THE EFFECTS OF A MUSCLE CALCIUM SENSITIZER ON EXERCISE PERFORMANCE IN MALE SPRAGUE-DAWLEY RATS

by

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Abstract

Skeletal muscle fatigue has a complex multifaceted etiology in which the ability to regulate intracellular calcium concentration ($[Ca^{2+}]_i$) and the myofibrillar response to elevated $[Ca^{2+}]_i$ are key components. Pertinent to this issue, a calcium sensitizer compound has been shown to increase contractile function via altered myofibrillar Ca^{2+}sensitivity in in-vitro preparations. We tested the hypothesis that a calcium sensitizer compound would increase the endurance capacity and VO_{2peak} in young male Sprague-Dawley rats above saline control values. The exercise tolerance test consisted of a progressive exercise test in which each rat initially ran at a speed of 25 m/min up a 10% grade for 15 min. Thereafter the treadmill speed was increased by 5m/min every 15 min until fatigue (i.e., the rat could no longer maintain pace with the treadmill). VO_{2peak} was determined according to previously established methods used in our laboratory. Each rat initially ran at 25 m/min up a 10% grade for 2-3 minutes. The speed of the treadmill was the increased progressively in a ramp-like manner until fatigue. VO_{2peak} was defined either as the point at which O_2 uptake did not further increase despite increases in treadmill speed or the highest VO_{2peak} prior to fatigue. A calcium sensitizing compound or saline was administered via gastric gavage. There was a significant increase ($P<0.05$) in endurance capacity with 10 mg/kg of the calcium sensitizer compound, but not at lower (0.5-5 mg/kg) or higher (20-40mg/kg) doses. This improvement in endurance capacity occurred in the absence of any changes in VO_{2peak}. The highest dose (40 mg/kg) produced a significant decrease ($P<0.05$) in the endurance capacity as well as VO_{2peak}. These data demonstrate that the in-vitro observations of increased $[Ca^{2+}]_i$ sensitivity and improved muscle function with a calcium sensitizing compound can translate to improved whole body exercise performance. Further studies need to be conducted to explore the efficacy of calcium sensitizing agents in animal models of chronic disease (i.e. CHF and diabetes). It is possible that a calcium sensitizer compound could be used as a potential ergogenic aid for patients whom enhanced physical capacity could be of significant therapeutic value, and lead to increases in activities of daily living and quality of life.

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Acknowledgements

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CHAPTER 1 - INTRODUCTION

Repeated, intense muscle contraction(s) leads to a decline in performance known as muscle fatigue. This phenomenon is reversible after a period of rest (Eberstein and Sandow, 1963; Allen *et al*., 2008). There have been multiple mechanisms identified that may contribute to muscle fatigue. These include changes in the action potential, altered regulation of extracellular and intracellular ions, and intracellular metabolites, as well as a reduction of force and shortening velocity, and/or prolongation of muscle relaxation (Allen *et al*., 2008; Allen *et al.,* 1995).

Muscles are activated by a complex pathway originating in the motor cortex that leads to excitation of the lower motor neurons in the spinal cord. From there, the axon of the lower motor neuron carries the action potential (AP) to the neuromuscular junction of the muscle. The action potential in the motor neuron causes the release of acetylcholine (Ach) into the synaptic cleft of the neuromuscular junction. Ach binds with receptors on the motor end-plate, producing an end-plate potential that leads to AP conduction down the transverse tubules (t-tubules). When the AP reaches the voltage gated calcium Ca^{2+} channels which connect with the sarcoplasmic reticulum (SR), Ca^{2+} is released into the sarcoplasm. The Ca^{2+} binds to troponin which causes a shift of tropomyosin to uncover the "active sites" located on actin. Following this, Adenosine Triphosphate (ATP) is broken down to adenosine diphosphate (ADP) + inorganic phosphate (P_i) + energy and the released energy is used to "cock" the myosin crossbridge. Then the "cocked" myosin crossbridge attaches to the active site on actin and pulls the actin molecule over the myosin. Next, the attachment of "fresh" ATP to the myosin crossbridge allows the crossbridge to detach from actin and the contraction cycle is repeated as long as Ca^{2+} (and ATP) is present. Finally the AP stops, the SR actively removes Ca^{2+} from the sarcoplasm and tropomyosin moves to its inhibitory position covering the active sites on the actin molecules. This process emphasizes the importance of Ca^{2+} in skeletal muscle contraction/relaxation.

The processes that occur inside the spinal cord and within higher neural pathways are generally defined as central, whereas the processes in the peripheral nerve, neuromuscular junction, and muscle are defined as peripheral in function (Allen *et al*., 2008). It is clear that fatigue can potentially arise at one or more points throughout this pathway, and as such can be

classified as central fatigue and/or peripheral fatigue. Some studies suggest that some degree of fatigue is due to central failure of activation (Noakes & Tucker, 2008; Marcora, 2008; Gandevia, 2001). However, the compelling weight of evidence indicates that fatigue arises primarily from within the muscles (peripheral), and therefore can be studied via either isolated muscle tissues or during whole body exercise (Allen *et al.*, 2008).

As discussed by Allen & Westerblad (1995) the reduction in isometric force can be divided into three components: (1) reduced maximum isometric force (i.e. force at saturating calcium concentration, F_{max}); (2) reduced myofibrillar Ca^{2+} sensitivity; and (3) reduced SR Ca^{2+} release, causing a decline in myoplasmic free calcium concentration ($[Ca^{2+}]$) during the tetanus (tetanic $[Ca^{2+}]_i$). One study concluded that fatigue caused by voluntary repeated short tetani in a human can lead to a 50% reduction in force production. (Bigland-Ritchie *et al.,* 1986). Of that 50% decline, it's suggested that ~10% is due to reductions in F_{max} due to increased P_i, and the remaining 40% is attributed to decreased Ca^{2+} sensitivity of contractile proteins and reduced sarcoplasmic reticulum (SR) release (Allen *et al.,* 2008).

Thus, a calcium sensitizing compound that may up regulate Ca^{2+} sensitivity of skeletal muscle may prevent or constrain the decreased contractile performance of contracting muscle(s). Pursuant to this issue, there are many studies that have shown that reduced tetanic $[Ca^{2+}]_i$ is an important factor for the force decline in fatigue (Allen et al., 1989; Westerblad & Allen, 1991; Westerblad & Allen, 1993). It is possible that a calcium sensitizing compound may work like other agents capable of facilitating and increased Ca^{2+} sensitivity and SR Ca^{2+} release, such as caffeine, by sensitizing the Ryanodine receptor (RYR) channels to activation of cystolic $[Ca^{2+}]$, leading to an increase in the open probability (P_o) of the channel (Eberstein and Sandow, 1963).

