). Ca2+ clearance and contractility in vascular smooth muscle: Evidence from gene-altered murine models. *Journal of Molecular and Cellular Cardiology*, *45*, 347–362. doi:10.1016/j.yjmcc.2008.05.024

Pacher, P., Beckman, J. S., & Liaudet, L. (2007). Nitric oxide and peroxynitrite in health and disease. *Physiological Reviews*, *87*(1), 315–424. doi:10.1152/physrev.00029.2006

Periasamy, M., & Kalyanasundaram, A. (2007). SERCA pump isoforms: their role in calcium transport and disease. *Muscle & Nerve*, *35*(4), 430–42. doi:10.1002/mus.20745

Pryor, W. A., & Squadrito, G. L. (1995). The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. *The American Journal of Physiology*, *268*(5 Pt 1), L699–722.

Radi, R., Cassina, A., & Hodara, R. (2002). Nitric oxide and peroxynitrite interactions with mitochondria. *Biological Chemistry*, *383*(3-4), 401–9. doi:10.1515/BC.2002.044

Radi, R., Cassina, A., Hodara, R., Quijano, C., & Castro, L. (2002). Peroxynitrite reactions and formation in mitochondria. *Free Radical Biology & Medicine*, *33*(11), 1451–64.

Rhodes, R. A., Packer, S., & Meiss, R. A. (1988). Pulmonary Vascular Smooth Muscle Contractility: Effect of Free Radicals. *Chest*, *93*, 94–95.

Sag, C. M., Wagner, S., & Maier, L. S. (2013). Role of oxidants on calcium and sodium movement in healthy and diseased cardiac myocytes. *Free Radical Biology & Medicine*, *63*, 338–49. doi:10.1016/j.freeradbiomed.2013.05.035

Schmidt, T., Zaib, F., Samson, S. E., Kwan, C.-Y., & Grover, A. K. (2004). Peroxynitrite resistance of sarco/endoplasmic reticulum Ca2+ pump in pig coronary artery endothelium and smooth muscle. *Cell Calcium*, *36*(1), 77–82. doi:10.1016/j.ceca.2003.12.002

Sharov, V., Dremina, E., Galeva, N., Williams, T. D., & Schoneich, C. (2006). Quantitative mapping of oxidation-sensitive cysteine residues in SERCA in vivo and in vitro by HPLC–electrospray-tandem MS: selective protein oxidation during biological aging. *Biochem. J*, *394*, 605–615. doi:10.1042/BJ20051214

Squier, T. C., & Bigelow, D. J. (2000). Protein oxidation and age-dependent alterations in calcium homeostasis. *Frontiers in Bioscience : A Journal and Virtual Library*, *5*, D504–26.

Stadtman, E. R. (1993). Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. *Annual Review of Biochemistry*, *62*, 797–821. doi:10.1146/annurev.bi.62.070193.004053

Stojanovic, M. O., Ziolo, M. T., Wahler, G. M., & Wolska, B. M. (2001). Anti-adrenergic effects of nitric oxide donor SIN-1 in rat cardiac myocytes. *American Journal of Physiology. Cell Physiology*, *281*(1), C342–9.

Suarez-Pinzon, W. L., Mabley, J. G., Strynadka, K., Power, R. F., Szabó, C., & Rabinovitch, A. (2001). An inhibitor of inducible nitric oxide synthase and scavenger of peroxynitrite prevents diabetes development in NOD mice. *Journal of Autoimmunity*, *16*(4), 449–55. doi:10.1006/jaut.2001.0507

Suarez-Pinzon, W. L., Szabó, C., & Rabinovitch, A. (1997). Development of autoimmune diabetes in NOD mice is associated with the formation of peroxynitrite in pancreatic islet beta-cells. *Diabetes*, *46*(5), 907–11.

Sutliff, R., Conforti, L., Weber, C. S., Kranias, E. G., & Paul, R. J. (2004). Regulation of the spontaneous contractile activity of the portal vein by the sarcoplasmic reticulum: evidence from the phospholamban gene-ablated mouse. *Vascular Pharmacology*, *41*, 197–204. doi:10.1016/j.vph.2004.11.004

Sutliff, R. L., Hoying, J. B., Kadambi, V. J., Kranias, E. G., & Paul, R. J. (1999). Phospholamban is present in endothelial cells and modulates endothelium-dependent relaxation: evidence from gene-ablated mice. *Circulation Research*, *84*, 360–364. doi:10.1161/01.RES.84.3.360

Suzuki, Y. J., & Ford, G. D. (1991). Inhibition of Ca (2+)-ATPase of vascular smooth muscle sarcoplasmic reticulum by reactive oxygen intermediates. *American Journal of Physiology- Heart and Circulatory Physiology*, *261*, H568–H574.

