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Mechanisms of Pronounced and Sustained Microvascular Vasoconstriction during Cryotherapy

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I would like to take some time to acknowledge all those involved in helping me get to this point. Being in school this long could not have been possible if it weren't for my parents and family inspiring me. I've been blessed to have a great number of excellent educators in my two decades in school, inspiring me to learn and achieve the most that I can. I greatly appreciate the effort and time put in by my college and graduate school mentors who helped introduce me to research and achieve great things with it. I couldn't have done all of this without the help of my research participants who helped sit for multiple hours and refrain from using the bathroom or eating breakfast.

Mechanisms of Pronounced and Sustained Microvascular Vasoconstriction during Cryotherapy

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Cryotherapy is the commonly used application of a cold compress to alleviate pain and swelling with injuries. However, while being one of the more well-known and commonly used therapies, there is limited systematic data collected in controlled environments showing benefits of cryotherapy. Many claims and perceived benefits of cryotherapy are based on empirical and anecdotal evidence, leaving much to be discovered. A typical cryotherapy protocol of 30 minutes can suppress blood flow, tissue temperature, and present with sustained vasoconstriction in the hours after treatment. This vasoconstriction can cause tissue death and necrosis, deemed non-freezing cold injuries, and results in roughly ~1,500 reported clinical cases per year in the United States. Furthermore, non-freezing cold injuries in the civilian population are a rare occurrence, but this type of injury, which relates to these investigations can reach epidemic proportions during times of war. Our primary findings from the research have implicated Rho kinase as being primarily involved in the formation of sustained vasoconstriction following cryotherapy treatment. Additionally, Rho kinase action impairs the temperature blood flow relationship that is necessary for abolishing much of the risk of ischemic injury. Secondary findings have

furthered our knowledge of mechanisms of locally induced vasoconstriction and the significance of the sympathetic nerves in returning blood flow towards basal conditions following cryotherapy treatment. Finally, these investigations have shown that there appears to be limited involvement of nitric oxide in the lower ranges of blood flow. This finding might suggest that during a pronounced vasoconstrictive state (60-80% reduction in basal blood flow) much of the action of Rho kinase drives its activity by enhancing adrenergic activity. Our findings are generally in agreement with previous literature investigating mechanisms of cold-induced vasoconstriction. However, these previous studies typically made measurements on much smaller skin surfaces and also used much more mild temperatures for cooling. Accordingly, these findings outlined in the current studies improve our understanding of the vascular kinetics of cryotherapy use. We conclude that there needs to be a greater understanding and awareness provided to layman users of cryotherapy, allowing them to understand the risks and appropriate methods of application.

Table of Contents

List of Figures

Chapter 1: Introduction

Cryotherapy is the use of cold as a therapeutic technique focused on localized cooling in attempts to reduce tissue damage, swelling, pain, bleeding, and inflammatory processes(84). Many of these benefits lie within the physiological benefit of low tissue temperature. Lowering tissue temperature (thermal effect) is considered to be the most advantageous benefit of cryotherapy, over the vascular responses, and neural benefits(4, 65, 85). It is a commonly utilized therapeutic approach to treating an injury or illness and is an almost essential follow up to many surgeries, soft tissue injuries, as well as a host of other clinical treatments. Cryotherapy application involves locally reducing tissue temperature of the affected area through the use of cold water (non-frozen; >0°C) or direct ice application. Clinically, there are many advantageous effects of cryotherapy (as outlined above), which vastly outweigh the potential deleterious effects. However, while beneficial for most individuals, cryotherapy is often associated with a number of negative side effects. For example, cryotherapy treatment has been linked to a variety of conditions including, tissue necrosis, neuropathy, and in the most extreme cases limb amputation. These conditions are likely the result of drastic reductions in local tissue temperature and the subsequent pronounced tissue ischemia following the period of cryotherapy, which can lead to tissue necrosis and nerve injury. The vasoconstrictive effects of cryotherapy extend beyond the treatment phase and persist for hours following the termination of the cooling.

Injury with cryotherapy is likely, at least in part, attributed to the pronounced local vasoconstriction which results in an inability to deliver blood and oxygen to the tissues for

a sustained period causing tissue ischemia, necrosis, and death. This ischemia can occur due to short (i.e. within 5 min) and prolonged (i.e. 30 min or longer) cryotherapy treatment, but also persists during the subsequent recovery phase. Cryotherapy can cause persistent vasoconstriction even after the cessation of cooling, which can lead to cell death if oxygen delivery is compromised to the tissues for a substantial duration of time. This persistence arises from enhanced modulation of the vasoconstrictor tone and inhibition of the vasodilatory pathway(s)(87), which have been previously elucidated(13, 32, 42, 45, 51, 56, 60, 66). Independently these responses aren't deleterious, however, once tissue rewarms, and vasoconstriction persists, profound ischemic stress develops(65). The underlying interplay between the two systems is currently not well understood, within in vivo models(87). The two pathways are mutually exclusive and each acts to offset or counterbalance the effect of the other, but their role, as it pertains to cryotherapy, is not yet known. The previous studies investigating the interplay or independent vascular pathways during skin surface cooling were highly controlled. While these studies are very informative and provide much mechanistic insight into vascular control, from a physiologically perspective they used conditions (see below) that likely do not adequately represent what occurs during cryotherapy treatment. For example in most research studies, the rate, magnitude and duration of cooling are very well controlled; however, these conditions do not necessarily reflect conditions imposed during cryotherapy treatment. For example the former involves much more rapid and dramatic cooling. Furthermore, these studies cooled a substantially smaller area of tissue than what is common during cryotherapy treatment (silver dollar vs. entire joints and muscle groups; respectively).

Therefore, understanding the role of these antagonistic pathways during a practical application such as cryotherapy treatment is necessary, to begin to understand ideal protocols for cryotherapy, with as few complications as possible.

Accordingly, the primary focus of my dissertation research is to investigate several candidate mechanisms involved in the control of vascular tone during skin surface cooling. This was accomplished by infusing vasoactive substances, via microdialysis, into the skin microvasculature. This approach allowed us to investigate the role of various mechanisms of vascular control within the skin while undergoing a typical cryotherapy protocol. Multiple treatment sites can be generated with the microdialysis technique. By monitoring the blood flow over each pharmacologically altered portion of the skin with laser Doppler probes, we assessed multiple components of these pathways as it pertains to ischemic injury with cryotherapy. With this approach, we investigated specific elements of the vasoconstrictor and vasodilator pathways with the focus on returning blood flow to baseline following treatment and eliminating the sustained vasoconstriction.

Chapter 2: Statement of the Problem

Cryotherapy is the use of cold as a therapeutic technique focused on localized cooling in attempts to reduce tissue damage, swelling, pain, bleeding, and inflammatory processes(84). Many of these benefits lie within the physiological responses to reductions in tissue temperature. Lowering tissue temperature (thermal effect) is considered to be the most advantageous benefit of cryotherapy, over the vascular responses, and neural benefits(4, 65, 85). It is a commonly utilized therapeutic approach to treating an injury or illness and is almost essential follow up to many surgeries, soft tissue injuries, as well as a host of other clinical treatments. Cryotherapy application involves locally reducing tissue temperature of the affected area through the use of cold water (non-frozen; >0°C) or direct ice application. Clinically, there are many advantageous effects of cryotherapy (as outlined above), which vastly outweigh the potential deleterious effects. However, while beneficial for most individuals, cryotherapy is often associated with a number of negative side effects. For example, cryotherapy treatment has been linked to a variety of conditions including, tissue necrosis, neuropathy, and in the most extreme cases limb amputation. These conditions are likely the result of drastic reductions in local tissue temperature and the subsequent pronounced tissue ischemia following the period of cryotherapy, which can lead to tissue necrosis and nerve injury. The vasoconstrictive effects of cryotherapy extend beyond the treatment phase and persist for hours following the termination of the cooling.

Injury with cryotherapy arises from the inability to deliver blood and oxygen to the tissues for a sustained period which can result in tissue ischemia, necrosis, and death. This

ischemia can occur due to acute and prolonged cryotherapy treatment, but also during the subsequent recovery phase. Cryotherapy can cause persistent vasoconstriction even after cooling terminates, which can lead to cell death if oxygen delivery is compromised to the tissues. This persistence arises from enhanced modulation of the vasoconstrictor tone and inhibition of the vasodilatory pathway(s)(87), which have been previously elucidated(13, 32, 42, 45, 51, 56, 60, 66). Independently these responses aren't deleterious, however, once tissue rewarms, and vasoconstriction persists, profound ischemic stress develops(65). The underlying interplay between the two systems is currently not well understood, within in vivo models(87). Cryotherapy has a wide range of uses clinically. It is often associated with a host of different modalities aimed at utilizing the benefits from cold in treating an underlying injury or medical concern. However, as previously mentioned, it is not without its potential complications. The purpose of this series of investigations is to understand better how cryotherapy can generate secondary ischemic damage within the treatment area due to the persistent vasoconstrictor tone. Therefore, in **study #1** we investigated one of the primary modulators of the vasoconstrictor pathway, Rho kinase, and its role in a cryotherapy protocol. Specific contributors of the Rho kinase pathway were teased out, pharmacologically, to investigate how this pathway modulates vascular tone while recovering from cryotherapy. Blood flow was assessed with laser Doppler flow probes, and specific parts of the constrictor pathway blocked utilizing the microdialysis technique to infuse drugs, pharmacologically, changing the vascular response. The aim for **study #2** was to identify the role of the adrenergic nervous system on blood flow during cryotherapy induced cooling as well as during passive and active rewarming. Due to the clear role that

the sympathetic nerves play in systemic vascular vasoconstriction as well on cutaneous vasodilation, we investigated the significance of adrenergic function within cryotherapy, utilizing the same methods as in study #1. However, following passive rewarming an acute active heating phase occurred by perfusing hot water on the treatment site to assess subsequent vasoreactivity. We blocked multiple aspects of the sympathetic pathway to investigate this issue both pre and post-synaptically. Finally, for the purposes of **study #3** we focused on one of the primary vasodilatory pathways, the nitric oxide pathway. Persistent vasoconstriction is thought to downregulate the nitric oxide pathway which, therefore, may contribute to the ischemic state independent / or in addition to the actual vasoconstrictive effects. The investigation into the role of nitric oxide during cryotherapy and recovery is, therefore, essential for returning blood flow to baseline conditions. ***Overall, we used these 3 studies to investigate the role of multiple steps of the two antagonistic systems, vasoconstriction, and vasodilation, are regulated as the skin undergoes a typical cryotherapy protocol.***

Chapter 3: Experimental Design

Systematic investigations into the mechanisms of vasoconstriction that mediate the profound state of vasoconstriction that occurs during cryotherapy requires a pharmacological approach. Drug manufacturing has come a long way and allows for specific blockade of receptors, pathways, and multiple isoforms of certain receptors, enzymes, or molecules, ultimately allowing for a systematic approach to understanding many aspects of vascular tone. Additionally, the microdialysis technique is a minimally-invasive technique able to deliver a drug challenge that can either enhance or inhibit pathways and molecular action. An additional advantage to microdialysis is the localized effect of these drug challenges, allowing the assessment of multiple drugs on several sections of treated skin simultaneously. We, therefore, utilized the microdialysis technique while assessing the action of three separate drug challenges with a typical cryotherapy protocol and commercially available cryotherapy unit. This protocol design allowed for the most translatable model towards either the clinical application of cold or the self-treating individuals in their homes. This approach provides us with the greatest chance of furthering the knowledge and understanding of cryotherapy treatment as a whole.

Chapter 4: Study #1

ABSTRACT

Cryotherapy is a therapeutic technique using ice or cold water applied to the skin to reduce local blood flow. While beneficial, there are some side effects such as pronounced vasoconstriction and tissue ischemia that is sustained for hours post-treatment. For example, cryotherapy treatment induces pronounced tissue ischemia that is sustained for hours even during a post-treatment rewarming period.

PURPOSE: To investigate the role of multiple components of the Rho kinase activation on cutaneous blood flow during 30 min of cryotherapy as well as during the subsequent 2 hr of passive rewarming. This study tested the hypothesis that this pronounced and sustained vasoconstriction is mediated by 1) Rho-kinase and/or 2) elevated oxidative stress and 3) neuropeptide-Y.

