THE INVOLVEMENT OF NOREPINEPHRINE, NEUROPEPTIDE Y, AND ENDOTHELIAL NITRIC OXIDE SYNTHASE ON CUTANEOUS VASCULAR RESPONSES TO LOCAL SKIN WARMING

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ABSTRACT

We investigated the role of endothelial nitric oxide synthase (eNOS), norepinephrine (NE), and neuropeptide Y (NPY) in cutaneous vasodilation in response to local skin warming. In a two part study, we used four treatment sites on the skin of the forearm for insertion of microdialysis fibers, and placement of local skin heaters and laser-Doppler probes. We allowed an hour and a half for needle trauma resolution. We recorded 10 min of baseline data, begin drug perfusion for 50 min to ensure full receptor antagonism (α, β, Y_1) and enzyme inhibition. In both parts of the study, the local warming protocol was such that local skin temperature was increased from 33 to 42 °C at 0.5 °C · 15 s^{-1}. In Part 1 of our study, we used three sites for drug treatment 1) L-NAA (eNOS inhibition), 2) Yohimbine (YOH) and Propranolol (PRO) (α- and β- receptor antagonism), 3) a combination site (L-NAA+YOH+PRO), 4) untreated site for control. Treatments resulted in a reduction of vasodilation ($P < 0.05$) that did not differ ($P > 0.05$) from each other. In study 2 the same test procedure was utilized, with four treatment sites: 1) L-NAA, 2) BIBP (antagonize Y_1-receptors), 3) L-NAA+BIBP, 4) control site. Treated sites resulted in a reduction ($P < 0.05$) of the vasodilator response when compared to control sites; again treatments did not differ ($P > 0.05$) from each other. These data indicate that NE and NPY are working via eNOS in cutaneous vasodilator response to local skin warming.

DEDICATION

This thesis is dedicated to the incredibly patient, supportive, and understanding individuals that have helped me throughout this process. I'd like to especially thank my parents and Dr. Hodges whose support and encouragement allowed me to endure the process and complete this project.

LIST OF ABBREVIATIONS AND SYMBOLS

α	Alpha
β	Beta
μm	Micrometer
cm	Centimeter
mm	Millimeter
LDF	Laser-Doppler flowmetry
NPY	Neuropeptide Y
NE	Norepinephrine
L-NAA	N^G-amino-L-arginine
SNP	Sodium Nitroprusside
NO	Nitric oxide
NOS	Nitric oxide synthase
eNOS	Endothelial nitric oxide synthase
YOH	Yohimbine
PRO	Propranolol
BIBP-3226	N^2-(diphenacetyl)-N-([4-hydroxyphenyl]methyl)-D-arginine amide
MAP	Mean arterial pressure
T_{loc}	Local skin temperature
T_{sk}	Whole body mean skin temperature

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CONTENTS

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The human cutaneous circulation plays an important role in thermoregulation. Under conditions of heat stress, skin blood flow increases in order to dissipate heat, while exposure to extreme cold elicits a reduction in skin blood flow to maintain temperature homeostasis. In response to increased local skin temperature (no change in core temperature), the skin blood flow response is characterized by an initial peak and a nadir, succeeded by a stable plateau. The initial peak can be abolished, or greatly reduced, under conditions of sympathetic and/or sensory nerve blockade (12, 28, 35, 40). The plateau phase is greatly dependent on endothelial nitric oxide synthase (eNOS) (23) and the sympathetic noradrenergic neurotransmitters norepinephrine (NE) and neuropeptide Y (NPY) (11, 12, 35). There appears to be little, if any, sensory nerve involvement in the plateau phase of the cutaneous vasodilator response to local skin warming (5, 28, 34).

Previously, we have shown that cutaneous sympathetic nerves and their neurotransmitters, NE and NPY, are involved in the cutaneous vasodilatation to local skin warming (5, 11, 12, 35). Furthermore, we also produced data that indicated that NE and NPY were working via NOS to elicit this vasodilator response (12). This notion was supported by *in vitro* work that shown had shown that the binding of NE and NPY to endothelial-based α_2- and Y_1-receptors increased the activity of eNOS (1, 6, 38). Since our previous finding, Kellogg et al (23, 25) found that the NOS isoform involved in cutaneous vasodilator response to local skin warming was the eNOS isoform. While it is generally accepted that the endothelium regulates vasculature tone independently of sympathetic function, our previous data, and more recently, that of Nausch et al (29) indicates that sympathetic nerves can influence endothelial function.

Our previous data indicated that the antagonism of α-, β- and Y_1-receptors decreased cutaneous vasodilation via the production of NO (11, 12). The current study directly investigated the inhibition of the eNOS isoform while also antagonizing, α-, β- and Y_1-receptors to determine if these systems are working in a series fashion to elicit cutaneous vasodilation to localized skin warming. In Part 1 we examined the role of NE and in Part 2 the role of NPY. We hypothesized that the attenuation of the vasodilator response to local skin warming would not differ among the treatments (inhibition of eNOS and antagonism of NE and NPY receptors), which would be the case if these systems were working in a series fashion. Furthermore, we also clarified the relative contributions of the 2 sympathetic neurotransmitters, NE and NPY in the cutaneous vasodilator response.

CHAPTER II

METHODS AND PROCEDURES

Participants

All studies were approved by the local Institutional Review Board, and all participants were fully informed of the methods and risks before consent was obtained. Seven participants (27 ± 2 yr, 175 ± 4 cm, 75 ± 2 kg) volunteered for both parts of this study. Participants were healthy, normotensive, non-smokers, and not taking any medications. All study participants visited the Human Performance Laboratory at the University of Alabama for two testing sessions. Participants reported to the laboratory after a 2-h fast, but well hydrated.