Pertinent to this issue, increased fatigability is a common accompaniment to many different disease states (such as CHF and diabetes) that affect skeletal muscle. Understanding the mechanisms involved with the structural and functional maladaptations of these diseases may help to cure or, at least, alleviate some of the symptoms associated with them, such as exercise intolerance.

The purpose of this present investigation was to test, non-invasively, if a calcium sensitizing compound affects exercise performance and peak oxygen uptake (VO_{2peak}) in male Sprague-Dawley rats. This study was designed to test the in-vivo efficacy of a calcium sensitizing compound in healthy animals as an essential precursor to evaluating its utility in the

treatment of chronic diseases such as CHF. We tested the hypothesis that the rats' demonstrated ability to generate more force in their locomotor muscles will translate into:

1) The ability to exercise at a greater maximal workload and use more O_2 (VO_{2peak})

2) A greater endurance capacity during a graded treadmill test to fatigue.

CHAPTER 2 - Review of Literature

The mechanisms of skeletal muscle fatigue have been studied for many years. Eberstein and Sandow (1963) first suggested that failure of excitation-contraction (EC) coupling contributed to muscle fatigue. The literature suggests that an array of mechanisms are involved with skeletal muscle fatigue. The challenge is to determine which mechanisms contribute to skeletal muscle fatigue under different circumstances. The majority of the mechanistic studies of fatigue have been performed in-vitro on isolated animal tissues. One important challenge is for scientists to take the knowledge generated from these studies, and place it within the context of fatigue in intact animals particularly during whole body exercise to help understand the etiology of exercise intolerance that is symptomatic of chronic human disease(s).

1) Excitation-Contraction Coupling in Skeletal Muscle

Excitation-contraction (EC) coupling in skeletal muscle is well understood (Dulhunty, 2006; Melzer *et al*., 1995; Stephenson *et al.*, 1998). Failure in one of the processes associated with EC coupling could contribute to fatigue. The AP of the muscle begins at the neuromuscular junction by release of Ach. The transmission of the AP along the surface membrane is dependent on the sodium (Na^+) channels. Potentially, AP transmission could be influenced by numerous factors including the membrane potential, the extracellular and intracellular Na^+ and Potassium (K^+) concentrations, the internal and external resistances, or the membrane resistance and capacitance (Allen *et al.*, 2008). From the myocyte surface, the AP is conducted down the t-tubules into the interior of the muscle (Bezanilla *et al*., 1972). The t-tubule has a high content of L-type Ca^{2+} channels which change their conformation during an AP, resulting in a charge movement. These voltage sensitive channels are in close contact with SR Ca^{2+} release channels, and lead to conformational change in the ryanodine (RYR) receptor and opening of the SR Ca^{2+} release channel (Schmeider & Chandler, 1973). SR Ca^{2+} release fails in many types of fatigue, and continues to be the focus of many scientific investigations.

Ca^{2+} released from the SR gives rise to a transient increase in myoplasmic free Ca^{2+}. This calcium binds to troponin C and instigates the movement of tropomyosin, which allows for the cycling of cross bridges, and therefore force development (Ashley & Mulligan, 1991).

The intramyocyte Ca^{2+} concentration depends on the coordinated activity of SR Ca^{2+} release, troponin C, parvalbumin, the SR Ca^{2+} pump, calmodulin, and ATP, which are calcium buffers (Baylor & Hallingsworth, 1998). Currently there are no measurements of Ca^{2+} buffering in muscle fatigue which could assess how the Ca^{2+} buffering is affected by the ionic and metabolic changes associated with fatigue; as a consequence, the assumption that changes in the Ca^{2+} transient reflect changes in SR Ca^{2+} release may be incorrect.

E-C coupling concludes with cross-bridge activation and the muscle relaxes as Ca^{2+} is pumped back into the SR by an ATP-driven SR Ca^{2+} pump, decreasing cytosolic or myoplasmic $[Ca^{2+}]$. These SR pumps are sensitive to many of the metabolic changes that are associated with fatigue such as changes in [ATP], [ADP], and/or magnesium concentrations $[Mg^{2+}]$, however the contribution of the changing pump properties to the slowing of relaxation in fatigue are still uncertain (Allen *et al.*, 2008).

2) Role of Inorganic Phosphate (P_i) in Skeletal Muscle Fatigue

The exchange of high energy phosphate between ATP and Phospho creatine (PCr) is catalyzed by creatine kinase (CK) according to the following reaction: $PCr + ADP + H^+ \rightarrow Cr$ (creatine) + ATP. During times of high energy demand, the ATP concentration remains constant while PCr breaks down into Cr and diprotonated P_i (Allen *et al.*, 2008). This P_i may cause a decrease of myofibrillar force production and reduced Ca^{2+} sensitivity as well as impaired SR Ca^{2+} release. Increased P_i is considered to be a major cause of fatigue (Westerblad *et al.*, 2002). This has been shown in mice lacking creatine kinase in their skeletal muscles ($CK^{-/-}$ mice). Fast-twitch skeletal muscle fibers of $CK^{-/-}$ mice display an increased myoplasmic P_i concentration at rest; furthermore, during fatigue there is no significant P_i accumulation. The maximum Ca^{2+} -activated force of unfatigued $CK^{-/-}$ fast-twitch fibers is markedly lower than that of wild-type fibers, which supports a force-depressing role of increased Pi (Dahlstedt *et al.*, 2001).

The current models of cross-bridge force production state that the myosin head is first bound weakly and then strongly to the actin filament (Allen *et al.*, 2008). Thereafter P_i is released, possibly resulting in a further decrease in force production (Takagi *et al*., 2004). This would imply that the transition to a high-force cross-bridge attachment is inhibited by increased P_i and fewer cross bridges would be in the high-force state when P_i increases during fatigue

mainly due to the breakdown of PCr. The effect of P_i on cross-bridge force production has proven difficult to test directly in intact muscle cells because altering myoplasmic P_i directly produces other metabolic changes. Again, the most recent experimental model that has been used to study these affects is the genetically modified mouse which completely lacks CK in the skeletal muscles ($CK^{-/-}$ mice) (Steeghs *et al.*, 1997). Skeletal muscle fibers of $CK^{-/-}$ mice display an increased myoplasmic P_i concentration at rest, and there is no significant P_i accumulation during fatigue. The $F_{ca,max}$ (tetanic Ca^{2+} at maximum force) of unfatigued $CK^{-/-}$ fast-twitch fibers is markedly lower than that of normal wild type fibers, and this can be partly explained by a P_i-induced depression of cross-bridge force production (Steeghs *et al.*, 1997). In conclusion, increased myoplasmic P_i can inhibit force production by direct action on the cross-bridge, and is likely responsible for the decrease in tetanic force.

