# MECHANISTIC INSIGHT INTO FUNCTIONAL SYMPATHOLYSIS IN HUMAN SKELETAL MUSCLE FEED ARTERIES

by

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# ABSTRACT

Through the use of *in vitro* methods, previous research in animal models has attempted to isolate the effects of local metabolic factors, normally associated with exercising skeletal muscle, on vascular reactivity and thus blood flow regulation. Utilizing an *in vitro* vessel approach and human skeletal muscle feed arteries, the purpose of these studies was to determine if local factors, such as heat and acidosis, could alter vascular reactivity and thus contribute to reduced sympathetically induced vasoconstriction (functional sympatholysis) and exercise hyperemia. Specifically, it was hypothesized that heat and acidosis will suppress vasocontracile function in human skeletal muscle feed arteries. The first study investigated the effect of temperature on $\alpha_1$-adrenergic receptor stimulation using phenylephrine (PE). Cooling to 35 °C or heating to 39 °C both resulted in an attenuated PE-induced vasocontraction, while smooth muscle function, as determined by KCl, remained unchanged. The goal of the second study was to determine if the potent vasodilator, nitric oxide (NO), could be a factor mediating the heat-induced attenuation in PE-induced contraction observed in the first study. Heating again reduced PE-induced vasocontraction, and was restored with NO blockade. Using molecular approaches, it was determined that heating did not change the density of $\alpha_1$-receptors, but increased endothelial nitric oxide synthase (eNOS) protein expression. The third study sought to determine the effect of acidosis on vascular reactivity. Increasing levels of acidosis resulted in graded reductions in $\alpha_1$- receptor-induced vasocontraction,

which was in part due to reduced smooth muscle function as assessed by KCl. Increasing acidosis maintained maximal vasorelaxation and suggested enhanced sensitivity to both the endothelium-dependent agonist acetylcholine (ACh) and the endothelium-independent NO donor sodium nitroprusside (SNP). Collectively, these results indicate that local factors normally associated with exercise-induced increases in skeletal muscle metabolism (i.e. heat and acidosis) are, in fact, capable of altering feed artery vascular reactivity in humans, and therefore likely play a role in sympatholysis *in vivo*.

TABLE OF CONTENTS

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# BACKGROUND

Energy production for movement and exercise relies heavily upon the delivery of reactants for metabolism such as oxygen, nutrients, as well as the removal of metabolic by-products from the active skeletal muscle. Blood flow is a key variable governing both the delivery of metabolic reactants and removal of waste. Thus, the ability to exercise is dependent upon the capacity to increase or maintain skeletal muscle blood flow as dictated by a given level of muscular activity and requisite metabolic rate.

It is well known that heart rate, blood pressure, cardiac output, blood flow, and oxygen consumption increase linearly with graded exercise from rest towards maximal effort (1, 27). The purpose of this dynamic cardiovascular response is to increase blood flow and delivery of oxygen and nutrients but also to remove accumulating metabolic by-products associated with muscular work such as heat, hydrogen ions, and carbon dioxide (27). In the face of the increased demand for skeletal muscle perfusion, a finite blood volume must be redistributed to maintain adequate blood pressure; failure to do so would result in decrement in exercise performance and more importantly, a loss of consciousness (40, 41).

At the onset of exercise, there is a global sympathetic activation, known as the exercise pressor reflex (2, 8, 41). The exercise pressor reflex is characterized by a global vasoconstriction prior to and at the onset of exercise (44). However, shortly thereafter, blood flow toward active muscle beds increases dramatically, despite systemic sympathetically induced vasoconstriction (46). This functional hyperemia has been attributed to many different mechanisms, such as the effect of local metabolites on vascular smooth muscle contraction, an alteration in the response to vasoconstrictor substances, or the mechanical effects of muscle contraction (19, 28, 41, 46).

The acute regulation of vascular smooth muscle tone and mean arterial pressure is largely achieved through sympathetically mediated adrenergic tone. Thus, the functional hyperemia at the onset of exercise, despite a significant sympathetic activation, is generally referred to as "functional sympatholysis," originally recognized by Remensynder et al. in 1962 (34). The term sympatholysis was coined based upon the original concept that during the initial phase of exercise, sympathetic tone was being "lysed" to allow increased blood flow to the now highly metabolically active muscle compartment.

One of the purported factors involved in sympatholysis is an elevation in local temperature, a consequence of increased metabolic demand (15). Previous research has provided evidence that intramuscular temperature can increase significantly at the onset of, and throughout, an acute exercise bout (12, 26). Cui et al. (5) found that passive heating reduced mean vascular responsiveness to the systemic administration of the $\alpha_1$-adrenergic agonist phenylephrine (PE). However, this study lacked the specificity to determine if increased temperature can exhibit a sympatholytic effect in arteries feeding skeletal muscle, as the drug was administered systemically and its consequences were measured via mean arterial pressure (5). Thus, the findings by Cui et al. (5) cannot truly be applied to the skeletal muscle vasculature, and the question if temperature could be a mechanism contributing to functional sympatholysis remains unanswered.

Functional sympatholysis during exercise appears to depend on a functional endothelium (10), suggesting a potential link between sympatholysis and nitric oxide (NO) (3). Indeed, Harris et al. (13) found that heat exposure significantly reduced the vasoconstrictory effects of PE in rat aortic rings, and this effect was abolished with NO

blockade. These findings implicate NO as a component of the sympatholytic effect of temperature, perhaps by offering increased endogenous opposition to vasoconstrictors. However, these findings have yet to be confirmed in human arteries.

Another local factor associated with skeletal muscle metabolism is increased hydrogen ion ($H^+$) production and subsequent reductions in pH. In 1880, Gaskell (11) originally proposed the hypothesis that lactate and the associated hydrogen ions exert a profound effect upon the vasculature. Not until the 1960s did another research group follow up on Gaskells' hypothesis. Specifically, Kontos and colleagues (6, 7, 22) performed an elegant series of *in vivo* experiments originally designed to understand the role of hypoxia and hypercapnia on hemodynamics. They determined that hypercapnia and the associated acidosis was a major stimulus for vasodilation (21, 23-25); however, it was unknown how acidosis induced this hyperemic response. Subsequent *in vitro* experiments have confirmed these findings indicating a vasodilatory effect of acidosis at baseline (9, 14, 17, 18, 29, 33), and in response to various agonists (4, 16, 20, 30, 31, 37, 42) in the animal model, although not all agree acidosis is vasodilatory (35, 36, 38).

Only one study, to date, has used human arteries to determine the effect of acidosis on vascular reactivity. Rohra et al. (39) obtained internal mammary arteries from coronary bypass graft surgery and studied them *in vitro* using isometric tension technique. This study determined that acidosis elicited a vasorelaxation from pre-contraction with PE or endothelin-1 (39). However, the question remains whether acidosis, such as that achieved during exercise, can alter the vascular reactivity in arteries that are capable of regulating skeletal muscle blood flow, such as the feed artery (43, 47). Most of the aforementioned studies have used cerebral (9, 21, 29, 31, 33), pulmonary (16,

31), coronary (14, 17, 18), mesenteric (42), or conduit level arteries (4, 20); only Faber and colleagues have studied the effect of acidosis on microvascular or resistance arteries in the skeletal muscle vasculature (30, 45). While these experiments were elegantly and carefully performed, the results from the cremaster muscle model used by Faber may not be applicable to locomotor muscles.

Recent work by Moore and colleagues (32) has provided evidence that challenges the classic dogma that proximal regulation of blood flow (e.g. feed artery) is controlled by $\alpha_1$-adrenergic receptors and distal regulation (e.g. terminal arterioles) is primarily controlled by $\alpha_2$-adrenergic receptors, such that only the $\alpha_2$- receptors should be suppressed by metabolic inhibition. In fact, Moore et al. (32) determined that adrenergic receptor function is rather heterogeneous in progression from larger arteries to the terminal arterioles. Thus, the effect of local factors (e.g. acidosis or heating) upon the potent $\alpha_1$-adrenergic receptors in human skeletal muscle feed arteries remains unknown.

Accordingly, the purpose of this dissertation will be to determine the effect of temperature on $\alpha_1$-adrenergic-mediated vasocontraction and the potential role for NO in mediating this temperature-induced sympatholysis. Additionally, we sought to determine the potential for local reductions in pH to reduce smooth muscle and $\alpha_1$-adrenergic mediated vasocontraction. The first study will determine, *in vitro*, if temperature exerts a sympatholytic effect on the $\alpha_1$-adrenergic receptor. The second study will determine, *in vitro*, the role of NO in mediating the sympatholytic effect of temperature on the $\alpha_1$-adrenergic receptor. The third study will determine, *in vitro*, the effect of local reductions in pH on $\alpha_1$-adrenergic mediated vasocontraction. Overall, these studies will provide insight into the role of local factors in functional sympatholysis.

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# HUMAN SKELETAL MUSCLE FEED ARTERIES STUDIED *IN VITRO:* THE EFFECT OF TEMPERATURE ON $\alpha_1$-ADRENERGIC RESPONSIVENESS

Reproduced with Permission from Ives, S.J., Andtbacka, R.H.I, Noyes, R.D., McDaniel J., Amann, M., Witman, M.A.H., Symons, J.D., Wray, D.W., & Richardson, R.S. (2011). Effect of temperature on alpha-adrenergic responsiveness in human arteries studied in vitro. *Experimental Physiology, 96*(9), 907-918.

## Abstract

Heat and cold exposure can decrease and increase total peripheral resistance, respectively, in humans. With unique access to human skeletal muscle feed arteries, we sought to both characterize these vessels and to determine the interaction between temperature and $\alpha_1$-adrenergic receptor responsiveness. We hypothesized that $\alpha_1$-mediated vasocontraction of human feed arteries would be attenuated in response to 39 or 35˚C. Skeletal muscle feed arteries were harvested from thirty-two human volunteers and studied using isometric techniques. Vessel function was assessed using potassium chloride (KCl), sodium nitroprusside (SNP), phenylephrine (PE), and acetylcholine (ACh) dose-response curves (DRCs) to characterize non-receptor- and receptor-mediated vasocontraction and vasorelaxation. Single doses of PE (1mM) and KCl (100mM) were administered at 37˚C and then, in a balanced design, repeated at both 35˚ and 39˚C. The KCl and PE DRCs elicited significant vasocontraction (2009±407; 1974±508 developed tension (mg), respectively), whereas SNP and ACh induced the expected vasorelaxation (102±6; 73±10 % relaxation, respectively). Altering temperature had no effect on inherent smooth muscle function (KCl response), but both a reduction (35˚C) and an increase (39˚C) in temperature decreased the vasocontractile response to 1mM PE (37˚C: 1478 ± 338, 35˚C: 546 ± 104, and 39˚C: 896 ± 202 mg; $p<0.05$), or across PE dose ($p<0.05$; 35 and 39˚C vs. 37˚C). Despite clear heterogeneity between both the human volunteers and the feed arteries themselves, this novel approach to the procurement of human vessels revealed a robust “inverted U” response to altered temperature, such that $\alpha_1$-mediated vasocontraction was attenuated with either warming or cooling.

## Introduction

In rodent skeletal muscle, it has been established that a primary control point for regulating *total* muscle blood flow during exercise is the feed artery (33, 39). Human skeletal muscle feed arteries, while relatively large compared to the equivalent vessels in rodents, are by human standards very small (1-2% of aortic diameter), and have a similar anatomical location. Therefore, human feed arteries also likely contribute significantly to blood flow regulation by varying vascular resistance prior to entry into the muscle bed. Although difficult to obtain, human skeletal muscle feed arteries can, in fact, be harvested during certain surgical procedures and studied *in vitro*. However, not only are the vasocontraction and vasorelaxation characteristics of human feed arteries not well characterized, the impact of temperature upon vasoconstrictor agents important in the regulation of muscle blood flow in response to stress, such as norepinephrine, is unknown.

The specific effect of temperature on vasoconstrictor responsiveness has been extensively studied *in vitro* in vessels from animals. However, potentially, at least in part, due to methodological differences, the results have been equivocal, with alterations in temperature leading to an increase (23, 28), a decrease (8, 17, 24, 26, 31, 37), or no change (16, 22, 34) in α-adrenergic mediated vasoconstriction. While there are no human *in vitro* studies with which to compare, prior *in vivo* animal and human studies on this topic reveal a general reduction in α-adrenergic vascular reactivity during local heating (40) or systemic increases in core temperature (2, 18, 25, 40). However, recent work performed by Keller et al. (15) suggested that adrenergic responsiveness is maintained during heat stress localized to the leg. Such *in vivo* measurements represent the

summation of the net vascular response and thus with such an approach it is currently not possible to differentiate the contribution of various levels of the arterial tree. An *in vitro* assessment of temperature and adrenergic responsiveness in human vessels recognized to regulate blood flow could provide greater clarity to this issue.

Thus, with the novel approach of harvesting human skeletal muscle feed arteries and an interest in $\alpha_1$-receptor responsiveness in humans, utilizing an isolated *in vitro* model, we sought to a) assess the vasocontraction and vasorelaxation characteristics of these human feed arteries and b) determine if changes in temperature exert an effect on the $\alpha_1$-adrenergic receptor responsiveness of these human feed arteries. Specifically, with implications for blood flow regulation in the face of both heating and cooling, we hypothesized that both an increase or a decrease in temperature from 37˚C would attenuate the vasocontractile response to the $\alpha_1$-agonist phenylephrine.

## Methods

*Subjects and General Procedures*

A heterogeneous group of 32 subjects (20 males and 12 females, 37-93 yrs old) agreed to have their vessels used in this study (Table 1). Although medical conditions and medications were noted, by means of medical records (Table 1), there were no exclusions based on this information. All subjects included in this study had not received chemotherapy, as this was a contraindication for surgery. All protocols were approved by the Institutional Review Boards of the University of Utah and the Salt Lake City VA Medical Center, and written informed consent was obtained by all subjects prior to vessel harvesting.

*Vessel Harvest*

Human skeletal muscle feed arteries from the axillary and inguinal regions were obtained during elective surgeries for melanoma at the Huntsman Cancer Hospital, University of Utah. Patients were anaesthetized using a standard protocol including propofol, fentanyl, benzodiazepines, and succinylcholine. After removal of sentinel lymph nodes, skeletal muscle feed arteries in the axillary (e.g. serratus anterior, or latissimus dorsi) or inguinal (e.g. hip adductors, or quadriceps femoris) regions were identified and classified as feed arteries based on entry into a muscle bed, structure, coloration, and pulsatile bleed pattern. The vessels were ligated, excised, and immediately placed in iced isotonic saline and brought to the laboratory within 15 minutes of harvesting. Vessels were dissected under a stereo microscope at room temperature in normal physiological saline solution (NPSS) (125 NaCl, 4.7 KCl, 1.2 $KH_2PO_4$, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 18 $NaHCO_3$, 0.026 $Na_2EDTA$, and 11.2 Glucose mM). All NPSS solutions and drugs were newly prepared on the morning of the experiment. Vessel internal diameter was measured using a calibrated micrometer eyepiece and reported in micrometers (μM). Perivascular adipose tissue was dissected from the feed arteries. NPSS was continuously aerated with carbogen gas (95% oxygen, 5% carbon dioxide), and pH was monitored at regular intervals and maintained at pH 7.35 – 7.45 by altering the amount of aeration (Orion 3 Star, Thermo Scientific, Waltham MA). Vessels were dissected into four rings measuring approximately 2 mm in length, and mounted in wire myography chambers (700 MO, DMT Systems, Aarhus, DK). Once mounted, vessel chambers were also aerated with the same carbogen gas mixture, and chamber NPSS was

exchanged at 10 minute intervals, except during cumulative drug dose responses. Vessel chambers were warmed to 37˚C over a 30 minute equilibration period prior to the protocol.

*Vessel Function Protocols*

The experimental timeline is illustrated in Figure 1. All vessel segments underwent length tension procedures at 37˚C to determine the length at which the vessels produced the greatest tension in response to a single dose of 100mM KCl ($L_{max}$) (35). $L_{max}$ was operationally defined as less than a 10% improvement in developed tension in response to 100mM KCl. The viability of all vessels was characterized using KCl (10-100mM), phenylephrine (PE; $10^{-9}$ – $10^{-3}$ M), and acetylcholine (ACh; $10^{-7}$ – $10^{-3}$ M) dose-response curves to determine non-receptor- and receptor-mediated vasocontraction and vasorelaxation at 37˚C. All vasorelaxation responses are expressed as percent relaxation (%) from PE precontraction. Temperature changes were achieved by changing myograph chamber and solution temperature utilizing multiple water baths set to the corresponding temperatures. Temperatures of 35, 37, and 39˚C were chosen as they can be achieved under physiologic conditions, either during cold exposure, normothermia, or during exercise (27, 32). All feed arteries started experimentation at 37˚C to confirm normal vessel function (via KCl, PE, and ACh dose-response curves) prior to further experimental procedures (Figure 1). Single doses of PE ($10^{-3}$ M) and KCl (100mM) were administered at 37˚C, and were then, in a balanced design, randomized to either 35 or 39˚C. All vessel segments were exposed to all temperatures, though to minimize the potential for an ordering effect, the temperature perturbations away from 37˚C were

balanced with an equal number of vessels starting with either 35 or 39˚C, and were then crossed over to the remaining temperature. Each experimental protocol was separated by 30 minutes. To determine endothelium-independent vasorelaxation, a sodium nitroprusside (SNP; $10^{-9}$ – $10^{-4}$ M) dose-response curve was performed in all vessels at 37˚C, and was conducted last in every case because of its known effect of abolishing vessel reactivity *in vitro*.

In addition to these main vessel function studies, three supplemental studies were performed. In the first, complete dose-response curves for PE were performed across each temperature in a longitudinal design, as in the main vessel function studies (Figure 1). In the second, complete dose-response curves for PE were again performed at 35˚, 37˚, and 39˚C, but this time in a cross-sectional design, where each vessel only experienced a single temperature. The developed tension (current tension – baseline tension) at each PE dose, for each condition, was expressed as a percent of the maximal tension developed at 37˚C. Finally, a third supplemental study examined stability of the preparation and the potential for tachyphylaxis with repeated contractions. Specifically, time control experiments were performed using repeated single doses of 1 mM PE and 100mM KCl, each separated by at least 30 minutes to replicate the timing employed in the main studies in which each experimental protocol was separated by 30 minutes. All the vasocontraction responses were expressed as percent of initial tension. For each of these supplemental studies, subject and vessel characteristics were also assessed.

