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ABSTRACT

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The purpose of this study was to characterize a novel technique to study neurogenic control of cutaneous vasodilation. We monitored skin blood flow (SkBF) during in-vivo electrical stimulation (e-stim) intended to activate cutaneous nerves and used intradermal microdialysis to deliver receptor antagonists to characterize their contribution to cutaneous vasodilation. We examined the role of acetylcholine receptors (R_{ACh}), nitric oxide (NO), and vasoactive intestinal peptide receptors (R_{VIP}) on the cutaneous vasodilation induced by e-stim in the absence of the sympathetic adrenergic nervous system. Six men and three women participated in the study. Three intradermal microdialysis probes were placed in the skin of the dorsal side of their forearm. The adrenergic nervous system was eliminated by delivery of a cocktail of phentolamine (0.01 mg/ml), propranolol (1 mM), and BIBP-3226 (10 μM). At one skin site atropine (0.1 mg/ml) was delivered to block R_{ACh}. At a second site we blocked nitric oxide synthase (NOS, 10 mM L-NAME) and $R_{ACh.}$ Finally at the third site, we blocked $R_{ACh,}$ NOS, and R_{VIP} (0.47 mg/ml VIP_{10-28}). The SkBF response to 1 minute stages of graded increases in frequency (0.2, 1, 2, 4, 8, and 32 Hz) at a current of 1.0 ± 0.1 mA was used to generate a stimulus-response curve before and after drug delivery. At skin site 1 R_{ACh} blockade decreased the area under curve (AUC) by 4% from 614 ± 279 to 591 ± 331 ($p>0.05$). Nitric oxide synthase and R_{ACh} blockade reduced the vasodilator response to e-stim by 23% from 1036 ± 457 to 801 ± 448 AUC ($p>0.05$). Combined NOS, R_{ACh}, and R_{VIP} blockade reduced the vasodilator response by 48% from 802 ± 412 to 418 ± 268 AUC ($p=0.07$). R_{ACh} blockade had no effect on the vasodilator response to e-stim. However, in these preliminary studies both NOS and R_{VIP} blockade provided some attenuation of the cutaneous vasodilator response associated with e-stim. Additional studies will focus on these two neurotransmitters as this novel method is refined. Advantages of e-stim include activating the sympathetic nervous system without activating local and humoral factors and studying neurotransmitters in an in-vivo setting during rest, thermal stress, or exercise.

Keywords: Cutaneous circulation, microdialysis, sympathetic, adrenergic, vasodilation, intradermal, nitric oxide synthase

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Introduction

Adjustments in the cutaneous circulation play a vital role in the regulation of body temperature during heat stress. The change in cutaneous circulation during heat stress is controlled by both local and reflex control systems (6). The local response relies on paracrine release of vasoactive substances from either the vascular endothelium lining (8) and/or other skin cells, such as keratinocytes (4). In addition, the local response may involve an axon reflex mediated release of neuropeptides following the activation of a cutaneous nociceptor (16). The reflex response is mediated by baro- and thermoceptor afferents (8) via activation of the sympathetic nervous system.

Many of the neurochemicals and paracrine substances involved in these local and reflex responses have been identified. The sympathetic nervous system controls skin blood flow primarily by the release of norepinephrine (NE), and its effects on alpha adrenergic receptors causing contraction of vascular smooth muscle (2, 10). However, sympathetic adrenergic nerves also release co-transmitters such as neuropeptide Y (19). Active cutaneous vasodilation is mediated by a sympathetic cholinergic nerve (18). Most scientists believe that the cutaneous sympathetic vasodilator system releases acetylcholine (ACh) and the co-transmitter, vasoactive intestinal peptide (VIP), which evokes cutaneous vasodilation (1, 11, 15). However, ACh receptor (R_{ACh}) and VIP receptor (R_{VIP}) blockade during heat stress attenuates, but does not abolish, cutaneous vasodilation (1). Thus, other neurochemicals must also be involved in mediating active cutaneous vasodilation. Nitric oxide (NO) is a likely candidate. Local heating produces NO in the cutaneous interstitial space (12), and blocking NO production during local heating attenuates the increase in skin blood flow (9). Recently, Kellogg et al. (13) demonstrated that neuronal nitric oxide synthase (nNOS) is involved the cutaneous dilation associated with

local heating. While this finding is novel and intriguing, recent work of Stewart, Medow, Minson and Taneja (20) failed to confirm this observation. It is likely that the complex nature of the homeostatic response to whole body heating, exercise, or even local skin heating may limit our ability to differentiate the role of various neurochemicals in the regulation of skin blood flow (5).

The current study proposes a novel method to investigate neurogenic control of cutaneous vasodilation. This method involves the placement of two thin stainless steel needles 2 mm deep into the skin to directly depolarize cutaneous nerves thereby producing the release of their respective neurochemicals. By combining cutaneous nerve stimulation with local receptor blockade using intradermal microdialysis and local monitoring of SkBF with laser Doppler velocemitry, we hope to characterize the contribution of various neurochemicals on skin blood flow control. Specifically, these series of experiments describe the cutaneous vascular responses to intradermal electrical stimulation following 1) adrenergic receptor blockade 2) combined adrenergic receptor and R_{ACh} blockade 3) combined adrenergic, R_{ACh} blockade, and non-selective inhibition of nitric oxide synthase (NOS) and 4) combined adrenergic, R_{ACh} blockade, NOS inhibition, and R_{VIP} receptor antagonism.

Methods

Nine subjects participated in this study, 6 men and 3 women, with an average age of 23 ± 1 years (1 SEM), height of 173.5 ± 3.6 cm, and weight of 74.6 ± 3.8 kg. All subjects were free of cardiovascular, renal, or metabolic diseases and abstained from alcohol, caffeine, and exercise for twelve hours prior to testing. Female subjects were tested during the first seven days of their menstrual cycle. Written informed consent was obtained before participating in the study which was approved by the Brigham Young University Institutional Review Board.

Upon arrival, subjects entered the environmental chamber (room temperature 28.2 ± 0.1 °C) and were fitted with a water-perfused suit that covered the entire body except the head, feet, hands, and right forearm. The subject then sat upright in a dental chair and was instrumented with ECG electrodes and a non-invasive automated brachial artery blood pressure cuff (Model STBP-780, Colin Medical Instruments, Komaki, Japan) on the left arm. The water circulating through the suit was set at 34 °C throughout the experiment. Next, without anesthesia, three intradermal microdialysis probes were placed 3-4 cm from the elbow at a depth of 1-2 mm in the skin of the dorsal aspect of the right forearm using a 27 gauge needle as a guide cannula. Approximately 4-5 cm separated each probe and sterile saline was perfused through the probes at 10 µl/min for the next 120 minutes to allow the skin to recover from the insertion trauma.

Specially made Plexiglas probe holders were placed directly over the path of the hollow fiber portion of the microdialysis probe using double-stick tape. A laser Doppler flow probe placed in the middle guide tube (directly over the microdialysis probe) monitored changes in skin blood flow (SkBF) using a VP12 flow probe connected to a moorLAB laser Doppler system (time constant set at 1.0 sec, sampling rate set at 20 Hz) (Moor Instruments, Devon, England) . On either side of the laser Doppler probe holder were two guide tubes where the stimulating electrodes (stainless steel acupuncture needles) were inserted 2 mm deep into the skin with a tip separation of < 4 mm. The stimulating electrodes were then connected to a constant current stimulator (Model DS7AH, Digitimer Ltd., West Garden City, England) whose frequency was driven by a Grass S88 stimulator (Model Grass S88 Stimulator, Grass Instrument Co., Quincy, MA). A thermocouple was placed between the Plexiglas probe holder and skin to measure local skin temperature.

The amplitude of the electrical current that activated the perivascular nerves surrounding the cutaneous blood vessels and elicited a vasomotor response was determined individually for each skin site. Initially, single pulses of the electrical stimulus were sent to the skin starting at 0.5 mA. The stimulus, when detected, typically felt like a "tapping" sensation on the skin. If the subject could not detect the stimulus pulse, the current level was increased by 0.1 mA until the stimulus was detected. If the stimulus was initially felt at a stimulus strength of 0.5 mA, the current was decreased 0.1 mA until the stimulus was not detected. A current level 0.1 mA above where the stimulus was not detected was set as the initial current stimulus. At this pre-determined current level, the stimulus frequency was then set at 8 Hz and the current increased 0.2 mA every 20 seconds until a significant change (increase or decrease) in SkBF occurred. A significant change in SkBF was defined as ± 0.5-0.7 V change in the voltage output from the laser Doppler system. After a five minute recovery period, the selected amplitude was retested by applying a minute stimulus at a frequency of 8 Hz to confirm that a significant change in SkBF occurred. If the response was not confirmed then the current amplitude was increased 0.2 mA and retested five minutes later. This method was used for all three sites to identify the current amplitude to be used at each skin site.

Based upon the current intensity that elicited a significant change in SkBF, we produced stimulus-response curves for each skin site by plotting SkBF as a function of the stimulus frequency. To generate the stimulus response curve we used a graded stepwise increase in stimulus frequency (0.2, 1, 2, 4, 8, and 32 Hz) with each step lasting 1 minute (Figure 1). After the last stimulus at each site, the subject rated the pain of the stimulus on a 11-point modified Borg scale from 6-16 with 6 indicating no pain at all and 16 indicating unbearably painful (14).

Experimental Protocol

The experimental protocol is outlined in Figure 2. Sixty minutes following the determination of the electrical current amplitude that elicited a significant change in SkBF, a cold challenge test (CCT) was administered to verify sympathetic mediated cutaneous vasoconstriction. The water circulating through the water-perfused suit was rapidly switched to a cold water bath (4 °C), and the subject was cooled for 3 minutes. SkBF was recorded continuously and blood pressure was recorded once every minute. Fifteen minutes after the end of the CCT the first dose response curve was performed. Blood pressure was measured every minute during the CCT and every seven minutes during remaining portions of the experimental protocol.

After performing the initial stimulus response curve, each skin site was treated with a cocktail of blocking agents. Every drug cocktail contained a basic mixture of adrenergic receptor blockers phentolamine (0.01 mg/ml) and propranolol (1 mM) and the neuropeptide Y receptor antagonist BIBP-3622 (10 µM). In addition to the adrenergic blocking combination, one drug cocktail contained atropine (0.1 mg/ml), one contained atropine (0.1 mg/ml) and nitro-L-arginine methyl ester (10 mM L-NAME), and one contained atropine (0.1 mg/ml), L-NAME (10 mM) and VIP_{10-28} (0.47 mg/ml). Each cocktail was delivered for 30 minutes at 3 µl/min using a Harvard infusion pump (Model PHD 2000, Harvard Apparatus, Holliston, MA). The drug cocktails were randomly assigned to different skin sites. Following the 30 minute perfusion period a second set of stimulus-response curves at each skin site was performed. Fifteen minutes after the last stimulus-response curve, a second CCT was performed to verify adrenergic blockade. Finally, 28 mM sodium nitroprusside (SNP) was perfused at 5 µl/min for 30 minutes to determine the maximum skin blood flow at each site.

Data Analysis

SkBF and local skin temperature were recorded continuously at 400 Hz using a PowerLab 16S (ADInstruments Pty Ltd., Castel Hill, Australia). The SkBF and skin temperature data were then averaged every 5 seconds for analysis. Cutaneous vasomotor response to the electrical stimulus is described by changes in cutaneous vascular conductance (CVC). Cutaneous vascular conductance was calculated by dividing the voltage output of the laser Doppler flow probe by the average mean arterial blood pressure. Cutaneous vascular conductance was expressed as a percent of maximal CVC obtained during administration of 28 mM SNP. During each graded stepwise increase in stimulus frequency the CVC response was characterized by the area under the CVC–time response curve (AUC) for each minute stimulus period. In the calculation of AUC, CVC was expressed as a percent of the CVC measured just prior to electrical stimulation (Figure 1).

The data were analyzed using a general linear model repeated measures ANOVA using SAS (SAS 9.2) with post hoc comparisons between groups performed using Tukey's MSD test. The stimulus response curves were also analyzed to determine the EC_{50} threshold and saturation level using non-linear sigmoidal dose-response curve fit (Prism, GraphPad Software Inc, San Diego, CA).

Results

Preliminary studies

Electrical stimulation of the skin produced two basic vascular responses: constriction or dilation (Figure 1). In preliminary experiments we verified that we were activating sympathetic nerves in the skin by using a biological assay. In this case we monitored local sweat production during similar stimulus conditions. Figure 3 illustrates the ability of our stimulus parameters to

activate sweating in a dose dependent manner. This sweating response is abolished by atropine and thus represents the effects of the stimulus to depolarize sympathetic sudomotor nerves and cause the release of ACh leading to sweat production.

Additionally, preliminary studies demonstrated that use of our graded staircase stimulus pattern did not bias our results. In these preliminary studies we compared the impact of performing each one minute frequency stimulation in random order spaced five minutes apart versus our staircase approach. The data shown in Figure 4 indicates identical stimulus-response curves for both experimental approaches. As such, we used the shorter staircase stimulus to evaluate the CVC response.

Figure 5 shows the time course of SkBF during the cold challenge test before and after perfusion of our adrenergic blocking drug cocktail. CVC levels dropped by 16 ± 3 % ($p<0.05$) during the cold challenge prior to blockade and decreased by 9 ± 2 % after adrenergic blockade. After review of each skin site response to CCT, it appeared that less than 50% of the sites demonstrated adequate adrenergic blockade: Atropine site, n=3; Atropine and L-NAME site, n=4; Atropine, L-NAME, and VIP_{10-28} site, n=6.

Figure 6 describes the average area under the CVC-time curve (AUC) for each stimulus frequency before (saline) and after each blocking cocktail. Before infusion of atropine, the highest stimulation frequency of 32 Hz caused AUC to increase to an average of 614 ± 279. After infusion of atropine, AUC increased to an average of 591 ± 331. Infusion of atropine and L-NAME appeared to decrease dilation, compared to saline, by 23% from 1036 ± 457 to 801 ± 448 AUC ($p>0.05$). Infusion of atropine, L-NAME, and VIP_{10-28} decreased dilation by 48% from 801 ± 412 to 418 ± 268 AUC ($p>0.05$). The stimulus-response curves for the three saline sites were similar and therefore pooled together to provide an average response (Figure 7). The

AUC for CVC increased significantly from 0.2 Hz at 2, 4, 8, and 32 Hz in the saline and atropine sites indicating dilation. In contrast, the AUC for CVC did not increase significantly with varying frequencies in the atropine & L-NAME or atropine, L-NAME, & VIP_{10-28} sites indicating no significant dilation. Calculated EC_{50} levels from the stimulus-response curves are shown in Figure 8. The EC_{50} averaged 3.1 ± 1.1 Hz during saline infusion and was unchanged during atropine infusion averaging 3.2 ± 1.2 Hz. Infusion of atropine and L-NAME causes a small increment in the EC_{50} to 5.6 ± 1.2 Hz while infusion of atropine, L-NAME, and VIP_{10-28} increased the EC_{50} to 6.1 ± 1.5 Hz (p=0.08).

Table 1 lists the average skin temperature, pain, and current amplitude for each of the three skin sites. There were no differences in these parameters for any skin site. Overall, the average of all three sites for skin temperature, rating of perceived pain, and current amplitude was 32.9 ± 0.2 °C, 7.8 ± 0.6 (on a 6-16 point scale), and 1.0 ± 0.1 mA, respectively.

Discussion

We developed a method of in-vivo electrical stimulation (e-stim) in an attempt to provide insight into the neural control of SkBF. This preliminary study provides compelling evidence that intradermal e-stim is a viable method to evaluate neurovascular control of SkBF. The first novel observation was that blockade of R_{ACh} with atropine had no impact on the magnitude of cutaneous vasodilation associated with e-stim. Combined blockade of R_{ACh} and NOS activity reduced dilation by 23% compared to no blockade. Although this difference was not statistically significant it suggests a small contribution of NO to this dilator response. This may be physiologically significant in the fact that NO's ability to reduce SkBF by 23% may have some effect in producing sufficient dilation to release heat from the body during thermal stress or exercise.