3) Lactate and H^+ and Their Roles in Skeletal Muscle fatigue

Historically, the accumulation of lactic acid in muscle has been considered a major cause of muscle fatigue (Fitts, 1994). Lactate and H^+ are produced by the muscle during heavy intense exercise, and, in humans, the intracellular lactate concentration may reach 30mM or more, driving the intracellular pH down by ~ 0.5pH units (Sahlin *et al.*, 1976). Although increased levels of lactate may reduce muscle performance (Hogan *et al*., 1995), it appears that its harmful effects have been considerably overestimated and that other beneficial effects have been overlooked (Westerblad *et al.*, 1997).

Experiments with skinned muscle fibers at constant ionic strength have shown that lactate concentrations up to 50mM have a relatively modest effect on force production by the contractile machinery, reducing maximum force (F_{max}) by < 5% and having little or no effect on Ca^{2+} sensitivity. Also, skinned fiber experiments with functional excitation contraction E-C coupling have shown that voltage-sensitive activation of Ca^{2+} release is little, if at all, affected and twitch and tetanic forces are virtually unchanged in the presence of 30mM lactate concentration ($[La^-]$) (Andrews *et al.*, 1996; Chase & Kushmerick, 1988; Posterino & Lamb, 2003). The research largely suggests that the intracellular accumulation of lactate is not the major factor in muscle fatigue (Hogan *et al.*, 1995).

Skeletal muscle pH at rest is ~ 7.05 and after exhaustive exercise may drop to as low as ~ 6.5 (Sahlin *et al.*, 1976; Spriet *et al.*, 1989). However, in other cases, pH decreases only

to 6.8 or 6.9 at the point of exhaustion (Bangsbo *et al.*, 1996; Hogan *et al.*, 1999). These results demonstrate that muscle fatigue can occur without large changes in hydrogen ion concentrations ($[H^+]$). At the onset of exercise or muscle stimulation, pH may initially increase due to the H^+ consumed during PCr breakdown, and conversely, pH can decrease as PCr is resynthesized (Sahlin & Ren, 1989). Studies have shown that when pH does drop to low levels in a fatigued muscle, upon ceasing the exercise or stimulation, force typically recovers much faster than pH, indicating that the low pH per se was not responsible for the force production deficit (Baker *et al.*, 1993; Cady et al., 1989; Sahlin & Ren, 1989; Thompson *et al.*, 1992). Furthermore, attenuating the decline in muscle pH during a stimulation period does not reduce fatigue in frog muscle fibers (Stary & Hogan, 2005).

It is pertinent that low pH also reduces Ca^{2+} sensitivity of the contractile machinery (Fabiato & Fabiato, 1978) likely due to the H^+ competing with Ca^{2+} binding to troponin C. This is often presumed to have major deleterious effects on muscle performance. However, low pH may also reduce the affinity of Ca^{2+} binding at other sites in the muscle fiber, in particular that of the SR Ca^{2+} pump which may lead to an increase in resting cytoplasmic free Ca^{2+} (Westerblad et al., 1993) which may actually favor and increase in force development. Thus, even though the affinity of troponin C for Ca^{2+} may be reduced, the total amount of Ca^{2+} binding to troponin C may not decrease (Allen *et al.*, 2008). In summary, it is now considered that low pH has far less severe inhibitory effects on the activation of the contractile machinery and Ca^{2+} release than previously suggested, and its affects on the SR Ca^{2+} pump may actually favor force development by increasing the amount of free $[Ca^{2+}]$.

3) Role of Reactive Oxygen Species

Within the scientific community, the interest in reactive oxygen species (ROS) has grown rapidly. There is strong evidence that ROS play a role in skeletal muscle fatigue. For example, exogenous ROS scavengers (such as N-acetylcysteine, superoxide dismutase (SOD), dimethyl sulfoxide (DMSO), catalase, Tempol) reduce the rate of fatigue in isolated muscles, intact animals, and in humans (Shindoh *et al.*, 1990; Reid et al., 1992; Moopanar & Allen, 2005). The most important ROS are superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-). In addition nitric oxide (NO) can interact with O_2^- to form peroxynitrate ($ONOO^-$), one of

many reactive nitrogen species that likely have a role in skeletal muscle fatigue (Reid & Durham, 2002).

The increase in ROS during exercise or muscle stimulation is well established, but the source of ROS is not as well understood. Mitochondria are generally thought to be the main source of ROS because of the direct production of O_2^-, especially during intense exercise when mitochondrial O_2 consumption increases up to 100-fold. It is thought that the excess O_2^- may form H_2O_2 and diffuse across the mitochondrial membrane into the myoplasm (Clanton *et al.*, 1999). Another possible source of ROS is nonphagocytic NAD(P)H oxidase (NOX). The current literature suggests that NOX may be associated with the SR or t-tubular membrane (Xia *et al.*, 2003; Hidalgo *et al.*, 2006; Javesghani *et al.*, 2002). These studies showed NOX-dependent O_2^- production, and inhibitors of NOX (diphenyleneiodonium, DPI) reduced O_2^- production by the muscle. Currently there is no consensus about the major source of ROS production within the muscle, but mitochondria and NOX seem probable contenders.

ROS such as O_2^- and H_2O_2, may decrease maximum Ca^{2+} activated force (Andrade *et al.*, 1998; Plant *et al.*, 2001). Specifically, O_2^- causes a 15% reduction in $F_{ca,max}$, and hydroxyl radicals reduce force by 44%. ROS may also decrease Ca^{2+} sensitivity (Andrade *et al.*, 2001; Andrade *et al.*, 1998). One study showed that longer exposure to large concentrations of H_2O_2 causes a large fall in Ca^{2+} sensitivity that is reversed by dithioreitol (DTT) which is a membrane-permeant thiol-specific reducing agent capable of converting disulphide bridges in proteins to sulphydryl groups. Interestingly, the administration of DTT caused a decrease in Ca^{2+} sensitivity when applied alone, suggesting alterations in the ROS-sensitive signaling pathway (Allen *et al.*, 2008). Lastly, it has also been established that ROS can inhibit the SR Ca^{2+}- ATPase and reduce the rate of Ca^{2+} re-uptake into the SR which has negative effects on skeletal muscle force production, and may contribute to muscle fatigue. (Scherer & Deamer, 1986).

