ABSTRACT

Coronary heart disease is one of the leading causes of death worldwide. After an acute myocardial infarction timely and effective reperfusion is one of the treatment strategies for limiting the size of the infarct. However, despite the clearly positive effect of reperfusion therapy like reduce mortality, limit infarct size, and improve functional parameters there are many detrimental consequences of myocardial reperfusion. Reperfusion injury is a serious pathological process that is induced by the restoration of blood flow to previously ischemic tissue. When ischemic myocardium is reperfused and oxygen reintroduced, there is a sudden burst of oxygen free radicals (OFR) production. OFR occurring during reperfusion injury have been implicated in the pathogenesis of myocardial stunning and progression of heart failure.

Hydroxyl radicals (OH*) are one of the most aggressive species of OFR that attack many molecules in the human body. These OH* are involved in the pathogenesis of ischemia reperfusion (IR) injury, which is observed in many clinical situations including acute heart failure, stroke, and myocardial infarction. It is documented that two different sub-cellular defects: deranged calcium handling and alterations of myofilament responsiveness contribute to the development of acute myocardial dysfunction. Although the phenotype of the acute injury response is rather well documented, the underlying mechanism and the relative importance of these two factors that is responsible for the
transient injury and subsequent partial recovery remains incompletely understood. Hence the specific aims presented in this thesis tries to delineate, in a time resolved manner, the contribution of altered calcium handling and myofilament responsiveness throughout the various stages of IR injury.

In order to develop novel treatment strategies against reperfusion injury of the heart, we need to unravel the basic mechanistic sequence of the mediators of this injury and their contribution at various stages during both the acute and the sustained injury. To elucidate the mechanism that are responsible for the phenotypical dysfunction after IR injury, we propose to test the following hypothesis:

**The mechanism of oxidative stress injury is the cumulative effect of a deranged calcium handling and changes in myofilament function, but they exert their maximal impact at distinctly different time-points during the injury phase.**

First, we set out to develop a method necessary to complete this project. We developed the in vitro model of OH* injury in mouse, rabbit and rat in which we can directly expose the papillary muscle/trabeculae with OH* comparable to those that occur in IR injury.

Then we wanted to look at the role of altered calcium handling. We investigated whether up-regulation of SR-Ca$^{2+}$ATPase function (SERCA), can attenuate OH* induced dysfunction. Small, contracting right ventricular papillary muscles from wild type, SERCA1a over-expressing and SERCA2a heterogeneous knock-out mice were directly exposed to OH*. We found out that modulation of SERCA activity can indeed alter the magnitude of effects of OH* injury on the heart. The OH* induced injury is substantially less in transgenic mice with higher SERCA activity, and aggravated in mice with a
Next we focused on the possible interactions of altered calcium handling and altered myofilament responsiveness at different time points of IR injury. To test our hypothesis that calcium overload plays a major role during acute/early OH* injury and altered myofilament response in the sustained/later part of the OH* injury. We temporarily delineated the impact of these two mechanisms by calibrating intracellular Ca^{2+} transients using ionophoretically loaded bis fura-2, potassium contracture were assessed in parallel in isolated rabbit trabeculae. After the exposure of OH*, initially there is a marked increase in diastolic force in parallel with an increase in diastolic calcium. We followed these parameters until a new steady state level was reached at about 45 minutes post-OH*-exposure. At this new baseline, diastolic calcium had returned to near-normal, pre-OH* levels, whereas diastolic force remained markedly elevated. Assessment of the myofilament responsiveness confirmed an increased calcium-sensitivity at the new baseline after OH*-induced injury as compared to the pre-OH* state. In conclusion the acute injury that occurs after OH* exposure is mainly, if not entirely is due to calcium overload, while the later sustained myocardial dysfunction is mainly due to the altered/increased myofilament responsiveness.
Dedicated to my parents: Laxman Das and Kavita Vaswani
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FIELDS OF STUDY

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2. Medicine
3. Cardiac contractile physiology
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<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<tr>
<td>CAMKII</td>
<td>Calcium-Calmodulin Kinase 2</td>
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<tr>
<td>cAMP</td>
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<tr>
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<td>Long Type</td>
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Chapter 1

INTRODUCTION

1.1 General Introduction

The cardiovascular system is composed of the heart and blood vessels which provides the body’s tissues with a continuous supply of oxygen, nutrients, and waste removal. The heart is the engine of the circulatory system. It is a hollow muscular pump in all vertebrates responsible for pumping blood through the blood vessels by repeated, rhythmic contractions. In humans, during each heartbeat, typically about 60 to 90 ml (about 2 to 3 oz) of blood is pumped out of the heart. The heart’s contractile function has been extensively studied for the last 100 years such that we now understand the basic mechanisms by which the heart contracts and relaxes.

Heart disease is the leading cause of death in the United States. Almost 2,000 Americans die of heart disease each day. Congestive heart failure (CHF) is a condition in which the heart can not pump enough blood to the body's other organs. In developing countries, around 2% of adults suffer from heart failure, but in those over the age of 65, this increases to 6-10% (Dickstein 2008). Both cardiac and systemic factors can impair cardiac performance and cause or aggravate heart failure. Though etiologies
vary, two of the most common types are ischemic cardiomyopathy and pressure overload (hypertrophic) cardiomyopathy (Cowie, Mosterd et al. 1997). Ischemic cardiomyopathy (CM) is the most common type of cardiomyopathy in the United States. It affects approximately 1 out of 100 people, most often middle-aged to elderly men. In Ischemic CM, the heart's ability to pump blood is decreased because the heart's main pumping chamber, the left ventricle, is enlarged, dilated and weak. This is caused by ischemia - an insufficient supply of blood to the heart muscle caused by coronary artery disease and heart attacks. Timely and effective reperfusion is one of the treatment strategies for limiting the size of the infarct. Timely reperfusion facilitates cardiomyocyte salvage and decreases cardiac morbidity and mortality.

1.2 Reperfusion Injury

Reperfusion of an ischemic area may result, however, in paradoxical cardiomyocyte dysfunction, a phenomenon termed "reperfusion injury" (Verma, Fedak et al. 2002). When a tissue is subjected to ischemia, a sequence of chemical events is initiated that may ultimately lead to cellular dysfunction and necrosis. If ischemia is ended by the restoration of blood flow, a second series of injurious events ensue producing additional injury. Thus, when there is an ischemia which leads to transient decrease or interruption of blood flow the net injury is the sum of two components - the direct injury occurring during the ischemic interval and the indirect or reperfusion injury which follows. Reperfusion injury results in myocyte damage through myocardial stunning, microvascular and endothelial injury, and irreversible cell damage or necrosis.
1.2.1 Mediators of Reperfusion Injury

When ischemic myocardium is reperfused and oxygen reintroduced, there is a sudden burst of OFR production. These radicals have been implicated in the pathogenesis of myocardial stunning and progression of heart failure. OFR such as superoxide anion, and OH* are produced within the first few minutes of reflow (Bolli, Jeroudi et al. 1989). During myocardial reperfusion, the acute ischemic myocardium is subjected to several abrupt biochemical and metabolic changes. These changes include mitochondrial reenergization, the generation of reactive oxygen species (ROS), intracellular Ca\(^{2+}\) overload, the rapid restoration of physiologic pH, and inflammation, all of which interact with each other to mediate the induction of cardiomyocyte hypercontracture and cardiomyocyte death.

ROS are central to cardiac injury during ischemia and reperfusion. ROS are ions or very small molecules that include oxygen ions, free radicals, and peroxides, both inorganic and organic. They are highly reactive due to the presence of unpaired valence shell electrons. There are numerous types of free radicals that can be formed within the body. The most common ROS include: the superoxide anion, the OH*, singlet oxygen, and hydrogen peroxide.

OH* are the most aggressive species of OFR that attack many molecules in the human body (Gao, Liu et al. 1996; Zeitz, Maass et al. 2002). These OH* interact with lipids, proteins, nucleic acids, and damage cell membranes and as a result impair cellular function. OH* are involved in the pathogenesis of reperfusion injury, which is observed in many clinical situations including acute heart failure, stroke, and myocardial infarction. Conditions of reperfusion injury are created routinely during heart surgery and
transplantation (Bolli and Marban 1999). OFR occurring during reperfusion injury have been implicated in the pathogenesis of myocardial stunning and progression of heart failure. However, the mechanism by which these ROS foster damage is poorly understood.

In the mitochondria 1–4% of available oxygen is normally incompletely reduced and leaks in the form of superoxide primarily at complexes I and III but this percentage increases at supranormal O₂ tensions or after mitochondrial injury. During myocardial reperfusion, ROS are generated not only by the reduction of molecular oxygen but by xanthine oxidase (mainly from endothelial cells) and NADPH oxidase (mainly from neutrophils). Myocardial reperfusion also leads to neutrophil activation and accumulation (Jordan, Zhao et al. 1999). In addition to the dysfunction of the sarcoplasmic reticulum and contribution to intracellular Ca²⁺ overload, there is an abrupt increase in intracellular Ca²⁺ damaging the cell membrane by lipid peroxidation, inducing enzyme denaturation, and causing direct oxidative damage to DNA. During myocardial reperfusion, the already Ca²⁺-overloaded cardiomyocyte is subjected to a further influx of Ca²⁺ through a damaged sarcolemmal membrane, ROS-mediated dysfunction of the sarcoplasmic reticulum, and reverse function of the Na⁺–Ca²⁺ exchanger (NCX)(Zeitz, Maass et al. 2002). During myocardial reperfusion, the rapid washout of lactic acid together with the function of the Na⁺–H⁺ and Na⁺–HCO₃ transporters mediate the rapid restoration of physiologic pH and cardiomyocyte hypercontracture.

1.2.2 Mechanism of Reperfusion Injury

It is documented that multiple sub-cellular defects contribute to the development
of acute myocardial dysfunction, predominantly intracellular calcium overload and alteration of myofilament responsiveness. The response of intact contracting papillary/trabeculae muscles in rat and rabbit to OH* mimics that of OH* exposure of the whole heart. Previous studies have demonstrated that intact contracting cardiac trabeculae from mouse, rat, and rabbit after acute exposure of OH* develop a rigor like contracture marked by an increase in diastolic tension, myofilament proteolysis, and overall decreased cardiac contractility, followed by a partial recovery (Zeitz, Maass et al. 2002). Although the phenotype of the acute injury response is rather well documented, the underlying mechanism and the relative importance of these two factors, deranged calcium handling versus altered myofilament function, that are responsible for the transient injury and subsequent partial recovery, remains incompletely understood.

1.2.3 Role of Calcium Handling

There are multiple mechanisms that are possibly responsible for calcium overload via sarcoplasmic reticulum damage, mitochondrial damage and changes in properties of NCX and L-type channels that have been implicated to play a role. The relative contributions of these factors are still unknown, specifically to what extent and how they relate to the sustained damage observed after OH* exposure. Previous studies have shown that there is a direct impairment of sarcoplasmic reticulum function due to OH* exposure (Flesch, Maack et al. 1999). There is a clear attenuation of the positive force frequency relationship after the exposure of OH* (Janssen, Zeitz et al. 1999; Zeitz, Maass et al. 2002). These studies indicate that there is impairment of sarcoplasmic reticulum function due to OH*. Reverse NCX (Zeitz, Maass et al. 2002) has also been reported as a
major cause of acute diastolic dysfunction due to OH* exposure. In addition, in many studies, calcium channel antagonists (Ehring, Bohm et al. 1992; Ferrari, Curello et al. 1993) have been proposed to prevent the myocardial stunning, which shows that the L-type calcium channel is the main source of intracellular calcium overload, but calcium antagonists are only effective if applied before or immediately after ischemia.

Combined, from these studies, it is clear that calcium overload plays a significant role in acute cardiac dysfunction due to OH* injury. In order to target novel treatment strategies for OH* injury on heart, a further analysis is required to assess the role of calcium overload at different time-points during the injury phase.

1.2.4 Role of Myofilament Responsiveness

In parallel to understanding when calcium handling is deranged, it is important to know when myofilament function is involved in the phenotype of OH*-induced injury. During IR injury there is an acute change in myofilament response, due to the metabolic acidosis and accumulation of different metabolites. Previous studies have shown that OH* exposure can lead to the proteolysis of different sub-units of TnIn different species (Zeitz, Maass et al. 2002). In rabbit myocardium, hydroxyl exposure leads to a degradation of troponin T (TnT) associated with increased calcium sensitivity, while in rat myocardium, troponin I (TnI) was degraded with intact TnT and unaltered calcium sensitivity (Zeitz, Maass et al. 2002). In humans, degradation of TnI has been observed in myocardium of bypass patients due to IR injury with intact TnT (McDonough, Labugger et al. 2001).

Because of the species specific differences regarding myofilament degradation in
response to OH*, a further analysis is required to assess the role of myofilament-specific proteolysis in OH* induced cardiac dysfunction as well as the exact extent of these myofilament alterations.

1.2.5 Interaction Between Myofilament Responsiveness and Calcium Handling

It is clearly known that there is a calcium overload involved in OH* injury. Due to this calcium overload, there is activation of calcium dependent proteases like calpain which in turn causes degradation(s) of the troponin complex (Di Lisa, De Tullio et al. 1995; Gao, Atar et al. 1997) and potentially alters the calcium sensitivity. Therefore, we aim to assess the possible interactions of these two mechanisms at different time points of IR injury. By doing so, we prove our hypothesis that calcium overload plays a major role during acute/early OH* injury and altered myofilament response in the sustained/later part of the OH* injury. By elucidating the relative importance of these two distinctly different injury pathways at various stages throughout the injury window, we may gain crucial insight in how to target hypothesis-driven novel treatment strategies for oxidative stress injury in the heart.

1.3 Excitation Contraction Coupling

Excitation-contraction coupling (ECC) is the process whereby an action potential triggers the myocyte to contract by myocyte depolarization and entrance of calcium ions into the cell.
1.3.1 Cardiac Action Potential

The momentary change in electrical potential on the surface of a cardiac muscle cell, that occurs when it is stimulated, results in the transmission of an electrical impulse. When an action potential passes over a cardiac muscle membrane, the action potential spreads to the interior of cardiac muscle fiber along the membranes of transverse tubules. All myocytes are connected at their long ends to other myocytes via electrically coupled gap junctions allowing action potentials to travel from cell to cell. When these cells are rapidly depolarized to a threshold voltage of about -70 mV by an action potential in an adjacent cell, there is a rapid depolarization that is caused by, the opening of voltage gated sodium channels. This leads to the entrance of calcium ions through L-type calcium channels located on the sarcolemma. The highest concentration of L-type calcium channels is located in the transverse tubules, near the intracellular calcium stores of the sarcoplasmic reticulum (SR). It is in the transverse tubules that the calcium entering through the L-type calcium channel interacts with the ryanodine receptor (RyR) of the SR causing the sudden release of calcium into the cytoplasm. Calcium ions entering through slow calcium channels prolong depolarization of the membrane, creating a plateau. Repolarization occurs when voltage gated potassium channels open, along with the inactivation of Ca^{++} channels. Once an action potential is initiated, there is a period of time that a new action potential cannot be initiated, the refractory period of the cell. During the refractory period, stimulation of the cell by an adjacent cell undergoing depolarization does not produce new, propagated action potentials. This acts as a protective mechanism in the heart by preventing multiple, compounded action potentials from occurring. This is important because at very high heart rates, the heart would be
unable to adequately fill with blood and therefore ventricular ejection would be reduced.

Ischemia/hypoxia causes an elevation in extracellular K\(^+\) due to the leaking of K\(^+\) through K\(_\text{ATP}\) channels (normally inhibited by ATP) and due to the decreased activity of the Na\(^+\)/K\(^+\)-ATPase pump. These changes occur because ATP levels decline in hypoxic cells. The increase in extracellular K\(^+\) cause membrane depolarization and inactivation of fast Na\(^{++}\) channels, leading to decreased conduction velocity. These changes can often produce arrhythmias that may require the use of antiarrhythmic drugs.

1.3.2 Calcium Transient in Myocyte

The calcium transient is the sharp rise and fall of intracellular calcium that occurs with each cardiac beat. To maintain calcium homeostasis, the amount entering from the extracellular space and the SR must be removed from the cell and pumped back into the SR respectively. Myocardial calcium handling differs between species, mainly in the relative contribution between the sources for activator calcium. Intracellular calcium decline after activation is predominantly accomplished by the SERCA, and secondarily facilitated through NCX. SERCA is regulated primarily by a neighboring SR protein phospholamban (PLB), which when bound to SERCA, lowers its affinity for calcium, decreasing its pumping rate. Dephosphorylated PLB inhibits the affinity of SERCA for Ca\(^{2+}\), whereas phosphorylation removes this inhibition. In smaller rodents like the rat 95-98% of activator Ca\(^{2+}\) is resequestered from the cytoplasm by SERCA, while in the rabbit, only 70% is resequestered, similar to human (Bers 2002; Bers 2002b).

There are multiple mechanisms that are possibly responsible for calcium overload in reperfusion injury; dysfunction of SR, dysfunction of the L-type calcium channel, leaks
in the sarcolemma, and increased calcium influx via reverse mode of the NCX (Bolli 1990; Xu, Zweier et al. 1997; Bolli and Marban 1999). It is known that the activity of the SERCA is reduced by OFR. Xu and coworkers (Xu, Zweier et al. 1997) presented evidence that the ATP binding site of the SERCA is attacked by OFR. In several studies, calcium channel antagonists prevented myocardial stunning (Ehring, Bohm et al. 1992; Ferrari, Curello et al. 1993; Massoudy, Becker et al. 1995). These data led to the hypothesis that increased open probability of the L-type calcium channel is the source of intracellular calcium overload in radical exposed myocardium (Bolli and Marban 1999). The relative contributions of these factors are still unknown, specifically to what extent and how they relate to the sustained damage observed after OH* exposures.

The SERCA pump, a 110-kDa transmembrane protein, is the major regulator of Ca\textsuperscript{2+} homeostasis and contractility in cardiac and skeletal muscle. There are five isoforms of SERCA genes that have been isolated. SERCA2a gene is the predominant SERCA isotype expressed in both normal and failing hearts. SERCA2a plays a central role in SR Ca\textsuperscript{2+} handling required for ECC in the heart. The expression levels and activity of SERCA2a are critical determinants of cardiac function. It transfers Ca\textsuperscript{2+} from the cytosol of the cell to the lumen of the SR at the expense of ATP hydrolysis during muscle relaxation.

PLB is a 52 amino acid protein of 6.1 kDa that forms a homopentamer. In the dephosphorylated state, it is an inhibitor of SERCA2a activity, but inhibition is relieved upon phosphorylation of PLB. During muscle contraction, PLB binds to the Ca\textsuperscript{2+} pump and prevents Ca\textsuperscript{2+} from being pumped back into the SR. During muscle relaxation, PLB is phosphorylated by Protein Kinase A (PKA) at Ser16 and by CaMKII at Thr17 which
removes the inhibition and restores low calcium levels in the cytoplasm. Previous studies have demonstrated a decrease in SERCA activity at increasing concentrations of OH* (Flesch, Maack et al. 1999) as well as a clear attenuation of the positive force-frequency relationship after the exposure of OH* (Schouten and ter Keurs 1986; Janssen, Zeitz et al. 1999; Zeitz, Maass et al. 2002). These studies indicate that there may be a direct impairment of sarcoplasmic reticulum function due to OH* exposure. Previous studies by us also indicate that after OH* exposure in mice trabeculae there is a marked decrease in the level of PLB phosphorylation at Ser16 (Hiranandani, Bupha-Intr et al. 2006). This indicates a specific role for the phosphorylation of PLB on basal contractile function, and represents a compensatory mechanism via which the calcium handling is normalized in presence of altered SERCA activity. We need to further explore the possible functional significance of SERCA and PLB in the mechanical recovery after IR injury.

The NCX is an antiporter membrane protein which removes calcium from cells. It uses the energy that is stored in the electrochemical gradient of sodium (Na+) by allowing Na+ to flow down its gradient across the plasma membrane in exchange for the counter transport of calcium ions. The NCX removes a single calcium ion in exchange for the import of three sodium ions. Since the transport is electrogenic (alters the membrane potential), depolarization of the membrane can reverse the exchanger's direction if the cell is depolarized enough, as may occur in reperfusion injury. It has been proposed that there is building of internal Na+ level in myocytes via Na+/H+ exchanger by intracellular acidosis in ischemia. During initial phase of reperfusion injury this Na+ is extruded out by reverse NCX and leads to the calcium overload as Na+/K-ATPase is impaired due to depletion of ATP.
In order to target novel treatment strategies for OH* injury in the heart, a further analysis is required to assess the role of calcium overload at different time-points during the injury phase.

