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Pronounced and Sustained Cutaneous Vasoconstriction during and Following Cyrotherapy Treatment: Role of Neurotransmitters Released from Sympathetic Nerves

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Abstract

Cryotherapy is a therapeutic technique using ice or cold water applied to the skin to manage soft tissue trauma and injury. While beneficial, there are some potentially detrimental side effects, such as pronounced vasoconstriction and tissue ischemia that are sustained for hours post-treatment. This study tested the hypothesis that this vasoconstriction is mediated by 1) activation of post-synaptic α-adrenergic receptors and/or 2) activation of post-synaptic neuropeptide Y1 (NPY Y1) receptors. 8 subjects were fitted with a commercially available cryotherapy unit with a water perfused bladder on the lateral portion of the right calf. Participants were instrumented with four intradermal microdialysis probes beneath the bladder. The following conditions were applied at the four treatment sites: 1) control (Ringer solution), 2) combined post-synaptic β-adrenergic receptors and neuropeptide (NPY) Y_1 receptors blockade (P+B site), 3) combined post-synaptic α-adrenergic receptor, β-adrenergic receptor, and NPY Y_1 receptor blockade (Y+P+B site), and 4) blockade of pre-synaptic release of all neurotransmitters from the sympathetic nerves (BT site). Following thermoneutral baseline data collection, 1 °C water was perfused through the bladder for 30 min, followed by passive rewarming for 60 min. Skin temperature (T_{skin}) fell from ~ 34 °C to ~ 18.5 °C during active cooling across all sites and there was no difference between sites (P>0.05 vs. control for each site). During passive rewarming T_{skin} rose to a similar degree in all sites (P>0.05

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Conflict of Interest

Patent applications for some aspects of this technology as applied to cryotherapy have been submitted by Dr. Khoshnevis and Dr. Diller to the United States Patent and Trademark Office. Ownership rights to this IP reside with The University of Texas System. Dr. Diller has served as an expert witness for both plaintiff and defendant counsel since 2000 in numerous legal cases regarding the safety and design of existing cryotherapy devices.

relative to the end of cooling). In the first 20 min of cooling %CVC was reduced at all sites however, this response was blunted in the BT and the Y+P+B sites (P>0.05 for all comparisons). By the end of cooling the degree of vasoconstriction was similar between sites with the exception that the reduction in %CVC in the Y+B+P site was less relative to the reduction in the control site. %CVC was unchanged in any of the sites during passive rewarming such that each remained similar to values obtained at the end of active cooling. These findings indicate that the initial vasoconstriction (i.e. within the 1st 20 min) that occurs during cryotherapy induced local cooling is achieved via activation of post-synaptic α-adrenergic receptors; whereas nonadrenergic mechanisms predominate as the duration of cooling continues. The sustained vasoconstriction that occurs following cessation of the cooling stimulus does not appear to be related to activation of post-synaptic α-adrenergic receptors or NPY Y1 receptor.

Keywords

vasoconstriction; ischemia; skin-surface cooling; cryotherapy; soft tissue injury

Introduction

Regulation of the cutaneous vasculature during local (i.e. direct) skin surface-cooling has been the topic of many previous research studies and reviews (Hodges et al., 2006; Johnson, 2007; Johnson and Kellogg, 2010; Thompson-Torgerson et al., 2007a; Thompson-Torgerson et al., 2007b; Yamazaki, 2010). Overall, the general consensus of these studies is that the pronounced vasoconstriction during local cooling is primarily mediated by a combination of adrenergic and nonadrenergic mechanisms (Hodges et al., 2006; Johnson, 2007; Johnson and Kellogg, 2010; Thompson-Torgerson et al., 2007a; Thompson-Torgerson et al., 2007b; Yamazaki, 2010). During the initial period of cooling (i.e. within the first 10 min) norepinephrine (NE) released from sympathetic nerves elicits vasoconstriction secondary to binding to α_2-adrenoreceptors (Ekenvall et al., 1988; Freedman et al., 1992; Pérgola et al., 1993). Whereas, during prolonged cooling the primary mechanism involved is a reduction in nitric oxide synthase (NOS) and a subsequent reduced nitric oxide (NO) bioavailability (Hodges et al., 2006; Johnson et al., 2005; Pérgola et al., 1993; Yamazaki et al., 2006).

