# ADENOSINE RECEPTORS IN CUTANEOUS THERMAL HYPEREMIA AND ACTIVE VASODILATION IN HUMANS

by

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## Abstract

Mechanisms underlying the cutaneous vasodilation response to local skin heating and whole body heating in humans remain unresolved. Although nitric oxide (NO) is known to contribute to these responses, it remains unclear as to the source of NO. Adenosine receptors induce vasodilation in many human tissues and may work, in part, through NO. As these receptors are also known to be located in the cutaneous vasculature, the studies contained in this thesis were designed to investigate a potential contribution of adenosine receptor activation to the rise in skin blood flow elicited by local skin and whole body heating.

The study presented in chapter IV was designed to determine a potential role for adenosine receptors in contributing to cutaneous thermal hyperemia. Four cutaneous microdialysis sites were randomly assigned one of four drug treatments designed to elucidate the contribution of $A_1/A_2$ adenosine receptors during local skin heating. Each site was locally heated from a baseline temperature of 33°C to 42°C at a rate of 1°C/10 s and skin blood flow was monitored via laser-Doppler flowmetry (LDF). The data obtained from these experiments suggest $A_1/A_2$ adenosine receptor activation directly contributes to cutaneous thermal hyperemia. These data further suggest a portion of the NO response may be explained by $A_1/A_2$ adenosine receptor activation; however, a substantial portion of the NO response is independent of the adenosine receptor contribution.

The study presented in chapter V was designed to determine a potential role for $A_1/A_2$ adenosine receptors in contributing to cutaneous active vasodilation. Four cutaneous microdialysis sites were randomly assigned one of four drug treatments, as above, and skin blood flow was monitored via LDF. Whole body heat stress, sufficient to raise oral temperature at least 0.8°C above baseline, was induced via water-perfused suits. The data obtained from these

experiments suggest $A_1/A_2$ adenosine receptor activation does not directly contribute to cutaneous active vasodilation; however, a role for $A_1/A_2$ adenosine receptor activation is unmasked when NO synthase is inhibited. The data from this study further suggest that $A_1/A_2$ adenosine receptor activation may be responsible for a portion of the known NO component of cutaneous active vasodilation.

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# Chapter 1 - Introduction

## Statement of the Problem

Regulation of human skin blood flow serves to protect the skin and core from extreme temperatures. Skin blood flow is increased in response to an increase in local skin temperature and an increase in core body temperature; however, the mechanisms governing these responses differ. The cutaneous vasodilation elicited by increases in local skin temperature, known as thermal hyperemia is mediated primarily by sensory nerves. This thermal hyperemic response protects the skin by moving the heat away from the heated area of the skin. In response to an increase in core body temperature, by contrast, humans exhibit a substantial increase in skin blood flow mediated by a sympathetic cholinergic active vasodilator system. This cutaneous active vasodilation along with a concurrent increase in sudomotor activity serves to increase heat loss to the environment, and is the primary autonomic means by which humans defend against increasing core temperatures.

The precise mechanisms mediating thermal hyperemia and active vasodilation are not yet fully understood. As such, the studies contained in this thesis aimed to provide a better understanding of the mechanisms mediating each of these responses. Specifically, the study described in Chapter IV of this thesis was designed to investigate a potential role for $A_1/A_2$ adenosine receptor activation in thermal hyperemia. The study described in Chapter V was similarly designed to investigate a role for $A_1/A_2$ adenosine receptor activation in cutaneous active vasodilation.

## Background

Thermal hyperemia, induced by local heating of the skin, is mediated in part by sensory nerves. Rapid, nonpainful, local heating of human skin elicits a thermal hyperemic response that is biphasic and characterized by a rapid rise in skin blood flow to an initial peak, followed by a brief nadir, and, finally, a more slowly established and prolonged plateau (Kellogg et al. 1999; Minson et al. 2001). The initial peak and nadir of cutaneous thermal hyperemia have been shown to be primarily mediated by an axon reflex via activation of transient receptor potential vanilloid type 1 (TRPV-1) channels located on C-fiber afferent neurons (Magerl and Treede, 1996; Minson et al. 2001; Wong & Fieger, 2010), and a modest contribution from nitric oxide (NO) (Kellogg et al. 1999; Minson et al. 2001). The secondary plateau phase of the local heating response, by contrast, consists of a sustained increase in skin blood flow that is approximately 70% dependent on NO (Minson et al. 2001). The sensory nerves involved may elicit vasodilation through the release of substance P and/or calcitonin gene-related peptide (CGRP). A recent study by Wong and Minson (2011) suggests neurokinin-1 ($NK_1$) receptors, which preferentially bind substance P, are involved in the thermal hyperemic response; however, direct evidence for substance P and/or CGRP is currently not available. Questions remain as to the mechanisms mediating thermal hyperemia and what other vasodilator substances may be involved.

Thermoregulatory skin blood flow, elicited by alterations in core body temperature, is uniquely controlled by a sympathetic adrenergic vasoconstrictor system and a sympathetic cholinergic active vasodilator system (Grant and Holling, 1938; Roddie et al.1957). The cutaneous vasoconstrictor system is tonically active, regulating subtle alterations in skin blood flow during periods of normothermia as well as causing increased cutaneous vasoconstriction during exposure to cold stress (Grant and Holling, 1938). As core temperature rises during heat stress, an initial rise in skin blood flow occurs via withdrawal of sympathetic adrenergic

vasoconstriction. With a further increase in temperature to a specific core temperature threshold, reflex cutaneous active vasodilaton occurs, concurrent with the onset of sweating (Grant and Holling, 1938; Roddie et al. 1957). The resulting increase in skin blood flow moves heat from the body's core to the periphery where heat is lost via convection from the blood to the environment through the skin. The simultaneous increase in sweating allows for evaporative heat loss and regulation of normal core body temperature.

The active vasodilator system is known to be mediated by sympathetic cholinergic nerves and is responsible for both the sweat and skin blood flow responses (Grant and Holling, 1938; Roddie et al. 1957; Fox and Hilton, 1958; Kellogg et al. 1995). By the current co-transmission theory, acetylcholine is released from sympathetic nerves and primarily mediates the sweat response, while cutaneous vasodilation is mediated by unknown co-transmitter(s) (Kellogg et., 1995). Additionally, nitric oxide (NO) has been shown to directly mediate 30-45% of this process and is required for full expression of active vasodilation (Kellogg et al. 1998; Shastry et., 1998; Wilkins et al. 2003). The co-transmitter(s) released from sympathetic vasodilator nerves to directly mediate cutaneous vasodilation and/or promote the production of NO remain unknown.

$A_1$ and $A_2$ adenosine receptors have been found to be located in the human skin microcirculation (Stojanov and Proctor, 1989) and activation of these receptors has been shown to mediate vasodilation in multiple organ systems (Abebe et al. 195l; Ray and Marshall 2009; Stojanov and Proctor, 1989). Microdialysis infusion of adenosine into the interstitial space of human skin has been shown to elicit vasodilation to an extent similar to that achieved during whole body heating (Shibaski et al. 2007). Furthermore, adenosine receptors have been shown to induce vasodilation, at least in part, by increasing NO production (Mortensen et al. 2009; Stewart et al. 2004; Vials and Burnstock, 1993). The studies described in Chapters IV and V were

designed based on this rationale to investigate a potential role for $A_1$ and $A_2$ adenosine receptors in the thermal hyperemic and cutaneous active vasodilator responses, respectively.

## Specific Aims

The studies discussed in this thesis were designed to address the following specific aims:

1. The purpose of the study presented in chapter IV of this thesis was to investigate a potential $A_1/A_2$ adenosine receptor-mediated component to the cutaneous thermal hyperemic response. Additionally, this study aimed to determine whether a portion of the NO-dependent component of this response may be explained by adenosine receptor activation.

2. The purpose of the study presented in Chapter V of this thesis was to determine whether adenosine receptor activation contributes to reflex cutaneous active vasodilation. Additionally, this study aimed to determine whether a portion of the NO component of cutaneous active vasodilation might be explained by $A_1/A_2$ adenosine receptor activation.

## Hypotheses

The studies presented in this thesis were designed to test the following two hypotheses:

1. The study described in Chapter IV was designed to test two main hypotheses. We hypothesized adenosine receptor blockade would result in an attenuated plateau response to local heat application. We further hypothesized simultaneous blockade of adenosine receptors combined with NOS inhibition would result in a greater attenuation of the NO-dependent secondary plateau.

2. The study described in Chapter V was designed to test two hypotheses. We hypothesized a direct role for adenosine receptor activation would be suggested by an attenuation of the skin blood flow response to heat stress in the presence of the non-selective $A_1/A_2$

adenosine receptor antagonist theophylline. In addition, we hypothesized $A_1/A_2$ adenosine receptor inhibition combined with NO synthase inhibition would further attenuate this response, which would suggest adenosine receptor activation may also contribute to cutaneous active vasodilation through the production of NO.

**Limitations**

1. Based on the experimental approach of the studies described in Chapters IV and V, we can only discuss the data in terms of $A_1/A_2$ adenosine receptor activation and not adenosine, per se, being involved in cutaneous thermal hyperemia and active vasodilation. Although adenosine is the most likely candidate to bind to, and activate, the $A_1/A_2$ adenosine receptors, the present data suggest an indirect role for adenosine, per se, and we cannot rule out the possibility that some other substance(s) bind to, and activate, the $A_1/A_2$ adenosine receptors.

2. In Chapters IV and V the use of theophylline, a competitive but non-selective inhibitor of $A_1$ and $A_2$ adenosine receptors, does not allow us to determine the specific contribution of the various isoforms of adenosine receptors to cutaneous thermal hyperemia and active vasodilation. Both A1 and A2 adenosine receptors have been localized in human skin (Stojanov and Proctor, 1989) and both isoforms have been shown to elicit vasodilation (Bryan and Marshall, 1999); however, it is difficult to determine and assign specific roles to each isoform and subtype as the distribution and mechanism of action is heterogeneous. Further complicating the matter is that selective adenosine receptor antagonists approved for human use are lacking. As such, we chose to use a competitive, but non-specific, $A_1/A_2$ adenosine receptor antagonist.

3. A 4 mM dose of theophylline was the maximum dose that could be administered via microdialysis in these studies without causing non-specific vasodilation. In preliminary experiments, concentrations higher than 4 mM resulted in substantial vasodilation, which would

have influenced and made interpretation of the results of this study difficult. The increase in baseline skin blood flow observed with higher concentrations of theophylline is most likely due to the phosphodiesterase inhibitor properties of theophylline, which would act to increase cAMP levels and result in smooth muscle relaxation (Taddei et al. 1991). Due to this effect of higher doses of theophylline on baseline skin blood flow, we chose to use a 4mM dose. Our data clearly indicate a direct role for $A_1/A_2$ adenosine receptor activation to cutaneous thermal hyperemia as well as a possible source of NO; however, it is possible our data underestimate the contribution of $A_1/A_2$ adenosine receptor activation to cutaneous thermal hyperemia.

# Chapter 2 - Review of the Literature

## Sensory Nerves and Axon-Reflex Mediated Vasodilation in the Cutaneous Vasculature

The cutaneous vasodilation resulting from direct heat application to the skin is mediated by cutaneous sensory nerves and an axon reflex mechanism. An initial discussion of an axon reflex in the cutaneous vasculature was presented by Bayliss in 1901, based on the finding that cutaneous vasodilation could be elicited by stimulation of the peripheral end of a dorsal root primary afferent nerve, after the nerve had been cut. As cutting the afferent nerve would have prevented the transmittal of sensory information to efferent ventral nerves, the end-organ response of cutaneous vasodilation was unexpected. To explain these findings, Bayliss (1901) suggested that the observed vasodilation resulted from a signal traveling down afferent nerves in an efferent direction, a phenomenon he termed antidromic vasodilation. Further, evidence of sensory nerve innervation in the cutaneous circulation was cited to propose that sensory nerves and an axon reflex might mediate this cutaneous vasodilation.

Subsequent studies supported the occurrence of axon reflex mediated vasodilation in the cutaneous circulation. Lewis and Grant (1924) and Lewis (1927) demonstrated that the flare reaction of local skin injury was prevented from spreading to nearby skin sites on which anesthesia was applied. Based on these results, these authors suggested that the flare response was mediated by an axon reflex, with a network of axons branching off of the afferent nerve being contained in the cutaneous circulation. Figure 2-1, below illustrates an axon reflex in the cutaneous cutaneous circulation, activated by a thermal stimulus.

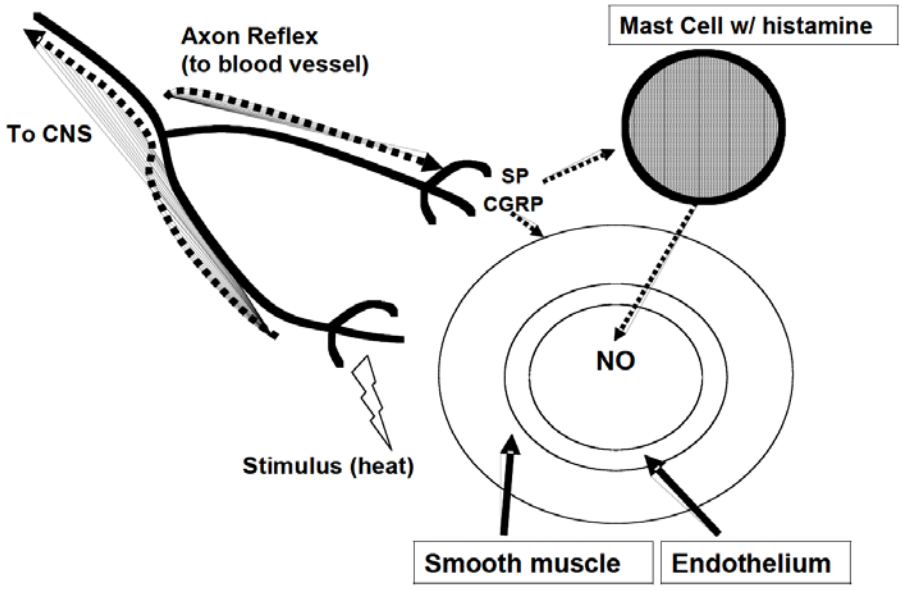


**Figure 2-1: Schematic Drawing of the Axon Reflex in Human Skin.**

## Mechanisms of Cutaneous Thermal Hyperemia

The direct application of heat to human skin is known to elicit cutaneous vasodilation. As early as 1911 Hewlett et al. reported increased blood flow in response to skin heating. Subsequent studies found that infrared radiation of an area of skin elicited cutaneous vasodilation that was capable of spreading to nearby areas of skin, similar to the spread of the flare response due to local skin injury (Cooper, 1950; Crockord and Hellon, 1959). Due to the simultaneous timing of the vasodilation in radiated and irradiated areas of the skin, Crockford and Hellon (1959), concluded that a neural mechanisms was responsible for initiating the observed cutaneous vasodilation; however, it remained unknown whether this neural mechanism involved the central nervous system or was an axon reflex. Alternatively, these authors later suggested a theory of electronic coupling as a mechanism by which vasodilation may spread to nearby regions of skin.

Following a several year gap in the literature, several studies provided strong evidence of axon-reflex mediated cutaneous vasodilation in response to skin heating. One such study was presented by Magerl and Treede in 1996. These authors recorded changes in skin blood flow via laser-Doppler flowmetry at varying distances from a point of local heat application to the skin. Data from this study demonstrated cutaneous vasodilation which occurred between 8 and 30mm away from the site of heat application. As the length constant of vascular electronic coupling is approximately 1-2mm, these data suggested another mechanism, such as an axon reflex, must be involved to mediate cutaneous vasodilation at sites farther from the application of heat.

More recently, Minson et al (2001), established that nonpainful heat application to nonglabrous human skin produces a biphasic thermal hyperemic response. This consistently produced response is characterized by a rapid rise in skin blood flow to an initial peak and nadir, attributed to an axon reflex, followed by a more slowly established and prolonged plateau (Kellogg et al. 1999; Minson et al. 2001). A series of experiments by Minson et al. (2001), which will be explained in more detail below, revealed that independent mechanisms mediate the two phases of this response. As demonstrated by these studies, the initial peak and nadir of cutaneous thermal hyperemia is primarily mediated by an axon reflex, while the more slowly formed and prolonged rise in skin blood flow is primarily mediated by NO. Figure 2-2, below represents this characteristic skin blood flow response to rapid, nonpainful direct heating of human skin.