1.3.3 Cardiac Myofilaments

Myofilaments are the contractile protein filaments whose highly organized arrangement results in the striations observed in the single muscle fiber. A sarcomere is the basic unit of a muscle's cross-striated myofibril. Myofilaments consist of two types of filament, thick and thin. The thick filament system is composed of myosin protein which is connected from the M-line to the Z-disc by Titin. It also contains myosin-binding protein C which binds to the thick filament at one end, and to actin at the other end. The thin filaments are assembled by actin monomers bound to nebulin, which also involves tropomyosin (a dimer which coils itself around the F-actin core of the thin filament). Tropomyosin (TM), along with the troponin complex, associates with actin in muscle fibers and regulate muscle contraction by regulating the binding of myosin. In a resting muscle, TM overlays the myosin binding sites on actin and is "locked" down in this position by TnT (TnT) (tropomyosin binding troponin) and TnI (TnI) (inhibitory troponin). Upon release of calcium from the sarcoplasmic reticulum, calcium binds to troponin C (TnC) (calcium binding troponin). This "unlocks" TM from actin, allowing it to move away from the binding groove. Myosin heads can now access the binding sites on the actin. Once one myosin head binds, TM completely displaced, allowing additional myosin heads to bind, initiating muscle shortening and contraction. Once calcium is pumped out of the cytoplasm and calcium levels return to normal, TM again binds to
actin, preventing myosin from binding. In the muscles’s resting state, calcium is not bound to TnC and myosin and actin have no force producing cross bridges. ATP is bound to the myosin heavy chain providing it with future energy for force production. The myosin ATPase hydrolyzes the ATP to an ADP plus a free organic phosphate both still bound to myosin. This energizes the myosin and equips it for the formation of a cross bridge. When calcium binds to TnC, the conformational change that subsequently occurs exposes the myosin binding site on the actin. This permits myosin to bind to actin and begin the so-called power stroke (the force generating action of the cross bridge), and releases the phosphate (keeping the ADP) in the process. With the myosin still bound to actin, a new ATP replaces the ADP on the myosin allowing for the interaction to break. With calcium still bound to TnC, the cycle will continue with the formation of a new cross bridge. If calcium as left TnC, the myofilaments stay in their resting state.

1.3.4 Myofilaments Cardiac Sensitivity

Myofilament calcium sensitivity reflects the contractile response of the myofilaments to a given calcium concentration at steady state. ECC is linked to both the rise and fall of the intracellular calcium concentration \([\text{Ca}^{2+}]_i\), and intrinsic properties of the myofilaments (Backx, Gao et al. 1995). The relation between \([\text{Ca}^{2+}]_i\), and steady state force development is known as force-pCa relationship. The myofilament calcium sensitivity curve is a sigmoid shaped curve showing that the cardiac myofilaments are activated by calcium, in a graded manner. There are three parameters of the myofilament calcium sensitivity curve which can provide information about the calcium responsiveness of the contractile system. The maximum force, or \(F_{\text{max}}\), is the maximum
force produced by the myofilaments that cannot be increased by adding more calcium. Other parameters include nHill and EC50. EC50 provides a very good measure to assess calcium sensitivity as it is the effective calcium concentration at which half maximal force is being developed. The other parameter is nHill or Hill coefficient which is the slope of force-pCa relation at EC50 point and quantifies the cooperativity of contractile processes.

There are many factors that alter the calcium sensitivity of the contractile proteins such as temperature, pH, myofilament protein phosphorylation, etc (Solaro, el-Saleh et al. 1989; Kentish, McCloskey et al. 2001) Thus, changes in the cooperative processes leading to force development and changes in calcium sensitivity can be assessed by myofilament calcium sensitivity curve under different conditions. In general, an increase in myofilament calcium sensitivity can result in greater force production and slower relaxation. Decrease in myofilament calcium sensitivity can cause decreased force production and faster relaxation.

The most widely studied modulation of myofilament calcium sensitivity is the phosphorylation of TnI at serines 23 and 24 by adrenergic response kinase PKA. Phosphorylation of TnI has been shown to decrease calcium sensitivity of the myofilaments in intact (Okazaki, Suda et al. 1990) and skinned muscle preparations (Herzig, Peterson et al. 1982). TnI phosphorylation at these sites can produce an increase in the EC₅₀ but no change in the Fₘₐₓ decreasing force production at non-saturating calcium concentrations.

Although the [Ca²⁺], and myofilament response are linked, changes in calcium and force production are decoupled in the time domain. myofilament calcium sensitivity
reflects the contractile response of the myofilaments to a given calcium concentration at steady state. Thus this sensitivity plays a major role in determining the contractile response to the intracellular calcium transient. Myofilament calcium sensitivity has traditionally been assessed in so-called skinned fibers where membranes are dissolved away and myofilaments are exposed to EGTA-buffered calcium solutions (Fabiato and Fabiato 1978). Skinned preparations have been very useful in defining calcium sensitivity changes with respect to mutations at the myofilament level; however, they can not be unambiguously compared with dynamic twitch contractions nor can they traditionally be done at physiological temperature due to technical limitations. First, after the preparations are demembranated, they can no longer respond to normal physiological stimuli such as neurohumoral modulators (which require receptors) and electrical stimulations (which require membrane action potentials). In addition, cytosolic factors and protein phosphorylations (and other posttranslational modifications, such as methylations), that occur physiologically and can potentially contribute to the myofilament calcium sensitivity, are generally not controlled in skinned fiber preparations (Gao, Backx et al. 1994). Although assessment of myofilament calcium sensitivity in intact muscle has been performed in the past, it has been limited to experiments at or below room temperature and in the presence of compounds that prevent sarcoplasmic reticulum cycling (CPA, thapsigargin, etc.) (Gao, Backx et al. 1994; Perez, Hashimoto et al. 1999). Experiments with an incapacitated SR induce contractures that are not reversible or cannot be repeated multiple times do not lend themselves to the assessment of calcium sensitivity. Therefore, data so obtained can easily be extrapolated to in vivo conditions. To eliminate these disadvantages, we used the technique recently developed in our lab (Varian, Raman et al.
based on iontophoresis coupled with K\(^+\) contractures to construct the steady-state relationship. This technique allowed for assessment of the dynamic force-[Ca\(^{2+}\)]\(_i\) relationship measurements at multiple times, and under different conditions all in the same trabeculae preparation (to eliminate intermuscle variation).

Previous studies have shown that OH\(^*\) exposure can lead to the proteolysis of different sub-units of TnI in different species (Zeitz, Maass et al. 2002). Despite a similar intracellular calcium overload, specific myofilament alterations or lesions may be one of primary causes of contractile dysfunction that sustains beyond the rigor-like initial insult after OH\(^*\) exposure. However, because of the differences between the species regarding myofilament degradation in response to OH\(^*\), a further analysis is required to assess the role of myofilament-specific proteolysis in OH\(^*\) induced cardiac dysfunction as well as the exact extent of these myofilament alterations.

1.4 Choice of Model

Thus far, studies have been done using the rat and the rabbit (Gao, Liu et al. 1996; McDonough, Labugger et al. 2001; Zeitz, Maass et al. 2002). For our initial studies we used mice papillary muscles. The mouse model allowed for experiments on transgenic animals. Because of the various models used, a straightforward species comparison using identical models is required to resolve this problem. As there are significant differences between rat and rabbit, results obtained in a transgenic mice will be more useful to unravel the mechanism by which these free radicals impair cardiac contractility and to develop the novel treatment strategy for OH\(^*\) injury on heart.

The rabbit is chosen for these aspects of the later studies as it is more similar to...
the human with regards to EC coupling and myofilament composition. For instance, 95-98% of activator Ca^{2+} is resequestered from the cytoplasm by SERCA in mice and the rat, while in rabbit only 70% is resequestered, similar to the human (Bers 2002; Bers 2002). In addition, myofilament isoforms like myosin heavy chain are much faster in mice and rat in comparison to rabbit and human (Tardiff, Hewett et al. 2000). Although there are significant differences in calcium handling and myofilament composition between mouse and rabbit, the OH* induced contracture was extremely similar in rabbit and mouse muscles (both in the acute and sustained phase). Therefore, we are confident we can interpret the data without significant species-dependent artifacts.

1.5 Introduction to Methods and Techniques

We used freshly harvested multicellular trabeculae for our experiments as there are distinct advantages of using these as compared to the whole heart and isolated myocytes (Bers 1997; Sys, De Keulenaer et al. 1998). The most physiologically relevant technique is whole heart experiments exemplified by those done on the Langendorff apparatus (Farkas, Acsai et al. 2008). The advantage of this method is that it is more relevant to the in vivo situation. However, one cannot assess myofilament function using this method because muscle length changes with every beat in the intact heart, changing both F_{max} and myofilament calcium sensitivity (due to the Frank-Starling mechanism). Also, measurement of intracellular calcium is not accurate and technically very difficult to measure (Hayashi, Kamanu et al. 2008).

Cardiac function can also be measured using isolated myocytes. This model gives a more accurate measurement of intracellular calcium than the whole heart because of the
controlled environment (no influence of other cells and connective tissue), and the ability to modify the proteome. However, assessing myofilament function is very difficult as myocytes shorten significantly when they contract, decreasing their length. In addition, there are no surrounding attachments to the extracellular matrix stretching the cell, their resting length is shorter than even a contracted myocyte in the whole heart. The principle model used in this project was intact ventricular trabeculae. Trabeculae are linear cardiac muscle preparations that can be found lining the inside of the right and left ventricular free wall. They provide a unique representation of intact myocardium that contains all cells of the normal heart (myocytes, fibroblasts, endothelial cells etc.). Using this model allowed for several benefits. First, this model will allow the assessment of different loading conditions of the heart (within and outside the physiological range). Second, these preparations allow for the application of the iontophoresis loading method (Backx and Ter Keurs 1993). Using this method, we can introduce the free salt form of fluorescent dye into a single myocyte within the trabeculae without hampering the calibration in comparison to loading the ester form of dye in intact heart and/or isolated myocytes which cause potentially incorrect calibration due to loading of dye in intracellular organelles. This method does have some limitations too. Since no two trabeculae are identical, inter-muscle variability can be a disadvantage. In addition, not every animal has suitable trabeculae for experimentation.

1.5.1 Induction of OH* Injury

The Fenton reaction is an iron-salt-dependent decomposition of dihydrogen peroxide, that generates the highly reactive OH*. It is currently one of the most powerful
oxidizing reactions available. The reaction involves hydrogen peroxide and a ferrous iron
catalyst. The peroxide is broken down into a hydroxide ion and a hydroxyl free radical.
The hydroxyl free radical is the primary oxidizing species and can be used to oxidize and
break apart organic molecules.

\[ \text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + \text{HO}^- + \text{HO}^* \]

One primary advantage of the Fenton's reaction is that it does not produce further organic
compounds or inorganic solids such as permanganate or dichromate, since there is no
carbon in the peroxide. The mechanism of reaction with respect to hydrogen peroxide is
very complex and may change with conditions of the reaction. We used the 3.75 mM and
10µM of H$_2$O$_2$ and Fe-NTA respectively in the organ bath. The amount of OH* generated
under these conditions is comparable to those that occur in IR injury (Zweier,
Kuppusamy et al. 1989; Zeitz, Maass et al. 2002). H$_2$O$_2$ is infused via a separate line
feeding directly into the organ bath at the level of the muscle to ensure that radical
formation took place right at the muscle where H$_2$O$_2$ meets Fe-NTA.

1.5.2 Measurement of Intracellular Calcium

Calcium overload plays a significant role in acute cardiac dysfunction due to OH*
injury. Measurement of calcium at different time points during reperfusion injury is
necessary to characterize the excitation contraction coupling during twitch contractions
more precisely.

Trabeculae were iontophorically injected with bis-fura-2 (Texas Fluorescence) as
described previously (Backx and Ter Keurs 1993; Janssen, Stull et al. 2002). Bis-fura-2
was chosen due to its higher signal to calcium buffering ratio (allowing for a loading of
the dye to 5–10 times background without impacting buffering) and slightly higher $K_d$ (390 nM in vitro) than fura-2, (allowing for still accurate diastolic values but a better resolution at higher $[\text{Ca}^{2+}]_i$). Iontophoretic loading of the dye was performed at room temperature (at body temperature, the rate of dye leak is higher). The very high background-to-signal ratio makes the AM form of the dyes extremely susceptible to calibration anomalies, and we will avoid this by using the free-salt form in combination with iontophoresis. However, iontophoresis in trabeculae has some disadvantages too: suitable muscles are not always found in each heart, and dye-leakage rates reduce the success rate of these experiments compared to intact hearts or isolated myocytes. Using this method, a single cell is punctured with the tip of the micropipette and the salt form of the dye is electrically driven into the cell. Gap junctions that connect myocytes and make their cytoplasm continuous allow for uniform spreading of the indicator throughout the trabeculae.

1.5.3 Measurement of Myofilament Calcium Sensitivity

Assessment of myofilament calcium sensitivity is crucial to understand the mechanism by which these free radicals impair cardiac contractility. Our lab has successfully devised a protocol to tetanize cardiac muscle at body temperature and preload by applying small KCl contracture by increasing the KCl concentration (142 mM) while lowering sodium (30 mM) in the buffer. Exposing cardiac muscle to high K$^+$ solutions causes a slow depolarization of myocyte membranes (Holubarsch 1983) due to the dependence of resting potential on the K$^+$ gradient and elicit a slow-forming contracture in the muscle resembling a twitch but occurring around 200–400 times
slower. The steady state phase of KCl contractures and slow rise of intracellular calcium and force, allowed us to construct the steady state curve under true physiological conditions.

1.6 **Objective of the Studies**

The goal of this project is to understand the mechanism by which myofilament degradation on one hand and intracellular calcium overload on other, contribute to impair cardiac contractility after OH* exposure. Understanding the basic characteristics of OH* injury and the mechanism by which these free radicals impair cardiac contractility is a prerequisite to understand what goes awry in the heart after OH* injury. In the studies presented we attempted to elucidate the relative importance of myofilament degradation and intracellular calcium overload at various stages throughout the OH* injury.

Accordingly, the specific aims of the studies presented in this thesis are to test the following hypothesis:

1. SERCA overexpression can reduce the OH*-induced contractile dysfunction in murine myocardium, whereas a reduced SERCA activity aggravates this injury.
2. Hyperthermia reduces the peak force development as well as β-adrenergic-induced inotropism in isolated myocardium.
3. Relation between SERCA activity and cardiac contractility is critically dependent on stimulation frequency.
4. Acute injury that occurs after OH* exposure is mainly if not entirely due to calcium overload in acute phase, and altered/increased myofilament response is mainly responsible for the sustained/later phase of the OH*-induced injury.
5. Both atrial and ventricular muscles develop a rigor like contracture after acute OH*-induced injury, but atrial muscles showed a lesser degree of contractile dysfunction which both reached its maximum and relaxed faster than in ventricular muscles.
Figure 1. Cellular and molecular mechanisms of radical generation (took from (Zweier and Talukder 2006))
Figure 2. Reversible changes in cytosolic cation control in ischemia-reperfusion. ATP = adenosine triphosphate; NCE = Na⁺/H⁺ exchanger. Took from (Piper, Meuter et al. 2003)
CHAPTER 2
SERCA OVEREXPRESSION REDUCES OH* INJURY IN MURINE MYOCARDIUM

2.1 Introduction

OH* are one of the most aggressive species of OFR that attack all molecules in the human body (Gao, Liu et al. 1996; Zeitz, Maass et al. 2002). These OH* are involved in the pathogenesis of IR injury, which is observed in many clinical situations including acute heart failure, stroke, and myocardial infarction. When ischemic myocardium is reperfused and oxygen reintroduced, there is a sudden burst of OFR production. This leads to the formation of damaging reactive species, such as OH*, hydrogen peroxide, and peroxynitrite (Bolli and Marban 1999). These reactive oxygen species -especially OH*- interact with lipids, proteins and nucleic acids and damage cell membranes and impair cellular function. Conditions of IR injury are created routinely during heart surgery and transplantation (Bolli and Marban 1999). OFR occurring during IR injury have been implicated in the pathogenesis of myocardial stunning and progression of heart failure.

There are two different sub-cellular defects that may contribute to the development of acute myocardial dysfunction: deranged calcium handling and alteration of myofilament responsiveness. Previous studies have demonstrated that intact contracting cardiac trabeculae from rat and rabbit after acute exposure of OH* develop a
rigor like contracture marked by an increase in diastolic tension, myofilament proteolysis and overall decreased cardiac contractility (Zeitz, Maass et al. 2002). During the acute phase, there are multiple mechanisms that are possibly responsible for calcium overload: sarcoplasmic reticulum damage, mitochondrial damage, changes in properties/activity of NCX and/or changes in L type channel activity. The relative contributions of these factors to the acute calcium overload are still unknown.

Previous studies demonstrated a decrease in SERCA activity at increasing concentrations of OH* (Flesch, Maack et al. 1999) as well as a clear attenuation of the positive force-frequency relationship after the exposure of OH* (Schouten and ter Keurs 1986; Janssen, Zeitz et al. 1999; Zeitz, Maass et al. 2002). These studies indicate that there may be a direct impairment of sarcoplasmic reticulum function due to OH* exposure. Reverse mode NCX (Zeitz, Maass et al. 2002) has also been reported as a major cause of acute diastolic dysfunction due to OH* exposure. In addition, calcium channel antagonists (Ehring, Bohm et al. 1992; Ferrari, Curello et al. 1993) has been proposed to (partially) prevent myocardial stunning showing that L-type calcium channel could be an important route of intracellular calcium overload. Combined, from these studies it is clear that calcium overload plays a significant role in acute cardiac dysfunction due to OH* injury.

The main goal of this study is to test our hypothesis that OH* injury can be attenuated via (partial) normalization of calcium handling via modulation of SERCA activity. Not only could the total absolute loss of function be attenuated by simply having more calcium pumps available, preserved Ca\(^{2+}\) handling (e.g. preserved SERCA activity) could potentially aid in removing excess Ca\(^{2+}\) that has entered the myocytes as a result of
OH*-induced damages. Our results indeed indicate that modulation of SERCA activity can indeed alter the magnitude of effects of OH* injury on the heart, the OH* induced injury is substantially less in transgenic mice with higher SERCA activity, and aggravated in mice with a reduction in SERCA activity.

2.2 Materials and Methods

2.2.1 Transgenic Mouse Models

All mouse models have been published previously. SERCA overexpressed mice (TG) are mice expressing the (skeletal) SERCA1a isoform (2.5-fold increase in the total amount of SERCA and a ~2-fold increase in SR Ca^{2+} uptake function) (Loukianov, Ji et al. 1998). We chose the SERCA1a overexpressed mice for this proof-of-principle study; SERCA1a possesses faster Ca^{2+} transport kinetics, but unaltered Ca^{2+} affinity, pH response, and PLB affinity, and therefore possible positive effects of enhanced SERCA activity (Sumbilla, Cavagna et al. 1999) would be most pronounced. SERCA2a heterozygous knock-out mice (HET) have a ~40% reduction in expression of SERCA2a, resulting in a decreased SR calcium re-uptake (Huke, Liu et al. 2003). The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

2.2.2 Muscle Preparation and Experimental Set-up

Adult mice (2-3 months old) were anesthetized with urethane ip (300 mg/1ml of .9% NaCl). Under deep anesthesia, after bilateral thoracotomy and intra-ventricular (via the apex) heparin administration (1000 U), the heart was rapidly removed and perfused
retrogradely through the aorta with Krebs-Henseleit solution containing (in mmol/L): 120 NaCl, 5KCl, 2 MgSO4, 1.2 NaH2PO4, 20 NaHCO3, 0.25 Ca²⁺ and 10 glucose (pH 7.4) in equilibrium with 95% O₂/5% CO₂ at 37°C. Additionally, 20 mmol/L 2,3-butanedione monoxime (BDM) was added to the dissection solution to stop the heart from beating and to prevent damage during dissection (Mulieri, Hasenfuss et al. 1989; Mulieri, Hasenfuss et al. 1992; Janssen and Hunter 1995). The effects of BDM after brief exposure, have been found to be reversible (Zweier, Kuppusamy et al. 1989; Janssen and Hunter 1995). After flushing the blood out of the heart, the right ventricle was carefully opened and small, suitable papillary muscles were dissected carefully as previously described and mounted in the experimental set-up (ter Keurs, Rijnsburger et al. 1980; Janssen, Stull et al. 2002; Raman, Kelley et al. 2005). In addition to the papillary muscle, the dissected specimen contained a small portion of tricuspid leaflet attached to one end and a block of tissue from right ventricular free wall or septum to the other end to facilitate mounting the muscle onto the experimental setup. Only very thin papillary muscles were used for the study, with a maximum diffusion distance of 100 µm to the core, so core hypoxia will be avoided and not affect the outcome of the results factor (Raman, Kelley et al. 2005). Using a dissection microscope, muscles were mounted in the chamber. The piece of tricuspid valve leaflet was connected to a hook like extension of micromanipulator and the cube of ventricular tissue rest in a platinum-iridium basket-shaped extension of the force transducer (KG7, Scientific Instruments GmbH, Heidelberg, Germany). This method has been shown to minimize the end-damage compliance of the muscle and prevents excessive loss of force throughout the experimental protocol (ter Keurs, Rijnsburger et al. 1980; de Tombe and ter Keurs 1990; Janssen and de Tombe 1997;
Layland and Kentish 1999; Janssen, Stull et al. 2002). The muscle was bathed in a continuous flow of oxygenated K-H solution (without the BDM). The dimensions (length, width and thickness) of the muscle were measured at 40X magnification (~ 10 µm resolution), and used to normalize force to cross-sectional area.

2.2.3 Protocol

After mounting the preparation in experimental setup Ca$^{2+}$ was raised to 1.5 mmol/L and electrically stimulated by 3 ms pulses at 4 Hz at a temperature of 37°C and stretched until maximal active force reached. This length is comparable to maximally attained length in vivo at the end of diastole (around 2.2 µm sarcomere length) (Rodriguez, Hunter et al. 1992). After the stabilization of contractile parameters (15-20 min), we exposed the muscle to OH* for 2 minutes. Twitch contractions were monitored until the transient acute dysfunction has subsided and diastolic and developed force had reached their new steady state (typically 45 min after OH* exposure).

