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The Effect of Local Heating on the Concentration of Interstitial ATP in Human Skin

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ABSTRACT

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Skin blood flow (SKBF) demonstrates a biphasic response to innocuous, local heating. Much about the mechanism of the first phase is unknown. A type of ion channel (TRPV3) sensitive to and increasingly activated by temperatures from ~33 to ~45°C may be involved. TRPV3 channels are abundantly located in the keratinocytes and are believed to elicit the release of ATP, a putative cutaneous vasodilator, upon activation. This study investigated the possibility that TRPV3 channels and ATP have a role in the first phase of the SBKF response to local heat.

Fifteen young, healthy subjects participated in the study. Two microdialysis probes were inserted into the dermis on the forearm. Using a peltier module, the skin above the probes (3cm x 3cm) was heated to 31, 35, 39, and 43°C to manipulate the level of activation of TRPV3 channels for eight minutes each. The probes were perfused with 0.9% saline at 2µl/min. Dialysate from each phase was analyzed for the concentration of ATP ($[ATP]_d$). Cutaneous vascular conductance (CVC), measured by laser Doppler flowmetry, was monitored throughout.

The $[ATP]_d$ decreased significantly when the skin was heated to temperatures known to strongly activate TRPV3 channels (i.e 39and 43°C). $[ATP]_d$ demonstrated no relationship with CVC and only a very weak relationship with peltier temperature ($r^2 = 0.02$, $p<0.05$). These data indicate that local heating and presumably heat-induced activation of the TRPV3 channels results in the decrease, not increase, of the release of ATP in human skin, and that the $[ATP]_d$ is not related to changes in skin blood flow.

Significant dilation was observed at 35°C. This threshold, which is several degrees lower than the threshold previously reported, suggests that the TRPV3 channels may be involved in the dilator response in some way independent of interstitial ATP.

Keywords: hyperemia, thermosensation, axon reflex

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Introduction

Skin blood flow (SKBF) plays a major role in maintaining thermal homeostasis when the body is under heat stress (21, 22, 39). Adjustments in SKBF in response to thermal stimuli are mediated by local and neural mechanisms (5, 22). While changes in whole-body temperature typically result in a whole-body response, the application of a heat source to a localized area of skin results in a localized increase in blood flow to the heated skin.

Under normal circumstances, a localized, biphasic increase in SKBF is observed when human skin is heated to innocuous temperatures (around 35°C to 44°C) (21, 33). The initial phase of the SKBF response is a prompt dilation around the heated area that peaks within 3-5 minutes followed by a rapid decrease in blood flow. A subsequent, gradual increase in blood flow around the heated area results in a long-lasting dilation that comes to a plateau between 20-40 minutes (5, 22, 33).

Only part of what mediates these two phases of localized dilation is understood. Much, but not all, of the initial phase appears to be the result of a rapid neural event known as an axon reflex (33). Many researchers (5, 33, 59) hypothesize that the initiation and termination of the axon reflex is dictated by the release of calcitonin gene-related peptide (CGRP) and substance P from local nerve endings in the skin. Although there is mounting evidence in support of this hypothesis, the evidence remains inconclusive (41, 53, 59). Recent findings (59) suggest that substance P indirectly inhibits the vasodilator properties CGRP by causing the release of proteases from nearby mast cells. When this inhibition of CGRP is eliminated, there is no nadir between the two phases of the dilator response. Thus, it appears that Substance P's indirect inhibition of CGRP is what accounts for the nadir typically observed after the axon reflex.

The axon reflex itself does not account for all of the dilation observed during the first phase. Blockade of the axon reflex by applying a topical anesthetic (EMLA cream) results in a blunted, yet persistent, response (33). This indicates that there are additional mechanisms involved in the first phase of dilation besides the axon reflex. Nitric oxide synthase (NOS) and its product nitric oxide (NO), as well as the neurotransmitter norepinephrine, (19) are also involved in this phase; however, the influence of these chemicals only accounts for part of the first increase in blood flow, leaving much more to be explained about the response (33). As such, all of the mechanisms involved in the first phase of local dilation are unknown at this time.

The second phase of the SKBF response is largely, but not entirely, dependent on NOS and NO (26, 33). While NOS accounts for a significant portion of the second phase, blockade of NOS activity results in the attenuation, not the elimination, of the dilator response (33). This indicates that there are other mechanisms independent of NOS that contribute to the second phase. Further investigation into the mechanism of the SKBF response to local heating is needed to gain a better understanding of both phases of the local hyperemic response to local heating.

The vanilloid family of temperature-sensitive ion channels, known as transient receptor potential ion channels (TRP), is a potential factor in the SKBF response to local heating. One member of the family of ion channels, type-1 (TRPV1), is already known to play a significant role in sensing the skin temperature and mediating both phases of the SKBF response (49, 58). With several other members of the family of channels being sensitive to the innocuous heat that is typically used to elicit the biphasic response, it seems reasonable that one or more of these ion channels have a role in the response. Aside from the TRPV1 in channels, the vanilloid type-3 TRP (TRPV3) ion channels are of particular interest when considering the hyperemic response due to reasons explained later.

TRPV1 ion channels are sensitive to temperatures above ~43°C and are also sensitive to the potent ingredient in chili peppers, capsaicin (40). In recent years scientists have used combinations of heat, capsaicin and TRPV1 antagonists to show that these ion channels do have a significant role in the SKBF response to local heating (49, 58). Most recently, Wong et al (58) delivered a TRPV1 antagonist, capsazepine, along with other chemicals known to manipulate SKBF, to the skin using microdialysis probes in order to test the role of the TRPV1 ion channels. SKBF during the first and second phases of the response to heating was significantly attenuated by the TRPV1 antagonist, indicating that the channels have a role (both upstream and independent of NO) in both phases. The role of the TRPV1 ion channels during the first phase was substantial, while their role during the second phase was only modestly significant.

With the TRPV1 ion channels already having been shown to be involved, it is possible that the TRPV3 ion channels, which are also sensitive to innocuous heat, are involved in the biphasic response as well.

TRPV3 ion channels are found in abundance within the keratinocytes of the skin, and have a temperature-threshold of activation (~ 33°C) that is several degrees lower than that of the TRPV1 ion channels (~43°C) (40). Interestingly, TRPV3 channels are increasingly activated as temperatures increase from 33°C up to 45°C (37). Unlike many other thermosensitive TRP ion channels, including TRPV1 channels, the TRPV3 ion channels are found only sparsely within the free nerve endings of the skin. The TRPV3 ion channels in the keratinocytes appear to have no direct connection with nervous tissue. Despite the lack of a connection to the nervous system, TRPV3 ion channels still communicate thermal information to the nervous system. TRPV3 channels embedded in the keratinocytes of mice relay thermal information to the nervous system in a paracrine fashion by releasing adenosine triphosphate (ATP). The released ATP then binds

to purinergic receptors on nearby sensory afferents, thereby delivering thermal information to the nervous system. This release of ATP from the keratinocytes appears to be proportional to the magnitude of the heat-induced activation of the TRPV3 ion channels (32). Another member of the vanilloid family, type 4 (TRPV4), which is sensitive to temperatures not typically associated with dilation (~ 24°C to ~34°C), also communicates thermal information from the keratinocytes of mouse skin in a similar fashion (32). Whether TRPV3 and TRPV4 ion channels employ this paracrine system in human skin is unknown at this point.

In addition to the fact that their TRPV1 counterparts are known to be involved in the SKBF response to local heating, there are several other reasons to suspect the involvement of the TRPV3 ion channels in biphasic hyperemia observed in response to innocuous, local heating. First, dilation in response to heating ensues at temperatures below the putative temperature threshold for the TRPV1 ion channels (31). The proposed threshold for dilation (~39°C) (19, 31) is a temperature which, to our knowledge, is only sensed by the TRPV3 ion channels (4, 40) thereby suggesting that TRPV3 channels may be responsible for initiating the response at the lower threshold for dilation. Second, infusion of ATP into human skin or large arteries has been shown to result in increased blood flow to skin (7, 57). As mentioned earlier, heating TRPV3 ion channels embedded within keratinocytes has been shown to elicit the release of ATP from the keratinocytes in a mouse model (32). Whether thermally activating TRPV3 ion channels in the keratinocytes of human skin results in a similar release of ATP remains to be seen, but the observations in mouse skin do provide grounds for investigating the possibility in human skin. Finally, a component of ATP, adenosine, plays a role in the first and second phases of the SKBF response to local heating (11). All together, the observations described above provide grounds

for investigating the possibility that TRPV3 ion channels and ATP are involved in the local hyperemia observed in response to local heating of the skin.

The purpose of this study was to investigate the possibility that TRPV3 ion channels and ATP play a role during the first phase of local hyperemia observed in response to innocuous, local heating of the skin. While TRPV3 channels and ATP may be involved in both phases of the biphasic response, in light of our own pilot data and the findings of Wong el al (58) that the TRPV1 ion channels had the greatest role during the first phase of the response, we chose to investigate the first phase only. The ideal way to determine if TRPV3 ion channels and ATP have a role in the SKBF response to local heating would be to heat the skin to temperatures that increasingly activate the TRPV3 ion channels and measure SKBF when the ATP receptors are inhibited and uninhibited. While ideal, this method remains impossible at the current time because no antagonist of ATP receptors is safe for human use (57). As such, this study was designed to test the role of TRPV3 ion channels and ATP during the first phase of the SKBF response to local heating by heating the skin to temperatures that vary in the magnitude of activation of TRPV3 ion channels while measuring changes in SKBF and the concentration of ATP released in interstitial fluid at each temperature. We hypothesized that the concentration of ATP present in the interstitial fluid of the skin during the first phase of the SKBF response to local heating would increase as the activation of the TRPV3 ion channels increased due to increased temperature. We also hypothesized that the changes in concentration of ATP in the interstitial fluid of the skin would correlate to the changes in SKBF at each temperature.

Methods

All methods and procedures for this study were approved by the Institutional Review Board at Brigham Young University prior to any recruiting of subjects or data collection.

Subjects

Fifteen healthy, non-smokers, 8 males and 7 females, between 19 and 31 years of age participated in this study. Subjects had no history of cardiovascular disease or diabetes. Subjects refrained from exercise and the consumption of alcohol and caffeine for 12 hours immediately before participating in the study. Subjects were also free from any prescription medication at the time of the study. Female subjects were tested during the first week after completion of the menstrual cycle to minimize the variation in body temperature and skin blood flow associated with fluctuations in hormone concentrations.

Procedures

After giving written consent, subjects were seated in a dental chair inside of an environmental chamber with the temperature set to 28 ± 1°C. Sterile, 27-gauge needles were used to insert, in parallel (within 1-2mm of each other), two microdialysis probes with hollow fibers of a molecular weight cutoff of 18,000 Daltons (Model 132295, InVivo microdialysis hollow fibers. Spectrum Laboratories Inc. Rancho Dominguez, CA), 1-2 mm deep into the skin at a site on the dorsal aspect of the forearm free of superficial veins. After the insertion of the probes, 0.9% sterile saline was perfused through the probes at a rate of 10 µl/min using a Harvard pump (Model PHD 2000, Harvard Apparatus, Holliston, MA) until the SKBF stabilized (minimum of 60 minutes). Blood pressure and heart rate were monitored in four-minute intervals throughout the experiment on the arm free of probes using a non-invasive automated

brachial artery cuff and three-lead EKG (Model 2120,Tango+ Stress BP. Suntech Medical. Morrisville, NC).

Immediately after placing the microdialysis probes in the skin, a peltier module (3 cm x 3 cm) was placed on the skin above the probes to maintain the skin at a temperature of 31°C. A laser Doppler probe (Model A25059,Moor Instruments, Devon, England) inserted through the middle of the peltier module was also placed on the skin at this time.

