Catecholamine Augmented RyR1 Ca^{2+} Release In Malignant Hyperthermia Sensitive Human B-Lymphocytes

A Dissertation

By

Lt. Colonel Susan M. Browder Perry, PhD-c,

MSN, USAF, NC, CRNA

Doctoral Candidate

Graduate School of Nursing

Uniformed Services University

Bethesda, Maryland

Committee Members:

Dr. Christine Kasper, PhD, Chair

Dr. Sheila Muldoon, MD

Dr. Rolf Bunger, PhD, MD

Dr. Michaela Shafer, PhD, RN

Distribution Statement

Distribution A: Public Release.

Dedication: To Anthony

In the past three years I have felt like a kite, sometimes enjoying the wind of discovery marveling at all I could find and sometimes afraid I would be caught up in the ebbs and flows of the wind currents and blown away. You, always on the ground, have kept just the right amount of tension on the string, enough slack to allow me to soar… enough tension to keep me safe.

Susan

ACKNOWLEDGEMENTS

First I would like to thank Dr. Christine Kasper, the Chair of my Dissertation Committee, for her unwavering support during this journey of discovery. Dr. Kasper was not only enthusiastic and supportive but demonstrated the freedom of imagination and engendered a joy of scientific discovery. I can never thank her enough for her patience and willingness to share her knowledge

Next, I would like to thank Dr. Rolf Bunger and Dr. Michaela Shafer. Dr. Bunger's experience with catecholamine and cardiac research and his extensive knowledge of physiology and statistical analysis were invaluable. I could not have completed this without his "word-smithing" and his willingness to identify steps I needed to take in my methodology in order to prove my principle. Dr. Shafer provided constant support and mentoring on the place of Nursing throughout this research. Her energy and joy of discovery sustained me on days when I was not sure I could do this.

The fact that I could even use the methodology for this research is a tribute to the effort of Dr. Bayarsaikhan Munkhuu, who worked with me on mastering the measurement of RyR1 calcium emissions and Dr. Andrei Blokhin, who tirelessly worked with me to explain Quantum Physics, laboratory methods and reagent re-constitution. I also thank Dr. Saiid Bina and Dr. Sambuughin for sharing their MH research experience with me.

My husband Anthony Perry is the unsung hero in this song of discovery. Providing me with all I needed and never once complaining about the long hours and times I was not there to participate in family life. He deserves all the love I can give for the rest of my life.

Finally, I would like to acknowledge Dr. Sheila Muldoon, a world-renowned researcher in the area of Malignant Hyperthermia (MH). While undoubtedly a woman of science, dedicated to research, Dr. Muldoon is also a clinical anesthesiologist full of grace and empathy. She always related the research to the patients who suffer from the effects of an MH episode, especially children. I believe she has the kindest nature and is perhaps the most humble and unaffected hero I will ever meet. With an Irish twinkle in her eye and a delightful enthusiasm for life and discovery, she was patient, kind, and supportive, while at the same time exacting precise and controlled science from her "graduate student". She has blessed so many in so many ways. If nothing else, working under her program of research has enhanced my life.

And, thank you God, creator and sustainer off all things. I hope I found what you wanted.

Susan

ABSTRACT

Purpose: To examine the possibility that activation of the stress response in Malignant Hyperthermia susceptible (MHS) individuals results in an adrenergic augmentation of intracellular Ca^{2+} release in a way that is different from patients that are not MHS and that this augmentation is involved in the triggering of Malignant Hyperthermia. **Background:** Malignant Hyperthermia (MH) is a threat that is ubiquitous to all anesthesia providers and remains an unpredictable intraoperative crisis that may result in patient death and significant morbidity for those that survive. The signs and symptoms are often insidious. MHS individuals may also experience this syndrome as a result intraoperative administration of inhalational agents or non-depolarizing muscle relaxants and also as a result of heat or emotional stress. If we can establish a link between abnormalities in the effect of catecholamines in MHS individuals, and if this link is demonstrated to be measurable by testing B-cells this could be added to the MH diagnostic criteria, perhaps saving lives and preventing disability.

Methods: Experimental model using Fura 2-am stained Epstein Barr Virus (EBV) immortalized Human B-lymphocytes from known MHS subjects as the experimental group and known MHN cells for comparison. Cell lines acted as their own controls. Cells were exposed to 1, 1.5 and 2mM doses of the Ryanodine (RyR1) agonist 4-CmC and 1uM norepinephrine to measure the effect on Fura Ca^{2+} emission ratios. There were three measurements used for this analysis, the baseline area (BA), the area under the emissions curve (AUC) and the peak emissions ratios (PE).

Data Analysis: A Gaussx Statistical program was used to analyze PE, AUC and BA emission ratio responses to 1 ⌠M, 1.5 ⌠M and 2.0 ⌠M doses of the RyR1 agonist 4 CmC and 1⌠M norepinephrine. Statistically significance ($P <. 05$) was assessed using ANOVA and paired t-test to look for difference between and within groups and multivariate regression analysis to examine model validity. **Results:** Differences between MHS and MHN AUC and PE ratios were significant (F=1.78; P < .03; df =112; F=1.94; P < 0.02; df =112 respectively). There was a statistical difference in all doses between the groups when BA, PE and AUC were analyzed (T=4.55, P < .01, df=54). At 1.0 ⌠M, 1.5 ⌠M and 2.0 ⌠M 4 CmC there was significant statistical difference between the MHS and MHN response (1⌠M: T=2.33, P < .04, df = 22; 1.5 ⌠M: T=2.24, P < .04; df= 17; 2.0 mM: T= 13.02, P < .01, df= 8). Regression analysis was significant with $R^2 = 0.69$; Durbin Watson statistic of 1.14 and a Heteroscedasticity of 0.023 for the three independent variables (Dose of 4 CmC: t = 3.63, P < .01; BA Ca^{2+} response t= 2.83; P < .02; and Norepinephrine: t=5.93, P < .01).

Conclusions: EBV immortalized Human B-lymphocytes display a statistically significant increased sensitivity to the catecholamine norepinephrine when compared to those cells from MHN individuals as measured by the increased fura 2 Ca^{2+} emission ratios in response to the known RyR1 agonist 4-CmC. This may indicate an abnormality in the adrenergic response to stress and lead to improved diagnostic and treatment for individuals with Malignant Hyperthermia Susceptibilty.

Keywords: Malignant Hyperthermia, Human B- lymphocytes, 4 CmC, Stress Response, Norepinephrine, Adrenergic Response

Table of Contents Page

Appendix A:

List of Figures and Tables

Page

List of Figures and Tables

Page

Tables

Chapter 1

Introduction

Malignant Hyperthermia (MH) is a threat that is ubiquitous to all anesthesia providers and remains an unpredictable intraoperative crisis that may result in patient death and significant morbidity for those that survive. In humans MH is an autosomal inherited disorder that predisposes to a life threatening hypermetabolic syndrome. In susceptible individuals the commonly used inhalational anesthetics and the depolarizing muscle relaxant succinylcholine may induce an accelerated metabolism that leads to a potentially fatal syndrome (Nelson & Flewellen, 1983). Figures place the incidence of intraoperative triggering between 1:15,000 in the pediatric population and 1:50,000 for adults undergoing general anesthesia (Nelson and Flewellen, 1983). However, this estimate is possibly understated, as it would only reflect reported cases of MH and only those displayed in a more fulminate manner.

A fulminate episode is characterized by tachypnea, hypercarbia, tachycardia, muscle rigidity, rhabdomyolysis, metabolic acidosis, hyperkalemia and hyperthermia. However, these are non-specific signs with no single feature being specifically diagnostic (Muldoon, et al. 2004). Further complicating the diagnosis is pharmacogenetic triggers may be administered to the susceptible individuals without causing an MH episode. Without the definitive treatment of Dantrolene Sodium and discontinuation of all triggering agents, mortality is in excess of 70% (Muldoon et al. 2004).

While most MH susceptible individuals are asymptomatic prior to triggering, others have a history of variable skeletal muscle abnormalities with documented basal increases in creatine kinase (Sambuughin et al.2001). This variation in clinical phenotype is hypothesized to be the consequence of unresolved genetic and environmental related factors (Muldoon et al. 2004). Identification prior to surgery is difficult and usually only possible if a family history of the disease is known, or the patient reports a prior suspicious metabolic response to general anesthetics. Currently the only definitive diagnosis of MHS requires a surgical biopsy of the vastus lateralis to measure the muscles response to Halothane and caffeine via the Caffeine Halothane Contracture Test (CHCT) (Larach, 1989). The CHCT has a diagnostic sensitivity of 97% and a specificity of 78% (Allen et al., 1998).

Genetic studies have demonstrated that the RyR1 gene, located on chromosome 19q13.1 is the primary locus responsible for MH susceptibility (McLennan et al., 1990). In skeletal muscle the RyR1 receptors function in the sarcoplasmic reticulum to trigger myofibril contraction by elevating the myoplasmic Ca^{2+} levels (Mackrill, 1998). Mutations in the RyR1 linked to MH cause the channel to respond with increased sensitivity to activators such as 4-chloro-m-cresol (4-CmC) and caffeine (Hamilton and Serysheva, 2009). In response to these activators, the RyR1 channel is unable to control intracellular levels of Ca^{2+}. Over 50% of MHS genetic linkages are estimated to be associated with the RyR1 gene. However, with more than 300 different mis-sense RyR1 mutations found in patients with positive CHCT, or presumptive clinical diagnoses for MH, genetic testing lacks the sensitivity and specificity to be used as a screening tool for this disease (Vukcevic et al. 2010; Yang et al. 2003).

Early research in MH focused on the adrenergic nervous system (ANS) as a primary trigger of the syndrome. This hypothesis was based on the initial signs and symptoms of MH such as tachycardia, cardiac arrhythmias, hypertension and signs of increased metabolism, in patients triggering to MH. These signs are not related to the RyR1 receptor in skeletal muscle, but are associated with the adrenergic receptor response. Supporting these early links between MH and the ANS were case reports from anesthesia providers who reported that patients who subsequently developed MH after a non-triggering previous anesthetic, had appeared unusually stressed prior to the surgical procedure in which they triggered. These finding all point to emotional or physiological stress, and the related ANS response to stress, as a potential explanation of the variability in MH triggering in the surgical population.

The Malignant Hyperthermia Center USU is the only center for the Armed Forces Malignant Hyperthermia Program**,** and one of only four MH diagnostic centers in the United States. Every year military patients with a family history or intra-operative event are referred to USU for a definitive diagnosis and or clinical and fitness for duty recommendations. This number has averaged 10-20 a year (Muldoon, 2004). Currently there is no diagnostic test that does not depend on a previous history of an MH episode in the individual, or an invasive biopsy based on family history of MH. In addition, case and exercise studies indicate an increased possibility of triggering an MH episode under conditions of increased heat and exercise load.

A false negative result can prove fatal to the patient during subsequent general anesthetics; therefore a high sensitivity for MH testing is required. On the other hand, **a** false positive test will have serious implications, potentially terminating the service member's career and depriving the military of that the individuals unique skills and training. Therefore the low specificity of the current CHCT can result in an unacceptably inflated number of patients being falsely or ambiguously labeled as MHS. Current Department of Defense (DoD) policy states that MH is a cause for rejection for appointment, enlistment or induction in the military service.

If identified as MHS after entry into the military, the service member is considered not worldwide qualified and may be determined to be non-deployable (Muldoon, 2004). Forward operating bases and special operations medical teams are not capable of providing effective treatment for troops who initiate a MH crisis as a result of anesthetic agents or heat stress. The need for treatment materials such as sufficient stock of Dantrolene Sodium, sterile water needed to mix the drug, cooling capability such as ice and chilled fluids may not be available. As advance practice nurses and anesthesia providers we may be precluded from providing effective diagnosis or treatment of this disorder in the forward field locations. The current environments under which our military troops are deployed place any undiagnosed MHS soldier at increased risk for death or disability.

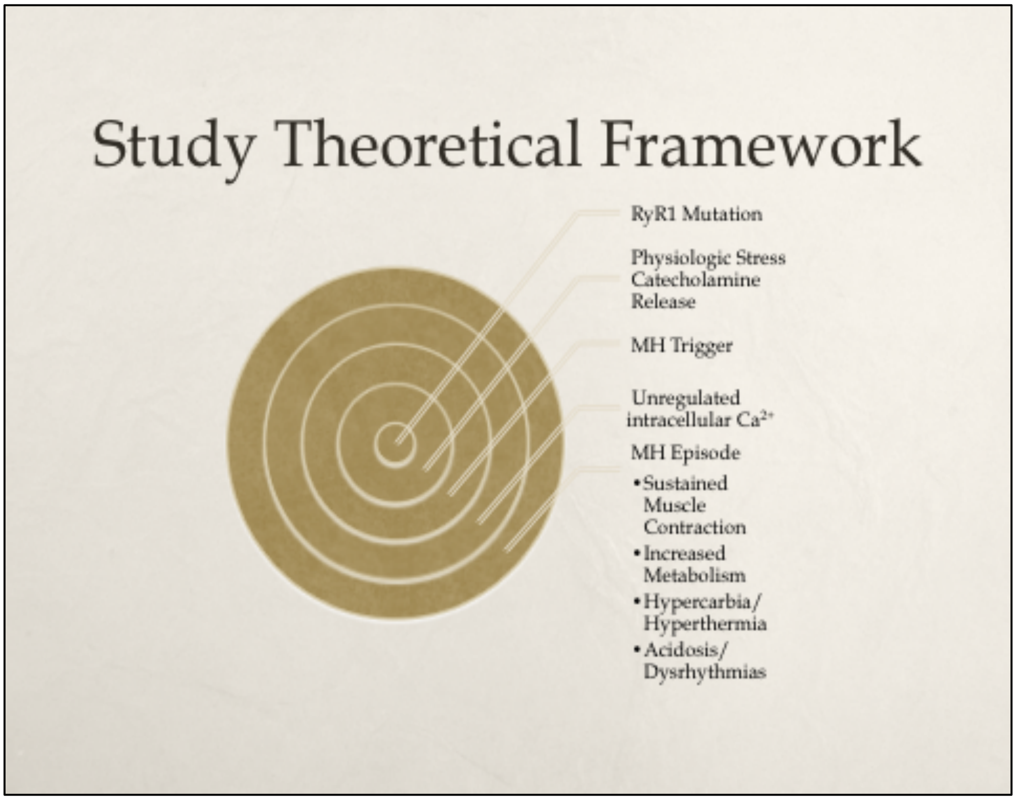
If we can establish a link between abnormalities in receptor sensitivity for, or release, re-uptake, or metabolism of catecholamines in MHS patient we may be able to use these as markers of the disease. The first step in this process is to establish a link between the ANS and Ca^{2+} release from the RyR1 receptor. The theoretical framework displaying this hypothesis is shown in Figure 1 below.

Purpose:

The purpose of this investigation is to examine the possibility that an abnormal sensitivity or response to the stress response in MH susceptible individuals results in an adrenergic augmentation of intra-cellular Ca^{2+} release from the RyR1 receptor in a way that is different from patients that are not MHS and that this augmentation is involved in the triggering of MH.

Hypothesis:

žImmortalized Human B-lymphocytes derived from a known MHS population, will display increased sensitivity to catecholamines, as evidenced by an increase in myoplasmic RyR1 Ca^{2+} accumulation, when compared to MHN controls.



Study Theoretical Framework

RyR1 Mutation

Physiologic Stress Catecholamine Release

MH Trigger

Unregulated intracellular Ca^{2+}

MH Episode

- Sustained Muscle Contraction
- Increased Metabolism
- Hypercarbia/ Hyperthermia
- Acidosis/ Dysrhythmias

Figure 1: Outline of Theoretical Framework for Hypothesis
Note: At the core triggering of an MHS individual to a fulminant MH episode lies the RyR1 genetic mutation. In the face of physiologic stress there is an increased release of catecholamines in response to the ANS activation of the stress response. When combined with a known MH trigger such as inhalational anesthetics, exertional or heat stress, the MHS individual experiences an MH episode which results in uncontrolled release of $Ca^{2=}$ from the intracellular stores of the sarcoplasmic reticulum. This results in sustained skeletal muscle contraction, increased metabolism, increased lactic acidosis, hypercarbia, hyperthermia, acidosis, rhabdomyolysis, dysrhythmias, DIC and death unless treated immediately by the discontinuation of triggering events and the treatment with Dantrolene Sodium.

CHAPTER 2

REVIEW OF LITERATURE

Literature Review: SNS Role in Porcine MH

Much of the early research involving the role of stress and the involvement of the autonomic nervous system in triggering of MH was done using the porcine model. This was possible because MH susceptible swine are homozygous for the genetic mutation in the RyR1 gene and can develop fulminant MH in response not only to general anesthetics and succinlycholine but also to environmental heat and stress (Porcine Stress Syndrome). A comparison of Porcine and Human MHS characteristics are shown in Figure 2.

In the porcine model Williams and colleagues developed a hypothesis implicating norepinephrine (NE) as a major factor in MH both in the basic abnormality and as a trigger. They postulated that the MH swine has a continually increased metabolism as a result of excessive NE effects upon an abnormal MH affected muscle. According to this hypothesis the MH pig constantly releases or controls heat production and metabolism by its own homeostatic mechanisms. However, when some factor such as general anesthetics, hot environment, fighting, coitus or stresses occur, the animal cannot control the additional heat production and acute fulminant MH occurs (Williams, 1988).

In the 1970 and 1980s researchers used the swine to measure differences in catecholamine levels in MHS vs MHN pigs in an attempt to elucidate the sequence of adrenergic involvement during a MH episode. These different research modalities are presented below.

Swine: Difference in Plasma Catecholamine Levels in MHS vs MHN Pigs

In 1976 and 1977 the research group of Lucke, Hall and Lister published several studies on porcine MH. In their first study, the MH susceptible swine were triggered with two doses of succinylcholine and halothane. The authors reported a significant increase in catecholamines in the MHS pigs after triggering. Control levels of catecholamines at baseline were measured as 1.6 ug/L and increased to 7.4 ug/L after the first dose of succinylcholine. Plasma catecholamines continued to increase and, in MHS swine, were measured at 44.6ug/L before death. The authors also reported a highly significant correlation between the log of the total plasma catecholamine and lactate levels (Lucke, et. al, 1976).

That same year Gronert et al. examined the effect of the SNS on MHS swine. They found that prior to introduction of the triggering agents, there was no significant difference in catecholamine levels between the groups prior to the introduction of the 1% Halothane (triggering agent). However, within 30 minutes of the Halothane administration, the MHS swine began to exhibit significantly higher levels of epinephrine and norepinephrine when compared with MHN controls. Within 60 minutes the differences in catecholamine levels in the MHS swine reflected a mean of 21.27 ng/ml +/- 6.45 for epinephrine in MHS swine vs 0.33 ng/ml +/- .03 in the MHN controls and norepinephrine levels were measured as 19.87 ng/ml +/- 7.10 in the MHS vs 0.26 ng/ml /-.07 in the MHN swine (Gronert et al., 1976).

In 1982 the research group of Davis, et al exposed MHS and MHN pigs to Halothane anesthesia and compared differences in catecholamine level using high performance liquid chromatography (HPLC). They found no increase in catecholamine response to Halothane in the MHN pigs (0.5ng/ml); however the MHS swine all exhibited increased levels of NE, beginning at approximately 30 minutes after the introduction of the triggering agent. Norepinephrine levels rose from a baseline of 0.5 ng/ml to a high of 14.9 ng/ml at 45 minute. The authors reported the findings the increase in MHS swine as high as "eight fold during times of intense peripheral vasoconstriction and body temperature rise" (Davis et. al. 1982)

Swine: Blocking of ANS Response

If in fact there were a disordered regulation of catecholamines in MHS swine, the next questions would be, could we block the resulting MH if the ANS or adrenal release of catecholamines is blocked. Research on this question is presented in this section.

In 1975 Kerr, et al. presented the results of a study using MHW swine. The MH susceptibility of the pigs was established by subjecting the swine to a Halothane challenge. If the pig triggered to an MH episode, they were labeled MHS, and allowed to recover before the next experimental phase was conducted.

Following the recovery period, the local anesthetic drug 2% lidocaine was used to establish an extradural block in the swine. The purpose of the epidural anesthetic was to block the sympathetic outflow from adrenal and post-ganglionic nerve terminals in the swine.

It was reported that the MHS swine with successful epidural anesthetic levels. as demonstrated by a complete motor and sensory blockade, did not develop MH when again subjected to Halothane anesthesia. The authors stated, "Malignant hyperthermia in susceptible swine was completely blocked by epidural anesthesia with lidocaine. These studies indicate the importance of the sympathetic nervous system in the triggering of malignant hyperthermia" (Kerr et al, 1975).

In 1976, another research group led by Lister again examined the effect of an adrenergic blockade in a group of MHS swine. In this study, one group of MHS swine was pre-treated with reserpine to obtain a “whole body” sympathetic blockade, by depletion of central and peripheral catecholamine stores. In a second group infusions of phentolamine, an 〈 adrenergic blocker, or propranolol, a ® adrenergic blocker were titrated to examine the effect of preventing MH.