Instrumentation

Intradermal microdialysis was used to deliver pharmacological agents into the skin (5, 8, 10-14, 35). Four intradermal microdialysis fibers were placed in the skin on the dorsal surface of the right forearm of each participant. These probes consisted of 2 cm of microdialysis tubing (inner diameter 200 μm, 18 kDa nominal molecular weight cutoff) attached at each end to polyimide tubing providing a 1 cm window for drug transfer between the fiber and skin. Before implantation, the area of skin was temporarily anesthetized by the application of an ice pack for 5 min (8). A 22-gauge needle was inserted superficially into the skin and ~2.5 cm across the skin before exiting. The microdialysis probe was introduced into the skin via the lumen of the needle, the needle was then removed, leaving the probe in place. All probes were placed in this manner, and ~1.5 h was allocated for the effects of the insertion trauma to subside (8).The four intradermal microdialysis fibers were placed ~3 cm apart and taped in place. The participant was supine throughout this procedure.

Measurements

All measurements were performed with the subjects resting in a supine posture. Skin blood flow was measured from the dorsal aspect of the forearm by laser-Doppler flowmetry (Moor Instruments Inc., Axminster, UK), and expressed as laser-Doppler flow (LDF) (20, 30). LDF has no known side-effects and is a reliable and accurate, non-invasive method for the measurement of blood flow in the skin and these measures are not contaminated by underlying skeletal muscle blood flow (31). LDF uses low-level laser light, Class 1, output power 2.5 mW max, and does not require additional certificates for health and safety. Local skin temperature (T_{loc}) control was achieved with heating probe holders (Moor Instruments Inc., Axminster, UK); these control surface temperature over an area of 133 mm^2 with the exception of a small aperture (7.1 mm^2) in the centre of the holder to enable placement of the laser-Doppler probe. T_{loc} can be precisely maintained within 0.1 °C. Blood pressure was recorded non-invasively by auscultation from the contralateral arm. Mean arterial pressure (MAP) was calculated as: MAP = (SBP + (2 * DBP))/3. Whole body skin mean temperature (T_{sk}) was recorded as the weighted mean from six thermocouples placed on the body surface and controlled by the use of a water-perfused suit (33).

Pharmacological treatments

Five pharmacological agents were administered via microdialysis from a syringe pump at a rate of 4 $\mu l \cdot min^{-1}$, and all agents were filtered sterilized with 0.2-µm micropore syringe filters (Acrodisc, Pall, Ann Arbor, MI) (14). All agents were dissolved in sterile lactated Ringer's solution. Antagonism of α-adrenergic receptors was achieved by the application of yohimbine (YOH) (Sigma-Aldritch, St. Louis, MO, USA) at a concentration of 5 mM (11, 35). YOH is

traditionally regarded as an α_2-adrenergic antagonist; however, prior work has successfully used YOH at this concentration and infusion rate in antagonizing all α-receptors (11, 12, 21, 35). Antagonism of β-adrenergic receptors was achieved by the application of propranolol (PRO) (Sigma-Aldritch, St. Louis, MO, USA) at a concentration of 1 mM (11, 12, 35). A 10 µM solution of the N^2-(diphenacetyl)-N-([4-hydroxyphenyl] methyl)-D-arginine amide (BIBP3226) (Sigma-Aldritch, St. Louis, MO, USA), was used to antagonize Y_1-receptors. This concentration and administration has proven effective in revealing NPY involvement in cutaneous vasoconstrictor responses (12, 32). Inhibition of eNOS (the enzyme that produces endothelial-derived NO) was achieved by the use of N^G-amino-L-arginine (L-NAA) at a concentration of 3 mM (23, 25).

To attain maximal skin blood flow levels, local skin temperature was raised and held at 42 °C, and sodium nitroprusside (SNP) (Sigma-Aldritch, St. Louis, MO, USA) was administered at a concentration of 58 mM, which previous work has shown to elicit maximal skin blood flow (5, 23-27).

Protocols

Part 1 assessed the contribution eNOS to the cutaneous vasodilator response from the sympathetic neurotransmitter, NE (Fig 1). Site one acted as an untreated control site and was perfused with lactated Ringer's only (vehicle). At microdialysis site 2, blockade of the α- and β-receptors was achieved by the infusion of YOH+PRO. At microdialysis site 3, inhibition of eNOS was achieved via the infusion of L-NAA. At site 4, a

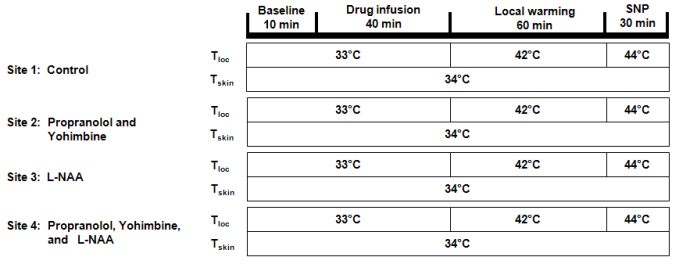

Figure 1. Schematic of the protocol designed to test Part 1. Four skin sites instrumented with microdialysis fibers, laser-Doppler probe and local heating probe holders. Whole body skin temperature (T_{sk}) thermoneutral at 34 °C. 10 min baseline with all site perfused with saline, site 2 – 4 treated with α- and β-receptor antagonism, endothelial nitric oxide synthase inhibition, and a combination, respectively. Local skin temperature (T_{loc}) increased from 33 to 42 °C. Max vasodilation achieved via infusion of sodium nitroprusside (SNP) and increase T_{loc} to 42 °C.

combination of the 3 agents was used. After the effects of the microdialysis fiber insertion trauma subsided (1.5 h) (8), drug infusion began as previously described for 40 min. Then local warming protocol was initiated at each of the laser-Doppler probe sites, increasing T_{loc} from 33 °C to 42 °C at a rate of 0.6 °C · min^{-1}. Once T_{loc} of 42 °C was reached, this temperature was held constant for 50 minutes to evaluate skin blood flow responses to the individual treatments. Then, all sites were warmed to 42 °C and infused with 58 mM SNP to elicit maximal cutaneous vasodilation (maximal skin blood flow). Blood pressure (BP) was taken by auscultation every 10 min.