Finally, R_{VIP} inhibition produced a greater attenuation (about 48%) of the CVC response to e-stim. Although the attenuated response was not statistically significant, the reduction of dilator response with R_{VIP} blockade is of interest. A decrease in almost 50% of CVC demonstrates that nearly half of the vasodilation occurring in skin may be contributable to ACh, NO, and VIP. Furthermore, VIP blockade nearly doubled the decrease in SkBF compared to NOS blockade. Physiologically, VIP may play a vital role in vasodilation. Increasing the power of the study by adding more subjects could help to identify a greater role for NO or R_{VIP} in this e-stim mediated dilation.

When performing our preliminary tests, we did verify that both sympathetic adrenergic and cholinergic nerves were activated by the e-stim. Blocking the release of sympathetic adrenergic neurotransmitters with bretylium tosylate completely eliminated the vasoconstrictor response; in fact, the SKBF response was transformed into dilation (Figure 10). Also, the e-stim protocol depolarized sympathetic sudomotor nerves causing the sweat rate to increase (Figure 3).

Another interesting observation was the large variability in SkBF response to e-stim at each site. One possible explanation for this observation is the great variability in C-nociceptor fiber innervation density of the skin. Our previous observations of activation of the sympathetic nervous system with e-stim suggest that our stimulus parameters were appropriate to activate sympathetic C-nociceptor fibers in the skin. Schmidt et al. (17) discovered many different sizes and shapes of mechanically activated C-nociceptor innervation territories. Activation of these C-nociceptor fibers during e-stim could potentially release vasodilator substances. During this study involving e-stim, sites expressed either an increase or decrease in SkBF with e-stim. Sites that expressed an increase in SkBF may have contained more of these C-nociceptor fibers than

sites that expressed a decrease in SkBF. The great variation in size and shape of C-nociceptor innervation territories could explain why we had varying results.

In-vivo electrical field stimulation may increase the release of VIP, a cutaneous vasodilator. Recently, Kellogg et al. (7) demonstrated that VIP was involved in cutaneous vasodilation. Bennett et al. (1) also found similar findings. Bennett et al. (1) used intradermal microdialysis probes to infuse VIP and VIP_{10-28}, a R_{VIP} antagonist, into human skin. During heating, the site with VIP_{10-28} alone caused blood flow to increase to around 27% of maximum blood flow. With VIP_{10-28} and atropine, blood flow levels increased to around 20% maximum blood flow. The blood flow in the control site increased to 35% of maximum blood flow (1). The findings of Bennett et al. provide evidence that VIP contributes to active cutaneous vasodilation associated with heat stress. Interestingly, Bennett et al. noted that when R_{VIP} blockade was combined with R_{ACh} blockade that cutaneous vasodilation was reduced from 35% max CVC (R_{ACh} blockade) to 20% max CVC (R_{VIP} blockade and R_{ACh} blockade). In the present study, we saw a 48% decrease in AUC when we delivered VIP_{10-28} supporting the work of Bennett et al., but we saw no added impact when combined with R_{ACh} blockade. The interpretation of our results is limited due to lack of statistical significance.

Because Bennett et al. (1) did not observe a complete elimination of dilation with VIP and atropine, we hypothesized that NO may have enabled the cutaneous vasodilation during heating. The importance of NO in increasing blood flow has clearly been demonstrated in previous studies. Kellogg et al. (9) inserted intradermal microdialysis probes in two sites in the forearm of subjects and perfused one site with nitro-L-arginine methyl ester (L-NAME) which is a nonselective blocker of nitric oxide synthase (NOS), and the other site was perfused with Ringer solution. In the untreated site when local skin temperature was raised to 38-38.5 °C, blood flow

rose to 44% of maximum blood flow and in the L-NAME site blood flow rose to 30% maximum blood flow (9). By blocking NOS, blood flow did not increase similarly to blood flow in skin with unblocked NOS. Nitric oxide production is clearly needed during local heating to induce increases in skin blood flow.

Recent evidence suggests that neuronal nitric oxide synthase (nNOS) produces the NO that causes reflex vasodilation during whole-body heating. Kellogg et al. (13) used 7-nitroindazole (7-IN), a selective antagonist of nNOS, to study nNOS involvement in thermoregulatory reflex vasodilation. One site was infused with 5% DMSO and Ringer solution, while the other site was infused with 2 mM 7-NI with 5% DMSO. In one protocol subjects core temperature was elevated by 1°C using a whole-body suit with the forearm being uncovered. In another protocol subject's forearms were locally heated to 41.5 °C. In the whole-body heating protocol, 7-NI significantly decreased skin blood flow when compared to the control site by 15%. However, in the protocol with local skin warming, no significant difference was seen between the 7-NI site and control site (13). Kellogg et al.'s findings show that nNOS is involved in the thermoregulatory reflex response during whole-body heating due to the attenuation seen with the blockade of nNOS.

When examining the role of NOS during e-stim we noted a small (23%) reduction in cutaneous vasodilation. The lack of significance might indicate that NO is not typically released in response to depolarization of nerves. Because e-stim did produce a small change in dilation with blockade of NO, nNOS may contribute to a small portion of cutaneous vasodilation as proposed by Kellogg et al. (13), but this has yet to be confirmed (20). Clearly, further refinement of our e-stim protocol is required to address relatively small effects of NOS on our cutaneous vasodilator response to e-stim. Our results also indicate the lack of complete blockade

of vasodilation during e-stim following infusion of ACh, NOS, and VIP antagonists. The continued presence of vasodilation during e-stim may be due to some other substance or substances. Possible vasodilator substances released from cells could have been prostanoids or histamine.

E-stim may have activated nociceptor fibers which release neuropeptides such as calcitonin gene-related peptide (CGRP) and substance P via an axon reflex. One way to indicate if these nociceptor fibers were activated is to determine the perceived pain felt by the subject during the stimulus. Subjects rated pain on a scale of 6-16 with 6 indicating no pain at all and 16 indicating unbearable pain. The average pain rating was between 7-8 for each site. Despite the low pain ratings, the nociceptors could have still been activated. In preliminary testing there was no significant axon reflex increase in SkBF during e-stim with the current levels or frequencies used. As such, we have no evidence that our stimulus parameters activated cutaneous nociceptors thereby eliciting an axon reflex. Furthermore, 5-10 minutes after e-stim SkBF returned to baseline levels (Figure 9). Generally, the activation of nociceptor fibers cause the release of neuropeptides such as CGRP which produce a large and prolonged vasodilation (3).

These observations indicate that CGRP and substance P were most likely not important factors causing the dilation seen during ACh, VIP, and NOS blockade. In preliminary studies the desensitization of nociceptors with chronic capsaicin application had little impact on the SkBF response to e-stim (Figure 11). In addition, blockade of the CGRP receptor with the CGRP (8-37) had little impact on the SkBF response to e-stim (Figure 11). However, these substances were not blocked in the current study and future studies with e-stim are required to verify this assumption.

Several key limitations existed in this study. Figure 3 shows poor adrenergic blockade. Following the administration of adrenergic blocking agents, SkBF decreased significantly from baseline levels during the cold challenge test indicating a poor adrenergic blockade. In addition, we were not confident that the CCT provided an adequate evaluation of adrenergic blockade. If the SkBF levels were too low before the CCT, then a false positive result would be observed indicating sufficient adrenergic blockade when in fact sufficient adrenergic blockade was not present. Our results in Figure 3 could be due to an insufficient concentration of the adrenergic drug cocktail. In follow-up work with higher concentrations of phentolamine, we noted a much more pronounced attenuation of the CCT. We recommend that future studies focus first on determining a strong enough concentration of phentolamine to produce an adequate adrenergic blockade. Second, low resting SkBF levels appeared to limit the effectiveness of the CCT response. When resting SkBF levels were low we could identify very little change in SkBF during the CCT regardless of whether adrenergic blockade was adequate. More recently, we have used the delivery of 6.3×10^{-6} M norepinephrine to the skin to evaluate adrenergic blockade. We recommend that future studies use this technique to verify adrenergic blockade.

In summary, in the absence of a functioning sympathetic adrenergic system, intradermal e-stim produced a graded dilation proportional to the frequency of a non-painful stimulus. Blocking of R_{ACh} by itself did not blunt the dilatory response seen by e-stim. In contrast, we noted sufficient attenuation of the dilator response (>23%) following NOS or R_{VIP} blockade to suggest that a refinement of this technique could identify the contribution of specific neurogenic vasodilator substances to active cutaneous neurogenic vasodilation. These data indicate the possible contribution of NOS and/or VIP in regulating vasodilation in the cutaneous circulation. Future studies using e-stim to identify specific neurogenic vasodilator substances may need a

greater concentration of blocking agents or other receptor systems may need to be removed such as nociceptors (with capsaicin) or prostanoids (with COX inhibitors). Several advantages of this technique will arise as this method is refined. One advantage of using in-vivo electrical field stimulation is the activation of the sympathetic nervous system without interacting with various humoral factors that are released during either exercise or whole body heating. Another potential advantage of this technique is the ability to activate the cutaneous sympathetic nerves in human skin under a variety of conditions to evaluate its interaction with other homeostatic control mechanisms.

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Figure Legends

Figure 1. Representative tracings of the cutaneous vascular conductance (CVC) responses to intradermal stimulation (top) and the dose-response curves generated from these data (bottom).

Figure 2. Timeline of Experimental Protocol. Each skin site was perfused with a cocktail of adrenergic blocking agents containing 0.01 mg/ml phentolamine, 1 mM propranolol, and 10 μM BIBB-36220. Each of the three solution (C through E) represents the combination of the adrenergic blocking cocktail and a blocking agent for a suspected dilator substance: Sol. C = 0.1 mg/ml atropine; Sol. D = 0.1 mg/ml atropine and 10 mM nitro-L-arginine methyl ester (L-NAME); and Sol. E = 0.1 mg/ml atropine, 10 mM (L-NAME), and 0.46 mg/ml Vasoactive Intestinal Peptide (VIP_{10-28}). SNP = 28 mM sodium nitroprusside, EFS = intradermal electrical field stimulation, CCT = cold challenge test.

Figure 3. Impact of graded levels of intradermal stimulation on local sweat rate.

Figure 4. Comparison of dose-response curves generated by a random application of 1 minute intradermal stimulation spaced 5 minutes apart and a graded staircase stimulation from 0.1 to 32 Hz at identical current intensity. Values represent the mean and SE of 4 subjects.

Figure 5. Change in cutaneous vascular conductance (CVC) expressed as a % of baseline during the three minute cold challenge test with and without the presence of the adrenergic blocking cocktail of 0.1 mg/ml phentolamine, 1 mM propranolol, and 10 μM BIBB-36220. Values represent the mean ± 1 SEM for n=27 skin sites. * $p<0.05$ Adrenergic Blockade different from Saline. † $p<0.05$ different from time = 0.

Figure 6. Staircase response curves before and after solution treatments. Area under the skin blood flow–time response curve (AUC) where skin blood flow is expressed as a percent of resting blood flow prior to electrical stimulation. The dose response curve therefore reflects the plot of stimulus frequency versus the AUC for skin blood flow.

Figure 7. Frequency effect on SkBF during in-vivo electrical field stimulation. Area under the SkBF–time response curve (AUC) where SkBF is expressed as a percent of resting blood flow prior to electrical stimulation. * $p<0.05$ different from 0.2 Hz.

Figure 8. Effects of vasodilator antagonists on the EC_{50} stimulus frequency for the area under the CVC–time response curve (AUC) where CVC is expressed as a percent of resting CVC just prior to electrical stimulation. EC_{50} represents the estimated frequency to produce 50% of the peak dilation at 32 Hz.

Figure 9. Example of SkBF recovery in one site from in-vivo electrical field stimulation. SkBF returns back to baseline levels 5-10 minutes after stimulus.

Figure 10. Effect of 100 mM bretylium tosylate on the skin blood flow response to e-stim. Area under the SkBF–time response curve (AUC) where SkBF is expressed as a percent of resting blood flow prior to electrical stimulation. The dose response curve therefore reflects the plot of stimulus frequency versus the AUC for skin blood flow.

Figure 11. The effect of chronic topical application of capsaicin or microdialysis delivered calcitonin gene-related peptide fragment CGRP (8-37) on SkBF during e-stim. Area under the SkBF–time response curve (AUC) where SkBF is expressed as a percent of resting blood flow prior to electrical stimulation.

Figure 1

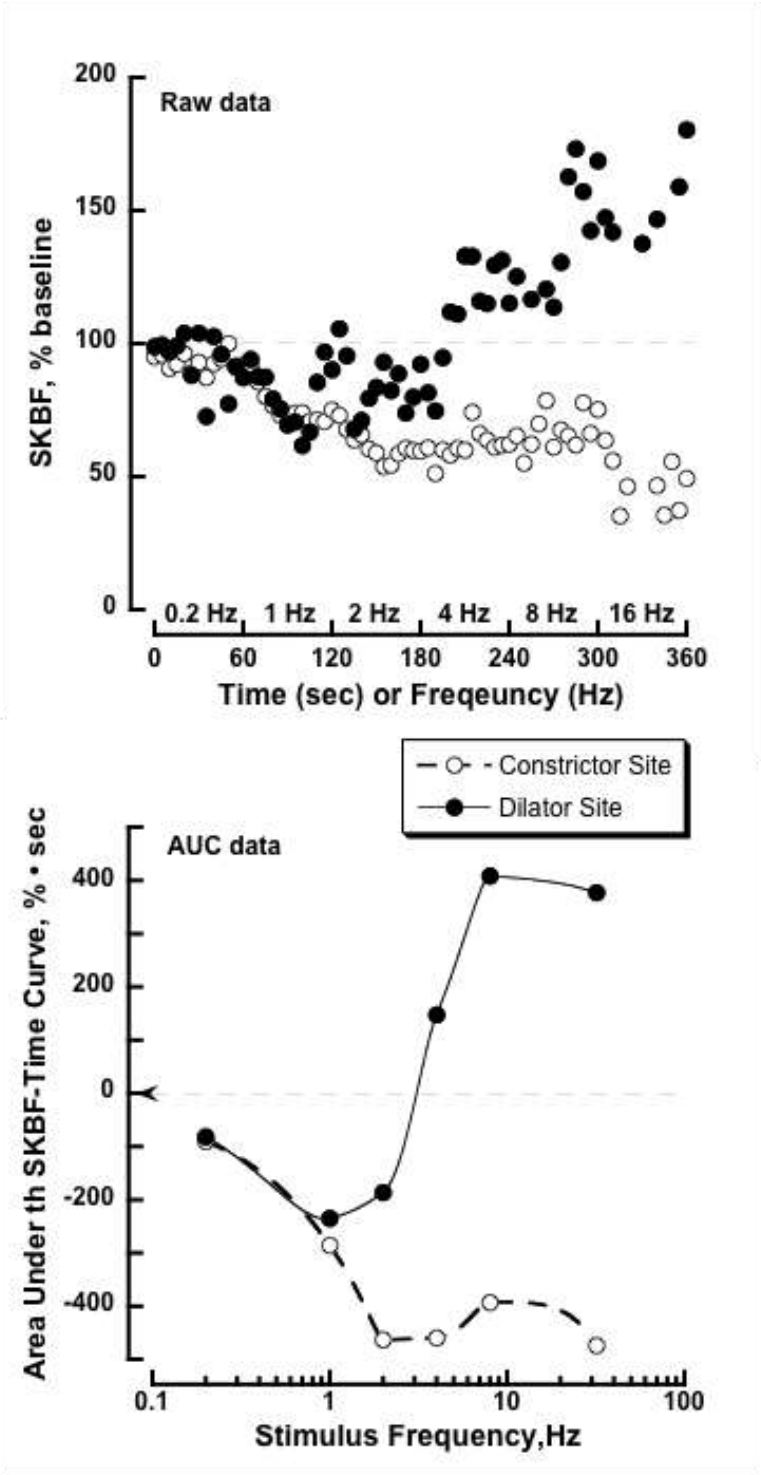
Raw data
SKBF, % baseline
200
150
100
50
0
0.2 Hz
1 Hz
2 Hz
4 Hz
8 Hz
16 Hz
0
60
120
180
240
300
360
Time (sec) or Freqeuncy (Hz)
- Constrictor Site
Dilator Site
AUC data
Area Under th SKBF-Time Curve, % • sec
400
200
0
-200
-400
0.1
1
10
100
Stimulus Frequency,Hz