It is clear that ROS are generated in active muscle and contribute to the process of fatigue. How ROS are produced and which ROS play a major role in skeletal muscle fatigue is less well defined.

4) SR Ca^{2+} Content and Its Role in Fatigue

The total amount of Ca^{2+} contained and released within muscle fiber is important to its overall function. In resting rat skeletal muscle, the total Ca^{2+} content in both fast- and slow- twitch fibers is ~ 1.1 mmol/kg, with most of this stored in the SR, equivalent to about 11 (fast) and 21 (slow) mM when expressed relative to the respective SR volume (Fryer & Stephenson, 1996; Gissel & Clausen, 1999; Owen *et al.*, 1997). Total Ca^{2+} in human muscle fibers is slightly lower (about 0.8 mmol/kg) (Overgaard *et al.*, 2004; Salviati *et al.*, 1982). The majority of the Ca^{2+} in the SR is bound to calsequestrin. If the total number of Ca^{2+} molecules in the SR drops substantially below its normal level, the amount of Ca^{2+} released by each action potential is reduced, thus reducing the force response (Posterino & Lamb, 2003). Specifically a 35% decrease in the SR Ca^{2+} content reduces peak tetanic force by 46% (Dutka *et al.*, 2005). This occurs despite the fact that the amount of Ca^{2+} remaining in the SR may be considerably higher than the number of Ca^{2+} binding sites on troponin C. Thus it is the reduction in the total amount of releasable Ca^{2+} in the SR that adversely affects Ca^{2+} release and impairs the force responses.

5) Slowing of Relaxation

Skeletal muscle fatigue is accompanied by a marked slowing of relaxation. This slowing of relaxation can limit performance during dynamic exercise where rapidly alternating movements are performed (Allen *et al.*, 1995). Relaxation of skeletal muscle cells is a complex process that involves the following steps: 1) SR Ca^{2+} release stops, 2) Ca^{2+} is taken up by the SR via ATP- driven pumps, 3) the resulting decline in $[Ca^{2+}]$ means that Ca^{2+} dissociates from troponin C, and 4) cross bridge cycling ceases. Potentially, any disturbance in these steps could contribute to the slowing of relaxation. The changes in ADP and P_i during fatigue directly affect myosin and SR Ca^{2+} -ATPase and could also contribute to the slowing of relaxation (Edwards *et al.*, 1975).

6) Conclusions

It is widely accepted that fatigue has a complex and a multi- focal etiology and that in different types of activity the mechanisms and quantitative importance of each mechanism may vary. The reduction in isometric force in fatigue can be divided into three components: 1)

reduced maximum isometric force, 2) reduced myofibrillar Ca^{2+} sensitivity, and 3) reduced SR Ca^{2+} release (Allen *et al.*, 1995). The major focus for the future should be to identify mechanisms that contribute to skeletal muscle fatigue during disease processes. Fatigue is the most frequent symptom reported in the primary health care setting (Hickie *et al.*, 1996). This occurs when the intended physical activity can no longer be continued or is perceived as involving excess effort and discomfort. This is particularly tragic because, often, those patients in whom physical activity could be of great therapeutic value (e.g., heart failure and diabetes patients) are the least capable of performing such activity.

Patients with CHF frequently report increased fatigue; intuitively one may presume that this fatigue is associated with poor cardiac performance and consequently impaired muscle O_2 delivery. However there is evidence that muscle dysfunction in CHF can be demarcated from O_2 delivery deficits per se. (Wilson, 1995). This implies that there may be crucial peripheral deficits in skeletal muscles in CHF, a conclusion that is supported by numerous studies which have reported important functional and biochemical changes in skeletal muscle cells in CHF (Harrington & Coats, 1997; Lunde *et al.*, 2001; Nicoletti *et al.*, 2003). These changes may affect fatigue development by acting both on myofibrillar function and SR Ca^{2+} handling (Lunde *et al*, 2001; Lunde *et al.*, 2006).

The aim of the current project is to determine the in-vivo efficacy of a calcium sensitizing compound using a healthy whole body exercise (running) animal model. The future goals of this project would include testing of calcium sensitizing compounds in models of chronic disease (i.e. CHF and diabetes).

CHAPTER 3 - Methods

Animals

Thirty three male Sprague-Dawley rats (initial age 2-4 months) were obtained from Charles River Laboratories (Boston, MA). Upon arrival at Kansas State University, all rats were housed two per cage in an Associations for Assessment and Acreditation of Laboratory Animal Care (AAALAC) approved facility, maintained on a 12:12 hour light/dark cycle, and provided with food and water *ad libitum*. All experimental procedures were approved by the Kansas State University Institutional Animal Care and Use Committee IACUC and performed according to the American Physiological Society's guidelines for animal handling and care.

Determination of Exercise Tolerance

Each rat was weighed before the initiation of the exercise tolerance protocol for determination of exercise endurance. The protocol consisted of a progressive exercise test in which each rat initially ran at a speed of 25 m/min up a +10 % grade for 15 min. Thereafter, the treadmill speed was increased by 5 m/min every 15 min until the rat could no longer maintain pace with the treadmill, despite encouragement to do so by applying manual bursts of high-pressure air aimed at the hind legs. Immediately after the end of each exercise test, each rat was removed from the treadmill and fatigue was confirmed by the loss of the animals' righting reflex. The time-to-fatigue was recorded to the nearest second and each rat was re-weighed upon completion of the test. The results reported herein reflect the rats' post-exercise test weight. Our laboratory has previously demonstrated that this protocol results in depletion of muscle glycogen stores and therefore elicits a true index of physiological fatigue (Musch *et al.*, 1988; Musch *et al.*, 1990).

