CUTANEOUS VASODILATION AT SIMULATED HIGH ALTITUDE: IMPACTS ON HUMAN THERMOREGULATION AND VASOCONSTRICTOR FUNCTION

by

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During acute altitude exposure, humans maintain higher skin temperature and lower core body temperature. However, the role of cutaneous vascular regulation in these thermoregulatory differences is unclear. Therefore, the purpose of these studies was to investigate the impact of altitude exposure on reflex control of skin blood flow and core temperature during cold exposure. In Chapter IV, the effects of hypoxia and hypocapnia on cutaneous vasoconstriction during mild cold exposure were investigated. We found that hypoxia stimulates cutaneous vasodilation in men whereas skin blood flow is unaltered in women. However, during whole body cooling skin blood flow is upward shifted in both sexes. The development of hypocapnia does not affect the vascular response to

hypoxia in either sex, but reduces the magnitude of cutaneous vasoconstriction during cold exposure by 50% in women. In Chapter V, we studied the timecourse of α-adrenergic blockade by yohimbine in the cutaneous circulation and how the duration of cold exposure modulates cotransmitter-mediated vasoconstriction during cold stress. We found that yohimbine produces functional α-adrenergic blockade within 30 minutes of initial delivery and completely abolishes reflex cutaneous vasoconstriction during mild cold stress. This latter finding was surprising, and an additional protocol demonstrated that cotransmitter-mediated vasoconstriction only participates in the vascular response to cold stress when the exposure is more prolonged. In Chapter VI, the effects of hypoxia on cutaneous vasoconstrictor mechanisms and core cooling rate were tested during more prolonged and severe cold stress. In contrast to our findings during brief cold exposure, we showed that cutaneous vasoconstriction during prolonged cold stress is potentiated by hypoxia and abolishes hypoxic vasodilation. Moreover, increased cotransmitter-mediated vasoconstriction appears to account for this response. Hypoxia had no effect on core cooling rate during severe cold exposure. The selective potentiation of cotransmitter-mediated vasoconstriction observed during hypoxia in Chapter VI provided the basis for Chapter VII. This study was designed to test the effect of hypoxia on cutaneous vascular responsiveness to peripherally stimulated sympathetic vasoconstriction. The results demonstrated that α-adrenergic vasoconstrictor transduction is not affected by hypoxia, and that stimulation of

adrenergic nerves with tyramine does not elicit cotransmitter-mediated vasoconstriction in skin.

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CHAPTER I

INTRODUCTION

Background

In thermoneutral or cool environments, maintenance of thermal balance in humans is dependent on two primary temperature gradients: a gradient between the core and skin and a gradient between the skin and ambient environment. Metabolic heat produced within the core tissues (~ 37.5°C) circulates in the blood down a temperature gradient to the skin (~ 33.0°C), and heat transferred to the skin is dissipated to the ambient environment (< 33.0°C). Thus, control of the circulation through the skin represents the primary physiological mechanism for thermoregulation in humans in neutral or cool environments.

When environmental temperature falls, skin blood flow is reduced in order to decrease heat transfer from the core to the periphery, thereby promoting heat conservation (Toner & McArdle, 1988). This thermoregulatory adjustment also decreases skin temperature, which narrows the gradient for cutaneous heat loss and is vital to the prevention of hypothermia in cold environments. The reduction in skin blood flow upon body cooling is regulated by cutaneous sympathetic adrenergic nerves, which contain both norepinephrine and other sympathetic cotransmitters (Kellogg *et al.*, 1989; Lundberg, 1996). As ambient temperature decreases, the activity of cutaneous adrenergic nerves increases, releasing

norepinephrine onto post junctional α-receptors (predominantly α_2-receptors). The binding of norepinephrine to post junctional α-receptors causes vasoconstriction, and this mechanism is responsible for approximately 60% of the reduction in skin blood flow during cold stress (Thompson & Kenney, 2004). The remaining ~ 40% of the cold induced vasoconstriction is mediated by adrenergic nerve cotransmission (Stephens *et al.*, 2001; Thompson & Kenney, 2004). The primary peptide thought to mediate sympathetic cotransmission in this setting is neuropeptide Y, which causes cutaneous vasoconstriction via post junctional neuropeptide Y_1-receptors (Stephens *et al.*, 2004).

When humans encounter cold temperatures at high altitude, lower oxygen levels and cold temperatures may have competing effects on skin blood flow. For example, hypoxia *per se* elicits a cutaneous hyperemia that is mediated at the tissue level (Anderson *et al.*, 1991; Simmons *et al.*, 2007). In this setting, the rate of core cooling is accelerated, and lower core temperatures are reached during cold exposure compared to sea level conditions (Cipriano & Goldman, 1975; Johnston *et al.*, 1996). While core temperature falls faster at altitude, skin temperature remains higher throughout cold exposure compared to sea levels conditions (Cipriano & Goldman, 1975; Blatteis & Lutherer, 1976). Taken together, these results indicate a less effective reduction in core-to-skin heat transfer during cold exposure at altitude. Such impairment in heat conservation could reflect an acute alteration in output from the thermoregulatory controller during hypoxia (central mechanism). Conversely, the mechanism responsible could be peripheral, affecting some aspect of cutaneous vascular regulation by

sympathetic adrenergic nerves. A peripheral impairment in cutaneous vascular control during cold stress could result from two different scenarios: first, the maintenance of higher skin temperature (and probably higher skin blood flow) could represent the summation of two environmental stimuli affecting the cutaneous circulation through completely separate mechanisms. In this scenario, the higher skin blood flow during cold stress (at altitude) would represent hypoxic vasodilation superimposed on reflex vasoconstriction. The second possibility is that low tissue oxygen tension could impair the transduction of sympathetic vasoconstrictor outflow into vascular resistance. In this scenario, hypoxic vasodilation would still be superimposed on cutaneous vasoconstriction, but the vasoconstriction would also be less effective. Therefore, in addition to the simple summation of two environmental stimuli, the cutaneous vascular response may also reflect the interaction of hypoxia and cold thermoregulatory effector mechanisms.

Statement of the problem

High altitude environments are characterized by reduced barometric pressure and inadequate oxygen supply. In this setting, a variety of species selectively regulate a lower body temperature, presumably favoring a protective reduction in metabolism. Some evidence suggests that humans exhibit a similar response in hypoxic environments, as discussed above. However, existing data can not adequately explain the mechanisms underlying changes in human thermoregulation at high altitude. For example, exposure of humans to hypobaric hypoxia reduces core temperature measured during 4 hours of cold

exposure, indicating more rapid heat loss to the environment. However, it is unclear from these observations whether augmented core cooling was caused by the reduction in barometric pressure or the maintenance of greater heat flow from the core to the skin. Barometric pressure *per se* is capable of altering the coefficient for heat transfer across the skin, which could increase cutaneous heat loss solely through biophysical mechanisms. Conversely, the cutaneous vasodilation induced by low oxygen levels in thermoneutral conditions could persist during cold exposure. This would counter the reduction in heat transfer away from the core, and would impact heat loss through hemodynamic mechanisms. Importantly, there are no reports in the literature of cutaneous perfusion measurements (in non-acral skin) during combined altitude and cold stress.

Therefore, the purpose of these experiments was to investigate the regulation of skin blood flow and core body temperature during combined hypoxia and cold exposure. The investigation described in Chapter IV had two main goals. First, we sought to study whether the magnitude of cutaneous vasodilation during hypoxia is affected by changes in blood carbon dioxide levels. Second, we sought to determine if cutaneous vasodilation persists during whole body cooling in a hypoxic environment. In Chapter V, we tested the timecourse over which two pharmacological agents affect the cutaneous vasculature in human skin. A second protocol in Chapter V tested the relationship between mean skin temperature and sympathetic cotransmitter-mediated (non-noradrenergic) vasoconstriction during prolong cold exposure. In Chapter VI we

first sought to study the function of both noradrenergic and non-noradrenergic cutaneous vasoconstriction during combined cold exposure and hypoxia. Second, we sought to determine if the persistent vasodilation observed during combined hypoxia and cold stress could explain the increased rate of core cooling in this setting described by others. Finally, the investigation in Chapter VII was designed to test post-junctional responsiveness to sympathetic vasoconstrictor stimuli during hypoxia in human skin.

Hypotheses

The series of studies contained in this dissertation were designed to test the following hypotheses:

1. In Chapter IV we hypothesized that cutaneous vasodilation during hypoxia is greater in magnitude when accompanied by hypercapnia, and that cutaneous vasodilation persists during mild whole body cooling regardless of carbon dioxide levels. Furthermore, we hypothesized that rapid exposure to hypoxia produces a greater cutaneous vascular response than slower, progressive hypoxic exposure.
2. In Chapter V we hypothesized that selective blockade of α-adrenergic and neuropeptide Y-Y_1 receptors in different skin sites reduces, but does not completely abolish, reflex cutaneous vasoconstriction during whole body cooling. We further hypothesized that these effects increase over time once drug delivery is begun. Lastly, we hypothesized that sympathetic non-noradrenergic vasoconstriction is selectively activated at lower skin temperatures during prolonged cold stress.

3. In Chapter VI we hypothesized that the upward shift in skin blood flow during whole body cooling is mediated through noradrenergic mechanisms, and that a tight relationship exists between the degree of upward shift in skin blood flow and the increase in core cooling rate during severe cold exposure in hypoxic conditions.
4. In Chapter VII we hypothesized that non-noradrenergic, but not noradrenergic cutaneous vasoconstrictor responsiveness is increased during systemic hypoxia in resting humans.

Significance

Environmental hypoxia and cold temperatures interact in some way to make the sojourner unusually susceptible to a decline in core body temperature (Cipriano & Goldman, 1975; Johnston *et al.*, 1996). Historically, military personnel have had the greatest risk of encountering cold temperatures and high altitude conditions simultaneously (Hamlet, 1988). However, as mountaineering has moved into the mainstream this risk has increased in civilian populations. Numerous skilled athletes die each year when attempting to summit high altitude peaks in cold temperatures. In view of the potentially lethal effects of simultaneous altitude and cold exposure, well controlled laboratory studies are needed to probe thermoregulatory adjustments to this unique environmental extreme.

CHAPTER II

REVIEW OF THE LITERATURE

Introduction

This review of the literature is organized into four sections that provide the background and context from which to understand the impetus for and design of these studies. The first section gives a brief introduction into differential vasomotor control mechanisms which influence acral (non-hairy) and non-acral (hairy) skin blood flow in humans. This section is not part of the formal literature review, but is necessary in order to understand how seemingly disparate observations made in different portions of the integument can be reconciled into a coherent control scheme. The second section begins the literature review by addressing cutaneous vascular responses to changes in blood oxygen and carbon dioxide levels. The third section reviews the work leading up to our current understanding of cutaneous vascular regulation in humans during cold exposure. The fourth and final section examines a body of literature leading to our current understanding of how high altitude hypoxia affects sympathetic vasoconstriction in human limbs.

Differential autonomic control of cutaneous blood vessels

Cutaneous vascular innervation by the sympathetic nervous system is variable across the integument, with nerve activity directed to acral and non-acral

portions responding differently during a variety of environmental stresses (e.g., chemoreceptor stimulation, thermal stress). The circulation to the acral skin of the hands, ears, nose, feet, etc., is innervated only by the adrenergic branch of the sympathetic nervous system. These are vasoconstrictor nerves, releasing norepinephrine (and sympathetic cotransmitters) from their varicosities which bind vascular alpha receptors, causing vasoconstriction (Fox & Edholm, 1963). These nerves increase their activity during cold stress (vasoconstriction) and decrease activity during body warming (passive vasodilation). The non-acral skin of the arms, legs, etc., possesses both the adrenergic vasoconstrictor system as well as a non-adrenergic active vasodilator system (Roddie *et al.*, 1957; Fox & Edholm, 1963). The non-adrenergic system mediates both sweating and active vasodilation via cholinergic nerve co-transmission (Kellogg *et al.*, 1995). Both sets of nerves are sympathetic in origin; however, each is activated in separate circumstances during thermal stress. In summary, cutaneous vascular regulation by the autonomic nervous system is different in acral and non-acral areas of the skin. Thus, responses to environmental stimuli in these two portions of the integument may be different.

Cutaneous vascular responses to changes in blood gas levels

Acral skin and oxygen

Around the start of the first world war, Schneider and Sisco (1914) published the first observations on the blood flow to the human hand during acute high altitude exposure. These investigators exposed 5 men to an altitude of 14,000 feet by transporting them from Colorado Springs, CO to Pike's Peak

(elevation: 14,109 ft). Their measurements were made using calorimetry, and therefore estimations of blood flow were based on the amount of heat transferred from the subject's hand to a water bath in which it was fully immersed. It was shown that exposure to high altitude caused a 30 to 76 % increase in hand blood flow, and this effect was partially reversed by the administration of supplemental oxygen. The authors concluded that low oxygen *per se* was responsible for these changes in hand blood flow because oxygen administration partially reversed the response. However, the possibility that low blood carbon dioxide levels (hypocapnia) or alkalosis may have mediated part or all of this response, secondary to increased alveolar ventilation at altitude, was not considered. The hypocapnia and alkalosis associated with hypoxic hyperpnea would also abate with the administration of supplemental oxygen, which would reduce alveolar ventilation by decreasing the firing of peripheral chemoreceptors. Nevertheless, these early observations established the possibility that some aspect of high altitude exposure is capable of increasing blood flow in the extremities of humans.

In a later study, Durand *et al.* (1969) measured blood flow in the hand over a range of local temperatures at both high altitude and sea level. Three sea level natives were studied at three different altitudes: sea level, 3,750 meters (12,300 ft), and 4,800 meters (15,750 ft). Blood flow to the hand was measured by the method of venous occlusion plethysmography with a water filled plethysmograph, and local temperature of the hand was affected by adjusting the temperature of the water filling this device. The results showed that hand blood flow was not

altered when local skin temperature was in the normal range (i.e., 33°C or below). However, as skin temperature was increased over a range of local temperatures reaching 43°C, blood flow was lower at higher altitudes, and this effect was scaled to the elevation of exposure such that it was most pronounced at 4,800 meters (Durand *et al.*, 1969). Because the hand circulation is directed mostly to skin and consists of relatively little muscle tissue, the authors concluded that high altitude causes cutaneous vasoconstriction. However, at least two issues related to this data set warrant mention. First, while this study included data from 38 subjects, some of them lowlanders and others native of high altitude, only 3 sea level natives participated in blood flow studies at each of the three altitudes. Therefore, the authors conclusions about cutaneous vasoconstriction during hypoxia are based on a sample of only 3 subjects. Second and perhaps related to the first issue, the authors failed to demonstrate an effect of local temperature on hand blood flow when water temperature was reduced below 33°C (in sea level conditions). Resting blood flow measured at thermoneutral skin temperature was low, and no further decrease was seen as local temperature was decreased to 7°C. This is contrary to the vasoconstrictor response expected during local cold exposure, and raises questions about whether relatively small but physiologically significant changes in perfusion could be detected in this study. For example, if exposure of the hand to a locally applied cold stimulus typically decreases local skin blood flow by 50% of the resting value (Johnson *et al.*, 2005), the failure to detect a difference in this study suggests that a cutaneous vascular response of equal magnitude (but possibly

opposite direction) could have occurred upon exposure to high altitude, and these changes would have been missed in this investigation. In summary, the combination of small sample size and the failure to demonstrate a decrease in hand blood flow during severe local cooling raise the possibility that a type II statistical error was made (i.e., no difference was detected in a situation where one does exist). Nevertheless, in view of the downward shift in hand blood flow observed with increasing altitude (at higher local temperatures), these data suggest there may be an interaction between local blood flow and altitude exposure in determining the cutaneous vascular response to high altitude in acral skin.

In 1983, the first simultaneous measurements of skin sympathetic vasoconstrictor nerve activity and skin blood flow were made in humans exposed to hypoxia (Kollai, 1983). This study evaluated the relationship between skin sympathetic nerve activity, recorded from the median nerve at the wrist, and finger pulse wave amplitude as measured by photoplethysmography. While hypoxic exposures were transient (90 seconds), the results demonstrated that peripheral chemoreceptor stimulation is capable of increasing sympathetic outflow directed to the skin, and that this neural activity has a measurable impact on the hand circulation. However, as noted by the author, it is unclear from these photoplethysmographic measurements which vascular segments (resistance vs. capacitance vessels) were affected by the increase in sympathetic nerve activity during hypoxia, and the 90 second exposure was too short to obtain an estimation of the net vascular response to steady state hypoxia. Nevertheless,

while these data did not address the steady state vascular response to hypoxia in the hand circulation, they demonstrated that hypoxia activates sympathetic nerve activity to the hand circulation, and that this produces vasoconstriction at some level of the vasculature.

It is important to note that the observations described above (Kollai, 1983) were made during hypoxia which also produced hypocapnia. Therefore, the possibility exists that the sympathetic or vascular responses (or both) measured were caused by the fall in blood carbon dioxide levels. Along these lines, sympathetic stimulation of forearm veins during mild hypoxia is abolished if the associated hypocapnia is corrected with supplemental CO_2 (Weil *et al.*, 1971). Inasmuch as forearm venoconstriction is confined solely to the cutaneous circulation, and is absent during a variety of stresses when the skin circulation is arrested (Zelis & Mason, 1969), these data would suggest that the hypocapnia associated with transient hypoxic exposure contributed to the increase in sympathetic nerve activity directed to the hand circulation.

The first study to address whether skin blood flow responses are different in acral vs. non-acral skin during hypoxia was performed in 1986 (Sagawa *et al.*). These investigators elegantly showed that changes in cutaneous blood flow during altitude exposure depend on the type of skin circulation being investigated, with finger blood flow (measured with venous occlusion plethysmography) and vascular conductance unchanged by 60 minutes at a simulated altitude of 5,600 meters (~ 18,400 ft). As discussed below, non-acral cutaneous vascular responses were divergent from those measured in the hand,

indicating that cutaneous vascular regulation during altitude exposure may vary across different portions of the integument (Sagawa *et al.*, 1986).

In 1998, a paper was published in *Clinical Science* in which the variability of cutaneous pulse wave amplitude was assessed at high altitude (16,300 ft) and at sea level by power spectral analysis of a photoplethysmographic signal obtained from the right index finger (Bernardi *et al.*, 1998). As with previous field studies (see above), no attempt was made to control for changes in CO_2, but these investigators controlled breathing frequency in hopes of clamping minute ventilation at altitude. Unfortunately, increases in minute ventilation occur almost exclusively via increases in tidal volume in this setting (Simmons *et al.*, 2007). Nevertheless, the results showed a reduction in "global variability" of skin blood flow, which the authors interpreted as evidence of marked cutaneous vasoconstriction at high altitude.

In summary, investigations of the acral skin vascular response to hypoxia over the past century have varied widely in both techniques used to quantify blood flow and chosen units in which to expresses vascular changes (blood flow vs. vascular conductance). None of these investigations have adequately addressed the role of hypoxic hyperpnea in measured vascular responses, nor has any attempted to account for changes in blood carbon dioxide levels as a potential mediator of these responses (none has regulated CO_2 levels). As mentioned above, increases in skin sympathetic nerve activity in the median nerve during hypoxia may indicate venoconstrictor or arteriolar vasoconstrictor signals. Furthermore, voluntary hyperventilation elicits a robust reduction in skin

blood flow of both the hands and feet, but it is unclear whether this response is mediated by low carbon dioxide levels, increased lung stretch, or elevated oxygen levels (Anderson *et al.*, 1991; Barker *et al.*, 1991). Nevertheless, the lack of evidence for sustained vasoconstriction in the hand during hypoxia may indicate that hypoxia *per se* opposes the pro-constrictive actions of either hypocapnia or hyperpnea (or both) in this circulation.

Non-acral skin and oxygen

It has been shown that the rise in blood flow to the human forearm, during either passive whole body heating or lower body dynamic exercise, is confined to the non-acral skin circulation (Detry *et al.*, 1972; Johnson & Rowell, 1975). Therefore, Rowell *et al.* (1982) measured the effect of simulated exposure to 4,500 meters on forearm vascular responses to upright cycle exercise in order to determine whether sympathetic vasoconstrictor outflow was activated toward the non-acral skin circulation. These authors hypothesized that if cutaneous vasoconstriction occurred during hypoxia, the rise in forearm blood flow during exercise would be blunted, and the forearm blood flow-esophageal temperature relationship would be rightward shifted with respect to internal temperature. The results from the 4 subjects tested in that study (*protocol 1*) did not indicate that any shift occurred in the forearm blood flow-esophageal temperature relationship during exercise. Therefore, these authors concluded that hypoxia had no appreciable effect on the cutaneous circulation. However, as discussed by the authors, any effect of hypoxia on the skeletal muscle circulation would have also affected forearm blood flow in this investigation, but this possibility was dismissed

because hypoxia was not thought to produce any net vascular response in human muscle. While the reasoning of Rowell *et al.* (1982) with respect to the influence of muscle blood flow on their data was sound, the data in support of their contention (that muscle blood flow is not increased during hypoxia) were equivocal at the time. It is now recognized that exposure to simulated high altitude elicits skeletal muscle vasodilation, although a portion of the response is masked by increased muscle sympathetic nerve activity and vasoconstriction (Leuenberger *et al.*, 1991; Weisbrod *et al.*, 2001; Moradkhan *et al.*, 2007). In addition, as discussed below, hypoxia also increases skin blood flow in the forearm, though this response is difficult to detect if small numbers of subjects are tested (Weisbrod *et al.*, 2001). Nevertheless, this response, together with hypoxic vasodilation in muscle, would further increase forearm blood flow during hypoxia, a response not observed by Rowell *et al.* (1982). Thus, it may be that exercise *per se*, or the > 20% increase in relative work rate caused by the simulated altitude of 4,500 meters, might have dominated the control of sympathetic effector responses in this investigation. With these additional factors affecting the circulation, added to the fact that responses of the study were negative, no definitive conclusions can be drawn from this investigation about regulation of non-acral skin blood flow at rest in humans.

As discussed above, Sagawa *et al.* (1986) were the first to address the concept of differential regional control of skin blood flow during hypoxia. These investigators simulated an altitude of 5,600 meters by inducing hypobaric hypoxia at an ambient temperature of 28°C. During both sea level and altitude exposure

blood flow was evaluated in the finger and the forearm (by venous occlusion plethysmography) over a range of ambient temperatures from 28°C to 38°C. These authors also addressed biophysical mechanisms of heat transfer between their subjects and the ambient environment, and this enabled estimation of the mean heat transfer coefficient between the skin and ambient for each subject both at altitude and sea level. Their results showed that forearm blood flow was upward shifted for any given change in forearm skin temperature at simulated high altitude, a response probably reflecting changes in blood flow to both skeletal muscle and non-acral skin. However, because the mean heat transfer coefficient was increased in hypobaric hypoxia, and this value was calculated using changes in skin temperature of the non-acral skin, the authors concluded that part of the forearm vasodilation represented an increase in non-acral skin blood flow. Coupled with their findings of unchanged finger vascular conductance (see above), these data provided the first demonstration that cutaneous vascular responses to hypoxia can vary across the integument.

The effects of hypobaric hypoxia were further studied by Kolka *et al.* (1987). These investigators studied the relationship between forearm blood flow and core temperature during 35 minutes of cycle exercise at 60% of the altitude specific peak VO_2. Three altitudes were tested (sea level, 2,596 meters, and 4,575 meters), and the highest altitude was associated with a reduction in the esophageal temperature-forearm blood flow relationship. These results disagreed with the findings of Rowell *et al.* (1982), and the hypobaric environment was suggested as a possible cause. Hypobaria *per se* increases

evaporative heat loss across the skin, and this effect probably played a major role in reducing skin temperature because subjects performed exercise which increased core temperature by ~ 0.60°C. Therefore, the reduced forearm blood flow for a given workload was likely mediated in part by the reduction in skin temperature during hypobaric hypoxia (Wenger et al, 1985).

The first investigation of the non-acral cutaneous vascular response to hypoxia in resting humans came in 1991. Two studies published in different journals (and with very different study aims) applied the laser Doppler flowmetry technique to investigate cutaneous microcirculatory responses during systemic hypoxia. Leuenberger *et al.* (1991) studied forearm and cutaneous vascular responses during 30 minutes of normobaric hypoxia producing a mean arterial oxygen saturation of 74%. During hypoxia, forearm blood flow rose (and vascular resistance fell) as did skin blood flow. The increase in skin blood flow was on the order of 20%, but this change failed to reach statistical significance likely due to sample size limitations (see below). Anderson *et al.* (1991) studied cutaneous vascular responses to a range of barometric pressures from 0.12 to 2.5 atmospheres absolute. Cutaneous vascular responses were studied in the skin of the calf, and an inverse relationship between ambient pressure and skin blood flow was observed. This study was important because it confirmed that cutaneous vasodilation during hypoxia is also present during hypobaria, indicating that the use of exercise and the associated high rates of evaporative heat loss probably contributed to reductions in skin temperature and blood flow observed previously during hypobaric hypoxia (Kolka *et al.*, 1987). In addition,

this study provided evidence in the leg that non-acral cutaneous blood flow varies inversely with the partial pressure of oxygen, and this observation was later confirmed in the human forearm skin (Yamazaki *et al.*, 2007).

The results of Leuenberger *et al.* (1991) were extended when Weisbrod *et al.* (2001) studied sympathetic vascular regulation during hypoxia using intra-arterial phentolamine to produce post-synaptic blockade of α-adrenergic receptors (thus inhibiting sympathetic vasoconstriction). During hypoxia, the area of skin pretreated with phentolamine displayed a greater vasodilation than the skin with intact adrenergic control (Weisbrod *et al.*, 2001). Based on these results, the authors concluded that sympathetic vasoconstriction masked a portion of hypoxic vasodilation in the cutaneous circulation as is the case in the skeletal muscle vasculature. However, Simmons *et al.* (2007) later showed that pretreatment of the skin with bretylium tosylate (for presynaptic inhibition of adrenergic nerves) did not affect the magnitude of hypoxic vasodilation, indicating that increases in sympathetic vasoconstrictor nerve activity do not oppose hypoxic vasodilation in the skin. The combined results from these two studies indicated the following: in the skin, blockade of sympathetic adrenergic nerves does not affect hypoxic vasodilation, but blockade of α-adrenergic receptors unmasks greater hypoxic vasodilation.

These seemingly disparate observations appear to be reconciled by consideration of the different loci at which phentolamine and bretylium block sympathetic vasoconstriction. For instance, phentolamine blocks postsynaptic α_1- and α_2- adrenergic receptors (Hoffman, 2001) while bretylium tosylate blocks

adrenergic nerve transmission presynaptically (Haeusler *et al.*, 1969). Blockade of α_1- and α_2- adrenergic receptors interferes with both humoral and neural adrenergic vasoconstriction, whereas only neural vasoconstriction is blocked by inhibition of sympathetic adrenergic fibers. Thus the results indicating that cutaneous vasodilation is unmasked by α-receptor blockade may indicate that humoral (adrenergic) vasoconstriction is present in the skin during hypoxia. This vasoconstriction would be sensitive to α-receptor blockade but not to presynaptic inhibition by bretylium. A likely candidate for such a circulating mediator of adrenergic vasoconstriction is epinephrine, which is present in the blood at higher concentrations during acute hypoxia (Weisbrod *et al.*, 2001). Epinephrine has been shown to cause cutaneous vasoconstriction when administered intra-arterially, intravenously, by subcutaneous injection, or by iontophoresis (Barcroft *et al.*, 1955; Cooper *et al.*, 1955; Roddie, 1983; Hoffman, 2001). Taken together, the combined observations of Weisbrod *et al.* (2001) and Simmons *et al.* (2007) suggest that adrenergic vasoconstriction of humoral origin (possibly epinephrine) may mask some of the cutaneous vasodilation during hypoxia, and adrenergic vasoconstriction of neural origin (i.e., sympathetic vasoconstrictor nerves) does not affect hypoxic vasodilation in human skin.

Acral skin and carbon dioxide

Diji (1959) first described the effect of carbon dioxide on hand blood flow in humans. Blood flow was measured by calorimetry while the hand was immersed in a water bath. When carbon dioxide was mixed into the bath, heat elimination to the water bath (index of blood flow) increased by an average of

40%. Because subjects breathed normal air and were therefore without chemoreceptor stimulation by CO_2, the effects on hand blood flow were assumed to be local in origin. Subsequent investigations probed the forearm vascular response to local administration of CO_2 as well as systemic effects mediated by central and peripheral chemoreflexes (Richardson *et al.*, 1961; Kontos *et al.*, 1967). However, in each of these investigations blood flow to the hand was occluded by inflation of a wrist cuff to suprasystolic pressure, and therefore it remains unknown if a competition exists between chemoreflex-mediated vasoconstrictor and locally-mediated vasodilator influences in the hand circulation during hypercapnia.

Non-acral skin and carbon dioxide

Carbon dioxide baths were in use for the treatment of peripheral vascular disease dating back to the late 1800s. However, the evidence for a beneficial effect caused by the direct action of CO_2 on the skin microcirculation was not demonstrated until 1942. In a simple study, Stein and Weinstein (1942) showed that the diffusion of CO_2 into the cutaneous microcirculation produced a limb hyperemia which ranged up to 150% of baseline blood flow. These results were obtained in the lower leg, and the data were the first to demonstrate that the beneficial effects of carbon dioxide bath treatment are due in part to a locally-mediated vasodilation in the skin microcirculation.

The results of Stein and Weinstein (1942) were later extended to the forearm circulation by Diji and Greenfield (1960). These authors administered subcutaneous injections of gas containing carbon dioxide concentrations ranging

from 12.5% to 100%. The results showed that skin temperature in the region of subcutaneous injection was elevated versus the temperature measured at a control site, and this response was present beginning with the lowest carbon dioxide concentrations used. After confirming that these responses were not an effect of subcutaneous gas administration, the authors concluded that the local action of carbon dioxide on the resistance vessels in the skin was responsible for local increases in blood flow. Importantly, these data were the first to demonstrate that physiological concentrations of carbon dioxide, approximated by the lowest dose of CO_2 administered (Thompson & Brown, 1960), could stimulate vasodilation in the forearm cutaneous circulation.

In order to investigate the degree to which sympathetically-mediated chemoreflex responses can oppose the local action of CO_2 on skin blood vessels during hypercapnia, Simmons *et al.* (2007) exposed 13 subjects to mild and moderate systemic hypercapnia elevating end-tidal PCO_2 by 5 and 9 torr, respectively. Cutaneous vascular responses were studied in the forearm in an area with intact vasoconstrictor control and an area in which adrenergic vasoconstriction was abolished by treatment with bretylium tosylate. The results demonstrated that both mild and moderate hypercapnia caused cutaneous vasodilation, but the mechanisms behind this response were not entirely clear. During mild hypercapnia, withdrawal of sympathetic vasoconstriction appeared to mediate most of the vasodilator response, whereas no effect of vasoconstrictor tone was observed in the moderate hypercapnia condition. Neither response was reproduced by voluntary hyperventilation when breathing frequency and

volume were matched to hypercapnic values. Thus, it appears that modest levels of arterial hypercapnia cause a slight increase in skin blood flow, and this hyperemia is mediated in part by withdrawal of sympathetic vasoconstriction. Also, a local effect of CO_2 may be present if the degree of hypercapnia is more severe, but this issue remains unclear.

In summary, current data support the notion that high concentrations of carbon dioxide administered locally to either acral or non-acral skin cause a substantial increase in cutaneous blood flow (Stein & Weinstein, 1942; Diji, 1959; Diji & Greenfield, 1960). Interestingly, it would appear that systemic induction of hypercapnia results in withdrawal of cutaneous sympathetic vasoconstrictor tone instead of the opposite response observed in the skeletal muscle circulation (Richardson *et al.*, 1961). During acute exposure to systemic hypercapnia in humans, sympathetic withdrawal appears to mediate the cutaneous vascular response to lower levels of hypercapnia, while a local effect emerges as the stress becomes more severe (see discussion in Simmons *et al.*, 2007).

Cutaneous vascular regulation during whole body cold stress

In response to whole body cold stress, skin blood flow is reduced by activation of sympathetic adrenergic nerves directed to cutaneous arterioles. The adrenergic nature of these nerves is demonstrated by the abolition of the response after pretreatment with bretylium (Kellogg *et al.*, 1989). For many years it was thought that only norepinephrine was released from cutaneous sympathetic adrenergic nerves, and that this norepinephrine mediated the whole of the vasoconstrictor response during cold stress by binding to post-synaptic α-

adrenergic receptors (Fox & Edholm, 1963; Kellogg, 2006). However, this dogma was slowly changed with the publication of numerous studies which together suggest that cutaneous vasoconstriction is mediated partly through sympathetic cotransmitter-mediated (non-noradrenergic) mechanisms.