Figure 2

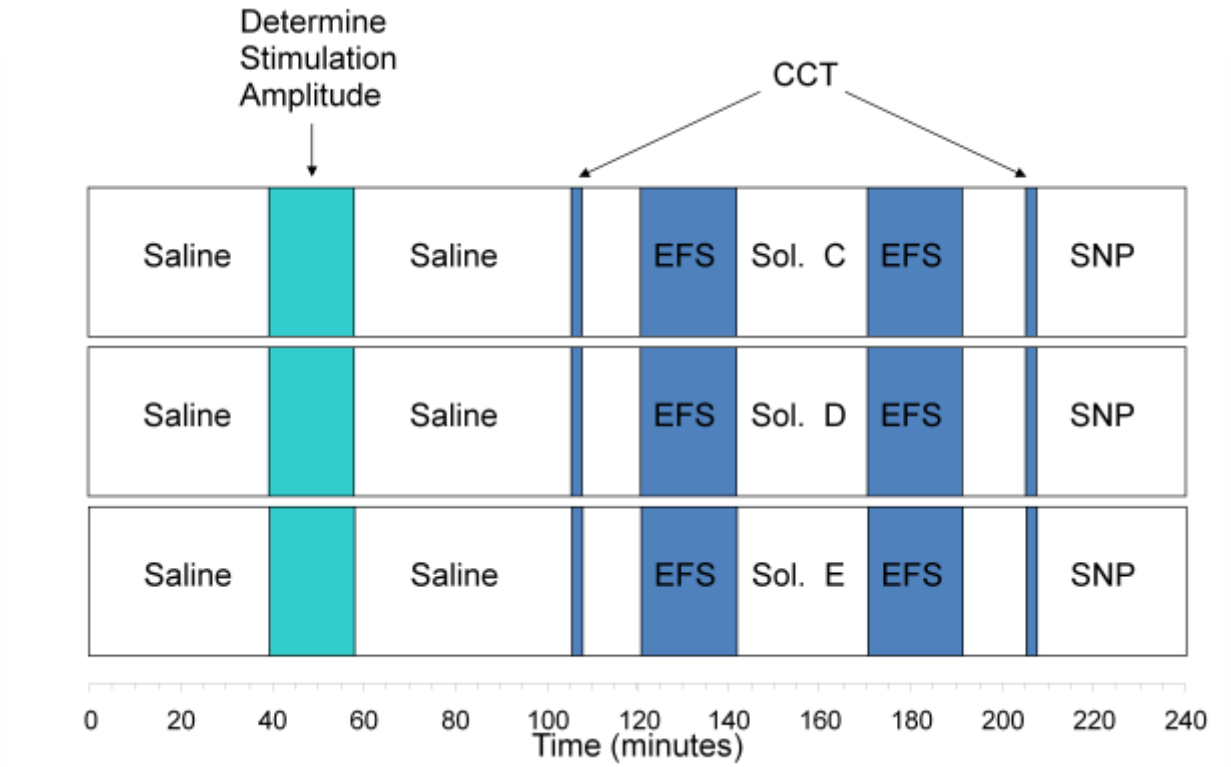
Determine
Stimulation
Amplitude
CCT
Saline
Saline
EFS
Sol. C
EFS
SNP
Saline
Saline
EFS
Sol. D
EFS
SNP
Saline
Saline
EFS
Sol. E
EFS
SNP
0
20
40
60
80
100
120
140
160
180
200
220
240
Time (minutes)

Figure 3

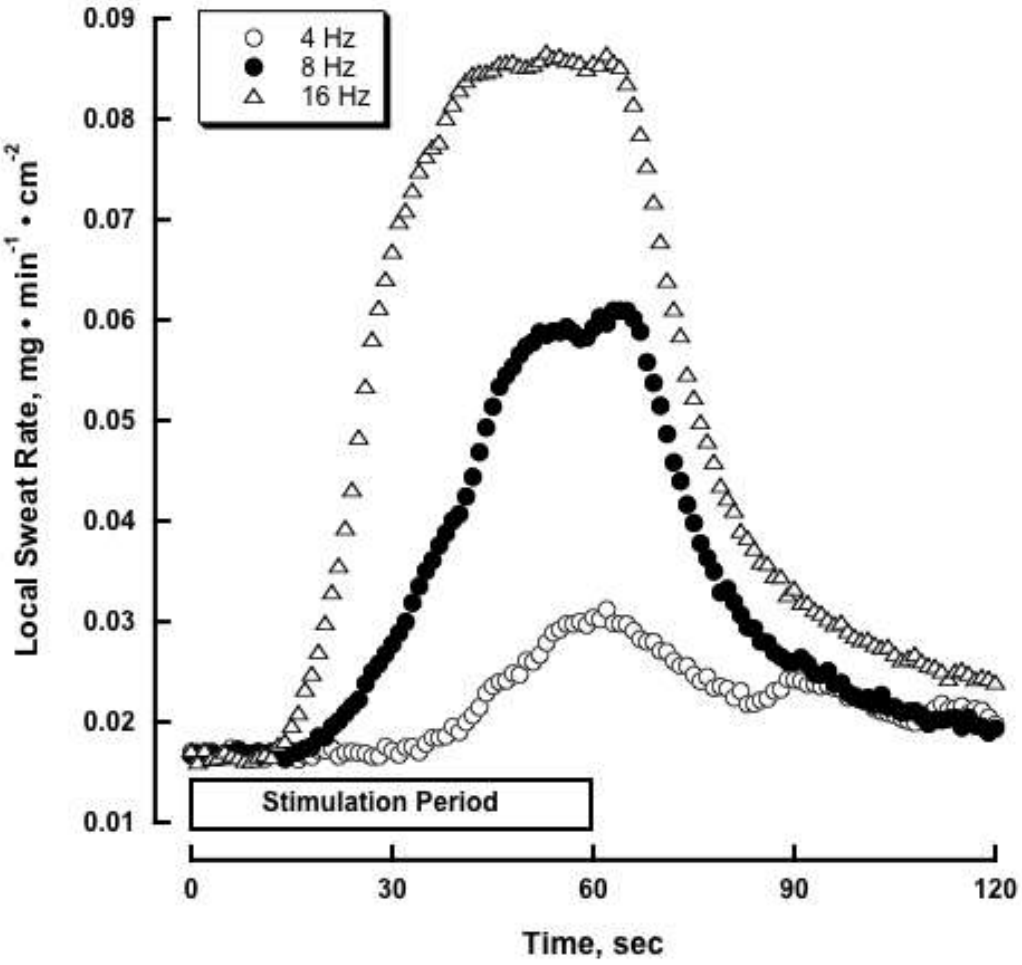
4 Hz
8 Hz
16 Hz
Stimulation Period
Local Sweat Rate, mg • min-1 • cm-2
Time, sec
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
30
60
90
120

Figure 4

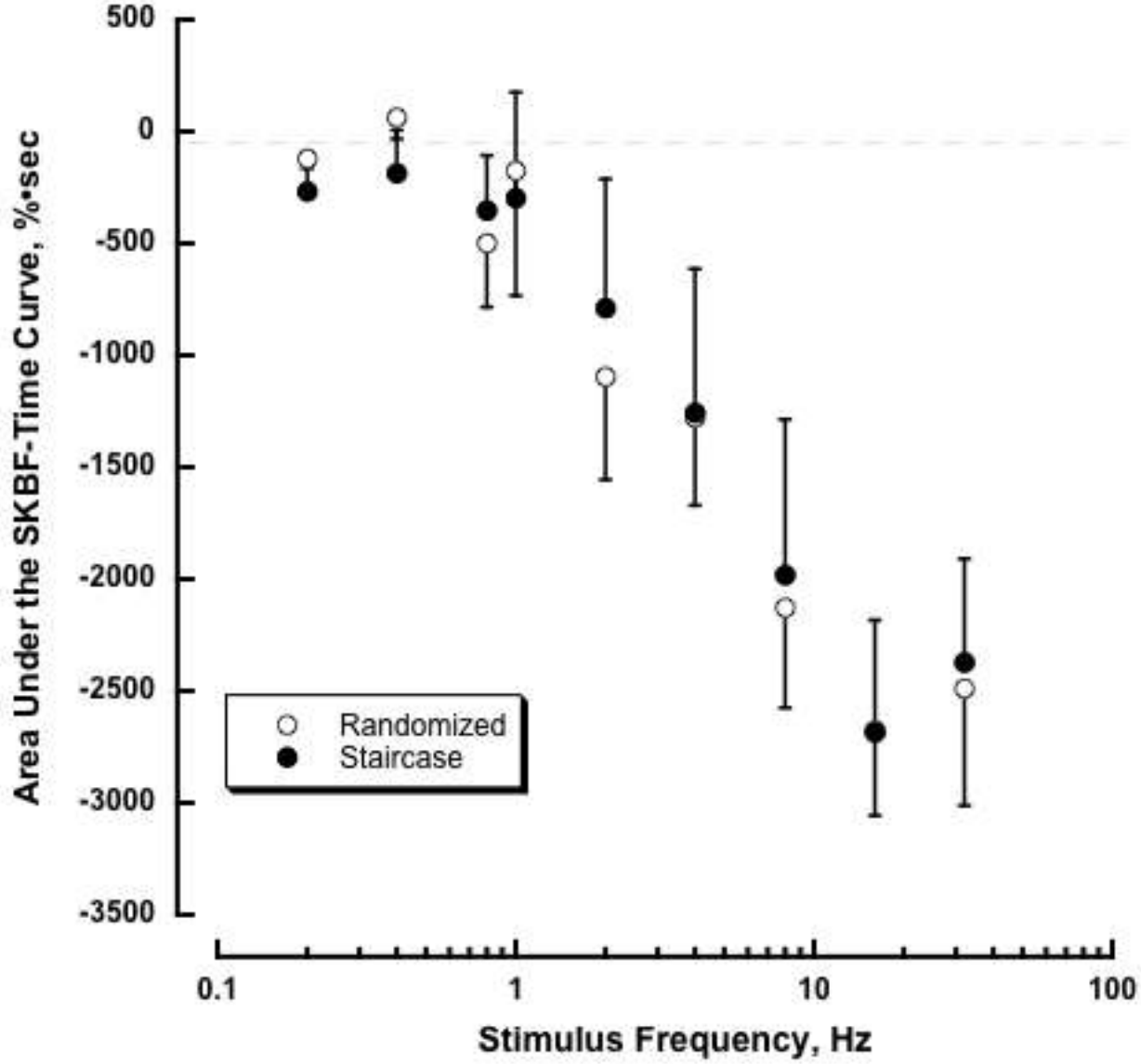
500
0
-500
-1000
-1500
-2000
-2500
-3000
-3500
Area Under the SKBF-Time Curve, %•sec
Randomized
Staircase
0.1
1
10
100
Stimulus Frequency, Hz

Figure 5

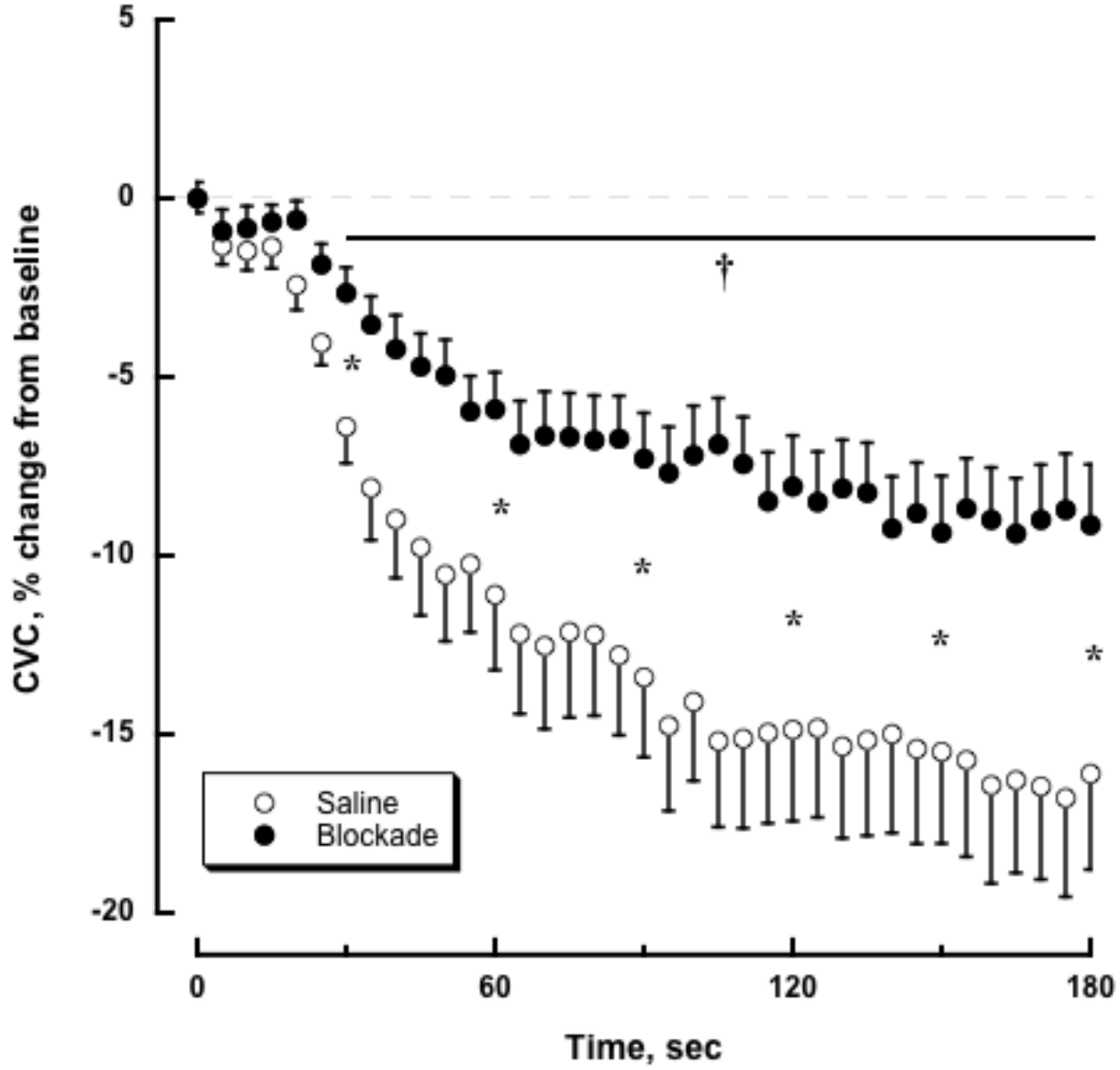
5
0
-5
-10
-15
-20
CVC, % change from baseline
Saline
Blockade
†
*
0
60
120
180
Time, sec