Determination of VO_{2peak}

Each rat was weighed before the initiation of the maximal exercise test for determination of VO_{2peak}. VO_{2peak} was determined according to previously established methods that have been used extensively in our laboratory (Musch *et al.*, 1988; Musch *et al.*, 1988b). This method employs a metabolic chamber (14.5 x 43 x 7 cm) designed to fit into one stall on the treadmill,

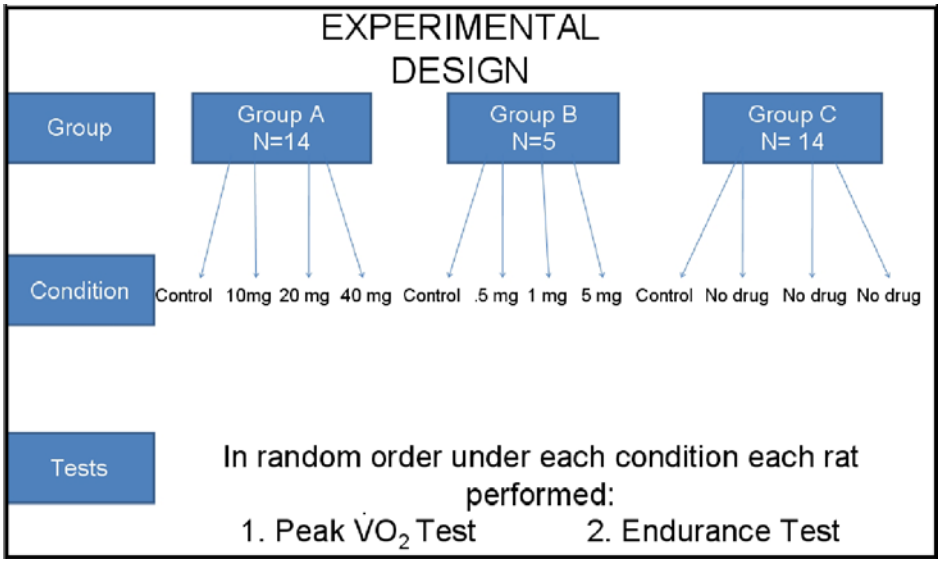
and utilizes the standard techniques described by Brooks and White (Brooks & White, 1978) and Musch et al. (Musch *et al.*, 1988) for determining O_2 and carbon dioxide production (CO_2). Gas analysis measurements were made via CO_2 and O_2 (CO_2: model CD-3A; O_2: model S-3A/I; AEI Technologies, Pittsburgh, PA) analyzers set in series. The analyzers were calibrated before and after each maximal exercise test using precision-mixed gases that spanned the expected range of gas concentrations based on previous investigations.

The maximal exercise test was initiated by having each rat run at 25 m/min up a 10% grade for 2-3 minutes which served both as a warm-up and familiarization phase. The speed of the treadmill was then increased progressively in a ramp-like manner until the rat could no longer keep up with the treadmill speed. VO_{2peak} was defined as the point at which O_2 did not further increase despite increases in treadmill speed, or the VO_2 at which the rat was no longer able/willing to run. In untrained rats it has previously been documented that either of these criteria produce similar VO_{2peak} values (Bedford *et al.*, 1979). Each rat was re-weighed upon termination of the maximal exercise test, and the results reported herein reflect the rats' post-exercise test weight. We have previously reported qualitatively that determination of VO_{2peak} utilizing this protocol is highly reproducible; however, quantitative measures of within-rat reproducibility have not been reported previously.

Experimental Protocol

All rats were familiarized with running on a motor-driven treadmill 5-10 min/day for 5 days at 25 m/min, +10% grade. Upon familiarization, and within a maximum of 3 days of the final acclimatization run, the experimental protocol was initiated in which each rat performed 5 submaximal endurance runs to fatigue for the determination of exercise tolerance, and 5 maximal exercise tests for determination of VO_{2peak}. No exercise tests, irrespective of nature, were completed on successive days. Prior to each run (~2 hours) rats were given a calcium sensitizing compound via gastric gavage. Our laboratory is experienced with this technique. The rats were divided randomly into three groups, which consisted of control (n=14), a High dose group (n=14), which performed each test at 3 different doses (10, 20, and 40 mg/kg), and a Low dose group (n=5) which performed each test at different doses (0.5, 1, and 5 mg/kg).

Experimental Design



Statistical Analysis

All data are presented as mean ± SE. Repeated measures ANOVA was used to compare data obtained on the exercise tolerance and VO_{2peak} tests across conditions. Where a significant difference was detected, Tukey's *post hoc* test was used to determine where the differences existed between runs. Pearson correlations were used to assess the possibility of correlations between variables. The significance level was set at $P < 0.05$.

CHAPTER 4 - Results

Body Weights

The average initial body weight of the rats was 352 ± 13 g (range 278 – 396 g) and increased to 473 ± 10 (range 396-513 g) by the end of the ~6 week experimental protocol.

Measurement of Endurance Exercise Tolerance

The average endurance running time is presented in Figure 1. The dose of 10 mg/kg significantly increased ($P<0.05$) running endurance. This effect was not present at either lower (0.5-5 mg/kg) or higher (20-40 mg/kg) doses. Indeed, the very high dose of the calcium sensitizing compound (40 mg/kg) had a deleterious effect on exercise performance ($P<0.05$). The individual responses from the high dose group are represented in figure 2. Once again, the data indicates a significant increase ($P<0.05$) in the endurance capacity at 10 mg/kg, and a marked decrease ($P<0.05$) in the endurance capacity at 40 mg/kg. Control group running times were not significantly different from each other ($P<0.05$)

Measurement of VO_{2peak}

The average VO_{2peak} for all six doses of the calcium sensitizing compound is presented in figure 3. As depicted, the increase in running capacity at 10 mg/kg occurred in the absence of any alterations in VO_{2peak}. The highest dose (40 mg/kg) showed a significant decrease in VO_{2peak} ($P<0.05$) compared to all other doses of the drug, including the control condition. Control Group VO_{2peak} was not significantly different from each other ($P<0.05$).