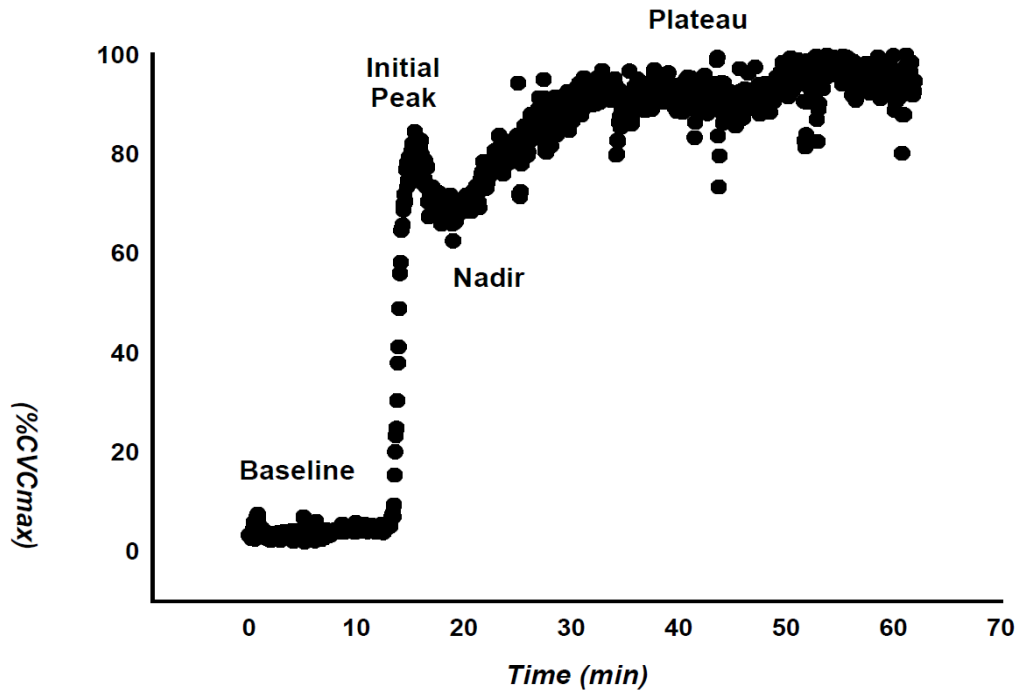


**Figure 2-2: Representative Tracing of the Cutaneous Thermal Hyperemic Response.**

As previously stated, the initial peak and nadir of the thermal hyperemic response are currently attributed to an axon reflex mechanism. As demonstrated by Minson et al. (2001), the initial peak and nadir components of the thermal hyperemic response are significantly attenuated when EMLA cream (topical anesthetic; 2.5% lidocaine and 2.5% prilocaine) is used to prevent the axon reflexes, while antebrachial nerve blockade does not alter this response. In this same study, the initial peak and nadir responses were also attenuated by NO synthase inhibition and further attenuated by combined NO synthase inhibition and EMLA cream. The attenuation resulting from combined NO synthase inhibition and EMLA cream, however, was not greater than that achieved by EMLA cream alone. Moreover, recent data from Wong & Fieger (2010) suggests that activation of TRPV-1 channels are required for full expression of the initial peak and activation of TRPV-1 channels, by heat, on sensory nerves may be responsible for

depolarization of cutaneous sensory nerves. Taken together, these data provide strong evidence that the initial peak and nadir components of skin blood flow response to local heating are mediated by an axon reflex, and nitric oxide contributes modestly to this response (Minson et al. 2001).

It has been suggested that the neuropeptides substance P and CGRP are involved in the axon reflex-mediated component of cutaneous thermal hyperemia. Specifically, the direct application of heat to the skin may stimulate the release of these substances from heat-sensitive C-fiber afferent nerves in human skin to mediate the axon reflex component of cutaneous thermal hyperemia. Substance P and/or CGRP are the most likely candidates as these have been shown to be involved in axon-reflex mediated vasodilation. A recent study by Wong & Minson (2011) demonstrates the desensitization of $NK_1$ receptors significantly alters the thermal hyperemic response and indirectly suggests substance P is involved in cutaneous thermal hyperemia; however, a role for these substances in the axon reflex component of cutaneous thermal hyperemia has not been directly investigated. Thus, while the initial peak and nadir components of the thermal hyperemic response have been shown to be mediated by an axon reflex, the neuropeptides involved in this process have not been specifically identified.

In contrast to the axon reflex component of thermal hyperemia, the secondary plateau phase of the local heating response consists of a sustained increase in skin blood flow that is largely dependent on NO, where inhibition of NO synthase greatly attenuates the plateau phase of thermal hyperemia (Kellogg et al. 1999; Minson et al. 2001). These studies have determined that nitric oxide contributes approximately 70% to the secondary plateau phase of thermal hyperemia in the skin. Kellogg and colleagues (2009) have recently provided evidence to suggest

endothelial NOS (eNOS) may be the NOS isoform responsible for the NO-dependent secondary plateau.

It remains unclear, however, as to the source of NO or what may stimulate the increase in eNOS activity during the local heating skin blood flow response. The contributions of $H_1$ receptor activation and the COX-1/COX-2 pathways to cutaneous thermal hyperemia have recently been investigated in independent studies (McCord et al. 2006; Wong et al. 2006). Inasmuch as these studies have demonstrated a minimal role for $H_1$ receptor activation (Wong et al. 2006) and no role for COX-1/COX-2 activation (McCord et al. 2006) to cutaneous thermal hyperemia, it appears these pathways are not sources of NO during local heating. Although there are likely to be several redundant vasodilator pathways involved in mediating cutaneous thermal hyperemia, important questions remain as to what substances may be involved in this response, either as a direct vasodilator or by increasing NO production.

## Sympathetic Nerves and the Cutaneous Vasculature

Thermoregulatory control of the cutaneous circulation is uniquely accomplished via dual innervation from the sympathetic nervous system. These two branches of the sympathetic nervous system in human skin regulate an adrenergic vasoconstrictor system and a sympathetic cholinergic active vasodilator system. Sympathetic adrenergic vasoconstrictor nerves release norepinephrine and the co-transmitter neuropeptide Y to result in vasoconstriction of cutaneous arterioles. By contrast, acetylcholine and one or more co-transmitters are released from cholinergic active vasodilator nerves. Each of these systems contribute to thermoregulatory control of skin blood flow as illustrated in Figure 2-3.

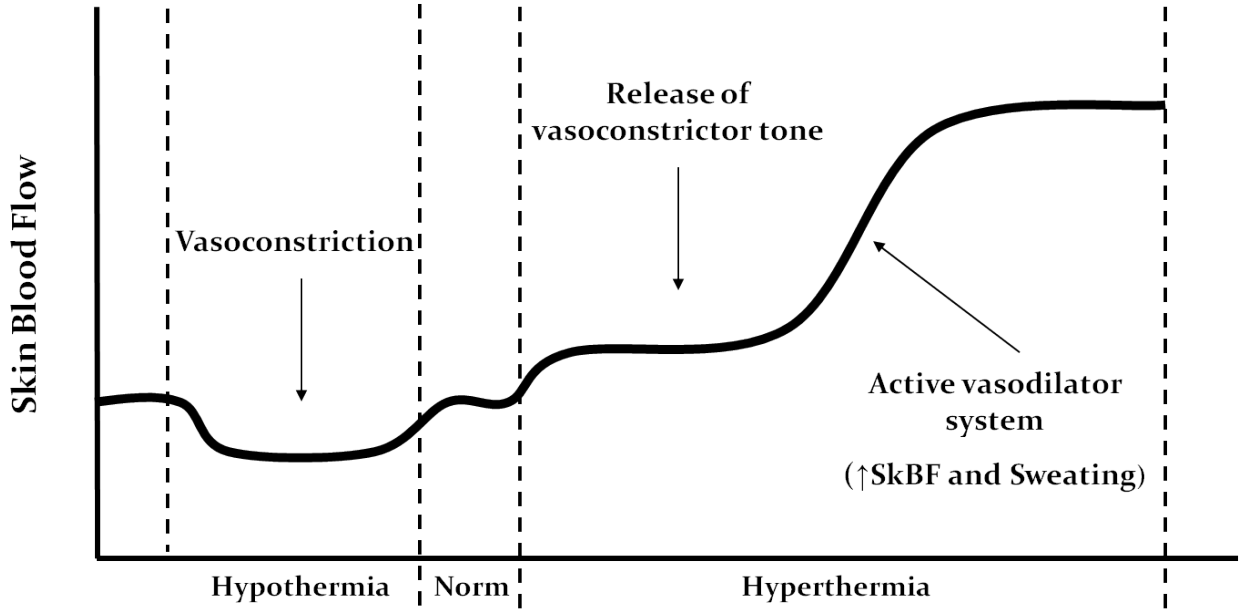


**Figure 2-3: Schematic Illustrating Thermoregulatory Control of Skin Blood Flow.**

The activity of these two systems regulates skin blood flow, and ultimately maintains core body temperature during different environmental conditions. Under resting normothermic conditions, the adrenergic vasoconstrictor system remains tonically active to regulate subtle alterations in skin blood flow and maintain vascular tone (Grant and Holling, 1938). Upon exposure to cold, the activity of this vasoconstrictor system is increased to elicit a higher degree of cutaneous vasoconstriction. The resulting decrease in skin blood flow shunts blood flow to the periphery, reducing the loss of the body's heat to the environment (Stephens et al. 2000). Upon exposure to a warm environment, by contrast, vasoconstrictor tone is withdrawn. The resulting vasodilation approximately doubles skin blood flow compared to normothermic conditions, and increases the dissipation of heat from the blood to the environment. (Roddie et al. 1957).

With a further increase in temperature to a specific core temperature threshold, reflex cutaneous active vasodilaton and sweating occur (Roddie et al. 1957; Kellogg et al. 1995). The

resulting increase in skin blood flow moves heat from the body's core to the periphery where heat is lost via convection from the blood to the environment through the skin. The simultaneous increase in sweating allows for evaporative heat loss and further maintenance of normal core body temperature. This active cutaneous vasodilation and increase in sudomotor activity are the primary autonomic means by which humans defend against increasing core temperatures.

## Mechanisms of Cutaneous Active Vasodilation

Documented evidence that skin blood flow is under thermoregulatory control, dates as early as 1900. A study by Hough (1900), documented observed changes in venous size in the hand, during exposure to a range of external temperatures. Hough reported increased skin blood flow in response to high external temperature, providing the earliest known documentation of thermoregulatory cutaneous vasodilation.

Subsequent studies provided evidence that increases in blood flow in response to heat challenges, may be reflexively mediated by sympathetic nerves. Gibbon and Landis (1932) demonstrated that immersion of the upper extremities in warm water produced vasodilation in the lower limbs. Furthermore, in the same study, occlusion of the heated limb was found to delay the appearance of vasodilation in the opposite limb, leading to the conclusion that the return of heated blood from the warmed extremity was the stimulus for vasodilation in the opposite limbs. This study corroborated the findings of a 1931 study by Lewis and Pickering, which further showed that vasodilation was prevented in a sympathectomized limb. Several later studies confirmed the involvement of sympathetic nerves in this response (Grant and Holling, 1938; Grant and Pearson, 1938; Barcroft et al, 1943). Together, these findings demonstrated that thermoregulatory increases in blood flow may be reflexively mediated by sympathetic nerves.

This observed vasodilation was subsequently attributed specifically to the cutaneous circulation. Using various measurement techniques, several studies showed that the increase in blood flow was confined to the skin with no involvement of underlying muscle (Cooper et al. 1954; Barcroft et al. 1955; Edholm et al;, 1956, Roddie et al. (1956). With the results of these studies, the cutaneous vasculature was identified as a major effector of thermoregulatory reflexes.

The contributions of the vasoconstrictor and vasodilator nerves to this thermoregulatory reflex were later determined. Roddie et al. (1957) provided evidence that the increase in skin blood flow in response to increased core temperature involved both a withdrawal of vasoconstriction and activation of the vasodilator nerves. Blood flow through the forearm was measured via venous occlusion plethysmography during an initial whole body cooling followed by a transition to whole body heating. During the transition to whole body heating, a small initial increase in blood flow occurred. This initial increase in blood flow was found to be similar in magnitude to that reported previously when the vasoconstrictor nerves in the skin have been blocked (Barcroft, 1943), and was not prevented by intra-arterial administration of atropine. As demonstrated by this study by Roddie et al. (1957), the initial increase in blood flow was followed by a larger and more rapid increase in skin blood flow that occurred simultaneous with the onset of sweating. The administration of intra-arterial atropine was found to delay this large increase in blood flow and abolish the sweat response. Taken together, these results led to the following conclusions: 1) during exposure to environmental heat, an initial increase in skin blood flow occurs due to the release of vasoconstrictor tone, and 2) the subsequent large increase in skin blood flow is linked to the onset of sweating and is mediated by cholinergic nerves.

A co-transmission theory has recently been proposed to explain the mechanisms mediating cutaneous active vasodilation, as well as the link between cutaneous vasodilation and the sweat response. By this theory, acetylcholine is co-released with other substances from sympathetic cholinergic nerves. Acetylcholine then primarily mediates the sweat response, while the involved co-transmitters are primarily responsible for mediating cutaneous vasodilation, as illustrated in Figure 2-4. Strong experimental evidence for this theory was provided by Kellogg et al. (1995). In this study, the use of botulinum toxin to presynaptically block cholinergic vessels prevented both cutaneous vasodilation and the sweat response, suggesting that cutaneous vasodilation and sweating are both mediated by sympathetic cholinergic nerves. As stated above, the use of atropine abolishes only the sweat response. Together, these data suggest that acetylcholine and one or more neurotransmitters are co-released from sympathetic cholinergic nerves to mediate the sweat response and cutaneous active vasodilation, respectively.

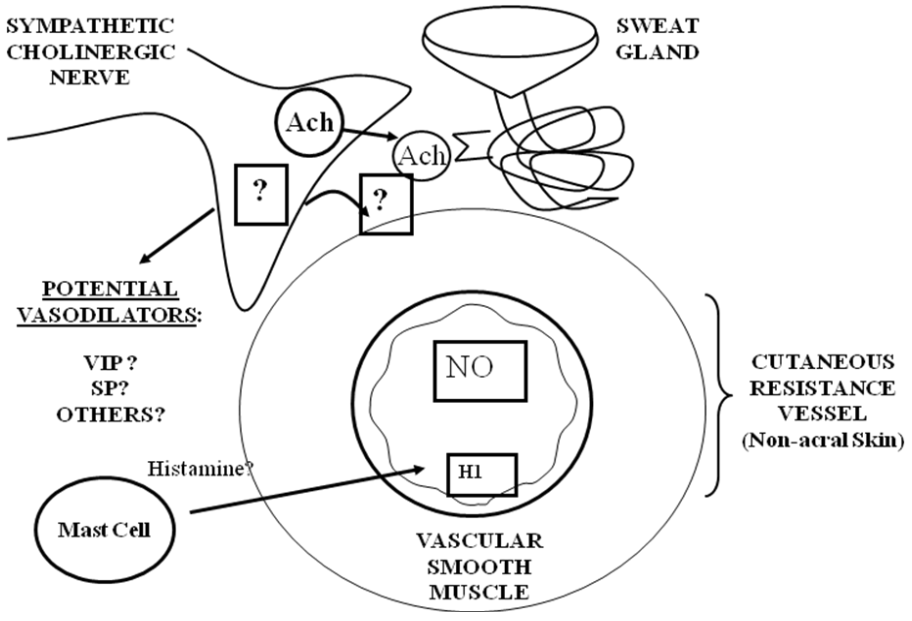


**Figure 2-4:Co-transmission theory of Cutaneous Active Vasodilation.**

Several substances have been proposed as potential co-transmitters involved in active vasodilation. One study by Bennet et al. (2003), suggested a possible role for the neuropeptide vasoactive intestinal polypeptide (VIP) in the skin blood flow response to whole body heating. These findings, however, have not been confirmed by subsequent studies (Wilkins et al. 2005). As such, a potential role for VIP in this response remains undetermined, and the identities of possible co-transmitters involved in cutaneous active vasodilation remain unknown.

In addition to the unknown co-transmitters involved in active vasodilation, a substantial role for NO has also been demonstrated in the response. Taylor and Bishop (1993) provided evidence of a role for nitric oxide, using the rabbit ear as a model for active vasodilation. In this study, NO synthase inhibition was found to abolish active vasodilation in the rabbit ear. One study by Dietz and colleagues (1994) was unable to determine a role for NO the active vasodilation occurring in human skin; however, using different experimental methods, several later studies have found that NO accounts for approximately 45% of reflex active vasodilation (Kellogg et al. 1995; Shastry et al. 1998).

Originally, this known role for nitric oxide in cutaneous active vasodilation was proposed to be permissive. Again, using the rabbit ear as a model for active vasodilation, Farrell and Bishop (1995), investigated the nature of the role for NO in this response. After blocking the production of NO in the rabbit ear, these investigators found that a low-dose infusion of the NO donor nitroprusside, given during hyperthermia, fully restored the blood flow response. By contrast, the same infusion of nitroprusside given during normothermia had no effect on blood flow in the rabbit ear. Given that the effects of nitroprusside were confined to the hyperthermic condition, and the hyperthermic blood flow response was fully restored by only a low-dose infusion of an NO donor, these investigators determined that NO held a permissive role in active

vasodilation. In other words, only a basal level of NO was believed to be required for full expression of active vasodilation.

In human skin, however, NO has since been found to contribute in a direct, rather than permissive manner to active vasodilation. In direct contrast to the findings of Farrel and Bishop (1995) in the rabbit ear, Wilkins et al. (2003) found that a low dose of exogenous NO in humans did not restore the skin blood flow response to whole body heating in microdialysis sites pre-treated with an NO synthase inhibitor. Furthermore, Kellogg et al. (2003) measured an increase in the concentration of NO in human skin during cutaneous active vasodilation. An increase in NO concentration would not be expected if NO was permissive in this response. These studies provide strong evidence that NO directly contributes to cutaneous active vasodilation.

While NO has been found to directly contribute by approximately 45% to the increase in skin blood flow during whole body heating, questions remain as to the source(s) of NO during this response. Kellogg and colleagues (2009) have recently provided evidence to suggest neuronal NO synthase (nNOS) may be the NO synthase isoform responsible for mediating NO generation during cutaneous active vasodilation. As nNOS inhibition, but not endothelial NO synthase inhibition, attenuates the skin blood flow response to whole body heat stress, this suggests a role for nNOS in this response.

Several possible sources may contribute to increased NO generation by nNOS during active vasodilation. Shibaski et al (2002) found acetylcholine may contribute to NO synthesis; however, this contribution appears to be isolated to the early stages of heat stress, where acetylcholine may not be a significant source of NO once significant vasodilation has occurred. Additionally, while $H_1$ histamine receptor activation and $NK_1$ receptor activation have both been shown to directly mediate a portion of the cutaneous vasodilation during hyperthermia, evidence

suggests these receptor types may also contribute to the NO component (Wong et al. 2004; Wong and Minson, 2006). Important questions remain as to what substances may be involved in cutaneous active vasodilation either as a direct vasodilator or by increasing NO production, and there are likely to be several redundant vasodilator pathways involved in mediating this response.

## Adenosine Receptors

The vasoactive effects of the purinergic receptors have been widely studied for several years. First recognized by Burnstock in 1978, these receptors were classified into two main groups: the P1 receptors, activated by adenosine; and the P2 receptors, activated by ATP. Several subtypes of receptors have subsequently been identified. Of these, both the $A_1$ and $A_2$ subtypes of adenosine receptors have been located in human skin (Stojanov and Proctor, 1989) and may exert vasoactive effects on the cutaneous circulation.