These previous studies provide valuable insight into mechanisms of cold-induced vasoconstriction. However, the skin-surface cooling utilized in most of these studies is typically performed on a relatively small skin surface area of approximately 6.3 cm^2, and the skin-surface is commonly cooled to 24 °C, which is approximately only 10 °C below baseline values, and is relatively modest (Hodges et al., 2006; Johnson, 2007; Johnson and Kellogg, 2010; Thompson-Torgerson et al., 2007a; Thompson-Torgerson et al., 2007b; Yamazaki, 2010).

Another commonly utilized method of skin-surface cooling is cryotherapy treatment which is often used in orthopedic or sports medicine settings to treat and/or reduce bleeding, inflammation, metabolism, pain, and swelling following soft tissue trauma and injury (Swenson et al., 1996). This is often accomplished using commercially available cryotherapy units that circulate ice water through a bladder that covers an area being treated

such as the shoulder, knee, thigh, or shin region (Babwah, 2011; Bassett et al., 1992). As a result, the skin-surface temperature drops to about 16 °C over a relatively large skin-surface area (Christmas et al., 2016; Khoshnevis et al., 2015a; Khoshnevis et al., 2015b). While beneficial for therapeutic purposes, cryotherapy treatment also carries the risk of side effects including tissue necrosis and neuropathy (Babwah, 2011; Bassett et al., 1992; Brown and Hahn, 2009; Lee et al., 2007; Moeller et al., 1997). These conditions are likely the result of profound reductions in local tissue temperature and the subsequent pronounced tissue ischemia during the period of cryotherapy and the sustained tissue ischemia that remains for up to 2 hr during passive rewarming despite skin temperature returning to near baseline (i.e. pre-cooling) values (Christmas et al., 2016; Khoshnevis et al., 2015a; Khoshnevis et al., 2015b). This reduced blood flow is therapeutic in that it aids in reducing the inflammatory cascade and edema formation; however, when sustained for sufficient time, the reduced supply of oxygen and cell nutrients in conjunction with the buildup of toxic metabolic byproducts may lead to the aforementioned detrimental side effects including tissue necrosis and neuropathies (Santilli and Santilli, 1999). In addition, a prolonged state of ischemia can lead to reperfusion injury when flow is reestablished (Jia and Pollock, 1999) and may be an agent of nonfreezing cold injury (Francis, 1984; Francis and Golden, 1985).

Whether or not the previously identified mechanisms of cold-induced vasoconstriction can be extrapolated to the vasoconstriction that occurs during cryotherapy treatment, which results in much cooler skin temperatures over a larger skin-surface area (Christmas et al., 2016; Khoshnevis et al., 2015a; Khoshnevis et al., 2015b) remains unknown. Furthermore, to our knowledge no studies have investigated mechanisms of sustained vasoconstriction following termination of the cooling stimulus (Christmas et al., 2016; Khoshnevis et al., 2015a; Khoshnevis et al., 2015b) particularly using commercially available cryotherapy units. Accordingly, this study utilized the intra-dermal microrddialysis technique to administer specific post-synaptic and pre-synaptic antagonists into the cutaneous circulation directly beneath the cryotherapy cooling pad. We hypothesized that cold-induced vasoconstriction during cryotherapy treatment would be mediated in large part via activation of post-synaptic α-adrenergic receptors. Furthermore, we hypothesized that the sustained vasoconstriction during passive rewarming, that we previously observed (Christmas et al., 2016; Khoshnevis et al., 2015a; Khoshnevis et al., 2015b) is also mediated in large part via activation of post-synaptic α-adrenergic receptors. Knowledge of the various vasoactive mediators involved in the pronounced and sustained vasoconstriction during cryotherapy treatment could provide valuable information to the scientific and clinical community. For example this information could be insightful in regards to the design of various intervention and/or treatment strategies to reduce the negative side effects of cryotherapy while maintaining the beneficial effects.