2.2.4 Generation of OH*

OH* were produced via the Fenton reaction through H$_2$O$_2$ + Fe$^{2+}$- NTA system (Zeitz, Maass et al. 2002). The concentration of the H$_2$O$_2$ and Fe-NTA (ferric nitriilotriaceticacetate) in the organ bath was 3.75 mmol/L and 10 µmol/L respectively. The amount of OH* generated under these conditions is comparable to those that occur in IR injury (Zweier, Kuppusamy et al. 1989; Zeitz, Maass et al. 2002). H$_2$O$_2$ was infused via a separate line feeding directly into the organ bath at the level of the muscle to ensure that radical formation took place as closely to the preparation as possible.
2.2.5 Protein Quantification

At the end of the experiment, stimulation is switched off and both valve and block ends are cut off on either side of muscle, or the central part of the muscle is quickly frozen in liquid nitrogen and stored at −80°C. For protein composition analysis, muscles (~0.1 mg or less) are homogenized in 32.5 µl of homogenization buffer (25 mM imidazole (pH 7.4), 300 mM sucrose, 1mM DTT, 20 mM sodium metabisulfite, protease inhibitor cocktail 10 µl/ml). Samples are applied to 14% PAGE. After electrophoresis and blotting on nitrocellulose membranes, blots are blocked with 5% non fat dry milk in TBS and Tween 20 overnight, and incubated with monoclonal antibodies against Calsequestrin, total PLB and PLB phosphorylation at Ser16 and Thr17 for 2 hours. Calsequestrin concentration was used for normalization. Antigen antibody complexes are visualized by peroxidase-conjugated anti-mouse antibodies using enhanced chemiluminescence (Pierce).

2.2.6 Data Analysis and Statistics

Data were collected and analyzed on- and offline using custom-written software in LabView (National Instruments). Data are expressed as mean ± SEM unless otherwise stated. Data were statistically analyzed using ANOVA, or Student’s t-tests (paired or unpaired) where applicable. A two-tailed value of P<0.05 was considered significant.
2.3 Results

2.3.1 Contractile Function after OH* Exposure

In accordance with previous studies in other species (Zweier, Kuppusamy et al. 1989; Gao, Liu et al. 1996; Flesch, Maack et al. 1999; Zeitz, Maass et al. 2002) acute OH* exposure in murine papillary muscles led to a rapid increase in diastolic force and a decrease in developed force. Figure 3a shows the raw record of force before, during and after exposure to OH* in a murine papillary muscle. As can be clearly seen, direct acute exposure of OH* for 2 minutes on papillary muscles of mice resulted in an increase of diastolic force and decrease of developed force. In figure 3b, the effect on diastolic and developed force for n=13 muscles after the exposure of OH* is depicted. Contractile parameters were observed until a new steady state level was reached. This new steady state level was marked by an elevated diastolic force and reduced developed force compared to pre-interventional values.

Diastolic force at peak of contracture (~ 12 minutes after OH* exposure) was increased to 485±49% (P<0.05) of its value before OH* exposure and after approximately 45 minutes returned to a new steady state level of 238±28% (P<0.05). Developed force at peak of contracture was decreased drastically to 11.3±2.8% (P<0.05) and returned to a new steady state level of 37.6±5.6% (P<0.05). In another set of control experiments contractile parameters remained unchanged when muscles were treated similarly but without OH* for the equivalent period of time (data not shown), indicating reliability and durability of the preparation, as well as showing that prolonged study of these isolated trabeculae is not complicated by an excessive loss of function over time.
2.3.2 Role of SERCA Expression on OH* Induced Injury

To determine the effect of SR calcium handling ability on the outcome of acute OH* injury, we repeated the above experiments in mice that expressed SERCA1a (higher SERCA activity than WT) as well as in mice with reduced levels of SERCA2a (HET, with a reduction in SERCA activity compared to WT). Figure 4a shows the effect of a 2 minute OH* exposure on the contractile parameters of the papillary muscles in WT, TG and HET mice. At baseline average developed force in TG, WT and HET was 35.9±2.4, 26.1±2.1, and 22.0±3.7 mN/mm² respectively. Initial diastolic forces were similar in all groups (~ 5-8 mN/mm²). As can be clearly seen, at baseline developed force is significantly higher in TG mice in comparison to WT and HET mice (P<0.05). In addition developed force is slightly lower in HET mice compared to WT controls (P=0.16). In accordance with the previous experiments, OH* exposure of murine papillary muscles led to a rapid decrease in developed force and increase in diastolic force. At peak of contracture, developed force went down from 35.9±2.4 to 14.7±1.3 mN/mm², 26.1±2.1 to 2.9±0.7 mN/mm² and 22.0±3.7 to 2.4±0.6 mN/mm² (POC=12 min) in TG, WT and HET mice respectively (all P<0.05). At baseline in Figure 4b average diastolic force was 6.6±1.7 mN/mm². At peak of contracture (occurring at ~12 min after OH* exposure in WT and HET, and earlier in TG mice, ~3 min) Ca²⁺ overload led to increase in diastolic force to 36.7±3.6 and 42.1±3.4 mN/mm² respectively but in TG mice diastolic force increased only to 19.2±1.6 mN/mm² (P<0.05, vs. HET and WT). Contractile parameters were observed until a new steady state level was reached. This new steady state was marked by a sustained dysfunction, with an elevated diastolic force and reduced developed force in both WT and HET. At this new steady state developed
force returns back to 10±2 and 7±1 mN/mm² in WT and HET mice respectively while diastolic force came back to 18±5 and 18±2 mN/mm². In contrast, the sustained dysfunction was significantly less in TG mice: Diastolic force and developed force returned back to 11±1 and 24±2 mN/mm² respectively in comparison to the pre-interventional values. Figure 5 shows the percentage effect of OH radicals on the contractile parameters of the papillary muscles in WT, TG and HET mice.

2.3.3 Phospholamban Phosphorylation after OH* Exposure

To determine the PLB phosphorylation levels after OH* exposure, small, intact contracting RV papillary muscles from WT, TG and HET were again directly exposed to OH*. At the peak of contractile dysfunction, the muscle is quickly frozen in liquid nitrogen and stored at –80°C. These samples, as well as non OH-exposed control samples, are analyzed for PLB expression and PLB phosphorylation levels via western blot (Figure 6). Our results shows that before exposing to OH*, TG hearts show a decrease in PLB protein, pPLBSer16 and pPLBThr17 while HET mice show a decrease in PLB protein and increase in pPLBSer16 and pPLBThr17 as compared to WT. After exposure to OH* there is decrease in pPLBSer16 in WT, TG and HET mice in comparison to control muscles but there was no or only little increase in the level of pPLBThr17 in all groups.

2.4 Discussion

2.4.1 OH* Induced Injury in Isolated Murine Myocardium

The acute response of murine contracting papillary muscles to OH* radicals is
very similar to what has been previously described for the rat (Gao, Liu et al. 1996) and rabbit (Zeitz, Maass et al. 2002). A transient, rigor-like contraction develops several minutes after OH* exposure. During the acute phase, this injury is marked by an increase in diastolic tension and a decreased developed force. During the recovery phase, diastolic force declines, but not all the way to pre-OH*radical levels, indicating a sustained injury. At this new baseline, diastolic force thus remained elevated, and developed force was decreased.

2.4.2 Attenuation of OH* Induced Injury by Increase SERCA Activity

The main goal of this study was to test our hypothesis that improved SERCA function can attenuate the acute OH* injury. The present study is the first to directly investigating the influence of altered SERCA activity on the contractile response of the cardiac muscle to OH*-induced injury. We observed that enhanced SR function in TG mice expressing SERCA1a (Loukianov, Ji et al. 1998) significantly attenuated the OH*-induced injury. Both diastolic and systolic performance of the myocardium was much less affected compared to the response in WT littermates. The protective effect of enhanced SERCA activity is potentially two-fold. First, an increase in the number of SR calcium pumps will increase the overall capacity to transport Ca^{2+} back into the SR during diastole (Lalli, Yong et al. 2001). If a number of pumps or even a percentage of this elevated capacity is affected, increased basal activity will still ensure a larger capacity of SR Ca^{2+} transport after injury compared to mice with normal basal SR calcium pump levels. This (albeit reduced compared to pre-injury baseline) capacity could potentially be sufficient to handle “normal” diastolic Ca^{2+} levels. Second, Ca^{2+}
overload can be dealt with more efficiently as well. Increased capacity may partially alleviate the high Ca\(^{2+}\) levels as they exist during acute injury. The functional capacity of the SR Ca\(^{2+}\) pumps after OH\(^{*}\) injury appears not large enough to completely prevent the contractile dysfunction, but a significant attenuation of acute dysfunction was observed. On the other hand, it may be that the capacity of the SR is simply insufficient to deal with the Ca\(^{2+}\) overload. It is likely that the total amount of calcium that has entered the cell during the injury exceeds the SR storage capacity, and thus SR Ca\(^{2+}\) pump level may not be the limiting factor anymore. In WT mice a limited SR capacity is clearly not the case, as increased SERCA activity in the TG animals does clearly result in a significant reduction in injury. For proof-of-principle that increased expression of SERCA is indeed solely responsible for the reduction in OH\(^{*}\)-induced injury, we repeated the experiments with mice (HET) in which SERCA2a expression and overall function was significantly reduced (Periasamy, Reed et al. 1999). Using the identical protocol, in HET mice the OH\(^{*}\)-induced injury was found to be aggravated; diastolic force rose significantly higher than in WT mice during the acute injury phase, while also several other contractile parameters likewise were worse or at best equal compared to WT mice. Thus, from the HET experiments we concluded that a reduced expression of SERCA2a aggravates the OH\(^{*}\)-induced injury. A reduced SR function is one of the hallmarks of end-stage cardiac failure (Arai, Alpert et al. 1993). Reduced SERCA activity contributes to both diastolic and systolic failure, and is one of the main changes compared to normal myocardium that is responsible for the alteration in force-frequency behavior (Schouten and ter Keurs 1986; Janssen, Zeitz et al. 1999) and β-adrenergic response (Flesch, Maack et al. 1999). Thus, a similar OH\(^{*}\)-induced injury would likely result in a greater amount of injury in
myocardium with low SERCA activity.

**2.4.3 Decrease in PLB Phosphorylation after OH* Exposure**

In close accordance to previous work, we show that before exposing to OH*, TG hearts show a decrease in PLB protein and PLB phosphorylation while HET mice show a decrease in PLB protein and increase in PLB phosphorylation compared to WT. The latter finding indicates a specific role for the phosphorylation of PLB on basal contractile function, and represents a compensatory mechanism via which the calcium handling is normalized in presence of altered SERCA activity induced by the transgene or knockdown. Our results indicate that after OH* exposure, all groups displayed a marked decrease in level of PLB phosphorylation at Ser16. The sustained cardiac dysfunction (rise in diastolic force and loss of developed force), was significantly less in TG in comparison to WT and HET. We conclude that under control conditions, PLB phosphorylation partially compensates for the decreased SERCA activity/expression in HET mice. After OH* exposure, dephosphorylation of PLB at Ser16 contributes to the contractile dysfunction in WT mice, while TG mice are less susceptible to the OH* induced dysfunction, as they already have low PLB phosphorylation levels prior to OH* exposure, and thus maintain their normal SR calcium uptake capacity. In sharp contrast, in HET mice the high levels of PLB phosphorylation at Ser16 that were present at baseline are now lost after OH* exposure, thereby unmasking the reduced SR calcium uptake capacity, and thereby aggravating the injury response to OH*. Overall, our results indicate PLB dephosphorylation at Ser16 contributes to the dysfunction observed after OH* exposure. Experiments in which we investigated phosphorylation of the Thr17 site
reveal that OH* exposure did not decrease the phosphorylation at Thr17.

2.4.4 Limitations of the Study

We used two different lines of mice in our studies. To show that a reduced SERCA activity aggravated the OH*-induced injury, we used the SERCA2a HET mouse, in which 1 allele is mutated resulting in reduced SERCA2a expression resulting in a decreased SR Ca\(^{2+}\) uptake activity compared to WT mice. To show the other end of the spectrum, we used the SERCA1a transgenic mouse line. This mouse expressed the SERCA1a isoform (~80% of total SERCA) in conjunction with the native cardiac isoform SERCA2a (~20%). The objective of our studies was to test whether enhanced calcium handling would partially prevent OH* induced injury, which according to our data was clearly the case. Although SERCA1a and SERCA2a are different isoforms, when expressed in the heart, apart from the higher activity, they have been reported to behave virtually identical. Ji and coworkers (Ji, Loukianov et al. 1999) showed that only the maximal velocity was changed, and that the apparent affinity for Ca\(^{2+}\), ATP affinity, Hill coefficient, pH dependence of Ca\(^{2+}\) uptake, and turnover rate were similar to SERCA2a. In addition, Lalli (Lalli, Yong et al. 2001) and coworkers found that SERCA1a can substitute both structurally and functionally for SERCA2a in the heart and that SERCA1a overexpression can be used to enhance SR Ca\(^{2+}\) transport and cardiac contractility. Thus, although technically the SERCA1a mouse has a reduced SERCA2a expression, it has an enhanced SERCA activity (because of SERCA1a expression) under the conditions studied, evident by the enhanced contractions at baseline. This model thus does not allow us to conclude that the great reduction in the injury response is due to
SERCA2a specifically, but it does allow to prove our main hypothesis in that SERCA activity modulated OH* induced injury.

In conclusion, the acute injury that occurs after OH* exposure is dependent on the level of SERCA activity and PLB dephosphorylation specifically at Ser16. Increased SERCA activity can partially rescue the heart from OH* induced injury, while a reduction in SERCA activity can aggravate the OH*-induced contractile dysfunction. Thus, interventions primarily aimed at restoration of depressed contractility in various cardiomyopathies via improvement of SR Ca^{2+} pumping (up-regulation of SERCA activity (Hajjar, Schmidt et al. 1997) and/or down-regulation/blocking of phospholamban) (Dieterle, Meyer et al. 2005) may also be beneficial during reperfusion injury situations by providing a means to more effectively deal with the calcium overload that results from the oxidative stress/reperfusion injury.
Figure 3 A: Raw record of force before, during and after exposure to OH radicals. Addition of OH radicals led to an increase in diastolic force and a loss of developed force in mice papillary muscle. B: Changes in diastolic and developed forces after a 2 minute OH* exposure in mice papillary muscles (n=13). New steady state level (~45 minutes after OH* exposure) was marked by an elevated diastolic force and reduced developed force compared with pre-interventional values. Stimulation frequency was 4 Hz and temperature was 37º C throughout the experiment.
Figure 4 A: Effect of 2 minute exposure of OH radicals on the contractile parameters of the papillary muscles in WT, TG and HET mice (n=8 each). Developed force is higher in TG mice in comparison to WT and HET mice throughout the contracture. At peak of contracture (occurring at ~12 min after OH* exposure In WT and HET) Ca$^{2+}$ overload led to significant increase in diastolic force and decrease in developed force in WT and HET mice in contrast to TG mice. New steady state was marked by significant elevated diastolic force and reduced developed force in both WT and HET, but again came back to near baseline in TG in comparison to pre-interventional values. Stimulation frequency was 4 Hz and temperature was 37º C throughout the experiment.
Figure 5: Percentage effect of OH radicals on the contractile parameters of the papillary muscles in WT, TG and HET mice (n=8 each group). At peak of contracture (occurring at ~12 min after OH* exposure in WT and HET) Ca\(^{2+}\) overload led to significant increase in diastolic force and decrease in developed force in WT and HET mice in comparison to TG mice. The new steady state was also marked by significant elevated diastolic force and reduced developed force in both WT and HET, but again came back near baseline in TG in comparison to pre-interventional values. Stimulation frequency was 4 Hz and temperature was 37\(^\circ\) C throughout the experiment. * indicates preservation of function vs. WT, P<0.05; ** indicates loss of function vs. WT, P<0.05.
Figure 6: Western blot analysis of total PLB and PLB phosphorylation at Ser 16 and Thr17 before and after exposure to OH*. A. Before exposing to OH*, TG hearts show a decrease in PLB protein and pPLBSer16 and pPLBThr17 while HET mice show a decrease in PLB protein and increase in pPLBSer16 and pPLBThr17 compared to WT. B. After OH* exposure all groups, WT exposed (WTe), TG exposed (TGe), and HET exposed (HETe) displayed a marked decrease in level of PLB phosphorylation at Ser16 but there was no significant change in PLB phosphorylation at Thr17 compared to unexposed controls. Calsequestrin concentration was used as an internal control for normalization.
CHAPTER 3
FRQUENCY-DEPENDENT CONTRACTILE STRENGTH IN MICE
OVER- AND UNDER- EXPRESSING THE SARCOPLASMIC RETICULUM
CALCIUM ATPase.

3.1 Introduction

The SERCA actively re-sequesters calcium ions from the cytoplasm. The total activity of all SERCA pumps is a strong determinant of myocardial contractility (Periasamy and Huke 2001); i.e. in steady state contractions the total amount of Ca\(^{2+}\) ions pumped back into the SR each beat under steady state conditions is both followed and preceded by an equivalent amount of calcium released via the ryanodine receptors upon activation of the next (and/or previous) beat. In end-stage heart failure, the total activity of the SERCA pumps is decreased (Arai, Alpert et al. 1993), be it via loss of number of pumps, or loss of activity of the individual pumps, or a combination thereof (Lehnart, Schillinger et al. 1998; Prestle, Dieterich et al. 1999).

Frequency-dependent inotropic modulation largely stems from an increase in SR load via established via increased total activity of SERCA. Within the physiological range, total SERCA activity generally increases with frequency, generally resulting in a higher contractility in healthy myocardium (Mulieri, Hasenfuss et al. 1992). This frequency-dependent activation not only can increase the force of contraction, it also
speeds up the relaxation (Janssen, Stull et al. 2002), so the cardiac muscle has sufficient
time in diastole to maintain adequate filling prior to the next beat (Pieske, Maier et al.
1999). The SERCA pump is mainly driven by the calcium concentration in the cytosol.
At high intracellular calcium concentrations, activity of the pump is increased. Thus, as it
pumps calcium ions back into the SR, it reduces the intracellular calcium transient, and
negatively feeds back on its own activity. As a result, in the time-domain SERCA pump
activity is cycling continuously, but with variable capacity. When inter-beat duration is
decreased (increased frequency), as a result of a time-average higher calcium
concentration, activity is higher per time-unit, resulting in an elevation of SR load during
first few beats following a frequency increase before a new homeostasis at with increased
SR calcium levels sets in.

In order to understand, and quantify, the frequency-dependent SERCA pump
function, we set out to investigate frequency-dependent activation in isolated cardiac
tissue with normal, increased, and reduced SERCA activity levels. Our working
hypothesis is that increased SERCA levels promote increased force, and because the
maximum force generating capacity is limited by the myofilaments, the increases in
SERCA level at low frequency may increase calcium handling and contractile force,
resulting in a reduced capacity to increase further, or possibly may result in such a high
baseline force that no increases are observed at higher frequencies. To test whether
SERCA function affect basal contractility to an extent that the cardiac reserve is
significantly compromised, we investigated isometric developed force generation in ultra-
thin trabeculae isolated from hearts of SERCA 1a over-expressing mice (higher baseline
SERCA function) (Baker, Hashimoto et al. 1998; Loukianov, Ji et al. 1998), and SERCA
2a heterozygous KO mice (reduced SERCA function) (Periasamy, Reed et al. 1999), as well as from respective wild-type animals. Our results indicate that enhanced baseline SR function leads to enhanced contractile strength. However, the higher baseline strength prohibits further increases in frequency-dependent inotropy observed normally in WT animals.

3.2 Methods

3.2.1 Transgenic Mouse Models

All mouse models have been published previously. SERCA overexpressed mice (TG) are mice expressing the (skeletal) SERCA1a isoform, and have a 2.5-fold increase in the total amount of SERCA and a ~2-fold increase in SR Ca$^{2+}$ uptake function (Loukianov, Ji et al. 1998). We chose the SERCA1a overexpressed mice for this proof-of-principle study; SERCA1a possesses faster Ca$^{2+}$ transport kinetics and therefore possible effects of enhanced SERCA activity (Baker, Hashimoto et al. 1998) would be possibly most pronounced. SERCA2a heterozygous knock-out mice (HET) have 1 allele mutated, and as a result have a ~40% reduction in expression of SERCA2a (Periasamy, Reed et al. 1999), resulting in a decreased SR calcium re-uptake (Periasamy, Reed et al. 1999; Ji, Lalli et al. 2000; Huke, Liu et al. 2003). Protein expression levels of SERCA 1a and SERCA 2a were regularly checked to ensure altered protein expression via standard western blotting techniques. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.
3.2.2 Muscle Preparation and Experimental Set-Up

Mice weighing 20-25 g were anesthetized with the inhalation anesthetic halothane. After bilateral thoracotomy and intra-ventricular heparin administration, hearts were rapidly excised and placed in Krebs-Henseleit buffer containing (mM): 120 NaCl, 5 KCl, 2 MgSO₄, 1.2 NaH₂PO₄, 20 NaHCO₃, 0.25 Ca²⁺, and 10 glucose (pH 7.4) equilibrated with 95%O₂ / 5% CO₂. Additionally, 20 mM BDM (2, 3–butanedione-monoxime) was added to the dissection buffer to prevent cutting injury. The effects of BDM after brief exposure have been found to be reversible (Mulieri, Hasenfuss et al. 1992; Janssen and Hunter 1995). Hearts were cannulated via the ascending aorta and retrograde perfused with the same buffer for several minutes. Blood was thoroughly washed out and thin, uniform, non-branched trabeculae from the right ventricle (along the tricuspid valve) were carefully dissected leaving a block of tissue at one end from the right ventricular free wall and a small part of the valve at the other end to facilitate mounting (Stull, Leppo et al. 2002). The dimensions of the muscles were measured using a calibration reticule in the ocular of the dissection microscope (40X, resolution ~10 µm). Cross-sectional area was calculated assuming an ellipsoid cross-sectional shape. Suitable trabeculae (thickness not exceeding 200 µm) were dissected and contractile parameters assessed in identical set-ups. However, only 1 muscle per heart was randomly selected to be included in the analysis of contractile properties. All experimental protocols conformed to institutional guidelines regarding the use and care of animals.