All data were acquired at 4Hz using an analog-to-digital data acquisition system (Biopac Systems, Goleta, CA) to monitor vessel tensions and allow later offline analyses. In the majority of cases (including supplemental studies), the data represent the average

response of vessel rings in four chambers (average number of baths was 3.2 ±0.4 for each patient; CV across rings = 10-14%).

*Statistical Analyses*

Statistical analyses were performed using commercially available software (SPSS v. 17, Chicago, IL). A non-linear slope analysis and one-way repeated-measures Analysis of Variance (ANOVA) were performed on the PE dose-response data to determine the effect of temperature on PE-induced vasocontraction across doses. Two-way Repeated-Measures ANOVA were utilized to determine if an interaction existed between order (2 levels; 35 or 39˚C) and temperature (3 levels; 37, 39, 35˚C) on baseline tension and in the vasocontraction response to phenylephrine and KCl. One-way repeated-measures ANOVA were used to determine if time control data differed over time and if vasocontraction was different between 35 and 39˚C when expressed as a percent change from 37˚C. Significant differences were further analyzed using Tukeys' Least Significant Difference *post hoc* test to make pairwise comparisons. Intraclass correlation coefficient analyses were performed on the time control experiments to determine the stability of the preparation for both PE- and KCl-mediated vasocontraction. The level of significance was set to $p < 0.05$. All data are reported as mean +/- standard error (SE).

## Results

*Subject Characteristics*

The subject characteristics, attained by medical records for all studies (main and supplemental), are listed in Table 1. The subjects were varied in terms of demographics

(e.g. age), and tended to be overweight and with evidence of systolic hypertension, although it should be noted that these blood pressures were obtained during preoperative examination. Given the blood chemistry and complete blood count (CBC) results (Table 1), which were on average within normal range except for creatinine, which was slightly elevated, the subjects appeared otherwise to be relatively healthy.

*Vessel Characteristics*

Thirty-two human arteries were successfully harvested (19 inguinal, 13 axial). No statistical differences in $L_{max}$, vasocontraction or vasorelaxation responses were observed in terms of anatomical location (axial vs. inguinal) or gender (male vs. female). Consequently, responses from all vessels were combined. The average unpressurized internal diameter for these feed arteries was 500 ± 200 µM. Baseline tensions prior to KCl and PE contractile function curves were 105 ± 29 mg and 106 ± 33 mg, respectively. Vessel function protocols revealed robust vasocontraction (peak KCl 2009 ± 407 mg and peak PE 1974 ± 508 mg) (Figure 2, panel A and B). Vessels were precontracted to 1460 ± 330 mg of tension prior to ACh and SNP dose-response curves, corresponding to approximately 70% of the maximal PE response, and from this point feed artery segments achieved significant vasorelaxation (max SNP 102 ± 6 and max ACh 73 ± 10 % relaxation) (Figure 2, panel C and D). Taken together, these results indicate the feed arteries had functional smooth muscle, $\alpha_1$-adrenergic receptors, and an intact endothelium. Interestingly, there was no relationship between $\alpha_1$-adrenergic responsiveness, KCl-induced contraction, or the ratio of these two variables and age.

*Temperature and $\alpha_1$-mediated Vasocontraction*

In the main protocol, baseline tensions were lower with a temperature of 35˚C (111± 42 mg) or 39˚C (85± 39 mg) as compared to 37˚C (158 ± 42 mg) ($p < 0.05$). There was a decreased vasocontractile response to a single dose of PE as a consequence of altering temperature from 37˚C (1478 ± 338 mg), to either 35˚C (546 ± 104 mg) or 39˚C (896 ± 202 mg) ($p < 0.05$ vs. 37˚C) (Figure 3, panel B). Cooling tended to attenuate vasocontraction more so than heating; however, this trend did not achieve statistical difference (PE-induced vasocontraction at 35 vs. 39˚C; $p = 0.07$). However, when expressed as percent change from 37˚C, the vasocontraction at 35˚C was significantly more attenuated than that at 39˚C ($p < 0.05$; 53.5 ± 7.1 vs. 20.6 ± 12.3 %, respectively). The supplemental studies also indicated that altering temperature prior to a phenylephrine dose-response curve significantly reduced $\alpha_1$-mediated vasocontraction at both 35 and 39˚C (Figure 4; $p < 0.05$ 35 and 39˚C vs. 37˚C) utilizing either a longitudinal (panel A) or cross-sectional (panel B) study design. Additionally, standard curve analysis revealed a reduced Hillslope at 35 and 39˚C, as compared to 37˚C, indicating a reduced $\alpha_1$-receptor sensitivity with altered temperature.

*Smooth Muscle Function*

In the main protocol, baseline tensions prior to KCl were lower with a temperature of 35˚C (127 ± 46mg) or 39˚C (107 ± 40mg), as compared to 37˚C (207± 35mg) ($p < 0.05$). The effects of temperature were not due to altered inherent smooth muscle function, as measured by KCl-mediated vasocontraction, evidenced by a lack of effect ($p = 0.96$) of changing temperature from 37˚C (Figure 3, panel A). Additionally,

the temperature effects did not appear to be the result of the passage of time, as the time control experiments revealed significant intraclass correlation coefficients for repeated single doses of KCl (0.964, $p < 0.001$) and PE (0.873, $p < 0.01$) over time (Figure 5), and responses were not different across time ($p > 0.10$). These time intervals were similar to those employed in the protocols that did reveal differences in PE-induced tension at different temperatures. Furthermore, all vessels achieved complete relaxation in response to SNP at the end of the experiment, providing additional evidence of intact inherent smooth muscle function after exposure to different temperatures. Taken together, these data provide evidence in support of the stability of the preparation, and indicate that the effect of temperature on $\alpha_1$-mediated vasoconstriction is not the result of altered inherent smooth muscle function.

## Discussion

The successful harvesting of human skeletal muscle feed arteries, their characterization, and subsequent use in an *in vitro* isolated vessel study documents the usefulness of this approach to better understand human vascular control. Beyond this novel approach, the main finding of this study was that a change in temperature from the homeostatic set point (37˚C) reduced $\alpha_1$-mediated vasocontraction in human skeletal muscle feed arteries. Of importance, this observation was determined to not be a consequence of altered inherent smooth muscle function. Thus, in combination, these data reveal an “inverted U” response, such that $\alpha_1$-receptor responsiveness in human skeletal muscle feed arteries is temperature dependent. The potential implications of this finding are discussed.

*Hyperthermia and Inherent Smooth Muscle Function*

Elevated temperature may alter inherent smooth muscle function, as some *in vitro* findings have also revealed an altered depolarization-induced contraction (23, 28), although not all agree (38). This clearly has the potential to confound findings that suggest heating results in altered receptor-mediated vasoconstriction. Although the current study did reveal an altered baseline tension, indicating an altered basal smooth muscle tone, this change was diminutive, particularly in light of such large reductions in $\alpha_1$-mediated vasocontraction, and stable KCl-mediated vasocontraction across temperature. In addition, the seminal works of Vanhoutte and Shepard (38) concluded that temperature does not exert its effect through reduced inherent smooth muscle contractility, which is in accordance with the results of the current study (Figure 3, panel A).

*Effects of Hyperthermia on Vasoconstrictor Agents*

Hyperthermia has previously been demonstrated to attenuate adrenergic mediated vasoconstriction *in vivo* in humans (2, 40) as well as in animal studies utilizing *in vivo* or *in vitro* approaches (18, 22-25, 30). However, not all agree, with recent evidence from Keller and colleagues (15) indicating that adrenergic responsiveness may be preserved during isolated heat stress in humans *in vivo*. Indeed, Keller et al. (2010) concluded that passive leg heating increases muscle blood flow and that, despite an apparent intact adrenergic sensitivity, whole limb vascular conductance remained significantly elevated with local hyperthermia in the face of clear sympathomimeticly-induced vasoconstriction in the femoral artery. In contrast, *in vitro*, utilizing the isolated rat femoral artery, Kluess

et al. (2005) found that heating had no effect on PE-induced vasocontraction, but reduced purinergic-mediated vasocontraction (16). As already indicated, Keller et al. (15) found no such effect, indicating, not surprisingly, that the femoral is not involved in the generation of resistance *in vivo*. In this context, the negative findings of Kluess and colleagues, again studying the femoral, but this time in rats, are also not surprising (16). In fact, the present study appears to be the first to indicate that local heating can reduce $\alpha_1$-adrenergic responsiveness in human feed arteries, an important site of blood flow regulation (33, 39). As the skin, although likely effected by differing mechanisms, has been extensively studied, it is interesting to note that the current data from feed arteries respond in a similar fashion to cutaneous vessels with respect to heating (40).

While not directly assessed in the current study, the effect of elevated temperature on agonist-receptor interaction may, in fact, be non-selective (17, 24), although not all agree (16). Indeed, Massett and colleagues (24) found, *in vitro*, in rat vessels that increasing temperature not only reduced norepinephrine mediated vasocontraction, but also the vasocontractile response to endothelin-1, and angiotensin II. Therefore, it is likely that heating results in an attenuation of receptor-mediated vasoconstriction that may be indiscriminant of receptor type. It appears that attenuated temperature-dependent receptor-mediated vasoconstriction, as demonstrated here in human feed arteries with phenylephrine (Figures 3 and 4), may serve as an autoregulatory mechanism that increases perfusion to metabolically active skeletal muscle.

*Temperature, $\alpha_1$-mediated Responsiveness, and Functional Sympatholysis*

The precise factor or combination of factors that contribute to functional sympatholysis has yet to be identified in humans. Indeed, during exercise, many variables likely contribute to functional sympatholysis (e.g. K+, nitric oxide, ATP, $H^+$, etc.); however, a local elevation in temperature is also a potential candidate (3, 16) which has not been thoroughly examined, especially not in human feed arteries. The increase in skeletal muscle metabolism associated with exercise releases copious amounts of heat as a by-product. Indeed, as much as 65% of the energy conversion in the metabolic pathways may be lost as heat (20), elevating intramuscular temperature to ~39˚C during moderate intensity exercise (21, 32). Mechanistically, increased temperature may contribute to functional sympatholysis through the direct or indirect attenuation of $\alpha_1$-adrenergic vasoconstriction, decreasing vascular resistance and enhancing blood flow to the active skeletal muscle (3, 16).

*Effect of Hypothermia on Vascular Responsiveness*

Previous *in vitro* animal studies have revealed that cooling alters receptor-mediated vasocontraction in both cutaneous veins and arteries in skeletal muscle (11, 13). Faber (1988) found that cooling appeared to exert a direct effect on basal vasomotor tone such that, upon cooling, vessels exhibited a transient vasorelaxation, revealing a suppressant effect of cooling on vascular tone. In terms of α-adrenergic stimulation, it appears that cooling selectively attenuates $\alpha_1$-mediated vasocontraction, whereas $\alpha_2$-induced vasocontraction remains intact or increased perhaps to compensate for reduced $\alpha_1$ responsiveness (11, 13). More recently, another elegant series of experiments

performed by Flavahan and colleagues (1, 4-6, 9, 10) has provided convincing evidence, in cells and cutaneous arteries, that cold exposure induces translocation of previously quiescent $\alpha_2$c-adrenergic receptors, in part mediated by reactive oxygen species, towards the cell surface which increases vasoconstriction to selective $\alpha_2$c-adrenergic agonists. However, this effect was not apparent with $\alpha_1$-adrenergic agonists (4), as the response to PE was unaffected by cold exposure which contrasts somewhat with the current finding of reduced $\alpha_1$-adrenergic responsiveness with cooling. Although *in vivo* cold exposure is well known to stimulate a reduction in cutaneous blood flow to maintain core temperature, very little is known regarding the specific effect of a fall in temperature on skeletal muscle blood flow.

Teleologically, and in a not too dissimilar scenario to voluntary exercise and heating, it is possible that cold-mediated sympatholysis plays a role in supporting the metabolic demand associated with shivering thermogenesis during cold stress (7). Indeed, cold exposure in humans has been documented to increase cutaneous vascular resistance, which resulted in a stroke volume-induced increase in cardiac output and prevented any decrement in MAP, despite a significantly reduced total peripheral resistance (29). While blood flow to the thermogenically active skeletal muscle was not measured in this study, the current data suggest that during cold exposure, blood flow to deep skeletal muscle may increase to support shivering thermogenesis, ultimately reducing resistance in the skeletal muscle vasculature. However, this observation may only be evident in arteries feeding deep skeletal muscle, such as those feed arteries used in the current study, and is likely only to be of significance under prolonged and severe cooling conditions that cannot be overcome by shivering thermogenesis, but these hypotheses need to be

confirmed *in vivo*. Potential explanations for the observed reduction in $\alpha_1$-adrenergic responsiveness in the current study (Figures 3 and 4) include reduced membrane permeability, decreased diffusional capacity, or a conformational change in the adrenergic receptor as a consequence of cooling (12, 19). Reduced adrenergic responsiveness could be a mechanism by which cold-induced vasodilation occurs (14) and may augment pathological hypotension during hypothermia (36).

*Origin of the Vessel*

The subjects who took part in this study were certainly heterogeneous in terms of age, gender, and health, but, although exhibiting a tendency to be overweight and some evidence of systolic hypertension (although obtained during preoperative examination), they were taking minimal medications and had normal blood chemistry and CBC data (Table 1). However, it should also be recognized that these subjects were undergoing prophylactic surgical treatment for melanoma and vessels were harvested during sentinel node dissection, although most lymph nodes were found to be negative for melanoma metastasis via Polymerase Chain Reaction analysis. In addition, it is of note the lactate dehydrogenase values (considered to be a crude indicator of metastasis), were within the normal range for all subjects and subjects were not undergoing any chemotherapy.

Despite rather varied origin, the novel approach of harvesting these human arteries during such surgeries yielded the expected receptor-mediated and non-receptor-mediated vasocontraction and vasorelaxation characteristics (Figure 2), suggestive of normal physiology. With our historical interest in limb specific vascular differences (41) and each harvest site (axial and inguinal) being associated with either the upper or lower

extremities, it was anticipated that vessel characteristics may vary by limb proximity. However, although the magnitude of within-site structural heterogeneity was of interest, this variation was too great to reveal any limb-specific information. Therefore, despite a group of heterogeneous subjects, varied vessel harvest location (i.e. axial and inguinal) and potential pathology, the notion that temperature exhibited a common effect speaks to the robust nature of this response as it relates to the regulation of sympathetically mediated vasoconstriction. Specifically, independent of age, gender, or disease status, altering the temperature from the homeostatic set-point significantly attenuates $\alpha_1$-mediated vasocontraction.

*Limitations*

While the novel approach of harvesting human skeletal muscle feed arteries affords a unique experimental paradigm, it was not achieved without limitation. The human feed arteries used in the current study were inarguably heterogeneous (i.e. harvest location, vessel size, age, gender, medications, health, etc.) and this could be considered a limitation of this study. However, a repeated measures design was employed specifically to reduce the impact of interindividual variation. Additionally, although relatively small, some tachyphylaxis was observed in the time control studies (Figure 5), and thus may have contributed to the reduction in PE-induced tension development observed during measurements at subsequent temperatures. However, in supplemental studies, when PE dose responses at the different temperatures were performed in a cross-sectional design, to avoid any time/tachyphylaxis effects, essentially the same temperature-dependent results were observed (Figure 4, panel B). Additionally, it is also acknowledged that in

these supplemental and time control studies tension development was, on average, lower than the main protocol. This was attributed to differences in vessel caliber and arterial ring length and an unavoidable consequence of limited human feed artery availability. However, despite these differences, it is important to note that the functional characteristics of the vessels included in these supplemental studies were not different from those utilized in the main protocol. Finally, a reduction in baseline tension over time was also observed, which may have altered the contractile response to PE. However, it should be noted that KCl-induced tension remained stable across temperature, and the magnitude of the baseline shift (~70 mg tension) is highly unlikely to account for the dramatic reduction in tension (~500 mg tension) observed during the temperature perturbations.

*Conclusion*

Altering temperature, by either cooling or heating, reduces the contractile response of human skeletal muscle feed arteries to the $\alpha_1$-adrenergic receptor agonist phenylephrine *in vitro.* Importantly, these findings were independent of alterations in inherent smooth muscle function, as measured by KCl-mediated vasocontraction and have implications for human blood flow regulation in both hyperthermic and hypothermic conditions.