METHODS: 13 subjects were fitted with a commercially available cryotherapy unit with a water perfused bladder on the lateral portion of the right calf. Four microdialysis membranes were threaded through the dermis of the skin directly underneath the water bladder. One site was perfused with lactated ringers solution (CON) which served as the control site, one with Fasudil (FAS) to locally block the Rho Kinase pathway, one with BIBP (BIBP) to block Neuropeptide Y mediated vasoconstriction, and one with vitamin C (VitC) to reduce cold-induced oxidative stress. Skin temperature (SkT) and skin vascular conductance (CVC) was measured at each site. Following instrumentation and baseline

data collection the subjects were exposed to 0 °C water perfused through the cryotherapy bladder for 30 min, followed by passive rewarming (i.e. water shut off) for 2 hr.

RESULTS: SkT fell from 34°C to 17.9°C during cold water application across all sites. CVC was reduced after 30 min of cooling with both FAS and VitC (71 and 53%, respectively; $p<0.05$) having attenuated vasoconstriction when compared to control (43%). Following 2 hr of passive rewarming CVC was elevated at the FAS site (69%) whereas it remained reduced at the CON and VitC sites (26 and 25%, respectively; $p<0.05$).

CONCLUSION: These findings indicate that the Rho-kinase and oxidative stress contribute to pronounced vasoconstriction during cryotherapy and that the Rho-kinase pathway contributes to sustained vasoconstriction during the subsequent rewarming period post treatment

INTRODUCTION

Cryotherapy application is a widely used therapeutic technique using ice or cold water on the skin and is used clinically to reduce local blood flow(17, 86, 94), decrease cellular metabolism(74), reduce pain(11), and attenuate edema formation(20). Cryotherapy treatment is commonplace therapeutically following surgery or injury to attenuate the associated swelling, pain, and damage that can occur within the injured tissue. Of the benefits of cryotherapy, the vascular effects are one of the three primary responses to skin cooling, which aids in reducing swelling and edema. Several investigators have identified the mechanisms of vasoconstriction responsible for this vascular response during application of local cooling(6, 7, 51, 72, 97), but still not understood is how these mechanisms pertain to cryotherapy treatment in clinical settings. Investigations into the mechanisms of cooling induced vasoconstriction have found the Rho kinase pathway(6) to be centrally involved in the cutaneous vasoconstrictor response. It is activated by the formation of mitochondrial reactive oxygen species in response to cold within the vascular smooth muscle(7) and exists as one of the most powerful modulators of vasoconstriction.

Rho kinase in response to local skin cooling induces translocation of α2-adrenoreceptors to the smooth muscle surface and also increases their sensitivity for norepinephrine(29, 47, 73, 93), with an additional inhibition of vasodilator pathways(42, 87). The Rho kinase pathway also downregulates eNOS(87) and thus reduces nitric oxide (NO) bioavailability during as well as for up to 2 hr post skin cooling(42). Skin cooling to 24 ºC presented with a 40% reduction in basal blood flow for two hours, which would imply pronounced constrictor tone. These findings make the Rho kinase pathway of

primary interest in regards to skin cooling and rewarming due to the changes in the NOS pathway and enhanced constrictor tone. In addition, previous studies have determined that subcutaneous application of Vitamin C attenuates cold-induced vasoconstriction, potentially due to a reduction in reactive oxygen species (ROS) formation in the mitochondria impairing Rho kinase activation.

Cryotherapy treatment typically involves cooling of a much larger skin surface area, a greater magnitude of skin cooling, and ultimately a greater depth of cooling within the tissue relative to the aforementioned studies that have investigated mechanisms of cold-induced vasoconstriction (39, 41, 97). Additionally, sustained vasoconstriction to local cooling at 24 ºC increases release of norepinephrine and neuropeptide-Y (NPY) from the sympathetic nerve terminals. To our knowledge, the relative contribution of NPY to cold-induced vasoconstriction remains relatively unknown.

Accordingly, the aim of this study was to investigate the role of neuropeptide-Y, Rho kinase, and reactive oxygen species on the vasoconstrictor mechanisms during cryotherapy mediated cooling and the subsequent recovery of blood flow with passive rewarming. It is currently unknown how a greater magnitude of cooling and colder temperature would augment the findings and the post treatment recovery of blood flow shown in previous studies. We hypothesized that with the application of microdialysis in combination with cryotherapy we can identify that the Rho kinase pathway is a significant mediator of vasoconstriction during cryotherapy and sustained vasoconstriction following skin cooling with Fasudil treatment. Additionally, we hypothesized that local infusion of vitamin C we would be able to attenuate vasoconstriction by interfering with reactive

oxygen species formation and the activation of Rho kinase. Finally, with the inhibition of neuropeptide-Y we hypothesized that we would attenuate the magnitude of vasoconstriction that occurs with local cooling and following.

METHODS

Ethical Approval. The Institutional Review Board at The University of Texas at Austin approved all study procedures and the consent process used in the present study. Subjects were given a verbal description of all procedures and informed of the purpose and risks involved in the study before providing their informed and written consent.

Subjects. 12 healthy young subjects (10 males) participated in this study. Average (mean ± SEM) subject characteristics were: age, 24 ± 1 years; height, 180 ± 1 cm; and weight, 81 ± 3 kg. Subjects were non-smokers, were not taking medications and were free from cardiovascular, neurological, or metabolic diseases. None of the subjects reported a history of knee injury or cryotherapy or other form of cold exposure in the lower extremities for at least a year prior to the experiment. All studies were conducted in the morning following an overnight fast (> 12 hr). Subjects refrained from strenuous exercise, alcoholic beverages, and caffeine for 24 hrs prior to the experimental trial. Additionally subjects underwent an overnight fast for at least 10 hr prior to the experimental trial, which was conducted in a temperature controlled laboratory (~24°C and 40% relative humidity).

Instrumentation and Measurements: All data were collected with the subject seated in a semi-recumbent position. Four microdialysis membranes (CMA 31 Linear Microdialysis Probe, 55 KDalton cut-off membrane; Harvard Apparatus, Holliston, MA) were inserted ~5 cm apart into the nonglabrous skin on the lateral side of the right calf. Following placement, each membrane was perfused with lactated Ringer's solution (Baxter,

Deerfield, IL) at a rate of 2 μL/min via a perfusion pump (Harvard Apparatus, Holliston, MA) while insertion trauma associated with membrane placement subsided (minimum 90 min). During this period, each membrane site was instrumented with an integrating laser Doppler flow probe (VP7a, Moor Instruments, Wilmington, DE) which provided a continuous index of skin blood flow. A thermocouple (Type T Thermocouple Probe, Physitemp Instruments INC, Cliffton, NJ) was placed immediately adjacent to the Doppler flow probe for the continuous assessment of local skin temperature. Following placement of the membranes, Doppler flow probes, thermocouples, and a commercially available cryotherapy cooling pad (Arctic Ice Universal Pad; Pain Management Technologies, Akron, OH) was applied overlying the instrumented area, and fixed in place using an Ace bandage. The cooling pad was connected to an Arctic Ice cryotherapy unit (Pain Management Technologies, Akron, OH) which allowed for manipulation of the underlying skin (Tskin) and tissue temperature according to the manufacturer's recommendation (see below for more detail). A cuff was placed around the left arm for intermittent blood pressure measurements from the brachial artery using electrosphygmomanometry (Tango, SunTech Medical Instruments, Raliegh, NC).

Study Protocol: After the hyperemic response associated with insertion trauma subsided (minimum of 90 min) each site was perfused with its respective vasoactive agent for a 45 min wash in period. One site received lactated Ringer solution (CON, Baxter, Deerfield, IL) which served as the control site. Inhibition of the Rho Kinase pathway was achieved with a 3 mM Fasudil infusion to a second site (FAS, ToCris Bioscience, Ellisville, MO).

Inhibition of NPY was achieved through a 10 μM solution of the NPY Y1 antagonist N2-(diphenylacetyl)-N-([4-hydroxyphenyl]methyl)-d-arginine amide (BIBP; Sigma, St Louis, MO, USA) at a third site and the last site received Ascorbic Acid (Vitamin C) (VitC, 10mM, Mylan Institutional LLC, Rockford, IL) to locally scavenge reactive oxygen species. Fasudil, BIBP and Ascorbic Acid were dissolved in sterile lactated Ringer solution. Each site was initially perfused at 52 μL/min for a 30 sec priming period after which the rate was reduced to 2 μL/min for the remainder of data collection. Following instrumentation the cooling pad was perfused with 34 ºC water for the entire hyperemic and drug infusion period for baseline data collection. This was followed by 30 min of active skin-surface cooling that was accomplished by circulating 0 - 1 ºC water through the cryotherapy unit and cooling pad. At the end of the cooling phase the cryotherapy unit was turned off for a 2 hr period of data collection during passive rewarming and disconnected from the cold water reservoir.

Data Analysis: Laser-Doppler flux and Tskin data was continuously collected at a sampling rate of 125 Hz via a data-acquisition system (Biopac System, Santa Barbara, CA). One min averages of these data were analyzed at the following time points: the final min of the 34 ºC baseline condition (baseline), min 5, 10, 15, 20, 25, and 30 of active cooling; and min 30, 60, 90, and 120 of passive rewarming. Mean arterial pressure (MAP) was calculated as 1/3 systolic pressure + 2/3 diastolic pressure and used for subsequent calculation of cutaneous vascular conductance (CVC) (Doppler-derived flux/MAP). All CVC data throughout active cooling and passive rewarming were normalized to value

obtained during the final min of 34 ºC baseline and expressed as %CVC. Hysteresis loops were created by comparing the temperature and %CVC relationship for each of the different drug treatments. The slope for cooling and passive rewarming was separately measured between treatments for comparison.

Statistical Analysis: Statistical analyses were performed using a statistical software package (SigmaPlot 12.5; Systat Software, Inc., San Jose, CA). Skin blood flow, %CVC, and skin temperature, Tskin, were both analyzed using separate two-way repeated measures ANOVAs comparing the control site to the other sites (i.e. CON vs. FAS; CON vs. VitC; and CON vs. BIBP) by time effects. When a main effect or interaction was found, a Tukey's posthoc analysis was performed to determine further differences. All data is shown as mean±SEM. For all tests, significance was found at $P < 0.05$.

RESULTS

Throughout the active cooling period Tskin decreased at all sites relative to the pre-cooling baseline. Average temps at the sites were; Baseline: 34±0.2 vs. Cooling: 26.9±1.2, 24.1±1.2, 21.3±1.2, 20.1±1.1, 18.6±1.1, 17.9±1.1, °C; 5, 10, 15, 20, 25, 30 min cooling, respectively; p=0.001) and recovered towards baseline with passive rewarming (30, 60, 120-min Rewarming: 23.6±0.4, 25.3±0.3, 27.5±0.3 °C, respectively; p=0.001), but still had not reached baseline levels even after 2 hrs of rewarming (Fig. 1a). Tskin were no different among the 4 trials at any time point ($p>0.05$).

Skin vascular conductance, %CVC, during the ~34°C baseline and throughout the cooling and reheating protocol is illustrated in Figure 1b. Local cold water application resulted in a significant decrease from baseline %CVC in all trials ($p<0.001$). During active skin-cooling CVC was reduced to a greater magnitude at the CON site relative to the VitC site (43±5 vs. 53±9 % of baseline, respectively; p =0.027) and FAS site (43±5 vs. 71±14 % of baseline, respectively; p =0.001); however, the reduction at the CON and BIBP sites were similar (43±5 vs. 44±24 % of baseline, respectively; $p>0.05$) (Fig. 1b). During passive rewarming, CVC at the control site was reduced relative to the FAS site (27±7 vs. 69±13 % of baseline, respectively; p=0.001), but CVC at the CON site was similar to both the VitC site and the BIBP site (26±5 vs. 25±3 vs. 25±12 % of baseline, respectively; $p>0.05$). In fact in these three sites CVC was reduced 2 hr post-cooling to a similar degree as at the end of 30 min skin-surface cooling (CON: 30-min Cooling: 29±2 vs. Rewarming @ 30, 60, 120: 21±3, 23±2, 35±6 % of baseline, respectively; p=0.86) (VitC: 30-min

Cooling: 36±4 vs. Rewarming @ 30, 60, 120: 23±2, 24±2, 30±2 % of baseline, respectively; p=0.89)

Hysteresis loops were broken down into cooling and passive rewarming. There was a significant difference between slopes during the cooling phase (CON: 4.16±0.3, VitC: 4.16±0.3, BIBP: 3.53±0.6, FAS: 2.7±0.3 %CVC/°C; p<0.048), and during passive rewarming (CON: 1.33±0.5, VitC: -0.12±0.5, BIBP: -0.44±1.1, FAS: 3.09±1.2 %CVC/°C; p=0.036), with posthoc analysis showing FAS being significantly different from BIBP (p=0.044) (Fig. 2). When all data was run as a two-way ANOVA (treatment vs. cooling/passive rewarming) there was a no significant effect of treatment (p=0.32), but there was an effect of cooling/passive rewarming (p<0.01) and a significant interaction between treatment and cooling/passive rewarming (p<0.01). Post hoc analysis shows that within each treatment there was a significant difference between their respective cooling and passive rewarming phases (CON: 3.53±0.6 vs. -0.44±1.1; p<0.01; VitC: 4.16±0.3 vs. -0.12±0.5; p < 0.01; BIBP: 4.16±0.3 vs. 1.3±0.5 %CVC/°C; p<0.01), except in the case of FAS, which was highly non-significant (2.7±0.3 vs. 3.1±1.2; p>0d.98).

