Effects of Neuronal Nitric Oxide Synthase Signaling On Myocyte Contractile Function

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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2011

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ABSTRACT

Nitric Oxide (NO) has been shown to be a key regulator of myocyte contractile function. NO is produced via an enzyme, NO synthase (NOS). Within cardiac myocytes, NO is produced endogenously via two constitutively active NOS isoforms: Neuronal NOS (NOS1) and Endothelial NOS (NOS3). In disease, NO signaling becomes altered leading to contractile dysfunction. In order to understand the alterations caused by NO signaling in disease, it is imperative to determine the molecular mechanisms by which different NOS isoforms regulate contraction. At present, NOS3 has been associated with regulation of the β-adrenergic (β-AR) receptor signaling pathway with no regulation of basal contractile function. However, it remains unclear the role played by NOS1 in regulation of contractile function. Thus, the purpose of the studies presented herein was to determine how NOS1 modulates myocyte contractile function.

We first investigated NOS1’s modulation of myocyte contractile function by investigating the Force-Frequency Response (FFR) in isolated cardiac trabeculae from NOS1 Knockout (NOS1−/−) and Wild-Type (WT) mice. NOS1−/− trabeculae exhibited a blunted FFR and prolonged relaxation compared to WT. We next reaffirmed these findings in NOS1−/− and WT isolated cardiac myocytes. NOS1−/− myocytes exhibited decreased contractile function compared to WT myocytes. Selective inhibition of NOS1
in WT myocytes via S-Methyl-L-thiocitrulline (NOS1 inhibitor) produced contractile dysfunction similar to that seen in NOS1\(^{-/-}\) myocytes. Interestingly, the effect of SMLT was absent in phospholamban knockout (PLB\(^{-/-}\)) myocytes suggesting NOS1 promotes contractile function in part via regulation of a key excitation-contraction coupling (ECC) protein, phospholamban (PLB).

We next examined the mechanism by which NOS1 regulates PLB. NOS1\(^{-/-}\) myocytes exhibited decreased PLB phosphorylation (PLB-P) at the serine 16 PKA site compared to WT. To further investigate NOS1’s regulation of PLB, we examined a functional measure of PLB-P state: sarcoplasmic reticulum (SR) calcium (Ca\(^{2+}\)) load. NOS1\(^{-/-}\) myocytes exhibited decreased SR Ca\(^{2+}\) load compared to WT myocytes. We next determined if the effects of NOS1\(^{-/-}\) could be reversed by increasing serine16 PLB-P via \(\beta\)-AR receptor stimulation. NOS1\(^{-/-}\) myocytes exhibited a decreased functional response to \(\beta\)-AR receptor stimulation compared to WT myocytes. However, \(\beta\)-AR receptor stimulation abolished the prolonged relaxation observed in NOS1\(^{-/-}\) myocytes. These data suggest NOS1 regulates PLB via phosphorylation at the PKA site, serine16 and \(\beta\)-AR receptor stimulation can abolish the prolonged relaxation observed in NOS1\(^{-/-}\) myocytes.

NO can either signal in a cyclic-GMP dependent or cyclic-GMP independent manner. Therefore, we investigated the signaling pathway thru which NOS1 signals. ODQ, an inhibitor of NO stimulation of guanylate cyclase, had no effect in NOS1\(^{-/-}\), WT or WT+SMLT myocytes, which suggested NOS1 does not signal in a cyclic-GMP dependent manner. Conversely, FeTPPS, a peroxynitrite (ONOO\(^{-}\)) decomposition catalyst, produced contractile dysfunction and decreased serine16 PLB-P in WT myocytes, but was without effect in the NOS1\(^{-/-}\) or WT + SMLT groups. Taken together,
these data suggest NOS1 signals in a cyclic-GMP independent manner via formation of peroxynitrite ONOO⁻.

An altered balance of reactive oxygen and nitrogen species (i.e. nitroso-redox imbalance) has been observed in NOS1⁻/⁻ myocytes, and has been shown to play a role in the contractile dysfunction present in NOS1⁻/⁻ myocytes. Therefore, we next determined if a novel superoxide scavenger, EMEPO, was able to correct this nitroso-redox imbalance, thus improving contraction in NOS1⁻/⁻ myocytes. EMEPO normalized superoxide (O₂⁻) and NO levels in NOS1⁻/⁻ myocytes to those observed in WT myocytes. These data suggest EMEPO restored the nitroso-redox balance in NOS1⁻/⁻ myocytes via decreasing O₂⁻ levels and increasing NO levels. Furthermore, we investigated the effect of EMEPO on myocyte contractile function. EMEPO increased myocyte contraction and accelerated the rate of relaxation at various stimulation frequencies. EMEPO improved the SR leak/load relationship in NOS1⁻/⁻ myocytes, suggesting increased RyR activity. In addition, EMEPO increased serine 16 PLB-P in NOS1⁻/⁻ myocytes. Interestingly, EMEPO’s effects on contraction in NOS1⁻/⁻ myocytes were significantly greater than those of a O₂⁻ scavenger or a NO donor. In addition, EMEPO potentiated the functional response of NOS1⁻/⁻ myocytes to β-AR receptor stimuli. These data suggest EMEPO is a novel O₂⁻ scavenger that improves contractile function via regulation of SR Ca²⁺ handling.

The results presented herein provide evidence that NOS1 signaling leads to positive inotropy and lusitropy in the heart. In addition, it appears EMEPO, a novel O₂⁻ scavenger, can restore nitroso-redox balance under conditions of oxidative stress.
Furthermore, the present work may have therapeutic implications for the treatment of cardiovascular disease, a pathological state in which NO signaling is altered.
DEDICATED TO THE MEMORIES OF RUTH AND JENNIFER TRAYNHAM
ACKNOWLEDGMENTS

After five long years of graduate school, I have finally reached the end of this long tenuous road. At this time, I feel it is prudent to thank the many people that have helped me along this journey. I would like to begin by thanking my advisor, Dr. Mark Ziolo. Over the years, Mark has been a constant source of guidance and encouragement. Mark has shown me how to become apt at experimental design and scientific thinking. After working in Mark’s lab, I consider him a valued friend and colleague. I also would like to thank both the former and current members of the Ziolo lab for their help and support over the years. Dr. Mark Kohr, Mark’s first graduate student, was and remains to this day an invaluable resource of knowledge when it comes to scientific thought and data interpretation. Dr. Honglan Wang has had many spirited conversations with me about experiments. Furthermore, Honglan has been critical to the completion of my projects. In addition, Lifei Tang, Steve Roof, Xin Huang, Dominic Catalano and Debra Wheeler have also aided in the completion of experiments presented herein. During my time at OSU, many faculty members have assisted me. I would like to thank the late Dr. Allen Yates for seeing my scientific potential and admitting me into IBGP. Dr. Yates always took time to talk to me about research and life in general. He will be dearly missed. I would like to thank Drs. Brandon Biesiadecki, Jonathan Davis and Erick Villamena for their
invaluable aid in experimental design. I would like to thank my dissertation advisory committee: Drs. Billman, Janssen and Gumina for their expertise in their respective areas of research and their guidance during this journey.

My graduate career has been one of the most trying periods of my life with the untimely passing of my sister, Jennifer Traynham and my grandmother, Ruth Traynham. With this said, I could not have gotten through these unfortunate events without the love and support of the people near and dear to me. My mother and father, Joan and James Traynham, have been amazing. I am truly blessed to have parents that are there for me through the good times and the bad. My sister, Deborah Vaughn, has always been there for me, willing to provide a laugh when I need one or an open ear when I need to talk. My friends (spanning from high school to Michigan State to Ohio State and beyond) have also proven invaluable during this journey. They know me better than I know myself, and they mean the world to me. During this journey, I have had many ups and downs along the way. However, I overcame it all and I stand prepared to take the next step. Without the love of my friends and family, I would not be the scientist I have become but more importantly, I would not be the man I have grown to be.
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ix
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1. *Emphasis*: Cell, Organ Systems, and Integrative Biology

2. Cardiac Physiology
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<td>AP</td>
<td>Action potential</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AV</td>
<td>Atrioventricular</td>
</tr>
<tr>
<td>β-AR</td>
<td>β-adrenergic</td>
</tr>
<tr>
<td>BH₄</td>
<td>Tetrahydrobiopterin</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>[Ca²⁺]ᵢ</td>
<td>Intracellular Ca²⁺ concentration</td>
</tr>
<tr>
<td>CAMKII</td>
<td>Calcium/calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>CICR</td>
<td>Ca²⁺ induced Ca²⁺ release</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic-AMP</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic-GMP</td>
</tr>
<tr>
<td>DMPO</td>
<td>5,5-dimethylpyrroline N-oxide</td>
</tr>
<tr>
<td>ECC</td>
<td>Excitation-contraction coupling</td>
</tr>
<tr>
<td>EDRF</td>
<td>Endothelium derived relaxing factor</td>
</tr>
<tr>
<td>EMEPO</td>
<td>2-(2-ethoxy-2-oxoethyl)-2-(ethoxycarbonyl)-3,4-dihydro-2H-pyrrole 1-oxide</td>
</tr>
<tr>
<td>EPR</td>
<td>Electron paramagnetic resonance</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
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<td>--------------</td>
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<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FDAR</td>
<td>Frequency dependent acceleration of relaxation</td>
</tr>
<tr>
<td>FeTPPS</td>
<td>5,10,15,20-tetrakis-(4-sulfonatophenyl)porphyrinato-iron(III)</td>
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<tr>
<td>FFR</td>
<td>Force-Frequency response</td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin mononucleotide</td>
</tr>
<tr>
<td>$G_s$</td>
<td>Stimulatory G-protein</td>
</tr>
<tr>
<td>HF</td>
<td>Heart failure</td>
</tr>
<tr>
<td>I/R</td>
<td>Ischemia-Reperfusion Injury</td>
</tr>
<tr>
<td>$I_{Ca,L}$</td>
<td>L-type Ca$^{2+}$ current</td>
</tr>
<tr>
<td>$I_{Ca,T}$</td>
<td>T-type Ca$^{2+}$ current</td>
</tr>
<tr>
<td>$I_f$</td>
<td>“Funny current”</td>
</tr>
<tr>
<td>$I_{K1}$</td>
<td>Resting membrane potential potassium current</td>
</tr>
<tr>
<td>$I_{Kr}$</td>
<td>Delayed rectifier potassium current</td>
</tr>
<tr>
<td>$I_{Ks}$</td>
<td>Delayed rectifier potassium current</td>
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<tr>
<td>MENO</td>
<td>Methyl-ester Nitrooxide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NCX</td>
<td>Sodium-calcium exchanger</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>NO synthase</td>
</tr>
<tr>
<td>NOS1</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NOS1$^{-/-}$</td>
<td>NOS1 knockout</td>
</tr>
<tr>
<td>NOS2</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>NOS3</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>$\text{O}_2^-$</td>
<td>Superoxide</td>
</tr>
<tr>
<td>ODQ</td>
<td>1H-[1,2,4] Oxadiazolo [4,3-a] quinoxalin-1-one</td>
</tr>
<tr>
<td>ONOO$^{-}$</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>PBN</td>
<td>$\alpha$-phenyl-tert-butyl-nitrone</td>
</tr>
<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
</tr>
<tr>
<td>PKA</td>
<td>cAMP-dependent protein kinase A</td>
</tr>
<tr>
<td>PKG</td>
<td>cGMP-dependent protein kinase G</td>
</tr>
<tr>
<td>PLB</td>
<td>Phospholamban</td>
</tr>
<tr>
<td>PLB$^{-/-}$</td>
<td>PLB knockout</td>
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<td>PLB-P</td>
<td>PLB phosphorylation</td>
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<tr>
<td>PP</td>
<td>Protein phosphatase</td>
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<tr>
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<td>Resting Cell Length</td>
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<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
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<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine receptor</td>
</tr>
<tr>
<td>SA</td>
<td>Sinoatrial</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarcoplasmic-endoplasmic reticulum Ca$^{2+}$ ATPase</td>
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<tr>
<td>SMLT</td>
<td>S-Methyl-L-thiocitrulline</td>
</tr>
<tr>
<td>SNAP</td>
<td>S-Nitro-N-acetyl-DL-penicillamine</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SR</td>
<td>Sarco-plasmic reticulum</td>
</tr>
<tr>
<td>TnC</td>
<td>Troponin C</td>
</tr>
<tr>
<td>TnI</td>
<td>Troponin I</td>
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</table>
TnT  Troponin T
WT  Wild-Type
XO  Xanthine oxidoreductase
CHAPTER 1

INTRODUCTION

1.1 General Introduction

The heart in combination with blood vessels constitute the cardiovascular system. The cardiovascular system is responsible for a multitude of tasks ranging from transport of de-oxygenated blood and metabolic waste products out of the body to efficient delivery of blood, oxygen, hormones, and nutrients to the periphery. Although much is known about the cardiovascular system as a whole, many questions remain with respect to the heart. The heart’s primary function is to contract in an effort to pump blood to the rest of the body. The mechanism that regulates the heart’s pumping function is referred to as, “Excitation-Contraction coupling” (ECC). Over time, the heart has developed several signaling mechanisms to tightly regulate ECC, namely the β-adrenergic (β-AR) receptor and Nitric Oxide (NO) signaling pathways. Although the heart is tightly regulated, it is possible for these signaling pathways to become dysregulated, transitioning the heart to a disease state. Thus, it is imperative to delineate how these signaling processes modulate myocyte contraction in the healthy myocardium in an effort to understand the heart’s progression to a disease state.
1.2 Excitation-Contraction Coupling

ECC is the process responsible for regulation of cardiac contractility (Bers, 2002; Bers, 2001). ECC is initiated by an action potential (AP) that enters the sarcolemmal plasma membrane, resulting in myocyte contraction (Figure 1). In the forthcoming sections, the specific steps involved in ECC will be detailed in an effort to provide sufficient background for the studies presented herein.

1.2.1 Cardiac AP

ECC is initiated by electrical activity that travels from nodal cells to the atria and finally to the ventricles, resulting in myocyte contraction. This electrical activity has been termed, the cardiac AP. The cardiac AP is initiated when pacemaker cells in the Sinoatrial (SA) node become depolarized. This wave of depolarization is then carried via gap junctions to the atria, resulting in atrial depolarization. This AP is carried to the ventricles via the atrioventricular (AV) node, the Bundle of His, and finally Purkinje fibers resulting in ventricular depolarization. It is important to note that the AV node serves to slow the wave of depolarization in an effort to allow the atria sufficient time to complete ejection of blood into the ventricles prior to ventricular depolarization. The cardiac AP differs greatly as this electrical activity travels throughout the heart, thus resulting in different cell types exhibiting distinct AP waveforms. This can be attributed to different ion channel gating properties and expression in each cell type (Nerbonne & Kass, 2005).

Nodal cells from the SA node exhibit an AP waveform and ion channel conductances that are very unique (Figure 2). SA Nodal cell AP waveforms are divided
into the following phases: Phase 4: Slow Depolarization; Phase 0: AP Upstroke; Phase 3: Repolarization. Phase 4 is referred as the “Slow Depolarization” because of the roles played by potassium channels and a unique channel current termed, “the funny current” ($I_f$) (Amin et al., 2009). $I_f$ is unique in that it opens at hyperpolarized membrane potentials, therefore conducting an inward depolarizing sodium current. In addition to $I_f$ current, membrane permeability for potassium decreases over time. Taken together, these two factors are largely responsible for the “Slow Depolarization” observed in Phase 4. Phase 0 is largely governed by Ca$^{2+}$ channels, specifically the L-type ($I_{Ca,L}$) and T-type ($I_{Ca,T}$) Ca$^{2+}$ channels. Both of these Ca$^{2+}$ channels exhibit increased inward conductance, thereby making the cell more positive leading to the upstroke of the AP (Lakatta et al., 2010). In order for the SA nodal cell to partially repolarize, potassium channels ($I_{kr}$, $I_{ks}$) increase their permeability. Due to the fact $I_{K1}$ is absent in nodal cells, these cells are never able to fully repolarize like other cardiac cell types (i.e. ventricular cells), thus allowing these cells to spontaneously excite. Therefore, the SA node APs are referred to as “pacemaker potentials”, thus, the SA node is a key regulator of heart rate (Amin et al., 2009).

In contrast, APs in ventricular myocytes are primarily regulated by Na$^+$, K$^+$, and Ca$^{2+}$. Furthermore, the cardiac AP can be separated into four phases based on the fluxes of the aforementioned ions: Phase 0: Rapid Depolarization; Phase 1: Transient Repolarization; Phase 2: The Plateau Phase; Phase 3: Rapid Repolarization; Phase 4: Resting Membrane Potential (Figure 2). Phase 0 is initiated when Na$^+$ channels open while potassium channels close ($I_{K1}$) resulting in an inward Na$^+$ current responsible for the upstroke of the AP. Shortly after the upstroke of the AP, potassium channels open
resulting in a transient period of repolarization termed, Phase 1. Phase 2 follows shortly thereafter with the opening of $I_{\text{Ca,L}}$ channels, resulting in a prolonged influx of $\text{Ca}^{2+}$ into the cell. $I_{\text{Ca,L}}$ channel inactivation is slow in nature, therefore these channels stay open for extended periods of time resulting in the plateau observed in the ventricular AP. Ventricular cells repolarize when potassium channels ($I_{\text{K}}, I_{\text{Kr}}$) open (Phase 3) returning the cell to its resting membrane potential (Phase 4). The electrical events that comprise the cardiac AP lead to the formation of the $[\text{Ca}^{2+}]_i$ transient ultimately leading to myocyte contraction.

### 1.2.2 $[\text{Ca}^{2+}]_i$ Transient

The formation of the $[\text{Ca}^{2+}]_i$ transient begins when $I_{\text{Ca,L}}$ channels open during ECC resulting in an influx of calcium ions into the myocyte. This “trigger calcium” binds to ryanodine receptors (RyRs) located on the SR, the main source of $\text{Ca}^{2+}$ within the myocyte, resulting in increased SR $\text{Ca}^{2+}$ efflux (Bers, 2002; Bers, 2001). Thus, this process is defined as calcium-induced-calcium-release (CICR). It is important to note that SR $\text{Ca}^{2+}$ load is a key determinant of myocyte contractile function (Bers, 2002). Furthermore, the amount of calcium released during CICR has been shown to be regulated by both trigger $\text{Ca}^{2+}$ and SR $\text{Ca}^{2+}$ load. Specifically, increases in trigger $\text{Ca}^{2+}$ and SR $\text{Ca}^{2+}$ load both lead to proportional increases in fractional release from the SR (Bassani et al., 1995). Calcium released during CICR makes possible myocyte contraction.

The decline of the $[\text{Ca}^{2+}]_i$ is largely regulated by the sarcoplasmic-endoplasmic reticulum $\text{Ca}^{2+}$ ATPase (SERCA)/PLB complex and sodium-calcium exchanger (NCX).
SERCA is a Ca\(^{2+}\) ATPase that pumps 2 Ca\(^{2+}\) ions into the SR per adenosine tri-phosphate (ATP) utilized (Reddy et al., 1996; Brini & Carafoli, 2009). In large mammals such as rabbits and humans, SERCA accounts for \(~ 70\%) of the [Ca\(^{2+}\)]\(_i\) decline while in small rodents such as mice and rats SERCA is responsible for \(~ 92\%) of [Ca\(^{2+}\)]\(_i\) decline (Bassani et al., 1994; Bers, 2001). SERCA, itself, can be regulated by a phospho-protein, PLB. In its native state, PLB is a pentameric protein that remains in close association with the SERCA pump, thereby inhibiting pump function (James et al., 1989; Thomas et al., 1998). Specifically, PLB in its dephosphorylated state decreases SERCA’s affinity for Ca\(^{2+}\), effectively serving as a “brake” on Ca\(^{2+}\) influx into the SR (Brittsan & Kranias, 2000). PLB’s inhibition of SERCA pump function can be relieved via phosphorylation at either its cAMP-dependent protein kinase A (PKA) site (serine 16) or its calcium/calmodulin-dependent protein kinase II (CAMKII) site (threonine 17), thereby increasing SR Ca\(^{2+}\) load (Simmerman et al., 1986). Furthermore, PKA-dependent phosphorylation of PLB has been shown to precede CAMKII phosphorylation during β-AR receptor stimulation (Wegener et al., 1989). As with all phospho-proteins, PLB can be dephosphorylated by a class of enzymes called protein phosphatases (PPs). Specifically, PP1 has been shown to account for \(~ 70\%) of serine 16 phosphatase activity with PP2A accounting for the remaining \(~ 30\%) (MacDougall et al., 1991). Similarly, PP1 was also found to be the major PP responsible for dephosphorylation of threonine 17 (\(~ 74\%)). NCX also plays a key role in the decline of [Ca\(^{2+}\)]\(_i\) transient. NCX is a membrane associated antiporter that in the forward mode pumps 3 Na\(^{+}\) ions into the cell for every 1 Ca\(^{2+}\) ion pumped out of the cell (Bers & Ginsburg, 2007). Similar to the SERCA pump, NCX’s contribution to [Ca\(^{2+}\) decline]\(_i\) differs across species. Specifically
in large mammals, NCX accounts for ~ 30% of the [Ca\(^{2+}\)]\(_i\) decline while in small rodents NCX is responsible for ~ 7% of [Ca\(^{2+}\)]\(_i\) decline (Bers, 2001). Interestingly, the mitochondria have also been suggested to play a role in [Ca\(^{2+}\)]\(_i\) decline (Dedkova & Blatter, 2008). Specifically in small and large mammals, mitochondrial Ca\(^{2+}\) uptake has been estimated at 1-2 % of total [Ca\(^{2+}\)]\(_i\) decline (Bers et al., 1996). Therefore, mitochondrial Ca\(^{2+}\) uptake is generally considered to play a minor role in [Ca\(^{2+}\)]\(_i\) decline. Taken together, the aforementioned Ca\(^{2+}\) sequesteration methods play a key role in myocyte relaxation.