*Acknowledgements*

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Table 1. *Characteristics of the subject population (n = 32).*

| | Mean ± SE | Normal Range |
|---|---|---|
| Age (yr) | 59.00 ± 4.2 | -- |
| Height (cm) | 174.00 ± 3.5 | -- |
| Weight (kg) | 89.50 ± 6.1 | -- |
| Systolic Blood Pressure | 135.60 ± 6.4 † | -- |
| Diastolic Blood Pressure | 75.10 ± 2.4 † | -- |
| Glucose (mg/dl) | 109.10 ± 9.3 | 65 – 110 |
| Blood Urea Nitrogen (mg/dl) | 16.39 ± 1.8 | 6 – 21 |
| Creatinine (mg/dl) | 1.03 ± 0.1 | 0.52 – 0.99* |
| Albumin (g/dl) | 4.17 ± 0.3 | 3.3 – 4.8 |
| Bilirubin (mg/dl) | 0.37 ± 0.2 | 0.2 – 1.3 |
| Lactate Dehydrogenase (U/L) | 494.45 ± 36.3 | 313 – 618 |
| Hemoglobin (g/dl) | 14.40 ± 0.7 | 12 – 16 |
| WBC (K/uL) | 8.12 ± 0.9 | 3.6 – 10.6 |
| RBC (M/uL) | 4.61 ± 0.2 | 4 – 5.2 |
| Platelets (K/uL) | 238.22 ± 20.1 | 150 – 400 |
| Hematocrit (%) | 41.90 ± 1.9 | 36 – 46 |
| Lymphocyte (%) | 30.30 ± 3.9 | 24 – 44 |
| Monocyte (%) | 7.97 ± 1.2 | 0 – 12 |
| Neutrophil (%) | 59.75 ± 3.0 | 36 – 66 |
| Eosinophil (%) | 2.07 ± 0.4 | 0 – 5 |
| Basophil (%) | 0.56 ± 0.1 | 0 – 5 |
| Medications (users/n) | | |
| Cardiovascular | | |
| Diuretic | 2/32 | |
| $Ca^{++}$ Channel Blocker | 2/32 | |
| Statin | 1/32 | |
| ACE inhibitor | 2/32 | |
| Beta Blocker | 1/32 | |
| Thyroid | | |
| Levothyroxine | 1/32 | |
| Other | | |
| Insulin | 1/32 | |
| Antibiotic | 1/32 | |

***** Average outside of normal range

† Data obtained during pre-operative examination

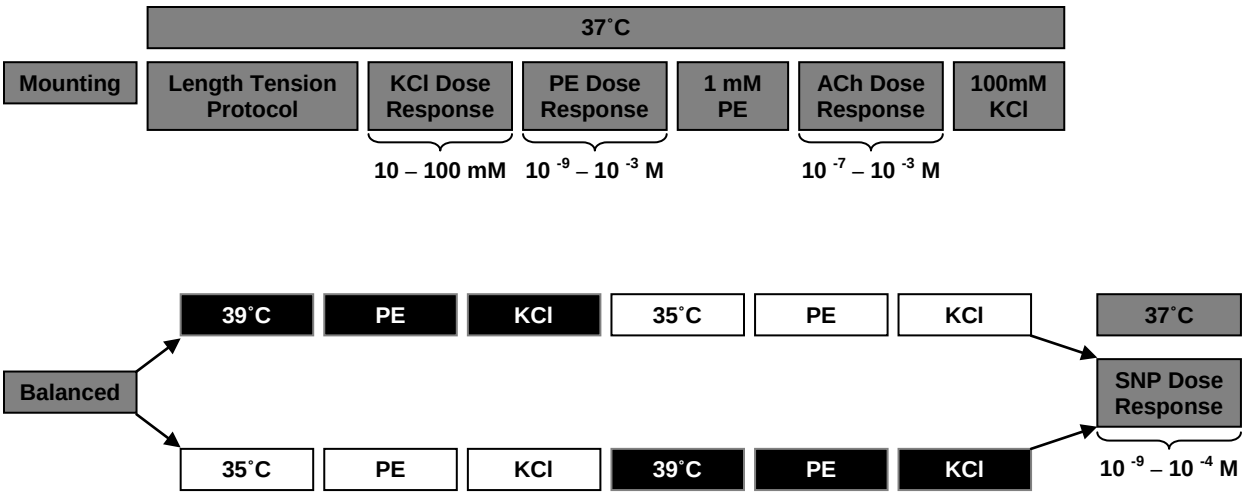


*Figure 1*. **Experimental Timeline**. Potassium Chloride (KCl), Phenylephrine (PE), Acetylcholine (ACh) & Sodium Nitroprusside (SNP) concentrations in millimolar (mM) or log molar ($10^{-x}$ M).

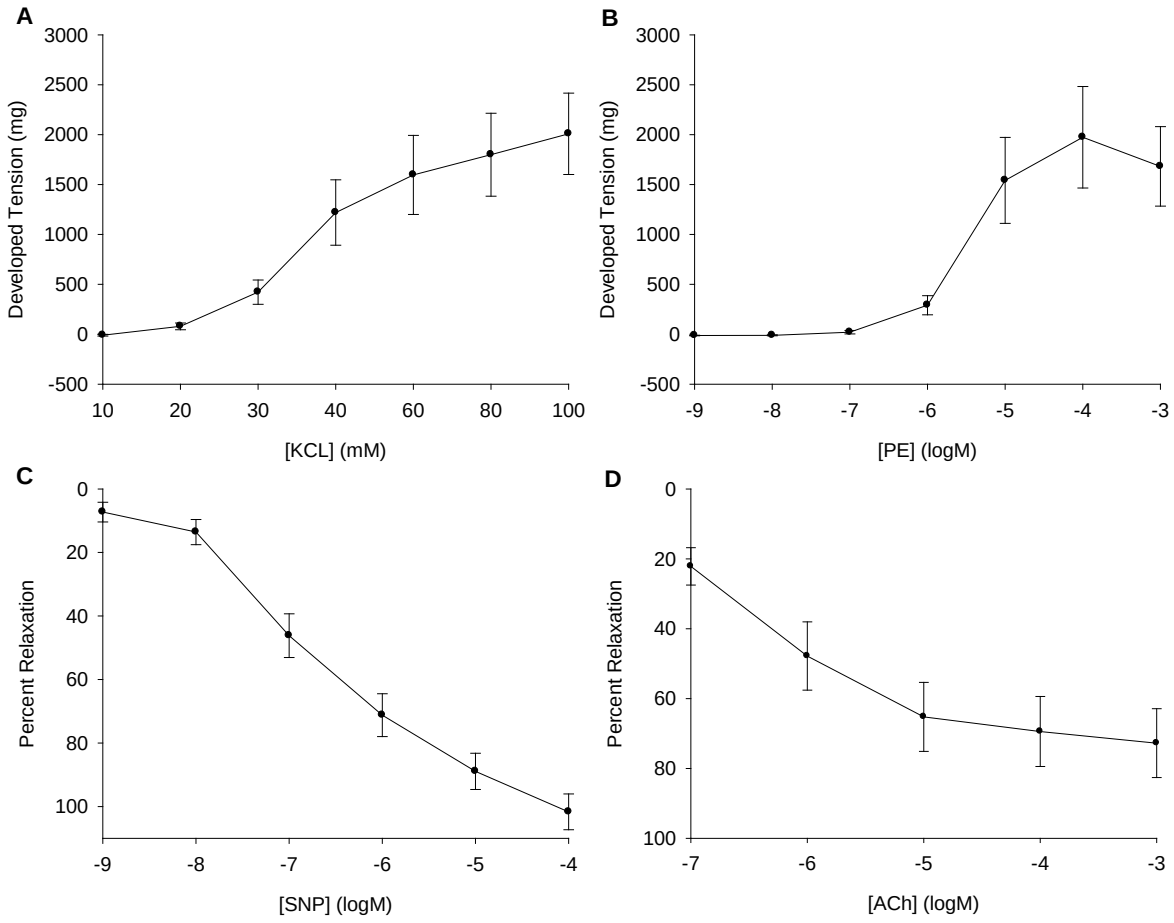


*Figure 2.* **Human skeletal muscle feed artery functional characteristics assessed at normothermia (37˚C)**. A) Non-receptor-mediated vasocontraction dose-response for KCl. B) Phenylephrine (PE) dose-response for $\alpha_1$-adrenergic mediated vasocontraction. C) Sodium Nitroprusside (SNP) dose-response (% relaxation from PE precontraction). D) Acetylcholine (ACh) dose response (% relaxation from PE precontraction). Data are presented as mean +/-SE (n=32).

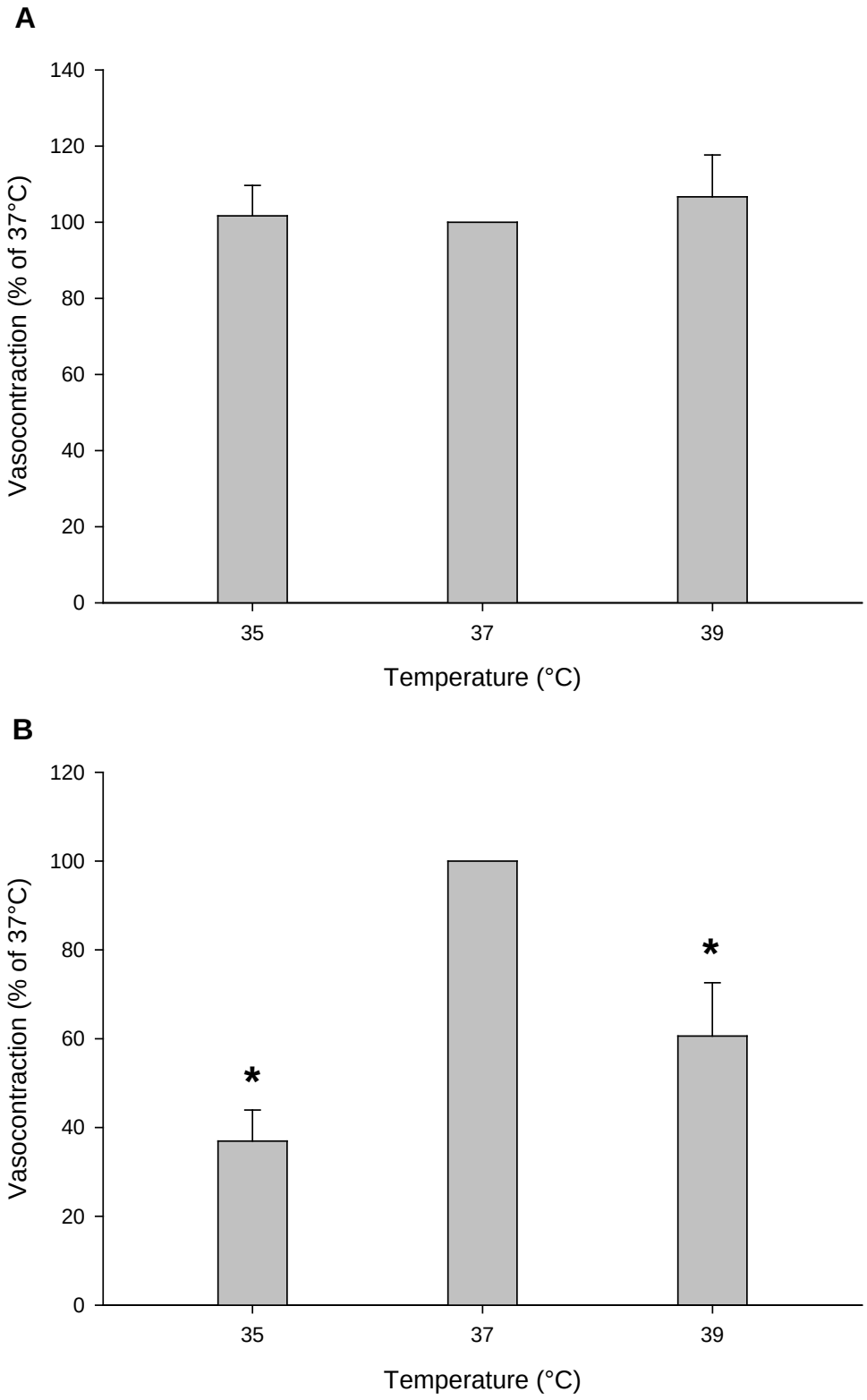


*Figure 3.* **The effect of temperature (35˚, 37˚, and 39˚ C) on the contractile response to (A) KCl and (B) a single dose of phenylephrine (PE) (1 mM) in human skeletal muscle feed arteries**. * $p < .05$ vs. 37˚C. Data are presented as mean +/-SE (n = 14).

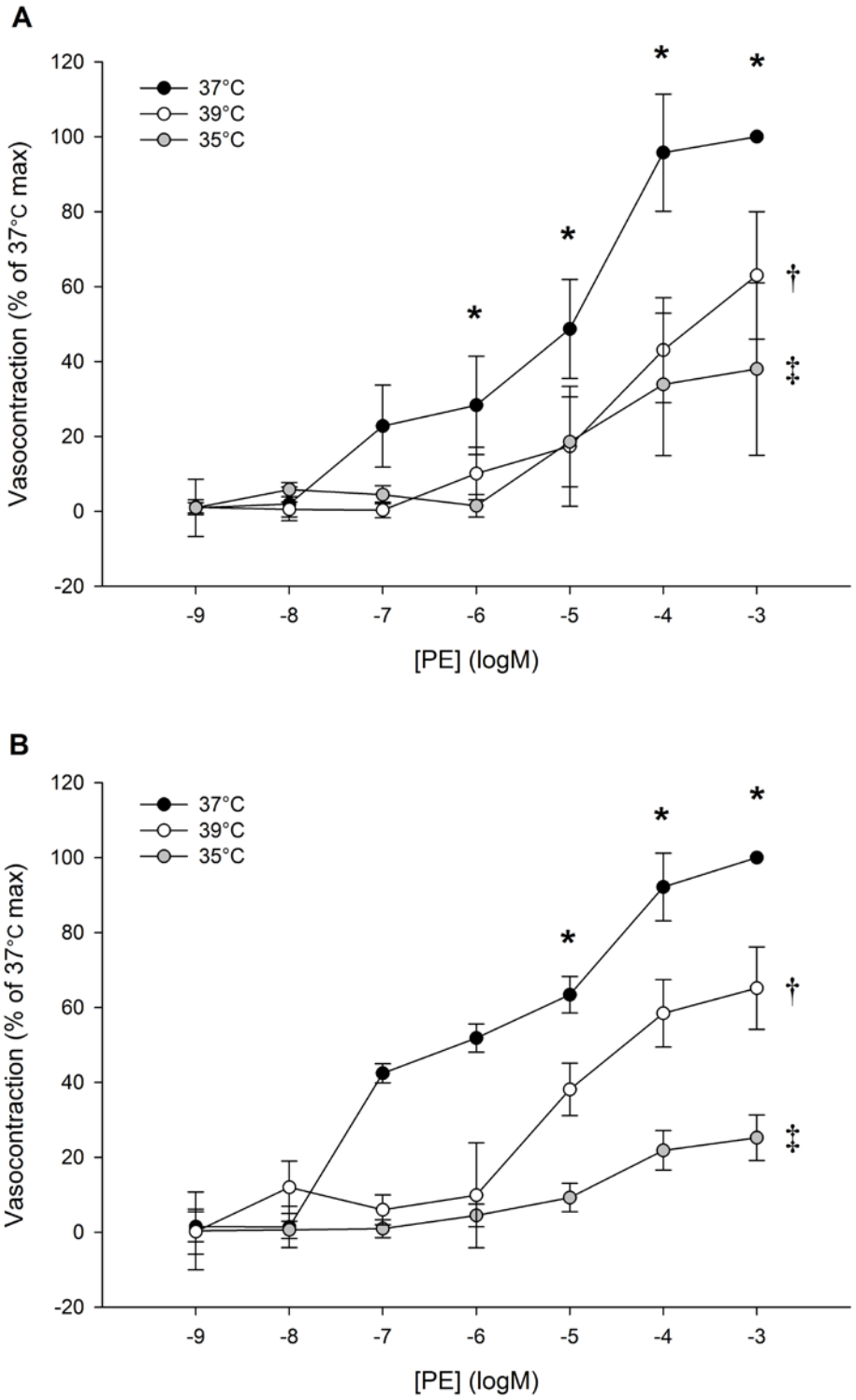


*Figure 4.* **Phenylephrine Dose-Response Curves at 35˚, 37˚, and 39˚C in human skeletal muscle feed arteries assessed in both a longitudinal (A) and cross sectional design (B).** (Temperature effect: $^{\dagger}p < 0.05$, 39 vs. 37˚C; $^{\ddagger}p < 0.05$, 35 vs. 37˚C; * dose effect: $p < 0.05$ vs. baseline). Note: These data represent an additional 6 feed arteries not represented in figures 3, or 5. Data are presented as mean +/-SE (n = 6 per panel).

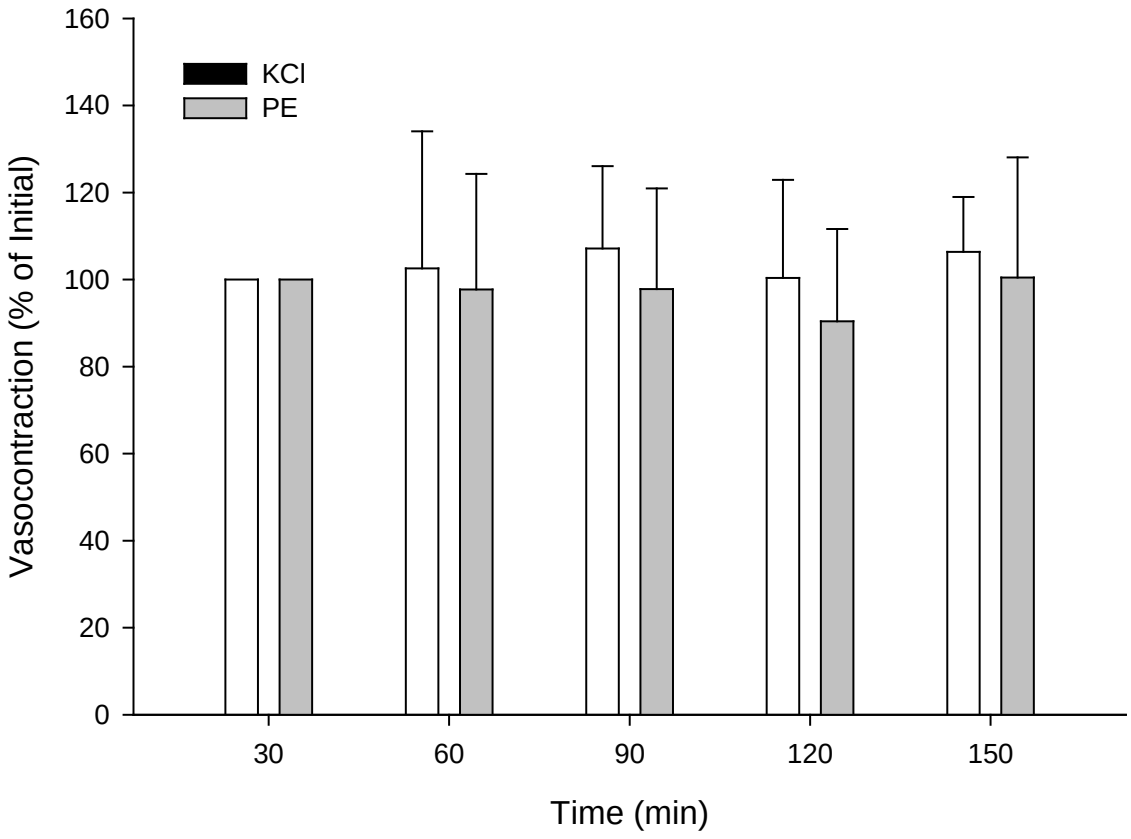


*Figure 5.* **Time control experiments for KCl and PE mediated vasocontraction expressed as developed tension (mg) at 37˚C in human skeletal muscle feed arteries**. Note: These data represent an additional 6 feed arteries not represented in figures 3, or 4. Data are presented as mean +/-SE (n = 6).