Once the SKBF stabilized, at least 60 minutes after needle insertion, the perfusion rate of sterile saline in the probes was slowed to 2 µl/min. At this point, the experimental protocol was commenced (Fig. 1). Temperatures included in the heating protocol (31, 35, 39, and 43°C) were chosen specifically to manipulate the activation of the TRPV3 ion channels from minimal activation (31°C) to near-maximal activation (43°C). During the heating protocol, skin temperature was increased above baseline (31°C) at a rate of 0.2°C per second, held at the stimulus temperature for 8 minutes and returned to baseline at a rate of 0.2°C per second. Following each 8-minute heating stimulus, the skin temperature was maintained at 31°C until SKBF returned to baseline. This required 16 minutes following heating 35°C and 32 minutes following heating to 39°C. The dialysate from both microdialysis probes was collected into a single 1.5 ml eppendorf tube during each 8-minute collection phase, which corresponded with the 8-minute intervals of the heating protocol. Eight-minute collection periods were chosen to allow enough dialysate to be collected to conduct the ATP assay in duplicate. Once a dialysate sample was collected it was immediately frozen on dry ice and stored in a freezer at -80°C. Following the last 8-minute heating phase (43°C), skin temperature was maintained at 43°C for an additional 24 minutes to elicit a peak SKBF. SKBF was then normalized as a percent of peak CVC for each person.

During each heating period, subjects provided a rating of perceived intensity of heat using the labeled magnitude scale developed by Green et al (14).

Analysis of concentration of ATP

The concentration of ATP in each dialysate sample was measured using a chemiluminensence assay (Enliten ATP Assay System. Promega. Madison, Wi). In brief, 10µl of each dialysate sample was placed in a well of a 96-well optiplate (Optiplate 96. Perkin Elmer. Waltham, Ma) with 100 µl of luciferase reagent. Each well was exposed to light for 10 seconds, and counts per second (CPS) for this complex was measured using a luminometer (Perkin Elmer, 1420 Multilabel Counter, Victor3, Finland). Using a standard curve of known concentrations of ATP, the counts per second for each dialysate sample was converted to concentration of ATP (moles/liter). The concentration of ATP in each dialysate sample ($[ATP]_d$) was measured in duplicate, while the standard curve was measured in triplicate. Samples were stored (frozen at -80°C) for no more than 7 days by the time they were used in the ATP assay.

Statistical Analysis

Major variables that were measured include skin temperature (°C), the concentration of ATP in the dialysate (moles/liter), heart rate (beats per minute), blood pressure (mmHg), and Laser Doppler flux (LDF, volts). Ratings of perceived thermal intensity were reported as percentages of the strongest imaginable heat sensation by dividing the distance of the rating from zero by the distance of the strongest imaginable label from zero. Vasomotor responses to heating were expressed as cutaneous vascular conductance (CVC) by dividing LDF by mean arterial pressure (MAP) ($MAP = \frac{(2* Diastolic\ Pressure)+Systolic\ Pressure}{3}$). CVC was normalized by

dividing by the peak CVC observed during minutes 24-32 of the 43°C heating phase and expressed as a percent of peak CVC.

Difference between means of ratings of perceived thermal sensation, MAP, and $[ATP]_d$ for each temperature were determined using one-way, repeated measures analysis of variance (ANOVA) followed by a Dunnet post hoc test. In order to test for significant differences between CVC (% peak) at baseline (30 seconds immediately prior to each heating phase) and CVC (% peak) at each 30-second interval of the heating phase, a one-way, repeated measures ANOVA was performed, followed by a Dunnett post-hoc test. Since the CVC response to skin heating displayed a biphasic pattern (early rise in CVC with a subsequent decline), we also calculated the area under the CVC (% peak) -time curve (AUC) for each heating period and compared the AUC's by repeated measures ANOVA and Tukey minimum significant difference post hoc test. The functional relationship between our outcome variables was evaluated with linear and non-linear, least squares regression analysis. Statistical significance declared when the p-values were less than 0.05. All statistical analyses were performed on PRISM 4 (Prism4 for Macintosh. Graphpad Software, Inc. La Jolla, Ca).

Results

Figure 1 illustrates the average CVC and $[ATP]_d$ response over the course of our skin-heating protocol. Baseline $[ATP]_d$ obtained during the first eight minutes when skin temperature was controlled at 31°C averaged $2.17 \times 10^{-8} \pm 6.09 \times 10^{-9}$ moles/liter. Duing local heating to 35°C the $[ATP]_d$ tended to be lower than the baseline but was not found to be significantly different than the baseline. The $[ATP]_d$ when the skin was heated to 39°C ($5.88 \times 10^{-9} \pm 1.68 \times 10^{-9}$ moles/liter) and 43 °C ($8.75 \times 10^{-9} \pm 3.44 \times 10^{-9}$ moles/liter) were both found to be significantly less than the baseline $[ATP]_d$, but not significantly different from each other. The

$[ATP]_d$ demonstrated a very weak, negative, linear correlation with peltier module temperature during the heating phases (first eight minutes when heated to 31, 35, 39, and 43°C) (R^2 = 0.02, P<0.05). While these data indicate that as skin temperature increased the concentration of ATP in the dialysate decreased, no correlation, linear or non-linear, was found between the magnitude of change (from baseline) in temperature and $[ATP]_d$ for each heating phase (R^2=0.04, P=0.19).

No significant relationship between CVC and $[ATP]_d$ was found to exist when considering all eight-minute intervals (including recovery) or even when considering just eight-mintue heating phases (the first eight minutes of 31, 35, 39, 43°C). The ratings of perceived thermal sensation showed a very weak correlation to $[ATP]_d$ when only heating phases were considered (first 8 minutes of 31, 35, 39, and 43°C). Ratings of perceived thermal sensation were found to have a negative linear correlation with $[ATP]_d$ observed at these times (R^2 = 0.07, P< 0.05). While significant, the correlations of $[ATP]_d$ with temperature and perceived thermal sensation may not be very meaningful.

Cutaneous vascular conductance (% peak) responded to heating in a dose-response fashion with the hotter temperatures eliciting a greater increase in CVC (% peak). The average CVC (% peak) for each 8-minute heating period is shown in Figure 1. When examining average area under the CVC (% peak)—time curve (AUC), the AUC of 35°C (50.92 ± 4.71), 39°C (210.2 ± 14.82) and 43°C (373.90 ± 19.90) heating phases were all significantly greater than 31°C (6.77 ±1.42), as well as each other (P< 0.05). When examining 30-second averages of CVC (% peak), significant dilation at 35°C occurred within 1 minute of the onset of heating (P < 0.05), while significant dilation at 39°C and 43°C occurred within 1.5 minutes after the onset of heating (P < 0.05) (Fig. 2). Mean arterial pressure was constant throughout the experiment

(79.27 ±0.27 mmHg), indicating that any change in CVC came as result of local factors and not as a consequence of a systemic response.

Ratings of perceived thermal sensation were strongly correlated with CVC (% peak) at the time of the rating (R^2 = 0.62, P <0.05).

Discussion

There were three novel findings in this study. First, innocuous local heating is associated with a decrease in the concentration of ATP in the interstitial fluid of the skin during the first phase of the SKBF response to local heating. Second, $[ATP]_d$ has no relationship with CVC during the first phase of the SKBF response to local heating. Third, significant dilation occurs when locally heating the skin from 31°C to 35°C.

The results of this study failed to show that ATP was released in a dose response fashion to increases in temperature/activation of the TRPV3 ion channels during the first phase of the SKBF response. In fact, our results very weakly demonstrated a negative, linear relationship between $[ATP]_d$ and skin temperature (R^2 = 0.02, P< 0.05). In general, $[ATP]_d$ was significantly less than the baseline when the skin was heated to temperatures that strongly activate the TRPV3 ion channels (i.e. 39 and 43°C) (Fig. 1).

These results indicate that in humans, the greatest concentration of ATP in the interstitial fluid of the skin is more closely associated with temperatures that activate the TRPV4 ion channels (31°C) than temperatures that activate the TRPV3 ion channels. Furthermore, the data indicate that temperatures that strongly activate the TRPV3 ion channels (i.e 39 and 43°C) are associated with the lowest concentrations of ATP in the interstitial fluid of the skin. Thus, it appears that the heat-activated release of ATP by the keratinocytes is more intimately tied to the

activation of the TRPV4 ion channels (sensitive to temperatures from ~ 24°C to ~34°C) (16) than the activation of the TRPV3 ion channels (sensitive to temperatures from ~ 33°C to ~ 45°C) (40, 61). Mandadi et al (32) reported that in addition to the TRPV3 ion channels, the TRPV4 ion channels in cultured keratinocytes of mice were also involved in the heat-induced release of ATP. However, the heat-induced activation of TRPV3 ion channels was related to a much greater release of ATP than the heat-induced activation of the TRPV4 ion channels, apparently contradicting our observations.

The disparity between our findings and those of Mandadi et al might stem from the use of two very distinct models. Mandadi et al used cultured, scrotal keratinocytes from mice, while we used an in vivo model with human skin of the forearm. It is possible that the TRPV3 ion channels function differently in mice scrotal skin than in human forearm skin. It is also possible that several factors that were present in our model may have influenced the concentration of ATP in the interstitial fluid that were not present in Mandadi's model (32). One such factor may have been the fluctuations in SKBF. Clough et al (6) reported that fluctuations in SKBF affect the ability of microdialysis probes to recover small molecules. Increases in skin blood flow, which were a part of our intervention, tend to dilute the interstitial fluid, decreasing the ability of the probes to recover small molecules by ~50% (compared to recovery during normal flow). Such dilution of the interstitial fluid may have been a factor in our study. We observed that $[ATP]_d$ was significantly less than baseline only when SKBF significantly increased above baseline (Fig. 1). It is unclear how much of an influence this dilution may have had on our results. Future studies may overcome the influence of dilation on the ability of microdialysis probes to recover molecules from the skin by treating the skin with a vasoconstrictor molecule, like norepinephrine, before the experiment in order to minimize the dilator response to heating.

The results of this study also indicate that no significant relationship exists between $[ATP]_d$ and CVC during the first phase of the SKBF response to local heating. This suggests that, contrary to what we hypothesized, an increase in the concentration of ATP in the interstitial fluid of the skin is not necessary for the increase in SKBF seen during the first phase of the SKBF response to local heating.

One limitation to our study is the possibility that the ATP obtained in the dialysate had degraded by the time we performed the ATP assay. We attempted to minimize degradation of the molecule by freezing the dialysate on dry ice immediately after collection and performing the ATP assay within seven days of being frozen, but significant degradation still may have occurred. Future studies attempting to measure ATP collected by microdialysis may benefit from measuring the degradation products of ATP as well.

When considering the data obtained during this experiment, we must accept the null hypothesis which states that greater temperature-induced activation of the TRPV3 ion channels does not result in greater release of ATP into interstitial fluid during the first phase of the SKBF response to local heating. We must also consider that greater activation of TRPV3 ion channels results in a decrease in release of ATP into the interstitial fluid. Furthermore, we must accept the null hypothesis that $[ATP]_d$ does not have a positive, linear relationship, or any relationship, with CVC during the first phase of the SKBF response to local heating.

While the results of this study failed to show a connection between $[ATP]_d$ and heat-induced activation of the TRPV3 ion channels or vasodilation, they do not rule out the possibility that the TRPV3 ion channels have a role in the biphasic, hyperemic response to local heating. In

fact, data obtained in this study, and other studies, regarding the temperature threshold for dilation suggests that the TRPV3 ion channels might be involved in this response.