1.2.3 Myocyte Contraction

The electrical activity that produces the Cardiac AP ultimately leads to ventricular depolarization and therefore myocyte contraction. Myocyte contraction occurs through the coordinated action of tropomyosin, troponin, and the actin and myosin filaments (Kobayashi & Solaro, 2005; Kobayashi et al., 2008). Troponin is a protein complex made up of three subunits: troponin I (TnI), troponin C (TnC), and troponin T (TnT). At low [Ca\(^{2+}\)]\(_i\), the thin and thick filaments are blocked from interaction by TnI and tropomyosin. As [Ca\(^{2+}\)]\(_i\) increases during ECC, TnC binds Ca\(^{2+}\) ions resulting in a conformational change in the troponin complex. Specifically, TnI dissociates from the actin filament and comes into tight association with TnC, leading to the movement of tropomyosin from actin’s myosin-binding site. These two events allow myosin to interact with actin leading to force production and myocyte shortening in the presence of ATP. As [Ca\(^{2+}\)]\(_i\) declines, myosin dissociates from actin resulting in myocyte relaxation.
1.3 Regulation of ECC

Over time, the heart has developed many mechanisms by which ECC can be modulated in response to external cues to increase cardiac output. Specifically, the FFR, β-AR receptor signaling and reactive nitrogen species (RNS) have all been shown to be key regulators of ECC.

1.3.1 FFR

Myocyte contraction can be modulated by frequency. This relationship is defined as the FFR. Specifically, FFR refers to the fact that as the rate of stimulation increases, Ca\(^{2+}\) transient amplitudes and therefore myocyte contraction increase (i.e. positive FFR) (Stull et al., 2002; Endoh, 2004). Positive FFRs have been correlated with increased SR Ca\(^{2+}\) load, and therefore increased SERCA Ca\(^{2+}\) uptake (Pieske et al., 1999). In addition, increased I\(_{\text{Ca,L}}\) current has indirectly been shown to play a role in the positive FFR (Stemmer & Akera, 1986). It is important to note that in some species such as rabbit, the FFR can become negative at higher frequencies. This has been attributed to core hypoxia in muscle preparations, altered Ca\(^{2+}\) handling, and alterations in intracellular pH (Morii et al., 1996; Layland & Kentish, 1999; Antoons et al., 2002; Raman et al., 2006). Similarly, increases in frequency can also increase the rate of relaxation in a phenomena known as force-dependent acceleration of relaxation (FDAR). The mechanism governing FDAR has been yet to be fully elucidated. However, it has been suggested that FDAR is in part regulated, by CAMKII-dependent stimulation of SR Ca\(^{2+}\) transport and decreased myofilament Ca\(^{2+}\) sensitivity (DeSantiago et al., 2002; Varian & Janssen, 2007).
together, FFR and FDAR exhibit functional effects similar to those observed in the β-AR receptor signaling pathway.

1.3.2 β-adrenergic receptor signaling

β-AR receptor signaling is a key regulator of ECC (Bers & Ziolo, 2001). The primary receptors involved in this pathway are β1 and β2. In the heart, β1 receptors comprise 75-80% of all β-AR receptors while β2 receptors account for roughly 20% (Rockman et al., 2002). β-AR receptor signaling is initiated when a receptor agonist (i.e. catecholamine) binds to a β-AR receptor, leading to activation of a G-protein coupled to the receptor. The primary G-protein associated with β-AR receptors is stimulatory G-protein (Gs). When active, Gs activates adenylate cyclase, thereby increasing cyclic AMP (cAMP) production. cAMP production leads to activation of PKA. In the heart, PKA phosphorylates a variety of ECC proteins including I_{Ca,L}, PLB, TnI, and others (Salazar et al., 2007). PKA-dependent phosphorylation of these ECC proteins produces many vital alterations in the contractile state of the myocyte. Upon PKA-mediated phosphorylation, I_{Ca,L} current increases. The junctional SR, which is in close proximity to these I_{Ca,L} channels, exhibit increased SR Ca^{2+} cycling. This increased cycling is seen functionally as an increase in Ca^{2+} transient amplitude, and is a direct result of PLB phosphorylation. Phosphorylation of PLB via β-AR signaling pathway occurs at its PKA-dependent phosphorylation site (serine 16) resulting in dissociation of PLB from SERCA and an increase in SERCA’s affinity for Ca^{2+}. This allows the SR to sequester Ca^{2+} at a faster rate (i.e. increase the rate of relaxation). In addition, SR Ca^{2+} load increases, making more Ca^{2+} available to increase myocyte contraction. Lastly, PKA modulates the troponin complex through phosphorylation of TnI, which also increases the rate of relaxation. In
summary, $I_{Ca,L}$ channel phosphorylation (i.e. increased $I_{Ca,L}$) and increased Ca$^{2+}$ transients (via PLB phosphorylation) increase contractility (i.e. positive inotropy) in the cardiac myocyte. In addition, the relaxation rate is increased (i.e. positive lusitropy) due to both TnI and PLB phosphorylation. Thus, the net results of β-AR receptor signaling are positive inotropic and lusitropic effects in the heart.

1.4. Reactive Nitrogen Species

Another key regulator of both ECC and the β-AR signaling pathway is the NO signaling pathway (Ziolo, 2008; Ziolo et al., 2008). NO is a highly diffusible gas that is largely recognized as the endothelium-derived relaxing factor (EDRF) (Palmer et al., 1987). In addition, NO has been shown to play key roles in synaptic transmission, immunological responses, and retention of memory (Mayer & Hemmens, 1997). Endogenous NO is produced via NOS in a two step oxidation of L-arginine to L-citrulline, forming N$^G$-hydroxy-L-arginine as a reaction intermediate (Stuehr et al., 1991; Andrew & Mayer, 1999). NO synthase functions as a dimer composed of two identical monomers. Each monomer consists of a reductase and oxygenase domain. The reductase domain consists of binding sites for nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN) while the oxygenase consists of binding sites for oxygen, L-arginine, tetrahydrobiopterin (BH$_4$) and heme. NOS also has a calmodulin binding site, referred to as the “latch domain” that lies between the oxygenase and reductase domains. In the presence of Ca$^{2+}$, the reductase domain via FAD and FMN transfers electrons from the oxidation of NADPH to the oxygenase domain. Specifically, calmodulin binds to the “latch domain” and transfers
electrons from FAD and FMN to the haem moiety of the oxygenase domain, thus allowing the oxygenase domain to bind oxygen leading to the formation of NO and \( L \)-citrulline (Su et al., 1995; Fleming & Busse, 1999). Due to the fact the reductase domain is unable to transfer electrons to the haem moiety of the oxygenase domain in the absence of \( Ca^{2+} \), NOS is considered a calcium-dependent enzyme (Busse & Mulsch, 1990).

NO has been shown to be a potent signaling molecule. NO can signal via either the cyclic GMP (cGMP) or cGMP independent pathways. In the cGMP-dependent signaling pathway, NO activates soluble guanylate cyclase, thereby increasing cGMP levels. This increase in cGMP levels leads to the activation of cGMP-dependent protein kinase G (PKG) or cGMP-regulated phosphodiesterases (PDE; cGMP-stimulated: PDE2; cGMP-inhibited: PDE3) leading to regulation of a variety of protein targets including \( I_{Ca,L} \), TnI, and RyR (Layland et al., 2002; Yang et al., 2007; Ziolo et al., 2008). NO can also signal via a cGMP independent pathway. This pathway has been primarily associated with a protein modification called S-nitrosylation, which is the addition of a nitrosyl group to a free thiol residue. Furthermore, cGMP independent signaling can also occur when NO reacts with various reactive oxygen species (ROS) to form new RNS such as peroxynitrite. Due to the fact NO can form other RNS and is able to signal via two distinct pathways, it is not surprising that NO has been shown to exhibit different effects on various ECC proteins. For example, NO has been reported to both increase and decrease \( I_{Ca,L} \) (Ono & Trautwein, 1991; Campbell et al., 1996; Sun et al., 2006). Interestingly, another caveat that has come to light with regard to these differential effects is the concept of NOS localization within the myocyte (Barouch et al., 2002; Ziolo & Bers, 2003).
1.4.1 Endogenous NO production

In recent years, a role for NOS has been elucidated in the heart (Kelly et al., 1996). Within cardiac myocytes, three isoforms of NOS are expressed. NOS1 and NOS3 are constitutively expressed while Inducible NOS (NOS2) is expressed due to environmental cues (i.e. cytokine production). NOS1 and NOS3 are calcium-dependent enzymes while NOS2 is calcium-independent (Cho et al., 1992). In addition, NOS1 and NOS3 produce low levels of NO while NOS2 produce high levels of NO. Interestingly, these NOS isoforms have been shown to produce differential effects on myocyte contraction, which may be attributable to distinct sub-cellular localization within the myocyte and different protein targets and levels of NO production.

1.4.2 NOS1 signaling

NOS1 was first identified in the brain (Knowles et al., 1989). However, NOS1 has been identified in cardiac myocytes. Specifically, NOS1 has been shown to be localized to the SR and co-immunoprecipitate with RyR (Xu et al., 1999; Barouch et al., 2002). Due to its sub-cellular localization, it was hypothesized that NOS1 was able to regulate myocyte contraction (Figure 5). In order to study NOS1’s effect on contraction, a NOS1−/− mouse was generated (Huang et al., 1993).

Since the development of NOS1−/− mice, many studies have investigated the role of NOS1 signaling in regulation of myocyte contraction. NOS1−/− myocytes have been shown to exhibit decreased basal and β-AR receptor stimulated myocyte contraction while relaxation is prolonged relative to control (Barouch et al., 2002; Khan et al., 2003).
Conversely, basal and β-AR receptor stimulated contraction have also been shown to be increased in NOS1−/− myocytes (Ashley et al., 2002; Sears et al., 2003). Taken together, these studies suggest that NOS1 can modulate ECC. However, it is unclear why differential contractile effects have been observed in NOS1−/− myocytes. It is possible this is due to differences in experimental conditions (i.e. temperature, frequency, muscle preparation). Thus, further studies are needed to confirm these hypotheses.

Although prior studies have observed seemingly divergent NOS1 mediated contractile effects, it is intriguing that all of these studies have observed prolonged relaxation in NOS1−/− myocytes, thereby suggesting PLB as an important protein target of the NOS1 signaling pathway (Ashley et al., 2002; Barouch et al., 2002; Khan et al., 2003; Sears et al., 2003; Zhang et al., 2008). PLB, in its native state, is capable of reducing SERCA’s affinity for Ca2+, therefore decreasing SR Ca2+ load and prolonging relaxation. When PLB is phosphorylated, this inhibition is relieved and SR Ca2+ uptake is increased. NOS1−/− myocytes exhibit decreased PLB phosphorylation (Zhang et al., 2008). Furthermore, the ratio of SERCA/PLB is increased in NOS1−/− myocytes (i.e. increased SERCA expression; decreased PLB expression) (Khan et al., 2003). With this said, it is not surprising that SR Ca2+ stores were also decreased in NOS1−/− myocytes (Khan et al., 2003; Gonzalez et al., 2007). Taken together, it appears PLB is a protein target of NOS1 derived NO. However, the identity of the NO-derived signaling molecule which regulates PLB has yet to be determined. In addition, the mechanism by which this NO-derived signaling molecule regulates PLB remains to be determined. Therefore, further studies will be needed to address these topics.
NOS1 has also been shown to modulate RyR. Specifically, NO via nitrosylation (i.e. the addition of a NO group to free thiol residues) has been shown to increase RyR open probability, thereby increasing SR Ca\(^{2+}\) release (Stoyanovsky et al., 1997). In NOS1\(^{-/-}\) myocytes, RyR S-nitrosylation levels are decreased relative to control (Gonzalez et al., 2007; Wang et al., 2010). Furthermore, this decrease in RyR S-nitrosylation translated into decreased RyR channel function (i.e. decreased open probability, reduced Ca\(^{2+}\) spark frequency and amplitude, decreased leak/load relationship) (Wang et al., 2010). Interestingly, RyR levels and I\(_{\text{Ca,L}}\) are increased in NOS1\(^{-/-}\) myocytes (Sears et al., 2003; Gonzalez et al., 2007). In ECC, RyR fractional release is graded based on “trigger calcium” and SR Ca\(^{2+}\) load (Bassani et al., 1995). Therefore, it is possible that these compensatory changes occur in an effort to increase contraction in the myocyte via increased SR Ca\(^{2+}\) release and ultimately increased SR Ca\(^{2+}\) load.

1.4.3 NOS3 signaling

NOS3 was first identified in the vasculature. Recently, NOS3 has been shown to be expressed in cardiac myocytes, and localized to a sarcolemmal micro-domain, the caveolae in tight association with caveolin-3 (Balligand et al., 1995; Feron et al., 1998; Barouch et al., 2002). Furthermore, it was hypothesized that due to its localization in caveolae in close approximation with I\(_{\text{Ca,L}}\) and aspects of the β-AR receptor signaling pathways, NOS3-derived NO may exert effects on contractile function (Figure 5) (Rybin et al., 2000; Balijepalli et al., 2006; George & Pitt, 2006). In an effort to study the effect of NOS3 on cardiac function, NOS3\(^{-/-}\) mouse have been generated (Huang et al., 1995).
Unlike NOS1, NOS3 primarily regulates β-AR receptor stimulated contraction with no effect on basal contraction (Barouch et al., 2002; Khan et al., 2003; Wang et al., 2008c). For example, NOS3\(^{+/−}\) myocytes exhibit an increased functional response to β-AR receptor stimuli compared to control myocytes. Interestingly, mice that overexpress NOS3 have been shown to exhibit a decreased functional response to β-AR receptor stimuli (Brunner et al., 2001; Massion et al., 2004). In addition, NOS3\(^{−/−}\) myocytes had an increased incidence of arrhythmogensis during β-AR receptor stimuli, observed as aftercontractions and early after depolarizations (EADS). Ablation of NOS3 has also been shown to promote hypertrophy (Wenzel et al., 2007). Taken together, these studies suggest that NOS3-derived NO 1) may be cardioprotective via regulation of hypertrophic signaling and 2) prevention of arrhythmia through buffering the β-AR receptor response.

A protein target that has been identified to play a role in this NOS3 mediated regulation of β-AR receptor functional response is I\(_{Ca,L}\). Specifically, NOS3\(^{+/−}\) myocytes exhibit increased I\(_{Ca,L}\) during β-AR receptor stimuli. The mechanism by which NOS3 regulates I\(_{Ca,L}\) has been shown to be quite complex due to compartmentalized signaling (Wang et al., 2009). Previous studies have demonstrated that phosphodiesterase (PDE) 5, a primary regulator of cGMP levels, is able to regulate basal and β-AR receptor stimulated I\(_{Ca,L}\) (Ziolo et al., 2003). With this said, it is not surprising that inhibition of PDE5 decreased myocyte shortening, Ca\(^{2+}\) transients and I\(_{Ca,L}\) while stimulation of guanylate cyclase only decreased I\(_{Ca,L}\) in WT myocytes. Interestingly, localization of PDE5 to z-bands is lost in NOS3\(^{−/−}\) myocytes (Takimoto et al., 2005a). Specifically in agreement with Takimoto et. al., inhibition of PDE5 did not lead to anti-adrenergic effects in NOS3\(^{−/−}\) myocytes while stimulation of guanylate cyclase led to decreased
myocyte shortening, \( \text{Ca}^{2+} \) transients and \( I_{\text{Ca,L}} \). Taken together, it appears NOS3 mediated regulation of \( \beta \)-AR receptor stimulated \( I_{\text{Ca,L}} \) is based largely on PDE5 mediated regulation of cGMP and therefore, compartmentalized signaling. However when NOS3 is disrupted, PDE5 becomes mislocalized, leading to diffuse effects of cGMP and ultimately the loss of the compartmentalized effect of PDE5 on \( I_{\text{Ca,L}} \). It is important to note that NOS3 may not regulate \( I_{\text{Ca,L}} \) solely via the cGMP-dependent signaling pathway. A prior study has shown that female NOS3\(^{-/-}\) hearts exhibit decreased \( I_{\text{Ca,L}} \) compared to male NOS3\(^{-/-}\) due to increased S-nitrosylation of the \( \alpha \)-subunit of \( I_{\text{Ca,L}} \) (Sun et al., 2006).

In summary, it appears NOS3-derived NO is a key regulator of \( I_{\text{Ca,L}} \), and it is possible NOS3 may regulate \( I_{\text{Ca,L}} \) via both the cGMP-dependent and cGMP-independent signaling pathways.

NOS3 has also been shown to regulate potassium (\( K^+ \)) channels (Bai et al., 2005). Specifically, NOS3-derived NO increased a portion of the delayed rectifier current, \( I_{\text{Ks}} \). Furthermore, NOS3-derived NO led to the shortening of the cardiac AP and inactivation of \( I_{\text{Ca,L}} \). Due to the fact \( K^+ \) and \( \text{Ca}^{2+} \) are both vital determinants of AP duration, it possible that this data provides further evidence for NOS3’s role in prevention of arrhythmogenesis.

Another protein target regulated by NOS3 is the myofilament. Specifically, NOS3-derived NO increased TnI phosphorylation, leading to a decrease in myofilament \( \text{Ca}^{2+} \) sensitivity (Kaye et al., 1999). Taken together, it appears NOS3-derived NO is able to regulate TnI and \( I_{\text{Ks}} \), in addition to \( I_{\text{Ca,L}} \).

### 1.4.4. NOS2 signaling
NOS2 was first identified in macrophages and has since been shown to be expressed in cardiac myocytes (Lowenstein et al., 1992; Balligand et al., 1994). NOS2 is a cytosolic protein that is induced during immunological responses (Schulz et al., 1992). In myocytes, induction of NOS2 decreased both basal and β-AR receptor stimulated contractile function, which was reversible with selective NOS2 inhibition (Schulz et al., 1995; Ziolo et al., 1998; Ziolo et al., 2001a; Ziolo et al., 2001b). Furthermore, NOS2 has been shown to regulate various protein targets including TnI, the SR, and I_{Ca,L} (Figure 6). NOS2 decreased basal shortening with no effect on Ca^{2+} transients, therefore suggesting a decrease in myofilament Ca^{2+} sensitivity via PKG phosphorylation of TnI (Yasuda & Lew, 1997). NOS2 also can regulate I_{Ca,L} via reduction of its current (Ziolo et al., 2001a). Lastly, NOS2 has been shown to regulate SR Ca^{2+} handling proteins, namely RyR and PLB. Specifically after treatment with β-AR receptor agonists, NOS2 decreased Ca^{2+} spark frequency via a cGMP-independent pathway (Ziolo et al., 2001c). Furthermore, NOS2 was also able to decrease serine16 PLB-P, therefore suggesting decrease SR Ca^{2+} uptake (Yu et al., 2005).