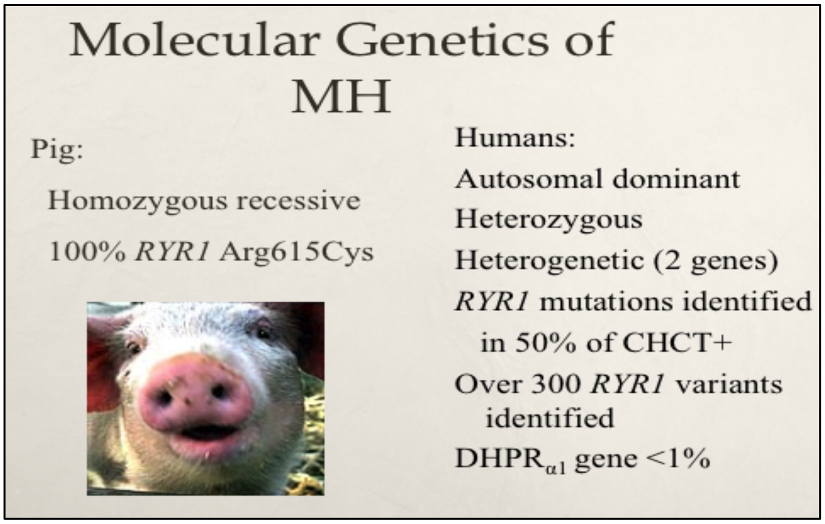
As expected, reserpinized pigs had decreased plasma catecholamines and successfully survived the succinylcholine challenge. However, it should be noted that many of the animals did not survive the pre-treatment with reserpine long enough to be used for the study.

The MHS pigs that received the 〈 adrenergic blocker phentolamine had an initial increase in catecholamines when the Halothane was administered and then their catecholamine levels decreased. Only the MHS pigs that received phentolamine in a dose of 50ug/kg/min survived the succinylcholine challenge. Lower doses of phentolamine were not successful at preventing a fatal episode of MH (Lister et al. 1976).

All MHS swine randomized to the group receiving the ® blocker, propranolol, triggered to a fatal MH event. This was true for all doses of propranolol. The authors stated, “It is evident that catecholamine depletion had occurred in the survivors, whereas the mean plasma concentrations in the pigs who died were similar to those of the untreated control group.” The authors wrote, “The important finding of this experiment was that although neither reserpine nor phentolamine altered the initial muscle response to suxamethonium, they prevented the further stimulation of muscle metabolism found in fatal MH” (Lister, et. al, 1976).

In 1977 this same research group again examined the importance of the SNS in the triggering of MH. Comparing the MHS Pietrain Pigs to the control group of MHN Large White pigs, they administered infusions of norepinephrine (NE), or the adrenergic agonist phenylephrine. They pre-treated a group of MHS and MHN swine with either the 〈 adrenergic blocker phentolamine or the ® adrenergic blocker, propranolol in order to examine the effect of adrenergic blockade in the model.

When the NE was administered to the MHN pigs, they developed a small increase in body temperature, but did not trigger to an MH episode. However, the MHS swine given infusions of NE or phenylephrine experienced a fatal hyperthermic event. They also reported that the ®-blocker propranolol was not successful in preventing the event. Which was consistent with the previous study (Hall et al., 1977).



Molecular Genetics of MH

Pig:

Homozygous recessive

100% *RYR1* Arg615Cys

Humans:

Autosomal dominant

Heterozygous

Heterogenetic (2 genes)

RYR1 mutations identified in 50% of CHCT+

Over 300 *RYR1* variants identified

$\text{DHPR}_{\alpha 1}$ gene <1%

Figure 2: Comparison of Molecular Genetics in Porcine MH and Human MH

Literature Review Supporting the Role of the SNS in MH in Humans

Other than exercise studies in MHS individuals, there is very little experimental literature on MH in humans. For ethical reasons, experimental research on the role of the ANS and triggering of MH in humans has been limited to examination of cAMP levels in skeletal muscle and plasma catecholamine levels. However, there are a few examples of research in the literature to support the role of stress and the ANS in the triggering of MH. These exist as case reports, exercise studies and the measurements of catecholamines and “Second Messengers” in the ANS stress response.

Human Case Reports

In 1981 Wingard published a series of case studies on 6 patients who he postulated had experienced possible MH episodes as a response to stress alone. Wingard suggested that frequently patients who are MHS do not trigger to anesthetics while they can demonstrate a hyper pyretic response and signs of MH without triggers (Wingard, 1981). However compelling the anecdotal nature of his case studies were in implicating stress as a component of MH triggering, no scientific conclusions were possible. The patients had not been positively identified as MHS by CHCT, genetic profiling was not yet possible, and the described episodes of MH were not validated by using a clinical grading scale (Wingard, 1981).

Supporting the professions of Dr. Wingard, in 1975 R.F.W. Moulds wrote a letter to The Lancet in which he outlined a case for MH being a “manifestation of a generalized stress syndrome” in both humans and pigs. His underlying theory was that “abnormalities in the calcium storing muscle cells could also be present in the catecholamine secretory cells”. He further wrote, “all the features necessary for the perpetuation of this cycle during malignant hyperpyrexia are probably exaggerations of normal responses rather than completely atypical responses” (Moulds, 1975).

In 1980, Gronert et al. presented the case of a 42-year-old male who reported stress-induced episodes of “fever, aching joints and sweats that lasted for several days”. According to the authors the symptoms were relieved with Dantrolene. They suggested that the patient was experiencing a hyper metabolic response to stress and that perhaps even the patient ‘s diabetic pattern was “secondary to increased circulating levels of catecholamines or adreno-cortical stimulation.” They also postulated that perhaps humans, when not under the influence of anesthesia more readily recognize the symptoms of impending MH and have the physiologically adaptive response to compensate by resting, thereby preventing a further escalation of a hyper-metabolic episode from triggering to fulminate MH (Gronert, et, al., 1980).

Britt, in a 1988 case report, described several family members in a MHS family who had been previously diagnosed by as MHS. She outlined a history of several anesthetic related and non -anesthetic related incidences. She proposed in her paper that stress was a triggering factor for several of the reactions, one of which progressed to a fatal outcome (Britt, 1988). .

Recently there was the report of a stress related triggering of a fatal hyperthermic event in a 12 year old male who had survived a previous anesthesia related MH episode. Several months following the initial intraoperative event, the child experienced a fatal hyperthermic event while playing football. Autopsy revealed no cardiac or other pathologic contributory conditions other than a RyR1 mutations causative for MH susceptibility (Tobin, 2001). The advent of molecular genetics and the successful linking of mutations in the RyR1 and dihydropyridine receptors to MH susceptibility in Humans have provided an avenue for improved identification of those MHS individuals who experience an MH episode in the absence of a known pharmacogenetic trigger. This may lead to the elucidation the role in stress related triggering of MH in humans.

Literature on Human Experimental Studies

In 1981 Campbell, Ellis and Evans used 9 MHS and 9 control patients. Patients had muscle biopsies performed the day following testing, so the researchers were blinded to the patient susceptibility during testing. Subjects were randomized to groups and exercised to examine the effects of light work on a bicycle ergometer, first in a fasting and then in a fed state. Findings included that MHS subjects, unlike the controls, did not exhibit dietary induced thermogenesis with the exercise. MHS individuals also exhibited higher insulin and triglyceride levels. Lactate levels rose to similar levels in both groups, however the MHS patient's levels failed to resolve when they were rested. Cortisol levels were also significantly lower in the fed and exercised MHS group. The authors summarize with a suggestion that there was an underlying abnormality in the sympathetic control mechanism of the patients in the MHS group (Campbell, et al., 1981).

Adrenergic Second Messengers (cAMP, Adenylate Cyclase) and MH

Wilner, Cerri and Wood published a study Investigations reporting the use of chemically skinned muscle fibers from MH survivors to explore the role of the ANS in MHS individuals. They measured the content of cyclic AMP (cAMP), adenylate cyclase (AC) and phosphodiesterase (PdE) in skeletal muscles of these MH survivors and relatives of survivors. Their sample consisted of 18 survivors of MHS episodes and 15 relatives of MH survivors. These researchers found "basal AC activity abnormally increased in muscle of MH reactors and those relative with abnormal single fiber responses to caffeine, compared to control muscle.

They also found that increased AC activity occurred in the muscles of MHS subjects when they were asymptomatic. The authors state, "The normal function of AC provides an explanation for this paradox…. although activity of AC can be readily measure in vitro, cAMP is synthesized at a relatively low rate in vivo and in the absence of physiological stimulants it is rapidly degraded by phosphodiesterase (PDE)." The usually asymptomatic myopathy associated with MH is therefore associated with abnormality of an enzyme that normally requires stimulation to be catalytically active. Catecholamine secretion and anesthetics could provide that stimulation" (Wilner, Cerri and Wood, 1981).

This same research group had performed a previous study in 1979 entitled, “Malignant Hyperthermia: Abnormal Cyclic AMP Metabolism”. In this study the researchers examined skeletal muscle biopsies of children who had exercise-provoked hyperthermia, or anesthesia provoked MH, and found increased cAMP content. They also reported that following an infusion of isoproterenol in these individuals, the differences in the cAMP increased. The authors suggested that these findings were supportive of the possibility that catecholamines, as well as halothane, could act in MHS humans to trigger “a genetic defect expressed in the plasmalemma” and that ”abnormal SR calcium transport could be a consequence of this defect” (Wilner, Cerri and Wood, 1979).

Stanec and Stefano performed an experimental study of 10 MHS and 10 non-MHS subjects given a Bruce Protocol treadmill test. Each individual was exercised to their maximum exertion level and then tested for levels of cAMP. Statistically significant results included the findings that MHS individuals reached their maximum predicted increase in HR and systolic blood pressure earlier than normal subjects and reported feeling “exhausted” earlier than non-MHS controls. When levels of cAMP were measured at baseline before exercise, the MHS sample had higher levels of cAMP but not to a statistically significant degree. However, cAMP increased to statistically significant higher levels in the MHS group during the exercise tests, and the duration of the increased in the cAMP levels in the MHS group, up to 2 hours after the test was completed, did reach statistically significant levels (Stanec and Stefano, 1984).

Literature Review Supporting Human B-Lymphocytes as a Model

In 1999 an new acceptable model was found for study of MH in humans when it was demonstrated that human B cells, from two cell lines, DAKIKI and human primary CD 19 (+), expressed RyR1**,** RyR2 and RyR3 in 56, 22, and 0% respectively, while T cells from the same samples (N=9), expressed RyR1, 2 or 3 in significantly lower levels. The RyR1 receptors present in these cell lines were identical with that found in skeletal muscle (Sei, et al. 1999). This was the first research finding which established the possibility that Human B-lymphocytes could be used as a model to examine RyR1 function in immune cells.

In 2002, Sei et al. demonstrated that B-lymphocytes from MHS individuals exhibited significantly increased Ca $^{2+}$emissions in response to the RyR1 agonists 4-CmC and Caffeine when compared to MHN controls. This supported that there was an altered Ca^{2+} homeostasis in MHS individuals and that this was related to the RyR1. Using 50mM caffeine or 400uM 4-chloro-m-cresol (4-CmC) in fluo-3 prepared B cells; the researchers reported a significantly increased fluorescence in B-cells from MHS patients when compared to controls with a correlation between response to agonists and Ca^{2+} release (Sei et al. 2001). Girard et al. (2001) demonstrated that immortalized Epstein Barr Virus (EBV) B-cells from a MHS patient with a defined mutation exhibited a slightly larger fraction of total releasable intracellular Ca^{2+} pool when exposed to 400 or 600 uM of 4-CmC as compared to controls.

McKinney et al, used Dakiki and PP normal human B cell lines, as well as lymphocytes obtained from MHS and normal pigs, to compare the effects of 4-CmC on eliciting RyR1mediated Ca^{2+} release. Ca^{2+} was measured fluorometrically with Fura -2 am for cells in suspension and with fluo-4 in single cells using confocal microscopy. ThyR1 mediated $^{Ca2+}$ release using caffeine or 4-CmC. They also reported that B-lymphocytes from MHS swine were more sensitive to the RyR1 agonist 4-CmC than normal swine and that this could be shown be measurement of fluorescent emissions using Fura-2 AM dye (McKinney et al., 2006).

These investigations successfully demonstrated that human B-lymphocytes provide a valid and acceptable scientific model which can be effectively used to explore the sensitivity of RyR1 agonist induced Ca^{2+} release between MHS and MHN individuals.

B-Lymphocytes and the ANS

In order to investigate the effects of ANS catecholamine release on human B-cells it is necessary that the B cells express adrenergic receptors. The presence of adrenergic receptors on human B-lymphocytes is well documented in the literature, with the largest type being ®2 (Rici et al.1999; Maisel et al, 1990, Williams et al. 1976).

Williams, et al. examined the effect of isoproterenol, epinephrine and norepinephrine on human b-lymphocytes and found that the ® adrenergic agonist isoproterenol was the most effective in stimulating the adenylate cyclase production in these immune cells. The authors stated that their results “provided an experimental approach to the study of states of altered sensitivity to catecholamines at the receptor level in man” (Williams et al, 1976).

In 1980 Tohmeh and Cryer reported on their finding that adrenergic agonists (isoproterenol and epinephrine) were responsible for the up-regulation of and subsequent increased receptor sensitivity of ® receptors to catecholamines (Tohmeh&Cryer, 1980) and Redwine et al. demonstrated that increased stress in individuals resulted in a change in the activity of, and density in, ®2- adrenergic receptors on human B- lymphocytes (Redwine et al., 1996).

Ricci et al. successfully demonstrated the expression of α1 receptors on human lymphocytes in 1999. This group used reverse transcription–polymerase chain reaction (RT-PCR), radio-ligand binding assay techniques, as well as the use of specific ⟨$_1$ antibodies to prevent the binding of the pharmacologic ⟨ specific agonist prazosin. They found the expression of three ⟨$_1$

receptor subtypes on human peripheral lymphocytes.

These studies support use of human B-lymphocytes from MHS and MHN controls to examine the sensitivity of RyR1 mediated Ca^{2+} release in response to pharmacologic reagents and catecholamines in MHS human B-lymphocytes.

Summary and Unanswered Questions

The analysis of literature supports the possibility of a link between hypersensitivity ANS response and RyR1 genetic pre-disposition in the triggering of MH in MHS individuals and further offers support for using the human B-lymphocyte model to examine this possibility. The failure to identify a common pathway for MHS individuals who trigger to a fulminant MH episode, the case reports on perceived stress in MHS individuals prior to triggering and the experimental data on human and animal models leave a large gap in our understanding of MH triggering and the involvement of the ANS.

As shown in the studies referenced above, inhalational anesthetic agents and succinylcholine do not always trigger a MHS patient to develop a fulminant MH episode. Furthermore, the literature suggests that humans can trigger to MH and MH like episodes in the absence of these identified triggers and that there appear to be differences in the effect of catecholamine, and second messengers, such as cAMP and adenylate cyclase, related to catecholamine stimulation in MHS individuals. These findings support a physiologic link between stress, the ANS and triggering of MH or MH like episodes and leave many questions unanswered. Primarily these questions can be summarized as follows:

-Which factors other than a RyR1 genetic pre-disposition are necessary or sufficient for a MHS individual to trigger to MH?

-What are the common biochemical and signaling pathways for MHS individuals who do trigger vs. those MHS individuals who do not trigger?

-What are the mechanistic links in terms of biochemical and signaling factors between the ANS and MH triggering?

Most literature reviews limit themselves to studies conducted within the last 10 years. I understand that this review does not meet that criterion. However, after an extensive literature search on the role of stress in MH, I was struck by the paucity of research performed this area after the mid 1980s. While there was a flow of research in this area in the late 1970s and 1980s, most research after 1980s exploring the issue was done primarily in the veterinary community. The connection between the RYR1 receptor and MH occurred in the mid 1980s. It is possible that once that connection was established there was simply a re-direction of MH research away from the stress/SNS link to triggering and progression.

Therefore the definitive answer as to what is the common pathway involved in the triggering of a MH event remains undefined. In their article entitled "Is there a Link between Malignant Hyperthermia and Exertional Heat Illness, Muldoon, Deuster, Brandom and Bunger examined the relationship between the two syndromes. In their overview they discuss variability as a "striking characteristic" of the disease MH. Several of the above case studies have demonstrated differences in MH susceptible individuals when subjected to exercise. These researchers suggest that MH "stems from an interaction between genes and environmental factors, and that "5-8% develop symptoms with exercise emotional stress and /or environmental heat exposure" (Muldoon, et al., 2004).

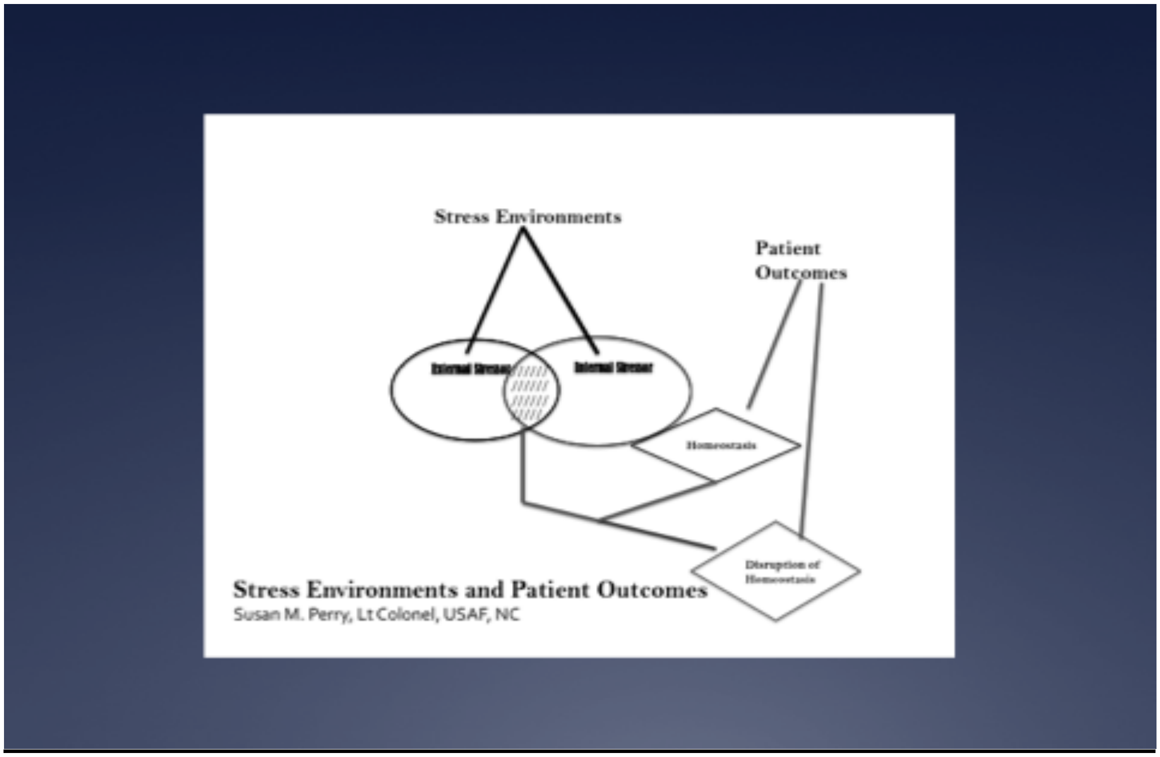
The studies on cAMP, though done over 20 years ago, are interesting in supporting a possibility that the relationship between MH and exertional heat illness is perhaps one of a continuum, which may involve many levels of increasing hyperthermic syndromes. If this is the case the early researchers such as Gronert, were correct in suggesting that in humans, the awareness of symptoms may work to control the onset of the hyperthermic events.

Although most of the direct studies on the role of the sympathetic nervous system and MH were published more than 30 years ago, more recent studies on the abnormalities in ryanodine receptors indicate a possible link between hyper-catecholaminemia and RYR1 and RYR2 hyperphosphorylation, possibly resulting in dissociation of FKBP12/12.6 and leading to increased leakiness in the RYR1 membrane (Gaburjakova, et al, 2001; Marx, et al, 2001, Chelu, et al, 2004; Lanner, et al, 2010; Dainese, M, et al. 2009; Dulhunty, A., et al, 2001)). These studies, combined with the previous research, leads to my hypothesis that an underlying abnormal response to catecholamines reduce the threshold for triggering cellular calcium flooding in skeletal muscle of patients with malignant hyperthermia genetic mutations.

Literature Review and Nursing Theory

Literature on Nursing Theoretical Framework for Study

As I was unable to identify a theoretical framework that would be applicable for this research, I proposed a theory that could be used for the underpinning of my study. This proposed framework is entitled: **Theory of Stress Environments and Outcomes** as shown in Figure 3 below.



Stress Environments and Patient Outcomes

Susan M. Perry, Lt Colonel, USAF, NC

Figure 3: Proposed " Theory of Stress Environments"

The constructs for this theory are "Stress Environment and "Patient Outcomes". The Axiom for the theory is that interactions between the Internal and External Stress Environments directly affect patient outcomes.

Construct of Stress Environments Theory

For the development of this theory "Stress Environment" is defined as the internal and external environments of an individual patient, organ, cell or organelle in which a stressor exists or may be introduced. The "External" and "Internal" stress environments are further differentiated. The External Stress Environment is that environment lying outside the external/internal interface (skin, organ, cell, or organelle membranes) in which the Internal Stress Environment exists.

A review of nursing's Grand Theories as outlined in the text, "Conceptual Models of Nursing" revealed that while many theorist discussed the importance of the environment (Nightingale, Orem, Roy, Neuman and Rogers), after reading Barbara Pieper's description and critique of Levine's Theory in Chapter 10, I found the approach proposed by "Myra Levine's Nursing Model: The Conservation Principles" came the closest to the description of the role of environment proposed in this model. Simplified, in Levine's Theory the internal environment is maintained in a state of non-static flow in which the physiologic and psychologic elements of the person are constantly adapting to factors in the external environment.

The environments in this proposed middle range theory are further delineated to involve not only the external and internal patient environments but also to be conceptualized as the external and internal environments of the cell, as well as the external and internal environments of intracellular organelles. In this way the theory may be utilized for multiple aspects of nursing research.

Patient Outcomes Defined

Outcome is defined by Webster's on-line dictionary as "Something that follows as a result or consequence". Patient Outcomes is defined as the results or consequence resulting from the patient's interaction with the Stress Environments. The optimum Outcome is the maintenance of Homeostasis. In this middle range theory the idea of "Outcome" is expanded throughout the patient system to include, organ, cell or cellular organelle and defined as the " result or consequence or the with disruption of homeostasis resulting in a negative patient outcome.