Activation of $A_1/A_2$ adenosine receptors has been shown to mediate vasodilation in multiple organ systems including the skin (Abebe et al, 1995; Ngai and Winn, 1993; Stojanov and Proctor, 1989). As further evidence of a vasodilatory role for $A_1/A_2$ adenosine receptor activation in the cutaneous circulation, microdialysis infusion of 2.8 mM adenosine into the interstitial space of human skin has shown to elicit vasodilation to an extent similar to that achieved during whole body heating (Shibaski et al. 2007). This vasodilation elicited by $A_1$ and $A_2$ adenosine receptors could occur through a variety of mechanisms by which adenosine receptors are known to mediate their effects.

Activation of $A_1/A_2$ adenosine receptors may contribute to the skin blood flow responses to local skin and/ or whole body heating directly or by contributing to the production of NO. For example, adenosine receptors have been found to be coupled to ATP-sensitive potassium channels ($K^+_{ATP}$) and have been found to induce NO and vasodilation through their actions on

these channels (Bryan & Marshall, 1999 a,b; Hein & Kuo, 1999). Additionally, adenosine-induced vasodilation and NO production may stem from an increase in prostaglandin formation. Recent data from Mortensen et al. (2009), for example, has shown adenosine contributes to exercise hyperemia in the human leg primarily by increasing prostaglandin and NO formation.

There is further evidence to suggest an interaction between histamine receptors and purinergic ($A_1/A_2$) receptors (Dickenson and Hill, 1994; Forsythe and Ennis, 1999) and between $NK_1$ receptors and purinergic receptors (Burnstock, 2009; Ralevic, 2009). Wong and colleagues (2006) have demonstrated that $H_1$ histamine receptor activation contributes to cutaneous active vasodilation while Wong & Minson have shown $NK_1$ receptor activation contributes to cutaneous thermal hyperemia (2011) and cutaneous active vasodilation (2004). As such, it is possible the effects of adenosine and adenosine receptor activation during hyperthermia may be manifest through the $H_1$ and $NK_1$ receptor activation components; however, these potential interactions await further investigation.

### *Potential Role for Adenosine Receptors in Cutaneous Thermal Hyperemia*

The cutaneous thermal hyperemic response to nonpainful local heating of the skin has been shown to be mediated by two independent mechanisms. The initial peak and nadir of this response is predominantly due to an axon reflex with a some contribution of NO, while the secondary plateau phase of this response is predominantly (~70%) mediated by NO (Minson et al. 2001). Important questions remain as to what substances may be involved in this response, either as a direct vasodilator or by increasing NO production. The study presented in Chapter IV was designed to investigate a possible $A_1/A_2$ adenosine receptor-mediated component to the cutaneous thermal hyperemic response. In addition, this study sought to determine whether a

portion of the NO-dependent component of this response may be explained by $A_1/A_2$ adenosine receptor activation.

### *Potential Role for Adenosine Receptors in Cutaneous Active Vasodilation*

As discussed earlier in this literature review, a co-transmission theory has been proposed to explain the mechanisms mediating cutaneous active vasodilation and the sweat response. By this theory, acetylcholine is co-released with other substances from sympathetic cholinergic nerves. The co-transmitters involved in mediating cutaneous vasodilation in response to whole body heating remain unknown; however, nitric oxide is known to directly mediate approximately 45% of this response (Kellogg et al. 1995; Shastry et al. 1998). As such, important questions remain as to what substances may be involved in cutaneous active vasodilation either as a direct vasodilator or by increasing NO production. The study presented in Chapter V of this thesis was designed to investigate a role for adenosine receptor activation in contributing to cutaneous active vasodilation directly or through the production of NO.

# Chapter 3 - Overview of Methodologies

## Ethical Approval

The Institutional Review Board of Kansas State University approved the protocols of both studies presented in this thesis. Subjects were recruited from the Kansas State University student population. Written, informed consent was obtained from each subject prior to participation and all protocols conformed to guidelines as set forth by the Declaration of Helsinki.

## Subjects

Thirteen subjects, including 10 men and 3 women between the ages of 20 and 28 participated in the studies described in this thesis. A general health screening was given prior to participation in these studies to ensure that all subjects were healthy, nonsmokers, without diabetes or a history of cardiovascular disease. Subjects were not taking medications aside from oral contraceptives. All subjects were asked to refrain from caffeine, alcohol, and exercise for 12 hours prior to each study.

## Subject Monitoring

Subjects were equipped with an electrocardiogram. Blood pressure was monitored beat-by-beat via photoplethysmography (NexfinHD; BMEYE, Amsterdam, The Netherlands), and verified via automated brachial auscultation (S/5 Light Monitor; Datex-Ohmeda, GE Healthcare; Madison, WI, USA) every 10 minutes. Skin blood flow measurements were made from the lateral aspect of the left forearm and subjects rested supine with the experimental arm at heart level for the entire protocol. All experiments were performed in a thermoneutral laboratory.

During the experiments which consisted of a period of local heating, local skin temperature was controlled by local skin heating devices (PF5020 local heating units and PeriFlux 5020 Temperature Unit; Perimed; Jarfalla, Sweden). The local heating units used in these experiments cover a 10mm area of the skin surface, and allowed for temperature increases of 0.1°C increments.

During the protocols which included a period of whole body heating, oral temperature was measured with a thermistor placed in the sublingual sulcus, and was used as an index of core body temperature. Oral temperature has previously been established as an accurate index of core temperature (Cranston, 1954; Mairiaux, 1983). Subjects' oral temperature was monitored for 5-10 minutes prior to, and for the duration of, the whole body heating period.

## Subject Instrumentation

### *Microdialysis Technique*

All subjects had four microdialysis fibers, which are small tubes containing a semi-permeable membrane, placed into the dermal layer of the skin of the left ventral forearm. The semi-permeable membrane in each fiber, as seen in figure 3-1(below), allows for the diffusion of compounds from an area of high concentration to an area of low concentration, resulting in the exchange of substances into and out of the probe from the extracellular fluid in the local area. In the experiments described in this thesis, microdialysis fibers were used to administer drugs to local areas of the skin on the forearm. The membranes of the microdialysis fibers were 10 mm in length with a 55-kDa molecular mass cutoff (CMA 31 Linear Probe; CMA Microdialysis, Sweden).

Fibers were placed approximately 3-5 cm apart on the ventral surface of the left forearm in the absence of anesthetics; however, ice was used to numb the skin prior to placement

(Hodges et al. 2009). Fiber placement was accomplished by first threading a 23-gauge needle through the skin at each desired site of microdialysis placement. A fiber was threaded through the lumen of the needle, and the needle was removed, leaving the membrane in place. Approximately 45-90 minutes were allowed for resolution of the trauma response induced by microdialysis fiber placement in the skin before the start of the protocol. During this time, all fibers were perfused with lactated Ringer's solution at a rate of 4 μl/min.

### *Laser-Doppler Flowmetry*

Skin blood flow was recorded as red blood cell (RBC) flux and measured by laser-Doppler flowmetry. Utilizing the Doppler shift of a laser as it is reflected off of red blood cells moving through the skin, this method of measurement provides a signal that is proportional to the speed and concentration red blood cells moving past the laser. Laser-Doppler flowmetry has been shown to be a reliable method of determining skin blood flow without influence from blood flow to the underlying muscle, and is widely used as a non-invasive method of obtaining a continuous index of skin blood flow. (Johnson et al, 1984; Saumet et al, 1988).

Each site on a subject's arm where a microdialysis probe was placed served as a separate recording site to examine the vasodilator response to local or whole body heating. A laser Doppler probe (PeriFlux 5010 laser-Doppler perfusion monitor; Perimed; Jarfalla, Sweden), was placed on the forearm at each microdialysis site, directly over the placement of each semi-permeable membrane. Local heating units (PF5020 local heating units and PeriFlux 5020 Temperature Unit; Perimed; Jarfalla, Sweden) were placed on the skin directly over each microdialysis membrane, and an integrated laser-Doppler probe (Probe 413; Perimed; Jarfalla, Sweden) was placed in the center of each local heating unit to measure RBC flux directly over

each microdialysis site. Figure 3-1, below, illustrates the placement of a microdialysis fiber and laser-Doppler flow probe.

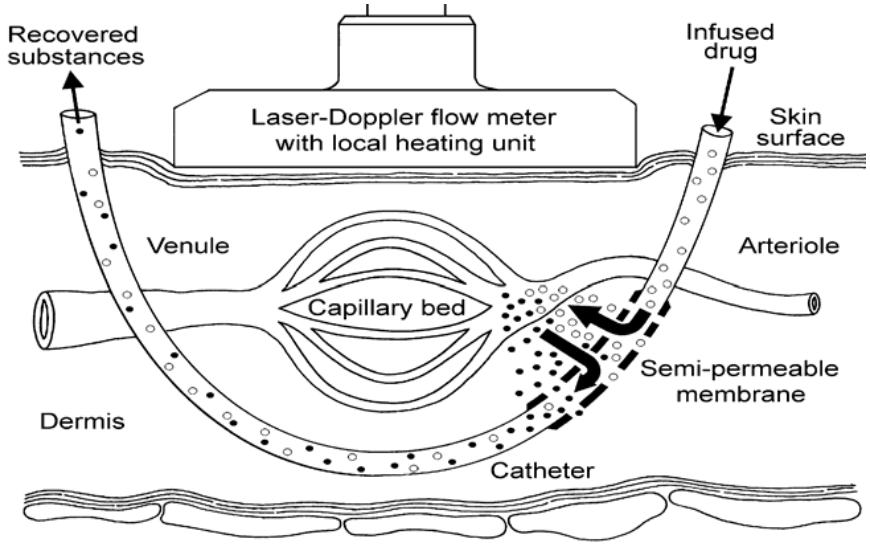


**Figure 3-1: Schematic drawing of the microdialysis technique and placement of a laser-Doppler flow probe.**

## Protocols

### *Local Heating*

Pre-infusion baseline skin blood flow measurements were recorded during a 5 minute period, with local heaters set at 33°C. Following baseline measurements, drug infusion through each microdialysis fiber was initiated. Following 45 minutes of drug infusion, the temperature of the local heaters was increased to 42°C at a rate of 1°C/10s (equivalent to 0.5°C/5 s; Minson et al. 2001; Wong et al. 2006). The temperature of the local heating units was maintained at 42°C until a plateau in skin blood flow was observed and maintained for 20-30 min.

### *Whole Body Heating*

The protocol for the study described in Chapter V involves a period of whole body heating. Subjects were outfitted in a water-perfused suit covering the entire body except for the

head, hands, feet, and the left forearm from which skin blood flow measurements were recorded. Thermoneutral conditions were maintained during the microdialysis trauma resolution period and baseline data collection period, by circulating thermoneutral water at approximately 33°C through the suit. During periods of whole body heating for the purpose of testing the cutaneous vasodilator response to heat stress, water at a temperature of approximately 50°C from a second water tank was circulated through the suit to raise $T_{or}$ at least 0.8°C above baseline. When a 0.8°C rise in $T_{or}$ had been achieved, subject's temperature was maintained at this level to acquire a stable 10 min plateau of skin blood flow. Once a stable 10 min period of skin blood flow was achieved, subjects were cooled by perfusing 33°C water through the suit and removing the plastic rain suit.

## Drugs Administered

### *Nitric Oxide Synthase Inhibition*

A 10mM dose of the L-arginine analog $N^G$-nitro-L-arginine methyl ester (L-NAME; EMD Biosciences; San Diego, CA, USA), dissolved in lactated Ringer's solution was used to inhibit NO synthase. This concentration of L-NAME has been previously shown to adequately inhibit NO synthase in human skin (Fieger and Wong, 2010; McCord et al. 2006; Minson et al. 2001; Wilkins et al. 2003; Wong and Minson, 2006; Wong et al. 2004). L-NAME was administered via microdialysis at a rate of 4 μl/min for 45 minutes prior to the beginning of the local and whole body heating protocols.

### *A1/A2 Adenosine Receptor Inhibition*

A 4mM dose of theophylline (Tocris Biosciences; Ellisville, MO) dissolved in lactated Ringer's solution was used as a competitive and non-selective $A_1/A_2$ adenosine-receptor antagonist. Pilot data from our laboratory demonstrated that 4mM theophylline was the highest

dose that could attenuate the local heating response without eliciting vasodilation (Fieger and Wong, 2010). Theophylline was administered via microdialysis at a rate of 4 μl/min for 45 minutes prior to the beginning of the local and whole body heating protocols.

### *Nitric Oxide Donor*

A maximal skin blood flow response was elicited at the end of every protocol via infusion of 28 mM sodium nitroprusside (SNP). This concentration of SNP has been previously shown to elicit maximal vasodilation in human skin (Kellogg, 1999; Minson, 2001).

## Data Collection and Analysis

Data were digitized and stored at 100 Hz on a personal computer. Data were analyzed offline using signal-processing software (Windaq; Dataq Instruments, Akron, OH, USA). Because of the transient and rapid nature of the initial peak and nadir responses (Chapter IV), a stable 30-90 second period of skin blood flow was used for analysis. For all other skin blood flow values reported in Chapters IV and V, a stable 3-5 minute period of skin blood flow was used for analysis. Skin blood flow data were converted to cutaneous vascular conductance (CVC), calculated as the ratio of skin blood flow to mean arterial pressure (RBC flux/mean arterial pressure). CVC data were expressed as a percentage of maximal vasodilation (%$CVC_{max}$) via SNP infusion and local heating to 43°C.

Phase of menstrual cycle or oral contraceptive use was noted for female subjects but not controlled for in these experiments. Due to the robust nature of the skin blood flow response, phase of menstrual cycle did not significantly alter the overall responses when data from all subjects was averaged. Further, due to the lower number of female subjects who participated in the studies presented in Chapter IV (n=2) and Chapter V (n=1), it was not possible to run a full statistical analysis on the data from female subjects alone.

The statistical analyses performed included the use of paired t-tests, one way ANOVA with repeated measures and two way ANOVA with repeated measures. For all ANOVAs, Student-Newman-Keuls post hoc analysis was used to determine where significance differences occurred. All statistical analyses were performed using SigmaStat 3.5 (Systat Software; Point Richmond, CA, USA). All values are presented as mean ± SEM, and P-values $< 0.05$ were considered to be significant.

# Chapter 4 - Adenosine Receptor Inhibition With Theophylline Attenuates the Skin Blood Flow Response To Local Heating In Humans [1]

## Introduction

Assessment of the thermal hyperemic response to local heating of the skin provides a convenient method of examining microvascular and endothelial function. The skin blood flow response to local heat application is commonly used to evaluate the vessels in various populations and disease states (Arora et al. 1998; Boignard et al. 2005; Stewart et al. 2004); however, the mechanisms underlying this heat-evoked cutaneous vasodilation are not completely understood. It is, therefore, important to investigate the mechanisms by which this response is mediated.

Rapid, nonpainful, local heating of human skin elicits a thermal hyperemic response that is biphasic and characterized by a rapid rise in skin blood flow to an initial peak, followed by a brief nadir, and, finally, a more slowly established and prolonged plateau (Kellogg et al. 1999; Minson et al. 2001). It has been suggested the initial peak and nadir of cutaneous thermal hyperemia is primarily mediated by an axon reflex via activation of C-fiber afferent neurons (Magerl and Treede, 1996; Minson et al. 2001). The sensory neurons may elicit vasodilation through the release of substance P and/or calcitonin gene-related peptide, although these theories have yet to be directly tested experimentally. Additionally, nitric oxide (NO) has been shown to contribute modestly to the initial peak of the local heating response (Kellogg et al.,

[1] Reprinted with permission from "Adenosine receptor inhibition with theophylline attenuates the skin blood flow response to local heating in humans". *Exp Physiol* 95: 946-954, 2010.

1999; Minson et al. 2001).

The secondary plateau phase of the local heating response, by contrast, consists of a sustained increase in skin blood flow and is ~70% dependent on NO, where inhibition of NO synthase (NOS) greatly attenuates the plateau phase of thermal hyperemia (Kellogg et al., 1999; Minson et al. 2001). Kellogg and colleagues (2009) have recently provided evidence to suggest endothelial NOS (eNOS) may be the NOS isoform responsible for the NO-dependent secondary plateau; however, Stewart and colleagues (2007) have provided evidence that neuronal NOS (nNOS) is the isoform responsible for the increase in NO during local heating of the skin. Thus, it remains unclear as to the source of NO or what may stimulate the increase in eNOS and/or nNOS activity. Further, the oxidative state of the system and the rate of NO metabolism influence the amount of bioavailable NO such that increases in NO production may not translate to increased NO-dependent vasodilation.

Several putative vasodilator pathways have been shown to induce smooth muscle relaxation, in part, through the production of NO. For example, $H_1$ histamine receptor activation and prostaglandins, by activation of the cyclooxygenase-1 and -2 (COX- 1 and -2) pathways, have both been shown to elicit vasodilation in part through a NO-dependent component (Brown and Roberts, 2001; Salvemini, 1997). The contribution of $H_1$ activation and the COX-1/COX-2 pathways to cutaneous thermal hyperemia has recently been investigated in independent studies (McCord et al. 2006; Wong et al. 2006). Inasmuch as these studies have demonstrated a minimal role for $H_1$ activation (Wong et al. 2006) and no role for COX-1/COX-2 activation (McCord et al. 2006) to cutaneous thermal hyperemia, it appears these pathways are not sources of NO during local heating. Although there are likely to be several redundant vasodilator pathways involved in mediating cutaneous thermal hyperemia, important questions

remain as to what substances may be involved in this response, either as a direct vasodilator or by increasing NO production.

Activation of adenosine receptors has been shown to mediate vasodilation in the heart, brain, and several other organ systems of both animals and humans, including human skin (Abebe et al. 1995; Ngai and Winn, 1993; Olsson and Pearson, 1990; Stojanov and Proctor, 1989). Further evidence suggests adenosine receptors may induce vasodilation, at least in part, by increasing NO production, and the presence of $A_1$ and $A_2$ adenosine receptors in human skin microcirculation has been established (Stojanov and Proctor, 1989). In view of these findings, it is reasonable to suggest adenosine-receptor activation may directly contribute to cutaneous thermal hyperemia as well as through an increase in NO production.