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 ± SD) 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 and alcoholic beverages for 24 hr and from consuming caffeine and food for 12 hr prior to the experimental trial that 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) for continuous assessment 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 continuous assessment of local skin temperature (T_{skin}). Following placement of the membranes, Doppler flow probes, and thermocouples, 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 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 (Baxter, Deerfield, IL) which served as the control site (Con). One site received a combination of 1mM propranolol and 10μM of N2-(diphenylacetyl)-N-([4-hydroxyphenyl]methyl)-D-arginine amide (BIBP-3226; 10μM) to block post-synaptic β-adrenergic receptors and neuropeptide Y_1 receptors respectively. This site (P+B) allowed for examination of the role of the post-synaptic α-adrenergic receptors. One site received a combination of 5mM yohimbine, 1mM propranolol, and 10μM of

BIBP-3226 (Y+P+B) to post-synaptically block α-adrenergic, β-adrenergic, and neuropeptide (NPY) Y_1 receptors respectively. This combination and concentrations of these vasoactive agents inhibit the reduction in cutaneous vascular conductance during whole-body cooling (Stephens et al., 2004). The last site received 10mM of bretylium tosylate to block the presynaptic release of neurotransmitters from the sympathetic nerves (BT, (Hodges et al., 2008; Johnson et al., 2005)). All vasoactive agents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were dissolved in lactated Ringer solution. Each site was initially perfused at 2 μL/min for a 30 sec priming period after which the rate was reduced to 2 μL/min for the remainder of data collection.

After the 45 min wash in period, the cryotherapy unit and cooling pad was perfused with 34 °C water for 15 min of baseline data collection. This was followed by 30 min of active skin-surface cooling which was accomplished by circulating 0 – 1 °C water through the cryotherapy unit and cooling pad. At the end of the cooling phase the cryotherapy unit was turned off for 60 min of data collection during passive rewarming. In attempt to not interfere with the quality of the data collection the cooling pad remained in place during the passive rewarming period.

Data Analysis

Laser-Doppler flux and T_{skin} 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 °C baseline condition, min 5, 10, 15, 20, 25, and 30 of active cooling and min 30 and 60 of passive rewarming. Mean arterial pressure (MAP), calculated as 1/3 systolic pressure + 2/3 diastolic pressure, was also measured during each of these time points and used for subsequent calculation of cutaneous vascular conductance (CVC) (Doppler-derived flux/MAP). All CVC and T_{skin} data throughout active cooling and passive rewarming were normalized to the value obtained during the final min of 34 °C baseline.

Statistical Analysis

Statistical analyses were performed using a statistical software package (SigmaPlot 12.5; Systat Software, Inc., San Jose, CA). CVC and T_{skin} were both analyzed using a two-way repeated measures ANOVA with main factors for treatment (Con, Y+P, Y+P+B, and BT) and time. When a significant interaction was identified post hoc analyses of multiple comparisons were performed using Bonferroni *t* tests. All data are shown as mean±SD. For all tests significance was found at $P < 0.05$.

Results

T_{skin} throughout the Different Phases of the Protocol

The T_{skin} responses throughout the different phases of the protocol were similar between the 4 different sites (Fig 1A; main effect of site: P=0.99).

Active Cooling—Throughout the active cooling period T_{skin} decreased at all sites relative to the pre-cooling baseline (Fig 1A; main effect of time: P<0.001). T_{skin} at pre-cooling

baseline was ~34.0 °C for each site (Fig 1A). During active cooling T_{skin} was immediately reduced to ~ 28.0 °C after the first 5 min (P<0.001 vs. pre-cooling baseline) for each site and continued to decline throughout active cooling reaching a minimum value of ~ 18.5 °C at the end of 30 min of cooling (Fig 1A; P<0.001 vs. pre-cooling baseline) for each site.