In this study we found that significant heat-induced vasodilation in the skin occurs at temperatures as low as 35°C (Fig. 2) when heating from 31°C. The threshold for initiation of dilation of 35°C is several degrees cooler than the 39.5°C threshold that Magerl et al reported under similar conditions (31). With 31°C being below what is considered normal skin temperature (33-34°C), it is possible that part of this increase in SKBF was brought about by a locally-mediated decrease in vasoconstriction as the temperature rises (1). Localized vasoconstriction in response to local cooling is mostly dependent on the inhibition of basal activity of NOS; enhancement of basal adrenergic activity also has a role, but to a lesser extent (17). The extent to which these local vasoconstrictor mechanisms are involved in regulating SKBF in response to such a mild cooling stimulus (31°C), and the temperature at which dilation transitions from being the result of inhibition of NO to being the result of the activation of an axon reflex is unknown at this time. Thus, it is difficult to eliminate the possibility that the increased blood flow we observed in response to 35°C was, in part, influenced by the activation dilator mechanisms and not just the inhibition constrictor mechanisms. In several subjects we saw, what visually appeared to be, the normal biphasic response to local heating when we heated their skin to 35°C. This observation lends credence to the idea that mechanisms similar to those used at higher temperatures may have been employed at 35°C to induce dilation.

If what we observed was in fact the typical biphasic response to local heating, the TRPV3 ion channels would probably be responsible for mediating that response, because 35°C is a temperature that appears to be sensed predominantly, if not exclusively, by the TRPV3 ion channels (40). Even if what we observed was solely the result of a decrease in vasoconstriction

and not the activation of vasodilator mechanisms, there is still reason to suspect that the TRPV3 ion channels have a role in mediating the biphasic dilation in response to local heating. The putative temperature threshold for the biphasic response when heating from thermoneutral conditions is between 37.8 and 39.5 °C (19, 31). TRPV1 ion channels are known to be involved in sensing the heat and conveying the response (58), but their threshold for activation does not come into play until ~43°C (4, 40). The fact that dilation occurs below the TRPV1 threshold of sensitivity exposes a hole in our understanding of what is sensing the localized heat and initiating the events that lead to dilation at 35°C or even 39°C. Currently, the only ion channels known to be sensitive to temperatures around these thresholds are the TPRV3 ion channels (~33-45°C) (40, 61). Thus, further investigation into the possible role of the TRPV3 ion channels in the biphasic hyperemia observed in response to local heating is warranted.

In summary, we observed that innocuous local heating is associated with significant decreases in in the concentration of ATP in the interstitial fluid of the skin. We interpret this to mean that the activation of TRPV3 ion channels in the keratinocytes of humans is not associated with the release of ATP as it is in mice (32). We also found that the concentration of ATP in the interstitial fluid of the skin is not related to SKBF during the first phase of the SKBF response to local heating. Finally, we observed that significant dilation occurs at a skin temperature as low as 35°C. We suggest that activation of TRPV3 ion channels may contribute to this dilation.

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Figures

Figure 1: Top panel: Heating protocol over time. Temperature was changed from one temperature to another at a rate of 0.2°C • second^{-1}. Middle panel: Average cutaneous vascular conductance (CVC % peak) for each phase during the heating protocol. Bottom panel: Dialysate concentration of ATP ($[ATP]_d$)for each phase during the heating protocol. Values represent mean ± 1 SEM for 15 subjects. *Significantly different from the baseline value obtained during the first eight minutes at 31°C.

Figure 2: Thirty-second averages of cutaneous vascular conductance (CVC % peak) for three different heating temperatures. Zero minutes represents baseline CVC (% peak) at 31°C immediately prior to each heating phase. *Significantly different from time 0. Note that area under the curve for 35, 39 and 43°C is greater than area under the curve for 31°C.

Figure 1:

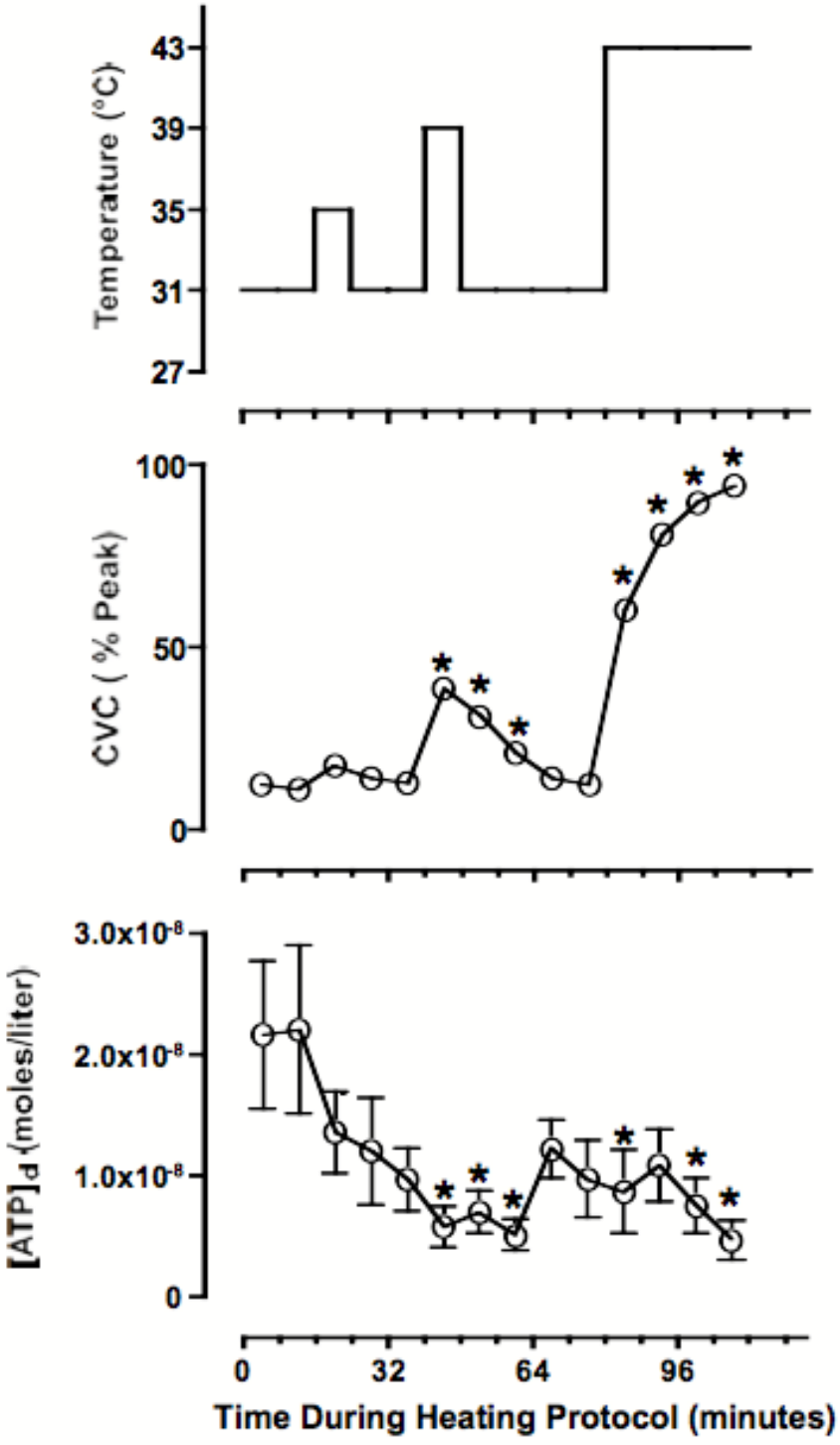
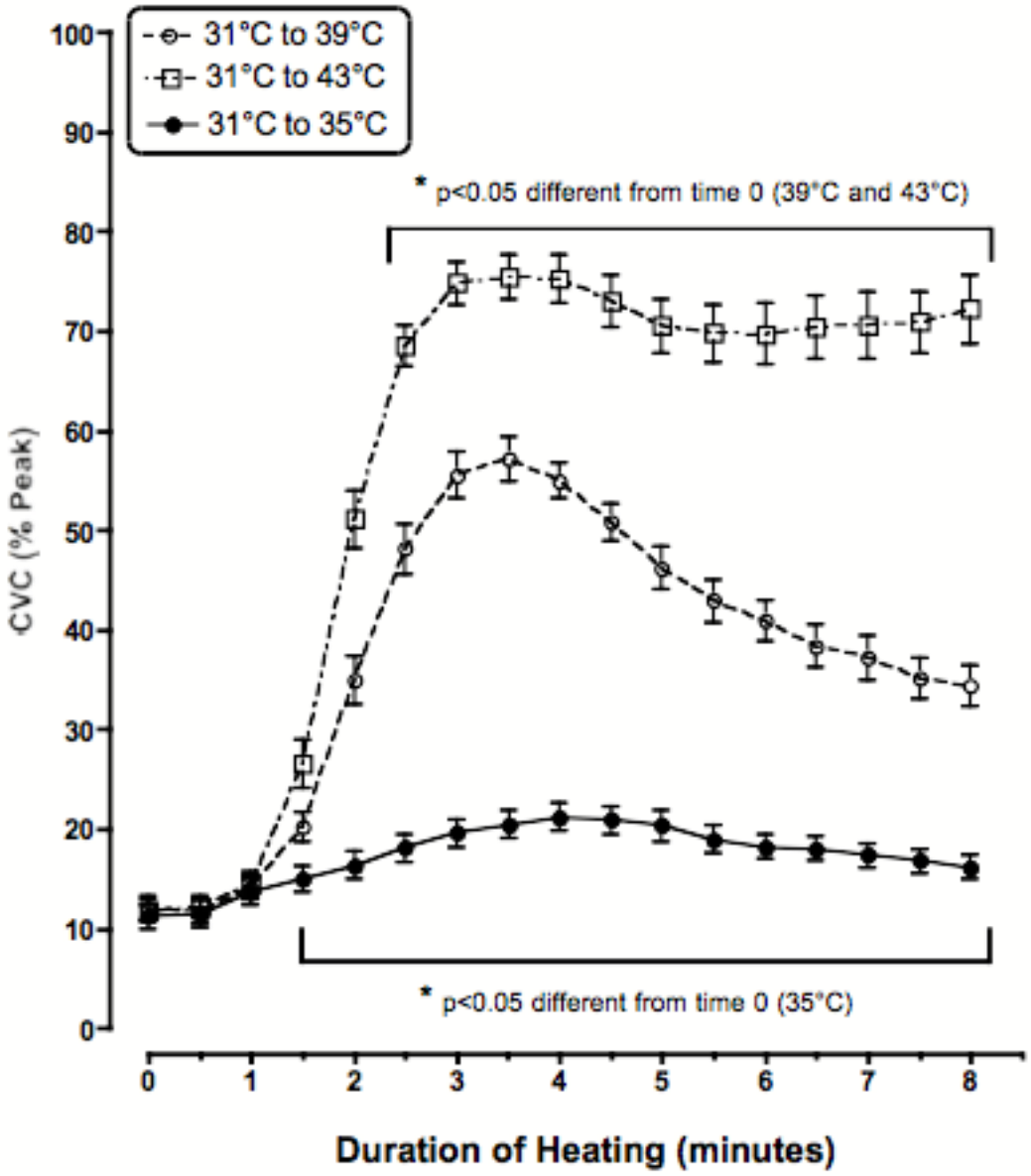


Figure 2:



Prospectus

Introduction

Skin blood flow (SKBF) plays a major role in maintaining homeostasis during heat stress (22, 39). Adjustments in SKBF to thermal stresses are mediated by neural reflexes and local mechanisms (5, 22). Changes in core temperature (Tcore) as well as changes in skin temperature (Tskin) elicit predictable responses in SKBF. Increases in either Tcore or Tskin typically result in an increase in SKBF in order to dissipate excess heat, while decreases in Tcore or Tskin typically results in decreases in SKBF to retain heat. Changes in temperature do not have to be global (over the whole body) to elicit changes in SKBF—for example, locally heating small areas of skin results in vasodilation of the cutaneous vasculature surrounding the area stimulated (33).