Figure 6

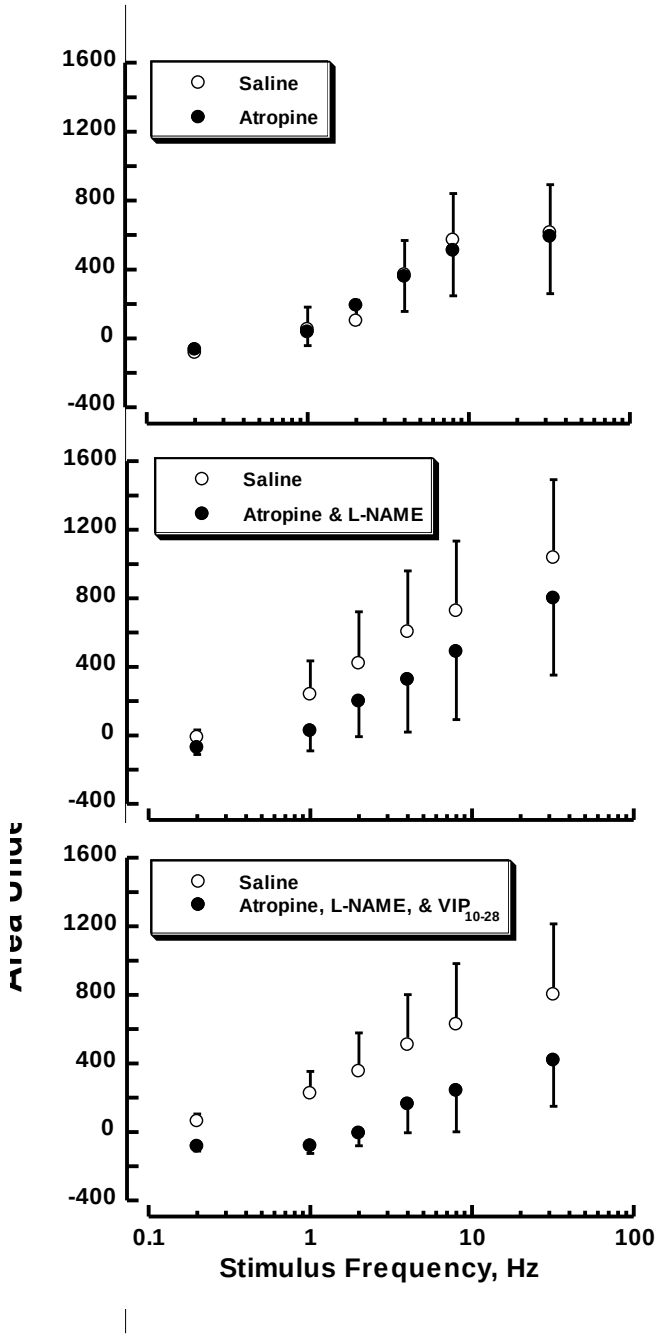
1600
1200
800
400
0
-400
Saline
Atropine
Saline
Atropine & L-NAME
Saline
Atropine, L-NAME, & VIP10-28
0.1
1
10
100
Stimulus Frequency, Hz

Figure 7

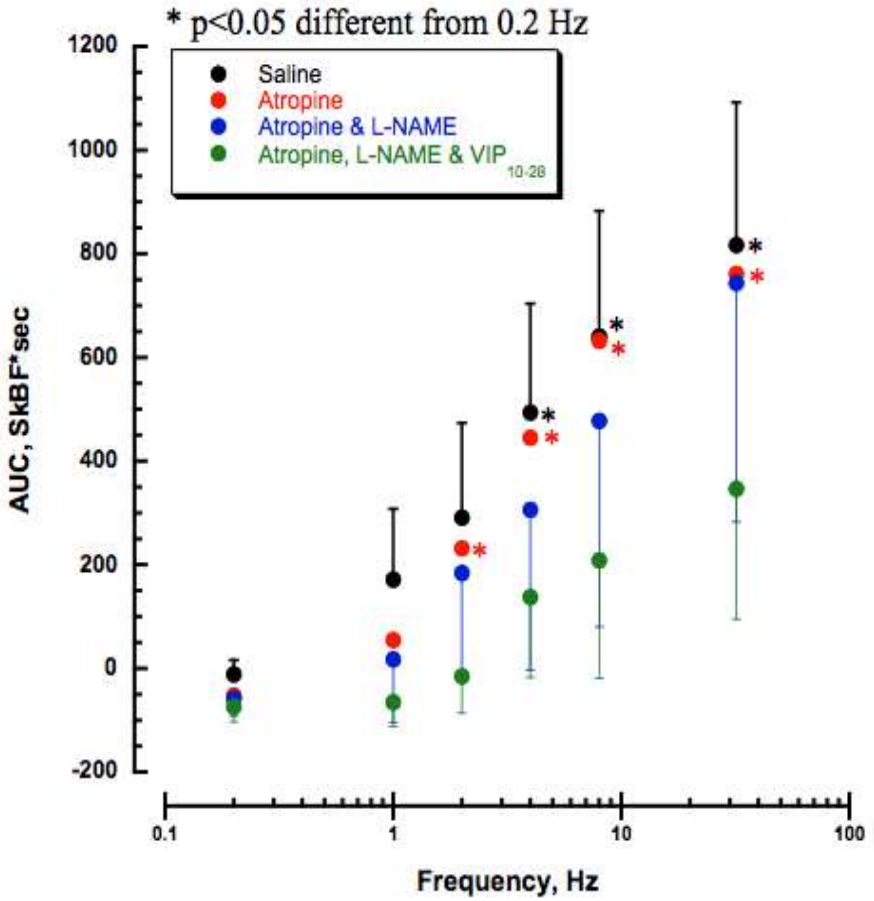
* p<0.05 different from 0.2 Hz
Saline
Atropine
Atropine & L-NAME
Atropine, L-NAME & VIP 10-28
AUC, SkBF*sec
1200
1000
800
600
400
200
0
-200
0.1
1
10
100
Frequency, Hz

Figure 8

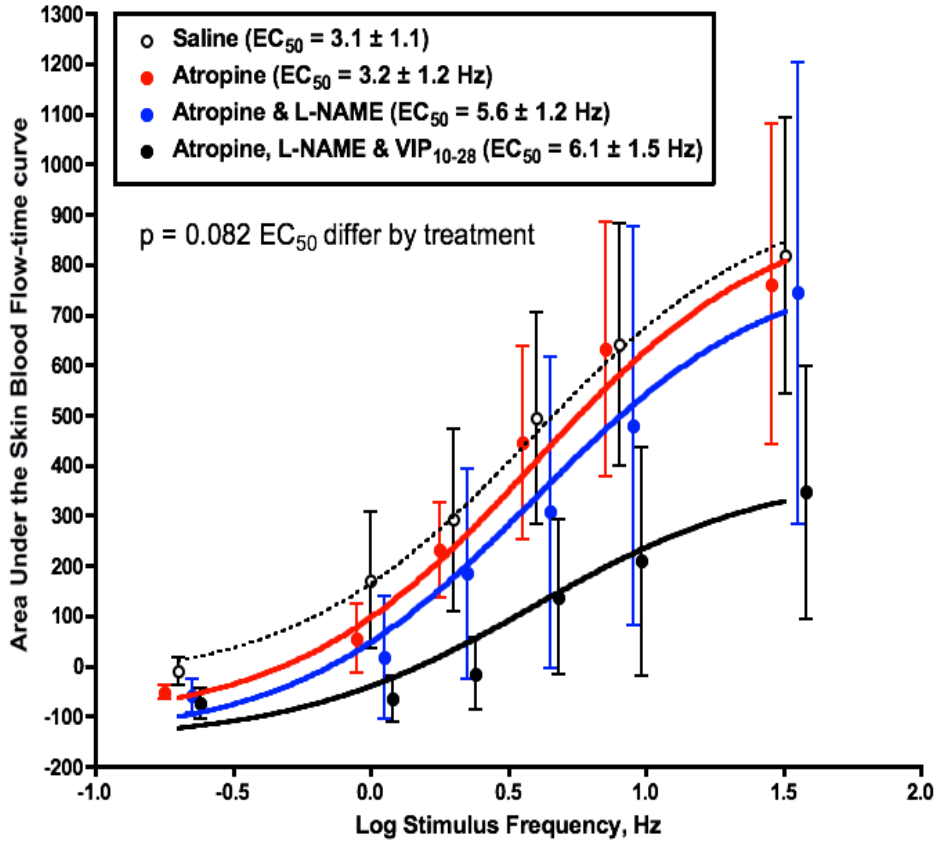
Saline (EC_{50} = 3.1 ± 1.1)
Atropine (EC_{50} = 3.2 ± 1.2 Hz)
Atropine & L-NAME (EC_{50} = 5.6 ± 1.2 Hz)
Atropine, L-NAME & VIP_{10-28} (EC_{50} = 6.1 ± 1.5 Hz)
p = 0.082 EC_{50} differ by treatment
Area Under the Skin Blood Flow-time curve
Log Stimulus Frequency, Hz
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
-100
-200
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0

Figure 9

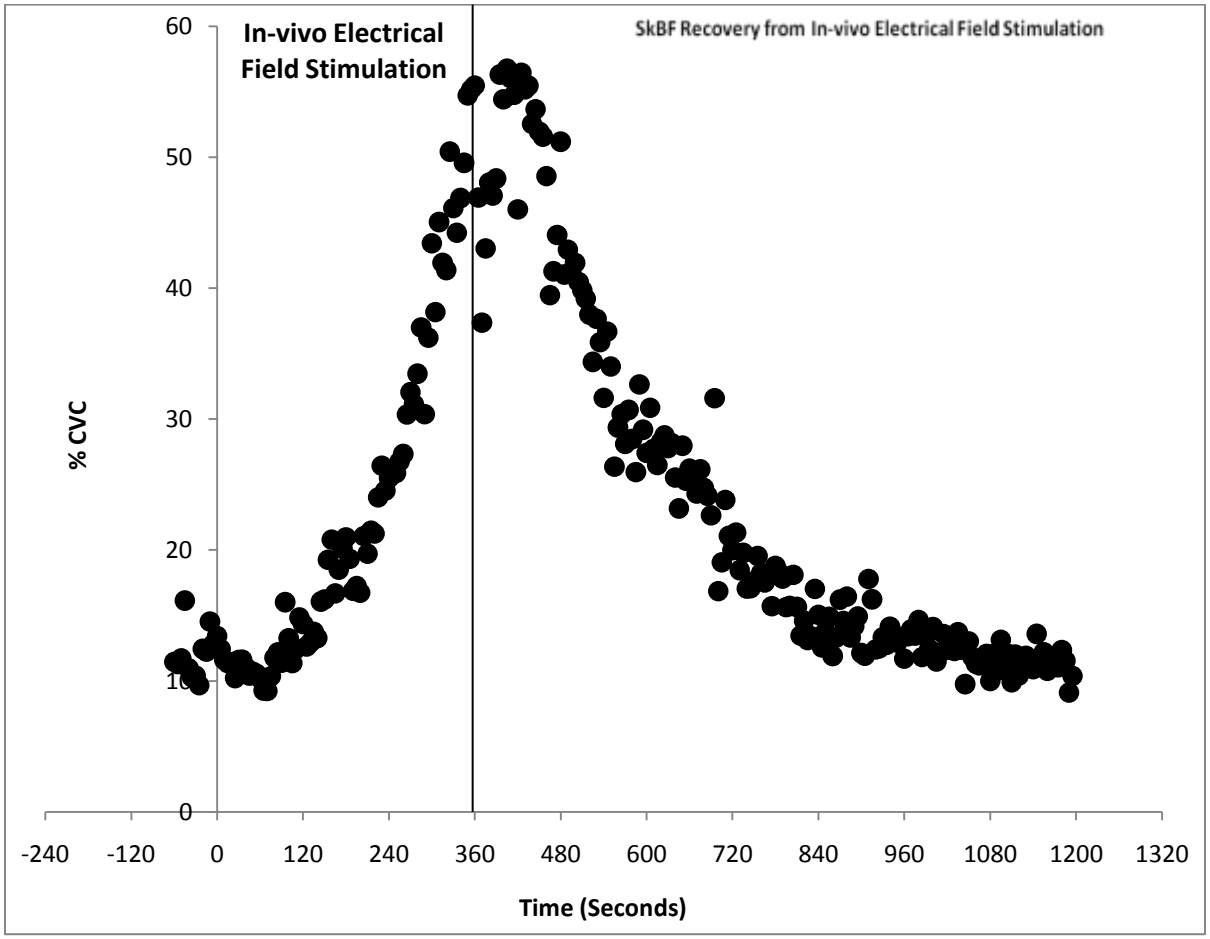
In-vivo Electrical Field Stimulation
SkBF Recovery from In-vivo Electrical Field Stimulation
% CVC
Time (Seconds)
60
50
40
30
20
10
0
-240
-120
0
120
240
360
480
600
720
840
960
1080
1200
1320

Figure 10

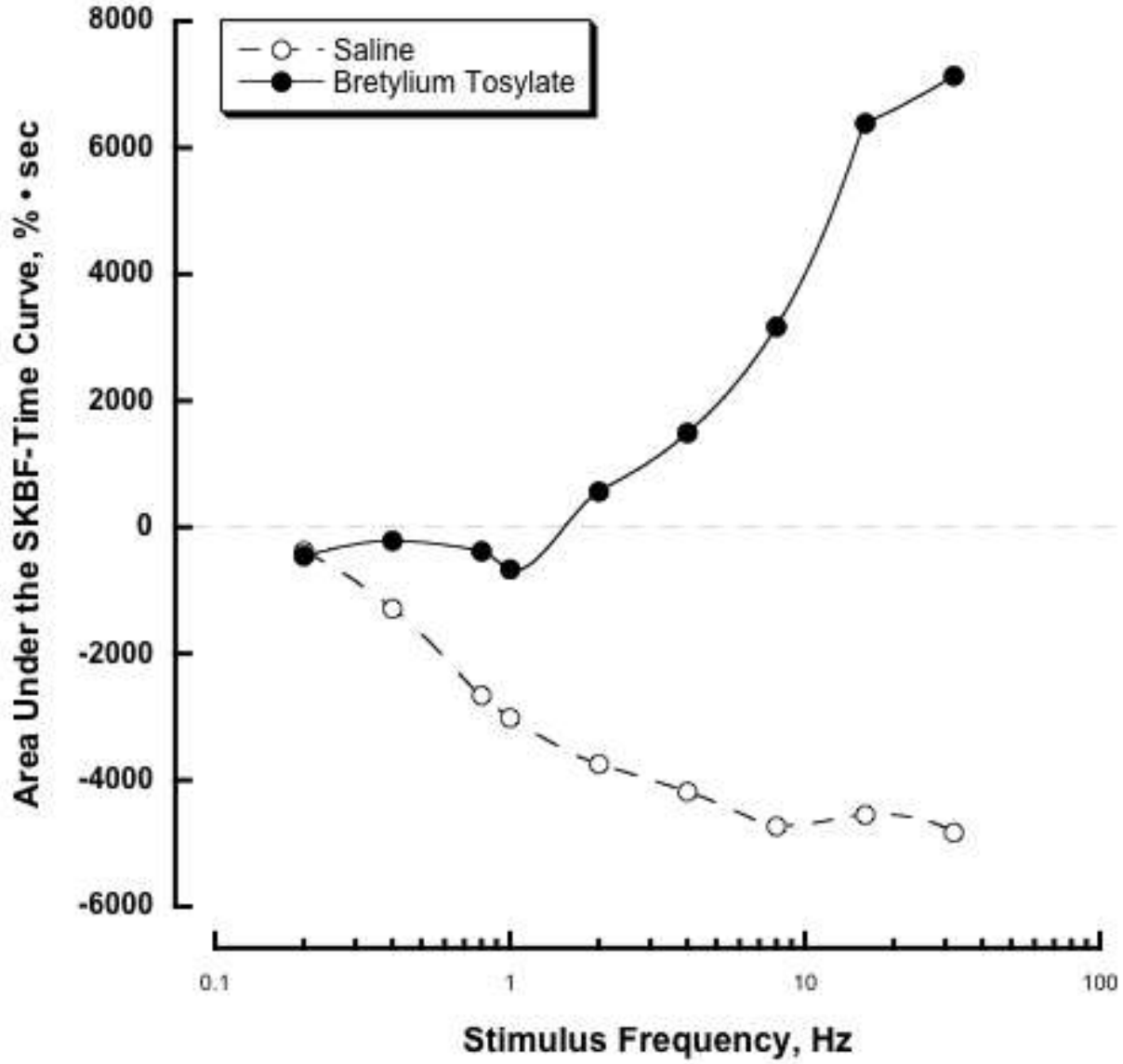
8000
6000
4000
2000
0
-2000
-4000
-6000
Saline
Bretylium Tosylate
Area Under the SKBF-Time Curve, % • sec
0.1
1
10
100
Stimulus Frequency, Hz