1.4.5 Nitroso-Redox Balance

In health, the myocardium possesses a unique characteristic known as nitroso-redox balance (Hare, 2004). That is, NO and superoxide (O_2^-) exist in a tightly regulated balance. O_2^- is a free radical species that is capable of producing contractile dysfunction in the heart (Zima & Blatter, 2006; Kuster et al., 2010). O_2^- is rapidly buffered by various enzymes (i.e. xanthine oxidoreductase (XO), NADPH oxidase, superoxide dismutase (SOD), the mitochondria and glutathione). Interestingly, the constitutively active NOS
isoforms (NOS1, NOS3) have been shown to play a role in this phenomenon. NOS1 has been shown to co-immunoprecipitate with XO. Due to this localization of NOS1 with XO, it is believed that NOS1 in WT myocytes serves as a “brake” on XO function. For example, O$_2^-$ levels have been shown to be increased in NOS1$^{-/-}$ myocytes, thus suggesting that when NOS1 is inhibited O$_2^-$ production becomes dysregulated (Khan et al., 2004; Kinugawa et al., 2005; Zhang et al., 2009). Furthermore, this increase in O$_2^-$ levels has been hypothesized to contribute to the contractile dysfunction observed in NOS1$^{-/-}$ myocytes. NOS3 has also been shown to co-localize with a key buffer of O$_2^-$ production, SOD (Brahmajothi & Campbell, 1999). It is likely that this localization drives NOS3 to signal via a cGMP-dependent mechanism in regulation of its various protein targets (i.e. I$_{Ca,L}$). Taken together, it appears nitroso-redox balance plays a significant role in NOS-dependent regulation of contractile function.

1.5 Heart Failure

The heart is not impervious to disease. When the heart transitions from health to disease, contractile dysfunction ensues due in part due to various alterations in ECC. Interestingly, nitroso-redox balance becomes altered as the myocardium transitions to disease thereby leading to changes in: 1) ECC; 2) β-AR receptor signaling; and 3) NO signaling.

ECC is a tightly regulated process. However in disease, many key ECC proteins, notably the SERCA/PLB complex, NCX, RyR, and I$_{Ca,L}$ become altered. In healthy myocardium, the SERCA/PLB complex and NCX serve to regulate [Ca$^{2+}$]i during relaxation. In heart failure (HF), SERCA expression decreases while PLB expression is
unaltered. In addition, it appears PLB-P is decreased in HF (Huang et al., 1999; Schwinger et al., 1999). These alterations are seen functionally as decreased SR Ca\(^{2+}\) load, prolonged relaxation, and ultimately decreased contractile function (Hobai & O'Rourke, 2001). As a result of this decreased SR Ca\(^{2+}\) uptake, NCX plays a larger role in relaxation (Pieske et al., 1999). Specifically in humans, NCX contribution to Ca\(^{2+}\) decline increased from ~ 30% to ~ 50% while SERCA contribution decreased from 70% to ~ 50%. In disease, RyR has also been shown to be altered. Specifically, RyR diastolic leak is increased in HF, which has in part been explained by increased RyR-P via PKA (Marx et al., 2000). Furthermore, this increased diastolic leak has been proposed as a possible explanation for the reduced SR Ca\(^{2+}\) load observed in HF (Belevych et al., 2007). With regard to I\(_{\text{Ca,L}}\), channel density and current have been shown to be unaltered in disease (Bito et al., 2008). The β-AR receptor signaling pathway is also altered in disease (Feldman et al., 2008). Specifically, β1 receptor density declines while β2 receptor density increases (i.e. 60% β1 vs. 40% β2) (Salazar et al., 2007). Furthermore, it has been suggested that β1 and β2 receptors are involved in a non-classical β-AR signaling pathway. Studies have demonstrated that β1 signaling can be deleterious to cardiac function via activation of apoptotic signaling cascades while β2 signaling may play a role in cardio-protection (Vatner et al., 2000; Lohse et al., 2003).

Nitroso-redox imbalance has been implicated in the contractile dysfunction observed in HF (Gonzalez et al., 2010.). That is, O\(_2^-\) levels are increased while NO bioavailability is decreased (Hare & Stamler, 2005). Specifically, NOS2 expression is increased, leading to increased production of NO. NOS2 induction has been shown to decrease both basal and β-AR receptor stimulated contraction (Ziolo et al., 1998; Ziolo et
NOS1 has been shown to translocate from the SR to the sarcolemma via interactions with caveolin-3 while NOS3 expression decreases (Damy et al., 2003; Damy et al., 2004). Furthermore, this translocation of NOS1 to the sarcolemma has been shown to decrease β-AR stimulated contraction (Bendall et al., 2004). NOS3 has also been shown to be uncoupled in disease, leading to increased O$_2^-$ levels (Takimoto et al., 2005b). The enzymes that regulate O$_2^-$ production in the myocyte also become altered. The contributions of the mitochondria, NADPH oxidase, and XO to myocardial O$_2^-$ production all increase in HF (Ide et al., 1999; Cappola et al., 2001; Heymes et al., 2003). SOD expression and activity decrease, suggesting a decreased level of O$_2^-$ intracellular buffering in HF (Sam et al., 2005). It is important to note that NO and O$_2^-$ can react rapidly to form peroxynitrite (ONOO$^-$). ONOO$^-$ production is increased in ischemia-reperfusion injury (I/R) and HF (Yasmin et al., 1997; Ferdinandy et al., 2000). High concentrations of either endogenous or exogenous ONOO$^-$ have been shown to decrease both basal and β-AR receptor stimulated contraction (Lopez et al., 1997; Kohr et al., 2008b). Furthermore, ONOO$^-$ has been implicated as a key regulator of the negative inotropic effects attributed to NOS2 induction in the myocardium (Ferdinandy et al., 2000). Taken together, these findings suggest further studies are needed to determine if it is possible to develop new therapeutic agents capable of correcting nitroso-redox imbalance.

1.6 Specific Aims, Objectives, and Rationales

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At the onset of my dissertation, the following specific aims and objectives were created. For each aim and objective, the appropriate rationale has been provided. As data was accumulated, hypotheses were reconsidered and conclusions were made.

1.6.1 **Determine the role of NOS1 signaling in myocyte contraction**

At present, the role of the NOS1 signaling pathway in regulation of ECC is unclear. Previous studies have shown that when NOS1 is genetically ablated, basal myocyte contraction is decreased, suggesting that NOS1-derived NO is a positive inotrope (Khan *et al.*, 2003). Conversely, NOS1−/− myocytes have also been reported to exhibit increased basal contraction, suggesting that NOS1-derived NO is a negative inotrope (Ashley *et al.*, 2002; Sears *et al.*, 2003). With this said, it is unclear why dissimilar results have been observed in NOS1−/− myocytes. It is possible these discrepancies are due to different experimental conditions (i.e. temperature, frequency, muscle preparation). To address this question, we performed FFR experiments in a more physiological model: isolated trabeculae from NOS1−/− and WT mice. Experiments were performed at 37 °C and in the mouse physiological frequency range (4-14 Hz). To confirm that these results, we repeated these experiments in NOS1−/− and WT myocytes ± SMLT. SMLT was utilized to confirm that our results were not due to genetic alterations present in NOS1−/− myocytes.

1.6.2 **Determine if NOS1 signaling regulates SR Ca^{2+} handling**

NOS1 is known to be localized to the SR. This led us to hypothesize that NOS1 signaling was able to regulate SR Ca^{2+} handling. To investigate this, we studied SR Ca^{2+}
load in NOS1\textsuperscript{−/−} and WT myocytes. These experiments were conducted to determine if either genetic ablation or pharmologic inhibition of NOS1 led to decreased SR Ca\textsubscript{2+} load compared to WT.

1.6.3 Determine a protein target by which NOS1 signaling regulates SR Ca\textsubscript{2+} handling

NOS1\textsuperscript{−/−} myocytes have also been shown to exhibit prolonged relaxation (Ashley et al., 2002; Khan et al., 2003; Zhang et al., 2008). A key regulator of [Ca\textsubscript{2+}]\textsubscript{i} and therefore SR Ca\textsubscript{2+} load is the SERCA/PLB complex. Specifically, PLB in its native state acts to inhibit SERCA function, thereby leading to decreased SR Ca\textsubscript{2+} load. However, this inhibition is relieved when PLB is phosphorylated. This led us to hypothesize that when NOS1 is ablated, PLB-P is decreased leading to the observed decreased SR Ca\textsubscript{2+} load. To confirm this, we investigated PLB-P at both serine 16 and threonine 17 in WT myocytes ± SMLT. Serine 16 is the PLB-P site associated with activation of the β-AR receptor signaling pathway. Therefore, we next wanted to determine if β-AR receptor stimulation could reverse the effects of NOS1\textsuperscript{−/−}. Taken together, these experiments were conducted to verify PLB is a protein target of the NOS1 signaling pathway.

1.6.4 Determine the mechanism by which NOS1 signaling regulates PLB

Although PLB was identified as a protein target of NOS1, it was unclear how it was being regulated. Therefore, we next determined if cGMP-dependent or cGMP-independent NO signaling was responsible for regulation of PLB. To test this, we used inhibitors of cGMP-dependent and cGMP-independent signaling to determine if we could reproduce the functional effects of NOS1\textsuperscript{−/−} (i.e. decreased contraction; prolonged
relaxation). Furthermore, we used a cGMP-independent inhibitor to determine if ONOO\(^-\) was able to modulate PLB. These experiments were conducted to verify the causal species responsible for NOS1 signaling and its associated mechanism of action.

1.6.5 **Determine the effect of EMEPO on nitroso-redox balance in NOS1\(^{-/-}\) myocytes**

\(\text{O}_2\)\(^-\) has been shown to drastically alter ECC (Zima & Blatter, 2006). Interestingly, \(\text{O}_2\)\(^-\) levels are increased and have been suggested to play a role in the contractile function observed in NOS1\(^{+/+}\) myocytes (Khan *et al.*, 2004; Kinugawa *et al.*, 2005; Zhang *et al.*, 2009). Ultimately, this leads to nitroso-redox imbalance in NOS1\(^{-/-}\) myocytes (i.e. increased \(\text{O}_2\)\(^-\), and decreased NO bioavailability) (Gonzalez *et al.*, 2007). A new class of therapeutics, nitrone spin traps, have been introduced that have potential to restore nitroso-redox balance. Specifically, nitrone spin traps are able to bind \(\text{O}_2\)\(^-\) leading to the release of NO as a reactionary byproduct (Locigno *et al.*, 2005). As a result, we hypothesized that a previously untested intracellular nitrone spin trap, EMEPO, would be able to restore nitroso-redox balance in NOS1\(^{-/-}\) myocytes. Experiments were conducted to measure both NO and \(\text{O}_2\)\(^-\) levels in NOS1 myocytes after EMEPO incubation. These experiments were conducted to investigate EMEPO’s effect on nitroso-redox balance (i.e. \(\text{O}_2\)\(^-\), NO levels).

1.6.6 **Determine the functional effects of EMEPO in NOS1\(^{-/-}\) myocytes.**

Due to the fact EMEPO restored nitroso-redox balance in NOS1\(^{-/-}\) myocytes, we hypothesized that EMEPO would increase myocyte contraction in NOS1\(^{+/+}\) myocytes. As a result, functional experiments were conducted to determine the effect of EMEPO on
myocyte contraction. β-AR stimulated contraction is also decreased in NOS1−/− myocytes (Barouch et al., 2002; Wang et al., 2008b). Therefore, we next determined if EMEPO could improve β-AR receptor stimulated contraction in NOS1−/− myocytes. β-AR receptor stimulation has also been shown to increase ROS production (Zhang et al., 2005; Frederico et al., 2009). Therefore, we wanted to investigate if EMEPO could potentiate the β-AR receptor functional response in WT via scavenging ROS.

1.6.7 **Determine the mechanism by which EMEPO increases NOS1−/− myocyte contraction.**

Two identified protein targets of the NOS1 signaling pathway are RyR and PLB (Wang et al., 2008b; Wang et al., 2010). Specifically, NOS1 has been shown increase RyR activity while also increasing serine16 PLB-P via ONOO− mediated regulation of PKA. Therefore, we hypothesized that EMEPO was able to increase NOS1−/− contractile function via regulation of RyR and PLB. These experiments were conducted to determine the mechanism by which EMEPO exerts its positive inotropic effects in NOS1−/− myocytes.

1.6.8 **Overall Goal & Significance.**

The experiments conducted in the forthcoming studies were performed in an effort to elucidate the role of NOS1-derived NO in ECC, and its associated mechanisms of action. Furthermore, a secondary intent of these studies was to characterize EMEPO as a potential therapeutic capable of restoring nitroso-redox balance in the heart. It is our
hope that the findings presented herein will have clinical implications with further study in conditions of disease (i.e. HF).
Figure 1. Excitation-contraction coupling in the cardiac myocyte (Bers, 2002). The present diagram illustrates the movement of Ca$^{2+}$ within the cardiac myocyte during excitation-contraction coupling. The red arrows represent Ca$^{2+}$ influx and the upstroke of the [Ca$^{2+}$]$_i$ transient, while the green arrows represent the decline of the [Ca$^{2+}$]$_i$ transient and Ca$^{2+}$ efflux.
Figure 2. The Cardiac Action Potential (Amin et al., 2009). Ventricular and SA nodal cells exhibit significantly different AP waveforms and therefore different ion channel conductances. SA Nodal cell AP waveforms are divided into the following phases: Phase 0: AP Upstroke; Phase 3: Repolarization; Phase 4: Slow Depolarization. Ventricular AP waveforms are distinguished by four phases: Phase 0: Rapid Depolarization; Phase 1: Transient Repolarization; Phase 2: The Plateau Phase; Phase 3: Rapid Repolarization; Phase 4: Resting Membrane Potential.
Figure 3. β-adrenergic receptor signaling pathway in the cardiac myocyte (Bers, 2002). The following diagram illustrates the downstream effects of the β-AR signaling pathway in the cardiac myocyte.
Figure 4. NOS structure (Andrew & Mayer, 1999). NOS functions as a dimer composed of two identical monomers. Each monomer consists of a reductase and oxygenase domain. The reductase domain consists of binding sites for NADPH, FAD, and FMN while the oxygenase consists of binding sites for oxygen, L-arginine, BH4 and heme. NOS also has a calmodulin binding site that lies between the oxygenase and reductase domains.
Figure 5. Constitutively Active NOS isoforms exert differential effects on myocyte contraction. NOS1 regulates both basal and β-AR receptor stimulated contraction while NOS3 only regulates β-AR receptor stimulated contraction.
Figure 6. NOS2 in the myocardium. NOS2 decreases both basal and β-AR receptor stimulated contraction in cardiac myocytes.
CHAPTER 2

NEURONAL NITRIC OXIDE SYNTHASE SIGNALING WITHIN CARDIAC MYOCYTES TARGETS PHOSPHOLAMBAN

2.1 Introduction

It is well established that nitric oxide (NO) is an important regulator of cardiac contractility in normal and diseased hearts (Ziolo & Bers, 2003). Two isozymes of NO synthase are constitutively expressed within cardiac myocytes: neuronal NO synthase (nNOS, NOS1) and endothelial NO synthase (eNOS, NOS3). Although NO is a highly diffusible signaling molecule, recent studies have suggested that signaling via NOS1 and NOS3 is compartmentalized. NOS1 is localized to the sarcoplasmic reticulum (SR), whereas NOS3 is localized to the caveolae (Xu et al., 1999; Barouch et al., 2002), and both modulate cardiac contraction differently (Barouch et al., 2002).

Contraction of cardiac myocytes occurs by excitation-contraction coupling (EC coupling) (Bers, 2002). EC coupling is initiated by \( \text{Ca}^{2+} \) entry through the L-type \( \text{Ca}^{2+} \) channel. This \( \text{Ca}^{2+} \) induces a larger \( \text{Ca}^{2+} \) release through the SR \( \text{Ca}^{2+} \) release channel (ryanodine receptor, RyR). During relaxation, \( \text{Ca}^{2+} \) is resequestered into the SR via the sarco(endo)plasmic reticulum \( \text{Ca}^{2+} \) ATPase (SERCA)-phospholamban complex. The rate of \([\text{Ca}^{2+}]_i \), and thus relaxation, is mainly determined by SR \( \text{Ca}^{2+} \) uptake in isolated unloaded myocytes. This is especially true in mouse myocytes, where \([\text{Ca}^{2+}]_i \) decline is \( \sim 92\% \) dependent on SERCA (Bers, 2001). SERCA activity can be regulated by many
factors, one of which is the SERCA/PLB ratio, such that an increase in the SERCA/PLB ratio should result in an enhanced rate of relaxation (Koss & Kranias, 1996). NOS1−/− hearts have an increase in the SERCA/PLB ratio (Khan et al., 2003; Sears et al., 2003). However, studies consistently observed that NOS knockout (or acute inhibition) lead to a slower [Ca²⁺]ᵢ decline and/or myocyte relengthening (Ashley et al., 2002; Khan et al., 2003; Sears et al., 2003). As with [Ca²⁺]ᵢ decline, an increase in the SERCA/PLB ratio should also result in enhanced SR Ca²⁺ load (Koss et al., 1997), which is an important influence on cardiac contractility (Bassani et al., 1995; Shannon et al., 2000; Bers & Ziolo, 2001). That is, the more Ca²⁺ that is stored in the SR, the more Ca²⁺ that will be released, leading to a larger contraction. However, studies have observed a reduced SR Ca²⁺ load, which contributes to a blunted force-frequency response (Khan et al., 2003; Gonzalez et al., 2007).

Besides decreased SERCA activity, slowed [Ca²⁺]ᵢ may theoretically be due to increased RyR activity (Bers, 2001). It has been found that the effects of NOS1 knockout are, in part, via increased oxidation of RyR leading to increased RyR open probability (Gonzalez et al., 2007). We suggest that reduced SR Ca²⁺ uptake may also be involved in NOS1−/− mice and with NOS1 inhibition in wild-type (WT) mice. In addition to changes in SERCA/PLB ratio, SERCA activity can be acutely regulated by PLB, such that PLB inhibits SERCA function. However, phosphorylation of PLB relieves this inhibition. For example, stimulation of the β-AR pathway leads to positive inotropy and lusitropy (Bers & Ziolo, 2001). These effects are mainly via cAMP-dependent protein kinase (PKA) phosphorylation of PLB at serine16, which leads to increased SR Ca²⁺ load, contraction, and an increased rate of relaxation (Chu et al., 2000). Thus, we propose that the slowed
[Ca$^{2+}$], decline and blunted contraction are due to, in part, decreased SR Ca$^{2+}$ uptake. Therefore, the purpose of this study is to investigate the role of PLB in the NOS1 mediated alterations in Ca$^{2+}$ handling.

2.2 Materials and Methods

2.2.1 Force-frequency experiment

Right ventricular ultra-thin trabeculae were isolated from NOS1 knockout (NOS1$^{-/-}$) and corresponding WT (C57/BL/6J) (Jackson Laboratories, Bar Harbor, ME) mice as previously described (Stull et al., 2002). Briefly, the heart was rapidly excised and immediately perfused with Krebs-Henseleit solution containing (in mM) 137 NaCl, 5 KCl, 1.2 NaH$_2$PO$_4$, 20 NaHCO$_3$, 10 glucose and 0.25 CaCl$_2$; 20 mM 2,3-butanedione monoxime was added to minimize cutting damage and to arrest the heart (Mulieri et al., 1989). The buffer solution was maintained in equilibrium with 95% O$_2$-5% CO$_2$. After the blood was flushed out of the hearts, the right ventricle was carefully opened and unbranched ultrathin trabeculae were carefully dissected and mounted onto the experimental setup. Baseline conditions were set at 37°C, 1.5 mM extracellular Ca$^{2+}$, at a stimulation frequency of 4 Hz. The muscles were allowed to stabilize for ~15-30 min, and then the muscle’s length was stretched until developed force was maximal or an increase in developed force was accompanied by a disproportional increase in diastolic force. This length corresponded to an end diastolic sarcomere length of ~2.2 µm. Data were obtained at stimulation frequencies of 4, 6, 8, 10, 12, and 14 Hz after force had stabilized at each frequency. All the animal protocols and procedures were performed in
accordance with National Institutes of Health guidelines and approved by the Institutional Laboratory Animal Care and Use Committee at the Ohio State University.