Passive Rewarming—Following 30 min of passive rewarming T_{skin} was elevated to ~ 22.4 °C (P<0.001 vs. end of cooling) for each site and continued to rise throughout passive rewarming reaching a value of ~ 24.1 °C at each site at the end of 60 min of passive rewarming (Fig 1A; P<0.001 vs. end of cooling). Despite this elevation during passive rewarming, T_{skin} at both time points remained below pre-cooling baseline values (Fig 1A; P<0.001 vs. pre-cooling baseline).

CVC throughout the Different Phases of the Protocol

Cutaneous vascular conductance, % CVC with respect to the baseline value, throughout the entire protocol is illustrated in Fig 1B and Fig 2.

Active Cooling—Local cold water application resulted in an immediate decrease in %CVC relative to the pre-cooling baseline with the exception of min 5 of cooling in the BT site (P<0.05; Fig 1B & 2). Throughout the remainder of active cooling, %CVC continued to decrease in all sites such that at the end of 30 min of cooling %CVC was reduced at each site relative to its respective pre-cooling baseline and 5 min of cooling value (Fig 1B & 2; P<0.001 for both comparisons). The magnitude of vasoconstriction throughout the first 20 min of active cooling was blunted in the BT and Y+P+B sites compared to the P+B and Control sites (P<0.05 for all comparisons). At the end of active cooling the reduction in %CVC was similar between the 4 sites with the exception that the reduction in %CVC in the Y+B+P site was less relative to the reduction in the control site (Fig 1B & 2; P=0.02).

Passive Rewarming—Throughout the duration of passive rewarming, %CVC at all sites did not return toward baseline values such that each remained similar to values obtained at the end of skin-surface cooling (Fig 1B & 2; P<0.05 for each comparison) despite skin temperatures being elevated relative to the end of skin-surface cooling (Fig 1A).

Discussion

This study extends previous research investigating mechanisms of local cooling induced vasoconstriction. The primary difference in the current protocol is that the cold application was designed to simulate conditions both in terms of cooling duration (~ 30 min) and cooling source (i.e. circulating ice water) using a commercially available cryotherapy unit that is commonly prescribed in orthopedic/sports medicine settings. As a result the surface area and magnitude of cooling mimic conditions that are utilized in real life scenarios. To the best of our knowledge, other than a previous publication from our group (Christmas et al., 2016), there is no other information regarding mechanisms of pronounced and sustained cold-induced vasoconstriction during and following cryotherapy treatment. Our primary findings are that the pronounced cold-induced vasoconstriction that occurs during 30 min of cryotherapy is primarily mediated by activation of post-synaptic α-adrenergic receptors. These results are in agreement with previous studies which assessed mechanisms of

vasoconstriction during a milder cooling stimulus in a much smaller surface area of skin in the arm (Hodges et al., 2006; Johnson, 2007; Johnson and Kellogg, 2010; Thompson-Torgerson et al., 2007a; Thompson-Torgerson et al., 2007b; Yamazaki, 2010). Additionally, the sustained vasoconstriction that persists during passive rewarming was similar between all four sites suggesting that release of neurotransmitters from sympathetic nerve endings does not contribute to this response.