# ALPHA ADRENERGIC SENSITIVITY AND TEMPERATURE IN HUMAN SKELETAL MUSCLE FEED ARTERIES: THE ROLE OF NITRIC OXIDE

## Abstract

Increases in local temperature have been found to exert a sympatholytic effect on the vasculature. We hypothesized that this attenuated $\alpha_1$-receptor sensitivity may actually be due to a temperature-induced increase in nitric oxide (NO) bioavailability, reducing the impact of the $\alpha_1$-adrenergic receptor agonist phenylephrine (PE). Thirteen human skeletal muscle feed arteries, obtained during surgeries, were dissected into 2mm rings and mounted on a wire myograph. All vessels underwent a stepwise length tension procedure to determine $L_{max}$ ($\leq$10% increase in developed tension (mg$\Delta$) to 100mM Potassium Chloride; [KCl]). Vessel function was characterized using KCl, sodium nitroprusside (SNP), PE, and acetylcholine (ACh) dose-response curves (DRCs) to determine nonreceptor- and receptor-mediated vasocontraction and vasorelaxation. PE DRCs were performed at 37 and 39˚C, both with and without the endothelial nitric oxide synthase (eNOS) inhibitor L-$N^G$-monomethyl-arginine (L-NMMA). A subset of arteries was exposed to 37 or 39˚C, and the protein content of eNOS and $\alpha_1$-adrenergic receptors analyzed by western blot. Maximal PE-induced vasocontraction ($PE_{max}$) at 39˚C was lower than at 37˚C (39±10 vs. 84±30 $\%KCl_{max}$). NO blockade restored vasocontraction at 39˚C to that achieved at 37˚C (80±26 $\%KCl_{max}$). Western blot analyses revealed increased eNOS protein, but no such change in $\alpha_1$-receptors. These findings reveal that NO blockade can restore the attenuated $\alpha_1$-mediated vasocontraction at 39˚C to that achieved at 37˚C, implicating NO-bioavailability as an important component of functional sympatholysis. Additionally, the study highlights the important role that vasodilators can play in the vascular response to vasoconstrictors.

## Introduction

Increases in temperature have previously been documented to significantly reduce $\alpha_1$ -mediated vasoconstriction (4, 15, 20, 24, 28, 31, 46). Unfortunately, it is unclear from these studies whether these thermal effects are due to changes in receptor-agonist affinity, an effect on postreceptor signal integration, or altered inherent smooth muscle function. Our group (14, 15) has recently demonstrated that, in the face of reduced $\alpha_1$-receptor responsiveness, inherent smooth muscle function in human feed arteries, as assessed by the response to potassium chloride (KCl), was unchanged with heating to 39˚C. Therefore, in human feed arteries, known to be important in the distribution of blood flow (48), it does appear that heat exerts an effect specific to receptor-mediated vasoconstriction, but it cannot be ruled out that this could also be the result of antagonistic vasodilation.

The pluripotent molecule nitric oxide (NO) exerts a significant vasodilatory effect, and is normally produced during exercise through shear stress stimulation of the endothelial NO synthase enzyme (eNOS) or catalyzed by neuronal nitric oxide synthase (nNOS) on the sarcolemma (2, 33, 41, 44). NO may directly reduce the impact of sympathetic nerves innervating smooth muscle (36) or relax the smooth muscle itself by stimulating guanyl cyclase, providing opposition to vasoconstriction (3, 5, 11, 29). As it appears that sympathetic discharge remains intact during exercise (27) and warming (6), a reduction in postjunctional receptor responsiveness to sympathetic activity is most likely responsible for the attenuated vasoconstriction under such conditions (8, 13, 24). Therefore, increased bioavailability of NO is a plausible mechanism by which heat mediates its effect, though relatively little attention has been paid to this issue, let alone

in humans.

Previous work by Kregel and colleagues investigated the role of NO during heating using an *in vivo* sinoaortic deafferented rodent model (19). This work revealed that eNOS inhibition using L-NAME significantly attenuated the reduction in hind limb resistance observed during heating, implicating NO as a factor responsible for heat-induced vasodilation. Using an *in vitro* approach, Harris et al. (2003) demonstrated that prior heat shock (42˚C) in rat aortas resulted in a significantly reduced vasocontractile response to PE, which was effectively restored with NOS blockade (12). Finally, in cell culture, heat exposure increased eNOS mRNA, protein expression, and activity, implicating heat as a stimulus for NO which could produce an antagonistic effect on receptor-mediated contraction (12). However, currently lacking, translational work is needed to determine if the aforementioned observations remain true in human arteries that were innervated and capable of producing vascular resistance (48).

Consequently, performed *in vitro*, in the absence of endogenous sympathetic influence, and other metabolites, the goal of this study was to determine if NO inhibition would restore the heat-induced reduction in $\alpha_1$-mediated vasocontraction in human skeletal muscle feed arteries, thus implicating NO as an important modulator of heat-induced vasocontraction. Additionally, using molecular approaches, we sought to determine if an upregulation in eNOS, or a downregulation of $\alpha_1$-receptors contribute to the attenuation of sympathomimetic-induced vasocontraction during heating. We hypothesized that: 1) increased temperature will attenuate $\alpha_1$-mediated vasocontraction, and effective blockade of eNOS will partially restore vasocontraction, and 2) increased temperature will also increase eNOS and/or reduce $\alpha_1$-receptor protein expression.

## Methods

*Subjects and General Procedures*

Thirteen heterogeneous subjects (5 males, 8 females, 49 ±18yrs, 36-85yrs) agreed to the use of their vessels in this study (Table 2). Although medical conditions and medications were noted, there were no exclusion criteria based on this information. All protocols were approved by the Institutional Review Boards of the University of Utah and the Salt Lake City VA Medical Center, and written informed consent was obtained by all subjects prior to vessel harvesting.

*Vessel Harvest and Preparation*

Feed arteries supplying muscles in the axillary and inguinal regions were obtained during elective surgeries for melanoma at the Huntsman Cancer Hospital, University of Utah. Patients were anaesthetized using a standard protocol including: propofol, fentanyl, benzodiazepines, and succinylcholine. During the process of accessing sentinel lymph nodes, skeletal muscle feed arteries in the axillary (e.g. serratus anterior, or latissimus dorsi) or inguinal (e.g. hip adductors, or quadriceps femoris) regions were identified and classified as feed arteries based on entry to a muscle, structure, coloration, and pulsatile bleeding pattern. These feed arteries typically emanated from the thoracodorsal artery or the medial circumflex branch of the femoral artery, in the axial and inguinal regions, respectively. The vessels were ligated, excised, and immediately placed in iced isotonic saline and brought to the laboratory within 15 minutes of harvesting. The feed arteries were dissected (perivascular adipose tissue, etc.) and cut into rings under a stereo microscope at room temperature in normal physiological saline solution (NPSS) (125 NaCl, 4.7 KCl, 1.2 $KH_2PO_4$, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 18

$NaHCO_3$, 0.026 $Na_2EDTA$, and 11.2 Glucose mM). The NPSS was continuously aerated with carbogen gas (95% oxygen, 5% carbon dioxide), and pH monitored at regular intervals and maintained at pH 7.35 – 7.45 (Orion 3 Star, Thermo Scientific, Waltham MA). Arteries were dissected into four 2 mm rings, and mounted in wire myography chambers (700 MO, DMT Systems, Aarhus, DK). Once mounted, vessel chambers continued to be aerated with the same carbogen gas mixture, and chamber NPSS was exchanged at 10-minute intervals, except during cumulative drug dose-responses when the NPSS exchange was lengthened according to the protocol. Vessel length and diameter measurements were obtained using a calibrated micrometer eyepiece. Prior to vessel diameter measurements, the mounting pins were adjusted to 10 mg of tension to ensure the pins were touching the vessel wall. Diameters were assessed using the outer portions of the pins which were in contact with the walls of the vessel. Diameters and length are reported in micrometers (μM). Vessel chambers were gradually brought up to 37˚C over a 30-minute equilibration period prior to the protocol.

*Vessel Function Protocols*

All vessels underwent length tension procedures to determine the length at which the vessels produced the greatest tension in response to a single dose of 100mM KCl ($L_{max}$) (39, 47). $L_{max}$ was operationally defined as less than a 10% improvement in developed tension in response to 100mM KCl. Vessel function was characterized using KCl (10-100mM), sodium nitroprusside (SNP; $10^{-9}$ – $10^{-4}$ M), phenylephrine (PE; $10^{-9}$ – $10^{-3}$ M), and acetylcholine (ACh; $10^{-7}$ – $10^{-3}$ M) dose-response curves to determine nonreceptor- and receptor-mediated vasocontraction and vasorelaxation. All contractile

responses were normalized as a percent of the individual ring maximal response to 100 mM KCl at 37˚C (%$KCl_{max}$), to normalize the adrenergic mediated vasocontraction to the maximal functional capacity of the arterial smooth muscle for each segment (38). All vasodilation responses are expressed as percent relaxation towards basal tone from a PE-induced precontraction, approximating 60-70% of the maximal response to PE.

To determine the role of NO in mediating the attenuation of PE-induced vasocontraction with heating, two of four myograph chambers were increased to 39˚C, to one of which the nitric oxide synthase inhibitor L-Nitro$^{G}$-monomethyl-arginine (L-NMMA, $10^{-3}$ M) was added prior to the phenylephrine dose-response series. The other two chambers remained at 37˚C to serve as controls, and again L-NMMA was added to one of these chambers. Effective NOS inhibition was confirmed by a lack of response to an acetylcholine challenge to the eNOS enzyme, with intact sodium nitroprusside relaxation after the challenge.

Each experimental protocol was separated by 30 minutes. Vessel tension data were acquired at 4Hz using an analog to digital data acquisition system (Biopac Systems, Goleta, CA) allowing real-time monitoring and offline analyses. In the majority of cases, the data represent the average response of vessel rings in four baths.

*Western Blot Analysis*

To further elucidate the role of eNOS, as a component of the mechanism by which heating alters vasocontraction, we performed an additional set of experiments examining protein content changes with heating. Specifically, in a subset of vessels with tissue remaining after dissection for wire myography, two vessel rings were placed in 2

mL cryovials containing pH balanced (~7.4), and well oxygenated NPSS. One vessel ring was exposed to 37 and the other to 39˚C for a period of 90 minutes, to determine if heating itself would alter eNOS or $\alpha_1$-adrenergic receptor protein expression. Vessels were then snap-frozen in liquid nitrogen for later analysis. The pH of the NPSS was measured again to confirm proper pH during the heat exposure, and was found to be unchanged.

Following the differing temperature exposures, the feed arteries were placed in tubes containing 200 μL of ice-cold homogenization buffer [(0.05M HEPES, 0.01M sodium pyrophosphate, 0.01M sodium fluoride, 0.002M EDTA, 0.002M sodium orthovanodate, 1% Triton X-100, 10% Glycerol, and 1:100 Sigma protease inhibitor cocktail (Sigma, St. Louis, MO)]. Samples were homogenized and centrifuged for 15 minutes at 13,800 g at 4°C. Supernatant then was collected and total protein concentrations determined [bicinchoninic acid (BCA) method] using a Synergy 4 Microplate Reader (Biotek, Winooski, VT) with bovine serum albumin (BSA) as a standard. Supernatants were stored at -80°C. Equal amounts of protein were suspended in Laemmli loading buffer (Bio-Rad, Hercules, CA), incubated for 5 minutes at 95-100°C, and then resolved in a SDS-polyacrylamide gel (SDS-PAGE). Resolved proteins were transferred to a polyvinylidene difluoride (PDVF) membrane (Millipore Immobilon-P Transfer membrane; Millipore Corporation, Billerica, MA) at 4°C. Following transfer, the membranes were blocked with 5% nonfat dry milk in phosphate buffered saline with 0.1% Tween-20 (PBST) for 1 hour at room temperature. Blocked membranes were probed with primary antibodies for eNOS, $\alpha_1$-adrenergic receptor, and β-actin (BD Transduction Laboratories, Franklin Lakes, NJ). The membranes were then washed with

TBST, incubated with the appropriate secondary antibody conjugated to horseradish peroxidase [Anti-rabbit IgG; Anti-mouse IgG (Amersham Biosciences, Piscataway, NJ], and visualized via enhanced chemiluminescence (ECL) (Pierce detection kit, Pierce Chemical Company, Rockford, IL) using a digital imaging system (BioRad Chemidoc XRS Imager, Bio-Rad Laboratories, Hercules, CA). Membranes were stained with Coomassie blue (BioRad Laboratories, Hercules, CA) for a loading control. Protein expression values at 39˚C were normalized as a percent of the 37˚C control.

*Statistical Analyses*

Statistical analyses were performed using commercially available software (SPSS v. 16, Chicago, IL). A repeated measures analysis of variance (ANOVA) was utilized to determine if there were differences in developed tension responses to cumulative doses of phenylephrine between conditions (37˚C, 39˚C, 37˚C+ L-NMMA or 39˚C + L-NMMA). Due to potential differences in kinetics, the individual $PE_{max}$ data across conditions were analyzed using a one-way ANOVA. Significant differences were followed up using Tukeys' Honestly Significant Difference test to make pair wise comparisons. The Wilcoxon signed ranks test was used to determine the effect of heating on eNOS and $\alpha_1$-adrenergic receptor protein expression. Significance was established to be $p < 0.05$. All data are reported as mean ± standard error (SE).

## Results

### *Subject Characteristics*

Subject characteristics are listed in Table 2. The subjects were varied in terms of demographics (e.g. age), but, as a whole, should be considered relatively healthy given the blood pressure and blood analysis results (Table 2), as the group average was within normal range for each assessment.

### *Vessel Characteristics*

The average internal diameter for these arteries was 888 ± 75 μM, and the average length was 2184 ± 141 μM. Baseline tensions prior to KCl and PE contractile function curves were 105 ± 23 mg and 104 ± 20 mg, respectively. Vessel function protocols revealed robust vasocontraction (peak KCl 1794 ± 279 mg and peak PE 1080 ± 190 mg). Vessels were precontracted using PE to 706 ± 261 mg of tension prior to ACh & SNP dose-response curves, corresponding to approximately 65% of the maximal PE response, and achieved significant vasorelaxation ($SNP_{max}$ 105 ± 7, & $ACh_{max}$ 68 ± 7 % relaxation). Taken together, these results indicate functional smooth muscle, the presence of $\alpha_1$-adrenergic receptors, and an intact endothelium (Figure 6).

### *Nitric Oxide, Temperature, and Vessel Function*

Basal tone was found to be statistically different between conditions, with heating resulting in an increased basal tone ($p < 0.05$), which was significantly augmented by eNOS blockade using L-NMMA in both normothermic and heated conditions (Figure 7). However, it is important to note that these changes represent approximately only 1.3 to

3.3 %$KCl_{max}$. There was a significant ($p < 0.05$) difference between conditions with regards to PE-induced vasocontraction at 37 and 39˚C, with, and without L-NMMA (Figure 8A). *Post hoc* pairwise comparisons indicated that heating significantly attenuated the response to phenylephrine at the last dose ($10^{-3}$ logM) of PE (39˚C, 37.28 ± 10 %$KCl_{max}$; $p< 0.05$); however, blockade of eNOS at 39˚C evoked a vasocontractile response similar to that of 37˚C (79.02 ± 27 vs. 82.77 ± 30 %$KCl_{max}$, respectively; Figure 8A). Blockade of eNOS also increased the response to PE at 37˚C (102.69± 30 %$KCl_{max}$; Figure 8A); however, the effect of the block was not statistically significant ($p > 0.05$). Additionally, $\alpha_1$-adrenergic sensitivity was reduced with heating as the log $EC_{50}$ was significantly reduced at 39˚C ($p < 0.05$), which was no longer different from 37˚C with eNOS blockade (Table 3).

The maximal PE responses were lower ($p < 0.01$) at 39˚C (38.66 ± 10 %$KCl_{max}$) as compared to 37˚C (84.45 ± 30 %$KCl_{max}$); however, NOS blockade restored vasocontraction at 39˚C to equal that of 37˚C (80.29 ± 27 %$KCl_{max}$) (Figure 8B). Blockade of eNOS also enhanced PE-induced vasocontraction at 37˚C (107± 31 %$KCl_{max}$), but not to the same extent as the heated condition (Figure 8B). When the maximal response to PE was expressed as percent increase from eNOS intact (basal) to eNOS blocked, the heated condition revealed a greater elevation in $\alpha_1$-mediated vasocontraction ($p < 0.05$), indicating an elevated basal nitric oxide tone with heating.

*Western Blot Analysis*

The western blot analyses revealed no significant difference in $\alpha_1$-adrenergic receptor protein expression across temperatures (Figure 9A; $p > 0.05$). However, for

vessel rings incubated at 39˚C, eNOS protein was significantly up-regulated compared to 37˚C (Figure 9B; $p < 0.05$).

## Discussion

The main finding of this study was that heating attenuated the vascular sensitivity to $\alpha_1$-adrenergic stimulation in human skeletal muscle feed arteries and this effect was reversed by NO blockade. At the molecular level, heating induced a significant increase in arterial eNOS protein expression, but no change in $\alpha_1$-adrenergic receptor protein expression. Thus, in an *in vitro* model devoid of sympathetic nerve activity and other skeletal muscle metabolites, heat-induced reductions in $\alpha_1$-mediated vasocontraction are, at least in part, due to an increase in NO bioavailability. Thus, the current data indicate that hyperemic stimuli, such as heat, may not actually induce sympatholysis, in the traditional sense, but at least in part, may be the result of increased vasodilatory stimuli (e.g. NO) and not reduced vasoconstriction *per se*.