Figure 11

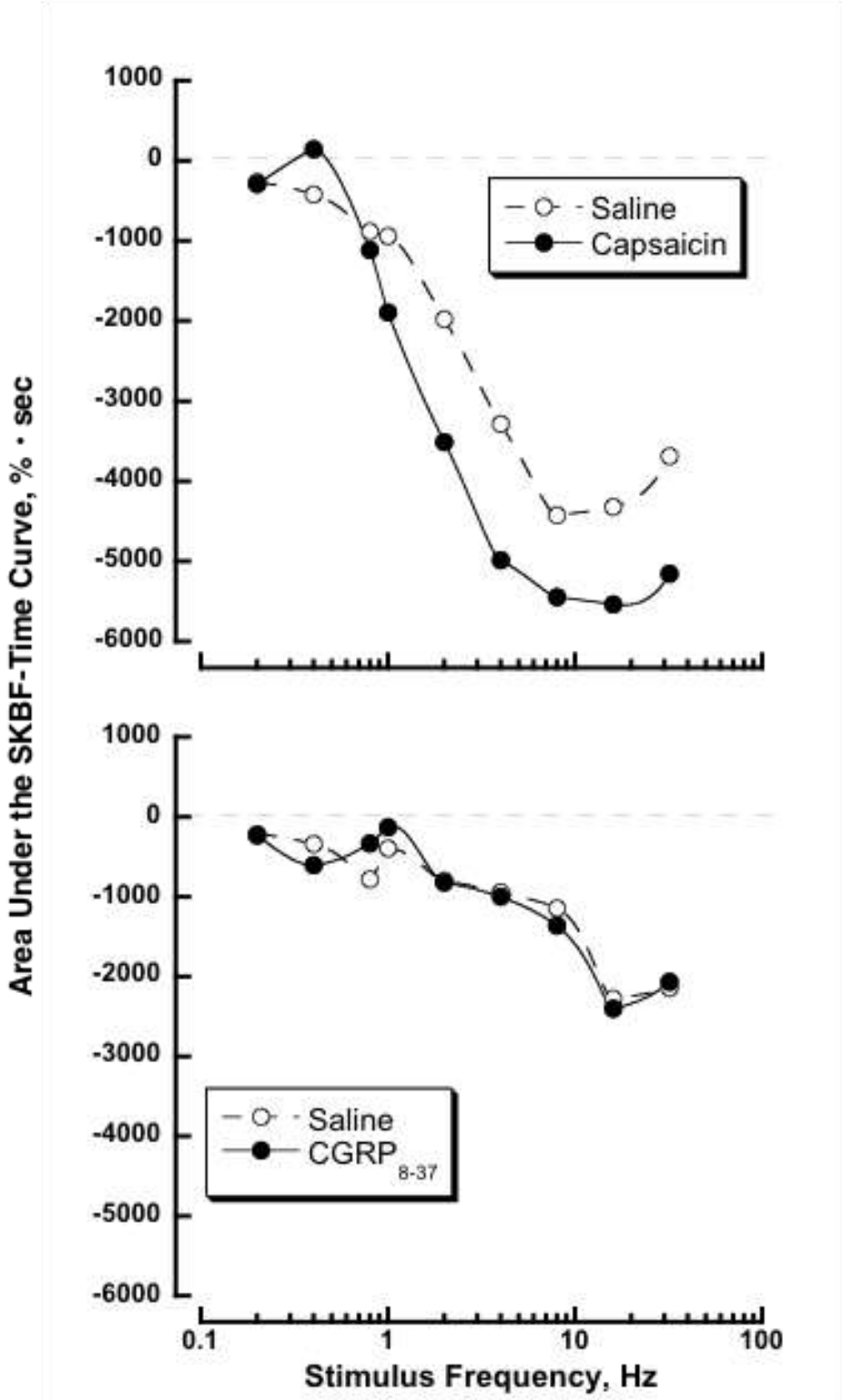
Area Under the SKBF-Time Curve, % • sec
1000
0
-1000
-2000
-3000
-4000
-5000
-6000
Saline
Capsaicin
1000
0
-1000
-2000
-3000
-4000
-5000
-6000
Saline
CGRP 8-37
0.1
1
10
100
Stimulus Frequency, Hz

Table 1

Mean averages of skin temperature, pain levels, and current amplitudes. Values are mean ± SEM for 9 subjects. Site location as referenced from the elbow.

Skin Site	Skin Temperature, °C	Stimulus Current, mA	Pain Rating (6-16)
Distal Site	32.8 ± 0.3	1.0 ± 0.1	7.6 ± 0.5
Middle Site	33.5 ± 0.2	1.1 ± 0.2	7.7 ± 0.6
Proximal Site	32.5 ± 0.2	0.9 ± 0.1	8.1 ± 0.6

Appendix A

Prospectus

Chapter 1

Introduction

Body temperature regulation during thermal stress and exercise allows the body to maintain homeostasis. Adjustments in the cutaneous circulation play a vital role in this regulation. The change in cutaneous circulation during exercise and/or thermal stress is controlled by both local and reflex systems (17). The local response relies on circulating pressor hormones and chemicals released from the vascular endothelium lining and the skin keratinocytes (18). The reflex response is controlled by cardiovascular, thermal, and nociceptor afferents (18). These afferent signals control the skin blood flow through activation of the sympathetic nervous system.

The sympathetic nervous system controls skin blood flow through sympathetic adrenergic and cholinergic nerves. The sympathetic adrenergic nerves release norepinephrine. Norepinephrine is the predominant neurochemical producing vasoconstriction in the blood vessels (4, 20). However, sympathetic-mediated vasodilation (active cutaneous vasodilation) is mediated by an unknown neurochemical. Most scientists believe that the cutaneous sympathetic vasodilator system releases acetylcholine (ACh) and a cotransmitter (vasoactive intestinal peptide, VIP) to evoke vasodilation (3, 22, 28). However, when ACh and VIP receptors are blocked during heat stress, a decrease in skin blood flow occurs, but the original vasodilation is not completely abolished (3). Thus, another neurochemical must be involved in mediating active cutaneous vasodilation. Nitric oxide (NO) is a likely candidate. NO is found readily in the cutaneous interstitial space during heating (23), and when NO is blocked during heating, the increase in skin blood flow is attenuated (19).

One way to examine the effects of ACh, VIP, and NO on control of skin blood flow during thermal stress is through the use of microdialysis probes and laser Doppler probes. Infusing antagonists into the dermal space of the skin, via microdialysis probes, allows the examination of local skin blood flow response to heating without systemic effects. Currently, the ability to differentiate the role of various neurochemicals on the skin blood flow response to thermal stress is limited by the numerous systemic effects imposed upon the body by either the thermal stress (high ambient temperature) or exercise used to induce the thermal stress (12).

In order to avoid the systemic effects produced during thermal stress and exercise, we have developed a novel method to investigate neurogenic control of skin blood flow by directly stimulating perivascular nerves. This method involves in-vivo electrical field stimulation. In-vivo electrical field stimulation uses electrodes (thin stainless steel acupuncture needles) placed 2 mm deep into the skin. A constant current applied to the electrodes causes depolarization of all perivascular nerves thereby producing the release of their respective neurochemicals. By combining intradermal electrical stimulation with measurements of the skin blood flow and intradermal microdialysis, the specific effect of each known neurochemical on skin blood flow should be differentiated.

Problem Statement

Using in-vivo electrical field stimulation and eliminating the action of norepinephrine released from sympathetic adrenergic perivascular nerves (vasoconstriction) with adrenergic blocking agents, we should be able to identify the contribution of ACh, NO, and VIP to vasodilation in the skin. Thus, the purpose of this study is to specifically quantify the influence of ACh, NO, and VIP released from perivascular nerves on skin blood flow in the forearm.

Hypotheses

First, blocking ACh and NO will reduce the increase in skin blood flow during electrical stimulation greater than only blocking ACh.

Second, blocking ACh, NO, and VIP will reduce the increase in skin blood flow during electrical field stimulation greater than only blocking ACh.

Third, blocking ACh, NO, and VIP will reduce the increase in skin blood flow during electrical field stimulation greater than blocking ACh and NO.

Null Hypotheses

First, skin blood flow will not be significantly different when blocking ACh and NO during electrical stimulation when compared to only blocking ACh.

Second, skin blood flow will not be significantly different when blocking ACh, NO, and VIP during electrical stimulation when compared to only blocking ACh.

Third, skin blood flow will not be significantly different when blocking ACh, NO, and VIP during electrical stimulation when compared to blocking ACh and NO.

Assumptions

Nociceptor nerves are not being activated due to low levels of pain exerted by the electrical field stimulation as determined by the perceived pain scale.

Delimitations

The findings will only be applicable to blood flow in the forearms.

Limitations

If nociceptor nerves are activated by the electrical stimulation, an axon reflex will occur away from the electrical stimulation site causing an increase in skin blood flow. However, because of limited laser Doppler probes, we will only measure skin blood flow directly over electrical stimulation sites.

Significance of the Study

The regulation of cutaneous circulation is a vital component in keeping core body temperature at viable levels. Understanding how skin blood flow is regulated will help determine ways to effectively treat individuals who may have impaired cutaneous circulation. No one has used the novel method of in-vivo electrical field stimulation to study skin blood flow. The results of this study will increase insight into the involvement of different neurochemicals released from the perivascular nerves on cutaneous circulation.

Chapter 2

Review of Literature

Cutaneous circulation is extremely important in regulating body temperature during exercise and during exposure to cold and heat. The ability of the body to respond rapidly to different thermal stimuli depends on a variety of signaling systems that include both reflex and local responses. Reflex neural control involves sensory information from thermal, cardiovascular, and nociceptor afferents (18). In particular to skin blood flow, the neural control involves perivascular nerves that surround the skin blood vessels which include adrenergic, cholinergic, sensory afferent, and nitrodergic neurons (18). Local control involves the action of circulating pressor hormones (angiotensin II, arginine vasopressin) and paracrine release of substances such as nitric oxide, prostaglandins, and histamine from the blood vessel endothelium or keratocytes (18). This review will focus on the role of perivascular nerves in the reflex control of skin blood flow.

Thermoregulatory Control of Skin Blood Flow

Skin blood flow is mainly affected by thermal stress. One of the original studies examining local thermal stress on forearm blood flow was done by Barcroft and Edholm (2). In their study, they immersed subject's forearms in water baths at different temperatures and found that temperatures above 35 °C caused vasodilation while forearm blood flow was significantly reduced at temperatures below 35 °C (2). Taylor, Johnson, O'Leary, and Park (35) found that when the skin was heated to 42 °C, forearm blood flow rose substantially. They discovered that maximal forearm skin blood flow averaged 21 ml•100 ml^{-1} •min^{-1} which when extrapolated to approximate whole-body skin blood flow equaled about 7.6 L•min^{-1} (35). These early studies

demonstrate the large increase in local skin blood flow that occurs during heat stress. In these studies, blood flow was measured in the arm that was heated or cooled. Because of this, the responses reflect a combination of local and reflex responses to temperature. Wenger, Stephenson, and Durkin (37) performed similar experiments following brachial plexus blockade. After nerve block they saw similar increases in forearm skin blood flow with or without nerve block. As such, most of the increase in forearm skin blood flow during local heating could be attributed to a local response. Despite the fact that these responses were mainly mediated by local factors, they demonstrated the significant change that occurs to skin blood flow during thermal stress.

Reflex thermoregulatory responses in the skin are derived from afferent thermal information sent to the hypothalamus where it is integrated and an appropriate effector organ response is generated (i.e., dilation or constriction). One efferent pathway used to control skin blood flow through the activation of thermal reflexes is the sympathetic nervous system. The sympathetic nervous system's involvement in regulating blood flow during heat and cold stress is extremely important. The efferent motor output of the sympathetic nervous system is composed of both sympathetic noradrenergic vasoconstrictor neurons and sympathetic cholinergic neurons. The sympathetic noradrenergic vasoconstrictor neurons innervate both glabrous skin (nonhairy skin) (10) and nonglabrous skin (hairy skin) (18) in humans. The sympathetic cholinergic neurons innervate nonglabrous skin, but they do not play a significant role in glabrous skin blood flow (10, 14, 15).

The sympathetic noradrenergic system controls reflex vasoconstriction in skin blood vessels in response to a variety of systems including thermal and cardiovascular reflexes. Early studies identified the involvement of sympathetic noradrenergic neurons through

sympathectomies of various neurons. To examine the vasoconstrictor activity in the forearm, Warren, Walter, Romano, and Stead (36) blocked the sympathetic ganglia of the upper right extremities by a paravertebral injection of novacaine. The nerve block caused a significant increase in blood flow in the right forearm compared to blood flow before the sympathectomy (36). Due to the increased blood flow with nerve blockade, it was concluded that a sympathetic vasoconstrictor system must have been active when the nerves were not blocked.

To examine the pharmacological control of blood vessels during activation of the sympathetic noradrenergic system, different adrenergic-receptor blocking agents have been used. One particular blocking agent that is effective in blocking the sympathetic noradrenergic system is bretylium tosylate. Blair et al., (4) studied the response of forearm blood flow after infusing bretylium during cold stress, heat stress, valsalva manoeuvre, and emotional stress. When bretylium was infused during cold stress and the valsalva manoeuvre, blood flow did not decrease as seen in the control arm. When bretylium was infused during heat stress and emotional stress, blood flow increased similarly in the control and bretylium arms (4). Because no vasoconstriction occurred during cold stress and the valsalva manoeuvre it was concluded that bretylium treatment blocked sympathetic noradrenergic vasoconstrictor activity. Furthermore, because the vasodilation occurred during heating and emotional stress with bretylium, the vasodilator system was not affected by bretylium. Bretylium effectively blocks the sympathetic noradrenergic system and not the sympathetic cholinergic system.

Skin blood flow in the forearm is also controlled by an active sympathetic vasodilator system. Johnson and Proppe (17) described active as meaning that "an increase in SkBF arises, either directly or indirectly, from increased efferent nerve activity." Early studies indicated that an increase in skin blood flow in the forearm was controlled by a sympathetic vasodilator

system. Barcroft, Bonnar, and Edholm (1) did unilateral upper limb sympathectomies on subjects. After the sympathectomies, they placed the subject's feet in 44-45 °C water and put blankets on them for 50 minutes. No significant increase in blood flow was seen in the sympathectomized forearm after heating, but a significant increase in blood flow was seen in the normal forearm (1). These results show that a sympathetic vasodilator system controls blood flow during heat stress.

Edholm, Fox, and Macpherson (9) confirmed the involvement of a sympathetic vasodilator. They blocked the cutaneous nerves with a 2% solution of lignocaine at the tip of the olecranon. Control subjects received injections of saline in this area. When the body core temperature was elevated to 38 °C, forearm blood flow in the anesthetized forearm did not increase. However, forearm blood flow in the normal arm injected with saline increased significantly (9). Furthermore, when lignocaine was injected during heating, blood flow decreased (9). Because blocking cutaneous nerves caused a decrease in forearm blood flow during heating, an increase in blood flow is due to some type of sympathetic nerve activity.

In order to study the active sympathetic vasodilator system without the involvement of the sympathetic noradrenergic system, bretylium tosylate has been used. As mentioned earlier, Blair et al. (4) determined that bretylium blocked the vasoconstrictor sympathetic nerves but did not block the sympathetic vasodilation nerves. However, his study used venous plethysmography to evaluate blood flow, and they infused bretylium throughout the body which may have caused systemic effects.