The purpose of this study was to investigate a possible adenosine receptor-mediated component to the cutaneous thermal hyperemic response and to determine whether a portion of the NO-dependent component of this response may be explained by adenosine receptor activation. We hypothesized adenosine receptor blockade would result in an attenuated plateau response to local heat application. We further hypothesized simultaneous blockade of adenosine receptors combined with NOS inhibition would result in a greater attenuation of the NO-dependent secondary plateau compared to either NOS inhibition or adenosine receptor blockade alone.

## Methods

### *Ethical Approval*

The Institutional Review Board of Kansas State University approved this study and subjects were recruited from the Kansas State University student population. Written, informed

consent was obtained from each subject prior to participation and all protocols conformed to guidelines as set forth by the Declaration of Helsinki.

### *Subjects*

Six subjects (4 men, 2 women) between the ages of 20 and 26 participated in the study. Power analysis of preliminary data indicated that 7-8 subjects would be required to reach significance. Initial power analysis was performed using mean and standard deviation for 6 mM and 8 mM theophylline. These higher concentrations resulted in greater variability and, as such, power analysis indicated more subjects would be required to reach significance. A 4 mM concentration of theophylline resulted in less variability and significance was achieved using six subjects. All subjects were healthy, nonsmokers, free of cardiovascular disease and diabetes, and were not taking any medications aside from oral contraceptives. All subjects were asked to refrain from caffeine, alcohol, and exercise for 12 hours prior to the study. Phase of menstrual cycle or oral contraceptive use was noted for female subjects but not controlled for in these experiments. Due to the robust nature of the thermal hyperemic response, phase of menstrual cycle did not significantly alter the overall responses when data from all subjects was averaged (see Data Analysis below).

### *Instrumentation and Measurements*

Skin blood flow measurements were made from the lateral aspect of the left forearm and subjects rested supine with the experimental arm at heart level for the entire protocol. Blood pressure was monitored beat-by-beat via photoplethysmography (NexfinHD; BMEYE, Amsterdam, The Netherlands), and verified via automated brachial auscultation (S/5 Light Monitor; Datex-Ohmeda, GE Healthcare; Madison, WI, USA) every 10 minutes. Four microdialysis fibers were placed into the dermal layer of the skin of the left ventral forearm.

Fibers were placed in the absence of anesthetics; however, ice was used to numb the skin prior to placement (Hodges et al. 2009). Fibers were placed approximately 3-5 cm apart. Fiber placement was accomplished by first threading a 23-gauge needle through the skin at each desired site of microdialysis placement. A fiber was threaded through the lumen of the needle, and the needle was removed, leaving the membrane in place. The membranes of the microdialysis fibers were 10 mm in length with a 55-kDa molecular mass cutoff (CMA 31 Linear Probe; CMA Microdialysis, Sweden). Approximately 1.5-2 hours were allowed for resolution of the trauma response induced by microdialysis fiber placement in the skin before the start of the protocol. During this time, all fibers were perfused with lactated Ringer's solution at a rate of 4 μl/min.

Skin blood flow was recorded as red blood cell (RBC) flux, measured by laser-Doppler flowmetry (PeriFlux 5010 laser-Doppler perfusion monitor; Perimed; Jarfalla, Sweden). Local heating units (PF5020 local heating units and PeriFlux 5020 Temperature Unit; Perimed; Jarfalla, Sweden) were placed on the skin directly over each microdialysis membrane, and an integrated laser-Doppler probe (Probe 413; Perimed; Jarfalla, Sweden) was placed in the center of each local heating unit to measure RBC flux directly over each microdialysis site.

### *Experimental Protocol*

Data collection began when skin blood flow was observed to stabilize over the areas of microdialysis insertion via laser Doppler flowmetry, indicating resolution of the trauma response. Pre-infusion baseline skin blood flow measurements were recorded during a 5 minute period, with local heaters set at 33°C. Following baseline measurements, drug infusion through each microdialysis fiber was initiated. Each microdialysis fiber was randomly assigned one of four treatments: 1) lactated Ringer's to serve as a control; 2) 4mM theophylline (Tocris Biosciences; Ellisville, MO, USA), a non-selective, competitive $A_1/A_2$ adenosine receptor

antagonist; 3) 10mM of the L-arginine analog NG-nitro-L-arginine methyl ester (L-NAME; EMD Biosciences; San Diego, CA, USA) to inhibit NOS; and 4) combined 4 mM theophylline and 10 mM-L-NAME, to simultaneously block adenosine-receptors and NOS, and to determine a potential interaction between adenosine receptors and NO. The concentration of theophylline was chosen based on extensive pilot work, which indicated 4 mM was the highest dose that could attenuate the local heating response without eliciting vasodilation. A 10 mM concentration of L-NAME has been shown previously to adequately inhibit NOS in human skin (Minson et al. 2001; McCord et al. 2006; Wong et al. 2006). All treatments were perfused at a constant rate of 4μl/min with a microinfusion pump (Bee Hive controller and Baby Bee Syringe Pumps; Bioanalytical Systems, West Lafayette, IN, USA). Pre-infusion baseline was taken as the last minute prior to drug infusion. Post-infusion baseline was taken as the last 1-2 minutes of infusion prior to local heating.

All drugs were infused for at least 45 minutes prior to commencement of the local heating protocol. Extensive pilot work indicated that at least 45 minutes of infusion of theophylline was required for maximal effect and previous studies have demonstrated this duration of infusion adequately inhibits NOS (Holowatz, et al. 2005; Minson et al. 2001; McCord et al. 2006; Wong et al. 2006). Following 45 minutes of drug infusion, the temperature of the local heaters was increased to 42°C at a rate of 1°C/10s (equivalent to 0.5°C/5 s; Minson et al. 2001; Wong et al. 2006). The temperature of the local heating units was maintained at 42°C until a plateau in skin blood flow was observed and maintained for 20-30 min. Once this stable plateau was established, a maximal skin blood flow response was elicited via infusion of sodium nitroprusside (SNP) at a rate of 4 μl/min and a simultaneous temperature increase to 43°C. This temperature increase and dose of SNP have been previously determined effective

in eliciting a maximal skin blood flow response (Holowatz, et al. 2005; Minson et al. 2001; McCord et al. 2006; Wong et al. 2006).

### *Data Collection and Analysis.*

Data were digitized and stored at 100 Hz on a personal computer. Data were analyzed offline using signal-processing software (Windaq; Dataq Instruments, Akron, OH, USA). Skin blood flow data were converted to cutaneous vascular conductance (CVC), calculated as the ratio of skin blood flow to mean arterial pressure (RBC flux/mean arterial pressure). CVC data were expressed as a percentage of maximal vasodilation (%CVCmax) via SNP infusion and local heating to 43°C.

Because of the transient and rapid nature of the initial peak and nadir responses, a stable 30-90 second period of skin blood flow was used for analysis. For the secondary plateau and maximal skin blood flow responses, a stable 3-5 minute period of skin blood flow was used for analysis. Due to the low number of female subjects who participated in this study (n = 2), a full statistical analysis could not be run to compare data from men and women. Any potential differences due to phase of menstrual cycle or oral contraceptive use would not be expected to significantly alter the data due to a low number of female subjects who participated in this study. Therefore, data from all subjects were averaged for statistical analysis. For each experimental site, a paired t-test was used to compare pre-drug infusion and post-drug infusion (before heating) baseline values.

The percent contribution of $A_1/A_2$ receptor activation, NO, and combined $A_1/A_2$ receptor activation + NO were calculated as:

[(%CVC max control – %CVC max treatment site) ÷ %CVCmax control)*100]

where “treatment site” is theophylline, L-NAME, or combined theophylline + L-NAME. The

percent contribution for each treatment site was calculated for the initial peak and secondary plateau phases of local heating. A one way repeated measures ANOVA was used to compare the effect of drug treatment between experimental sites. The relative contribution of adenosine-receptor activation and NO as well as the interaction between adenosine-receptor activation and NO was determined with the use of a one-way ANOVA with repeated measures. Percent contribution for each treatment site was compared using a one-way ANOVA with repeated measures. Maximal CVC values for each site were compared using a one-way ANOVA. For all ANOVAs, Student-Newman-Keuls post hoc analysis was used to determine where significance differences occurred. All statistical analyses were performed using SigmaStat 3.5 (Systat Software; Point Richmond, CA, USA). P-values $<0.05$ were considered to be significant and all data presented are mean ± SEM.

## Results

The administration of theophylline, L-NAME, or theophylline + L-NAME did not alter baseline skin blood flow values ($p=0.210$). That is, there was no significant difference between pre-infusion and post-infusion (before heating) baseline values within any of the treatment sites. There was no significant difference in baseline values between treatment sites (figure 4-1). Maximal absolute CVC responses did not differ between treatment sites (Table 4-1). The group mean data for the initial peak response in the four treatment sites is shown in figure 4-2. Compared to control (81 ± 2%CVCmax), the initial peak was significantly reduced in theophylline (68 ±2%CVCmax, $p<0.01$), L-NAME (54 ± 5%CVCmax, $p<0.001$), and theophylline + L-NAME (52 ± 5CVCmax, $p<0.001$) sites. The magnitude of reduction of the initial peak was significantly

greater in L-NAME and theophylline + L-NAME sites compared to theophylline only ($p<0.01$ for all conditions). There was no significant difference between L-NAME and theophylline + L-NAME.

| Treatment Site | Maximal CVC Values |
|---|---|
| Control | 1.78 ± 0.20 |
| Theophylline | 2.01 ± 0.24 |
| L-NAME | 1.67 ± 0.11 |
| Theophylline + L-NAME | 1.61 ± 0.10 |

Values are mean ± SE. There was no difference in the raw maximal CVC values between treatment sites. CVC, cutaneous vascular conductance (RBC flux/MAP). There was no difference between sites.

**Table 4-1: Absolute Maximal CVC Values.**

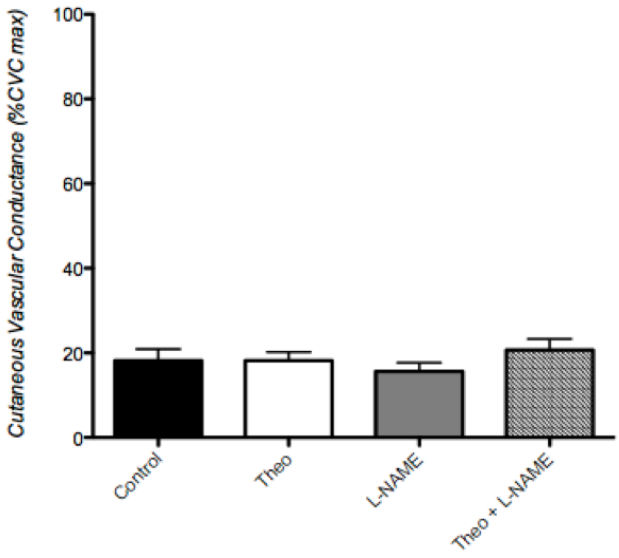


**Figure 4-1: Effect of drug treatment on baseline CVC.** Baseline CVC values were not different between treatment sites. Pre-drug infusion CVC was not significantly different compared to post-drug infusion CVC (not shown). Theo, theophylline (adenosine receptor antagonist); LNAME, NOS inhibition.

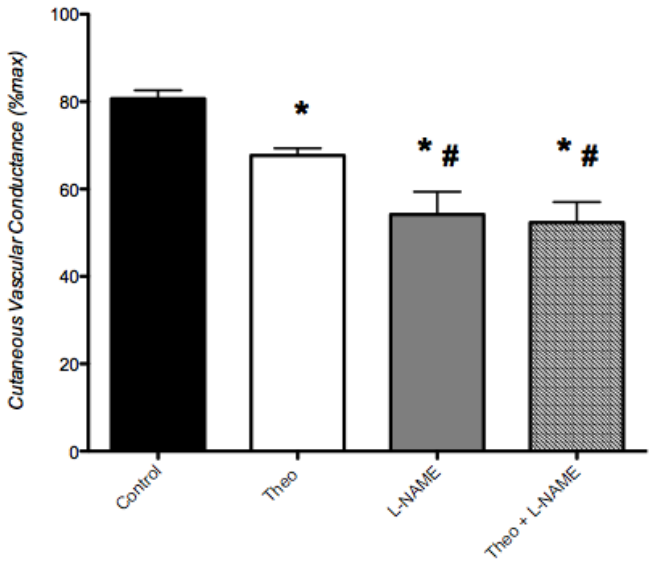


**Figure 4-2: Effect of drug treatment on the initial peak response to local heating.** The initial peak was significantly reduced in Theo, L-NAME, and Theo + L-NAME sites compared to control. L-NAME and Theo + L-NAME sites were significantly attenuated compared to Theo but were not significantly different from each other. Drug treatment notation same as in figure 4-1. *,$p < 0.05$ vs. control; # $p < 0.05$ vs. Theo.

The nadir in control sites averaged 58 ± 4%CVCmax. The nadir was reduced in the LNAME (36 ± 4%CVCmax) and theophylline + L-NAME sites (32 ± 1%CVCmax) compared to control ($p<0.01$ for both sites); however, no significant reduction in the nadir was observed in the theophylline only site (52 ± 6%CVCmax) compared to control. L-NAME significantly reduced the nadir compared to theophylline ($p<0.05$). Theophylline + L-NAME significantly reduced the nadir compared to theophylline only ($p<0.05$) but not compared to L-NAME. Group mean data for the nadir in all four treatment sites is depicted in figure 4-3.

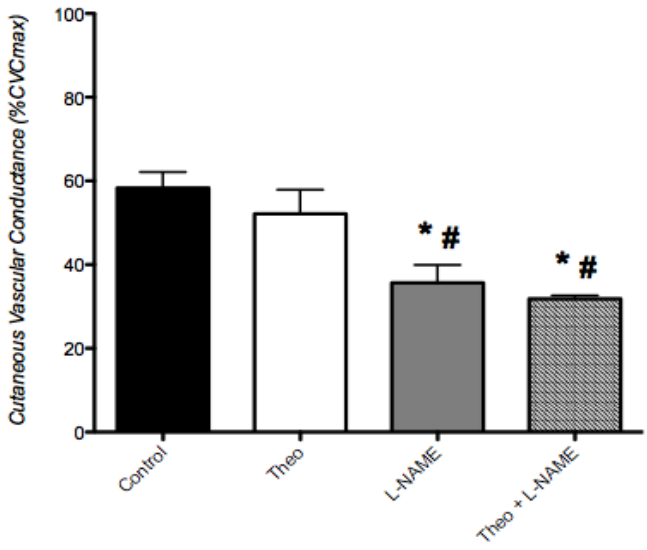


**Figure 4-3: Effect of theophylline and L-NAME on the nadir response to local heating.** Compared to control, Theo had no effect on the nadir. L-NAME and Theo + L-NAME significantly reduced the nadir compared to control and Theo but were not significantly different from each other. Drug treatment notation same as in figure 4-1. * $p < 0.05$ vs. control; # $p < 0.05$ vs. Theo.

The secondary plateau averaged 94 ± 2%CVCmax in control. Compared to control, the secondary plateau was reduced in theophylline (77 ± 2%CVCmax), L-NAME (60 ± 2%CVCmax), and theophylline + L-NAME sites (53 ± 1%CVCmax, $p<0.001$ for all conditions). Compared to theophylline, the secondary plateau was reduced in L-NAME sites ($p<0.001$). The co-infusion of L-NAME + theophylline further reduced the secondary plateau compared to theophylline ($p<0.001$) and compared to L-NAME ($p<0.05$). These data are shown in figure 4-4. Data showing the percent contribution of $A_1/A_2$ receptor activation, NO, and combined $A_1/A_2$ receptor activation + NO are shown in figure 4-5A (initial peak) and figure 4-5B (secondary plateau). The contribution of $A_1/A_2$ receptor activation to the initial peak was 16 ± 1%CVCmax, NO contributed 33 ± 1%CVCmax, and combined $A_1/A_2$ receptor activation + NO contributed 36 ± 2%CVCmax to the initial peak (figure 4-5A).

The contribution of combined $A_1/A_2$ receptor activation + NO and NO alone was significantly greater than the contribution of $A_1/A_2$ receptor activation alone ($p < 0.001$ for both

conditions); however, there was no significant difference between the contribution of combined $A_1/A_2$ receptor activation + NO and NO alone. For the secondary plateau phase (figure 4-5B), the percent contribution of $A_1/A_2$ receptor activation (18 ± 1%CVCmax) was significantly less than the contribution of NO alone (36 ± 1%CVCmax) and combined $A_1/A_2$ receptor activation + NO (44 ± 1%CVCmax; $p < 0.001$ for both conditions). Further, the contribution from NO alone was significantly less than the contribution of combined $A_1/A_2$ receptor activation + NO ($p < 0.001$).

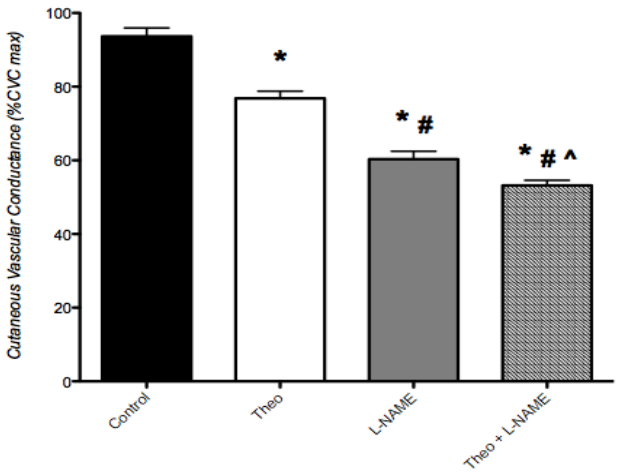


**Figure 4-4: Effect of theophylline and and L-NAME on the plateau phase to local heating.** The plateau phase was significantly attenuated in all treatment sites compared to control. L-NAME and Theo + L-NAME attenuated the plateau phase compared to control and Theo. Theo + L-NAME further reduced the plateau phase compared to L-NAME. Drug treatment notation same as in figure 4-1. * $p < 0.05$ vs. control; # $P < 0.05$ vs. Theo; ^ $p < 0.05$ vs. Theo + L-NAME.