2.2.2 Cardiac myocyte isolation

Ventricular myocytes were isolated from NOS1/−, their corresponding WT, and PLB knockout (PLB−/−) mice, as previously described (Wang et al., 2008c). Briefly, the heart was mounted on a Lagendorff apparatus and perfused with modified MEM (Sigma, St. Louis, MO, 37°C, bubbled with 95% O2-5% CO2). Blenzymane Type IV (0.077 mg/ml) (Roche Applied Science, Indianapolis, IN) was then added to the perfusate. After 7-20 min, the heart was taken down, the ventricles minced, and myocytes dissociated by trituration. Subsequently, the myocytes were filtered, centrifuged, and resuspended in MEM containing 200 µM Ca2+. Myocytes were used within 6 h after isolation.

2.2.3 Simultaneous measurement of Ca2+ transients and myocyte shortening.

Myocytes were loaded at 22°C with Fluo-4 AM (10 µM, Molecular Probes, Eugene, OR) for 30 min and washed out, and then an additional 30 min were allowed for intracellular deesterification. The instrumentation used for cell fluorescence measurement was a Cairn Research (Faversham, UK) epifluorescence system. Myocytes were stimulated via platinum electrodes connected to a Grass S48 stimulator at a frequency of 0.5 Hz. [Ca2+]i was measured by Fluo-4 epifluorescence with excitation at 480±20 nm and emission at 535±25 nm. The illumination field was restricted to collect the emission of a single cell. Data were expressed as F/F0, where F was the fluorescence intensity and F0 was the intensity at rest. Simultaneous measurement of shortening was also performed.
by use of an edge-detection system (Crescent Electronics, Sandy, UT). Data were expressed as percentage of resting cell length (%RCL). Measurements were performed at room temperature.

2.2.4 SR Ca\textsuperscript{2+} load

SR Ca\textsuperscript{2+} load was measured by rapid application of 10 mM caffeine for 10s. The amplitude of the caffeine-induced Ca\textsuperscript{2+} transient was used as an index of SR Ca\textsuperscript{2+} load (Trafford et al., 1999).

2.2.5 Western Blot

Western blots were performed on isolated myocytes or whole heart homogenates. Total phospholamban and phosphorylated phospholamban were measured using antibodies to phospholamban (Zymed, Invitrogen; Carlsbad CA), phosphorylated serine 16 and threonine 17 (Badrilla, UK). We also measured the expression of NOS1 (BD Biosciences, San Diego, CA, USA) in PLB\textsuperscript{-/-} and WT (CF1) cardiac homogenates.

2.2.6 Solution and drugs

Normal tyrode solution consists of (in mM) 140 NaCl, 4 KCl, 1 MgCl\textsubscript{2}, 1 CaCl\textsubscript{2}, 10 glucose, 5 HEPES, 1 L-arginine, pH 7.4 adjusted with HCl. S-methyl-L-thiocitrulline (SMLT, Calbiochem, La Jolla, CA), a specific NOS1 inhibitor; isoproterenol (Iso, 1 \mu M), a nonselective β-AR agonist; 1H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one (ODQ, 25 \mu M), an inhibitor of NO stimulation of guanylate cyclase; and 5,10,15,20-tetrakis (4-sulfonatophenyl) prophyrinato iron (III) (FeTPPS, 50 \mu M, Calbiochem), a peroxynitrite
decomposition catalyst were prepared fresh each experimental day. All chemicals were from Sigma except where indicated.

2.2.7 Statistics

Results were expressed as means ± SE. Statistical significance (P < 0.05) was determined by ANOVA (followed by Newman-Keuls test) for multiple groups. Paired or unpaired t-tests were used for comparison between two groups.

2.3 Results

2.3.1 Effect of NOS1 knockout on force-frequency response.

We performed force-frequency experiments on trabeculae isolated from WT and NOS1 knockout (NOS1−/−) mouse hearts. Shown in Fig. 7A are representative traces at various stimulation frequencies in a WT and NOS1−/− trabeculae. Data are summarized in Fig. 7B. WT trabeculae showed the typical positive force-frequency response (4 Hz: 14.0 ± 0.8 mN/mm²; 14 Hz: 15.4 ± 1.6 mN/mm²). However, trabeculae from NOS1−/− hearts displayed a negative force-frequency response (4 Hz: 14.5 ± 0.4 mN/mm²; 14 Hz: 9.1 ± 0.3 mN/mm²). In addition, trabeculae from NOS1−/− hearts exhibited a slower relaxation observed as an increased time to 50% relaxation (RT₅₀). Thus, these data show that NOS1−/− trabeculae have a blunted force-frequency response as well as slowed relaxation.

2.3.2 Effect of NOS1 knockout or inhibition on basal cardiac function.

Since we observed a decrease in force production and slowed relaxation in trabeculae, we investigated the mechanism in isolated myocytes. Contractile function was
tested in WT and NOS1−/− myocytes by simultaneously measuring Ca^{2+} transients and cell shortening. Shown in Fig. 8A are representative examples of cell shortening and Ca^{2+} transient traces in a NOS1−/− and WT (±SMLT) myocyte. Data are summarized in Fig. 8B. In NOS1−/− vs. WT myocytes, we found a significantly decreased basal cardiac function observed as a decrease in cell shortening amplitude (3.6 ± 0.7 vs. 7.0 ± 1.0%RCL; P < 0.05), Ca^{2+} transient amplitude (0.31 ± 0.04 vs. 0.66 ± 0.10 ΔF/F₀; P < 0.05), and slower decline in [Ca^{2+}]_i measured as RT₅₀ (409 ± 29 vs. 252 ± 17 ms; P < 0.05). These results are analogous to our trabeculae data. We also measured basal cardiac function in WT myocytes in the presence of SMLT (300 nM), a specific NOS1 inhibitor (Narayanan & Griffith, 1994). Fig. 8 shows that, similar to NOS1 knockout, acute NOS1 inhibition with SMLT in WT myocytes significantly decreased shortening amplitude (3.2 ± 0.4% RCL; P < 0.05 vs. WT), decreased Ca^{2+} transient amplitude (0.44 ± 0.06 ΔF/F₀; P < 0.05 vs. WT), as well as slowed the decline in [Ca^{2+}]_i (374 ± 23 ms; P < 0.05 vs. WT). In addition, SMLT had no effect in NOS1−/− mice (data not shown). Thus, these data show that NOS1 knockout or inhibition has negative inotropic and lusitropic effects.

2.3.3 Effect of NOS1 inhibition in PLB−/− mice.

Myocyte contraction and rate of [Ca^{2+}]_i decline are determined by many factors, one of which is PLB function. We investigated the effects of NOS1 signaling on PLB by performing functional studies in PLB−/− myocytes in the presence or absence of the specific NOS1 inhibitor SMLT. Shown in Fig. 9A are our summary data. Acute inhibition with SMLT in PLB−/− myocytes did not change basal contractility compared with control PLB−/− myocytes (cell shortening amplitude, 10.4 ± 1.2 vs. 12.2 ± 1.2% RCL,
Ca\(^{2+}\) transient amplitude, 2.6 ± 0.2 vs. 2.3 ± 0.2 ΔF/F\(_0\), Ca\(^{2+}\) transient RT\(_{50}\), 126 ± 7 vs. 113 ± 5 ms). The effects of NOS1 inhibition in WT vs. PLB\(^{-/-}\) can be more clearly seen in Fig. 9B. Since there were increased baseline features with PLB knockout, we compared the effect of SMLT on cell shortening amplitude, Ca\(^{2+}\) transient amplitude, and Ca\(^{2+}\) transient RT\(_{50}\), as the percent of control (without SMLT). The effects of SMLT were only observed in WT myocytes compared with PLB\(^{-/-}\) myocytes on cell shortening amplitude (-54 ± 7 vs. -13 ± 13% change from – SMLT), Ca\(^{2+}\) transient amplitude (-33 ± 13 vs. 13 ± 16% change from – SMLT), and [Ca\(^{2+}\)]\(_i\) decline (48 ± 14 vs. 12 ± 8% change from – SMLT). This lack of effect of SMLT was not due to a change in NOS1 expression, since our Western blot data showed that in PLB\(^{-/-}\) hearts, compared with WT, there were no differences in the expression of NOS1 (144 ± 5 vs. 130 ± 5 AU, n= 4 hearts). Thus, these data suggest that PLB is an important end target of NOS1 signaling.

2.3.4 Effect of NOS1 on PLB phosphorylation.

Since our data revealed that PLB is an important end target of NOS1 to regulate myocyte contraction and [Ca\(^{2+}\)]\(_i\) decline, we investigated the basal phosphorylation status of PLB in isolated myocytes. We examined PLB phosphorylation at the PKA site serine 16 (normalized to total PLB) in WT myocytes (±SMLT). Fig. 10A shows that acute inhibition decreased basal serine 16 PLB phosphorylation (phosphorylated serine 16/total PLB: 0.62 ± 0.04 vs. 0.87 ± 0.11, P < 0.05). We also investigated the phosphorylation status at the Ca\(^{2+}/\)CAM-dependent protein kinase site, threonine 17. Figure 10B shows that acute NOS1 inhibition with SMLT did not modify the phosphorylation at threonine 17 [phosphorylated threonine 17/total PLB: 0.35 ± 0.05 vs. 0.36 ± 0.05, P = not significant
These results suggest that NOS1 mediates PLB function via alterations in its phosphorylation status at serine 16. Thus the acute NOS1 inhibition-induced decrease in PLB serine 16 phosphorylation should decrease myocyte contraction and slow \([Ca^{2+}]_i\), decline, which we did observe.

### 2.3.5 Effect of NOS1 on SR \(Ca^{2+}\) load.

The decrease in PLB serine 16 phosphorylation should lead to a decrease in SR \(Ca^{2+}\) load. Thus, SR \(Ca^{2+}\) load was measured by the caffeine-induced \(Ca^{2+}\) transient amplitude in NOS1\(^{-/-}\) and WT (±SMLT) myocytes. Shown in Fig. 11, specific NOS1 inhibition with SMLT in WT myocytes lead to a significant decrease in SR \(Ca^{2+}\) load vs. WT myocytes (1.7 ± 0.2 vs. 2.8 ± 0.3 \(\Delta F/F_0\); \(P < 0.05\)). NOS1\(^{-/-}\) myocytes also had a decreased SR \(Ca^{2+}\) load (2.0 ± 0.2 \(\Delta F/F_0\); \(P < 0.05\) vs. WT). In addition, SMLT had no effect on NOS1\(^{-/-}\) SR \(Ca^{2+}\) load (1.6 ± 0.2 \(\Delta F/F_0\)). Our data indicate that NOS1 signaling is able to increase SR \(Ca^{2+}\) load. Thus, under our experimental conditions, the negative force-frequency response and the decrease in shortening and \(Ca^{2+}\) transient amplitudes with NOS1 knockout and/or inhibition are likely due to the decrease SR \(Ca^{2+}\) load. The majority of the caffeine–induced \(Ca^{2+}\) transient decline is due to \(Ca^{2+}\) extrusion via \(Na^+/Ca^{2+}\) exchanger (NCX) and can be used as an indirect measure of NCX activity. We observed no change in the caffeine-induced \(Ca^{2+}\) transient \(RT_{50}\) between NOS1\(^{-/-}\) and WT myocytes (2,228 ± 237 vs. 2,050 ± 108 ms, \(P= NS\)). These data suggest that NOS1 signaling does not regulate NCX function.

### 2.3.6 Effect of NOS1 knockout on the response to \(\beta\)-AR stimulation.
We next examined whether increasing PLB serine 16 phosphorylation via β-adrenergic (β-AR) stimulation can reverse the effects of NOS1 knockout. The effects of β-AR stimulation were tested in NOS1−/− and WT myocytes by measuring Ca2+ transients and cell shortening at 1 Hz. After reaching steady state, myocytes were perfused with Iso (β-AR agonist, 1 µM). Data are summarized in Fig. 12. We found that NOS1−/− myocytes had a significantly decreased β-AR stimulated Ca2+ transient amplitude compared with WT myocytes (0.56 ± 0.05 vs. 1.01 ± 0.17 ΔF/F0, P < 0.05). Cell shortening (data not shown) paralleled the Ca2+ transient data. Interestingly, the Iso-stimulated Ca2+ transient RT50 in NOS1−/− myocytes was similar to that of WT myocytes (107 ± 4 vs. 110 ± 4 ms, P= NS). These data suggest that NOS1−/− myocytes have a decreased Ca2+ transient and cell shortening response to β-AR stimulation, but ISO abolished the slower [Ca2+]i in NOS1−/− myocytes.

2.3.7 NOS1 signaling pathway

We investigated the NOS1 signaling pathway (cGMP dependent or independent) by using ODQ (an inhibitor of NO stimulation of guanylate cyclase) (Garthwaite et al., 1995). ODQ (25 µM) did not have any effect in WT, NOS1−/− or WT + SMLT myocytes (Fig. 13A; WT: Ca2+ transient amplitude 0.60 ± 0.06 vs. 0.60 ± 0.7 ΔF/F0; Ca2+ transient RT50 226 ± 12 vs. 226 ± 13 ms; NOS1−/−: Ca2+ transient amplitude 0.40 ± 0.06 vs. 0.39 ± 0.04 ΔF/F0; Ca2+ transient RT50 278 ± 10 vs. 279 ± 11 ms; WT + SMLT: Ca2+ transient amplitude 0.42 ± 0.04 vs. 0.44 ± 0.04 ΔF/F0, Ca2+ Transient RT50 284 ± 10 vs. 283 ± 8 ms; all P = NS). Cell shortening (data not shown) paralleled the Ca2+ transient data.
These data suggest that NOS1 signaling regulating myocyte contraction and $[\text{Ca}^{2+}]_i$ decline is via the cGMP-independent pathway.

We further examined the cGMP-independent pathway by using FeTPPS, a peroxynitrite decomposition catalyst (Misko et al., 1998). Perfusion of myocytes with FeTPPS (50 $\mu$M) resulted in decreased cell shortening amplitude (data not shown), decreased $\text{Ca}^{2+}$ transient amplitude, and slowed $[\text{Ca}^{2+}]_i$ decline in WT myocytes (Fig. 13B; $\text{Ca}^{2+}$ transient amplitude, $0.64 \pm 0.04$ vs. $0.75 \pm 0.05 \Delta F/F_0$; $\text{Ca}^{2+}$ transient RT$_{50}$ 255 $\pm$ 11 vs. 230 $\pm$ 11 ms, all $P<0.05$). However, FeTPPS did not have any effect in NOS1$^{-/-}$ or WT + SMLT myocytes (NOS1$^{-/-}$: $\text{Ca}^{2+}$ transient amplitude, $0.36 \pm 0.03$ vs. $0.37 \pm 0.03 \Delta F/F_0$; $\text{Ca}^{2+}$ transient RT$_{50}$ 280 $\pm$ 7 vs. 279 $\pm$ 7 ms; WT + SMLT: $\text{Ca}^{2+}$ transient amplitude, $0.40 \pm 0.06$ vs. $0.42 \pm 0.06 \Delta F/F_0$; $\text{Ca}^{2+}$ transient RT$_{50}$ 293 $\pm$ 9 vs. 295 $\pm$ 9 ms; all $P = \text{NS}$). Thus, FeTPPS mimicked the effects of NOS1 knockout or inhibition on myocyte contraction and $[\text{Ca}^{2+}]_i$ decline, which suggests that NOS1 signals, in part, via the formation of peroxynitrite.

We also examined the effects of FeTPPS on PLB serine 16 phosphorylation (normalized to total PLB) in WT myocytes ($\pm$ SMLT). Figure 13C shows that FeTPPS decreased basal serine 16 PLB phosphorylation (phosphorylated serine 16/total PLB; $0.42 \pm 0.10$ vs. $0.92 \pm 0.20$, $P < 0.05$) in WT myocytes. However, in WT myocytes with acute NOS1 inhibition, FeTPPS had no effect on serine 16 PLB phosphorylation (phosphorylated serine 16/total PLB; $0.55 \pm 0.17$ vs. $0.78 \pm 0.12$, $P = \text{NS}$). These data indicated that peroxynitrite decomposition by FeTPPS decreases PLB serine16 phosphorylation, which could account for the decreased myocyte contraction and slowed $[\text{Ca}^{2+}]_i$ decline in WT myocytes with FeTPPS.
2.4 Discussion

Our results demonstrate that NOS1 knockout blunts the force-frequency response manifested as decreased force production and slowed relaxation. Similar results were obtained in isolated myocytes. Specifically, NOS1 knockout or inhibition decreased shortening and Ca\(^{2+}\) transient amplitudes and slowed the decline of [Ca\(^{2+}\)]. This was associated with a reduced SR Ca\(^{2+}\) load. However, in PLB\(^{-/-}\) mice, there was no effect of NOS1 inhibition. Acute inhibition also decreased PLB serine 16 phosphophorylation. Peroxynitrite decomposition by FeTPPS in WT myocytes mimicked the functional effects of NOS1 knockout or inhibition (i.e. decreased cell shortening and Ca\(^{2+}\) transient amplitudes and slowed the decline of [Ca\(^{2+}\)]). FeTPPS also decreased PLB serine 16 phosphorylation. Therefore, these findings demonstrate that NOS1 signals, in part, by the formation of peroxynitrite, which modulates PLB phosphorylation leading to changes in inotropy and lusitropy.

2.4.1 Functional Effects of NOS1 knockout or inhibition.

Previous studies have found conflicting results on cardiac myocyte function with NOS1 knockout or inhibition. It has been reported that specific NOS1 knockout or inhibition leads to an increase in myocyte basal contractility and the response to β-AR stimulation (Ashley et al., 2002; Sears et al., 2003; Martin et al., 2006; Zhang et al., 2008). However, other studies have shown that NOS1 knockout or inhibition decreases the response to β-AR stimulation and decreases myocyte contraction evidenced as a blunted force-frequency response (Barouch et al., 2002; Khan et al., 2003; Gonzalez et
These discrepancies may arise due to the use of unloaded myocytes and/or variations in frequency range.

Hence, we performed experiments on trabeculae isolated from WT and NOS1\(^{-/-}\) mice. Using trabeculae, we were able to study NOS1 in experiments in which we can impose a load on the muscle and use stimulation frequencies that encompass the physiological range of the mouse and perform the protocol at body temperature (37 °C). Our data indicate that trabeculae from NOS1\(^{-/-}\) have a blunted force-frequency response (Fig. 7) compared with WT trabeculae, which performed normally compared with previous studies (Stull et al., 2002; Hiranandani et al., 2006; Janssen & Periasamy, 2007). Even though previous studies have found opposite effects of NOS1 knockout or inhibition on myocyte contraction, these studies have consistently found a slowed rate of \([\text{Ca}^{2+}]_i\) decline or myocyte relengthening (Ashley et al., 2002; Khan et al., 2003; Sears et al., 2003; Zhang et al., 2008). Consistent with these findings, our NOS1\(^{-/-}\) trabeculae also exhibited a slowed decline in force (Fig. 7). Interestingly, it has been found that increasing developed force generally results in a slowed relaxation (Janssen & Hunter, 1995). Thus with the increased force production in WT trabeculae we should have observed a slower RT\(_{50}\) (vs. NOS1\(^{-/-}\)), which was not the case. Thus considering this force-dependent factor, our data suggest that the isolated effect of NOS1 knockout alters relaxation to an even greater extent than quantified in our experiments. However, few studies have investigated the mechanism(s) responsible for the decreased inotropy and lusitropy in NOS1 knockout mice.

We used isolated myocytes from WT and NOS1\(^{-/-}\) hearts to investigate the mechanisms of the NOS1-induced effects on cardiac function. Consistent with our
trabeculae data, we found decreased basal contraction (shortening and Ca\(^{2+}\) transient amplitudes) and slowed \([\text{Ca}^{2+}]_i\) decline (Fig. 8).

We also observed similar functional effects in our WT myocytes with acute inhibition of NOS1 with SMLT as with NOS1\(^{-/-}\) myocytes (Fig. 8). Under our experimental conditions SMLT was a specific NOS1 inhibitor, since we did not observe an effect of SMLT in NOS1\(^{-/-}\) myocytes. Also, we did not observe any upregulation of NOS3 expression in NOS1\(^{-/-}\) myocytes (data not shown), which confirms previously published results (Ashley et al., 2002; Barouch et al., 2002; Kinugawa et al., 2005). Thus, we confirm here that NOS1 signaling regulates myocyte contraction and \([\text{Ca}^{2+}]_i\) decline.