In the 1980s, Ekblad *et al.* (1984) and Pernow *et al.* (1987) demonstrated that another putative neurotransmitter – neuropeptide Y – is commonly colocalized with norepinephrine in sympathetic perivascular nerves. These investigators used immunofluorescence to determine that neuropeptide Y is contained in the neuronal terminals of sympathetic perivascular nerves. These authors suggested that neuropeptide Y may be released with norepinephrine and play a role in regulation of local blood flow. This idea was later confirmed by the combined results of Eckberg *et al.* (1988) and Pernow *et al.* (1989a). Eckberg *et al.* (1988) demonstrated that release of neuropeptide Y into the plasma was strongly correlated ($r = 0.87$) with directly measured sympathetic nerve activity over a range of blood pressures. Next, Pernow *et al.* (1989a) showed that intra-arterial infusion of neuropeptide Y into the human forearm causes a robust vasoconstrictor response. Taken together, these combined studies demonstrated that neuropeptide Y is present in sympathetic perivascular neurons, is released by sympathetic nerves when they are activated by the autonomic nervous system, and causes vasoconstriction in the human limb circulation (Ekblad *et al.*, 1984; Pernow *et al.*, 1987; Eckberg *et al.*, 1988; Pernow *et al.*, 1989a).

In 2001, Stephens *et al.* first demonstrated that cutaneous vasoconstriction during cold stress involves a sympathetic nerve cotransmission component. These investigators administered yohimbine via intradermal injection to produce post-synaptic blockade of alpha-adrenergic receptors in the forearm skin of seven men. A separate area of forearm skin received saline and served as a control. Although yohimbine is classically thought of as an α_2 receptor antagonist, previous work has shown that it functions as a non-specific α-blocker when administered at high concentrations, and numerous studies have recently used yohimbine in the 5mM range to block both α_1- and α_2- adrenergic receptors in the cutaneous microcirculation (Stephens *et al.*, 2001; Stephens *et al.*, 2002; Stephens *et al.*, 2004; Thompson & Kenney, 2004). After sites on the skin were pretreated with either yohimbine or saline, subjects were exposed to whole body cold stress for 15 minutes while blood flow was measured over both sites using laser Doppler flowmetry. The results showed that cutaneous vasoconstriction persists in the presence of post-synaptic α-blockade, with ~ 40% of the response apparently mediated by sympathetic cotransmission (Stephens *et al.*, 2001). These same authors later showed that cotransmitter-mediated vasoconstriction was also present in women taking oral contraceptives, but only during the high hormone phase of contraceptive use (Stephens *et al.*, 2002).

In 2004, the possibility that sympathetic cotransmitter-mediated vasoconstriction involves the binding of post-synaptic neuropeptide-Y receptors was tested. Stephens *et al.* (2004) repeated the protocol in which cotransmitter-mediated vasoconstriction was demonstrated by blocking adrenergic

vasoconstriction with yohimbine. This time another skin site was included in which both adrenergic receptors as well as neuropeptide Y receptors were blocked. The authors used BIBP-3226 to selectively block neuropeptide Y-Y_1 receptors (Doods *et al.*, 1996) because this receptor subtype was previously shown to mediate vasoconstriction in the splenic and renal vascular bed of the swine (Modin *et al.*, 1991) and the mesenteric artery of the rat (Zukowska-Grojec *et al.*, 1996). The skin blood flow responses during cold stress were further blunted in the presence of neuropeptide Y receptor antagonism, and this abolished virtually all of the cotransmitter-mediated vasoconstrictor response.

In summary, the dogma which held that cutaneous vasoconstriction is mediated solely through adrenergic vasoconstriction has been revised over the past decade. It is now understood that approximately 40% of the vasoconstrictor response is non-adrenergic in origin, but appears to be sensitive to blockade of neuropeptide Y receptors. Thus, both norepinephrine and neuropeptide Y are released from sympathetic adrenergic nerves during cold stress. Norepinephrine causes cutaneous vasoconstriction through α_1- and α_2- receptors, while neuropeptide Y affects vasoconstriction via neuropeptide Y-Y_1 receptors.

Hypoxia and sympathetic vasoconstriction

Long standing interest has centered on the possibility that tissue hypoxia, through effects on the chemical milieu within the muscle, can alter the transduction of sympathetic vasoconstrictor outflow into vascular resistance (Dinenno, 2003). While the focus of this review is on the cutaneous circulation, vasoconstrictor transduction during hypoxia is relatively under investigated in the

skin compared to the skeletal muscle circulation. Therefore, pivotal studies from this body of research will be cited here in order to provide a base from which to understand the discussion of cutaneous vasoconstrictor responses in this dissertation, and how these responses might be altered by hypoxia.

A number of complex issues have obscured a clear understanding of sympathetic vasoconstrictor transduction for many decades. For example, the measured variable in most studies of vasoconstrictor transduction is either a change in blood flow, vascular resistance, or vascular conductance. However, when the environmental stress being considered (hypoxia) alters baseline blood flow, the expression of vasoconstrictor responses can be obscured by presenting values as a percentage of baseline parameters (i.e., the law of initial values). Furthermore, vascular resistance and conductance are inversely related such that when blood flow is high, a considerable change in vascular conductance produces only a small change in vascular resistance. Therefore, complications as simple as how to express data have impeded progress in this area over the years.

In addition to methodological complications, the central versus peripheral actions of hypoxia have made experimental results difficult to interpret with respect to vasoconstrictor transduction. For example, hypoxia is known to impact sensory integration within the central nervous system (Steiner *et al.*, 2002). However, the peripheral vascular effects of hypoxia can also result in functional hemodynamic changes (Heistad & Abboud, 1980). Therefore, a common limitation to some experiments in this area is that the central and

peripheral effects of hypoxia can not be disassociated from one another. Fortunately, some experimental models have controlled for this issue, and select studies from this body of literature are presented below.

In 1970, Heistad and Wheeler published the first report addressing sympathetic vasoconstrictor function during hypoxia in humans. These authors tested forearm vascular responses to a variety of sympathoexcitatory stimuli as well as intra-arterial infusions of vasoconstrictor substances. In this comprehensive approach, vasoconstrictor responses were first tested during physiologic excitation of the sympathetic nervous system and subsequently retested during infusions of exogenous norepinephrine. Lower body negative pressure and the application of ice to the forehead were used to activate centrally integrated sympathetic vasoconstrictor responses. The results demonstrated that vasoconstrictor responses to lower body negative pressure as well as ice on the forehead were reduced when subject breathed a 12% oxygen mixture. In addition, forearm vascular responses to intra-arterial infusions of norepinephrine were reduced during hypoxia, indicating that a peripheral effect of hypoxia may play a role in these altered vasoconstrictor responses (Heistad & Wheeler, 1970). Therefore, the authors concluded that sympathetic vasoconstriction is blunted in the forearm circulation during hypoxia, and a peripheral effect on vasoconstrictor transduction is a least partly responsible for this phenomenon. These authors also tested the role of hypocapnia in these responses and demonstrated that hypoxia was the primary culprit responsible for their findings.

These same authors later tested both forearm and hand vascular responses to the same sympathoexcitatory stimuli (not intra-arterial infusions) during carbon monoxide breathing. This experimental approach induces tissue hypoxia without affecting arterial oxygen tension, and so peripheral chemoreflex activation is absent or minimal with this approach. The results confirmed the author's previous findings that sympathetic vasoconstriction is reduced in the forearm, and demonstrated that similar effects are seen in the hand circulation (i.e., acral skin). These data demonstrated that reduced vasoconstriction in the limbs during hypoxia is not the result of central interactions between the peripheral chemoreflex and other major homeostatic reflexes (i.e., baroreflexes, thermoregulatory reflexes, etc), and showed that vasoconstrictor transduction in the skin circulation is also affected by hypoxia (Heistad & Wheeler, 1972). Importantly, while a role for central reflex interactions was ruled out by these experimental results, the effects of hypoxia on central integration of afferent and efferent stimuli within the major reflexes (e.g., thermoregulatory reflexes) was not addressed by this study (see paragraph above).

In 1990, Rowell and Seals measured forearm vascular responses to graded lower body negative pressure during both normoxia and hypoxia. During hypoxia, absolute changes in vascular resistance were smaller at every level of lower body negative pressure, but the percent change from baseline was the same owing to a decrease in resting vascular resistance (Rowell & Seals, 1990). These results highlight the problems with interpreting results as a percentage of baseline values when resting hemodynamics is altered. However, a comparison

of these results with those obtained by Heistad and Wheeler (1970) allows some tentative conclusions to be drawn. For example, when Heistad and Wheeler (1970) exposed subjects to hypoxia they observed no change in resting forearm vascular resistance. Therefore, the data from that study represent an opportunity to compare vascular responses during hypoxia without the confounding effects of a change in baseline blood flow. Because the percent changes in forearm vascular resistance measured by Heistad and Wheeler (1970) were diminished by hypoxia, and the absolute values measured by Rowell and Seals (1990) were similarly diminished, it appears that hypoxia is capable of decreasing sympathetic vasoconstrictor responses, at least during the application of lower body suction.

As described above, Heistad and Wheeler (1970) showed that the reduction in sympathetic vasoconstriction during hypoxia is at least partly explained by blunted vascular transduction, as indicated by reduced vasoconstrictor responses to exogenous norepinephrine. However, Dinenno *et al.* (2003) recently demonstrated that forearm vascular responses to intra-arterial infusion of tyramine were not altered by three different levels of hypoxia. In contrast to intra-arterial infusion of norepinephrine, tyramine causes endogenous release of norepinephrine from sympathetic terminals. Therefore, the discrepancy between peripheral vascular responses noted by Heistad and Wheeler (1970) and Dinenno *et al.* (2003) may relate to the exogenous versus endogenous nature of the adrenergic stimulus. For example, norepinephrine clearance is increased by hypoxia, an effect that may be mediated by enhanced axonal norepinephrine reuptake (Leuenberger *et al.*, 1991). Therefore, one

possible explanation for differing responses to exogenous and endogenous norepinephrine is that infused norepinephrine is cleared and metabolized faster during hypoxia, thus reducing the amount of catecholamine to reaches post-junctional α-adrenergic receptors. However, the mechanisms which stimulate increased clearance of norepinephrine during hypoxia are poorly understood, and the degree to which intraluminal and extraluminal (abluminal) norepinephrine are affected is unknown.

In contrast to human studies, experiments in rats consistently report blunted sympathetic vasoconstriction during hypoxia in skeletal muscle (Coney & Marshall, 2003, 2007). These studies have yielded important information because it is possible to electrically stimulate post-ganglionic sympathetic nerves in animal preparations, thereby overcoming the limitations of central integration of afferent stimuli during reflex activation. Results from these studies indicate that α-adrenergic receptors are sensitive to inhibition by hypoxia, whereas neuropeptide Y receptors are resistant to this effect (Coney & Marshall, 2007). This recent finding has significant implications for previous research in humans, and raises the possibility that discrepant findings from past studies may be explained by differential contributions from cotransmitter-mediated vasoconstrictor pathways. In support of this possibility are data demonstrating that neuropeptide Y release into human plasma is correlated with muscle sympathetic nerve activity during baroreflex-mediated changes in sympathetic outflow (Eckberg *et al.*, 1988). Therefore, different degrees of sympathetic activation in previous human studies, and thus different contributions of

cotransmitter-mediated vasoconstriction, may partially explain some of the disparate findings regarding vasoconstrictor transduction during hypoxia.

CHAPTER III

OVERVIEW OF METHODOLOGIES

Subjects

These studies were approved by the institutional review board of the University of Oregon, and each subject gave written, informed consent before participation.

Forty seven healthy, nonsmoking, normotensive subjects (25 men, 22 women), age 23 ± 3 yr, participated in this series of studies [height 175 ± 13 (SD) cm, weight 72.2 ± 15.9 kg, body mass index 23.2 ± 2.8 kg/m^2]. Subjects were taking no medications, except for oral contraceptives, and none had been to altitude (>1,500 m) within 5 months. Women in Chapters IV, V, and VII were studied either during the early follicular phase of the menstrual cycle or during the placebo phase of oral contraceptive use. In Chapter VI, only women who were using oral contraceptives were studied, and these women were tested during the high hormone phase of oral contraceptive use (Stephens *et al.*, 2002). All female subjects had a negative urine pregnancy test within 1 hour of participation.

Instrumentation

In the studies described within Chapters IV, V, and VII, all data collection took place with the subject laying supine with the exception of the drug infusion

period in the study described in Chapter VII. During the drug infusion period in that study, subjects sat semi-recumbent with their arm positioned at heart level and were later moved to the supine position for the last half of the study. Two body positions were used in that study because the duration of the protocol (7.5 hours) made it difficult for subjects to remain still in the supine position during the final hours of data collection. In the study described within Chapter VI, all data was collected with the subject seated in the semi-recumbent position inside of a custom modified jacuzzi bathtub. As with the other studies contained in this dissertation, the subject's arms were placed at heart level.

The studies described in Chapters V, VI, and VII made use of the microdialysis technique as described below. When the subject arrived on the study day, three microdialysis fibers (model MD 2000, Bioanalytical Systems, West Lafayette, IN) were placed in the skin of the ventral forearm. The microdialysis fiber (or probe) consists of a length of fine tubing impermeable to diffusion except for a 10 mm membrane that allows the diffusion of compounds no larger than 20 kDa. Fibers were placed by first inserting a 25-gauge needle through the dermis of the skin to create a path for insertion. The fiber was then threaded through the internal lumen of the needle, and the needle was withdrawn, leaving the membrane in the skin. The fiber was taped in place and then connected to a syringe via a luer tip connector at the proximal end. The fiber was then perfused with lactated Ringer's solution at a rate of 4 μl/min with a microinfusion pump (CMA/102, CMA Microdialysis, Stockholm, Sweden). Sites were at least 5 cm apart.

Pharmacological interventions

Each of the study compounds used in this dissertation has either been approved for human use by the United States Food and Drug Administration or has been administered to human volunteers previously in the research setting in the exact format used here. The mechanism of action as well as the theoretical basis for the use of each compound is described below and throughout the individual chapters. In addition, specific mixing instructions for target drug concentrations have been included in the separate appendices.

Yohimbine HCl was used throughout these studies to block both α_1- and α_2-adrenergic receptors. Yohimbine is classically thought of as a selective α_2-adrenergic receptor antagonist, and this is the main pharmacological effect when administered systemically. However, yohimbine can be delivered locally into the skin via intradermal injection or microdialysis and this method allows the administration of much higher concentrations of the drug in a small area of skin. At high local concentrations (5mM), yohimbine acts as a non-selective blocker of α-adrenergic receptors and abolishes the vasoconstrictor response to both norepinephrine and phenylephrine (α_1-receptor agonist) administration (Stephens *et al.*, 2001; Stephens *et al.*, 2002; Stephens *et al.*, 2004; Thompson & Kenney, 2004). Therefore, yohimbine has recently been used by numerous investigators to block the adrenergic vasoconstrictor effects of norepinephrine in the skin (Stephens *et al.*, 2001; Stephens *et al.*, 2002; Stephens *et al.*, 2004; Thompson & Kenney, 2004).

BIBP-3226 is a neuropeptide Y-receptor antagonist selective for the neuropeptide Y-Y_1 receptor. This drug selectively binds the neuropeptide Y-Y_1 receptor and displaces the ligand, exerting no intracellular signaling effects (i.e., competitive inhibition). *In vitro,* BIBP-3226 significantly displaces the neuropeptide Y binding curve to the right in both human and animal vascular tissues. *In vivo*, intravenous infusion of BIBP-3226 produces a dose dependent abolition of the pressor response to neuropeptide Y in the rat (Doods *et al.*, 1996). In conscious humans, BIBP-3226 delivered via intradermal microdialysis (10.5 μM) was previously shown to block sympathetic cotransmitter-mediated (non-noradrenergic) vasoconstriction during whole body cooling (Stephens *et al.*, 2004). Therefore, a similar dose of BIBP-3226 was used throughout these studies with the goal of blocking sympathetic vasoconstriction mediated through neuropeptide Y-Y_1 receptors.

Tyramine was used in the study described in Chapter VII to stimulate the release of neurotransmitters from cutaneous sympathetic adrenergic nerves. Tyramine is selectively taken up by adrenergic nerve terminals and displaces intraneuronal transmitters from their storage vesicles, causing a transient release of neurotransmitter into the extracellular space. Tyramine is considered an indirect acting sympathomimetic agent insofar as it possesses no direct vasoconstrictor properties on vascular smooth muscle (Lundberg *et al.*, 1989b; Hoffman, 2001). Previous research has provided mixed results regarding which neurotransmitters are released from sympathetic terminals upon stimulation with tyramine. For example, Lundberg *et al.* (1989b) showed that tyramine

stimulation of the pig spleen *in vivo* caused vasoconstriction that was associated with an increase in norepinephrine outflow but no change in neuropeptide Y outflow in the venous effluent draining the isolated organ. Later, Takiyyuddin *et al.* (1994) demonstrated that intravenous infusion of tyramine caused an increase in arterial pressure and plasma norepinephrine, while plasma neuropeptide Y was unaltered. In contrast, Cheng *et al.* (1987) demonstrated that tyramine releases both norepinephrine and neuropeptide Y from sympathetic terminals of the rabbit jejunum, but that neuropeptide Y is only released at higher concentrations of tyramine. In addition, tyramine was shown to cause a dose dependent rise in neuropeptide Y release from sympathetic terminals of the rat vas deferens (Cheng & Shen, 1987). Therefore, it appears that tyramine stimulates the release of neuropeptide Y with norepinephrine in certain tissues, whereas norepinephrine is released alone in others. Another aspect that may affect the release of neuropeptide Y by tyramine is the mode of delivery to the target tissue. For example, the studies which have documented neuropeptide Y release from sympathetic terminals have delivered tyramine to neuronal tissues via the extracellular fluid. In contrast, those studies indicating that tyramine does not stimulate neuropeptide Y release have delivered tyramine through intraluminal infusion (arterial or venous supply). Therefore, the intraluminal versus abluminal delivery of tyramine may cause different outcomes regarding neurotransmitter release from sympathetic terminals, in agreement with studies on other vasoactive compounds (Marshall, 2007). Tyramine was delivered in

Chapter VII by microdialysis so as to introduce it directly into the cutaneous interstitium.

Propranolol was used in Chapters V and VI to block β-adrenergic receptors, which have been identified in the skin circulation of humans (Crandall *et al.*, 1997). Propranolol is a non-selective β-adrenergic receptor antagonist that possesses no α-adrenergic blocking properties (Hoffman, 2001).

Environmental stress

Cold exposure

Cold stress was achieved via three different methods in this series of studies. The three cooling methods were similar insofar as cold stress was applied to a large portion of the body to induce reflex cutaneous vasoconstriction. Local cold exposure in the region of blood flow measurement was prevented either by confining the cold exposure to other regions of the body or by local heating, or both. The cutaneous vascular response to local cooling of the skin induces a vasoconstrictor response which is mediated by different mechanisms than the reflex response initiated when a larger portion of the body is cooled (Kellogg, 2006). The local cooling response involves an early phase in which vasoconstriction is mediated through α-adrenergic receptors and dependent on sympathetic vasoconstrictor nerves, and a latter phase in which vasoconstriction is non-neural, non-noradrenergic, and appears to be mediated through an effect of cold temperature on intracellular mechanisms within the vascular smooth muscle (Kellogg, 2006; Thompson-Torgerson *et al.*, 2008).

In Chapters IV and part of Chapter V, subjects wore a water-perfused suit that covered the entire body surface except for the head, hands, feet, and forearm where measurement of vascular responses was performed. To induce cold stress, the water circulating through the whole body suit was cooled either progressively or in a ramp protocol (see Chapters IV and V). Because the suit makes contact with the skin surface of the subject, the contribution of conductive heat transfer away from the skin surface using this technique is relatively high compared to ambient temperature cooling. In the second protocol contained within Chapter V, whole body cold stress was induced by progressively decreasing the temperature of the environmental chamber in which the subject lay supine (i.e., ambient temperature cooling). Because the surface on which the subject lay was not being directly cooled, and air movement was considerable within the chamber, the mechanisms through which heat loss were increased were largely convective and radiative (although conductive heat loss to the bed likely increased somewhat). In the study described within Chapter VI, cold exposure was induced by cooling a tub of water in which the subject was immersed to the sternum. Water was used to cool the subject in Chapter VI because the goal of the second phase of the study was to induce a decline in core body temperature. Conscious humans are resistant to changes in core body temperature owing to robust thermoregulatory reflexes including peripheral vasoconstriction and shivering. Therefore, the use of water for body cooling is necessary if a rapid decline in body temperature is desired because water increases the rate of heat transfer from the skin surface by 20 times (Santee &

Gonzalez, 1988). The water bathing the subject was cooled by the addition of ice, and a jacuzzi circulation system maintained the homogeneity of water temperature within the tub.

Changes in blood gases

To achieve control of respiratory gases, subjects breathed from a custom built breathing circuit using a scuba mouthpiece and nose clip. The inspiratory mixture provided at the mouthpiece originated from 3 gas tanks – air (20.93% O_2), nitrogen (100% N_2), and carbon dioxide (100% CO_2) – connected to the inspiratory line by flow regulators. The fraction of inspired oxygen (FIO_2) and carbon dioxide ($FICO_2$) were varied by adjusting the flowrate from each of the gas tanks, and the inspired air was mixed as it filled a 6 liter tube which served as an inspiratory reservoir. The 6 liter tube connected to the breathing circuit proximal to the mouthpiece, and its distal end was open to room air. Total flowrate through the breathing circuit was always greater than the subjects' minute ventilation, and thus excess gas delivered through the inspiratory line escaped to the room through the 6 liter tube. The mouthpiece consisted of two one-way valves (Rudolph) to insure inspiration from the breathing circuit and expiration to room air, except in the study described in Chapter VI. In that study, expiratory gases were passed through a mixing chamber integrated with a mass spectrometry system for the measurement of whole body oxygen consumption.

Measurements

Heart rate

Heart rate was monitored throughout each protocol using a 3-lead electrocardiogram (Cardiocap/5, Datex-Ohmeda, Madison, WI).

Arterial pressure

Systolic (SBP) and diastolic (DBP) arterial pressure were measured from the brachial artery via oscillometry (Cardiocap/5, Datex-Ohmeda, Madison, WI) throughout each protocol. Mean arterial pressure was calculated as DBP + ((SBP-DBP)/3). In addition, arterial pressure was measured on a beat to beat basis by photoplethysmography (Finometer, Finapres Medical Systems BV, Arnhem, the Netherlands) in the study described within Chapter IV. However, during this study it was determined that this method fails to adequately track changes in arterial pressure during cold stress, likely due to a reduction in distal extrernity blood flow. Therefore, these measurements were not included in any of the analyses for Chapter IV.

Arterial oxygen saturation

Arterial O_2 saturation was measured during the studies described in Chapters IV, VI, and VII via pulse oximetry (Cardiocap/5, Datex-Ohmeda, Madison, WI) on the earlobe.

End-tidal PCO_2

The partial pressure of carbon dioxide (PCO_2) in the alveoli was estimated by measuring end-tidal PCO_2 via infrared capnography (Cardiocap/5, Datex-Ohmeda, Madison, WI) or mass spectrometry (Marquette MGA 1100, MA Tech

Services, St. Louis, MO) throughout these protocols. Infrared capnography makes use of the fact that CO_2 absorbs light in the infrared (IR) range, and the amount of CO_2 in a sample is determined relative to the amount of IR light absorbed when a beam is passed through the sample. Mass spectrometry measures the mass to charge ratio of molecules ionized and accelerated in a vacuum. Therefore, CO_2 in a given sample can be separated and expressed as a percentage or partial pressure of the total gas sample.

Minute ventilation

Tidal volume was measured throughout these protocols using a turbine pneumotach (VMM-400, Interface Associates, Laguna Niguel, CA). Peak values of the inspiratory volume tracing were identified and determined along with their interval length using CODAS software (WinDaq, Dataq Instruments, Akron, OH). Minute ventilation was calculated as the product of tidal volume and respiratory frequency and expressed in liters of inspired volume per minute.

Whole body oxygen consumption

Whole body VO_2 was assessed during normoxia and hypoxia via indirect open circuit calorimetry using the following equation: $VO_2 = V_E ((1 - (F_EO_2 + F_ECO_2)) / F_IN_2 \times F_IO_2 - F_EO_2)$ where VO_2 is whole body oxygen consumption, V_E is minute ventilation, F_EO_2 and F_ECO_2 are the fractions of expired oxygen and carbon dioxide, and F_IN_2 and F_IO_2 are the fractions of inspired nitrogen and oxygen, respectively. V_E was measured as described above, F_EO_2 and F_ECO_2 were measured by passing the expired respiratory gases through a mixing chamber (Parvomedics, Sandy, UT) integrated with a mass spectrometry system

(Marquette MGA 1100, MA Tech Services, St. Louis, MO), F_IN_2 is constant, and F_IO_2 was measured from the peak of the tidal PO_2 tracing on a minute by minute basis.

Skin blood flow

Skin blood flow was measured in each of these studies by laser-Doppler flowmetry on the ventral surface of the forearm. This technique measures skin blood flow by directing low power light from a monochromatic stable laser at the skin surface. The emitted light is scattered by moving red blood cells in the cutaneous microvasculature and photodetected to yield a perfusion unit termed 'flux'. The flux value measured is proportional to the product of the average speed and number of red blood cells moving through the cutaneous microvasculature to a depth of approximately 1mm (measured tissue volume ~ 1 mm^3). This value has been validated against measurements of absolute skin blood flow during thermal stress, and correlations of 0.94 to 0.98 were observed between the cutaneous vascular response to whole body heating as measured by laser-Doppler flowmetry and venous occlusion plethysmography (Johnson *et al.*, 1984). Skin blood flow was divided by mean arterial pressure to calculate cutaneous vascular conductance, and measurements were scaled to values recorded during baseline conditions (Thompson & Kenney, 2004).

Skin temperature

Weighted mean skin temperature was measured using copper-constantan thermocouples on representative areas of the skin (see Chapters V and VI). Thermocouples measure skin temperature based on the non-linear relationship

that exists between temperature and the flow of voltage between two wires that are fastened in contact and insulated (copper and constantan). The signal obtained from the thermocouples was first linearized and then transferred to the data acquisition system for recording. The temperature of the water bath in the study described within Chapter VI was also measured using a thermocouple. All thermocouples were calibrated prior to the study with a two point calibration using water baths of known temperature.

Core temperature

Internal body temperature was measured using a CoreTemp (HQInc, Palmetto, FL) wireless core body temperature telemetry based monitoring system. In this method, core temperature is measured by an ingestible pill that is designed for human use (approved by the FDA) and is accurate to 0.1°C. The subjects ingested the pill at least 6 hours before the study, or the night before if the study began early in the morning. This system allowed for core temperature to be directly recorded in real time, and this method has been validated against rectal temperature measurements during interval running trials (Gant *et al.*, 2006). In validation studies intestinal pill temperature measurements have given readings ~ 0.15°C higher than the rectal temperature probe, but changes during heating and cooling are tracked well by this method.

Data acquisition and analysis

The majority of data collected during these protocols were digitized with signal-processing software (WinDaq, Dataq Instruments, Akron, OH) and analyzed off-line. Notable exceptions were core temperature measurements and

brachial artery blood pressures, which were recorded on paper during the experiment. Statistical analyses were performed with SAS statistical software using PROC MIXED (SAS v9.1.3, SAS Institute, Cary, NC). Details of specific analyses are provided within the individual chapters and depended on the design of the study. A theme throughout these analyses was the univariate analysis of variance (ANOVA), performed with one, two, or three factors depending on the circumstance. The paired t-test was also used for certain analyses when appropriate.

CHAPTER IV

EFFECT OF CO_2 REGULATION ON THE CUTANEOUS VASCULAR RESPONSE TO HYPOXIA AND MILD COLD STRESS

Introduction

It is well established that low oxygen levels exert a relaxing effect on peripheral blood vessels in the human limb circulation (Schneider & Sisco, 1914; Sagawa *et al.*, 1986; Anderson *et al.*, 1991; Leuenberger *et al.*, 1991; Weisbrod *et al.*, 2001; Simmons *et al.*, 2007). In the skeletal muscle circulation and likely the circulation to the hand, locally and humorally-mediated hypoxic vasodilation is opposed by increases in sympathetic vasoconstrictor nerve activity (Kollai, 1983; Saito *et al.*, 1988; Weisbrod *et al.*, 2001). However, we have recently shown that hypoxic vasodilation in the non-acral skin of the forearm is not restrained by neurally-mediated sympathetic vasoconstriction (Simmons *et al.*, 2007). It was suggested that this "unchecked" cutaneous vasodilation may have important thermoregulatory consequences inasmuch as relatively small changes in resting skin blood flow can have large impacts on convective heat loss.

During whole body cold stress, skin blood flow is reduced in order to decrease heat transfer from the core to the periphery, thereby promoting heat conservation (Hamlet, 1988). This thermoregulatory adjustment is vital to the prevention of hypothermia in cold environments. However, when cold and

hypoxia are encountered simultaneously, the rate of core cooling is accelerated, and lower core temperatures are reached compared to sea level conditions (Cipriano & Goldman, 1975; Johnston *et al.*, 1996). While core temperature falls faster at altitude, skin temperature remains higher throughout cold exposure compared to sea levels conditions (Cipriano & Goldman, 1975; Blatteis & Lutherer, 1976). Taken together, these results indicate a less effective reduction in core-to-skin heat transfer during cold exposure at altitude. Importantly, we are aware of no studies which have directly measured skin blood flow during combined hypoxia and whole body cold stress.

Previous studies of the cutaneous vascular response to hypoxia have failed to address the potential role of carbon dioxide in mediating these changes. The vasodilator action of carbon dioxide on cutaneous blood vessels was first demonstrated by Stein and Weinstein (1942) who showed that adding carbon dioxide to a water bath increased blood flow in the immersed calf by 60%. Diji and Greenfield (1960) later extended these observations to encompass the peripheral effects of physiological levels of carbon dioxide. More recently, we have shown that systemic hypercapnia elicits cutaneous vasodilation at levels commonly encountered in patients with respiratory disease (Simmons *et al.*, 2007). However, these responses may have been dominated by the systemic actions of hypercapnia rather than local vascular effects. Conversely, the hypocapnia associated with hypoxia exposure has been shown to mediate both arterial dilation and venous constriction in human limbs (Black & Roddie, 1958;

Weil *et al.*, 1971). The degree to which *hyper*capnia or *hypo*capnia affects the cutaneous vascular response to hypoxia remains unknown.

With the above information as a background, this study was designed around three main goals. First, we sought to test the effect of changes in carbon dioxide on the cutaneous vascular response to hypoxia. We hypothesized that the magnitude of hypoxic vasodilation is directly related to the blood carbon dioxide level (i.e., *hypo* vs. *hyper*capnia). The second study aim was to determine whether hypoxic vasodilation persists during whole body cooling. We hypothesized that skin blood flow is upward shifted by hypoxia throughout the duration of whole body cold exposure. Lastly we sought to determine if the rate of fall in blood oxygen levels during exposure to hypoxia affects the magnitude of vascular response in the skin. This last question was stimulated by pilot work in our laboratory, which suggested that a greater vasodilation is observed if hypoxia is induced over a short timecourse (a few minutes) rather than over ten minutes or more. Therefore, we tested the hypothesis that rapid induction of hypoxia (in < 2 minutes) produces a greater cutaneous vascular response than progressive hypoxic exposure.

Methods

This study was approved by the institutional review board of the University of Oregon, and each subject gave written, informed consent before participation.

Subjects

Fourteen healthy, nonsmoking, normotensive subjects (7 men, 7 women), age 23 ± 3 yr, participated in this study [height 178 ± 12 (SD) cm, weight 75.5 ±

19.5 kg, body mass index 23.6 ± 3.5 kg/m^2]. Subjects were taking no medications, except for oral contraceptives, and none had been to altitude (>1,500 m) within 5 months. Women were studied either during the early follicular phase (1–4 days after the onset of menstruation) of the menstrual cycle or during the placebo phase of oral contraceptive use to minimize the potential effects of female hormones on these responses. All female subjects had a negative urine pregnancy test within 1 hour of participation.

Instrumentation and measurements

Experiments were performed in thermoneutral conditions with the subject supine, wearing a whole body suit perfused with 34°C water, except during cold stress. The water-perfused suit covered the entire body surface except for the head, hands, feet, and forearm where measurement of vascular responses was performed. After donning the water perfused suit, subjects were instrumented for the measurement of heart rate via electrocardiography (Cardiocap/5, Datex-Ohmeda, Madison, WI), ventilation via turbine pneumotach (VMM-400, Interface Associates, Laguna Niguel, CA), arterial pressure via brachial artery oscillometry (Cardiocap/5) and photoplethysmography (Finometer, Finapres Medical Systems BV, Arnhem, the Netherlands), arterial O_2 saturation via pulse oximetry (Cardiocap/5), and end-tidal PO_2 and PCO_2 via mass spectrometry (Marquette MGA 1100, MA Tech Services, St. Louis, MO). Isocapnia/eucapnia was defined as the mean end-tidal PCO_2 (nasal cannula) during a 5-minute period of quiet breathing. To obtain an index of skin blood flow, cutaneous red blood cell flux was measured on the ventral forearm by laser-Doppler flowmetry (DRT4, Moor

Instruments, Devon, UK) with integrated laser-Doppler probes fixed to the skin with adhesive tape. Skin blood flows were expressed as cutaneous vascular conductance (red blood cell flux/mean arterial pressure) and normalized to baseline values as described previously (Simmons *et al.*, 2007).