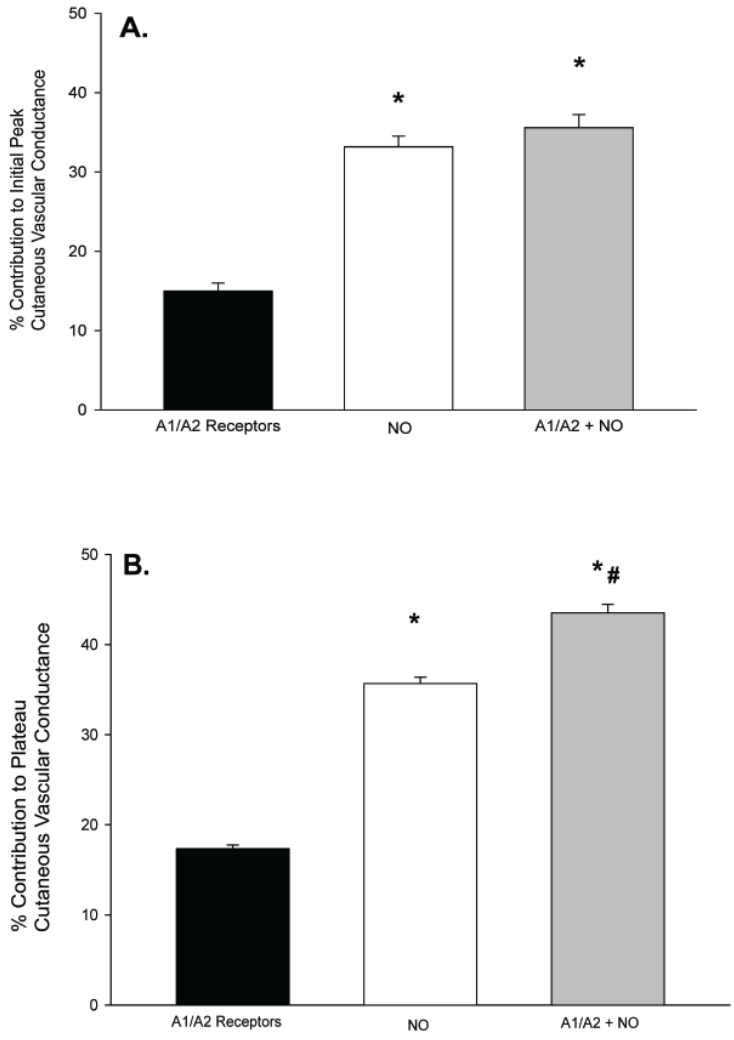


**Figure 4-5: Percent contribution of adenosine receptor activation, NO, and combined adenosine receptor activation + NO to the initial peak and secondary plateau.** A: Percent contribution to the initial peak; B, Percent contribution to the secondary plateau. $A_1/A_2$ receptors, contribution of adenosine receptors (theophylline). * $p < 0.05$ vs. adenosine receptor activation; #, $p < 0.05$ vs. NO.

| Concentration | Average RBC Flux Increase | Average %CVCmax Increase | # Sites |
|---|---|---|---|
| 6 mM | 31 ± 12 | 24 ± 7 | n = 3 |
| 8 mM | 53 ± 14 | 33 ± 10 | n = 4 |

RBC Flux, red blood cell flux (unitless). Values are averages from the last 3-5 minutes of drug infusion. # Sites is the number of microdialysis sites that received each concentration of theophylline. Values are mean ± SE.

**Table 4-2: Vasodilation to Increasing Doses of Theophylline.**

## Discussion

Two primary findings resulted from this study. First, adenosine receptor inhibition attenuated the initial peak and plateau of the local heating response, indicating adenosine receptor activation directly mediates a portion of the thermal hyperemic response. Second, combined inhibition of adenosine receptors and NOS further reduced the magnitude of the NO-dependent secondary plateau. Inasmuch as the combined effect of adenosine receptor inhibition and NOS inhibition further diminished, but did not abolish, the sustained thermal hyperemia, suggests adenosine receptor activation may account for a portion of the NO component of the local heating response, but is not the sole mechanism of NO production (Figures 4-4 and 4-5B). In addition, as demonstrated in Figures 4-5A and 4-5B, the percent contribution of $A_1/A_2$ receptor activation to both the initial peak and secondary plateau phases of cutaneous thermal hyperemia is relatively modest (16% and 18% contribution, respectively) whereas the NO contribution to both the initial peak and secondary plateau is substantially greater than the contribution of $A_1/A_2$ receptor activation.

Adenosine receptor inhibition attenuated the thermal hyperemic response, which provides evidence of a direct role for adenosine receptors in mediating this response. The adenosine receptor-mediated component may be a result of direct action of these receptors on smooth muscle cells (Bryan and Marshall, 1999b; Baker and Sutton 1993; Skinner and Marshall, 1996). Studies suggest adenosine-induced vasodilation may work through NO-independent mechanisms such as through its effects on adenylate cyclase, inositol phosphates, KATP channels, and/or changes in $Ca^{2+}$ and $K^+$ fluxes (Long et al. 1987; Bryan and Marshall 1999b; Marshall et al. 1993; Hein and Kuo, 1999). Clearly, more research is required to further elucidate these ideas as possible pathways involved in cutaneous thermal hyperemia.

In the present study, simultaneous inhibition of adenosine receptors and NOS diminished, but did not abolish, the local heating plateau. These data suggest adenosine receptor activation may be one source of NO yet the majority of NO produced during local heating of the skin is from sources other than adenosine receptor activation (figures 4-4 and 4-5B). Several in vivo and in vitro studies have suggested a close interaction between adenosine receptor activation and NO in a host of tissues and vascular beds (Li et al. 1998; Martin et al. 2006, Mortenson et al, 2009; Sobrevia et. al, 1997; Vials and Burnstock, 1993) and adenosine receptor activation could mediate NO production through a variety of mechanisms such as through alterations in $Ca^{2+}$ flux and/or $K^{+}_{ATP}$ channels (Bryan and Marshall, 1999 a,b; Hein and Kuo, 1999).

It is also possible adenosine-induced vasodilation and NO production stems from an increase in prostaglandin formation. Recent data from Mortensen et al. (2009) has shown adenosine contributes to exercise hyperemia in the human leg primarily by increasing prostaglandin and NO formation. In the context of cutaneous thermal hyperemia in humans, McCord et al. (2006) found inhibition of the COX-1 and COX-2 pathways did not alter the skin blood flow response to direct local heating and combined inhibition of NOS and COX-1/COX-2 had no further effect compared to NOS inhibition alone, suggesting the COX-1/COX-2 pathways are not involved in the thermal hyperemic response and COX-1/COX-2 are not a source of NO. Although the data from McCord et al. (2006) strongly suggest COX-1/COX-2 (prostaglandin production) are not involved in the cutaneous thermal hyperemic response, we cannot completely rule out that a portion of the adenosine receptor component observed in this study may be explained by activation of the COX pathway.

The data from the present study further suggest a large portion of the NO component

cannot be explained by adenosine receptor activation. Several possibilities have been proposed to explain the large increase in NO production during local heating. It is possible application of heat to the skin may directly cause endothelial production of NO via a shear stress mechanism; however, reactive hyperemia, a condition which would presumably increase shear stress, appears to be independent of NO in human skin (Wong et al. 2003; Zhao et al. 2004). It is also possible the direct effect of heat directly enhances eNOS activity or increases the binding of heat shock protein 90 (HSP90) to eNOS thereby resulting in greater NO production. The direct effect of heat enhancing eNOS activity appears to be unlikely (Venturini et al. 1999) and inhibition of HSP90 appears to account for a modest portion of the NO response (Shastry and Joyner, 2002). Multiple and redundant pathways are undoubtedly involved in this response and it is unlikely that any one pathway will completely account for the increase in NO production, yet experiments designed at investigating mechanisms underlying the large increase in NO production in response to local skin heating remain important areas of future research.

### *Experimental Considerations and Limitations*

While the data from the present study suggest involvement of adenosine receptors in the thermal hyperemic response to local heating, there are at least four limitations to be addressed. First, use of a competitive, but non-selective, adenosine-receptor antagonist, theophylline, does not allow for specific roles of adenosine receptor subtypes during local heating of skin to be distinguished. The existence of both $A_1$ and $A_2$ adenosine receptors has been demonstrated in human skin (Stojanov and Proctor, 1989). Activation of both $A_1$ and $A_2$ adenosine receptors have been shown to induce vasodilation (Bryan and Marshall, 1999b; Danialou et al. 1997; Sobrevia et al. 1997; Vials and Burnstock, 1993); however, the specific roles and contributions of each of these receptor subtypes appears to differ according to the type of tissue and conditions in which

they are studied. For example, Bryan and Marshall (1999 a, b) have demonstrated $A_1$ receptors are primarily responsible for adenosine-mediated vasodilation in skeletal muscle under resting, hypoxic conditions whereas Ray and Marshall (2009) have shown $A_{2A}$ receptor activation induces vasodilation in skeletal muscle in response to tetanic contractions. Both $A_1$ and $A_2$ isoforms of the adenosine receptor have been found in human skin and may display discrepant activation patterns similar to skeletal muscle. As such, we chose to use theophylline to antagonize both $A_1$ and $A_2$ receptor subtypes.

Second, based on the experimental approach, we can only discuss the data in terms of an adenosine receptor activation component and not adenosine, per se, being involved in cutaneous thermal hyperemia. Although adenosine is the most likely candidate to bind to, and activate, $A_1/A_2$ receptors, the present data suggest an indirect role for adenosine, per se, and we cannot rule out the possibility that some other substance(s) bind to, and activate, $A_1/A_2$ receptors.

Third, a 4 mM dose of theophylline was the maximum dose that could be administered via microdialysis without causing non-specific vasodilation (Table 4-2). In preliminary experiments, concentrations higher than 4 mM resulted in substantial vasodilation. This nonspecific vasodilation of adenosine receptor antagonists is not unique to human skin. Casey et al. (2009; 2010) have demonstrated increases in forearm blood flow and vascular conductance under baseline conditions in response to infusion of a similar non-specific adenosine receptor antagonist, aminophylline. The increase in baseline skin blood flow observed with higher concentrations of theophylline is most likely due to the phosphodiesterase inhibitor properties of theophylline, which would act to increase cAMP levels and result in smooth muscle relaxation

(Taddei et al. 1991). It is possible our data underestimate the contribution of adenosine receptor activation to cutaneous thermal hyperemia; however, our data clearly indicate a direct role for adenosine receptor activation to cutaneous thermal hyperemia as well as a possible source of NO.

Fourth, theophylline and aminophylline are the most common adenosine receptor antagonists used in human-based experiments. It is possible results may have been different if aminophylline was used instead of theophylline. This seems unlikely inasmuch as Radegran and Calbet (2001) and Mortensen et al. (2009) have observed significant reductions in leg blood flow during exercise using theophylline and Casey et al. (2009; 2010) have observed similar reductions in forearm blood flow during hypoxic exercise using aminophylline. Thus, it appears theophylline and aminophylline are similarly effective at antagonizing $A_1$ and $A_2$ adenosine receptors.

### *Summary and Conclusions*

While the data from this study indicate adenosine receptor activation may directly mediate a portion of the cutaneous vasodilation and may account for a portion of the NO component, there is still a substantial degree of vasodilation for which adenosine receptor activation cannot account. Mechanisms attending cutaneous thermal hyperemia are undoubtedly complex and redundant and, as such, inhibition or blockade of one or two potential vasodilator pathways is unlikely to completely abolish this robust vasodilation. As such, current and future research aimed at investigating potential mechanisms involved in cutaneous thermal hyperemia will have to be taken with the understanding that it is unlikely one or more vasodilator pathways are simply “additive” and the redundant nature of the system may unmask roles for other unexamined vasodilator pathways when one or two pathways are blocked. For example, although

McCord et al. (2006) observed no role for the COX pathway when COX was inhibited either independently or combined with inhibition of NOS, it is possible a role for the COX pathway may be unmasked when combined with adenosine receptor inhibition.

In conclusion, this study provides evidence for the direct involvement of adenosine receptors in the cutaneous thermal hyperemic response to local heating as observed by the reduced initial peak and plateau phases of thermal hyperemia in theophylline sites. Inasmuch as combined inhibition of adenosine receptors and NOS unmasked a larger reduction in skin blood flow than either adenosine inhibition or NOS inhibition alone, these data suggest adenosine receptor activation can account for a portion of the NO component of thermal hyperemia but a large portion of the NO component is due to sources other than adenosine receptor activation. Moreover, although these data provide evidence of a direct role for adenosine receptor activation, this represents a relatively minor contribution compared to NO.

# Chapter 5 - Does Adenosine Receptor Activation Contribute To Cutaneous Active Vasodilation in Humans?

## Introduction

In response to an increase in core body temperature, humans exhibit a substantial increase in skin blood flow and sweating. This cutaneous vasodilation and increase in sudomotor activity is the primary autonomic means by which humans defend against increasing core temperatures. As core temperature rises during heat stress, an initial rise in skin blood flow occurs via withdrawal of sympathetic adrenergic vasoconstriction. With a further increase in temperature to a specific core temperature threshold, reflex cutaneous active vasodilaton occurs, concurrent with the onset of sweating (Grant and Holling, 1938; Roddie et al. 1957). Active vasodilation accounts for 85-95% of the substantial increase in skin blood flow that occurs during heat exposure.

The active vasodilator system is known to be mediated by sympathetic cholinergic nerves and is responsible for both the sweat and skin blood flow responses. By the current co-transmission theory, acetylcholine is released from sympathetic nerves along with one or more unknown neurotransmitters to mediate sweating and cutaneous vasodilation, respectively. Kellogg and colleagues (1995) demonstrated acetylcholine is primarily responsible for the sweat response, whereas cutaneous vasodilation is, therefore, mediated by the co-transmitter(s) released with acetylcholine. The responsible co-transmitter(s) remain unknown; however, several substances and vasodilator pathways have been shown to play a role in cutaneous active vasodilation, including vasoactive intestinal peptide (VIP) (Bennet et al, 2004; Wilkin et al, 2005), $H_1$ histamine receptor activation (Wong et al. 2004), neurokinin-1 ($NK_1$) receptor activation (Wong and Minson, 2006), and vasoactive prostanoids (McCord et al. 2006).

Additionally, nitric oxide (NO) has been shown to directly mediate 30-45% of active vasodilation (Kellogg et al. 1998; Shastry et al. 1998; Wilkins et al. 2003). In contrast to the permissive role of NO in active vasodilation occurring in the rabbit ear (Farell and Bishop, 1995), multiple studies have provided evidence of a direct role for NO in active vasodilation in human skin (Kellogg et al. 2003; Wilkins et al. 2003). In the skin of the human forearm, Kellogg and colleagues (2003) measured increases in cutaneous interstitial concentrations of NO that occurred along with increases in skin blood flow following the onset of active vasodilation. Additionally, Wilkins et al. (2003) demonstrated that low-dose infusion of sodium nitroprusside, an exogenous source of NO, does not fully restore the skin blood flow response to whole body heating following NO synthase inhibition. Taken together, these data suggest an increase in NO production, rather than the presence of a basal level of NO, is required for full expression of cutaneous active vasodilation, and indicate NO is not permissive but directly mediates a portion of cutaneous active vasodilation. Data from Wilkins et al. (2003) further suggest NO may act synergistically with another vasoactive substance during cutaneous active vasodilation, in addition to having a direct role as a vasodilator,

While NO is known to directly mediate a significant component of the increase in skin blood flow during heat stress, it remains unclear as to the source of NO, what may cause the increase in NO synthase activity, or the NO synthase isoform involved in cutaneous active vasodilation. Kellogg and colleagues (2009) have recently addressed the question as to which NO synthase isoform may be involved in cutaneous active vasodilation. The data from these investigators suggests neuronal NO synthase (nNOS) may be the NO synthase isoform responsible for mediating NO generation during cutaneous active vasodilation, as nNOS

inhibition, but not endothelial NO synthase inhibition, attenuates the skin blood flow response to whole body heat stress.

Several possible sources may contribute to the increased NO generation by nNOS during active vasodilation. Shibaski et al (2002) found acetylcholine may contribute to NO synthesis; however, this contribution appears to be isolated to the early stages of heat stress, where acetylcholine may not be a significant source of NO once significant vasodilation has occurred. Additionally, while $H_1$ histamine receptor activation and $NK_1$ receptor activation have both been shown to directly mediate a portion of the cutaneous vasodilation during hyperthermia, evidence suggests these receptor types may also contribute to the NO component (Wong and Minson, 2006; Wong et al. 2004). Important questions remain as to what substances may be involved in cutaneous active vasodilation either as a direct vasodilator or by increasing NO production, and there are likely to be several redundant vasodilator pathways involved in mediating this response.

Adenosine $A_1$ and $A_2$ receptors have been shown to be located in human skin microcirculation and activation of these receptors has been shown to mediate vasodilation in multiple organ systems including the skin (Stojanov and Proctor, 1989). Furthermore, microdialysis infusion of 2.8 mM adenosine into the interstitial space of human skin has shown to elicit vasodilation to an extent similar to that achieved during whole body heating (Shibaski et al. 2007) and adenosine receptors have been shown to induce vasodilation, at least in part, by increasing NO production (Mortensen et al. 2009; Stewart et al. 2004; Vials and Burnstock, 1993). In the context of human skin, we have recently demonstrated adenosine receptor activation contributes to the rise in skin blood flow elicited by local heating of the skin both directly and through the production of NO (Fieger and Wong, 2010); however, to date, there

have been no studies investigating a potential role for adenosine and/or adenosine receptor activation to cutaneous active vasodilation.