### 2.4.2 Effects of NOS1 inhibition in PLB\(^{-/-}\) myocytes.

Since PLB is an important regulator of cardiac contractility (MacLennan & Kranias, 2003), we examined the role of PLB in NOS1 signaling. Specific inhibition of NOS1 with SMLT had no effect on PLB\(^{-/-}\) myocyte shortening amplitude, Ca\(^{2+}\) transient amplitude, or Ca\(^{2+}\) transient RT\(_{50}\) (Fig. 9A). Figure 9B shows that NOS1 inhibition only had an effect in WT myocytes compared with PLB\(^{-/-}\) myocytes. A previous study also found that acute NOS1 inhibition in PLB\(^{-/-}\) myocytes did not affect \([\text{Ca}^{2+}]_i\) decline, but no data on the effects on shortening and Ca\(^{2+}\) transient amplitudes were reported (Zhang et al., 2008). We have extended this initial observation by showing that there is also no effect of acute NOS1 inhibition on shortening and Ca\(^{2+}\) transient amplitudes. Thus our data suggest that PLB is an end target of NOS1 signaling leading to increased myocyte contraction and faster \([\text{Ca}^{2+}]_i\) decline. Previous studies have shown a decrease in PLB expression in NOS1\(^{-/-}\) mice (Khan et al., 2003; Sears et al., 2003; Zhang et al., 2008), and
we suggest that the decrease in PLB expression may be compensatory to try to increase myocyte contraction. Accordingly, the change in SERCA/PLB ratio should have increased the Ca\textsuperscript{2+} affinity and SR Ca\textsuperscript{2+} transport, leading to accelerated relaxation and increased contraction in NOS1\textsuperscript{−/−} mice, but neither was observed in previous studies or in our present results.

2.4.3 NOS1 and PLB phosphorylation.

Since PLB is an end target of NOS1 signaling, we examined the effect of NOS1 inhibition on PLB phosphorylation levels. Our data showed that acute NOS1 inhibition in WT myocytes reduced basal serine 16 phosphorylation (Fig. 10). We hypothesize that these changes in PLB phosphorylation translate into significant effects on [Ca\textsuperscript{2+}], decline, and relengthening. In mice, SERCA is responsible for \(\sim92\%\) of the [Ca\textsuperscript{2+}], decline (Bers, 2001). PLB inhibits SERCA activity, and when phosphorylated, this inhibition is relieved. Thus any changes to PLB phosphorylation will have effects on the decline of the Ca\textsuperscript{2+} transient. In addition, NCX is responsible for \(\sim7\%\) of the Ca\textsuperscript{2+} transient decline (Bers, 2001). The decline of the caffeine-induced Ca\textsuperscript{2+} transient data, an indirect measure of NCX, indicated that NOS1 knockout does not cause a change in NCX activity.

2.4.4 NOS1 effects on SR Ca\textsuperscript{2+} load.

Decrease PLB serine 16 phosphorylation, which decreases SR Ca\textsuperscript{2+} uptake, should result in decreased SR Ca\textsuperscript{2+} load. A major determinant of cardiac contractility is the SR Ca\textsuperscript{2+} load (Bassani et al., 1995; Janczewski et al., 1995; Shannon et al., 2000). Our data showed a reduced SR Ca\textsuperscript{2+} load in NOS1\textsuperscript{−/−} myocytes and WT myocytes with
NOS1 inhibition (Fig. 11), which is consistent with the slowed \([\text{Ca}^{2+}]_i\), decline and decreased PLB serine 16 phosphorylation. Consistent with our observed decrease in SR \text{Ca}^{2+} load, a previous study found that NOS1\(^{-/-}\) cardiac homogenates had decreased SR \text{Ca}^{2+} uptake compared with WT cardiac homogenates (Zhou et al., 2002). Thus under, our experimental conditions, the blunted force-frequency response in trabeculae as well as decreased myocyte shortening and \text{Ca}^{2+} transient amplitudes can be explained by a reduced SR \text{Ca}^{2+} load.

2.4.5 NOS1 effects on the response to \(\beta\)-AR stimulation.

Since we observed a decrease in PLB phosphorylation at the PKA site serine 16, we investigated whether increasing PLB serine 16 phosphorylation could reverse the effects of NOS1 knockout. Thus NOS1\(^{-/-}\) and WT myocytes were perfused with the nonspecific \(\beta\)-AR agonist Iso. Our data show that NOS1\(^{-/-}\) myocytes have a blunted response to \(\beta\)-AR stimulation. This was observed as a decrease in shortening (data not shown) and \text{Ca}^{2+} transient amplitudes (Fig. 12). Interestingly, however, with \(\beta\)-AR stimulation the \text{Ca}^{2+} transient RT\(_{50}\) were similar in NOS1\(^{-/-}\) and WT myocytes. It has been previously shown that PLB serine 16 phosphorylation and \([\text{Ca}^{2+}]_i\) decline were similar in \(\beta\)-AR stimulated NOS1\(^{-/-}\) and WT myocytes (Zhang et al., 2008). This study also showed that there was no difference in \(\beta\)-AR- stimulated myocyte shortening between NOS1\(^{-/-}\) and WT myocytes. Our results were somewhat different in that although our \([\text{Ca}^{2+}]_i\) decline was similar between the two groups, the NOS1\(^{-/-}\) myocytes still had a blunted response (shortening and \text{Ca}^{2+} transient amplitudes) to \(\beta\)-AR stimulation. These data suggest that there is another target of NOS1 signaling. It has been reported that the SR
Ca\(^{2+}\) release channel (RyR) is altered in NOS1\(^{-/-}\) myocytes (Gonzalez et al., 2007), which could contribute to our observed blunted β-AR response. We may not have observed the effects of NOS1 signaling on RyR in PLB\(^{+/−}\) myocytes because of their supercontractile phenotype and compensatory adaptations (i.e. reduced RyR levels) (Chu et al., 1998), which may have obscured the effects of NOS1 signaling on RyR.

2.4.6 NOS1 signaling pathway.

NO can signal via two general pathways: cGMP dependent or independent (Ziolo, 2008). We first determined which general pathway NOS1 signals through by using ODQ, an inhibitor of NO stimulation of guanylate cyclase. ODQ did not have an effect on shortening amplitude, Ca\(^{2+}\) transient amplitude, or [Ca\(^{2+}\)]\(_i\) decline in WT myocytes, NOS1\(^{-/-}\) myocytes, and WT myocytes with acute NOS1 inhibition (Fig. 13). These data suggest that NOS1 signals via the cGMP-independent pathway, consistent with a previous study (Zhang et al., 2008). NOS1 co-immunoprecipitates with xanthine oxidoreductase (XOR) (Khan et al., 2004). XOR is a major source of superoxide radicals, which can react with NO to form peroxynitrite. We further tested the NOS1 signaling pathway by using FeTPPS, a peroxynitrite decomposition catalyst. Our data showed that FeTPPS in WT myocytes resulted in decreased shortening and Ca\(^{2+}\) transient amplitudes and slowed decline of [Ca\(^{2+}\)]\(_i\) (Fig. 13). However, FeTPPS had no effect in NOS1\(^{-/-}\) myocytes or WT myocytes with acute NOS1 inhibition (Fig. 13). Furthermore, perfusion with FeTPPS in WT myocytes decreased PLB serine 16 phosphorylation. The effects of FeTPPS mimicked the effects of NOS1 knockout or inhibition. Thus our data suggest that NOS1 signals through the cGMP-independent pathway via, in part, the formation of
peroxynitrite. These data are consistent with our previous study that showed that low levels of exogenous peroxynitrite (produced from SIN-1) increased basal Ca\(^{2+}\) transient and shortening amplitude in murine myocytes. Interestingly, similar to NOS1 inhibition, the contractile effects of SIN-1 were absent in PLB\(^{-/-}\) myocytes (Kohr et al., 2008a). However, it still remains to be determined whether NO itself or other reactive nitrogen species are also part of the NOS1 signaling pathway.

A previous study (Zhang et al., 2008) also found NOS1 knockout or inhibition decreased PLB serine 16 phosphorylation similar to our study. These authors concluded that the reduced PLB phosphorylation was responsible for the slowed [Ca\(^{2+}\)]\(_i\) decline. Upon further investigation they demonstrated that there was increase protein phosphatase1 (PP1) and 2a (PP2a) activity in NOS1\(^{-/-}\) myocytes, which was responsible for the decreased PLB phosphorylation and slowed relaxation. The redox environment in NOS1 knockout (or with acute inhibition) is conducive to phosphatase activation. Specifically, our study demonstrated that NOS1 signals, in part, via peroxynitrite formation; it has been shown that low levels of peroxynitrite can inhibit phosphatase activity (Delgado-Esteban et al., 2007). Also, superoxide, which is increased in NOS1\(^{-/-}\) myocytes (Khan et al., 2004; Kinugawa et al., 2005), increases phosphatase activity (Sommer et al., 2002). Zhang et. al (Zhang et al., 2008) observed increased myocyte shortening and Ca\(^{2+}\) transient amplitudes. Their previous study showed that this was due to an increase in SR Ca\(^{2+}\) load resulting from enhanced L-type Ca\(^{2+}\) current (Sears et al., 2003). We may not have observed this positive inotropic effect of NOS1 knockout in our trabeculae and isolated myocyte studies owing to different experimental conditions. Yet consistent with our data, other studies have shown that increased PP1 or PP2a activity,
which decreased PLB serine 16 phosphorylation, leads to decreased myocyte shortening, Ca\(^{2+}\) transient amplitude and prolonged relaxation (Carr et al., 2002; Gergs et al., 2004). In addition to the enhanced phosphatase activity, PLB can have decreased phosphorylation because of reduced PKA activity. Zhang et al. observed that PKA inhibition slowed myocyte relaxation in WT myocytes but had no effect in NOS1\(^{-/-}\) myocytes, suggesting decreased PKA activity in NOS1\(^{-/-}\) myocytes. It has been demonstrated within cardiac myocytes that S-nitroso-N-acetyl-DL-penicillamine (nitrosylating agent) is able to stimulate adenylate cyclase, increase cAMP production, and activate PKA, resulting in a positive inotropic effect (Vila-Petroff et al., 1999). It is known that PKA is a redox-sensitive protein (First & Taylor, 1989). Thus, NOS1 signaling may also directly activate PKA. Alterations in any of these pathways will change PLB phosphorylation levels. Further studies are needed to identify other NOS1-mediated pathways that would modify PLB phosphorylation.

### 2.4.7 Conclusion.

In summary, our results demonstrate that NOS1 knockout or inhibition blunted the force frequency response, decreased shortening and Ca\(^{2+}\) transient amplitudes, and prolonged relaxation and [Ca\(^{2+}\)]\(_i\) decline. This was associated with a decrease in PLB serine 16 phosphorylation and a reduced SR Ca\(^{2+}\) load. FeTPPS mimicked the functional effects of NOS1 knockout or inhibition. In addition, acute NOS1 inhibition had no effect in PLB\(^{-/-}\) myocytes. Thus NOS1 signaling, in part, via peroxynitrite targets PLB phosphorylation regulating the inotropic and lusitropic state of the myocyte.
Figure 7. NOS1 knockout blunts the force-frequency response in trabeculae. A: Representative force traces at different stimulation frequencies in a WT (left) and NOS1 knockout (right) trabeculae. Numbers (4-14) correspond to stimulation frequency (Hz).

B: Summary data (mean±s.e.m.) of force (left) and time to 50% relaxation (RT$_{50}$) (right).

*P<0.05 vs WT at similar stimulation frequency. n= 3 trabeculae/3 hearts for WT (dashed line), n= 4 trabeculae/4 hearts for NOS1$^{-/-}$ (solid line).
Figure 8. NOS1 knockout or inhibition decreases basal shortening and Ca\(^{2+}\) transient amplitudes, and slows [Ca\(^{2+}\)]\(_i\) decline. A: Individual, steady-state cell shortening (top) and Ca\(^{2+}\) transient (bottom) traces measured in wildtype (WT, black), WT + SMLT (dashed) and NOS1 knockout (NOS1\(^{-/-}\), gray) myocytes. B: Summary data (mean±s.e.m.) of the effects of NOS1 knockout (NOS1\(^{-/-}\)) or specific inhibition with SMLT (300 nmol/L) in wildtype (WT + SMLT) myocytes on shortening (top) and Ca\(^{2+}\) transient (middle) amplitudes, and [Ca\(^{2+}\)]\(_i\) decline measured as time to 50% relaxation (RT\(_{50}\)) (bottom). * P<0.05 vs WT. n=22 cells/8 hearts for WT, n=21 cells/6 hearts for WT + SMLT, n=18 cells/7 hearts for NOS1\(^{-/-}\).
Figure 9. No effect of NOS1 inhibition in PLB knockout myocytes. A: Summary data (mean±s.e.m.) of the effects of NOS1 inhibition (SMLT, 300 nmol/L) on cell shortening (left) and Ca\(^{2+}\) transient (middle) amplitudes, and [Ca\(^{2+}\)]i decline measured as time to 50% relaxation (RT\(_{50}\)) (right). B: Summary data (mean±s.e.m.), expressed as a % change from myocytes without SMLT, of the effects of NOS1 inhibition (SMLT) on wildtype (WT, black bars) and PLB knockout (PLB\(^{-/-}\), gray bars) myocytes on cell shortening (left) and Ca\(^{2+}\) transient (middle) amplitudes, and Ca\(^{2+}\) transient RT\(_{50}\) (right). n=26 cells/4 hearts for PLB\(^{-/-}\), n=27 cells/4 hearts for PLB\(^{-/-} +\) SMLT.
Figure 10. NOS1 inhibition decreases PLB phosphorylation. A: Summary data (mean±s.e.m.) for PLB serine 16 phosphorylation normalized to total PLB in isolated WT myocytes ±SMLT. Inset is a representative Western blot, serine 16 phosphorylation (top) and total PLB (bottom). B: Summary data (mean±s.e.m.) for PLB threonine 17 phosphorylation normalized to total PLB in isolated WT myocytes ±SMLT. Inset is a representative Western blot, threonine 17 phosphorylation (top) and total PLB (bottom). * P<0.05 vs -SMLT, n=8 hearts.
Figure 11. NOS1 knockout or inhibition decreases SR Ca$^{2+}$ load. Summary data (mean±s.e.m.) of the effects of NOS1 knockout (NOS1$^{-/-}$) or specific inhibition with SMLT (300 nmol/L) in wildtype (WT + SMLT) myocytes on SR Ca$^{2+}$ load. * P<0.05 vs all other groups. n=32 cells/10 hearts for WT, n=46 cells/8 hearts for WT + SMLT, n=34 cells/8 hearts for NOS1$^{-/-}$, n=29 cells/6 hearts for NOS1$^{-/-}$ + SMLT.
Figure 12. Effects of NOS1 knockout on the response to β-AR stimulation. LEFT) Summary data (mean±s.e.m.) of Ca\(^{2+}\) transient amplitude before and after β-AR stimulation (ISO) in WT (clear bars) and NOS1\(^{-/-}\) (filled bars) myocytes. RIGHT) Summary data (mean±s.e.m.) of [Ca\(^{2+}\)]\(_i\) decline measured as time to 50% relaxation (RT\(_{50}\)) before and after β-AR stimulation (ISO) in WT (clear bars) and NOS1\(^{-/-}\) (filled bars) myocytes. * P<0.05 vs WT CONT, ** P<0.05 vs WT ISO. n=23 cells/11hearts for WT, n=28 cells/13hearts for NOS1\(^{-/-}\).
Figure 13. NOS1 signals, in part, via peroxynitrite. A: Summary data (mean±s.e.m.) of the effects of ODQ on myocyte Ca\textsuperscript{2+} transient amplitude (left) and RT\textsubscript{50} (right) in WT, NOS1\textsuperscript{-/-}, and WT (+SMLT) myocytes. B: Summary data (mean±s.e.m.) of the effects of FeTPPS on myocyte Ca\textsuperscript{2+} transient amplitude (left) and RT\textsubscript{50} (right) in WT, NOS1\textsuperscript{-/-}, and WT (+SMLT) myocytes. C: Summary data (mean±s.e.m.) for PLB Serine16 phosphorylation normalized to total PLB in isolated WT myocytes ±FeTPPS. Inset is a representative Western blot, Ser16 phosphorylation (top) and total PLB (bottom).

*P<0.05 vs WT CONT (paired t-test); **P<0.05 vs WT CONT (unpaired t-test). ODQ experiments: n=13 cells/3hearts for WT, n=11 cells/3hearts for NOS1\textsuperscript{-/-}, n=15 cells/3hearts for WT+SMLT. FeTPPS experiments: n=23 cells/6hearts for WT, n=29 cells/5hearts for NOS1\textsuperscript{-/-}, n=15 cells/6hearts for WT+SMLT. PLB Phosphorylation experiments: n=8 hearts.
Figure 13 continued.

C

PLB Ser16 Phosphorylation in WT Myocytes

Serine16/Total PLB

FeTPPS

-FeTPPS +FeTPPS

Ser16-PLB

PLB

*
3.1 Introduction

Despite recent advances in treatment strategies, heart failure (HF) is a growing epidemic that still presents with poor clinical prognosis. Thus, the development of new therapeutic agents is of vital importance. Recently, therapies have been developed to target superoxide (O$_2^-$) and nitric oxide (NO) (Taylor et al., 2004; Baldus et al., 2006). Both of these signaling molecules are key regulators of myocyte contraction and exist in a precise balance known as nitroso-redox equilibrium (Hare, 2004; Zimmet & Hare, 2006). This equilibrium not only depends on the concentration of these ROS and RNS (production and degradation) but also on their spatiotemporal distribution. In various cardiomyopathies, this relationship between O$_2^-$ and NO is altered due to changes in concentration and/or spatiotemporal distribution. This nitroso-redox imbalance has been found to contribute to both the contractile dysfunction and adverse remodeling in various cardiomyopathies. Specifically, O$_2^-$ production is increased in HF via NADPH oxidase, xanthine oxidase, and/or mitochondria while degradation is decreased via a reduction in superoxide dismutase activity (Ide et al., 1999; Cappola et al., 2001; Heymes et al., 2003; Sam et al., 2005). In hypertrophy, there is increased production of O$_2^-$ due to uncoupling
of NOS3 (Takimoto et al., 2005b). During ischemia/reperfusion (I/R) injury, there is a burst in \( \text{O}_2^- \) production from mitochondria (Ambrosio et al., 1993). As a result, inhibitors of the enzymes that produce \( \text{O}_2^- \) and scavengers of \( \text{O}_2^- \) have been developed and used as therapeutic agents. Unfortunately, in a clinical trial, the xanthine oxidase inhibitor oxypurinol did not lead to clinical benefits in HF patients (Hare et al., 2008). This type of therapy may not have been beneficial since reducing \( \text{O}_2^- \) levels by itself will not restore the altered nitroso-redox balance since there are also changes in NO bioavailability (Thomson et al., 2009; Nediani et al., 2010). For example, this occurs in HF, (translocation of NOS1, expression of NOS2), I/R injury (expression of NOS2) and hypertension (uncoupling of NOS3) (Wang & Zweier, 1996; Wildhirt et al., 1999; Damy et al., 2003; Damy et al., 2004; Ziolo et al., 2004; Takimoto et al., 2005b). Thus, a therapy is needed that will equalize both \( \text{O}_2^- \) and NO levels, thereby restoring nitroso-redox balance.

Spin traps have been used as reagents to detect and identify transient radicals including \( \text{O}_2^- \) using electron paramagnetic resonance (EPR) spectroscopy in chemical and biological systems. Nitrone spin traps such as 5,5-dimethylpyrroline N-oxide (DMPO), \( \alpha \)-phenyl-tert-butyl-nitrone (PBN) and its sulfonyl derivative, NXY-059 have shown pharmacological activity against I/R injury in the heart and brain (Zuo et al., 2009), neurodegeneration (Kamat et al., 2008) and cancer (Nakae et al., 2004). Moreover, PBN and DMPO exhibited NO-releasing capabilities (Locigno et al., 2005), showed therapeutic properties against stroke (Floyd & Hensley, 2000) and improvement of cerebral blood flow (Inanami & Kuwabara, 1995) in animal models. Our recent work has shown DMPO to be cardioprotective in hearts undergoing I/R injury (Tosaki et al., 2005).
Although nitrones are known to exhibit cardioprotective effects, the mechanism of their action is not fully understood. In particular, their role in nitroso-redox balance and calcium handling are not known. A novel ester derivative of DMPO, 2-(2-ethoxy-2-oxoethyl)-2-(ethoxycarbonyl)-3,4-dihydro-2H-pyrrole 1-oxide (EMEPO) (Fig. 14), was therefore synthesized. Due to its intracellular target specificity, EMEPO is expected to impart better pharmacological activity compared to other treatments.