Using the dissection microscope, muscles were mounted between a platinum-iridium basket-shaped extension of a force transducer (tissue block end) and a hook (valve end) connected to a micromanipulator. This method has been shown (ter Keurs,
Rijnsburger et al. 1980; de Tombe and ter Keurs 1990; Janssen and de Tombe 1997; Layland and Kentish 1999; Janssen, Stull et al. 2002) to minimize end-damage compliance of the muscle and to prevent excessive loss of force throughout the experimental protocols. Muscles were superfused with the same buffer at 37 °C as above (with the exception that BDM was omitted) and stimulated at 4 Hz. $[\text{Ca}^{2+}]_o$ was raised to 1.5 mM and muscles were allowed to stabilize for at least 30 minutes before the experimental protocol was initiated. Muscles were stretched to a length where a small increase in length resulted in about equal increases in resting tension and active developed tension. This is a length (optimal length) that is slightly below the length where active force development is maximal, and was selected to be comparable to the maximally-attained length in vivo at end of diastole (around 2.2 µm sarcomere length) (Rodriguez, Hunter et al. 1992).

### 3.2.3 Determination of Contractile Properties

In order to assess the effect of muscle length on developed force and relaxation time, the following protocol was performed; first, slack length (length of muscle without any pre-load) and optimal length was determined. The difference was divided into 3 equal steps and the muscle was stretched sequentially, with each step increasing the length of the muscle until the baseline length was achieved. Parameters were recorded when the muscle had stabilized at each length. Thereafter, we assessed the effects of increasing stimulation frequencies between 2 and 14 Hz. At each frequency, forces were allowed to reach steady state before data were recorded. The effects of β-adrenergic stimulation were assessed in a subset of muscles by a concentration-response curve with
isoproterenol (10^{-9} - 10^{-6} M) at a baseline stimulation frequency of 4 Hz. In a subset of experiments, at the \textit{in vivo} resting frequency of 8-10 Hz (Vornanen 1992; Kass, Hare et al. 1998; Georgokopoulos and Kass 2001), the effect of maximal isoproterenol stimulation was also tested. In order to assess the size of SR calcium load rapid cooling contracture (RCC) amplitude was measured. After the stabilization of muscle, stimulation was stopped and muscles were rapidly cooled to 0°C in less than 1 second. After a steady force was reached the temperature was switched back to 37.5 °C. This protocol was repeated at different frequencies. After stabilization at baseline of 4 Hz, in a set of muscles we measured the post rest potentiation (PRP) as it is an important determinant of force frequency response. In this protocol stimulation interval was sequentially set to 2, 4, 6, 10, 15, 30, 60 and 120 s. from the basic frequency of 4 Hz.

\textbf{3.2.4 Data Analysis and Statistics}

In all the experiments performed, the parameters of developed force and diastolic force were determined, and normalized to the cross-sectional area of the muscle. Additionally, as a model-independent parameter of force decay kinetics, time from peak force to 50\% relaxation (RT_{50\%}) was determined. Parameters were calculated off-line and also on-line to facilitate immediate judgment of preparation quality. Preparations that displayed excessive rundown of developed force (>10\%/hour) were excluded. In muscles that underwent the length, force-frequency, isoproterenol and RCC or PRP protocol, total protocol time was nearly 4 hours.
One way repeated ANOVA was used to determine significant differences between the groups, with Newman-Keuls post-hoc t-test when appropriate. A two-sided P-value of < 0.05 was considered significant. All values are expressed as mean ± SEM.

### 3.3 Results

First we verified the protein phenotype of our mice. In Figure 7, we depict that in the SERCA1a mouse, this protein is indeed robustly expressed, whereas in the SERCA2a HET mice the SERCA2a protein level was reduced compared to the respective wild-type. This is in close agreement to the initial studies on these mice that show nearly identical protein expression patterns as we observe in these generations (Loukianov, Ji et al. 1998; Periasamy, Reed et al. 1999). It is already known that phospholamban (PLB) levels are unchanged in SERCA1a mice (Loukianov, Ji et al. 1998). Likewise, it is published that in the SERCA2a HET mouse PLB is decreased, whereas PLB-P at Ser16 is increased (Hiranandani, Bupha-Intr et al. 2006). We very recently verified several of these key protein expression patterns (Hiranandani, Bupha-Intr et al. 2006) and again specifically verified phospholamban phosphorylation in the mice used for this study, and our results were consistent with the previously published protein levels. The respective phosphorylation status of Ser16 and Thr17 is depicted in Figure 7B, and indicated Ser16 was increased in SERCA2aHET and unchanged in SERCA1a mice, while no significant differences were found in levels of Thr17 phosphorylation.

Next, we examined the contractile response of isolated cardiac trabeculae under isometric conditions at 37.5 °C, at pre-load resembling end-diastolic values. We observed that compared to the respective WT muscles, at a stimulation frequency of 4 Hz,
SERCA1a expressing trabeculae displayed a significant higher level of active developed force (36.4 ± 5.1 vs. 19.2 ± 3.5 mN/mm², P<0.05). This was accompanied by a faster relaxation: time from peak force development to 50% relaxation was significantly faster in SERCA1a expressing trabeculae (21.8 ± 1.3 vs. 26.8 ± 2.2 ms, P<0.05). Similarly, time to peak tension was faster in SERCA1a muscles (47.2 ± 2.1 vs. 54.5 ± 1.8 ms, P<0.05). Another often used index of contractility and relaxation, the first derivative of force, mirrored these findings; the maximum and minimum dF/dt values were indicative of increased contractility 1500 ± 199 vs. 1092 ± 333 mN/mm²/s and 1210 ± 165 vs. –977 ± 299 mN/mm²/s respectively.

Next, we investigated the contractile response to increased stimulation frequencies. Starting from the 4 Hz baseline, the frequency was increased in steps of 2 Hz up to 14 Hz. At each frequency, the contractile parameters were allowed to stabilize before they were recorded and the next frequency was applied. In figure 8, active developed force for frequencies ranging from 4 to 14 Hz is depicted; raw force tracings from individual experiments at 4, 8, and 12 Hz (left panel), as well as average values at 4-14 Hz (right panel). At the low end of the range, 4 and 6 Hz, the SERCA1a muscles are significantly stronger (SERCA 1a 36.4±5.1 and 35.7±5.3 vs. WT 19.2±3.5 and 21.7±4.2 mN/mm² at 4 and 6 Hz resp.) At 8 Hz the difference still persists but is no longer significant, and from 10-14 Hz there is no significant difference compared to the muscles. The response to isoproterenol in SERCA1a expressing muscles is depicted in figure 9. With increasing isoproterenol concentrations, force development increases. At concentrations of isoproterenol 3*10⁻⁸ M and above, the difference between the two groups is no longer significant. Thus, the absolute response to isoproterenol is greater in
WT muscles, due to the lower baseline contractility. However, the levels of developed force under maximal isoproterenol stimulation are similar between the two groups.

SERCA1a expression did not only elevate basal contractility, it also accelerated the contractions. At 4 Hz, time from peak force to 50% relaxation was significantly shorter in SERCA1a muscles versus WT muscles (Figure 10). At the highest stimulation frequency used, 14 Hz, or in presence of 1 μM isoproterenol, the difference between the two groups was no longer present.

Using the identical protocol, we proceeded to investigate the same contractile parameters in mice SERCA2a (SERCA2a HET) where one of the two alleles is mutated. The expression level of SERCA2a in these muscles is reduced by ~40%. The background strain for these mice is C57/BL6. This is a different strain than the SERCA1a mice (FVBN), and hence we used different WT controls. We recently reported (Stull, Hiranandani et al. 2006) that within the physiological frequency range 3 different strains possessed very similar contractile properties. This was also the case in this study. However, similarly to our previous study, at lower frequency ranges, strain differences can be observed. We indeed observed a significantly higher basal contractility in the WT of the SERCA2a HET background strain versus the SERCA1a background strain.

In SERCA2a HET muscles, basal contractility at 4 Hz appeared reduced, but this was not quite significant (P=0.11). As frequency was increased, both SERCA2a HET and WT mice behaved similarly, up to 12 Hz. At 14 Hz, which is the upper limit of the in vivo murine heart rate (Georgokopoulous and Kass 2001), the difference was significant P<0.05), SERCA2a HET mice had a lower developed force compared to WT littermates, 16.2 ± 3.5 vs. 24.4 ± 2.2 mN/mm² (Figure 11). The isoproterenol response, at a baseline
frequency of 4 Hz was not different between the groups (Figure 12). Relaxation kinetics was slightly slower in SERCA2a HET muscles, but this was not significant. Similar results regarding relaxation times were observed for 14 Hz and 1 µM Isoproterenol (Figure 13).

Calcium handling was tested using SR constant estimations via rapid cooling contraction and a post-rest potentiation protocol. There was no change in rapid cooling contracture amplitude between SERCA2a HET and WT mice at baseline frequency of 4 Hz or at the higher frequency (12 Hz) (Figure 14A). Figure 14B shows the post rest potentiation (PRP) behavior in SERCA2a HET and WT mice. During the longest of rest durations (60-120 s) the amount of PRP is significantly higher in SERCA2a HET as compared to WT, but for the short durations, PRP behavior is similarly positive for both groups.

3.4 Discussion

The present study clearly demonstrates that any correlation between the SERCA activity and contractility is critically dependent on stimulation frequency. Previous studies (Periasamy and Huke 2001) showed that SERCA is one of the decisive determinants of cardiac contractility. A decrease in SERCA pump expression and activity were observed in a variety of pathological conditions. It has been postulated that decreased SERCA activity reduces the SR Ca\(^{2+}\) content and contractility (Periasamy, Reed et al. 1999; Ji, Lalli et al. 2000) while increased SERCA expression improve SR Ca\(^{2+}\) content and hence the myocardial function and contractility (He, Giordano et al. 1997; Baker, Hashimoto et al. 1998). At the lower frequency range (4-6 Hz) trabeculae
expressing SERCA1a indeed revealed significantly higher active developed force and relaxation rate as compared to WT but at the higher frequency range (~10-12 Hz) there was no persistent difference between WT and SERCA1a trabeculae. These data indicate that in normal WT mice, the enhanced stimulation of SERCA appears sufficient to elicit a maximal contractile response at high frequency. In the mouse, with increasing frequency, calcium transients increase (Stull, Leppo et al. 2002), and the time-averaged increase in intracellular calcium directly stimulates the SERCA pump (Periasamy and Huke 2001), whereas additional regulatory mechanisms that enhance SERCA activity, phospholamban-phosphorylation and/or -dissociation, can further enhance SERCA activity. The fact that SERCA 1a trabeculae do not show an enhanced contractile response compared to WT at the highest frequencies may thus indicate that at these conditions, a normal level of SERCA expression is not limiting force development.

At a baseline frequency of 4 Hz, SERCA2a HET mice show slightly (non-significant) reduced basal contractility and relaxation kinetics, and a similar isoproterenol response but this difference became significant when frequency was in the range of 12-14 Hz, compared to WT. Clearly, interpretation of whether, and to what extent, SERCA expression changes contractility is critically dependent on frequency or range of frequencies.

At sub-physiological frequencies, SERCA1a over expressed trabeculae possessed a higher basal contractility, and a faster relaxation rate compared to WT. These findings are consistent with previous studies (Loukianov, Ji et al. 1998; Lalli, Yong et al. 2001; Xu, Zhang et al. 2005). Interestingly at higher (physiological) frequencies response of SERCA1a over expressed trabeculae were not significantly different anymore versus the
WT trabeculae. This likely indicates that at lower frequencies, due to higher SERCA activity in the SERCA 1a trabeculae, there is increased reuptake of Ca$^{2+}$ into the SR thereby decreasing the TTP and relaxation time. At higher frequencies there is little or no room for any an increase compared to wild type, as at these higher frequencies the wild type mice SERCA activity is enhanced per se. Previous studies showed that greater SERCA expression can limit the calcium transient by competing with troponin C for Ca binding and by curtailing its peak (Loukianov, Ji et al. 1998; Teucher, Prestle et al. 2004). We know that the SERCA pump can increase the SR Ca$^{2+}$ load, but only up to a certain limit. Once that SR calcium storage limit is reached, SERCA pump activity cannot increase the SR Ca$^{2+}$ load further (Shannon and Bers 1997; Ginsburg, Weber et al. 1998; Shannon, Ginsburg et al. 2002). This further possibly explains that an increase in contractility in SERCA1a over expressed trabeculae is observed mainly at sub physiological frequencies where the maximal SR load has not yet been reached.

At a 4 Hz stimulation rate, relaxation was significantly faster (shorter) in SERCA1a muscles versus WT but at the high frequency range this difference was no longer present. As we learned from previous studies (Hunter 2000), there are mainly two factors responsible for cardiac relaxation; decrease of intracellular [Ca$^{2+}$], and myofilament properties. These findings support that at sub-physiological frequencies relaxation rate may mainly depend on calcium reuptake by SERCA, while during physiological frequencies intrinsic myofilament properties are the major determinant of relaxation rate. Reuptake of Ca$^{2+}$ into the SR may thus possibly act as a rate-limiting step in relaxation at low stimulation frequencies, however, at physiologically frequencies
myofilament properties likely govern the relaxation rate, in accordance with our previous work (Janssen, Stull et al. 2002).

At baseline frequency of 4 Hz, SERCA2a HET mice show a slightly lower developed force compared to WT, and this difference becomes significant at higher frequencies. In line with results from previous studies, (Stull, Leppo et al. 2002; Stull, Hiranandani et al. 2006) our results shows that WT trabeculae show a positive force frequency response between 4 and 8 Hz, while in SERCA2a HET mice the contractile response compared to WT was impaired at high frequency. At baseline, developed force was less; decreased SERCA expression at the low end of the frequency range causes reduced SR Ca$^{2+}$ uptake. At higher frequencies there is less time to transport calcium, therefore decreased capacity of SR Ca$^{2+}$ reuptake and SR Ca$^{2+}$ loading become more prominent. Previous studies have shown that this alteration in force frequency response can be due to an in decrease in calcium transient with increased frequency (Pieske, Kretschmann et al. 1995; Pieske, Maier et al. 1999). In our study, the isoproterenol response was similar in WT and SERCA2a HET mice, indicating no significant change in β-adrenergic response due to decreased SERCA activity. This is consistent with previous studies which show that in SERCA2a HET mice there is no altered activation of the sympathetic system as levels of NE in plasma and cardiac was not changed (Ji, Lalli et al. 2000).

Although in all mammals generally a positive force-frequency relationship (FFR) is observed under physiological conditions, it is well known that this response is most prominent in larger mammals. Although in rats and mice this FFR is positive as well under physiological conditions, it is often extremely small (Georgokopoulos and Kass
FFR plays a prominent role in large mammals, the difference between resting heart rate and maximal heart rate is 300-400% in humans, whereas this is only 30-50% at the most in mice and rats resp (Kass, Hare et al. 1998). Moreover, in isolated myocardium, it is only positive in mice in ultra-thin trabeculae where core-hypoxia is virtually absent. Previous studies have shown that in isolated rat trabeculae, a hypoxic core unavoidably develops at a muscle thickness of 250 µm at room temperature (Schouten and ter Keurs 1986), and already at a muscle thickness exceeding 150 µm at body temperature at 8 Hz (Raman, Kelley et al. 2005). Thus for mice that even have faster heart rates, core-hypoxia may potentially occur at even thinner muscles. Although in this study we used ultra-thin muscles, with diameters around 150 µm, we cannot altogether exclude some minimal levels of core hypoxia, and this may be a limitation of our studies in the high frequency ranges. The levels of developed force however indicate that this possible core hypoxia was rather modest in these studies; even at the most demanding inotropic conditions (maximum isoproterenol response), force development was in the range of 40-50 mN/mm², which is significantly larger (up to exceeding a full order of magnitude) than regularly reported values for murine papillary muscles. The decrease in contractile force at the higher frequencies in all groups may be partially due to insufficient energetic supply. However, this is not the only reason, as at these very high frequencies, the extremely rapid calcium removal from the cytosol may decrease the time that calcium levels are above TnC threshold, limiting contractile activation. This latter process may thus partially, and likely mainly, be responsible for the reduced contractile forces at 10-12 Hz.
3.5 Conclusion

In conclusion, the relation between SERCA activity and cardiac contractility is critically dependent on stimulation frequency. Although at sub-physiological frequencies SERCA1a over expressed trabeculae exhibited a higher basal contractility, relaxation rate, and isoproterenol response, there is a limit after which there will be no further increase in calcium transient and results in a contractile response similar to wild type mice. Therefore at higher frequencies there is no room for further increases in contractility due to reduction in the contractile reserve as basal contractility was already increased. Small increases in SERCA expression may thus improve cardiac function but too much SERCA activity may lead to loss of contractile reserve and could cause negative effects on cardiac contractility.
Figure 7 A: SERCA1a mice express SERCA1a, whereas no SERCA1a is expressed in the WT mice. In SERCA2a HET animals, levels of SERCA2a protein are reduced by about 40%. B: Phosphorylation of phospholamban at Ser16 is increased in SERCA2a HET mice, but unchanged in SERCA1a mice (*, P<0.05, n=3/group). Phosphorylation of phospholamban at Thr17 appeared similar in all mice.
Figure 8 A: Representative tracings at 4, 8, and 12 Hz from SERCA 1a and WT mice. B: Comparison of average force frequency relationship between SERCA 1a (n=15) and WT (n=12) murine muscles. At low frequency (4-6 Hz) developed force is significantly higher in SERCA1a muscles but at the higher range of frequency there is no difference as compared to WT muscles. Temperature was 37ºC throughout the experiment.
Figure 9 Difference in isoproterenol response between SERCA 1a (n=14) and WT (n=11) mice. At base frequency developed force is higher in SERCA 1a compared to WT but as the concentration of isoproterenol increases difference is no longer significant.

Temperature was 37°C throughout the experiment. * indicates a difference of P<0.05 between the groups.
Figure 10. Changes in RT50% at different frequency (A) and with isoproterenol (B) in SERCA 1a and WT mice. At baseline frequency of 4 Hz, RT50% is significantly shorter in SERCA 1a muscles but at the higher frequency and with isoproterenol the difference between the two groups is no longer present. Temperature was 37°C throughout the experiment. * indicates a difference of P<0.05 between the groups, ** indicates a difference of P<0.05 between conditions in the same group.
Figure 11. Comparison of force frequency relationship between SERCA2a HET (n=8) and WT (n=9) murine muscles. At low frequency (4-6 Hz) developed force appeared reduced in SERCA2a HET muscles but not quite significant. With the increase in frequency both groups showed positive FF response, but at high range of frequency this difference became significant and SERCA2a HET mice had a lower developed force as compared to WT mice. Temperature was 37 °C throughout the experiment.
Figure 12. Comparison of isoproterenol response between SERCA 2a HET (n=8) and WT (n=10) mice. At base frequency of 4 Hz there is no difference in isoproterenol response between SERCA 2a HET and WT mice. Temperature was 37°C throughout the experiment.
Figure 13. Changes in RT50% at different frequency (A) and in presence of isoproterenol (B) in SERCA 2a HET and WT mice. Relaxation kinetics is slightly slower in SERCA 2a HET mice but this was not significant. Temperature was 37°C throughout the experiment. ** indicates a difference of P<0.05 between conditions in the same group.
Figure 14. A: Change in the rapid cooling contracture amplitude with frequency in SERCA2a HET and WT mice. At base frequency of 4 Hz and at higher frequency (12 Hz) there is no difference in RCC amplitude between SERCA2a HET and WT mice. Temperature was 37°C throughout the experiment. B: Change in post rest behavior in SERCA2a HET and WT mice trabeculae. With the increase in time period both group showed positive post rest potentiation response, but at high range of time period SERCA2a HET mice had a higher PRP as compared to WT mice. Temperature was 37°C throughout the experiment. * indicates a difference of P<0.05 between the groups.
CHAPTER 4
FREQUENCY-DEPENDENT CONTRACTILE RESPONSE OF ISOLATED CARDiac trabeculae under hypo-, normo-, and hyper-thermic conditions

4.1 Introduction
Thermoregulation of the body occurs under normal physiological conditions, such that a normal body temperature is maintained even under most pathological states. However, the body is from time to time exposed to non-normothermic conditions; both hypo- and hyperthermia can occur as a result of external (exposure, exercise, environment) or internal (pathogens, allergens) stressors. As vital organs need to be kept functioning even when temperature of the body drops below or increases above the optimal temperature, delivery of nutrients and oxygen are a must under hyper- and hypo-thermic conditions, and thus cardiac contractile function needs to be adapted to provide an adequate circulation.