The proposition is that there is a direct relationship between the interaction of Stress Environments and Patient Outcomes

Concepts and Variables

There are 4 Concepts in the model. They are "External Stressors", "Internal Stressors", "Homeostasis" and "Disruption of Homeostasis". In 1976, Hans Seyle in his text on "Stress in Health and Disease" defined stress as "The body's non-specific response to a demand placed on it" (p.17). Stressors are varied and depend on the specific setting in which the patient and nurse interact. These can be defined as concepts in relation to those existing in the external environment and the internal environment. The result of the stressors may lead an indentified variable "stress response" which may be effectively measured. The definition of the theory concepts is given below.

External Stressors: Those stressors existing in or introduced into to the External Stress Environment.

Internal Stressors: Those stressors existing in or introduced into the Internal Stress Environment.

In Chapter 6 of their text, "Integrated Knowledge Development in Nursing", Chin and Kramer discuss the importance of defining expected outcomes early in the process of theory development. They state that "…theory suggest goals or outcomes that the profession values and …the fundamental purpose for using the theory is to achieve those goals" (p155). Therefore, specific outcomes applied to this theory will, by definition be presented as concepts and will vary depending on the nursing practice or research setting for which it is being adapted. Within each of these settings there was varied outcomes measures serving as dependent variables. In the peri-anesthetic setting, for example, there are many recognized outcome variables. The concepts of Homeostasis and Disruption of Homeostasis are defined below.

Homeostasis: Positive Outcome: Optimization of adaptation to stressors in the External and/or Internal Stress Environments

Disruption of Homeostasis: Negative Outcome: Negative adaptation to stressors in the External and/or Internal Stress Environments.

The proposition is that there is a direct relationship between External Stressors, Internal Stressors and the Maintenance of Homeostasis or Disruption of Homeostasis. Postulates for this theory are that External Stressors and Internal Stressors exist in, or are introduced into the External Stress Environment and there is a direct relationship between the maintenance or disruption of homeostasis and Patient Outcomes. For the purpose of this theory, variables or empirical indicators are defined in the context of the research/practice area being studied using the Stress Environments and Outcomes Model.

Application of "Stress Environments and Patient Outcomes" to Dissertation

My dissertation research focus is on the effect of stress on the triggering of Malignant Hyperthermia. In humans Malignant Hyperthermia (MH) is an autosomal inherited disorder that predisposes to a life threatening hypermetabolic syndrome. In susceptible individuals the commonly used inhalational anesthetics and the depolarizing muscle relaxant, succinlycholine may induce an accelerated metabolism that leads to a potentially fatal syndrome. Without the definitive treatment of Dantrolene Sodium and discontinuation of all triggering agents, mortality is in excess of 70% (Muldoon et al. 2004).

My original interest in this research dealt with the question of why some patients with a known genetic mutation causative for MH did not develop MH even in the face of a known triggering anesthetic while others immediately exhibited fulminant hypermetabolic responses. Even more confusing were the statistics that 53% of those developing MH had, in fact, undergone on average 2 other anesthetics without triggering to MH (Newmark et al. 2007; Larach et al. 2010). The theory I developed to shape my dissertation proposal was that there had to be a "stressor" introduced into the patient's peri-anesthetic environment that acted as a co-factor with the existing genetic stressor to trigger MH.

In order to begin the research on the effect of stress on the triggering of MH, I examined the intracellular reaction of the RyR1 receptor to a known stress hormone, epinephrine and norepinephrine to see if these physiologic stressors increased the reactivity of the RyR1 receptor in B-lymphocytes from MHS individuals. It is easier to study this in the human B-lymphocyte model with has been successfully used to show variable Ca^{2+} release in response to the RyR1 agonist 4CmC, which varies in regards to MH susceptibility in the same manner as skeletal muscle (Sei et al. 2002; Girard et al. 2001; McKinney et al, 2006).

Constructs specific to Research using Stress Environments and Outcomes

The constructs of "Stress Environments" for this study exists in the context the inter-relationship between the External and Internal Stress Environments of the endoplasmic and/or sarcoplasmic reticulum and the outcome of Malignant Hyperthermia. The Axiom being that there is a relationship between the External and Internal Stress Environments and the patient outcome known as Malignant Hyperthermia.

Specific Concepts for the Study

The concepts for the study are "Stress", "RyR1 Genetic Mutations" and "Calcium Homeostasis". The postulate linking the concepts to the construct is that an increase in "Stress" from the External Stress Environment, impact the existing "RyR1 Genetic Defect", which exists in the Internal Stress Environment, and this directly has the outcome of increasing the amount of Ca^{2+} released from the endoplasmic reticulum.

Many studies and case reports support this proposition and implicate emotional and exercise induced stress or pain as causes of MH separate from the known anesthetic triggers (Moulds, 1975; Katz, et al. 1976; Gronert, et al., 1980; Wingard, 1981; Grinberg, 1983; Montegi, et al., 1996; Muldoon, et al., 2004).

Stress in the study is expressed as the physiologic response of stress introduced into the External Stress Environment of the Cell in the form of catecholamines such as epinephrine/norepinephrine, which are known adrenergic agonists, released in response to stress (Morgan & Mikhail, p. 219).

Cyclic AMP (cAMP) is an intracellular marker, which is formed following activation of adrenergic receptor activation by catecholamines. Researchers have found significantly higher levels of cAMP in MHS vs. MHN patients both after exercise and isoproterenol infusions (Stanec and

Stefano, 1984, Wilner et al., 1979 & 1981). This indicates that there may be an alteration in the autonomic nervous system in patients with RyR1 defects.

The second concept is the “RyR1 Genetic Defect”. The RyR1 is the largest calcium ion channel in the body and is attached to the sarcoplasmic reticulum in skeletal muscle. In 1990, McLennan et al. performed the sentinel study that found that the RyR1 gene located on the intracellular sarcoplasmic reticulum in skeletal muscle, located on chromosome 19q13.1 was the primary locus responsible for MH susceptibility. Mutations in the RyR1 linked to MH cause the channel to respond with increased sensitivity to activators such as 4-chloro-m-cresol (4CmC) and caffeine (Hamilton and Serysheva, 2009). In response to these activators, the RyR1 channel is unable to control intracellular levels of Ca^{2+}. These studies provide the necessary substantial of a link between the RyR1 and Ca^{2+} release.

The third concept is “Intracellular Ca^{2+} Release”. The acute syndrome known as MH has been linked to an abnormally sustained increase of Ca^{2+} in the cytoplasm of skeletal muscle. Different mechanisms have been suggested to account for this, but at present enhanced release of Ca^{2+} from sarcoplasmic reticulum by way of the Ryanodine Receptor (RyR1) is considered causative (Mackrill, 1998).

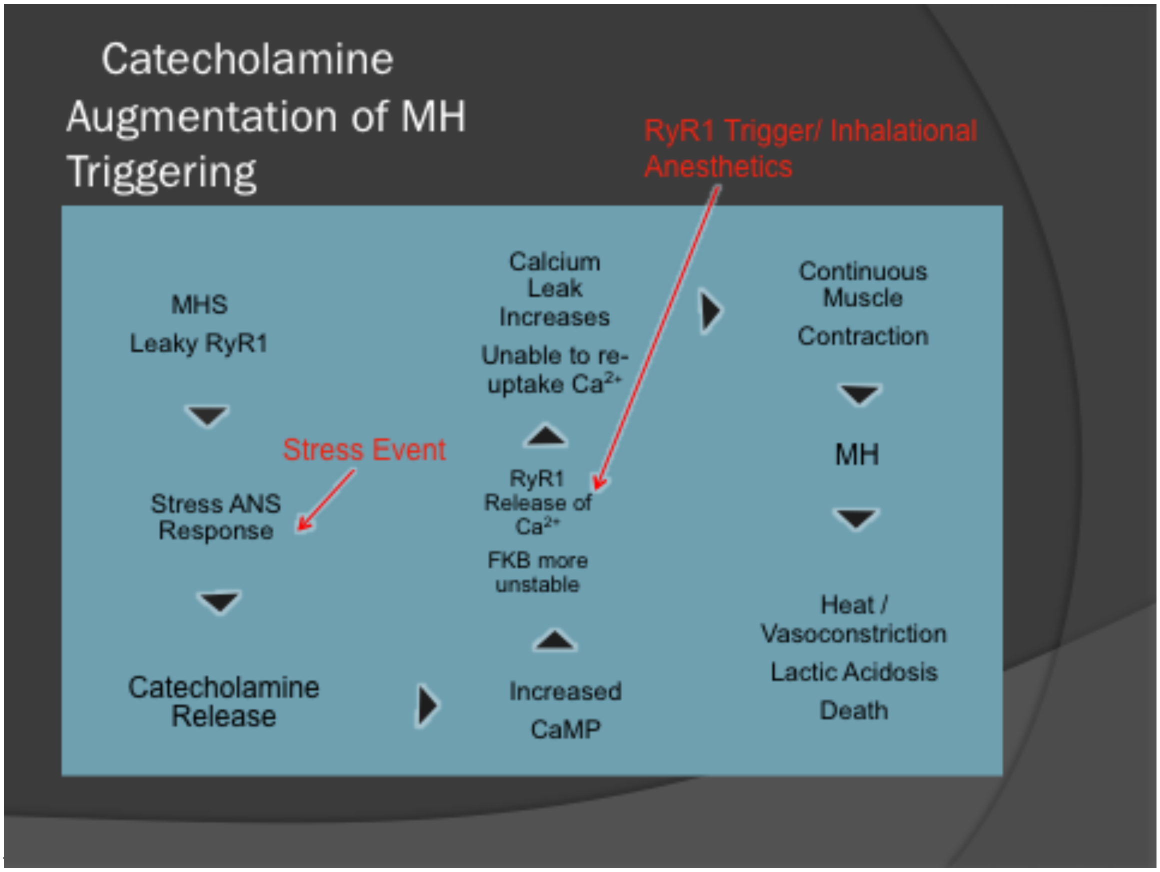
The proposition is that the presence of stress acts as a factor in patients with RyR1 genetic mutations to trigger higher levels of intracellular Ca^{2+} release in B-lymphocytes from MHS patients when compared to controls. This completes the theoretical analysis of the model.

Referential or Variable

At the operational level of the theory we begin with variables, which Dulock and Holzemer defined as “actual elements or experimental conditions” linked by the hypothesis. (84). The hypothesis for this study is that žEBV Immortalized Human B-lymphocytes derived from a known MHS population, will display increased sensitivity, (EC50 dose response) to the RyR1 agonist, 4-CmC in the presence of alpha or beta-adrenergic agonist, as evidenced by an increase in myoplasmic RyR1 Ca^{2+} accumulation when compared to MHN controls. Therefore the two variables for this study are EC50 Dose Response and Myoplasmic Ca^{2+} Accumulation with a comparison between two groups, MHS and MHN. The transformational statement linking the Variables to the Concepts is that the EC 50 in response to the RyR1 agonist was lower in B-lymphocytes derived from MHS vs MHN patients and the total increase in myoplasmic Ca^{2+} was significantly increased in MHS samples.

Measures/Scores/Values/Data Analysis

The measure for this study was the difference in Ca^{2+} released from the endoplasmic reticulum in response to varying doses of the RyR1 agonist 4CmC in the presence or absence of catecholamines. Differences were presented and dose responses and florescent curves of Ca^{2+} emissions using the radiometric dye Fura 2. Data was presented as continuous data with paired t-test and analysis of variance used to explore differences in mean values. This measure and analysis has been used in multiple studies to measure the differences in MHS and MHN lymphocyte Ca^{2+} responses to RyR1 agonists (McKinney et al. 2004). For an adaptation of the theory specific to this research see Figure 4. below.



Catecholamine Augmentation of MH Triggering

RyR1 Trigger/ Inhalational Anesthetics

MHS
Leaky RyR1

Stress Event

Stress ANS Response

Catecholamine Release

Increased
CaMP

FKB more unstable

RyR1 Release of Ca^{2+}

Calcium Leak Increases

Unable to re-uptake Ca^{2+}

Continuous Muscle
Contraction

MH

Heat / Vasoconstriction
Lactic Acidosis
Death

Figure 4. Theorectical Framework for Catecholamine Augmentation of RyR1 Calcium Release in MHS Human B – Lymphocytes.

CHAPTER 3

MATERIALS AND METHODS

Experimental Research Design and Protocol Overview

This experimental in-vitro design attempted to quantitatively evaluate observable differences in the RyR1 response to the pharmacological RyR1 agonist 4-CmC in the absence and presence of adrenergic agonist and antagonists. The experimental and control groups consisted of Epstein Barr immortalized B-lymphocyte cell lines generated from MH susceptible (MHS) and MH-negative (MHN) patients. RYR1 responses to 4CmC from MHS and MHN individuals were measured as the intracellular release of Ca $^{2+}$.

MHS and MHN Lymphocyte Samples

Existing immortalized B-Cells were obtained from an approved protocol at the Uniformed Services University, "The Molecular Basis of RyR1 Myopathies: Core: C" under a grant from the National Institutes of Health No: G180BO. The USU Anesthesiology MH referral center at the Uniformed Services University Department of Anesthesia had an adequate sample of existing MHS and MHN immortalized B-cell lines available for use in this investigation.

The existing samples were obtained in the following manner: Following IRB approval, letters were sent to members of the USU MH registry requesting participation in the investigation. After obtaining informed consent, approximately 30 ml of blood was obtained from MHS and MHN individuals clinically characterized by CHCT genotyping at the USU MH Center. Blood was then prepared and transported to the Tissue Culture Facility at The University of North Carolina at Chapel where they were immortalized using the EBV (Epstein-Bar Virus). The immortalized B cells were returned to the Uniformed Services University in dry ice, and maintained in liquid nitrogen. Patient names and any other identifiers were removed prior to their inclusion and each sample was assigned a new code, MHS 1-7 and MHN 1-7. These codes were used for the research outlined below. The PI was blinded to any personal identifiable data.

The following criteria were used to assign cells to the MHS group: 1) positive Caffeine Halothane Contracture test in the patient, unequivocal for MH positive results and 2) known genetic mutations in the RYR1 or the DHCPR genes previously linked to clinical MH episodes. The criteria for assigning cells to the control MHN group were as follows: 1) negative Caffeine halothane contracture test in the subject and 2) no genetic mutation linkable to MH in the RYR1 or the DHCPR gene. MHS and MHN cells were then re-assigned experimental codes of MHS 1to5 and MHN 1to5. No patient-specific identifying data were included in the numbering and categorizing of the groups for the experimental cell-line work. The outline of the Protocol followed by the MH Center is shown in Figure 5.

In order to calculate an appropriate sample size a power analysis was performed based on a similar investigation performed by Sei et al. who examined calcium control in immortalized B-lymphocytes in MHS cells relative to MHN controls. Using their results (Mean = 33.0+/- 3.6 vs. 15.5 +/- 3.1 P<0.005) for MHS and control respectively, it was calculated that this investigation would reach 100% power with more than 2 samples per experimental group (DSS Research). However, as an investigation examining the possible effects of catecholamines on the RyR1 response in Human B- lymphocytes has not been performed previously, we decided to use a sample size of 14 cell lines, 7 MHS and 7 MHN. This sample size allowed for the possibility of premature cell line failure.

A statistical blocking technique was used to assist in ensuring validity of the study. The blocking was performed by the PI and was primarily performed to decrease the risk to the internal validity concerns such as changes in environmental temperature over the period of data collection (10 months), potential variations in laboratory conditions, variance in lot numbers from reagents, etc. The blocking also allowed for MHS and MHN cell lines to be grown under the same conditions in the incubator. See Table 1 for the blocking outline. In addition, each cell line acted as it's own control and effect of the adrenergic agonists and blockers were compared against the previous controls in each experiment on the same day.

Cell Line Group		BlockingTable	
First Group		MHS 1	MHN 3
Second Group		MHN 2	MHS 4
Third Group		MHS 2	MHN 5
Fourth Group		MHS 3	MHS 5
Fifth Group		MHN 4	MHN 6
Sixth Group		MHS 6	MHN 7

TABLE 1: MHS and MHN Cell lines were assigned a number from 1-7 and then randomly chosen for inclusion in the groups outlined above. Cell lines from the same group were removed from liquid nitrogen on the same day and grown at the same time under the same temperature/humidity conditions. Experiments for cells in the same group were performed within the same period of time. Data was collected and not compared until the end of data collection period.

Validation Measures

The USU Anesthesiology Department had all the necessary equipment, expertise and personnel required to reproducibly apply a Fura-2 fluorescence-based intracellular Ca^{2+} level assessment, both in response to RyR1 agonists such as 4-CmC and in the absence and presence of catecholamines. It could be argued that the immortalized B-cell line may not provide the best model to investigate the possible effects of catecholamines on the RyR1 sensitivity in the real clinical case of MHS. Clearly, our immortalized B cell line is only an in-vitro model the clinical relevance of which is not established yet and requires additional investigations. Nevertheless, lymphocyte B-cells have both catecholamine and RyR1 receptors (Rici et al.1999; Maisel et al, 1990, Williams et al. 1976). We thus considered the immortalized B-cell lines as an acceptable model for this study.

Reagents and buffer solutions were made freshly each day of experimentation. Catecholamines were dissolved in the presence of 1 % ascorbic acid and prepared in red light conditions, then stored in a cool, dark environment during experimentation in order to prevent auto-oxidation.

Fura-2 AM Dye technique

Fura 2, a polyamino carboxylic acid, is a ratio-metric dye typically used for assessing intracellular calcium level changes in a variety of cell systems, including B-cells (Lambert, 1999). Fura 2 has the fluorescent properties that make it an ideal dye for measuring emissions following 4-CmC induced RyR1 Ca^{2+} release from intra-cellular stores such as the endoplasmic or sarcoplasmic reticulum (Hirst et al, 1999). Fura-2 is excited by “near ultraviolet (UV) wavelengths between 340-380 nm” (Simpson, 1999.) When an agonist is introduced and elicits an increase in free intracellular Ca2+ levels, this change in cellular free Ca^{2+} is recorded as an increase in the fluorescence ratio. Commercial prepared Fura 2-AM is a form of Fura 2 formulated to contain an Acetoxymethyl ester. Formulating the Fura-2 with an ester linkage provides it with the lipophilicity necessary to allow the dye to permeate the plasma membrane. Once inside the cell, esterases effectively cleave the AM groups, leaving only the Fura 2 contained in the cytoplasm (Lambert, 1999, section 2).

To minimize the compartmentalization of the Fura 2 within the organelles the cells were maintained at room temperature for a minimum of 40 minutes prior to Ca^{2+} measurement experiments (Simpson, 1999, Section 2.6 & 3.2). According to Simpson et al. temperature plays a major role in reducing the sequestering of the dye in organelles by reducing exoctytosis (Simpson, et al, 1999,Section 2.6). After a minimum of 40 minutes at room temperature the cells were spun at 5000 RPM X 30 seconds and the supernatant was removed from the cell pellet. The pellet was then re-suspended in dye-free buffer prior to placing them into the spectrofluorometer for measurement of emissions (McKinney, 2009).

Calibration of the fluorescent ratio in terms of actual mean intracellular calcium level was not performed. The total releasable levels of intracellular Ca$^{2+\text{ as}}$ measured by Thapsigargin were used as a control. This is a limitation of this study.

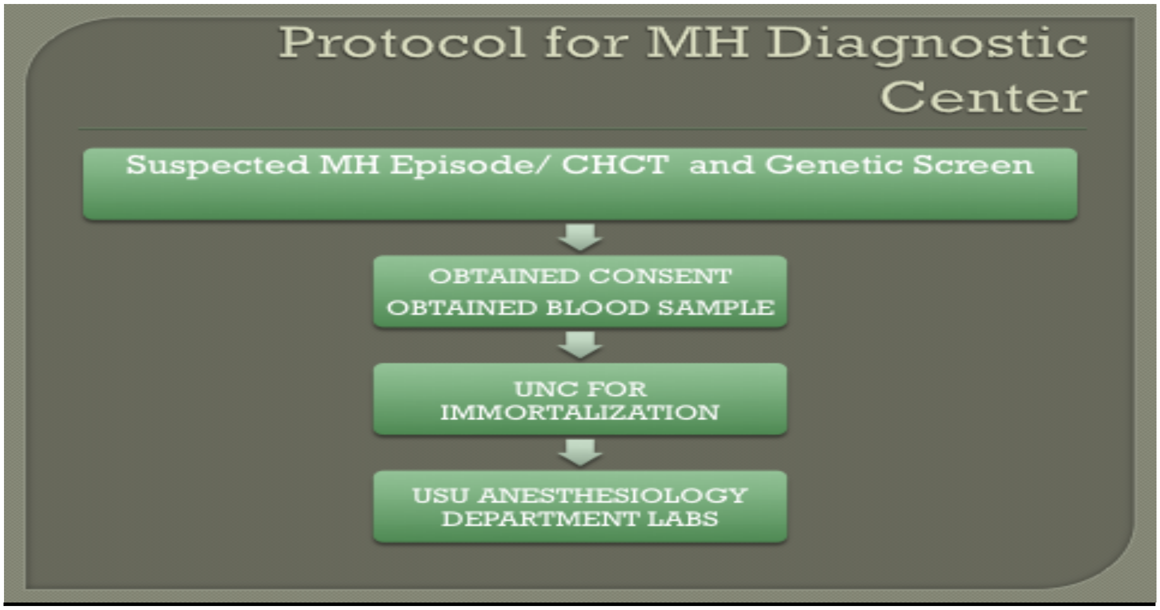
Spectrofluoroscopy and Ca^{2+} Measurements

A Quantamaster® 40 Spectrofluorometer (Photon Technology International) was used to excite the cells at 340/380 nm and measure fluorescent emissions at 510 nm. The manufacturers of this instrument use state of the art technology to address threats to precise fluorescent measurements such as photo-bleaching, stray light, excitation and emissions errors. These are reduced by the integration of specific software at the factory that automatically auto-calibrates the machine based on the protocol selected by the researcher (Technical Manual, PTI, 2011).