Terentyev, D., Viatchenko-Karpinski, S., Gyorke, I., Terentyeva, R., & Gyorke, S. (2003). Protein phosphatases decrease sarcoplasmic reticulum calcium content by stimulating calcium release in cardiac myocytes. *The Journal of Physiology*, *552*(Pt 1), 109–18. doi:10.1113/jphysiol.2003.046367

Thiyagarajan, M., Kaul, C. L., & Sharma, S. S. (2004). Neuroprotective efficacy and therapeutic time window of peroxynitrite decomposition catalysts in focal cerebral ischemia in rats. *British Journal of Pharmacology*, *142*(5), 899–911. doi:10.1038/sj.bjp.0705811

Touyz, R., & Schiffrin, E. (2004). Reactive oxygen species in vascular biology: implications in hypertension. *Histochemistry and Cell Biology*, *122*, 339–352. doi:10.1007/s00418-004-0696-7

Toyoshima, C. (2009). How Ca2+-ATPase pumps ions across the sarcoplasmic reticulum membrane. *Biochimica et Biophysica Acta*, *1793*(6), 941–6. doi:10.1016/j.bbamcr.2008.10.008

Toyoshima, C., Asahi, M., Sugita, Y., Khanna, R., Tsuda, T., & MacLennan, D. H. (2003). Modeling of the inhibitory interaction of phospholamban with the Ca2+ ATPase. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(2), 467–72. doi:10.1073/pnas.0237326100

Toyoshima, C., Nakasako, M., Nomura, H., & Ogawa, H. (2000). Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 A resolution. *Nature*, *405*, 647–655.

Trebak, M., Ginnan, R., Singer, H. A., & Jourd'heuil, D. (2010). Interplay between calcium and reactive oxygen/nitrogen species: an essential paradigm for vascular smooth muscle signaling. *Antioxidants & Redox Signaling*, *12*(5), 657–674.

Tsuji, T., Del Monte, F., Yoshikawa, Y., Abe, T., Shimizu, J., Nakajima-Takenaka, C., … Takaki, M. (2009). Rescue of Ca2+ overload-induced left ventricular dysfunction by targeted ablation of phospholamban. *American Journal of Physiology. Heart and Circulatory Physiology*, *296*(2), H310–7. doi:10.1152/ajpheart.00975.2008

Vandecaetsbeek, I., Trekels, M., De Maeyer, M., Ceulemans, H., Lescrinier, E., Raeymaekers, L., … Vangheluwe, P. (2009). Structural basis for the high Ca2+ affinity of the ubiquitous SERCA2b Ca2+ pump. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(44), 18533–8. doi:10.1073/pnas.0906797106

Ver Heyen, M., Heymans, S., Antoons, G., Reed, T., Periasamy, M., Awede, B., … Wuytack, F. (2001). Replacement of the muscle-specific sarcoplasmic reticulum Ca(2+)-ATPase isoform SERCA2a by the

nonmuscle SERCA2b homologue causes mild concentric hypertrophy and impairs contraction-relaxation of the heart. *Circulation Research*, *89*(9), 838–46.

Verboomen, H., Wuytack, F., De Smedt, H., Himpens, B., & Casteels, R. (1992). Functional difference between SERCA2a and SERCA2b Ca2+ pumps and their modulation by phospholamban. *The Biochemical Journal*, *286 ( Pt 2*, 591–5.

Verboomen, H., Wuytack, F., Van den Bosch, L., Mertens, L., & Casteels, R. (1994). The functional importance of the extreme C-terminal tail in the gene 2 organellar Ca(2+)-transport ATPase (SERCA2a/b). *The Biochemical Journal*, *303 ( Pt 3*, 979–84.

Viner, R. I., Ferrington, D. A., Williams, T. D., Bigelow, D. J., & Schöneich, C. (1999). Protein modification during biological aging: selective tyrosine nitration of the SERCA2a isoform of the sarcoplasmic reticulum Ca2+-ATPase in skeletal muscle. *The Biochemical Journal*, *340 ( Pt 3*, 657–69.

Viner, R. I., Hühmer, A. F., Bigelow, D. J., & Schöneich, C. (1996). The oxidative inactivation of sarcoplasmic reticulum Ca(2+)-ATPase by peroxynitrite. *Free Radical Research*, *24*(4), 243–59.