Previously, our lab and others have studied the contractile function of myocytes isolated from neuronal nitric oxide synthase knockout (NOS1\(^{-/-}\)) mice. Specifically, myocytes from NOS1\(^{-/-}\) mice exhibit decreased basal contraction and slowed relaxation compared to wild-type (WT) myocytes (Khan et al., 2003; Gonzalez et al., 2007; Wang et al., 2008a). In addition, NOS1\(^{-/-}\) myocytes also exhibit a decreased functional response to β-AR receptor stimulation (Barouch et al., 2002; Wang et al., 2008a). Interestingly, O\(_2^-\) and NO levels are also altered, thus suggesting nitroso-redox imbalance is in part responsible for the contractile dysfunction observed in NOS1\(^{-/-}\) myocytes (Khan et al., 2004; Kinugawa et al., 2005; Zhang et al., 2009). Due to this observed nitroso-redox balance, NOS1\(^{-/-}\) myocytes mimic a disease state, and therefore, were selected as our model of study. We hypothesize that EMEPO will improve contractile function in NOS1\(^{-/-}\) myocytes via restoration of nitroso-redox balance leading to increased myocyte contraction.

### 3.2 Materials and Methods

#### 3.2.1 Synthesis of EMEPO
The synthesis of EMEPO is shown in Scheme 1 according to the procedure previously described (Han et al., 2009). The synthesis of diethyl 2-nitrosuccinate from 1 and 2 was carried out according to published procedures (Schipchandler, 1979; Diez-Barra et al., 1994) and its spectral data was in agreement with the literature (Schipchandler, 1979; Diez-Barra et al., 1994). TEA (0.2 mL) was slowly added to a cooled (10 °C) solution of 7 (1.8 g, 8.2 mmol/L) and acrolein (0.66 mL, 9.9 mmol/L) in CH$_3$CN (5mL). The mixture was stirred for 2 h at room temperature and then concentrated under reduced pressure to give the crude product 4. The crude product 4 was dissolved in THF (90 mL) at -10 °C bath and a solution of NH$_4$Cl (2.8 g) in 20 mL water was added followed by Zn dust (2 g) which was added within 0.5 h portion-wise at -10 °C. The mixture was stirred for another 2 h at -10 °C, then solid NaCl was added until saturated and extracted with Et$_2$O. The collected organic phase was dried over anhydrous MgSO$_4$ and filtered. The solvent was removed in vacuo and the residue was purified by flash column chromatography using CH$_2$Cl$_2$/MeOH (97:3 v/v) as eluent to give EMEPO as yellow oil (1.2 g, 60%). $^1$H NMR (400 MHz, CDCl$_3$) δ 1.29 (overlap of two triplets,
6H), 2.71 (m, 1H), 2.76 (m, 3H), 3.05-3.33 (AB system, 2H), 4.13-4.27 (m, 4H), 6.97 (t, 1H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 14.3, 14.5, 26.9, 30.2, 38.0, 61.3, 63.0, 79.9, 136.3, 169.1, 170.0. IR (neat, cm$^{-1}$) ν 3440, 2983, 1733, 1586, 1207, 1183, 1076, 1027. GC-MS calcd for C$_{11}$H$_{17}$NO$_5$ m/z 243.1, found 243.0. HRMS calcd for (M+Na) C$_{11}$H$_{17}$NO$_5$Na m/z 266.1004, found 266.0995.

3.2.2 Cardiac myocyte isolation.

As previously described, myocytes were isolated from 4-6 month old NOS1 Knockout (NOS1$^{-/-}$) and their corresponding wild-type (WT, C57BL/6J) (Jackson Laboratories, Bar Harbor, Maine) mice (Wang et al., 2008a). Mice were anesthetized with sodium pentobarbital (i.p. injection of 50 mg/kg). Hearts were cannulated and perfused on a Langendorff apparatus at 37°C with Ca$^{2+}$ free normal Tyrode solution (in mmol/L: 140 NaCl, 4 KCl, 1 MgCl$_2$, 10 Glucose, 5 HEPES, pH 7.4) for four minutes. After which time, the flow was switched to tyrode solution containing Liberase Blendzyme IV (Roche Diagnostics) and 2.5 X 10$^{-5}$ mol/L CaCl$_2$. Digestion with Blendzyme IV lasted three to five minutes. The heart was removed from the Langendorff, atria trimmed off, and the heart minced. Tissue was triturated, filtered and spun down to produce cell pellets. The pellets were washed and re-suspended in normal Tyrode solution supplemented with 2 X 10$^{-4}$ mol/L CaCl$_2$. Isolated myocytes were used experimentally within 4-6 hours of their isolation. All the animal protocols and procedures were performed in accordance with National Institutes of Health guidelines and approved by the Institutional Laboratory Animal Care and Use Committee at The Ohio State University.
3.2.3 Simultaneous measurement of Ca\(^{2+}\) transients and myocyte shortening

Simultaneous measurement of Ca\(^{2+}\) transients and myocyte shortening was performed as previously described (Wang et al., 2008a). Myocytes were loaded at 22 °C with Fluo-4 AM for 30 min, washed out, and then 30 min was allowed for intracellular de-esterification. Myocytes were incubated with EMEPO (1 mmol/L) for 30 min concurrently with Fluo-4 AM. The instrumentation used for cell fluorescence measurement was a Cairn Research Limited (Faversham, UK) epifluorescence system. \([\text{Ca}^{2+}]_{\text{i}}\) was measured by Fluo-4 epifluorescence with excitation at 480±20 nm and emission at 535±25 nm. The illumination field was restricted so the emission of a single cell could be collected. Data was expressed as F/F\(_0\), where F is the fluorescence intensity and F\(_0\) is the intensity at rest. Myocytes were field stimulated at 1 Hz at room temperature (22°C) via Grass S48 stimulator (West Warwick, RI, USA). Simultaneous measurement of shortening was performed using an edge detection system (Crescent Electronics, Sandy, UT). Data were expressed as % resting cell length (%RCL).

3.2.4 Force-frequency experiment

Force-frequency experiments were performed in isolated myocytes from NOS1\(^{-/-}\) and WT mice. Data were obtained in myocytes stimulated at 0.2, 0.5, and 1 Hz at room temperature. Data were presented as the % of 0.2 Hz.

3.2.5 Measurement of SR Ca\(^{2+}\) leak/load relationship

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SR Ca\(^{2+}\) leak was measured as the tetracaine (RyR2 inhibitor)-induced shift in diastolic [Ca\(^{2+}\)], (Shannon et al., 2002; Knollmann et al., 2006; Picht et al., 2007).

Myocytes were loaded at 22°C with Fluo-4 AM (10 µM, Molecular Probes, Eugene, OR, USA) for 30 min, washed out, and then an additional 30 min was allowed for intracellular de-esterification. Then the cells were stimulated for 1 min in normal Tyrode solution. After reaching steady state, the solution was rapidly switched to 0 Na\(^+\), 0 Ca\(^{2+}\) Tyrode solution (Na\(^+\) was replaced by Li\(^+\)) plus 1 mM tetracaine (Sigma) for 30 s. Afterwards, the solution was rapidly switched to 0 Na\(^+\), 0 Ca\(^{2+}\) Tyrode solution for 20 s. Tetracaine, by blocking RyR2 and therefore SR Ca\(^{2+}\) leak, caused a decrease in diastolic [Ca\(^{2+}\)]. The shift in diastolic [Ca\(^{2+}\)], upon the removal of tetracaine was used as a measure of RyR2-dependent SR Ca\(^{2+}\) leak. Since SR Ca\(^{2+}\) leak is also dependent upon SR Ca\(^{2+}\) load, SR Ca\(^{2+}\) load of each cell was measured. Measurements were performed at room temperature. Data was expressed as the ratio of SR Ca\(^{2+}\) leak equalized to its accompanying SR Ca\(^{2+}\) load (i.e. leak/load).

3.2.6 Western blot

Western blots were performed on isolated myocyte homogenates. Total PLB and phosphorylated PLB were measured by using antibodies to PLB (Zymed, Invitrogen; Carlsbad, CA) and phosphorylated PLB at serine16 (Badrilla, Leeds, UK).

3.2.7 Measurement of O\(_2^−\) levels

Lucigenin-enhanced chemiluminescence was used to measure O\(_2^−\) levels in cardiac myocyte homogenates from WT and NOS1\(^{+/−}\) mice, as previously described.
(Khan et al., 2004). Hearts were homogenized in Homogenization Buffer (in mmol/L): 1 Tris pH 7.5, 4 NaCl, 0.1 sodium pyrophosphate, 1 sodium vanadate, 1 benzamidine, 0.1 M NaF, 100% Tergitol NP-40). The homogenates were transferred into an opaque 96-well microplate containing 5 µmol/L lucigenin (bis-N-methylacridinium nitrate). Chemiluminescence was recorded by a Centro XS3 LB 960 luminometer (Berthold Technologies, Bad Wildbad, Germany) reporting relative light units (RLU) emitted over a 30-min period. \( \text{O}_2^\cdot^- \) levels were reported as the chemiluminescence of lucigenin-containing buffer with tissue minus background. \( \text{O}_2^\cdot^- \) production was expressed as RLU per µg/µl of protein.

### 3.2.8 Measurement of NO levels

Intracellular NO imaging was performed using an Olympus Fluoview 1000 laser scanning confocal microscope equipped with Olympus oil objective (60X, 1.4 NA). NO levels were measured in isolated cardiac myocytes from NOS1\(^{-/-}\) and WT mice. Myocytes were incubated concurrently with DAF-FM diacetate (10 µmol/L, Molecular Probes, Eugene, OR) and EMEPO for 20 min and then 20 min was allowed for de-esterification. DAF-FM diacetate was excited at the 488nm line of an argon ion laser, and fluorescence was acquired at >510 nm in the X-Y scan mode of the confocal system at the rate of 5.2 s per scan. Maximum fluorescence, as a measure of myocyte dye loading, was obtained by superfusing the myocyte with 1 mM spermine NONOate. The magnitude of fluorescent signals was quantified in terms of average pixel intensity. Data were expressed as the % of DAF fluorescence maximum (i.e. the ratio of basal DAF fluorescence divided by spermine NONOate DAF fluorescence multiplied by 100).
3.2.9 Solutions and drugs

Normal Tyrode supplemented with 1 mmol/L CaCl₂ was used for all functional experiments. DMPO (1 mM), EMEPO, Methyl-ester Nitrooxide (MENO, 10 µM, cell-permeable O₂⁻ scavenger), S-Nitro-N-acetyl-DL-penicillamine (SNAP, 1 µM, NO donor), Isoproterenol (Iso, 1 µM, a non-selective β-AR receptor agonist; Sigma) and Spermine NONOate stocks were prepared fresh each experimental day.

3.2.10 Statistics

Results were expressed as means ± SE. Statistical significance (P < 0.05) was determined by ANOVA (followed by Newman-Keuls test) for multiple groups. Paired or unpaired t-tests were used for comparison between two groups.

3.3 Results

3.3.1 Effect of EMEPO on basal contractile function.

We determined the functional effects of EMEPO on NOS1⁻/⁻ myocyte contraction by simultaneous measurement of myocyte shortening and Ca²⁺ transients. Shown in Figure 15A are representative shortening and Ca²⁺ transient traces in the presence or absence of EMEPO. As shown in Figures 15B, NOS1⁻/⁻ myocytes that were incubated with EMEPO had significantly increased shortening amplitude (1.7 ± 0.1 vs. 4.3 ± 0.6 % RCL; P<0.05) and Ca²⁺ transient amplitude (0.7 ± 0.1 vs. 1.4 ± 0.1 Δ F/F₀; P<0.05) compared to control NOS1⁻/⁻ myocytes (i.e., not incubated with EMEPO). In addition, EMEPO was able to enhance the rate of relaxation measured as the time to 50%
relaxation (RT$_{50}$) (relengthening RT$_{50}$: 370 ± 25 vs. 268 ± 20 msec; P<0.05, Figure 15C) and the Ca$^{2+}$ transient decline (RT$_{50}$: 294 ± 10 vs. 232 ± 8 msec; P<0.05, Figure 15C).

These data suggest that EMEPO can improve inotropy and lusitropy in NOS1$^{-/-}$ myocytes.

Since WT myocytes have a normal nitroso-redox balance, we next determined the effect of EMEPO on WT contractile function. Shown in Figure 15B, EMEPO had no effect on WT myocyte shortening amplitude (2.4 ± 0.3 vs. 2.8 ± 0.4 % RCL, P=NS), Ca$^{2+}$ transient amplitude (0.9 ± 0.1 vs. 0.9 ± 0.1 Δ F/F$_{0}$, P=NS). There was also no effect of EMEPO on the rate of relaxation (relengthening RT$_{50}$: 266 ± 15 vs. 244 ± 16 msec, P=NS; Figure 15C) or Ca$^{2+}$ transient decline (RT$_{50}$: 234 ± 9 vs. 244 ± 10 msec, P=NS; Figure 15C). These data suggest EMEPO does not affect contractile function in myocytes which have a preserved nitroso-redox balance.

It is possible that frequency may play a role in EMEPO’s observed effects on NOS1$^{-/-}$ myocyte contraction. To address this, we performed force-frequency experiments (0.2, 0.5, and 1 Hz) in NOS1$^{-/-}$ and WT myocytes incubated with EMEPO. EMEPO did not alter the FFR in NOS1$^{-/-}$ and WT myocytes. Interestingly as shown in Figure 16, EMEPO increased Ca$^{2+}$ transient amplitudes in NOS1$^{-/-}$ myocytes at 0.2 Hz (94.2 ± 15.1 vs. 214.9 ± 20.9 % of 0.2 Hz; P<0.05), 0.5 Hz (98.9 ± 11.1 vs. 154.2 ± 12.5 % of 0.2 Hz; P<0.05) and 1.0 Hz (70.2 ± 6.6 vs. 127.9 ± 12.0; P<0.05) compared to EMEPO incubated WT myocytes. Cell shortening (data not shown) paralleled the Ca$^{2+}$ transient data. These data suggest that the positive inotropic effects of EMEPO occur across various stimulation frequencies.

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Nitrone spin traps have been shown to release NO as a reactionary byproduct of their reaction with $O_2^-$. With this said, it is possible that nitrone spin traps produce more robust effects on myocyte contraction than either a $O_2^-$ scavenger or a NO donor. To test this hypothesis, we compared the effects of EMEPO, SNAP (NO donor) and MENO (cell permeable $O_2^-$ scavenger) on NOS1$^{-/-}$ myocyte contraction. EMEPO, SNAP and MENO all significantly increased NOS1$^{-/-}$ myocyte contraction. Interestingly, EMEPO incubated myocytes exhibited a larger percent increase in Ca$^{2+}$ transient amplitude ($126.0 \pm 10.2$ (MENO); $158.2 \pm 13.9$ (SNAP) vs. $197.4 \pm 14.3 \%$ of control, $P<0.05$; Figure 17) and shortening amplitude ($129.4 \pm 14.0$ (MENO); $159.5 \pm 25.6$ (SNAP) vs. $257.6 \pm 37.2 \%$ of control, $P<0.05$; Figure 17) compared to MENO or SNAP perfused NOS1$^{-/-}$ myocytes. These data suggest that EMEPO produces functional effects unique from other treatments.

DMPO, the parent molecule of EMEPO, lacks ester groups and should poorly permeate the cell membrane. Thus, we determined the effect of DMPO on NOS1$^{-/-}$ myocyte contraction. Shown in Figures 15B-C, DMPO had no effect on NOS1$^{-/-}$ myocyte shortening amplitude ($1.7 \pm 0.1$ vs. $1.8 \pm 0.1 \%$RCL, $P=\text{NS}$), Ca$^{2+}$ transient amplitude ($0.7 \pm 0.1$ vs. $0.8 \pm 0.1 \Delta F/F_0$, $P=\text{NS}$), the rate of relaxation (relengthening $R_{T50}$: $370 \pm 25$ vs. $323 \pm 15$ msec, $P=\text{NS}$), or Ca$^{2+}$ transient decline ($R_{T50}$: $294 \pm 10$ vs. $286 \pm 10$ msec, $P=\text{NS}$). These data provide further evidence that EMEPO exerts effects on NOS1$^{-/-}$ contractile function unique from other nitrone spin traps.

### 3.3.2 Effect of EMEPO on the SR Ca$^{2+}$ Leak/Load Relationship.
NOS1 has been shown to localize to the SR (Xu et al., 1999). Furthermore, NOS1\(^{-/-}\) myocytes have been shown to have depressed SR Ca\(^{2+}\) stores (Khan et al., 2003; Wang et al., 2008b; Wang et al., 2010). As a result, we next determined the effect of EMEPO on the SR Ca\(^{2+}\) leak/load relationship. In agreement with previous studies, the leak/load relationship was decreased in NOS1\(^{-/-}\) myocytes compared to WT myocytes (0.004 ± 0.001 vs. 0.012 ± 0.003, P< 0.05; Figure 18). EMEPO significantly increased the leak/load relationship in NOS1\(^{-/-}\) myocytes (0.004 ± 0.001 vs. 0.007 ± 0.001, P<0.05; Figure 18), but was without effect in WT myocytes (0.011 ± 0.004 vs. 0.012 ± 0.003, P=NS, Figure 18). Taken together, these data suggest that EMEPO increases RyR activity.

### 3.3.3 Effect of EMEPO on PLB.

Interestingly, EMEPO was unable to fully restore the leak/load relationship in NOS1\(^{-/-}\) myocytes. Therefore, we hypothesized that EMEPO increased myocyte contraction via regulation of multiple protein targets. Previous studies have demonstrated that NOS1 can regulate SR Ca\(^{2+}\) handling in part via PLB phosphorylation (Wang et al., 2008b). Specifically, NOS1\(^{-/-}\) myocytes exhibit decreased serine 16 PLB phosphorylation compared to WT myocytes. Therefore, we next determined if EMEPO could increase contraction via increased PLB phosphorylation. EMEPO increased serine16 PLB phosphorylation in NOS1\(^{-/-}\) myocytes (0.2 ± 0.07 vs. 0.4 ± 0.04, P< 0.05; Figure 19). These data suggest that EMEPO is able to improve the leakeload relationship in part via increased PLB phosphorylation.
3.3.4 Effect of EMEPO on β-AR stimulation.

PLB is phosphorylated at serine 16 by PKA, a kinase activated in the β-AR receptor signaling pathway. Therefore, we next determined if EMEPO could regulate the β-AR receptor signaling pathway. Previously it has been shown that NOS1−/− myocytes exhibit a decreased functional response to β-AR receptor stimulation (Khan et al., 2003; Wang et al., 2008a). Shown in Figure 20, EMEPO incubation significantly increased Ca\(^{2+}\) transient amplitude in NOS1−/− myocytes (2.3 ± 0.2 vs. 3.3 ± 0.2 Δ F/F\(_0\); P<0.05; Figure 20), compared to control NOS1−/− myocytes. Cell shortening (data not shown) paralleled the Ca\(^{2+}\) transient data. These data suggest EMEPO can improve β-AR receptor stimulated contractile function in NOS1−/− myocytes.

Prior studies have provided evidence that both acute and chronic administration of β-AR agonists can lead to increased O\(_2\)− production (Zhang et al., 2005; Frederico et al., 2009). Therefore, we next determined if EMEPO could potentiate the functional response of WT myocytes to β-AR receptor stimulation. These data suggest a trend of increased β-AR receptor stimulated Ca\(^{2+}\) transient amplitude in EMEPO incubated WT myocytes (3.0 ± 0.3 vs. 3.7 ± 0.3, Δ F/F\(_0\); P=0.08; Figure 20), compared to control WT myocytes. Cell shortening (data not shown) paralleled the Ca\(^{2+}\) transient data. These data suggest that EMEPO may also potentiate the functional response to β-AR receptor stimulation in WT myocytes.