Protocol

After instrumentation, subjects were familiarized with a custom built breathing circuit equipped with scuba mouthpiece and nose clip. When subjects were comfortable breathing from the mouthpiece, an initial cold stress was performed while subjects breathed normoxic gas and isocapnia was maintained. To induce cold stress, the water circulating through the whole body suit was cooled at a rate of 2°C min^{-1} from 34°C to 14°C during a 10 minute period. This 10 minute cooling period was preceded by 5 minutes of baseline data collection, and subjects were removed from the breathing apparatus and re-warmed upon completion of the cold stress. A resting period lasting 20 minutes followed this initial cold stress. Pilot data was collected in three subjects to insure that the cutaneous vascular response to 10 minutes of aggressive whole body cooling is reproducible after 20 minutes. During these pilot studies, cutaneous vascular conductance decreased to 66 ± 5.7 % baseline and 69.5 ± 1.7 % baseline before and after 20 minutes of supine rest (P = 0.563 for pre vs. post supine rest).

Following the first cold stress and 20 minute resting period, subjects were exposed to systemic hypoxia 4 different times in random order, and each exposure was separated by another 20 minutes of quiet supine rest. Pilot data was also collected to insure that the cutaneous vascular response to hypoxia is

reproducible after 20 minutes. During these pilot studies, arterial oxygen saturation decreased to 80.5 ± 0.9 % and 82.7 ± 0.2 % and cutaneous vascular conductance increased to 127.5 ± 1.7 % baseline and 129.3 ± 2.1 % baseline before and after 20 minutes of supine rest (n = 2).

One of the 4 hypoxic exposures was designated as "rapid", during which arterial O_2 saturation was decreased to 80% within 2 minutes. This exposure lasted 10 minutes and was preceded by 5 minutes of baseline data collection. Carbon dioxide levels were not controlled during the rapid hypoxic exposure (i.e., poikilocapnia). The other 3 hypoxic exposures were identical to one another with the exception of how carbon dioxide levels were controlled. During the 3 identical hypoxic exposures, 5 minutes of baseline data was collected initially. Hypoxia was then induced by decreasing the fraction of inspired oxygen (FIO_2) progressively over a 10 minute period. The rate of decrease in FIO_2 was designed to produce a fall in arterial O_2 saturation of ~ 2% min^{-1} down to 80%. After the target level of hypoxia was reached, 5 minutes of steady state data were recorded before a cold stress lasting 10 minutes was performed exactly like the first. Subjects were re-warmed and allowed to breathe room air after whole body cooling was complete. This hypoxic exposure (with cold stress) was performed once while end-tidal PCO_2 was allowed to fall during hypoxia (poikilocapnia), once while end-tidal PCO_2 was clamped at resting levels (isocapnia), and once while end-tidal PCO_2 was elevated by 3 mmHg (hypercapnia). All values were recorded continuously prior to and throughout

each simulated environmental stress. The exception was brachial artery blood pressure which was recorded every 5 minutes.

Control of breathing mixture

To achieve control of tidal gases, subjects breathed from a custom built breathing circuit using a scuba mouthpiece and nose clip. The inspiratory mixture provided at the mouthpiece originated from 3 gas tanks – air (20.93% O_2), nitrogen (100% N_2), and carbon dioxide (100% CO_2) – connected to the inspiratory line by flow regulators. The fraction of inspired oxygen (FIO_2) and carbon dioxide ($FICO_2$) were varied by adjusting the flowrate from each of the gas tanks, and the inspired air was mixed as it filled a 6 liter tube which served as an inspiratory reservoir. The 6 liter tube connected to the breathing circuit proximal to the mouthpiece, and its distal end was open to room air. Total flowrate through the breathing circuit was always greater than the subjects' minute ventilation, and thus excess gas delivered through the inspiratory line escaped to the room through the 6 liter tube. The mouthpiece consisted of two one-way valves (Rudolph) to insure inspiration from the breathing circuit and expiration to room air.

Data acquisition and analysis

Data were digitized with signal-processing software (WinDaq, Dataq Instruments, Akron, OH) and analyzed off-line. All data (except brachial blood pressures) were averaged over 2.5 minute intervals and analyzed with SAS statistical software using PROC MIXED (SAS v9.1.3, SAS Institute, Cary, NC). Because clear differences were apparent in the responses of males and females

to hypoxia and cold stress, these two groups were separated for data analysis. Within sex, results were analyzed with a 2 way ANOVA testing for the effects of time and condition.

Because changes in baseline skin blood flow can affect interpretation of cutaneous vascular responses during cold stress (Hodges *et al.*, 2007), we expressed decreases in cutaneous vascular conductance during whole body cooling in terms of the "absolute" change as well as the "relative" change. The "absolute" decrease in cutaneous vascular conductance was defined as the magnitude of drop during cold stress expressed as a function of normoxic, isocapnic baseline values (recorded before hypoxic exposure). The "relative" decrease in cutaneous vascular conductance was defined as the magnitude of drop during cold stress expressed as a function of steady state hypoxic values recorded immediately prior to initiation of whole body cooling. In this way, any effect of a baseline shift in cutaneous vascular conductance (during hypoxia) on the interpretation of vasoconstrictor responses during cold stress should be apparent in our results. Differences were considered statistically significant when $P < 0.05$. All values are presented as means ± SE unless otherwise indicated.

Results

Effect of CO_2 on responses to progressive hypoxia

Figure 1 shows cutaneous vascular and cardiorespiratory responses to the three progressive hypoxia conditions in male subjects. As planned, oxyhemoglobin saturation decreased to ~ 80% with only slight differences in the

kinetics of this response between conditions ($P < 0.001$ for condition x time interaction). End-tidal PCO_2 rose by ~ 2 mmHg during hypercapnic hypoxia and fell by ~ 5 mmHg during poikilocapnic hypoxia, with only minimal changes from pre-exposure baseline during isocapnic hypoxia ($P < 0.001$ for condition x time interaction). Importantly, small decreases in end-tidal PCO_2 during isocapnic hypoxia do not represent a deviation from eucapnic values, but rather from slightly elevated values at baseline during mouthpiece breathing. Minute ventilation and heart rate both increased the most during hypercapnic hypoxia, while minute ventilation did not change during poikilocapnic hypoxia (condition x time interaction: $P < 0.001$ for both minute ventilation and heart rate). Cutaneous vascular conductance increased in all three conditions during progressive hypoxia, with blood flow most consistently elevated during the isocapnic condition ($P = 0.045$ for condition main effect, $P = 0.063$ for condition x time interaction). Mean arterial pressure was increased by hypercapnic (88.6 ± 4.5 vs. 95.3 ± 4.8 mmHg; $P = 0.020$) but unchanged by poikilocapnic (87.4 ± 3.9 vs. 90.9 ± 4.0 mmHg; $P = 0.150$) and isocapnic hypoxia (89.2 ± 3.3 vs. 89.6 ± 4.3 mmHg; $P = 0.857$; $P = 0.012$ for condition x time interaction).

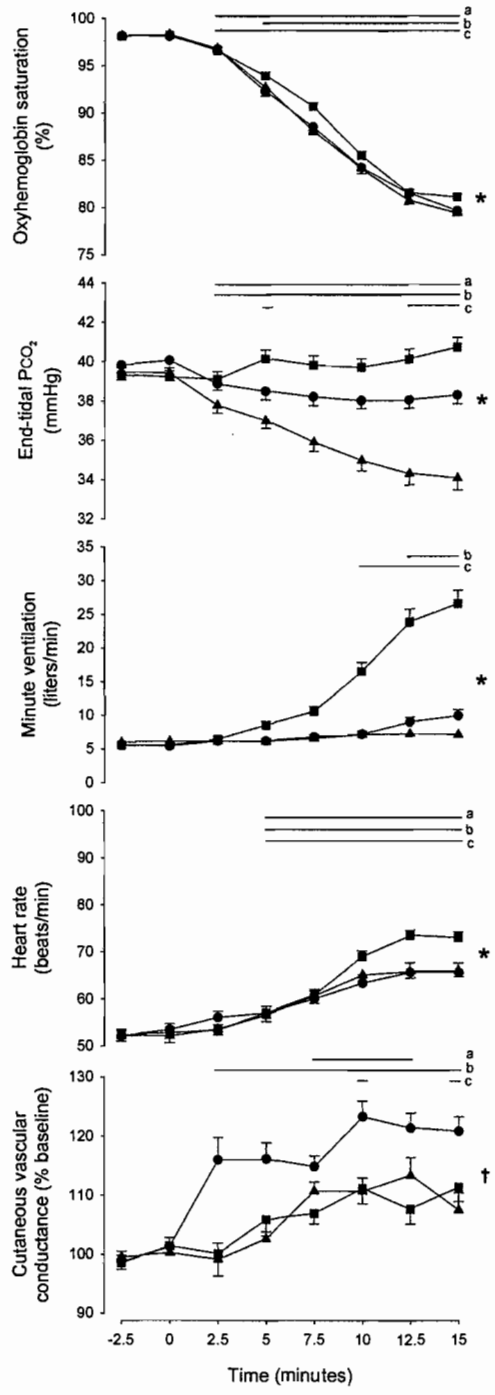

Figure 1. Cutaneous vascular and cardiorespiratory responses to progressive hypoxia in male subjects. Triangles = poikilocapnic hypoxia, circles = isocapnic hypoxia, squares = hypercapnic hypoxia. Values are means ± SE; $n = 7$. Horizontal lines indicate time points significantly different from time 0 ($P < 0.05$ for [a] poikilocapnic, [b] isocapnic, and [c] hypercapnic hypoxia). † $P < 0.05$ for condition main effect; * $P < 0.05$ for condition x time interaction effect.

Figure 2 shows cutaneous vascular and cardiorespiratory responses to the three progressive hypoxia conditions in female subjects. Cardiorespiratory responses were similar to those displayed in figure 1. However, cutaneous vascular conductance was unchanged by progressive hypoxia during any of the conditions ($P = 0.178$, $P = 0.624$, and $P = 0.941$ vs. baseline for poikilocapnic, isocapnic, and hypercapnic hypoxia).

Cutaneous vasoconstrictor responses during whole body cooling

Figure 3 shows cutaneous vascular responses to whole body cooling in males (A) and females (B) during normoxia and poikilocapnic, isocapnic, and hypercapnic hypoxia. In male subjects, the decline in cutaneous vascular conductance during whole body cooling was upward shifted across all time points in the isocapnic hypoxia condition ($P < 0.05$ vs. normoxia at all time points; $P = 0.018$ for condition main effect). However, during both poikilocapnic and hypercapnic hypoxia, cutaneous vascular conductance was not significantly different vs. normoxia at any point during the cold stress (all $P > 0.05$). In female subjects, cutaneous vascular conductance during the cold stress was higher than the normoxic condition in all three hypoxic conditions between minutes 5 and 7.5 (all $P < 0.05$), while this trend only remained through the end of the cold stress in the poikilocapnic condition ($P = 0.004$ vs. normoxia; $P < 0.001$ for condition x time interaction).

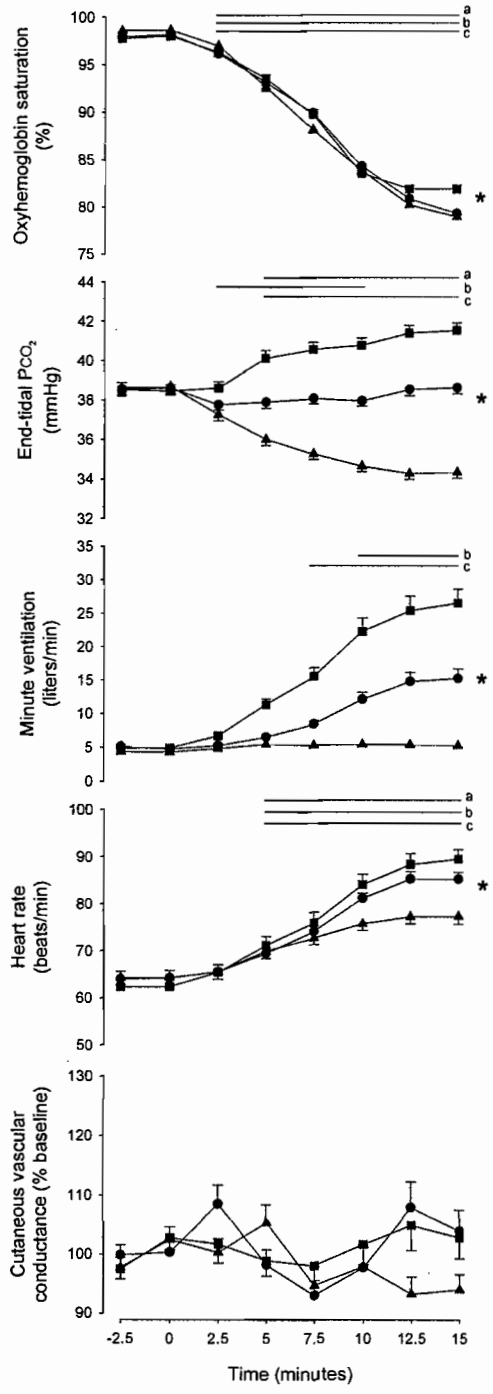

Figure 2. Cutaneous vascular and cardiorespiratory responses to progressive hypoxia in female subjects. Triangles = poikilocapnic hypoxia, circles = isocapnic hypoxia, squares = hypercapnic hypoxia. Values are means ± SE; $n = 7$. Horizontal lines indicate time points significantly different from time 0 ($P < 0.05$ for [a] poikilocapnic, [b] isocapnic, and [c] hypercapnic hypoxia). * $P < 0.05$ for condition x time interaction effect.

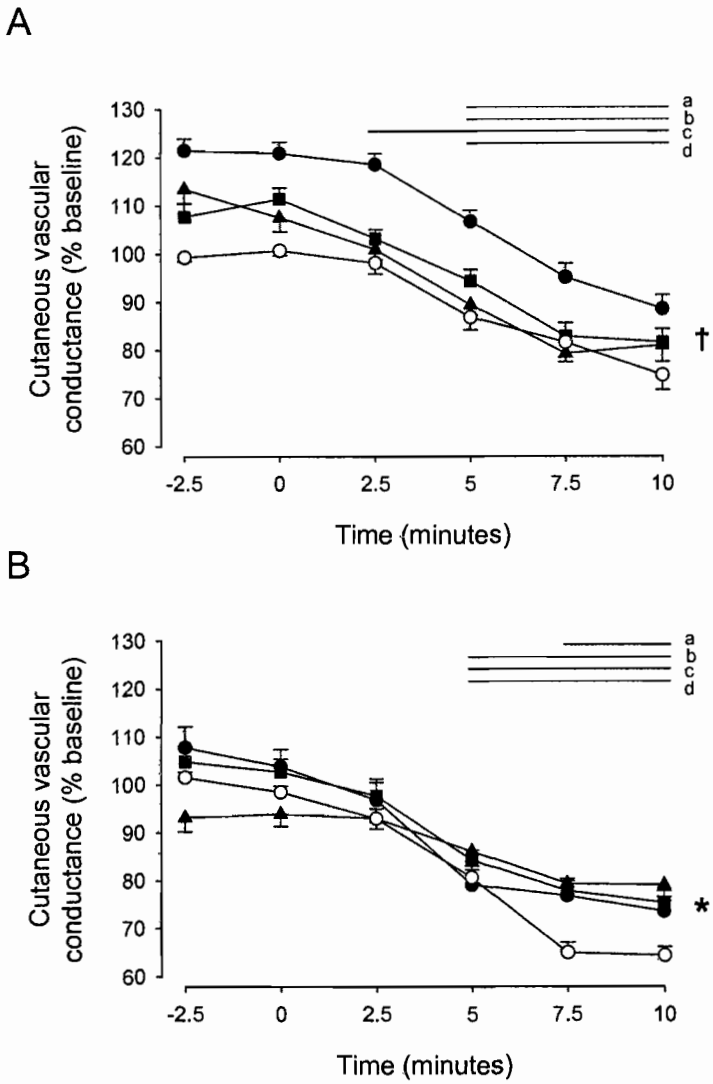

Figure 3. Cutaneous vascular responses to whole body cooling during normoxia (open circles) and poikilocapnic (triangles), isocapnic (filled circles), and hypercapnic (squares) hypoxia. Responses from male subjects (A) are displayed on the top panel and female responses (B) are displayed on the bottom panel. Values are means ± SE for 7 males and 7 females. Horizontal lines indicate time points significantly different from time 0 ($P < 0.05$ for [a] poikilocapnic hypoxia, [b] isocapnic hypoxia, [c] hypercapnic hypoxia, and [d] normoxia). † $P < 0.05$ for condition main effect; * $P < 0.05$ for condition x time interaction effect.

Figure 4 displays the reduction in cutaneous vascular conductance in response to cold stress during normoxic and hypoxic conditions in male (A) and female (B) subjects. Responses during hypoxia are contrasted with those during

normoxia, and are displayed as the "absolute" and "relative" change (see *methods*). In men, cutaneous vascular conductance was reduced by ~ 25 – 30% during cold stress with no differences between conditions (all $P > 0.05$). In women, cutaneous vascular conductance was reduced by ~ 25 – 35% during cold stress, except in the poikilocapnic condition when the magnitude of vasoconstriction was reduced by approximately half ($P = 0.031$ and $P = 0.023$ vs. normoxia for absolute and relative changes, respectively). Cardiorespiratory responses during whole body cooling in each condition are displayed in table 1.

Rapid vs. progressive hypoxic exposure

Cutaneous vascular responses to rapidly induced poikilocapnic hypoxia are contrasted with those to progressive poikilocapnic hypoxia in figure 5. The mean time taken to achieve target oxyhemoglobin saturation (80%) was 105.6 seconds (min: 69 sec, max: 152 sec) during rapid hypoxia and 588.8 seconds (min: 509 sec, max: 741 sec) during progressive hypoxia. In men, a sustained elevation in cutaneous vascular conductance was observed after the induction of rapid hypoxia ($P = 0.025$ vs. baseline). However, the elevation in cutaneous vascular conductance observed during the induction of progressive hypoxia (see figure 1) was not sustained through 5 minutes of steady state measurement ($P = 0.262$ vs. baseline for final time point before cold stress). In women, cutaneous vascular conductance was not significantly different from baseline after the induction of rapid or progressive poikilocapnic hypoxia ($P = 0.934$ and $P = 0.314$ vs. baseline for rapid and progressive hypoxia, respectively). While only men responded to hypoxia with vasodilation, the direction of the trends observed

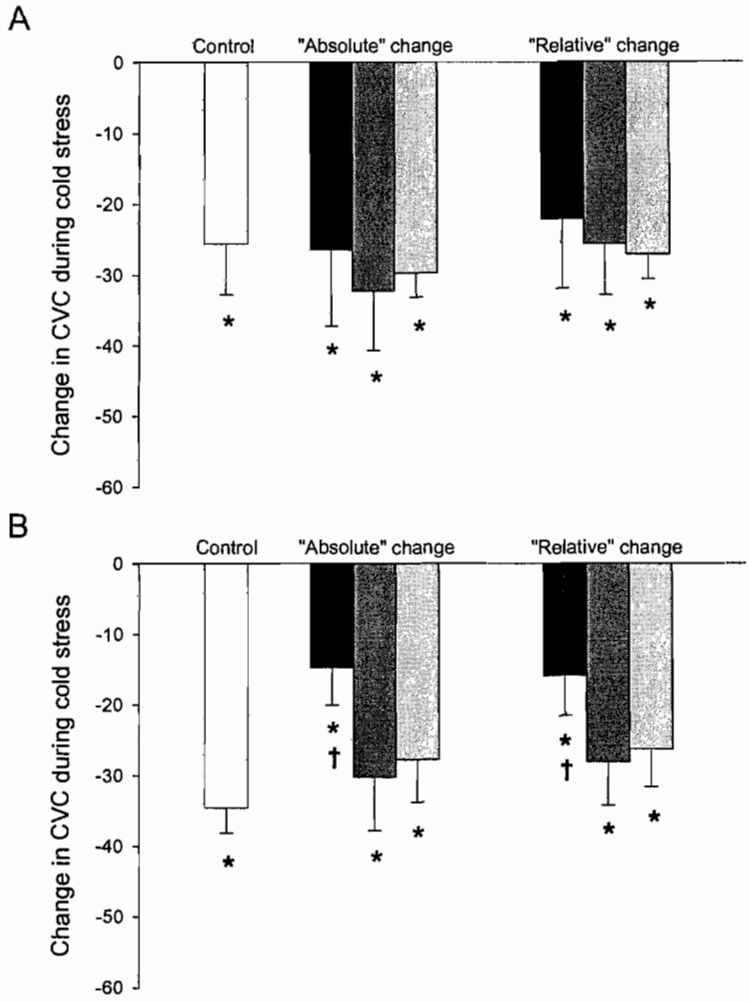

Figure 4. Vasoconstrictor responses to whole body cooling during hypoxia expressed as a function of two different baselines. White bars = normoxia (i.e., control), black bars = poikilocapnic hypoxia, dark grey bars = isocapnic hypoxia, light grey bars = hypercapnic hypoxia. Responses from male subjects (A) are displayed on the top panel and female responses (B) are displayed on the bottom panel. Values are means ± SE for 7 males and 7 females. * indicates significant reduction in CVC during whole body cooling ($P < 0.05$). † $P < 0.05$ vs. control response. See *methods* for definitions of "absolute" and "relative" change in CVC.

Table 1. Cardiorespiratory responses to whole body cooling during normoxia and poikilocapnic, isocapnic, and hypercapnic hypoxia. Values are means ± SE for 7 males and 7 females. * $P < 0.05$ vs. thermoneutral within sex and condition. [a] $P < 0.05$ vs. normoxia within time and sex. [b] $P < 0.05$ vs. poikilocapnic hypoxia within time and sex. [c] $P < 0.05$ vs. isocapnic hypoxia within time and sex.

	Normoxia		Poikilocapnic hypoxia		Isocapnic hypoxia		Hypercapnic hypoxia	
	Thermoneutral	Cold	Thermoneutral	Cold	Thermoneutral	Cold	Thermoneutral	Cold
End-tidal P_{O_2} (mmHg)								
Men	101.3 ± 0.8	108.7 ± 1.4*	44.5 ± 0.5[a]	46.8 ± 0.7*[a]	45.6 ± 0.4[a]	46.8 ± 0.4[a]	47.1 ± 0.4[ab]	46.7 ± 0.0[a]
Women	103.8 ± 1.0	108.0 ± 0.7*	47.6 ± 0.4[a]	48.1 ± 0.5[a]	47.9 ± 0.5[a]	48.7 ± 0.4[a]	51.0 ± 0.8[ab]	50.0 ± 0.4[a]
End-tidal P_{CO_2} (mmHg)								
Men	39.8 ± 0.3	39.2 ± 0.4	34.1 ± 0.6[a]	31.1 ± 1.2[a]	38.3 ± 0.4[ab]	38.4 ± 0.4[b]	40.7 ± 0.5[bc]	41.1 ± 0.4[abc]
Women	38.9 ± 0.3	38.5 ± 0.3	34.3 ± 0.3[a]	32.9 ± 0.8[a]	38.6 ± 0.3[b]	38.9 ± 0.3[b]	41.5 ± 0.4[abc]	41.6 ± 0.4[abc]
Oxyhemoglobin saturation (%)								
Men	98.2 ± 0.2	98.6 ± 0.2	79.4 ± 0.3[a]	82.3 ± 0.8*[a]	79.6 ± 0.3[a]	80.7 ± 0.3[a]	81.1 ± 0.3[a]	80.6 ± 0.6[a]
Women	98.5 ± 0.2	98.7 ± 0.2	78.9 ± 0.2[a]	80.9 ± 0.2*[a]	79.3 ± 0.2[a]	80.5 ± 0.2*[a]	81.9 ± 0.4[abc]	82.1 ± 0.4[a]
Ventilation (L/min)								
Men	5.08 ± 0.32	6.38 ± 0.34	7.11 ± 0.43	11.47 ± 1.48[a]	9.90 ± 0.95[a]	11.89 ± 1.15[a]	26.57 ± 2.00[abc]	30.94 ± 2.10[abc]
Women	4.28 ± 0.17	4.92 ± 0.22	5.24 ± 0.18	7.02 ± 0.74[a]	15.23 ± 1.41[ab]	19.06 ± 2.21[ab]	26.52 ± 2.06[abc]	29.10 ± 2.44[abc]
Heart rate (beats/min)								
Men	58.2 ± 1.4	54.3 ± 1.5*	66.0 ± 1.3	68.8 ± 1.2[a]	65.6 ± 2.0[a]	64.2 ± 1.5[a]	73.1 ± 1.2[a]	74.5 ± 1.7[ac]
Women	65.9 ± 1.8	63.6 ± 1.9*	77.3 ± 1.7[a]	75.4 ± 1.7[a]	85.2 ± 1.5[ab]	82.3 ± 1.2*[a]	89.4 ± 2.0[ab]	85.9± 1.9*[a]
Mean arterial pressure (mmHg)								
Men	86.3 ± 4.4	89.4 ± 5.1	90.9 ± 4.0[a]	97.4 ± 6.4[a]	89.6 ± 4.3	95.3 ± 6.1[a]	95.3 ± 4.8[ab]	102.0 ± 6.5[abc]
Women	81.7 ± 2.1	88.3 ± 2.5*	89.4 ± 3.0[a]	93.3 ± 3.0[a]	90.3 ± 3.3[a]	94.4 ± 3.6[a]	93.3 ± 3.1[a]	99.0 ± 3.4*[abc]

within sex between rapid and progressive hypoxic exposure were strikingly similar (see figure 5). Therefore, data from both sexes were combined in order to analyze the effect of rate of hypoxic exposure on the cutaneous vascular response to steady state hypoxia. This analysis provided evidence for an upward shift in cutaneous vascular conductance when the rate of fall in arterial oxygen saturation was faster ($P = 0.059$ for CVC during rapid vs. progressive hypoxia).

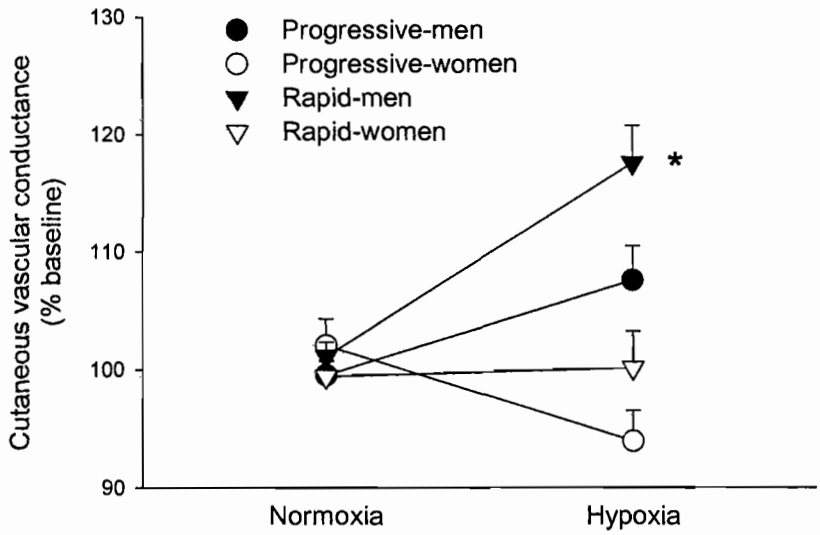

Figure 5. Cutaneous vascular responses to rapid vs. progressive poikilocapnic hypoxia. Values are means ± SE for 7 males and 7 females. * $P < 0.05$ vs. normoxia within condition and sex.

Discussion

This study yielded several new findings. First, a change in blood carbon dioxide does not impact the cutaneous vascular response to hypoxia in thermoneutral conditions. Second, the magnitude of vasoconstriction elicited by whole body cooling is not altered by hypoxia *per se*. An exception to this rule is

the response in women when hypocapnia is concomitant with hypoxia. In this group, the hypocapnia secondary to hypoxic hyperventilation reduces the magnitude of cutaneous vasoconstriction by approximately 50%. The combined results in both men and women indicate that hypoxic vasodilation generally persists during whole body cold stress, even in skin that is not dilated by hypoxia in thermoneutral conditions. Third, a faster reduction in blood oxygen levels results in higher skin blood flow once steady state hypoxia is achieved. And finally, men and women may exhibit different cutaneous vascular responses to acute hypoxia (see below).

We have previously demonstrated that isocapnic hypoxia causes vasodilation that is not restrained by sympathetically-mediated vasoconstriction in non-acral skin (Simmons *et al.*, 2007). However, we are aware of no previous studies addressing the role of hypocapnia in the cutaneous vascular response to hypoxia. Therefore, we sought to test the effect of hypocapnia on the magnitude of cutaneous vasodilation during hypoxia. We found that cutaneous vascular responses to hypoxia were not altered by changes in carbon dioxide regulation (poikilocapnic versus isocapnic hypoxia). This provides strong evidence that cutaneous vascular responses recorded while breathing hypoxic gas mixtures can be extrapolated to the high altitude setting.

At first glance it seems that the failure of hypercapnia to potentiate hypoxic vasodilation in this study is at odds with previous research demonstrating the vasodilator actions of carbon dioxide in the skin circulation (Stein & Weinstein, 1942; Diji & Greenfield, 1960; Simmons *et al.*, 2007). However, it is important to

view these data in the context of the degree of hypercapnia imposed (~ 2 – 3 mmHg above eucapnia). This hypercapnic stimulus was sufficient to increase ventilation from 5.2 liters min^{-1} to 26.5 liters min^{-1} during equivalent hypoxic exposures, likely due to the synergistic effect of combined hypoxia and hypercapnia on peripheral chemoreflex activation (Somers *et al.*, 1989). However, the sensitivity of cutaneous blood vessels to carbon dioxide is probably much less than that of peripheral chemoreceptors and therefore a much greater hypercapnic stimulus is necessary to affect these vessels. Previous studies which demonstrated a local effect of carbon dioxide on skin blood flow used either water baths at high CO_2 concentrations (4 g of CO_2 $liter^{-1}$) or subcutaneous injections of 12.5 – 100% CO_2 gas (Stein & Weinstein, 1942; Diji & Greenfield, 1960). In addition, our previous work indicating that hypercapnia causes sympathetically-mediated vasodilation used end-tidal PCO_2 values of 5 and 9 mmHg above eucapnia, and these responses were modest in magnitude (Simmons *et al.*, 2007). At these levels of hypercapnia, we were not able to detect a local effect of CO_2 on cutaneous blood vessels. Taken together, these data indicate that the lack of effect of hypercapnia on hypoxic cutaneous vasodilation should not be viewed as evidence against the impact of CO_2 on skin blood flow, but likely resulted from the use of a hypercapnic stimulus too weak to exert peripheral vascular effects in the cutaneous circulation.

In male subjects, we found that the magnitude of vasoconstriction during whole body cooling was unaffected by either hypoxia or changes in carbon dioxide. This resulted in an upward shift in skin blood flow throughout whole

body cooling during isocapnic hypoxia. However, the magnitude of this shift was somewhat diminished by any change in carbon dioxide levels (hypercapnia or hypocapnia). Nevertheless, the data indicate that hypoxia *per se* is responsible for the upward shift in blood flow during cold stress in men, and this is consistent with our earlier findings in thermoneutral conditions (Simmons *et al.*, 2007). These data also provide the first evidence that elevated skin temperature in men exposed to cold stress at high altitude is the result of increased perfusion of the skin (Cipriano & Goldman, 1975; Blatteis & Lutherer, 1976). Importantly, the higher skin temperatures observed during combined hypoxia and cold stress were associated with a faster fall in core temperature compared to normoxia (Cipriano & Goldman, 1975). Taken together, these data suggest that hypoxic vasodilation persists during cold exposure, and that this results in higher core-to-skin heat transfer and ultimately greater core heat loss.

The cold-induced vasoconstrictor responses in women were different from those observed in men in two important ways. First, the upward shift in skin blood flow seen during the late stages of whole body cooling occurred despite a lack of hypoxic vasodilation in thermoneutral conditions. In this case, differences in skin blood flow between normoxic and hypoxic conditions only became apparent during whole body cooling. The mechanism underlying this response pattern is unclear. It may be that sympathetic vasoconstriction is affected in some way in females so as to reduce the efficacy of vasoconstrictor transduction when nerve firing frequency is above tonic levels. This would explain why vasodilation was only seen once reflex vasoconstriction was stimulated. Along

these lines, sympathetic vasoconstrictor nerves are known to release additional neurotransmitters (i.e., sympathetic cotransmitters) at higher firing frequencies (Eckberg *et al.*, 1988). Therefore, it may be that some additional substance released from sympathetic vasoconstrictor nerves is affected by hypoxia in women.