Cyrotherapy to treat pain associated with a variety of soft tissue trauma and injury has been used for centuries (Swenson et al., 1996) and is very common in a variety of clinical and sports medicine settings. The beneficial effects of cryotherapy treatment are proposed to be related to a variety of mechanisms including reductions in the bleeding, inflammation, cellular metabolism, and swelling that accompany traumatic or surgical injury (Swenson et al., 1996). While beneficial, cryotherapy treatment is associated with various negative side effects including, tissue necrosis, and neuropathy (Babwah, 2011; Bassett et al., 1992; Brown and Hahn, 2009; Lee et al., 2007; Moeller et al., 1997). These side effects are likely secondary to the pronounced vasoconstriction and thus tissue ischemia that occurs at the site of cooling. Furthermore, this vasoconstriction persists for up to 2 hr following cessation of the cooling stimulus (Christmas et al., 2016; Khoshnevis et al., 2015a; Khoshnevis et al., 2014; Khoshnevis et al., 2015b). As a result, during this period of tissue ischemia there is a reduction in oxygen and cell nutrient supply accompanied by a build-up of metabolic byproducts which may contribute to the aforementioned detrimental side effects and may also contribute to reperfusion injury (Jia and Pollock, 1999) and nonfreezing cold injury (Francis, 1984; Francis and Golden, 1985).

The mechanisms of cold-induced vasoconstriction have been previously assessed in a number of systematic investigations (Hodges et al., 2006; Johnson, 2007; Johnson and Kellogg, 2010; Thompson-Torgerson et al., 2007a; Thompson-Torgerson et al., 2007b; Yamazaki, 2010). Below we will discuss the aforementioned mechanisms as they pertain to the pronounced and sustained vasoconstriction associated with cryotherapy.

At the onset of cooling, the initial vasoconstriction is predominantly mediated by activation the sympathetic vasoconstrictor system (Hodges and Johnson, 2009; Hodges et al., 2008; Johnson and Kellogg, 2010; Johnson et al., 2005). This is evidenced by a number of studies that demonstrate that the initial vasoconstrictor response to local cooling is abolished when pre-synaptic release of neurotransmitters from the sympathetic nerves is prevented via local infusion of bretylium tosylate or when the post-synaptic β- and α-adrenergic receptors are blocked with local infusion of propranolol and yohimbine respectively (Hodges et al., 2008; Johnson et al., 2005). This vasoconstriction at the onset of cooling is the result of activation of cold sensitive receptors which result in reflex mediated increase in sympathetic nerve activity as well as activation of local sensory nerves which stimulate norephinephrine release from the sympathetic nerves (Johnson et al., 2005; Pérgola et al., 1996; Pérgola et al., 1993). The sympathetic nerves also secrete additional neurotransmitters including neuropeptide Y (NPY) which has been implicated as a factor involved in cold stress induced tissue ischemia and non-freezing cold injury (Maturi et al., 1989). Stephens et al. reported that approximately 30% of the reflex vasoconstrictor response to whole-body cooling is dependent on activation of post-synaptic NPY Y1 receptors (Stephens et al., 2004).

Furthermore, this reflex vasoconstriction is essentially eliminated when the NPY Y1 receptors and the post-synaptic β- and α-adrenergic receptors are blocked (Stephens et al., 2004). In contrast to whole-body cooling, blockade of NPY Y1 receptors has no effect on the vasoconstrictor response to local cooling (Johnson et al., 2005). In the current study the degree of local cooling induced vasoconstriction was significantly blunted within the first 20 – 25 min of cooling at a site where the post-synaptic β- and α-adrenergic receptors and NPY Y1 receptors were blocked (Y+P+B site). A similar pattern was observed when pre-synaptic release of all neurotransmitters from the sympathetic nerves was prevented with bretylium tosylate (BT site). In contrast when only the post-synaptic β-adrenergic receptors and NPY Y1 receptors were blocked the pattern and degree of vasoconstriction was similar to that observed in the control site (P+B site). These findings are in line with previous reports in that the degree of cryotherapy treatment induced vasoconstriction, particularly at the onset of cooling, is primarily dependent on activation of post-synaptic α-adrenergic receptors with little contribution of either post-synaptic β-adrenergic receptors or NPY Y1 receptors.

