Peripheral Vascular Effects of Sulfhydryl-containing Antihypertensive Pharmacotherapy on Microvessels in Humans

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Study Protocol and Statistical Analysis Plan

PENNSTATE

Protocol for Human Subject Research with Use of Test Article(s)

Protocol Title: Peripheral vascular effects of sulfhydryl-containing antihypertensive pharmacotherapy on microvascular function and vessel remodeling in hypertensive humans (IRB#3224)

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1.0 Objectives

1.1 Study Objectives

Essential hypertension is a complex pathology and clinical guidelines (JNC8) strongly support angiotensin converting enzyme inhibitors (ACEi) for the treatment of essential hypertension. It is increasingly recognized that secondary peripheral vascular effects of systemic ACEi significantly contribute to reduced mortality in hypertension-associated disease. A specific class of ACEi containing a sulfhydryl group is purported to exert a beneficial peripheral effect through their highly lipophilic targeting to the endothelium where they act through hydrogen sulfide (H_2S)-dependent mechanisms. With the emergence of the importance of H_2S as a modulator in hypertension, there is an immediate need to explore the therapeutic potential related to these signaling mechanisms in humans. The proposed work has the potential to uncover novel vascular signaling mechanisms related to the gasotransmitter H_2S in humans and better understand target-based therapeutics in hypertension associated vascular dysfunction.

Note: This project covers Specific Aim 2 of the grant.

Specific Aim: To determine the peripheral vascular effects of sulfhydryl (SH)-containing antihypertensive pharmacotherapy on *in vivo* and *in vitro* indices of microvascular function and vessel remodeling (longitudinal intervention). We will utilize a 16-wk randomized intervention comparing and contrasting the effects of (1) a SH-ACEi, (2) a non-SH containing ACEi, and (3) a thiazide-type diuretic (nonvascular therapeutic control).

Hypothesis a. Both SH-ACEi and ACEi treatments will improve endothelial-dependent vasodilation in response to physiological and pharmacological stimuli (*in vivo*) and improve *in vitro* markers of vasodilator responsiveness (pVASP/VASP) compared to diuretic treatment.
Hypothesis b. SH-ACEi will be more effective at reducing end-organ vasoconstrictor responsiveness to adrenergic stimuli than ACEi and diuretic treatments.

Hypothesis c SH-ACEi will improve *in vivo* and *in vitro* indices of microvessel remodeling compared to ACEi and diuretic treatment.
Hypothesis d. SH-ACEi will improve exogenous H_2S mediated-vasodilation compared to ACEi.
Hypothesis e. SH-ACEi will [synergistically] improve endothelium-dependent vasodilation through both H_2S and NOS-dependent mechanisms (cross talk).

1.2 Primary Study Endpoints
The study ends when enough subjects have completed the protocol to adequately test the above hypotheses. Outcomes include blood pressure (BP), cutaneous vascular conductance (DVD), flow mediated vasodilation (FMD), rate of reactive oxygen species (ROS) production, H_2S production, protein concentration, and hydrogen sulfide (H_2S), acetylcholine (ACh), norepinephrine (NE) dose responses.

1.3 Secondary Study Endpoints
NA

2.0 Background
2.1 Scientific Background and Gaps
Essential hypertension is a complex pathology characterized by systemic inflammation, altered neurovascular control, including microvascular dysfunction characterized by a loss of endothelium-dependent signaling pathways), and vessel remodeling. Clinical outcomes data forming the basis for the Eight Joint National Committee (JNC8) guidelines strongly support angiotensin converting enzyme inhibitors (ACEi) for the first line treatment of essential hypertension. Importantly, reduced mortality attributable to ACEi therapy is strongly related to their secondary peripheral vascular effects.

Emerging data indicate that ACEi containing a sulfhydryl group (SH-ACEi) exert beneficial peripheral vascular effects via lipophilic targeting to the endothelium where they act through hydrogen sulfide (H_2S)-dependent mechanisms. H_2S is a gasotransmitter that acts as an endothelium-derived hyperpolarizing factor (EDHF) and is synthesized in the vasculature enzymatically by cystathionine-γ-lyase (CSE) and mercaptopyruvate sulfurtransferase, (MPST). Because of the convergence of H_2S on multiple endothelial signaling pathways including NO, examining H_2S signaling is critical to elucidating (1) the mechanisms of vascular dysfunction in hypertension, and (2) its viability as a therapeutic target for SH-based pharmacologics.