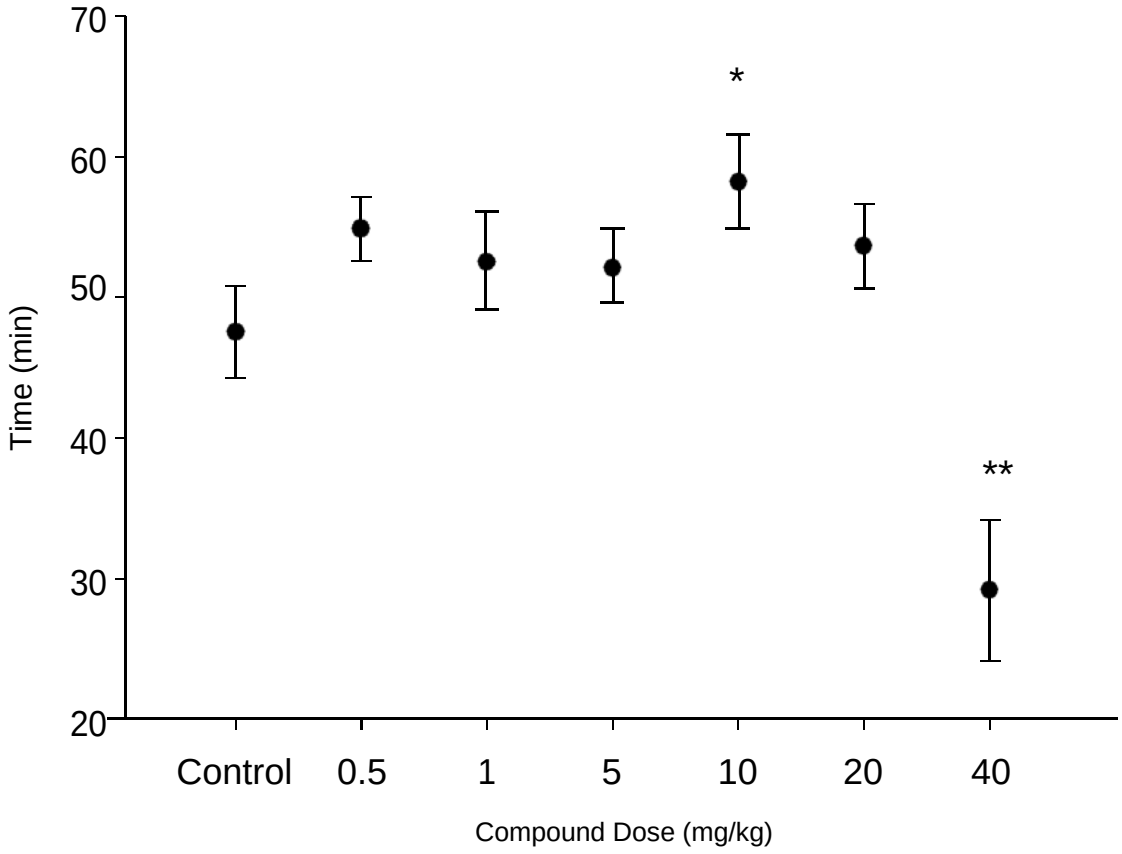


Figure 4.1. Endurance running times for all six doses of the calcium sensitizing compound. Mean ± SE. * P< 0.05, ** P< 0.05 vs. all other compound doses.

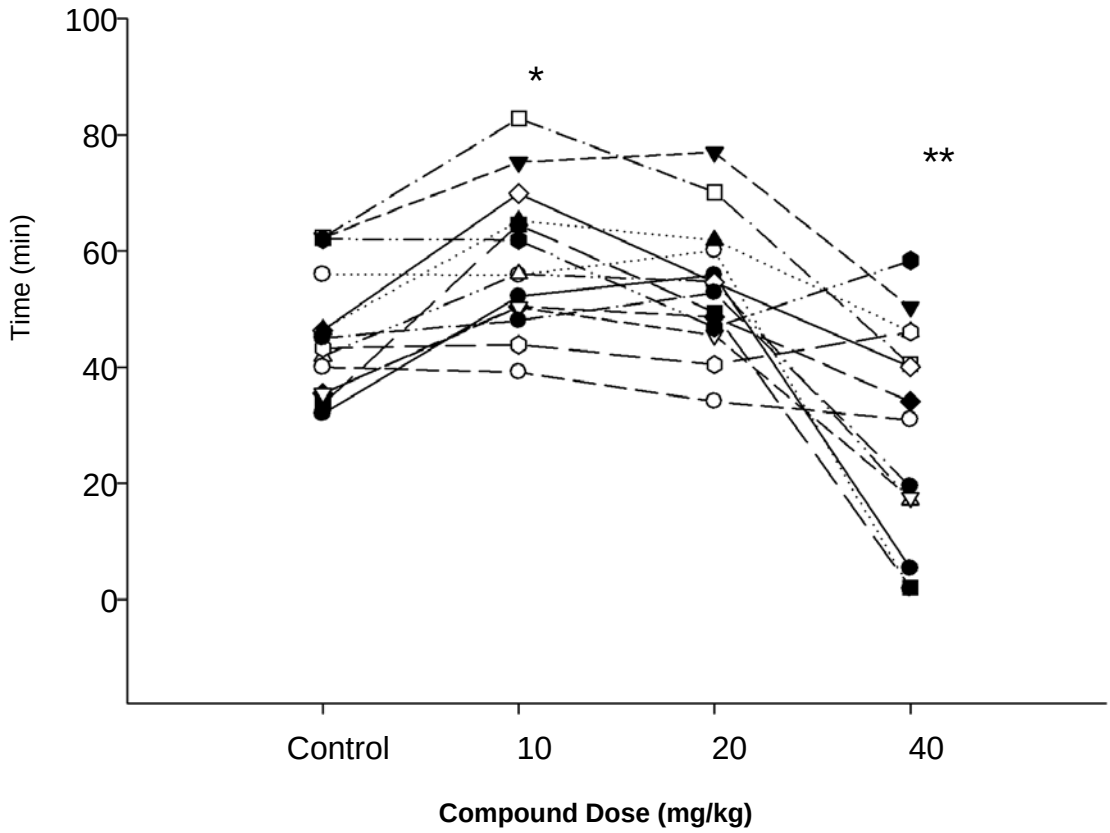


Figure 4.2. Individual responses from high dose group for endurance running time. * P< 0.05, ** P< 0.05 vs. all other drug doses.