DISCUSSION

Our primary finding is that the pronounced vasoconstriction that occurs during 30 min of skin-surface cooling and the sustained vasoconstriction that persists in the subsequent 2 hr passive rewarming period was significantly blunted when the Rho Kinase pathway is blocked. Additionally, vasoconstriction during skin-surface cooling was blunted following antioxidant supplementation; however this treatment did not have an effect on the sustained vasoconstriction during passive rewarming. Contrary to our hypothesis blockade of NPY receptors had no effect on vasoconstriction during either the cooling or rewarming periods. Together these data suggest a disconnect between skin temperature and blood flow during rewarming, and a unique role for ROS and the Rho kinase pathway in the vasoconstrictor response during and after cryotherapy application (Fig 2).

Our data agrees with findings that suggest local cooling mediated vasoconstriction is directly impacted by Rho kinase(6). Fasudil treatment inhibited the Rho kinase pathway and was responsible for roughly 40% of the total vasoconstrictor response over CON. Thompson-Torgerson et al. performed a similar protocol by cooling skin from 34-24°C and showed a similar response by inhibiting the Rho kinase pathway while control sites fell 77% (88). We experienced a greater degree of vasoconstriction while infusing Fasudil despite a similar level of vasoconstriction in the control site, which could be attributed to the magnitude of skin cooling, the rate of cooling, and the application of an ice slurry (0-1°C) rather than a small surface cooling elements (24°C) or suggest a larger contribution of local adrenergic mechanisms with our protocol(88).

Since the production of mitochondrial ROS has been proposed as a critical step in the signaling mechanism for vasoconstriction, we chose to investigate this mechanism during cryotherapy. Other studies have shown that vitamin C application with microdialysis attenuates vasoconstriction when using smaller skin surface cooling devices and cooling to 24°C(97). Despite these minor differences in cooling protocol, our results support a similar attenuation in the rate of vasoconstriction with vitamin C. However, as vitamin C was not able to fully attenuate the vasoconstrictor response and formation of Rho kinase we believe this data suggests incomplete blockade. This is based on the fact that the VitC and FAS treatments were significantly different. One shortcoming with the use of vitamin C it is non-specific once introduced, and may require a more specific anti-oxidant to abolish the ROS formation within the mitochondria.

Statistically, there is little to be said about the hysteresis loops as the posthoc analysis was not able to show any differences between treatments. However, Fasudil treatment was the only treatment to show a similar temperature to blood flow relationship between cooling and passive rewarming. This suggests that the inability of the cold vasoconstricted tissue to return towards basal levels of blood flow is primarily a function of Rho kinase. This is the strongest evidence towards Rho kinase mediating the sustained vasoconstriction due to the similar rate of cooling and rewarming with Fasudil treatment.

Some experimental considerations to take into account are that the 3 mM fasudil used in this study may not have fully blocked the Rho kinase pathway. At larger doses, fasudil starts to have significant action on multiple kinases also responsible for vasoconstriction, but not the Rho kinase pathway(88). Previous studies have shown a high

degree of blockade with 3 mM. However, our study utilized a larger magnitude of cooling limiting the comparability between studies. Additionally, since the application of Y27632 is currently not FDA approved fasudil remains the only complete inhibitor of the Rho kinase pathway FDA approved for human use.

In summary, fasudil treatment and inhibition of Rho kinase explains a large portion of the vasoconstrictor tone that occurs during and following a typical cryotherapy protocol. This conclusion is supported by both previous and present findings that fasudil mediates a large portion of the vasoconstrictor response to local cooling. The results of this study also implicate Rho kinase as the mediator of sustained and pronounced vasoconstriction and adds to our understanding of non-freezing cold injuries in both civilian and military populations.

TABLES AND FIGURES

Figure 1. Temperature and blood flow responses to cryotherapy and the subsequent recovery

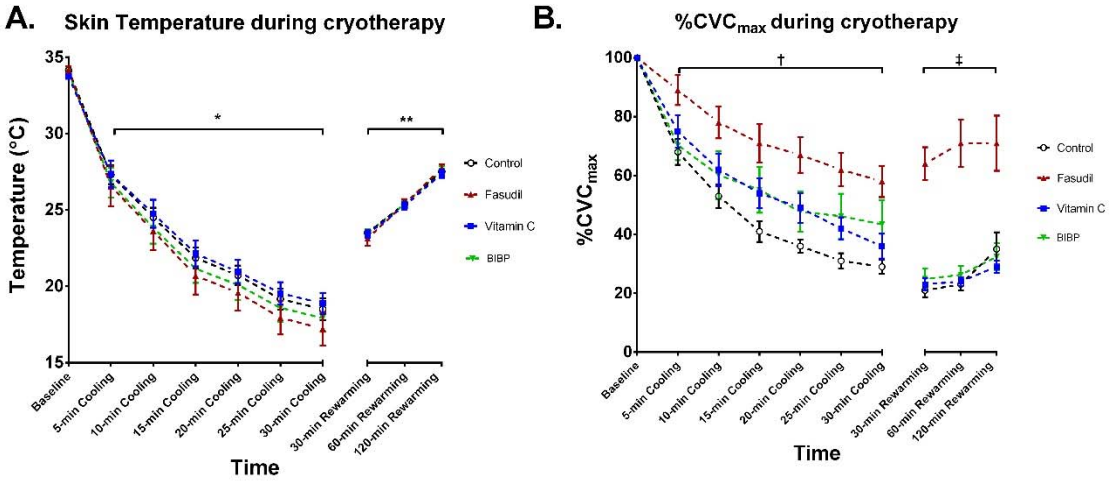

Figure 1. - Panel A) Skin temperature during a typical cryotherapy protocol and the subsequent 2-hour recovery. * denotes significantly different than baseline $p<0.01$. ** denotes significantly different than the end of the 30 min cryotherapy period $p<0.01$. Panel B) † denotes significantly different than baseline %CVC $p<0.01$. ‡ denotes FAS was statistically different from CON, VitC, and BIBP $p<0.01$.

Figure 2. Hysteresis loops during cryotherapy treatment and the subsequent recovery

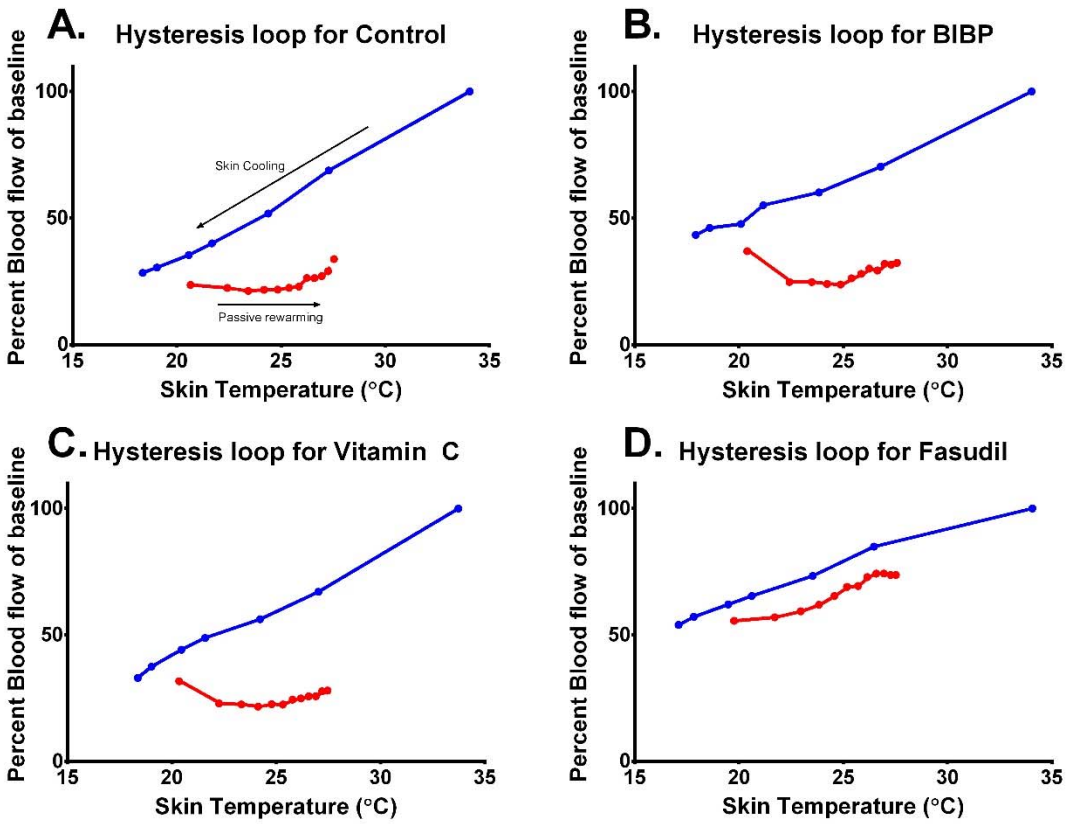

Figure 2 – Panel D) was found to have a significantly different slope to active cooling when compared to CON, VitC, and BIBP. Additionally, in panels A, B, and C each treatment was found to have a significantly different slope for cooling and passive rewarming, but not for FAS (Panel D).

Chapter 5: Study #2

ABSTRACT

Cryotherapy application is a widely clinically used therapeutic technique using ice or cold water applied to the skin surface to reduce local blood flow. While beneficial, cryotherapy treatment does have the potential to induce some detrimental physiological side effects. For example, cryotherapy treatment induces pronounced tissue ischemia that is sustained for hours even during a post-treatment rewarming period.

PURPOSE: To investigate the role of adrenergic receptor activation on cutaneous blood flow during 30 min of cryotherapy as well as during the subsequent 1 hr of passive rewarming. We hypothesized that adrenergic-mediated vasoconstriction is involved in pronounced and sustained cutaneous vasoconstriction that occurs during cooling and passive rewarming.

METHODS: A commercially available cryotherapy unit with a water perfused bladder was used. The bladder was placed on the lateral portion of the calf. Four microdialysis membranes were threaded through the dermis of the skin directly underneath the water bladder. One site was perfused with lactated ringers solution (CON), one with bretylium tosylate (PRE) to block the presynaptic release of neurotransmitters from the sympathetic nerve terminals, one with propranolol+yohimbine+BIBP (POST) to block the postsynaptic beta-adrenoreceptors, alpha-receptors, and neuropeptide Y receptors, and one with propranolol+BIBP (P+B), to block the postsynaptic beta-adrenoreceptors, and neuropeptide Y receptors. Skin temperature (SkT) and skin vascular conductance (CVC)

was measured at each site. Subjects had 0 °C water perfused through the cryotherapy bladder for 30 min, followed by passive rewarming (i.e. water shut off) for 1 hr, followed by direct heating with 46 °C for 10 min.