The second distinction between male and female vasoconstrictor responses was that, in females, the development of hypocapnia was associated with a 50% reduction in the magnitude of vasoconstriction during whole body cooling. The comparison of this response to that observed during isocapnic hypoxia suggests that this effect was mediated by hypocapnia. However, the mechanism by which hypocapnia reduces cutaneous vasoconstriction is unclear. The preponderance of evidence from animal literature indicates that *hyper*capnia rather than *hypo*capnia reduces sympathetic vasoconstriction at the tissue level (McGillivray-Anderson & Faber, 1990). Therefore, it is unlikely that this blunted vasoconstrictor response in women is mediated through a peripheral effect of decreased PCO_2. Another possibility is that sympathetic vasoconstrictor outflow in response to cold stress is diminished by hypocapnia in women. During hypoxia, males and females exhibit similar activation of the sympathetic nervous system, however, the effect of hypocapnia on these responses is poorly understood (Jones *et al.*, 1999). Thus, hypocapnia may selectively impact central integration of sensory cold stimuli in women. More work in this area is necessary to uncover the mechanisms responsible for blunted vasoconstriction during hypocapnia in women.

The lack of vasodilation in female subjects during hypoxia is seemingly at odds with a previous study from our lab in which no sex differences were noted in hypoxic vasodilator responses during isocapnic hypoxia (Simmons *et al.*, 2007). However, this may be explained by differences in study design and, more specifically, between the effects examined during statistical analysis. For example, our previous study was concerned with vascular responses to two hypoxic conditions (85 and 80% SaO_2), and there was a condition effect in the current study as well (level of CO_2). However, the additional effect in this study (besides sex) in the initial 3 way ANOVA was time. This is contrasted with our previous study in which drug site (bretylium versus saline) was the additional effect in the 3 way ANOVA. The inclusion of this drug effect in the ANOVA dictated that the statistical analysis be run on percent changes from baseline during each condition, rather than perfusion values with respect to time. We speculate that these differences in statistical analysis, which were dictated by the study design, may explain why no sex differences were previously observed in the cutaneous vascular response to hypoxia. We are aware of no other studies which have examined this phenomenon.

There exists no standardized protocol for the induction of arterial hypoxemia in the study of limb vasodilator responses in humans. A number of laboratories perform such studies, but the method for hypoxic exposure varies. Some investigators induce hypoxia by immediately switching the inspired gas from air to 10 – 12% oxygen (Somers *et al.*, 1989; Somers *et al.*, 1991; Leuenberger *et al.*, 1999), while others gradually reduce the fraction of inspired

oxygen over 10 – 15 minutes or more (Weisbrod *et al.*, 2001; Dinenno *et al.*, 2003; Wilkins *et al.*, 2006). Our laboratory has employed both of these techniques in the past, and we have noted some evidence that more rapid hypoxic exposure induces a greater vascular response. Therefore, in addition to the three conditions separated by differing carbon dioxide regulation, subjects were exposed to both rapid and progressive hypoxia in this study (both poikilocapnic exposures). The results demonstrate that, although vasodilation was only observed in male subjects during hypoxia, skin blood flow was upward shifted by the faster rate of decline in SaO_2 in both sexes. Combination of these data sets (males and females) for analysis revealed that more rapid induction of hypoxemia is associated with greater vasodilator responses.

Exposure to hypoxia alters resting skin blood flow and therefore has the potential to confound interpretation of cutaneous vasoconstrictor responses when expressed relative to baseline values (Hodges *et al.*, 2007). To overcome this potential limitation, we measured cutaneous vascular conductance continuously throughout the transition between normoxic, hypoxic, and whole body cooling conditions. Therefore, we were able to use values recorded during normoxia as pseudo absolute units which were not subject to the effect of hypoxia on baseline blood flow. When these values are used to express changes occurring during hypoxia (e.g., during cold stress) they represent absolute units of change, even though they are expressed as a percentage change (i.e., from values measured before hypoxia). Using this in addition to the conventional method of expressing

changes during cold stress as a percentage of pre-cooling baseline, we showed that this issue does not affect the interpretation of results from this study.

A limitation of this study is that arterial/tissue PCO_2 and pH were not measured. Therefore, while we document large excursions in end-tidal PCO_2 during poikilocapnic hypoxia, and would expect subsequent alkalosis, we can only speculate as to the magnitude of these changes at the tissue level.

In summary, we have shown that the cutaneous vascular response to hypoxia is not affected by carbon dioxide, but may be affected by sex. A heterogeneous sample of females studied during the early follicular phase of the menstrual cycle (not controlled for oral contraceptive use) displayed no vasodilation during hypoxia in thermoneutral conditions but maintained higher skin blood flow during whole body cooling. This resulted in a characteristic upward shift in skin blood flow during whole body cooling that occurred in both sexes. The magnitude of cutaneous vasoconstriction during brief whole body cooling was not affected by hypoxia, but was decreased 50% by hypocapnia in women.

Chapter IV entitled "Effect of CO_2 regulation on the cutaneous vascular response to hypoxia and mild cold stress" yielded several novel findings, two of which were particularly germane to the study of thermoregulation at high altitude. First, acute hypoxia caused an upward shift in skin blood flow during whole body cooling. Second, hypocapnia reduced the magnitude of cutaneous vasoconstriction during whole body cooling by 50% in women, while this response was unaffected in men. Collectively, these data indicated that hypoxic vasodilation persists during cold stress, and that reflex cutaneous vasoconstriction may be selectively affected by hypocapnia in women. These findings provided the basis for the study in Chapter VI. However, two pilot studies were necessary in order to clarify the timecourse over which study drugs need to be infused and to better characterize cutaneous non-noradrenergic vasoconstriction so that the contribution of this alternate pathway to whole body cooling responses during hypoxia could be studied. Therefore, in Chapter V entitled "Lack of non-noradrenergic cutaneous vasoconstriction during short duration whole body cooling" we studied the timecourse over which yohimbine and BIBP-3226 exert pharmacologic action on skin blood vessels and also investigated the dependence of non-noradrenergic cutaneous vasoconstriction on the duration of cold stress.

CHAPTER V

LACK OF NON-NORADRENERGIC CUTANEOUS VASOCONSTRICTION DURING SHORT DURATION WHOLE BODY COOLING

Introduction

Yohimbine is classically thought of as a selective α_2-adrenergic receptor antagonist, and this is the main pharmacological effect when administered systemically. However, yohimbine can be delivered locally into the skin via intradermal injection or microdialysis and this method allows the administration of much higher concentrations of the drug in a small area of skin. At high local concentrations (5mM), yohimbine acts as a non-selective blocker of α-adrenergic receptors and abolishes the vasoconstrictor response to both norepinephrine and phenylephrine (α_1-receptor agonist) administration (Stephens *et al.*, 2001). Therefore, yohimbine has recently been used by numerous investigators to block noradrenergic vasoconstriction in the skin (Stephens *et al.*, 2001; Stephens *et al.*, 2002; Stephens *et al.*, 2004; Thompson & Kenney, 2004).

While yohimbine has become increasingly common for use in studies of cutaneous vascular regulation, the timecourse of drug delivery varies widely between studies, ranging from 30 minutes to 75 minutes prior to protocol initiation. However, some investigators have found that even 60 minutes of continuous infusion is not sufficient to produce full blockade of sympathetic

vasoconstriction with other adrenergic blocking agents (Simmons *et al.*, 2005). Therefore, we felt it important to test the timecourse over which the antagonistic properties of yohimbine develop.

BIBP-3226, a neuropeptide Y-Y_1 receptor specific antagonist, has been shown to block the non-noradrenergic component to reflex cutaneous vasoconstriction during whole body cooling (Stephens *et al.*, 2004). For this reason, we used BIBP-3226 in Chapters VI and VII at the same concentrations used by Stephens *et al.* (2004). The cost of this drug, coupled with the unavailability of a specific neuropeptide Y-Y_1 receptor agonist for human use and the extremely high cost of neuropeptide Y for human use, effectively limited the amount of pilot work performed with BIBP-3226 throughout these dissertation studies. However, we included BIBP-3226 in this initial pilot study because only one previous study used this drug in the skin during whole body cooling, and little rationale was provided for the timecourse of drug delivery in that study.

The purpose of these pilot protocols was to better understand the timecourse over which selective blockers of cutaneous vasoconstrictor pathways exert their influence in the skin. A further goal was to test the hypothesis that non-noradrenergic cutaneous vasoconstriction is activated at relatively lower skin temperatures than noradrenergic vasoconstriction during whole body cooling in humans.

Methods

This study was approved by the institutional review board of the University of Oregon, and each subject gave written, informed consent before participation.

Subjects

Nine healthy, nonsmoking, normotensive subjects (6 men, 3 women) participated in this study. Subjects were taking no medications, except for oral contraceptives, and none had been to altitude (>1,500 m) within 5 months. Women were studied either during the early follicular phase of the menstrual cycle or during the placebo phase of oral contraceptive use to minimize the potential effects of female hormones on these responses. All female subjects had a negative urine pregnancy test within 1 hour of participation.

Instrumentation and measurements

Experiments were performed in thermoneutral conditions except for periods of cold stress, and subjects either wore a whole body water perfused suit (protocol 1) or rested inside of a purpose built environmental chamber (Tescor, Inc; protocol 2). When the subject arrived on the study day, three microdialysis fibers (model MD 2000, Bioanalytical Systems, West Lafayette, IN) with a membrane length of 10 mm and molecular mass cutoff of 20 kDa (<5 μl dead space) were placed in the skin of the ventral forearm. Fibers were placed by inserting a 25-gauge needle through the dermis of the skin while the subject lay in the supine position. The fiber was then threaded through the internal lumen of the needle, and the needle was withdrawn, leaving the membrane in place. The fiber was taped in place and perfused with lactated Ringer's solution at a rate of 4 μl/min with a microinfusion pump (CMA/102, CMA Microdialysis, Stockholm, Sweden). Sites were at least 5 cm apart.

Immediately after microdialysis fiber insertion, subjects rested while the local hyperemia subsided at each microdialysis site. To obtain an index of skin blood flow, cutaneous red blood cell flux was measured over the microdialysis sites by laser-Doppler flowmetry (MoorLAB, Moor Instruments, Devon, UK) with integrated laser-Doppler probes fixed to the skin with adhesive tape. Blood pressure was monitored via brachial artery oscillometry, and skin blood flow was expressed as cutaneous vascular conductance (red blood cell flux/mean arterial pressure) and normalized to baseline values as described previously (Simmons *et al.*, 2007).

Protocol 1

The purpose of this protocol was to test the timecourse over which yohimbine and BIBP-3226 develop full inhibition of their respective receptor targets in the skin. The original design of this protocol called for serial infusions of norepinephrine and [D-Arg^{25}]-NPY to selectively stimulate α-adrenergic receptors and neuropeptide Y-Y_1 receptors, respectively. The hypothesis was that vasoconstrictor responses to these agents would eventually be abolished when their receptor blockers (yohimbine and BIBP-3226) built up sufficient local concentration to competitively inhibit ligand binding. However, as mentioned above, there is currently no selective neuropeptide Y-Y_1 receptor agonist (including [D-Arg^{25}]-NPY) available for use in humans. Therefore, we tested cutaneous vascular responses to repeated cold stresses in skin sites treated with yohimbine and BIBIP-3226. Our rational was that as each drug began to inhibit the ligand binding of its target receptor(s), the magnitude of vasoconstriction

would be reduced over time until a plateau was reached, and the timepoint at which this plateau was reached would represent the amount of time necessary to administer the drug before full blockade is achieved.

Six subjects participated in protocol 1. After resolution of the local hyperemia (~1.5 h), skin blood flow was monitored for 5 minutes in resting conditions. Subjects were supine for the entire protocol. Once baseline skin blood flow was recorded, one microdialysis site was perfused with 5mM yohimbine (Sigma, St. Louis, MO) in lactated Ringer's solution for the purpose of α-adrenergic blockade. A second site was perfused with the neuropeptide Y Y_1-receptor antagonist BIBP-3226 (10.5μM; Sigma, St. Louis, MO) in lactated Ringer's, and this concentration was based on previous studies (Stephens *et al.*, 2004). The third microdialysis fiber received only lactated Ringer's solution and was designated a control site.

After the initiation of drug infusions, the remainder of the protocol occurred in continuous 30 minute intervals. During the first 20 minute stage of each interval (thermoneutral conditions), 34°C water circulated through the water perfused suit. During the last 10 minutes of each interval, 10°C water was circulated through the suit to induce cold stress. A total of five 30 minute intervals were conducted, resulting in each subject being exposed to 5 cold stresses. Earlier pilot data indicated that cutaneous vascular responses to 10 minutes of cold stress were reproducible after 20 minutes of rest in thermoneutral conditions (see Chapter IV). The results of protocol 1 indicated that yohimbine

treatment blocked all of the vasoconstrictor response to whole body cold stress, and this led to the design of protocol 2.

Protocol 2

Because no vasoconstriction was observed in the skin sites treated with yohimbine in protocol 1, protocol 2 was designed to (1) confirm that our failure to demonstrate non-noradrenergic cutaneous vasoconstriction was not due to the exclusion of propranolol from that protocol, and (2) to test the hypothesis that non-noradrenergic cutaneous vasoconstriction during whole body cooling is related to the severity of the cold stimulus.

Three men participated in protocol 2, and each lay supine in a temperature controlled environmental chamber (28.5°C) wearing only shorts. After resolution of the local hyperemia associated with microdialysis fiber insertion (~1.5 h), one site was perfused with 5mM yohimbine in lactated Ringer's solution for the purpose of α-adrenergic blockade. A second site was perfused with yohimbine (5mM) and propranolol (1mM; Sigma, St. Louis, MO) for the purpose of combined α- and β- adrenergic blockade. The third microdialysis fiber received only lactated Ringer's solution and was designated a control site. Study drugs were infused for 60-75 minutes prior to beginning the protocol on the basis of our pilot work with propranolol. During this time, 4 thermocouples were placed over the skin for the continuous measurement of weighted mean skin temperature (Sawka & Wenger, 1988).

Once study drugs had been infused for 60-75 minutes, baseline skin blood flow and skin temperature were recorded for 5 minutes. Subjects were then

exposed to a progressive cold stress by decreasing the ambient temperature from 28.5°C to 15°C over 30 minutes. Blood pressure was monitored every 3 minutes during this period, and local skin temperature was clamped at 33°C.

Data acquisition and analysis

Data were digitized with signal-processing software (WinDaq, Dataq Instruments, Akron, OH) and analyzed off-line. In protocol 1, cutaneous vascular responses at every site were first analyzed to determine if vasoconstriction occurred during each cold stress. Next, vasoconstrictor responses were analyzed with a two way repeated measures ANOVA to test the effects of drug treatment and time (cold stresses 1 – 5). In protocol 2, means for cutaneous vascular conductance are presented but these data were not analyzed statistically because only 3 subjects were tested. Statistical analyses were performed with SAS statistical software using PROC MIXED (SAS v9.1.3, SAS Institute, Cary, NC). Differences were considered significant when $P < 0.05$. All values are presented as means ± SE, unless otherwise indicated.

Results

Protocol 1

During each whole body cold stress, cutaneous vascular conductance was consistently reduced in both the control site and the site receiving BIBP-3226 (all $P < 0.05$ vs. baseline for both sites), and the magnitude of this reduction was not different between sites (all $P > 0.05$). In the yohimbine site, cutaneous vascular conductance was unchanged by cold stress at any time during the study (all $P > 0.05$ vs. baseline). In addition, vasoconstrictor response magnitude was

unchanged by time (i.e., cold stresses 1 – 5) in any of the skin sites (all $P > 0.05$ within site; $P = 0.202$ for main effect of time). These data have been summarized in figure 6, with responses averaged over the first and second hour of the protocol.

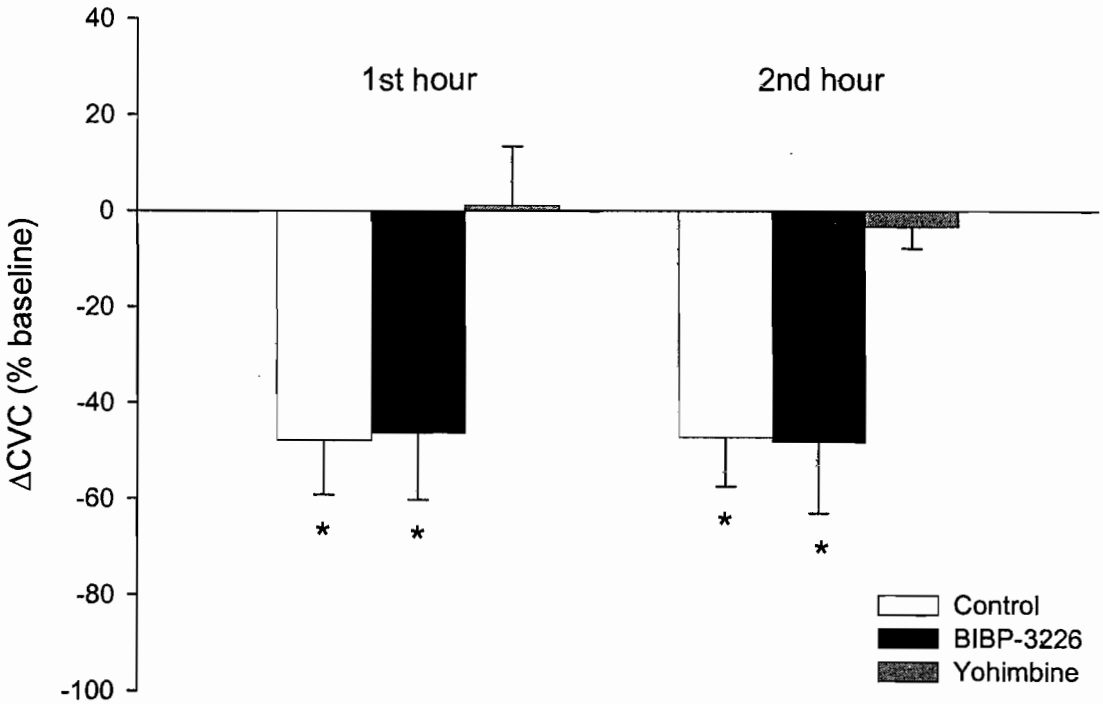

Figure 6. Average cutaneous vasoconstrictor responses to 10 minutes of whole body cooling in skin sites treated with lactated ringers, yohimbine, or BIBP-3226. Averages represent two vasoconstrictor responses, which were measured every 20 minutes. * $P < 0.05$ vs. baseline for vasoconstrictor responses measured during that hour. See text for details; n = 6.

Protocol 2

In protocol 2 the 30 minute reduction in ambient temperature caused a fall in weighted mean skin temperature from 32.1°C to 28.3°C. As a result, cutaneous vascular conductance fell to 59.6%, 90.5%, and 85.4% of baseline in control, yohimbine, and combination yohimbine + propranolol treated sites. The responses in yohimbine and combination yohimbine + propranolol sites were both reduced in magnitude and shifted to lower skin temperatures compared to control responses (see figure 7).

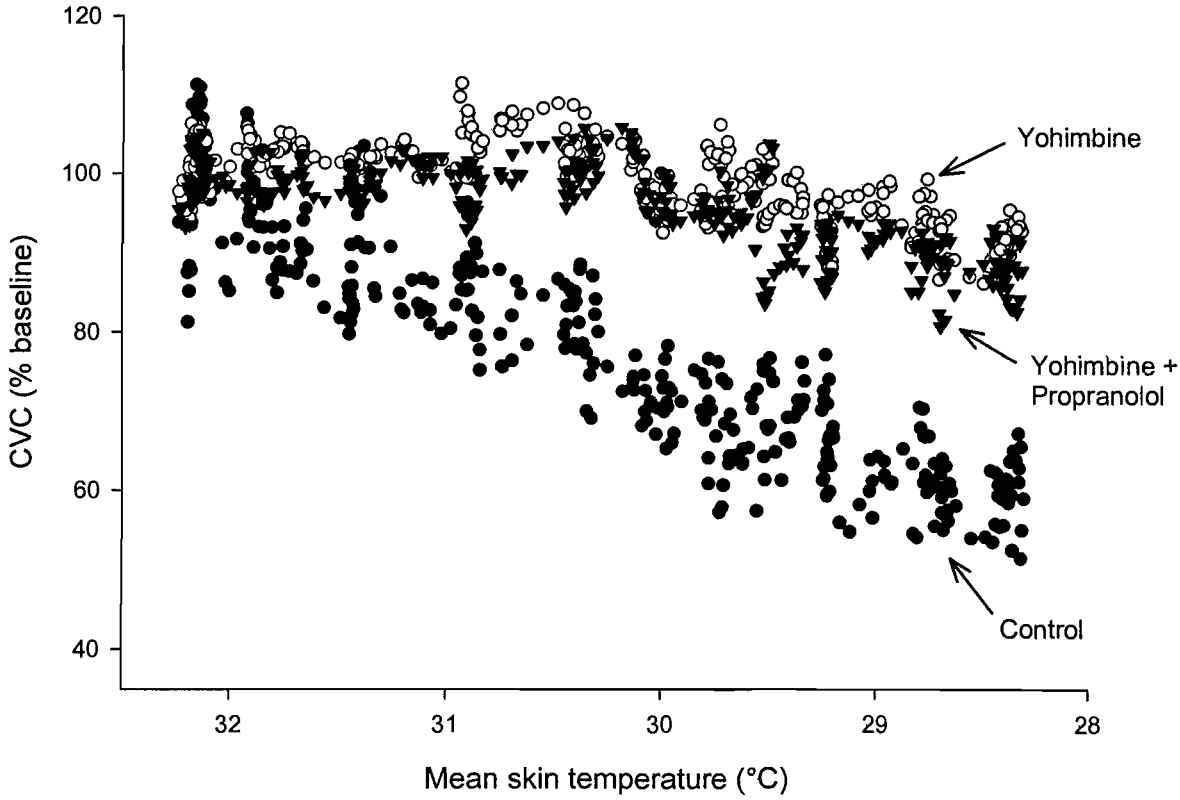

Figure 7. Cutaneous vascular responses to a progressive reduction in ambient temperature measured in sites treated with yohimbine (5mM), combinded yohimbine (5mM) + propranolol (1mM), and lactated ringers (control). Ambient temperature was reduced from 28.5°C to 15.5°C over 30 minutes. Data are means from 3 subjects.

Discussion

These pilot studies yielded several new findings. First, the action of yohimbine on α-adrenergic receptors is very fast when administered at 5mM via microdialysis (within 30 minutes). Second, there appears to be no residual vasoconstriction during ten minutes of whole body cooling when α-adrenergic receptors are blocked. However, when cold stress is prolonged, non-noradrenergic vasoconstriction is activated and a reduction in skin blood flow is observed in skin where α-adrenergic receptors are blocked. This response appears to occur at lower skin temperatures than noradrenergic vasoconstriction. Finally, any effect of propranolol treatment on cutaneous vasoconstrictor responses during α-receptor blockade is small and unlikely to explain the lack of non-noradrenergic cutaneous vasoconstriction observed during ten minutes of whole body cooling in protocol 1.

Yohimbine (5mM) delivered via cutaneous microdialysis abolished all of the vasoconstrictor response to whole body cooling in protocol 1. These results are seemingly at odds with numerous previous studies which demonstrated persistent vasoconstrictor responses in skin sites treated with comparable yohimbine concentrations (Stephens *et al.*, 2001; Stephens *et al.*, 2002; Stephens *et al.*, 2004; Thompson & Kenney, 2004). However, it appears that this discrepancy can be explained by the length of cold exposure used in these different studies. In protocol 1, whole body cooling was performed for ten minutes at a time so that multiple cold exposures could be performed during the 150 minute protocol. Each of these cold exposures was performed by reducing

the temperature of water circulating through the water perfused suit to 10°C, and this is similar to the magnitude of cold stress used in other studies. However, the duration of cold stress used in previous studies was longer, lasting 15 minutes to 45 minutes (Stephens *et al.*, 2001; Thompson & Kenney, 2004). Therefore, because skin temperature continues to fall when exposed to a constant ambient temperature below skin temperature (Grant H. Simmons, unpublished observations), it is likely that the fall in skin temperature in these previous studies was greater than in protocol 1. This would result in a greater input to the thermoregulatory centers in the hypothalamus during such a cold stress because the reflex cutaneous vasoconstriction is driven entirely through changes in skin temperature (core temperature does not change). Therefore, it is possible that the lack of non-noradrenergic cutaneous vasoconstriction observed in protocol 1 resulted from the use of a weaker cold stimulus than previous studies.

The mechanism by which non-noradrenergic cutaneous vasoconstriction is sensitive to the magnitude of cold stress is unclear. However, there is considerable evidence to suggest that the release of cotransmitters from sympathetic vasoconstrictor nerves is related to the frequency and/or burst characteristics when these nerves are activated. For example, Eckberg *et al.* (1988) demonstrated that release of neuropeptide Y into plasma is correlated with the increase in sympathetic nerve activity during experimental changes in blood pressure in humans. Importantly, neuropeptide Y is thought to be the most likely candidate for a sympathetic cotransmitter mediating non-noradrenergic cutaneous vasoconstriction (Stephens *et al.*, 2004). In the dog gracilis muscle,

neuropeptide Y is only released into the venous circulation at high rates of experimental nerve stimulation, whereas norepinephrine is released over a broader range including low level stimulation (Pernow *et al.*, 1989b). Taken together, these studies suggest that neuropeptide Y is released from sympathetic nerves only when the frequency of nerve firing is raised above some threshold level. Therefore, the observation of non-noradrenergic cutaneous vasoconstriction in previous studies using a greater magnitude of cold stress could be explained by the generation of stronger afferent input to the hypothalamus and a larger subsequent efferent response, manifesting in greater activation of sympathetic vasoconstrictor nerves and the release of cotransmitters with norepinephrine.

In order to test this hypothesis, protocol 2 was performed to study the occurrence of non-noradrenergic cutaneous vasoconstriction as a function of mean skin temperature during more prolonged cold exposure. In agreement with our hypothesis, non-noradrenergic vasoconstriction was absent during the first half of the 30 minute cooling period and over the range of skin temperatures likely induced during protocol 1. However, figure 7 shows a clear inflection at a mean skin temperature of ~ 30.5°C after which skin blood flow falls steadily in sites where α-adrenergic receptors were blocked. These data provide strong support for the idea that non-noradrenergic cutaneous vasoconstriction is absent during mild cold exposure, but becomes activated as the increase in sympathetic outflow is more pronounced (i.e., at lower skin temperatures).

There is some evidence to suggest that functional β-adrenergic receptors exist in the cutaneous microcirculation and play a small but potentially important role in modulating vasoconstriction during whole body cooling (Crandall *et al.*, 1997; Stephens *et al.*, 2001). For example, Stephens *et al.* (2001) demonstrated that when α-adrenergic receptors were blocked with intradermal injection of yohimbine, transient vasodilation was seen in a small percentage of subjects (20%) during whole body cooling. When propranolol treatment was included with yohimbine to block both α- and β- adrenergic receptors the percentage of subjects displaying transient vasodilation during cold stress was reduced to 14%, but fewer total subjects were tested. Finally, when these investigators compared the effect of propranolol treatment and saline on vasoconstrictor responses during cold stress, no effect of propranolol was demonstrated on the magnitude of cutaneous vasoconstriction. Taken together, these data suggest that sparsely distributed β-adrenergic receptors can be activated during whole body cooling, but that any effects on cutaneous vasoconstrictor responses are likely to be small and relatively uncommon in the population. Nevertheless, we tested the possibility that non-noradrenergic cutaneous vasoconstriction was masked by superimposed β-adrenergic vasodilation in protocol 1. We found that the inclusion of propranolol with yohimbine in protocol 2 did not profoundly alter the timecourse or magnitude of non-noradrenergic cutaneous vasoconstriction during prolonged whole body cooling. This suggests that the lack of non-noradrenergic cutaneous vasoconstriction in protocol 1 was not due to β-adrenergic vasodilation overriding the cotransmitter-mediated vasoconstrictor signal.

Perspectives

The current results support the notion that brief cold exposure activates reflex cutaneous vasoconstriction that is entirely mediated by noradrenergic pathways. Interestingly, the brief cold exposures in protocol 1 (10 minutes) were similar to those used in our previous study of cutaneous vasoconstriction during hypoxia (see chapter IV). One difference, however, was that water perfused suit temperature was ramped down to 10°C in the current study whereas it was decreased gradually (to 14°C) in our previous investigation. Therefore, the cold exposure in the current study was in fact more aggressive, which further supports the idea that reflex vasoconstrictor responses in our previous investigation were mediated solely through noradrenergic pathways.

In summary, these studies provide strong evidence that non-noradrenergic cutaneous vasoconstriction is selectively activated at lower skin temperatures during more prolonged cold exposure. In addition, these data support the notion that cutaneous vasoconstriction during a brief ten minute cold exposures is mediated entirely through noradrenergic pathways.

The main findings from Chapter V entitled "Lack of non-noradrenergic cutaneous vasoconstriction during short duration whole body cooling" were that (1) blockade of α-adrenergic receptors with yohimbine completely abolished reflex cutaneous vasoconstriction during a ten minute cold stress, and that (2) during more prolonged cold exposure, non-noradrenergic cutaneous vasoconstriction was absent at higher skin temperatures but was activated when lower skin temperatures were reached (during more prolonged cooling). These results suggest that cutaneous vasoconstriction during brief cold exposure is mediated entirely through adrenergic pathways. Thus, inasmuch as Chapter VI entitled "Increased contribution from non-noradrenergic cutaneous vasoconstriction abolishes hypoxic vasodilation during prolonged cold stress" was designed to investigate both noradrenergic and non-noradrenergic cutaneous vasoconstrictor pathways during combined hypoxia and cold exposure, a 30 minute cold exposure was used in this chapter. An additional goal of Chapter VI was to test the contribution of blunted cutaneous vasoconstriction to the decline in core body temperature during severe cold exposure in hypoxia.

CHAPTER VI

INCREASED CONTRIBUTION FROM NON-NORADRENERGIC CUTANEOUS VASOCONSTRICTION ABOLISHES HYPOXIC VASODILATION DURING PROLONGED COLD STRESS

Introduction

When humans undergo cold stress in low oxygen environments the normal decline in skin temperature is upward shifted – even abolished – suggesting less effective redistribution of blood away from the periphery (Cipriano & Goldman, 1975; Blatteis & Lutherer, 1976). We have recently shown that skin blood flow remains elevated in this setting, confirming the role of cutaneous vasodilation in the associated redistribution of body temperature (see Chapter IV). However, it is unclear how individual vasoconstrictor pathways are affected to bring about this net shift in cutaneous perfusion during combined hypoxia and cold stress.

The reduction in skin blood flow during whole body cooling is regulated by cutaneous sympathetic adrenergic nerves, as demonstrated by the sensitivity of this response to bretylium treatment (Kellogg *et al.*, 1989; Thompson & Kenney, 2004). As ambient temperature decreases, the activity of cutaneous adrenergic nerves increases causing the release of norepinephrine and sympathetic cotransmitters. The binding of norepinephrine to post-junctional α-adrenergic

receptors (predominantly α_2-receptors) causes vasoconstriction, and this mechanism is responsible for approximately 60% of the reduction in skin blood flow during cold stress (Stephens *et al.*, 2001; Thompson & Kenney, 2004). The remaining ~40% of the cold induced vasoconstriction is mediated by adrenergic nerve cotransmission, and recent evidence has implicated neuropeptide Y acting through post-junctional neuropeptide Y Y_1-receptors in this response (Stephens *et al.*, 2004).

When humans are exposed to cold temperatures at high altitude, the rate of core cooling is accelerated, and lower core temperatures are reached compared to sea level conditions (Cipriano & Goldman, 1975; Johnston *et al.*, 1996). One mechanism suggested to account for this disparity is the increase in respiratory heat loss associated with hypoxic hyperventilation. However, Johnston *et al.* (1996) calculated that the increase in heat loss due to hypoxic hyperventilation (~ 11.5 kcal/hr) would explain less than 40% of the increased core cooling rate observed in their study. Furthermore, those data were collected during a hypoxic exposure in which carbon dioxide was added to the breathing mixture to prevent the development of hypocapnia. Therefore, total ventilation and respiratory heat loss were likely greater in that study than would occur in the high altitude setting (see Chapter IV).

Cipriano and Goldman (1975) suggested the maintenance of higher peripheral blood flow could explain increased core cooling during hypoxia through a blunted reduction in core-to-skin heat transfer. Our recent demonstration that cutaneous vasodilation persists during whole body cooling in

hypoxic conditions supports this idea (see Chapter IV). However, the cold exposure in that study lasted only ten minutes and was not sufficient to alter core body temperature. One difficulty in studying skin blood flow during induced core cooling is that the shivering associated with such extreme cold exposure obscures cutaneous vascular measurements. To overcome this problem, we designed a two phase protocol to investigate the relationship between cutaneous vascular responses to progressive cooling and core temperature responses to extreme cooling during normoxic and hypoxic conditions.

