Tetrahydrobiopterin does not affect end-organ responsiveness to norepinephrine-mediated vasoconstriction in aged skin

James A. Lang,1 Lacy A. Holowatz,1 and W. Larry Kenney1,2

1The Pennsylvania State University, Noll Laboratory, Department of Kinesiology and 2Graduate Physiology Program, University Park, Pennsylvania

Submitted 25 February 2010; accepted in final form 30 September 2010

Lang JA, Holowatz LA, Kenney WL. Tetrahydrobiopterin does not affect end-organ responsiveness to norepinephrine-mediated vasoconstriction in aged skin. Am J Physiol Regul Integr Comp Physiol 299: R1651–R1655, 2010. First published October 6, 2010; doi:10.1152/ajpregu.00138.2010.—We have recently demonstrated that tetrahydrobiopterin (BH4) augments reflex vasoconstriction (VC) in aged skin. Although this appears to occur through its role in norepinephrine (NE) biosynthesis, the extent with which vascular conductance (CVC) was attenuated VC response to gradual whole body cooling (Tsk = 30.5°C) in aged skin (24, 25). BH4 is synthesized naturally in cultured sympathetic neurons, induction of oxidative stress reduced BH4 ~90%, resulting in a ~75% reduction in catecholamine biosynthesis (28). Elevated oxidative stress in aged skin may deplete BH4 and compromise TH function, thereby contributing to the attenuated VC response in aged skin; however, it is unclear whether or not BH4 additionally affects other postsynaptic mechanisms during reflex VC.

In addition to its role in preventing nitric oxide (NO) synthase (NOS) uncoupling, BH4 readily scavenges several oxidants, including superoxide and peroxynitrite (13, 16, 19). This may be particularly relevant to aged skin considering recent evidence indicating that rho-kinase, which is directly stimulated by ROS, mediates ~50% of the reflex VC response in older adults (26). Additionally, BH4 minimizes NOS-derived ROS by preventing enzymatic uncoupling (19, 29). Thus, during cutaneous VC, BH4 may affect end-organ responsiveness to norepinephrine (NE) by altering the redox state in the cutaneous vasculature or by affecting sympathetic mechanisms such as increasing NO bioavailability.

Although we have demonstrated that BH4 does not affect the VC response to a supraphysiological concentration of NE (10−2 M), it is probable that this dose elicits a constriction where blood flow through the vessel approximately zero, which limits the ability to make inferences regarding the presence of postsynaptic effects related to BH4 supplementation (24, 25). Thus, to fully treat this mechanism, we have utilized bretylium iontophoresis to eliminate any extant neural effects and a full NE dose-response curve, which includes more physiological doses of NE [i.e., 10−8–10−12 M doses (14, 15, 27, 30)], to determine whether or not BH4 alters the VC response through postsynaptic mechanisms. We hypothesized that localized BH4 supplementation would minimally affect VC responsiveness to exogenous NE, thereby suggesting that the effects of BH4 in aged skin are mechanistically isolated to the nerve terminal as opposed to the cutaneous vasculature.

METHODS

Subjects. With Pennsylvania State University Institutional Review Board approval and after verbal and written informed consent, 10 young (Y group; 22 ± 1 yr, 5 men, 5 women) and 10 older (O group; 73 ± 3 yr; 4 men, 6 women) subjects participated in the study. Young women were tested in the early follicular phase (days 1–7) of the menstrual cycle, and older women were postmenopausal and not taking hormone replacement therapy. All subjects were healthy, nonobese, normotensive, normal cholesterolemic, nonsmokers, and not taking any medications or vitamin supplements that would other-
wise alter cardiovascular or thermoregulatory function. All procedures conformed to the standards set by the Declaration of Helsinki.

Instrumentation. Subjects arrived at the laboratory between 07:00 – 09:00 and remained in a semisupine position with the experimental forearm at heart level throughout the protocol. Two sites were marked with ink on the left ventral forearm and spaced at least 4.0 cm apart to prevent cross-reactivity of pharmacological agents between sites. At each marked site, 10 mM bretylium was iontophoresed (USP to prevent cross-reactivity of pharmacological agents between sites. forearm at heart level throughout the protocol. Two sites were marked 09:00 and remained in a semisupine position with the experimental

After microdialysis fibers were threaded through the needle, the skin (17). For each fiber, a 25-gauge needle was inserted horizontally such that entry and exit points were 2.5 cm apart. After microdialysis fibers were threaded through the needle, the needle was withdrawn, leaving the membrane in place. All fibers were taped in place, and lactated Ringer solution was initially perfused to test the integrity of the fiber during the resolution period following needle-insertion trauma. Microdialysis sites were then randomly assigned with respect to position on the forearm and were perfused with J) lactated Ringer solution serving as control and 2) 5 mM BH4. The BH4 dosage was determined from a previous study in our laboratory (24).