RESULTS: SkT fell from 34°C to 18.8°C during 30 min of cold water application across all sites. CVC at CON was reduced to 26% of baseline at the end of 30 min of cooling and was not significantly different from P+B ($p>0.05$). Skin CVC decrease across 30 min of cryotherapy was attenuated at the PRE and POST sites while cooling (5-30min) compared the control (CVC: BRE = 65%, POST = 62%, CON 41% of baseline ($p<0.05$ for each comparison)). The CVC response to 10 min of active warming and the 20 min following was lower in PRE site than CON and POST (CVC: 39% vs. 52% and 52%, respectively; ($p<0.05$). **CONCLUSION:** Our primary finding was that adrenergic function mediates the withdrawal of vasoconstriction that occurs with active heating following a cryotherapy protocol. This is in line with previous literature investigating the adrenergic mechanisms of vasoconstriction with skin cooling.

INTRODUCTION

We have previously reported a significant role of the Rho kinase pathway in the pronounced vasoconstriction that occurs during skin surface cooling as well as the sustained vasoconstriction that occurs following cessation of skin-surface cooling. One of the mechanisms of Rho kinase is the elevated activity of the α_2C adrenoceptor density and sensitivity(34-38). This activity would enhance the other local vasoconstrictor pathway driven by the short local loop sympathetic neurons. Investigation of the potential contributions of the local adrenergic sympathetic nervous system is, therefore, significant especially with the possible action of neuropeptide-Y and heightened sensitivity to norepinephrine.

Findings by other investigators have illustrated that the local adrenergic activity explains ~95% of the vasoconstrictor tone on the skin, but with mild skin cooling(34-31 ºC)(51). As local skin temperature starts to dip lower, the adrenergic vasoconstrictor tone becomes less significant, and vasoconstriction is driven by Rho kinase(59, 88). No study has fully investigated the activity of the adrenergic action to local cooling or the temperatures reached during typical cryotherapy protocol.

Due to Rho kinase activity causing increased α_2C adrenoceptor density and sensitivity we believe it plays a significant role in triggering the sustained vasoconstriction that occurs due to local cooling. We hypothesized that the local adrenergic sympathetic neurons would be principally involved in the sustained vasoconstriction that occurs after skin cooling. Finally, we hypothesized by comparing pre-synaptic and post-synaptic blockade we will investigate the potential for further cotransmission.

METHODS

Ethical Approval. The Institutional Review Board at The University of Texas at Austin approved all study procedures and the consent process used in the present study. Subjects were given a verbal description of all procedures and informed of the purpose and risks involved in the study before providing their informed, written consent.

Subjects. 9 healthy young subjects (6 males) participated in this study. Average (mean ± SEM) subject characteristics were: age, 27 ± 2 years; height, 178 ± 1 cm; and weight, 77 ± 3 kg. Subjects were non-smokers, were not taking medications and were free from cardiovascular, neurological, or metabolic diseases. None of the subjects reported a history of knee injury or cryotherapy or other form of cold exposure in the lower extremities for at least a year prior to the experiment. All studies were conducted in the morning following an overnight fast (> 12 hr). Subjects refrained from strenuous exercise, alcoholic beverages, and caffeine for 24 hrs prior to the experimental trial. Additionally subjects underwent an overnight fast for at least 10 hr prior to the experimental trial, which was conducted in a temperature controlled laboratory (~24°C and 40% relative humidity).

Instrumentation and Measurements: All data were collected with the subject seated in a semi-recumbent position. Four microdialysis membranes (CMA 31 Linear Microdialysis Probe, 55 KDalton cut-off membrane; Harvard Apparatus, Holliston, MA) were inserted ~5 cm apart into the nonglabrous skin on the lateral side of the right calf. Following placement, each membrane was perfused with lactated Ringer's solution (Baxter,

Deerfield, IL) at a rate of 2 μL/min via a perfusion pump (Harvard Apparatus, Holliston, MA) while insertion trauma associated with membrane placement subsided (minimum 90 min). During this period, each membrane site was instrumented with an integrating laser Doppler flow probe (VP7a, Moor Instruments, Wilmington, DE) which provided a continuous index of skin blood flow. A thermocouple (Type T Thermocouple Probe, Physitemp Instruments INC, Cliffton, NJ) was placed immediately adjacent to the Doppler flow probe for the continuous assessment of local skin temperature. Following placement of the membranes, Doppler flow probes, thermocouples, and a commercially available cryotherapy cooling pad (Arctic Ice Universal Pad; Pain Management Technologies, Akron, OH) was applied overlying the instrumented area, and fixed in place using an Ace bandage. The cooling pad was connected to an Arctic Ice cryotherapy unit (Pain Management Technologies, Akron, OH) which allowed for manipulation of the underlying skin (Tskin) and tissue temperature according to the manufacturer's recommendation (see below for more detail). A cuff was placed around the left arm for intermittent blood pressure measurements from the brachial artery using electrosphygmomanometry (Tango, SunTech Medical Instruments, Raliegh, NC).

Study Protocol: After the hyperemic response associated with insertion trauma subsided (minimum of 90 min) each site was perfused with its respective vasoactive agent for a 90 min wash in period. One site received lactated Ringer solution (CON, Baxter, Deerfield, IL) which served as the control site. Postsynaptic receptor blockade was achieved at one site, which received 1 mM propanolol, 5mM yohimbine, 10 mM BIBP (POST).

Presynaptic blockade of neurotransmitter release from the sympathetic nerve terminal was achieved with a treatment at a third site with 10 mM bretylium tosylate (PRE). Isolation of the role of α receptors was achieved with infusions of a mixture of 1 mM propranolol and 10 mM BIBP (P+B) to block postsynaptic beta-adrenoreceptors and NPY receptors respectively. All drugs were obtained from Sigma-Aldrich (St Louis, MO, USA). All solutions were dissolved in sterile lactated Ringer solution. Each site was initially perfused at 52 μL/min for a 30 sec priming period after which the rate was reduced to 2 μL/min for the remainder of data collection. Following instrumentation the cooling pad was perfused with 34 ºC water for the entire hyperemic and drug infusion period for baseline data collection. This was followed by 30 min of active skin surface cooling that was accomplished by circulating 0 - 1 ºC water through the cryotherapy unit and cooling pad. At the end of the cooling phase, the cryotherapy unit was turned off for a 1 hr period of data collection during passive rewarming and disconnected from the cold water reservoir. Following passive rewarming for 1 hr a brief, 10 min, active heating phase takes place by perfusing 46 ºC water through the cryotherapy pad. After this 10 min heating period, the pump was turned off again, disconnected from the hot water reservoir for a final 20 min period of passive rewarming.

Data Analysis: Laser-Doppler flux and Tskin data were continuously collected at a sampling rate of 125 Hz via a data-acquisition system (Biopac System, Santa Barbara, CA). One min averages of these data were analyzed at the following time points: the final min of the 34 ºC baseline condition (baseline), min 5, 10, 15, 20, 25, and 30 of active cooling;

min 30, 60, of passive rewarming; min 10 active rewarming; and 10 and 20 minutes post active heating. Mean arterial pressure (MAP) was calculated as 1/3 systolic pressure + 2/3 diastolic pressure and used for subsequent calculation of cutaneous vascular conductance (CVC) (Doppler-derived flux/MAP). All CVC data throughout active cooling and passive rewarming were normalized to value obtained during the final min of 34 ºC baseline and expressed as %CVC. Hysteresis loops were created by comparing the temperature and %CVC relationship for each of the different drug treatments. The slope for cooling and passive rewarming was separately measured between treatments for comparison.

Statistical Analysis: Statistical analyzes were performed using a statistical software package (SigmaPlot 12.5; Systat Software, Inc., San Jose, CA). Skin blood flow, %CVC, and skin temperature, Tskin, were both analyzed using a two-way repeated measures ANOVA comparing treatment (CON vs. POST vs. PRE vs. P+B) by time effects. When a main effect or interaction was found, a Tukey's posthoc analysis was performed to determine further differences. All data is shown as mean±SEM. For all tests, significance was found at $p<0.05$.

RESULTS

Skin temperature throughout the trial is shown in fig 3a and decreased with skin cooling from baseline due to skin cooling. Average temps at the sites were; Baseline: 33.8±0.2 vs. Cooling: 28.5±0.5, 24.7±0.3, 22.4±0.3, 20.6±0.3, 19.5±0.3, 18.5±0.3 °C; 5, 10, 15, 20, 25, 30 min cooling, respectively; $p<0.001$) and recovered towards baseline with passive rewarming when compared to 30 min cooling (30 and 60 Rewarming: 22.4±0.2, 24.2±0.2 °C, respectively; $p=0.001$), but still had not reached baseline levels even after 1 hr of rewarming. Skin temperature increased from 60 min passive rewarming and remained elevated in the 10 and 20 minutes in response to active warming (31.6±0.5, 31.2±0.3, 30.8±4 °C, respectively; $p<0.001$). Skin temperatures were no different among the 4 treatments at any time point ($p>0.05$).

There was no effect of drug treatment during the 34 baseline for CON, PRE, or Y+B ($p>0.05$), however, POST was significantly higher than its predrug baseline (76±3 vs. 100±0 %, respectively; $p<0.03$). Skin blood flow during the ~34°C baseline and throughout the cooling and reheating protocol is illustrated in Figure 3b. Local cold water application resulted in a significant decrease from baseline %CVC in all trials ($p<0.001$). The reduction in the CON and P+B sites were significantly greater than both POST and PRE sites (41±4, 46±4 vs. 65±3, 62±4 % of baseline, respectively; $p<0.001$) in the 30 min while cooling. During the passive rewarming period all treatments were not found to be significantly different across time or between treatments (Con: 24±5, POST: 35±5, PRE: 27±4, Y+B: 30±5 % of baseline; $p=0.27$). Active heating significantly increased %CVC

over the passive recovery phase for all treatments and in the 20 minutes following (60 min passive: 27±4 vs. 10 min active heating: 61±6, 10 min post active heating: 66±6, 20 min post active heating: 61±5 % of baseline; $p<0.001$). Additionally, PRE was found to be significantly lower than CON, POST, and Y+B in response to active heating and the 20 minutes following (42±5 vs. 61±4, 56±6, 58±7 % of baseline, respectively; $p<0.05$) as determined by posthoc analysis.

Hysteresis loops were broken down into cooling, and passive rewarming, and then active rewarming separately and were similar between treatments and across time.

DISCUSSION

Our primary finding is that the adrenergic vasoconstrictor nerves in the cutaneous circulation partially contributes to the vasodilatory response of cold vasoconstricted skin back towards basal blood flow values. Specifically, comparing the responses between PRE and POST to active rewarming it appears that there may be cotransmission of an additional neurotransmitter from the sympathetic vasoconstrictor nerves that is sensitizing tissue to temperature by withdrawing the a portion of the vasoconstrictor tone or dilation.

To our knowledge, this is the first investigation to show that the adrenergic vasoconstrictor nerves are involved in the vasodilation of chronically vasoconstricted skin. Previous investigations by Hodges et al. had shown that by the co-treatment with bretylium and L-NAME abolished all vasodilatory responses to local rewarming when heating locally cooled and chronically vasoconstricted skin(42). Based on our finding here that roughly 40% of the vasodilatory response to active rewarming is masked by the effects of bretylium we would hypothesize that this is mediated by a vasodilator neurotransmitter, perhaps stimulating NO, but its release is blocked. However, it is not quite fully understood to us what may be occurring within the sympathetic vasoconstrictor nerves that facilitate part of this response. Hodges et al. in a separate set of studies has shown that by treating the skin with bretylium and rapidly heating the skin you can attenuate the early phase vasodilatory action that occurs in the skin suggesting some role for the vasoconstrictor nerves in facilitating vasodilation(42). However, this finding partially conflicts with other studies that show inhibition of the vasoconstrictor

nerves with bretylium does not alter vasodilatory responses to local heating(72). This may be due to the fact that this study made its comparisons based on the percentage of max CVC rather than percent of baseline, the rate of cooling and max temperature attained.

Many of our other findings are in support of previous literature on the role of the adrenergic vasoconstrictor responses to cooling. We were able to show similar levels of vasoconstriction between both pre and post synaptic blockades(51), suggesting appropriate blockade of the vasoconstrictor response. However, we experienced a larger decrease in blood flow with bretylium treatment than Johnson et al. who showed less than 10% decrease after 30 min versus our 70% decrease(51). Much of this is due to the nature of their model, which utilizes mild skin cooling rather than our much more dramatic localized cooling. Local cooling from 34-24°C with bretylium treatment shows a 40% decrease in blood flow, which is still 30% higher than the control. When compared to our data where at the end of 30 min of cryotherapy POST and PRE were nonsignificantly different from CON it would appear that adrenergic activity ceases between skin temperature of 24 and 18°C. However, this may require direct measurement of neural activity to confirm fully. It appears that as skin temperature is depressed more severely the contribution of vasoconstriction shifts towards being more dependent upon Rho kinase and less so upon adrenergic regulation, which is supported by the smaller effect of bretylium treatment as cooling becomes more dramatic.