As the duration of the cooling stimulus extends there is a gradual transition to nonadrenergic mechanisms of vasoconstriction (Hodges et al., 2008; Johnson and Kellogg, 2010; Johnson et al., 2005; Thompson-Torgerson et al., 2007a; Yamazaki et al., 2006). These findings are generally in agreement with the current study in that the magnitude of vasoconstriction at the end of the cooling phase was similar in all sites. The exception was that the decrease in CVC was slightly, but significantly, less in the site where the post-synaptic β- and α-adrenergic receptors and NPY Y1 receptors were blocked relative to the control site. However, the reduction at this site was similar to the site where pre-synaptic neurotransmitter release was prevented and post-synaptic β-adrenergic receptors and NPY Y1 receptors were blocked. While this study was not designed to investigate these other mechanisms leading to vasoconstriction during prolonged cooling, reports indicate that it appears to be more dependent on a disruption of NO mediated vasodilation secondary to reduced activity of NOS as well as at a downstream signaling level such that the vasodilator response to NO is also impaired (Hodges et al., 2006; Johnson et al., 2005; Pérgola et al., 1993; Yamazaki et al., 2006). The mechanisms resulting in this process are likely multifactorial but are in part related to cold induced increase in reactive oxygen species (ROS) primarily from the vascular smooth muscle mitochondria (Bailey et al., 2005). Elevated ROS reduces NO bioavailability via various pathways including increasing available arginase which competes L-arginine thus uncoupling endothelial NOS (eNOS) (Holowatz et al., 2006), oxidation of tetrahydrobiopterin, the essential cofactor for eNOS (Stanhewicz et al., 2012), as well as a directly scavenging NO (Holowatz and Kenney, 2007). The ROS generated during local cooling also activates the Rho kinase pathway which enhances vascular tone by 1) stimulating translocation of α_{2c}-adrenoreceptors to the smooth muscle cell surface (Bailey et al., 2004; Bailey et al., 2005; Chotani et al., 2000; Thompson-Torgerson et al., 2007a), 2) by increasing smooth muscle sensitivity to intracellular Ca^{2+} (Bailey et al., 2004; Thompson-Torgerson et al., 2007b), and 3) by downregulating the eNOS pathway thus reducing NO bioavailability (Bivalacqua et al., 2004; Ming et al., 2002; Takemoto et al., 2002). To this end we have previously demonstrated that pretreatment of a skin site with ascorbic acid to reduce oxidative stress and another site with Fasudil to block the Rho Kinase pathway

blunted cold-induced vasoconstriction during a similar cryotherapy protocol as utilized in the current study (Christmas et al., 2016).

Following cessation of the cooling stimulus T_{skin} remained below pre-cooling values throughout 60 min of passive rewarming; however, there was warming during this period as evidenced by a significant increase in T_{skin} relative to the end of cooling. Despite this increase in T_{skin} there remained a sustained vasoconstriction that persisted for at least 60 min in all 4 sites. This hysteresis loop between skin blood flow is in agreement with prior work from our group, using a similar cryotherapy cooling protocol, that has already been described in greater detail (Khoshnevis et al., 2015a). While multiple studies have examined the mechanisms of vasoconstriction during local cooling, there are limited studies that have monitored vasoconstriction following the cessation of the cooling stimulus. CVC in the forearm returned to baseline following the cessation of local cooling to 24 °C (~10 °C below baseline) in a control site as well as a site pretreated with ascorbic acid (Yamazaki, 2010). Potential differences in the magnitude of CVC recovery (and thus lack of hysteresis) between the studies include differences in: 1) anatomical locations, 2) magnitude of cooling, 3) skin surface area being cooled, and 4) the sites were actively warmed as opposed to passively warmed to restore local T_{skin} (Yamazaki, 2010). Our group has also used active rewarming following cryotherapy cooling to restabilize CVC (Khoshnevis et al., 2014).