This study was designed to accomplish two main goals. The first was to test the effect of hypoxia on the noradrenergic and non-noradrenergic components of cutaneous vasoconstriction during progressive cold stress, and the second was to assess whether persistent vasodilation during cold stress contributes to more rapid core cooling in hypoxic conditions. We tested the hypothesis that the increase in core cooling rate during hypoxia is directly related to the upward shift in skin blood flow during whole body cooling.

Methods

This study was approved by the institutional review board of the University of Oregon, and each subject gave written, informed consent before participation.

Subjects

Thirteen healthy, nonsmoking, normotensive subjects participated in this study. However, one subject was unable to tolerate the entire experimental procedure, and withdrew from the study voluntarily. Therefore, 12 subjects (6 men, 6 women), age 22 ± 3 yr, completed the study. Subject anthropometric

characteristics were as follows: height 172 ± 12 (SD) cm, weight 63.4 ± 9.7 kg, body mass index 21.4 ± 1.3 kg/m^2, sum of 4 skin folds 46.6 ± 15.4 mm (Durnin & Womersley, 1974). Subjects were taking no medications, except for women who were taking oral contraceptives, and none had been to altitude (>1,500 m) within 5 months. Because non-noradrenergic cutaneous vasoconstriction occurs in women during the high hormone phase of oral contraceptive use, but not the low hormone (placebo) phase, women in this study were tested during the high hormone phase of oral contraceptive use (Stephens *et al.*, 2002). As an extra precaution, all female subjects had a negative urine pregnancy test within 1 hour of participation.

Experimental procedures

Experiments were performed in a temperature controlled laboratory with the ambient temperature maintained at 25°C (average: 25.0 ± 0.7°C). Subjects visited the laboratory on three separate days, the first of which was an initial interview. During this initial visit, anthropometric data were obtained and subjects were familiarized with the laboratory and all experimental procedures. The following two laboratory visits were identical except that one day involved exposure to simulated high altitude and the other was a sham exposure in which the subject breathed room air from the breathing circuit. The two experimental days were performed in random order, and each took place at the same time of day because cutaneous vasoconstrictor responses to whole body cooling are altered by time of day (Aoki *et al.*, 2003).

The two experiments were performed at least 72 hours apart. Men were instructed to wear swimming shorts, while women wore either soccer shorts with a sports bra or a two piece swim suit. In addition, each subject was given two ingestible pills for the measurement of core temperature (HQInc, Palmetto, FL) and instructed to take one the night before each study. When the subject arrived on each study day, they immediately changed and lay in the supine position. After the application of ice to the ventral forearm, three microdialysis fibers (model MD 2000, Bioanalytical Systems, West Lafayette, IN) with a membrane length of 10 mm and molecular mass cutoff of 20 kDa (<5 µl dead space) were placed in the skin of the numbed area. Fibers were placed by inserting a 25-gauge needle through the dermis of the skin while the subject lay in the supine position. The fiber was then threaded through the internal lumen of the needle, and the needle was withdrawn, leaving the membrane in place. The fiber was taped in place and perfused with lactated Ringer's solution at a rate of 4 µl/min with a microinfusion pump (CMA/102, CMA Microdialysis, Stockholm, Sweden). Sites were at least 5 cm apart.

With microdialysis fiber secured in place, subjects rested in the supine position while the local hyperemia subsided at each rnicrodialysis site and study drugs were prepared. After 70 to 90 minutes, one microdialysis site was perfused with 5mM yohimbine + 1mM propranolol (Sigma, St. Louis, MO) in lactated Ringer's solution for the purpose of α- and β- adrenergic blockade. Although yohimbine has traditionally been thought of as an α_2-adrenergic receptor antagonist, numerous recent studies have demonstrated that at high

concentrations yohimbine (5mM) produces non-specific blockade of both α_1- and α_2-adrenergic receptors in human skin (Stephens *et al.*, 2001; Thompson & Kenney, 2004). Pilot work in our lab has confirmed this dose (see Chapter V), and the time course of drug delivery in this protocol was based on pilot work with propranolol performed in our laboratory. A second site was perfused with the neuropeptide Y Y_1-receptor antagonist BIBP-3226 (10.5μM) + 1mM propranolol (Sigma, St. Louis, MO) in lactated Ringer's. This concentration of BIBP-3226 abolishes the non-noradrenergic component of reflex cutaneous vasoconstriction during whole body cooling in humans (Stephens *et al.*, 2004). The third microdialysis fiber received only 1mM propranolol in lactated Ringer's solution and was designated a control site. This study design aimed to produce one skin site in which vasoconstrictor function was normal, one site in which only noradrenergic vasoconstriction was functional, and one site which isolated non-noradrenergic cutaneous vasoconstriction (presumably mediated through neuropeptide Y Y_1-receptors).

After 60 – 75 minutes of continuous study drug infusion, subjects were allowed to use the restroom and were instrumented for the measurement of heart rate via electrocardiography (Cardiocap/5, Datex-Ohmeda, Madison, WI) and mean skin temperature via thermocouples placed on the head, chest, upper arm, forearm, abdomen, thigh, and calf (Sawka & Wenger, 1988). Once EKG and thermocouples were placed, subjects were helped into a custom modified jacuzzi bathtub in which they sat semi-recumbent, and rested their arms on machine fabricated arm rests positioned out of the water and at heart level. The water

level in the tub was adjusted so that the subject was submerged to the level of the sternum. Once in the tub and comfortable, subjects were instrumented for the measurement of arterial pressure via brachial artery oscillometry (Cardiocap/5) and arterial O_2 saturation via pulse oximetry (Cardiocap/5). Constant rate infusion of study drugs was continued as soon as the subject was seated in the tub. To obtain an index of skin blood flow, cutaneous red blood cell flux was measured on the ventral forearm by laser-Doppler flowmetry (MoorLAB, Moor Instruments, Devon, UK) with integrated laser-Doppler probes placed over the microdialysis sites. Skin blood flow was expressed as cutaneous vascular conductance (red blood cell flux/mean arterial pressure) and normalized to baseline values as described previously (Simmons *et al.*, 2007). Laser-Doppler flow probes were housed in local skin heaters (Moor Instruments, Devon, UK), and these local heaters were set to maintain a constant skin temperature of 33°C throughout the protocol.

When the subject was comfortable in the tub, head gear was donned which secured a mouthpiece connected to inspiratory and expiratory tubes. The mouthpiece apparatus incorporated a one-way, non-rebreathing valve system to insure inspiration and expiration from separate tubes. A nose clip was placed to prevent nasal breathing, and a gas sampling line connected to the mouthpiece for monitoring of end-tidal PO_2 and PCO_2 (Cardiocap/5, Datex-Ohmeda, Madison, WI). The inspiratory mixture provided at the mouthpiece originated from 2 gas tanks – air (20.93% O_2) and nitrogen (100% N_2) – connected to the inspiratory line by flow regulators. The fraction of inspired oxygen (FIO_2) was varied by

adjusting the flowrate from each of the gas tanks, and the inspired air was first humidified and then mixed as it filled a 6 liter tube which served as an inspiratory reservoir. The 6 liter tube connected to the breathing circuit proximal to the mouthpiece, and its distal end was open to room air. Total flowrate through the breathing circuit was always greater than the subjects' minute ventilation, and thus excess gas delivered through the inspiratory line escaped to the room through the 6 liter tube. Expired gas was collected and the fraction of expired oxygen (FEO_2), carbon dioxide ($FECO_2$), and minute ventilation were measured via a pneumotach and mixing chamber (Parvomedics, Sandy, UT) integrated with a mass spectrometry system (Marquette MGA 1100, MA Tech Services, St. Louis, MO). These data were combined with inspiratory values (FIO_2 and $FICO_2$) from the tidal O_2 and CO_2 tracing to calculate whole body O_2 uptake on a minute by minute basis. Shivering onset was determined from the O_2 uptake data as described by others (Johnston *et al.*, 1996).

Protocol

The purpose of this study was two fold. First, this study was designed to probe the effects of simulated high altitude exposure on cutaneous reflex vasoconstrictor mechanisms during progressive cooling. Second, the functional importance of a previously observed upward shift in skin blood flow during combined whole body cooling and hypoxia was investigated. Toward this end, subjects underwent two identical study visits, one in which simulated high altitude exposure was induced and one which simulated sea level conditions. The experimental protocol is depicted in figure 8.

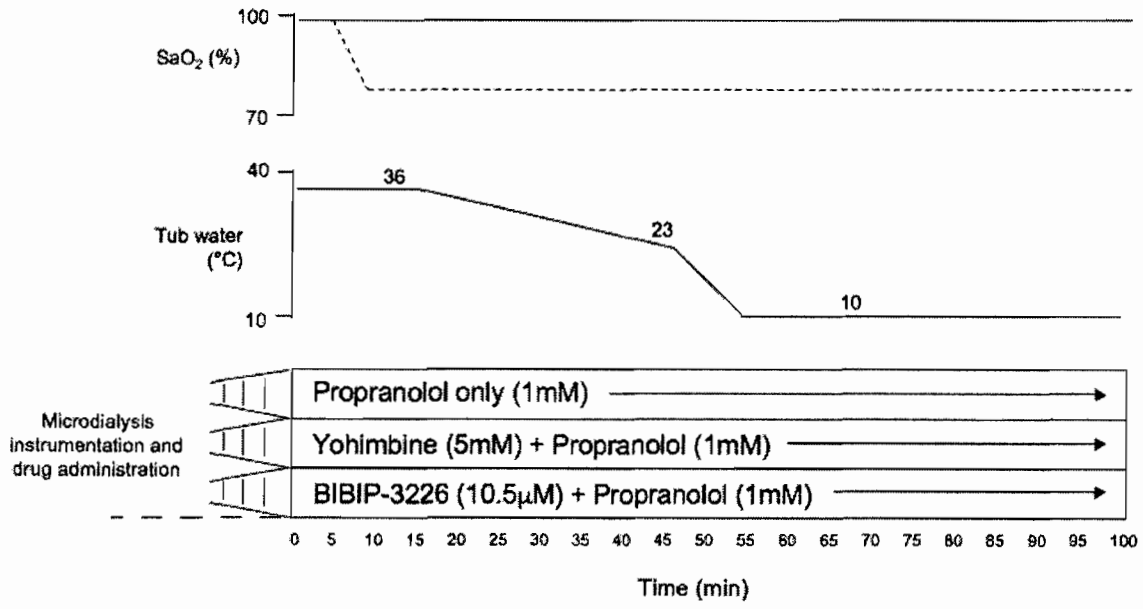

Figure 8. Schematic representation of chapter VI experimental protocol. Experiments took place on two study days, one in which normoxia was maintained (solid line at top) and one during exposure to systemic hypoxia (dashed line at top). Alpha-adrenergic and neuropeptide Y (NPY) receptor antagonists were infused continuously throughout the protocol.

The protocol began with 5 minutes of rest in a water temperature of 36°C, during which baseline data values were recorded. Water temperature was maintained by circulating the jacuzzi tub water through an extensive copper coil (heat exchanger) submerged in a constant temperature water circulating bath (Polyscience, Niles, IL). This system was used to heat the water above room temperature (to 36°C), but was inactivated once cooling began because it lacked the capacity to affect a change in water temperature over the desired time course. Ice was added to the water bath in order to reduce the water temperature during cooling. To maintain a homogeneous temperature within the tub, water circulation continued during the cooling period, and the heat

exchanger was simply removed from the constant temperature circulating bath. Throughout the protocol, water temperature was measured by a thermocouple placed 9 inches below the surface near the subject.

After baseline data were collected, subjects were exposed to either poikilocapnic hypoxia (simulated high altitude) or a sham gas mixture (normal air) during a transition period lasting 5 minutes. Once the target level of hypoxia (80% arterial O_2 saturation) or sham (time control) was reached, steady state data were recorded in this condition for 5 minutes. Immediately following steady state data collection, the temperature of the water bath was progressively reduced from 36°C to 23°C over a 30 minute period. This temperature and time course was chosen because it typically produced maximal vasoconstriction in pilot experiments without inducing uncontrollable shivering (obscuring vascular measurements). All variables were recorded continuously during this period, except core temperature and blood pressure which were recorded every 5 minutes. After 30 minutes of progressive cooling, water temperature was rapidly reduced to 10°C by the addition of more ice to the water bath. This target water temperature was reached within 7-10 minutes. Once water temperature was steady at 10°C, a timer was started and subjects remained in the water bath until either the timer reached 45 minutes or their internal temperature reached 35°C. Core temperature was measured at 2 minute intervals during this period.

After termination of the cooling protocol, the subject was disconnected from all measurement devices and helped out of the tub and into a climate controlled environmental chamber (Tescor, Inc) maintained at 35°C and

equipped with infrared radiant heat lamps. Subjects were immediately towel dried, wrapped in a blanket, and laid in the supine position. Core temperature was monitored and microdialysis fibers removed during this time. Once the subject was comfortable and able to maintain a stable gait, they were given access to a laboratory shower to finish the rewarrning process.

Data acquisition and analysis

Data were digitized with signal-processing software (WinDaq, Dataq Instruments, Akron, OH) and analyzed off-line. Cutaneous vasodilator, vasoconstrictor, and core temperature responses were first analyzed with a two or three-way ANOVA (depending on whether drug site was a factor) to test the effect of sex on these responses. There were no effects of sex in this protocol (all $P > 0.05$). Thus data from men and women were pooled for all subsequent analyses. Skin blood flow was not monitored during drug infusion in this protocol because microdialysis instrumentation and initial drug infusions were not performed in the jacuzzi bathtub, around which most of the necessary equipment was built. However, we and others have shown that neither yohimbine nor BIBP-3226 alters baseline skin blood flow at concentrations used in this study (see chapter 5; (Stephens *et al.*, 2004). Furthermore, each microdialysis fiber received propranolol and thus any potential effect of propranolol on baseline skin blood flow would be equally expressed in all skin sites. Statistical analyses were performed with SAS statistical software using PROC MIXED (SAS v9.1.3, SAS Institute, Cary, NC). Differences were considered significant when $P < 0.05$. All values are presented as means ± SE, unless otherwise indicated.

Results

Cardiorespiratory variables

Table 2 displays water bath temperature and cardiorespiratory responses during the three thermal conditions on both experimental days. As planned, water bath temperatures were not different between experimental days within thermal condition. Heart rate was higher and oxyhemoglobin saturation was lower at each timepoint on the hypoxia day (both $P < 0.05$ vs. normoxia day). Mean arterial pressure was increased during progressive and severe cooling on both experimental days ($P < 0.05$ vs. thermoneutral for both timepoints on both days) while ventilation was only increased on the normoxic day ($P < 0.05$ vs. thermoneutral for both timepoints). End-tidal PCO_2 fell slightly during severe cooling on the hypoxia day ($P = 0.010$ vs. thermoneutral) but was not different from normoxia at any timepoint (all $P > 0.05$ vs. normoxia day).

Cutaneous vascular responses

Figure 9 displays cutaneous vascular conductance in propranolol only, BIBP-3226 + propranolol, and yohimbine + propranolol treated sites measured after 5 minutes of steady state hypoxia or sham exposure (control day). Cutaneous vascular conductance was increased in all sites during hypoxia relative to air breathing (all $P < 0.05$), and this effect was greater in the site treated with combined yohimbine + propranolol ($P = 0.006$ vs. control site). BIBP-3226 had no effect on the cutaneous vascular response to hypoxia ($P = 0.462$ vs. control). During progressive cooling, cutaneous vascular conductance in the control site was reduced to 54.2 ± 4.1 and 62.8 ± 5.9 % normoxic baseline

Table 2. Cardiorespiratory responses to progressive and severe cold exposure during normoxia and hypoxia. Values are means ± SE for 6 males and 6 females. * $P < 0.05$ vs. thermoneutral, † $P < 0.05$ vs. progressive cooling, and ‡ $P < 0.05$ vs. normoxia.

	Thermoneutral	Progressive cooling	Severe cooling
Water temperature (°C)			
Normoxia	35.9 ± 0.2	23.4 ± 0.4*	9.9 ± 0.1*†
Hypoxia	36.0 ± 0.1	23.4 ± 0.5*	10.2 ± 0.1*†
End-tidal P_{CO_2} (mmHg)			
Normoxia	36.0 ± 0.7	35.7 ± 1.2	35.0 ± 0.9
Hypoxia	35.8 ± 0.7	35.2 ± 0.9	34.2 ± 0.7*
Oxyhemoglobin saturation (%)			
Normoxia	98.9 ± 0.2	98.8 ± 0.3	98.8 ± 0.3
Hypoxia	81.3 ± 0.9‡	83.3 ± 0.7*‡	82.6 ± 0.5‡
Ventilation (L/min)			
Normoxia	7.63 ± 0.41	8.78 ± 0.53*	8.75 ± 0.73*
Hypoxia	8.62 ± 0.50	8.91 ± 0.58	10.12 ± 1.19
Heart rate (beats/min)			
Normoxia	69.2 ± 3.8	61.1 ± 3.2*	62.9 ± 3.8*
Hypoxia	78.5 ± 4.4‡	72.2 ± 4.8‡	73.4 ± 4.7‡
Mean arterial pressure (mmHg)			
Normoxia	82.5 ± 1.4	92.2 ± 1.7*	93.5 ± 1.8*
Hypoxia	83.6 ± 2.7	93.2 ± 2.8*	95.2 ± 2.3*

on the normoxia and hypoxia days, respectively ($P = 0.177$). In the BIBP-3226 + propranolol treated site cutaneous vascular conductance was reduced to 55.1 ± 4.5 and 64.3 ± 8.1 % normoxic baseline on the normoxia and hypoxia days ($P = 0.239$), while cutaneous vascular conductance in the yohimbine + propranolol site was reduced to 89.6 ± 6.6 and 114.9 ± 12.7 % normoxic baseline on the normoxia and hypoxia days ($P = 0.034$).

Figure 10 contrasts the magnitude of vasoconstriction in each skin site during progressive cooling in normoxic and hypoxic conditions. Hypoxia caused a similar increase in the magnitude of cutaneous vasoconstriction in control, BIBP-3226 + propranolol, and yohimbine + propranolol treated sites (all $P < 0.05$). Neither normoxic nor hypoxic vasoconstrictor responses were affected by

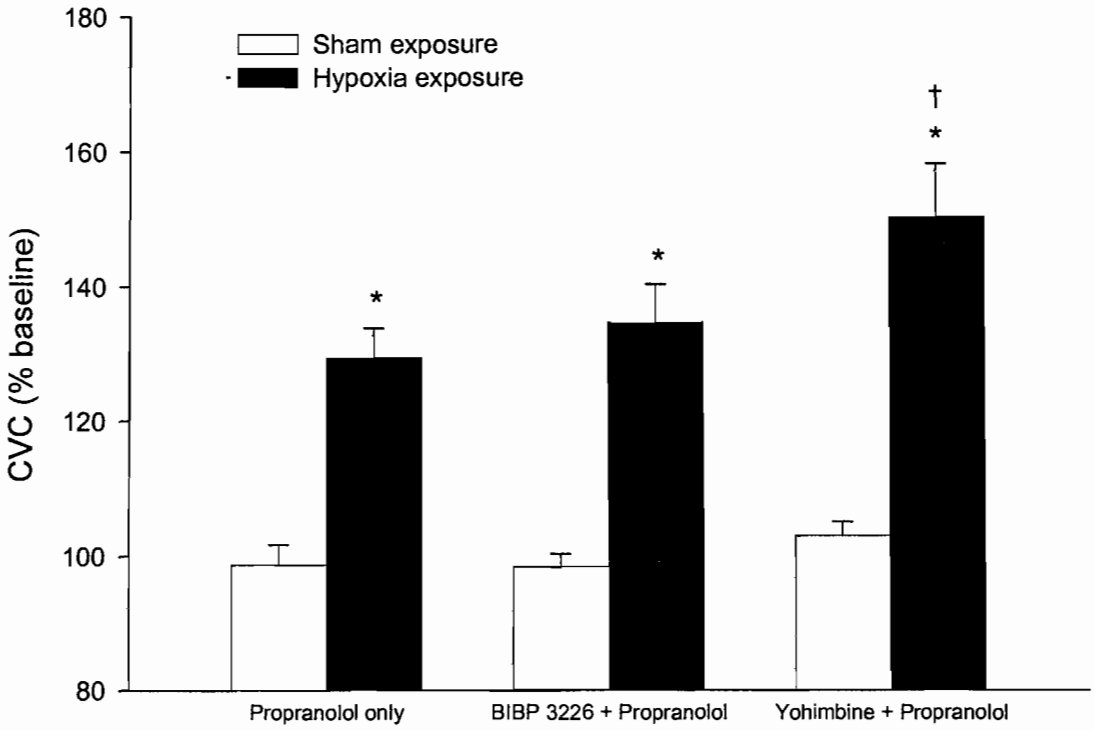

Figure 9. Cutaneous vascular responses to hypoxic exposure (experimental day) and sham exposure (control day) during partial immersion in 36°C water (i.e., thermoneutral). Values are means ± SE for 6 males and 6 females. * $P < 0.05$ vs. sham exposure; † $P < 0.05$ vs. control site (propranolol only) within condition.

BIBP-3226 ($P > 0.05$ vs. control in both conditions). In contrast, yohimbine treatment reduced the magnitude of vasoconstriction during both normoxic and hypoxic conditions ($P < 0.05$ vs. control in both conditions).

Body temperature responses

Mean skin temperature (n = 8) was not significantly altered by exposure to hypoxia (34.9 ± 0.2 vs. 34.9 ± 0.2°C) or air breathing (34.5 ± 0.2 vs. 34.4 ± 0.2°C; both $P > 0.05$), but was reduce by progressive cooling to 29.7 ± 0.2 and 29.3 ± 0.2°C on hypoxia and sham days, respectively (both $P > 0.05$ vs. pre-cooling).

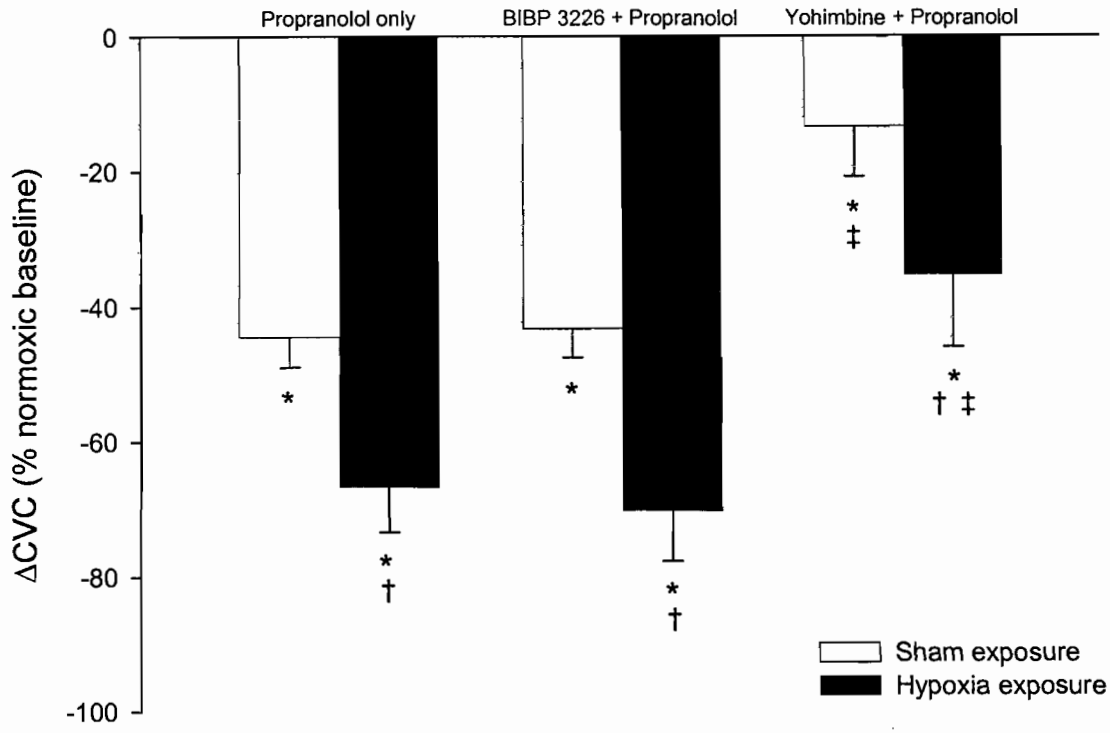

Figure 10. Cutaneous vascular responses to 30 minutes of progressive water cooling in normoxic (sham) and hypoxic conditions. Water temperature bathing the subject was reduced from 36°C to 23°C with the addition of ice to the water bath. Values are means ± SE for 6 males and 6 females. * $P < 0.05$ vs. pre-cooling baseline; † $P < 0.05$ vs. sham exposure; ‡ $P < 0.05$ vs. control site within condition.

There were no differences in mean skin temperature between hypoxia and normoxia days at any time point (all $P > 0.05$ between conditions). Core body temperature (n = 12) also was not altered by exposure to hypoxia or air breathing (both $P > 0.05$ vs. baseline). However, progressive cooling exerted differential effects, increasing core body temperature from 37.29 ± 0.06 to 37.39 ± 0.06°C during normoxia ($P = 0.004$) but having no effect on the hypoxia day (37.35 ± 0.09 vs. 37.35 ± 0.11°C; $P = 0.972$, $P = 0.009$ for condition x time interaction).

One of our study cessation criteria was a core temperature reading of 35°C during severe cold exposure (10°C water). This temperature was reached in 3 of the 12 subjects who completed both study days, resulting in early termination of the study. However, only one of these subjects reached this core temperature on both study days, and the other two subjects only reached 35°C on one of the study days. Therefore, core temperature data were analyzed by calculating the core cooling rate, defined as the slope of the relation between core temperature and time while the subject was immersed in 10°C water. In this way, all 12 subjects were included in the analysis and differences between hypoxic and normoxic cooling rates are not masked by similar final core temperature readings in a subject who, for example, reached 35°C much faster on one experimental day versus another. This analysis demonstrated that core cooling rates during partial immersion were not different between normoxia and hypoxia ($P = 0.511$; see figure 11).

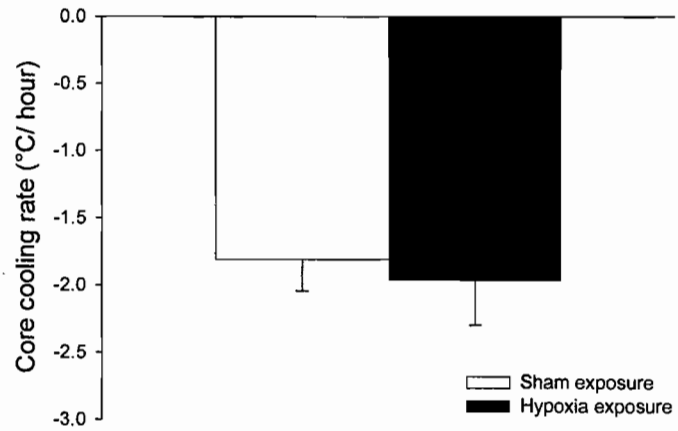

Figure 11. Core temperature response during 45 minutes of partial immersion in water at 10°C. Cooling rates were determined from the slope of the core temperature – time relationship beginning when water temperature reached 10°C. Values are means ± SE for 6 men and 6 women.

Figure 12 shows individual relationships between the effects of hypoxia on cutaneous vasoconstriction and core cooling rate. Regression analysis of this relationship yielded an r^2 value of 0.163 (P = 0.193; i.e., ~ 16% of the change in core cooling rate is explained by the effects of hypoxia vasoconstriction). Therefore, it appears that changes in core cooling rate during hypoxia are not related to the effects of hypoxia on cutaneous vasoconstriction.

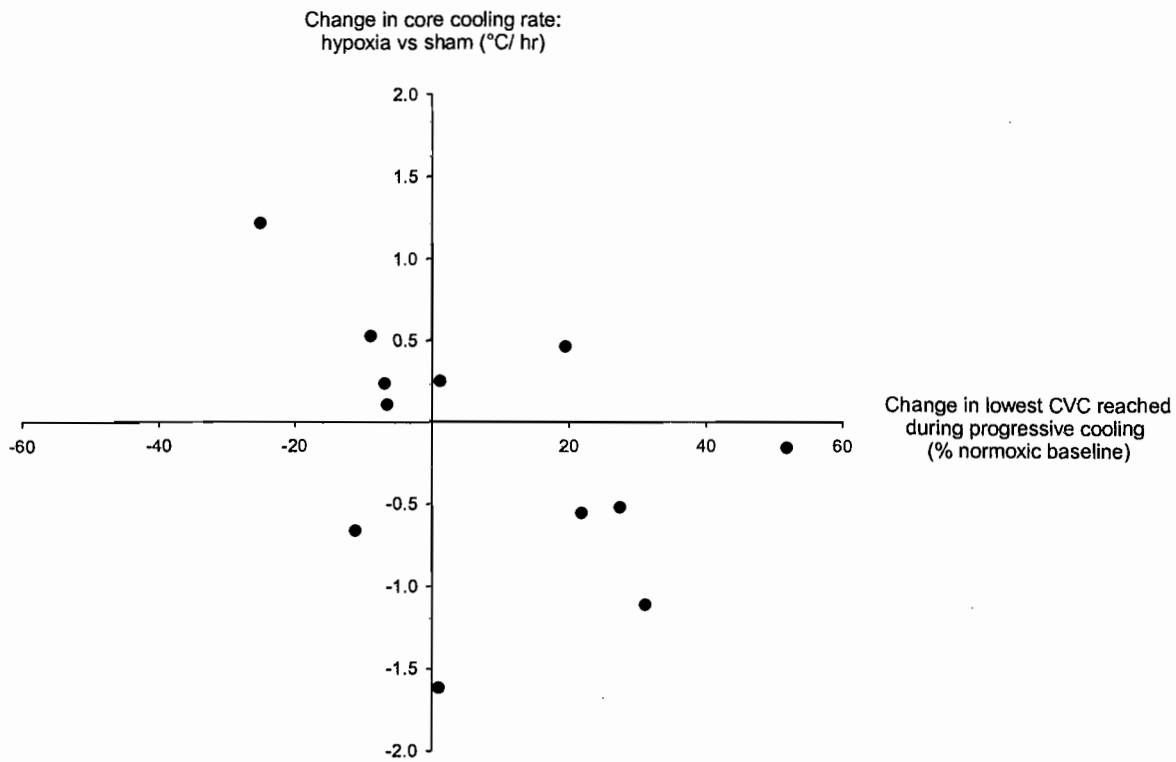

Figure 12. Scatter plot of individual data relating the effects of hypoxia on core cooling rate and peak vasoconstriction. Positive values on x-axis represent an upward shift in peak vasoconstriction during hypoxia. Positive values on y-axis represent slower core cooling during hypoxia. Data are from 6 males and 6 females.

Discussion

This study yielded several new findings. First, post-synaptic blockade of α-adrenergic receptors increased the magnitude of cutaneous vasodilation during hypoxia. Second, the magnitude of vasoconstriction during prolonged whole body cooling was increased by hypoxia, and this was sufficient to abolish the upward shift in skin blood flow that occurred in thermoneutral conditions. Third, the increased vasoconstrictor response during hypoxia was present (and equal in magnitude) when α-adrenergic vasoconstriction was blocked, suggesting a role for non-noradrenergic cutaneous vasoconstriction in this response. And finally, hypoxia did not affect resting core temperature or the rate of decline in core temperature during 45 minutes of partial immersion in 10°C water. Furthermore, analysis of individual responses demonstrated no relationship between the effects of hypoxia on cutaneous vasoconstriction and core body cooling during severe cold stress.

Traditionally the peripheral vascular response to systemic hypoxia has been viewed as a competition between chemoreflex control, mediated primarily through the sympathetic nervous system, and local vasodilator influences (Heistad & Abboud, 1980; Rowell, 1986). Therefore, the net change in perfusion observed during hypoxia represents the summation of two opposing vasoactive processes: vasodilation mediated through locally produced and/or circulating chemical mediators, and vasoconstriction mediated through the actions of sympathetic vasoconstrictor nerves and/or circulating constrictor substances (e.g., catecholamines, angiotensin II, etc). In forearm skin, previous work has

shown that blockade of α-adrenergic receptors with phentolamine unmasks greater vasodilation during acute hypoxia (Weisbrod *et al.*, 2001; Moradkhan *et al.*, 2007). However, we have recently shown that pre-synaptic blockade of sympathetic vasoconstrictor nerves does not affect the hypoxic response in the same area of skin (Simmons *et al.*, 2007). From these combined observations, it was suggested that a circulating catecholamine (epinephrine) may exert an adrenergic vasoconstrictor influence in non-acral skin, and this was supported by data showing elevated plasma epinephrine in previous studies which documented the effects of post-synaptic α-adrenergic blockade (Weisbrod *et al.*, 2001). Our current observations contribute further evidence to this concept, and overcome previous concerns about the non-specific effects of phentolamine on the cutaneous microcirculation (see discussion in Simmons *et al.*, 2007). Importantly, the true test of this hypothesis awaits an investigation directed specifically at this question in which the effects of pre- and post- synaptic adrenergic blockade are tested in the same subjects, at the same time, and with interstitial sampling of epinephrine during hypoxia.