Of additional mention is that despite any type of drug treatment there was no recovery of blood flow during the passive rewarming phase. This is consistent with our

previous investigation that showed the only mediation of the passive recovery phase came from active Rho kinase inhibition. Additionally, local skin cooling studies have previously mentioned that Rho kinase makes up roughly 60% of the local vasoconstrictor tone, but based on this and our previous investigation I would surmise that dropping skin temperature below 20 ºC becomes predominantly driven by the Rho kinase pathway, but this would require a separate investigation to support adequately.

Some experimental considerations are that in this study we infused bretylium throughout the entire protocol. While this doesn't directly impose an issue with our findings, it does contrast to some of the literature that primarily pre-treated with bretylium and infused saline throughout the remainder of the trail(42). Additionally, analysis of our hysteresis loops lacked significance, which may have suffered from a limited number of data points during the passive cooling phase.

In summary, we have shown that the adrenergic vasoconstrictor nerves are involved in returning blood flow towards baseline in chronically vasoconstricted skin. This may suggest a role for withdrawal of vasoconstrictor tone or a dilatory response. Additionally, we've shown that the most appropriate method of restoring the temperature-blood flow relationship and abolishing the effects of sustained vasoconstriction is active local heating with hot water exposure.

TABLES AND FIGURES

Figure 3. Skin temperature and blood flow responses to cryotherapy and the subsequent passive and active recovery

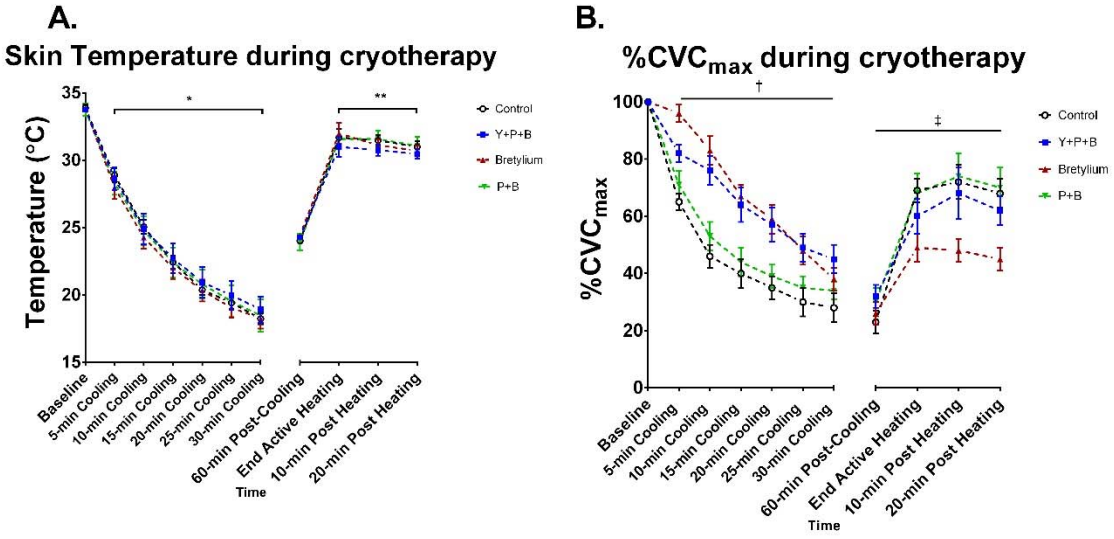

Figure 3 - Panel A) Skin temperature during a typical cryotherapy protocol and the subsequent 1 hour recovery, 10 min active heating and 20 min recovery. * denotes significantly different than baseline $p<0.01$. ** denotes significantly different than the end of the 60 min passive recovery period $p<0.01$. Panel B) † denotes significantly different than baseline %CVC $p<0.01$. ‡ denotes PRE had a statistically different response to active warming than CON, POST, and P+B $p<0.01$.

Figure 4. Hysteresis loops during cryotherapy treatment and the subsequent recovery

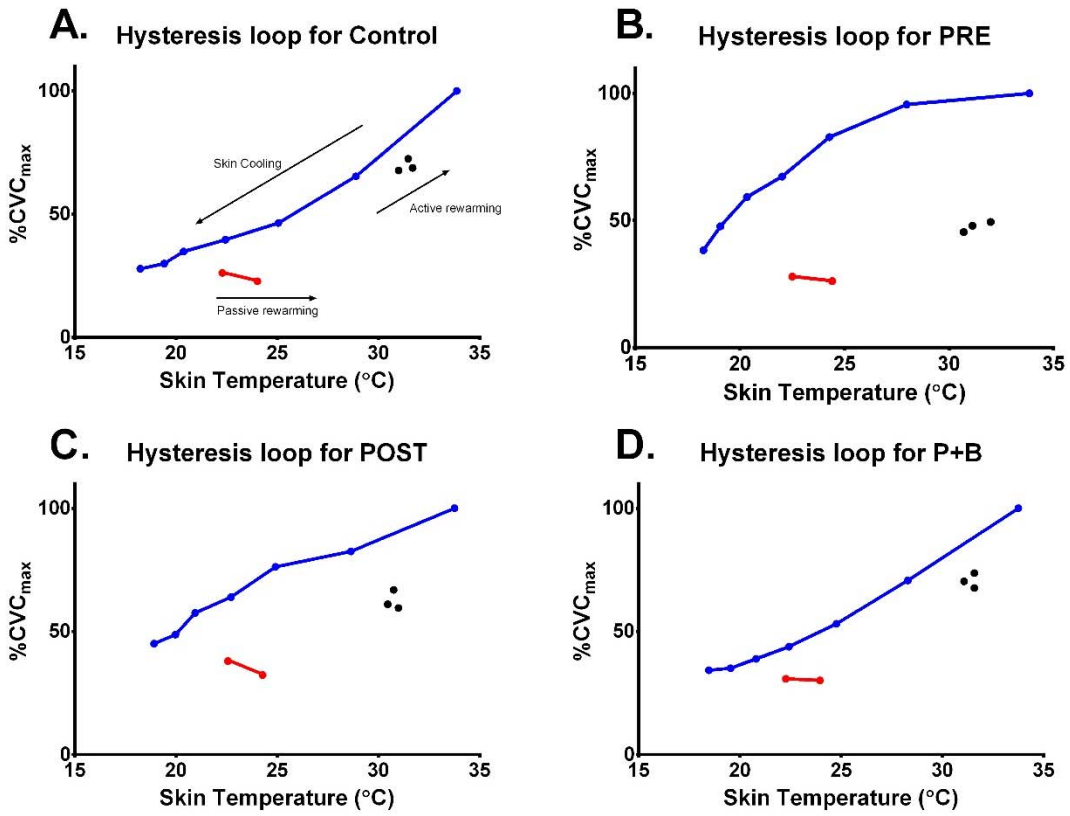

Figure 4 - Hysteresis loops for the adrenergic assessment of the blood flow/temperature relationship. No significant effects were found between treatments or across time.

Chapter 6: Study #3

ABSTRACT

Cryotherapy application utilizes rapid skin cooling to reduce the local blood flow response and can manifest with sustained and pronounced vasoconstriction. This vasoconstriction is mediated by Rho kinase that has known mechanisms to downregulate and impair nitric oxide function in the skin.

PURPOSE: To investigate the role of nitric oxide on cutaneous blood flow during 30 min of cryotherapy as well as during the subsequent 1 hr of passive rewarming. We hypothesized that a reduction in nitric oxide bioavailability is involved in the pronounced and sustained cutaneous vasoconstriction that occurs during cooling and passive rewarming in a typical cryotherapy protocol.

METHODS: A commercially available cryotherapy unit with a water perfused bladder was used. The bladder was placed on the lateral portion of the calf. Four microdialysis membranes were threaded through the dermis of the skin directly underneath the water bladder. One site was perfused with lactated ringers solution (CON), one with L-NAME, one with NPLA, and one with LNIO. Skin temperature (SkT) and skin vascular conductance (CVC) was measured at each site. Subjects had 0 °C water perfused through the cryotherapy bladder for 30 min, followed by passive rewarming (i.e. water shut off) for 1 hr, followed by direct heating with 46 °C for 10 min.

RESULTS: SkT fell from 34°C to 18.7°C during 30 min of cold water application across all sites. CVC at CON was reduced ~75% after 30 min of cooling and was not significantly

different from LNAME, NPLA, or LNIO (78, 75, 77%, respectively; $p>0.05$). The CVC response to 10 min of active warming and the 10 min following was no different between CON and LNAME, NPLA, or NLIO (CVC: 38% vs. 35%, 33%, 32%, respectively; ($p<0.05$). **CONCLUSION:** Our primary finding was that nitric oxide is not involved in the vasoconstrictor response or recovery of blood flow during a typical cryotherapy application or active heating. Additionally, our data suggests that active heating of chronically vasoconstricted skin is necessary for the return of the blood flow/temperature relationship.

INTRODUCTION

The Rho-kinase pathway is a potent mediator of vasoconstriction in the skin in response to local cooling. Rho kinase enhances vasoconstrictor tone in part by the down-regulation of nitric oxide synthase (NOS)(70, 79), which is involved in the formation of nitric oxide (NO) and vasodilation. The Rho-kinase and NOS pathways mediating vasoconstriction and vasodilation, are mutually inhibitory; cGMP-dependent protein kinase (PKG), involved in NO production, inhibits Rho-kinase activation, and Rho-kinase mediates phosphorylation of MLCP and Rho-kinase can downregulate endothelial NOS (eNOS)(70, 79). This interplay between the two pathways is only partially understood, but it is likely significant as it pertains to cryotherapy treatment.

As NO is a primary contributor to local heating induced vasodilation(68) and partially contributes to the vasoconstrictor response that occurs to local cooling(42), we believe it may contribute to sustained vasoconstriction as well. Additionally, it has previously been identified that eNOS specifically contributes to this dilatory response to local cooling(55) but has had some conflicting results suggesting nNOS activity within the leg of individuals with postural orthostatic tachycardia syndrome(82).

Therefore, this study examined the role of NO production by the various isoforms of NOS on the vasoconstrictor response during a typical cryotherapy protocol and during the sustained vasoconstriction that occurs during the subsequent period of passive rewarming. We hypothesized that NOS inhibition would mediate the vasoconstrictor response and active rewarming phase. Additionally, we hypothesized that the effects of vasodilation would occur due primarily to intact eNOS activity.

METHODS

Ethical Approval. The Institutional Review Board at The University of Texas at Austin approved all study procedures and the consent process used in the present study. Subjects were given a verbal description of all procedures and informed of the purpose and risks involved in the study before providing their informed, written consent.

Subjects. 8 healthy young subjects (6 males) participated in this study. Average (mean ± SEM) subject characteristics were: age, 25 ± 1 years; height, 179 ± 1 cm; and weight, 79 ± 2 kg. Subjects were non-smokers, were not taking medications and were free from cardiovascular, neurological, or metabolic diseases. None of the subjects reported a history of knee injury or cryotherapy or other form of cold exposure in the lower extremities for at least a year prior to the experiment. All studies were conducted in the morning following an overnight fast (> 12 hrs). Subjects refrained from strenuous exercise, alcoholic beverages, and caffeine for 24 hrs prior to the experimental trial. Additionally subjects underwent an overnight fast for at least 10 hrs prior to the experimental trial, which was conducted in a temperature controlled laboratory (~24°C and 40% relative humidity).