Kellogg et al. (20) avoided systemic problems by delivering bretylium tosylate to the skin through intradermal microdialysis probes. They found that with bretylium tosylate delivery to the skin, no cutaneous vasoconstriction occurred during cold stress. However, during heat stress

the bretylium tosylate failed to abolish the rise in blood flow (20). By effectively infusing bretylium in one site without affecting the active vasodilator system, an effective way for studying the active vasodilator by itself has been found without any possible systemic confounders.

Evidence for Sweating and Vasodilation

One possible theory that may explain how vasodilation occurs is the link between sweating and vasodilation. Sweat glands are innervated by sympathetic cholinergic sudomotor nerves. When these nerves activate sweat glands, a vasodilator substance called bradykinin is also released. Fox and Hilton (11) examined the relationship between bradykinin formation in humans and vasodilation. When the body was heated, subcutaneous bradykinin levels were five times higher compared to resting levels. The increase in bradykinin levels correlated with the onset of vasodilation. Sweating occurred when the bradykinin activity reached its peak (11). Because vasodilation corresponded with increased levels of bradykinin, bradykinin was thought to be a factor in skin vasodilation.

However, more recent evidence suggests that bradykinin blockade does not alter active cutaneous vasodilation. Kellogg, Liu, McAllister, Friel, and Pergola (21) used 40 μM of the bradykinin B_2-receptor antagonist HOE-140 to see if bradykinin caused vasodilation in heated subjects. Skin temperature rose 1°C and vasodilation occurred both in the untreated sites and HOE-140 treated sites. No significant difference in vasodilation between the two sites happened. The authors concluded that bradykinin did not play a role in cutaneous vasodilation with heat stress because bradykinin blockade did not decrease vasodilation (21).

Other evidence supporting the idea that sweat glands may play a role in vasodilation in the skin is seen in patients without sweat glands. Brenglemann, Freund, Rowell, Olerud, and Kraning (5)

studied vasodilation in the forearm of subjects with hereditary anhidrotic ectodermal dysplasia (AED). Subjects with AED do not have functioning sweat glands. In their experiment, subjects were clothed in water-perfused suits, and their oral temperature was increased 1.4-1.7 °C above resting body core temperature. Measuring blood flow with venous occlusion plethysmography, they did see evidence of sweating and did not find a significant increase in blood flow (5). These findings indicate that sweat glands play some role in active vasodilation in the skin.

Evidence against Sweating and Vasodilation

Despite the evidence supporting a link between sweating and vasodilation, evidence also exists that does not support it. One idea that contradicts the role of sweating and vasodilation is the different timing associated with sweating and vasodilation. If sweating was essential for vasodilation then the onset of sweating should always precede the onset of cutaneous vasodilation. When examining vasodilation and sweating, Johnson and Park (16) did not see a similar shift in sweat rate and vasodilation during exercise and rest. Subjects were heated with water-perfused suits to 38-38.5 °C for 60-75 minutes in supine and upright positions. Bicycle exercise in the upright and supine positions was done for 20-30 minutes. The threshold esophageal temperature (body core temperature) at which cutaneous vasodilation occurred was delayed during exercise, but the onset of sweating was unchanged (16). Because sweat rate did not change similarly to forearm skin blood flow during exercise, sweating may not be the most important factor contributing to vasodilation in the skin.

Evidence against sweating and vasodilation is obtained when sweating is blocked with atropine, but cutaneous vasodilation still occurs. Kolka, Stephenson, Allan, and Rock (27) examined the effects of atropine on vasodilation. Subjects started in a cool environment of 22 °C. Two separate trials were done on individuals such that one day they received an atropine

treatment and another day they received a saline treatment. Subjects exercised for 30 minutes at 55% of their $\dot{V}O_2$ peak. During exercise, forearm sweating decreased significantly with atropine treatment compared to control trials. However, despite the decrease in sweating, forearm skin blood flow increased to a similar degree in control and atropine treated subjects (27). Because forearm blood flow increased when blocking sweat gland output, sweating may not play as significant a role in skin vasodilation as earlier proposed.

Cotransmitter Vasodilator System

One possible explanation of the increased blood flow despite the blocking of ACh's action on sweat glands may be related to the release of a cotransmitter from sympathetic cholinergic nerves. Kellogg et al. (22) determined that active cutaneous vasodilation is caused by the release of neurotransmitters from a cholinergic nerve in humans. To determine this, an area of 0.6 cm^2 on the nonglabrous skin of the forearm was injected with 5 U of botulinum toxin. Botulinum toxin acts presynaptically on the cholinergic nerves and blocks the release of ACh and any other neurotransmitters found in the cholinergic nerves. When compared to an untreated site, the site with botulinum toxin did not have a significant increase in blood flow or sweat rate (22). Because no vasodilation or sweating occurred with the presence of botulinum toxin, active vasodilation is dependent on the release of a neurotransmitter from the cholinergic nerves.

One promising cotransmitter found in cholinergic nerve terminals is vasoactive intestinal peptide (VIP). Using immunohistochemical staining, Lundberg et al. (28) found significant amounts of VIP and ACh present in cholinergic nerves controlling exocrine glands in cats. Bennett et al. (3) used intradermal microdialysis probes to infuse VIP and VIP (10-28) fragment, a VIP antagonist, into human skin. 7.5 μM of VIP caused cutaneous dilation of similar magnitude as seen during heating in humans. When VIP (10-28) was infused at levels of 214

μM with and without atropine, a significant decrease in vasodilation occurred. During heating, the site with VIP (10-28) alone caused blood flow to increase to around 27 % of maximum blood flow. With VIP (10-28) and atropine, blood flow levels increased to around 20 % maximum blood flow. The blood flow in the control site increased to 35 % of maximum blood flow (3). The findings of Bennett provide evidence that VIP could play a role in active vasodilation in the skin in humans.

Despite evidence of the importance of VIP in cutaneous vasodilation, other findings show that VIP may not play an important role. Savage, Brengelmann, Buchan, and Freund (30) studied subjects with cystic fibrosis. Using immunohistochemical analysis, they found that these subjects had very little VIP present in their nerve endings. Despite a relative lack of VIP released, subjects with cystic fibrosis had similar increases in blood flow when compared to control subjects without cystic fibrosis (30). Even though VIP was almost absent in patients with cystic fibrosis, vasodilation still occurred during heating. As such, other substances besides VIP may also contribute to cutaneous vasodilation during thermal stress.

Nitrogen Oxide and Vasodilation

Besides the involvement of VIP and ACh in causing vasodilation, NO may also play an important role. Nitric oxide is made from arginine by different nitric oxide synthase (NOS) enzymes that are found in endothelial cells surrounding blood vessels or in cholinergic nerve endings and sweat glands (7). The main NOS enzymes involved in skin blood flow regulation are endothelial NOS (eNOS) (24) and neuronal NOS (nNOS) (25). One way that NOS is activated is through shear stress on the blood vessels (8). Another way that NOS is activated is from the release of ACh from nerve endings that bind to endothelial cells (6). Nitric oxide synthase enzymes are also activated by action potentials that cause NOS to make NO from

arginine and release NO from the nerve endings (6). Nitric oxide has a short half-life and therefore influences cells close to its release site (13).

Because NO is located around blood vessels, it may be a key player in the control of skin blood flow during heat stress. Researchers have reported an increase in intradermal NO concentration during heat stress. Kellogg et al. (23) placed a NO-selective, amperometric electrode in the forearm to measure concentrations of NO before and during heating. Significant increases in blood flow and NO occurred during whole-body heating. When ACh was infused in the skin, significant increases in skin blood flow and NO levels also occurred (23). The increase in NO levels concomitantly with increasing temperature demonstrates that NO is involved in the vasodilation and may actually be the substance that causes vasodilation.

One way to investigate NO's role in reflexive control of blood flow is to block NO synthase (NOS). Kellogg et al. (19) inserted intradermal microdialysis probes in the forearm of subjects and perfused one site with nitro-L-arginine methyl ester (L-NAME) which is a nonselective blocker of NOS, and the other site was perfused with Ringer solution. In the untreated site when local skin temperature was raised to 38-38.5 °C, blood flow rose to 44 % of maximum blood flow and in the L-NAME site blood flow rose to 30 % maximum blood flow (19). By blocking NOS, blood flow did not increase similarly to blood flow in skin with unblocked NOS. Nitric oxide production is clearly needed during local heating to induce increases in skin blood flow.

Other studies using the NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA) report similar results in attenuating blood flow. Shastry, Dietz, Halliwill, Reed, and Joyner (31) heated subjects using a water-perfused suit and increased body core temperature by 1°C. N^G-monomethyl-L-arginine was infused after heating into the blood through a brachial artery

catheter at 5 mg/min for 21 minutes. Using laser Doppler flowmetry, they found that skin blood flow levels decreased by 14 % during heating in the treated forearm when L-NMMA was infused. This was significantly lower than the pre-L-NMMA values (31). The decreased levels of blood flow following L-NMMA treatment demonstrate that NO is likely involved in active cutaneous vasodilation. However, NOS inhibition did not completely decrease blood flow to baseline levels. Either the dose of NOS inhibitor was insufficient to completely block NO production or other substances must be involved in the process of vasodilation.

If NO is important for cutaneous vasodilation, then the question that arises is how does ACh affect NO release, and do they need each other for vasodilation to occur or are they additive to each other? Shastry, Minson, Wilson, Dietz, and Joyner (32) investigated this question by heating subjects in water-perfused suits to increase body core temperature by 1°C. After heating subjects, 58 μM of atropine were infused for 30 minutes and then 10 mM of L-NAME was infused for 30 minutes. During the infusion of atropine, subjects showed little change in skin blood flow. However, during the administration of L-NAME, skin blood flow decreased significantly compared to the control site which remained constant throughout the infusion. They concluded from these results that ACh is not necessary to maintain vasodilation during heating and that NO is probably not a by-product of ACh's action on other tissues (32).

Shibasaki, Wilson, Cui, and Crandall (33) performed their infusions at the beginning of heating instead of during full vasodilation. They tested if ACh from cholinergic nerves caused vasodilation through NO at the beginning of heating. Three microdialysis sites were chosen on the forearm. One site was a control site infused with Ringer solution while another site was perfused with neostigmine, an acetylcholinesterase inhibitor, and the third site with neostigmine and L-NAME. They found that during early heat stress vasodilation occurred significantly

sooner in the site with neostigmine. However, the site with neostigmine and L-NAME did not vasodialate earlier. Throughout the experiment, blood flow in the L-NAME site was lower than at other sites (33). The results of Shibasaki et al.'s experiment provide evidence that ACh operates through a NO dependent mechanism in early stages of heating due to a decrease in the onset of vasodilation when NO was blocked.

Recent evidence suggests that nNOS produces the NO that causes reflex vasodilation seen during whole-body heating. Kellogg et al. (25) used 7-nitroindazole (7-IN) a selective antagonist of nNOS to study nNOS involvement in thermoregulatory reflex vasodilation. One site was infused with 5 % DMSO and Ringer solution, while the other site was infused with 2 mM 7-NI with 5 % DMSO. In one protocol subjects core temperature was elevated by 1°C using a whole-body suit with the forearm being uncovered. In another protocol subject's forearms were locally heated to 41.5 °C. In the whole-body heating protocol, 7-NI significantly decreased skin blood flow when compared to the control site by 15 %. However, in the protocol with local skin warming, no significant difference was seen between the 7-NI site and control site (25). Kellogg et al.'s findings clearly show that nNOS is involved in the thermoregulatory reflex response during whole-body heating due to the attention seen with the blockade of nNOS.

More evidence supporting the involvement of nNOS in reflex response to whole-body heating has been done by studying the combined effects of eNOS and nNOS blocking agents on skin blood flow. Kellogg, Zhao, and Wu (26) infused the eNOS antagonist N^{ω}-amino-L-arginine (LNAA) and the nNOS antagonist N^{ω}-propyl-L-arginine (NPLA) separately and in combination. During whole-body heating, the site with NPLA had significantly attenuated skin blood flow, but the site with NPLA and LNNA had no significant change from the NPLA site (26). The authors concluded that because no difference in blood flow occurred when blocking both eNOS and

nNOS compared to blocking with only nNOS during whole-body heating that nNOS is the main contributor of NO during reflex thermoregulation.

Axon Reflex Control

Another way that the body controls skin blood flow is through a local axon reflex. This system involves the sensory nociceptors in the skin which release neuropeptides, such as CGRP and Substance P, which cause neurogenic vasodilation. Some researchers have used transcutaneous electrical stimulation to study the effect of activating these nociceptors on skin blood flow.

Early work with transcutaneous electrical stimulation was done to test nociceptor or pain fiber functions in patients. Westerman et al. (38) tested the effects of electrical stimulation on nociceptor nerves in the skin. Using an anode and a cathode electrode placed on top of the skin, they applied 2, 4, 8, 16, and 32 electrical impulses to the skin at 2 Hz frequency with 150 volts at 0.5-1.0 ms duration per pulse All subjects felt pain at the cathode site. Six subjects received a capsaicin treatment to block the nociceptor fibers while the others did not receive a treatment. Cutaneous skin blood flow was measured with a laser Doppler probe. With increasing number of electrical impulses, an increase in blood flow was seen with and without capsaicin, but the treatment with capsaicin was considerably less (38). Also, the level of perceived pain was less in those who received the capsaicin treatment (38).

The stimulation was performed at a level where pain was perceived so that the nociceptors were known to be active. Furthermore, the reduction in the magnitude of neurogenic vasodilation with capsaicin treatment indicates that either the nociceptors were only partially blocked or that other substances and/or nerve fibers were activated that contributed to the dilation.

Other studies have used surface electrical stimulation to stimulate neurogenic vasodilation without invoking pain in subjects. Petrofsky, Hinds, Batt, Prowse, and Suh (29) used rubber surface electrodes to stimulate activation of blood flow in the skin without invoking pain. The electrodes were placed 4 cm apart on the quadriceps muscle. Skin blood flow increased significantly during electrical stimulation. Between the electrodes, epinephrine and L-NAME were delivered to the skin through iontophoresis and blood flow decreased significantly prior to electrical stimulation but blood flow did not differ significantly during the electrical stimulation (29). They concluded that the increase in blood flow seen during electrical stimulation was caused mainly by NO because L-NAME treatment caused resting blood flow to decrease and the increase in blood flow during electrical stimulation was abolished (29).

Conclusion

Despite our current knowledge of how skin blood flow is controlled, there are still numerous questions about the exact mechanism and the interaction of the various control systems in regulating skin blood flow. Heating and cooling the body activate thermosensitive neurons that change blood flow. The sympathetic nervous system (SNS) causes vasoconstriction during cold stress through the action of sympathetic adrenergic nerves. The SNS also causes vasodilation through the action of a sympathetic cholinergic dilator nerve (unknown neurotransmitter or cotransmitter). The different neurochemicals from the cholinergic nerves that may cause this vasodilation are ACh and VIP. However, conflicting evidence remains on the importance of each one, and how they are involved with each other.

Beside the involvement of the SNS in reflex skin blood flow, NO is an important local factor in controlling skin blood flow. The presence of NO causes vasodilation and may be a part of the pathway that ACh uses to increase skin blood flow. Finally, an axon reflex can be

initiated when nociceptors are activated. These activated nociceptors release CGRP which increase skin blood flow. The axon reflex has been simulated through the use of surface electrical stimulation however it is unclear if this reflex response is activated during skin warming. More research needs to be done in an in-vivo setting to help gain a greater knowledge of how these regulatory pathways interact to control skin blood flow. Skin blood flow is a vital component in maintaining livable body temperature during exercise and temperature changes. Other diseases such as Raynaud's disease and diabetes where cutaneous circulation are impaired could significantly benefit from a greater understanding of the factors controlling cutaneous circulation.

In this proposal we describe a novel approach to understanding the control of skin blood flow by perivascular nerves. In this model we will use graded intradermal electrical stimulation to depolarize perivascular nerves and monitor the local skin blood flow response. By simultaneously applying a variety of receptor antagonists via intradermal microdialysis, we hope to eliminate the actions of many known chemical modulators of skin blood flow and quantify their contribution to either vasoconstrictor or vasodilator responses.