We have previously shown that vasoconstrictor magnitude was unchanged by hypoxia and that as a result skin blood flow was increased during whole body cooling in the hypoxic setting (see Chapter IV). These results were demonstrated during a ten minute cold stress, a response which our previous data suggest is mediated entirely through noradrenergic mechanisms (see Chapter V). However, hypoxic vasodilation was abolished in the current study during a more prolonged cold exposure lasting 30 minutes. Moreover, the

magnitude of increased vasoconstriction in the α-adrenergic blockade site was sufficient to explain all of the net increase in vasoconstriction observed during hypoxia. This conclusion is based on a comparison of vasoconstrictor responses in control and α-adrenergic blocked sites. Importantly because these two sites responded differently to hypoxia, it is possible that the difference in magnitude of the hypoxic response limits our ability to draw conclusions through comparison of response magnitudes. However, we feel that this comparison is justified for at least three reasons. First, the cold responses are expressed in units measured during normoxic baseline conditions. Therefore, the shift in cutaneous vascular conductance during hypoxia – which was greater in the α-adrenergic blocked site – did not influence the calculation of the magnitude of these responses. Second, the difference in the responses between control and α-adrenergic blocked sites was ~ 20% of baseline cutaneous vascular conductance, and we have previously shown that a shift in baseline of this magnitude does not affect interpretation or comparison of vasoconstrictor responses regardless of how they are calculated (see Chapter IV). And third, we (see Chapter VII) and others (Stephens *et al.*, 2004) have shown that yohimbine treatment does not alter baseline skin blood flow in agreement with previous findings using other sympatholytic drugs (Houghton *et al.*, 2006). Furthermore, any effect of propranolol on baseline cutaneous vascular conductance would have been evenly distributed between skin sites inasmuch as it was included in the control rnicrodialysis fiber as well. Taken together, these factors suggest that comparison of the vasoconstrictor responses between control and α-adrenergic blocked sites is warranted.

Therefore, we interpret these data as evidence that hypoxic vasodilation is overcome by reflex vasoconstriction during prolonged cold stress such that no upward shift in skin blood flow persists in this setting. The mechanism through which this occurs is a selective increase in non-noradrenergic cutaneous vasoconstriction.

The increase in non-noradrenergic cutaneous vasoconstriction during hypoxia may be explained by central or peripheral mechanisms. For example, a potentiation of the output from thermoregulatory centers would increase sympathetic neural frequency leading to greater cotransmitter release (Eckberg *et al.*, 1988). Conversely, an increase in vascular responsiveness to sympathetic cotransmitters could be stimulated by local hypoxia. Unfortunately, the present data can not distinguish between these potential mechanisms. However, we believe that a local effect of hypoxia on non-noradrenergic cutaneous vasoconstriction is the more likely explanation for these results for two reasons. First, a wide variety of species respond to low oxygen environments by selectively regulating a lower body temperature, and this phenomenon appears to be regulated centrally within the hypothalamus (Steiner & Branco, 2002). Therefore, it is unlikely that subjects in this study would display the opposite response and generate a larger efferent response to equivalent afferent input (as demonstrated by unchanged mean skin temperature in the two conditions). The second reason which argues against a central mechanism regulating the increased non-noradrenergic cutaneous vasoconstriction is that neuropeptide Y-mediated vasoconstrictor responses in rats, stimulated peripherally by activation

of the lumbar sympathetic chain (bypassing central integration), are robust to the effects of hypoxia whereas α-adrenergic-mediated vasoconstriction is not (Coney & Marshall, 2007). These data suggest that non-noradrenergic cutaneous vasoconstriction is affected differently by hypoxia than noradrenergic vasoconstriction, and support the idea that increased vasoconstriction during cold stress in hypoxia is caused by a selective upregulation of vascular responsiveness to sympathetic cotransmitters.

A main goal of this study was to evaluate the relationship between two previously demonstrated phenomena which were thought to be related. One of these phenomena was an upward shift in skin blood flow and the other was a faster core cooling rate, both observed during acute hypoxia (see Chapter IV; (Cipriano & Goldman, 1975; Johnston *et al.*, 1996). Unexpectedly, neither of these phenomena occurred on a consistent basis in this study as evidenced by the group responses. However, we also analyzed individual relationships between these two phenomena because a perfect correlation ($r^2 = 1.00$) could exist between upward shifted skin blood flow and faster core cooling during cold stress even if group means for both responses are not different between normoxia and hypoxia. For example, if a straight line could be drawn through all of the data points in figure 12, with half of the data points distributed in the upper left quadrant and the other half in the bottom right quadrant, then this would produce a scenario where a strong relationship exists but is masked by the analysis of mean data. Nevertheless, analysis of the individual data, which

represents the true test of our hypothesis, indicate that changes in core cooling rate during hypoxia are not explained by persistent cutaneous vasodilation.

The lack of an effect of hypoxia on the core temperature response to severe cold exposure disagrees with some but not all previous human studies in this area. For example, Cipriano and Goldman (1975) found that core temperature was shifted downward by hypoxia during cold stress while core cooling rate increased early in the cooling period. In addition, Johnston *et al.* (1996) found that core cooling rate during post-exercise water immersion was increased by 33% n hypoxic conditions. However, Blatteis and Lutherer (1976) found no evidence of altered core cooling during high altitude exposure in a field study. Our results agree with those of Blatteis and Lutherer (1976) and suggest that acute hypoxia does not increase the rate of core cooling during severe cold exposure. The reason for conflicting results in this area is unclear, but may be related to differences in methodology between studies. In the study of Cipriano and Goldman (1975), hypoxia was superimposed on cold stress mid-way through a 4 hour cold exposure. Therefore, it may be that the interaction between hypoxia and cold is different depending on which environmental stress is initiated first. Conversely, the more prolonged (hours) hypoxic and cold exposures in that study may have resulted in less vasoconstriction, as evidenced by the stabilization of mean skin temperature at higher values during cooling. An important consideration in the study of Johnston *et al.* (1996) is that core cooling responses were measured immediately after cessation of dynamic exercise. This undoubtedly provided a greater range over which to study core temperature

responses inasmuch as exercise increased esophageal temperature by ~ 0.7°C. However, thermoregulatory control is mechanistically different post-exercise, and may be affected by relative work rate during the exercise period (Kenny *et al.*, 2003). Therefore, the possibility that the different relative work rate during normoxia and hypoxia contributed to altered post-ex thermoregulatory control can not be ruled out.

We did not include a site where sympathetic adrenergic nerves were blocked in this study. Therefore, the assumption that increased non-noradrenergic vasoconstrictor responses during hypoxia are mediated through adrenergic nerve cotransmission relies on the observations of previous studies in this area (Stephens *et al.*, 2001; Thompson & Kenney, 2004). However, these previous studies were performed in normoxic conditions, and so there may be vasoconstrictor systems activated by combined hypoxia and cold stress that were not addressed in the studies that identified adrenergic nerves as mediators of sympathetic cotransmission. Therefore we can not rule out the contributions of circulating vasoconstrictor substances to the increase in cutaneous vasoconstriction observed during combined hypoxia and cold stress in the current study.

The delivery of 10.5 μM BIBP-3226 via microdialysis did not reduce cutaneous vasoconstriction during whole body cooling, even though non-noradrenergic cutaneous vasoconstriction was activated (see figure 10). This observation is at odds with a previous investigation in which the same concentration of BIBP-3226 blocked most of the non-noradrenergic cutaneous

vasoconstrictor response during whole body cooling (Stephens *et al.*, 2004). In this case, both our laboratory and others (Lacy A. Holowatz, personal communication) have been unable to reproduce these results. It is unclear whether this is due to inaccurate reporting of the original concentration of BIBP-3226 used by Stephens *et al.* (2004) or to some other methodological differences. Regardless, this issue has proved difficult to resolve because of the high cost of BIBP-3226 and the higher cost of research grade neuropeptide Y. Finally, there is currently no selective neuropeptide Y-Y_1 receptor agonist approved for use in humans. Therefore, future studies will be necessary to resolve this issue.

In summary, we show for the first time that the magnitude of cutaneous vasoconstriction is increased during prolonged cold stress in a hypoxic environment. This effect is sufficient to abolish hypoxic vasodilation present during shorter cooling bouts, and it appears to be mechanistically linked to increased non-noradrenergic cutaneous vasoconstriction. Acute hypoxia does not alter the kinetics of core temperature measured during to severe cold exposure, and individual responses demonstrated no evidence for a relationship between the effects of hypoxia on cutaneous vasoconstriction and core body cooling during severe cold stress.

Chapter VI entitled "Increased contribution from non-noradrenergic cutaneous vasoconstriction abolishes hypoxic vasodilation during prolonged cold stress" yielded several novel findings. Most striking was the observation that cutaneous vasoconstriction was greater in magnitude when cold exposure was performed in hypoxic conditions. Moreover, the increase in vasoconstrictor magnitude was mediated entirely through non-noradrenergic pathways. These findings suggest that during acute hypoxia, either a central upregulation of vasoconstrictor outflow during cold stress increases cotransmitter release from sympathetic nerve terminals, or vascular responsiveness to sympathetic cotransmitters is selectively increased. Therefore, Chapter VII entitled "Does hypoxia affect post-junctional vasoconstrictor responsiveness in human skin?" was designed to test cutaneous vascular responsiveness to locally released sympathetic vasoconstrictor stimuli. Cutaneous sympathetic vasoconstriction was stimulated peripherally so as to control for possible central effects of hypoxia on sensory integration (i.e., during cold exposure).

CHAPTER VII

DOES HYPOXIA AFFECT POST-JUNCTIONAL VASOCONSTRICTOR RESPONSIVENESS IN HUMAN SKIN?

Introduction

The reduction in skin blood flow during whole body cooling is regulated by cutaneous sympathetic adrenergic nerves, as demonstrated by the sensitivity of this response to bretylium treatment (Kellogg *et al.*, 1989; Thompson & Kenney, 2004). As ambient temperature decreases, the activity of cutaneous adrenergic nerves increases causing the release of norepinephrine and sympathetic cotransmitters. The binding of norepinephrine to post-junctional α-receptors (predominantly α_2-receptors) causes vasoconstriction, and this mechanism is responsible for approximately 60% of the reduction in skin blood flow during cold stress (Stephens *et al.*, 2001; Thompson & Kenney, 2004). The remaining ~40% of the cold induced vasoconstriction is mediated by adrenergic nerve cotransmission, and recent evidence has implicated neuropeptide Y acting through post-junctional neuropeptide Y_1-receptors in this response (Stephens *et al.*, 2004).

Acute exposure to systemic hypoxia causes cutaneous vasodilation in non-acral skin, and this response is not restrained by neurally-mediated sympathetic vasoconstriction (Simmons *et al.*, 2007). We recently demonstrated

that this hypoxic vasodilation persists during whole body cooling of short duration (10 minutes; see Chapter IV). However, when cold exposure is more prolonged, the upward shift in skin blood flow is abolished and cutaneous perfusion is not different between hypoxic and normoxic conditions (see Chapter VI). Our previous work suggests the mechanism mediating vasoconstriction during short duration cold stress is entirely noradrenergic, while more prolonged cooling elicits both noradrenergic and non-noradrenergic cutaneous vasoconstriction (see Chapter V). During hypoxia, the increase in non-noradrenergic cutaneous vasoconstriction accounts for all of the increase in net vasoconstriction in response to whole body cooling. Therefore, it appears that non-noradrenergic cutaneous vasoconstriction is selectively upregulated during hypoxia, and this effect overcomes hypoxic cutaneous vasodilation during cold exposure (see Chapter VI).

The mechanism by which non-noradrenergic cutaneous vasoconstriction is upregulated during hypoxia is unclear. For example, a central effect of hypoxia could alter the sensory integration within the pre-optic area of the hypothalamus (POAH), thereby shifting the magnitude of sympathetic outflow for a given combination of temperature inputs (Steiner & Branco, 2002). Conversely, vascular responsiveness to sympathetic cotransmitters could be increased, thereby eliciting greater vasoconstriction in response to equal neurotransmitter release. In order to test this latter possibility, we stimulated cutaneous vasoconstriction locally to eliminate a possible central influence of hypoxia on sympathetic vasoconstrictor outflow.

The original design of this study involved the selective stimulation of separate cutaneous vasoconstrictor pathways by intradermal administration of norepinephrine (α-receptor agonist) and [D-Arg^{25}]-NPY (neuropeptide Y_1-receptor agonist). However, neither [D-Arg^{25}]-NPY nor any other specific neuropeptide Y_1-receptor agonist is currently approved for use in humans. Therefore, because both noradrenergic and non-noradrenergic cutaneous vasoconstriction are stimulated by sympathetic adrenergic nerves, we stimulated vasoconstriction pre-synaptically using tyramine. We reasoned that presynaptic stimulation of sympathetic vasoconstrictor nerves would lead to both norepinephrine and cotransmitter release, because previous studies have demonstrated that neuropeptide Y is colocalized with norepinephrine in sympathetic perivascular nerves (Ekblad *et al.*, 1984; Pernow *et al.*, 1987). Furthermore, we reasoned that individual vasoconstrictor pathways could be isolated by selective post-synaptic blockade of either pathway in discrete regions of the skin. This is supported by our previous work (Chapter VI) and the work of others (Stephens *et al.*, 2004).

Therefore, the purpose of this study was to test post-junctional vasoconstrictor responsiveness in non-acral skin during exposure to acute hypoxia. We tested the hypothesis that non-noradrenergic cutaneous vasoconstriction is selectively potentiated during hypoxia, while noradrenergic vasoconstriction is unaffected.

Methods

This study was approved by the institutional review board of the University of Oregon, and each subject gave written, informed consent before participation.

Subjects

Twelve healthy, nonsmoking, normotensive subjects (6 men, 6 women), age 23 ± 2 yr, participated in this study [height 176 ± 14 (SD) cm, weight 77.7 ± 18.6 kg, body mass index 24.7 ± 3.5 kg/m^2]. Subjects were taking no medications, except for oral contraceptives, and none had been to altitude (>1,500 m) within 5 months. Women were studied either during the early follicular phase of the menstrual cycle or during the placebo phase of oral contraceptive use to minimize the potential effects of female hormones on these responses. All female subjects had a negative urine pregnancy test within 1 hour of participation.

Instrumentation and measurements

Experiments were performed in thermoneutral conditions inside of a purpose built environmental chamber (Tescor, Inc) with ambient temperature maintained at 24°C. When the subject arrived on the study day, three microdialysis fibers (model MD 2000, Bioanalytical Systems, West Lafayette, IN) with a membrane length of 10 mm and molecular mass cutoff of 20 kDa (<5 μl dead space) were placed in the skin of the ventral forearm. Fibers were placed by inserting a 25-gauge needle through the dermis of the skin while the subject lay in the supine position. The fiber was then threaded through the internal lumen of the needle, and the needle was withdrawn, leaving the membrane in place. The fiber was taped in place and perfused with lactated Ringer's solution at a rate of 4 μl/min with a microinfusion pump (CMA/102, CMA Microdialysis, Stockholm, Sweden). Sites were at least 5 cm apart.

Immediately after microdialysis fiber insertion, subjects were moved to a reclined chair and sat in the semi-recumbent position while the local hyperemia subsided at each microdialysis site. Subjects were instrumented for the measurement of heart rate via electrocardiography (Cardiocap/5, Datex-Ohmeda, Madison, WI), ventilation via turbine pneumotach (VMM-400, Interface Associates, Laguna Niguel, CA), arterial pressure via brachial artery oscillometry (Cardiocap/5), arterial O_2 saturation via pulse oximetry (Cardiocap/5), and end-tidal PO_2 and PCO_2 via mass spectrometry (Marquette MGA 1100, MA Tech Services, St. Louis, MO). Isocapnia/eucapnia was defined as the mean end-tidal PCO_2 (nasal cannula) during a 5-min period of quiet breathing. To obtain an index of skin blood flow, cutaneous red blood cell flux was measured on the ventral forearm by laser-Doppler flowmetry (MoorLAB, Moor Instruments, Devon, UK) with integrated laser-Doppler probes fixed to the skin with adhesive tape. Skin blood flows were expressed as cutaneous vascular conductance (red blood cell flux/mean arterial pressure) and normalized to baseline values as described previously (Simmons *et al.*, 2007).

After resolution of the local hyperemia (~1.5 h), one microdialysis site was perfused with 5mM yohimbine (Sigma, St. Louis, MO) in lactated Ringer's solution for the purpose of α-adrenergic blockade. Although Yohimbine has traditionally been thought of as an α_2-adrenergic receptor antagonist, numerous recent studies have demonstrated that at high concentrations yohimbine (5mM) produces non-specific blockade of both α_1- and α_2-adrenergic receptors in human skin (Stephens *et al.*, 2001; Thompson & Kenney, 2004). Pilot work in our lab

has confirmed this dose (see Chapter V), and the time course of drug delivery in this protocol was based on previous studies (Stephens *et al.*, 2004; Hodges *et al.*, 2008). A second site was perfused with the neuropeptide Y Y_1-receptor antagonist BIBP-3226 (10.5μM; Sigma, St. Louis, MO) in lactated Ringer's. This concentration of BIBP-3226 abolishes the non-noradrenergic component of reflex cutaneous vasoconstriction during whole body cooling in humans (Stephens *et al.*, 2004). The third microdialysis fiber received only lactated Ringer's solution and was designated a control site. This study design aimed to produce one skin site in which vasoconstrictor function was normal, one site in which only noradrenergic vasoconstriction was functional, and one site which isolated cotransmitter-mediated vasoconstriction (presumably mediated through neuropeptide Y Y_1-receptors). Study drugs were infused for 60-75 minutes prior to beginning the protocol.

Protocol

After the initial drug infusion period, subjects were allowed to use the restroom, and were moved from the semi-recumbent to the supine position. The rest of the protocol was completed in the supine position.

To achieve control of tidal gases, subjects breathed from a custom built breathing circuit using a scuba mouthpiece and nose clip. The inspiratory mixture provided at the mouthpiece originated from 3 gas tanks – air (20.93% O_2), nitrogen (100% N_2), and carbon dioxide (100% CO_2) – connected to the inspiratory line by flow regulators. The fraction of inspired oxygen (FIO_2) and carbon dioxide ($FICO_2$) were varied by adjusting the flowrate from each of the gas

tanks, and the inspired air was first humidified, and then mixed as it filled a 6 liter tube which served as an inspiratory reservoir. The 6 liter tube connected to the breathing circuit proximal to the mouthpiece, and its distal end was open to room air. Total flowrate through the breathing circuit was always greater than the subjects' minute ventilation, and thus excess gas delivered through the inspiratory line escaped to the room through the 6 liter tube. The mouthpiece consisted of two one-way valves (Rudolph) to insure inspiration from the breathing circuit and expiration to room air.

This study was designed to test the effect of hypoxia on post-junctional vasoconstrictor responsiveness in human skin. Toward this end, cutaneous vasoconstrictor responses to intradermal Tyramine (Sigma, St. Louis, MO) administration were tested twice in random order: once during normoxia and once during isocapnic hypoxia. Tyramine is selectively taken up by adrenergic nerve terminals and displaces intraneuronal transmitters from their storage vesicles, causing a transient release of neurotransmitter into the extracellular space (Hoffman, 2001). In human skin, tyramine has recently been shown to increase interstitial norepinephrine levels when administered through cutaneous microdialysis (Leis *et al.*, 2004). However, we are aware of no published reports of vascular responses to intradermal tyramine administration.

A schematic representation of the experimental protocol is shown in figure 13. Tyramine infusions were separated by 90 minutes. The timing of Tyramine infusions was based on pilot studies in our lab. Extensive pilot work was performed to determine a dose of Tyramine which elicits vasoconstriction similar

in magnitude to that recorded during aggressive whole body cooling. A further requirement was that skin blood flow return to baseline levels soon enough that two doses of Tyramine could be delivered on the same study day. Finally, it was essential that consecutive Tyramine infusions elicit quantitatively similar vasoconstrictor responses. After experimenting with a variety of regimens, we found that a 3.5 minute infusion of 30 μg/ml Tyramine (173 μM) at a rate of 4 μl/min satisfied the necessary requirements. This infusion regimen delivers 420 ng of Tyramine through the microdialysis membrane over a short time course.

Before each Tyramine infusion, steady state data were recorded during mouthpiece breathing in either normoxia or isocapnic hypoxia. Isocapnic hypoxia was induced by decreasing the fraction of inspired oxygen (FIO_2) progressively over a 10 minute period. The rate of decrease in FIO_2 was designed to produce a fall in arterial O_2 saturation of ~ 2% min^{-1} down to 80%. After the target level of hypoxia was reached, 5 minutes of steady state data were recorded before the infusion of Tyramine began. For each tyramine infusion, all three microdialysis fibers were perfused with a solution of Tyramine at 30 μg/ml (173 μM) for 3.5 minutes (constant infusion rate: 4 μl/min). Tyramine solutions included the drug blockers that were already perfusing each microdialysis site, and solutions containing only these blockers were reintroduced immediately after the Tyramine infusion was stopped. The full extent of Tyramine-evoked vasoconstriction was typically manifest around 20 minutes after cessation of the infusion. Therefore, in addition to recording baseline data prior to Tyramine infusion, data were recorded continuously for at least 20 minutes after the infusion was stopped.

Care was taken to insure that a nadir in skin blood flow had been reached prior to removing the subject from the breathing circuit.

Data acquisition and analysis

Data were digitized with signal-processing software (WinDaq, Dataq Instruments, Akron, OH) and analyzed off-line. Vasoconstrictor responses were first analyzed with a three-way ANOVA (effects: sex, drug site, O_2 levels) to test the effect of sex on cutaneous vascular responses. There was no effect of sex on cutaneous vasoconstrictor responses in this study ($P = 0.985$ for sex main effect). Thus data from men and women were pooled for all subsequent analyses. Baseline skin blood flow was recorded after the initial drug infusion period of 60-75 minutes, prior to moving the subject to the supine position. Cutaneous vascular conductance was calculated in arbitrary units (Keller *et al.*, 2006), and these data were analyzed with a one-way repeated measures ANOVA to test the effect of drug treatment on baseline skin blood flow. Pretreatment with these study drugs did not significantly alter baseline cutaneous vascular conductance ($P = 0.588$ vs. control for yohimbine site, $P = 0.679$ vs. control for BIBP-3226) as shown previously by others (Stephens *et al.*, 2004). Statistical analyses were performed with SAS statistical software using PROC MIXED (SAS v9.1.3, SAS Institute, Cary, NC). Differences were considered significant when $P < 0.05$. All values are presented as means ± SE, unless otherwise indicated.

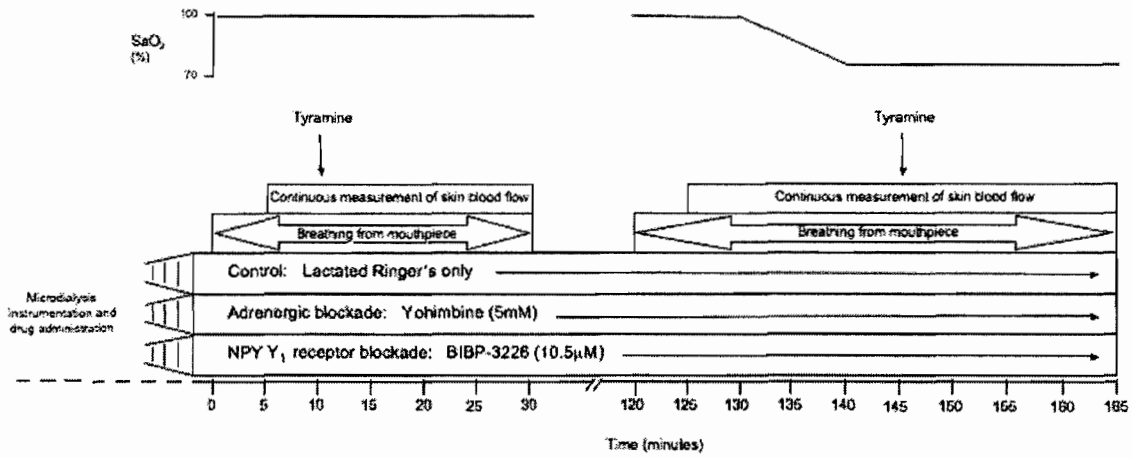

Figure 13. Schematic representation of chapter VII experimental protocol. Alpha-adrenergic and neuropeptide Y (NPY) receptor antagonists were infused continuously throughout the protocol. Tyramine was infused at a dose of 30 μg/ml (173 μM) for 3.5 minutes (constant infusion rate: 4 μl/min).

Results

Cardiorespiratory variables

Table 3 displays the cardiorespiratory data collected during normoxia and hypoxia. During hypoxia, heart rate was elevated while arterial oxygen saturation was reduced (both $P < 0.05$). Neither end-tidal PCO_2 nor arterial pressure were changed by hypoxia (both $P > 0.05$), while ventilation increased slightly ($P = 0.065$).

Cutaneous vascular responses

Cutaneous vascular responses to tyramine administration are displayed in figure 14. During normoxia, tyramine reduced cutaneous vascular conductance in both the control site and the site receiving BIBP-3226 by equal magnitudes (both $P < 0.05$ vs. pre-tyramine; $P = 0.445$ vs. between sites). However, vascular conductance in the site receiving yohimbine did not change ($P = 0.398$ vs. pre-tyramine). During hypoxia, responses to tyramine followed a similar trend as

Table 3. Cardiorespiratory data during normoxia and isocapnic hypoxia. Values are means ± SE for 6 males and 6 females. * $P < 0.05$ vs. normoxia.

	Normoxia	Hypoxia
End-tidal PCO_2 (mmHg)	35.7 ± 0.6	36.0 ± 0.5
Oxyhemoglobin saturation (%)	98.4 ± 0.2	82.5 ± 0.6*
Ventilation (L/min)	6.01 ± 0.40	7.51 ± 0.62
Heart rate (beats/min)	60.4 ± 1.7	68.8 ± 2.0*
Mean arterial pressure (mmHg)	86.1 ± 2.3	88.4 ± 2.4

during normoxia, with vascular conductance decreased by equal magnitudes in the control site and the site receiving BIBP-3226 (both $P < 0.05$ vs. pre-tyramine; $P = 0.814$ between sites). Again, cutaneous vascular conductance was unchanged in the site receiving yohimbine ($P = 0.732$ vs. pre-tyramine). There was no effect of hypoxia on vasoconstrictor responses at any site (all $P > 0.05$ vs. normoxia).

Discussion

The major new findings of this study are that (1) sympathetic vasoconstriction evoked by intradermal tyramine administration is mediated entirely through α-adrenergic mechanisms, and (2) post-junctional α-adrenergic vasoconstrictor responsiveness in non-acral skin is not affected by systemic hypoxia.

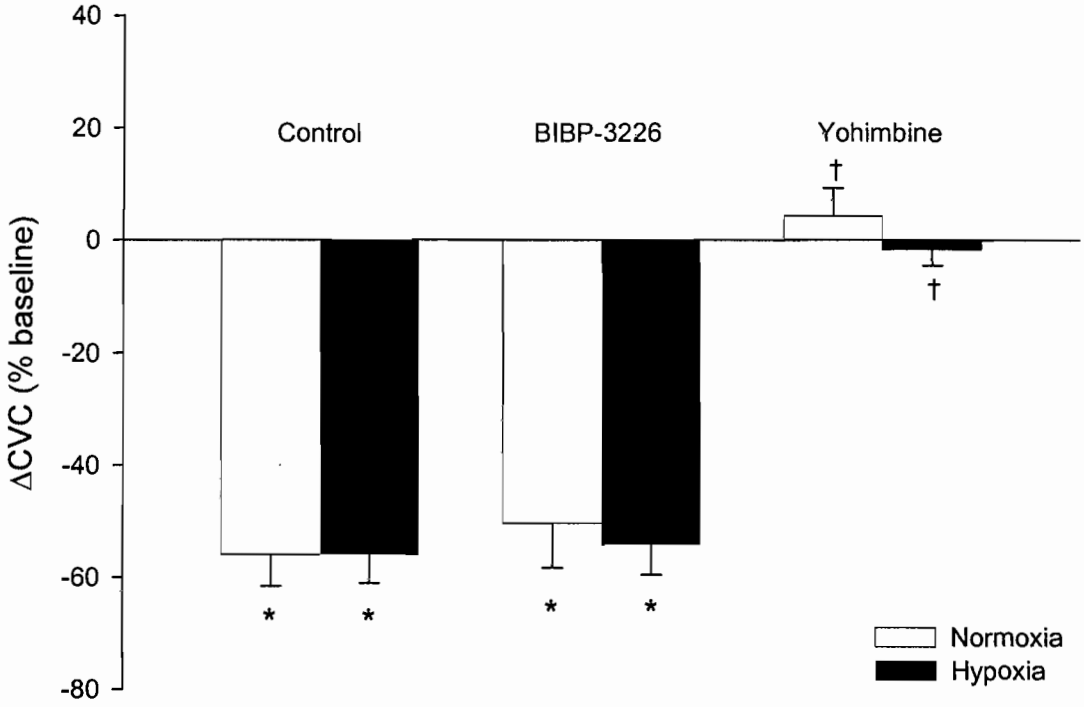

Figure 14. Cutaneous vasoconstrictor responses to intradermal tyramine administration during normoxia and isocapnic hypoxia. Control site received only lactated ringers; BIBP-3226 and yohimbine were infused at constant doses of 10.5μM and 5mM, respectively. Values are means ± SE for 6 males and 6 females. * $P < 0.05$ vs. baseline; † $P < 0.05$ vs. control response.

Our laboratory has previously shown that systemic hypoxia selectively increases the magnitude of non-noradrenergic cutaneous vasoconstriction during prolonged cold exposure (see Chapter VI). However, it was unclear from those findings whether increased non-noradrenergic cutaneous vasoconstriction was due to central neural or peripheral vascular effects of hypoxia (Rowell & Blackmon, 1987). Therefore, in the current study we stimulated vasoconstriction peripherally using intradermal tyramine administration. Peripheral stimulation of sympathetic vasoconstriction circumvents the central integration associated with

reflex sympathetic activation and therefore eliminates the central nervous system as a possible site for hypoxic-mediated effects. Because tyramine selectively stimulates sympathetic adrenergic nerve endings, we reasoned that its application to the cutaneous interstitium would liberate vasoconstrictor substances from these nerves in a similar fashion to that observed during whole body cooling. This reasoning is supported by the fact that sympathetic cotransmitters, including neuropeptide Y, are co-stored with norepinephrine in sympathetic perivascular nerves in both animals and humans (Lundberg *et al.*, 1983; Ekblad *et al.*, 1984; Pernow *et al.*, 1987). However, the application of tyramine caused no cutaneous vasoconstrictor response in the presence of α-adrenergic blockade. Therefore, these results suggest that tyramine does not stimulate non-noradrenergic vasoconstriction in human skin.

The failure of tyramine to stimulate non-noradrenergic cutaneous vasoconstriction could be explained by two possibilities. First, it may be that tyramine does not induce the release of sympathetic cotransmitters (specifically neuropeptide Y) upon uptake into adrenergic nerve endings. This possibility is supported by some (Lundberg *et al.*, 1989b; Takiyyuddin *et al.*, 1994) but not all (Cheng & Shen, 1987; Cheng *et al.*, 1987) previous studies in this area. For example, Lundberg *et al.* (1989b) showed that tyramine stimulation of the pig spleen *in vivo* caused vasoconstriction that was associated with an increase in norepinephrine outflow but no change in neuropeptide Y outflow in the venous effluent draining the isolated organ. Later, Takiyyuddin *et al.* (1994) demonstrated that intravenous infusion of tyramine caused an increase in arterial

pressure and plasma norepinephrine, while plasma neuropeptide Y was unaltered. In contrast, Cheng *et al.* (1987) demonstrated that tyramine releases both norepinephrine and neuropeptide Y from sympathetic terminals of the rabbit jejunum, but that neuropeptide Y is only released at higher concentrations of tyramine. In addition, tyramine was shown to cause a dose dependent rise in neuropeptide Y release from sympathetic terminals of the rat vas deferens (Cheng & Shen, 1987). Therefore, it appears that tyramine stimulates the release of neuropeptide Y with norepinephrine in certain tissues, whereas norepinephrine is released alone in others. Thus, one interpretation of our results is that sympathetic cotransmitter release is not stimulated by tyramine in human skin. A second possibility is that neuropeptide Y is present in cutaneous adrenergic nerve terminals but at concentrations too low to exert vasoactive effects. For example, separate analysis of the neuronal cell body (sympathetic ganglia) and axon terminal (peripheral tissue) of sympathetic neurons displaying neuropeptide Y-like immunoreactivity yielded norepinephrine:neuropeptide Y ratios of 150:1 and 10:1 for axonal cell bodies and peripheral terminals, respectively (Fried *et al.*, 1985). These data indicate that NPY content is higher in cell bodies, and lower in axon terminals of sympathetic neurons. Furthermore, Fried *et al.* (1985) provided evidence that replenishment of neuropeptide Y to the nerve terminal occurs via slower axonal transport mechanisms. Taken together, these data suggest that neuropeptide Y is present (with norepinephrine) in the terminal endings of sympathetic vasoconstrictor nerves, but at relatively lower concentrations which may not exert vascular effects. However, during intense

stimulation of sympathetic activity, axonal transport mechanisms are activated which provide neuropeptide Y to the nerve terminal for subsequent release (Lundberg *et al.*, 1990).