The protocol for *Part 2* is illustrated in figure 2. This protocol determined the contribution of eNOS to the cutaneous vasodilator response from the sympathetic neurotransmitter, NPY. Site one acted as an untreated control site and was perfused with Ringer's only. At microdialysis site 2, blockade of Y_1-receptors was achieved by the infusion of BIBP3326. At microdialysis site 3, inhibition of eNOS was achieved via the infusion of L-NAA. At site 4, a combination of the 2 agents was used. The sequence and timing was the same as described for *Part 1*.

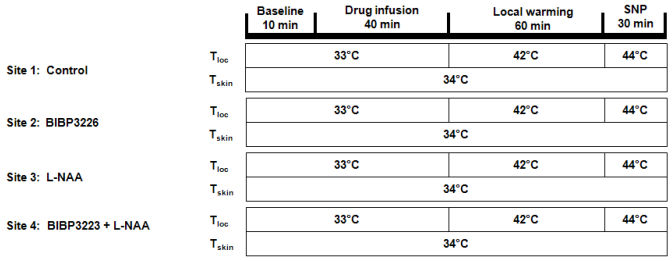

Figure 2. Schematic of the protocol designed to test Part 2. Four skin sites instrumented with microdialysis fibers, laser-Doppler probe and local heating probe holders. Whole body skin temperature (T_{sk}) thermoneutral at 34°C. 10 min baseline with all site perfused with saline, site 2 – 4 treated with Y_1-receptor antagonism (BIBP3326), endothelial nitric oxide synthase inhibition (L-NAA), and a combination, respectively. Local skin temperature (T_{loc}) increased from 33 to 42°C. Max vasodilation achieved via infusion of sodium nitroprusside (SNP) and increase T_{loc} to 44°C.

Data and statistical analysis

Blood pressure was recorded by auscultation every 10 min. All other variables (LDF and T_{loc} at each skin site, and T_{sk}) were collected at 100 Hz and stored for offline analysis (Biopac, MP150). Skin blood flow is expressed as cutaneous vascular conductance (CVC; LDF/MAP)

and given as a percentage of maximal CVC (achieved in response to 58 mM SNP). Thus, the data is normalized to the CVC values obtained from the local skin temperature of 42 °C combined with SNP infusion. Stable 5 min sections of baseline and plateau data were used for comparisons. Due to the rapid and transient nature of the initial peak response, 30 s periods of data were used for analysis of this section. To assess involvement of NE and eNOS in the vasodilator responses observed, we compared the PRO+YOH, L-NAA to one another, while we also analyzed all 3 treated sites with the control site. To assess the involvement of NPY in the vasodilator responses observed, we compared the BIBP3226, L-NAA, and combination treated sites to the control site. For both *Parts* differences among the 4 treatments were evaluated by a mixed-model two-way repeated measures analysis of variance. Planned comparison tests, including Bonferroni *post-hoc* tests, were performed where appropriate to determine where differences occurred. Statistical analyses were performed using SAS (v9.2, SAS Institute inc., USA). Sample size was determined using nQuery (v7.0, Statistical Solutions, Cork, Ireland). Statistical significance was set at $P < 0.05$ and data were expressed as mean ± standard deviation.

CHAPTER III

RESULTS

3.1. Contribution of eNOS and NE to cutaneous vasodilation in response to local skin heating.

Baseline

Inhibition of eNOS with L-NAA significantly reduced baseline CVC compared to control (6 ± 3 *vs.* 15 ± 3 CVC%max) ($P < 0.05$) (Fig 3). By contrast, antagonism of α- and β-receptors with YOH+PRO significantly increased baseline CVC when compared to the untreated control (21±3 *vs.* 15 ± 3 CVC%max) ($P < 0.05$). The combined treatment of L-NAA, YOH, and PRO did not change ($P > 0.05$) baseline CVC (12 ± 4 CVC %max) when compared to the control skin sites.

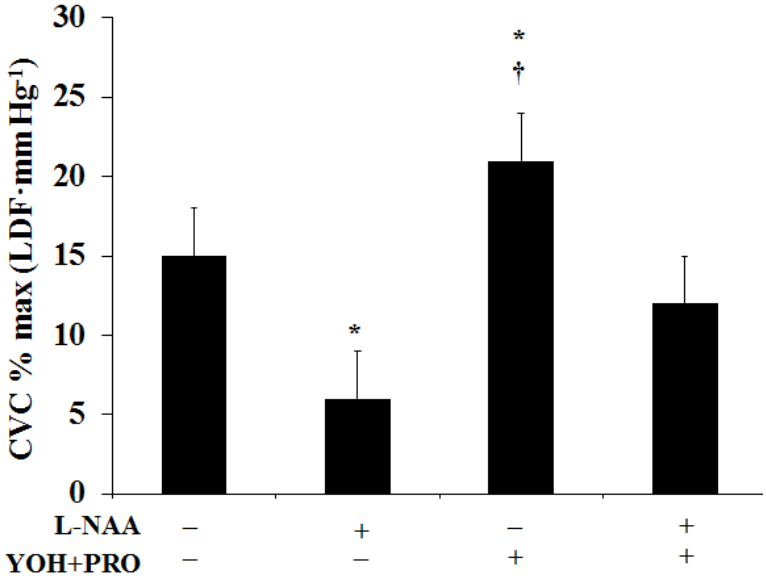

Figure 3. The effect of drug treatment on baseline in *part 1*. Inhibition of eNOS with L-NAA, significantly decreased baseline CVC. Antagonism of α- and β-receptors increased baseline CVC. The + and − indicate sites were or were not treated with antagonists, respectively. Values are means ± SD; $n = 7$ subjects. *$P < 0.05$ vs. untreated site; †$P < 0.05$ vs. L-NAA treated site.