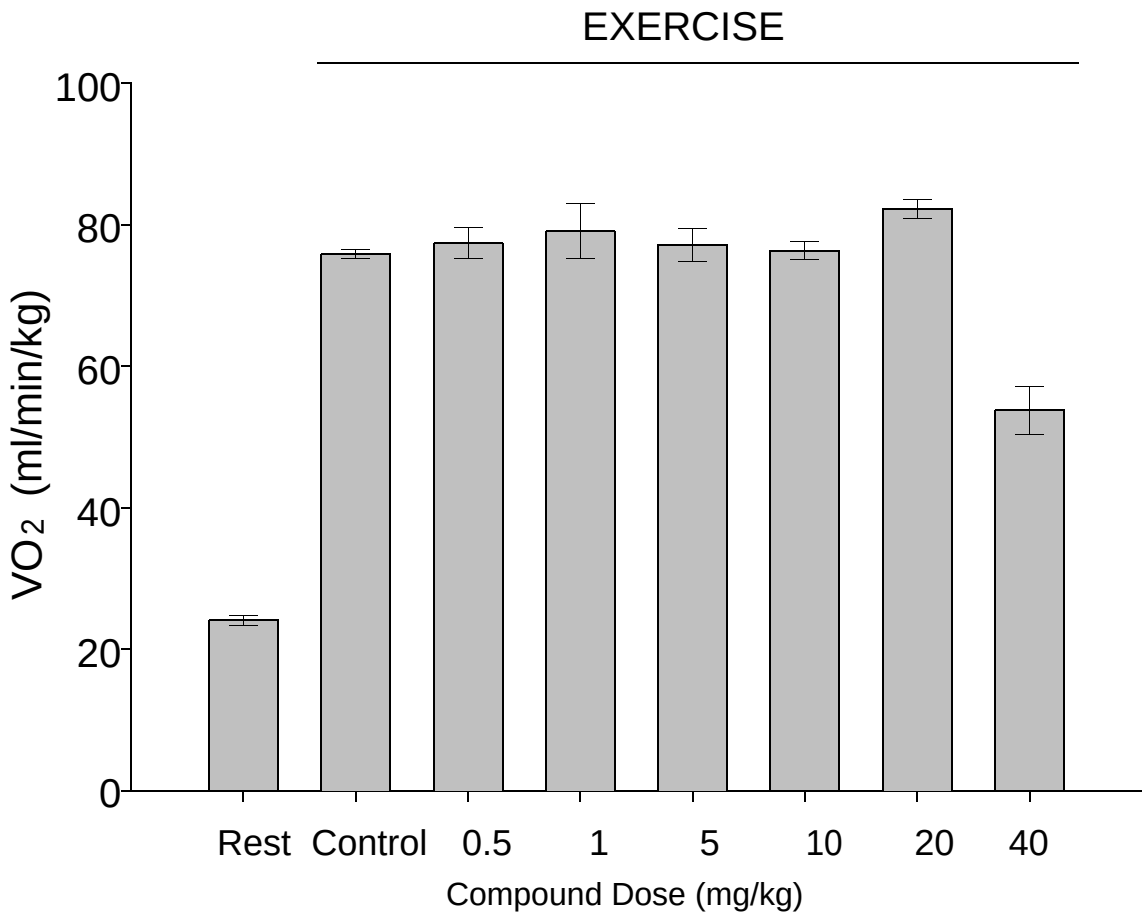


Figure 4.3. VO_{2peak} responses measured at all six doses of the calcium sensitizing compound along with VO_{2peak} measured under resting conditions. Mean ± SE. * P< 0.05 from all other doses of the compound.

CHAPTER 5- Discussion

The key finding of this investigation was that acute administration of calcium sensitizing compound, at a dose of 10 mg/kg, effectively increased the running endurance in young male Sprague-Dawley rats. This effect was not present at either lower (0.5-5 mg/kg) or higher (20-40 mg/kg) doses. The improvement in running capacity occurred in the absence of any alterations in VO_{2peak}.

Putative Mechanism(s) of Action

The physiological mechanism behind how the calcium sensitizing compound used in this investigation works remains unknown to our investigative team at this time. However, this calcium sensitizing compound is postulated to have a role in increasing Ca^{2+} sensitivity of skeletal muscle. A reduction in maximum isometric force is one expression of fatigue. There are at least two metabolic changes that contribute to a decline of maximum isometric force: acidosis (due to lactic acid accumulation) and accumulation of P_i (due to PCr breakdown). It is well established that acidosis and increased P_i reduce the maximum isometric force in both skinned and intact muscle fibers (Fabiato & Fabiato, 1978; Sahlin *et al*., 1989; Westerblad & Allen 1993; Pate & Cooke, 1989; Dantzig *et al*., 1992). Acute administration of the compound used in our investigation may have increased cystolic $[Ca^{2+}]$ during fatigue. As the muscle becomes more fatigued there is a decrease in cystolic $[Ca^{2+}]$. This is partially due to increased P_i, which ultimately leads to a decreased Ca^{2+} in the SR ($[Ca^{2+}]_{SR}$). The compound used in our study may increase the amount of Ca^{2+} available as the $[Ca^{2+}]_{SR}$ declines during fatigue perhaps preserving function.

The reduction in myofibrillar Ca^{2+} sensitivity associated with fatigue can occur either via competition for the Ca^{2+} -binding sites on troponin C or via a reduction in the strength of cross bridge attachment (Allen *et al*., 2008). The classical view for acidosis is that an increase in cystolic $[H^+]$ inhibits Ca^{2+} binding to troponin C via competition between H^+ and Ca^{2+} (Blanchard *et al*., 1984). The compound used in our study may have increased the amount of free $[Ca^{2+}]$ and allow more Ca^{2+} to bind to troponin C, and also increased the affinity of troponin C for $[Ca^{2+}]$ which in turn increases force production.

Reduced SR Ca^{2+} release, caused by a decrease in the cystolic $[Ca^{2+}]_i$ is the final component of the reduction in isometric force. Impaired SR Ca^{2+} release is a well-established mechanism of fatigue (Allen et al., 2008; Favero,1999). The combined effects of reduced $[Ca^{2+}]_{SR}$, secondary to CaP_i precipitation, the inhibitory effect of high $[Mg^{2+}]_i$, and lowered [ATP] on the RyR all may contribute to reduced Ca^{2+} release. It also is postulated that there is a failure of the AP in the T-tubules (Allen *et al*., 2008). The compound used in our study may work like other agents capable of facilitating SR Ca^{2+} release, such as caffeine, by sensitizing the RYR channels to activation of cystolic $[Ca^{2+}]$, leading to an increase in the open probability (P_o) of the channel (Eberstein and Sandow, 1963).

The compound used in our study was able to increase the rats' endurance capacity in the absence of altered VO_{2peak}. Therefore, the increase in endurance capacity was not due to changing the exercise intensity domain per se for any given state of the test.

Comparison with Current Literature

The data suggest that a calcium sensitizing compound can improve performance and increase exercise tolerance, but only at the specific dose of 10 mg/kg for the compound we used in this investigation. Administration of the compound at a dose of 10 mg/kg increased exercise tolerance 22% (from 47.54 ± 3.12 min, control – to 58.28 ± 3.30 min, $P<0.05$). The data also suggest that treatment with the compound at high doses (40 mg/kg) may actually be deleterious to exercise performance. The findings of this study clearly warrant additional experiments to test the efficacy of this compound in animal models of chronic disease (i.e. CHF and diabetes). These individuals could benefit from such a calcium sensitizing agent and increase their exercise tolerance. This enhanced physical capacity could be of great therapeutic value in helping to alleviate symptoms associated with different disease states (i.e. CHF and diabetes).