Chapter 3

Methods

Experimental Design

A randomized control trial will be used to examine the influence of acetylcholine (ACh), nitric oxide (NO), and vasoactive intestinal peptide (VIP) released from the sympathetic perivascular nerves during electrical field stimulation on skin blood flow. Treatments will be randomly assigned to different sites on the dorsal aspect of the forearm of each subject.

Subjects

Fifteen male or female subjects 18-35 years old will be recruited to participate in the study. Subjects will be recruited from classes at Brigham Young University. Subjects will apparently be healthy and not taking any medications. Subjects with a history of renal or cardiovascular disease will be excluded from the study. All subjects will complete a health questionnaire to determine if they meet the inclusion criteria. Female subjects will be tested during the first seven days of their menstrual cycle to avoid fluctuations in sex hormones. Subjects will be asked to avoid consumption of alcohol and caffeine and avoid exercise 12 hours prior to participation in the study. The study will be approved by the Brigham Young University Institutional Review Board prior to data collection. All subjects will provide written informed consent prior to participation. Subjects will be told they can quit at any time with no consequence.

Environmental Conditions

Each subject will be fitted with a water-perfused suit that covers the entire body except the head, feet, hands, and left forearm that is being tested. The water perfusing through the suit will be set at 34 °C throughout the experiment. The upper and lower parts of the water-perfused suit will be connected with interlocking tubing and connected to a manifold that allows switching the inlet water from either a cold or warm water bath. The warm water bath will be set to 34 °C and the cold water bath will be set at 4 °C. The suit will be perfused with warm water throughout the experiment except when testing for reflex constriction of the skin blood vessels which will be produced by switching the suit to the cold water bath for three minutes. The room temperature will be set to 28 $\pm$ 0.5 °C throughout the experiment.

Subject Preparation

Subjects will be seated upright in a dental chair throughout the procedure. Three probe sites will be chosen on the dorsal side of the left forearm 3-4 cm from the elbow. Each site will be approximately 4-5 cm away from each other. Scissors will be used to cut arm hair close to the skin at each site to minimize skin irritation. Iodine and alcohol will be applied to the skin to sterilize insertion and exit sites of the microdialysis probes.

After the skin is prepared, intradermal microdialysis probes will be inserted across the forearm into each site. Microdialysis probes will be constructed in the biochemistry lab (Appendix A). A 27 gauge needle will be inserted underneath the skin at a depth of 1-2 mm for a length of 3 cm and then exit out of the skin. A microdialysis probe will be threaded through the needle, and the needle will be removed. The hollow fiber of the tube will be adjusted so that it is directly under the skin. All three sites will be perfused at 10 µl/min with a sterile, 0.9 % saline solution for the first 15 minutes of the experiment.

Specially made laser Doppler probe holders will be placed on the skin directly over the path of the hollow fiber portion of the microdialysis probe using double-stick tape. The laser Doppler probe holder has one hole directly in the center where a laser Doppler flow probe will be placed. The laser Doppler probe (Moor Instruments, Devon, England) will be used to measure skin blood flow. On either side of the laser Doppler probe hole are two guide tubes where electrical stimulating electrodes (stainless steel acupuncture needles) will be inserted 2 mm deep into the skin. The tips of the stimulating electrodes will be 4-5 mm apart. The guide tube prevents the stimulating electrodes from penetrating the skin more than 2 mm. The stimulating electrodes are connected to a constant current stimulator (Model DS7AH, Digitimer Ltd., West Garden City, England). Local skin temperature will be measured using a thermocouple wire placed between the probe holder and the skin.

Heart rate and blood pressure will be measured using an automated blood pressure machine (Model STBP-780, Colin Medical Instruments, Komaki, Japan). The blood pressure cuff will be put on the upper right arm of subjects one inch above the antecubital space and underneath the water-perfused suit. Two electrodes used to measure heart rate will be placed at the midclavicular point below the clavicle on the left and right side of the chest. The other electrodes will be placed in fifth intercostal space at the midclavicular line on the left side of the torso. Blood pressure will be measured five minutes before the cold pressure test and every minute during the cold pressor test. Also, heart rate and blood pressure will be measured five minutes before electrical field stimulation and every minute during electrical field stimulation.

Determination of Stimulus Amplitude

The experiment will invoke an electrical stimulus that activates the perivascular nerves surrounding the cutaneous blood vessels. The typical response of electrical stimulation on the

perivascular nerves over a period of 60 seconds is a decrease in skin blood flow (Figure 1). The magnitude of the electrical stimulus is determined for each subject using the following procedure. A Grass stimulator (Model Grass S88 Stimulator, Grass Instrument Co., Quincy, MA) controlling the frequency and a digitimer constant current stimulator (Model DS7AH, Digitimer Ltd., West Garden City, England) controlling the amplitude will initially be set at a frequency of 8 Hz and an amplitude of 0.5 mA, respectively. The stimulus should feel like a "tapping" sensation on the skin. If the subject does not feel the stimulus at 0.5 mA, then the amplitude will be increased 0.1 mA every five seconds until the stimulus is detected. If the stimulus is felt at 0.5 mA, the amplitude will be decreased 0.1 mA every five seconds until the stimulus is not detected. The amplitude 0.1 mA above where the stimulus was not detected will be used.

At the determined initial amplitude and at a frequency of 8 Hz, the electrical stimulus will be turned on, and the current will be increased 0.2 mA every 20 seconds until a significant increase or decrease in skin blood flow occurs. A significant change will occur when skin blood flow readings measured by laser Doppler probes (Moor Instruments, Devon, England) change from baseline levels by 0.5-0.7 V as recorded on the computer program chart. Once a significant increase or decrease in skin blood flow is seen, the stimulus will be stopped. Five minutes later the selected amplitude will be retested for one minute at a frequency of 8 Hz to make sure that a significant change occurs. If a significant change of 0.5-0.7 V happens, then this amplitude will be used as the determined experimental amplitude. If no significant response is seen, then the amplitude will be increased 0.2 mA and retested five minutes later for one minute. The amplitude will be increased in this manner until a significant change results.

Subjects will be asked to rate the pain experienced by the electrical field stimulation using a 120 mm visual analog scale with anchors at 0 (no pain) and 100 mm (unbearable pain).

Staircase Stimulus Protocol

A dose response curve invoking increasing stimulus frequencies will be performed at each treatment site and skin blood flow will be measured. The predetermined stimulating current will remain the same throughout the rest of the experiment. The frequency will be changed in a staircase manner from 0.2 to 32 Hz. The starting frequency of 0.2 Hz will last one minute and then the frequency will increase to 1 Hz which will last one minute. This pattern will continue with 2 Hz, 4 Hz, 8 Hz, 16 Hz, and 32 Hz. The total time to go through the five different frequencies will be seven minutes. For each stimulus frequency the skin blood flow response will be characterized by the area under the skin blood flow–time response curve (AUC) for one minute where skin blood flow is expressed as a percent of resting blood flow prior to electrical stimulation. The dose response curve therefore reflects the plot of stimulus frequency versus the AUC for skin blood flow.

Evidence Supporting Staircase Stimulus Protocol

During preliminary testing, five subjects were tested using the staircase method, and five subjects were tested by randomly assigning different frequencies and waiting five minutes between each stimulus. The frequencies tested ranged from 0.2-32 Hz. The average response of each method was similar for both the staircase and randomized frequency protocols (Figure 2). Both protocols produced similar results so the staircase frequency protocol will be used because of its shorter time period.

Bretylium Tosylate Blockade of Sympathetic Noradrenergic System

100 mM bretylium tosylate (Appendix B) will be delivered to the skin during a 90 minute perfusion period. Bretylium is taken up by the adrenergic neurons and blocks the release of norepinephrine from the axons. When this system is blocked vasoconstriction of the cutaneous blood vessels will not be invoked when the cutaneous sympathetic nerves are activated during a whole-body cold challenge. The absence of a cold induced cutaneous vasoconstriction will be used to verify adequate delivery of bretylium tosylate to the skin. In addition, preliminary studies indicate that electrical field stimulation of cutaneous perivascular nerves typically produces the activation of sympathetic adrenergic nerves and vasoconstriction. This effect is typically prevented by application of bretylium tosylate, again verifying the blockage of sympathetic noradrenergic nerves (Figure 3). Bretylium tosylate is in short supply at the moment. In the case that bretylium tosylate is not available, phentolamine and propranolol will be used to block alpha and beta-adrenergic receptors and BIBB-3622 to block neuropeptide Y (34).

Bretylium Infusion

Bretylium will be infused at 5 µl/min with a Harvard infusion pump (Model PHD 2000, Harvard Apparatus, Holliston, MA) for 120 minutes in all three sites and be considered the control condition. After 120 minutes, the electrical field stimulation will be done on all three sites and skin blood flow response will be recorded. Bretylium's effects last 4-5 hrs. A post trial cold pressor test will be used to verify blockade.

ACh Blockade

0.1 mg/ml atropine sulfate (Appendix B), an antagonist of ACh receptors, will be delivered to two of the three skin sites. Infusion of atropine will block the action of ACh during

electrical stimulation on ACh receptors in the cutaneous vasculature. Atropine will be infused for 30 minutes at 5 µl/min. The electrical field stimulation staircase protocol will be used to examine atropine's effect on skin blood flow.

ACh and NO Blockade

In one of the sites where ACh was blocked, a solution of 0.1 mg/ml atropine and 10 mM nitro-L-arginine methyl ester (L-NAME) (Appendix B) will be infused for 30 minutes at a rate of 5 µl/min. Nitro-L-arginine methyl ester is a nonselective antagonist of NO synthase and will be used to prevent NO production. After infusion, the electrical field stimulation staircase protocol will be done.

ACh, NO, and VIP Blockade

In the other site where ACh was also previously blocked, 200 µM of the VIP receptor antagonist (p-Chloro_D-Phe6,Leu17)-VIP or (10-28)-VIP, 10 mM L-NAME, and 0.1 mg/ml atropine will be infused in the site (Appendix B). The drug infusion will last 30 minutes and be infused at a rate of 5 µl/min. The electrical field stimulation staircase protocol will be done at the end of infusion.

Maximum Blood Flow

Following the final stimulation period, maximum skin blood flow will be determined by application of 28 mM sodium nitroprusside (SNP) (Appendix B). All drugs will be delivered by intradermal microdialysis at a rate of 5-10 µl/min with a Harvard infusion pump (Model PHD 2000, Harvard Apparatus, Holliston, MA) for 30-45 minutes.

Outline of Specific Protocol

Subjects will have an orientation meeting for 15-30 minutes where the experiment will be explained and a health questionnaire and consent formed will be filled out. At the next meeting, subject's height, weight and age will be recorded. Subjects will have avoided eating any caffeine, drinking any alcohol or exercising 12 hours prior.

Microdialysis probes will be inserted and probe holders, laser Doppler probes, and stimulating electrodes will be set up. Approximate set-up time is 30 minutes. Sterile saline solution will be pumped at a rate of 10 µl/min for 15 minutes. At the end of 15 minutes, a cold pressor test will be done for three minutes. After that the experimental current amplitude for each site will be determined for 10-30 minutes. Bretylium tosylate will then be infused for 90 minutes, and then a cold pressor test will be done for three minutes. After the cold pressor test, bretylium will continue to be infused for 30 minutes.

After 120 minutes of bretylium infusion in all three sites, the first electrical staircase stimulation will be performed. Five minutes after stimulation atropine will be infused in the two of the three sites. After 30 minutes of infusion, another staircase stimulation will be done in all three sites. Five minutes after stimulation, atropine and L-NAME will be infused in one site and atropine, L-NAME, and VIP will be infused in another site. After infusion for 30 minutes, a staircase will be performed in all three sites. Five minutes after the staircase stimulus a cold pressor test will be done to confirm blockade of sympathetic noradrenergic neurons. Five minutes later SNP will be infused for 30-45 minutes at all skin sites to obtain maximum skin blood flow levels (Figure 4).

Data Acquisition

All data from laser Doppler probes, thermocouples, and electrical stimulus will be recorded with Powerlab - 16 channel A/D converter (Powerlab/16 SP, ADInstruments Pty Ltd., Castel Hill, Australia) at 400 Hz using the computer program chart.

Data Analysis

Comparisons between treatments will be done using repeated measures analysis of variance (ANOVA). Statistical significance will occur when *p* values are less than 0.05. Specific comparisons between treatment sites will be performed using the Tukey minimum significant difference post-hoc test. Major variables to be measured are local skin temperature (°C), skin blood flow (v), heart rate (bpm), blood pressure (mmHg), and perceived pain levels. Skin blood flow will be converted to cutaneous vascular conductance (CVC) by taking SkBF (V)/ Mean Arterial Pressure (mm HG)*100. Cutaneous vascular conductance will be expressed as a percent of maximum CVC. Changes from baseline will be calculated by taking the baseline average one minute before stimulus minus the value during electrical field stimulation. Area under the curve will be calculated from the sum of the second-by-second differences from baseline for each stimulus frequency.

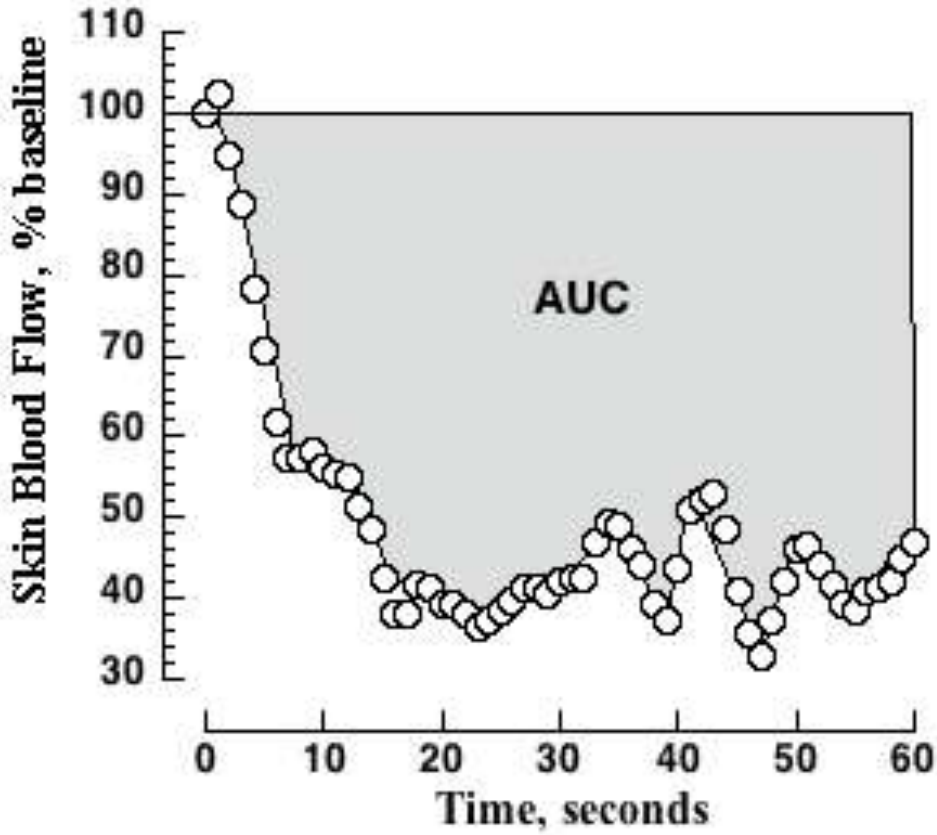

Figure 1. Skin blood flow response to 60 seconds of electrical stimulation. AUC = area under the skin blood flow–time response curve where skin blood flow is expressed as a percent of resting blood flow prior to electrical stimulation. The dose response curve therefore reflects the plot of stimulus frequency versus the AUC for skin blood flow.