Initial Peak

The initial peak response to the increase in local skin temperature from 33 to 42°C caused a significant increase in CVC at all skin sites ($P < 0.05$) (Fig. 4). Treatment with L-NAA caused a modest reduction ($P < 0.05$) in the initial peak relative to the untreated control site (62 ± 6 *vs.* 76 ± 5 CVC%max) (Fig. 4). As seen in our previous studies (12, 35), under conditions of

YOH+PRO treatment, there was a marked reduction in the initial peak, with CVC%max at these skin sites reaching only 49 ± 4 CVC%max, significantly reduced compared to both control and L-NAA (both $P < 0.05$). Interestingly, skin sites treated with the combination of L-NAA, YOH, and PRO, while significantly reduced when compared to control and L-NAA ($P < 0.05$), were not different when compared to the YOH+PRO skin sites (47 ± 4 *vs.* 49 ± 4 CVC%max).

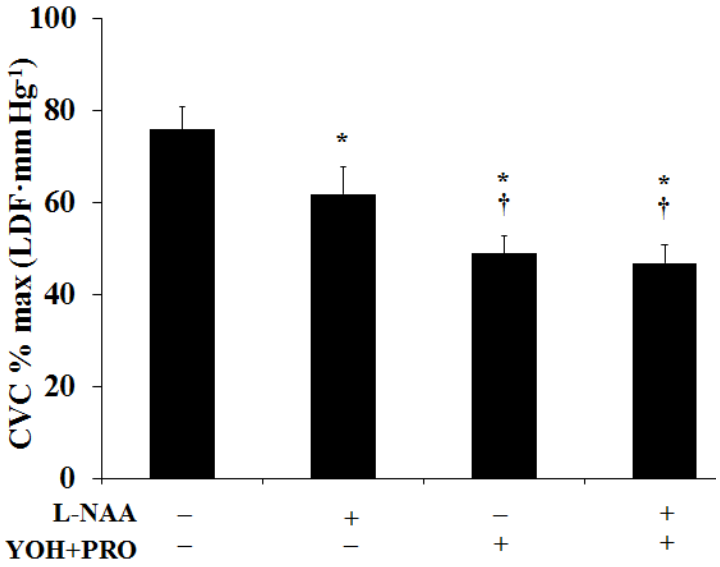

Figure 4. The initial peak responses to local skin warming in *part 1*. Inhibition of eNOS with L-NAA, significantly decreased the response. Antagonism of α- and β-receptors further reduced the initial peak response. Combination treatment was not different to only YOH_PRO treatment. The + and − indicate sites were or were not treated with antagonists, respectively. Values are means ± SD; $n = 7$ subjects. *$P < 0.05$ vs. untreated site; †$P < 0.05$ vs. L-NAA treated site.

Plateau

A stable plateau in CVC was reached across all skin sites at 42 °C ($P < 0.05$ vs. 32 °C). The plateau achieved at skin sites treated with L-NAA (64 ± 3 CVC%max), YOH+PRO (62 ± 3 CVC%max), and combination of L-NAA+YOH+PRO (61 + 4 CVC%max) were attenuated to similar extents compared to CVC at control sites (87 ± 5 CVC%max) (Fig. 5). CVC at all treated sites did not differ ($P > 0.05$).

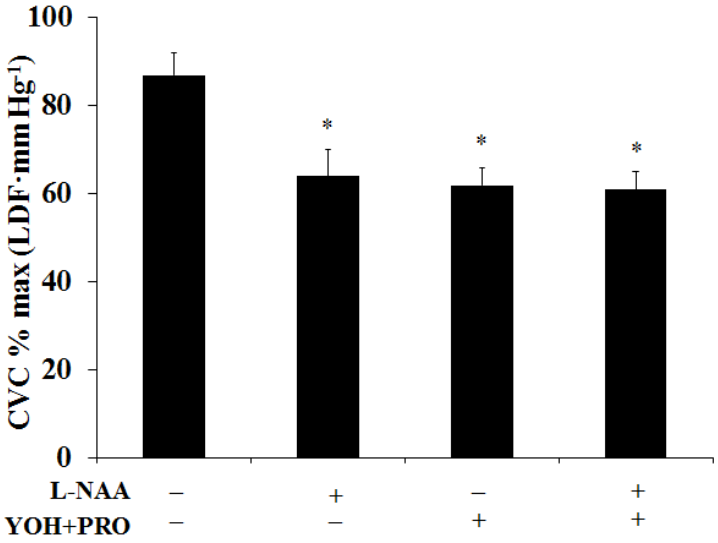

Figure 5. The plateau phase responses to local skin warming in *part 1*. All treatments reduced the vasodilator response similarly. The + and − indicate sites were or were not treated with antagonists, respectively. Values are means ± SD; $n = 7$ subjects. *$P < 0.05$ vs. untreated site.

3.2. Contribution of eNOS and NPY to cutaneous vasodilation response to local skin heating

Baseline

CVC averaged 13 ± 2 CVC%max at control sites,7±3 CVC%max at L-NAA treated sites, 14 ± 4 CVC%max at BIBP treated sites, and 8±5 CVC%max at L-NAA+BIBP combination treatment sites. Inhibition of eNOS with L-NAA significantly reduced baseline CVC compared to control and BIBP (both $P < 0.05$). CVC at BIBP treatment sites differed significantly from L-NAA treatment sites ($P < 0.05$), but did not differ from control sites or L-NAA+YOH+PRO (both $P > 0.05$). CVC for L-NAA+BIBP treated sites were not significantly different from other treatment sites ($P > 0.05$).

Initial Peak

The initial peak response to the increase in local skin temperature from 33 to 42°C caused a significant increase in CVC at all skin sites ($P < 0.05$). These CVC values were 73 ± 5 CVC%max at control sites, with significantly reduced responses in CVC at the L-NAA (60 ± 4 CVC%max), BIBP (52 ± 5 CVC%max), and L-NAA+BIBP treated sites (50 ± 6 CVC%max) (all

$P < 0.05$ compared to control). CVC values at sites treated with L-NAA, BIBP, and L-NAA+BIBP were attenuated to similar extents compared with control sites (all $P < 0.05$).