Instrumentation and Measurements: All data were collected with the subject seated in a semi-recumbent position. Four microdialysis membranes (CMA 31 Linear Microdialysis Probe, 55 KDalton cut-off membrane; Harvard Apparatus, Holliston, MA) were inserted ~5 cm apart into the nonglabrous skin on the lateral side of the right calf. Following placement, each membrane was perfused with lactated Ringer's solution (Baxter,

Deerfield, IL) at a rate of 2 μL/min via a perfusion pump (Harvard Apparatus, Holliston, MA) while insertion trauma associated with membrane placement subsided (minimum 90 min). During this period, each membrane site was instrumented with an integrating laser Doppler flow probe (VP7a, Moor Instruments, Wilmington, DE) which provided a continuous index of skin blood flow. A thermocouple (Type T Thermocouple Probe, Physitemp Instruments INC, Cliffton, NJ) was placed immediately adjacent to the Doppler flow probe for the continuous assessment of local skin temperature. Following placement of the membranes, Doppler flow probes, thermocouples, and a commercially available cryotherapy cooling pad (Arctic Ice Universal Pad; Pain Management Technologies, Akron, OH) was applied overlying the instrumented area, and fixed in place using an Ace bandage. The cooling pad was connected to an Arctic Ice cryotherapy unit (Pain Management Technologies, Akron, OH) which allowed for manipulation of the underlying skin (Tskin) and tissue temperature according to the manufacturer's recommendation (see below for more detail). A cuff was placed around the left arm for intermittent blood pressure measurements from the brachial artery using electrosphygmomanometry (Tango, SunTech Medical Instruments, Raliegh, NC).

Study Protocol: After the hyperemic response associated with insertion trauma subsided (minimum of 90 min) each site was perfused with its respective vasoactive agent for a 50 min wash in period. One site received lactated Ringer solution (Baxter, Deerfield, IL) which served as the control site (CON), one site received 5mM *N*5-[Imino(propylamino)methyl]-L-ornithine hydrochloride (NPLA, blockade of nNOS,

Tocris Cookson, Bristol, U.K.), one site received 10 mM *N*5-(1-Iminoethyl)-L-ornithine dihydrochloride (NLIO, blockade of eNOS, Tocris Cookson, Bristol, U.K.), and the last received 20 mM Nω-Nitro-L-arginine methyl ester hydrochloride (LNAME, NOS blockade, St Louis, MO, USA). All solutions were dissolved in sterile lactated Ringer solution. Each site was initially perfused at 52 μL/min for a 30 sec priming period after which the rate was reduced to 2 μL/min for the remainder of data collection. Following instrumentation the cooling pad was perfused with 34 ºC water for the entire hyperemic and drug infusion period for baseline data collection. This was followed by 30 min of active skin surface cooling that was accomplished by circulating 0 - 1 ºC water through the cryotherapy unit and cooling pad. At the end of the cooling phase, the cryotherapy unit was turned off for a 1 hr period of data collection during passive rewarming and disconnected from the cold water reservoir. Following passive rewarming for 1 hr a brief, 10 min, active heating phase takes place by perfusing through the cryotherapy pad 46 ºC water. After this 10 min heating period, the pump was turned off again, disconnected from the hot water reservoir for the final 20 min passive rewarming period.

Data Analysis: Laser-Doppler flux and Tskin data were continuously collected at a sampling rate of 125 Hz via a data-acquisition system (Biopac System, Santa Barbara, CA). One min averages of these data were analyzed at the following time points: the final min of the 34 ºC baseline condition (baseline), min 5, 10, 15, 20, 25, and 30 of active cooling; min 30, 60, of passive rewarming; min 10 active rewarming; and 10 and 20 minutes post active heating. Mean arterial pressure (MAP) was calculated as 1/3 systolic pressure + 2/3

diastolic pressure and used for subsequent calculation of cutaneous vascular conductance (CVC) (Doppler-derived flux/MAP). All CVC data throughout active cooling and passive rewarming were normalized to value obtained during the final min of 34 ºC baseline and expressed as %CVC. Hysteresis loops were created by comparing the temperature and %CVC relationship for each of the different drug treatments. The slope for cooling and passive rewarming was separately measured between treatments for comparison.

Statistical Analysis: Statistical analyzes were performed using a statistical software package (SigmaPlot 12.5; Systat Software, Inc., San Jose, CA). Skin blood flow, %CVC, and skin temperature, Tskin, were both analyzed using a two-way repeated measures ANOVA comparing treatment (CON vs. NPLA vs. LNIO vs. LNAME) by time effects. When a main effect or interaction was found, a Tukey's posthoc analysis was performed to determine further differences. All data is shown as mean±SEM. For all tests, significance was found at $P < 0.05$.

RESULTS

Skin temperature throughout the trial is shown in Fig 5a and decreased with skin cooling from baseline due to skin cooling (Baseline: 33.5±0.3 vs. Cooling: 27.9±0.6, 24.3±0.6, 22.1±0.7, 20.6±0.7, 19.4±0.7, 18.6±0.7 °C; 5, 10, 15, 20, 25, 30 min cooling, respectively; $p<0.001$) and recovered towards baseline with passive rewarming when compared to 30 min cooling (30 and 60 Rewarming: 22.5±0.4, 24.1±0.3 °C, respectively; $p<0.001$), but still had not reached baseline levels even after 1 hr of rewarming. Skin temperature increased during passive rewarming and remained elevated during the 10 min following active warming (31±0.7, 30.6±0.4 °C, respectively; $p<0.001$). Skin temperatures were no different among the 4 treatments at any time point ($p>0.05$).

There was no effect of drug treatment on CVC during the 34℃ baseline for CON, NPLA, or LNIO ($p>0.05$), however, LNAME was found to be significantly lower than its predrug baseline (100±0 vs. 70±6 %, respectively; $p<0.01$). Skin blood flow during the ~34°C baseline and throughout the cooling and reheating protocol is illustrated in Figure 5b. Local cold water application resulted in a significant decrease from baseline %CVC in all trials ($p < 0.001$). However, there was no effect of the drug treatments across the 30 min of cryotherapy. During the passive rewarming period all treatments were not found to be significantly different across time or between treatments (Con: 13±1, LNAME: 15±2, NPLA: 13±2, LNIO: 15±1 % of baseline; $p>0.05$). Active heating significantly increased %CVC over the passive recovery phase for all treatments and in the 10 min following (60

min passive: 13±2 vs. 10 min active heating: 34±8, 10 min post active heating: 34±8 % of baseline; $p<0.001$). There was no effect of treatment during the active heating stage.

Hysteresis loops were broken down into cooling, passive rewarming, and then active rewarming, separately, with a significant impact on phases of cooling, but not treatments and is shown in Figure 6. Posthoc analysis revealed passive skin cooling had a significantly different slope than both skin cooling and active warming (-0.55±1.34, 4.31±1.23, 4.02±2.68 %CVC/°C, respectively; $p<0.01$), however, there was no difference between skin cooling and active warming ($p>0.97$).

DISCUSSION

Our primary finding is that when assessing the skin blood flow and temperature relationship that only active heating restored this relationship and was found to be augmented during passive rewarming. To our knowledge, we are not aware of any previous investigations that have shown with hysteresis loops, an impaired dilatory response with cryotherapy, which was restored by active heating. Local skin cooling that activates the Rho kinase pathway has multiple mechanisms for enhancing constriction and potentially leading to sustained vasoconstriction. We show a clear difference in the CVC/temperature response during passive recovery that only appears to be corrected with the application of hot water to the surface of the skin. This has significant implications for the use of cryotherapy in a clinical setting.

Our assessment of the effects of NO on skin cooling was unable to identify a role for NO during cryotherapy. While previous investigations have identified a mechanism for Rho kinase to downregulate and inhibit NO(87), we were unable in our current investigation to show any action on NO. While LNAME was able to lower skin blood flow when compared to its predrug baseline, but not significantly different from any other drug treatments during the application of cryotherapy. Our heating protocol only increased skin blood flow from 13% to 34% it may be that within this range of vasoconstriction there is limited action of nitric oxide and may have yielded different results if skin was heated back to basal temperatures ~34°C. Hodges et al. showed in his investigation that during slow local cooling a portion of the vasoconstrictor response is driven by decreased NOS

activity(42). It may be that because of the rate of cooling with cryotherapy we were unable to show any action of nitric oxide.

Some experimental considerations are that the heating protocol we used in this study was identical to the previous study #2. However, despite no differences in skin temperature at any point in the protocol, active heating led to a 30% lower CVC value for control treatments. Since several of the participants in this investigation were also in the previous investigation, it's likely not driven by population differences. Perhaps, there is an effect of heat acclimation as these studies were done at different times of the year and perhaps have a lower blood flow response to our given protocol.

In summary, we've shown that nitric oxide has no significant role in the vasoconstrictor response during a typical cryotherapy protocol and subsequent passive and active recovery phases. Additionally, it appears that the sustained vasoconstriction that occurs during cryotherapy is most aptly corrected with skin surface heating back towards basal temperatures.

TABLES AND FIGURES

Figure 5. Temperature and blood flow responses to cryotherapy and the subsequent recovery

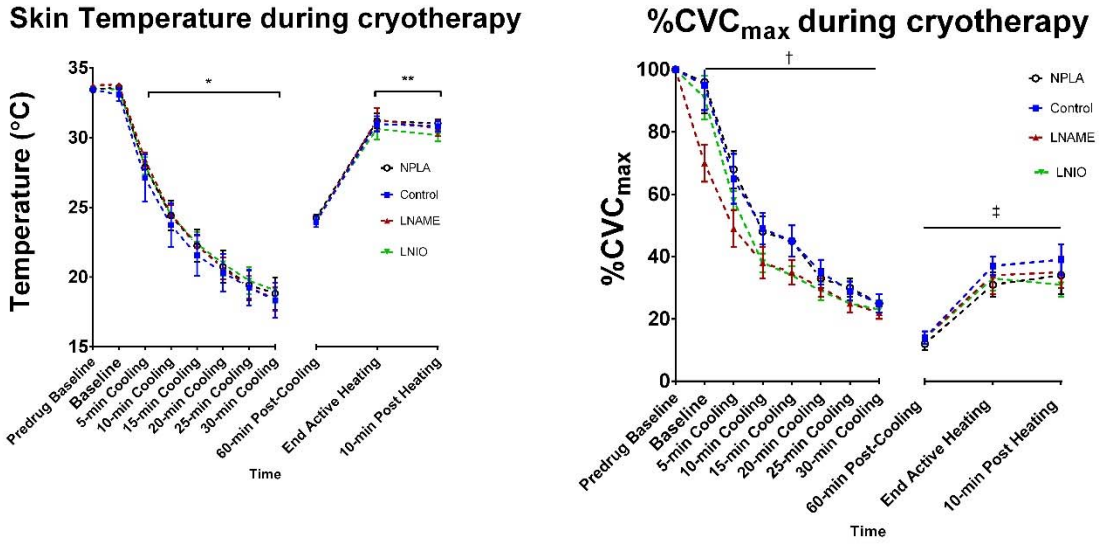

Figure 5 - Panel A) Skin temperature during a typical cryotherapy protocol and the subsequent 1-hour recovery, 10 min active heating, and 20 minutes following. * denotes significantly different than baseline $p<0.01$. ** denotes significantly different than the end of the 60 min passive recovery period $p<0.01$. Panel B) † denotes significantly different than baseline %CVC $p<0.01$. ‡ denotes an effect across time in response to active heating $p<0.01$.