In view of these findings, the purpose of this study was to determine whether adenosine receptor activation contributes to reflex cutaneous active vasodilation and whether a portion of the NO component of cutaneous active vasodilation might be explained by $A_1/A_2$ adenosine receptor activation. We hypothesized a direct role for adenosine receptor activation would be suggested by an attenuation of the skin blood flow response to heat stress in the presence of the non-selective $A_1/A_2$ receptor antagonist theophylline. In addition, we hypothesized adenosine receptor inhibition combined with NO synthase inhibition would further attenuate this response, which would suggest adenosine receptor activation may also contribute to cutaneous active vasodilation through the production of NO.

## Methods

### *Ethical Approval*

Subjects were recruited from the Kansas State University student population and written informed consent was obtained from each subject prior to participation. This study was approved by the Institutional Review Board of Kansas State University and conformed to the guidelines as set forth by the Declaration of Helsinki.

### *Subjects*

Seven subjects (6 men, 1 woman; ages 21-28) participated in this study. All subjects were healthy, nonsmokers, free of cardiovascular disease and diabetes, and were not taking any medications, including oral contraceptives. All subjects were asked to refrain from caffeine, alcohol, and exercise for 12 hours prior to the study.

### *Instrumentation and Measurements*

All experiments were performed in a thermoneutral laboratory. Skin blood flow measurements were made from the lateral aspect of the left forearm and subjects rested supine with the experimental arm at heart level for the entire protocol. Blood pressure was monitored beat-by-beat via photoplethysmography (NexfinHD; BMEYE, Amsterdam, The Netherlands), and verified via automated brachial auscultation (S/5 Light Monitor; Datex-Ohmeda, GE Healthcare; Madison, WI, USA) every 10 minutes.

Four microdialysis fibers were placed into the dermal layer of the skin of the left ventral forearm. Fibers were placed in the absence of anesthetics; however, ice was used to numb the skin prior to placement (Hodges et al. 2009). Fibers were placed approximately 3-5 cm apart. Fiber placement was accomplished by first threading a 23-gauge needle through the skin at each desired site of microdialysis placement. A fiber was threaded through the lumen of the needle, and the needle was removed, leaving the membrane in place. The membranes of the microdialysis fibers were 10 mm in length with a 55-kDa molecular mass cutoff (CMA 31 Linear Probe; CMA Microdialysis, Sweden). Approximately 1.5-2 hours were allowed for resolution of the trauma response induced by microdialysis fiber placement in the skin before the start of the protocol. During trauma resolution, all fibers were perfused with lactated Ringer's solution at a rate of 4 μl/min. Skin blood flow was recorded as red blood cell (RBC) flux, measured by laser-Doppler flowmetry (PeriFlux 5010 laser-Doppler perfusion monitor; Perimed; Jarfalla, Sweden). Local heating units (PF5020 local heating units and PeriFlux 5020 Temperature Unit; Perimed; Jarfalla, Sweden) were placed on the skin directly over each microdialysis membrane, and an integrated laser-Doppler probe (Probe 413; Perimed; Jarfalla, Sweden) was placed in the center of each local heating unit to measure RBC flux directly over each microdialysis site.

Subjects wore a water-perfused suit (Allen Vanguard; Ottawa, ON, Canada) to control whole-body temperature, which covered the entire body except the head, hands, feet and experimental forearm. Oral temperature was measured by placing a thermistor in the sublingual sulcus and used as an index of core temperature. Subjects' oral temperature was monitored for 5-10 minutes prior to, and for the duration of, the whole body heating period. Thermoneutral water (33°C) was perfused through the suit during the trauma resolution period following microdialysis placement, and during the baseline data collection and drug infusion periods. During the whole-body heating period, subjects were covered with a water-impermeable rain suit to minimize evaporative heat loss, and hot water (50°C) was perfused through the suit to raise subjects' oral temperature at least 0.8°C above baseline.

### *Experimental Protocol*

Following the trauma resolution period, baseline data were collected for 10 min with thermoneutral water pumped through the suit to maintain subjects' core temperature. After baseline measurements, drug infusion through each microdialysis fiber was initiated, and each site was randomly assigned one of four treatments: 1) lactated Ringer's to serve as a control; 2) 4mM theophylline (Tocris Biosciences; Ellisville, MO, USA), a non-selective, competitive $A_1/A_2$ adenosine-receptor antagonist; 3) 10mM of the L-arginine analog *NG*-nitro-L-arginine methyl ester (L-NAME; EMD Biosciences; San Diego, CA, USA) to inhibit NOS; and 4) combined 4 mM theophylline and 10 mM-L-NAME, to simultaneously block adenosine-receptors and NO synthase, and to determine a potential interaction between adenosine receptors and NO. Data from our laboratory demonstrated that 4 mM theophylline was the highest dose that could attenuate the local heating response without eliciting vasodilation (Fieger and Wong, 2010). A 10 mM concentration of L-NAME has been shown previously to adequately inhibit NO

synthase in human skin (Fieger and Wong, 2010; McCord et al. 2006; Minson et al. 2001; Wilkins et al. 2003; Wong and Minson, 2006; Wong et al. 2004) . All treatments were perfused at a constant rate of 4μl/min with a microinfusion pump (Bee Hive controller and Baby Bee Syringe Pumps; Bioanalytical Systems, West Lafayette, IN, USA). Drug infusion began 45 minutes prior to whole body heating and continued through the duration of the heating period. We previously demonstrated that at least 45 minutes of theophylline infusion was required for maximal effect on adenosine receptors (Fieger and Wong, 2010). Previous studies have also demonstrated this duration of L-NAME infusion adequately inhibits NO synthase (Fieger and Wong, 2010; McCord et al. 2006; Minson et al. 2001; Wilkins et al. 2003; Wong and Minson, 2006; Wong et al. 2004).

Following 45 minutes of drug infusion, 50°C water was circulated through the suit to begin the whole body heating period. The heating period raised subjects' oral temperature at least 0.8°C and was 35-50 minutes in duration. When a 0.8°C rise in $T_{or}$ had been achieved, subject's temperature was maintained at this level to acquire a stable 10 min plateau of skin blood flow. Once a stable 10 min period of skin blood flow was achieved, subjects were cooled by perfusing 33°C water through the suit and removing the plastic rain suit. A maximal skin blood flow response was elicited via infusion of 28 mM sodium nitroprusside (SNP) at a rate of 4 μl/min and a simultaneous temperature increase to 43°C at a rate of 1°C/10s (equivalent to 0.5°C/5 s; (Fieger and Wong, 2010; Minson et al. 2001) This temperature increase and dose of SNP have been previously determined effective in eliciting a maximal skin blood flow response (Minson et al. 2001).

### *Data Collection and Analysis*

Data were acquired, digitized, and stored at 100 Hz (Windaq; Dataq Instruments; Akron, OH, USA) on a personal computer. Data were analyzed offline using signal-processing software (Windaq). Skin blood flow data were converted to cutaneous vascular conductance (CVC), calculated as the ratio of skin blood flow to mean arterial pressure (RBC flux/mean arterial pressure). The CVC data were expressed as a percentage of maximal vasodilation (%CVCmax) via SNP infusion and local heating to 43°C. A stable 5 min period of skin blood flow was used for analysis of baseline, whole body heating plateau, and maximal skin blood flow. To determine the magnitude of increase in CVC for a given increase in oral temperature, skin blood flow during the final minute of each 0.1°C increase in oral temperature from baseline ($\Delta T_{or}$ 0.0°C) to the end of heat stress ($\Delta T_{or}$ 0.8°C) was used for analysis. The percent contribution of $A_1/A_2$ receptor activation, NO, and combined $A_1/A_2$ receptor activation + NO were calculated as:

[(%CVC max control – %CVC max treatment site) ÷%CVCmax control)*100]

where "treatment site" is theophylline, L-NAME, or combined theophylline + L-NAME.

Data from all subjects were averaged for statistical analysis as there was only one female subject who participated in this study. For each experimental site, a paired t-test was used to compare pre-drug infusion and post-drug infusion (before heating) baseline values. A one way repeated measures ANOVA was used to compare the effect of drug treatment (i.e., post-drug infusion baseline) between experimental sites. The effect of drug treatment on the increase in CVC during hyperthermia was compared with the use of a one-way ANOVA with repeated measures. A two-way ANOVA with repeated measures was used to compare the effect of drug treatment on the increase in CVC for each 0.1°C rise in oral temperature (drug treatment x CVC

x $\Delta T_{or}$). Percent contribution for each treatment site was compared using a one-way ANOVA with repeated measures. Maximal CVC values for each site were compared using a one-way ANOVA. For all ANOVAs, Student-Newman-Keuls post hoc analysis was used to determine where significance differences occurred. All statistical analyses were performed using SigmaStat 3.5 (Systat Software; Point Richmond, CA, USA). P-values < 0.05 were considered to be significant and all data presented are mean ± SEM.

## Results

The administration of theophylline, L-NAME, or theophylline + L-NAME did not alter baseline skin blood flow values. That is, there was no significant difference between pre-infusion and post-infusion (before heating) baseline values within any of the treatment sites (Table 5-1). Although there was a tendency for baseline CVC to be elevated in the theophylline sites, there was no significant difference in post-drug infusion baseline values between treatment sites (Figure 5-1). Maximal absolute CVC responses did not differ between treatment sites (Table 5-2).

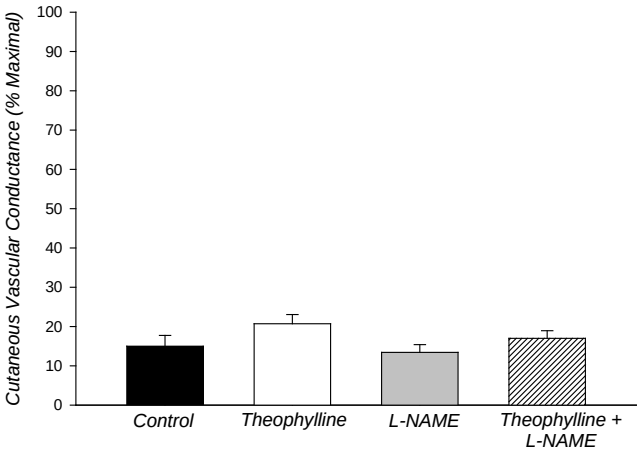


**Figure 5-1: Effect of drug treatment on baseline skin blood flow.** Although there was a tendency for a higher baseline in theophylline sites, this did not reach significance versus all other treatment sites. In all, post-drug infusion baseline was not significantly different between sites. Theophylline, $A_1/A_2$ adenosine receptor inhibition; L-NAME, NO synthase inhibition.

Figure 5-2 depicts the effect of drug treatment on the CVC response to whole body heat stress with a 0.8°C increase in oral temperature. In the control sites, CVC increased during whole body heating to 63 ± 4%CVCmax. CVC was significantly reduced in L-NAME sites (48 ± 2%CVC max; $p < 0.05$) compared with control sites. There was no effect of theophylline on the CVC response to heat stress (61 ± 5%CVC max) compared to the control sites; however, theophylline plus L-NAME sites were significantly reduced (39 ± 3%CVC max) compared to control sites ($p < 0.001$) and compared to L-NAME only sites ($p < 0.05$).

| | Pre-Infusion | Post-Infusion | P Value |
|---|---|---|---|
| Control | 14 ± 3 | 15 ± 3 | 0.4 |
| Theophylline | 17 ± 6 | 22 ± 8 | 0.1 |
| L-NAME | 15 ± 5 | 13 ± 3 | 0.5 |
| Theophylline + L-NAME | 17 ± 4 | 17 ± 2 | 0.9 |

Values are mean ± SEM. There was no significant difference between pre-drug infusion and post-drug infusion baseline values. Theophylline: $A_1/A_2$ adenosine receptor inhibition; L-NAME: NO synthase inhibition.

**Table 5-1: Pre- and Post- Drug Infusion Baseline Values.**

| | Maximal CVC |
|---|---|
| Control | 2.11 ± 0.7 |
| Theophylline | 2.16 ± 0.5 |
| L-NAME | 1.9 ± 0.7 |
| Theophylline + L-NAME | 2.5 ± 0.8 |

Values are mean ± SEM. There was no significant difference in maximal CVC values between sites.

**Table 5-2: Absolute Maximal CVC Values.**

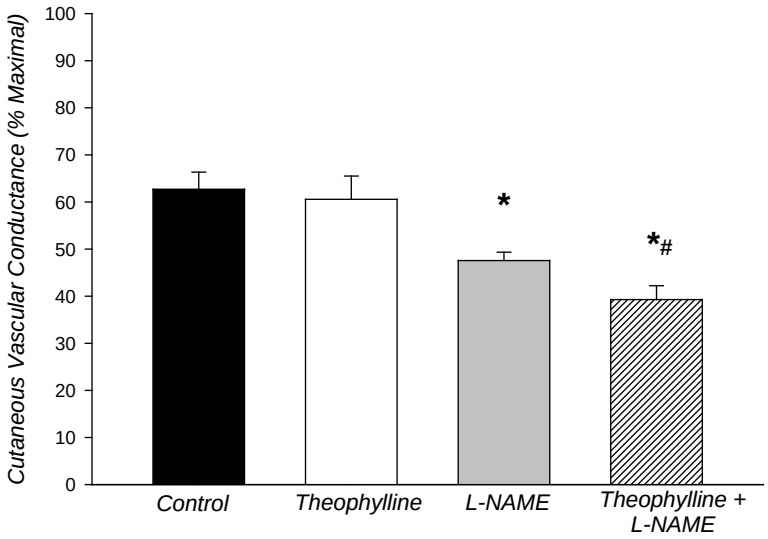


**Figure 5-2: Effect of A1/A2 adenosine receptor inhibition and NO synthase inhibition on the skin blood flow response to whole body heat stress.** Inhibition of $A_1/A_2$ receptors with theophylline (open bars) had no effect on cutaneous active vasodilation compared to control (black bars). Inhibition of NO synthase (L-NAME; gray bars) and combined $A_1/A_2$ + NO synthase inhibition (theophylline + L-NAME; hatched bars) attenuated active vasodilation compared to both control and theophylline. Values are mean ± SEM. *, $p < 0.05$ vs. control; #, $p < 0.05$ vs. L-NAME.

Figure 5-3 (below) depicts the increase in CVC in each of the treatment sites as a function of increasing oral temperature (CVC vs. $\Delta T_{or}$). In control (thick solid lines; filled circles) and theophylline (thick dashed lines; open circles) sites, CVC began to increase with a 0.2°C increase in oral temperature (*, $p < 0.05$ vs. baseline, $\Delta T_{or} = 0.0$). In contrast, CVC in L-NAME (thin solid lines; filled squares) and L-NAME + theophylline (thin dashed lines; open squares) were not significantly different from baseline ($\Delta T_{or} = 0.0$) until a 0.4°C increase in oral temperature was achieved. The L-NAME and L-NAME + theophylline sites were significantly attenuated compared to control and theophylline only sites at all increments of $\Delta T_{or} \geq 0.2$°C. Lastly, L-NAME + theophylline sites were significantly reduced at all increments of $\Delta T_{or} \geq 0.5$°C compared to L-NAME only sites.

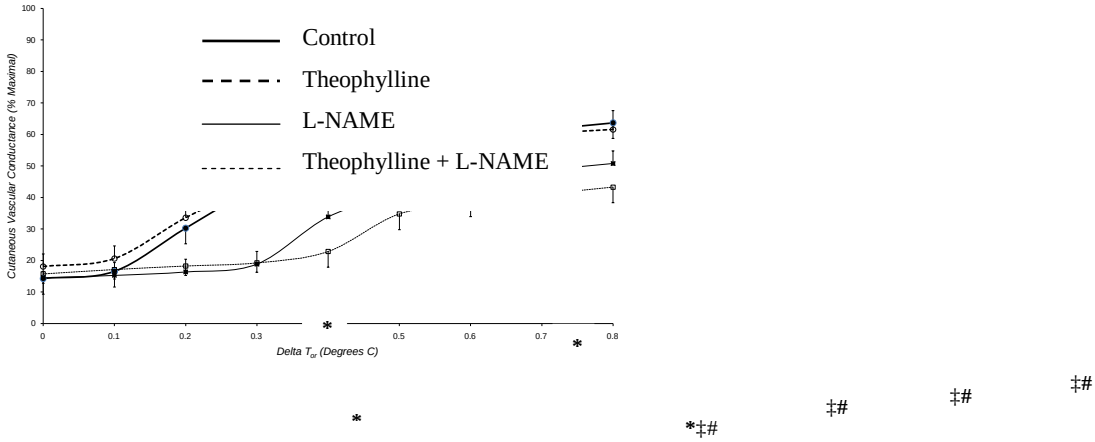


**Figure 5-3: Increase in CVC during whole body heating as a function of increasing oral temperature.** The rise in CVC during heat stress was plotted against increasing oral temperature in 0.1°C increments. There was no difference between control and theophylline sites in terms of the magnitude of increase in CVC or in terms of the onset of vasodilation. The magnitude of increase in CVC during hyperthermia was significantly attenuated in L-NAME and combined theophylline + L-NAME sites compared to both control and theophylline only sites. Similarly, the onset for vasodilation L-NAME and combined L-NAME sites was shifted to a higher oral temperature compared to control and theophylline only sites. Values at each 0.1°C increment in oral temperature are mean ± SEM. *, $p < 0.05$ vs. baseline (i.e., $\Delta T_{or} = 0.0°C$); ‡, $p < 0.05$ vs. control and theophylline only sites; #, $p < 0.05$ vs. L-NAME only sites.

The percent contribution of $A_1/A_2$ adenosine receptors, NO, and combined $A_1/A_2$ adenosine receptors + NO to cutaneous active vasodilation is shown in Figure 5-4. The percent direct contribution of $A_1/A_2$ receptor activation (1.0 ± 1%) was significantly less than the percent contribution of NO (32± 2%) and combined NO + $A_1/A_2$ receptor activation (53 ± 2%; $p < 0.001$ for both conditions) to the plateau in CVC during hyperthermia. In addition, the percent contribution of NO was significantly less than the percent contribution from combined NO + $A_1/A_2$ receptor activation ($p < 0.001$).