### *Heat and Vascular Reactivity*

Previous studies have demonstrated a reduction in mean arterial pressure, and/or reduced vasoconstrictor responsiveness during passive heating *in vivo* (8, 19, 20, 24, 30, 32, 37), despite potentially significant increases in sympathetic nervous system activity (7, 18), and intact baroreflex sensitivity (8, 25). A common explanation for these results is a reduction in postjunctional receptor responsiveness. Using an *in vitro* approach, we (15) and others have documented a sympatholytic effect of heating on sympathetically mediated vasocontraction (4, 12, 28, 45, 46). Thus, the sympatholytic effect of heating

has been suggested to be specific to receptor-mediated vasocontraction, as previous studies have indicated that direct depolarization of the smooth muscle was not, in fact, altered by heating (15, 20). In contrast, in the current study, we observed an increase in basal tone with heating (Figure 7) providing some evidence of enhanced basal smooth muscle function. However, the magnitude of this effect was very small, compared to the effect on vasocontraction observed during the PE dose-response (<1% of the difference in PE-induced developed tension between conditions). Thus, at rest, under passive tension, heating does result in a general increase in smooth muscle activity which can enhance basal tone (23). However, in contrast to a previous study (23), this augmentation of basal smooth muscle activity does not carry over to active tension development using an $\alpha_1$-receptor agonist or KCl (15), an observation that is in agreement with the seminal studies of Vanhoutte and colleagues (45, 46). Specifically, Vanhoutte and Shephard (46) concluded that the effects of temperature on venocontraction induced by agonists or direct electrical stimulation were not due to a direct thermal effect on the "contractile machinery" itself. Therefore, the suppressant effect of heating to 39˚C on $\alpha_1$-adrenergic receptor-mediated vasocontraction observed in the present study (Figure 8) does not appear to be the result of smooth muscle dysfunction.

*Temperature and Nitric Oxide*

The NOS enzyme has been determined to be a major promoter of exercise hyperemia, in part, by counteracting sympathetic nervous system activity (1, 9, 33-35, 40-42). The dependency of enzymes upon temperature is a well described phenomenon, thus it is not surprising that NOS activity may also be potentiated by heat (12, 19).

Further, eNOS, itself, has previously been demonstrated to be temperature-dependent, with heating increasing NO and inducing vasodilation that opposes vasoconstriction (12, 16, 17, 19), qualitatively the same as the results of the current study (Figure 8). However, not all studies have demonstrated an NO-dependent vasodilation and reduction in vasoconstrictor response with heating (22, 32) or during exercise (10). However, the complex processes and redundant mechanisms within the *in vivo* model, coupled with methodological considerations, may have confounded previous results which have left the impact of NO on vasoconstriction, during heat stress, somewhat equivocal.

A comprehensive study by Harris et al. (12) investigated the role of heat shock on the regulation of eNOS in a rodent model. These researchers found that heating not only increased heat shock protein (HSP) expression, but also increased the eNOS protein signal (mRNA), protein expression, eNOS activation/phosphorylation, and eNOS functional activity. Therefore, it is highly likely that the increased vasodilator function associated with heating reduces the functional consequence of vasoconstrictors. Indeed, Harris et al. (12) found that heat stress reduced the maximal response to phenylephrine, an effect which was reversed with eNOS blockade. In agreement with this prior work (12), the current study revealed that heating increased eNOS protein (Figure 9), in whole artery homogenate, and documented greater eNOS activity with heating, as evidenced by the greater effect of L-NMMA at 39˚C compared to 37˚C, indicating heightened basal NO bioavailability with heat (Figure 8).

*Sympatholysis as a "Tug of War"*

Historically, functional sympatholysis has been viewed under an analytical lens that infers, as the name implies, a "lysing" of sympathetic activity. Indeed, researchers have focused on the concept of reduced vasoconstrictor responsiveness, rather than a change in smooth muscle signal integration. However, the current data emphasize the potential for increased vasodilatory capacity, rather than reduced vasoconstrictor potency as a *bone fide* mechanism responsible for reduced $\alpha_1$-mediated vasoconstriction with heat, resulting in the concept of a "tug of war" between vasodilators and vasoconstrictors. This vasodilatory component of the process is likely postjunctional, as muscle sympathetic nerve activity (MSNA) and norepinephrine spillover remain intact during exercise (21, 26). Specific to heat, in a rodent model, increased MSNA did not prevent a fall in hindlimb resistance during passive heating (19), but blockade of the eNOS enzyme significantly attenuated this vasodilation, implicating NO, and more accurately heat-induced changes in NO, in the observed sympatholysis. In support of this concept, Shibasaki et al. (37) demonstrated that NO attenuated norepinephrine-induced cutaneous vasoconstriction *in vivo* in humans. Moreover, various studies in animals have documented a NO-dependent abatement of sympathetic vasoconstriction (41, 43). Thus, based upon such prior work and the current data, it appears that a component of functional sympatholysis is likely an increased abundance of vasodilators, such as NO, rather than simply an insufficiency of vasoconstrictors, which 'tip the balance' in favor of vasodilation and subsequent hyperemia in the tug of war of vasodilators and vasoconstrictors during exercise.

*Limitations*

The subjects who took part in this study were certainly heterogeneous in terms of age, gender, and health (Table 2). Additionally, vessels were harvested in proximity to sentinel node regions in individuals receiving prophylactic surgical treatment for melanoma. However, it should be noted that most lymph nodes of these patients were found to be negative for melanoma metastasis via Polymerase Chain Reaction analysis. It should also be noted that the novel approach of harvesting these human arteries during axial and inguinal surgeries yielded the expected receptor-mediated and nonreceptor-mediated vasocontraction and vasorelaxation characteristics (Figure 6), suggestive of somewhat uniform and normal physiology, despite potential pathology and rather varied origin. Therefore, despite both a group of heterogeneous subjects and potential pathology, the fact that temperature and eNOS blockade exhibited a common effect across subjects speaks to the robust nature of this response as it relates to the regulation of sympathetically mediated vasocontraction. Specifically, independent of age, gender, or disease status, heating significantly attenuates $\alpha_1$-mediated vasocontraction and this phenomenon can be overcome by reducing NO bioavailability by eNOS blockade.

*Conclusion*

This study provides evidence that heat increases NO bioavailability and opposes $\alpha_1$-mediated vasocontraction in human skeletal muscle feed arteries. Thus, with the capacity of feed arteries to regulate skeletal muscle blood flow, heat produced, as a consequence of muscle contraction, has the potential to stimulate eNOS, overriding $\alpha_1$-mediated vasoconstriction and thereby increasing skeletal muscle blood flow.

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Table 2. *Characteristics of the subject population (n =13).*

| | Mean +/- SE | Normal Range |
|---|---|---|
| Age (yr) | 49 ± 17.6 (36-85) | -- |
| Height (cm) | 172 ± 13.4 | -- |
| Weight (kg) | 83 ± 19.5 | -- |
| Systolic Blood Pressure | 133 ± 17.2 † | -- |
| Diastolic Blood Pressure | 75 ± 7.6 † | -- |
| Glucose (mg/dl) | 98 ± 19.7 | 65 – 100 |
| Blood Urea Nitrogen (mg/dl) | 16.4 ± 5.0 | 6 – 21 |
| Creatinine (mg/dl) | 0.92 ± 0.2 | 0.52 – 0.99 |
| Albumin (g/dl) | 4.25 ± 0.3 | 3.3 – 4.8 |
| Bilirubin (mg/dl) | 0.42 ± 0.2 | 0.2 – 1.3 |
| Lactate Dehydrogenase (U/L) | 440 ± 86.9 | 313 – 618 |
| Hemoglobin (g/dl) | 14.20 ± 1.8 | 12 – 16 |
| White Blood Cells (K/uL) | 6.78 ± 2.4 | 3.6 – 10.6 |
| Red Blood Cells (M/uL) | 4.58 ± 0.6 | 4 – 5.2 |
| Platelets (K/uL) | 239 ± 52.5 | 150 – 400 |
| Hematocrit (%) | 41.3 ± 5.0 | 36 – 46 |
| Lymphocyte (%) | 28 ± 6.2 | 24 – 44 |
| Monocyte (%) | 7.7 ± 2.2 | 0 – 12 |
| Neutrophil (%) | 62.8 ± 6.2 | 36 – 66 |
| Eosinophil (%) | 1.72 ± 0.8 | 0 – 5 |
| Basophil (%) | 0.54 ± 0.2 | 0 – 5 |
| Medications (users/n) | | |
| Cardiovascular | | |
| Diuretic | 2/13 | |
| $Ca^{++}$ Channel Blocker | 1/13 | |
| Statin | 2/13 | |
| ACE inhibitor | 1/13 | |
| Beta Blocker | 1/13 | |
| Thyroid | | |
| Synthroid | 1/13 | |
| Other | | |
| Insulin | 1/13 | |
| Metformin | 1/13 | |

† Data obtained during pre-operative examination

Table 3. *Phenylephrine dose response characteristics at 37˚ and 39˚C and in the presence and absence of the eNOS inhibitor L-$N^G$-monomethyl-arginine (L-NMMA).*

| Condition | Max | Hillslope | $EC_{50}$ | Log $EC_{50}$ |
|---|---|---|---|---|
| 37˚C | 84.45±29 | 1.05±0.03 | -4.19±0.02 | $-6.49^{-5}$ |
| 37˚C + L-NMMA | 107.00±31 | 1.53±0.21 | -4.25±0.07 | $-5.61^{-5}$ |
| 39˚C | 38.66±10* | 1.96±0.11 | -4.50±0.07 | $-3.14^{-5}$ * |
| 39˚C + L-NMMA | 80.29±27 | 1.32±0.07 | -4.31±0.04 | $-4.90^{-5}$ |

* $p < 0.05$ vs. 37˚C

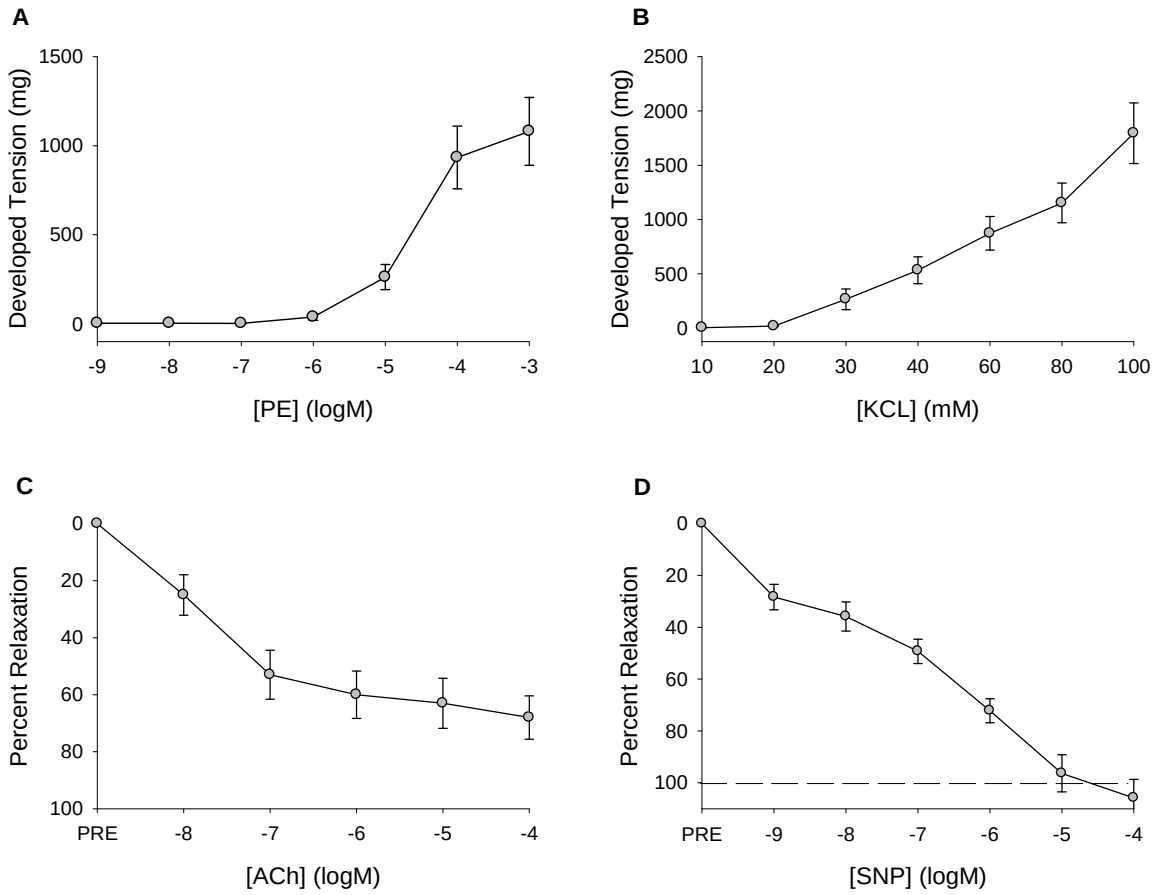


*Figure 6.* Vessel function characteristics. A) Phenylephrine (PE) dose response. B) Potassium Chloride (KCl) dose response. C) Acetylcholine (ACh) dose response (% relaxation from PE precontraction). D) Sodium Nitroprusside (SNP) dose response (% relaxation from PE precontraction) (n=13).

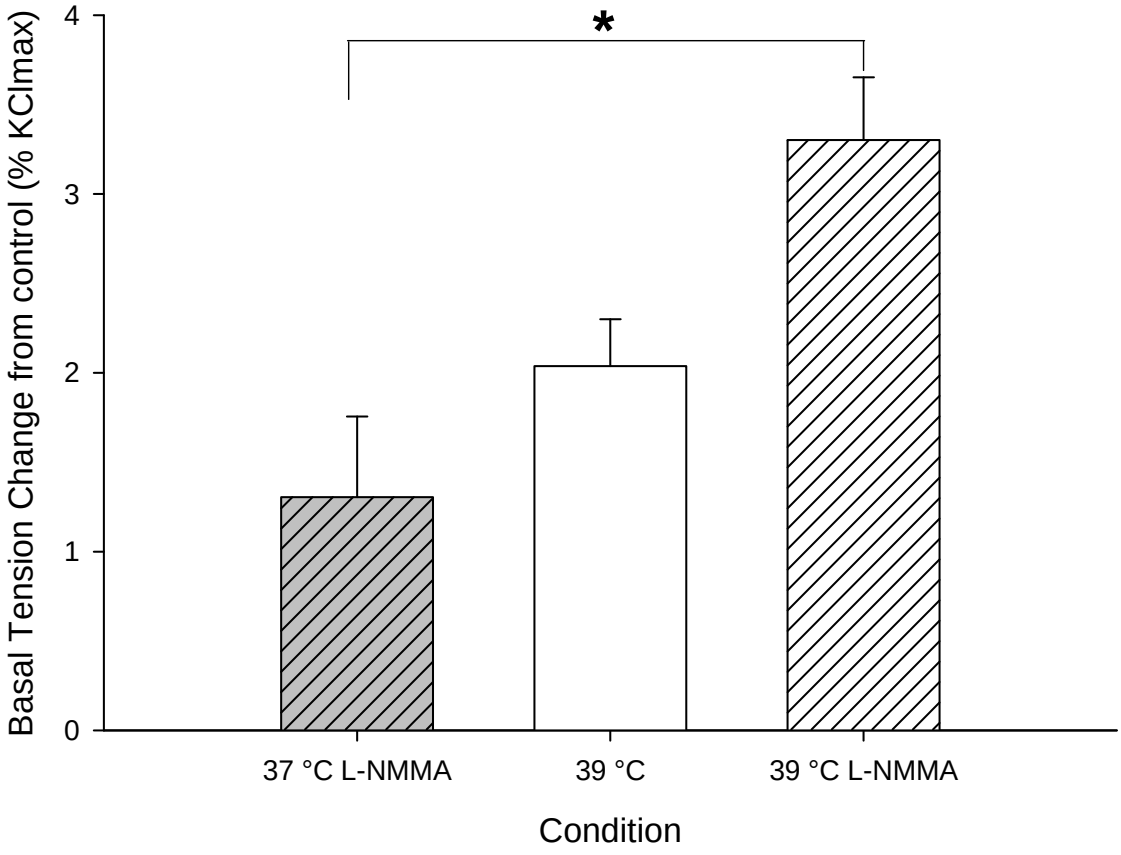


*Figure 7*. Effect of eNOS blockade and temperature on basal tension.