Figure 6. Hysteresis loop during cryotherapy and the subsequent passive and active recovery

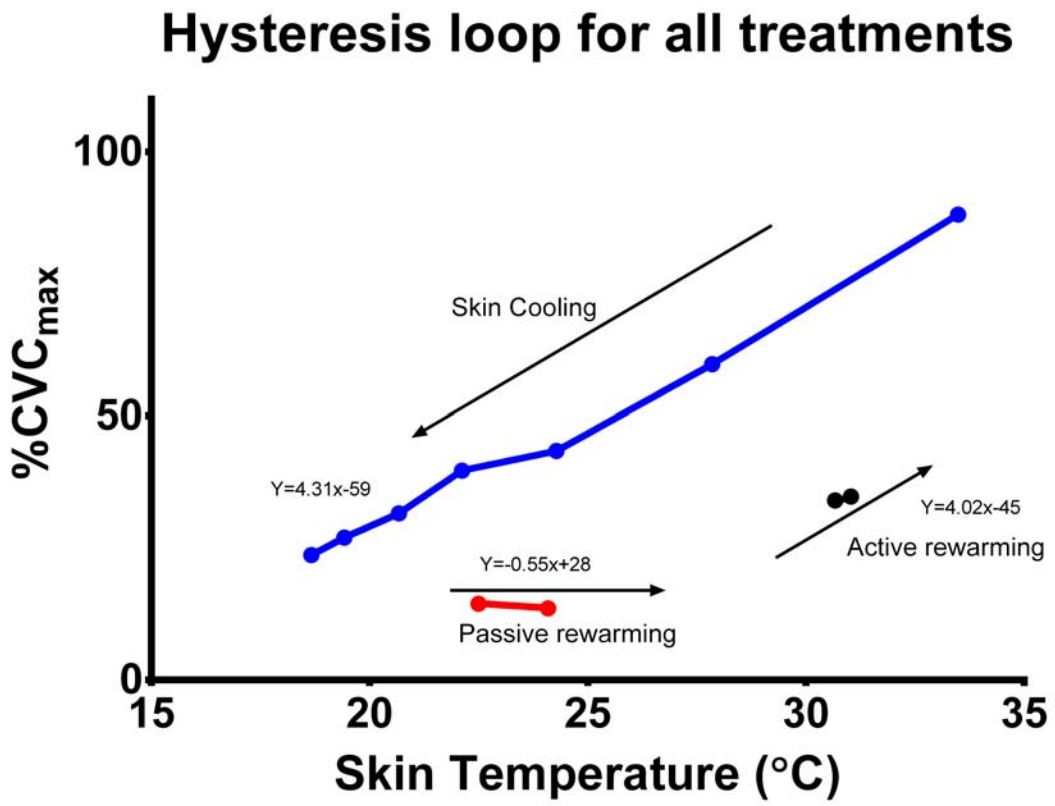

Figure 6 – Hysteresis loops during a typical cryotherapy protocol and the subsequent passive and active rewarming phases. Passive rewarming was found to be significantly different from both active warming and active cooling ($p<0.05$).

Chapter 7: Review of the Literature

BACKGROUND PHYSIOLOGY

Historically investigations into thermoregulation have often led researchers down the path of heat stress. This is partly because our bodies have a finite limit for heat storage before an individual's ability to perform work in strenuous environments is compromised. This was the primary reason for the Harvard Fatigue Lab arising from within the basement of the Harvard business school. At the time 14 workers had died and fallen due to heat stress, this required scientific inquiry as to improve work conditions and save lives. Bruce Dill and John Talbott were both sent to the Hoover dam with the focus at running biochemical and physiological studies on themselves and healthy volunteers. They concluded that the workers needed cooler sleeping quarters and the addition of copious amounts of salt in their meals(5), which improved work performance in the heat. Alternatively, in regards to cold stress, many investigations were directed at shielding the body from the low temperatures. Initially this aided the military as non-freezing cold injuries occur in civilian populations within manageable limits, but can reach epidemic proportions during war times(34).

Regulating cardiac output towards the skin is the primary method of regulating our internal core temperature with an effort to maintain thermoneutrality. Dramatic increases in core temperature can increase skin blood flow from almost zero in conditions of cold stress to as much as 8-10 L/min and becomes vital for maintaining a safe core temperature(50). The ability for blood to reduce the elevated thermal stress in the body lies

in the conductive and convective properties of heat dissipation in the skin. By dramatically increasing the blood flow to the skin and taking advantage of the evaporative properties of sweat, the rate of increase in core temperature can be minimized, significantly(15). Alternatively, during conditions of cold stress the blood flow can fall to near zero. The body attempts to maintain core temperature by inducing a state of profound vasoconstriction in the skin and periphery, which results in shunting of the blood towards the internal core, thus reducing the loss of heat in the extremities. The effect of tissue cooling can be further amplified by adding thermally conductive fluid to the surface of the skin enhancing the heat extraction over cold air. This is a benefit in regards to accelerating cooling with cryotherapy, but a serious medical concern with regards to hypothermia if the magnitude cooling becomes too much.

VASOCONSTRICTION

The mechanisms of vasoconstriction lie under the control of local factors and neural reflex mechanisms focused on optimizing the vasoconstrictor response. The neural components of the vasoconstrictor response were first theorized by Claude Bernard(13), a well-known physiologist, respected for his work on vasomotor nerves and for discovering glycogen(12). His conclusions laid the groundwork for later research, confirming that sympathetic neurons handled not only the resting vessel tone but also for the vasoconstriction observed during skin/body cooling(31, 36). The initial response to the exposure to cold stress, minimizing the convective heat loss, lies under the regulation of

local and reflex pathways. These systems can independently produce vasoconstriction, but cooperatively enhance vasoconstriction through multiple mechanisms. Local reflex vasoconstriction is mediated by perivascular sympathetic adrenergic nerves, which release norepinephrine (NE) and co-transmitters including neuropeptide Y (NPY) and is a graded response that can increase with intensity until a basement plateau is reached, where the vasculature becomes physically constrained(80, 81). Reflex vasoconstriction occurs when the skin temperature drops below 34°C due to convective or conductive heat loss to the environment(39) or by decreasing the core temperature, despite apparent changes in skin temperature(10). While skin cooling directly applies to the application of cryotherapy a reduction in core temperature without a change in mean skin temperatures is a rare occurrence, which most often presents under special clinical circumstances(14).

With reflex vasoconstriction, the primary neurotransmitter, NE, is synthesized in the cell body of noradrenergic neurons from L-tyrosine, with a rate limiting enzyme *tyrosine hydroxylase,* and its essential cofactor *tetrahydrobiopterin*(BH4)(37, 83). While BH4 may be prone to oxidation and subsequently deactivated(69), NE synthesis is rarely abnormal in healthy young populations with healthy systemic redox states(59). Nerve blockade and/or the subcutaneous application of adrenergic receptor antagonists have revealed that approximately 60% of the overall vasoconstrictor response to sympathetic activity is attributed directly to NE(53, 80, 81). Isolation of different post-synaptic receptors by pharmacological blockade along with findings from in vivo models suggests that other transmitters released from the sympathetic neurons alongside NE also contribute to the overall tone. Both neuropeptide-y (NPY) and ATP have been identified as

cotransmitters with NE(38, 62), which are considered to be the only mediators of vasoconstriction released by adrenergic neurons.

Also, in vitro models with direct stimulation of cutaneous vessels has provided evidence for Rho-kinase as the primary mediator in enhancing vasoconstrictor tone independent of adrenergic stimulation(27, 72). Local cooling of the skin stimulates the production of superoxides from the mitochondria, resulting in elevated reactive oxygen species (ROS), which acts as a stimulator of the Rho-kinase pathway(7). This pathway is responsible for modulating vasoconstrictor tone through two mechanisms: 1) inhibition of the myosin light chain phosphatase (MLCP), allowing for passive phosphorylation in the absence of calcium influx (enhanced constriction for any given stimulus); and 2) cAMP mediated translocation of α_2c-adrenoceptors from the trans-Golgi to the surface of the cell. The latter mechanism results in a 5-fold increase in α_2c-adrenoceptor density on the cell surface and thus enhances NE mediated vasoconstriction(6, 7, 16, 26, 48). These two mechanisms dramatically improve end-organ function within the vasculature in response to cooling as a way to enhance the neural reflex mechanisms(88). The Rho-kinase pathway also enhances vasoconstrictor tone in part by the down-regulation of nitric oxide synthase (NOS)(70, 79), which handles the formation of nitric oxide (NO) and vasodilation. The Rho-kinase and NOS pathways mediating vasoconstriction and vasodilation, respectively, are mutually inhibitory; cGMP-dependent protein kinase (PKG), involved in NO production, inhibits Rho-kinase activation, and Rho-kinase mediates phosphorylation of MLCP and Rho-kinase can downregulate endothelial NOS (eNOS)(70, 79). This interplay

between the two pathways is only partially understood, but it is likely significant as it pertains to cryotherapy treatment.

During local cooling, similar to what would occur with cryotherapy, the initial response (0-10 min) is mediated primarily by reflex vasoconstriction via NE release which binds to α_2 receptors(27) and is dependent upon the functionality of the sensory nerves(41). Treatment with bretylium tosylate, which prevents the post-synaptic release of neurotransmitters from the sympathetic neurons, results in a complete abolishment of vasoconstriction with local cooling during this time period with the outcome being a slight vasodilatory response(53). If the cooling persists beyond 10 minutes, vasoconstriction persists through the addition of non-adrenergic mechanisms directed at reducing the dilatory function of the vessel by selective downregulation of eNOS(42, 51, 72) and enhancement of end-organ vasoconstriction by Rho-kinase mediated pathways(88)(as described above). However, these mechanisms appear to be slightly delayed and don't seem to occur in the initial 10 min. Following 10-min vasoconstriction persists through the independent reflex neural mechanisms and local modulators. Additional mechanisms involved in vasoconstriction involves the reduced breakdown of NE (via MAO and COMT; enzyme breakdown), increased viscosity and aggregated platelets that release 5HT and thromboxane A_2(85). While these processes take place in healthy young adults, advanced aging provides significant insight into the role of Rho-kinase as a local modulator to vasoconstriction.

Aged skin experiences impaired mechanisms of vasoconstriction, which often results in higher average blood flows during cold exposure and further compromising their

susceptibility to hypothermia(21, 56, 92). Much of the recent work has culminated in a model whereby sympathetic neurotransmission is decreased along with local intracellular signals suggesting more generalized age-associated vascular dysfunction(90). In 1977, KJ Collins and colleagues noted the higher incidence of hypothermia in the aged population which presented a growing medical concern(18). This elevated prevalence of 'accidental hypothermia' is not a function of falls, injuries, or illness as both fit, and unfit elderly people are prone to hypothermia. This rise in hypothermia is a natural hazard of advanced aging(18). Part of the impaired thermoregulatory function with aging has been attributed to lower neural stimulation with a given cold stress. Sympathetic nerve activity was recorded across young, middle ages, and elderly subjects, with the elderly population, experienced a 60% depression in bursts/min when compared to the other groups(35). Also reduced NE release in aged skin(92), it has been suggested that aged individuals also have reduced neurotransmitter synthesis(19, 24, 33). While in the young population vasoconstriction is mediated ~ 60% by NE and ~ 40% by co-transmitters, within the elderly population there is an almost a full dependency upon NE with a complete loss of NPY mediated vasoconstriction. Aged skin experiences blunted end-organ vascular function(57, 91, 92). By direct administration of serial doses of NE and NPY, investigators have shown reduced sensitivity to both NE and NPY, but also a reduced maximal response(91, 92). Additionally, within aged rodent arterioles similar findings have been shown using ATP to assess end-organ vasoconstrictor function(57). In contrast to reflex vasoconstriction, local cold-induced vasoconstriction is also affected by aging as the magnitude of vasoconstriction from local mechanisms remains no different despite the reduced

adrenergic tone(89). This is partially due to a shift from an adrenergic control within the aged skin to having a larger dependence on the Rho-kinase pathway becoming the predominant contributor to constrictor tone(88). While the magnitude of local response to cooling does not change in the aged skin, the increased dependence on the Rho-kinase pathway in the aged skin may provide some insight into a host of vascular changes and diseases. Greater dependence on the Rho-kinase pathway results in an upregulation of the Rho-kinase pathways and is often associated with atherosclerosis, hypertension, vascular remodeling, erectile dysfunction, and diabetes(70). This may suggest that the change Rho-kinase is a function of aging rather than disease.

With normal healthy, young vessels the balance between Rho-kinase and NO (constrictor and dilator pathways) is slightly in favor of NO-mediated dilation. However, in the aged skin this is reversed to having a more predominate constrictor tone with a loss in endothelial dilation and enhancement in Rho-kinase mediated vasoconstrictor function. This is likely due to age and cold related increases in ROS production. Age-associated production of ROS and increased arginase activity both reduce the bioavailability of NO(43).

Raynaud's Disease is a vascular disorder where tissues (ears, lips, fingers, nose, toes) undergo vasospasms in response to cold exposure, which exists as an abnormal version of the bodies normal physiological response. Raynaud's can be idiopathic in origin or can arise from mechanical, immunological, or chemical stressors within the blood vessels in the skin(70). The understanding of the underlying pathophysiology of Raynaud’s is incomplete; however, it has been suggested that the same mechanisms underlying local

vasoconstriction mediate the effects, but perhaps to a greater sensitivity(70). Because women of childbearing age and postmenopausal women taking just estrogen therapy reflect the largest incidence of Raynaud's, estrogen has a likely role underlying the mechanisms(6, 26). This may be because observations suggest estrogen is a strong stimulator of the α_2 species receptors and would enhance end-organ response. However, these were made in the absence of cold stimulation(26). This would perhaps open the door for potential therapies like statins, which inhibit Rho kinase, in at-risk populations(70). In regards to female aging, there appears to be no contribution of age is no longer a predictor of prevalence or severity. This is a contrast to men where age is considered to be a risk factor for the onset of the disease(26).