During hyperthermia (or heat-stress), it is long known that heart rate increases, and sympathetic responses are upregulated. Faster heart rates, at least in healthy subjects, result in an increase in myocardial force production (Hasenfuss, Holubarsch et al. 1994). All mammals, within their own physiological range of rates (i.e. adult human ~1.3 Hz, rat ~5-9 Hz) display a so-called positive force-frequency relationship (Bowditch effect) (Bowditch 1871). As heart rate increases, so does contractile strength in humans.
(Hasenfuss, Holubarsch et al. 1994), as well as other mammals (Schouten and ter Keurs 1986; Kass, Hare et al. 1998; Layland and Kentish 1999). In addition to the increased force production at higher heart rates, the entire contraction is accelerated, both time to peak pressure (or force) and relaxation kinetics is speeding up in synergy with heart rate. The faster kinetics allows the heart to maintain a fast pacing rate without immediate diastolic fusion of successive contractions that can potentially cause a diastolic dysfunction.

Temperature has also a profound effect on myocardial function. In isolated myocardium, at constant frequency (or heart rate), increasing the temperature from room temperature to body temperature results in a profound loss of peak contractile force, while twitch kinetics speed up with temperature (Janssen, Stull et al. 2002). However, changes in force production and twitch kinetics do not occur in a parallel. Between 22.5 and 30 °C the loss of peak twitch force is minimal, while twitch kinetics speed up by a factor of 2-3. In contrast, between 30 and 37 °C force drastically declines by a factor 2-3 but twitch kinetics only speed up by a small amount. Little however is known regarding contractile performance of the myocardium under hyperthermic conditions. In the whole heart, an increase above normal temperatures induced negative inotropism (Saeki, Goto et al. 2000). However, cardiac output can be reduced by loss of myocardial contractility and/or insufficient filling. It remains unknown whether and how basic contractile performance of myocardial tissue is affected by hyperthermic conditions. Isolated cardiac muscle function has been extensively investigated under hypothermic conditions, but isolated myocardial contraction data obtained under hyperthermic conditions is altogether lacking. We hypothesize that when temperature of the myocardium is increased above
normal body temperature, twitch kinetics will accelerate and force development will decrease. Accordingly, we set out to investigate the influence of hyperthermia on the regulation of basic myocardial contraction, including at high heart rates. We observed that in isolated myocardium subjected to 42 °C, peak force development as well as β-adrenergic induced inotropism is reduced, while the force-frequency relation shifts rightward to a higher frequency optimum.

4.2 Material and Methods

4.2.1 Muscle Preparation

Male LBNF1 rats (175-225g) were anesthetized using intraperitoneal injection of Sodium Pentobarbital (60 mg/kg). The chest cavity was opened by bilateral thoracotomy and 1000 U of heparin was injected into the heart at the apex. The heart was removed and immediately perfused with Krebs Henseleit (KH) solution containing (in mM) 137 NaCl, 5 KCl, 1.2 MgSO4, 1.2 NaH2PO4, 20 NaHCO3, 10 glucose, and 0.25 CaCl2. 20 mM 2,3-butanedione monoxime (BDM) was added to the perfusate to minimize cutting damage, and to arrest the heart (Mulieri, Hasenfuss et al. 1989). The buffer solution was oxygenated continuously by bubbling with a mixture of 95% O2 / 5% CO2, this resulted in a pH of 7.32 at 37 °C, with minimal change at different temperatures (7.30 at 32 °C, and 7.34 at 42 °C). After flushing the blood out of the heart, the right ventricle was carefully opened and un-branched, ultra-thin trabeculae were carefully dissected as previously described (ter Keurs, Rijnsburger et al. 1980; Janssen, Stull et al. 2002; Raman, Kelley et al. 2005). A small portion of the tricuspid valve was left attached to one end, and a block of ventricular tissue remained attached to the other end of the trabeculae.
to facilitate mounting the preparation onto the experimental setup. Muscle dimensions were measured (Raman, Kelley et al. 2005) at 40x magnification (~10 µm resolution), and were 142 ± 16 µm wide, 82 ± 8 µm thick, and 1.70 ± 0.16 mm long (n=9). It is imperative that these preparations are ultra-thin; experimentally it has been show that at a temperature of 25 °C, core-hypoxia develops at preparations exceeding 200 µm (Schouten and ter Keurs 1986), and at a temperature of 37 °C this has recently been determined to be only 150 µm (Raman, Kelley et al. 2005). We realize that at even higher temperature/frequency this could size limitation be even smaller, and extrapolation of the previous two studies would potentially indicate that a hypoxic core could develop in preparations exceeding ~130 µm. In order to avoid any potential complications of core-hypoxia, we remained well below 100 µm for the thickness of our preparations (average of 82 µm), so they were in the order of 4 to 5 myocytes thick and thus had a maximal diffusion distance to the core of no more than 3 myocytes.

4.3 Protocols

After mounting the preparation at 37 °C in the experimental system, [Ca²⁺] was raised to 1.5 mM, and point-stimulation (via hook and basket) was initiated by 2 ms wide pulses at 120% threshold voltage (~4-5 V) at an initial frequency of 4 Hz. The muscle was allowed to stabilize, and thereafter the muscle length was stretched until developed force was maximal, or an increase in developed force was accompanied by a disproportional increase in diastolic force. After stabilization (15-25 minutes), muscle length was adjusted if needed, and the experimental protocol was initiated when contractile parameters were stable. Only healthy muscles, defined by a positive force-
frequency between 4 and 8 Hz, as well as a minimal isometric developed force of at least 20 mN/mm² at 32 ºC (4 Hz), were used for this study.

First, temperature was lowered to 32 ºC. After force had stabilized, data were collected. After collecting data at 6 and 8 Hz thereafter (again after forces had stabilized), frequency was returned to 4 Hz. Temperature was rapidly switched to 37 ºC, the muscle was allowed to stabilize at this new temperature, and this protocol was repeated, up to 10 Hz, and thereafter again at 42 ºC, with frequencies up to 12 Hz.

To test for cardiac reserve and β-adrenergic responsiveness at 42 ºC, a separate set of trabeculae (n=7) was subjected to an isoproterenol concentration-response protocol. Isoproterenol concentration was increased from 1 nM to 1 µM in semi-log steps, and force responses were recorded at each concentration.

In a third set of experiments, intracellular calcium transients were measured after iontophoresis of the calcium indicator bis-Fura-2 into the preparation. The dye loading technique and protocol that allows for assessment of calcium transients at body temperature has been described previously (Backx and Ter Keurs 1993; Georgokopoulos and Kass 2001; Janssen, Stull et al. 2002; Stull, Leppo et al. 2002) . Briefly, at room temperature, a micropipette filled with bis-Fura-2 is introduced into a single myocyte near the center of the preparation, and a small negative current is applied to introduce the dye into the cytoplasm. Via gap-junctions, this dye spreads to neighboring cells. Once the dye has uniformly spread, fluorescence intensity at 510 nm is measured under various conditions (temperature, frequency) during alternate exposure to 340 and 380 nm excitation light. Intracellular calcium transients were recorded at various combinations of temperature and frequency.
Briefly, in 2 additional experiments we tested whether the response to acidosis was preserved during hyperthermia. After assessment of a force-frequency relation at 42 °C, the pH of the perfusate was lowered to from 7.34 to 7.00. At this lower pH, the force-frequency relationship was again measured.

Twitch contractions were continuously recorded throughout the experiment. Force development was normalized to the cross sectional area of the trabeculae to allow for comparison between muscles of different diameters, including those from previously published work. Twitches were recorded at each experimental condition upon stabilization. Data were collected and analyzed on- and offline using custom-written software (LabView, National Instruments). Data are expressed as mean ± SEM unless otherwise noted. Data were statistically analyzed using ANOVA, or Student’s t-tests (paired or unpaired) where applicable. A two-tailed value of P<0.05 was considered significant.

4.4 Results

Figure 15a shows the twitch force development at a stimulation frequency of 4 Hz under hypo-, normo-, and hyperthermic conditions. As can be clearly seen, peak twitch force development (developed force) diminishes as temperature increases. In figure 15b, the average active isometric force development for n=9 trabeculae is depicted, as well as the kinetic parameters. Both time to peak tension (TTP) and time from peak tension to 50% relaxation (RT50) time are reduced upon elevation of temperature.

The optimum of the force-frequency relation depends highly on temperature (Figure 16a). At 32 C, a small increase is observed in developed force from 4 to 6 Hz, but
a clear and significant loss of developed force occurs between 6 and 8 Hz. At 8 Hz, contractions are too slow for force to completely return to un-stimulated (diastolic) values, resulting in a slightly elevated diastolic force, thereby reducing developed force. At body temperature, the frequency optimum is at ~8 Hz. At this rate of contraction, relaxation is fast enough to allow a complete relaxation. At an even faster rate of 10 Hz (supra-physiological), a significant decline in developed force is observed. At hyper-thermic conditions, developed force does not significantly decline between 8 and 10 Hz. In fact, even at a stimulation rate of 12 Hz, the decline is not significant. In several preparations developed force continues to increase when frequency was elevated from 10 to 12 Hz. Under all conditions, both an increase in temperature or frequency results in faster kinetics (reduction in RT50). However, at 42 C, this reduction in RT50, both relative and absolute, is less than at 32 and 37 °C. In Fig 16c it can be more clearly seen that the optimal frequency shifts towards higher values when temperature is increased. For each temperature, forces are depicted as a fraction of the ones measured at 4 Hz. In Figure 17 the twitch tracings of a typical force-frequency protocol are given under hyperthermic conditions, from 4-8 Hz force development significantly increases, and thereafter remains fairly stable. Even at the supra-physiological frequency of 12 Hz, diastolic force is hardly elevated. In fact, even when stimulated at even higher rates, up to 20 Hz, only a small elevation of diastolic force is observed, tension easily relaxed below 25% of maximal (not shown). In figure 17 it can be clearly seen that even up to 10 Hz, no elevation of diastolic force is found, indicating that the muscle can completely relax prior to the next twitch. At 12 Hz, a very small amount for diastolic force is observed, but this is much smaller than that at 10 Hz at 37 °C.
To test to what extent β-adrenergic stimulation can increase isometric force under hyperthermic conditions; a concentration response relationship was measured. In Figure 18 a can be seen that at the higher concentration (maximal effect), of 1 µM isoproterenol, developed force is clearly enhanced, while twitch kinetics are accelerated. In Figure 18b the response of developed force and RT50 shows the average values obtained for n=7 trabeculae.

To test whether the observed changes in force development are a result of altered calcium handling, intracellular calcium transients were assessed at various temperature and frequency. As can be seen in figure 19a, the amplitude of the calcium transient at a fixed frequency (depicted here is 6 Hz), is not much different, but the kinetics are faster; the decline of the calcium transient is greatly accelerated at higher temperatures. In panel B, both calcium transient and force development of trabeculae at 42 °C at 4 and 12 Hz is plotted. Clearly, the amplitude of the calcium transient is much higher at 12 Hz, while its kinetics is also faster. The resulting force traces reflect the same: a stronger contraction with a faster relaxation.

Lastly, we tested whether changes in pH could have an impact on cardiac contractility at 42 °C. We observed the expected effect, a loss of force development combined with an increase in twitch kinetics. Interestingly, we did observe a very clear frequency-dependent effect of the influence of pH; the loss of force and acceleration of kinetics were much more profound at lower frequencies (4-8 Hz) than at higher ones (10 and 12 Hz).
4.5 Discussion

We set out to study the mechanical function of isolated cardiac tissue under hyperthermic conditions. Isolated muscle preparations allowed us to study the contractile capacity of the heart (Bers 1997; Sys, De Keulenaer et al. 1998) without being hampered by altered loading conditions as prevail during hyper- and hypothermia. We found that the contractile force of isolated myocardium is significantly reduced when studied at a fixed pacing rate. In addition, we observed that the kinetics of the twitch contractions accelerate, and response to β-adrenergic stimulation is maintained.

It has long been known that under hypothermic conditions, isolated preparations display increased inotropy at the same pacing rate. In fact, most of the published data on contractility of isolated muscle preparations is carried out at temperatures below the physiological range, and its temperature dependency between room temperature and body temperature is well documented. In isolated trabeculae, we (Janssen, Stull et al. 2002) and others (Layland and Kentish 1999) have previously shown that although between 22.5 and 30 °C little or no loss of inotropy (at 1 Hz) occurs, between 30 and 37 °C a significant loss of developed force was found. We now add the finding that upon further increasing temperature this negative inotropism persists. We did not only observe this at very low stimulation rates, but also at rates within the physiological range: at any frequency spanning the physiological rate for the rat, between 5 and 9 Hz, increasing temperature led to a loss of force development. To date, virtually no information is however available on the contractile response of isolated myocardium under hyperthermic conditions. In this study we observed lower force development at constant rate which is in line with *in vivo* and whole heart studies that have observed negative
However, as force is lost with increased temperature, the contractions accelerate, allowing for a faster relaxation. As a result, at higher frequencies the isolated myocardium can reach higher rates without diastolic dysfunction. In our data, we observed an increase in contractile strength when stimulation frequency was increased. This positive force-frequency behavior is observed across mammalian species including rat (Layland and Kentish 1999; Janssen, Stull et al. 2002) and mouse (Georgokopoulos and Kass 2001), provided the frequencies tested fall within each species’ normal in vivo range. The magnitude of frequency-induced gain in contractility is also correlated to the size of the animal, and is therefore relatively small in the rat and mouse, but nonetheless positive. However, when frequencies are used outside the animal’s normal heart rate range, negative staircases can be obtained. Indeed, even when large mammals are subjected to very low twitch frequencies, they too can show a negative staircase behavior, but this process should ideally not be termed force-frequency behavior, as it is not reflecting the normal physiological response of mammalian myocardium to increase heart rate, but in stead be regarded as a display of an inverse post-rest behavior (Pieske, Sutterlin et al. 1996). In our data, the accelerate twitch kinetics that were observed at higher temperature allowed the already positive force-frequency to be extended to higher ranges, resulting in increased development of force even when frequencies well exceeded the rates usually observed under normothermic conditions. Thus, the loss of contractile force can be (partially) offset by the ability of the myocardium to achieve higher rates under hyperthermic conditions. This would be in close accordance to the response of the heart under hyperthermic condition; it has long been known that when core body
temperature increases, so does heart rate, especially under strenuous conditions. The positive force-frequency effect is thus clearly preserved, and even more pronounced at high temperature, while the fast relaxation allows the heart to sufficiently relax in between beats. Interestingly, in our experiments, at the very highest pacing rates, twitch kinetics were only slightly affected, between 8 and 12 Hz (and even as high as 20 Hz, which is well beyond the physiological rate), little further acceleration of the twitch kinetics was observed. Possibly, at these very high rates, cross-bridge cycling rates are approaching a maximal rate, imposing the rate-limiting step on cardiac relaxation.

Because of the loss of developed force at any given frequency at 42 °C, when isoproterenol, a β-adrenergic agonist was applied, a significant gain of force development was observed. However, even at the highest and maximal concentrations 10^{-7}-10^{-6} M, the force development did not reach the level that could be achieved at lower temperatures. Twitch kinetics further accelerated in presence of isoproterenol, with 50%-relaxation times near, and even below, 20 ms. These very rapid contractions may be the underlying factor in twitch force-production reduction; the intracellular calcium transients is only a few milliseconds above the level needed for thin filament activation, and full activation of the thin filament can simply not be achieved in that time period.

The underlying mechanism of hyperthermia-induced loss of inotropy has likely to be sought in the altered calcium handling. Our calcium transient measurements indicated faster kinetics, likely reducing the time for myofilament activation. Unfortunately, within the short time span intracellular calcium transients can be performed at supra-physiological temperatures (~10-15 minutes) due to fast leakage of the dye, we could not calibrate the calcium signals, which takes >40 minutes. Thus, we can only use the data as
illustrative and indicative of kinetic direction. Although Kd and on-off rates of the dye may depend on temperature, at a given temperature in the same preparation, a faster fluorescence transient will always be indicative of faster calcium kinetics. There are numerous studies that have studied the kinetics of calcium handling processes in myocardium. A common denominator in these studies is that hypothermia-induced gain of inotropy mainly stems from a slowed calcium transient, increasing the time for myofilament activation, and resulting in not only increased force production, but also in a prolongation of contraction. Indeed, in our data this is very clearly visible as well, the hypothermia data clearly shows an increase in force (at a given frequency), in concert with slowed twitch kinetics. Our data now extends these previous observations to hyperthermic conditions. As temperature increases, kinetics further speed-up, and result in a further loss of inotropy in combination with faster twitch kinetics.

In addition to the acceleration of the twitch and loss of peak force due to the temperature itself, lowering the pH of the perfusate had a similar, but additive, effect; when pH was lowered from 7.34 to 7.00, at the lower end of the frequency rage (4-8 Hz), twitch kinetics were slightly faster, and loss of developed force was pronounced. This is in line with the observations that lowering pH has a desensitizing effects on the myofilaments (Fabiato and Fabiato 1978; Jacobus, Pores et al. 1982). Interestingly, at 10 and 12 Hz this acidosis-induced loss of contractility was much less, and although these stimulation rates are supra-physiological under normo-thermic conditions, they could occur during hyperthermia, where prevailing heart rates are higher. At increasing frequency, relaxation rates are faster, and the molecular basis for frequency dependent acceleration of relaxation are incompletely understood. It is conceivable that
desensitization of the myofilaments is one of the underlying factors. Desensitization of the myofilaments would abbreviate relaxation, similar to the effect of β-adrenergic stimulation induced desensitization of the myofilaments. This desensitizing effect may aid in providing adequate relaxation at higher frequencies, and thus the pH-induced depression of myofilament calcium sensitivity may be less due to a common pathway to achieve this desensitization at higher frequencies.

In conclusion, under hyperthermic conditions myocardial contractile performance is markedly reduced compared to body temperature; isometric force development is lower at all frequencies spanning the physiological range. Both twitch and calcium transient kinetics are accelerated, while the positive force-frequency relationship is preserved, and has shifted its optimum to higher frequencies, thereby being able to produce adequate force at the higher heart rates that generally prevail during hyperthermia.
Figure 15. A. Twitch peak force development (developed force) is reduced as temperature increases. At a stimulation rate of 4 Hz, developed force is reduced while twitch kinetics are accelerated. B. Average data for developed force, time to peak tension (TTP), and time form peak tension to 50% relaxation (RT50) at a frequency of 4 Hz and at various temperatures. * denotes a significant (P<0.05) increase versus 37 °C, ** a decrease versus 37 °C data, n=9, mean ± SEM.
Figure 16.  A. Twitch peak force development (developed force) varies with frequency and temperature. At 32 °C, developed force is optimal at 6 Hz, whereas this is 8 Hz at 37 °C, and 10 Hz at 42 °C. Diastolic force starts developing at 8 Hz (32 °C), 10 Hz (37 °C), and 12 Hz (42 °C) respectively, data not shown. B. Time from peak tension to 50% relaxation (RT50) accelerates with both frequency and temperature. At 42 °C however, little acceleration is observed between 8 and 12 Hz. C. When expressed as fraction of developed force at 4 Hz at each respective temperature, more clearly can be seen that the higher the temperature, the more positive the force-frequency relationship is. It can be seen that at 32 °C, the optimal frequency is 8 Hz, where it is 10 and 12 Hz for 37 and 42 °C respectively. * denotes a significant (P<0.05) increase versus 37 °C, ** a decrease versus 37 °C data, n=9, mean ± SEM.
Figure 17. Original force recording of rat cardiac trabeculae at 42 °C at various frequencies, spanning the in vivo range. An increase in frequency results in an increase in developed force, and in an acceleration of twitch kinetics. Only at 12 Hz a very mild diastolic force is observed.
Figure 18.  

A. Isoproterenol increases both force development and twitch kinetics in isolated rat cardiac trabeculae at 42 °C. B. Concentration dependent increase in developed force (closed circles) and acceleration of time form peak tension to 50% relaxation (RT50, open circles) in cardiac trabeculae at 42 °C, n=7, mean ± SEM.
Figure 19.  

A. Intracellular calcium transients at various temperatures at a stimulation rate of 6 Hz. The amplitude of the calcium transient is only slightly increased at higher temperature, but the acceleration is clearly visible. B. At 42 °C, both the calcium transient and force development are increased in amplitude and accelerated.
Figure 20. At a temperature of 42 °C, the response to lowering of the pH has an additive negative inotropic effect on twitch contractions in the lower and mid-frequency range. Force development was significantly lower at pH 7.0 vs. pH 7.34 at stimulation rates of 4-10 Hz, while simultaneously the time from stimulation to 50% relaxation was abbreviated, indicative of faster twitch kinetics. At a rate of 12 Hz, which is supra-physiological, the loss of developed force and acceleration of twitch kinetics were smaller or absent, n=2-3.
CHAPTER 5

IMPACT OF OH*-INDUCED INJURY ON CALCIUM HANDLING AND MYOFILAMENT SENSITIVITY IN ISOLATED MYOCARDIUM

5.1 Introduction:

Reperfusion injury is a serious pathological process that occurs when the heart, or part of it, is re-oxygenized after a period of partial or complete ischemia. OFR occurring during reperfusion injury have been implicated in the pathogenesis of myocardial stunning and progression of heart failure. OH* (OH*) are one of the most aggressive species of OFR that attack many molecules in the human body (Gao, Liu et al. 1996; Zeitz, Maass et al. 2002). These OH* are involved in the pathogenesis of IR injury, which is observed in many clinical situations including acute heart failure, stroke, and myocardial infarction.