To obtain an index of skin blood flow, red blood cell flux was continuously measured with a laser Doppler flowmetry probes (Moor-LAB Temperature Monitor SH02; Moor Instruments, Devon, UK). Laser Doppler flowmetry probes were placed in the center of local heaters and positioned directly over each microdialysis fiber site. Local skin temperature was clamped at a thermoneutral temperature (34°C) throughout the experiment. Arterial blood pressure was measured every 5 min throughout the experiment via brachial auscultation. Mean arterial pressure was calculated as the diastolic blood pressure plus one-third of the pulse pressure. Cutaneous vascular conductance (CVC) was calculated as the ratio of laser Doppler flowmetry flux to mean arterial pressure and expressed as percent change from baseline values (%ΔCVCbaseline).

Protocol. After instrumentation with microdialysis fibers at bretylium-pretreated sites, local hyperemia was allowed to resolve for ~90 min, while sites were perfused with their assigned pharmacological agent. All drugs (IND No. 103180) were mixed just prior to use, dissolved in lactated Ringer solution, sterilized using syringe microfilters (Acrodisc; Pall, Ann Arbor, MI), and perfused at 2 µl/min (Bee Hive controller and Baby Bee microinfusion pumps; Bioanalytical Systems).

After baseline measurements, NE (preserved with 1 mg/ml of ascorbic acid) was perfused for 5 min at all sites followed by immediate washout with its designated pharmacologic (i.e., lactated Ringer solution or BH4) for ~20–30 min to allow skin blood flow to recover to initial baseline values (model 110 Liquid Switch; CMA Microdialysis). This protocol was repeated for each NE dose (10–12, 10–10, 10–8, 10–6, 10–4, 10–2 M). The initial three concentrations (10–12, 10–10, 10–8 M) were performed in random order followed by sequential perfusion of the final three more concentrated doses (10–6, 10–4, 10–2 M). Full resolution of the VC to the more potent concentra-

trations of NE prevented the randomization of these final doses. Finally, 28 mM sodium nitroprusside was perfused through all sites at a rate of 4 µl/min in combination with local heating of the skin to 43°C for ~30 min at each microdialysis site to ensure that vascular responsivity remained intact.

Data acquisition and analysis. Data were collected at 40 Hz, digitized, recorded, and stored in a personal computer until data analysis (Windaq; Dataq Instruments, Akron, OH). CVC data were averaged over 3-min intervals during baseline. During NE perfusion, the VC response was defined as the lowest CVC 1-min average observed during the washout period. Typically, the maximal VC response for a given NE dose was achieved ~2–3 min into its washout. Data were analyzed using three-way, mixed-model, repeated-measures ANOVA (i.e., NE dose × age × drug treatment) and Student’s paired t-test for subject characteristics (version 9.1.3; SAS, Cary, NC). The level of significance was set at α = 0.05 for main effects. Tukey post hoc tests were performed when appropriate to determine where age and drug treatment differences occurred. Values are expressed as mean ± SE.

RESULTS

Subject characteristics are presented in Table 1. Age groups were well matched with regard to height, weight, body mass index, mean arterial pressure, blood glucose, and cholesterol ratio (total cholesterol:HDL cholesterol). There were no significant differences in absolute baseline CVC, calculated as laser Doppler flux * mmHg−1, between age groups or between the control (Y: 0.18 ± 0.03, O: 0.22 ± 0.05; P = 0.50) and BH4 site (Y: 0.24 ± 0.05 P = 0.38 vs. control, O: 0.22 ± 0.04 P = 0.97 vs. control).

The efficacy of presynaptic adrenergic blockade with bretylium tosylate was tested with whole body cooling. Compared with nontreated sites (Y: 27.9 ± 2.2, O: 16.8 ± 0.3%ΔCVCbaseline), no VC was observed at bretylium-treated sites during whole body cold stress (Y: 1.1 ± 0.5, O: 0.1 ± 0.3%ΔCVCbaseline; P < 0.01). Figure 1 illustrates VC in response to six different concentrations of NE (10−12, 10−10, 10−8, 10−6, 10−4, 10−2 M) in young and older subjects. The VC response was attenuated at all doses in aged skin (P < 0.01).