Following 10 min of a cold exposure it has been reported that NPY plasma concentration is elevated by ~ 300% and remains elevated following cessation of the stimulus (Johnson, 2007; Stephens et al., 2004). Accordingly, we speculated that blockade of presynaptic release of NPY from the sympathetic nerves or post-synaptic blockade of the NPY Y1 receptors would result in a return of CVC during passive rewarming. However, as evidenced in Fig 1B & 2 this was not the case suggesting that NPY is not involved in the sustained vasoconstriction that persists following cryotherapy treatment. We are currently not able to speculate on the mechanisms involved in the sustained vasoconstriction despite T_{skin} being elevated relative to the values obtained at the end of cooling. This area warrants further research.

Methodological Considerations

The T_{skin} measurements were obtained at the surface of the skin, whereas the cutaneous perfusion measures were assessed within an interrogation volume focused at an average tissue depth of 0.5 mm (Clough et al., 2009). Furthermore, due to technical limitations we were not able to assess either temperature or perfusion at an intramuscular level. In this regard previous studies using direct or simulated temperature measurements during both cooling and passive rewarming have suggested that differential monitoring depths for temperature and perfusion do not influence the overall findings (Diller, 2015; Khoshnevis et al., 2015a; Merrick et al., 1993; Myrer et al., 1998).

Clinical/Practical Significance

Localized cooling is a common therapeutic practice in orthopedic or sports medicine settings to treat and/or reduce conditions associated with soft tissue trauma and injury. While beneficial for therapeutic purposes, cryotherapy treatment also carries the risk of side effects

including, tissue necrosis, and neuropathy (Babwah, 2011; Bassett et al., 1992; Brown and Hahn, 2009; Lee et al., 2007; Moeller et al., 1997). These conditions are likely the result of profound reductions in local tissue temperature and the subsequent pronounced tissue ischemia during the period of cryotherapy and the sustained tissue ischemia that remains for up to 2 hr during passive rewarming despite skin temperature returning to near baseline (i.e. pre-cooling) values (Christmas et al., 2016; Khoshnevis et al., 2015a; Khoshnevis et al., 2015b). Despite the widespread therapeutic use of localized cooling and cryotherapy the effect of cryotherapy on alteration of local blood flow and its time course is relatively unknown. The findings of the current study provide mechanistic insights into the control of vascular perfusion during cryotherapy that may be insightful in regards to development of intervention/treatment strategies for effective healing of soft tissue injuries with minimal risk of ischemic side effects.

Conclusion

The findings indicate that the initial vasoconstriction (i.e. within the 1st 20 min) that occurs during local cooling using a commercially available cryotherapy unit is predominantly achieved via activation of post-synaptic α-adrenergic receptors; however, as the duration of cooling continues nonadrenergic mechanisms predominate the vasoconstrictor response. The sustained vasoconstriction that occurs following cessation of the cooling stimulus does not appear to be related to activation of post-synaptic α-adrenergic receptors or NPY Y1 receptors.

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Highlights

- Cryotherapy treatment is used in treatment after surgery and in sports medicine.
- Cryotherapy causes pronounced and sustained vasoconstriction in the cooled area.
- This ischemia can cause side effects such as tissue necrosis and neuropathy.
- The initial vasoconstriction is due to activation of α-adrenergic receptors.
- Post-cooling vasoconstriction is not due to α-adrenergic or NPY Y1 receptors.