While much is understood about the control of SKBF in response to changes in Tcore (5, 22), how the body controls SKBF in response to innocuous local heating remains somewhat enigmatic (11, 34). Understanding what determines the SKBF response to local heating may help develop strategies to improve cutaneous perfusion in conditions that warrant such an increase, such as the thermoregulatory response to exercise in the heat and wound healing (5, 13, 22).

The Skin Blood Flow Response to Local Heating

The SKBF response to innocuous local heating is typically a biphasic increase in SKBF around the site of heat application (31, 33) directly proportional to the increase in Tskin (51). The initial phase of the SKBF response to the application of local heat is prompt dilation that peaks within 3-5 minutes followed by a rapid decrease in blood flow. Subsequently, a second,

gradual incline results in a long-lasting dilation that comes to a plateau between 20-40 minutes (5, 22, 33).

The increase in SKBF due to innocuous local heating occurs by mechanisms that are distinct from responses to whole-body heating. Unlike dilation elicited by whole-body heating, the dilation consequent to local heating occurs independently of the sympathetic nervous system (33). Current research indicates that the initial response is largely the result of a cascade of events triggered by a local neural response known as an axon reflex (33). The second response is largely mediated by the gas Nitric Oxide (NO) (5, 22, 33).

While the axon reflex and NO explain a large part of the observed response, they do not explain everything. When both the axon reflex and NO production are blocked, the SKBF response to heating is blunted, not prevented. (33). While adenosine is believed to have a small role in the response, independent of NO (11), very little is known about what else mediates this persistent response. Understanding what controls local cutaneous vasodilation in response to innocuous local heating, independently of the axon reflex and NO, can lead to advancements in vascular research.

Transient Receptor Potential Ion Channels: A Possible Link between Thermosensation and Local Hyperemia

In recent years a group of ion channels, known as transient receptor potential (TRP) ion channels, have been identified that are sensitive to the temperature of their environment. These ion channels are thought to be highly involved in regulating the body's responses to changes in temperature (40, 52). While the exact mechanism of their thermosensitivity is unknown, it is apparent that there are several different types of thermosensitive TRP ion channels that are each responsible for sensing different ranges of temperatures (2, 40). Thus, these TRP ion channels,

which among other places can be found in free nerve endings of cutaneous nerves in the skin, provide the ability to discriminate between different temperatures (40).

Among the multiple, distinct families of TRP ion channels, the vanilloid family (TRPV), more specifically type I (TRPV1), type III (TRPV3), and type IV (TRPV4) ion channels, are largely responsible for sensing temperatures in the range involved with typical, innocuous, local heating of the skin (25-45°C) (2, 40). Evidence shows that TRPV1 ion channels play a significant role in the SKBF response to local heat (31). Research regarding the role of TRPV3 and TRPV4 ion channels in the SKBF response to local heat is incomplete and inconclusive (11, 31, 32).

TRPV1 ion channels are sensitive to noxious temperatures above 43°C (4) and vanilloid ligands like capsaicin, the pungent ingredient in chili peppers (3, 4). These ion channels are primarily located in free nerve endings and are particularly abundant in the free nerve endings of the skin (40).

Research using capsaicin, which uniquely stimulates TRPV1 ion channels, indicates that the channels are involved in the SKBF response to local heating, particularly the axon reflex portion of the response (36, 38, 49). Acutely applying capsaicin stimulates a SKBF response, presumably through the TRPV1 ion channels (49). Chronically applying capsaicin results in an attenuated SKBF response to local heating which is presumably mediated by desensitization of the TRPV1 ion channels (36, 38).

TRPV3 ion channels are activated by temperatures greater than or equal to 33°C, and experience increased activation as temperatures increase up to45°C (37). TRPV4 ion channels are sensitive to temperatures from 24-36°C (2).

Among other places throughout the body, TRPV3 and TRPV4 are abundantly found in the keratinocytes of the skin. These ion channels are not found abundantly in the free nerve endings of the skin like their TRPV1 counterpart (37, 40, 61). With no direct attachment to nervous tissue, TRPV3 and TRPV4 ion channels appear to communicate with the nervous system through paracrine signaling (37). Upon heating adenosine triphosphate (ATP) is released from the keratinocytes, apparently through activation of TRPV3 and, to a lesser extent, TRPV4 ion channels (32). When ATP receptors are blocked, thermosensation is significantly impaired. As such, ATP appears to be a putative chemical messenger involved in communicating information from the TRPV3 and possibly TRPV4 ion channels to the nervous system (32, 37). It is interesting to note that ATP has been shown to elicit cutaneous vasodilation (57). The effect of ATP released by the TRPV3 rich keratinocytes on SKBF has yet to be investigated.

The role of TRPV3 and TRPV4 ion channels in the SKBF response to local heating remains scarcely investigated. One reason is that there is no known substance that exclusively desensitizes TRPV3 or TRPV4 ion channels as there is with TRPV1 channels and capsaicin (37, 40). The lack of an exclusive inhibitor has made the study of TRPV3 and TRPV4 ion channels in the SKBF response to local heating difficult. In one of the few studies targeting temperatures in the TRPV3 and TRPV4 range, Magerl et al (31) observed that vasodilation occurred at an average threshold of 39°C. This threshold is below the TRPV1 range of sensitivity. In addition, unpublished observations from our own laboratory have consistently shown dilation to initiate at lower temperatures than were reported by Magerl, which would indicate even less of a possible role for TRPV1 channels in this part of the response. All together, these observations indicate a role for the TRPV3 ion channels in dilation.

If dilation occurs at temperatures within the TRPV3 range, how is it mediated? With no direct connection to nervous tissue, it seems likely that TRPV3 ion channels stimulate dilation through a chemical messenger system, like the one used to transmit thermosensation from the ion channels in the keratinocytes to the nervous system (32, 37).

In an attempt to find the putative chemical signals involved in TRPV3 induced vasodilation, I propose investigating the role of ATP. In addition to being intimately involved in the transmission of thermosensation from the TRPV3 ion channels (32), ATP has also been shown to be released in abundance by TRPV3 dense keratinocytes when heated (32). Furthermore, ATP has been shown to induce vasodilation when infused into the interstitial space of the skin (57). In addition, inhibition of receptors for adenosine, a component of ATP, has been shown to decrease the SKBF response to local heating partially independent of NO (11). All together these data indicate a role for ATP in the cutaneous dilation seen in response to local heating.

To investigate the possible role of ATP in the cutaneous dilator response to local heating I propose measuring the amount of ATP released in response to heating at different temperatures and comparing the ATP concentrations to SKBF observed for each temperature. Since TRPV3 ion channels appear to release the most ATP, temperatures will be selected to increasingly activate the ion channels from no activation to maximal activation. While it would be more effective to block the ATP receptors and then measure the SKBF response, there is no ATP receptor antagonist that has been deemed safe for human use (57). Consequently, measuring ATP released in response to heating is the next best option.

Purpose Statement

Much of what mediates the SKBF response to local heating remains unknown. ATP released from TRPV3 ion channels in the keratinocytes of the skin appears to have a role in the SKBF response. As such, the purpose of this study is to quantify the amount of ATP released into the interstitial fluid of the skin when heated to temperatures that increasingly activate TRPV3 ion channels, from no activation to maximal activation. A second purpose of the study is to compare the ATP released at each temperature to the SKBF response to each temperature. With this information the role of ATP in the SKBF response to local heating, as well as the role of TRPV3 ion channels in releasing ATP in response to heating, will be able to be deciphered.

Hypotheses

First, ATP release will increase as the skin is heated to temperatures that increasingly activate TRPV3 ion channels (TRPV3 activation increases as the temperature goes from 35°C to 45°C).

Second, release of ATP from the keratinocytes will be proportional to the magnitude of activation of TRPV3 ion channels by heating.

Third, SKBF will be directly proportional to the amount of ATP present in the interstitial fluid of the skin at each temperature.

Null Hypotheses

First, ATP release will not be associated with increasing TRPV3 ion channel activation.

Second, release of ATP from the keratinocytes will not be proportional to the magnitude of activation of the TRPV3 ion channels.

Third, SKBF will not be directly proportional to the amount of ATP present in the interstitial fluid of the skin at each temperature.

Assumptions

First, ATP found in interstitial fluid in response to heating is coming from keratinocytes.

Second, heating to 31°C will only result in activation of TRPV4 ion channels and not the activation of TRPV3 ion channels.

Third, heating to 35°C will result in activation of TRPV3 ion channels.

Fourth, heating to 39°C will result in greater activation of TRPV3 ion channels than heating to 35°C.

Fifth, heating to 43°C will result in greater activation of TRPV3 ion channels than heating to 39°C, in addition to activating TRPV1 ion channels.

Delimitations

The findings of this study will only be applicable to the keratinocytes located in the forearm of healthy persons.

The results of this study will only be applicable to the first 8 minutes of the SKBF response to local heating.

Limitations

The role of TRPV3 ion channels in the SKBF response to local heating and ATP release will not be able to be separated from the role of TRPV1 ion channels when the skin is heated to 43°C.

Significance of the Study

Understanding what determines the SKBF response to different thermal stimuli may help develop strategies to improve cutaneous perfusion in conditions that warrant such an increase, such as wound healing and thermoregulatory responses to exercise in heat.

In addition, further insight into the role and function of TRP ion channels, which are present throughout the body, will be gained.

Review of Literature

Introduction

Skin blood flow (SKBF) plays a major role in human thermoregulation during exercise, when exposed to cold and heat (22, 39), and with wound healing. The distribution of blood between the core and skin of the body changes in response to fluctuations in core temperature (Tcore) and skin temperature (Tskin). While much is known about the control mechanisms of SKBF, many things remain enigmatic. Understanding what determines the SKBF response to different thermal stimuli may help develop strategies to improve cutaneous perfusion in conditions that warrant such an increase, such as thermoregulatory response to exercise in the heat (13) and wound healing. This review will focus on the mechanisms for controlling SKBF, with particular emphasis placed on current and potential research regarding the control mechanisms for SKBF in response to the application of local heat.

Control of Skin Blood Flow

In order to understand the SKBF response to local heating, one must first appreciate the control mechanism for the SKBF responses to whole-body heating and cooling. SKBF in response to whole-body, thermal stimulation is controlled by reflex (neural) and local factors (5, 12, 22). Reflex control is sensed by cardiovascular, thermal, and nociceptor afferents. The afferent information is subsequently sent to the hypothalamus and is then primarily effected by the sympathetic nervous system (5, 25). Local control factors of SKBF include circulating pressor hormones like angiotensin II, (12) and paracrine release of prostanoids and histamine released from nearby vascular endothelial lining and skin keratinocytes, respectively (22).

The skin can be classified into two distinct types: glabrous (hairless) and non-glabrous (hairy) (5, 22). The control of SKBF differs between these two types of skin. In glabrous skin, like the skin of the palm of the hands, cutaneous arterioles—which largely influence SKBF—are solely innervated by noradrenergic, sympathetic vasoconstrictor nerves (5, 22, 39). In non-glabrous skin, like that of the dorsal aspect of the forearm, cutaneous arterioles are innervated by sympathetic, noradrenergic vasoconstrictor, and sympathetic cholinergic, active vasodilator nerves (5, 22, 39). This review will focus on SKBF in non-glabrous skin.