The VC response to NE in BH4 pretreated sites is displayed in Fig. 2. Figure 2A demonstrates that BH4 had no effect on the VC response in young subjects (10−12: P = 0.68; 10−10: P = 0.49; 10−8: P = 0.81; 10−6: P = 0.40; 10−4: P = 0.81; 10−2: P = 0.25). Similarly, BH4 did not significantly affect NE-mediated VC in older subjects (Fig. 2B) (10−12: P = 0.10;

Table 1. Subjects’ characteristics

<table>
<thead>
<tr>
<th>Variable</th>
<th>Young</th>
<th>Older</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, M/F</td>
<td>5/5</td>
<td>4/6</td>
</tr>
<tr>
<td>Age, yr</td>
<td>22 ± 1</td>
<td>73 ± 3*</td>
</tr>
<tr>
<td>Height, cm</td>
<td>172 ± 3</td>
<td>166 ± 2</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>73 ± 5</td>
<td>68 ± 3</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>24 ± 1</td>
<td>24 ± 1</td>
</tr>
<tr>
<td>Resting MAP, mmHg</td>
<td>82 ± 2.2</td>
<td>86.7 ± 1.7</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>87 ± 2</td>
<td>89 ± 2</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>151 ± 6</td>
<td>191 ± 7*</td>
</tr>
<tr>
<td>HDL, mg/dl</td>
<td>51 ± 2</td>
<td>67 ± 4*</td>
</tr>
<tr>
<td>LDL, mg/dl</td>
<td>82 ± 7</td>
<td>107 ± 6*</td>
</tr>
<tr>
<td>Cholesterol ratio, total/HDL</td>
<td>3.0 ± 0.3</td>
<td>3.0 ± 0.2</td>
</tr>
</tbody>
</table>

Values are means ± SE for young (n = 10) and older (n = 10) men (M) and women (F). BMI, body mass index; MAP, mean arterial pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein. *P < 0.05.
DISCUSSION

The primary finding from this study was that localized BH4 supplementation did not affect the VC response to NE in bretylium-pretreated skin of young or older subjects, which indicates that BH4 has a negligible effect on the cutaneous vasculature during VC. In conjunction with our previous findings (24), this suggests that the primary site of action of BH4 during cutaneous VC is isolated to the axon terminal of adrenergic nerves. Thus, supplementation of BH4 did not alter the VC response postsynaptically via sympatholytic mechanisms, thereby indicating that BH4 acts independently of NOS coupling and increases in NO bioavailability during cutaneous VC. Additionally, we verified that VC in aged skin is blunted at various physiological (10^{-12}-10^{-8} M) and supraphysiological (10^{-4}-10^{-2} M) concentrations of NE (36, 40). This result occurred even when controlling for endogenous NE release with bretylium (40).

We have previously demonstrated that localized BH4 and/or L-tyrosine infusion in aged skin offset the attenuated VC response to both physiological (whole body cooling) and pharmacologically (tyramine)-mediated VC (24, 25). In sympathetic adrenergic nerve terminals, BH4 is important to NE biosynthesis because it acts as a cofactor for the rate-limiting enzyme TH. During neuronal activation, the affinity of TH for its cofactor markedly increases (20, 41). BH4 subsequently reduces the iron moiety of TH, thereby catalytically activating the enzyme for hydroxylation of tyrosine (9, 22, 38). However, in the relative absence of BH4, NE biosynthesis, and storage may be compromised resulting in blunted adrenergic VC. Reduced BH4 concentration is apparent in aged tissues, and this may be secondary to elevated oxidative stress (6, 39). Additionally, the number of transporters for NE in synaptosomes decreases with age (33). Thus, reduced BH4 or tyrosine bioavailability may result in suboptimal TH function, thereby compromising the available pool of NE required to fully express the VC response in aged skin.

In addition to its putative role in adrenergic nerve terminals, BH4 also affects vascular (i.e., postsynaptic) mechanisms by 1) its ability to couple NOS and increase NO bioavailability and 2) its antioxidant properties. Both exogenous and endogenous sources of NO have been demonstrated to attenuate cutaneous VC (10, 31, 32). Furthermore, NOS inhibition has the contrasting effect of augmenting the VC response (2, 31). Additionally, BH4 readily neutralizes ROS, in some cases demonstrating greater reactivity than ascorbate (13, 16, 19, 23). In fact, BH4 is 6–10 times more effective than ascorbate or reduced thiols in binding peroxynitrite (23). Lastly, BH4 minimizes NOS-derived ROS by preventing enzymatic uncoupling (19, 29). Although BH4 has an established role in the vasculature, these mechanisms did not appear to affect the cutaneous VC response to various concentrations of NE. Thus, the present study was an important follow-up to our previous findings (24, 25) because it indicates that the nerve terminal is required to observe the BH4-mediated augmentation of the VC response in aged skin.