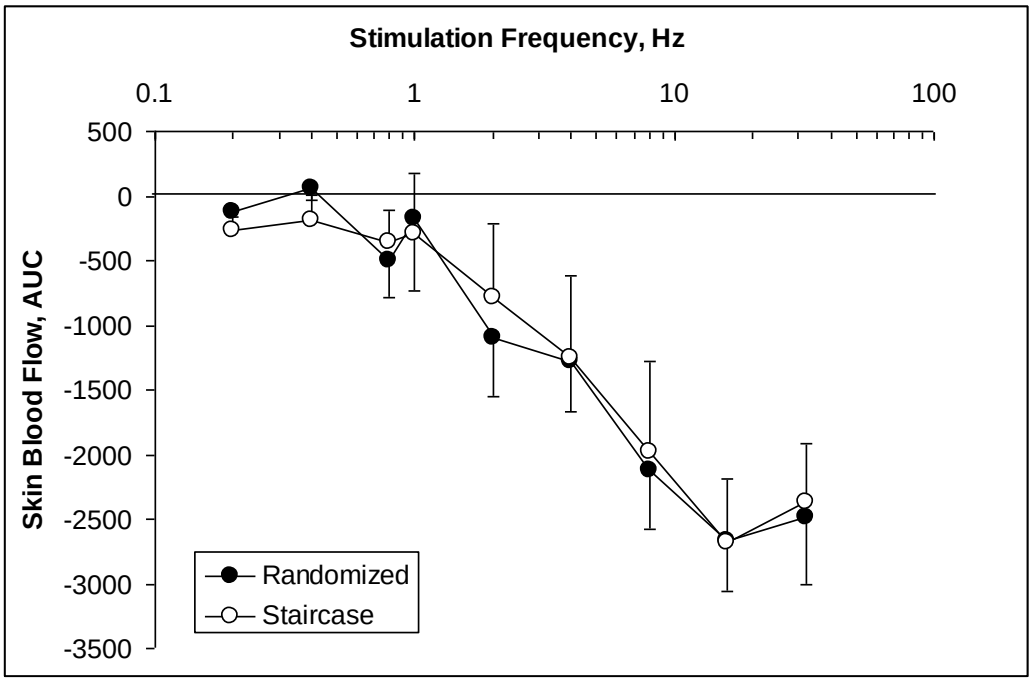

Figure 2. Dose Response Curve. AUC = area under the skin blood flow–time response curve where skin blood flow is expressed as a percent of resting blood flow prior to electrical stimulation. The dose response curve therefore reflects the plot of stimulus frequency versus the AUC for skin blood flow. Both randomized and staircase procedures produced similar responses at the same frequencies.

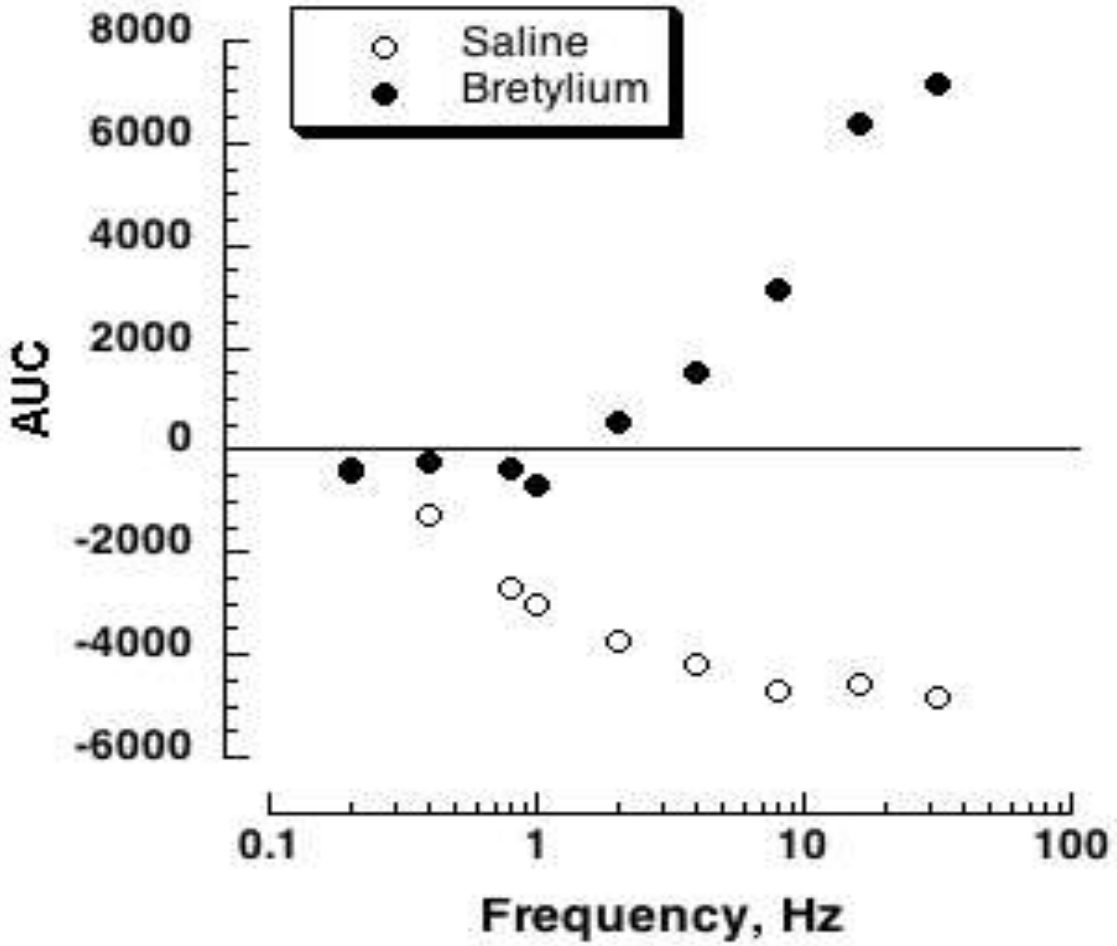

Figure 3. Bretylium effects on skin blood flow. AUC = area under the skin blood flow–time response curve where skin blood flow is expressed as a percent of resting blood flow prior to electrical stimulation. The dose response curve therefore reflects the plot of stimulus frequency versus the AUC for skin blood flow. Skin blood flow response to electrical stimulation with saline and with 100 mM Bretylium Tosylate. Bretylium effectively inhibits the sympathetic noradrenergic system causing only the sympathetic dilator system to be active.

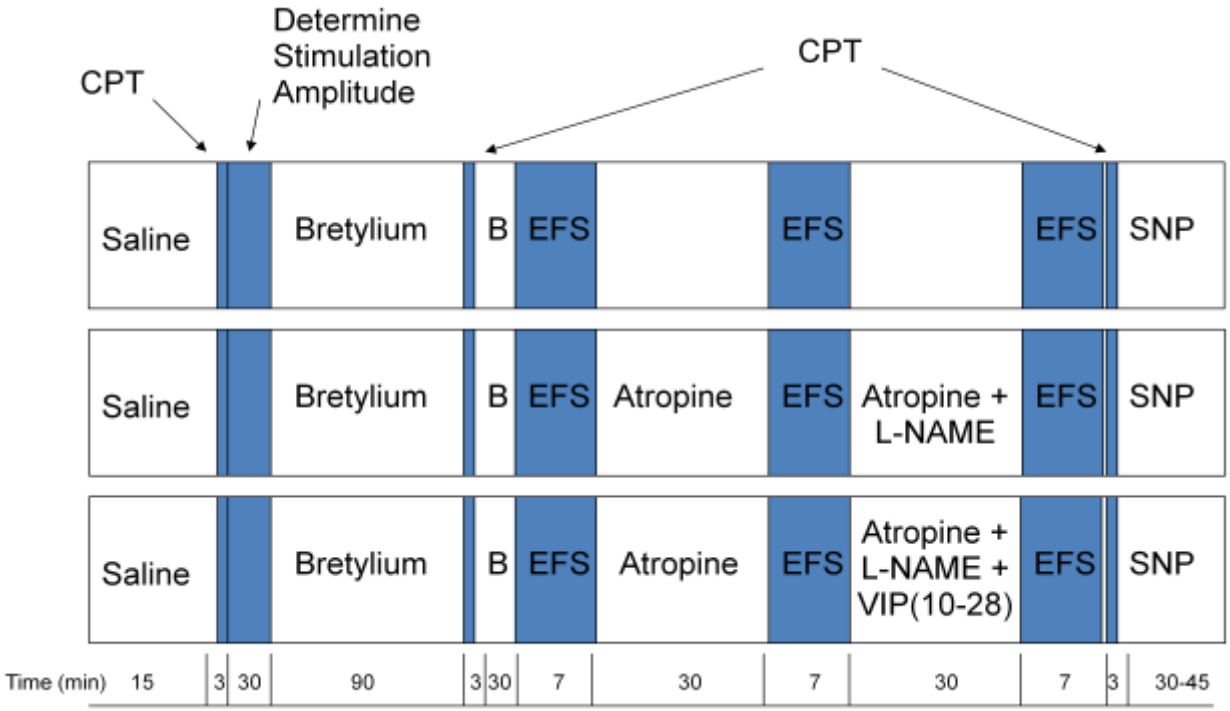

Figure 4. Experiment timeline. B stands for bretylium, SNP stands for sodium nitroprusside, EFS stands for electrical field stimulation, CPT stands for cold pressor test, VIP (10-28) stands for Vasoactive intestinal peptide antagonist, and L-NAME stands for nitro-L-arginine methyl ester. Estimated total time is 4 hours 45 minutes.

Appendix A -- Construction and insertion of microdialysis probes

Microdialysis Probe Materials

Two microdialysis probes will be inserted into the dorsal side of the forearm of the subjects approximately 3-5 cm from the elbow. Microdialysis probes are constructed in the biochemistry lab. A 45 cm stainless steel wire (Alan Baird Ind., Ho-Ho-Kus, NJ) composes the skeleton of the microdialysis probe. A 25 gauge luer stub adapter was attached to 4 cm long polyethylene (PE) 50 tubing. Six cm of PE 10 tubing was inserted 2 cm into the PE 50 tubing. Inserted into the PE 10 tubing approximately 2 cm was the 22 cm long polyimide tubing (A-M Systems, Inc). The 4.5 cm long hollow fiber dialysis membrane (Spectra-Por regenerated cellulose microdialysis hollow fibers) went one inch over the polyimide tubing. At the opposite end of the 4.5 cm hollow fiber, polyimide tubing was inserted into the hollow fiber one inch. Two and a half cm of hollow fiber was unobstructed to allow chemicals to diffuse freely through the hollow membrane.

Insertion of Microdialysis Probes

1. Skin preparation for insertion of the microdialysis probes consists of marking a 3 cm distance on the dorsal forearm where the microdialysis hollow fiber will go under the skin.
2. An alcohol pad will clean the two sides of the 3 cm line where a 27 gauge needle will be inserted and will leave the skin.
3. After letting the alcohol dry for a few seconds, a cotton swab will be dipped in iodine and then rubbed in a circular motion on the two ends of the line where the alcohol swab was used. Do this two times. Let it dry on the skin. Skin is now ready for needle insertion.

4. Open a sterile pad and place it on a flat surface in preparation to put the microdialysis probe and needle on it.
5. Carefully open the microdialysis probe package being sure not to touch the part of the probe that enters the skin.
6. Using a syringe with saline, check to make sure that the microdialysis probe has no leaks. You can touch the stub adapter and insert the syringe. Slowly infuse saline through the microdialysis probe and look at the joints of the probe to make sure saline is not leaking.
7. Fix joints as needed or get a new microdialysis probe.
8. Place microdialysis probe on sterile pad.
9. Open 27 gauge needle package and place on sterile pad without touching needle.
10. Put on sterile gloves. Only touch the inside with hands. Outside of gloves only to be touched with the gloves themselves.
11. Insert needle from medial side of marked line so that it leaves the lateral side of arm. Take the needle and while pulling down skin to make the skin tight insert the needle into the skin.
12. Do not go too deep into the skin but 1-2 mm below the skin surface. You should see a slight protuberance that indicates where the needle is.
13. Poke needle through skin when you get to the end of the 3 cm line.
14. Insert microdialysis probe into the needle until the end of the hollow fibers gets to the end of the needle.
15. Pull needle through skin.
16. Adjust microdialysis probe to where the hollow fiber is underneath the skin between the ends of the marked line.

17. Place a piece of tap where the microdialysis probe enters and leaves the skin so it does not move.
18. Mark on the tape with a straight line where the microdialysis probe enters and leaves skin.
19. Infuse saline through sites until electrical stimulation amplitude is determined.

Appendix B -- Preparation of drugs

Bretylium Tosylate

a. MW = 414.4 g/mole.

b. Make a 100 mM solution of Bretylium Tosylate.

c. 100 mM = 0.1 M = 0.1 moles/L.

d. Amount needed: 0.1 moles/L x 414.4 g/mole = 41.44 g/L = 41.44 mg/ml.

e. Weigh out Bretylium close to 41.44 mg in a sterile eppendorph tube.

f. Record weight of Bretylium.

g. Take measured (mg)/41.44 mg/ml to determine how much saline is needed for the solution.

h. Put sterile saline in eppendorph with Bretylium.

i. Mix well (vortex).

j. Draw solution in a syringe and then push solution through a um sterile filter into another syringe.

k. Label syringe and place in package.

l. Make 3 syringes with Bretylium Tosylate.

Atropine Dose = 0.1 mg/ml

a. Make a 0.1 mg/ml atropine solution.

b. M_{Stock} = 0.4 mg/ml.

c. V_{Stock} = ?.

d. $M_{dilution}$ = 0.1 mg/ml.

e. $V_{dilution}$ = 1 ml.

f. $M_{dilution}\ V_{dilution} = M_{Stock}\ V_{Stock}$.

g. 0.1 mg/ml x 1 ml = 0.4 mg/ml x ?.

h. V_{Stock} = (.01 mg/ml x 1 ml)/ 0.4 mg/ml = 0.25 mL.

i. Combine 0.25 mL of stock with 0.75 mL saline.

L-NAME

a. MW = 269.69 g/mole.

b. Make a 10 mM solution.

c. 10 mM = .01 M = .01 moles/L.

d. Amount needed: 269.69 g/mole x .01 mole/L = 2.6969 g/L = 2.6969 mg/ml.

e. Weigh out L-NAME close to 2.6969 mg in sterile eppendorph tube.

f. Record weight of L-NAME.

g. Take measured (mg)/2.6969 mg/ml to determine how much saline is needed for the solution.

h. Put saline in eppendorph tube with L-NAME.

i. Put solution in a syringe and then put it through a cold filter into another syringe to sterilize solution.

j. Put syringe in package and label.

Sodium Nitroprusside (SNP)

a. MW = 297.95 g/mole.

b. Make 28 mM solution.

c. 25 mg/ml solution.

d. What is 25 mg/ml concentration (mM)?.

e. 25 mg/ml x 1 g/1000 mg x 1 mol/295.95 g x 1000 mmoles/1 mole x 1000 mL/1 L = 84.47 mM.

f. M_{Stock} = 84.47 mM.

g. V_{Stock} = ?.

h. $M_{dilution}$ = 28 mM.

i. $V_{dilution}$ = 0.5 mL.

j. $M_{dilution}$ $V_{dilution}$ = M_{Stock} V_{Stock}.

k. 28 mM x .5 mL = 84.47 mM x ?.

l. V_{Stock} = (28 mM x .5 mL)/84.47 mM = .17 mL.

m. Combine .17 mL of stock with .33 mL saline.

Saline

a. Clean top of saline bottle with alcohol pad.

b. Insert needle in top and fill syringe.

c. Fill three syringes with saline.

VIP receptor antagonist- 200 μM (p-Chloro_D-Phe6,Leu17)-VIP.

Antagonists needed if Bretylium Tosylate is not available (34).

NPY antagonist- 10.5 μM BIBP-3226.

Beta-adrenergic antagonist

1 mM Propranolol.

Alpha-adrenergic antagonist

0.5 mM Phentolamine **- Nonselective alpha-adrenergic receptor antagonist**

or

5 mM Yohimbine – **Selective Alpha-1-adrenergic receptor antagonist**

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