In addition to the auto-calibration, the instrument used was calibrated by the department prior to use under the direction of the manufacturer using the standard technique of Water RAMAN spectroscopy. The specific Fura 2 Emissions Protocol available on the software integrated into the machine was used for this study. Company specifications list the sensitivity of the instrument in the following way (taken from Quantamaster specifications manual):

- Temporal Resolution: 1⌠s
- Wavelength Activity: +/- 0.5 nM
- Data Acquisition rate up to: 300 points/second
- Signal to Noise Ratio: 3000:1
- Stray Light rejection: 10^{-8}

All data are presented as Fura 2 Ca^{2+} emission ratios either as Baseline Area (BA), Area under the Curve (AUC) or Peak Emissions Ratios (PE). The experimental methods for Fura 2 measurements were adapted from those developed and validated by McKinney et al in 2006. This group explored differences in the in vitro fluorescence signal “ 0.7-7.7, corresponding to 0-350nm Ca^{2+}” and found that 1mm of 4-CmC made no significant difference in the dissociation constant of fura 2 for Ca^{2+} (McKinney, 20076. They reported that the leak from Fura-2 was “completely inhibited” by reducing the temperature from 37° to room temperature. During our experiments it was also noted that the in vitro Fura 2 Ca^{2+} fluorescence signal from our cells were between 0.7 and 7.7 in the presence of 4-CmC. Therefore our data validated that of McKinney et al. No additional calibration was indicated as all of our data were within these parameters.



Protocol for MH Diagnostic Center

Suspected MH Episode/ CHCT and Genetic Screen

OBTAINED CONSENT
OBTAINED BLOOD SAMPLE

UNC FOR IMMORTALIZATION

USU ANESTHESIOLOGY DEPARTMENT LABS

Figure 5: Protocol used by the MH Diagnostic Center at Uniformed Services Uniformed Services University for collection and immortalization of Human B-lymphocytes. Patients referred to the MH Center at the Uniformed Services University were enlisted for the research study following informed consent. CHCT and genetic screening were performed and blood was sent for B-Cell immortalization using the Epstein Barr Virus. These cell lines were returned to the MH Center and maintained in liquid nitrogen until use.

Methods: Preliminary Phase I and II Studies

The first groups of preliminary experiments were performed to examine the effect of catecholamines alone on baseline Fura 2 Ca^{2+} emissions in human B-lymphocytes examine the dose response to catecholamines on Fura 2Ca^{2+} baseline emissions in the absence and presence of 4-CmC.

Experiments were then performed to determine the effect of Ca^{2+} in the buffer. This was not planned during the protocol proposal, however, during the first phase it was noted that the catecholamines worked in the presence of Ca^{2+} but not in the absence of Ca^{2+}. This will be discussed in the results chapter. These preliminary findings were used to determine the least effective dose, buffer type and specific catecholamine to use in the hypothesis-testing phase of the study. Figure 6 shows an outline of the Protocol used for Phase I and II.

Figure 6: Phase I and II Time-line Overview: An overview of the time line used for experiments in Phase I/II

Phase I and II Protocol

Cells were maintained in RPMI 1640 L-Glutamate with 5ml of Bovine Serum and 5.5 ml of streptomycin and Penicillin (PCN). Cells in RPMI were maintained in an incubator at 37 ° Celsius in a 5% Carbon Dioxide and 95% Oxygen environment. A second stage regulator maintained the pressure at 10 mmHG PSI. The incubator humidity was maintained at 80 to 90%. This was accomplished by ensuring that the tray at the bottom of the incubator had water in the tray.

Cell Preparation Procedure Day of Study

B-lymphocytes samples were centrifuged at 2000 RPM X 2 minutes and RPMI growth medium was suctioned from flask. Cells were washed twice with HBSS containing Fetal Bovine Serum (albumin) and re-suspended in 10ml HBSS containing 0.1G Fetal Bovine Serum and stained with a final concentration of 5.0 µm Fura-2 AM dye and incubated for 40 minutes at 37 ° C in a dark water bath.

After 40 minutes, cells were removed from the water bath and centrifuged X 2 minutes at 2000 RPM and re-suspended in HBSS and placed in the dark at room temperature (≈22 ° C) for a minimum of 40 minutes prior to beginning first emission's measurements. Prior to being placed into the spectrofluorometer, cells were pipetted in 1ml aliquots, re-spun at 5000 RPM X 30 seconds and re-suspended at a density of 2 X 10^{-6} /ml in a 3-ml cuvette using either 1ml Krebs Ca^{2+} free or containing buffer. An additional 1ml of buffer was added to cuvette prior to placement of the 1ml cells in suspension. Buffer was maintained at 37 °C during procedure. Cuvettes with magnetic stirrer magnets were pre-warmed at 37 ° C. Cells in final volume of 2ml were placed in a Quantamaster fluorometer (Photon Technology, Inc., Monmouth, NJ) thermostated at 37 ° C and fluorescence measurements was taken approximately every 0.5 seconds using a dual excitation wavelength (ratio 340/380nm).

Epinephrine and norepinephrine were catecholamines used for this phase of the study. Concentrations for the reagents were increased in a stepwise fashion (10^{-7} to 10^{-6}) for Phase I of the study and then used in 1⌠M final concentrations. 4-Chloro-m-Cresol (4-CmC) was the RyR1 agonist that was used for this experiment. Concentrations of 4-CmC were increased from 1⌠M to 2⌠M in a stepwise fashion to distinguish dose response. Thapsigargin was used in a 100nM concentration.

Reagents for the study were constituted in solution fresh prior to each experiment on the day of the measurements in order to address validity of the study. The reagents were as follows:

1. 4-Chloro-m-cresol and Thapsigargin (Sigma Aldrich) were solubilized in dimethyl sulfoxide (DMSO, Sigma Aldrich).
2. Epinephrine and norepinephrine (Sigma Aldrich) were prepared in an ascorbic acid solution with 0.59 G ascorbic acid /100 ml double distilled water in red light conditions to prevent auto-oxidation. Once in solution 1ml of this was added to a buffer solution and maintained in an opaque bottle to protect from light. The catecholamines in solution were prepared immediately prior to the experiment and maintained in a cool/dark environment.

<u>Test Solutions/Reagents:</u> Test solutions were obtained from the following locations:

1. HBSS 1X (Gibco Invitrogen).
2. RPMI with L- Glutamine (Quality Biological).
3. Fetal Bovine Serum (Invitrogen)

4. Fura-2 cell permeant special packaging (Invitrogen)

5. Penicillin/Streptomycin (Invitrogen)

6. Free Ascorbic Acid (Sigma)

7. Ca^{2+} free and Ca^{2+} containing Krebs Buffer was made at the beginning of the experiments and stored in a refrigerator at 4 ° C. Prior to the beginning of the experiments a test emissions scan was run using the buffers prepared by the investigator and buffer solutions prepared by the previous research scientist who trained the investigator. No differences were found in results. This was done in order to verify the buffer solution was validated.

Final Phase III Study: Hypothesis-Testing

ž **Hypothesis Re-stated:** Immortalized Human B-lymphocyte cell lines derived from a known MHS population, will display increased sensitivity to RYR1 agonist 4-CmC in the presence of the catecholamine norepinephrine; this increased sensitivity will be demonstrated by an increase in cytoplasmic fura-2 fluorescence in the presence of various concentrations of the RyR1 agonist 4-CmC when pre-incubated with a near- maximum effective norepinephrine concentration.

Phase III Protocol Measurements

Based on the preliminary findings from Phase I and II, the final experimental protocol was developed to examine differences in Fura 2 Ca^{2+} emissions between MHN and MHS Human B-lymphocytes in the absence and presence of norepinephrine. The experiments were designed to compare the following:

1. Measurements of Fura 2 Ca^{2+} emission ratios in response to the RyR1 agonist 4-CmC in the absence of norepinephrine used at concentrations of 1μM, 1.5μM and 2.0 μM, respectively.

2) Measurements of Fura 2 Ca^{2+} emission ratios in response to 4-CmC 1 mM, 1.5 mM and 2.0 mM in the presence of norepinephrine in concentrations of 1.0 μM;

3) Measurements of Fura 2 Ca^{2+} emission ratios in response to 4-CmC in the presence of 1 μM norepinephrine; following prior incubations with specific adrenergic blockers phentolamine (alpha-adrenergic blockade) and propranolol (beta-adrenergic blockade) in concentrations of 2 μM each.

4) Measurement of the size of the internal stores of Fura 2 Ca^{2+} by using thapsigargin, which served to validate cell line viability on day of measurements

Phase III Experimental Protocol

Cells were maintained in RPMI 1640 L-Glutamate with 5ml of Bovine Serum and 5.5 ml of streptomycin and Penicillin (PCN). Cells in RPMI were maintained in an incubator at 37 ° Celsius in a 5% Carbon Dioxide and 95% Oxygen atmosphere. Incubator pressure was set at 10 mmHG PSI. The incubator humidity was 80 to 90%.

Cell Preparation Procedure Day of Study

B-lymphocytes samples were centrifuged at 2000 RPM X 2 minutes and RPMI growth medium was suctioned from the flask. Cells were washed twice with HBSS containing fetal bovine serum (albumin) and re-suspended in 10ml HBSS containing 0.1G Fetal Bovine Serum and stained with Fura-2 AM dye in a final concentration of 5μM and incubated for a minimum of 40 minutes at 37 ° C in a dark water bath. After 40 minutes cells were removed from the water bath and centrifuged X 2 minutes at 2000 RPM and re-suspended in HBSS and placed in the dark at room temperature

(≈22 ° C) for a minimum of 40 minutes prior to beginning first emission's measurements. This delay was needed to allow for the removal of the ester linkage from the Fura 2 in order to allow for the free dye to be sequestered and distributed inside the cellular cytoplasm.

After a minimum of 40 minutes 1ml aliquots of cells were re-spun at 5000 RPM X 30 seconds to remove any extra-cellular dye from solution and re-suspended in a 3-ml quartz cuvette using 1ml Krebs buffer containing Ca^{2+}. An additional 1ml of buffer was added to each cuvette prior to placement of the 1ml cells in suspension. Buffer was maintained at 37 °C during procedure. Cuvettes with magnetic stirrer magnets were pre-warmed at 37 ° C. Cells in suspension in final volumes of 2ml were placed in the Quantamaster fluorometer (Photon Technology, Inc., Monmouth, NJ) thermostated at 37 ° C and fluorescence measurements was taken approximately every 0.5 seconds using a dual excitation wavelength (Figure 7 below).

Fluorescent measurements were taken in stepwise fashion for the effect of 1 ⌠M norepinephrine on Ca^{2+} mobilization responses to 4-CmC for each sample cell line. Resulting emission curves were exponentially smoothed to remove noise. Baseline area (BA) was calculated by measuring the first 50 seconds of emission prior to the addition of 4-CmC. AUC was measured from the time of 4-CmC dosing (50 seconds into experiment) and extended for 250 seconds. This period to measure the AUC was chosen because Phase I and II data showed that after 250 seconds the MHS cells started to become unstable.

Measurements were taken and calculated using PTI Software Package ® by accessing the math applications. Fura 2 Baseline Measurements (BA), Area Under the Curve (AUC) and Fura 2 Peak ratio emissions (PE) were calculated by the PTI® software and entered into a spreadsheet.

Gaussx Statistical Program was used to analyze emissions ratio response to 1 ⌠M, 1.5⌠M and 2.0 ⌠M doses of the RyR1 agonist 4-CmC in the absence and presence of 1 ⌠M norepinephrine.

Statistical significance was defined as P <. 05, using ANOVA and paired t-tests measure differences within and between groups. A multivariate regression analysis was used to examine model validity and the effect of catechlamines on 4-CmC induced Ca^{2+} release. An overview of the Hypothesis Testing Phase is shown below in Figure 5 and an example of screen photograph of Fura-2 Emission Ratio measurements in a MHS cell line experiment is shown in Figure 7 below.

Following ANOVA analysis a multivariate analysis (linear regression model) was performed to examine the model validity. Multivariate regression was used to examine the significance of the independent variables of Baseline Area, Dose of 4-CMC, and effect of norepinephrine on the Dependent Variable of AUC. Separate analyses were done for the MHN group and the MHS group. The models were forced through zero (assuming that at zero Ca2+ there would be no emission from Fura-2); the goodness of fit of these models was examined using the Durbin Watson algorithm (test for serial errors and autocorrelations between the independent variables in the model) and the heteroscedasticity routine (test for randomness of errors around fitted data). Because serial errors and lack of randomness of variances was indicated in the linear multivariate models, the fit routine applied the Newey-West algorithm of Gaussx 9 software to account for possible errors in the estimated SEM's of the coefficients of the independent variables. Without such corrections the SEM's of the coefficients of the independent variables typically overstate the precision of the fit and cannot be trusted.

Figure 7: Photograph of photomultiplier screen display showing Fura 2 emission curves from MHS Human B-lymphocytes response to the RyR1 agonist 4-CmC (1.5 mM) in the absence/presence of 1 ⌠M norepinephrine and 1 ⌠M norepinephrine blocked with phentolamine. 50-second baseline area measurement is highlighted in grey.

CHAPTER 4

Results

Phase I and II Epinephrine Trials/Absence of Ca^{2+} in Buffer

Phase I of the study consisted of measurements of baseline Ca^{2+} emissions from the EBV immortalized Human B-lymphocyte cell lines in the presence or absence of the catecholamine epinephrine HCL. No RyR1 agonist was used in this phase. The purpose was to ascertain if the catecholamine had any effect on emissions in the absence of a RyR1 agonist. Based on previous studies using immortalized B-lymphocytes, a Ca^{2+} free Krebs Buffer was used for the suspension medium for the cells during the measurement cycle. Figure 8 is a screen image photograph taken directly from the results of the baseline measurements on Fura 2 emissions in response to increasing doses of epinephrine. In Ca^{2+} free Krebs Buffer, following 7 samples (4 MHN and 3 MHS), the mean Fura 2 emissions BA results for MHN samples was 48.36 +/-1.04 in the absence of epinephrine and 47.88 +/- 1.25 in the presence of epinephrine. MHS samples showed a mean of 47.28 +/- 1.89 in the absence of epinephrine and 47.91 +/- 1.72 in the presence of epinephrine. There was no statistical difference between MHS and MHN samples ($P > .05$) on baseline effect of epinephrine in terms of Ca^{2+} emission at baseline in the absence of a RyR1 agonist (See Figures 9 and 10 below). Performing baseline and RyR1 agonist portions of the study con-currently made most effective use of the B-cell line samples. Baseline effects were determined by the first 50 seconds of emissions; then the experiment was paused 4-CmC was added to measure for AUC and PE ratios.

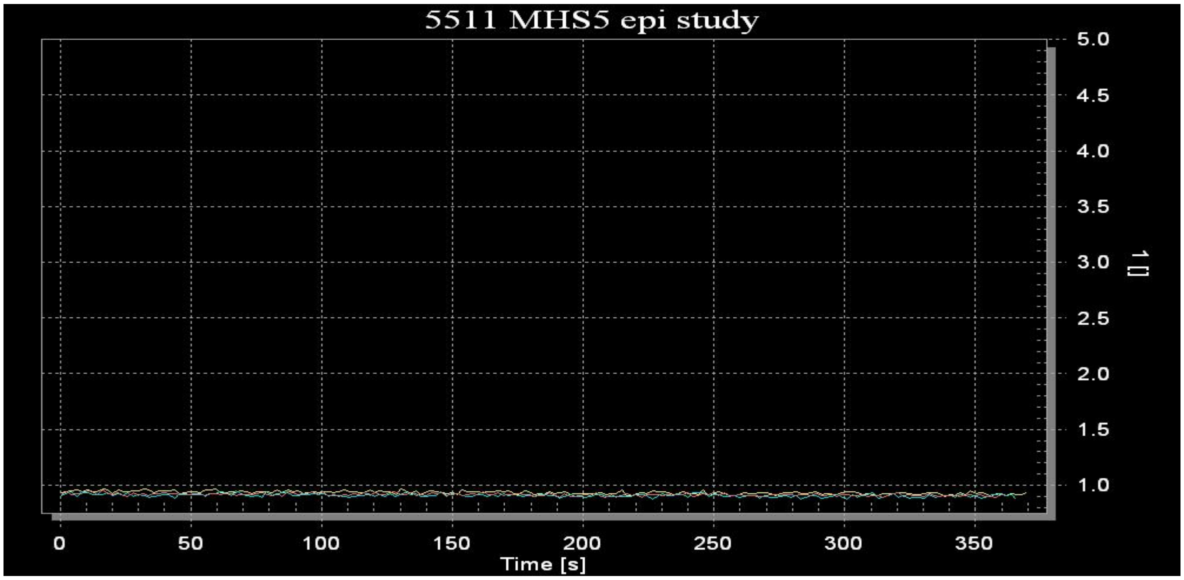


Figure 8: Photomultiplier Screen Photograph Example of BA Effect of Epinephrine on Ca^{2+} emissions on MHS sample: Epinephrine HCL was added to Human B-lymphocytes from a MHS cell line and baseline Fura 2 Ca^{2+} emissions were measured to examine the effect of the catecholamine of the model in the absence of any RyR1 agonist. Legend: Red Emissions line: Epinephrine 10.0 uM added at 0 seconds

Blue Emissions line: Epinephrine 1.0uM added at 0 seconds

Yellow Emissions line: Control (no Epinephrine added)

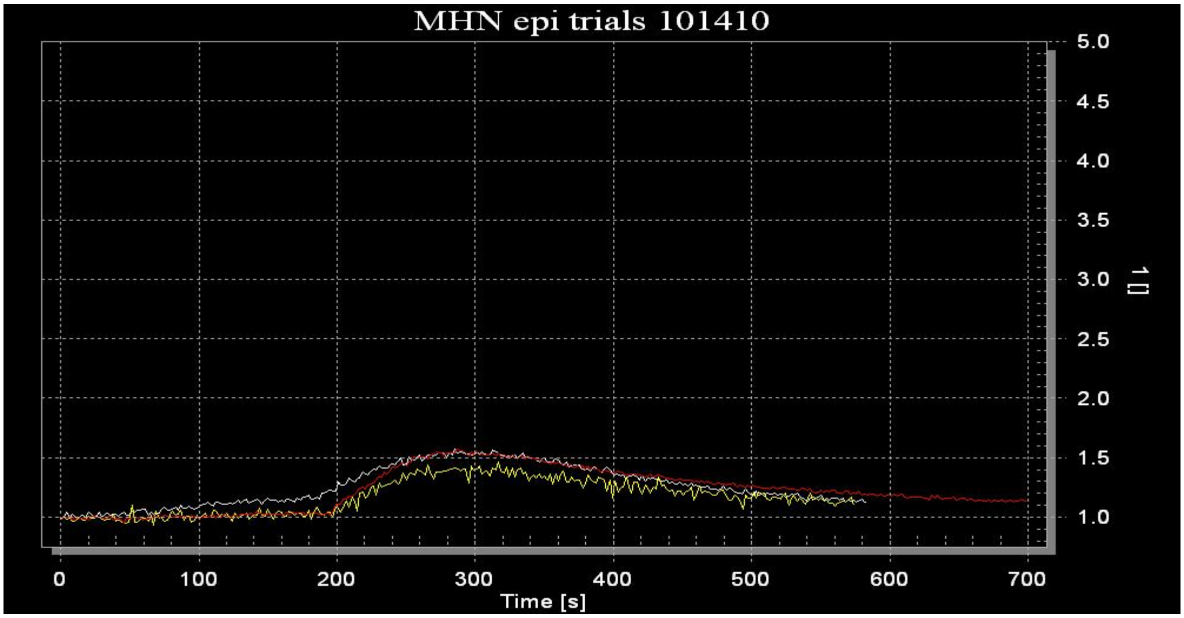


Figure 9: Photomultiplier Screen Photograph Example of Effect of Epinephrine and 4-CmC on Fura 2 Ca^{2+} emissions on MHN Sample. Legend: Aqua Emissions Curve: 1mM 4-CmC alone added at 200 seconds

Yellow Emissions Curve: 1uM epinephrine at 10 seconds and 1mM4-CmC added at 200 seconds

Red Emissions Curve: 1uM epinephrine at 0 seconds and 1mM 4-CmC added at 200 seconds.