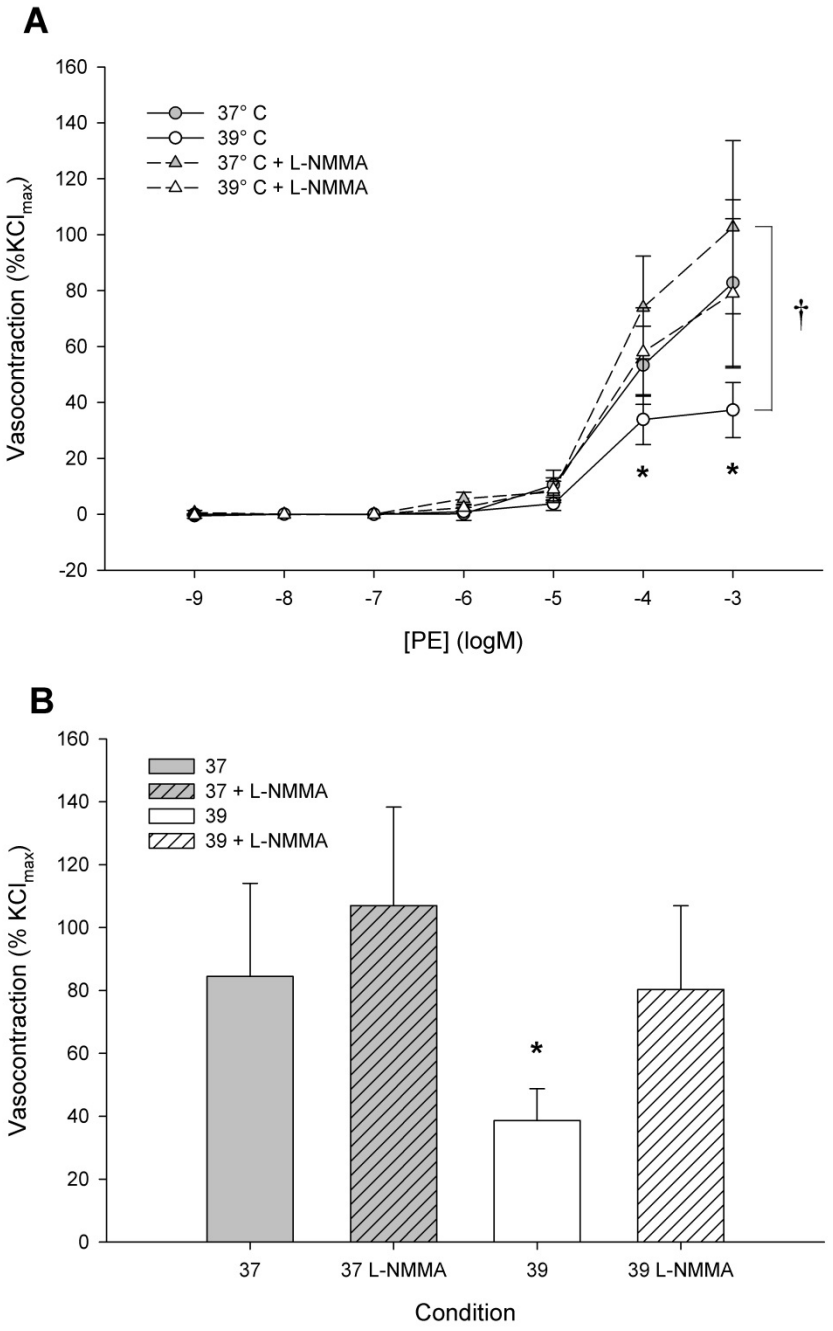


*Figure 8.* A) Phenylephrine dose responses at 37 and 39˚C in the presence of the eNOS inhibitor L-N$^{G}$-monomethyl-arginine (L-NMMA), vasocontraction expressed as a percent of $KCl_{max}$ (n=13). * Dose effect $p < 0.01$ vs. baseline, † Condition effect $p < 0.01$. B) Maximal Phenylephrine induced vasoconstrictor responses at 37, and 39˚C ± L-NMMA, expressed as a percent of $KCl_{max}$. * Condition effect, $p < 0.05$ (n=13).

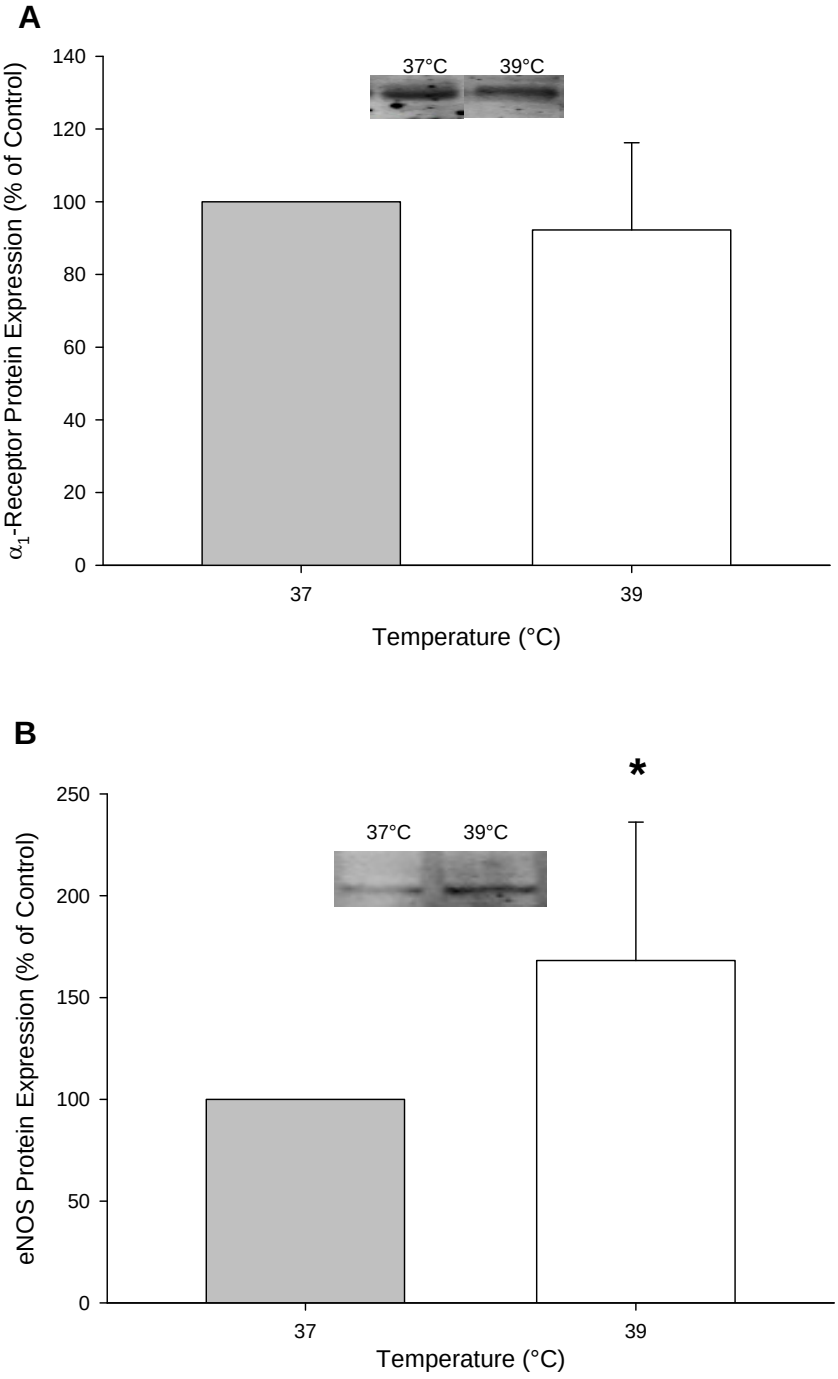


*Figure 9.* Western Blot Analyses for $\alpha_1$-adrenergic receptors (Panel A); and eNOS (Panel B), in an additional set of vessels where half of each vessel was exposed to 37 or 39˚C for 90 minutes. * $p < 0.05$ 37 vs. 39 (n = 4).

# ALPHA$_1$-ADRENERGIC RECEPTOR SENSITIVITY IN HUMAN SKELETAL MUSCLE FEED ARTERIES: THE IMPACT OF REDUCTIONS IN EXTRACELLULAR PH

## Abstract

Graded exercise results not only in the modulation of adrenergic-mediated smooth muscle tone and a preferential increase in blood flow to the active skeletal muscle termed "functional sympatholysis," but is also paralleled by metabolically induced reductions in pH. Therefore, we sought to determine if pH attenuates $\alpha_1$-adrenergic receptor sensitivity in human feed arteries. Feed arteries (560±31 μM ID) were harvested from 22 humans (24-86 yrs, 10 males, 12 females) and studied using the isometric tension technique. Vessel function was assessed using potassium chloride (KCl), phenylephrine (PE), acetylcholine (ACh), and sodium nitroprusside (SNP) dose-response curves (DRCs) to characterize nonreceptor- and receptor-mediated vasocontraction as well as endothelium-dependent and independent vasorelaxation, respectively. PE, KCl, ACh, and SNP DRCs were conducted with a pH of: 7.4, 7.1, 6.8, and 6.5. Increasing acidification reduced maximal PE-induced vasocontraction (pH 7.4 = 85 ± 19; 7.1= 57 ± 16; 6.8 = 34 ± 15; 6.5 = 16 ± 5 $\%KCl_{max}$), which was partially due to reduced smooth muscle function, as assessed by KCl, (pH 7.4 = 88 ± 13; 7.1= 67 ± 8; 6.8= 67 ± 9; 6.5= 58 ± 8 $\%KCl_{max}$). Graded acidosis had no effect on maximal vasorelaxation. In summary, these data reveal that reductions in extracellular pH attenuate $\alpha_1$-mediated vasocontraction, which is partially explained by reduced smooth muscle function, although vasorelaxation to ACh and SNP remained intact. These findings support the concept that local acidosis could be contributing to functional sympatholysis and exercise hyperemia by opposing sympathetically-mediated vasoconstriction and by not impacting vasodilation.

## Introduction

In 1880, Gaskell originally proposed that acidosis could exert a suppressant effect on vasculature tone (10), and this hypothesis has since been confirmed by other researchers who found, *in vivo*, that hypercapnic acidosis was capable of producing significant hyperemia (7, 8, 20-22). Interestingly, exercise of sufficient intensity results in acidosis within the active muscle bed (32, 38) and is also correlated with profound hyperemia (3). In parallel, exercise is associated with a reduction in vascular responsiveness to both endogenous sympathetic nerve activity (31), or exogenous sympathomimetics (48), termed "functional sympatholysis" (31).

In a series of *in vitro* studies, Faber and others (25, 37, 40) sought to determine if the acidosis-induced hyperemia previously observed *in vivo* was the result of altered vascular reactivity to sympathetic neurotransmitters, as in functional sympatholysis during exercise. In the *in vitro* rodent model, it was determined that arteriolar $\alpha_2$-adrenergic function was disrupted with acidosis, leaving $\alpha_1$-receptor function intact (18, 25, 27, 40, 41). However, other studies that have used a similar approach to determine if acidosis could attenuate agonist-induced vasoconstriction are equivocal, revealing an increase (33, 34), decrease (14, 25, 27, 30, 34, 37), or no change (18, 25, 27, 40) in the maximal response or sensitivity to an agonist (e.g. norepinephrine or PE). Additional studies have determined that the disparate effect of pH upon vasocontraction may depend upon species (27), rodent strain (34), vascular location and caliber (12-15, 24), and experimental model (6, 33, 36).

With the knowledge that species and vascular location/caliber alter the response to acidosis, the specific effect of acidosis on human arteries remains uncertain. An *in*

*vitro* study by Rohra and colleagues (36) using human internal mammary artery found that acidosis did, indeed, attenuate the response to a single dose of PE and KCL. The attenuated vasocontraction was likely the result of altered vasoconstrictor function; although not addressed in that study, enhanced vasodilator function could be contributing to the reduced vasocontraction (36). Indeed, extracellular acidosis has been demonstrated to induce hyperpolarization, via ion channels, which results in a reduction in vascular wall tension and likely opposes subsequent vasocontraction (5, 14, 24, 30, 33-36). However, it has yet to be tested if agonist-induced vasorelaxation, and thus the balance between vasocontraction and vasorelaxation, are altered during acidosis.

In rodent skeletal muscle it has been established that a primary control point for regulating *total* muscle blood flow during exercise is the feed artery (43-47). Human skeletal muscle feed arteries, while relatively large compared to the equivalent vessels in rodents, are by human standards very small (1-2% of aortic diameter) and have a similar anatomical location. Therefore, human feed arteries also likely contribute significantly to blood flow regulation by varying vascular resistance prior to entry into the muscle bed. Although difficult to obtain, human skeletal muscle feed arteries can, in fact, be harvested during certain surgical procedures and studied *in vitro* (17). It remains unknown if acidosis, a consequence of exercise, plays a significant role in modulating vasocontraction of human skeletal muscle feed arteries, considered to be a point of blood flow regulation.

Accordingly, with the novel approach of harvesting human skeletal muscle feed arteries, the purpose of this study was to determine the effect of pH on vascular reactivity in these arteries. Specifically, we sought to determine the effect of acidosis on $\alpha_1$-

adrenergic receptor responsiveness and the role of smooth muscle and endothelial function in mediating this process. We tested 3 hypotheses: Reductions in the pH of the medium surrounding human skeletal muscle feed arteries will 1) attenuate $\alpha_1$-adrenergic receptor responsiveness, 2) attenuate inherent smooth muscle function, and 3) enhance endothelium dependent vasodilation. If confirmed, such findings would implicate acidosis as a contributor to the local regulation of skeletal muscle blood flow by the feed artery and offer insight into the mechanisms responsible for functional sympatholysis during exercise.

## Methods

*Subjects and General Procedures*

A heterogeneous group of 22 subjects (10 males and 12 females, 54 ± 4 yrs, range 24-86 yrs) agreed to have their vessels used in this study (Table 1). Although medical conditions and medications were noted, there were no exclusions based on this information. All protocols were approved by the Institutional Review Boards of the University of Utah and the Salt Lake City VA Medical Center, and written informed consent was obtained from all subjects prior to vessel harvesting.

*Vessel Harvest*

Human skeletal muscle feed arteries from the axillary and inguinal regions were obtained during elective surgeries for melanoma at the Huntsman Cancer Hospital, University of Utah. Patients were anaesthetized using a standard protocol including: propofol, fentanyl, benzodiazepines, and succinylcholine. After removal of sentinel

lymph nodes, skeletal muscle feed arteries in the axillary (e.g. serratus anterior, or latissimus dorsi) or inguinal (e.g. hip adductors, or quadriceps femoris) regions were identified and classified as feed arteries based on entry into a muscle bed, structure, coloration, and pulsatile bleed pattern. The vessels were ligated, excised, and immediately placed in iced isotonic saline and brought to the laboratory within 15 minutes of harvesting. Vessels were dissected under a stereo microscope at room temperature in normal physiological saline solution (NPSS) (125 NaCl, 4.7 KCl, 1.2 $KH_2PO_4$, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 18 $NaHCO_3$, 0.026 $Na_2EDTA$, and 11.2 Glucose mM). All NPSS solutions and drugs were newly prepared on the morning of the experiment. Vessel internal diameter was measured using a calibrated micrometer eyepiece and reported in micrometers (μM). Perivascular adipose tissue was dissected from the feed arteries. NPSS was continuously aerated with carbogen gas (95% oxygen, 5% carbon dioxide), and pH was monitored at regular intervals and maintained at pH 7.35 – 7.45 by altering the amount of aeration (Orion 3 Star, Thermo Scientific, Waltham MA). Vessels were dissected into four rings measuring approximately 2 mm in length, and mounted in wire myography chambers (700 MO, DMT Systems, Aarhus, DK) to be studied using the isometric tension technique (29), as employed previously by our group (17). Once mounted, vessel chambers were also aerated with the same carbogen gas mixture, and chamber NPSS was exchanged at 10-minute intervals, except during cumulative drug dose responses. Vessel chambers were brought up to 37˚C over a 30-minute equilibration period prior to the start of a protocol.

*Characterization of Vessel Function*

All vessel segments underwent length tension procedures at 37˚C to determine the length at which the vessels produced the greatest tension in response to a single dose of 100mM KCl ($L_{max}$) (39). $L_{max}$ was operationally defined as less than a 10% improvement in developed tension in response to 100mM KCl. The viability of the vessels was characterized using KCl (10-100mM), PE ($10^{-9}$ – $10^{-3}$ M), SNP ($10^{-9}$ – $10^{-4}$ M), and ACh ($10^{-7}$ – $10^{-3}$ M) dose-response curves to determine nonreceptor- and receptor-mediated vasocontraction and vasorelaxation. All vasocontractile responses are expressed as a percent of the individual maximal response to 100mM KCl (%$KCl_{max}$) as described previously (42). All vasorelaxation responses are expressed as percent relaxation (%) from PE precontraction.

*pH and Vascular Reactivity*

Changes in pH were achieved by adding specific volumes (13 – 38 μL) of hydrochloric acid (1N HCl) to the myograph chamber, an approach that has been utilized previously (6, 36). Pilot work was performed, without vessels, to determine the appropriate volume of HCl necessary to achieve a target pH. Specifically, 30 different volumes of HCl were added separately to the myograph chamber containing a bicarbonate free medium and, after a similar time to that required to perform a cumulative dose-response, the pH was measured and a linear regression between pH and HCl volume was constructed. This pH to volume relationship was subsequently tested to determine efficacy and pH was confirmed *post hoc* in each myograph chamber after each protocol. Myograph chamber pH of 7.4 (control), 7.1, 6.8, and 6.5 were chosen as they

can be achieved during exercise (32). Under these conditions, the PE and KCl dose-responses were performed, in a balanced manner, to determine the effect of acidosis on vasocontraction. To determine the effect of acidosis on vasorelaxation or the sensitivity to the vasodilatory substances ACh (endothelium-dependent; $10^{-7}$ – $10^{-3}$ M) and SNP (endothelium-independent vasorelaxation; $10^{-9}$ – $10^{-4}$ M), dose-response curves were constructed. The SNP trials were conducted last in every case because of the known effect of SNP to irreversibly abolish vascular reactivity *in vitro*. All data were acquired at 4Hz using an analog to digital data acquisition system (Biopac Systems, Goleta, CA) to monitor vessel tensions and allow later offline analyses.

*Statistical Analyses*

Statistical analyses were performed using commercially available software (SPSS v. 16, Chicago, IL). Two-way Repeated-Measures ANOVA were utilized to determine if an interaction existed between pH (4 levels; 7.4, 7.1, 6.8, 6.5) and dose in the vasoreactivity to each agonist (PE, KCl, ACh, SNP) with varying pH. Due to potential individual differences in kinetics, the individual maximal response for each agonist (KCl, PE, ACh, SNP) was analyzed using a one-way repeated-measures ANOVA. Significant differences were assessed using Tukeys' Least Significant Difference *post hoc* test to make pairwise comparisons. To determine if increasing acidosis altered the sensitivity to an agonist, standard curve analyses were performed using commercially available software (BioDatafit v. 1.02, Castro Valley, CA). The level of significance was established at $p < 0.05$. All data are reported as mean ± standard error (SE).

## Results

*Vessel Characteristics*

Twenty-two human skeletal muscle feed arteries were successfully harvested (Table 4). No statistical differences in $L_{max}$, vasocontraction or vasorelaxation responses were observed in terms of anatomic location (axial vs. inguinal) or sex. Consequently, responses from all vessels were combined. The average internal diameter with minimal tension for these feed arteries was 560 ± 31 μM, and measured 1650 ± 75 uM in length. Vessel function protocols revealed robust vasocontraction in response to PE and KCl (83 ± 19; 87 ± 13 $\%KCl_{max}$, respectively; Figure 10A and B). Vessels were preconstricted to approximately 70% of the maximal PE response prior to ACh and SNP dose-response curves, and from this point, feed artery segments achieved significant vasorelaxation (102 ± 16; 78 ± 8 % relaxation, respectively) (Figure 10C and D). Taken together, these results indicate the feed arteries had functional smooth muscle, $\alpha_1$-adrenergic receptors, and an intact endothelium.