We propose to perform a comprehensive examination of the impact of different classes of antihypertensive pharmacotherapy on indices of eutrophic microvessel remodeling. The degree of vessel remodeling in hypertension is negatively associated with clinical outcomes. The proposed studies will provide insight into the potential benefits of utilizing available SH-containing antihypertensive agents on validated clinical outcomes. The research team is well-positioned to translate laboratory findings into [an *in vivo* human model and determine the effects of target-based antihypertensive treatments.

In summary, the proposal represents an integrative and innovative effort to (1) provide mechanistic evidence about the putative benefits of specific classes of antihypertensive agents, and (2) identify possible target-based treatment strategies in humans.

2.2 Previous Data
This study continues the research and focus of the study, “Cutaneous vascular effects of Hydrogen Sulfide,” (IRB# 43891).

Published data from our laboratory[20, 22] demonstrate that essential hypertensive humans have microvascular dysfunction characterized by attenuated endothelium-dependent vasodilation to both pharmacological and physiological stimuli, increased adrenergic responsiveness, and evidence of eutrophic microvessel remodeling[1, 2]. These data are further supported by biochemical data obtained from the analysis of human skin tissue showing reduced pro-vasodilator markers including phosphorylated vasoactive simulator protein (pVASP/VASP).

We have developed and validated *in vivo* methodology to examine H_2S-mediated vasodilation in human subjects (N=11). Our exogenous H_2S dose-response protocol, in which two different commonly utilized H_2S donors were delivered locally to the cutaneous microcirculation to elicit vasodilation, demonstrated that there was no difference in functional responsiveness or measured concentration of H_2S generated from the NaHS or Na_2S (R_2=0.97)[3].

In addition, we have published data determining the downstream mechanisms by which exogenous H_2S induces vasodilation in the human cutaneous vasculature[3]. We tested the efficacy of K_{Ca} channel inhibitors to elucidate the specific mechanisms underlying H_2S-mediated vasodilation. The data suggest that K_{Ca} channels, and not K_{ATP} channels, mediate exogenous H_2S-induced cutaneous vasodilation. Our published *in vivo* data are novel with respect to mechanistic investigation of H_2S-mediated vasodilation in humans[3]. *[Note, while more specific K channel inhibitors are available for use in animal studies, to date, the specific K_{Ca} and K_{ATP} inhibitors used in that study are the only available inhibitors available for use in humans. If others become available, we would test the efficacy upon IRB and FDA approval.]*

We have also published studies with appropriate NOS and COX blockade to examine potential cross-talk mechanisms involved in H_2S-mediated vasodilation[3]. The cutaneous vasodilation response to exogenous H_2S during both single and double blockade protocols showed that, similar to animal models[4, 5], there is cross talk between H_2S, NOS, and COX pathways in humans (N=8).

Pilot data have been collected on hypertensive subjects naïve to pharmacotherapy (resting MAP=109±5 mmHg). In support of hypothesis 1a, these data show decreased H_2S-mediated vasodilation, illustrating decreased peripheral vascular responsiveness specific to H_2S [that occurs, in part, through crosstalk with NOS systems.

In further support of hypothesis 1a, we have performed dose-response experiments to the endothelium-dependent agonist acetylcholine in the presence of the H_2S enzymatic inhibitor aminooxyacetic acid (AOAA). *NOTE: AOAA is the only available CSE inhibitor available for human use.* We first verified the efficacy of this inhibitor by utilizing an *in vitro* assay. *In vivo* data demonstrate that H_2S inhibition with AOAA attenuated acetylcholine-induced endothelium dependent cutaneous vasodilation in healthy adults (83±9), indicating that H_2S contributes to agonist mediated endothelium-dependent vasodilation healthy adults. In contrast, H_2S-mediated endothelium dependent vasodilation was reduced in 3 hypertensive adults (MAP 105±5) as the enzymatic H_2S inhibitor AOAA did not alter the response to Ach. These pilot data demonstrate that endogenous H_2S-mediated vasodilation is reduced in hypertensive humans.

In support of our *in vivo* data, *in vitro* approaches for examining H_2S-dependent mechanisms have been refined and optimized. Protein expression data for the main enzymatic sources of H_2S in our target tissue, CSE and MPST (N=7) demonstrate the presence of H_2S-generating enzymes in the human cutaneous microcirculation. The inhibition of CSE by AOAA was verified in these experiments.