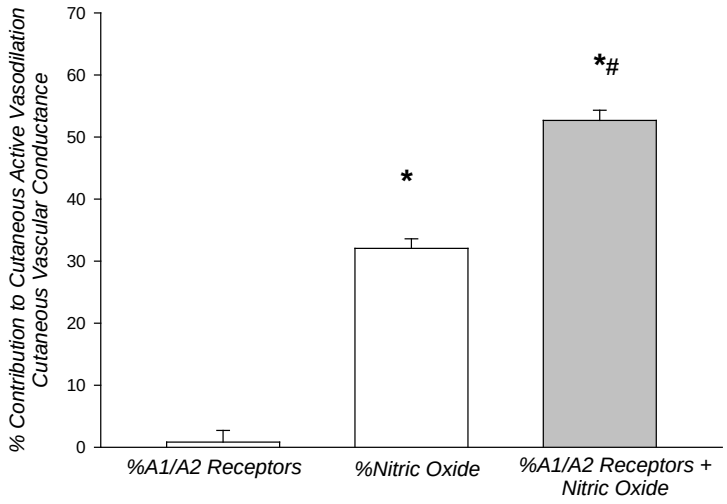


**Figure 5-4: Percent contribution of A1/A2 receptor activation, NO, and combined A1/A2 receptor activation + NO to cutaneous active vasodilation.** $A_1/A_2$ adenosine receptor activation made little to no direct contribution to cutaneous active vasodilation. The contribution of NO was ~30% while the combined contribution of $A_1/A_2$ adenosine receptor activation + NO was ~50%. Values are mean ± SEM. *, $p < 0.05$ vs. $\%A_1/A_2$ receptor activation; #, $p < 0.05$ vs. % Nitric oxide.

## Discussion

The aim of this study was to determine a potential role for $A_1/A_2$ adenosine receptor activation to reflex cutaneous active vasodilation. Specifically, this study was designed to test the hypotheses that 1) adenosine receptor activation directly contributes to cutaneous active vasodilation; and 2) adenosine receptor activation contributes to a portion of the known NO component of this response to heat stress. In contrast to our first hypothesis, we found adenosine receptor inhibition with theophylline did not attenuate the skin blood flow response to whole body heating, suggesting no direct contribution of adenosine receptor activation to reflex cutaneous active vasodilation. A role for adenosine receptors became evident, however, when $A_1/A_2$ receptor inhibition was combined with NO synthase inhibition. That is, inhibition of NO synthase with L-NAME appeared to unmask a role for adenosine receptor activation that was not observed when only adenosine receptors were blocked with theophylline, suggesting any direct contribution of adenosine receptor activation to cutaneous active vasodilation is modest, at best,

and masked by the vasodilator actions of NO and, presumably, other vasodilators (Figure 5-4). Inhibition of adenosine receptor activation did not alter the core temperature threshold for the onset of cutaneous active vasodilation compared to control sites (Figure 5-3). Taken together, these data suggest there is no direct role for adenosine receptor activation contributing to either the onset or magnitude of cutaneous active vasodilation.

In support of our second hypothesis, our data suggest adenosine receptor activation mediates a portion of the known NO component of cutaneous active vasodilation. This was evidenced by the further reduction in skin blood flow in sites treated with theophylline + L-NAME compared to sites treated with L-NAME only (Figures 5-2, 5-3, and 5-4). Inasmuch as the combined effect of L-NAME and theophylline was found to diminish but not abolish the skin blood flow response to heat stress, these data further suggest a substantial portion of the NO component cannot be explained by adenosine receptor activation. In addition, the onset for cutaneous active vasodilation was shifted to a higher oral temperature in theophylline + L-NAME sites ($\Delta T_{or}$ 0.5°C) compared to control and theophylline sites ($\Delta T_{or}$ 0.2°C) as well as compared to L-NAME only sites ($\Delta T_{or}$ 0.4°C). The observation that combined theophylline + L-NAME shifted the onset of cutaneous active vasodilation to a higher oral temperature compared to L-NAME only sites suggests not only that adenosine receptor activation may mediate a portion of the increase in NO but also that adenosine receptor activation may be partially responsible for the increase in NO during the early stages of hyperthermia.

The combined effects of theophylline and L-NAME in this study indicate adenosine receptors are activated during whole body heat stress to induce NO production and subsequent vasodilation, which is consistent with previous studies that have demonstrated $A_1/A_2$ receptors can induce vasodilation through the production of NO. By one proposed mechanism, $A_1/A_2$

receptor activation may increase NO synthesis through changes in calcium or potassium fluxes (Bryan and Marshall, 1999; Ishibashi, 1998). Adenosine receptors may also be coupled to ATP-sensitive potassium channels ($K^+_{ATP}$) and have been found to induce NO and vasodilation through their actions on these channels (Bryan and Marshall, 1999; Danialou et al. 1997; Hein and Kuo, 1999; Marshall et al. 1993; Mubagwa and Flameng, 2001); however, in the context of cutaneous active vasodilation, a role for $K^+_{ATP}$ channels has not yet been established.

An additional mechanism by which adenosine receptors may contribute to the NO component of reflex vasodilation may include an interaction between adenosine, NO, and prostaglandins. Previous studies indicate adenosine may cause vasodilation, at least in part, by stimulating release of both NO and prostaglandins from the vascular endothelium, where the release of NO caused by the action of adenosine receptors may be prostaglandin dependent (Danialou et al. 1998; Mortensen et al. 2009; Ray and Marshall, 2009). In the context of cutaneous active vasodilation, McCord et al. (2006) found that the cylooxygenase pathway, and presumably COX-derived prostaglandins, contributes to cutaneous active vasodilation; however, this cyclooxygenase contribution was found to be independent of NO. Nevertheless, it is possible there is an interaction between prostaglandins and adenosine.

There is further evidence to suggest an interaction between histamine receptors and purinergic ($A_1/A_2$) receptors (Dickensen and Hill, 1994) and between $NK_1$ receptors and purinergic receptors (Burnstock, 2009; Ralevic, 2009). Inasmuch as Wong and colleagues (2004) and Wong and Minson (2006) have demonstrated that $H_1$ histamine receptor activation and $NK_1$ receptor activation, respectively, contributes to cutaneous active vasodilation, it is possible the effects of adenosine and adenosine receptor activation during hyperthermia are

being manifest through the $H_1$ and $NK_1$ receptor activation components; however, these potential interactions awaits further investigation.

### *Limitations*

There are at least three limitations to this study that need to be addressed. First, as we observed no effect of theophylline on cutaneous active vasodilation, it is possible theophylline is either ineffective at inhibiting $A_1/A_2$ receptors or our concentration of theophylline was inadequate. We have previously demonstrated that during local heating of the skin, adenosine receptor activation contributes to the skin blood flow response both directly and through NO (Fieger and Wong, 2010). The cutaneous vasodilation elicited via local heating is, on average, greater in magnitude than that achieved with whole body heat stress. In our previous study we used a 4 mM concentration, as this was the highest possible concentration that could be used without eliciting non-specific vasodilation. The non-specific vasodilation of theophylline, or the similar antagonist, aminophylline, is not exclusive to human skin (Casey et al. 2009) and is most likely due to the phosphodiesterase inhibitor properties of these compounds (Taddei et al. 1991). Thus, our previous data demonstrating an attenuated cutaneous vascular response to local heating with 4 mM theophylline would argue against the notion theophylline is ineffective at inhibiting $A_1/A_2$ receptors and/or that the concentration used was inadequate.

Second, we did not directly measure adenosine and, as such, our data do not allow us to speak directly as to whether adenosine *per se* is responsible for the observed $A_1/A_2$ component when NOS is inhibited. Shibasaki et al. (2007) and Wingo et al. (2010) have both demonstrated 2.8 mM adenosine infused into the cutaneous interstitial space via microdialysis elicits vasodilation to an extent similar to that achieved during whole body heat stress, suggesting adenosine, at relatively low concentrations, is vasoactive in human skin. Several studies have

demonstrated an increase in skeletal muscle adenosine concentration during exercise (Frandsen et al. 2000; Hellsten et al. 1998; Mortensen et al. 2009); however, to our knowledge there have been no studies measuring changes in interstitial adenosine concentration from human skin. Although theoretically possible, a potential technical limitation to recovering and measuring adenosine from human cutaneous interstitial fluid is the reported half-life of adenosine which is approximately <1 second (Moser et al. 1989). Inasmuch as peak CVC during heat stress requires, on average, approximately 45 minutes in most subjects, it is highly probably that any adenosine produced during heat stress would be degraded during recovery of cutaneous interstitial dialysate.

Third, theophylline is a competitive but non-selective inhibitor of $A_1$ and $A_2$ adenosine receptors; this does not allow us to determine the specific contribution of the various isoforms of adenosine receptors to cutaneous active vasodilation. Both $A_1$ and $A_2$ adenosine receptors have been localized in human skin (Stojanov and Proctor, 1989) and both isoforms have been shown to elicit vasodilation (Bryan and Marshall, 1999). Further, both $A_1$ and $A_2$ isoforms have several subtypes (e.g., $A_{2B}$ subtype). It is difficult to determine and assign specific roles to each isoform and subtype as the distribution and mechanism of action is heterogeneous. For example, vasodilation in skeletal muscle during resting, hypoxic conditions has been shown to be due to $A_1$ receptor activation (Bryan and Marshall, 1999a,b) whereas $A_{2A}$ receptors are responsible for skeletal muscle vasodilation during tetanic contractions (Ray and Marshall, 2009). In addition, Ansari et al. (2007) recently observed that adenosine-mediated vasodilation in the aorta is due to $A_{2B}$ receptor activation working, in part, through NO. Further complicating the matter is that selective adenosine receptor antagonists approved for human use are lacking. As such, we chose to use a competitive, but non-specific, $A_1/A_2$ adenosine receptor antagonist.

Despite the aforementioned limitations, our data suggest a role for $A_1/A_2$ adenosine receptor activation when NOS is inhibited. Clearly, future research using isoform-specific adenosine receptor antagonists combined with recovery and measurement of interstitial adenosine during whole body heat stress is warranted.

### *Conclusion*

Data from this study suggest the primary contribution of $A_1/A_2$ adenosine receptor activation to cutaneous active vasodilation is to increase the production of NO. Our data further suggest that in the presence of NO, $A_1/A_2$ receptor activation does not directly contribute to cutaneous active vasodilation; however, a role for $A_1/A_2$ receptor activation was unmasked when NO synthase was inhibited. Inhibition of adenosine receptors combined with NO synthase inhibition results in a significant attenuation in skin blood flow that is greater than the effect of NO synthase inhibition alone, suggesting $A_1/A_2$ adenosine receptor activation may be responsible for a portion of the known NO component of cutaneous active vasodilation.

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# Appendix A - Informed Consent Form: Mechanisms of Cutaneous Thermal Hyperemia in Humans

This is an important document. Please read it carefully. This form explains what you need to know about this study. If you agree to take part in this research study, you need to sign this form. Your signature indicates you have been told about the study and the risks of participating in this study have been explained to you. Your signature on this form also indicates that you want to take part in this study.

Project Title: Mechanisms of Cutaneous Thermal and Reactive Hyperemia in Humans

Primary Investigator: Brett Wong, Ph.D.

Approved by Institutional Review Board (IRB): June 29, 2009

Expiration Date of IRB Approval: June 29, 2011

Protocol Number: 5092

Sponsor of Project: Kansas State University

## Purpose of This Research Project:

The purpose of the research described in this form is twofold. First, we seek to better understand how humans increase blood flow to the skin when mild heat is applied to a small area of skin. When mild heat is applied to the skin there is an increase in skin blood flow that is termed “thermal hyperemia”. The increase in blood flow is a protective mechanism. The blood flow “picks up” the heat and moves it away from the area being heated in order to prevent damage to the skin. Application of mild heat to the skin and observing the ensuing blood flow response is frequently used to test the overall health of the blood vessels in a number of populations, such as patients with diabetes and cardiovascular disease. It is also frequently used to test blood vessel health in healthy, older individuals. However, it is still unknown how skin

blood flow increases in response to local heat which minimizes the utility of this response as a clinical tool. The first purpose of our research is to better understand how blood flow increases to the skin when mild heat is applied to the skin.

The second goal of this research is to better understand how blood flow to the skin increases following a period of arterial occlusion. Blood flow to a limb, such as the forearm, can be occluded (stopped) for a period of time by inflating a blood pressure cuff placed on the upper arm. This stoppage is termed an "arterial occlusion." When the cuff pressure is decreased, there is a rapid and large increase in blood flow to the limb that is termed "reactive hyperemia." This response is frequently used to test the overall health of the blood vessels in healthy older humans and in patients with diabetes and cardiovascular disease. However, we do not completely understand what causes the increase in blood flow following an arterial occlusion and this limits the usefulness of reactive hyperemia as a clinical test of blood vessel health. We hope to better understand how blood flow increases to the skin following an arterial occlusion.

## Procedures or Methods To Be Used In The Study:

1. You will arrive at Dr. Wong's laboratory in room 8B Ahearn Hall in the Department of Kinesiology at Kansas State University to participate in the experimental protocol. This testing will take approximately 5 hours to complete. You will complete a brief health history form that will include your age, height, weight, allergies, etc. You will be instrumented with a cuff on your right finger so we can measure your blood pressure and small adhesive patches that will be connected to cables so we can monitor your heart rate (electrocardiogram or ECG). You will then be outfitted with a nylon jacket that has small tubing sewn into it. We will pump water through the suit to control your body temperature (you will not get wet). All subjects have the right to have a member of the same sex collect information on the health

history form and in the laboratory during data collection. For female subjects, Sarah Fieger is the designated female lab personnel and will be contacted upon your request.

2. You will have 4 small tubes called microdialysis fibers placed in the skin of your forearm. These tubes are smaller than the size of a pencil lead. To place the small tubes, we will first numb the area of skin with ice for about 5 minutes. We will then place a small needle just under the surface of your skin in four different locations on your forearm (one needle for each of the 4 small tubes). The small tubes will then be placed inside the needle, the needles will be removed, and the small tubes will be left under your skin for the duration of the experiment.
3. We will need to wait approximately 1-2 hours after the insertion of the small tubes to allow the insertion trauma (redness of your skin around the small tubes) to go away. During this time, we will place a small probe (laser-Doppler probe) over the skin where the small tubes were placed so that we can measure the skin blood flow over the small tubes.
4. During the experiment, we will inflate the cuff around your right finger to measure your blood pressure. If the cuff inflation becomes uncomfortable at any point, let the investigator know and it will be turned off for a few minutes.
5. We will maintain your body temperature at normal levels by pumping thermoneutral (32°C or ~90°F) water through the suit. This is a temperature of water that should maintain your body temperature at normal levels (37°C or ~98.6°F). We want you to remain comfortable (not too hot or too cold) so if the water temperature is too cold or too warm, inform the investigator and the temperature will be adjusted accordingly.
6. You will undergo one of the following experimental procedures (only the one circled):

a. "Slow" Local Heating: Small heaters about the size (diameter) of a quarter will be placed on your forearm skin with double sided tape. The heaters will be set at a temperature of 33°C (~91°F), which is the normal temperature of your skin when your body temperature is ~98.6°F. We will slowly increase the temperature of the heaters from 33°C (91°F) to 42°C (~108°F) at a rate of 1°C every 10 minutes. You may feel a slight warm sensation on your skin but you should not feel any pain associated with the heating. A temperature of 42°C (108°F) is well below the temperature that can become painful (~110°F) as well as the temperature that can burn the skin (~113°F). However, if the heating becomes painful for even a short period of time, inform one of the investigators and the temperature will be lowered or turned off.

b. "Rapid" Local Heating: With this procedure, the small heaters will be heated from 33°C (91°F) to 42°C (108°F) at a rate of 1°C every 10 seconds. This heating protocol may cause a slight stronger warm sensation on your forearm skin but should not cause any sensation of pain. However, if there is even a brief sensation of pain or burning, inform one of the investigators and the temperature will be either lowered or turned off.

c. Reactive Hyperemia: A blood pressure cuff will be placed on your right upper arm. We will stop blood flow to your arm three (3) times for a period of 5 minutes with at least 10 minutes of recovery between each blood flow occlusion.

7. During the experimental procedure, we will place very small doses of drugs through the small tubes in your skin. The drugs will cause the blood vessels in your skin to either open up or become narrow. The drugs have been used safely in humans in previous experiments,

have been approved and sterilized for human use, and will stay localized in the skin (i.e., the microdialysis fiber will prevent the drugs from circulating throughout the body or causing a systemic, whole-body effect). You should not feel anything when the drugs are infused through the small tubes. However, it is possible you may feel a slight tingling sensation in the area. This is normal but you should still inform the investigator.