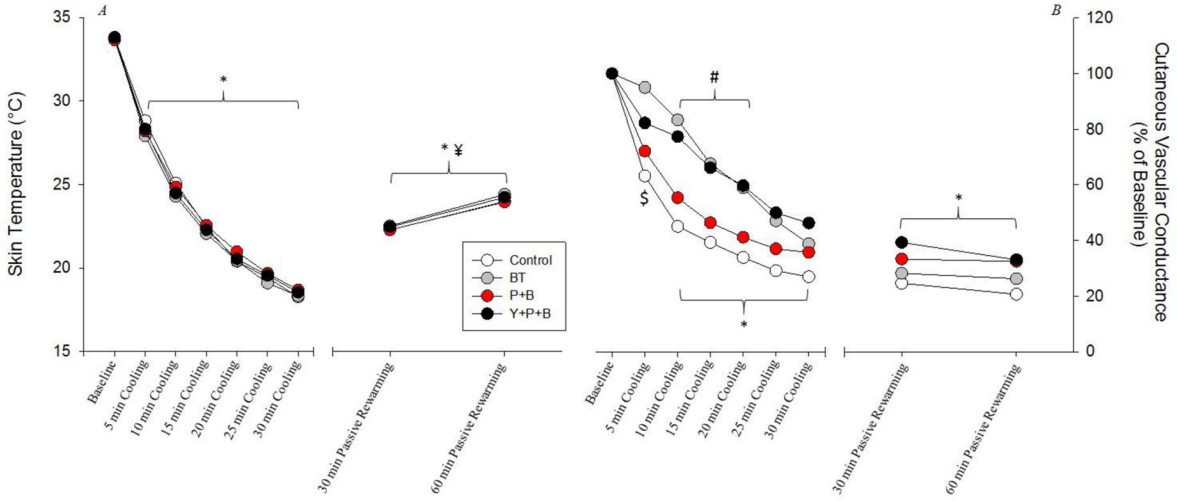

Figure 1. T_{skin} and %CVC Responses throughout Skin Surface Cooling and Passive Rewarming

The T_{skin} response is illustrated in panel A whereas the %CVC response is illustrated in panel B. Clear circles represent the control condition, grey circles represent the BT condition, red circles represent the P+B condition, while black circles represent the Y+P+B condition. All data presented as means ± SD. *: *P*<0.001 for each condition relative to pre-cooling baseline. ¥: *P*<0.001 for each condition relative to 30-min of cooling. #: P<0.001 for magnitude of constriction in Control & P+B sites relative to BT & Y+P+B sites. $: P<0.01 for Control, P+B, and Y+P+B sites relative to pre-cooling baseline. For clarity purposes error bars have been removed from both panels.

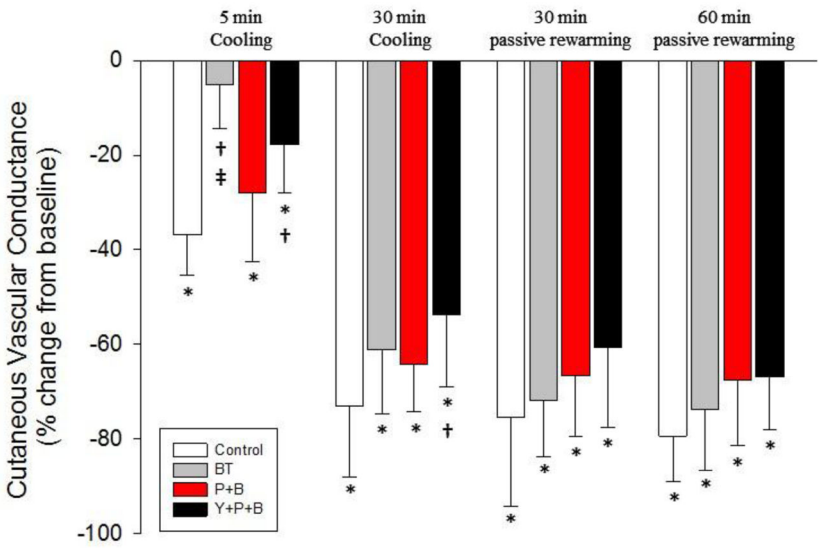

Figure 2. %CVC Responses throughout Skin Surface Cooling and Passive Rewarming

Clear bars represent the control condition, grey bars represent the BT condition, red bars represent the P+B condition, while the black bars represent the Y+P+B condition. All data presented as means ± SD. *: $P<0.001$ for each condition relative to pre-cooling baseline. †: $P<0.01$ relative to the control site. ‡ $P<0.01$ relative to the P+B site.