Pilot data in support of hypothesis 2b data show CSE and MPST are expressed in human skin samples. We also show that enzymatic production of H_2S is increased after treatment with the non-SH donating ACEi Lisinopril to normalize blood pressure. The pilot data demonstrate the beneficial peripheral vascular effects of systemic ACE-inhibition on these indices.

We have also conducted a preliminary cross-sectional study to examine the peripheral vascular effects of different classes of antihypertensive pharmacotherapy (N=10, MAP=96±4mmHg). These pilot data demonstrate moderate effectiveness of systemic ACEi to improve cutaneous endothelial measures. These improvements are due, in part, to an increase in both EDHF- and NOS-dependent signaling. Consistent (with regard to these classes of antihypertensive agents) with hypotheses 2a and 2b, we observe improvements in the function of endothelial mediators in subjects prescribed ACEi but do not observe any change in indices of eutrophic vessel remodeling. These data highlight the need to conduct a well-controlled interventional study testing the efficacy of antihypertensive pharmacotherapy -- including SH-ACEi, ACEi, and diuretics -- on indices of eutrophic vessel remodeling.

In addition to the pilot and preliminary data shown here, our research team has extensive experience conducting pharmacological interventional studies in human subjects. We have published extensively on the pleiotropic effects of systemic statin therapy[6, 7, 8] and systemic supplementation of essential cofactors and signaling intermediates to improve vascular function in adults with pre-clinical CVD[9, 10]. Importantly, these studies, published in high impact clinical and physiology journals, utilize similar methodologies to those proposed herein.

Our fellow investigator, Dr. Gail Thomas, has utilized a similar experimental design to take advantage of between (irbesartan vs chlorthalidone) and within (nebivolol vs. metoprolol) class differences in antihypertensive agents to gain insight into the central and peripheral mechanisms causing enhanced vasoconstriction in human subjects with essential hypertension[11, 12]. Consistent with hypothesis 2b antihypertensive agents with greater peripheral vasodilatory potential are more effective at reducing vasoconstrictor responses[114].

2.3 Study Rationale

We developed and validated the human cutaneous microcirculation as a powerful model for the *in vivo* examination of novel signaling mechanisms mediating microvascular dysfunction in hypertensive adults. The emerging importance of H_2S as an endothelial signaling modulator and inhibitor of eutrophic vessel remodeling in hypertension highlights the need to examine target-based intervention strategies related to these mechanisms. SH-ACEi have been extensively prescribed for the secondary treatment of cardiovascular disease with highly effective clinical outcomes; however, the precise mechanisms that underlie the therapeutic benefit of SH-ACEi in primary hypertension are unknown. We propose to elucidate the mechanisms underlying attenuated H_2S signaling and the mechanistic effects of lipophilic SH-ACEi in hypertensive humans in two specific aims. We will utilize *in vivo* and *in vitro* approaches to address the following aims to determine the peripheral vascular effects of SH-containing antihypertensive pharmacotherapy on indices of microvascular function and vessel remodeling in an interventional study with SH-based antihypertensive therapies.

3.0 Inclusion and Exclusion Criteria

3.1 Inclusion Criteria

- women and men
- 40-65 years
- blood pressure: Normotensive <120/80 mmHg
  Hypertensive ≥140/90 mmHg and <160/110 mmHg
- HbA1C of <6.5%
- Women are post-menopausal and not taking hormone replacement therapy, or have normal cycles and are tested in the early follicular phase. Subjects may or may not be taking one doctor-prescribed drug to lower blood pressure (e.g. diuretic, ACE inhibitor).
  - Must be able to stop physician-prescribed antihypertensive drug for the duration of the subject's participation in the study (with the approval of their personal physician).

3.2 Exclusion Criteria

Relevant to all subjects:

- current medications which could conceivably alter the cardiovascular or thermoregulatory control or responses (e.g. beta blockers, calcium channel blockers, angiotensin receptor blockers)
- allergy to test substances
- allergy to latex
- nicotine use (smoking, chewing tobacco, etc.)
- illegal/recreational drug use
- pregnancy or breastfeeding
- diabetes
- men taking medication for erectile dysfunction

Relevant to all hypertensive subjects:

- contraindication for all three pharmocotherapy drugs used in this study
    - Note: Subjects who have a contraindication (e.g. a condition, medication with a known interaction, known allergy) to only one or two of the three pharmacotherapy drugs, may be assigned one of the pharmocotherapy drugs that is not contraindicated.
- kidney problems
- liver problems
- history of heart disease or failure
- history of blood clots or stroke
- angioedema
- electrolyte imbalance
- planned surgery requiring general anesthesia during the pharmacotherapy period
- peripheral vascular disease

Relevant only to hypertensive subjects who are currently taking a physician-prescribed antihypertensive drug (e.g. diuretic, ACE inhibitor).