In our previous studies (24, 25), we have attempted to address the question of whether or not any postsynaptic effects of BH4 may be obfuscating our explanation of its role on TH during cutaneous VC. In aged skin, the BH4-mediated increase in the VC response was unaffected by NOS inhibition or by a
supraphysiological dose (10^{-2} \text{ M}) of NE (24, 25). However, it is likely, particularly with the 10^{-2} \text{ M} of NE, that a basement effect (i.e., where the vessel is closed or nearly so due to a maximal level of VC) occurred. This would ultimately reduce or eliminate the signal gain such that no further VC, or differences in the VC response between age groups, could be detected. As a result, we have utilized various physiological concentrations of NE (i.e., 10^{-12}-10^{-8} \text{ M}) to eliminate this confounding effect and to fully characterize whether or not the underlying mechanism for BH4 during VC is isolated to sympathetic adrenergic nerve terminals.

The importance of noradrenergic function is particularly evident in aged skin since this is the only mechanism responsible for eliciting thermoregulatory reflex VC (i.e., cotransmitter-mediated VC is functionally absent) (24, 37). Deficits in noradrenergic function have also been identified in adrenergic receptor and second messenger systems of vascular smooth muscle (7, 18, 26, 34, 36). Conflicting accounts exist regarding the effect of age on the cutaneous VC response to NE: one study showed no difference (40) and another demonstrated a blunted VC response in aged skin (36). Compared with these studies, we extended the dose-response range to include the 10^{-12} \text{ M} concentration and verified that the VC response to these doses of NE was attenuated in aged skin even after controlling for presynaptic adrenergic function with bretylium tosylate. Thus, these data collectively suggest that in response to a given physiological dose of NE, adrenoreceptor sensitivity is functionally blunted in aged skin.

Because BH4 normalizes adrenergic VC and couples NOS to increase NO bioavailability (24, 29), which putatively improves vasodilator function and minimizes superoxide generation, future studies may be warranted in assessing the effects of acute and chronic oral supplementation of this cofactor on vascular and thermoregulatory function. Acute oral BH4 improves flow-mediated dilation in older sedentary men by 45% (12). Four-week administration of BH4 (800 mg/day) reversed endothelial dysfunction and oxidative stress in hypercholesterolemic humans (4). Whether or not the effects of oral BH4 enhance thermoregulatory function in healthy older subjects remains unknown.

**Limitations.** Bretylium tosylate inhibits adrenergic function presynaptically only after an initial release in neurotransmitter substance (1, 8). This may result in acute desensitization of adrenoreceptors. However, bretylium administered at concentrations that cause presynaptic blockade does not affect exogenous NE-mediated VC (1). Furthermore, iontophoresis may induce a current-related vasodilatation (11). However, baseline absolute CVC was not different from those observed from previous protocols where iontophoresis was not used. Also, these limitations were minimized by the -3-h interval between iontophoresis of the final site and infusion of the first NE dose. Finally, a minimal concentration of ascorbate (1 mg/ml) was added as a preservative to prevent the degradation of NE. This concentration has been used in previous studies and did not alter the VC response (35, 36).

In summary, the present study indicates that the effects of localized BH4 supplementation on cutaneous VC are primarily localized to adrenergic nerve terminals. This corroborates our previous study that demonstrated an augmentation in the physiological (whole body cooling)- and pharmacological (tyramine)-induced VC in aged skin. Thus, reduced NE biosynthe-

**ACKNOWLEDGMENTS**

The authors gratefully acknowledge the subjects who participated in this study. We separately acknowledge Rebecca Bruning for her assistance in long hours of data collection and interpretation. We also especially thank Jane Pierzga, Anna Staniewicz, and Susan Beyerle for their technical and data collection assistance and the General Clinical Research Center for medical assistance.

**GRANTS**

This research was supported by the National Institutes of Health Grants RO1-AQ-07004-19 and MO1-RR-10732 (to General Clinical Research Center).

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**REFERENCES**


ROLE OF BH4 IN THE SKIN VASCULATURE DURING VASOCONSTRICTION