Cutaneous Vasoconstriction in Response to Whole-Body Cooling

The cutaneous vasoconstrictor mechanism is stimulated in response to sub-homeostatic Tcore and Tskin, and is largely controlled by the actions of the sympathetic nervous system. It was originally thought that the cutaneous vasoconstrictor system relied only on the neurotransmitter norepinephrine (NE), but a study by Stephens and associates (48) indicates that cotransmitters are likely involved as well. Using bretylium tosylate to blockade all noradrenergic nerves (which prevents release of neurotransmitters from the terminal end of an axon) prevented the vasoconstrictor response to whole body cooling, while complete and simultaneous blockade of alpha 1, alpha 2, and beta receptors did not. This indicates the existence of vasoconstriction-causing cotransmitters that bind to places other than the alpha and beta receptors. Neuropeptide Y (NPY) is believed to be such a cotransmitter. In 2004 , Stephens et al (50) found that the NPY receptor antagonist BIBP-3226 successfully attenuated the vasoconstrictor response to whole-body cooling. Stephens (50) also found that NPY blockade accompanied with alpha and beta receptor blockade completely prevented the vasoconstrictor response to whole body cooling.

Thus, it appears that the reflex vasoconstrictor response depends on NE, and NPY. Therefore, the appropriate steps to block a vasoconstrictor response would be to either use bretyllium tosylate (48) to block the release of all neurotransmitters, or to use a cocktail of drugs to block all influential receptors, including alpha, beta, and NPY receptors (50).

Cutaneous Vasodilation in Response to Whole-Body Heating

When the Tskin or Tcore increases above homeostatic levels, as is typical during exercise, the body responds by sympathetically dilating the cutaneous vasculature in order to dissipate excess heat (5, 22). Sympathetic, cholinergic nerves appear to be largely responsible for active vasodilation; however acetyl choline (ACh) does not appear to act alone in this mechanism. Research currently implicates a cotransmitter system (22) involving ACh and vasoactive intestinal polypeptide (VIP) as a key cotransmitter (33, 44, 56).

Whatever the sympathetic cotransmitter is, vasodilation appears to be, at least in part, stimulated by the production of Nitric Oxide gas (NO) (22, 33, 42). NO levels have been found (23) to increase in the cutaneous interstitial space during heating along with active vasodilation. In addition, blockade of NO producing enzyme, Nitric Oxide Synthase (NOS), with the N^{D}-nitro-L-arginine methyl ester (L-NAME) significantly attenuates the vasodilator response, as measured by laser Doppler flowmetry, to whole-body heating (24), and local application of heat (33). Also, NO donors like sodium nitro prusside (SNP) dilate the cutaneous vasculature (10, 22, 33) further implicating NO in cutaneous vasodilation. Nevertheless, NO does not seem to be the only factor involved in the vasodilator response. Shastry et al (42) found that NO blockade with L-NAME attenuated only a small portion of the SKBF increasing during whole-body heating. Clearly other vasodilator substances are involved.

Vasodilation in Response to Application of Local Heat

In addition to the sympathetic control of SKBF described above, SKBF can be influenced by local temperature changes (5, 33). Applying a heat source directly to the skin results in a biphasic increase in SKBF around the site of application (31, 33) directly proportional to the increase in temperature (51). The initial phase of the SKBF response to the application of local heat is prompt dilation that peaks within 3-5 minutes followed by a brief nadir. Subsequently, a second, gradual incline results in a long-lasting dilation that comes to a plateau between 20-40 minutes (5, 22, 33).

When investigating the SKBF response to local heating, precaution must be taken when selecting or comparing data between subjects, because SKBF responses to local heat are known to be altered with age (34) and with hormone levels related to the menstrual cycle and certain birth control medications (5). Any study attempting to examine the cutaneous blood flow in response to local heating should account for these variables as best as possible.

In 2001, Minson et al showed that both vasodilator responses to local heating are controlled in a different manner than the responses to whole-body heating, functioning independently of the central nervous system (CNS), more specifically the sympathetic nervous system. In this study Minson et al found that blockade of the cutaneous nerves with 0.5% bupivacaine had no effect on the SKBF response to local heating. These results confirm work by Wenger et al (55) that dilation associated with local heating is independent of the CNS. This indicates that both the initial peak and secondary plateau are controlled by local factors, the peripheral nervous system, or both. The next few sections will discuss the possible control mechanisms for the initial and secondary responses.

Local Heat and the Initial Phase

Given that the response is independent of the sympathetic nervous system, many researchers suggest that the initial response is neurally mediated through an axon reflex (19, 22, 33). An axon reflex in the skin is an efferent aspect of the mechanoinsenstive, afferent C-fibers that typically results in an inflammatory response, often termed neurogenic inflammation, in the area around the stimulated site (18, 29). When these C-fibers are stimulated by heat, ultraviolet light, or chemical stimuli, a signal is propagated antidromically to other afferent branches of the nerve, before being relayed back to the dorsal root ganglion or CNS (15). The propagated signal to the afferent branches causes the release of vasoactive neuropeptides like calcitonin-gene-related polypeptide (CGRP) and substance P (SP) (15, 18, 28). Interestingly an axon reflex can occur even without conscious perception of pain (31).

Minson and associates (33) suggested that the initial increase in SKBF in response to local heat is mediated by an axon reflex. They used EMLA cream (a lidocaine-based, topical anesthetic) to block the local cutaneous sensory nerves that mediate the axon reflex. Sites treated with EMLA had a blunted initial peak in SKBF in response to local heating indicating that the response is largely mediated by an axon reflex. Nevertheless, the initial increase was not completely abolished by the topical anesthetic. As such, we may assume that either the EMLA cream failed to completely block all cutaneous afferent fibers or that another mechanism contributes to the initial peak in SKBF. Minson et al verified the cutaneous sensory blockade by lack of sensation to pin prick, or stroking of the skin. In addition, the inflammatory flare response to painful heat was also abolished indicating that EMLA cream was effective in producing sensory blockade in the skin. Therefore, some other mechanism, independent of the axon reflex must also contribute to the initial peak in SKBF.

Minson et al (33) demonstrated that the initial peak is attenuated when NO production is blocked with L-NAME. However, when sensory blockade (EMLA) was undertaken, the application of L-NAME made no further reductions in the initial SKBF response to local heating. It is possible that the axon reflex stimulates NO production, indicating that the effect of L-NAME is downstream of the initial activation of the axon reflex. Alternatively, NO may participate in activating the axon reflex (18). As such, blockade of nitric oxide synthase by L-NAME may have reduced nitric oxide-stimulated axon reflex activity.

Local Heat and the Secondary Plateau Phase

The second, more prolonged response to local heat appears to be largely influenced by NO production by the endothelium (21, 22, 26, 27, 33). Minson et al (33) used L-NAME to block NO production and observed a marked reduction in SKBF (from 87% to 40% max SKBF) during the secondary phase of the dilator response. These data indicate that the secondary response is largely NO dependent. Despite the decrease in the secondary response being rather large, the 40% max SKBF observed after NOS inhibition is still far above the baseline measurement of 14% max SKBF, indicating that there are nitric oxide independent mechanisms that contribute to the secondary response.

Holes in Research Regarding SKBF Response to Local Heating

While Minson's study (33) appears to explain a large portion of how the SKBF response to local heat is controlled, there are still unknown factors that contribute to the response. Even when the axon reflex and NO production are blocked, there is still a vasodilator response, albeit attenuated, to the application of local heat. This indicates that there is a mechanism for controlling SKBF in response to local heating that functions independently of NO and the axon

reflex. Further investigation into the underlying mechanisms of thermosensation and the SKBF response to local heating are needed to explain the persistence of the response.

Transient Receptor Potential Ion Channels: A Basis for Thermosensation

In recent years a group of ion channels, known as transient receptor potential (TRP) ion channels, have been identified that are sensitive to the temperature of their environment. These ion channels are thought to be highly involved in regulating the body's responses to changes in temperature (40, 52). While the exact mechanism of their thermosensitivity is unknown, it is apparent that there are several different types of thermosensitive TRP ion channels that are each responsible for sensing different ranges of temperatures (2, 40). Thus, these TRP ion channels, which among other places can be found in free nerve endings of cutaneous nerves in the skin, provide the ability to discriminate between different temperatures (40).

Among the multiple, distinct families of TRP ion channels, the vanilloid family (TRPV), more specifically type I (TRPV1), type III (TRPV3), and type IV (TRPV4) ion channels of the TRPV family, are largely responsible for sensing temperatures in the range involved with typical local heating of the skin (25-50°C) (2, 40). Evidence shows that TRPV1 ion channels play a significant role in the SKBF response to local heat (31). Research regarding the role of TRPV3 and TRPV4 ion channels in the SKBF response to local heat is incomplete and inconclusive (11, 31, 32). The following sections will discuss current knowledge of TRPV1, TRPV3, TRPV4 ion channels in regards to thermosensation and the SKBF response to local heating.

TRPV1 Ion Channels

TRPV1 channels are Ca^{2+} permeable, non-selective cation channels present in free nerve endings of cutaneous nerves and are activated by noxious temperatures above 43°C (4, 40). TRPV1 ion channels have also been found at various locations in the cerebral vasculature (8).

As well as being sensitive to heat, TRPV1 channels can be stimulated by hydrogen ions, and vanilloids like capsaicin, the pungent ingredient in chili peppers (3, 4, 40) and the chemical camphor (60). The temperature threshold of TRPV1 channels to heat is influenced by the other stimuli that the channel has recently experienced. For example, applying capsaicin or injury to the area will decrease the temperature threshold for these channels (2, 31) making the channels sensitive to lower temperatures.

Capsaicin, the pungent ingredient in chili peppers, has been found to be very useful when studying TRPV1 ion channels. Acute application of capsaicin results in increased SKBF (30, 31, 49) and enhanced sensitivity to other painful stimuli like heat (3, 20). Prolonged or chronic application results in a period of desensitization to the painful stimuli (e.g. heat, capsaicin) that can last hours or an entire lifetime depending on the method of application (3, 30, 45). Thus, chronic application of capsaicin can be used to decrease sensitivity to local heating. TRPV1 channels are also sensitive to and can be desensitized by the chemical camphor (60).

TRPV3 Ion Channels

TRPV3 channels are similar to TRPV1 channels but are sensitive to different conditions. TRPV3 ion channels are activated by temperatures greater than or equal to 33°C, and experience increased activation as temperatures increase up through at least 45°C (37). In addition, TRPV3 ion channels appear to be sensitive to noxious temperatures above 50°C (40). Repeated heating of the skin tends to increase the sensitivity of TRPV3 channels (2, 37, 46). The chemical camphor stimulates these ion channels, as well as other TRP ion channels. Exogenous carvacrol, a component of oregano, has also been found to activate TRPV3 channels (9). Unlike TRPV1 ion channels, TRPV3 ion channels show no response to the chemical capsaicin (40).

TRPV3 ion channels can be desensitized by the chronic application of camphor, or similar substances (43). However, when trying to isolate the role of TRPV3 ion channels, desensitization through chronic camphor application is not useful because camphor also desensitizes TRPV1 ion channels (60). A chemical that exclusively desensitizes TRPV3 ion channels would be useful for studying the role of TRPV3 ion channels, but such a substance has yet to be discovered.