- Taking more than one physician-prescribed drug to lower blood pressure
- Unable or unwilling to stop the physician-prescribed antihypertensive drug for the duration of the subject's participation in the study (with the approval of their personal physician).
- Unable or unwilling to obtain the personal physician's approval to stop the physician-prescribed antihypertensive drug for the duration of the subject's participation in the study.
- patients who are taking clonidine
- African Americans who are taking a calcium channel blocker (Norvasc)
- Potential subjects who are taking physician-prescribed drugs for the control of hypertension will be evaluated and excluded when necessary by the principal investigator and/or Dr. Fragin on a case by case basis.

3.3 Early Withdrawal of Subjects

3.3.1 Criteria for removal from study

Participants may withdraw at any time. We may end the participant's role in the study without her/his consent if the researcher deems that her/his health or behavior adversely affects the study or increases the risks beyond those approved by the Institutional Review Board and agreed upon by her/him in the informed consent, or we develop new exclusion criteria.

Relevant only to hypertensive subjects who are currently taking a physician-prescribed antihypertensive drug.

Subjects discontinue the physician-prescribed antihypertensive drug for a 14-day washout period prior to participating in the first experiment. During the 14 days, they self-check their blood pressure with a blood pressure monitor (supplied by us) every 6-8 hours. If the subject's blood pressure exceeds 140 mmHg systolic or 90 mmHg diastolic during the 14 days, the subject notifies Susan Slimak, RN (the research nurse), resumes taking the physician-prescribed antihypertensive drug, and withdraws from the study.

3.3.2 Follow-up for withdrawn subjects

We follow up with subjects that withdraw from the study within one week of removal or withdrawal. The research nurse and/or project-affiliated physician follows up on withdrawals that are related to medical concerns. We contact them by phone or e-mail. Subjects are replaced by recruitment of new subjects.

Relevant only to hypertensive subjects who are currently taking a physician-prescribed antihypertensive drug.

We instruct subjects who withdraw from the study due to excessive blood pressure during the 14-day washout (see 3.3.1) to notify their personal physicians. The research nurse or study physician follows up with the subject within 24 hours. The research nurse contacts the subject 10 days later for additional follow-up. We advise subject to follow up with their personal physicians.

4.0 Recruitment Methods

4.1 Identification of subjects

We advertise for subjects. Interested persons contact us. We also avail ourselves of lists of potential participants maintained by the CRC and our lab. The people on these lists have screened for other studies and indicated that they wished to be maintained on a list of potential participants to be contacted in the event that they may qualify for addition studies.

4.2 Recruitment process

Research Match sends out emails to those who meet our eligibility criteria. If the potential subject chooses to click "yes" in the email, we receive the contact info for that subject allowing us to contact the potential subject. Also, we advertise for subjects (see below). Interested persons contact us. Using the IRB-approved "phone-script" or "ad," we telephone or email subjects, respectively, from the lists of potential subjects (see above). We obtain permission before using a listserv that we determine may aid us in recruitment. We discuss the study's purpose and protocol as well as the qualifications for the study with potential subjects. We tell them about the study and, if they are interested in participating, give them a chance to come to lab for a screening.

4.3 Recruitment materials

Research Match
Studykik
Newspaper/magazine ads *(print and/or online)*
Letters/Emails to potential participants
Flyers/posters *(permission to post obtained when needed)*
Script - Verbal *(i.e., telephone, face-to-face, classroom)*
Brochures *(describes the general research foci of the lab group)*
Web Sites *(website and initial screening form collect the same basic pre-screening information)*
Listserv *(on and off campus that target under-represented groups (e.g. FOBA) as well as listservs belonging groups (e.g. Elks) and others as we become aware of them; permission obtained)*
Note: We have a recruiting website through the Kinesiology department that we have used for most of our studies. The uploaded phone script and/or ad are used for additional recruiting purposes such as classroom announcements and emails.