We have previously shown that the magnitude of cutaneous vasoconstriction during ten minutes of whole body cooling is not altered by systemic hypoxia (see Chapter IV). In a subsequent pilot study, we demonstrated that vasoconstriction during ten minutes of whole body cooling is mediated entirely through α-adrenergic mechanisms (see Chapter V). Taken together, these data suggest that the noradrenergic component of cutaneous vasoconstriction is not altered during whole body cooling in humans. However, a limitation of this evidence is that opposing central neural (Farkas & Donhoffer, 1973) and peripheral vascular (Heistad & Wheeler, 1970) effects of hypoxia may have masked one another, thereby producing no net change in cutaneous vasoconstriction during cold stress. The current study overcomes this limitation by stimulating cutaneous vasoconstriction locally and bypassing any central effect of hypoxia. Furthermore, no non-noradrenergic cutaneous vasoconstriction was stimulated by tyramine either during normoxia or hypoxia (see above). Therefore, it appears that the vasoconstrictor responses presented here are mediated entirely through noradrenergic mechanisms, and we conclude that cutaneous vascular responsiveness to noradrenergic vasoconstrictor stimuli is not altered by systemic hypoxia.

The maintenance of cutaneous vascular responsiveness to α-adrenergic vasoconstriction agrees with some (Dinenno, 2003; Dinenno *et al.*, 2003) but not

all (Heistad & Wheeler, 1970, 1972) previous research in this area. Heistad and Wheeler (1970) showed that both hand and forearm vasoconstrictor responses to sympathetic stimulation were reduced during hypoxia. This effect was at least partly explained by blunted vascular responsiveness, as indicated by reduced vasoconstrictor responses to exogenous norepinephrine. However, Dinenno *et al.* (2003) recently demonstrated that forearm vascular responses to intra-arterial infusion of tyramine are not altered by hypoxia. The reason for discrepant findings during norepinephrine and tyramine infusion is unclear. One possibility is that forearm vascular responses to intraluminal and extraluminal norepinephrine differ because the diffusion distance for norepinephrine to the post-junctional receptors is shorter when released from sympathetic nerve terminals. Therefore, decreased vasoconstrictor responses to intra-arterial infusion of norepinephrine could be the result of increased norepinephrine clearance during hypoxia (Leuenberger *et al.*, 1991) and a subsequent reduction in the amount of catecholamine reaching post-junctional α-adrenergic receptors. Future studies in which exogenous norepinephrine is introduced into the interstitium by microdialysis (in both skin and muscle) during hypoxia may resolve this issue.

Perspectives

As discussed above, the results of this study contribute further to a body of evidence indicating that post-junctional α-adrenergic vasoconstrictor responsiveness in human skin is not altered by hypoxia. When viewed in the context of our previous work (see Chapter VI), these data support that notion that

increased cutaneous vasoconstriction during combined hypoxia and cold stress is mediated through non-noradrenergic pathways, suggesting a role for sympathetic cotransmission. Further evaluation of these hypotheses awaits the approval for human use of pharmacological tools that selectively inhibit and stimulate sympathetic cotransmitter-mediated vasoconstrictor pathways.

In summary, we have shown that sympathetic vasoconstriction evoked by intradermal tyramine is mediated entirely through α-adrenergic mechanisms. Furthermore, these noradrenergic vasoconstrictor mechanisms appear to be unaltered by systemic hypoxia in the cutaneous vasculature.

CHAPTER VIII

CONCLUSIONS

High altitude environments are characterized by reduced barometric pressure and inadequate oxygen supply. In this setting, a variety of species selectively regulate a lower body temperature, presumably favoring a protective reduction in metabolism (Wood, 1991; Steiner & Branco, 2002). Some evidence suggests that humans exhibit a similar response in hypoxic environments. For example, some (Cipriano & Goldman, 1975) but not all (Blatteis & Lutherer, 1976) studies report a downward shift in resting core temperature in humans exposed to cold temperatures in hypoxic environments. Conversely, numerous studies report that skin blood flow (and presumably cutaneous heat loss) is increased by hypoxia, although some report this response is masked by sympathetic vasoconstriction (Schneider & Sisco, 1914; Kollai, 1983; Sagawa *et al.*, 1986; Anderson *et al.*, 1991; Leuenberger *et al.*, 1991; Weisbrod *et al.*, 2001; Simmons *et al.*, 2007). In agreement with the blood flow data, mean skin temperature – reflecting a balance between heat transfer from core to skin and from skin to air – is increased both at rest and during cold exposure in hypoxic environments (Cipriano & Goldman, 1975; Blatteis & Lutherer, 1976). Moreover, one study found that the expected reduction in skin temperature (caused by cutaneous vasoconstriction) during 4 hours of cold exposure was abolished at a

simulated altitude of 5000 meters (Cipriano & Goldman, 1975). Taken together, these observations suggest that control of skin blood flow during thermoneutral conditions and cold exposure is altered by systemic hypoxia. Furthermore, these changes in thermoregulatory control of skin blood flow may adversely affect core temperature regulation at high altitude. The focus of this review is the impact of acute hypoxia on cutaneous vascular regulation in thermoneutral and cold environments.

Cutaneous vascular control during hypoxia in thermoneutral conditions

In thermoneutral conditions, skin blood flow is increased by 25 – 30% upon exposure to acute hypoxia. This response is present in both the upper and lower limbs and occurs in the absence of changes in arterial pressure, suggesting it is caused by vasodilation (Anderson *et al.*, 1991; Simmons *et al.*, 2007). Furthermore, this response is not mediated by hyperventilation and persists even if hypocapnia develops secondary to hypoxic hyperventilation (see Chapter IV; Simmons *et al.*, 2007). Therefore, it appears that hypoxemia *per se* elicits cutaneous vasodilation during exposure to systemic hypoxia, and this response is not affected by secondary changes in ventilation or blood carbon dioxide levels.

Traditionally, the peripheral vascular response to systemic hypoxia has been viewed as a competition between chemoreflex control, mediated primarily through the sympathetic nervous system, and local vasodilator influences (Heistad & Abboud, 1980; Rowell, 1986). Therefore, the net change in perfusion observed during hypoxia represents the summation of two opposing vasoactive

processes: vasodilation mediated through locally produced and/or circulating chemical mediators, and vasoconstriction mediated through the actions of sympathetic vasoconstrictor nerves and/or circulating constrictor substances (e.g., catecholamines, angiotensin II, etc). Weisbrod *et al.* (2001) previously dissected the components of this complex vascular control system in the skeletal muscle vasculature. These authors showed that, during hypoxia, increases in sympathetic vasoconstrictor nerve activity "mask" significant vasodilation, which is mediated partly through local and partly through circulating chemical mediators (Weisbrod *et al.*, 2001). Importantly, the control elements of the vascular response to hypoxia may differ among peripheral vascular beds.

In the cutaneous circulation, the behavior of sympathetic vasoconstrictor nerve activity is more difficult to document than in the skeletal muscle vasculature (Vallbo *et al.*, 2004). The reason for this is that the cutaneous circulation is innervated by two branches of the sympathetic nervous system: an adrenergic vasoconstrictor system and a cholinergic vasodilator system (Roddie *et al.*, 1957; Roddie, 1983; Johnson, 1986; Kellogg *et al.*, 1989; Kellogg *et al.*, 1995). Owing to this dual sympathetic innervation, microneurographic recordings of cutaneous sympathetic nerve activity can not adequately distinguish changes in vasoconstrictor and vasodilator nerve activity. Thus, investigators are forced to find pharmacological means to study the cutaneous vasculature under the influence of varying degrees of adrenergic inhibition.

Our group has twice used pharmacological methods to study the contribution of adrenergic vasoconstriction to the net cutaneous vascular

response to hypoxia. The results from these two investigations initially stimulated different conclusions regarding adrenergic vasoconstriction in the skin during hypoxia. In 2001, Weisbrod *et al.* administered intra-arterial phentolamine to produce post-synaptic blockade of alpha-adrenergic receptors in the forearm, thus inhibiting sympathetic vasoconstriction. During hypoxia, the area of skin pretreated with phentolamine displayed a greater vasodilation than the skin with intact adrenergic control (Weisbrod *et al.*, 2001). Based on these results, the authors concluded that sympathetic vasoconstriction masks a portion of hypoxic vasodilation in the cutaneous circulation as is the case in the skeletal muscle vasculature. However, Simmons *et al.* (2007) later showed that pretreatment of the skin with bretylium tosylate (for presynaptic inhibition of adrenergic nerves) did not affect the magnitude of hypoxic vasodilation, indicating that increases in sympathetic vasoconstrictor nerve activity do not oppose hypoxic vasodilation in the skin.

These seemingly disparate observations may be reconciled by consideration of the different loci at which phentolamine and bretylium interfere with sympathetic vasoconstriction. For instance, phentolamine blocks postsynaptic α_1- and α_2- adrenergic receptors (Hoffman, 2001) while bretylium tosylate blocks adrenergic nerve transmission presynaptically (Haeusler *et al.*, 1969). Blockade of α_1- and α_2- adrenergic receptors interferes with both neural and humoral adrenergic vasoconstriction, whereas only neural vasoconstriction is blocked by inhibition of sympathetic adrenergic fibers. Thus the results indicating that cutaneous vasodilation is unmasked by α-receptor blockade may indicate

that humoral (adrenergic) vasoconstriction is present in the skin during hypoxia. This vasoconstriction would be sensitive to α-receptor blockade but not to presynaptic inhibition by bretylium. We have recently verified the results obtained during intra-arterial infusion of phentolamine using the same model in which bretylium treatment was shown not to affect the hypoxic response (see Chapter VI). The results of this study confirmed that hypoxic vasodilation is augmented in the presence of α-adrenergic blockade, and provided further evidence that humoral vasoconstriction opposes hypoxic vasodilation in the skin. A likely candidate for such a circulating mediator of adrenergic vasoconstriction is epinephrine, which is present in the blood at higher concentrations during acute hypoxia (Weisbrod *et al.*, 2001). Epinephrine has been shown to cause cutaneous vasoconstriction (humoral) when administered intra-arterially, intravenously, by subcutaneous injection, or by iontophoresis (Barcroft *et al.*, 1955; Cooper *et al.*, 1955; Roddie, 1983; Hoffman, 2001). Taken together, these combined observations suggest that adrenergic vasoconstriction of humoral origin (possibly epinephrine) masks some of the cutaneous vasodilation during hypoxia, and adrenergic vasoconstriction of neural origin (i.e., sympathetic vasoconstrictor nerves) does not affect hypoxic vasodilation in human skin.

The regulatory mechanisms underlying hypoxic vasodilation in non-acral skin are currently unknown. As mentioned above, the non-acral cutaneous vasculature is innervated by two branches of the sympathetic nervous system: an adrenergic vasoconstrictor system and a cholinergic vasodilator system (Roddie *et al.*, 1957; Roddie, 1983; Johnson, 1986; Kellogg *et al.*, 1989; Kellogg *et al.*,

1995). While current understanding indicates that the adrenergic vasoconstrictor system is not involved in cutaneous vasodilation during hypoxia, the active vasodilator system may play a role. For example, Weisbrod *et al.* (2001) showed that blockade of nitric oxide production in the forearm reduces hypoxic vasodilation in the skin, suggesting that nitric oxide plays a role in cutaneous vasodilation during hypoxia. Nitric oxide contributes to cutaneous vasodilation during thermal stress via direct actions on vascular smooth muscle and synergistic activity with an unknown neurotransmitter associated with the active vasodilator system (Wilkins *et al.*, 2003). Thus the nitric oxide dependence of cutaneous vasodilation during hypoxia may indicate a role for active vasodilation. Conversely, nitric oxide may represent a pathway through which local vasodilator mechanisms act, as has been suggested in skeletal muscle during hypoxia (Blitzer *et al.*, 1996; Halliwill, 2003).

Cutaneous vascular control during combined hypoxia and cold stress

When environmental temperature falls, skin blood flow is reduced in order to decrease heat transfer from the core to the periphery, thereby promoting heat conservation (Toner & McArdle, 1988). This thermoregulatory adjustment also decreases skin temperature, which narrows the gradient for heat loss from the skin and is vital to the prevention of hypothermia in cold environments. The reduction in skin blood flow upon body cooling is regulated by cutaneous sympathetic adrenergic nerves, which contain both norepinephrine and other sympathetic cotransmitters including neuropeptide Y (Lundberg *et al.*, 1983; Ekblad *et al.*, 1984; Fried *et al.*, 1985; Pernow *et al.*, 1987; Kellogg *et al.*, 1989).

As ambient temperature decreases, the activity of cutaneous adrenergic nerves increases, releasing norepinephrine onto post junctional α-receptors (predominantly α_2-receptors). The binding of norepinephrine to post junctional α-receptors causes vasoconstriction, and this mechanism is responsible for approximately 60% of the reduction in skin blood flow during cold stress (Thompson & Kenney, 2004). The remaining ~40% of the cold induced vasoconstriction is mediated by adrenergic nerve cotransmission (Stephens *et al.*, 2001; Thompson & Kenney, 2004). The primary peptide thought to mediate sympathetic cotransmission in this setting is neuropeptide Y, which causes cutaneous vasoconstriction via post junctional neuropeptide Y Y_1-receptors (Stephens *et al.*, 2004).

During hypoxemia, Heistad and Wheeler (1972) have shown that vasoconstrictor responses to adrenergic stimulation (intra-arterial infusion of norepinephrine) are blunted in the hand circulation. Cipriano and Goldman (1975) and Blatteis and Lutherer (1976) later showed that mean skin temperature is elevated by hypoxia during whole body cold stress. Taken together, these data suggest that cutaneous vasoconstrictor responses during cold exposure may be altered by systemic hypoxia.

Simmons *et al.* (see Chapter IV) recently tested this idea by studying cutaneous vascular responses during combined hypoxia and cold stress. When cold exposure was induced gradually over a ten minute period, the final reduction in skin blood flow was upward shifted during hypoxia compared to normoxia, suggesting the maintenance of increased cutaneous heat loss. Furthermore, a

subsequent protocol demonstrated that cutaneous vasoconstriction during ten minutes of whole body cooling is mediated entirely through α-adrenergic mechanisms, whereas more prolonged cold exposure evokes sympathetic non-noradrenergic vasoconstriction (see Chapter V). These observations are likely to be explained by increased sympathetic vasoconstrictor outflow during more prolonged cold stress, inasmuch as the release of cotransmitters (e.g., neuropeptide Y) from sympathetic vasoconstrictor nerves is tightly linked to their frequency of activation (Eckberg *et al.*, 1988; Lundberg *et al.*, 1989a; Pernow *et al.*, 1989b). Therefore, the combined results above suggest that α-adrenergic vasoconstriction is functional during hypoxia, but that this system does not effectively reduce skin blood flow to the same levels during hypoxia as seen during normoxic cold stress.

As described above, when cold exposure is more prolonged, sympathetic non-noradrenergic vasoconstriction contributes to the net reduction in skin blood flow. In order to test the contribution of non-noradrenergic vasoconstriction to the cutaneous vascular response during combined hypoxic and cold exposure, Simmons *et al.* (see Chapter VI) exposed subjects to 30 minutes of progressive cold stress while measuring cutaneous vascular responses in functionally intact and α-adrenergic blocked sites on the forearm. These authors showed that the magnitude of cutaneous vasoconstriction during cold exposure was increased by hypoxia. This increase in vasoconstriction was sufficient to abolish cutaneous vasodilation during hypoxia, and the proportion of the total vasoconstrictor response accounted for by non-noradrenergic mechanisms increased from 30%

to > 50% during hypoxia. Furthermore, comparison of responses in control and α-adrenergic blocked sites indicated that increased vasoconstriction could be explained entirely by a greater non-noradrenergic cutaneous vasoconstrictor response (see figure 15). Therefore, it appears that non-noradrenergic cutaneous vasoconstriction is increased by hypoxia during prolonged cold stress, and this effect is sufficient to abolish hypoxic vasodilation observed in thermoneutral conditions.

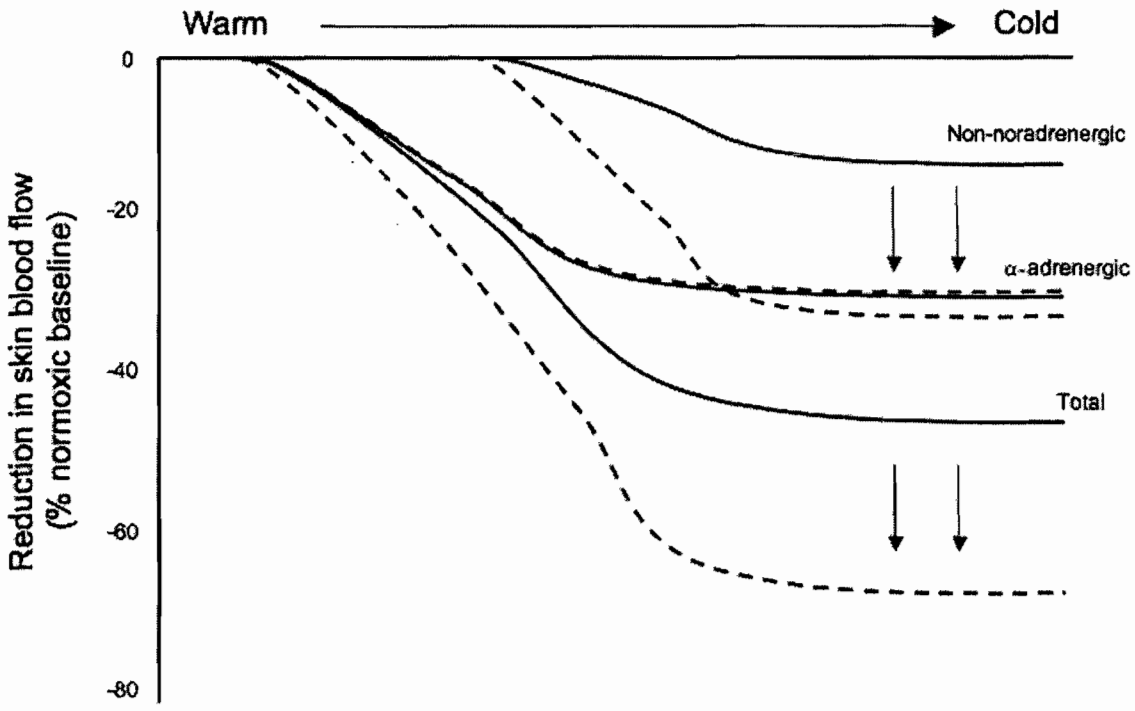

Figure 15. Proposed model explaining the noradrenergic and non-noradrenergic contributions to cutaneous vasoconstrictor responses during combined hypoxia and cold stress. Responses observed during hypoxia are indicated by dashed lines.

The effects of hypoxia on cutaneous vasoconstriction during prolonged cooling could be explained by central or peripheral mechanisms (see figure 16). However, the combined data thus far do not point to a clearly defined central mechanism. For example, during brief cold stress, baseline skin blood flow is increased by hypoxia but α-adrenergic vasoconstriction is unchanged, resulting in an upward shift in the cutaneous vascular response to cold in this setting (see Chapter IV). Interestingly, this effect is observed even if no hypoxic vasodilation is present (e.g., in women). Taken together, this suggests a possible central upregulation of cutaneous heat loss (i.e., increased thermoregulatory set point). However, during more prolonged cold exposure when both noradrenergic and non-noradrenergic vasoconstrictor mechanisms are activated, non-noradrenergic vasoconstriction is potentiated, resulting in a greater vasoconstrictor response and the abolition of hypoxic vasodilation (see Chapter VI). The increased vasoconstrictor response in this setting would suggest an opposite effect on central regulation of cutaneous heat loss (i.e., decreased thermoregulatory set point). Moreover, recent measurements of core temperature at rest and during aggressive cooling indicate no effect of hypoxia (see below). Therefore, data currently do not support a central effect of hypoxia on cutaneous vasoconstriction during cold exposure.

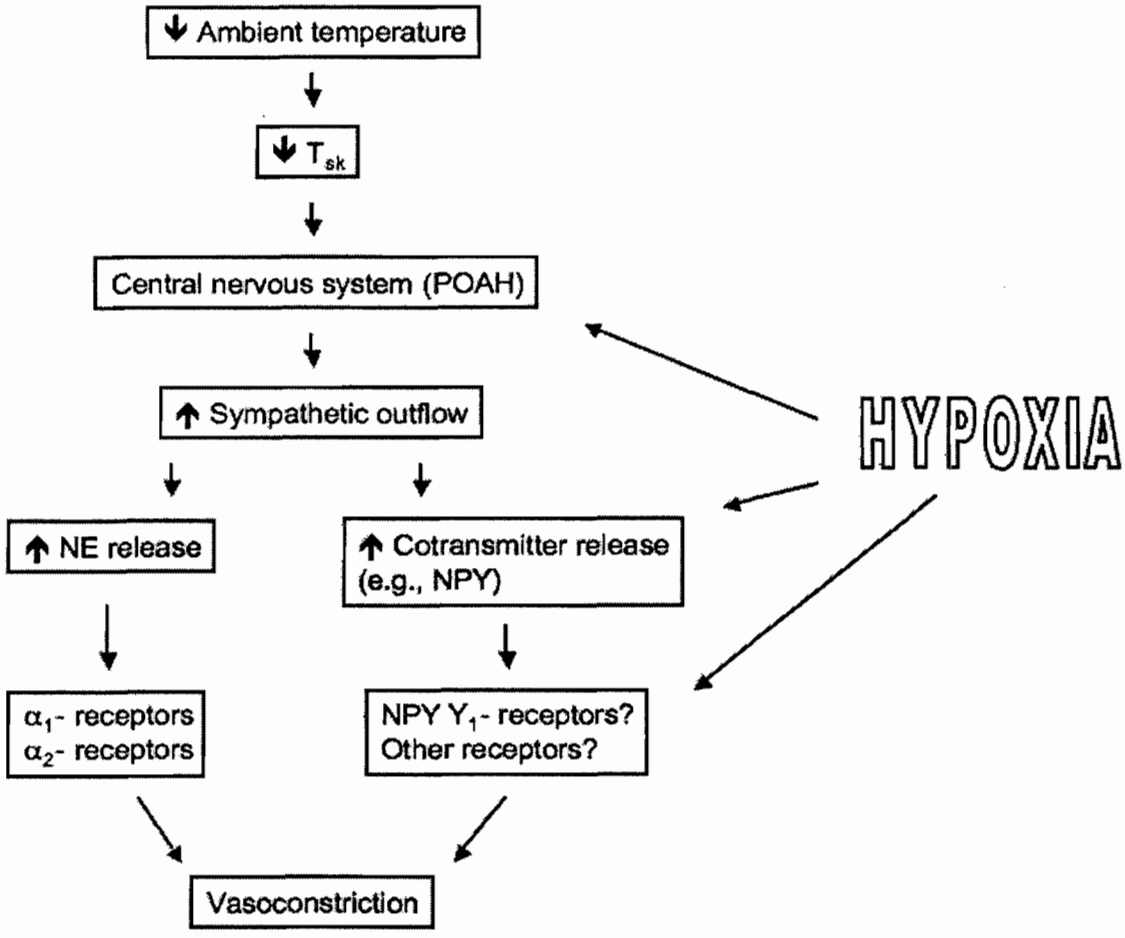

Figure 16. Possible mechanisms through which hypoxia affects the cutaneous vascular response to cold exposure. T_{sk} = Skin temperature; POAH = Pre-optic anterior hypothalamus; NE = Norepinephrine; NPY = Neuropeptide Y.

In order to investigate whether increased non-noradrenergic cutaneous vasoconstriction occurs through the peripheral effects of hypoxia, Simmons *et al.* (see Chapter VII) stimulated cutaneous vasoconstriction locally using microdialysis of tyramine. Tyramine has been shown to stimulate the release of both norepinephrine and neuropeptide Y from adrenergic nerves in some (Cheng & Shen, 1987; Cheng *et al.*, 1987) but not all preparations (Lundberg *et al.*,

1989b; Takiyyuddin *et al.*, 1994). Apparently the effect of tyramine on sympathetic cotransmitter release is dependent on the tissue examined as well as the mode of drug delivery (intraluminal versus abluminal). Therefore, because neuropeptide Y is colocalized with norepinephrine in perivascular nerve endings (Lundberg *et al.*, 1982; Lundberg *et al.*, 1983; Ekblad *et al.*, 1984; Pernow *et al.*, 1987) and post junctional neuropeptide Y receptors participate in reflex cutaneous vasoconstriction during whole body cooling in humans (Stephens *et al.*, 2004), Simmons *et al.* reasoned that intradermal tyramine delivery via microdialysis would stimulated both noradrenergic and non-noradrenergic cutaneous vasoconstrictor responses. Cutaneous vascular responses to tyramine were tested in control and α-adrenergic blocked sites during normoxia and hypoxia. Contrary to their hypothesis, however, tyramine elicited no vasoconstrictor response in the presence of α-adrenergic blockade. These data suggest that intradermal tyramine administration, at least at concentrations near those used by Simmons *et al.* (30 μg/ml [173 μM] x 3.5 minutes @ 4 μl/min), does not elicit vasoactive levels of sympathetic cotransmitter release in human skin (see discussion in Chapter VII). In agreement with the findings during brief cold stress, post-junctional α-adrenergic vasoconstrictor responses were not altered by hypoxia.

Perspectives

Previous studies have shown that mean skin temperature is elevated in humans exposed to cold stress during acute environmental hypoxia (Cipriano & Goldman, 1975; Blatteis & Lutherer, 1976). In addition, some evidence suggests

that increased skin temperature is associated with faster core heat loss in this setting (Cipriano & Goldman, 1975). This line of research stimulated the hypothesis that skin blood flow during cold exposure is upward shifted by hypoxia (as occurs in thermoneutral conditions), and that this response ultimately contributes to faster rates of core heat loss. Initial experiments conducted during brief cold exposure lasting ten minutes supported this hypothesis (see Chapter IV), demonstrating that hypoxic vasodilation persists through the end of cold exposure (i.e., skin blood flow was upward shifted). However, in a two phase protocol during which cutaneous vascular responses to progressive cold exposure and core cooling responses to severe cold exposure were tested back to back, no correlation was detected between the cutaneous vascular effects and core temperature effects of hypoxia in young healthy volunteers (Chapter VI). Therefore, it appears that the mechanisms of cutaneous vascular regulation during cold stress are altered by hypoxia, but these effects alone do not predispose young healthy persons to hypothermia.

In contrast to young healthy individuals, the aged are not able to adequately defend core temperature during prolonged mild cold stress. In the aged, core temperature is reduced and cutaneous vasoconstriction blunted during mild cold exposure compared to young volunteers (Degroot & Kenney, 2007). The effect of aging on the reduction in skin blood flow during cold exposure occurs through a selective loss of cotransmitter-mediated (non-noradrenergic) vasoconstriction (Thompson & Kenney, 2004). Therefore, inasmuch non-noradrenergic cutaneous vasoconstriction is potentiated by

hypoxia and eliminates the upward shift in skin blood flow during cold exposure (see Chapter VI), the aged may be significantly impacted by combined hypoxic and cold stress because they lack functional non-noradrenergic vasoconstrictor pathways.

APPENDIX A

INFORMED CONSENT FORM FOR CHAPTER IV

TITLE: Cutaneous vasodilation at high altitude: basic mechanisms and impacts on human thermoregulation (Protocol 1)

INVESTIGATOR: Dr. J. R. Halliwill and Colleagues

APPROVED BY INSTITUTIONAL REVIEW BOARD: July 27, 2007

This is an important form. Please read it carefully. It tells you what you need to know about this study. If you agree to take part in this research study, you need to sign the form. Your signature means that you have been told about the study and what the risks are. Your signature on this form also means that you want to take part in this study.

Why is this research study being done?

When humans breathe, we take in oxygen from the air and expel carbon dioxide that our bodies have made. When we travel to high altitudes, we fail to take in enough oxygen and we expel excess amounts of carbon dioxide. Conversely, some respiratory diseases are associated with failure to expel enough carbon dioxide produced within the body. Too little oxygen and too much or too little carbon dioxide may alter the distribution of blood in the limbs, increasing the amount of blood circulating in the skin. Unfortunately, we do not understand how these changes in blood distribution take place, or how they affect heat loss from the body. The purpose of this study is to provide more information on what causes the changes in blood flow when we take in too little oxygen or expel too little or too much carbon dioxide.

What will happen in the study?

Study visit

1) You will arrive at Dr. Halliwill's laboratory in Esslinger Hall at the University of Oregon on the study day. The study will last approximately 2 hours. You will meet with one of the investigators of the study to discuss the project, to see the laboratory, and to read this form. Your height and weight will be measured. You will need to refrain from consuming caffeine (for example, coffee, tea, red bull, coke, etc.) or medications (except oral contraceptives) for 12 hours prior to the study and abstain from alcohol or exercise for 24 hours prior to the study. In addition, you will need to refrain from eating for at least

2 hours prior to the study visit. If you use oral contraceptives, you should take this when you normally do so.

2) If you are a woman who can still become pregnant, you will be asked to undergo a pregnancy test. For this test, you will be asked to collect a sample of urine in the women's restroom near the physiology lab. If the test is "positive," indicating that you are pregnant, you will not be allowed to participate and will be advised to see your physician or the University of Oregon Health Center.

3) During the study visit, your heart rate will be monitored by electrocardiogram electrodes placed on your skin. If you are a woman, the adhesive patches used for this test will be placed on you by one of the women in the lab. Your blood pressure will be measured at periodic intervals by the inflation of a blood pressure cuff around your arm or around your middle finger.

4) During the study visit, two small probes (laser-Doppler probes) will be placed over your skin so that we can measure skin blood flow.

5) For a period of up to 10 minutes, your expired gas will be collected through a nasal cannula to assess the resting carbon dioxide levels in your blood. After removal of the nasal canulla, you will be asked to breathe air that has low oxygen content either with or without high carbon dioxide content for up to 30 minutes. During this time, you will breathe through a scuba mouthpiece with nasal breathing prevented by a nose clip. These procedures will increase your rate of breathing and heart rate and are similar to what happens when you travel to higher altitude. You will undergo several of these breathing procedures during the study.

6) During the study, we will inflate a small cuff that is placed on your middle and ring fingers of one of your hands to measure your blood pressure (Finometer device). If the cuff becomes uncomfortable, let the investigator know and they will turn it off for a few minutes.

7) You should notify the investigator immediately if you feel any significant discomfort or concern about your well-being at any time during the initial visit or the study visit. Some examples of discomfort include shortness of breath, light-headedness, and nausea.

How long will I be in the study?

You will be in the study for two hours.

What are the risks of the study?

1. Breathing air with a low oxygen content with/without high carbon dioxide content: During these procedures, it is possible you will feel distress, anxiety, shortness of breath, or feel lightheaded. These symptoms resolve rapidly

when you return to breathing room air. In addition, these breathing procedures have the potential to induce fainting. You should keep the investigator informed of your feelings and you should not attempt to prolong the breathing experiment if you do not feel well. You are free to stop at any time.

2. Laser-Doppler Probes: These probes send a small light into your skin. You will not feel anything except the probe touching your skin. The only risk associated with this procedure is that you may have some slight skin irritation (redness) to the adhesive tape used to hold the probe on your skin. There are no major risks associated with this device.

3. Finger blood pressure: In some people, this blood pressure cuff causes their finger to become red and uncomfortable after a long period (over 40 minutes). We will not inflate the cuff for longer than 40 minutes during the study. If your finger becomes uncomfortable during the study, let the investigator know and they will turn it off for a few minutes. There are no major risks associated with this device.

May I participate if I am pregnant or breast-feeding?

This study may be harmful to an unborn or breast-fed child. There is not enough medical information to know what the risks might be to a breast-fed infant or to an unborn child in a woman who takes part in this study. Breast-feeding mothers are not able to take part in this study. Women who can still become pregnant must have a negative pregnancy test no more than 24 hours before taking part in the study. If the pregnancy test is positive (meaning that you are pregnant), you will not be able to take part in the study. There is no cost for the pregnancy test.

Are there benefits to taking part in this study?

This study will not make your health better.

What other choices do I have if I don't take part in this study?

This study is only being done to gather information. You may choose not to take part in this study.

What are the costs of tests and procedures?

You will not need to pay for any tests or procedures that are done just for this research study. You will get $20 for participating in this study. This money is for the inconvenience and time you spent in this study. If you start the study but stop before the study has ended, the amount of money you receive will be pro-rated at a rate of $10 per hour of the study you complete.