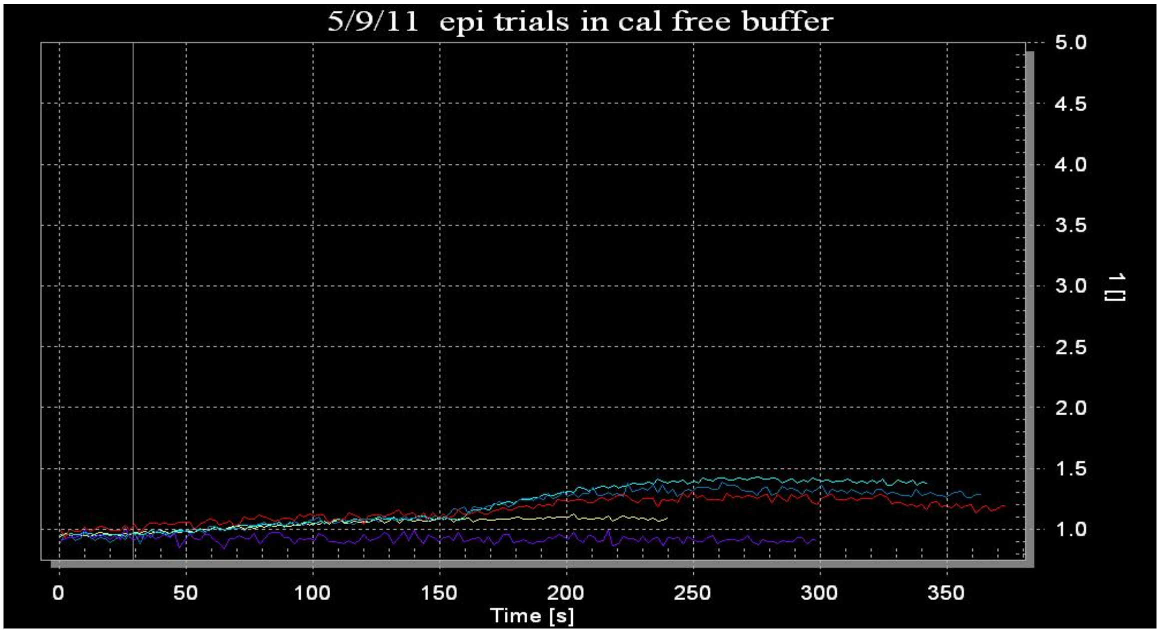


Figure 10: Photomultiplier Screen Photograph: Fura 2 Ca $^{2+}$Emissions in an MHS Cell line Response to Epinephrine in Ca^{2-}free buffer. Legend: Yellow Emissions Curve: Baseline Emissions in the absence of epinephrine or 4-CmC; Purple Emissions Curve: 10uM epinephrine for 30 minutes prior to measurement Red Emissions Curve: Incubated with 1uM epinephrine for 30 minutes; 1mM 4-CmC at 100 seconds
Blue Emissions Curve: 1mM epinephrine added at 0 seconds; 1mM 4 CmC added at 100 seconds
Aqua Emissions Curve: Control (1mM 4 CmC in the absence of epinephrine).

Results of Epinephrine Response Studies

There was no statistically significant difference in Fura 2 Ca^{2+} emissions in the presence or absence of epinephrine on BA, or in augmentation of 4-CmC agonism of the RyR1 receptor in this model as determined by measurement of AUC or PE Mean differences. Peak Emission Ratios were 1.49 +/- 0 .04 vs 1.52 +/- 0.07 in the absence or presence of epinephrine, respectively (P > .05). The effect of epinephrine on the AUC following administration of 4-CmC showed no significant differences in emission with AUC mean of 271.20 (SEM 3.73) vs 287.38 (SEM = 17.34; P > .05). Finally, there was no statistically significant difference between Fura 2 BA emissions (M=47.08; SEM = .66 vs M = 47.44; SEM = .52) in the absence and then in the presence of epinephrine (P > .05).

Comparison between MHS and MHN samples for differences in mean effects showed no statistical significance ($P > .05$) with MHN mean for AUC without epinephrine at 276.34 (SEM =5.30) vs MHS sample means of 264.34 (SEM= 0.0). Independent Samples two-tailed t-test for comparison of AUC measurement of Fura 2 Ca^{2+} emission in the presence of epinephrine was calculated as M= 312.55 (SEM = 23.3) vs M= 253.82 (SEM= 5.84) for MHN vs MHS cell lines.

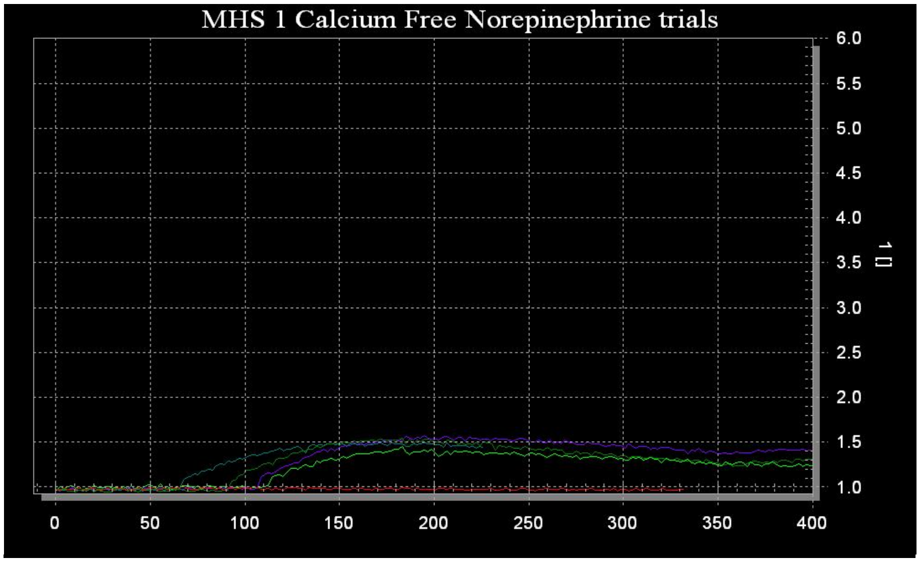
Phase I and II Norepinephrine Trials/Absence of Ca^{2+} in Buffer

Following the Epinephrine studies, comparisons of all the same parameters were undertaken to determine the effect of the catecholamine norepinephrine on augmentation of RyR1 Ca^{2+} emissions (Figure 11 below). Results described below showed no statistically significant difference in Fura 2 Ca^{2+} emissions in AUC, BA or PE in the presence or absence of norepinephrine:

BA: Results for experiments conducted on MHS and MHN EBV immortalized Human B-lymphocytes to examine the effects of norepinephrine on BA ratios showed the mean differences of 50.27 +/- 5.05 vs 51.54 +/- 7.51 for measurements in the absence or presence of norepinephrine respectively ($P > .05$).

PE: PE results for measurements in the absence of norepinephrine were 1.70 +/- .027 vs 1.74 +/- .22 for those in the presence of epinephrine ($P > .05$).

AUC: AUC measurements following 4- CmC dosing were 302.94 (SEM 19.93) vs 303.12 (SEM= 25.35) in the absence or presence of norepinephrine respectively ($P > .05$).



<u>Figure 11:</u> Example of Photomultiplier Fura 2 Ca2^{+} emissions in a MHS Sample in Ca^{2} free Buffer
Legend: Red Emissions Curve: Baseline emissions in the absence of 4 CmC or norepinephrine
Lime Emissions Curve: 1uM norepinephrine and 1mM 4 CmC
Purple Emissions Curve: 10 uM norepinephrine and 1mM 4 CmC
Green Emissions Curve: 1mM 4 CmC in the absence of norepinephrine
Teal Emissions Curve: Repeat of 1uM norepinephrine and 1mM 4 CmC.

<u>Phase I and II Trials Results: Presence of Ca^{2+} in Buffer</u>

Subsequently comparison studies were conducted to compare the effect of Ca^{2+} in the buffer and the effect this would have on catecholamine augmentation on RyR1 agonist 4- CmC on Ca^{2+} PE and BA emission ratios. This was the result of reading on lymphocytes and the effect of a Ca^{2+} free environment on emissions in this model. In addition, as we are expecting an effect from catecholamine attachment to adrenergic receptors on the cell membrane effecting the cAMP accumulation and subsequent phosphorylation prior to RyR1 release of Ca^{2+} stores from the endoplasmic reticulum (McKinney, 2006). Figure 12 is a representative screen photograph showing emissions in the presence of Ca^{2+} containing buffer.

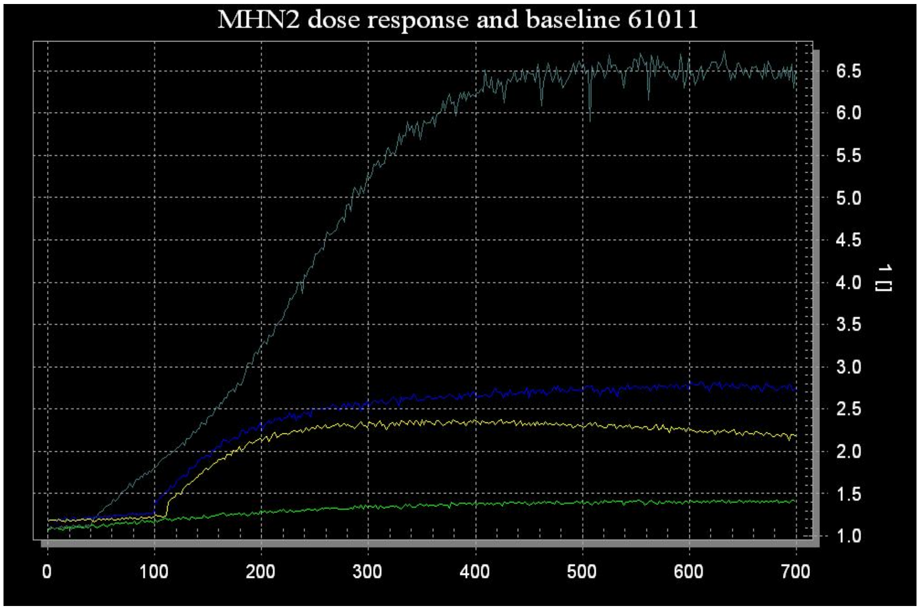


Figure 12: Example of Fura 2 Ca2^{+} emissions from MHN sample in Ca^{2+} containing buffer
Legend: Blue Emission Curve: 1uM norepinephrine with 1mM 4-CmC added at 100 seconds
Yellow Emissions Curve: 1mM 4-CmC in the absence of norepinephrine
Teal Emissions Curve: Thapsigargin 40nM
Green
Emissions Line: Baseline emissions 1uM Norepinephrine with no 4-CmC added

Buffer Study Results/Norepinephrine and 4-CmC

Studies on buffer types were performed to explore the possible effect of the presence or absence of Ca^{2+} in the buffer on RyR1 mediated myoplasmic Ca^{2+} levels. For the effect on buffer type and norepinephrine the effect on baseline Fura 2 emissions were statistically significant (CFB M = 51.68, SEM = 2.75 vs CCB M= 65.50, SEM=3.74; $P > .05$).

The presence of Ca $^{2+}$ in the buffer did make a significant difference in the 4-CmC Fura 2 Ca $^{2+}$ emissions ratios as determined by mean differences in AUC and PE in the presence of norepinephrine ($P < .01$). Peak Emissions Ratio means were 1.74 (SEM = .10) for CFB and 3.58 for CCB (SEM= .34). Mean AUC Fura 2 Emission ratios for CFB were 305.21 (SEM=15.38) vs 578.84 (SEM= 56.99) for CCB.

Buffer Study Results/Epinephrine and 4-CmC

Difference in the effect of the catecholamine epinephrine on RyR1 release of Ca $^{2+}$ in the presence of CCB VS CFB was also measured. Peak Emission means of Fura 2 Ca^{2+} emission ratios were statistically significant (CFB M=1.53; SEM .05 vs CCB M = 2.86;SEM = .03; $P < .01$) even when equal variance was not assumed. The AUC for this group also reached statistical significance (CFB M = 286, SEM=13.2) vs CCB M = 513.69, SEM= 11.5; $P < .01$).

Buffer Study Results: 4-CmC in the Absence of Catecholamines

Measuring the difference the effect of 4-CmC alone to elicit Fura 2 emissions ratio differences in the different buffers was also determined. In the absence of any catecholamine there remained a difference in effect of buffer on emissions. Both the AUC and PE ratio measurements showed Mean differences on emissions from cells suspended in CFB vs CCB. (AUC: CFB M = 269.34, N=5, SEM = 6.8 vs CCB M=505.51, N= 4, SEM = 75.9; PE: CFB M= 1.49, N=5, SEM = .02; CCB M= 2.94, N=4, SEM= .4). Using Levine' Test for Equality of Variances none of the mean differences reached Homogeneity significance ($P > .05$). However, even not meeting statistical significance, with such a large difference in the mean there obviously was an effect of buffer type the response to 4-CmC on the RyR1 Fura 2 Ca^{2+} emission ratios.

Phase III: Hypothesis Testing Results

The last phase of the study was undertaken to test the hypothesis that immortalized Human B-lymphocytes derived from a known MHS population would display increased sensitivity to the catecholamine, norepinephrine, as evidenced by an increase in myoplasmic RyR1 Ca^{2+} accumulation in response to the RyR1 agonist, 4 CmC, when compared to MHN controls.

Originally the plan was to measure the EC 50 as a percentage of Thapsigargin release from the cells. However, using a buffer with contains Ca^{2+} precluded this from being an effective measure. In addition, it was found that different MHS cell samples had very varied results in the face of catecholamines. For that reason we elected to use paired statistical t-test and split the results so that each sample could act as it’s own control and these were then compared for analysis. See Figure 13 for an outline of the Hypothesis Testing Timeline.

Figure 13: Hypothesis Testing Time Line

<u>Thapsigargin Results</u>

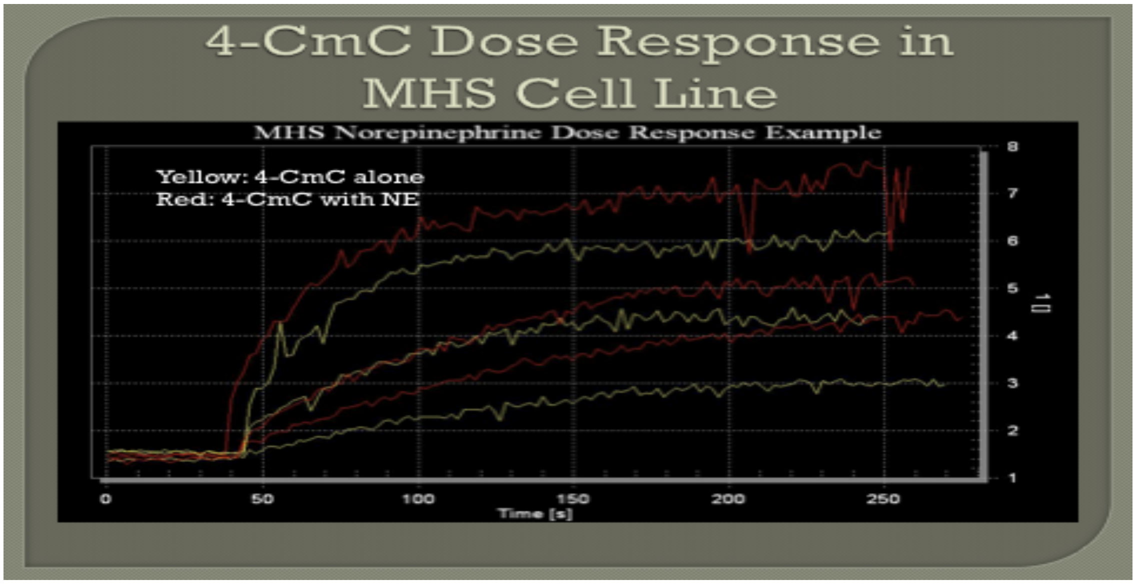
Although results were not measured as a percentage of Thapsigargin release we did elect to compare the results of the Thapsigargin measurements to compare total releasable Ca^{2+} stores between cell lines in order to demonstrate cell line viability and presence of total Ca^{2+} in the lines. The results showed no statistical difference (Mean MHN= 2.18 +/-.26 vs MHS = 2.22 +/- .21;P > .05) between cell lines (See figure 14 below).

Figure 14: Thapsigargin Results MHS vs MHN Cell Lines: Thapsigargin measurements to compare total releasable Ca^{2+} stores between cell lines in order to demonstrate cell line viability and presence of total Ca^{2+} in the lines. The results showed no statistical difference (Mean MHN= 2.18 +/-.26 vs MHS = 2.22 +/- .21;P > .05) between cell lines.

MHS vs MHN Emission Dose Response Results

Results for this study looked at Human B-cells Fura 2 Ca $^{2+}$ emissions ratios in response to 1⌈M, 1.5⌈M and 2.0 ⌈M doses of the RyR1 agonist 4-CmC and 1⌈M norepinephrine (NE) to determine statistically significant (P <. 05) differences between MHS vs MHN samples. There were three areas that were used for this analysis, the baseline area (BA), the area under the emissions curve (AUC) and the Peak Emissions Ratios (PE). First a general Analysis of Variance (ANOVA) was run using Gaussx Statistics Program. Descriptive Statistics for each group are displayed in Table 1-3. ANOVA was performed to analyze three-dose response to 4 CmC at 1⌈M, 1.5 ⌈M and 2.0 ⌈M of 4 CmC in the presence and absence of Norepinephrine in a final concentration of 1⌈M. A representative screen shot of MHN vs MHN are shown in Figures 15 and 16 below.

Figure 15: Photomultiplier Screen Photograph showing an MHN cell line of Human B-lymphocytes Fura 2 Ca^{2+} emission ratios exhibiting a dose response to 1.0 mM (lowest curves), 1.5mM (middle curves) and 2.0mM 4-CmC (highest curves) in the presence and absence of 1⌠M norepinephrine. Red: In the presence of norepinephrine; Yellow: 4-CmC in the absence of norepinephrine.



4-CmC Dose Response in MHS Cell Line

Figure 16: Photomultiplier Screen Photograph showing a MHS cell line Human B-lymphocytes Fura 2 emission ratio curves exhibiting a Dose Response to 1.0 mM (lowest curves), 1.5mM (middle curves) and 2.0mM (highest curves) 4-CmC in the presence and absence of 1⌠M norepinephrine. Red: In the presence of norepinephrine; Yellow: 4-CmC in the absence of norepinephrine.

ANOVA Results: MHS vs MHN Cell lines in the Presence/Absence of Norepinephrine

žMHS vs MHN groups demonstrated statistically significant differences in MHS vs MHN cell lines in response to norepinephrine augmentation of 4-CmC induced Fura 2 Ca^{2+} emissions when comparing Base Area (BA), Peak Emissions (PE) and Area Under Curve (AUC) ($P < .05$; Effect Size = 0.96).

žFinding that Fura 2 Baseline Ca^{2+} emissions means (BA) from MHS vs MHN cell lines were significantly different in the presence of norepinephrine ($P< .05$), this area was subtracted from each cell line AUC measurement prior to determining dose response.

AUC minus BA comparison showed a statistically significant difference in Fura 2 Ca^{2+} emissions between MHS and MHN cell lines in response to the RyR1 agonist 4-CmC both in the absence and presence of norepinephrine ($P < .05$).

Significant differences in Peak Emission Ratios between MHS and MHN cell lines were also demonstrated ($P = 0.01$). Statistical representation of AUC and PE results are illustrated in Figures 17 and 18 below.

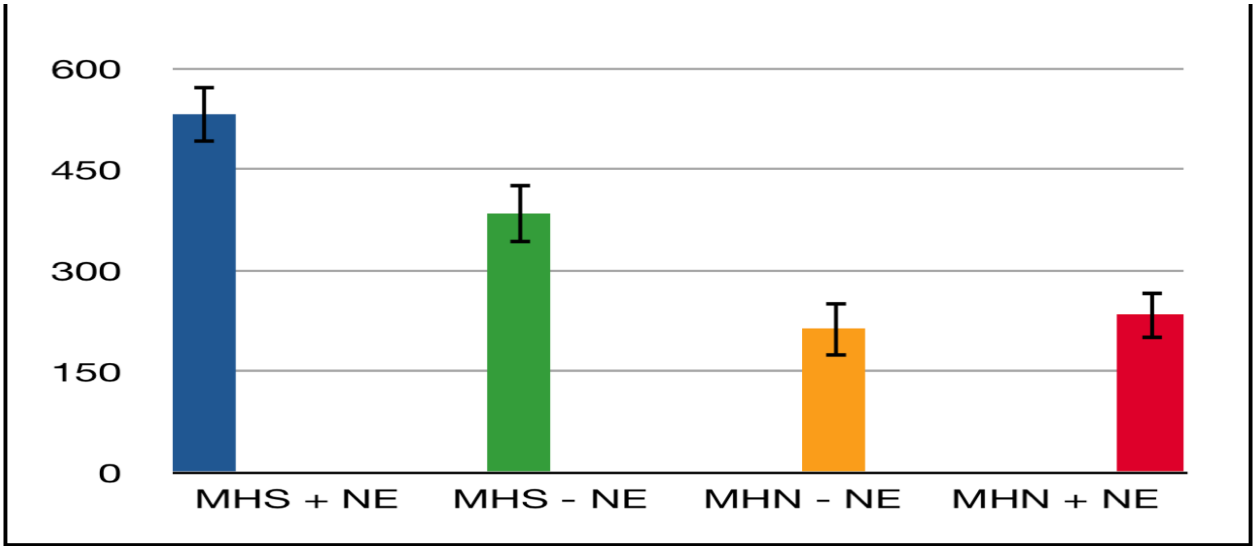


Figure 17: AUC Comparison for Fura 2 Ca^{2+} Emissions in MHS vs MHN Human B. Lymphocytes Response to RyR1 Agonist 4- CmC in the Presence or Absence of 1uM Norepinephrine. Legend: * No Significant Results; ** Significant Results. *Comparison of MHN cell lines response to 4-CmC in the absence/presence of norepinephrine (P > .05). ** Comparison of MHS cell lines response to 4-CmC in the absence/presence of norepinephrine (P < .05). ** Comparison of MHS vs MHN B-cell lines response to 4-CmC in the absence/ presence of norepinephrine (P < .05).

Figure 18: PE Comparison for Fura 2 Ca^{2+} Emissions in MHS vs MHN Human B. Lymphocytes Response to RyR1 Agonist 4- CmC in the Presence or Absence of 1uM Norepinephrine. Legend: * No Significant Results ** Significant Results Note: *Comparison of MHN cell lines response to 4-CmC in the absence/presence of norepinephrine (P > .05). ** Comparison of MHS cell lines response to 4-CmC in the absence/presence of norepinephrine (P < .05) ** Comparison of MHS vs MHN B-cell lines response to 4-CmC in the absence/ presence of norepinephrine (P < .05).

Dose Response Results Comparing MHS vs MHN Cell Lines

A Dose Response analysis of AUC, BA and PE ratios in responses to 4-CmC at three doses (1.0 ⌠M, 1.5 ⌠M and 2.0 ⌠M) were examined to look for differences between the MHN and MHS groups in the presences or absence of norepinephrine. Descriptive statistics are shown below in Tables 1, 2 and 3 below. Paired t-test analysis revealed that there were significant differences between MHS and MHN cell lines Fura 2 Ca^{2+} emission measurements for PE, AUC and BA in the presence of 1 ⌠M norepinephrine at each dose. Results are presented in Tables 2 and 3 below.