Plateau

A stable plateau in CVC was reached across all sites at 42 °C ($P < 0.05$ vs. 32 °C). These CVC values were 88 ± 6 CVC%max at control sites, 62 ± 3 CVC%max at L-NAA treated sites, 60 ± 6 CVC%max at BIBP treated sites, and 61 ± 3 CVC%max at L-NAA+BIBP combination treatment sites (Fig. 6). CVC at L-NAA treatment sites significantly differed from control sites ($P < 0.05$). CVC attenuation at L-NAA treatment sites did not differ from BIBP or L-NAA+BIBP combination treated sites ($P > 0.05$). CVC at BIBP treated sites significantly differed from control sites ($P < 0.05$) but did not differ from L-NAA or L-NAA+BIBP treatment sites ($P > 0.05$). Treatment with L-NAA+BIBP caused significant attenuation in CVC compared to control sites ($P > 0.05$). Interestingly L-NAA+BIBP treatment did not cause a significant attenuation in CVC when compared to L-NAA and BIBP treatment ($P > 0.05$).

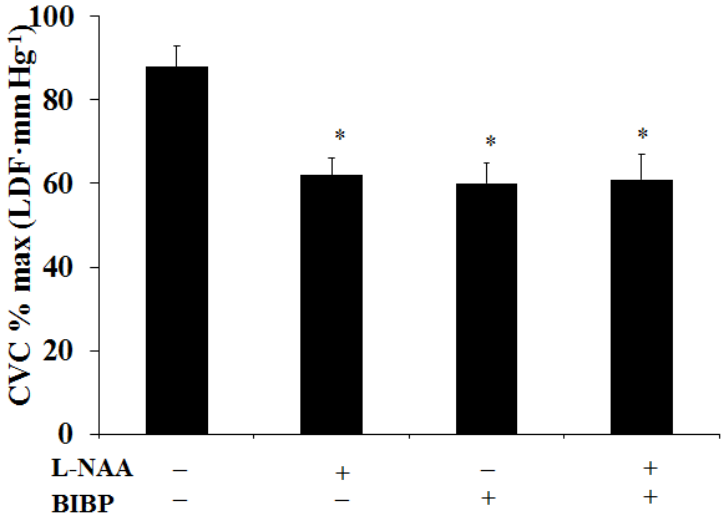

Figure 6. The plateau phase responses to local skin warming in *part 2*. All treatments reduced the vasodilator response similarly. The + and − indicate sites were or were not treated with antagonists, respectively. Values are means ± SD; $n = 7$ subjects. *$P < 0.05$ vs. untreated site.

CHAPTER IV

DISCUSSION

The present studies were designed to investigate further the involvement and relationship of eNOS, NPY, and NE in generating vasodilation in the cutaneous vasculature of the forearm to local skin warming. Previously, Hodges and co-workers (5, 9, 11, 12, 35) reported that the classical vasoconstrictors, NE and NPY, were involved in the vasodilation response to local skin warming. Additionally, prior work by Kellogg et al. (23) differentiated between NOS isoforms that were involved in the cutaneous vasodilator response to local skin warming, finding eNOS, but not nNOS, was involved. We chose to investigate the involvement of eNOS, NPY, and NE in conjunction to further characterize the roles of each in cutaneous vasodilation. Such an approach allowed investigation of each systems involvement in vasodilation individually, while also enabling determination of whether these mechanisms respond in series fashion or in parallel. We reasoned that if eNOS, NE, and NPY functioned in a series fashion this would result in identical attenuation of CVC responses from combination or separate enzyme inhibition and receptor antagonism. Indeed, we found this to be the case.

The major finding of these studies is that NE and NPY elicit cutaneous vasodilation via eNOS. We make this conclusion based on the lack of significant differences in CVC during plateau phases under separate inhibition of eNOS, antagonism of NE and NPY receptors, or the combined treatments. While the notion that NE and NPY can elicit vasodilation is counter-intuitive with respect to the classical view of these neurotransmitters, there are *in vitro* data that show NE and NPY bind to α_{2}- and Y_1-receptors on endothelial cells and stimulate eNOS production (1, 6, 38). These observations further elucidate the importance of NE and NPY in the production of NO in cutaneous vasodilation and also confirm that NE and NPY must be present

for a full vasodilation response to occur (plateau phase vasodilation). The findings of these studies suggest that NE and NPY work via eNOS in a series fashion, as we originally hypothesized.

How do NE and NPY elicit both vasoconstriction and vasodilation in skin? It is now well established that the vasoconstrictor responses to local skin cooling are mediated almost entirely by the cooling-induced activation of Rho-kinase A producing a translocation of α_{2C}-receptors from the Golgi apparatus to the cell surface, thus increasing the number of available receptors (2, 3, 36, 37). This is further supported by *in vitro* and isolated systems that indicates the cooling of skin vessels decreases the synthesis, release, and reuptake of NE and NPY (4, 7, 19, 39); consequently, it seems unlikely that the adrenergic component of the cutaneous vasoconstriction is due to a net increase in the concentration of norepinephrine in the subsynaptic cleft, and thus vascular responses to skin cooling are elicited by the extant neurotransmitter available. Indeed, Hodges et al., (11) produced an intriguing, if only indirect *in vivo* finding, which indicated that in response to local skin warming, the release of sympathetic neurotransmitters was increased. Thus, the increased release of NE and NPY during local skin warming might lead to the increased binding to endothelial-based receptors. Whether skin warming changes the expression of endothelial-based α- or Y_1-receptors is currently not known.