VASODILATION

Thermal control of body temperature is heavily dependent upon the compliance and vascular response to an increase in skin and/or core temperature, by increasing skin blood flow to values several fold higher over rest. The tremendous capacity for the skin to increase blood flow and optimize benefits from evaporative heat loss from sweat as well as convective cooling. During basal conditions the vasodilator systems aren't under regulation, but with the input of thermal stress the vasodilation occurs initially via removal of tonic sympathetic mediated vasoconstriction and then later via cholinergic nerve transmission from sympathetic vasodilator nerves. The potential neurotransmitters involved in sympathetically mediated vasodilation include, nitric oxide and an unknown transmitter, potentially: histamine and neurokinin receptor activation(96), vasoactive

intestinal peptide (VIP)(9), calcitonin gene-related peptide (CGRP)(76) and substance P(96).

Within healthy young skin, the thermoregulatory response during heat stress is to increase the activity of sympathetic cholinergic vasodilator nerves, which corelease acetylcholine, for sweating, and an unknown transmitter mediating the vasodilator response(64). Direct warming of the skin causes the dilation of local vascular beds to the degree and speed of local warming(8, 40). When local heating results in a rapid increase in skin temperature to 42°C and maintained for a period, local skin blood flow increases to its maximum level(49). Post-synaptic blockade of the muscarinic receptor attenuates the initial rise in skin blood flow, but not plateau, while completely abolishing the cutaneous sweat response. The presynaptic blockade, removing the ability for the cholinergic nerves to release any neurotransmitters completely abolishes the sweating and vasodilatory response(54). This suggests that the corelease of acetylcholine mediates part of the initial rise in blood flow and not just sweat response, but is unable to change the vessels maximal dilatory capacity. Isolation of the specific neurotransmitter responsible for the dilatory response is highly complex and remains elusive. Many of the proposed co-transmitters all play some role in the dilatory response and wholly may overlap becoming redundant. This is because acetylcholine(78), VIP(95), and substance P(96) all contribute to the vasodilatory pathway, stimulating NO release. Many of these chemicals contribute to NO-dependent vasodilation during different phases of the rise in skin blood flow. For example, disruption of the cholinergic component in active vasodilation is unable to change the final plateau phase(77).

Regardless of which, co-transmitter is the primary mediator of active vasodilation, nitric oxide is an essential component of this mechanism. Augmentation of (NOS) can handle 30-40% of the total maximal vasodilator response in healthy intact skin. In conjunction with NO, cyclooxygenase (COX)-dependent pathways also contribute to active vasodilation(63). Both NO and COX mediate dilation, but to what extent the two pathways are interactive has not yet been elucidated. Selective blockade of both pathways would suggest that their effects are additive in nature and not uniquely dependent upon each other. COX can be activated by the acetylcholine mechanism and mediates both early and late formation of prostanoids contributing to active vasodilation(44). The late release of prostanoids during active vasodilation during higher flow rates occurs through the stimulation of sheer stress on the endothelial layer, further enhancing the dilatory mechanisms(63). Furthermore, COX can alternatively be stimulated by NK1 receptor activation, increasing the calcium concentration in endothelial cells, stimulating the NOS and COX pathways(96).

While many of the mechanisms underlying these impaired neurotransmission and loss of NPY function have not fully been elucidated recent research has identified that de novo synthesis of BH4 and recycling pathways are vulnerable to oxidation from ROS. It has been established that aging is associated with elevated oxidation due to a shift in the redox state of the cell towards producing more reactive oxygen species(25). It is thought that this oxidation of BH4 explains the decreased adrenergic function in aged skin and the reduced bioavailability of BH4(23, 58). In aging models supplementation with BH4,

reverses the age-associated vasodilatory dysfunction by increasing BH4 bioavailability in the vascular endothelium(23, 28).

CRYOTHERAPY

Cryotherapy is the use of cold water or ice to reduce the temperature of the skin, surrounding tissues, and often applied to aid in tissue recovery. The known benefits of applying cold water to the body date back to the ancient Greeks when it was used to reduce an inflammatory response to injury or trauma. Historical methods of cryotherapy involved the use of snow, ice or water, but in more recent years more advanced methods have allowed for more accessible methods of reducing pain and inflammation. Commercially available devices can now be fitted with water perfused bladders, connected with a water pump and water circulated to allow for the benefit of cryotherapy, with greater ease. This in conjunction with the known benefits of cryotherapy has led to an increase in availability and comfort of treatment at home. It is been used to aid in the recovery following a range of traumas, post-surgical recovery, and a multitude of other clinical environments. For example, cryotherapy is shown to have an additive response in conjunction with narcotics following surgery by providing added analgesic effects to reduce pain. However, despite the high prevalence and knowledge of the benefits of cold application to the skin, much of the underlying mechanisms mediating the benefits are only partially understood. Trauma and tissue injury are heterogeneous in nature that include different tissues with different responses, with can fluctuate in the magnitude of injury, as well as kinetically over time.

This has led to the popular knowledge of cryotherapy based upon the conclusions from clinical and empirical findings leaving much of the population unable to fully grasp the potentially deleterious effects of cryotherapy. While acute cooling can be very useful and can significantly reduce swelling, inflammation, and pain; prolonged cryotherapy has the potential to amplify tissue damage by impairing the cellular activity and vascular responses resulting in necrosis and cell death. With the use of cold compress, as the length of time and magnitude of cooling increase in magnitude or length the potential for injury to go up.

Cryotherapy has become popularized due to its three main tissue responses; analgesia, depression of metabolism, and its vascular responses. Additionally, the depression of nerve temperature results in decreased nerve conduction until it is ultimately blunted altogether(2) and may prevent muscle spasms generating secondary ischemia injury or perhaps by reducing sensory nerve action(1). In regards to the injury, some investigators consider the effects of cold on metabolism to be the most important at reducing secondary injury from trauma(1, 65). Following most tissue injuries the cellular damage results in an inflammatory response, which causes swelling and tissue ischemia over 24 hours following the initial insult(52). Mclean clearly describes that the decreases in enzymatic activity that occurs with cryotherapy explains why tissue injury is attenuated by reducing the ischemic stress and lowering the cellular oxygen demand(65). With the initial insult causing cell damage, increased inflammatory mediators triggering elevated capillary permeability, increasing the edema. Furthermore, the release of intracellular proteins from damaged cells elevates the osmotic pressure within the extracellular space pulling water from the circulation into the site of injury. This local increase in fluid build-

up results in exponentially increasing the diffusion distance for oxygen generating hypoxic pockets within tissues. The following inflammation induced ischemia can impair tissue recovery and long-term improvements following an injury.

This reinforces the importance of understanding cryotherapies robust anti-inflammatory response when involved in the initial treatment of acute injuries. Direct cold application to the skin has previously shown that the permeability, magnitude of inflammation or swelling, and cellular response changes directly based on the tissue temperature(46). This supports the notion that RICE, i.e. rest, ice, compression, and elevation, should be an essential and immediate response to injury. This is an effective treatment and a safe way of limiting the extent of the injury, reducing the inflammatory stress as well as offering effective pain management(22). Cryotherapy is often the initial treatment following a sprain, tear, strains, or other inflammatory processes. This is partly due to the fact that cold application has been previously shown to reduce the inflammatory response with experimentally induced ligament injuries(30, 46). Cryotherapy results in increased permeability of the lymph vessels and can often lead to elevated swelling following cryotherapy(71). This has led to some controversy due to the fact that some instances of cryotherapy use has also been associated with increased inflammation as well(46). This may partially be explained that clinically the formation of edema following cryotherapy hasn't been recreated due to the elevation and compression that typically follows or is associated with cryotherapy. Precautions clinically involve monitoring time, length of cooling, and magnitude of tissue cooling for safety. While the exact temperature where cryotherapy becomes deleterious is not known temperatures around 15°C, where the

permeability of the lymph vessels increases could result in adverse inflammatory responses enhancing swelling(67). Precautions should be taken with the use of cold temperatures and may result in damaging effects. Additionally, a lesser known phenomena with cryotherapy application is that following use, passive warming of the tissue fails to stimulate the return of blood flow. The tissue can't remove the underlying constrictor tone, the temperature continues to rise, enzyme activity and oxygen demand return, but blood flow remains blunted (~70% reduced). If this occurs following an injury with inflammatory induced ischemic stress, it may exacerbate the ischemic injury and result in greater cell/tissue loss.

Understanding the vascular effects of cold application happen to be more complex and involves neural, end target receptor changes, smooth muscle, platelet and endothelial mechanisms. The initial phase of cooling is often the most rapid and causes reflex vasoconstriction from sympathetic neurons decreasing local blood flow and is followed by increased density of α_2 receptors enhancing end-organ function. The thermal effects reduce the metabolic activity of noradrenaline metabolizing enzymes (MAO and COMT), the increased blood viscosity due to constriction and aggregated activated platelets, releasing 5HT and thromboxane A2, all enhance the vasoconstrictor tone.

Chapter 8: General Discussion and Future Directions

Cryotherapy treatment is perhaps one of the most often utilized therapies in the United States. However, despite how commonly used, there are much fewer studies verifying and supporting its use and even fewer detailing its shortcomings and potential detrimental effects. While these studies haven't changed the importance of cryotherapy they've introduced a better understanding towards the possible ramifications of unsupervised or inappropriate cryotherapy treatment. Specifically, these studies highlight the significance of reheating tissue temperatures following "treatment."

This is based on our findings that show impaired blood flow/temperature responses for 2 hrs following cryotherapy and was only corrected by inhibition of Rho kinase or by actively heating the treated skin. This impaired blood flow/temperature response, we believe, is a direct result of the action of Rho kinase by enhancing vasoconstriction. Based on our findings we feel that there is not enough evidence to suggest that the impairment of nitric oxide function contributes to the manifestation of a profound and sustained vasoconstriction with cryotherapy use. This would propose the action of Rho kinase is primarily due to its enhanced end-organ sensitivity(70) and function(6).

Of unique interest in our investigations was the finding that despite a similar protocol and similar temperatures reached in the skin, blood flow responses to cold were augmented. While there isn't substantial evidence to suggest that the microcirculation can exhibit changes in vasoconstrictor tone with heat acclimation, there is little else that could explain such large differences in %CVC despite similar subjects and protocol. There have

been investigations showing the microvascular changes that occur with heat acclimation in trained cyclists(61), but a comparison of results is difficult due to their assessment during local heating rather than cooling. However, our findings would still support the notion of heat acclimation by having lower %CVC for a given temperature.

The future focus of research should be to understand better the significance of the Rho kinase pathway. It appears after these investigations that the Rho kinase pathway is the ultimate contributor to sustained vasoconstriction and as such a potent modulator of vasoconstriction important in the development of non-freezing cold injuries. Independent of cryotherapy the significance of understanding Rho kinase extends towards cardiovascular disease as a whole due to its effects on vascular tone and proliferation of vascular smooth muscle cells(3, 75). Furthermore, due to the fact that as adrenergic activity, neurotransmitter release, and sensitivity decrease with age, resulting in a greater reliance on the action of Rho kinase, the significance upon aging should be investigated more significantly(89, 90). Due to the enhanced activity of Rho kinase and its ability to induce profound and sustained vasoconstriction when activated, it lends the aged population at greater risk for all potential deleterious effects of cryotherapy or vascular health. It has been suggested that because of the greater dependence upon Rho kinase with aging that there's a shift from a state of youthful vasodilatory action towards an aged vasoconstrictor tone(59). This may partially contribute with the age-associated development of cardiovascular disease.

Our investigations illustrated, to some degree, that nitric oxide plays a no role in a typical cryotherapy protocol. This would suggest that much of what occurs during

cryotherapy is vasoconstriction dependent rather than a withdrawal of dilatory tone, within our population and model. This would suggest that much of the action of Rho kinase is mediated by heightened sensitivity to norepinephrine and increased vasoconstriction due to the inhibition of MLCP.

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