3.3.5 Effect of EMEPO on O\(_2\)− levels.

We next examined if the increased contraction observed with EMEPO incubation was due to normalized O\(_2\)− levels. Consistent with previous studies (Khan et al., 2004;
Kinugawa et al., 2005; Zhang et al., 2009), NOS1−/− myocytes had increased O$_2^-$ levels compared to WT myocytes (22.4±8.4 vs. 1.2±0.6 RLU, P<0.05; Figure 21). Furthermore, NOS1−/− myocytes incubated with EMEPO had significantly lower O$_2^-$ levels compared to control NOS1−/− myocytes (i.e. no EMEPO incubation) (22.4±8.4 vs. 1.1±0.4 RLU, P<0.05; Figure 21). In fact, there was no difference in O$_2^-$ levels between EMEPO incubated NOS1−/− myocytes and WT myocytes, suggesting near complete O$_2^-$ scavenging. These data suggest EMEPO is capable of scavenging O$_2^-$ in NOS1−/− myocytes.

3.3.6 Effect of EMEPO on NO levels.

To fully restore the nitroso-redox equilibrium, it is not sufficient to simply scavenge O$_2^-$, thus it is necessary to also increase NO bioavailability. Therefore, we measured NO levels in NOS1−/− myocytes. As shown in Figure 22, NOS1−/− myocytes incubated with EMEPO had an increased % of maximum DAF fluorescence vs. control NOS1−/− myocytes (i.e. no EMEPO incubation) (84.5 ± 2.3 vs. 98.9 ± 0.7 % of maximum DAF fluorescence; P<0.05). Furthermore, we also observed that EMEPO incubation had no effect on % of maximum DAF fluorescence in WT myocytes (91.6 ± 1.8 vs. 92.0 ± 0.8 % of maximum DAF fluorescence; P=NS). Taken together, these data suggest EMEPO is able to produce NO as a reactionary byproduct of its reaction with O$_2^-$ in NOS1−/− myocytes.

3.4 Discussion
Our current study demonstrates that a novel O$_2^-$ scavenger, EMEPO, can improve contractile function in isolated myocytes under conditions of nitroso-redox disequilibrium. Specifically, in NOS1$^{-/-}$ myocytes, a model of NO/ O$_2^-$ imbalance, EMEPO increased basal contractile function, observed as increased Ca$^{2+}$ transient and shortening amplitudes and a faster rate of relaxation, effects which were maintained across various stimulation frequencies. EMEPO’s contractile effects were much greater than either MENO, a cell permeable O$_2^-$ scavenger, or SNAP, a NO donor. In addition, EMEPO improved the SR Ca$^{2+}$ leak/load relationship in NOS1$^{-/-}$ myocytes via regulation of RyR and PLB. This was accomplished by scavenging excess O$_2^-$ while increasing NO bioavailability. There was no effect of EMEPO on WT basal contractile function, which was expected due to the low O$_2^-$ levels in WT myocytes. DMPO, an extracellular spin trap, was without effect in NOS1$^{-/-}$ myocytes suggesting that the observed effects in NOS1$^{-/-}$ myocytes are unique to EMEPO. Furthermore, EMEPO potentiated the functional effects of β-AR receptor stimulation in NOS1$^{-/-}$ myocytes. Taken together, these findings suggest EMEPO is a novel O$_2^-$ scavenger, which may have therapeutic implications with further study.

3.4.1 Nitroso-Redox Balance in Disease

In healthy myocardium, O$_2^-$, which is produced via mitochondria and enzymes such as xanthine oxidase and NADPH oxidase, is rapidly buffered by glutathione and degraded by superoxide dismutase (SOD). However, in many diseases there is an increase in O$_2^-$ levels. In HF, xanthine oxidase and NADPH oxidase activity increases while SOD activity decreases (Ide et al., 1999; Cappola et al., 2001; Heymes et al., 2003;
Sam et al., 2005). In hypertrophy, NOS3 becomes uncoupled which will lead to production of $O_2^-$ (Takimoto et al., 2005b). There is also an increase in $O_2^-$ levels in I/R injury due to mitochondrial production (Ambrosio et al., 1993). High levels of $O_2^-$ have been shown to alter the function of a variety of vital cardiac contractile proteins, notably RyR, NCX and the SERCA pump, leading to contractile dysfunction (Zima & Blatter, 2006; Zissimopoulos & Lai, 2006; Kuster et al., 2010). As a result, treatments have been developed to combat this oxidative damage. For example, allopurinol and oxypurinol (inhibitors of xanthine oxidase) have been shown to increase contractile function and promote cardioprotection in failing and I/R injured hearts (Werns et al., 1986; Perez et al., 1998; Kogler et al., 2003; Tan et al., 2009). Inhibitors of NADPH oxidase have also been shown to improve cardiac function in models of heart failure (Liu et al., 2010). In addition, SOD mimetics have been shown to improve contractile function in models of increased $O_2^-$ production (Nojiri et al., 2006). However, it has been shown that the success of antioxidants is dependent upon NO bioavailability (Saavedra et al., 2002). Specifically, when there were low levels of NO (caused by NOS inhibition), allopurinol was shown to be ineffective. Unfortunately in disease, there is altered NO production and an atypical spatiotemporal pattern. Specifically in HF, there is altered NO production due to the expression of NOS2 (in ~70% hearts) and translocation of NOS1 from the sarcoplasmic reticulum to the caveolae (Damy et al., 2003; Damy et al., 2004; Ziolo et al., 2004). In hypetrophy, the uncoupling of NOS3 will lead to a decrease in NO production (Takimoto et al., 2005b). In fact, it has recently been shown that it is the altered NO bioavailability and higher $O_2^-$ levels that contribute to the cardiac dysfunction in HF (Gonzalez et al., 2010.). Thus, the nitroso-redox disequilibrium is a major
contributor to the contractile dysfunction and altered remodeling present in many cardiomyopathies (Hare & Stamler, 2005).

3.4.2 EMEPO Structure and Function

Although cyclic nitrones are structurally simple molecules, they possess rich chemistries and biological properties that make them relevant pharmacological agents. For example, relevant to this study, nitrones can 1) act as oxidizing and reducing agents by virtue of their oxidation state (Villamena, 2010); 2) react with a variety of free radicals (Villamena et al., 2005); and 3) decompose to NO after reacting with O$_2^-$ (Locigno et al., 2005; Villamena, 2009). While the extracellular, non-target specific DMPO exhibited cardioprotective properties in isolated rat heart during I/R (Zuo et al., 2009), we anticipated that cell membrane permeable nitrones may affect intracellular events such as calcium handling much more efficiently. Under I/R conditions, where O$_2^-$ production is upregulated, NO bioavailability can be compromised due to the high rate of O$_2^-$ reaction with NO approaching its diffusion controlled limit to form peroxynitrite. Nitrones being both scavengers and NO donors (Locigno et al., 2005) may exhibit pharmacological activity against cardiac mechanical dysfunction caused by nitroso-redox disequilibrium.

3.4.3 NOS1$^{-/-}$ myocytes as a Model of Study

Genetic deletion of NOS1 leads to decreases in both NO production and bioavailability. Previous studies have also shown that when NOS1 signaling is disrupted, O$_2^-$ levels increase (Khan et al., 2004; Kinugawa et al., 2005; Zhang et al., 2009). Increased O$_2^-$ levels and decreased NO bioavailability (i.e., nitroso-redox imbalance)
contribute to the contractile dysfunction observed in NOS1−/− mice. For example, a previous study demonstrated that allopurinol was capable of partially restoring the force-frequency response in NOS1−/− myocytes (Khan et al., 2004). Interestingly, after myocardial infarction, NOS1−/− mice have increased mortality and adverse remodeling due to an imbalance between O2− and NO (Dawson et al., 2005; Saraiva et al., 2005). Thus, NOS1−/− myocytes present an ideal model for a proof of principle study. The intent of our study was to determine if EMEPO could improve the contractile dysfunction observed in NOS1−/− myocytes.

3.4.4 EMEPO increases basal contraction in NOS1−/− myocytes

Previous studies have demonstrated that NOS1−/− myocytes exhibit decreased basal contractile function (Khan et al., 2003; Gonzalez et al., 2007; Wang et al., 2008a; Wang et al., 2010). As a result, we investigated if EMEPO, an intracellular target specific nitrone, could improve NOS1−/− myocyte contractile function. As hypothesized, EMEPO increased Ca2+ transient and shortening amplitudes and enhanced the rate of relaxation (Figure 15A-C). In addition, EMEPO improved contraction across a variety of stimulation frequencies. In a recent study from our lab, we demonstrated that a NO donor, S-Nitro-N-acetyl-DL-penicillamine (SNAP), was able to improve NOS1−/− contractile function (Wang et al., 2010). Interestingly, the observed effects of EMEPO on NOS1−/− contractile function were much greater than those observed with SNAP (Figure 17). Furthermore, we also studied the effect of MENO, a cell permeable O2− scavenger, on NOS1−/− contractile function (Figure 17). As with SNAP, the effects of EMEPO were greater than those observed with MENO. It is our belief that the greater effect of EMEPO
on contractile function can be attributed to the fact that EMEPO acts as both a NO donor and a $O_2^-$ scavenger, thus speaking to its novelty.

As a control, we studied the effect of EMEPO on WT contractile function. EMEPO did not exhibit an effect on WT basal contractile function (Figure 15B-C). This result was expected since WT myocytes have a maintained nitroso-redox balance.

The extracellular spin trap, DMPO, the parent molecule of EMEPO, differs from EMEPO in structure due to its lack of ester groups within its structure. However due to the lack of an ester group, it is unlikely that DMPO would be able to permeate the myocyte cell membrane. Thus we hypothesized that DMPO would have no effect on NOS1$^{-/-}$ myocyte function. Indeed, DMPO did not exhibit an effect on contractile function (i.e Ca$^{2+}$ transient and shortening amplitudes; rate of relaxation) in NOS1$^{-/-}$ myocytes (Figure 15B-C), suggesting EMEPO’s effects on contraction differ from those of other nitrone spin traps.

3.4.5 EMEPO increases myocyte contraction via regulation of RyR and PLB

NOS1 has been shown to localize to the SR (Xu et al., 1999). Furthermore, NOS1$^{-/-}$ myocytes have been shown to have depressed SR Ca$^{2+}$ handling (Khan et al., 2003; Wang et al., 2008b; Wang et al., 2010). A protein that actively regulates SR Ca$^{2+}$ handling is RyR. As a result, we next determined if EMEPO could improve myocyte contraction in NOS1$^{-/-}$ myocytes via regulation of RyR. EMEPO increased the leak/load relationship in NOS1$^{-/-}$ myocytes, but was without effect in WT myocytes suggesting increased RyR activity (Figure 18). The WT leak/load relationship was not statistically different from EMEPO incubated NOS1$^{-/-}$ myocytes, suggesting EMEPO partially
restored the leak/load relationship in NOS1\(^{-/-}\) myocytes. Surprisingly, EMEPO was unable to fully restore the leak/load relationship to the level of WT. This result was unexpected because a previous study, observed that S-nitroso-N-acetyl-DL-penicillamine (nitrosylating agent) could normalize the leak/load relationship in NOS1\(^{-/-}\) myocytes (Wang et al., 2010). Furthermore, EMEPO increased [Ca\(^{2+}\)]\(_i\) in NOS1\(^{-/-}\) myocytes to levels higher than those observed in WT myocytes. Therefore, we expected EMEPO would either normalize the leak/load relationship to the level of WT or increase it beyond the level of WT. With this said, there appears to be a discrepancy between the effects of EMEPO on [Ca\(^{2+}\)]\(_i\) levels and the leak/load relationship in NOS1\(^{-/-}\) myocytes. These data suggest that RyR is a protein target of EMEPO, but more protein targets may be involved in EMEPO’s mechanism of action, which may account for this discrepancy. In addition, it is necessary to identify the post-translation modification by which EMEPO increases RyR activity.

Another protein that regulates SR Ca\(^{2+}\) handling and is a known NOS1 signaling protein target is PLB. PLB is a vital protein that via phosphorylation can increase SERCA’s affinity for Ca\(^{2+}\) leading to increased SR Ca\(^{2+}\) load. Previous studies have shown that PLB-P is decreased in NOS1\(^{-/-}\) myocytes, due to increased protein phosphatase activity (Zhang et al., 2008). Therefore, we determined if EMEPO could increase PLB-P in NOS1\(^{-/-}\) myocytes. EMEPO incubation increased PLB-P in NOS1\(^{-/-}\) myocytes compared to control NOS1\(^{-/-}\) myocytes (Figure 19). It is possible that increased PLB-P may, in part, may account for the discrepancy between [Ca\(_i\)] levels and the leak/load relationship in EMEPO incubated NOS1\(^{-/-}\) myocytes. We believe that EMEPO increased PLB-P as a result of scavenging O\(_2^-\) levels leading to decreased protein
phosphatase activity. Further studies will be needed to confirm this hypothesis. Taken together, these data suggest EMEPO regulates SR Ca\(^{2+}\) handling via both RyR and PLB working in concert. It should be noted that EMEPO may have protein targets that may not be SR-associated. Recent studies have suggested that NOS1 may regulate [Ica], (Sears et al., 2003). Therefore, it is possible that additional protein targets may also be involved in EMEPO’s contractile effects.

### 3.4.6 EMEPO potentiates β-AR receptor stimulated myocyte contraction

PLB is a protein target that is actively regulated by the β-AR receptor signaling pathway. Previous studies have shown that β-AR receptor stimulated contraction is decreased in NOS1\(^{-/-}\) myocytes. Due to the fact EMEPO increased PLB-P in NOS1\(^{-/-}\) myocytes, we hypothesized that EMEPO would improve β-AR stimulated contraction in NOS1\(^{-/-}\) myocytes. As hypothesized, EMEPO potentiated the effects of non-specific β-AR receptor stimulation in NOS1\(^{-/-}\) myocytes (Figure 20). Prior studies have provided evidence that both acute and chronic administration of β-AR agonists can lead to increased O\(_2\)\(^{-}\) production (Zhang et al., 2005; Frederico et al., 2009). Therefore, we next determined if EMEPO could potentiate the functional response of WT myocytes to β-AR stimulation. Surprisingly, there is a trend towards EMEPO potentiating the β-AR receptor response in WT myocyte, which was unexpected because the time course of our experiments is much shorter than previously reported acute administration of β-AR receptor agonists (Figure 20). Further studies will be needed to determine the mechanism by which EMEPO potentiates β-AR receptor stimulated contraction.
3.4.7 EMEPO restores nitroso-redox balance in NOS1−/− myocytes.

Nitroso-redox balance is altered in NOS1−/− myocytes (Khan et al., 2004; Kinugawa et al., 2005; Zhang et al., 2009). Specifically, O₂− levels are increased and NO bioavailability are decreased. Therefore, we next determined if EMEPO increased myocyte contraction via restoration of nitroso-redox balance. EMEPO normalized O₂− and NO levels to the level of WT (Figure 21-22). These data confirm that EMEPO is able to act as both a NO donor and a O₂− scavenger.

3.4.8 Conclusion

In summary, EMEPO is a novel intracellular nitrone spin trap that can act as both a NO donor and a O₂− scavenger. This was seen functionally as increased basal contraction across various frequencies, accelerated relaxation and an increased leak/load relationship with no effect observed in WT myocytes. EMEPO’s effects on contraction were associated with increased RyR activity and PLB-P. In addition, EMEPO’s effects were unique from those observed with other treatments. Furthermore, EMEPO potentiated β-AR receptor stimulated contraction in NOS1−/− myocytes. Taken together, these data suggest EMEPO restores the nitroso-redox balance and increases myocyte contraction via regulation of SR Ca²⁺ handling. With further study, EMEPO may prove useful in the treatment of various cardiomyopathies in which nitroso-redox balance is altered.
Figure 14. Structure of DMPO and EMEPO
Figure 15. EMEPO increases contraction in NOS1<sup>−/−</sup> myocytes with no effect observed in WT myocytes or NOS1<sup>−/−</sup> myocytes incubated with DMPO. A: Individual, steady-state cell shortening (top) and Ca<sup>2+</sup> transient (bottom) traces measured in (NOS1<sup>−/−</sup>, black) and EMEPO incubated NOS1<sup>−/−</sup> (EMEPO, gray) myocytes. B: Summary data (mean±s.e.m.) of the effects of EMEPO and DMPO on shortening (top left) and Ca<sup>2+</sup> transient (top right) amplitudes. C: Summary data (mean±s.e.m.) of the effects of EMEPO and DMPO on rate of relaxation (bottom left) and [Ca<sup>2+</sup>]<sub>i</sub> decline measured as time to 50% relaxation (RT<sub>50</sub>) (bottom right). * P<0.05 vs NOS1<sup>−/−</sup>. Control myocytes in black, and EMEPO incubated myocytes in gray. n=25 cells/5 hearts for WT, n=22 cells/3 hearts for WT + EMEPO, n=6 cells/2 hearts for NOS1<sup>−/−</sup>, n=18 cells/2 hearts for NOS1<sup>−/−</sup> + EMEPO, and n=21 cells/3 hearts for NOS1<sup>−/−</sup> + DMPO (gray, hatched).
Figure 16. EMEPO increases contraction at various stimulation frequencies in NOS1−/− myocytes. Summary data (mean±s.e.m.) of the % of 0.2 Hz Ca2+ transient amplitude in EMEPO incubated NOS1−/− and WT myocytes. * P<0.05 vs WT + EMEPO. n=37 cells/5 hearts for WT + EMEPO (black,solid) and 37cells/6 hearts for NOS1−/− + EMEPO (gray, solid).
Figure 17. EMEPO increases contraction in NOS1−/− myocytes greater than MENO and SNAP. Summary data (mean±s.e.m.) of % of control in shortening and Ca^{2+} transient amplitudes. ANOVA was used to determine statistical significance between the three treatments. * P<0.05 EMEPO vs. MENO and SNAP. n=17 cells/5 hearts for NOS1−/− + MENO (black), n=25 cells/5 hearts NOS1−/− + SNAP (white) and n=18 cells/2 hearts for NOS1−/− + EMEPO (gray).
Figure 18. EMEPO increases the SR Ca\(^{2+}\) leak/load relationship in NOS1\(^{-/-}\) myocytes. Summary data (mean±s.e.m.) of SR Ca\(^{2+}\) leak/load relationship in NOS1\(^{-/-}\) and WT myocytes. * P<0.05 vs NOS1\(^{-/-}\). Control myocytes in black, and EMEPO incubated myocytes in gray. n=9 cells/4 hearts for WT, n=16 cells/5 hearts for WT + EMEPO, n=10 cells/6 hearts for NOS1\(^{-/-}\), and n=19 cells/6 hearts.
Figure 19. EMEPO increases serine 16 PLB-P in NOS1⁻/⁻ myocytes. Summary data (mean±s.e.m.) of EMEPO’s effect on serine 16 PLB-P/ Total PLB in NOS1⁻/⁻ hearts. * P<0.05 vs NOS1⁻/⁻. n=4 hearts for NOS1⁻/⁻ (black) and n=5 hearts for NOS1⁻/⁻ + EMEPO (gray).
Figure 20. EMEPO potentiated the β-AR receptor response. Summary data (mean±s.e.m.) of EMEPO’s effect on β-AR stimulated Ca\(^{2+}\) transient amplitude in NOS1\(^{-/-}\) and WT myocytes. Unpaired t-test was used to determine statistical significance between ISO and ISO+ EMEPO in NOS1\(^{-/-}\) and WT myocytes. * P<0.05 NOS1\(^{-/-}\) + EMEPO + ISO vs NOS1\(^{-/-}\) + ISO. P=0.08 WT + EMEPO + ISO vs. WT + ISO. Control myocytes in black, Isoproterenol (ISO) in white, EMEPO incubated myocytes in gray (solid), and EMEPO incubated myocytes + ISO in gray (hatched) n=16 cells/6 hearts for WT, n=22 cells/6 hearts for WT + EMEPO, n=13 cells/5 hearts for NOS1\(^{-/-}\) and n=10 cells/4 heart.
Figure 21. EMEPO normalizes $O_2^\cdot$ levels in NOS1$^{-/-}$ myocytes. Summary data (mean±s.e.m.) of the effect of EMEPO on $O_2^\cdot$ levels. ANOVA was used to determine statistical significance between the three groups. * $P<0.05$ vs NOS1$^{-/-}$. n=4 hearts for WT (white), n=3 hearts for NOS1$^{-/-}$ (black), and n=3 hearts for NOS1$^{-/-}$ + EMEPO (gray).
Figure 22. EMEPO normalizes NO levels in NOS1−/− myocytes. Summary data (mean±s.e.m.) of the effect of EMEPO on NO levels. * P<0.05 vs NOS1−/−. Control myocytes in black, and EMEPO incubated myocytes in gray. n=12 cells/3 hearts for WT, n=15 cells/3 hearts for WT + EMEPO, n=20 cells/3 hearts for NOS1−/−, and n=17 cells/3 hearts for NOS1−/− + EMEPO.
CHAPTER 4

DISCUSSION

4.1 Principle Findings

This section summarizes the most novel findings of chapters 2 and 3.