The important secondary finding of the studies are the different roles NE and NPY perform during baseline and in the initial stages of cutaneous vasodilation. Antagonism of α- and β- receptors produced a marked increase in skin blood flow at rest compared to control; by contrast, Y_1-receptor antagonism had no effect. This finding supports reports in rats that show there is no role for NPY in basal control of skeletal muscle blood flow (16-18). Furthermore, antagonism of α- and β- receptors caused a pronounced reduction in the initial peak compared to

both the untreated control response and that achieved under conditions of eNOS inhibition. However, while Y_1-receptor antagonism also reduced the initial peak, this was to a lesser extent than α- and β- receptor antagonism, and similar to that which was achieved with eNOS inhibition. This is interesting that these differences are not present during the overall vasodilator response (plateau phase), in which the plateau phase is equally suppressed under conditions of α- and β-receptor and Y_1-receptor antagonism. Previous studies by our group have determined antagonism of NE and NPY in combination did not cause greater attenuation than separate treatment (12, 35); as to why this is the case is currently not clear.

Kellogg et al. (23-25) investigated two specific isoforms of NOS, eNOS and nNOS, to determine if a particular isoform is more or less active in local and whole body warming. The authors determined eNOS is responsible for vasodilation in local skin warming, whereas nNOS is active in whole body warming, with both functioning in these responses exclusively. Hodges et al. (12) determined that antagonism of α-, β-, and Y_1-receptors significantly reduces CVC responses to local skin warming. The present investigation verifies the importance of eNOS in cutaneous vasodilation, while also determining the roles of NE and NPY. The current data strongly suggest that NE and NPY work via eNOS to elicit vasodilation in a series fashion.

An interesting tertiary finding of this study indicates basal eNOS activity may play an important role in normothermic cutaneous blood flow control. CVC was significantly lower at L-NAA treated sites compared to the control skin sites during the baseline period (Fig. 3). This suggests endothelial-derived NO may play an important role in maintaining cutaneous blood flow under normothermic conditions. Future work in this area should involve activities downstream from the endothelial layer but may include how eNOS activity affects other mechanisms involving substance P, reactive oxygen species, or guanylyl cyclase.

Because the isoform specificity of NOS antagonists declines with increasing concentration, we performed studies that defined the lowest L-NAA concentration that attenuated the vasodilator response to exogenous acetylcholine that is in part NO dependent and mediated by eNOS (15, 22, 23, 25). These studies demonstrated that 3 mM was the lowest L-NAA concentration that would reliably attenuate endothelium-dependent vasodilation caused by exogenous acetylcholine and yet also be the least likely to inactivate nNOS.

In summary, we found that inhibiting eNOS and antagonizing α-, β-, and Y_1 receptors significantly attenuated cutaneous vasodilation responses in the human forearm. We determined that NE and NPY elicit cutaneous vasodilation via eNOS. Secondarily, we also determined that the initial peak response is primarily mediated via NE and NPY but not NO. These findings indicate that the cutaneous vasculature has multiple, redundant systems responsible for eliciting vasodilation.

CHAPTER V

REFERENCES

1. **Angus JA, Cocks TM, and Satoh K**. Alpha 2-adrenoceptors and endothelium-dependent relaxation in canine large arteries. *Br J Pharmacol* 88: 767-777, 1986.

2. **Bailey SR, Eid AH, Mitra S, Flavahan S, and Flavahan NA**. Rho kinase mediates cold-induced constriction of cutaneous arteries: role of alpha2C-adrenoceptor translocation. *Circ Res* 94: 1367-1374, 2004.

3. **Bailey SR, Mitra S, Flavahan S, and Flavahan NA**. Reactive oxygen species from smooth muscle mitochondria initiate cold-induced constriction of cutaneous arteries. *Am J Physiol Heart Circ Physiol* 289: H243-250, 2005.

4. **Boels PJ, Verbeuren TJ, and Vanhoutte PM**. Moderate cooling depresses the accumulation and the release of newly synthesized catecholamines in isolated canine saphenous veins. *Experientia* 41: 1374-1377, 1985.

5. **Carter SJ, and Hodges GJ**. Sensory and sympathetic nerve contributions to the cutaneous vasodilator response from a noxious heat stimulus. *Exp Physiol* 96: 1208-1217, 2011.

6. **Cocks TM, and Angus JA**. Endothelium-dependent relaxation of coronary arteries by noradrenaline and serotonin. *Nature* 305: 627-630, 1983.

7. **Flavahan NA**. The Role of Vascular Alpha-2-Adrenoceptors as Cutaneous Thermosensors. *News in Physiological Sciences* 6: 251-255, 1991.

8. **Hodges GJ, Chiu C, Kosiba WA, Zhao K, and Johnson JM**. The effect of microdialysis needle trauma on cutaneous vascular responses in humans. *J Appl Physiol* 106: 1112-1118, 2009.

9. **Hodges GJ, Jackson DN, Mattar L, Johnson JM, and Shoemaker JK**. Neuropeptide Y and neurovascular control in skeletal muscle and skin. *Am J Physiol Regul Integr Comp Physiol* 297: R546-555, 2009.

10. **Hodges GJ, Kosiba WA, Zhao K, Alvarez GE, and Johnson JM**. The role of baseline in the cutaneous vasoconstrictor responses during combined local and whole body cooling in humans. *Am J Physiol Heart Circ Physiol* 293: H3187-3192, 2007.

11. **Hodges GJ, Kosiba WA, Zhao K, and Johnson JM**. The involvement of heating rate and vasoconstrictor nerves in the cutaneous vasodilator response to skin warming. *Am J Physiol Heart Circ Physiol* 296: H51-56, 2009.

12. **Hodges GJ, Kosiba WA, Zhao K, and Johnson JM**. The involvement of norepinephrine, neuropeptide Y, and nitric oxide in the cutaneous vasodilator response to local heating in humans. *J Appl Physiol* 105: 233-240, 2008.

13. **Hodges GJ, Traeger JA, 3rd, Tang T, Kosiba WA, Zhao K, and Johnson JM**. Role of sensory nerves in the cutaneous vasoconstrictor response to local cooling in humans. *Am J Physiol Heart Circ Physiol* 293: H784-789, 2007.

14. **Hodges GJ, Zhao K, Kosiba WA, and Johnson JM**. The involvement of nitric oxide in the cutaneous vasoconstrictor response to local cooling in humans. *J Physiol* 574: 849-857, 2006.