	1 mM 4 CmC *	1.5mM 4 CmC**	2mM 4 CmC***
MHN - NE	412.56 SEM 9.37	488.58 SEM 17.11	648.46 SEM 43.84
MHN + NE	428.08 SEM 9.82	503.60 SEM 28.32	649.13 SEM 40.83
MHS - NE	509.56 SEM 44.55	641.85 SEM 76.76	1322.50 SEM 391.21
MHS + NE	619.59 SEM 57.17	803.70 97.01	1539.70 SEM 368.75

Table 2: MNS vs MHN AUC Fura 2 Ca^{2+} 4-CmC Dose Response Emissions in the Presence or Absence of Norepinephrine 1uM. Paired t-test compared MHS vs MHN AUC mean differences in Ca2+ emissions response to the presence or absence of norepinephrine to 1.0 mM, 1.5 mM or 2.0 mM 4 CmC. Results were: *1 mM: $P < 0.05$; **1.5 mM: $P < 0.05$; ***2.0 mM: $P < .01$

	1 mM 4 CmC *	1.5mM 4 CmC **	2mM 4 CmC***
MHN - NE	2.39 SEM 0.72	2.83 SEM 0.13	3.73 SEM 0.21
MHN + NE	2.51 SEM 0.06	2.93 SEM 0.20	3.84 SEM 0.20
MHS - NE	2.97 SEM 0.26	4.02 SEM 0.67	8.23 SEM 0.67
MHS + NE	3.72 SEM 0.31	5.09 SEM 0.64	9.23 SEM 0.64

Table 3: MHS vs MHN Peak Fura 2 Ca^{2+} Emission Ratios 4-CmC Dose Response in the Presence or Absence of Norepinephrine 1uM. Paired t-test compared MHS vs MHN PE mean differences in Ca^{2+} emission response to the presence or absence of norepinephrine to 1.0 mM, 1.5 mM or 2.0 mM 4-CmC.ž Results were: *1 mM: $P < 0.05$, df = 22; **1.5 mM: $P < 0.05$; ***2.0 mM: $P < 0.02$

Multivariate Analysis

Following ANOVA analysis a multivariate analysis was performed to examine the model validity. Multivariate regression was used to examine the significance of the independent variables of Baseline Area, Dose of 4-CMC, and effect of norepinephrine on the Dependent Variable of AUC. Separate studies were performed on the MHN group and the MHS groups. The models were forced through zero and the Durbin Watson algorithm was used to test for autocorrelations between the independent variables in the model.

For the MHN cell lines the regression yield $R^2 = 0.69$ with a Durbin Watson statistic of 1.14 and a Heteroscedasticity of 0.023. All independent variables were found to be statistically significant. (Dose of 4 CmC: $t = 5.37$, $P < .01$; BA: $t=5.85$, $P < .01$; Presence of Norepinephrine: $t= 3.12$, $P <. 01$).

The MHS model retained validity with a regression yield $R^2 = 0.62$, DW = 1.32 and Heteroscedasticity = 0.021. All 3 independent variable retained their significance with Dose of 4-CMC: $P < .01$; Baseline Ca^{2+} response, $P < .02$; and norepinephrine, $P < .01$.

The significance of norepinephrine in the model was greater in the MHS cell lines than the MHN cell lines, supporting the hypothesis of the study that MHS cells would be more sensitive to the effects of catecholamines.

From the two regression fits we estimated that the effect of NE to be responsible for approximately a 30% increase in RyR1 sensitivity to 4-CMC as demonstrated by increase in ACU Fura 2 Ca^{2+} emissions.

Phase IV: Adrenergic Blocker Study Results

Following experiments on the effect of the adrenergic agonist norepinephrine on augmentation of Fura 2 Ca^{2+} emissions elicited by the RyR1 agonist 4-CmC, we examined the effect of adrenergic blockers on the emissions measurements in this model. Although this was not a separate aim of the study, it was determined that it would be important to examine the effect of adrenergic blockers to begin to look at whether the effect was 〈 or ®-adrenergic stimulation.

Cells were again grown IAW the protocol use in Phase III above. Three different groups of test were run. All adrenergic agonist and blockers were constituted just prior to the experiment and the methods were the same as those in Phase III. Blockers chosen were phentolamine HCL to block the effect 〈 effect of the norepinephrine and propranolol to block the ® effect of norepinephrine. Isoproterenol was used to examine the pure ® effect on the model and propranolol was used to examine the ® blocker effect in the model. Paired Student's t-test were performed using Graphpad Software® with a statistical significance set at P < .05. The Norepinephrine/Propranolol Blocking protocol is outlined in Figure 19 below.

Norepinephrine Propranolol Results

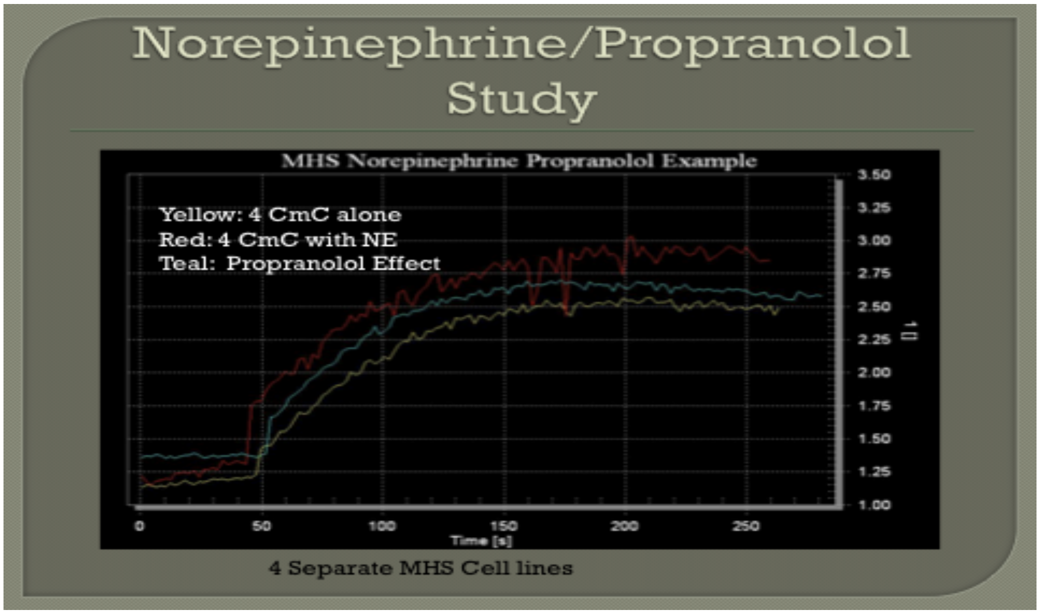
For this study 4 MHS groups of cells were selected based on previous response to norepinephrine in Phase III of the study. For blocker effect, 2 ⌠M (final concentration/2 ⌠L) propranolol was added 30 minutes prior to emissions measurements. Just prior to measurement, cells were spun at 5000 RPM X 30 seconds and fresh buffer was added for a total volume of 2mL to which 1uM norepinephrine was added. Emissions baseline measurements were taken at 50 seconds. 4-CmC was added at this point and the AUC and PE Peak were read at 250 seconds (200 seconds after the 4 CmC was added). All results were taken using PTI software.

Figure 19: Norepinephrine/Propranolol Protocol Outline

There remained a statistically significant difference in the AUC emissions (BA removed) in the absence or presence of norepinephrine in the cells in this study (M= 452.01 +/- 53.18 vs 553.05 +/- 66.48; P < .02). Comparisons between AUC emissions in cells incubated with propranolol prior to augmentation with norepinephrine were also statistically significant from those in the absence of norepinephrine (M=482.29 +/- 62.93 vs 452.01 +/- 53. 18; P < .03). However, although there was a decrease in means there was no statistically significant difference between the emissions from the cells treated with norepinephrine and those incubated with propranolol prior to norepinephrine (M= 553.05 +/- 66.48 vs 482.29 +/- 62.93; P > .05).

Overall analysis showed that in this model, B-lymphocytes exposed to 2⌠M of propranolol for 30 minutes prior to exposure to 1⌠M norepinephrine and then the RyR1 agonist 4-CmC exhibited between a 48% and 83% reduction in AUC Ca^{2+} emissions.

B-Lymphocytes from MHN controls were also examined to ascertain the effects of propranolol on norepinephrine augmented RyR1 $Ca^{2=}$ emissions. Although there was a decrease in AUC and Peak emissions after cells were incubated with propranolol HCL for 30 minutes, the results were not statistically significant (P > .05). Figure 20 shows an example of a Norepinephrine/Propranolol cell line sample result screen photograph.



Norepinephrine/Propranolol Study

4 Separate MHS Cell lines

Figure 20: Photomultiplier Screen Photograph displaying Fura 2 Ca^{2+} emission ratio dose response curves from an MHS human B-lymphocyte cell line Legend: is 1.5mM 4-CmC in the Presence of 1uM Norepinephrine; Yellow Curve is 1.5mM 4-CmC in the Absence of Norepinephrine and Teal colored emissions curve is from cells incubated with 2uM of Propranolol for 30 minutes prior to administration of 1uM Norepinephrine and 1.5 mM 4 CmC. (4 CmC was placed at 50 seconds).

Norepinephrine/ Phentolamine Results

We next examined the effect of phentolamine (an 〈-adrenergic blocker) on the norepinephrine augmentation of 4-CMC induced Fura 2 Ca^{2+} emissions in the MHS B-lymphocyte model. The same protocol was used as described above in the propranolol blocking experiments. The time line is shown below in Figure 21 below. Initial test comparing AUC emissions (with BA emissions removed) and PE from the effect of 1.5 ⌠M 4-CmC in the presence and absence of 1 ⌠M norepinephrine demonstrated a statistically significant difference (P = 0.01). Results are shown in Figure 22 and 23 below. Figure 24 shows a representative photomultiplier screen photograph of Norepinephrine/Phentolamine cell line result.

MHN cells showed no statistically significant difference in the effect of phentolamine on the Ca^{2+} emissions augmentation from norepinephrine (M= 442.54 +/- 42.32 vs 439.52 +/- 31; P > .05). There were also no statistically significant differences in MHN peak emission ratios (P >.05).

Figure 21: Norepinephrine/Phentolamine Protocol Outline

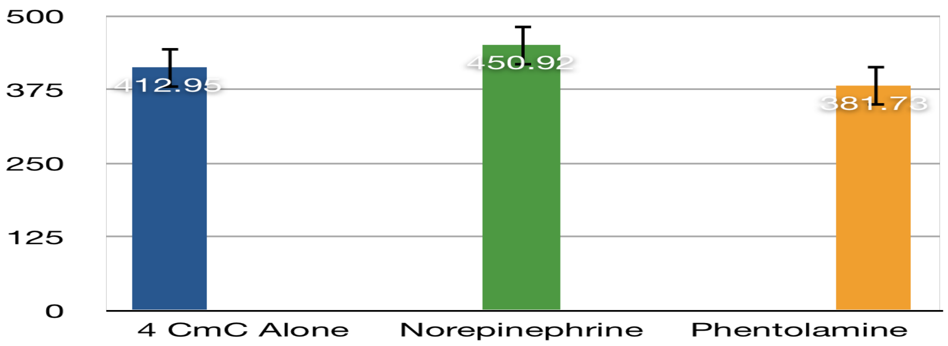


Figure 22: Effect of Phentolamine Incubation on response to norepinephrine and 4-CMC: AUC Ca $^{2+}$ emissions (Blue= MHS/ Green= MHN) <u>Results</u>: Paired t-test comparison of Ca^{2+} emission AUC Fura 2 ratios for MHS cell line response to 1.5mM 4-CMC in the presence or absence of norepinephrine and norepinephrine blocked with phentolamine. Results: Mean of the 4-CmC alone group (blue bar): 412.95 (SD =60.34) vs the norepinephrine group (green bar) at 450.92 (SD = 60.72). There remained a statistically significant difference between the norepinephrine (green bar) and phentolamine (yellow bar) groups (M= 450.92 vs 381.77; SD = 59.11; P = 0.01); however there was no statistical difference between AUC emissions in response to 1.5 ⌠M 4 CmC between the 4 CmC alone (blue bar) and phentolamine (yellow bar) group (P < .05; SD=13.13).

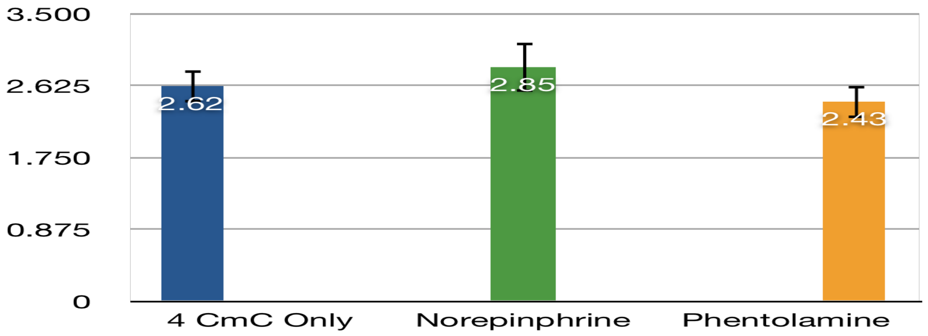
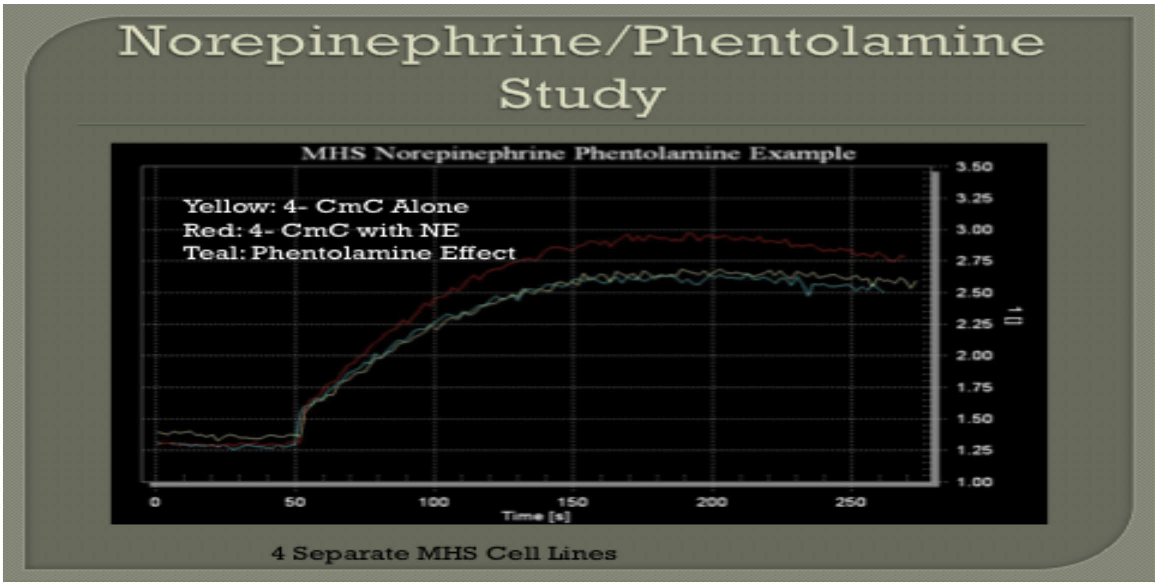


Figure 23: Effect of Phentolamine Incubation on Peak Ca $^{2+}$ emissions on MHS vs MHN Human B- Lymphocytes prior to Norepinephrine Augmentation of RyR1 agonist 1.5mM 4-CmC administration.
Note: Paired t-test comparison of Ca^{2+} emission PE Fura 2 ratios for MHS cell line response to 1.5mM 4-CMC in the presence or absence of norepinephrine and norepinephrine blocked with phentolamine. MHS cells also showed a statistically significant difference in PE between the norepinephrine and 4-CmC alone groups in response to 1.5 ⌠M 4-CmC (M=2.6 +/- .21 vs 2.85 +/- .21, P =. 02). The effect on emissions following incubation with phentolamine HCL for 30 minutes showed a decreased in PE below those seen with 1.5 ⌠M 4-CmC alone (2.62 vs 2.43) but this was not statistically significant (P< .05). However, there was a statistically significant difference in the PE when compared the phentolamine group vs those in the norepinephrine group. (M=2.43 vs 2.85, P = 0.01).



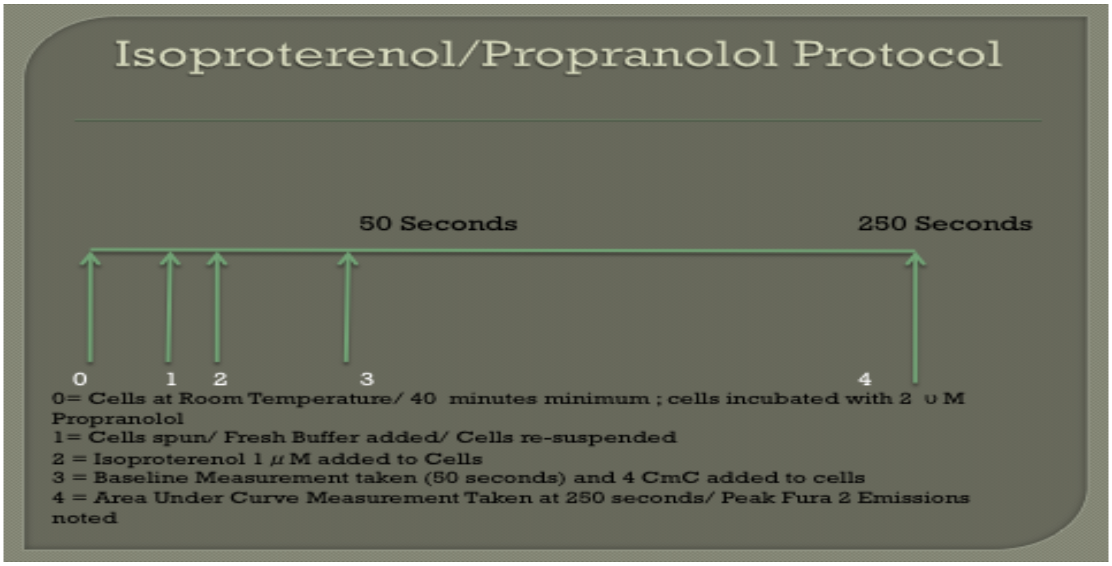
Norepinephrine/Phentolamine Study

4 Separate MHS Cell Lines

Figure 24: Screen Photograph of MHS Phentolamine Emissions Study
Legend: Red Curve is 1.5mM 4-CmC in the Presence of 1uM Norepinephrine; Yellow Curve is 1.5mM 4-CmC in the Absence of Norepinephrine and Teal Curve is Phentolamine 2uM response to 1and1.5mM 4-CmC in the Presence of 1uM Norepinephrine.

Isoproterenol/Propranolol Results

Results were next obtained for the effect of the ® agonist isoproterenol on the model to identify the pure ® effect on the Ca^{2+} emissions and the ability of the ®-blocker, propranolol to block the effect of the isoproterenol. This protocol is outlined in Figure 30 below.



Isoproterenol/Propranolol Protocol

0= Cells at Room Temperature/ 40 minutes minimum ; cells incubated with 2 υ M Propranolol
1= Cells spun/ Fresh Buffer added/ Cells re-suspended
2 = Isoproterenol 1 μ M added to Cells
3 = Baseline Measurement taken (50 seconds) and 4 CmC added to cells
4 = Area Under Curve Measurement Taken at 250 seconds/ Peak Fura 2 Emissions noted

Figure25: Isoproterenol/Propranolol Protocol Timeline

When BA was removed from the AUC results our analysis failed to show a statistically significant difference in the emissions AUC for RyR1 agonist 4-CmC in the absence or presence of isoproterenol (M=362.65+/- 55 vs 365.03 +/- 42.83; P > .05, df=3). However, in cells incubated with propranolol the AUC was decreased when compared with both of the other groups (330.28 +/- 41.42) and although this difference did not reach statistical significance (P > .05), it was interesting that the effect of propranolol did decrease the effect of 1.5 ⌠M 4-CmC alone to almost at statistically significant level.

There were no statistically significant differences in PE responses (4 CmC alone: M = 2.29 +/- .31; Isoproterenol + 4-CmC: M= 2.33 +/- 0.28; Propranolol prior to Isoproterenol and 4-CmC: M =

2.11 +/-.26 P >.05)). Again PE almost reached statistical significance (P = .06) when the 4-CmC alone and 4-CmC with the Propranolol group were compared (M=2.29 +/-.31 vs 2.11 +/- 0.13). See Figure 26 for a representative photomultiplier screen photograph.

Figure 26: Photomultiplier Screen Photograph of Fura 2 Ca $^{2+-}$ emissions curves from a MHS Human B-lymphocyte cell line showing an example of results from an Isoproterenol/Propranolol Emissions Study. Legend: Red Curves is 1.0, 1.5mM 4-CmC in the Presence of 1uM isoproterenol; Yellow Curves are1.0mM1.5mM 4-CmC in the Absence of Isoproterenol and Teal Curves are responses to 1.0uM and 1.5mM 4- CmC and 1uM Isoproterenol following incubations with 2uM Propranolol for 30 minutes prior to dosing

Chapter 5

Discussion

Overview

This research is the first successful demonstration of increased sensitivity of MHS cells to the adrenergic effect of a stress hormone. This supports the hypothesis of this experimental study, that Human B-lymphocytes from MHS individuals would display increased sensitivity to the RyR1 agonist 4-CmC in the presence of an adrenergic agonist when compared cells from MHN individuals.