1. NOS1 knockout or inhibition blunted the force frequency response, decreased shortening and Ca\(^{2+}\) transient amplitudes, and prolonged relaxation and [Ca\(^{2+}\)]\(_i\) decline.

2. Decreased contraction in NOS1\(^{-/-}\) myocytes is associated with decreased SR Ca\(^{2+}\) load and serine16 PLB-P.

3. Scavenging of ONOO\(^-\) via FeTPPS mimicked the effects of NOS1\(^{-/-}\), suggesting that NOS1 signaling increases myocyte contraction, in part, via ONOO\(^-\) mediated regulation of PLB phosphorylation.

4. EMEPO is a novel intracellular spin trap that can act as both a NO donor and a O\(_2^-\) scavenger.

5. EMEPO increased basal contraction across various frequencies, accelerated relaxation and Ca\(^{2+}\) decline, in NOS1\(^{-/-}\) myocytes with no effect observed in WT myocytes.

6. EMEPO increased contraction in NOS1\(^{-/-}\) myocytes via increased RyR activity and serine 16 PLB phosphorylation.
7. EMEPO potentiated β-adrenergic stimulated contraction in NOS1\(^{-/-}\) myocytes.

4.2 Physiological Implications of NOS1 signaling in the Myocardium

In Chapter 2, we determined the role of NOS1 signaling in murine myocardium. We examined the force frequency response in NOS1\(^{-/-}\) and WT trabeculae. NOS1\(^{-/-}\) trabeculae exhibited a blunted force frequency response and prolonged relaxation compared to WT. To elucidate the mechanism by which NOS1 signaling regulates contraction, we evaluated myocyte contraction via simultaneous measurement of Ca\(^{2+}\) transient and shortening amplitudes in NOS1\(^{-/-}\) and WT myocytes. In agreement with previous studies, NOS1\(^{-/-}\) myocytes exhibited decreased Ca\(^{2+}\) transient and shortening amplitudes and prolonged relaxation compared to WT (Barouch et al., 2002; Gonzalez et al., 2007). Furthermore, acute inhibition of NOS1 in WT myocytes recapitulated the functional effects observed in NOS1\(^{-/-}\) myocytes.

Since PLB is a key regulator of myocyte contraction and [Ca\(^{2+}\)] decline, we evaluated the functional effects of NOS1 signaling on PLB. Acute inhibition of NOS1 had no effect in PLB\(^{-/-}\) myocytes, suggesting PLB as a protein target of the NOS1 signaling pathway. PLB is primarily regulated via phosphorylation; therefore we next determined the effect of NOS1 signaling on PLB phosphorylation. Acute inhibition of NOS1 in WT myocytes decreased basal serine 16 PLB-P compared to control WT myocytes with no effect observed on threonine 17 PLB-P. This is in agreement with a prior study that found NOS1\(^{-/-}\) myocytes exhibit decreased serine 16 PLB-P compared to
WT via increased protein phosphatase activity (Zhang et al., 2008). When PLB is phosphorylated, SERCA Ca\(^{2+}\) uptake increases leading to increased SR Ca\(^{2+}\) load. Furthermore, NOS1\(^{-/-}\) cardiac homogenates have been shown to exhibit decreased SR Ca\(^{2+}\) uptake compared to WT cardiac homogenates (Zhou et al., 2002). Therefore, we evaluated the effect of NOS1 signaling on SR Ca\(^{2+}\) load. NOS1\(^{-/-}\) myocytes exhibited decreased SR Ca\(^{2+}\) load compared to WT myocytes. These data correspond with our observed decreased myocyte contraction and prolonged relaxation. Taken together, these data suggest that NOS1 signaling exerts positive inotropic and lusitropic effects via regulation of SR Ca\(^{2+}\) handling.

Since we observed decreased phosphorylation of PLB at its PKA-phosphorylation site, serine 16, we examined the functional effects of NOS1 signaling on \(\beta\)-AR receptor stimulated contraction. In agreement with previous studies, NOS1\(^{-/-}\) myocytes exhibited decreased \(\beta\)-AR receptor stimulated contraction (i.e. Ca\(^{2+}\) transient and shortening amplitude) compared to WT (Barouch et al., 2002). Interestingly, \(\beta\)-AR receptor stimulation normalized relaxation in NOS1\(^{-/-}\) myocytes to the level of WT. Due to the fact \(\beta\)-AR receptor stimulation is unable to normalize contraction in NOS1\(^{-/-}\) myocytes, it is possible that NOS1 signaling regulates more protein targets than PLB. Due to its localization to the SR, it is possible that RyR may be an additional protein target of NOS1 signaling. However, further studies will be needed to confirm this hypothesis.

NO can signal either in a cGMP-dependent or a cGMP-independent manner (Ziolo et al., 2008). Therefore, we next determined the pathway by which NOS1 signaling exerts its effects on myocyte contraction. We repeated functional experiments in WT myocytes with pharmacological inhibitors of the NO regulated cGMP-dependent
or cGMP independent pathways. ODQ, an inhibitor of guanylate cyclase and therefore cGMP dependent NO signaling, was without effect in WT myocytes. Conversely, FeTPPS, a ONOO\textsuperscript{-} scavenger, exerted functional effects similar to those observed in NOS1\textsuperscript{-/-} myocytes (i.e. decreased Ca\textsuperscript{2+} transient and shortening amplitudes, and prolonged [Ca\textsuperscript{2+}]; decline). Furthermore, FeTPPS was without effect in NOS1\textsuperscript{-/-} myocytes. To test the hypothesis that ONOO\textsuperscript{-} is the causal species of NOS1 signaling, we examined the effect of FeTPPS on serine 16 PLB-P in WT myocytes. As expected, FeTPPS decreased serine 16 PLB-P in WT myocytes.

NOS1 is known to co-localize with xanthine oxidase (a key regulator of O\textsubscript{2-} levels) (Khan et al., 2004). Thus, it is likely that NOS1-derived NO reacts with O\textsubscript{2-} to produce endogenous ONOO\textsuperscript{-}. Our present data suggests that this endogenously produced ONOO\textsuperscript{-/-} is then able to modulate PKA to increase contraction. In agreement with this conclusion, a prior study showed that PKA inhibition prolonged relaxation and decreased serine 16 PLB-P in WT myocytes with no effect observed in NOS1\textsuperscript{-/-} myocytes, inferring decreased PKA activity in NOS1\textsuperscript{-/-} myocytes. This is not unexpected because PKA is known to be a “redox sensitive” enzyme (First & Taylor, 1989). Furthermore, prior studies have shown that low levels of SIN-1 (a ONOO\textsuperscript{-} donor) are able to increase myocyte contraction (i.e. Ca\textsuperscript{2+} transient and shortening amplitudes) and accelerate the rate of relaxation via cAMP-independent activation of PKA (Kohr et al., 2008a; Kohr et al., 2010). In addition, SIN-1 has been shown to regulate SR Ca\textsuperscript{2+} handling via increasing serine 16 PLB-P. Although our present study identified ONOO\textsuperscript{-} as the causal species of NOS1 signaling, the mechanism by which ONOO\textsuperscript{-} modulates PKA remains elusive. A prior study demonstrated that SNAP, a nitrosylating agent, increased cAMP levels and
PKA activity leading to increased myocyte contraction (Vila-Petroff et al., 1999). Thus, more studies will be needed to determine if nitrosylation or an additional post translational modification is responsible for the PKA mediated effects of NOS1 signaling.

With regard to the pathological setting, NOS1 has been shown to translocate from the SR to the sarcolemma (Damy et al., 2004). This event in itself may lead to decreased myocyte contraction similar to that observed in NOS1−/− myocytes. In addition, it is known that ONOO− production increases dramatically due to expression of NOS2 (Ferdinandy et al., 2000). These higher levels of ONOO− have been associated with detrimental contractile effects (Ziolo et al., 2001b; Ziolo et al., 2004). With this said, our study suggests that all ONOO− is not detrimental to cardiac function. It is possible that ONOO− is held in a tightly regulated balance via NOS1 in the healthy myocardium. However when the heart transitions to disease, this system of regulation is altered leading to hazardous levels of ONOO− being produced. Thus, regulation of ONOO− levels in the diseased myocardium may have potential as a therapeutic treatment in HF.

4.3 Therapeutic Implications of EMEPO in Myocardium

In Chapter 3, we investigated EMEPO’s effect on nitroso-redox balance and therefore myocyte contraction. In the healthy myocardium, a precise balance exists between O2− and NO, known as nitroso-redox balance. As the heart transitions to disease, this balance is lost leading to nitroso-redox imbalance. NOS1 has been shown to co-localize with xanthine oxidase, suggesting regulation of O2− levels by NOS1 (Khan et al., 2004). Specifically, O2− levels are known to be increased in NOS1−/− myocytes compared to WT. Due to increased levels of O2− and decreased NO bioavailability, NOS1−/−
myocytes mimic the nitroso-redox imbalance present in disease. With this said, a new class of compounds, nitrone spin traps, have been used to combat the damage caused by nitroso-redox imbalance. These compounds are unique in that they have been shown to act as both NO donors and O$_2^-$ scavengers. Therefore, we determined if EMEPO, an intracellular nitrone spin trap, could restore nitroso-redox balance in NOS1$^{-/-}$ myocytes leading to increased myocyte contraction.

We first determined the effect of EMEPO on myocyte contraction via simultaneous measurement of Ca$^{2+}$ transient and shortening amplitudes in NOS1$^{-/-}$ and WT myocytes. EMEPO significantly increased Ca$^{2+}$ transient and shortening amplitudes and accelerated the rate of relaxation in NOS1$^{-/-}$ myocytes with no effect observed in WT myocytes. To confirm that this result was not due to our stimulation frequency (1 Hz), we performed additional experiments at 0.2 and 0.5 Hz. EMEPO significantly increased Ca$^{2+}$ transient and shortening amplitudes in NOS1$^{-/-}$ myocytes at both 0.2 and 0.5 Hz compared to EMEPO incubated WT myocytes. We next examined if EMEPO exerts functional effects unique from other treatments by repeating functional experiments in NOS1$^{-/-}$ myocytes with MENO, a O$_2^-$ scavenger, SNAP, a NO donor, and DMPO, an extracellular nitrone spin trap. EMEPO, MENO, and SNAP all increased myocyte contraction in NOS1$^{-/-}$ myocytes. However, the effect of EMEPO was significantly greater than either MENO or SNAP suggesting that EMEPO performs a function different from that of a O$_2^-$ scavenger or a NO donor. In addition, DMPO was without effect in NOS1$^{-/-}$ myocytes suggesting that EMEPO exerts effects unique from other nitrone spin traps.
We next investigated the mechanism by which EMEPO increases contraction in NOS1\(^{-/-}\) myocytes. NOS1 has been shown to be localized to the SR (Xu \textit{et al.}, 1999). Furthermore, NOS1 has been shown to increase contraction via regulation of RyR and PLB (Wang \textit{et al.}, 2008b; Wang \textit{et al.}, 2010). Therefore, we determined if EMEPO increased contraction in NOS1\(^{-/-}\) myocytes via regulation of RyR and PLB. EMEPO increased the leak/load relationship in NOS1\(^{-/-}\) myocytes with no observed effect in WT myocytes, suggesting increased RyR activity. In addition, EMEPO increased serine 16 PLB-P in NOS1\(^{-/-}\) myocytes. Taken together, these data suggest EMEPO increases contraction, in part, via regulation of RyR and PLB. Interestingly, EMEPO partially restored the leak/load relationship in NOS1\(^{-/-}\) myocytes to the level of WT. This result was unexpected because SNAP, a nitrosylating agent, was capable of normalizing the leak/load relationship in NOS1\(^{-/-}\) myocytes (Wang \textit{et al.}, 2010). Since EMEPO in theory is both a O\(_2^-\) scavenger and a NO donor, it was expected that RyR activity would either be normalized or increased above the level of WT in NOS1\(^{-/-}\) myocytes. Therefore, it is possible EMEPO may have protein targets that are not SR-associated. Recent studies have suggested that NOS1 may regulate [Ica]\(_i\) (Sears \textit{et al.}, 2003). Thus, further studies will be needed to determine if EMEPO is able to target non SR-associated protein targets.

PLB phosphorylation is primarily regulated by the \(\beta\)-AR receptor signaling pathway. Thus, we next determined if EMEPO regulated this signaling pathway. As seen in Chapter 2, NOS1\(^{-/-}\) myocytes exhibited a blunted response to \(\beta\)-AR receptor stimulation compared to WT. EMEPO was able to normalize the \(\beta\)-AR receptor response in NOS1\(^{-/-}\) myocytes to the level of WT myocytes. Interestingly, our data suggest EMEPO may potentiate the \(\beta\)-AR receptor response in WT myocytes. Previous studies
have shown that acute and long term administration of β-AR receptor agonists can lead to increased $O_2^-$ production (Zhang et al., 2005; Frederico et al., 2009). Therefore, it is possible that EMEPO potentiates the β-AR receptor response via $O_2^-$ scavenging. Further studies will be needed to confirm this hypothesis.

Nitroso-redox imbalance has been implicated in the contractile dysfunction observed in NOS1−/− myocytes (Khan et al., 2004). Thus, we determined if EMEPO increased contraction via restoration of nitroso-redox balance. We first measured $O_2^-$ levels in NOS1−/− and WT cardiac myocyte homogenates. As previously reported, NOS1−/− myocytes exhibited increased $O_2^-$ levels compared to WT (Khan et al., 2004; Kinugawa et al., 2005; Zhang et al., 2009). EMEPO incubation normalized $O_2^-$ levels in NOS1−/− myocytes to the level of WT suggesting near complete scavenging of $O_2^-$. To fully restore nitroso-redox balance, it is necessary to normalize both $O_2^-$ and NO levels. Therefore, we examined if EMEPO could regulate NO levels in NOS1−/− myocytes. EMEPO increased NO levels in NOS1−/− myocytes with no effect observed in WT myocytes. Taken together, these data suggest EMEPO like other nitrone spine traps (i.e. PBN, DMPO) can release NO as a reactionary byproduct of $O_2^-$ scavenging (Locigno et al., 2005). However, EMEPO is unique in that it is intracellularly targeted unlike PBN and DMPO.

In HF, xanthine oxidase and NADPH oxidase activity increases while SOD activity decreases (Ide et al., 1999; Cappola et al., 2001; Heymes et al., 2003; Sam et al., 2005). In hypertrophy, NOS3 becomes uncoupled which will lead to production of $O_2^-$ (Takimoto et al., 2005b). There is also an increase in $O_2^-$ levels in I/R injury due to mitochondrial production (Ambrosio et al., 1993). High levels of $O_2^-$ have been shown to
alter the function of a variety of vital cardiac contractile proteins, notably RyR, NCX and
the SERCA pump, leading to contractile dysfunction (Zima & Blatter, 2006;
Zissimopoulos & Lai, 2006; Kuster et al., 2010). NO bioavailability is also altered in HF.
Specifically, NOS1 translocates from the SR to the sarcolemma and NOS2 is induced
(Ziolo et al., 2001b; Damy et al., 2004). This nitroso-redox imbalance has been
implicated in the contractile dysfunction observed in HF (Gonzalez et al., 2010.). With
this said, treatments have been developed to combat the effects of nitroso-redox
imbalance. Unfortunately, in a clinical trial, the xanthine oxidase inhibitor oxypurinol did
not lead to clinical benefits in HF patients (Hare et al., 2008). This type of therapy may
not have been beneficial since reducing O$_2^-$ levels by itself will not restore the altered
nitroso-redox balance. In our present study, we observed that EMEPO restores nitroso-
redox balance in a model of oxidative stress leading to improved contraction. Therefore,
it is possible that EMEPO has potential as a therapeutic agent in HF.

4.4 Future Directions

The results presented herein provide evidence that NOS1-derived NO can
modulate myocyte contraction. However, our present studies do leave several questions
to be answered. In Chapter 2, we determined that ONOO$^-$ is the causal species of NOS1
signaling and increases contraction in part via increasing serine 16 PLB-P. Although we
identified ONOO$^-$ as the causal species, it remains unclear how ONOO$^-$ regulates PKA to
increase serine 16 PLB-P. PKA has two free cysteine residues (First & Taylor, 1989). It
is possible ONOO$^-$ modifies PKA via S-nitrosylation of a free cysteine residue to
increase PKA activity and therefore serine 16 PLB-P. With regard to PKA activity, it is
unclear if PKA activity differs between NOS1\(^{-/-}\) and WT myocytes. A prior study demonstrated inhibition of PKA in WT myocytes produced effects similar to those observed in NOS1\(^{-/-}\) myocytes (i.e. prolonged relaxation and decreased serine 16 PLB-P) (Zhang et al., 2008). These findings lead us to hypothesize that PKA activity is decreased in NOS1\(^{-/-}\) myocytes compared to WT myocytes. Our present study supports the notion that NOS1-derived NO regulates PLB. However, recent studies have suggested that NOS1-derived NO may regulate \([i_c]\) and the sarcolemmal calcium pump (Sears et al., 2003; Oceandy et al., 2007). We hypothesize that due to NOS1 primarily being localized to the SR, NOS1-derived NO will have minimal effects on either of these protein targets.

In Chapter 3, we determined that EMEPO increased myocyte contraction in NOS1\(^{-/-}\) myocytes via restoration of nitroso-redox balance. Due to the fact this was a pilot study to determine EMEPO’s function, many questions remain unanswered. EMEPO increased contraction, in part, via increased serine 16 PLB-P. In NOS1\(^{-/-}\) myocytes, PP activity is known to be increased due to increased susceptibility of PP’s to higher \(O_2^-\) levels (Sommer et al., 2002; Zhang et al., 2008). As a result of EMEPO’s effects on \(O_2^-\) levels, we hypothesized that EMEPO would decrease PP activity leading to its observed effects on serine 16 PLB-P. Conversely, PKA, the kinase which phosphorylates PLB at serine 16, is also a “redox-sensitive” enzyme (First & Taylor, 1989). Therefore, it is our hypothesis that EMEPO may also increase PKA activity via \(O_2^-\) scavenging. We also found that EMEPO increased contraction via increased RyR activity. RyR activity can be regulated via phosphorylation, nitrosylation or oxidation (Stoyanovsky et al., 1997; Marx et al., 2000; Terentyev et al., 2008). Due to EMEPO’s unique characteristics (i.e. \(O_2^-\) scavenger, NO donor), it is possible EMEPO can increase RyR activity via 1) decreased
oxidation of RyR, 2) increased S-nitrosylation of RyR or 3) increased RyR phosphorylation. Thus, more studies are needed to determine the post-translational modification by which EMEPO regulates RyR function. Interestingly, EMEPO did not normalize the leak/load relationship in NOS1−/− myocytes to the level of WT myocytes. Thus, it is must be determined if EMEPO regulates more protein targets than RyR and PLB. Lastly, EMEPO has potential to restore nitroso-redox balance and increase contraction in HF. Therefore, experiments must be conducted to determine EMEPO’s therapeutic potential in a more clinically relevant model of HF.

4.5 Final Remarks

Nitric Oxide is a potent regulator of cardiac contractile function. The results presented herein provide evidence that NOS1-derived NO is a positive inotrope in the heart. Furthermore, these results report the novel finding that an intracellular nitrone spin, EMEPO, can increase contractile function via restoration of nitroso-redox balance in a model of oxidative stress. These findings suggest that reactive nitrogen species are important regulators of cardiac function in both health and disease. With further study, the results presented herein may help aid in the development of new therapeutic agents to treat heart failure.
LIST OF REFERENCES