There is a broad range of strategies available that may improve exercise tolerance, such as the administration of sodium bicarbonate ($NaHCO_3$), antioxidant supplementation, carbohydrate (CHO) loading, and the ingestion of caffeine. The efficacy of these will be considered below.

The evidence suggesting that $NaHCO_3$ ingestion can be beneficial and may increase work capacity is varied. Mckenzie *et al*. (1986) demonstrated that the administration of

$NaHCO_3$ increased time-to-fatigue by 4%. The subjects in the study performed six 60-s cycling bouts, at a work rate corresponding to 125% VO_{2peak}, with 60 s recovery between work bouts. The sixth work bout was continued until the pedal rate dropped below 50 rev min. Another study suggested that $NaHCO_3$ could increase mean peak power by 2% (Lavender *et al*., 1989). Subjects in this latter study completed six trials; each trial consisted of 10 ten-second sprints on a cycle ergometer with 50 seconds recovery between each sprint. $NaHCO_3$ acts as a buffer to help sequester hydrogen ions and forestall the decrement in blood and muscle pH. $NaHCO_3$ binds with H^+ to form carbonic acid, which subsequently dissociates to carbon dioxide (CO_2) and water (H_2O) (Matson *et al.,* 1993). In general, $NaHCO_3$ is thought not to have a major impact on endurance performance due to the fact that endurance exercise is supported mostly by oxidative metabolism.

The data suggesting that antioxidant supplementation has an effect on exercise performance is also varied. It has been demonstrated that antioxidants help protect against the effects of exercise-induced muscle damage (Sen, 2001). Kaminiski and Boal (1992) demonstrated that antioxidant supplementation reduced muscle soreness by >33%. Schröder *et al*. (2000) observed a decrease of 13.6% in the lipoperoxide levels in athletes who were supplemented with a three-compound antioxidant regimen, and an increase in the oxidative status. Therefore the question still remains whether individuals need antioxidant supplements to prevent oxidative damage from exercise or to help them to recover from that damage.

The use of caffeine as an ergogenic aid was stimulated by a study examining the effect of caffeine ingestion prior to cycling to exhaustion (Costill *et al*., 1978). The cyclists increased their time to exhaustion by 28% (75 min placebo vs. 96 min caffeine ingestion). A second study also demonstrated the ergogenic potential of caffeine by increasing the amount of work performed in 2 hours of isokinetic cycling exercise (80 rpm) by 20% (Ivy *et al*., 1979). It has also been shown that caffeine can increase lypolysis 23% which may lead to a glycogen sparing within the skeletal muscle via stimulating adrenaline secretion which mobilizes free fatty acids which in turn causes an increase in fat utilization thereby retarding glycogen depleting (Costill *et al*., 1978). In addition to reducing glycogenolysis caffeine increases $[Ca^{2+}]_i$ via mobilization of Ca^{2+} from the SR (Juhn, 2002; Graham, 2001).

It now widely accepted that several days of elevated CHO intake following a glycogen depleting bout of exercise will super-compensate glycogen levels and help improve exercise performance, especially in exercise lasting longer than 90 minutes but, also during very high intensity exercise. Diets high in complex or simple CHO have been shown to increase running endurance by 23 and 26% (Brewer *et al*., 1988; Lamb *et al*., 1991). Another study showed that 312 grams of carbohydrate ingested 4 hours prior to moderately intense intermittent endurance exercise increased performance by 15% (Sherman *et al*., 1989).

The commonality between the nutritional ergogenic aids discussed, and the compound used in this study is that they all result in an increase in exercise performance, and some may also decrease the damage caused by strenuous exercise, allowing the individual to exercise for longer durations at high intensity. The calcium sensitizing compound used in this study was able to increase endurance tolerance by 22%. This value is impressive when compared to other so-called ergogenic aids evaluated in the scientific literature, and reinforces the therapeutic potential that calcium sensitizing agents may have for individuals with chronic disease (i.e. CHF and diabetes).

Methodological Considerations

In the rat, a broad range of treadmill running protocols exists for submaximal exercise endurance tests. It is surprising, therefore, that no rigorous analysis of exercise performance reproducibility has been performed in the rat, particularly considering the diversity of protocols, equipment, and running technique employed in different laboratories (Bedford *et al*.,1979; Musch *et al*., 1988; Hilty *et al*., 1989; Dubouchaud *et al*.,1999; Kim *et al*., 2003; Georgieva & Boyadjiev, 2004). The design of the treadmill protocol in the present investigation ensured that fatigue occurred within a reasonable time and maximizes both aerobic and so-called anaerobic energy systems. Using the present protocol Copp *et al.* (2009) demonstrated that, over time, exercise tolerance and VO_{2peak} are highly reproducible with repeated testing coefficients of variation < 10%. In this regard, important considerations for experimental design include the introduction of increasing variability as more exercise tolerance test are performed. In addition, there is the potential for confounding influence of increases in body mass during the experimental protocol that might affect VO_{2peak}. However, our results and our experimental design provide confidence that this did not influence the results of the present investigation.

Conclusions

As stated earlier, there are many ergogenic aids directed towards athletes and "weekend warriors" that can potentially improve exercise performance. What is less appreciated is that individuals who suffer from chronic diseases (i.e. CHF, and diabetes) can also benefit greatly from the administration of ergogenic aids to improve exercise performance, and their quality of life. In the case of CHF, most pharmacological interventions have been aimed at improving some of the central components to offset fatigue, for example enhancing heart function and reducing the increase in sympathetic system stimulation. A novel approach to combat CHF may be to target the periphery (i.e. muscle) and reverse the pathologic changes in the skeletal muscle itself possibly through the administration of calcium sensitizing compounds.

The compound used in this study was able to significantly increase endurance capacity of normal rats at a dose of 10 mg/kg. Indeed, this improvement was not accompanied with increases in VO_{2peak}. Further studies need to be conducted to explore the efficacy of this compound in animal models of chronic disease (i.e. CHF and diabetes). The compound used in our investigation may potentially be an ergogenic aid for patients whom enhanced physical capacity could be of great therapeutic value, and lead to increases in activities of daily living and quality of life.

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