Like TRPV1 channels, TRPV3 channels are found in cutaneous nerve endings and at various locations in the cerebral vasculature (8), but unlike TRPV1 channels, TRPV3 channels are abundantly expressed in keratinocytes of the skin (37, 61). There appears to be no direct connection between the TRPV3 ion channels in keratinocytes and the nervous system. Recently, Mandadi et al (32) found that activation of the TRPV3channels in keratinocytes with warm stimuli causes the release of ATP, which in turn communicates with the nervous system. TRPV4 ion channels also appear to release ATP in response to similar stimuli, although to a much lesser extent than their TRPV3 counterparts. Mandadi et al also found that blockade of the ATP receptors, purinergic 2 receptors, resulted in no sensitivity to heat in TRPV1 knockout mice, indicating that ATP plays a vital role in the thermosensitivity of TRPV3 ion channels.

The exact effect these channels located in keratinocytes have on the SKBF response to local heating is unknown. Nevertheless, there is reason to suspect that these ion channels have a role in the cutaneous dilation observed in response to local heating. Although the role of the ATP released in response to activation of the TRPV3 ion channels on SKBF has not been examined, it is interesting to note that ATP is known to elicit vasodilation in cutaneous vasculature (57).

TRPV4 Ion Channels

TRPV4 ion channels are sensitive to temperatures ranging from 24-36°C (2, 40), but do not appear to be directly activated by heat (54). Watanabe et al (54) suggests that because TRPV4 channels only function in cell-attached mode, they may be activated by a ligand released by heating. Similar to TRPV3 ion channels, TRPV4 ion channels may also release ATP when stimulated; however the amount released by TRPV4 channels is thought to be much less than the amount released by TRPV3 channels (32). The thermosensitivity of these channels is heightened in hypotonic solutions (16). TRPV4 channels are also sensitive to arachidonic acid (AA) and anadamide, but again they are only sensitive to these substances in the cell-attached mode (40).

Like TRPV3 ion channels, TRPV4 channels are present in many different tissues including keratinocytes (8, 16, 40). The function of these ion channels in the keratinocytes remains unclear, but they appear to provide a redundant mechanism for sensing temperatures in the lower end of the TRPV3 range (35) and possibly contribute to intercellular junction formation in keratinocytes (47). TRPV4 ion channels are also known to be in the endothelial cells of the aorta (54), and the smooth muscle cells of the aorta, cerebral, and mesenteric arteries (8). The TRPV4 ion channels in these parts of the circulatory system appear to help regulate vasoconstriction and vasodilation in response to arachidonic acid and possibly changes core temperature (8). The role of TRPV4 ion channels in the SKBF response to local heating is unknown.

TRPV1, TRPV3, and TRPV4 Ion Channels Interact

The actions of TRPV1, TRPV3, and TRPV4 ion channels appear to overlap and influence the activity of each other. TRPV1 and TRPV3 ion channels have been found to coexist on the same nerve endings (61) and when collocated with a TRPV3 receptor, TRPV1 receptors appear

to be more sensitive to stimuli (40, 46). In addition TRPV3 knockout mice have been found to be less sensitive to temperatures within the TRPV1 range than their wild-type counterparts (35). Thus, one type of receptor appears to influence the function of the other.

The functions of TRPV3 and TRPV4 ion channels also possibly overlap. Moqrich et al (35) placed both TRPV3 knockout mice and wild mice on a surface with a temperature gradient between 15 and 55°C for two hours and monitored their behavior. Wild-type mice quickly and overwhelmingly settled on staying in the 30-38°C area of the gradient, while the TRPV3 KO mice did not show any preference during the first hour of observation. This indicates an impaired sensitivity to such temperature, but their sensitivity was not completely absent. During the second hour of observation, TRPV3 KO mice behaved just like their wild-type counterparts and overwhelmingly preferred the 30-38°C area of the gradient. This suggests that a delayed mechanism, possibly TRPV4 ion channels, acts redundantly to provide temperature information for this range.

TRPV Ion Channels and the Skin Blood Flow Response to Local Heating

The role of some TRPV ion channels in the SKBF response to local heating has been studied more than others. TRPV1 ion channels have been studied extensively in relation to local heating (38, 49). Research in relation to TRPV3 has been less extensive (31). Research regarding TRPV4 ion channels and the SKBF response to local heat is virtually non-existent. The dearth of research involving TRPV4 ion channels is possibly due to the fact that these channels sense temperatures that are usually below the scope of study with local heating.

The fact that the presence of one ion channel influences the activity of the others has made it difficult to separate the functions of each one. This necessitates more detailed research into the role of each ion channel both independent and in the presence of the rest. The

following sections will address the role of TRPV1, TRPV3 and TRPV4 ion channels in the SKBF response to local heat.

TRPV1 Ion Channels and the SKBF Response to Local Heat

In 2001, Stephens et al (49) stimulated TRPV1 ion channels with topical capsaicin and then observed changes SKBF. SKBF was found to increase in response to topical application of capsaicin, indicating that TRPV1 ion channels, the only ion channels sensitive to capsaicin, are involved in the SKBF response to local heating. Stephens et al (49) also found that application of capsaicin to a site prior to the application of local heat tended to decrease the temperature threshold for the vasodilator response. This indicates that the TRPV1 ion channels are sensitized by the acute application of capsaicin.

In 2003, Munce et al (36) chronically applied capsaicin to the skin of the forearm of 18-30 year olds for 7 days to desensitize the capsaicin-sensitive TRPV1 ion channels. Once desensitization was verified, Munce et al measured CVC in response to heating at desensitized sites and untreated sites. The desensitized sites had a significantly lower initial peak than the control site (53.9% vs. 74.4% CVCmax). The secondary plateau remained unaffected by chronic application of capsaicin. These findings are in agreement with those of Roberts et al (38). These findings indicate that the desensitization of the TRPV1 ion channels affects the SKBF response to local heat, especially the axon reflex portion of the response. Therefore, desensitization of TRPV1 ion channels with chronic application of capsaicin can be used to investigate the role of TRPV1 ion channels in the SKBF response to local heat. This same desensitization may possibly be used to uncover the role of TRPV3 ion channels by attenuating the effects of the TRPV1 ion channels.

TRPV3 and TRPV4 Ion Channels and the SKBF Response to Local Heat

The role of TRPV3 and TRPV4 channels in the SKBF response to local heat is unclear and still under investigation. In 1996, Magerl and associates devised a method to test the role of warm fibers, presumably populated by TRPV3 and TRPV4 ion channels, in the SKBF response to local heat. In this study Magerl increased local skin temperature from 30-35°C and 35-37.4°C and measured changes in SKBF around the site. The temperature range was selected in order to isolate the role of warm fibers, presumably containing TRPV3 and TRPV4 channels, and avoid the interference of nociceptors (TRPV1 channels).

When the skin was stimulated as described, no change in SKBF was observed around the site as is typical at higher TRPV1-stimulating temperatures. Dilation was not observed until a temperature around 39°C was reached. Magerl et al (31) interpreted this to mean that warm fibers play no role in axon reflex dilation. These conclusions should not be extrapolated to say that TRPV3 ion channels play no role in the SKBF response to local heating. While Magerl did find that warm heat (30-37.4°C), which would stimulate TRPV3 and TRPV4 ion channels (40) did not affect SKBF, he did not test, the possible role of warm receptors and TRPV3 ion channels) at temperatures greater than 37.4°C which are still within the range of sensitivity of the TRPV3 ion channels. Furthermore, unpublished observations from our own laboratory have consistently indicated a dilator response to temperatures lower than Magerl's prescribed threshold of 39°C. As such, at least the TRPV3 ion channels cannot be deemed absolutely inactive in the SKBF response to local heating.

The limitations of Magerl's study (31) highlight a common roadblock in studying the role of TRPV3 ion channels in humans. With no known chemical that exclusively desensitizes TRPV3 ion channels it is difficult to separate the effects of TRPV3 ion channels from other ion channels, like TRPV1. Consequently, studies investigating the role of TRPV3 ion channels have

been limited. However, desensitization of TRPV1 ion channels with capsaicin possibly offers a way to overcome this common limitation. Chronic application of capsaicin may blunt the function of TRPV1 ion channels enough to give insight into what TRPV3 ion channels do in response to local heating.

The role of the TRPV4 ion channels in the SKBF response seems to be minimal at best because of the fact that the cutaneous vasodilator response to local heating has typically been found to occur between 34°C and 39°C (31, 33, 49), and the proposed upper limit of sensitivity for the TRPV4 ion channels is 34°C (40). This suggests that the TRPV4 ion channels have a minimal role, if any, in the response.

ATP: A Possible Link between TRPV3 Ion Channels and SKBF

ATP is known to elicit vasodilation. In 2009, Wingo et al. showed that infusion of ATP into the interstitial space of the skin via microdialysis probes results in significant vasodilation. Wingo also showed that the infusion adenosine (a component of ATP) also results in significant vasodilation (57).

TRPV4 and especially TRPV3 ion channels are a possible source of ATP in the interstitial space of the skin. As mentioned earlier, Mandadi et al (32) found that, upon heating, keratinocytes release significant amounts of ATP. ATP release from TRPV3 deficient keratinocytes was much more attenuated than ATP release from TRPV4 and TRPV1 deficient keratinocytes, indicating that TRPV3 ion channels are most intimately involved in the ATP release by keratinocytes in response to heat. In the same study Mandadi et al also found that when ATP receptors, purinergic type 2 receptors (P2), are blocked, the dorsal root ganglia of TRPV1 knockout mice showed no response to heating. These data indicate that TRPV3 and, to

a much lesser extent, TRPV4 ion channels release ATP in response to heating, and that ATP is intimately involved in thermosensation.

Recent work in humans by Fieger and Wong (11) indicates that adenosine ligands, possibly derived from the ATP released by TRPV3 and TRPV4 ion channels in the keratinocytes, may play a role in the SKBF response to local heating. Fieger et al (11) blocked adenosine 1 (A1) and adenosine 2 (A2) receptors with the infusion of theophylline, and then measured cutaneous vascular conductance (CVC) in response to local heating. CVC was decreased during the initial peak (13% decrease) and the plateau phase (22% decrease) under A1/A2 blockade compared to the unblockaded control site. Thus, A1/A2 receptors account for 16% of the initial peak and 18% of the plateau phase.

Much, but not all, of the A1/A2 receptors' effect on SKBF appears to be mediated by increasing NO concentrations in the area. In the same study mentioned above, Fieger et al (11) found that NOS blockade accompanied with A1/A2 blockade did not decrease the initial peak more than NOS blockade alone. This indicates that the A1/A2 receptors mediate their effect on the initial peak through NO. The plateau phase, however, was further decreased when NOS blockade was accompanied by A1/A2 blockade, indicating that part of the A1/A2 mechanism is independent of NO. A1/A2 receptor activation only accounts for part of the release of NO and SKBF response.

While adenosine may only account for a small fraction of the persistent SKBF response to local heating observed by Minson et al (33), the findings of Fieger et al (11), and Wingo et al (57) combined with the findings of Mandadi et al (32) suggest that ATP released by TRPV3 and aTRPV4 ion channels in the keratinocytes may also explain another portion of the persistent

SKBF response. Further investigation into the possible role of ATP released from TRPV3 and TRPV4, ion channels in the keratinocytes in the SKBF response to local heating is required.

Conclusion

While a great deal is understood about the controls of skin blood flow, there are still many things that remain enigmatic. Of all the SKBF control mechanisms, the control mechanisms for biphasic response to local heat remain among the most nebulous. Understanding what determines the SKBF response to different thermal stimuli may help develop strategies to improve cutaneous perfusion in conditions that warrant such an increase, such as wound healing and the thermoregulatory response to exercise in heat.

The initial cutaneous dilator response to local heating is partially, but not entirely, regulated by an axon reflex, while it appears that the second response is largely, but not entirely, influenced by the production of NO (33). Despite the current explanations for the biphasic response to local heat, there are still underlying mechanisms that contribute to SKBF response to local heat.