4.4 Eligibility/screening of subjects

We use the phone questionnaire and the website documents to determine eligibility before obtaining informed consent. We inform subjects that the information they provide is kept confidential. The pre-screening information is provided by the subject either verbally over the phone or via electronically via REDCap. If this occurs via phone, the investigator records this information on the telephone interview form. The data from people who qualify or do not qualify for the study is stored until end of study, then destroyed. We request permission from participants who qualify or do not qualify for the study to maintain the information and to contact for future studies

Relevant to hypertensive subjects who are currently taking a physician-prescribed antihypertensive drug.

We fax a letter and completed HIPAA form (see uploaded documents) to the subject's physician requesting the physician's approval for the subject to stop taking the physician-prescribed drug for the duration of the subject's participation in the study. We only include a subject in the study when her/his personal physician returns the letter indicating the physician's permission.

5.0 Consent Process and Documentation

5.1 Consent Process

5.1.1 Obtaining Informed Consent

5.1.1.1 Timing and Location of Consent

When a potential participant reports to Noll Lab or CRC, she/he signs the informed consent before screening procedures begin.

5.1.1.2 Coercion or Undue Influence during Consent

If subjects are not able to understand the protocol and instructions for any reason, written or verbal, they are not included in the study. Subjects are informed throughout the consenting, screening, and conduction of the study that their participation is voluntary, and they may discontinue their participation at any time. It is possible that a person enrolled in the study could be a student or employee. We tell the person that participation in the study is voluntary and no aspect of their participation or non-participation has an effect on their class or grade, employment or salary, respectively. The person conducting the screening and consenting is not a professor from any of the student's classes or the employee's supervisor.

Relevant to hypertensive subjects who are currently taking a physician-prescribed antihypertensive drug.

The HIPAA form informs subjects that their treatment, payment, or enrollment in any health plans or eligibility for benefits is not affected by their declining to sign the HIPAA form.

5.1.2 Waiver or alteration of the informed consent requirement

NA

5.2 Consent Documentation

5.2.1 Written Documentation of Consent

All participants sign the informed consent document. After the participant signs the consent, we photocopy it and give it to the participant.

5.2.2 Waiver of Documentation of Consent

We are requesting a waiver of documentation of consent for the screening procedures only. It will be a verbal consent on the phone, or an implied consent if the screening is done in Redcap. These procedures present no more than minimal risk. Due to COVID-19, one subject who has her own blood pressure monitor at home will provide verbal consent to: (1) monitor her blood pressure at home, (2) report it to the investigators by telephone, and (3) will have her medications mailed to her residence directly from the pharmacy. This participant is confirmed post-menopausal and there for a pregnancy test is not required to refill her medication.

5.3 Consent – Other Considerations

5.3.1 Non-English Speaking Subjects

All participants will be able to understand written and verbal English and give consent in English.

5.3.2 Cognitively Impaired Adults

This research does not include cognitively impaired adults.

5.3.3 Subjects who are not yet adults (infants, children, teenagers)

NA

6.0 HIPAA Research Authorization and/or Waiver or Alteration of Authorization

NA

6.1 Authorization and/or Waiver or Alteration of Authorization for the Uses and Disclosures of PHI

Check all that apply:

X Authorization will be obtained and documented as part of the consent process.

If a potential subject is taking a personal physician-prescribed antihypertensive drug, the subject may participate if her/his personal physician gives permission for the subject to discontinue the antihypertensive drug for the duration of the subject's participation in the study. In this case, the subject signs a HIPAA form (see related uploaded documents).

☐ Partial waiver is requested for recruitment purposes only *(Check this box if patients' medical records will be accessed to determine eligibility before consent/authorization has been obtained)*

☐ Full waiver is requested for entire research study *(e.g., medical record review studies)*
☐ Alteration is requested to waive requirement for written documentation of authorization

6.2 Waiver or Alteration of Authorization for the Uses and Disclosures of PHI
6.2.1 Access, use or disclosure of PHI representing no more than a minimal risk to the privacy of the individual
6.2.1.1 Plan to protect PHI from improper use or disclosure
6.2.1.2 Plan to destroy identifiers or a justification for retaining identifiers
6.2.2 Explanation for why the research could not be practicably be conducted without access to and use of PHI
6.2.3 Explanation for why the research could not practicably be conducted without the waiver or alteration of authorization
6.3 Waiver or alteration of authorization statements of agreement

7.0 Study Design and Procedures

7.1 Study Design

We recruit subjects from central Pennsylvania using resources including the Penn State Center for Healthy Aging and our clinical translational research center (CTRC). We over-sample minority populations using currently active strategies approved by NIH. Because JNC8 has different treatment recommendations for Black hypertensive patients, concerted efforts are made to recruit all races, and secondary analyses are conducted to examine race differences.