It is documented that multiple sub-cellular defects contribute to the development of acute myocardial dysfunction, predominantly intracellular calcium overload and alteration of myofilament responsiveness. Previous studies have demonstrated that intact contracting cardiac trabeculae from mouse, rat, and rabbit after acute exposure of OH* develop a rigor like contracture marked by an increase in diastolic tension, myofilament proteolysis and overall decreased cardiac contractility, which is followed by a partial recovery (Gao, Liu et al. 1996; Hiranandani, Bupha-Intr et al. 2006). Although the phenotype of the acute injury response is rather well documented, the underlying mechanism and the relative importance
of these two factors, deranged calcium handling versus altered myofilament function, that are responsible for the transient injury and subsequent partial recovery remains incompletely understood. Hence, the goal of this study is to delineate, in a time resolved manner, the contribution of altered calcium handling and altered myofilament responsiveness throughout the various stages of experimental IR injury.

There are multiple mechanisms that are possibly responsible for calcium overload via sarcoplasmic reticulum damage, mitochondrial damage, changes in properties of NCX, and L-type channels have all been implicated to play a role (Ehring, Bohm et al. 1992; Heusch 1992; Gao, Liu et al. 1996; Bolli and Marban 1999; Janssen, Zeitz et al. 1999; Zeitz, Maass et al. 2002; Hiranandani, Bupha-Intr et al. 2006). Although the relative contributions of these factors are still unknown, specifically to what extent and how they relate to the sustained damage observed after OH* exposure. It is known that impaired sarcoplasmic reticulum (SR) function is one of the main pathways through which calcium overload is mediated. Previous studies have shown that there is a direct impairment of sarcoplasmic reticulum function due to OH* exposure and SERCA overexpression can reduce the OH*-induced contractile dysfunction in murine myocardium whereas decreased SERCA activity aggravates this injury (Flesch, Maack et al. 1999; Hiranandani, Bupha-Intr et al. 2006; Talukder, Kalyanasundaram et al. 2007). These studies clearly indicate that calcium overload plays a significant role in cardiac dysfunction due to OH*-induced processes. Previous studies also showed that OH* exposure can lead to the proteolysis of different sub-units of TnIn different species (Zeitz, Maass et al. 2002). Although intracellular calcium overload is present, myofilament alteration may be the primary cause of contractile dysfunction by OH* exposure. The goal of this study is to understand the
relative magnitude by which myofilament responsiveness on one hand and deranged calcium handling (increased diastolic calcium levels and reduced calcium transient amplitude) on the other, impair cardiac contractility after acute OH* exposure. In this study we have focused on the possible interactions of these two mechanisms at different time points of IR injury to test our hypothesis that calcium overload plays a major role during acute/early OH*-induced injury and altered myofilament response in the sustained/later part of the OH*-induced injury. Elucidating the relative importance of these two distinctly different injury pathways at various stages throughout the injury window will gain crucial insight in how to target hypothesis-driven novel treatment strategies for oxidative stress injury in the heart. Our results shows that calcium overload is mainly responsible for acute diastolic dysfunction after OH*-induced injury, while sustained myocardial dysfunction is mainly due to the alterations in myofilament responsiveness.

5.2 Methods:

5.2.1 Preparation of Trabeculae and Overall Protocol

New Zealand White Rabbits (2 kg, approximately 3 months old) were anesthetized with 50 mg/kg pentobarbital sodium (IV) and injected with 5,000 units/kg heparin. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The heart was rapidly removed and perfused retrogradely through the aorta with Krebs Henseleit solution containing (in mmol/L): 120 NaCl, 5 KCl, 2 MgSO₄, 1.2 NaH₂PO₄, 20 NaHCO₃, 0.25 Ca²⁺ and 10 glucose (pH 7.4) in equilibrium with 95%O₂/5%CO₂ at body temperature (37°C). Additionally, 20 mmol/L 2,3-butanedione
monoxime (BDM) was added to the dissection solution to stop the heart from beating and to prevent damage during dissection (Mulieri, Hasenfuss et al. 1989; Mulieri, Hasenfuss et al. 1992; Janssen and Hunter 1995). The effects of BDM after brief exposure, have been found to be reversible (Mulieri, Hasenfuss et al. 1992; Janssen and Hunter 1995). Suitable trabeculae from the right ventricle were dissected carefully without touching the central part of the muscle and mounted in the bath. In addition to the muscle, the dissected specimen contained a small portion of tricuspid leaflet attached to one end and a block of tissue from right ventricular free wall to the other end to facilitate mounting the muscle onto the experimental setup (ter Keurs, Rijnsburger et al. 1980; de Tombe and ter Keurs 1990; Janssen and Hunter 1995; Janssen and de Tombe 1997; Layland and Kentish 1999). We used this technique because it allows for attachment of the muscle in the experimental chamber without damaging the muscle. Only thin muscles were used for the study, with a maximum diffusion distance of 100 µm to the core, so core hypoxia would be avoided and not affect the outcome of the results (Raman, Kelley et al. 2005). Using a dissection microscope, muscles were mounted in the chamber. The piece of tricuspid valve leaflet was connected to a hook like extension of micromanipulator and the cube of ventricular tissue rest in a platinum-iridium basket-shaped extension of the force transducer. By using this method we can minimize the end-damage compliance of the muscle and to prevent excessive loss of force throughout the experimental protocol (ter Keurs, Rijnsburger et al. 1980; de Tombe and ter Keurs 1990; Janssen, Zeitz et al. 1999; Janssen, Stull et al. 2002). The muscle was bathed in a continuous flow of oxygenated K-H solution (without the BDM). The muscle was stimulated at 2 Hz at a temperature of 37°C, and the calcium concentration was raised to 1.5 mM and the muscle stretched until maximal active force
was attained. This length is comparable to maximally attained length in-vivo at the end of
diastole (around 2.2 µm sarcomere length) (Rodriguez, Hunter et al. 1992). After the
stabilization of contractile parameters (15-20 min), we exposed the muscle to OH* for 2
minutes. Twitch contractions were monitored until the transient acute dysfunction has
subsided and diastolic and developed force had reached their new steady state (typically 45
min after OH* exposure).

**5.2.2 Intracellular Calcium Measurements**

After the stabilization of contractile parameters, we stopped the stimulation and
trabeculae were iontophoretically loaded with bis-fura-2 (Texas Fluorescence) as described
previously (Backx and Ter Keurs 1993; Janssen, Stull et al. 2002; Varian, Raman et al.
2006). As bis-fua-2 is a ratiometric dye it automatically cancels out confounding variables,
such as variable dye concentration due to leakage or inactivation of dye by radicals and
cell thickness. Bis-fura-2 was chosen for its higher signal to calcium buffering ratio
(allowing for a loading of the dye to 5–10 times background without impacting buffering),
its slightly higher K_d (390 nM in vitro) than fura-2, allowing for still accurate diastolic
values but a better resolution at higher [Ca^{2+}]_i, and the speed of the indicator (Monasky,
Varian et al. 2008). Iontophoretic loading of the dye was performed at room temperature
(at body temperature, the rate of dye leak is higher). We loaded the bis-fura-2 until the
photomultiplier output at baseline 380 nm excitation was between 6 and 10 times over
background. After loading and spreading of the dye was completed at room temperature,
the system was returned to 37°C. The muscle was stimulated to contract at 2 Hz while
force and fluorescent emission measurements (excitation, 340 and 380 nm) were recorded.
5.2.3 OH* Exposure

After the stabilization of contractile parameters, we exposed the muscle to OH* for 2 minutes and measured force and fluorescence (excitation at 340 nm and 380 nm). As fura 2 is a ratiometric dye we can avoid the problems caused by the inactivation of dye by OH*. Contractions were monitored until diastolic and developed force reached their new steady state (typically 45 min). In this study OH* was produced via the Fenton reaction through H₂O₂ + Fe³⁺-NTA (ferric nitrilotriacetic acid) system (Zeitz, Maass et al. 2002):

\[ \text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + \text{HO}^- + \text{HO}^* \]

The reaction involves hydrogen peroxide and a ferrous iron as a catalyst. The concentration of the H₂O₂ and Fe-NTA in the organ bath is 3.75 mM and 10µM respectively. The amount of OH* generated under these conditions is comparable to those that occur in IR injury (Zweier, Kuppusamy et al. 1989; Zeitz, Maass et al. 2002). As the half life of H₂O₂ is short, it was infused via a separate line feed directly into the organ bath at the level of the muscle to ensure that OH-radical formation took place right in the muscle and muscle bath where H₂O₂ meets Fe-NTA. We also performed control experiments by exposing these muscles with H₂O₂ or Fe-NTA alone. In agreement with previous studies (Zweier, Kuppusamy et al. 1989; Zeitz, Maass et al. 2002) using this protocol, exposure of H₂O₂ alone or Fe-NTA alone did not cause any change in the contractile parameters suggesting that the contractile dysfunction is specifically due to the formation of radicals, and not H₂O₂ or Fe-NTA.

To further confirm that the myocardial dysfunction occurring after exposing the muscle with H₂O₂ and Fe-NTA is actually due to specific OH* exposure, we used a
modified K-H solution containing 1 mM (Sigma) allopurinol (Kinugasa, Ogino et al. 2003) instead of normal K-H solution in these experiments. Allopurinol is a xanthine oxidase inhibitor and thereby has potential clinical application in the prevention of reperfusion injury, as myocardial injury occurs primarily during reperfusion with the generation of OFR via the hypoxanthine-xanthine oxidase reaction (Berry and Hare 2004). After the stabilization of contractile parameters, we assessed a force-frequency protocol by increasing the frequency from 2 Hz to 5 Hz, then we exposed the muscle to OH* for 2 minutes. Twitch contractions were monitored until the new steady state (typically 45 min after OH* exposure). We repeated the force-frequency protocol at new steady state (typically 45 min after OH* exposure).

5.2.4 Potassium Contractures

To obtain levels of steady state force development, which are needed to construct the steady state force–[Ca^{2+}]i relationship we wanted to tetanize cardiac muscle reversibly while measuring calcium at physiological conditions. At room temperature, cardiac muscle can be tetanized by rapidly pacing the muscle (at 15 Hz). However, at a physiological temperature, even at pacing rates as fast as 20 Hz, healthy, well-perfused muscles almost fully relax at each beat. We thus resorted to K+ contractures before and after OH*-induced injury to obtain levels of steady-state force development, which are needed to construct the steady-state, force-[Ca^{2+}]i relationship. After ionophoretically loading right ventricular trabeculae with bis-fura 2, the superfusion solution was switched to a modified K-H solution containing (in mM) 142 KCl, 0 NaCl, and 3 CaCl2. Because of the very thin size of trabeculae, fast diffusion of dye and slowly forming contracture, all the cells in the
muscle tissue will depolarize at the same time. The rest of the contents were identical to that of the normal K-H buffer. Excitation wavelengths were switched back and forth between 340 and 380 nm so that the 340 to 380 ratio could be taken at given points during the contracture. The high K\(^+\) solution was applied for 20 s and then washed out. We plotted the curve between ratio and force to the peak of K\(^+\) which was typical of a myofilament calcium sensitivity curve.

### 5.2.5 Data Analysis and Statistics

Data were collected and analyzed on- and off-line using custom-written software in LabView (National Instruments). Data are expressed as means ± SE unless otherwise stated. Data were statistically analyzed using ANOVA or Student's t-tests (paired or unpaired) where applicable. A two-tailed value of P < 0.05 was considered significant.

### 5.3 Results

#### 5.3.1 Contractile Function after OH\(^*\) Exposure

In accordance with previous studies in other species, acute OH\(^*\) exposure in rabbit trabeculae muscles led to a rapid increase in diastolic force and a decrease in developed force. Figure 21a shows the effect on diastolic and developed force for (n=8) trabeculae after the exposure of OH\(^*\) in rabbit trabeculae. Direct acute exposure for 2 minutes of OH\(^*\) resulted in an increase of diastolic force and decrease of developed force. Contractile parameters were observed for 45 minutes till a new steady state level was reached. This new steady state level was marked by an elevated diastolic force and reduced developed force compared to pre-interventional values.
Diastolic force at the peak of contracture (~12 minutes after OH* exposure) was increased to 279±8% (P<0.05) of its value before OH* exposure, and after approximately 45 minutes returned to a new steady state level of 133±9% (P<0.05). Developed force at peak of contracture was decreased drastically to 36±2% (P<0.05) and returned to a new steady state level of 70±3% (P<0.05). In another set of control experiments contractile parameters remained unchanged when muscles were treated similarly but without OH* for the equivalent period of time (data not shown), indicating reliability and durability of the preparation, as well as showing that prolonged study of these isolated trabeculae is not complicated by an excessive loss of function over time. There was no change in contractile parameters in control experiments (not shown) with either H$_2$O$_2$ or Fe-NTA alone, confirming that formation of OH* occurs only when meets H$_2$O$_2$ with Fe-NTA and that this OH* is the initiator of the injury response.

Elevated diastolic force and reduced developed force at the new steady state level was also accompanied by a slower relaxation: Figure 22 shows that RT$_{50\%}$ and RT$_{90\%}$-RT$_{50\%}$ were significantly slower after OH*-induced injury as compared to before injury. Interestingly, there was no significant difference in time to peak tension and another often-used index of contractility and relaxation, the first derivative of force (dF/dt), between the two groups, possibly indicating a specific impairment of relaxation kinetics, both in the early (RT$_{50\%}$) and late relaxation phase (RT$_{90\%}$-RT$_{50\%}$).

5.3.2 Force-Frequency Relationship

It is known that the activity of the SR calcium ATPase is reduced by OFR. An important aspect of characterizing SR function is the deviation of the force frequency
relationship from normal. Figure 23 not only shows that there is significantly decrease in developed force after OH*-induced injury at all frequencies, also the increase in developed force from 1 to 3 Hz before injury is significant (p<0.05) as compared to after injury. An increase in stimulation frequency from 2 to 5 Hz induced an increase in developed force before exposure (normal positive response) but after OH* exposure, there is clear attenuation of this positive force frequency relationship. A flat or negative force frequency relationship is viewed as an indicator of impairment of SR function, and is one of the classic hallmarks of congestive heart failure. Force frequency measurements were performed in RV rabbit trabeculae before and after the OH* exposure by using the same exact experimental setup.

5.3.3 Effect of Allopurinol on OH*-Induced Injury

To confirm that the observed myocardial dysfunction is due to OH* we used the modified K-H solution containing allopurinol for the whole experiment. Figure 24A shows that there is no change in the contractile parameters after the OH* exposure with allopurinol. Contractile parameters were observed until a new steady state level was reached. There was no difference in contractile parameters and force-frequency relationship before and after OH* exposure with allopurinol.

5.3.4 Role of Intracellular Calcium Overload in OH*-Induced Injury

To temporarily delineate the impact of intracellular calcium overload during OH*-induced injury, we measured the diastolic level, developed amplitude, and kinetics of calibrated intracellular Ca\(^{2+}\) transients and twitch contractions simultaneously using
ionophoretically loaded bis fura-2. Figure 25 shows the relationship between intracellular calcium and diastolic force after the exposure of OH* in rabbit trabeculae. Direct acute exposure of OH* resulted in an increase in diastolic calcium in parallel to the increase in diastolic force. Contractile parameters were observed until a new steady state level was reached. At this new steady state level, diastolic calcium returned to near-normal levels, whereas diastolic force remained significantly elevated compared to pre-interventional values.

Initially, there was a marked increase in diastolic force (on average from 6.2 to 46.4 mN/mm²) in parallel with an increase in diastolic calcium (on average from 137 to 779 nM). After the peak injury response, diastolic calcium levels returned to near-normal levels, whereas diastolic force development remained significantly elevated.

5.3.5 Role of Myofilament Calcium Sensitivity in OH*-Induced Injury

The elevated tension in absence of a significantly elevated calcium level could be explained by an increase in the sensitivity of the myofilament for calcium. We tested these directly by using potassium contractures. To elucidate the impact of altered myofilament responsiveness on sustained injury, we tracked [Ca²⁺]i (via bis-fura 2 ratio) and force throughout potassium contractures before and after OH*-induced injury. Figure 26A shows the raw force-calcium data from a typical experiment. After the OH*-induced injury force was increased to more than 2 fold. An upward and leftward shift of the sensitivity curve from before to after OH*-induced injury was observed. Figure 26B shows that there is a disproportional increase in force for a given calcium concentration and a sensitization of the myofilaments is responsible for the sustained increase in
diastolic force at the new steady state after OH*-induced injury.

5.4 Discussion

The results of this study show that direct acute exposure of rabbit trabeculae to clinically relevant levels of OH* is marked by a characteristic acute increase in diastolic force in parallel with the diastolic calcium with the sustained increase in diastolic force at new steady state (after 45 min). At this new steady state, there is an increase in myofilament calcium sensitivity as compared to before OH*-induced injury. These data indicate calcium overload is mainly if not entirely responsible for the acute myocardial dysfunction after OH*-induced injury, while the sustained dysfunction is mainly due to the alteration in myofilament responsiveness.

5.4.1 OH* Induced Injury in Isolated Rabbit Myocardium

The acute response of OH* radicals on rabbit trabeculae muscles is similar to what has been previously described in different species (Zeitz, Maass et al. 2002; Hiranandani, Bupha-Intr et al. 2006). A transient, rigor-like contraction develops several minutes after OH* exposure. During the acute phase, this injury is marked by an increase in diastolic tension and a decreased developed force. During the recovery phase, diastolic force declines somewhat, but does not nearly recover to pre-OH*radical levels, indicating a sustained injury. There was no change in the contractile parameters after the OH* exposure when we used modified K-H solution with allopurinol in it. At this new baseline, diastolic force thus remained elevated, and active developed force remains decreased. Assessments of RT50% and RT90%-RT50% indicate that myofilament relaxation kinetics was
prolonged after the injury. This prolongation might be due to Ca\textsuperscript{2+} reabsorption delay by the sarcoplasmic reticulum. The depressed contractile state was accompanied by the attenuation in the force-frequency response, one of the classic hallmarks of heart failure, after the OH*-injury.

Previous studies also demonstrated a decrease in SERCA activity at increasing concentrations of OH* (Flesch, Maack et al. 1999) as well as a significant attenuation of the positive force-frequency relationship after the exposure of OH* (Schouten and ter Keurs 1986; Janssen, Zeitz et al. 1999; Zeitz, Maass et al. 2002). These studies indicate that there is a direct impairment of sarcoplasmic reticulum function due to OH* exposure. Combined, from these studies and findings from this study it is clear that calcium overload plays a significant role in acute cardiac dysfunction due to OH*-induced injury.

5.4.2 Calcium Overload in Acute OH*-Induced Injury

One of the main goals of this study is to assess the potential role of the calcium overload at different time-points during the injury phase. In order to address the clinical problem of ischemia-reperfusion injury effectively in future stages, it is imperative we understand at what time point in the injury calcium handling is responsible for the derangement of contractile function. The present study is the first to directly observe the influence of altered calcium handling activity on the contractile response of the cardiac muscle to OH*-induced injury. To test our hypothesis that deranged calcium handling (increased diastolic calcium levels and reduced calcium transient amplitude) plays a major role during acute/early OH*-induced injury and impair cardiac contractility, we measured the amplitude and kinetics of calibrated intracellular Ca\textsuperscript{2+} transients and twitch
contractions simultaneously in rabbit trabeculae after OH* exposure. After acute OH*-induced injury there is a marked increase in resting tension in parallel with an increase in diastolic calcium. We observed these parameters until the transient acute dysfunction subsided and diastolic and developed force had reached their new steady state (45 min after OH* exposure). At this new baseline, diastolic calcium returned to near-normal levels to pre-OH*radical levels whereas diastolic force declines, but not all the way to pre-OH*radical levels, indicating a sustained injury. Previous studies also indicate administration of calcium antagonists and inhibition of reverse NCX during the early reperfusion period can prevent the cardiac dysfunction and myocardial stunning (Ehring, Bohm et al. 1992; Ferrari, Curello et al. 1993; Massoudy, Becker et al. 1995; Wei, Zhou et al. 2007). This indicate that calcium overload is mainly if not entirely responsible for acute diastolic dysfunction after OH*-induced injury.

5.4.3 Myofilament Sensitivity in Acute OH*-Induced Injury

In parallel to understanding when calcium handling is deranged, it is important to know when myofilament function is involved in the phenotype of OH*-induced injury. Although it is clear that during IR injury there is an acute change in myofilament response, due to the metabolic acidosis and accumulation of different metabolites. However, the interaction between calcium overload and myofilament responsiveness at different time points is not clear. To test our hypothesis that altered myofilament responsiveness plays a major role in late/sustained myocardial dysfunction after OH*-induced injury, we observed an alteration in myofilament calcium sensitivity before and after OH*-induced injury. Using the potassium contraction technique to assess myofilament calcium sensitivity we
recently developed (Varian, Raman et al. 2006), we explained the observed disproportional increase in force for a given calcium concentration in the dynamic relaxation phase after the OH* as compared to the pre- OH* level. Thus, we concluded that a sensitization of the myofilaments is partially, or perhaps exclusively accounts for the increase in force during the sustained injury phase. This indicates that the acute injury that occurs after OH* exposure is mainly, if not entirely, due to calcium overload in acute phase while sustained myocardial dysfunction is mainly due to the altered/increased myofilament responsiveness.