Viner, R. I., Williams, T. D., & Schoneich, C. (1999). Peroxynitrite modification of protein thiols: oxidation, nitrosylation and s-glutathilation of functionally important cysteine residue(s) in the sarcoplasmic reticulum Ca-ATPase. *Biochemistry*, *38*, 12408–12415.

Viner, R. I., Williams, T. D., & Schöneich, C. (1999). Peroxynitrite modification of protein thiols: oxidation, nitrosylation, and S-glutathiolation of functionally important cysteine residue(s) in the sarcoplasmic reticulum Ca-ATPase. *Biochemistry*, *38*(38), 12408–15.

Walia, M., Samson, S. E., Schmidt, T., Best, K., Kwan, C. Y., & Grover, A. K. (2003). Effects of peroxynitrite on pig coronary artery smooth muscle. *Cell Calcium*, *34*(1), 69–74. doi:10.1016/S0143-4160(03)00023-X

Walia, M., Samson, S. E., Schmidt, T., Best, K., Whittington, M., Kwan, C. Y., & Grover, A. K. (2003). Peroxynitrite and nitric oxide differ in their effects on pig coronary artery smooth muscle. *American Journal of Physiology. Cell Physiology*, *284*(3), C649–57. doi:10.1152/ajpcell.00405.2002

Wang, P., & Zweier, J. L. (1996). Measurement of nitric oxide and peroxynitrite generation in the postischemic heart. Evidence for peroxynitrite-mediated reperfusion injury. *The Journal of Biological Chemistry*, *271*(46), 29223–30.

Watanabe, A., Arai, M., Yamazaki, M., Koitabashi, N., Wuytack, F., & Kurabayashi, M. (2004). Phospholamban ablation by RNA interference increases Ca2+ uptake into rat cardiac myocyte sarcoplasmic reticulum. *Journal of Molecular and Cellular Cardiology*, *37*(3), 691–8. doi:10.1016/j.yjmcc.2004.06.009

Webb, R. C. (2003). Smooth muscle contraction and relaxation. *Advances in Physiology Education*, *27*(1-4), 201–6.

Wray, S., & Burdyga, T. (2010). Sarcoplasmic reticulum function in smooth muscle. *Physiological Reviews*, *90*(1), 113–78. doi:10.1152/physrev.00018.2008

Wu, K., Bungard, D., Lytton, J., & Lyt-, J. (2001). Regulation of SERCA Ca 2 + pump expression by cytoplasmic [ Ca 2 + ] in vascular smooth muscle cells Regulation of SERCA Ca 2 ∂ pump expression by cytoplasmic [ Ca 2 ∂ ] in vascular smooth muscle cells. *American Journal of Physiology. Cell Physiology*, *280*, C843–C851.

Xu, K. Y., Zweier, J. L., & Becker, L. C. (1997). Hydroxyl Radical Inhibits Sarcoplasmic Reticulum Ca2+-ATPase Function by Direct Attack on the ATP Binding Site. *Circulation Research*, *80*(1), 76–81. doi:10.1161/01.RES.80.1.76

Yin, X., Shan, Q., Deng, C., & Bourreau, J.-P. (2002). Effect of SIN-1 in rat ventricular myocytes: interference with beta-adrenergic stimulation. *Life Sciences*, *71*(3), 287–97.

Ying, J., Sharov, V., Xu, S., & Jiang, B. (2008). Cysteine-674 oxidation and degradation of sarcoplasmic reticulum Ca 2+ ATPase in diabetic pig aorta. *Free Radical Biology …*, *45*(6), 756–762. doi:10.1016/j.freeradbiomed.2008.05.029

Zalba, G., Beaumont, J., & José, G. S. (2000). Vascular oxidant stress: molecular mechanisms and pathophysiological implications. *Journal of Physiology …*, *56*(1), 57–64.

Zhang, P., Xu, X., Hu, X., van Deel, E. D., Zhu, G., & Chen, Y. (2007). Inducible nitric oxide synthase deficiency protects the heart from systolic overload-induced ventricular hypertrophy and congestive heart failure. *Circulation Research*, *100*(7), 1089–98. doi:10.1161/01.RES.0000264081.78659.45

Zhang, R., Zhao, J., Mandveno, A., & Potter, J. D. (1995). Cardiac troponin I phosphorylation increases the rate of cardiac muscle relaxation. *Circulation Research*, *76*(6), 1028–35.