You will receive the following drugs in your skin (only the ones circled by the investigator):

a. Sodium Nitroprusside: This produces a molecule called nitric oxide and causes the blood vessels in your skin to open. This solution is similar to nitroglycerin tablets that patients with heart disease place under their tongue when they experience chest pain.

b. Lactated Ringer's Solution or Saline Solution: These are solutions that are used to mimic the fluid in your body that surrounds the cells of your skin.

c. Propylene Glycol or Dimethyl Sulfoxide (DMSO): This is a solution that is used to dissolve some of the drugs that will be infused into your skin. This solution is commonly used to dissolve drugs that are given to patients through an i.v. DMSO is used similar to propylene glycol. DMSO is frequently used to treat muscle soreness and joint pain in athletes.

d. L-NAME: This is a drug that will block the production of a molecule called nitric oxide and will cause the blood vessels in your skin to close.

e. Capsazepine or JNJ 17203212: These are synthetic forms of capsaicin, which is the active ingredient in hot chili peppers. Both compounds will block microscopic openings found on sensory nerves in your skin (TRPV-1 receptors).

f. Calcitonin Gene-Related Peptide (CGRP) & CGRP 8-37: CGRP is a peptide (short protein) that is found in the nerve cells in your skin and in other nerves of your body. CGRP will cause your skin vessels to open and increase blood flow. CGRP 8-37 is a

fragment (small piece) of the normal CGRP peptide and is used to block the effect of the normal CGRP peptide.

g. Substance P: This is a peptide (short protein) that is found in the nerve cells in your skin and other places in the body. Substance P will cause your skin vessels to open and blood flow to increase.

h. Glibenclamide & PNU 37883: These are drugs that are often prescribed to diabetic patients and blocks small, microscopic openings (called ATP-sensitive potassium channels, or $K^{+}_{ATP}$ channels) and may cause your skin vessels to narrow and blood flow to decrease.

i. Theophylline & PSB 1115: These compounds will be used to block a molecule called adenosine, which is naturally produced by your body. These compounds may cause your skin vessels to narrow and blood flow to decrease. These compounds are similar to caffeine.

j. Rp-cGMPs: This substance blocks the effects of nitric oxide in your skin and may cause the skin vessels to narrow and blood flow to decrease.

k. Rp-cAMPs: This substance blocks the effects of CGRP and substance P in your skin and may cause your skin blood vessels to narrow and blood flow to decrease.

l. 8-Br-cGMP & 8-Br-cAMP: These compounds act similar to nitric oxide (8-Br-cGMP) and CGRP and substance P (8-Br-cAMP) and both may cause your blood vessels to open and blood flow to increase.

m. MY-5445 & T 0156: These compounds are called “phosphodiesterase inhibitors.” Your body naturally destroys nitric oxide to prevent it from working too long. These compounds prevent your body from breaking down nitric oxide. Sildenafil, or Viagra, is the most well-known form of these compounds. Your skin blood vessels may open and blood flow may increase.

8. We will heat the area of skin over each microdialysis fiber to 43°C (~109°F). This will be done at the same time sodium nitroprusside is infused. Both will be done in order to produce maximal (100%) blood flow in each area of skin. This will allow us to present the data as a percentage of 100%. If the temperature is uncomfortable, inform one of the investigators and the temperature will be turned down or off.
9. At the end of the experiment, we will clean the area of the skin where the small tubes are located, the tubes will be removed, and a bandage will be placed over the area of skin where the tubes were placed.

## Length of Study:

You will be in the study for only one day (about 5 hours).

## Anticipated 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 areas clean for a day or two following the study. Risk is considered minimal.
2. Local Skin Heating: The local skin heaters may cause some minor skin discomfort. The goal is to warm the area of skin to a temperature that has been determined to be below the threshold for pain. If the local heating becomes painful, you should tell the investigator and the temperature of the local heater will be lowered. There is a slight risk of burning the skin

at this sight, so it is important that you tell the investigators of any pain you are feeling. The heating device may be removed at any time if you experience any discomfort. The risks are considered to be minimal.

3. Experiment Drugs: We will be infusing very small doses of each drug, and only into a very small area of your skin. All of the drugs have been approved for use in humans. You will not have any systemic (whole body) effects of these drugs, and they will not alter your blood pressure in the small doses given in this study. There is a minimal risk of an allergic reaction to the prescription drugs.
4. Laser-Doppler Probes: These probes send a small light into your skin. You will not feel anything except the probe touching your skin. There are no major risks associated with this procedure.
5. Blood Pressure Cuff: In some people, inflation of the blood pressure cuff can become uncomfortable. Many times this is because the cuff has been placed around your finger too tightly or because it has been on for a long time. If the inflation of the blood pressure cuff becomes uncomfortable at any time, let the investigator know and they will deflate the cuff and check the placement of the cuff on your finger. There are no major risks associated with this device or procedure.
6. Blood Flow Occlusion: The inflation of the blood pressure cuff to stop blood flow may cause a slight tingling sensation. The sensations with longer occlusions are similar to the sensations when a limb has "fallen asleep." If, at any time, you experience any discomfort you may request that the blood pressure cuff be either loosened or removed. When the cuff is inflated, you may have the pressure released and the cuff deflated at any time should you experience any discomfort. There are no major risks associated with this procedure.

## Anticipated Benefits of This Study:

This study is done to gather information only and will not benefit your health or fitness. However, the information gathered from this study has the potential to help those individuals who are most prone to poor blood vessel health, such as the elderly and those with chronic diseases (diabetes, cardiovascular disease).

## Cost of Tests and Procedures:

You will not need to pay for any tests or procedures that are done for this research study.

You will be paid at a rate of $10.00 per hour spent in the 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, you will get part of this money.

## Your Rights If You Decide To 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 Kansas State University.

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. Any complications or adverse reactions will be immediately reported to the University Research Compliance Office at Kansas State University.

Kansas State University is not able to neither offer financial compensation nor absorb the costs of medical treatment should you be injured as a result of participation in this research. If such complications arise, the researchers will assist you in obtaining appropriate medical treatment, which will be provided at the usual charge.

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. You will be told of important new findings or any changes in the study or procedures that may happen.

## Extent of 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. Wong's office. All files with subject names and identification numbers will be destroyed after all data has been collected and analyzed and for a period of one year after the results from the study have been published.

Your social security number (SSN) and mailing will be collected so that you may be paid. Your SSN and mailing address is required by Kansas State University to mail you a check for your participation in this research. Your SSN and mailing address will be kept locked in a file cabinet until a check has been mailed to you and once it has been verified that you have cashed or deposited the check. Once the check has been mailed and cashed/deposited, your SSN and mailing address will be destroyed (shredded).

## Terms of Participation:

I understand this project is research and that my participation is completely voluntary. I also understand that if I decide to participate in this study, I may withdraw my consent at any time, and stop participating at any time without explanation, penalty, or loss of benefits, or academic standing to which I may otherwise be entitled.

I verify that my signature below indicates that I have read and understand this consent form, and willingly agree to participate in this study under the terms described, and that my signature acknowledges that I have received a signed and dated copy of this consent form.

Participant Name (Please Print): _____

Signature of Participant: _____ Date: _____

Signature of Project Staff: _____ Date: _____

# Appendix B - Informed Consent Form: Mechanisms of Cutaneous Active Vasodilation

This is an important document. Please read it carefully. This form explains what you need to know about this study. If you agree to take part in this research study, you need to sign this form. Your signature indicates you have been told about the study and the risks of participating in this study have been explained to you. Your signature on this form also indicates that you want to take part in this study.

Project Title: Sensory Nerves and Histamine Receptors in Cutaneous Active Vasodilation in Humans

Primary Investigator: Brett Wong, Ph.D.

Approved by Institutional Review Board (IRB): 20 January 2009

29 June 2009 (revised protocol approved)

Expiration Date of IRB Approval: 26 February 2012

Protocol Number: 4547.0

Sponsor of Project: Kansas State University

## Purpose of This Research Project:

The purpose of the research described in this form is to better understand how humans increase blood flow to the skin during periods of increased environmental temperatures. When humans are exposed to high environmental temperatures, our internal (core) body temperature also increases. The primary way in which humans defend against large increases in core body temperature is to increase blood flow to the skin and to sweat. The increase in blood flow to the skin will move warm blood to the skin where the heat can be released by evaporation of sweat.

Although it is clear that increasing skin blood flow during heat stress is important, we do not fully understand how humans increase blood flow to the skin.

In order to better understand these problems, we will address the following questions in this study:

- Do transient receptor potential voltage type 1 (TRPV-1) receptors contribute to the rise in skin blood flow during heat stress? (TRPV-1 receptors are small, microscopic openings located on the nerves in the skin.)
- Does histamine (a substance released from the cells in your skin and involved in seasonal allergies) contribute to the rise in skin blood flow during heat stress and in response to locally applied heat?
- Do sensory nerves in the skin (the nerves that sense itch, heat, cold, etc) contribute to the increase in skin blood flow during heat stress?

## Procedures or Methods To Be Used In The Study:

You will arrive at Dr. Wong's laboratory in room 8B Ahearn Hall in the Department of Kinesiology at Kansas State University to participate in the experimental protocol. This testing will take approximately 5 hours to complete. You will complete a brief health history form that will include your age, height, weight, allergies, etc. You will be instrumented with a cuff on your right arm so we can measure your blood pressure and small adhesive patches that will be connected to cables so we can monitor your heart rate (electrocardiogram or ECG). You will then be outfitted with a nylon suit that has small tubing sewn into it. We will pump water through the suit to control your body temperature (you will not get wet). All subjects have the right to have a member of the same sex collect information on the health history form and in the

laboratory during data collection. For female subjects, Ms. Sarah Fieger is the designated female lab personnel and will be contacted upon your request.

1. You will have 4 small tubes called microdialysis fibers placed in the skin of your forearm. These tubes are smaller than the size of a pencil lead. To place the small tubes, we will first numb the area of skin with ice for about 5 minutes. We will then place a small needle just under the surface of your skin in four different locations on your forearm (one needle for each of the 4 small tubes). The small tubes will then be placed inside the needle, the needles will be removed, and the small tubes will be left under your skin for the duration of the experiment.
2. We will need to wait approximately 1-2 hours after the insertion of the small tubes to allow the insertion trauma (redness of your skin around the small tubes) to go away. During this time, we will place a small probe (laser-Doppler probe) over the skin where the small tubes were placed so that we can measure the skin blood flow over the small tubes.
3. During the experiment, we will periodically inflate the cuff around your right arm to measure your blood pressure. This cuff will only be inflated for about 30 seconds. However, if the cuff inflation becomes uncomfortable at any point, let the investigator know and it will be turned off.
4. We will warm your body by pumping warm water through the nylon suit you are wearing until your body temperature is increased from about 98.6°F (normal body temperature) to about 101°F. It will take about 50-70 minutes to increase your body temperature to 101°F. To heat your body faster, we will cover your body with a plastic rain suit. During the heating period, we will monitor your heart rate (ECG) and your body temperature and we will periodically ask you how you are feeling. A small temperature-sensing wire will be placed

under your tongue to measure your body temperature during the experiment. At the end of the heating period, we will remove the plastic rain suit and pump cool water through the nylon suit you are wearing until your body temperature returns to normal.

5. We will heat a small area of your skin with a small heater to 43°C (about 107°F) to maximally open the blood vessels in your skin. This temperature is below the temperature where heating becomes painful (about 110°F) and well below the temperature that may burn your skin (about 113°F). However, if the heater is becoming painful, inform the investigator and the temperature will be lowered or the heater turned off.
6. During the experiment, we will place very small doses of drugs through the small tubes in your skin. The drugs have been used safely in humans in previous experiments, have been approved and sterilized for human use, and will stay localized in the skin (i.e., the microdialysis fiber will prevent the drugs from circulating throughout the body or causing a systemic, whole-body effect). The drugs will cause the blood vessels in your skin to either open up or become narrow. You should not feel anything when the drugs are infused through the small tubes. However, it is possible you may feel a slight tingling sensation in the area. This is normal but you should still inform the investigator.

    You will receive the following drugs in your skin (only the ones circled by the investigator):

    a. <u>EMLA Cream:</u> This is an anesthetic cream that is used to numb a small area of your skin. It will be placed on the surface of the skin and covered with a sterile bandage for a period of 1 hour. After one hour, the bandage will be removed, the cream wiped off, and a new application of the cream will be placed on your skin

for an additional 1 hour. This cream is frequently used in clinics and hospitals for procedures such as i.v. placement.

b. Histamine: This is a molecule that is found in cells in your body and is involved in seasonal allergies (runny nose, itchy eyes and skin associated with "hay fever"). This will cause the vessels in your skin to open.

c. Ciproxifan, Clobenpropit, or Thiperamide: These are drugs that block a form of the histamine receptor ($H_3$). Histamine receptors are involved in seasonal allergies ("hay fever"). Blocking the $H_3$ receptor, which is a microscopic opening located on nerves in your skin, may cause your skin blood vessels to narrow.

d. Pyrilamine: This is a drug that blocks the effects of histamine. It will prevent the vessels of your skin from opening when histamine is present.

e. Sodium Nitroprusside: This produces a molecule called nitric oxide and causes the blood vessels in your skin to open.

f. Lactated Ringer's Solution: This is a solution that is used to mimic the fluid in your body that surrounds the cells of your skin.

g. L-NAME: This is a drug that will block the production of a molecule called nitric oxide and will cause the blood vessels in your skin to close.

h. Capsazepine or JNJ 17203212: These are synthetic forms of capsaicin, which is the active ingredient in hot chili peppers. Both compounds will block microscopic openings found on sensory nerves in your skin (TRPV-1 receptors).

i. Imetit: This will activate the H3 histamine receptor and may cause your skin vessels to open.

j. Yohimbine: This will block small openings on the vessels of your skin and may cause them to close.

k. Propylene Glycol or Dimethyl Sulfoxide (DMSO): This is a solution that is used to dissolve some of the drugs that will be infused into your skin. This solution is commonly used to dissolve drugs that are given to patients through an i.v. DMSO is used similar to propylene glycol. DMSO is frequently used to treat muscle soreness and joint pain in athletes.

l. Glibenclamide & PNU 37883: These are drugs that are often prescribed to diabetic patients and blocks small, microscopic openings (called ATP-sensitive potassium channels, or $K^{+}_{ATP}$ channels) and may cause your skin vessels to narrow and blood flow to decrease.

m. Theophylline & PSB 1115: These compounds will be used to block a molecule called adenosine, which is naturally produced by your body. These compounds may cause your skin vessels to narrow and blood flow to decrease. These compounds are similar to caffeine.

n. Rp-cGMPs: This substance blocks the effects of nitric oxide in your skin and may cause the skin vessels to narrow and blood flow to decrease.

o. Rp-cAMPs: This substance blocks the effects of CGRP and substance P in your skin and may cause your skin blood vessels to narrow and blood flow to decrease.

p. 8-Br-cGMP & 8-Br-cAMP: These compounds act similar to nitric oxide (8-Br-cGMP) and CGRP and substance P (8-Br-cAMP) and both may cause your blood vessels to open and blood flow to increase.

q. MY-5445 & T 0156: These compounds are called “phosphodiesterase inhibitors.” Your body naturally destroys nitric oxide to prevent it from working too long. These compounds prevent your body from breaking down nitric oxide. Sildenafil, or Viagra, is the most well-known form of these compounds. Your skin blood vessels may open and blood flow may increase.

7. At the end of the experiment, we will clean the area of the skin where the small tubes are located, the tubes will be removed, and a bandage will be placed over the area of skin where the tubes were placed.

## Length of Study:

You will be in the study for only one day (about 5 hours).

## Anticipated 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 areas clean for a day or two following the study.
2. Whole Body Heating***:*** The heat exposure can be physically demanding and can cause light-headedness, low blood pressure, fatigue, nausea, or cramps. Therefore, you should keep the investigator informed of your feelings and you should not attempt to prolong the heating experiment if you do not feel well. You are free to stop at any time.
3. Local Skin Heating***:*** The local skin heaters may cause some minor skin discomfort. The goal is to warm the area of skin to a temperature that has been determined to be below the threshold for pain. If the local heating becomes painful, you should tell the investigator and the temperature of the local heater will be lowered. There is a slight risk of burning the skin

at this sight, so it is important that you tell the investigators of any pain you are feeling. The heating device may be removed at any time if you experience any discomfort.

4. Experiment Drugs***:*** We will be infusing very small doses of each drug, and only into a very small area of your skin. All of the drugs have been approved for use in humans. You will not have any systemic (whole body) effects of these drugs, and they will not alter your blood pressure in the small doses given in this study. There is a minimal risk of an allergic reaction to the prescription drugs.
5. Laser-Doppler Probes***:*** These probes send a small light into your skin. You will not feel anything except the probe touching your skin. There are no major risks associated with this procedure.
6. Blood Pressure Cuff***:*** In some people, inflation of the blood pressure cuff can become uncomfortable. Many times this is because the cuff has been placed around your arm too tightly. If the inflation of the blood pressure cuff becomes uncomfortable at any time, let the investigator know and they will deflate the cuff and check the placement of the cuff on your arm. There are no major risks associated with this device or procedure.

## Anticipated Benefits of This Study:

This study is done to gather information only and will not benefit your health or fitness. However, the information gathered from this study has the potential to help those individuals who are most prone to heat-related illnesses and heat-related deaths, such as the elderly and those with chronic diseases (diabetes, cardiovascular disease).

## Cost of Tests And Procedures:

You will not need to pay for any tests or procedures that are done for this research study.

You will be paid at a rate of $10.00 per hour spent in the 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, you will get part of this money.

## Your Rights If You Decide To 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 Kansas State University.

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. Any complications or adverse reactions will be immediately reported to the University Research Compliance Office at Kansas State University.

Kansas State University is not able to neither offer financial compensation nor absorb the costs of medical treatment should you be injured as a result of participation in this research. If such complications arise, the researchers will assist you in obtaining appropriate medical treatment, which will be provided at the usual charge.

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. You will be told of important new findings or any changes in the study or procedures that may happen.

## Extent of 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. Wong's office. All files with subject names and identification numbers will be destroyed after all data has been collected and analyzed and for a period of one year after the results from the study have been published.

## Terms of Participation:

I understand this project is research and that my participation is completely voluntary. I also understand that if I decide to participate in this study, I may withdraw my consent at any time, and stop participating at any time without explanation, penalty, or loss of benefits, or academic standing to which I may otherwise be entitled.

I verify that my signature below indicates that I have read and understand this consent form, and willingly agree to participate in this study under the terms described, and that my signature acknowledges that I have received a signed and dated copy of this consent form.

Participant Name (Please Print): _____

Signature of Participant: _____ Date: _____

Signature of Project Staff: _____ Date: _____

# Appendix C - Proof of Copyright Clearance

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9. Should any provision of this Agreement be held by a court of competent jurisdiction to be illegal, invalid, or unenforceable, that provision shall be deemed amended to achieve as nearly as possible the same economic effect as the original provision, and the legality, validity and enforceability of the remaining provisions of this Agreement shall not be affected or impaired thereby.

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11. This Agreement may not be assigned (including by operation of law or otherwise) by you without WILEY's prior written consent.

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14. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall prevail.

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