5.4.4 Interaction Between Myofilament responsiveness and Deranged Calcium Handling

Although it is clear that despite the intracellular calcium overload, myofilament alterations are the prominent cause of contractile dysfunction by OH*, the possible interactions of these two mechanisms at different time points of IR injury remain unclear. During myocardial reperfusion, ROS are generated by xanthine oxidase (mainly from endothelial cells) and NADPH oxidase (mainly from neutrophils). In addition to the dysfunction of the sarcoplasmic reticulum and contributing to intracellular Ca\(^{2+}\) overload, there is an abrupt increase in intracellular Ca\(^{2+}\) damaging the cell membrane by lipid peroxidation, inducing enzyme denaturation, and causing direct oxidative damage to DNA. Previous studies have shown that there is a accumulation of Na\(^{+}\) in myocytes via the Na\(^{+}/\)H\(^{+}\) exchanger through intracellular acidosis in ischemia. During the initial phase of reperfusion injury, Na\(^{+}\) is extruded out of the myocyte by reverse-mode Na\(^{+}/\)Ca\(^{2+}\) exchange and leads to the calcium overload, especially since the Na\(^{+}/\)K-ATPase is impaired due to depletion of ATP. In the acute phase of injury, this calcium overload is mainly responsible for the cardiac dysfunction. In later injury phase, when the SR is again
supplied with ATP, Na⁺/K-ATPase will start working again and SR will remove all the excess Ca from the cytoplasm and calcium returns to near normal levels. This calcium overload in acute phase of reperfusion injury has been attributed to initiate a degradation of proteins like TnI and alpha actinin due to activation of calcium dependent proteases such as calpain (Di Lisa, De Tullio et al. 1995; Gao, Atar et al. 1997). This targeted protein degradation potentially alters the calcium sensitivity which we show is mainly responsible for the sustained myocardial dysfunction. This in agreement with a study that shows that the addition of the calpain inhibitor protects the isolated heart from IR injury when given during reperfusion (Atsma, Bastiaanse et al. 1995). In skinned fibres, addition of exogenous calpain causes a decrease in rigor tension, loss of alpha actinin (Reddy, Etlinger et al. 1975) and TnI degradation (Gao, Atar et al. 1997), similar to IR injury. Previous studies implicated different subunits of troponin as a site of damage after IR injury depends on the species. In a study of dog hearts, loss/degradation of TnI (Gao, Atar et al. 1997) was observed, while in a study with rabbits the degradation of TnT (Zeitz, Maass et al. 2002) was reported. The possibility that this increased myofilament responsiveness is due to direct effect of OH* can be ruled out by the half life of OH*. As the half life of OH* is too short and changes in myofilament responsiveness only started in later part of the injury. Further studies are needed to show what impact the alterations of myofilament proteins have on the contractile behavior over an extended period, and how these myofilament alterations are dependent on the species.

In conclusion, by tracing intracellular calcium while monitoring contractile function, we show that acute injury that occurs after OH* exposure is mainly if not entirely due to calcium overload in acute phase, and altered/increased myofilament response is
mainly responsible for the sustained/later phase of the OH*-induced injury. Unraveling the temporal resolution of these distinctly different mechanisms that depress contractile function in this study may help target novel treatment strategies for oxidative stress injury in the heart.
Figure 21A: Effect of 2 minute exposure of OH radicals on the contractile parameters of the RV trabeculae. (n=10). 21B: Percentage effect of OH radicals on the contractile parameters of the RV trabeculae (n=10). At peak of contracture (occurring at ~12 min after OH* exposure) there is significant increase in diastolic force and a decrease in developed force. New steady state was marked by significant elevated diastolic force and reduced developed force. Stimulation frequency was 2 Hz and temperature was 37º C throughout the experiment.
Figure 22 A: Time from peak tension to 50% relaxation (RT$_{50\%}$), B: Time from 50% relaxation to 90% relaxation (RT$_{90\%}$ - RT$_{50\%}$), before and after OH*-induced injury are significantly different. At baseline frequency of 2 Hz, RT$_{50\%}$ and (RT$_{90\%}$ - RT$_{50\%}$) are significantly longer after injury as compared to before frequency. * indicates a difference of P < 0.05 between conditions in the same group.
Figure 23: Force-frequency relationship before and after 2 min of OH-radical exposure on RV trabeculae (n=8). The new steady state was marked by the flat force-frequency relationship as compared to positive force-frequency relationship (p<0.05) before application of OH radicals.
Figure 24 A: Effect of allopurinol on 2 minute exposure of OH radicals on the contractile parameters of the RV trabeculæ. (n=7). 24B: Effect of allopurinol on force frequency relationship before and after 2 min of OH radicals exposure on RV trabeculæ (n=7). There was no difference in contractile parameters and force frequency relationship before and after OH* exposure with allopurinol.
Figure 25 A: Relationship between intracellular calcium and diastolic force after the exposure of OH* in RV rabbit trabeculae. Raw record of diastolic force and intracellular calcium before, during and after exposure to OH -radicals. 25B: Percentage effect of OH- radicals on the intracellular calcium and diastolic force (n=8). At peak of contracture (occurring at ~12 min after OH* exposure) there is a significant increase in diastolic force and a decrease in diastolic calcium. New steady state was marked by a significant elevated diastolic force but diastolic calcium returned to near pre-interventional values.
Figure 26 A: Assessment of myofilament responsiveness before and after the OH*-induced injury. Using the K⁺ contracture protocol, a steady-state force-[Ca²⁺] relationship was measured before and OH*-induced injury. This shows that the curve shifts up and left after the injury. B: Shows that EC50 decreased after OH*-induced injury. *Difference at $P < 0.05$ between conditions in the same group.
CHAPTER 6
DIFFERENTIAL EFFECTS OF OH* INJURY IN ATRIAL AND VENTRICULAR MYOCARDIUM

6.1 Introduction

Ischemic heart disease is one of the leading causes of death in developed countries. Timely and effective reperfusion is one of the treatment strategies for limiting the size of the infarct. However, despite optimal reperfusion therapy, there are many detrimental consequences of myocardial reperfusion. Reperfusion injury is a serious pathological process that is induced by the restoration of blood flow to previously ischemic tissue. When ischemic myocardium is reperfused, and oxygen reintroduced, there is a sudden burst of oxygen free radical production. Of these oxygen free radical, the OH* (OH*) is one of the most aggressive species, and is involved in the pathogenesis of IR injury (IR injury). IR injury is observed in many clinical situations including acute heart failure, stroke, and myocardial infarction (Gao, Liu et al. 1996; Zeitz, Maass et al. 2002). There is abundant information available regarding the ventricular myocardial response to OH*, but little is known about the contractile response of atrial myocardium to OH*-induced injury.

Previous studies by us and others have shown that intact contracting cardiac ventricular trabeculae from rat and rabbit after acute exposure of OH* develop a rigor like contracture marked by an increase in diastolic tension, myofilament proteolysis, and
overall decreased cardiac contractility (Zeitz, Maass et al. 2002; Hiranandani, Bupha-Intr et al. 2006).

Because it is well known that atrial tissue differ from ventricular tissue morphologically, electrophysiologically, and also in pharmacological responses, we conducted this study to compare the OH*-induced injury response between atrial and ventricular muscles. Previous studies have shown that there are differences in the small and large mammals in response to OH* (Zeitz, Maass et al. 2002). However, it is currently unknown if there are differences between large mammals in response to OH*-induced injury. As rabbit and dog are similar to human regarding EC coupling and myofilament composition, we compared the OH* response between RA and RV trabeculae both in rabbit and dog under identical experimental conditions.

Our results showed that like RV muscles, RA muscles also showed a contractile dysfunction caused by a transient exposure to OH*. Exposure of OH* in both dog and rabbit eventually leads to increase in diastolic tension and overall decreased cardiac contractility. At a new steady state contractility level that has stabilized roughly 45 minutes after the transient rigor-like contracture, we observed elevated diastolic force and reduced developed force. In addition to the decreased contractility after the OH* exposure, there is a decrease in frequency-dependent activation, often denoted as a hallmark of heart failure. Atrial muscles exhibited a less severe contractile dysfunction that reached its peak earlier, and recovered faster compared to ventricular muscles, and dog muscles showed a similar injury response than rabbit one, albeit the injury was slower to develop.
6.2 Materials and Methods

6.2.1 Preparation of Rabbit Trabeculae and Overall Protocol

New Zealand White Rabbits (2 kg, approx 3 months old) were anesthetized with 50 mg/kg pentobarbital sodium (IV) and injected with 5,000 units/kg heparin. The heart was rapidly removed and perfused retrogradely through the aorta with Krebs Henseleit solution containing (in mmol/L): 120 NaCl, 5 KCl, 2 MgSO₄, 1.2 NaH₂PO₄, 20 NaHCO₃, 0.25 Ca²⁺ and 10 glucose (pH 7.4) in equilibrium with 95%O₂/5%CO₂ at body temperature. Additionally, 20 mmol/L 2,3-butanedione monoxime (BDM) was added to the dissection solution to stop the heart from beating and to prevent damage during dissection (Mulieri, Hasenfuss et al. 1989; Mulieri, Hasenfuss et al. 1992; Janssen and Hunter 1995). The effects of BDM after brief exposure, have been found to be reversible (Mulieri, Hasenfuss et al. 1989; Janssen and Hunter 1995). Suitable RV and RA trabeculae were dissected carefully without touching the central part of the muscle and mounted in the bath and mount the muscle onto the experimental setup (ter Keurs, Rijnsburger et al. 1980; de Tombe and ter Keurs 1990; Janssen and de Tombe 1997; Layland and Kentish 1999; Janssen, Stull et al. 2002). We have adopted this technique because it allows for attachment of the muscle in the experimental chamber without damaging the muscle. Only thin muscles were used for the study, with a maximum diffusion distance of 100 µm to the core, so core hypoxia will be avoided and not affect the outcome of the results factor (Raman, Kelley et al. 2005). Using a dissection microscope, muscles were mounted in the chamber. The piece of tricuspid valve leaflet was connected to a hook like extension of micromanipulator and the cube of ventricular tissue rest in a platinum-iridium basket-shaped extension of the force transducer. This
method has been shown to minimize the end-damage compliance of the muscle and to prevent excessive loss of force throughout the experimental protocol (ter Keurs, Rijnsburger et al. 1980; de Tombe and ter Keurs 1990; Janssen, Zeitz et al. 1999; Janssen, Stull et al. 2002). The muscle was bathed in a continuous flow of oxygenated K-H solution (without the BDM). Then muscle was stimulated at 2 Hz at a temperature of 37°C, calcium concentration was raised to 1.5 mM in the KH solution and stretched until maximal active force is reached. This length is comparable to maximally attained length in-vivo at the end of diastole (around 2.2 µm sarcomere length)(Rodriguez, Hunter et al. 1992). After the stabilization of contractile parameters, we assessed the force-frequency relationship by increasing the frequency from 2 Hz to 5 Hz, then we exposed the muscle to OH* for 2 minutes. Twitch contractions were monitored until 45 min after OH* exposure. We repeated the force-frequency protocol at this time of the injury response.

6.2.2 Preparation of Dog Trabeculae

Myocardium from normal mongrel dogs (45 pounds approx, male/female; 2–3 years of age) was obtained in a collaborative study, where the heart was freshly harvested, and we obtained a portion of the RV free wall as well as the right atrium. From these tissues, suitable trabeculae were carefully dissected. We used the exact same procedure as we did for rabbit trabeculae except the baseline frequency was set to 1 Hz. Previous studies by us and others (Janssen, Zeitz et al. 1999; Zeitz, Maass et al. 2002; Hiranandani, Bupha-Intr et al. 2006) showed that rabbit and mouse trabeculae reached to a new steady state after 45 min of OH*-induced injury. In this study, as we are comparing rabbit and dog RA and RV trabeculae, we likewise measured the twitch contractions for
45 min after OH* exposure. After 45 min, we assessed the force frequency protocol again, by increasing the frequency from 1 Hz to 5 Hz, then we exposed the muscle to OH* for 2 minutes. Twitch contractions were monitored till 45 minutes after OH* exposure and thereafter repeated the force frequency protocol.

6.2.3 OH* Exposure

After the stabilization of contractile parameters, we exposed the muscle to OH* for 2 minutes. Contractions were monitored until diastolic and developed force reached their new steady state (typically 45 min). In this study OH* was produced via the Fenton reaction through H₂O₂ + Fe²⁺-NTA system (Zeitz, Maass et al. 2002):

\[ \text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + \text{HO}^- + \text{HO}^{*} \]

The reaction involves hydrogen peroxide and a ferrous iron as a catalyst. The hydrogen peroxide is broken down into a hydroxide ion and a hydroxyl free radical. The concentration of the H₂O₂ and Fe-NTA in the organ bath is 3.75 mM and 10\(\mu\)M respectively. The amount of OH* generated under these conditions is comparable to those that occur in IR injury (Zweier, Kuppusamy et al. 1989; Zeitz, Maass et al. 2002). H₂O₂ is infused via a separate line feed directly into the organ bath at the level of the muscle to ensure that radical formation took place right in the muscle and muscle bath where H₂O₂ meets Fe-NTA. Control studies, performed for this study verified that either Fe-NTA alone, or H₂O₂ alone in the concentrations used did not have any effect on contractile parameters, and thus the assessed effects are results of products of the Fenton
reaction specifically.

6.2.4 Data Analysis and Statistics

Data were collected and analyzed on- and off-line using custom-written software in LabView (National Instruments). Data are expressed as means ± SE unless otherwise stated. Data were statistically analyzed using ANOVA or Student's t-tests (paired or unpaired) where applicable. A two-tailed value of P < 0.05 was considered significant.

6.3 Results

After acute exposure of OH*, intact cardiac trabeculae from RA and RV from dog and rabbit heart develop a rigor like contracture marked by increase in diastolic and systolic tension and overall decreased contractility. This injury is initiate by OH* radicals, as exposure to either Fe-NTA alone, or H$_2$O$_2$ alone, did not exert any effect on contractility. Since the OH* radical is very short lived, the contractile effects are not a direct result of OH* exposure, but of the cascade of events that follows OH* exposure. In Figure 27 we show the contractile response of a muscle under baseline conditions followed by a 2 minute exposure with OH* radicals in the RA and RV cardiac trabeculae in rabbit and dog. We measured these contractile parameters for 45 min after OH* exposure. In rabbit trabeculae, after 45 min, contractile parameters came to steady state, but in dog trabeculae contractile parameters had not (yet) come to steady state. 45 minutes post OH* exposure was marked by an elevated diastolic tension, and depressed force development compared to the pre-interventional values in both rabbit and dog RA and RV trabeculae.
Figure 28 shows the effect of acute OH* exposure on diastolic and developed force. After the OH* there is an increase in diastolic force and decrease in developed force. The development of the contracture was much faster in atrial muscles compared to those isolated from the RV. The amplitude of the contractures were also different in RA and RV muscles. At the peak of contracture, atrial muscles show less contractile dysfunction as compared to ventricular muscles.

Figure 29 shows the data expressed as the percentage effect of OH* exposure on diastolic and developed force. In rabbit RV muscles diastolic force at the peak of contracture (at ~ 12 min after OH* exposure) was increased to 240±7% (p<0.05) and developed force was decreased to 23±3% (p<0.05) and partially recovered 125±8% (p<0.05) and 65±4% (p<0.05) respectively. In rabbit RA muscles at the peak of contracture (occurring at ~7 min post exposure) developed force was decreased to 19±2% (p<0.05) while diastolic force was increased to 217±20% (p<0.05), and thereafter recover at the 45 minute mark to 38±8% (p<0.05) and 141±11% (p<0.05) respectively.

In dog myocardium, the peak of OH*-induced injury contracture occurred much later than in rabbit muscles. In dog RV muscles, diastolic force at the peak of contracture (occurring ~ 25 min after OH* exposure) was increased to 202±33% (p<0.05) and developed force was decreased to 16±3% (p<0.05) and recovered to 188±40% (p<0.05) and 17±3% (p<0.05) respectively at the 45 minute mark. In dog RA muscles at the peak of contracture (at ~25 min), developed force was decreased to 50%±7 (p<0.05) while was diastolic force was increased to 159±27% (p<0.05), and recovered to 59±8% (p<0.05) and 149±23% (p<0.05) respectively after 45 min of 2 min OH* exposure. This shows that the sustained contractile dysfunction was much higher in RA muscles as compared to RV
muscles in rabbit and dog. Also the sustained dysfunction was much higher in dog trabeculae as compared to rabbit because dog trabeculae didn’t come to steady state after 45 min as the frequency of stimulation in dog muscles was half than the rabbit trabeculae.

The observation of elevated diastolic force and reduced developed force at the 45 minute mark was accompanied by a slower relaxation. Both RT50% and RT90% were significantly slower in RA and RV muscles in both rabbit and dog after OH*-induced injury compared to before injury. Compared to RT50%, RT90% was much slower in ventricular muscles as compared to atrial muscles possibly indicating a specific impairment of relaxation kinetics in later part of relaxation. There was no significant difference in time to peak and the first derivative of force (dF/dt) between atrial and ventricular muscles.

Figure 32 shows the force frequency relationship before and after OH*-induced injury in atrial and ventricular muscles. An increase in stimulation frequency from 2 to 5 HZ in rabbit and 1 to 4 Hz in dog cardiac trabeculae shows an increase in developed force before OH* but a flat or negative force frequency was viewed after the OH*-induced injury. As the deviation of force frequency is a characterization of SR function. This change in force frequency relationship from positive to flat/negative after OH* exposure shows an impairment of SR function, which is one of the hallmarks of congestive heart failure.

6.4 Discussion

The results of this study shows that direct acute exposure of OH* to clinically relevant levels of reperfusion injury leads to a transient rigor like contracture in both
atrial and ventricular myocardium. During the acute phase there is a marked increase in diastolic force in parallel with decrease in developed force. During the recovery phase, diastolic force declines and developed force increased somewhat, but these parameters do not nearly recover to the pre-OH* levels. Although this result is similar to what has been previously described in different species in ventricular muscles (Zeitz, Maass et al. 2002; Hiranandani, Bupha-Intr et al. 2006), we did also observed significant differences between atrial and ventricular tissue in their response to OH*-induced injury. In atrial muscles, the development of the injury contracture occurred much faster compared to the response in ventricular muscles. At the peak of contracture, atrial muscles show less contractile dysfunction as compared to ventricular muscles. Assessment of RT90% indicate that relaxation kinetics were prolonged after OH*-induced injury, as well as and much faster in atrial muscles compared to ventricular ones. This prolongation of the twitch force might be due to a calcium reabsorption delay by sarcoplasmic reticulum. As it is known that with the increasing concentration of OH* there is decrease in SERCA activity (Flesch, Maack et al. 1999). This decrease in SERCA activity leads to slower resequestration of calcium in SR resulting in slower relaxation after OH* injury. The depressed contractile function was also accompanied by a flat/negative force frequency response, one of the classic hallmarks of heart failure (Ferrari, Curello et al. 1993; Janssen, Zeitz et al. 1999; Zeitz, Maass et al. 2002), after the OH*-induced injury. These results indicate that both atrial and ventricular muscles develop a rigor like contracture after acute OH*-induced injury but in atrial muscles the contractile dysfunction is less severe in magnitude, and reached its maximum effect faster than in ventricular muscles.

It has long been known that the action potential in atrial muscles is much shorter
in comparison to ventricular muscles, along with faster rate of contraction and relaxation
(Korecky and Michael 1974; Asgrimsson, Johannsson et al. 1995). Instead of different
relaxation kinetics calcium sensitivity and tension kinetics were same in atrial and
ventricular muscles (Vannier, Veksler et al. 1996). These studies indicate that these
atrioventricular differences in relaxation kinetics is mainly due to the SR calcium
handling. It is also known that there is a decrease in SERCA activity with increasing
concentration of OH* (Flesch, Maack et al. 1999) as well as clear attenuation of force
frequency response after OH* (Schouten and ter Keurs 1986; Janssen, Zeitz et al. 1999)
exposure. These studies also indicate that direct impairment of SR function and calcium
overload plays a significant role in acute cardiac dysfunction due to reperfusion injury.

Atrial muscles also have a lower phospholamban to SERCA ratio which leads to
higher SR calcium uptake and faster resequestration of calcium in SR resulting in faster
contraction and relaxation in atria as compared to ventricle (Koss, Ponniah et al. 1995).
In accordance with these studies our data also showed that after acute OH* exposure at
clinically relevant levels atrial muscles develop a transient rigor like contracture similar
to ventricular muscles but this development of contracture and relaxation was much faster
in atrial muscles in dog and rabbit as compared to ventricular muscles.

We previously showed that SERCA over-expression can reduce the OH*-induced
contractile dysfunction in murine myocardium, whereas a reduced SERCA activity
aggravates this injury (Hiranandani, Bupha-Intr et al. 2006). As there is higher expression
of SERCA 2a in atria as compared to ventricular muscles (Minajeva, Kaasik et al. 1997),
at the peak of contracture atrial muscles show less contractile dysfunction as compared to
ventricular muscles.
In dog muscles the development of the injury contracture was much slower and sustained function was higher as compared to the response in rabbit muscles. As twitch contractions are mainly dependent on the baseline frequency and the baseline frequency for dog is nearly half as that of the rabbit. In this study, as we compared rabbit and dog RA and RV trabeculae, we measured the twitch contractions for 45 min after OH* exposure at 1Hz in dog and 2 Hz in rabbit. After 45 min, contractile parameters came to a new steady state in rabbit trabeculae, but in dog trabeculae contractile parameters had not (yet) come to steady state. This may be the possible reason for late peak of contracture and higher sustained dysfunction in dog after 45 min as compared to rabbit.