This study also reports the first use of EBV immortalized human B-lymphocytes to examine differences in sensitivity to adrenergic agonists/blockers on Fura 2 Ca^{2+} emissions related to a specific pathologic condition. This same method can be used to examine adrenergic sensitivity differences in other immunologic pathologies.

ANS and MH

This research supports the findings of the early researchers in the area of Malignant Hyperthermia in their hypothesis that the Autonomic Nervous System (ANS) played a part in the triggering of MH. Early work by the previous researchers referenced in Chapter 2 of this dissertation, all espoused the belief that perhaps there was an important link between “stress” and the triggering or outcome of MH. The success of this research was precluded by the important work that came before. What was not possible until this time was an acceptable model, from which we could begin to examine the effect of catecholamines on MHS individuals. The other difference in the question raised between the previous researchers and this research was not the question of if there were higher levels of catecholamines in MHS individuals or animals, but if there was an increased sensitivity to the same level of catecholamines. Increased adrenergic receptor sensitivity was demonstrated for the first time by the results of this research.

Discussion/Importance of Method

Currently the only recognized method for the determination of an individual's susceptibility to MH requires a Caffeine Halothane Contracture Test (CHCT). The CHCT is only performed on approximately 10% of eligible patients due to the cost of the procedure, it's invasive nature and the requirement that the patient travel to one of only 4 medical centers in the United States that have the ability to perform the test (Bina et al., 2010).

One goal of the MH investigations currently underway at the Uniformed Services University is to develop a minimally invasive test with the sensitivity and specificity required to effectively test for MH. The identification of the RyR1 in Human B-Lymphocytes has been an important step in this direction. With the exception of one group of "control" cells, all of the cell groups that composed the MHN and the MHS components of this study were comprised of cells from individuals, or first degree relatives of probands, who had been referred to the MH Center at the University. Each of these individuals were believed to have experienced an intraoperative MH event and had undergone CHCT and genetic testing for MH susceptibility. Therefore, those individuals with cells selected for the MHN group for this study were ruled MHN based on a CHCT test. Prior to the results of the CHCT it was believed the individual had a high index of suspicion for being MHS. The ability of this method to provide a statistically significant differences in the release of Ca^{2+} emissions from the control and the experimental group is an indication that after further development, this method could be effective in identifying MHS vs MHN individuals.

One recommendation for further research will be to develop a prospective, double blind experimental study to combine genetic, CHCT, and B-Lymphocyte testing simultaneously on individuals and first degree probands referred to the University for MH testing. The aim would be to validate the ability of this Ca^{2+} emission protocol to identify MHS in individuals and to determine the sensitivity and specificity of the testing.

During this study it was noted that MHS cells had a propensity to "clump" more than MHN cells. In addition some of the MHS cells took up to 7 days longer to reach a density required for testing. Once cells were removed from the liquid nitrogen and place in growth medium (RPMI-1640) approximately 14 days was needed to reach the appropriate cellular density necessary to perform the measurements. However, if cells were not used within approximately 7 days from that time, it was noted that they did not respond to 4 CmC as well. Approximately 3% of the cells in the first set of baseline studies were not able to demonstrate stable baseline emissions or respond to 4-CmC. This was true for MHN as well and MHS cells. If it was determined that the cells were not able to give stable baseline or 4-CmC emissions curves they were not used for the study and were discarded. Additional cells from these groups were used for the studies were re-incubated and used for comparisons.

In addition, it was noted that the cells became unstable after approximately 2 hours at room temperature if Ca^{2+} was used in the buffer (less if buffer was Ca^{2+} free). For this reason all dose response experiments were completed within the 2-hour window. The decision to measure emissions at 250 seconds was based on observations over the first year, that after this time the MHS and MHN cells reached a plateau of emissions. If individual studies went past this time, we would be limited on the comparisons we could make due to the cells becoming unstable and experiencing wash out from the fura dye, both which would impact the results. In addition, limiting this time tenable us to use each group of cells, as it's own control without risking the loss of viable data. Therefore, another area for further study is to perform validation studies on the method to determine the reproducibility of the findings over time.

Importance of Ca2+ in Extracellular Buffer

The first finding in our study related to the importance of the presence of Ca^{2+} in the extracellular buffer. The original method that had been used for previous RyR1 research had been performed to show the presence, and reactivity of the RyR1 receptor in the B- lymphocytes. For this purpose it was necessary to eliminate any exogenous Ca^{2-} from the emissions.

However, in our model we could demonstrate no effect in the absence of exogenous Ca^{2+}. The transmitters used for this study were catecholamines, hydrophilic hormones, which rely on a transmembrane second messenger system. After catecholamines bind to the receptor they activate the enzyme adenylate cyclase, which in turn produces the second messenger cyclic adenosine monophosphate, (cAMP). It is the cAMP that acts as the messenger to effect the action initiated by norepinephrine. Once Ca^{2+} was added to the Krebs buffer it was apparent that the model was more appropriate for examining the effect to catecholamines on the augmentation of Ca^{2+} release in response to the RyR1 agonist, 4 CmC and immediately we began to observe statistically significant difference between results.

In human B-lymphocytes the importance of extracellular Ca^{2+} is very necessary for proper function of the cells in the immune process. Calcium not only acts in signal transduction, but in the function of cellular support structures and the initiation and cessation of calcium release itself. The importance of maintaining a close balance between extracellular and cytosolic Ca^{2+} concentrations is paramount to the survival of the cell (Engleke, et al, 2007). In order for the B-Lymphocytes to respond to stimulation by a hormone or other antigen interaction with a second messenger system is required.

In addition, once we added Ca^{2+} to the buffer we were able to identify a statistically significant difference in baseline Ca^{2+} emission ratios in MHS vs MHN Human B-Lymphocytes even in the absence of any reagents (M= 65.37 vs 60.93; t= 2.57; df=51; SE = 1.75). This supports findings of other MH researchers that individuals with MH susceptibility have increased resting levels of Ca^{2+} release at baseline.

Norepinephrine Effect

All measures of $Ca^{2}+$ emissions (AUC, BA and PE) demonstrated statistically significant differences when comparing MHS and MHN B-Lymphocytes in the presence of norepinephrine. Early researchers in the field of MH had reported finding increased levels of adenylate cyclase and cAMP in cells from MHS vs MHN individuals (Wilner, et al, 1979,1981). They postulated that while it was difficult to measure catecholamines due to their rapid metabolism; that the higher levels of the enzyme and second messenger were possibly related to higher levels of circulating catecholamines in these individuals.

In our study we have demonstrated that larger doses of catecholamines are not necessary to affect an intracellular increase RyR1 release of Ca^{2+}. Cells in this study were all subjected to the same 1⌠M concentration. While this study could not show an increased release of, or difference in metabolism of catecholamines in MHS individuals, our results do suggest that increased the cAMP may represent an increased sensitivity to normal physiologic levels of catecholamines in MHS patients. Future studies to measure the intracellular level of cAMP are now possible and provide an interesting possibility for exploring this in a subsequent study.

While it is known that B-lymphocytes from the MHS experimental group had both positive CHCT tests and a positive genetic profile for MHS, what is not know is why the adrenergic augmentation would increase the differences in the sensitivity to 4 CmC. The analysis of the model used showed that between 62 and 69% of the variance in the groups studied could be explained by the baseline response, the dose of 4 CmC and the presence or absence of norepinephrine. This leaves an additional 30% unexplained by our model.

Recent studies on MHS B-Lymphocytes have implicated a possible role for cytokines in the pathologic MH response. Girard et al., in a study published in The Journal of Biological Chemistry (2001) report that EBV immortalized B-Cells" produced more interleukin (IL)-1b after treatment with the RyR activators caffeine and 4-chloro-m-cresol". They go on to suggest that perhaps the symptoms seen during an MH episode may be related to the effects of the production of IL-1b (Girard, 2001).

It is the role of the immune cells, such as B-Lymphocytes, to produce antibodies and cytokines to protect the body from antigens and provide a competent immune system. The function of the adrenergic nervous system (ANS) is discussed in a paper presented by Kohm and Sanders in Pharmacological Reviews in 2001. The authors present an extensive overview of the ANS and the effect of norepinephrine effect on the ®-2 receptors in the immune system. In discussing the role of IL-1b, the author's state, "a significant number of studies have investigated the role of IL-1b in regulating the level of norepinephrine release in vivo (Kohn and Sanders 2001, p. 497). ®-adrenergic stimulation in B-cells results in activation of adenyl cyclase and the accumulation of cAMP stimulating the up-regulation of membrane surface proteins on the B-lymphocytes and influencing the production and actions of IgG and IgE. This has an implication in pathophysiologic conditions such as Down's syndrome, rheumatoid arthritis and multiple sclerosis (Kohn and Sanders, 2001,p. 519).

Therefore, it is possible that some of the un-explained increase in Ca^{2+} emissions found in our model was related to the effect of norepinephrine on cytokines in the B-lymphocytes and the possibility of different expression of these cytokines in MHS individuals. Further studies could include identification of cytokines and their effects on Ca^{2+} emissions in MHS B-lymphocytes.

Other possibilities to explain the variance include downstream effects from increased levels of adenylate cyclase and cAMP; effects from aberrant protein kinase (PKA) activity; or alterations in phosphorylation processes. In recent investigations, researchers have used skeletal muscle, knock in mice, and B-Lymphocyte models to investigate genetic differences in the function of the FK506 binding protein FKB12, Calsequestirin, and ATP levels in MHS groups (Gaburjakova, et al, 2001; Marx, et al, 2001, Chelu, et al, 2004; Lanner, et al, 2010; Dainese, M, et al. 2009; Dulhunty, A., et al, 2001). Many of these studies suggest that differences in phosphorylation processes involving multiple areas related to the stabilization of the RyR1 receptor, may be involved in the pathogenesis of MH and other RyR1 related disorders.

Blocker Discussion

The adrenergic blocker portion of this study was undertaken in order to describe the differences between the ⟨ and ®-adrenergic actions of norepinephrine in the model. The results were mainly examined in the MHS cells, where the largest effect from norepinephrine was demonstrated in the previous experiments in this study. First we looked at the effect of blocking the ® adrenergic effect of norepinephrine with the ® antagonist propranolol HCL. The results showed that only a portion of the increased in Ca^{2+} emissions increase in AUC and PE could be effectively blocked with propranolol. This ranged between 48% and 83% depending on the MHS cell group. This was similar to the finding that the difference in increased emissions ratios in response to 4-CmC in the presence or absence of norepinephrine in the MHS group overall was widely varied. However, in each of the individual cell groups the emissions ratios were decreased, and overall there was a statistically significant difference from the 4-CmC alone emissions and the 4-CmC with norepinephrine emissions, a significant difference in the 4-CmC and propranolol group, as well as smaller statistically significant difference in the norepinephrine and propranolol group. Therefore, even though the mean AUC and PE ratios were reduced when cells were incubated with propranolol prior to norepinephrine, only a portion of the Ca^{2+} emission increase was affected. This may indicate that the action of norepinephrine was mixed 〈 and ® in nature.

Examining the beta-adrenergic response by the use of isoproterenol to elicit the increased in Ca^{2+} emissions in MHS groups showed that there was no statistically significant differences in AUC emissions from cells in the absence or presence of isoproterenol when 4-CmC was administered to the cells at 1.5 ⌠M dosing; however there was a significant difference in PE between the isoproterenol group and the propranolol group. There has been research published on the effect of the adrenergic nervous system and the effect of propranolol on the proliferation of ® lymphocytes and of ®2 adrenergic receptors specifically (Aaron, et al, 1980, Maisel, 1990, Redwine, 1996, Kohm, 2001).

In addition, although there was no significant differences in BA between the groups (P > .05), we did note that in all groups, there was an increase in BA in those cells incubated with propranolol for 30 minutes prior to measurement and prior to 4-CmC dosing (4 CmC M= 53.19 +/- 3.63; isoproterenol M=57.42 +/-4.1 and propranolol with isoproterenol M=61.38+/- 8.13). The large variance in the MHS response to propranolol prior to isoproterenol was possibly the reason this BA comparison did not reach significance. Again, investigating the effect of propranolol on MHS cells is an area we recommend for further investigation.

Examining the 〈 adrenergic effects by using phentolamine to block the effects of norepinephrine were very successful. Phentolamine administered to cells 30 minutes prior to application of 1⌠M Norepinephrine and 1.5 ⌠M 4-CmC effectively blocked the increase in Ca^{2+} emissions ratios in AUC and PE measurements while there was no statistically significant difference in BA between any of the groups. This indicates that perhaps the 〈 effect of norepinephrine was the most significant adrenergic factor in the action of norepinephrine in MHS human B-Lymphocytes.

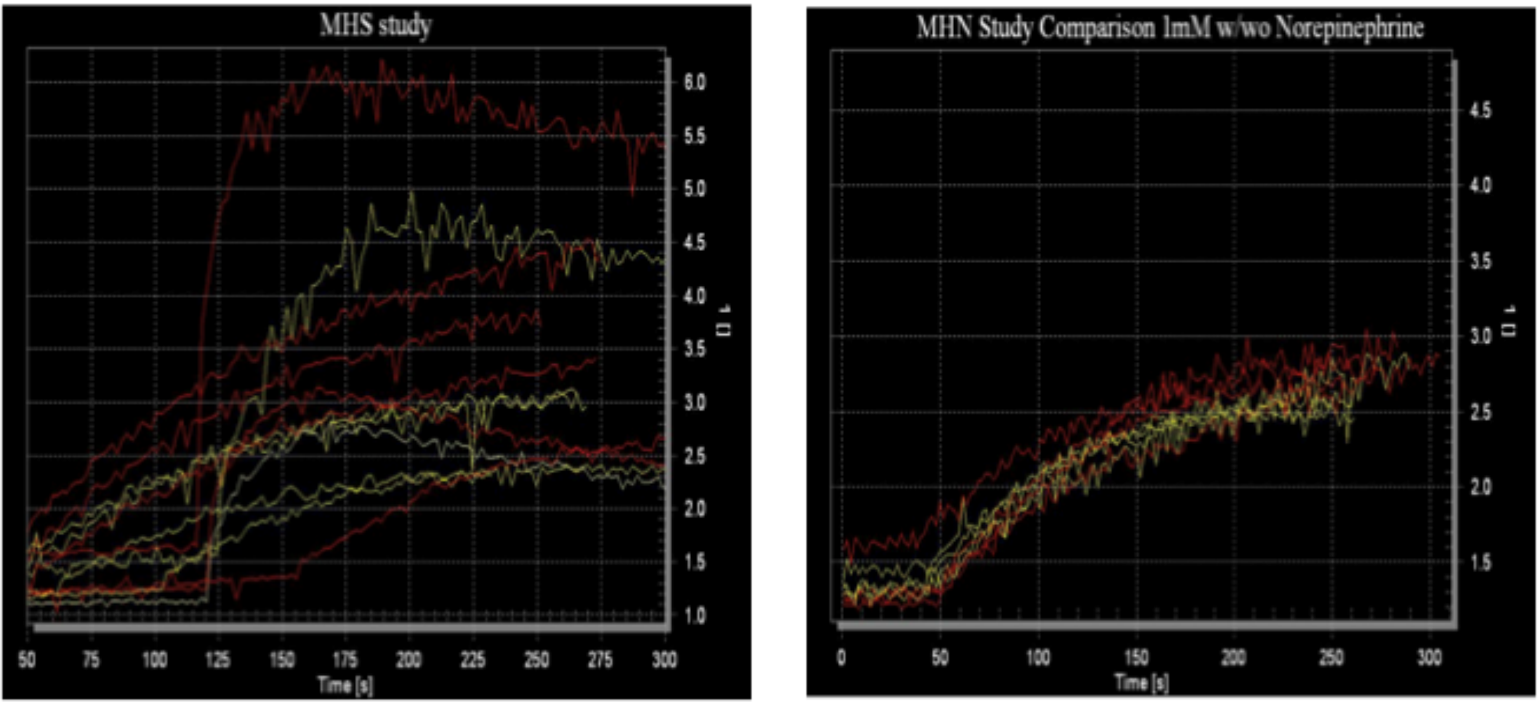
Summary

Taken in aggregate, the findings of this study demonstrate support of early research on the effect of the adrenergic system as a co-factor in the triggering of Malignant Hyperthermia. In 1976, the research group of Lucke, Hall and Lister undertook several studies in an attempt to investigate the effect of the adrenergic nervous system on the triggering of MH in swine and were the first to report a large increase in circulating catecholamines in MHS swine during an MH episode. Reporting a significant correlation between the plasma catecholamine and plasma lactate levels ($r= 0.84$; $P < .0001$), they found that the largest correlation was that contributed by norepinephrine ($r= 0822$; $P < 0.001$). The same research team subsequently were successful in blocking succinylcholine induced MH in MHS swine by the use of phentolamine, but not propranolol and a year later compared effect of phenylephrine and isoproterenol on MHS swine and found that those treated with phenylephrine became hyperthermic and died and those given isoproterenol did not develop hyperthermia. They also reported success in blocking the effect of norepinephrine in swine by using phentolamine, reporting that the pigs did trigger, but recovered without dying; however when they tried to block the effect of norepinephrine with propranolol, the pigs triggered to MH and died. Our findings also support the study by Dyer who reported from his study " The affinity of the 〈 adrenergic receptor in stress susceptible pigs was 2.6 fold greater than that of the control pigs." $P<0.01$ (Dyer, 1982).

Swine breeds that are susceptible to MH are homozygous and all carry the same mutations on the RyR gene Arg615Cys; while humans are heterozygous and display over 300 known mis-sense mutations in the RyR1 gene and display variable penetrance. However, in both swine and humans there now appear to be similar support for the ANS as an important factor in MHS. As noted in Chapter 2,one of the earliest letters regarding the possibility of a link between Malignant Hyperthermia and the Autonomic Nervous System. In a letter to The Lancet in 1975, R.F.W. Moulds proposed "abnormalities in the calcium storing muscle cells could also be present in the catecholamine secretory cells". He further wrote, "all the features necessary for the perpetuation of this cycle during malignant hyperpyrexia are probably exaggerations of normal responses rather than completely atypical responses" (Moulds, 1979). I believe we have successfully shown that the same Ca2^{+} storage organelles in Human B- Lymphocytes and that these responses are exaggerations of normal response.

Our results clearly demonstrate that norepinephrine administered to the MHS vs MHN EBV immortalized Human B-Lymphocytes caused a statistically significant increase in Fura 2 Ca^{2+} emission ratios in response to three different doses of the RyR1 agonist 4- CmC. That this increase was successfully blocked by phentolamine, but not completely by propranolol and that isoproterenol did not cause a significant increase in those same Ca^{2-} emission ratios in MHS vs MHN cells.

Another important finding of this study was related to the fact that there was so much variability in the response of the MHS group to the same dose of norepinephrine (1⌠M). While the MHN groups responded in a very predictable manner, the cells from the MHS group were very variable. (See Figures 27 and 28 below).



Figures 27 and 28: Aggregate Screen Photograph of 5 different MHS samples vs 5 MHN immortalized Human B-Lymphocyte samples of Fura 2 $Ca^{2=}$ Emission Curves. MHS on the left show large variance in response to 1mM 4CmC added in the presence of 1uM Norepinephrine; MHN Cells on the right show a very uniform response.

Reasons for this variability remain unclear, but perhaps represent the variability in MH presentation noted during intraoperative triggering of MH. A retrospective analysis of the various MHS individuals who had cells selected for the study could be undertaken in order to look for differences in how they responded to the CHCT contracture tests as well as what their clinical presentation was when they triggered to MH prior to biopsy. In fact, there was so much variability in the Ca^{1+} emissions in the MHS group, that had the control group not existed, the model would have been thought to be invalid.

It is important to point out that the model used was not skeletal muscle, the tissue normally associated with the disease of MH. It will be important to find a model that will allow the study of the effect of the catecholamines on skeletal muscle to see if the response in the B-lymphocytes also results in increased Ca^{2+} response from the RyR1 in muscle tissue. However, it is also important to remember that the model that was used potentially has implications for previously undescribed immune disorders in MHS individuals; as well as the possibility that the immune response contributes to the hyperthermia seen during an MH crisis. All these areas provide important avenues for new research.

Study Validity

Threats to internal validity of our model do exist. First, we used EBV immortalization of our lymphocytes used in this model rather than fresh lymphocytes. Fresh lymphocytes have very limited life-span and would not be reasonable to use for this type of study where we depend on the ability to use the cells for their own controls in varied dose response studies. However, there is the possibility that the immortalization process influenced how the cells responded. Future studies on how to stabilize the fresh lymphocytes would remove this threat. If in fact the EBV did affect the results, it remains that the differences in the groups were statistically significant, so the difference was, in effect still demonstrated.

The small number of groups used to for the antagonists study, particularly those with propranolol could have affected the statistical analysis validity. As we almost reached statistical significance, and because there was an increase in BA emissions in cells incubated with propranolol, it is recommended that future studies looking at the ® effects of propranolol be supported. Given the findings of others on increased levels of cAMP it is possible that there was an effect of ® blockade on the cells other than that demonstrated by this study.

This is the first time this method has been developed and used for this study; therefore it is possible that the results became more consistent as the study progressed. Anticipating this threat to validity, the cell samples were blocked so that an equal number of MHS vs MHN cells would be worked on within the same period of time. This was also done to avoid differences in seasonal temperature variations or medium differences. The use of each cell group as it's own control also addressed this threat.

All catecholamines and reagents were prepared immediately prior to each day's testing and discarded at the end of the day. Concentrations were validated with other research scientist prior to incorporation into the methods. Care was taken to not perform experiments when other researchers were in close proximity to the Quantamaster while fluorescent measurements were being taken. All data was smoothed prior to using the PTI ®software to obtain data. Not all measurements could be taken at exactly the same second so there could be some small difference in time between one group and another. This was off set by determining the time from administration of 4-CmC and the time to 250-second measurement. BA measurements were removed prior to calculating AUC statistics to avoid baseline differences from affecting the measurement responses to 4- CmC alone.