One possible factor in the biphasic response might be vanilloid type III transient receptor potential ion channels (TRPV3). Vanilloid type I transient receptor potential ion channels (TRPV1) have already been implicated in the initial peak (36, 49), and research suggests that the sensitivity of TRPV1 receptors is influenced by the presence of TRPV3 receptors (40, 46), which are often collocated on the same free nerve endings (61).

Also increasing suspicion of TRPV3 ion channels' possible role in the SKBF response to local heating is the fact that they have been found to release ATP from the keratinocytes, and that ATP and it's precursor, adenosine, have been found to propagate thermosensation and cutaneous

vasodilation (11, 32, 57). As such, the possible role of TRPV3 ion channels in regulating the SKBF response to local heat should be investigated.

In this proposal I describe a novel method for investigating the role of TRPV3 ion channels in the SKBF response to local heating. In this model I will compare ATP released into the interstitium of the skin in response to heating at various temperatures to SKBF at the same temperatures. The temperatures will be selected to activate TRPV3 ion channels in an increasing amount from no stimulation to maximal stimulation, thereby providing an opportunity to investigate a dose-response correlation between TRPV3 activation, ATP release and SKBF.

Procedures

Experimental Design

A quasi-experimental design will be used to examine the role of adenosine triphosphate (ATP) and vanilloid type transient receptor potential ion channels (TRPV) in the cutaneous dilation observed in response to local heating.

Prior to any recruitment or data collection, this study will be approved by the Brigham Young University Institutional Review Board (IRB).

Subjects

Fifteen male and female subjects 18-35 years old will be recruited to participate in this study. Subjects will be recruited from the Brigham Young University campus using IRB-approved flyers. Subjects will complete a health questionnaire (Appendix A) to verify eligibility for the study. Only apparently healthy subjects, with no history of cardiovascular disease or diabetes, who are not taking any medications at the time, will be accepted into the experiment. Due to fluctuations in core temperature and skin blood flow associated with different phases of the female menstrual cycle (5), all female subjects will be tested within the first seven days

following menstruation. In addition, subjects will be asked to avoid all voluntary exercise, and consumption of caffeine, and alcohol for 12 hours before the experiment.

After filling out the questionnaire, subjects who qualify for the experiment will also provide informed, written consent and will be instructed that they may withdraw from the study at any point with no consequence.

Environmental Conditions

The experiment will be performed while subjects remain seated in a dental chair located in the environmental chamber of the Human Performance Research Center (HPRC) at Brigham Young University. The environmental chamber will be maintained at a temperature of 28 ±1°C during the entirety of the experiment.

Subject Preparation

Upon arriving to the HPRC, subjects will have their height and weight measured after voiding their bladder. Subjects will then be seated upright in a dental chair inside the environmental chamber. Subjects will remain seated throughout the entirety of the experiment.

Two microdialysis probes will then be inserted as follows. A site on the dorsal aspect of the right forearm free of superficial veins will be selected. Entry and exit sites for the probe will be measured and marked with a marker 3 cm apart from each other. Scissors will then be used to carefully cut away arm hair around the site, avoiding as much skin irritation as possible. Alcohol and iodine will then be applied twice to skin in order to sterilize the entry and exit sites.

After the selected site is prepared and sterilized, a sterile intradermal microdialysis probe will be inserted through the selected entry and exit sites. The microdialysis probe itself will be manufactured and sterilized in the HPRC (Appendix B). To insert the probe, a 27-gauge needle will be inserted in the entry site marked on the skin, and passed 1-2 mm below the surface of the

skin, until it comes out at the predetermined exit site (3 mm from entry site). The sterile microdialysis probe will then be threaded through the needle, and the needle will then be removed, leaving the microdialysis probe entering and exiting the skin at the predetermined sites. This process will be repeated with a second probe, which will run parallel with the other, within 1-2 mm of the first probe. The hollow fiber of each probe will then be adjusted so that it is directly under the skin. Once each hollow fiber is properly situated, both probes will be perfused with sterile, 0.9% saline solution at a rate of 10 µl/min using a Harvard pump (Model PHD 2000, Harvard Apparatus, Holliston, MA) until blood flow recovers from the trauma induced by the needle. Once blood flow has recovered, the Harvard pump will be set to perfuse at a rate of 2 µl/min.

Immediately after commencing perfusion of the saline, a peltier module heated to 31°C will be placed over the site. A laser Doppler probe (Moor Instruments, Devon, England) will be inserted through a hole in the middle of the peltier module to monitor skin blood flow throughout the experiment. The site will be allowed to recover from the trauma of needle insertion for at least 90 minutes or until the SKBF has returned to a constant baseline level.

Heart rate (HR) and blood pressure (BP) will be measured at 5-minute intervals throughout the intervention using an automated blood pressure machine (Model STBP-780, Colin Medical Instruments, Komaki, Japan). The blood pressure cuff will be placed on the arm free of microdialysis probe, one inch above the antecubital space. HR will be measured by placing three electrodes on the chest of the subject. The first electrode will be placed at the midclavicular point below the right clavicle, while the second electrode will be placed at the midclavicular point below the left clavicle. The third electrode used to measure HR will be placed where the fifth intercostal space intersects with the midclavicular line of the left clavicle.

Procedures for Heating and Taking Sample of Dialysate

Once SKBF has stabilized after at least 90 minutes from the insertion of needles, several stages of heating and cooling will take place. Dialysate for each stage will be collected. The site will first be heated to a baseline temperature of 31°C for 8 minutes. A baseline temperature of 31°C has been selected, because it is a temperature that should not activate TRPV3 ion channels. Dialysate from both probes will be collected in the same container throughout the stage. Once the first stage of heating to 31°C has ended, the dialysate collected during that stage will be immediately frozen in liquid nitrogen and then stored at -80°C (Kendro Laboratory Products, Isotemp Basic, U86-25D35, North Carolina, USA) to minimize degradation of the contents of the dialysate.

Following the first stage, the temperature of the peltier module will be raised at a rate of 0.2°C/s until reaching a temperature of 35°C . The temperature will then be maintained at 35°C for 8 minutes while SKBF is measured and the dialysate is collected. After 8 minutes the temperature will be lowered back down to 31°C at a rate of 0.2°C/s. The temperature will then be maintained at 31°C for 16 minutes to allow the site to recover from the last stage. Similar protocols will be followed for 39°C and 43°C. However, the skin will be held at 43°C for 32 minutes in order to obtain peak SKBF and peak [ATP]. A more detailed diagram of the heating protocol is included below (Figure **1**).

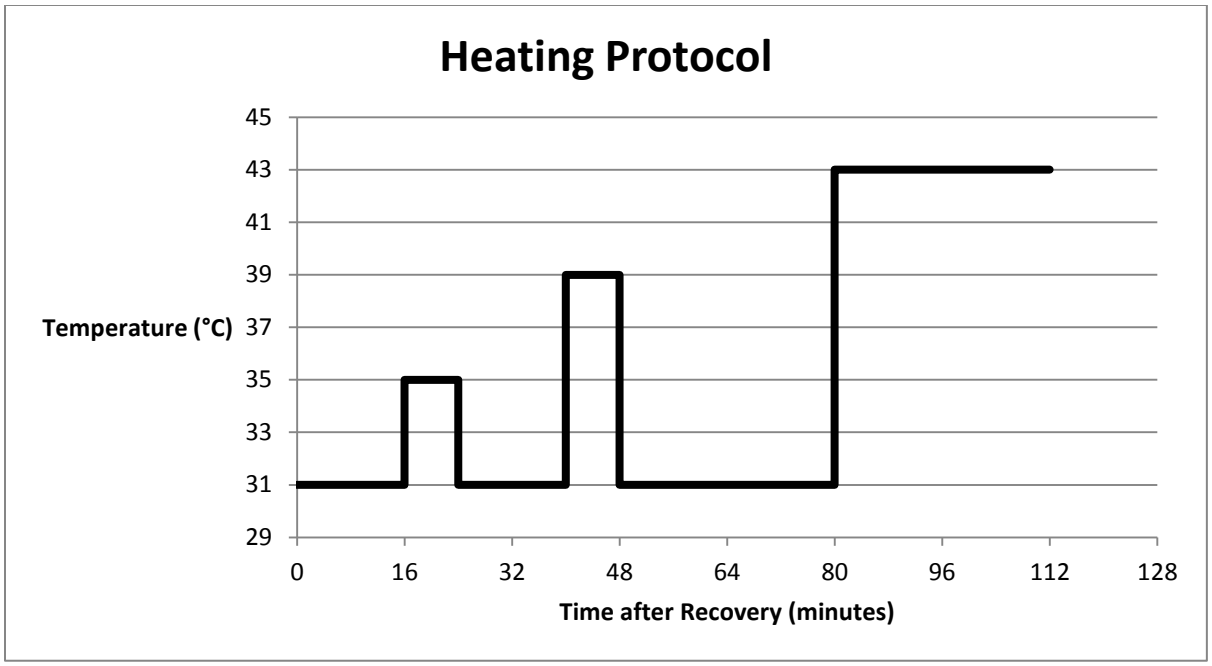


Figure 1: Description of Heating Protocol. When changing temperature, the temperature will be raised or lowered at a rate of 0.2°C/s. Dialysate will be collected and stored in 8-minute intervals to have the dialysate for each temperature in a separate container.

At the end of each heating phase, the subject will give a rating of the perceived sensation of heat associated with that temperature using a labeled magnitude scale devised by Green and associates (14) (Appendix C). For safety reasons, the subject will be equipped with an emergency shut-off switch that he or she can use to turn off the peltier heater if the temperature becomes painful at any point during the experiment.

Procedure of ATP Assay

The concentration of ATP in each dialysate sample will be measured using a luciferase-based ATP concentration determination kit (Enliten ATP Assay System. Promega. Madison, Wisconson, USA). In brief, 10µL of each dialysate sample will be placed in a well plate with 100 µL of luciferase reagent. Each well will be exposed to light for 10 seconds., and counts per second (CPS) for this complex will then be measured using a luminometer (Perkin Elmer, 1420 Multilabel Counter, Victor3, Finland). Using a standard curve, the counts per second for each

dialysate sample will be converted to concentration of ATP (Moles/Liter). The concentration of ATP in each dialysate sample will be measured in triplicate.

Data Collection and Statistical Analysis

All data from the peltier module, laser Doppler probe, and the automated blood pressure machine will be recorded with Powerlab—16 channel A/D converter (Powerlab/16 SP, ADInstruments Pty Ltd, Castel Hill, Australia) at 40 Hz using the computer program Chart (Chart v5.4.2 for Mac).

Major variables that will be measured are skin temperature (°C), interstitial ATP concentration (moles/liter) ([ATP]), heart rate (bpm), blood pressure (mmHg), and SKBF area under the curve (Volts). Pain scores will be reported as percentages of the strongest imaginable pain by dividing the distance of the pain rating from zero by the distance of the strongest imaginable label from zero Area under the curve (AUC) for SKBF at each temperature will be determined by taking the sum of second-by-second differences from the baseline value. Skin blood flow AUC will be converted to cutaneous vascular conductance (CVC) by using the following formula $CVC = \left(\frac{SKBF}{MAP}\right) * 100$. CVC will then be reported as a percent of the peak CVC observed when the skin is heated to 43°C. A rating of perceived heat pain for each heating phase will also be measured using a labeled magnitude scale developed by Green et al. (14)

Differences between mean CVC AUC and [ATP] for each temperature will be determined using repeated measures analysis of variance (ANOVA) followed by a Tukey minimum significant difference post hoc test. The relationships between CVC AUC, [ATP], and ratings from the labeled magnitude scale will then be investigated by using linear, and non-linear, least squares regression analysis. Statistical significance will occur when the p-values are less than 0.05.