Previous studies showed that in absence of SR function positive force frequency relationship is maintained in large mammal (rabbit) as compared to small mammal (rat) (Monasky and Janssen 2009). In this study we looked at the force frequency response between rabbit and dog before and after OH* injury to find if decrease in SR function can maintain the positive force frequency response in large mammals. Our results showed that after OH* induced injury depressed contractile function was also accompanied by negative force frequency response in dog, but in rabbit positive response was maintained. This also could be due to baseline frequency we used for dog and rabbit.

In conclusion, we show that direct acute exposure of OH* to clinically relevant levels of reperfusion injury leads to transient rigor like contracture marked by an increase in diastolic contraction and overall decrease contractility with partial recovery in atrial and ventricular muscles. But this acute contractile dysfunction was much less in atrial muscles which reached to its peak and relaxed faster as compared to ventricular muscles. Higher SERCA 2a expression and lower phospholamban to SERCA ratio in atria which
leads to higher SR calcium uptake and faster resequestration of calcium in SR could be the main modulator of lower acute contractile dysfunction and faster and shorter contracture in atrial muscles as compared to ventricular muscles in rabbit and dog.
Figure 27 RA and RV muscles from rabbit and dog developed a rigor like contracture after the exposure of OH*. After the acute OH* exposure there is significant increase in diastolic force and systolic force. New steady state was marked by significant elevated diastolic and systolic force. Stimulation frequency was 2 Hz in rabbit and 1 Hz in dog and temperature was 37º C throughout the experiment.
Figure 28 Effect of 2 minute exposure of OH radicals on the contractile parameters of the cardiac trabeculae. At peak of contracture there is significant increase in diastolic force and a decrease in developed force. New steady state was marked by significant elevated diastolic force and reduced developed force. Stimulation frequency 2 Hz in rabbit and 1 Hz in dog and temperature was 37°C throughout the experiment.
Figure 29 Comparison of percentage effect of OH radicals on the contractile parameters of the RV and RA trabeculae in rabbit and dog. Stimulation frequency 2 Hz in rabbit and 1 Hz in dog and temperature was 37º C throughout the experiment.
Figure 30 Difference between the RA and RV trabeculae contracture after OH* exposure regarding its magnitude and timing in rabbit and dog.
Figure 31 Time from peak tension to 50% relaxation (RT$_{50\%}$) and Time from peak tension to 90% relaxation (RT$_{90\%}$), before and after OH*-induced injury are significantly different. At baseline frequency, RT$_{50\%}$ is significantly longer after injury as compared to before injury in RA and RV trabeculae in rabbit and dog. * indicates a difference of $P < 0.05$ between conditions in the same group.
Figure 32 Force Frequency relationships before and after 2 min of OH-radical exposure on cardiac trabeculae. New steady state was marked by the flat force-frequency relationship as compared to positive force-frequency relationship before application of OH radicals in both RA and RV trabeculae.
CHAPTER 7
DISCUSSION

7.1 Principle Findings

This section summarizes the most important findings from chapter 2 to chapter 6.

1. SERCA overexpression can reduce the OH*-induced contractile dysfunction in murine myocardium, whereas a reduced SERCA activity aggravates this injury in mice papillary muscles. Loss of pPLB levels at Ser16 and increase in pPLB at Thr17 likely amplifies the differences observed in injury response.

2. In rat right ventricular trabeculae, at a physiological normal stimulation rate of 6 Hz, developed force decreases at 42°C compared with 37°C. In addition, twitch timing characteristics also accelerate, allowing for a faster relaxation.

3. Compared with normal levels, increased SERCA activity in mice enhanced force development only at subphysiological frequencies, while a reduction in SERCA activity showed a depression of force at the higher frequency range. Thus, generalizations regarding the correlation between SERCA activity and contractility can be highly ambiguous because this relationship is critically dependent on other factors including stimulation frequency.

4. The acute injury that occurs after OH* exposure is mainly, if not entirely, due to calcium overload, while the later or sustained myocardial dysfunction is mainly due to the altered/increased myofilament responsiveness.
5. Both atrial and ventricular muscles develop a rigor like contracture after acute OH*-induced injury, but atrial muscles showed a lesser degree of contractile dysfunction, which both reached its maximum and relaxed faster than in ventricular muscles. The probable reason may be due to lower PLB to SERCA ratio in atria as compared to ventricles.

7.2 Invitro Model of OH* Induced Injury

In preparation for this study, first, I developed the invitro model of OH* injury in mice, rat, rabbit and dog in which we can directly expose the papillary muscle/trabeculae with OH*. Using this method, we successfully exposed the cardiac muscles with same amount of OH* as comparable to those that occur in ischemia-reperfusion injury (Zweier, Kuppusamy et al. 1989).

7.2.1 Fenton Reaction

We used the Fenton reaction to produce OH* in our experiments. This reaction involves hydrogen peroxide and a ferrous iron as a catalyst. The hydrogen peroxide is broken down into a hydroxide ion and a hydroxyl free radical. The concentration of the H$_2$O$_2$ and Fe-NTA in the organ bath was 3.75 mM and 10µM respectively. The amount of OH* generated under these conditions is comparable to those that occur in IR injury (Zweier, Kuppusamy et al. 1989; Zeitz, Maass et al. 2002). H$_2$O$_2$ was infused via a separate line feed directly into the organ bath at the level of the muscle to ensure that radical formation took place right on the muscle in the muscle bath where H$_2$O$_2$ meets Fe-NTA. Control studies performed for this study verified that neither Fe-NTA alone nor
H₂O₂ alone in the concentrations used had any effect on contractile parameters, and thus, the assessed effects are results of products of the Fenton reaction specifically.

7.3 Role of Deranged Calcium Handling in OH* Induced Injury

There is an accumulation of Na⁺ in myocytes via the Na⁺/H⁺ exchanger through intracellular acidosis in ischemia. During the initial phase of reperfusion injury, Na⁺ is extruded out of the myocyte by reverse-mode Na⁺/Ca²⁺ exchanger and leads to the calcium overload, especially since the Na⁺/K-ATPase is impaired due to depletion of ATP. In the acute phase of injury, this calcium overload is mainly responsible for the cardiac dysfunction. There are multiple mechanisms that are possibly responsible for calcium overload; sarcoplasmic reticulum damage, mitochondrial damage, and changes in properties of NCX and L-type channels have all been implicated to play a role. Although the relative contributions of these factors are still unknown, specifically, to what extent and how they relate to the sustained damage observed after OH* exposure, it is known that impaired SR function is one of the main pathways through which calcium overload is mediated.

7.3.1 Role of SERCA in OH* Induced Injury

To assess the role of deranged calcium handling in OH* induced injury, in chapter 2, we measured the OH* -induced contractile injury in a model of depressed and enhanced SR calcium handling. For our studies, we used mice papillary muscles as the use of mice allowed us to do experiments on transgenic animals. This study was the first to directly investigate the influence of altered SERCA activity on the contractile response of the
cardiac muscle to OH*-induced injury. Reduced SERCA activity contributes to both diastolic and systolic failure and is one of the main changes compared with normal myocardium that is responsible for the alteration in force-frequency behavior (Schouten and ter Keurs 1986; Janssen, Zeitz et al. 1999) and β-adrenergic response (Flesch, Maack et al. 1999). Thus, a similar OH*-induced injury would likely result in a greater amount of injury in myocardium with low SERCA activity. Our results indicate that modulation of SERCA activity can indeed alter the magnitude of effects of OH* on the heart; the OH*-induced injury is substantially less in transgenic mice with higher SERCA activity and aggravated in mice with a reduction in SERCA activity. Not only could the total absolute loss of function be attenuated by simply having more calcium pumps available, but preserved Ca^{2+} handling (e.g., preserved SERCA activity) could also potentially aid in removing excess Ca^{2+} that has entered the myocytes as a result of OH*-induced damages.

7.3.2 Role of PLB in OH* Induced Injury

In chapter 2, we showed that under control conditions, the phosphorylation of PLB plays a specific role on basal contractile function, and represents a compensatory mechanism via which the calcium handling is normalized in presence of altered SERCA activity induced by the transgene or knock-down. After OH* exposure, dephosphorylation of PLB at Ser16 contributes to the contractile dysfunction in WT mice, whereas TG mice are less susceptible to the OH*-induced dysfunction because they already have low PLB phosphorylation levels before OH* exposure, and thus, maintain their normal SR calcium uptake capacity. In sharp contrast, in HET mice, the high levels of PLB phosphorylation at Ser16 that were present at baseline are now lost after OH*
exposure, resulting in unmasking the reduced SR calcium uptake capacity, and thereby aggravating the injury response to OH*.

7.4 Altered Myofilament Function in OH* Induced Injury

At present, it is important to determine to what extent calcium overload and impaired calcium responsiveness play a major role in the genesis of myofilament lesion responsible for stunning the mechanism that produces such lesions, and how contractile dysfunction is associated to impaired calcium responsiveness. Elevated [Ca$^{2+}$]$_i$ during ischemia and during the initial reperfusion period have long-lasting after effects by activating Ca$^{2+}$-dependent proteases (Kusuoka and Marban 1992) and cross-linking between troponins and other cardiac proteins (Gorza, Menabo et al. 1996). Unraveling the precise nature of myofilament dysfunction at various stages throughout the injury window will gain crucial insight in how to target hypothesis-driven novel treatment strategies for oxidative stress injury in the heart.

7.4.1 Role of Myofilament Sensitivity in OH* Induced Injury

Although the concentration of cytosolic calcium increases after the ischemia, there is no evidence of impairment in myofilament calcium responsiveness (Miller, McDonald et al. 1996; Van Eyk, Powers et al. 1998) or the proteolytic degradation of the contractile protein machinery (Gao, Atar et al. 1997) during the ischemic phase. Thus, reperfusion appears to be necessary for calcium overload to induce the mechanical abnormalities responsible for stunning. This may be due to the fact that the effects of calcium overload are prevented by the acidosis associated with ischemia, whereby the increased concentration of protons effectively competes with calcium for intracellular
Chapter 5 showed that direct acute exposure of OH* resulted in an increase in diastolic calcium in parallel to the increase in diastolic force. But after 45 min, diastolic calcium levels returned to near-normal levels, whereas diastolic force development remained significantly elevated. This elevated tension in the later part of the injury was accompanied by an increase in the sensitivity of the myofilament for calcium. Thus, we concluded that a sensitization of the myofilaments is partially, or perhaps exclusively accountable for the increase in force during the sustained injury phase. This calcium overload in the acute phase of reperfusion injury has been attributed to initiate a degradation of proteins like TnI and alpha actinin due to the activation of calcium dependent proteases such as calpain. Calpains are enzymes that cleave other proteins when cell calcium is elevated. They are widely distributed in cells from many tissues, including the myocardium (Mellgren 1980). This targetted protein degradation potentially alters the calcium sensitivity which we show is mainly responsible for the sustained myocardial dysfunction.

7.4.2 Role of Myofilament Alterations in OH* Induced Injury

Although the factors responsible for this change in myofilament Ca\(^{2+}\) responsiveness in stunned myocardium are not yet clear. There may be structural injury to one or more contractile proteins due to elevated [Ca\(^{2+}\)], during ischemia and during the initial reperfusion period that may have long-lasting after effects by activating Ca\(^{2+}\)-dependent proteases, which could then partially degrade the contractile proteins. Previous studies showed that OH* exposure can lead to the proteolysis of different sub-units of
TnI in different species (Zeitz, Maass et al. 2002). In rabbit myocardium, hydroxyl exposure leads to a degradation of TnT associated with increased calcium sensitivity, while in rat, TnI was degraded with intact TnT and unaltered calcium sensitivity (Zeitz, Maass et al. 2002). On the other hand in humans, degradation of TnI has been observed in myocardium of bypass patients due to IR injury with intact TnT (McDonough, Labugger et al. 2001). Despite a similar intracellular calcium overload, specific myofilament alterations or lesions may be one of the primary causes of contractile dysfunction that sustains beyond the rigor-like initial insult after OH* exposure. Because of the differences between the species regarding myofilament degradation in response to OH*, a further analysis is required to assess the role of myofilament-specific proteolysis in OH* induced cardiac dysfunction as well as the exact extent of these myofilament alterations.

7.4.3 Interaction between Deranged Calcium Handling and Altered Myofilament Function

Although it is clear that, despite the intracellular calcium overload, myofilament alterations are the prominent cause of contractile dysfunction by OH*, the possible interactions of these two mechanisms at different time points of IR injury remains unclear. There is an accumulation of Na⁺ in myocytes via the Na⁺/H⁺ exchanger through intracellular acidosis in ischemia. During the initial phase of reperfusion injury, this Na⁺ is extruded out of the myocyte by reverse-mode Na⁺/Ca²⁺ exchanger and leads to the calcium overload, especially since the Na⁺/K-ATPase is impaired due to the depletion of ATP. In the acute phase of injury, this calcium overload is mainly responsible for the cardiac
dysfunction as there is no evidence of impairment in myofilament calcium responsiveness (Miller, McDonald et al. 1996; Van Eyk, Powers et al. 1998) or the proteolytic degradation of the contractile protein machinery (Gao, Atar et al. 1997) during the ischemic phase. In later injury phase, when the SR is again supplied with ATP, Na⁺/K-ATPase will start working again, and the SR will remove all the excess calcium from the cytoplasm allowing calcium to return to near normal levels.

This calcium overload in the acute phase of reperfusion injury has been attributed to initiate a degradation of proteins like TnI and alpha actinin due to the activation of calcium dependent proteases such as calpain (Di Lisa, De Tullio et al. 1995; Gao, Atar et al. 1997). This attribution is in agreement with a study that shows that the addition of the calpain inhibitor protects the isolated heart from IR injury when given during reperfusion (Atsma, Bastiaanse et al. 1995). In skinned fibers, the addition of exogenous calpain causes a decrease in rigor tension, loss of alpha actinin (Reddy, Etlinger et al. 1975) and TnI degradation (Gao, Atar et al. 1997), which is similar to IR injury. Previous studies implicated that, different subunits of troponin as a site of damage after IR injury depends on the species. In a study of dog hearts, loss/degradation of TnI (Gao, Atar et al. 1997) was observed, while in a study with rabbits, the degradation of TnT (Zeitz, Maass et al. 2002) was reported. The possibility that this increased myofilament responsiveness is due to the direct effect of OH* can be ruled out by the half life of OH*. As the half life of OH* is too short and changes in myofilament responsiveness only started in the later part of the injury. Further studies are needed to show what impact the alterations of myofilament proteins have on the contractile behavior over an extended period and how these myofilament alterations are dependent on the species.
In chapter 5, by tracing intracellular calcium while monitoring contractile function, we show that acute injury that occurs after OH* exposure is mainly, if not entirely, due to calcium overload in the acute phase, and altered/increased myofilament response is mainly responsible for the sustained/later phase of the OH*-induced injury. Unraveling the relative contribution of these distinctly different mechanisms that depress contractile function in this study may help target novel treatment strategies for oxidative stress injury in the heart.

### 7.5 Frequency Dependency of SERCA Level on Contractility

The SERCA pump actively re-sequesters calcium ions from the cytoplasm. The total activity of all SERCA pumps is a strong determinant of myocardial contractility. In end-stage heart failure, the total activity of the SERCA pumps is decreased via the loss of a number of pumps, a loss of activity of the individual pumps, or a combination thereof. In chapter 3, we investigated the frequency-dependent activation in isolated cardiac tissue with normal, increased, and reduced SERCA activity levels. According to our hypothesis, increased SERCA levels promote increased force, and because the maximum force generating capacity is limited by the myofilaments, the increase in SERCA level at low frequency may increase calcium handling and contractile force, resulting in a reduced capacity to increase further or possibly resulting in such a high baseline force that no increase was observed at higher frequencies.

Our data indicate that in normal WT mice, the enhanced stimulation of SERCA appears sufficient to elicit a maximal contractile response at high frequency but fails to enhance the contractile response in SERCA 1a trabeculae at the highest frequencies. This indicates that at these conditions, a normal level of SERCA expression is not limiting
force development. At sub-physiological frequencies, SERCA1a over expressed trabeculae possessed a higher basal contractility, and a faster relaxation rate compared to WT. This likely indicates that at lower frequencies, due to higher SERCA activity in the SERCA 1a trabeculae, there is increased reuptake of Ca\(^{2+}\) into the SR thereby decreasing the TTP and relaxation time. At higher frequencies, there is little or no room for an increase compared to wild type, because at these higher frequencies the wild type mice SERCA activity is enhanced per se. Small increases in SERCA expression may thus improve cardiac function, but too much SERCA activity may lead to loss of contractile reserve and could cause negative effects on cardiac contractility (Teucher, Prestle et al. 2004).

7.6 Body Temperature and Cardiac Contractility

The body is from time to time exposed to non-normothermic conditions; both hypo- and hyperthermia. Although cardiac output regulation under hyperthermic conditions has been studied, the mechanical response of basic contractile function of the myocardium itself is incompletely understood. During hyperthermia (or heat-stress), it is long known that heart rate increases, and sympathetic responses are upregulated. Faster heart rates, at least in healthy subjects, result in an increase in myocardial force production and faster kinetics, which allows the heart to maintain a fast pacing rate without immediate diastolic fusion of successive contractions that can potentially cause a diastolic dysfunction. However, it remains unknown whether and how basic contractile performance of myocardial tissue is affected by hyperthermic conditions. In chapter 4, we
investigated the influence of hyperthermia on the regulation of basic myocardial contraction, including at high heart rates.

We observed that in isolated myocardium subjected to 42 °C, not only the peak force development decreased but there was also an increase in contraction kinetics allowing for faster relaxation. As a result, at higher frequencies, the isolated myocardium can reach higher rates without diastolic dysfunction. The accelerated twitch kinetics that were observed at a higher temperature allowed the already positive force-frequency to be extended to higher ranges, resulting in an increased development of force even when frequencies well exceeded the rates usually observed under normothermic conditions. Thus, we were able to produce adequate force at the higher heart rates that generally prevail during hyperthermia.

7.7 OH* Injury in Atrial and Ventricular Myocardium

Despite the widespread use of ventricular tissue in the investigation involving cardiac reperfusion injury, little is known about the impact on atrial myocardium. In chapter 6, we compared the OH* (OH*)-induced injury response between RA and RV muscles from both rabbits and dogs. Although the injury response showed similarities between atrial and ventricular myocardium, there were significant differences as well. Our results in chapter 6 indicate that both atrial and ventricular muscles develop a rigor like contracture after acute OH*-induced injury, but atrial muscles showed a lesser degree of contractile dysfunction by both reaching its maximum and relaxing faster than in ventricular muscles. Comparison of dog versus rabbit tissue showed that the response was similar in magnitude, but slower to develop in canine tissue. The possible reason for
this is baseline frequency because baseline frequency we used for dog is nearly half as that of the rabbit.

**7.8 Future Directions and Final reflections**

The data presented in this document raises several very important questions that need to be answered in future projects. There are still discrepancies in the literature as to the precise nature of myofilament dysfunction which may be due to proteolytic injury to the contractile proteins and/or to oxyradical-mediated covalent modifications. Most of this work only addresses the role of calcium handling and myofilament responsiveness at different time points during the reperfusion injury. This project provides interesting insight about the extent to which calcium overload and impaired calcium responsiveness play a major role in the genesis of myofilament lesion responsible for stunning. We know that in the acute phase of the reperfusion injury, calcium overload is mainly responsible for myocardial dysfunction, but sustained myocardial dysfunction is mainly due to change in myofilament sensitivity.

In the near future this project must be expanded. Future experiments should be done to assess the role of myofilament-specific proteolysis in OH* induced cardiac dysfunction as well as the exact extent of these myofilament alterations. The muscles have already been freezeed at various timepoints after OH* injury. Western blots and 2D proteomics need to be performed on these muscles to reveal information regarding protein alterations like phosphorylation, isoform changes and posttranslational modifications and to gain insight into how the alterations of myofilament proteins impact on the contractile behavior over an extended period and how these myofilament
alterations are dependent on the species.

There are multiple mechanisms that are possibly responsible for calcium overload; sarcoplasmic reticulum damage, mitochondrial damage, and changes in properties of NCX and L-type channels have all been implicated to play a role. The relative contributions of these factors are still unknown, specifically to what extent and how they relate to the sustained damage observed after OH* exposure.

Our lab has successfully developed a technique of culturing isolated trabeculae for several days without a significant drop in contractile force showing that prolonged study of these isolated trabeculae is not complicated by an excessive loss of function over time. By culturing these multicellular preparations isolated from the heart for 3-5 days, we can follow the changes in the contractile parameters over an extended period after OH* exposure in cardiac preparations. In addition to the contractile parameters, we can freeze the muscle after culturing for 3-5 days and measure the protein composition of trabeculae. Through this, we can correlate the contractile function with changes in protein expression over time.

I believe this project provides interesting insights about the role of deranged calcium handling and myofilament responsiveness at different time points during the reperfusion injury along with some interesting things about the differences between the effect of reperfusion injury on atrial and ventricular muscles. For me, it has raised many interesting questions that I will hopefully have the opportunity to address later in my career.
LIST OF REFERENCES


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