15. **Holowatz LA, Thompson CS, Minson CT, and Kenney WL**. Mechanisms of acetylcholine-mediated vasodilatation in young and aged human skin. *J Physiol* 563: 965-973, 2005.

16. **Jackson DN, Milne KJ, Noble EG, and Shoemaker JK**. Gender-modulated endogenous baseline neuropeptide Y Y1-receptor activation in the hindlimb of Sprague-Dawley rats. *The Journal of physiology* 562: 285-294, 2005.

17. **Jackson DN, Milne KJ, Noble EG, and Shoemaker JK**. Neuropeptide Y bioavailability is suppressed in the hindlimb of female Sprague-Dawley rats. *The Journal of physiology* 568: 573-581, 2005.

18. **Jackson DN, Noble EG, and Shoemaker JK**. Y1- and alpha1-receptor control of basal hindlimb vascular tone. *American journal of physiologyRegulatory, integrative and comparative physiology* 287: R228-R233, 2004.

19. **Janssens WJ, and Vanhoutte PM**. Instantaneous Changes of Alpha-Adrenoceptor Affinity Caused by Moderate Cooling in Canine Cutaneous Veins. *American Journal of Physiology* 234: H330-H337, 1978.

20. **Johnson JM** editor. *The cutaneous circulation.* 1990, p. 121-139.

21. **Johnson JM, Yen TC, Zhao K, and Kosiba WA**. Sympathetic, sensory, and nonneuronal contributions to the cutaneous vasoconstrictor response to local cooling. *Am J Physiol Heart Circ Physiol* 288: H1573-1579, 2005.

22. **Kellogg DL, Jr., Zhao JL, Friel C, and Roman LJ**. Nitric oxide concentration increases in the cutaneous interstitial space during heat stress in humans. *J Appl Physiol* 94: 1971-1977, 2003.

23. **Kellogg DL, Jr., Zhao JL, and Wu Y**. Endothelial nitric oxide synthase control mechanisms in the cutaneous vasculature of humans in vivo. *Am J Physiol Heart Circ Physiol* 295: H123-129, 2008.

24. **Kellogg DL, Jr., Zhao JL, and Wu Y**. Neuronal nitric oxide synthase control mechanisms in the cutaneous vasculature of humans in vivo. *J Physiol* 586: 847-857, 2008.

25. **Kellogg DL, Jr., Zhao JL, and Wu Y**. Roles of nitric oxide synthase isoforms in cutaneous vasodilation induced by local warming of the skin and whole body heat stress in humans. *J Appl Physiol* 107: 1438-1444, 2009.

26. **Kellogg DL, Jr., Zhao JL, Wu Y, and Johnson JM**. Antagonism of Soluble Guanylyl Cyclase Attenuates Cutaneous Vasodilation During Whole Body Heat Stress and Local Warming in Humans. *J Appl Physiol* 2011.

27. **Kellogg DL, Jr., Zhao JL, Wu Y, and Johnson JM**. VIP/PACAP receptor mediation of cutaneous active vasodilation during heat stress in humans. *J Appl Physiol* 109: 95-100, 2010.

28. **Minson CT, Berry LT, and Joyner MJ**. Nitric oxide and neurally mediated regulation of skin blood flow during local heating. *J Appl Physiol* 91: 1619-1626, 2001.

29. **Nausch LWM, Bonev AD, Heppner TJ, Tallini Y, Kotlikoff MI, and Nelson MT**. Sympathetic nerve stimulation induces local endothelial Ca2+ signals to oppose vasoconstriction of mouse mesenteric arteries. *Am J Physiol-Heart C* 302: H594-H602, 2012.

30. **Öberg PA**. Laser-Doppler flowmetry. *Crit Rev Biomed Eng* 18: 125-163, 1990.

31. **Saumet JL, Kellogg DL, Jr., Taylor WF, and Johnson JM**. Cutaneous laser-Doppler flowmetry: influence of underlying muscle blood flow. *J Appl Physiol* 65: 478-481, 1988.

32. **Stephens DP, Saad AR, Bennett LA, Kosiba WA, and Johnson JM**. Neuropeptide Y antagonism reduces reflex cutaneous vasoconstriction in humans. *Am J Physiol Heart Circ Physiol* 287: H1404-1409, 2004.

33. **Taylor WF, Johnson JM, Kosiba WA, and Kwan CM**. Cutaneous vascular responses to isometric handgrip exercise. *J Appl Physiol* 66: 1586-1592, 1989.

34. **Tew GA, Klonizakis M, Moss J, Ruddock AD, Saxton JM, and Hodges GJ**. Role of sensory nerves in the rapid cutaneous vasodilator response to local heating in young and older endurance-trained and untrained men. *Exp Physiol* 96: 163-170, 2011.

35. **Tew GA, Saxton JM, Klonizakis M, Moss J, Ruddock AD, and Hodges GJ**. Aging and aerobic fitness affect the contribution of noradrenergic sympathetic nerves to the rapid cutaneous vasodilator response to local heating. *J Appl Physiol* 2011.

36. **Thompson-Torgerson CS, Holowatz LA, Flavahan NA, and Kenney WL**. Cold-induced cutaneous vasoconstriction is mediated by Rho kinase in vivo in human skin. *Am J Physiol Heart Circ Physiol* 292: H1700-1705, 2007.

37. **Thompson-Torgerson CS, Holowatz LA, Flavahan NA, and Kenney WL**. Rho kinase-mediated local cold-induced cutaneous vasoconstriction is augmented in aged human skin. *Am J Physiol Heart Circ Physiol* 293: H30-36, 2007.

38. **Vanhoutte PM, and Miller VM**. Alpha 2-adrenoceptors and endothelium-derived relaxing factor. *Am J Med* 87: 1S-5S, 1989.