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Appendix A: Health/Medical History Questionnaire

Health/Medical History Questionnaire

Thank you for volunteering to be a subject for a study to be conducted at the Human Performance Research Center at BYU. It is important that we have an accurate assessment of your present health status to assure that you have no medical conditions that would make the tests especially dangerous for you. *Please complete the health history questionnaire as accurately as you can.*

THIS MEDICAL HISTORY IS CONFIDENTIAL AND WILL BE SEEN ONLY BY THE INVESTIGATORS.

Name:_____ Date:_____

Date of Birth:_____ ☐ Male ☐ Female Present Age:_____yrs

Height:_____cm Weight:_____kg

Ethnic Group:
- ☐ White
- ☐ Black
- ☐ Hispanic
- ☐ Asian
- ☐ Pacific Islands
- ☐ American Indian
- ☐ Other_____

HOSPITALIZATIONS AND SURGERIES

If you have ever been hospitalized for an illness or operation, please complete the chart below. Do not include normal pregnancies, childhood tonsillectomy, or broken bones.

YEAR	OPERATIONS OR ILLNESS

Are you under long-term treatment for a protracted disease, even if presently not taking medication? ☐ Yes ☐ No

If yes, please explain:_____
_____
_____

Name:_____ Medical History, page 2

MEDICATIONS

Please list all medications that you have taken within the past 8 weeks (include prescriptions, vitamins, over-the-counter drugs, nasal sprays, aspirins, birth control pill, etc):

☐ Check this box if you have not taken any medications.

MEDICATION	REASON YOU ARE PRESENTLY TAKING THIS

ALLERGIES

Please list all allergies you have (include pollen, drugs, alcohol, food, animals, etc.):

☐ Check this box if you have no allergies

1._____
2._____
3._____
4._____

Have you been given an anesthetic by injection (for example at the dentist)? ☐ Yes ☐ No
If yes, did you have any adverse reactions? ☐ Yes ☐ No

PROBLEMS AND SYMPTOMS

Please place an "X" in the box next to any of the following problems or symptoms that you have had:

General

☐ Mononucleosis If yes, when _____
☐ Recent weight loss while not on a diet
☐ Recent weight gain
☐ Thyroid disease
☐ Fever, chills, night sweats
☐ Diabetes
☐ Arthritis
☐ Heat exhaustion or heat stroke
☐ Abnormal chest x-ray

Name:_____ Medical History, page 3

Problems and Symptoms (cont'd)

- ☐ Pain in chest (persistent and/or exercise related)
- ☐ Heart attack
- ☐ Coronary artery disease
- ☐ High blood pressure
- ☐ Rheumatic fever
- ☐ Peripheral vascular disease
- ☐ Blood clots, inflammation of the veins (phlebitis)
- ☐ Asthma, emphysema, bronchitis
- ☐ Shortness of breath ☐ At rest ☐ On mild exertion
- ☐ Discomfort in chest on exertions
- ☐ Palpitation of the heart; skipped or extra beats
- ☐ Heart murmur, click
- ☐ Other heart trouble Please explain_____
- ☐ Lightheadedness or fainting
- ☐ Pain in legs when walking
- ☐ Swelling of the ankles
- ☐ Need to sleep in elevated position with several pillows
- ☐ High cholesterol If yes, what was the last measured value?_____

G.I. Tract

- ☐ Eating disorder (e.g. anorexia, bulimia)
- ☐ Yellow jaundice If yes, when?_____
- ☐ Hepatitis If yes, when?_____
- ☐ Poor appetite
- ☐ Frequent indigestion or heartburn
- ☐ Intolerance of fatty foods
- ☐ Changes in bowel habits
- ☐ Persistent constipation
- ☐ Frequent diarrhea
- ☐ Rectal bleeding
- ☐ Unusually four smelling or floating stools
- ☐ Pancreatitis

G.U. System

- ☐ Get up at night to urinate
- ☐ Frequent thirst
- ☐ History of kidney stones, kidney disease

Name:_____ Medical History, page 4

Problems and Symptoms (cont'd)

Nervous System

- ☐ Alcohol Problem
- ☐ Frequent or severe headaches
- ☐ Stroke
- ☐ Attacks of staggering, loss of balance, dizziness
- ☐ Persistent or recurrent numbness or tingling hands or feet
- ☐ Episode of difficulty in talking
- ☐ Prolonged periods of feeling depressed or "blue"
- ☐ Difficulty in concentrating
- ☐ Suicidal thoughts
- ☐ Have had psychiatric help

Musculoskeletal

- ☐ Recent soft tissue injury (deep bruises, charley horse, muscle strain)
- ☐ Recent or chronic joint sprain If yes, which joint(s)?_____
- ☐ Unstable joint If yes, which joint(s)?_____
- ☐ Limited range of motion If yes, which joint(s)?_____
- ☐ Patellofemoral syndrome
- ☐ Rheumatoid arthritis
- ☐ Ankylosing spondylothesis
- ☐ Back or neck pain, sacroiliac pain
- ☐ Pain radiating from back down limbs

Please give details for any items checked (when, severity, treatment):

_____
_____
_____
_____
_____
_____
_____

Other

Have you ever passed out during or after exertion? ☐ Yes ☐ No

Do you have a family history of coronary artery disease? ☐ Yes ☐ No

If yes, who? _____

Name:_____ Medical History, page 5

Do you use (complete the information if your answer is yes):

Alcohol	☐ Yes	☐ No	Drinks per week_____
Caffeine	☐ Yes	☐ No	Drinks per day (coffee, cola, tea, etc.)_____
Tobacco	☐ Yes	☐ No	Cigarettes ____ per day____ Pipe ____ per day____ Cigars ____ per day____

****

I, _____, have completed this medical/health history questionnaire honestly and completely as possible.

_____ _____
Subject Signature Date

Reviewed and approved for participation:

_____ _____
Principal Investigator Date

_____ _____
Examining Physician (if needed) Date

Appendix B: Instructions for Microdialysis Probe Construction

Instructions for Microdialysis Probe Construction

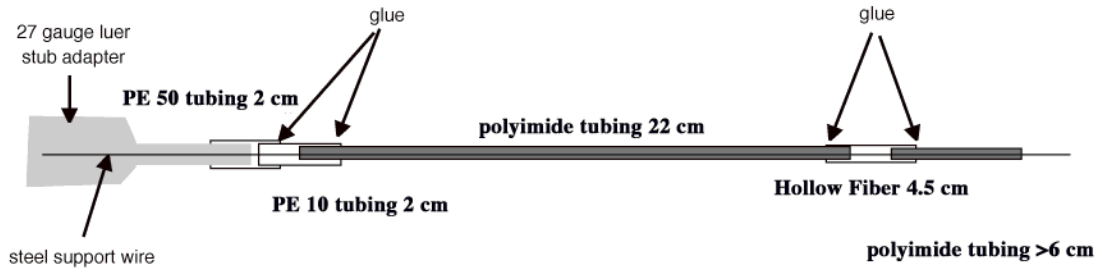
Supplies required.

1. Steel wire- 0.002 in spring temper stainless steel wire – Alan Baird Ind. Ho-Ho-Kus, NJ, special order item - ≈ $400-500/spool of 3000 ft
2. Polyimide tubing – OD = 0.0045in ID, 0.00050 in wall thickness, Catalog # 823400, A-M Systems, Inc
3. ***Hollow fiber dialysis membrane – Spectra-Por regenerated cellulose microdialysis hollow fibers, OD = 170µm, ID = 150 µm, Cat # 132 295, $70.98 per pkg
4. PE 10 tubing –
5. PE 50 tubing
6. 25 gauge luer stub adapter
7. Super glue – 5
8. Super glue - GEL

*** hollow fibers storage requirements: place package of hollow fibers in a sealed (ziplock) bag store in cardboard box in 4^{o}C refrigerator to prevent drying of the glycerol

Equipment require for construction

1. cm ruler
2. fine scissors
3. Tweezers with 90^{o} tip
4. Surgical Blade
5. 1 cc syringe w/ 27 g needle (for application of glue to connections)
6. Dissecting microscope with wide angle lens and 10x objectives



Instructions:

1. Cut individual pieces to the following lengths:

**** HINT** cut polyimide tubing on an angle with surgical blade to allow for easy insertion into hollow fiber membrane**

Steel wire	45 cm	
Polyimide tubing (dialysate side)	6 cm	
Polyimide tubing (perfusion side)	22 cm	
PE 10 tubing	2 cm	
PE 50 tubing	2 cm	
Hollow fiber	4.5 cm	**note: cut as used, do not leave hollow fibers exposed to air in order to prevent drying of glycerol

2. String PE 50, PE10 and 6cm polyimide tubing on steel wire and connect using the aid of the dissecting microscope (if necessary) (PE 10 should fit tightly into PE50, insert up to 1cm, and insert the polyimide ~1 cm into the PE10).
3. Place a drop of glue onto a piece of cardboard paper (it will sit and thicken for a few minutes before use).
4. Adhere the tubing at each junction using the needle tip to apply the "thickened" glue (capillary action will disperse the glue in between the 2 pieces of tubing, view this using the dissecting microscope to ensure glue doesn't perfuse past the end of the inner tube)
   ** use super glue GEL for the PE 10 - PE50 junction; it is very tight and the gel will not disperse in between the 2 tube, it will just create a seal on the outside.
   GEL can also be applied to the outside of the junction between the PE 10 and polyimide to reinforce the seal.

Note: Make the first half of 5 or 6 probes and then add the remaining pieces. This allows less air exposure to the hollow fiber and while the glue is drying it allows you to continue working. However, you may like to make one probe at a time - that is fine.

5. Remove hollow fibers from the sealed bag and cut into 4.5 cm pieces (only cut the number you will need, ie. if you are making 6 probes cut 6 pieces).
6. String the hollow fiber and polyimide tubing onto the steel wire
7. Connect the hollow fiber to the polyimide tubing on both sides with the help of the dissecting microscope. Work on a white surface that has a 2.5-cm long line within the visual field.
8. Push each piece of polyimide tubing approximately 1cm into the hollow fiber
9. Put a drop of glue on to a piece of paper.
10. Place connected pieces on 2.5 cm line and begin to withdraw polyimide tubing from hollow fiber membrane. Note the mid point and retract equal amount on both sides until precisely 2.5 cm of hollow fiber membrane is present.
11. CAREFULLY drape the probe over tweezers (to elevate junctions for gluing).
12. "Dab" the 27 g needle into the glue (super glue-5) to get a small drop onto the end.
13. Glue the polyimide tubing to the hollow fiber tubing by allowing a small drop of glue to touch the junction (this is the site where the outer hollow fiber is seen over the polyimide tubing).
14. Watch to see the glue creep, by capillary action into the joint.

*** make sure glue doesn't creep up too far and close off the end of the tube, also be very careful not to get any glue on the outside of the 2cm window of hollow fiber

(this will clog the pores and the probe will be unusable).

15. Wipe off the tip of the needle. I also scrape the needle off with a razor blade to clean off dried glue (when necessary).
16. Blot off any excess glue using the cleaned needle tip.
17. Repeat gluing procedure on the other joint. AVOID moving probe!
18. Allow glued joints to dry for 5 minutes minimum.
19. Insert a 23 gauge luer stub adapter into the PE 50 tubing. You should no more than 0.5 cm of PE 50 tubing between the luer stub end and the PE 10 tubing
20. Place entire probe inside a sterilization bag and seal. Be very careful handling the probe.
21. Label the bag with the date and the length of the exposed hollow fiber (ie. 2.5 cm)
22. Finally, gas sterilize the probes

Appendix C: Labeled Magnitude Scale of Sensation of Heat (Green et al. 1993)