*pH and Vasocontraction*

Reducing extracellular pH significantly attenuated vascular tension development in response to both the $\alpha_1$-adrenergic agonist PE, and KCl (Figure 11A and B). Reducing pH significantly attenuated KCl induced vasocontraction (pH effect $p < 0.05$; dose effect $p = 0.000$), but there was no difference in the maximal responses (Figure 11C). However, there were differences in the sensitivity to KCl, as the $EC_{50}$ and $logEC_{50}$ were altered with acidosis, though not in a graded manner as with PE (Table 5). The suppressant effect of incremental acidosis was evident in response to cumulative doses of PE (pH x dose $p$ =

0.000; pH effect $p = 0.000$; dose effect $p = 0.000$; Figure 11A). In addition, the $EC_{50}$ and $logEC_{50}$ for PE were clearly increased, indicating a reduced sensitivity of the arterial rings in acidotic conditions exposed to the same dose of PE (Table 5). When expressed as percent change from control (pH 7.4), the effect of pH on the arteries was more pronounced in response to PE, when compared to KCl (Figure 11C).

*pH and Vasorelaxation*

The ACh (endothelium-dependent) and SNP (endothelium-independent) vasorelaxation responses were performed with graded reductions in pH to determine if net relaxation or relaxation kinetics were altered by acidosis. There was no significant effect of graded reductions in pH on the maximal vasorelaxation in response to either ACh or SNP (Figure 12). The sensitivity of the smooth muscle to the vasodilatory effects of SNP, assessed by $EC_{50}$ and $logEC_{50}$, was reduced from pH 7.4, at pH 7.1 and 6.5, suggestive of reduced smooth muscle function (Table 5). However, graded acidosis significantly enhanced the sensitivity to ACh, again as assessed by the $EC_{50}$ and $logEC_{50}$ (Table 5), indicating heightened endothelial sensitivity.

## Discussion

The main finding of this study was that graded reductions in extracellular pH resulted in significant and progressive decreases in the response to sympathomimetic stimulation. This attenuated response to the $\alpha_1$-adrenergic agonist PE cannot be fully explained by the much smaller concomitant suppression of smooth muscle function. In stark contrast to the detrimental impact of acidosis on vasocontraction, there was no

significant effect on endothelium-dependent or -independent maximal vasorelaxation, with some evidence of enhanced sensitivity to the endothelium-dependent agonist ACh. These results reveal, for the first time in human skeletal muscle feed arteries, that not only does acidosis suppress vasocontractile capacity, but this phenomenon may also be enhanced by unaltered or even enhanced vasodilatory function. Therefore, these findings imply that acidosis, associated with skeletal muscle metabolism, could be a contributing factor in the reduction of sympathetically mediated vasoconstriction, or functional sympatholysis, observed during exercise by reducing vasocontractile function yet leaving vasodilatory function intact, ultimately producing significant reductions in vascular resistance.

*pH and Vasocontraction*

As already indicated, *en masse*, the results of studies that have attempted to determine the effect of acidosis on agonist-induced vasoconstriction remain unresolved. Although early studies focused on the receptor-mediated responses (25), later studies acknowledged that any observed reduction in agonist-induced vasocontraction could be, at least in part, mediated by altered inherent smooth muscle function (40). Most studies (1, 2, 5, 15, 30) now agree that acidosis exerts a direct effect on the vascular smooth muscle, via ion channels (ATP sensitive $K^+$ channels in particular), which may act in conjunction with reduced receptor sensitivity resulting in an attenuated maximal receptor-mediated responsiveness. In light of this, Rohra et al. (36) used the receptor-independent agonist KCl and receptor agonist PE to determine the effects of acidosis on vasocontractile function in the human internal mammary artery. Their findings indicated

that acidosis reduced KCl and, to a much greater extent, PE-induced vasocontraction (36). In agreement with Rohra and colleagues (36), the current findings reveal that $\alpha_1$-alpha adrenergic receptor function is suppressed in a proportional fashion by reductions in pH (Figure 11A), which persisted across a range of doses, and is, at least in part, mediated by reduced smooth muscle function (Figure 11B). Also in agreement with this prior work in a human artery, it appears that in human feed arteries, the receptor mediated vasocontraction, induced by the sympathomimetic PE, is far more susceptible to the effects of acidosis than the nonreceptor -mediated KCl-induced vasocontraction (Figure 11C).

The effect of pH may, or may not, be receptor selective as Kluess et al. (18) documented, in the rat femoral artery, that purinergic ($P_2X_1$) and not $\alpha_1$-adrenergic receptor-mediated vasocontraction was impaired during acidosis. Although the majority of studies indicate acidosis is inhibitory despite the agonist employed (36), some of the variation in the effect of acidosis can be explained by differences in species (27), genetic strain (34), vascular location and caliber (12-15, 24), and experimental model (6, 33, 36). For example, an *in vitro* rodent study determined that rat strain alone created a divergent response resulting in either a suppressed or enhanced $\alpha_1$-mediated vasoconstriction in the face of acidosis (34). Vessel location appears to be another important determinant of the effect acidosis may have upon vascular tension development as cerebral and coronary vessels seem highly sensitive to acidosis (6, 12, 27, 30), whereas the pulmonary artery appears to be less so (14, 27). Additionally, vessel order appears to alter the vascular sensitivity to acidosis, meaning that it has been documented that smaller arterioles appear to be more sensitive to acidosis than larger arterioles (25, 40), which has implications for

blood flow regulation and redistribution. This also likely explains the negative findings of Kluess et al. (18) where there was no effect of acidosis on PE-induced vasocontraction in the rat femoral artery, an unlikely site of blood flow regulation. Utilizing an artery more likely to be involved in the regulation of skeletal muscle blood flow, our findings indicate the acidosis does, in fact, reduce $\alpha_1$-mediated vasocontraction.

It should also be noted that studies which determined that acidosis suppressed $\alpha_2$- and not $\alpha_1$-adrenergic responsiveness using selective and nonselective adrenergic agonists employed the rat cremaster muscle model (25, 40). This model has recently been suggested to be unrepresentative of locomotor muscles (28), and challenges the dogma that terminal arterioles are primarily under $\alpha_2$-receptor control and more susceptible to "metabolic inhibition," while proximal arteries are under $\alpha_1$-receptor control (4, 25, 26). While the classic dogma was logical and certainly an attractive hypothesis, the work of Moore and colleagues (28) in essence challenges the applicability of prior findings from the cremaster model, as they found terminal arterioles in locomotor muscle were, in fact, primarily under $\alpha_1$-receptor control, but also varied in prevalence down the arterial tree. In this context, and in light of the potential for the feed artery to be a locus of skeletal muscle blood flow regulation (43-47), the findings from the current study support the suppressant effect of acidosis on $\alpha_1$-mediated vasoconstriction, which appears to be due to reduced receptor sensitivity, and inherent smooth muscle function.

*pH and Vasorelaxation*

The vasodilatory effects of acidosis have been well described both *in vitro* (6, 12, 13, 15, 16, 19, 24, 30), and *in vivo* in animals (7-9, 23), yet the question remains if these

results translate into humans. Early work in humans indicated that hypercapnia or the infusion of hypercapnic saline elicited a profound hyperemia (20-22). The prior human work, although quite impressive for the era, was not able to determine the mechanism by which acidosis elicited a vasodilatory effect. Subsequent animal work (6, 24) determined that acidosis elicited vasodilation in a dose-dependent manner, and endothelial denudation or pharmacological blockade reduced endothelial sensitivity to acidosis, but not the maximal response. The results of the current study were similar to the findings of these previous studies (6, 24), where the maximal response to ACh or SNP was unchanged (Figure 12), but the endothelial sensitivity was enhanced with graded acidosis (Table 5). Acidosis also reduced the sensitivity to the endothelium independent vasodilator SNP, but certainly not as clearly as the effect of acidosis on the ACh response. Thus, the effect of acidosis on vasorelaxation does not appear to be significantly impacted by altered smooth muscle cyclic-GMP function. To our knowledge, there has not been a single study that has investigated the effect of acidosis on agonist-induced vasorelaxation in either animals or humans. Therefore, this study appears to be the first to demonstrate that vasorelaxation function, both endothelial-dependent and -independent, remains intact when exposed to physiological levels of acidosis.

*pH and the Balance between Vasoconstrictors and Vasodilators:*

*Implications for Functional Sympatholysis*

There is a growing recognition that functional sympatholysis may actually be the alteration in the balance between sympathetic vasoconstriction and vasodilation induced

by metabolic by-products from muscle (11). In this context, we sought to determine the effect of acidosis on vasoreactivity and the varying contribution of vasorelaxation and vasocontractile function in this response. Additionally, utilizing receptor-dependent and receptor-independent agonists allowed the comparison of receptor versus smooth muscle function for both vasocontraction and vasorelaxation. Specifically, we determined the percent change in receptor-mediated function associated with acidosis (%Δ pH 7.4-6.5) for both PE and ACh; performing the same calculation for KCl and SNP, we then subtracted the percent change attributable to vascular smooth muscle for both vasocontraction (%ΔPE - %ΔKCl) and vasorelaxation (%ΔACh - %ΔSNP). This provides a theoretical approach to understand the effect of acidosis on receptor-mediated function for both vasocontraction and vasorelaxation, using a common unit of measure (%Δ). Using this subtraction approach, it is apparent that acidosis had a more profound effect upon receptor-mediated vasocontraction compared to vasorelaxation (Figure 13). In the context of exercise and functional sympatholysis, these results provide support that acidosis can indeed elicit a sympatholytic effect, but that the impact of this effect would be augmented by the concomitant maintenance of endothelium mediated vasodilation. It is likely that *in vivo,* these contrasting responses to acidosis act in concert to override sympathetic activity and facilitate hyperemia during exercise.

*Conclusions*

This study has demonstrated that local changes in pH significantly reduce $\alpha_1$-adrenergic receptor function and this observation is due, at least in part, to reduced smooth muscle function. Interestingly, endothelium-mediated vasodilatory capacity tends

to be unchanged while sensitivity is increased which may act to augment the impact of attenuated sympathetically mediated vasocontraction. These findings support the concept that local acidosis could be a contributing factor in functional sympatholysis and exercise hyperemia by opposing sympathetically mediated vasoconstriction and by not impacting or increasing endothelium dependent vasodilation.

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Table 4. *Characteristics of the subject population (n =22).*

| | Mean ± SE |
|---|---|
| Age (yr) | 55 ± 4 |
| Height (cm) | 161 ± 9 |
| Weight (kg) | 88 ± 10 |
| Systolic Blood Pressure | 131 ± 7 † |
| Diastolic Blood Pressure | 75 ± 8 † |
| Medications (users/n) | |
| Cardiovascular | |
| Diuretic | 2/22 |
| $Ca^{++}$ Channel Blocker | 1/22 |
| Statin | 1/22 |
| ACE inhibitor | 0/22 |
| Beta Blocker | 1/22 |
| Other | |
| Insulin | 1/22 |

† Data obtained during pre-operative examination

Table 5. *Dose response curve characteristics with increasing acidosis (mean±SE).*

| Drug - pH | Max (%$KCl_{max}$) | $EC_{50}$ | Log $EC_{50}$ |
|---|---|---|---|
| PE 7.4 | 85±19 | $3.0^{-5}$ | - 4.5±0.2 |
| 7.1 | 56±16* | $6.1^{-5}$* | - 4.2±0.1* |
| 6.8 | 34±15* | $6.7^{-5}$* | - 4.2±0.1* |
| 6.5 | 16±5* | $1.4^{-4}$* | - 3.8±0.1* |
| KCl 7.4 | 88±13 | $6.4^{59}$ | 59.8±0.6 |
| 7.1 | 67±8 | $1.3^{40}$* | 40.1±0.6* |
| 6.8 | 67±9 | $8.2^{59}$ | 59.9±0.5 |
| 6.5 | 58±8 | $1.5^{60}$ | 60.2±0.4 |
| ACh 7.4 | 81±8 | $1.8^{-5}$ | - 4.5±0.4 |
| 7.1 | 77±12 | $3.2^{-6}$* | - 5.5±0.3* |
| 6.8 | 67±7 | $8.6^{-6}$* | - 5.2±0.2* |
| 6.5 | 68±6 | $9.3^{-7}$* | - 6.0±0.2* |
| SNP 7.4 | 103±17 | $4.6^{-7}$ | - 6.3±0.3 |
| 7.1 | 110±9 | $1.6^{-7}$* | - 6.8±0.3* |
| 6.8 | 107±4 | $5.6^{-7}$ | - 6.2±0.3 |
| 6.5 | 93±7 | $1.3^{-6}$* | - 5.9±0.2* |

* Significantly different from pH 7.4 ($p < 0.05$)

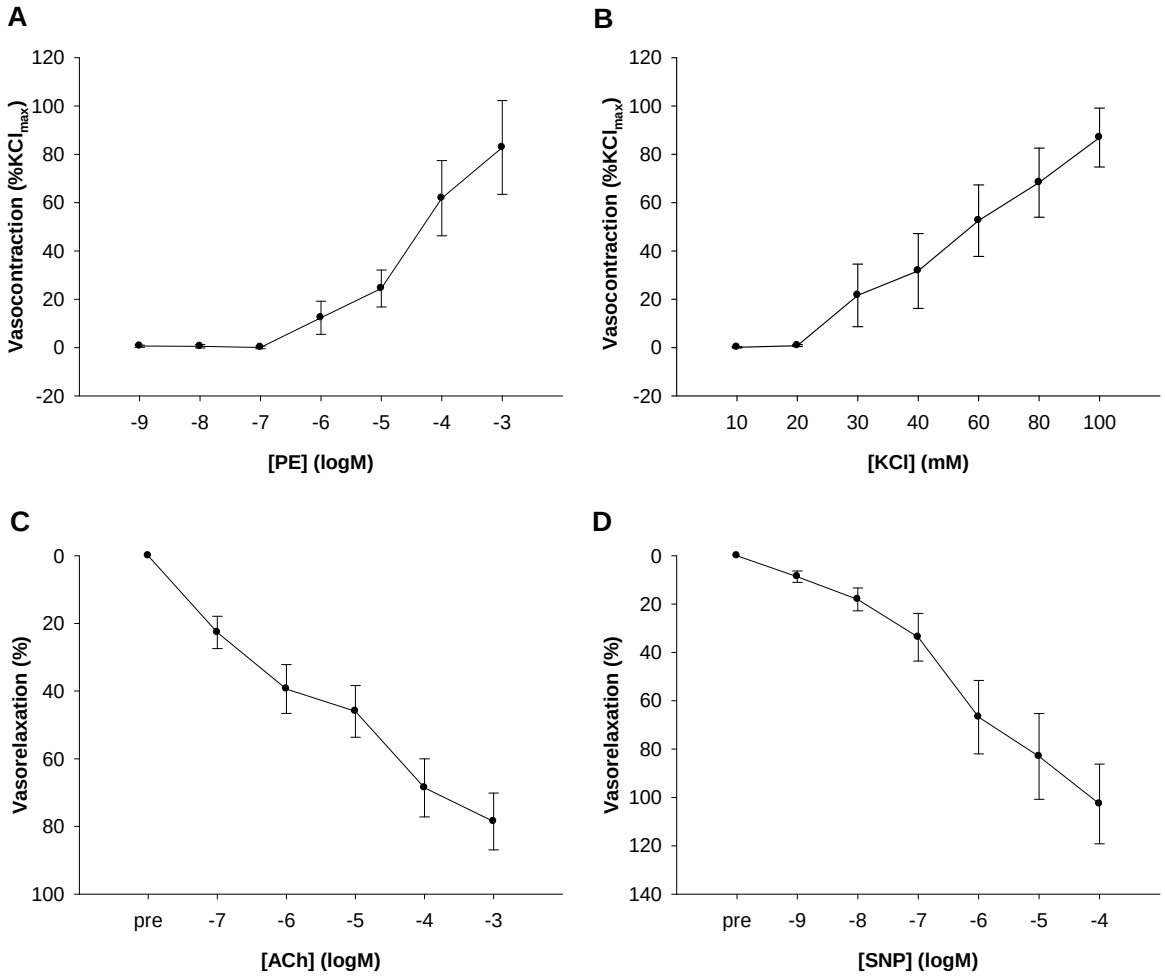


*Figure 10.* Human skeletal muscle feed artery functional characteristics. A) Phenylephrine (PE) dose-response for $\alpha_1$-adrenergic-mediated vasocontraction. B) Nonreceptor-mediated vasocontraction dose-response for KCl. C) Acetylcholine (ACh) dose response (% relaxation from PE precontraction). D) Sodium Nitroprusside (SNP) dose response (% relaxation from PE precontraction). Data are presented as mean ± SE.