Finally, the age of the individual donating the B-lymphocytes and the stage of cell development could have influenced the outcome of this study. In the future the ability to cohort samples based on age, genetic mutation, and CHCT response in a larger, double blind study would be useful in offsetting this threat.

Military Practice Significance

As previously discussed, humans are capable of experiencing awake episodes of MH that are unrelated to the pharmacogenetic triggers usually associated with an episode. In a recent commentary by Gronert, Tobin and Muldoon was published in Biochimica et Biophysica Acta. This commentary outlined several authenticated cases of MHS individuals who experienced awake episodes of MH, who were found to be positive for MH via either genetic or CHCT testing. The authors state, “There is indisputable evidence that humans susceptible to MH have stress related abnormal responses in the absence of exposure to triggering agents” (Gronert, Tobin and Muldoon, 2011). In two recent article published by researchers at the Uniformed Services University, a relationship was explored between exertional heat illness (EHI), rhabdomyolysis and MH. In these articles the researchers described case reports of military troops and their family members who had been victims of EHI, linked to MH either through post event testing, or a family history. The researchers describe multiple pathophysiologic similarities between the disorders and discuss the limitations of current test to differentiate between the two (Muldoon, et al., 2008; Capacchione and Muldoon, 2009).

Given current deployment environments and the physiologic and psychologic stress placed on members of the military the link between EHI and MH is of vital concern. Additional research and a test to differentiate between and effectively predict and treat these patients are needed.

Appendix

Abbreviations

4-CmC: 4 Chloro-m-Cresol

AMP: Adenosine monophosphate

ANS: Autonomic Nervous System

ATP: Adenosine triphosphate

AUC: Area Under the Curve

BA: Base Area

B-cells: Human B-lymphocytes

Ca^{2+}: Calcium

CACNA1: Voltage-dependent calcium channel alpha 1

cAMP: cyclic Adenosine monophosphate

CCB: Calcium Containing Buffer

CFB: Calcium Free Buffer

CHCT: Caffeine Halothane Contracture Test

CK: Creatine Kinase

DoD: Department of Defense

EBV: Epstein-Barr virus

EC: Excitation-contraction

ECG: Electrocardiograph

ER Endoplasmic reticulum

FKBP12: 12 kDa FK-506-binding protein

HCL: Hydrochloride

IAW: In accordance with

IgE: Immunoglobulin E

IgG: Immunoglobulin G

IL: Interleukin

IP3: Inositol 1, 4, 5-trisphosphate

MH: Malignant hyperthermia

MHN: Malignant hyperthermia normal

MHS: Malignant hyperthermia susceptible

NE: Norepinephrine

PdE: Phosphodiesterase

PE: Peak Emissions

PK: Protein kinase

PKA: Protein kinase A

PTI: Photon Technology International

RyR: Ryanodine receptor

SERCA: Sarcoplasmic/endoplasmic reticulum Ca2+-ATPase

SNS: Sympathetic Nervous System

SR: Sarcoplasmic reticulum

USU: Uniformed Services University

References

Aarons, R.D., Nies, A.S., Gal, J., Hegstrand, L.R. Molinoff, P.B. (1980). Elevation of ®-adrenergic receptor density in human lymphocytes after propranolol administration, Journal *of Clinical Investigations*, 65, 949-957.

Acikel, M., Buyukokuroglu, M.E., Erdogan, F., Aksoy, H., Bozkurt, E., Senocak, H. (2005). Protective effects of dantrolene against myocardial injury induced by isoproterenol in rats: biochemical and histological findings. *International Journal of Cardiology*, 98, 389-394.

Adeola, O., Ball, O., House, J.D., O'Brien, P.J. (1993). Regional brain neurotransmitter concentrations in stress-susceptible pigs. *Journal of Animal Science*, 71, 968-974.

Allen, G.C., Larach, M.G., Kunselman, A.R. (1998). The sensitivity and specificity of the caffeine-halothane muscle contracture test. North American Malignant Hyperthermia Group. *Anesthesia & Analgesia*, 69, 579-588.

Ayling, J.H., Ellis, F.R., Halsall, P.J., Currie, S. (1985). Thermoregulatory responses to cooling in patients susceptible to malignant hyperpyrexia. British Journal of Anaesthesia. 57 (10), 983-990.

Bardsley, M.E., Wheatley, A.M., Fowler, C.J., McCrodden, J.M., McLoughlin, J.V., Tipton, K.F (1982). Metabolism of monoamines in malignant hyperthermia susceptible pigs. *British Journal of Anaesthesia*, 54, 1313-1318.

Beutner, G., Sharma, V.K., Lin, L., Ryu, S.Y., Dirksen, R.T., Sheu, S.S. (2005). Type 1 ryanodine receptor in cardiac mitochondria: transducer of excitation-metabolism coupling. *Biochimica ET Biophysica Acta*. 1717 (1), 1-10.

Blum, A. (1988). *Plant Breeding for Stress Environments.* New York, NY: Springer Publishing Company, Inc.

Britt, B.A. (1988). Combined anesthetic and stress-induced malignant hyperthermia in two offspring of malignant hyperthermic-susceptible parents. *Anesthesia & Analgesia*, 67, 393-396.

Callanan, K.M., Keenan, A.K. (1984). Differential effects of D600, nifedipine and dantrolene sodium on excitation-secretion coupling and presynaptic Beta- adrenoceptor responses in rat atria. *British Journal of Pharmacology*. 83, 841-847.

Capacchione, J.F., Muldoon, S.M. (2009). The relationship between exertional heat illness, exertional rhabdomyolysis and Malignant Hyperthermia, *Anesthesia and Analgesia,* 10:10, 1-5 doi:101213/ane.Ob013e3181a9d8d9.

Chelu, M.G., Danila, C.I., Gilman, C.P., Hamilton, S.L. (2004). Regulation of ryanodine receptors by FK506 binding proteins. *Trends in Cardiovascular Medicine*, 14 (6), 227-234, 2004.

Chin, P.L. & Kramer, M.K. (2004). *Integrated Knowledge Development in Nursing, 6th ed.* St. Louis, MO: Mosby.

Cohen, J., Gutman, Y. (1979). Effects of verapamil, dantrolene and lanthanum on catecholamine release from rat adrenal medulla. *British Journal of Pharmacology*, 65, 641- 645.

Danila, C.I., Hamilton, S.L. (2004). Phosphorylation of ryanodine receptors. *Biological Research* 37 (4), 521-525.

Davis, T.P., Gehrke, C.W., Williams, C.H., Gehrke, C.W., Gerhardt, K.O. (1982). Pre- column derivatization and high-performance liquid chromatography of biogenic amines in blood of normal and malignant hyperthermic pigs. *Journal of Chromatography*, 228, 113-122, 1982.

Ducreux, S., Zorzato, F., Muller, C., Sewry, C., Muntoni, F., Quinlivan, R., Restagno, G., Girard, T, Treves, S. (2004). Effect of ryanodine receptor mutations on interleuin-6 release and intracellular calcium homeostasis in human myotubes from malignant hyperthermia susceptible individuals and patients affected by central core disease, *Journal of Biochemistry*, 279, 43838- 43846.

Dulhunty, A.F. Laver, D.R., Gallant, E.M., Casarotto, M.G., Pace, S.M., Curtis, S. (1999). Activation and inhibition of skeletal RyR channels by a part of the skeletal HPR II- III Loop: effects of DHPR Ser ^{687} and FKBP12. *Biophysical Journal*, 77,189- 203.

Dyer, D.C. (1982). Evidence for differences in 〈-adrenergic receptor affinity in stress susceptible swine, *Experientia*, 38, 1343-1344.

Engelke, M., Engels, N., Dittmann, K., Stork, B., Wienands, J. (2007). Ca^{2+} signaling in antigen receptor-activated B-lymphocytes. *Immunological Reviews*, 218, 235-246.

Fitzpatrick, J. J. & Whall, A.L. (1989). *Conceptual Models of Nursing: Analysis and Application*. East Norwalk, CT: Appleton and Lange Publishing.

Fitzpatrick, J. J. (Ed). (1998). *Encyclopedia of Nursing Research*. New York, NY: Springer Publishing Company, Inc.

Gaburjakova, M., Gaburjakova, J., Reiken, S., Huang, F., Marx, S.O., Rosemblit, N., Marks, A.R. (2001). FKBP12 binding modulates ryanodine receptor channel gating. *Journal of Biological Chemistry,* 276 (20), 16931-16935.

Grinberg, R., Edelist, G., Gordon, A. (1983). Post-operative malignant hyperthermia episodes in patients who received a safe anesthetic. *Canadian Anaesthesia Society Journal* 30:3:1, 273-276.

Girard, T., Cavagna, D., Padovan, E., Spagnoli, G., Urwyler, A. Zorzato, F. Treves, S. (2001). B- lymphocytes from malignant hyperthermia-susceptible patients have an increased sensitivity to skeletal muscle ryanodine receptor activators. The *Journal of Biological Chemistry*, 276:51, 48077-48082.

Gronert, G.A., Milde, J.H., Theye, R.A. (1976). Porcine malignant hyperthermia induced by halothane and succinylcholine: failure of treatment with procaine or procainamide. *Anesthesiology* 44:2, 124-132.

Gronert, G.A., Theye, R.A. (1976). Halothane-induced porcine malignant hyperthermia: metabolic and hemodynamic changes. *Anesthesiology* 44:1 36-43.

Gronert, G.A., Milde, J.H., Theye, R.A. (1977). Role of sympathetic activity in porcine malignant hyperthermia. *Anesthesiology* 47:5 411-41.

Gronert, G.A., Milde, J.H., Taylor, S.R. (1980). Porcine muscle responses to carbachol and 〈 and ® adrenoceptor agonists, halothane or hyperthermia. *Journal of Physiology*. 307, 319- 333.

Gronert, G.A., Thompson, R.L., Onofrio, B.M. (1980). Human malignant hyperthermia: awake episodes and correction by dantrolene. *Anesthesia and Analgesia,* 59:5, 377- 378.

Gronert, G.A., White, D. .A. (1988). Failure of norepinephrine to initiate porcine malignant hyperthermia. *Pflugers Arch* 41:1 226-228.

Gronert, G.A., Tobin, J.R. Muldoon, S. (2011). *Biochimica et Biophysica Acta,* doi:10.1016/j.bbamcr.2011.08.001, 1-2.

Haggendal, J., Jonsson, L., Johansson, G, Bjurstrom, S, Carlsten, J. (1988). Disordered catecholamine release in pigs susceptible to malignant hyperthermia. *Pharmacology & Toxicology*, 63, 257-261.

Hall, G.M., Lucke, J.N., Lister, D. (1977). Porcine malignant hyperthermia V: fatal hyperthermia in t he pietrain pig, associated with the infusion of 〈-adrenergic agonists. *British Journal of Anaesthesia*, 49, 855-863.

Hamilton, S. L., Serysheva, I. I. (2009). Ryanodine receptor structure: progress and challenges. *The Journal of Biological Chemistry*, 284, 4047-4051.

Hosoi, E., Nishizaki, C., Gallagher, K.L., Wyre, H.W. Matsuo, Y., Sei, Y. (2001). Expression of the ryanodine receptor isoforms in immune cells. *The Journal of Immunology*, 167, 4887-4894.

Huckell, V.F., Staniloff, H.M., Britt, B.A., Wasman, M.B., Morch, J.E. (1978). Cardiac manifestations of malignant hyperthermia susceptibility. *Circulation* 58:5, 918-925.

Huckell, V.F., Staniloff, H.M., Britt, B.A., Morch, J.E. (1982). Electrocardiographic abnormalities associated with malignant hyperthermia susceptibility.*Journal of Electrocardiology* 15:2, 137-142.

Jonsson, L, Haggendal, J, Johansson, G, Thoren-Tolling, K, Bjurstrom, S, Carlsten, J. (1989). Cardiac manifestation and blood catecholamine levels during succinylcholine induced stress of malignant hyperthermia in pigs. *Journal of Veterinary Medicine,* 36, 772-782.

Katz, J.D., Krich, L.B. (1976). Acute febrile reaction complicating spinal anaesthesia in a survivor of malignant hyperthermia. *Canadian Anaesthesia Society Journal,* 23:3, 285-289.

Kerr, D.D., Wingard, D.W., Gatz, E.E. (1975). Prevention of porcine malignant hyperthermia by epidural block. *Anesthesiology* 42:3 307-31.

Kohm, A.P., Sanders, V.M. (2001). Norepinephrine and ®2-adrenergic regulation of $CD4^+$ T and B lymphocyte function, *Pharmacological Reviews*, 53:4, 488-519.

Lambert, D.G., editor. (1999). *Clinical Signaling Protocols.* Totowa, New Jersey. Humana Press

Larach, M.G. (1989). Standardization of the caffeine halothane muscle contracture test. North American Malignant Hyperthermia Group. *Anesthesia and Analgesia*, 69, 511-515.

Levy, B., et al. (2008). Increased aerobic glycolysis through ®2 stimulation is a common mechanism involved in lactate formation during shock states. *Shock*, 30:4, 417-421.

Lister, D., Hall, G.M., Lucke, J.N.(1976). Porcine malignant H

hyperthermia III: adrenergic blockade. *British Journal of Anaesthesia*, 48, 831-838.

Lucke, J.N., Hall, G.M., Lister, D. (1976). Porcine Malignant Hyperthermia. I: Metabolic and Physiological Changes. *British Journal of Anaesthesia*, 48, 297-304.

Mackrill, J. J. (1998). Possible regulation of the skeletal muscle ryanodine receptor by a polyubiquitin binding subunit of the 26S proteasome. *Biochemical and Biophysical Communications,* 245, 428-429.

Maisel, A.S. Harris, T., Rearden C.A., Michel, M. C. (1990). Beta-adrenergic receptors in lymphocyte subsets after exercise. Alterations in normal individuals and patients with congestive heart failure. *Circulation,* 82, 2003-2010.

Meissner, G. (2002) Regulation of mammalian ryanodine receptors. *Frontiers in Bioscience*, Nov 1: 7, d 2072-2080.

Marx, S.O., Reiken, S., Hisamatsu, U.Y., Jayaraman, T., Burkhoff, D., Rosemblit, N., Marks, A.R. (2000). PKA phoshorylation dissociates FKBP12.6 from the Ca 2+ release channel (ryanodine receptor): defective regulation in failing hearts. *Cell*, 101, 365-376.

McKinney, L.C., Butler, T., Mullen, S.P., Klein, M.G. (2006). Characterization of ryanodine receptor-mediated Ca 2+ release in human B cells: relevance to diagnostic testing for malignant hyperthermia. *Anesthesiology*, 104: 1191-201.

McLennan, D.H., Duff, C., Zorzato, F., Fujii, J., Phillips, M., Korneluk, R.G. (1990). Ryanodine receptor gene is a candidate for predisposition to malignant hyperthermia. *Nature*, 343, 559-561.

Motegi, Y., Shirai, M., Arai, M., Kato, S., Kawana, .Y, Goto, F. (1996). Malignant hyperthermia during epidural anesthesia. Journal of Clinical Anesthesia, 8, 157-160.

Morgan, G.E., Mikhail, M.S., Murray, M.J. (2002). *Clinical Anesthesiology*. New York, NY. McGraw-Hill Medical Publishing.

Moulds, R.F.W. (1975). Malignant hyperthermia. *Lancet,* March, 618, 1975.

Muldoon, S., Deuster, P., Brandom, B., Bunger, R. (2004). Is there a link between malignant hyperthermia and exertional heat illness? *Exercise and Sports Science Reviews* 3 2, 174-179.

Nelson, T.E., Flewellen, E. (1983). Current concepts. The malignant hyperthermia syndrome. *New England Journal of Medicine*. 309, 416-418.

Ording, H., Brancadoro V., Cozzolino, S., Ellis, F.R., Glauber, V., Gonano, E.F., Halsall, P.H.J., Hartung, E., Heffron, J.J., Heytens L., Kozak-Ribbens, G., Kress, H., Krivosic-Horber, R., Lehmann-Horn, F., Mortier, W., Nivoche, Y., Ranklev-Twetman, E.,

Sigurdsson, S., Snoeck, M., Stieglitz, P., Tegazzin, V., Urwyler, A., Wappler, F. (1997). In

vitro contracture test for diagnosis of malignant hyperthermia following the protocol of the European MH Group: results of testing patients surviving fulminant MH and unrelated low-risk subjects. *Acta Anaesthesiology Scandanavia*, 41: 955-966.

Perez, C.F., Lopez, J.R., Allen, P.D. (2005). Expression levels of RyR1 and RyR3 control resting free Ca 2+ in skeletal muscle. *American Journal of Physiology: Cell Physiology*. 288 (3): C640-649.

Peterson S.J. & Bredow, T.S. (2009). *Middle Range Theories: Application to Nursing Research* (2nd Ed.). Philadelphia, PA. Lippincott, Williams & Wilkins.

Redwine, L., Jenkins, F., Baum, A. (1996). Relation between beta-adrenergic receptor density and lymphocyte proliferation associated with acute stress, *International Journal of Behavioral Medicine,* 3:4, 337-353.

Reynolds, A.C., Reynolds, E.V., Henschel, E. O. (1981). Physical exercise in malignant hyperthermia screening. *The Lancet*, August, (3), 303.

Rici, Bronzetti, E., Conterno, A., Greco, S., Mulatero, P., Schena, M., Schiavone, D., Tayebati, S.K., Veglio, F., Amenta, F.: (1999). Alpha 1 adrenergic receptor subtypes in human peripheral blood lymphocytes. *Hypertension,* 33, 708-712.

Rosenberg, H., Antognini, J.F., Muldoon, S. (2002). Testing for malignant hyperthermia. *Anesthesiology,* 96:1, 232-237.

Rosenberg, H., Davis, M., James, D., Pollock, N., Stowell, K. (2007). Malignant hyperthermia. *Journal of Rare Disease*, 2:21 Published online; http: //www.ncib.nlm.nih.gov/pmc. 1-27

Scholz, J., Roewer, N., Run, U., Schmitz, W., Schotz, H., Schulte am Esch, J. (1991). Effects of caffeine, halothane, succinlycholine, phenylephrine and isoproterenol on myocardial force of contraction of malignant hyperthermia susceptible swine. *Acta Anaesthesiology Scandanavia*, 35, 320-325.

Sei, Y., Gallagher, K.L., Daly, J.W. (2001). Multiple effects of caffeine on Ca++ release and influx in human B lymphocytes. *Cell Ca 2+,* 29 (3), 149-160.

Sei, Y., Brandom, B.W., Bina, S., Hosoi, E., Gallagher, K.L., Wyre, H.W., Pudimat, P.A., Holman, S.J., Venzon, D.J., Daly, J.W., Muldoon, S. (2001). Patients with malignant hyperthermia demonstrate an altered Ca 2+ control mechanism in B-lymphocytes. *Anesthesiology,* 97, 1052- 1058.

Sambuughin, N., Sei, Y., Gallagher, K.L., Wyre, H.W., Madsen, D., Nelson, T.E., Fletcher, J.E., Rosenberg, H., Muldoon, S. (2001). Screening of the Ryanodine receptor gene and Identification of novel mutations. *Anesthesiology*, 95:3, 594-599.

Short, C.E., Paddleford, R.R., McGrath, C.J., Williams, ClH. (1976). Pre-anesthetic evaluation and management of malignant hyperthermia in the pig experimental model. *Anesthesia & Analgesia*, 55:5, 643-653.

Stanec A., Stefano, G. (1984). Cyclic AMP in normal and malignant hyperpyrexia susceptible individuals following exercise. *British Journal of Anaesthesiology*. 56, 1243-1246.

Vukcevic, M., Broman, M., Ilander, G., Bodelsson, M., Ranklev-Twetman, El, Muller, C.R., Treves, S. (2010). Functional properties of RyR1 mutations identified in Swedish patients with malignant hyperthermia and central core disease. *Anesthesia & Analgesia*, 10: 10, www.anesthesia-analgesia.org, 1-6.

Wehrens, X.H., Lehnart, S.E., Huang, F., Vest, J.A., Reiken, S.R., Mohler, R.J., Sun, J, Guatimosim, S., Song, L. Priori, S.G., Lederer, W.F. (2003). Marks, A.R.: FKBP12.6 deficiency and defective Ca 2+ release channel (ryanodine receptor) function linked to exercise induced sudden cardiac death. *Cell* 113 (7), 829-840.

Williams, C.H. (editor) (1988). *Experimental Malignant Hyperthermia.* Springer-Verlag, New York, Berlin, Heidelberg.

Willner, J.H., Cerri, C.J., Wood, D.S. (1979) Malignant hyperthermia: abnormal cyclic AMP metabolism in skeletal muscle. *Neurology* 29, 557.

Willner, J.H., Cerri, C.G., Wood, D.S. (1981). High skeletal muscle adenylate cyclase in malignant hyperthermia. *Journal of Clinical Investigations*. 68, 1119-1124.

Williams, J.H., Barnes, W.S. (1989). The positive inotropic effect of epinephrine on skeletal muscle: a brief review. *Muscle & Nerve* 12, 968-975.

Williams, L.T., Snyderman, R., Lefkowitz, R. J. (1976). Identification of beta-adrenergic receptors in human lymphocytes by (-) (3H) Alprenolol binding. *The Journal of Clinical Investigation,* 57, 149-155.

Wingard, D.W. (1981). Familial stress syndrome. Inherited disease and anaesthesia; Elsevier North Holland Biomedical Press, Chapter 8. 201-209.

Yang, T., Ta, T.A., Pessah, I.N., Allen, P.D. (2003). Functional defects in six ryanodine receptor isoform-1 (RyR1) mutations associates with malignant hyperthermia and their impact on skeletal excitation-contraction coupling. *The Journal of Biological Chemistry,* 278 (28), 25722-25730.

.