Who can answer my questions?

You may talk to Dr. John Halliwill at any time about any question you have on this study. You may contact Dr. Halliwill by calling the Department of Human Physiology at (541) 346-5425.

What are my rights if I take part in this study?

Taking part in this research study is your decision. You do not have to take part in this study, but if you do, you can stop at any time. Your decision whether or not to participate will not affect your relationship with the University of Oregon.

You do not waive any liability rights for personal injury by signing this form. All forms of medical diagnosis and treatment whether routine or experimental, involve some risk of injury. In spite of all precautions, you might develop medical complications from participating in this study.

The investigators may stop you from taking part in this study at any time if it is in your best interest, if you do not follow the study rules, or if the study is stopped.

If you are physically injured because of the project, you and your insurance company will have to pay your doctor bills. If you are a UO student or employee and are covered by a UO medical plan, that plan might have terms that apply to your injury.

If you experience harm because of the project, you can ask the State of Oregon to pay you. If you have been harmed, there are two University representatives you need to contact. Here are their addresses and phone numbers:

General Counsel
Office of the President
University of Oregon
Eugene, OR 97403
(541) 346-3082

Office of Human Subjects Compliance
University of Oregon
Eugene, OR 97403
(541) 346-2510

A law called the Oregon Tort Claims Act limits the amount of money you can receive from the State of Oregon if you are harmed. The most you could receive would be $100,000, no matter how badly you are harmed. If other people are also harmed by the project, all of you together could only receive $500,000.

What about confidentiality?

Any information that is obtained in connection with this study and that can be identified with you will remain confidential and will be disclosed only with your permission. Subject identities will be kept confidential by assigning you a "subject identification number". The names associated with each subject identification number will be kept in a locked file cabinet in Dr. Halliwill's office area.

The Office for Responsible Conduct of Research and/or authorized representatives of the Food and Drug Administration (FDA) or National Institute of Health (NIH) may need to review records of individual subjects. As a result, they may see your name, but they are bound by rules of confidentiality not to reveal your identity to others.

The list of names will be destroyed when study results are published or 24 months after your participation, whichever comes first. Other information may be stored by the researchers indefinitely.

I have had an opportunity to have my questions answered. I have been given a copy of this form. I agree to take part in this study.

If you have questions regarding your rights as a research subject, contact Office for Protection of Human Subjects, 5237 University of Oregon, Eugene, OR 97403, (541) 346-2510.

Your signature indicates that you have read and understand the information provided above, that you willingly agree to participate, that you may withdraw your consent at any time and discontinue participation without penalty, that you will receive a copy of this form, and that you are not waiving any legal claims, rights or remedies.

_____ _____

(Date) (Signature of Participant)

_____ _____

(Date) (Signature of Individual Obtaining Consent)

APPENDIX B

INFORMED CONSENT FORM FOR CHAPTERS V & VII

TITLE: Cutaneous vasodilation at high altitude: basic mechanisms and impacts on human thermoregulation (Protocol 2)

INVESTIGATOR: Dr. J. R. Halliwill and Colleagues

APPROVED BY INSTITUTIONAL REVIEW BOARD: December 10, 2007

This is an important form. Please read it carefully. It tells you what you need to know about this study. If you agree to take part in this research study, you need to sign the form. Your signature means that you have been told about the study and what the risks are. Your signature on this form also means that you want to take part in this study.

Why is this research study being done?

When humans breathe, we take in oxygen from the air and expel carbon dioxide that our bodies have made. When we travel to high altitudes, we fail to take in enough oxygen and we expel excess amounts of carbon dioxide. Conversely, some respiratory diseases are associated with failure to expel enough carbon dioxide produced within the body. Too little oxygen and too much or too little carbon dioxide may alter the distribution of blood in the limbs, increasing the amount of blood circulating in the skin. Unfortunately, we do not understand how these changes in blood distribution take place, or how they affect heat loss from the body. The purpose of this study is to provide more information on what causes the changes in blood flow when we take in too little oxygen or expel too little or too much carbon dioxide.

What will happen in the study?

1) You will arrive at Dr. Halliwill's laboratory in Esslinger Hall at the University of Oregon on the study day. The study will last approximately 4 hours. You will meet with one of the investigators of the study to discuss the project, to see the laboratory, and to read this form. Your height and weight will be measured, and you will be asked to put on a black woven suit covered with small tubes through which water will be circulated (a "water perfused suit"). You will need to refrain from consurning caffeine (for example, coffee, tea, red bull, coke, etc.) or medications (except oral contraceptives) for 12 hours prior to the study and abstain from alcohol or exercise for 24 hours prior to the study. In addition, you will need to refrain from eating for at least 2 hours

prior to the study visit. If you use oral contraceptives, you should take this when you normally do so.

2) If you are a woman who can still become pregnant, you will be asked to undergo a pregnancy test. For this test, you will be asked to collect a sample of urine in the women's restroom near the physiology lab. If the test is "positive," indicating that you are pregnant, you will not be allowed to participate and will be advised to see your physician or the University of Oregon Health Center.

3) During the visit, your heart rate will be monitored by electrocardiogram electrodes placed on your skin. If you are a woman, the adhesive patches used for this test will be placed on you by one of the women in the lab. Your blood pressure will be measured at periodic intervals by the inflation of a blood pressure cuff around your arm or around your middle finger.

4) You will have 4 small tubes (these are called "microdialysis fibers", and are smaller than the lead of a pencil) placed in the skin of your forearm. First we will numb the area of skin by placing a bag of ice over the area for 5 minutes. Then a small needle will be placed just under the surface of your skin and will exit back out about 1½ inches from where it entered your skin. The small tubes will be placed inside the needle, and the needle will be withdrawn, leaving the small tubes under your skin. These will remain in your skin throughout the rest of the study.

5) We need to wait about 1-2 hours after the small tubes are placed in your skin to let the insertion trauma (redness of your skin around the small tubes) to go away. During this time, a small probe (laser-Doppler probe) will be placed over each area of skin where the small tubes are so that we can measure skin blood flow over the small tube.

6) Microdialysis infusions: We will put small doses of several drugs through the small probes in your skin. The drugs you may receive include the following:

    a. L-NAME: this stops nitric oxide from being produced and causes the skin vessels to narrow
    b. Bretylium Tosylate: this substance inhibits the release of transmitters from the nerves in your skin and causes vessels in the skin to open
    c. Yohimbine: this substance blocks the effect of transmitters in your skin and causes vessels to open
    d. Propranolol: this substance blocks the effect of transmitters in your skin and causes vessels to narrow
    e. BIBP-3226: this substance blocks the effect of transmitters in your skin and causes vessels to open
    f. Tyramine: this substance stimulates the release of transmitters from the nerves in your skin and causes vessels in the skin to narrow

7) For a period of up to 10 minutes, your expired gas will be collected through a nasal cannula to assess the resting carbon dioxide levels in your blood. After removal of the nasal canulla, you will be asked to breathe air that has low

oxygen content either with or without high carbon dioxide content for up to 30 minutes. During this time, you will breathe through a scuba mouthpiece with nasal breathing prevented by a nose clip. These procedures will increase your rate of breathing and heart rate and are similar to what happens when you travel to higher altitude. You will undergo several of these breathing procedures during the study.

8) During the study, we will inflate a small cuff that is placed on your middle and ring fingers of one of your hands to measure your blood pressure (Finometer device). If the cuff becomes uncomfortable, let the investigator know and they will turn it off for a few minutes.

9) For a period of up to 5 minutes, cold water will be circulated through the water perfused suit in order to decrease your skin temperature. After this simulated cold exposure, warm water will be circulated through the suit immediately in order to rewarm your skin. During the cold exposure, your breathing may become rapid and you may shiver briefly, and this is similar to what happens when you enter a cold environment. You will undergo several of these cold exposures during the study.

10) After the study, we will remove the small tubes in your skin and a bandage will be placed over the area of skin where the tubes were placed. You will then be sent home.

11) You should notify the investigator immediately if you feel any significant discomfort or concern about your well-being at any time during the initial visit or the study visit. Some examples of discomfort include shortness of breath, light-headedness, and nausea.

How long will I be in the study?

You will be in the study for four hours.

What are the risks of the study?

1. Skin Microdialysis: There may be some discomfort during the insertion of the small tubes in your skin. Once the needle is in place, the pain should subside. Infusions through the fibers should not be painful, and there should only be minor swelling at the site. At the end of the study, the fibers will be withdrawn and a sterile dressing will be applied. Any swelling or redness after the study should be gone a few hours after completion of the study. Although the small tubes are sterile, there is a slight risk of infection at the sites where the small tubes were placed in your skin. You will be instructed how to keep the area clean for a day or two following the study.
2. Study drugs administered to the skin: We will be infusing very small doses of each drug, and only into a very small area of your skin. The drugs are only infused into the small area of skin where the microdialysis tubes are placed. Therefore, these drugs will not directly cause any systemic (whole body) changes in blood pressure. However, as with any infusions or medications, there is the possibility that you are allergic to the drug and may have an

allergic reaction to the drug including changes in blood pressure and difficulty breathing.

3. Breathing air with a low oxygen content with/without high carbon dioxide content: During these procedures, it is possible you will feel distress, anxiety, shortness of breath, or feel lightheaded. These symptoms resolve rapidly when you return to breathing room air. In addition, these breathing procedures have the potential to induce fainting. You should keep the investigator informed of your feelings and you should not attempt to prolong the breathing experiment if you do not feel well. You are free to stop at any time.
4. Laser-Doppler Probes: These probes send a small light into your skin. You will not feel anything except the probe touching your skin. The only risk associated with this procedure is that you may have some slight skin irritation (redness) to the adhesive tape used to hold the probe on your skin. There are no major risks associated with this device.
5. Finger blood pressure: In some people, this blood pressure cuff causes their finger to become red and uncomfortable after a long period (over 40 minutes). We will not inflate the cuff for longer than 40 minutes during the study. If your finger becomes uncomfortable during the study, let the investigator know and they will turn it off for a few minutes. There are no major risks associated with this device.
6. Cold exposure: The duration and magnitude of cold exposure in the current study do not have the potential to alter internal temperature. Therefore, you will not become hypothermic during this procedure. However, cooling of the skin does have the potential to be discomforting. There are no major risks associated with this procedure.

May I participate if I am pregnant or breast-feeding?

This study may be harmful to an unborn or breast-fed child. There is not enough medical information to know what the risks might be to a breast-fed infant or to an unborn child in a woman who takes part in this study. Breast-feeding mothers are not able to take part in this study. Women who can still become pregnant must have a negative pregnancy test no more than 24 hours before taking part in the study. If the pregnancy test is positive (meaning that you are pregnant), you will not be able to take part in the study. There is no cost for the pregnancy test.

Are there benefits to taking part in this study?

This study will not make your health better.

What other choices do I have if I don't take part in this study?

This study is only being done to gather information. You may choose not to take part in this study.

What are the costs of tests and procedures?

You will not need to pay for any tests or procedures that are done just for this research study. You will get $40 for participating in this study. This money is for the inconvenience and time you spent in this study. If you start the study but stop before the study has ended, the amount of money you receive will be pro-rated at a rate of $10 per hour of the study you complete.

Who can answer my questions?

You may talk to Dr. John Halliwill at any time about any question you have on this study. You may contact Dr. Halliwill by calling the Department of Human Physiology at (541) 346-5425.

What are my rights if I take part in this study?

Taking part in this research study is your decision. You do not have to take part in this study, but if you do, you can stop at any time. Your decision whether or not to participate will not affect your relationship with the University of Oregon.

You do not waive any liability rights for personal injury by signing this form. All forms of medical diagnosis and treatment whether routine or experimental, involve some risk of injury. In spite of all precautions, you might develop medical complications from participating in this study.

The investigators may stop you from taking part in this study at any time if it is in your best interest, if you do not follow the study rules, or if the study is stopped.

If you are physically injured because of the project, you and your insurance company will have to pay your doctor bills. If you are a UO student or employee and are covered by a UO medical plan, that plan might have terms that apply to your injury.

If you experience harm because of the project, you can ask the State of Oregon to pay you. If you have been harmed, there are two University representatives you need to contact. Here are their addresses and phone numbers:

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Office of Human Subjects Compliance
University of Oregon
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(541) 346-2510

A law called the Oregon Tort Claims Act limits the amount of money you can receive from the State of Oregon if you are harmed. The most you could receive would be $100,000, no matter how badly you are harmed. If other people are also harmed by the project, all of you together could only receive $500,000.

What about confidentiality?

Any information that is obtained in connection with this study and that can be identified with you will remain confidential and will be disclosed only with your permission. Subject identities will be kept confidential by assigning you a "subject identification number". The names associated with each subject identification number will be kept in a locked file cabinet in Dr. Halliwill's office area.

The Office for Responsible Conduct of Research and/or authorized representatives of the Food and Drug Administration (FDA) or National Institute of Health (NIH) may need to review records of individual subjects. As a result, they may see your name, but they are bound by rules of confidentiality not to reveal your identity to others.

The list of names will be destroyed when study results are published or 24 months after your participation, whichever comes first. Other information may be stored by the researchers indefinitely.

I have had an opportunity to have my questions answered. I have been given a copy of this form. I agree to take part in this study.

If you have questions regarding your rights as a research subject, contact Office for Protection of Human Subjects, 5237 University of Oregon, Eugene, OR 97403, (541) 346-2510.

Your signature indicates that you have read and understand the information provided above, that you willingly agree to participate, that you may withdraw your consent at any time and discontinue participation without penalty, that you will receive a copy of this form, and that you are not waiving any legal claims, rights or remedies.

_____ _____

(Date) (Signature of Participant)

_____ _____

(Date) (Signature of Individual Obtaining Consent)

APPENDIX C

INFORMED CONSENT FORM FOR CHAPTER VI

TITLE: Cutaneous vasodilation at high altitude: basic mechanisms and impacts on human thermoregulation (Protocol 2)

INVESTIGATOR: Dr. J. R. Halliwill and Colleagues

APPROVED BY INSTITUTIONAL REVIEW BOARD: August 5, 2008

This is an important form. Please read it carefully. It tells you what you need to know about this study. If you agree to take part in this research study, you need to sign the form. Your signature means that you have been told about the study and what the risks are. Your signature on this form also means that you want to take part in this study.

Why is this research study being done?

When humans breathe, we take in oxygen from the air and expel carbon dioxide that our bodies have made. When we travel to high altitudes, we fail to take in enough oxygen and we expel excess amounts of carbon dioxide. Conversely, some respiratory diseases are associated with failure to expel enough carbon dioxide produced within the body. Too little oxygen and too much or too little carbon dioxide may alter the distribution of blood in the limbs, increasing the amount of blood circulating in the skin. Unfortunately, we do not understand how these changes in blood distribution take place, or how they affect heat loss from the body. The purpose of this study is to provide more information on what causes the changes in blood flow when we take in too little oxygen or expel too little or too much carbon dioxide, and how these changes affect responses to cold temperature.

What will happen in the study?

1) You will have an initial meeting with the study investigator prior to any experimentation. During this meeting, you will discuss the project, see the laboratory, and to read this form. In addition, you will be given two temperature pills and instructed to ingest one of these pills the night before each study day. Lastly, your height, weight, and skin fold thickness will be measured. Following this initial meeting, you will arrive at Dr. Halliwill's laboratory in Esslinger Hall at the University of Oregon on 2 separate study days. Each study will last approximately 5 hours. During each of these study days, you will need to refrain from consuming caffeine (for example, coffee,

tea, red bull, coke, etc.) or medications (except oral contraceptives) for 12 hours prior to the study and abstain from alcohol or exercise for 24 hours prior to the study. In addition, you will need to refrain from eating for at least 2 hours prior to the study visit. If you use oral contraceptives, you should take this when you normally do so.

2) You will be asked to bring water approved apparel with you on each study day. If you are a man, you will be asked to bring shorts or a swim suit to the experiment so that you can wear these during the partial immersion in a bath tub of water. If you are a woman, you will be asked to bring either shorts or a swim suit bottom, and additionally you will need to bring either a swim suit top or a sports bra. You will be allowed to change into these items in the privacy of a personal bathroom.

3) If you are a woman who can still become pregnant, you will be asked to undergo a pregnancy test. For this test, you will be asked to collect a sample of urine in the women's restroom near the physiology lab. If the test is "positive," indicating that you are pregnant, you will not be allowed to participate and will be advised to see your physician or the University of Oregon Health Center.

4) During the visit, your heart rate will be monitored by electrocardiogram electrodes placed on your skin. If you are a woman, the adhesive patches used for this test will be placed on you by one of the women in the lab. Your blood pressure will be measured at periodic intervals by the inflation of a blood pressure cuff around your arm or around your middle finger.

5) You will have 4 small tubes (these are called "microdialysis fibers", and are smaller than the lead of a pencil) placed in the skin of your forearm. First we will numb the area of skin by placing a bag of ice over the area for 5 minutes. Then a small needle will be placed just under the surface of your skin and will exit back out about 1½ inches from where it entered your skin. The small tubes will be placed inside the needle, and the needle will be withdrawn, leaving the small tubes under your skin. These will remain in your skin throughout the rest of the study.

6) We need to wait about 1-2 hours after the small tubes are placed in your skin to let the insertion trauma (redness of your skin around the small tubes) to go away. During this time, a small probe (laser-Doppler probe) will be placed over each area of skin where the small tubes are so that we can measure skin blood flow over the small tube. In addition, you will be placed in a bath tub with platforms on which you can rest your arms out of the water. The water will be lukewarm at this time.

7) While you are being moved into the bathtub, you will have additional electrodes place on your back and upper arm for the measurement of muscle activity. You will also have 9 thermocouples placed over your skin to measure your skin temperature.

8) Microdialysis infusions: We will put small doses of several drugs through the small probes in your skin. The drugs you may receive include the following:
    a. L-NAME: this stops nitric oxide from being produced and causes the skin vessels to narrow
    b. Bretylium Tosylate: this substance inhibits the release of transmitters from the nerves in your skin and causes vessels in the skin to open
    c. Yohimbine: this substance blocks the effect of transmitters in your skin and causes vessels to open
    d. Propranolol: this substance blocks the effect of transmitters in your skin and causes vessels to narrow
    e. BIBP-3226: this substance blocks the effect of transmitters in your skin and causes vessels to open
    f. Tyramine: this substance stimulates the release of transmitters from the nerves in your skin and causes vessels in the skin to narrow
9) For a period of up to 10 minutes, your expired gas will be collected through a nasal cannula to assess the resting carbon dioxide levels in your blood. After removal of the nasal canulla, you will be asked to breathe air that has low oxygen content either with or without high carbon dioxide content for up to 100 minutes. During this time, you will breathe through a scuba mouthpiece with nasal breathing prevented by a nose clip. These procedures will increase your rate of breathing and heart rate and are similar to what happens when you travel to higher altitude. You will undergo several of these breathing procedures during the study.
10) During the study, we will inflate a small cuff that is placed on your middle and ring fingers of one of your hands to measure your blood pressure (Finometer device). If the cuff becomes uncomfortable, let the investigator know and they will turn it off for a few minutes.
11) For a period of up to 90 minutes, the water in the bath tub will be cooled in order to decrease your body temperature. This cooling will be slow at first, targeting a water temperature of 86 degrees Fahrenheit (30°C) at the end of the first 30 minutes. Then, water temperature will be decreased to 50 degrees Fahrenheit (10°C) over the course of 15 minutes. This temperature will be held for the final 45 minutes of the study. After this cold exposure, you will be removed from the bath tub, towel dried, and placed in a slightly warm environmental chamber with a blanket. During the cold exposure, your breathing may become rapid and you may begin to shiver, and this is similar to what happens when you enter a cold environment. You will undergo a cold exposure on each of the 2 experimental days.
12) After the study, we will remove the small tubes in your skin and a bandage will be placed over the area of skin where the tubes were placed. You will then be sent home.
13) You should notify the investigator immediately if you feel any significant discomfort or concern about your well-being at any time during the initial visit

or the study visit. Some examples of discomfort include shortness of breath, light-headedness, and nausea.

How long will I be in the study?

You will be in the study for 5 hours on each study day, and 1 to 2 hours for the initial lab visit.

What are the risks of the study?

1. Skin Microdialysis: There may be some discomfort during the insertion of the small tubes in your skin. Once the needle is in place, the pain should subside. Infusions through the fibers should not be painful, and there should only be minor swelling at the site. At the end of the study, the fibers will be withdrawn and a sterile dressing will be applied. Any swelling or redness after the study should be gone a few hours after completion of the study. Although the small tubes are sterile, there is a slight risk of infection at the sites where the small tubes were placed in your skin. You will be instructed how to keep the area clean for a day or two following the study.
2. Study drugs administered to the skin: We will be infusing very small doses of each drug, and only into a very small area of your skin. The drugs are only infused into the small area of skin where the microdialysis tubes are placed. Therefore, these drugs will not directly cause any systemic (whole body) changes in blood pressure. However, as with any infusions or medications, there is the possibility that you are allergic to the drug and may have an allergic reaction to the drug including changes in blood pressure and difficulty breathing.
3. Breathing air with a low oxygen content with/without high carbon dioxide content: During these procedures, it is possible you will feel distress, anxiety, shortness of breath, or feel lightheaded. These symptoms resolve rapidly when you return to breathing room air. In addition, these breathing procedures have the potential to induce fainting. You should keep the investigator informed of your feelings and you should not attempt to prolong the breathing experiment if you do not feel well. You are free to stop at any time.
4. Laser-Doppler Probes: These probes send a small light into your skin. You will not feel anything except the probe touching your skin. The only risk associated with this procedure is that you may have some slight skin irritation (redness) to the adhesive tape used to hold the probe on your skin. There are no major risks associated with this device.
5. Finger blood pressure: In some people, this blood pressure cuff causes their finger to become red and uncomfortable after a long period (over 40 minutes). We will not inflate the cuff for longer than 40 minutes during the study. If your finger becomes uncomfortable during the study, let the

investigator know and they will turn it off for a few minutes. There are no major risks associated with this device.

6. Cold exposure: The duration and magnitude of cold exposure in the current study are designed to slightly decrease internal body temperature. However, you will not become hypothermic during this procedure (defined by the US Center for Disease Control and Prevention as a core body temperature below 35°C [95°F]). Nevertheless, whole body cooling does have the potential to be discomforting. Common responses to whole body cold exposure include an initial increase in breathing rate and depth, an increased heart rate (initially), and the occurrence of shivering. You will not be discouraged from shivering as needed during this study. You will be monitored closely by a trained physiologist throughout the cold exposure, and rewarming will occur inside of a temperature controlled room immediately after the study ends.
7. Core temperature: Core temperature is measured by an ingestible pill that is designed for human use (approved by the FDA). You will ingest the pill at least 6 hours before the study, or the night before if the study begins early in the morning. This pill will harmlessly pass through your intestinal tract. The pill is not recovered and is disposable. There are no major risks associated with this device.
8. Skin temperature: Skin temperature will be measured at multiple sites on the surface of your skin. The temperature sensors (thermocouples) are passive sensing devices consisting of small wires with a rubber tip. These sensors will be taped to your skin and relay the temperature of the skin surface to a data acquisition computer. There are no major risks associated with this device.

May I participate if I am pregnant or breast-feeding?

This study may be harmful to an unborn or breast-fed child. There is not enough medical information to know what the risks might be to a breast-fed infant or to an unborn child in a woman who takes part in this study. Breast-feeding mothers are not able to take part in this study. Women who can still become pregnant must have a negative pregnancy test no more than 24 hours before taking part in the study. If the pregnancy test is positive (meaning that you are pregnant), you will not be able to take part in the study. There is no cost for the pregnancy test.

Are there benefits to taking part in this study?

This study will not make your health better.

What other choices do I have if I don't take part in this study?

This study is only being done to gather information. You may choose not to take part in this study.

What are the costs of tests and procedures?

You will not need to pay for any tests or procedures that are done just for this research study. You will get $120 for participating in this study. This money is for the inconvenience and time you spent in this study. If you start the study but stop before the study has ended, the amount of money you receive will be pro-rated at a rate of $10 per hour of the study you complete.

Who can answer my questions?

You may talk to Dr. John Halliwill at any time about any question you have on this study. You may contact Dr. Halliwill by calling the Department of Human Physiology at (541) 346-5425.

What are my rights if I take part in this study?

Taking part in this research study is your decision. You do not have to take part in this study, but if you do, you can stop at any time. Your decision whether or not to participate will not affect your relationship with the University of Oregon.

You do not waive any liability rights for personal injury by signing this form. All forms of medical diagnosis and treatment whether routine or experimental, involve some risk of injury. In spite of all precautions, you might develop medical complications from participating in this study.

The investigators may stop you from taking part in this study at any time if it is in your best interest, if you do not follow the study rules, or if the study is stopped.

If you are physically injured because of the project, you and your insurance company will have to pay your doctor bills. If you are a UO student or employee and are covered by a UO medical plan, that plan might have terms that apply to your injury.

If you experience harm because of the project, you can ask the State of Oregon to pay you. If you have been harmed, there are two University representatives you need to contact. Here are their addresses and phone numbers:

General Counsel
Office of the President
University of Oregon
Eugene, OR 97403
(541) 346-3082

Office of Human Subjects Compliance
University of Oregon
Eugene, OR 97403
(541) 346-2510

A law called the Oregon Tort Claims Act limits the amount of money you can receive from the State of Oregon if you are harmed. The most you could receive would be $100,000, no matter how badly you are harmed. If other people are also harmed by the project, all of you together could only receive $500,000.

What about confidentiality?

Any information that is obtained in connection with this study and that can be identified with you will remain confidential and will be disclosed only with your permission. Subject identities will be kept confidential by assigning you a "subject

identification number". The names associated with each subject identification number will be kept in a locked file cabinet in Dr. Halliwill's office area.

The Office for Responsible Conduct of Research and/or authorized representatives of the Food and Drug Administration (FDA) or National Institute of Health (NIH) may need to review records of individual subjects. As a result, they may see your name, but they are bound by rules of confidentiality not to reveal your identity to others.

The list of names will be destroyed when study results are published or 24 months after your participation, whichever comes first. Other information may be stored by the researchers indefinitely.

I have had an opportunity to have my questions answered. I have been given a copy of this form. I agree to take part in this study.

If you have questions regarding your rights as a research subject, contact Office for Protection of Human Subjects, 5237 University of Oregon, Eugene, OR 97403, (541) 346-2510.

Your signature indicates that you have read and understand the information provided above, that you willingly agree to participate, that you may withdraw your consent at any time and discontinue participation without penalty, that you will receive a copy of this form, and that you are not waiving any legal claims, rights or remedies.

_____ _____

(Date) (Signature of Participant)

_____ _____

(Date) (Signature of Individual Obtaining Consent)

APPENDIX D

MIXING PROCEDURE FOR STUDY DRUGS IN CHAPTER V

Lactated Ringers (before subject arrives)

- Fill four 1ml syringes with Lactated Ringers and label R

Yohimbine, BIBP-3226 (after fibers are placed)

1. Mass out 19.5 mg Yohimbine in sterilized vial and label Y
2. Fill a 10 ml syringe with 10 ml Lactated Ringers and another 10 ml syringe with 1ml Lactated Ringers (eject from a 10 ml syringe)
3. Label the syringe with 10 ml "Y" and the other "10.5 B"
4. Empty Y syringe and tiny stir bar into Yohimbine vial; place on hot plate
5. Place 19 gage needle in thawed BIBP vial and draw contents into clean 10 ml syringe (label B)
6. Eject contents of B syringe until plunger lines up with the 9 ml mark
7. Eject remaining 9 ml from B syringe into lactated ringers syringe labeled 10.5 B
8. Shake well and place red needle on syringe tip
9. Fill a 1 ml syringe with contents of 10.5 B syringe, label 10.5 B
10. Suck heated Yohimbine solution back into Y syringe and align plunger at 10 ml
11. Use fresh 10 ml syringe (red needle) to fill rest of Y syringe with Lactated Ringers
12. Shake Y syringe well
13. Place sterilization filter and red needle on Y syringe and fill a 1 ml syringe with contents of Y syringe (label this syringe Y)

APPENDIX E

MIXING PROCEDURE FOR STUDY DRUGS IN CHAPTER VI

Yohimbine + Propranolol and BIBP-3226 + Propranolol

1. Mass out 19.5 mg Yohimbine, tare scale, and add 3 mg of Propranolol (Y+P)
2. In a separate vial, mass out 3 mg Propranolol alone (label P)
3. Fill two 10 ml syringes with EXACTLY 10 ml Lactated Ringers
4. Label "Y+P" and "P"
5. Empty 10 ml syringes into vials and place on hot plate for 20-30 minutes
6. Place 19 gage needle in thawed BIBP vial and draw contents into clean 10 ml syringe
7. Shake well and fill two 1 ml B+P syringes
8. Suck heated solutions back into both syringes and align plunger at 10 ml
9. Use two fresh 10 ml syringes to fill rest of these syringes w/Lactated Ringers
10. Shake well and fill two 1 ml Y+P syringes and two 1 ml P syringes

APPENDIX F

MIXING PROCEDURE FOR STUDY DRUGS IN CHAPTER VII

15 minutes before study begins:

1. Take BIBP-3226 out of freezer to thaw
2. Turn on hot plate
3. Fill three 1 ml syringes with Lactated Ringers

To mix the Yohimbine, BIBP-3226, and Tyramine

1. Fill two 1 ml syringes with Lactated Ringers and label R
2. Fill two 1 ml syringes with 0.6 ml Lactated Ringers, then eject the contents through needle until plunger is aligned with the 0.5 ml mark (label R)
3. Mass out 21.7 mg Yohimbine in sterilized vial and label Y
4. Mass out 3 mg Tyramine in sterilized vial and label T
5. Fill 2 10 ml syringes with 10 ml Lactated Ringers (eject from a 10 ml syringe)
6. Label one 10 ml syringe "Y" and another "300 T"
7. Fill a third 10 ml syringe with 9 ml lactated ringers and label it "30 T" (red needle)
8. Empty Y syringe and tiny stir bar into Yohimbine vial and place on hot plate
9. UNDER HOOD, empty 300 T syringe in Tyramine vial and dissolve drug
10. Suck 10 ml Tyramine solution back into 300 T syringe and attach red needle
11. Eject Tyramine from 300 T syringe until plunger lines up at 9 ml, then transfer 1 ml from this syringe into the 30 T syringe

12. Shake 30 T syringe and transfer ~0.5 ml into a 1 ml syringe and label 30 T
13. Align plunger in two 1 ml syringes at .6 ml and fill with contents of 300 T syringe
14. Eject ~ 0.1 ml from both 1 ml syringes so plunger rests at 0.5 ml (label both "T")
15. Place 19 gage needle in BIBP vial and draw contents into 10 ml syringe (label B)
16. Replace gray needle on B syringe with red needle
17. Align plunger in two 1 ml syringes at 0.6 ml and fill with contents of B syringe
18. Eject ~ 0.1 ml from both 1 ml syringes so plunger rests at 0.5 ml (label both "B")
19. Reattach grey needle, and eject contents of 10 ml B syringe until plunger lines up with the 8 ml mark
20. Obtain two 6 ml syringes and label one "10.5 B" and the other "10.5 B + T"
21. Into 10.5 B syringe, eject 4 ml from 10 ml B syringe and the contents of a 1 ml B syringe and a 1 ml R syringe (filled with 0.5 ml Lactated Ringers)
22. Shake well and fill two 1 ml syringes, label them "10.5 B"
23. Into 10.5 B + T syringe, eject 4 ml from 10 ml B syringe and the contents of a 1 ml B syringe and a 1 ml T syringe
24. Shake well and transfer 0.5 ml to a 1 ml syringe, label it "10.5 B + T"
25. Suck heated Yohimbine solution back into Y syringe and align plunger at 10 ml
26. Use fresh 6 ml syringe (red needle) to fill rest of Y syringe with Lactated Ringers
27. Shake Y syringe well
28. Replace gray needle on Y syringe with red needle
29. Align plunger in two 1 ml syringes at 0.6 ml and fill with contents of Y syringe

30. Eject ~ 0.1 ml from both 1 ml syringes so plunger rests at 0.5 ml (label both "Y")
31. Replace red needle on 10 ml Y syringe with gray needle
32. Eject contents of 10 ml Y syringe until plunger lines up with the 8 ml mark
33. Obtain two 6 ml syringes (w/ red needle) and label 1 "5 Y" and the other "5 Y + T"
34. Into 5 Y syringe, eject 4 ml from 10 ml Y syringe and the contents of a 1 ml Y syringe and a 1 ml R syringe (filled with 0.5 ml Lactated Ringers)
35. Shake well and fill two 1 ml syringes (use sterilization filter), label them "5 Y"
36. Into 5 Y + T syringe, eject 4 ml from 10 Y syringe and the contents of a 1 ml Y syringe and a 1 ml T syringe
37. Shake well and push a few milliliters through the sterilization filter to clear it out
38. Transfer 0.5 ml to a 1 ml syringe and label it "5 Y + T"

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