39. **Vanhoutte PM, and Verbeuren TJ**. Depression by local cooling of 3H-norepinephrine release evoked by nerve stimulation in cutaneous veins. *Blood Vessels* 13: 92-99, 1976.

40. **Wong BJ, and Minson CT**. Neurokinin-1 receptor desensitization attenuates cutaneous active vasodilatation in humans. *J Physiol* 577: 1043-1051, 2006.

APPENDIX

Office for Research

Institutional Review Board for the Protection of Human Subjects

April 7, 2011

THE UNIVERSITY OF ALABAMA RESEARCH

Gary J. Hodges, Ph.D.
Department of Kinesiology
College of Education
The University of Alabama

Re: IRB Protocol # 11-007-ME
"Involvement of Endothelial Nitric Oxide Synthase in Sympathetic-Mediated Cutaneous Vasodilation"

Dr. Hodges:

The University of Alabama Medical IRB has received the revisions requested by the full board on 3/25/11. The board has reviewed the revisions and your protocol is now approved for a one year period. Please be advised that your protocol will expire one year from the date of approval, March 10, 2011.

Should you need to submit any further correspondence regarding this proposal, please include the assigned IRB application number. Please use reproductions of the IRB approved informed consent form to obtain consent from your participants.

Good luck with your research.

Sincerely,

John C. Higginbotham, Ph.D., MPH
Medical IRB Chair
The University of Alabama

IRB Project #: 11-007-ME

UNIVERSITY OF ALABAMA
INSTITUTIONAL REVIEW BOARD FOR THE PROTECTION OF HUMAN SUBJECTS
REQUEST FOR APPROVAL OF RESEARCH INVOLVING HUMAN SUBJECTS

I. **Identifying information**

	Principal Investigator	Second Investigator	Third Investigator
Names:	Gary J. Hodges		
Department:	Kinesiology		
College:	Education		
University:	University of Alabama		
Address:	Box 870312		
Telephone:	348-2151		
FAX:	348-0867		
E-mail:	ghodges1@bamaed.ua.edu		

Title of Research Project: Involvement of Endothelial Nitric Oxide Synthase in Sympathetic-Mediated Cutaneous Vasodilation

Date Submitted: 2/15/11
Funding Source: UA Internal

Type of Proposal ☒ New ☐ Revision ☐ Renewal (Please attach a renewal application) ☐ Completed ☒ Exempt

Please attach a continuing review of studies form

Please enter the original IRB # at the top of the page

UA faculty or staff member signature: _____

II. NOTIFICATION OF IRB ACTION (to be completed by IRB):
Type of Review: ✓ Full board _____ Expedited

IRB Action:
___ Rejected Date: _____
___ Tabled Pending Revisions Date: _____
___ Approved Pending Revisions Date: _____
✓ Approved-this proposal complies with University and federal regulations for the protection of human subjects.
Approval is effective until the following date: 3-10-12 g.s.
Items approved: ___ Research protocol (dated_____)
___ Informed consent (dated_____)
___ Recruitment materials (dated_____)
___ Other (dated_____)

Approval signature _____ Date 4-7-11

March 22, 2012

Office for Research

Institutional Review Board for the
Protection of Human Subjects

THE UNIVERSITY OF
ALABAMA
RESEARCH

Gary J. Hodges, Ph.D.
Department of Kinesiology
College of Education
The University of Alabama

Re: IRB Protocol # 11-007-ME-R1
"Involvement of Endothelial Nitric Oxide Synthase in Sympathetic-Mediated Cutaneous Vasodilation"

Dr. Hodges:

The University of Alabama Medical IRB recently met to consider your renewal application. The IRB voted to approve your protocol for a period of one year.

Your application will expire on March 8, 2013. You will receive a notice of the expiration date 90 days in advance. If your research will continue beyond this date, complete the renewal portions of the FORM: IRB Renewal Application. If you need to modify the study, please submit FORM: Modification of An Approved Protocol. Changes in this study cannot be initiated without IRB approval, except when necessary to eliminate apparent immediate hazards to participants. When the study closes, please complete the FORM: Request for Study Closure.

Please use reproductions of the IRB approved stamped consent form to obtain consent from your participants.

Should you need to submit any further correspondence regarding this application, please include the above application number.

Good luck with your research.

Sincerely,

John C. Higginbotham, Ph.D., MPH
Medical IRB Chair
The University of Alabama

IRB Project #: 11-007-ME-/21

MAR 01 2012 PM02:52

UNIVERSITY OF ALABAMA
INSTITUTIONAL REVIEW BOARD FOR THE PROTECTION OF HUMAN SUBJECTS
REQUEST FOR APPROVAL OF RESEARCH INVOLVING HUMAN SUBJECTS

I. Identifying information

	Principal Investigator	Second Investigator	Third Investigator
Names:	Gary J. Hodges		
Department:	Kinesiology		
College:	Education		
University:	Alabama		
Address:	133 Russell Hall		
Telephone:	2053482151		
FAX:	2053480687		
E-mail:	ghodges1@bamaed.ua.edu		

Title of Research Project: Involvement of endothelial nitric oxide synthase in sympathetic-mediated cutaneous vasodilation.

Date Submitted: 2/27/2012
Funding Source: None

Type of Proposal	☐ New	☐ Revision	☒ Renewal Please attach a renewal application	☐ Completed	☐ Exempt
			Please attach a continuing review of studies form		
			Please enter the original IRB # at the top of the page		

UA faculty or staff member signature: _____

II. NOTIFICATION OF IRB ACTION (to be completed by IRB):
Type of Review: ✓ Full board _____ Expedited

IRB Action:
___ Rejected Date: _____
___ Tabled Pending Revisions Date: _____
___ Approved Pending Revisions Date: _____
✓ Approved-this proposal complies with University and federal regulations for the protection of human subjects.
Approval is effective until the following date: 3-8-13 6.5
Items approved: ___ Research protocol (dated_____)
___ Informed consent (dated_____)
___ Recruitment materials (dated_____)
___ Other (dated_____)

Approval signature _____ Date 3/22/12