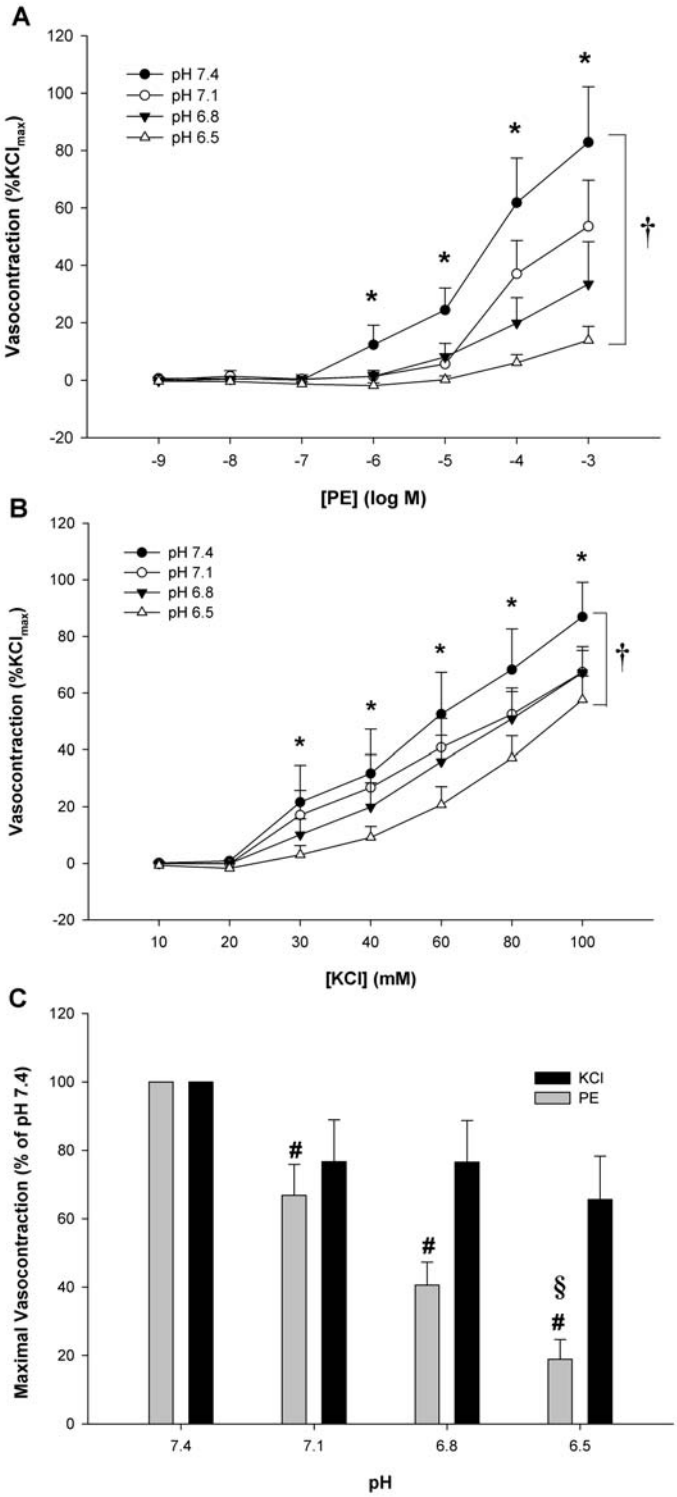


*Figure 11.* Vasocontractile Function and Acidosis. A) PE dose-responses across varying levels of acidosis. †main effect of pH, $p < 0.05$, *main effect of dose, significantly different from baseline ($p < 0.05$). B) KCl dose-response curves across varying levels of acidosis. C) Maximal PE and KCl responses expressed as a percent of control condition (pH 7.4). $^{\#}$ $p < 0.05$ vs. pH 7.4, $^{\S}$ $p < 0.05$ vs. pH 7.1. Data are presented as mean ± SE.

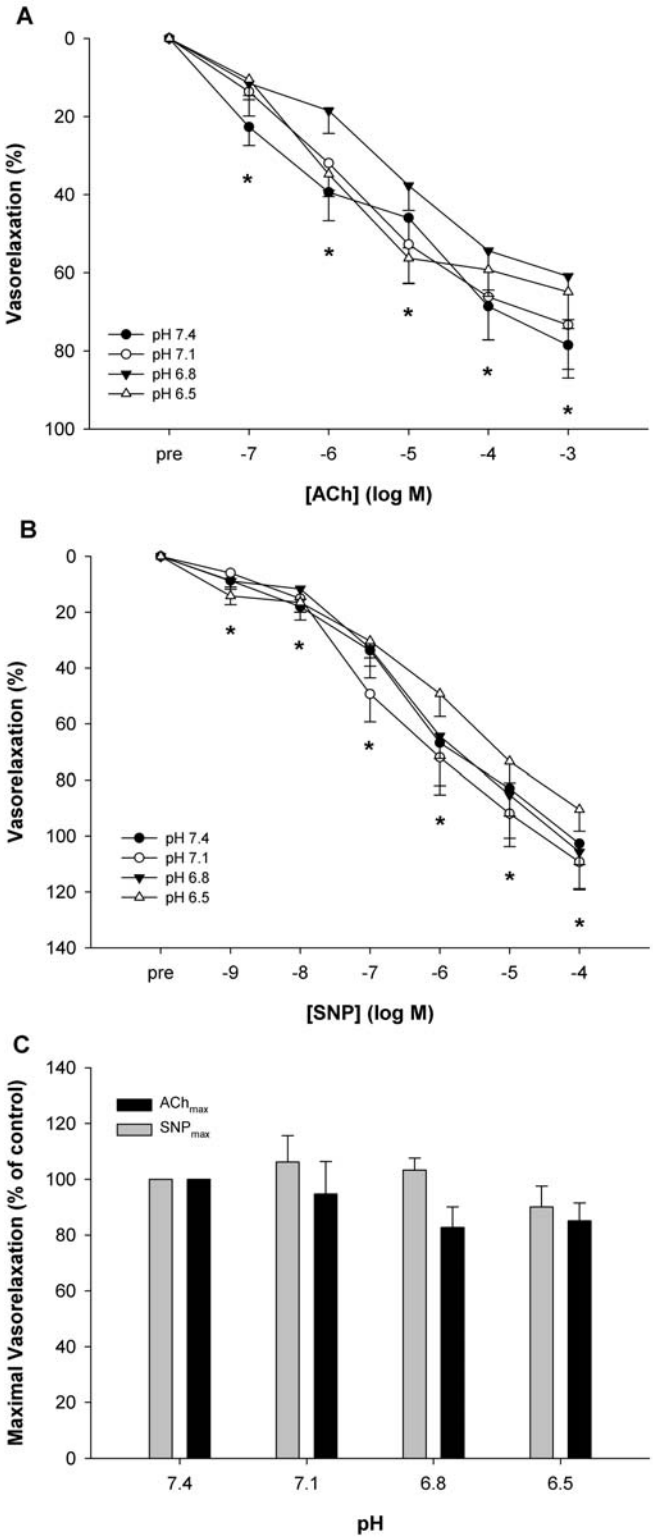


*Figure 12*. Vasorelaxation Function and Acidosis. A) ACh Dose-response curves across varying levels of acidosis. *main effect of dose, $p < 0.05$ vs. predrug. B) SNP Dose-response curves across varying levels of acidosis. *main effect of dose, $p < 0.05$ vs. predrug. Data are presented as mean ± SE.

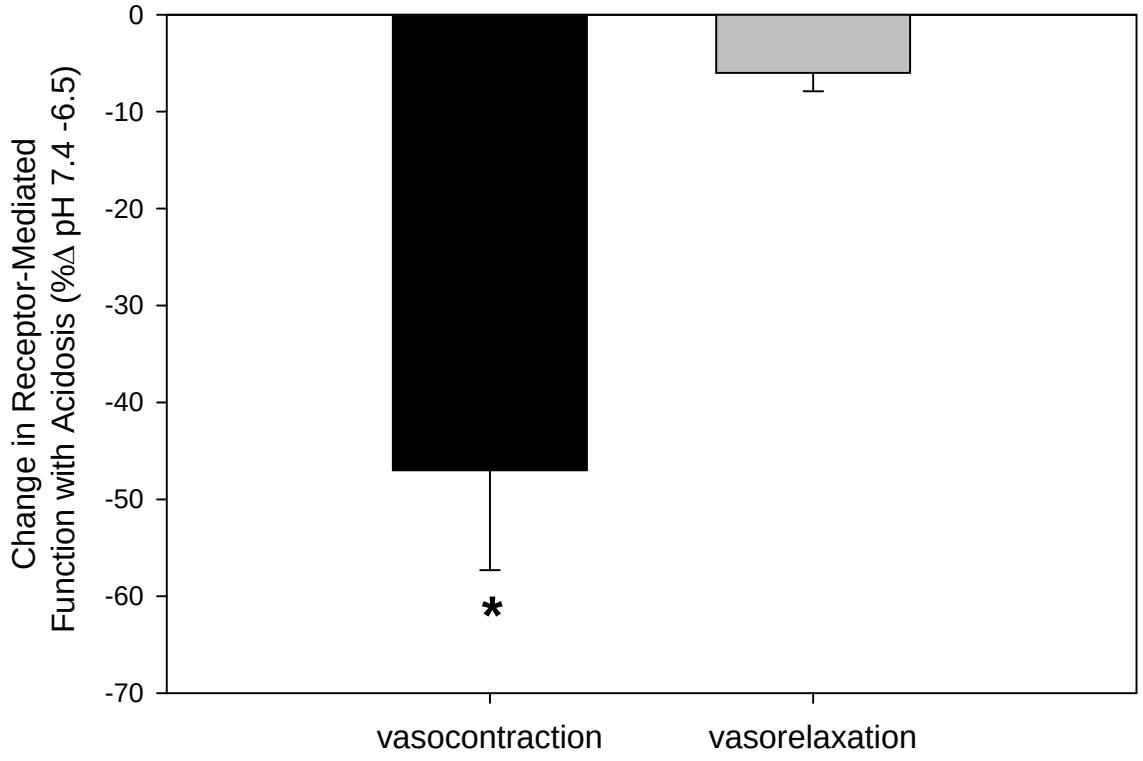


*Figure 13*. Changes in receptor-mediated function unaccounted for by changes in vascular smooth muscle function. Data are presented as mean ± SE.

# CONCLUSION

Using a novel approach of obtaining and studying human skeletal muscle feed arteries *in vitro*, these studies aimed to determine if physical factors, such as heat and acidosis, at physiological levels achievable during exercise could modulate the response to the $\alpha_1$-adrenergic sympathomimetic, phenylephrine. The results from these studies revealed that factors normally released in significant quantities as a consequence of skeletal muscle metabolism (e.g. heat and hydrogen ions) or their downstream effectors (e.g. NO) are certainly capable of inducing a sympatholytic effect upon the $\alpha_1$-receptors, a primary target for norepinephrine, the neurotransmitter released as a result of both feedback and feed-forward mechanisms in response to exercise. The consequence of the reductions in $\alpha_1$-mediated vasocontraction observed in these studies would likely prevent increases in vascular resistance in response to norepinephrine released as a consequence of exercise-induced increases in muscle sympathetic nerve activity. In addition, reduced vascular responsiveness associated with metabolic perturbation has implications for the maintenance of mean arterial pressure in emergency or pathological situations, such as hyperthermia, or metabolic acidosis.

In the first study, we aimed to determine the effect of temperature on vascular responsiveness. To achieve this, we performed *in vitro* vessel wire myography experiments in response to cooling (35°C) and heating (39°C) within a physiological range. The major mechanism involved in the acute regulation of vascular tone and blood flow is the sympathetic nervous system; thus, we sought to determine if heating or cooling would alter the response to phenylephrine, an $\alpha_1$-adrenergic agonist with a relatively greater affinity for the $\alpha_1$-receptor than the $\alpha_2$-adrenergic receptor. We chose to focus on the $\alpha_1$-adrenergic receptor, because prior work has demonstrated a graded

sympatholytic effect of exercise on $\alpha_1$-receptor responsiveness, whereas the $\alpha_2$-receptors appear to be lysed immediately, upon the commencement of exercise even with unloaded knee extensor exercise (4). Recognizing the potential for temperature to alter inherent smooth muscle function, we utilized potassium chloride (KCl) to determine if temperature would, in fact, alter smooth muscle function. If so, the responses to phenylephrine could have been normalized to the temperature-induced alteration in basal smooth muscle function. Additionally, given the lack of reference values for vascular reactivity of these human skeletal muscle feed arteries, and to rule out individual vascular dysfunction as a mediator in the response to temperature, we performed dose-response curves to characterize receptor- and nonreceptor-mediated vasocontraction using PE and KCl, respectively. To characterize the endothelium-dependent and endothelium-independent vasorelaxant function of these vessels, dose-response curves were performed for acetylcholine (ACh) and sodium nitroprusside (SNP), respectively.

The dose response curves for PE, KCl, ACh, and SNP revealed that these vessels are capable of producing significant force in response to PE and KCl, and the vasodilators ACh and SNP induced appropriate levels of vasorelaxation, indicating the vessels employed in these studies were functional and had an intact endothelium. We found that both heating (39°C) and cooling (39°C) reduced the vasocontractile response to a single dose or across a spectrum of doses of PE, performed either in a cross-sectional or longitudinal manner. Interestingly, the response to a single dose of KCl remained intact despite the temperature perturbation. These results indicate that the attenuating effect of perturbing temperature are not the result of the smooth muscle, *per se*, and other factors are instigating the reduction in the $\alpha_1$-adrenergic receptor response observed with

the changes in temperature. Regardless of a specific mechanism, it appears temperature could be a potent regulator of vascular tone in human skeletal muscle feed arteries, a vessel that is considered to be a point of regulation of skeletal muscle blood flow (3).

To ascertain the mechanism behind the attenuating effect of heating upon skeletal muscle feed artery response to the $\alpha_1$-receptor agonist, dose-response curves were again performed in normothermic (37 °C) and hyperthermic (39 °C) conditions, but now in the absence and presence of the endothelial nitric oxide synthase (eNOS) inhibitor, L-NMMA. We hypothesized that heating would increase the enzyme kinetics of eNOS, and increase NO bioavailability opposing the PE-induced increase in vascular tone. Thus, effective blockade of eNOS would partially restore the contractile effects of PE towards that observed at 37°C. Employing the same methodology as the first study, human skeletal muscle feed arteries were again obtained and studied using isometric force technique. To rule out potential vascular dysfunction dose-responses for PE, KCl, ACh, and SNP were performed to characterize the receptor- and nonreceptor-mediated vasocontraction and vasorelaxation, respectively. Additionally, we exposed a subset of arteries to normo- and hyperthermic conditions, to determine if the effect of heating was due to an upregulation of eNOS protein or downregulation of $\alpha_1$-adrenergic receptor protein, using standard molecular techniques.

In agreement with the first study, we found that temperature exerted an attenuating effect upon $\alpha_1$-mediated vasocontraction. Remarkably, blockade of eNOS during heating to 39°C restored the vasocontractile response to PE to that of the normothermic condition. Blockade of eNOS also tended to increase the response to PE at 37°C; however, the percent increase was not significant as compared to the hyperthermic

condition. Curve analysis revealed heating significantly reduced the sensitivity to the $\alpha_1$-agonist, PE. In support of these findings, the western blot analyses revealed an upregulation of eNOS, but no change in $\alpha_1$-adrenergic receptor protein. Taken together, these findings suggest that heating increases the bioavailability of NO, which acts to oppose the effects of $\alpha_1$-mediated vasocontraction. These data again reveal the potential for temperature to be an important regulator of blood flow, via reduced $\alpha_1$-receptor mediated vasocontraction, but also highlights the role for vasodilators (e.g. NO) in modulating vasoconstrictor responsiveness. In essence, functional sympatholysis may not be a result of reduced sympathetic efficacy as in the traditional sense, but the consequence of increased vasodilatory opposition to such sympathetic stimuli.

The third study investigated the role of increased hydrogen ion concentration and the parallel reductions in pH on vascular reactivity. Again, utilizing the *in vitro* approach, which allowed the strict control of the metabolic *milieu*, we were able to create graded levels of acidosis, in the absence of other metabolic disturbances. Again, human skeletal muscle feed arteries were obtained, and studied using the isometric tension technique. We performed dose-response curves for PE, KCl, ACh, and SNP to determine receptor- and nonreceptor-mediated vasocontraction and vasorelaxation characteristics, respectively. Each of these dose-response curves were performed at normal pH (7.4), and across increasing levels of acidosis, pH 7.1, pH 6.8, and pH 6.5. These pH values were chosen because these represent levels attainable in the skeletal muscle (1) and interstitial spaces (2) during graded exercise. We hypothesized that reductions in pH, within a physiological range, would be paralleled by reductions in $\alpha_1$-receptor-mediated vasocontraction. However, we also expected that the reductions in vasocontraction could be, at least in

part, due to altered smooth muscle function. Additionally, we hypothesized that the enhanced vasorelaxation would contribute to the suppressant effect of acidosis and thus the vasorelaxation response to ACh and SNP will be enhanced with acidosis.

We determined that increasing levels of acidosis resulted in parallel reductions in PE-induced vasocontraction which are partially explained by a concomitant reduction in KCl-mediated vasocontraction. However, it appears that the suppressant effect of acidosis on $\alpha_1$-adrenergic receptor function cannot be fully explained by reduced inherent smooth muscle function, as assessed by KCl. With the goal of determining if altered vasorelaxation contributed to the observed reduction in $\alpha_1$-receptor mediated vasocontraction, ACh and SNP dose-response curves were constructed at each pH. Interestingly, in the face of acidosis, there was no difference across ACh and SNP dose or maximal dilation. However, the curve analyses revealed that increasing acidosis resulted in significant increases in the sensitivity to the endothelial dependent dilator ACh, as assessed by $EC_{50}$ and log $EC_{50}$ analyses. Together, these results suggest that acidosis, such as that achievable during exercise, is capable of suppressing vasoconstrictor responsiveness which was likely the summation of reduced smooth muscle, $\alpha_1$-adrenergic receptor function, and maintained vasorelaxation capacity.

The attainment of human skeletal muscle feed arteries, is both novel and germane to understanding how factors such as temperature or acidosis could alter sympathetically mediated vasocontraction, via the $\alpha_1$-adrenergic receptor, and subsequently the regulation of skeletal muscle blood flow in humans. Collectively, these results indicate that local metabolic factors, such as heat and $H^+$, are capable of inducing a sympatholytic effect on human skeletal muscle feed arteries, and therefore likely contribute to exercise

hyperemia. These studies are the first to characterize human skeletal muscle feed artery function, and determine if these arteries are “metabolically-sensitive.” We also demonstrate that the $\alpha_1$-adrenergic receptor is not immune to metabolic-inhibition, as traditionally demonstrated in animal models. These findings may also have clinical implications for understanding factors that contribute to hypotension in emergent and pathological situations, such as heat stroke or metabolic acidosis. In summary, we conclude that metabolic by-products such as heat and hydrogen ions alter vascular reactivity in humans, which may have broad application for the understanding of human physiology.

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