Upon initial screening and again within a week of testing, all subjects have an assessment of 24-hour ambulatory BP. The research nurse and the Penn State CTRC medical staff perform the screening which includes anthropometry, and a chemical and lipid profile, liver and renal function. We analyze the blood samples of enrolled subjects for ACE activity, Ang II, Ang 1-7, oxLDL subfraction particles, ApoB, and assymetrical dimethyl L-arginine (ADMA) concentrations (ELISA), and plasma inflammatory cytokine panel (IL-6, sIL-6α receptors, CRP, TNFα, and gp130). Subjects also undergo an assessment of conduit vessel endothelial and vascular smooth muscle function with brachial artery flow-mediated vasodilation (FMD) and sublingual nitroglycerin, respectively.

We include normo- and hypertensive subjects. We recruit hypertensive subjects who may or may not be taking a physician-prescribed antihypertensive drug (e.g. diuretic, ACE inhibitor). Subjects taking a physician-prescribed antihypertensive drug cease taking the drug (with their physician's documented permission) for 14 days prior to the first experiment. They do not take their physician-prescribed antihypertensive drug for the duration of their participation in the study.

The study is a single blind randomized control trial consisting of 16 weeks of antihypertensive pharmacotherapy. (Pharmacokinetic and dynamic data from the literature suggest that blood pressure lowering and peripheral vascular effects are maximized by 12 weeks of antihypertensive therapy. Investigators are blinded to the condition, but the physician and nursing oversight are not. Captopril (SH-ACEi), enalapril (ACEi), or hydrochlorothiazide (HCTZ) are used for hypertensive therapy as required by the subject to target BP of less than 135/85 mmHg. Titration and doses are performed in accordance with FDA-approved labeling. If blood pressure is not within the target range after 12 weeks, we recruit additional subjects. A group of age- and sex-matched normotensive time control subjects are included (no pharmacotherapy).

Subjects undergo initial microdialysis (MD) experiments, and biopsy sampling. Then we randomly assign hypertensive subjects to a treatment group. Our nurse coordinator monitors blood pressure every 4 weeks and performs weekly compliance checks (telephone and/or email). The nurse performs 24-hour ambulatory blood pressure monitoring at week 8 to determine the efficacy of antihypertensive treatment and to inform dosing titration. Pharmacokinetic and dynamic data from the literature indicate that blood pressure lowing and peripheral vascular effects are maximized by 12 weeks of antihypertensive therapy and maintained

thereafter. After 16 weeks of the assigned intervention, subjects repeat the 24-hour BP monitoring, FMD/sublingual nitroglycerin trials, and MD experiments. We obtain post-pharmacotherapy cutaneous biopsies and repeat the venous blood sampling/analysis.

The normotensive time-control subjects undergo the series of experiments and then repeat the experiments about 16 weeks later.

7.2 Study Procedures

Screening: The subjects drink only water and do not eat after 10 PM the evening before the visit.
The research nurse and/or Clinical Research Center staff perform the screening procedures. The screening includes blood pressure, heart rate, height, waist circumference, and weight. If the research nurse is unavailable for an extended period of time, the CRC performs the full screening. The CRC clinician reviews the medical history. The CRC uses their admission form to admit potential participants for screening. Women who are not postmenopausal submit urine samples for pregnancy tests. The nurse draws about 30 ml (2 Tbsp) of blood from a vein in the subject's arm. We send the blood to labs for some analyses. If the subject takes thyroid hormone, we draw an extra 3.5 ml (0.2 Tbsp) to check the level of thyroid hormone. Screening tests performed on blood: CBC, Lipid panel, and blood chemistry, Interleukin Panel (IL), folic acid and B12, homocysteine, oxidized LDL, total antioxidant capacity. We do not perform genetic analyses on the blood nor look for presence of disease (e.g. HIV).

Subjects with hypertension or suspected of being hypertensive undergo ambulatory 24 hour blood pressure monitoring.

If a potential subject is taking a single, personal physician-prescribed antihypertensive drug (e.g. diuretic, ACE inhibitor), the subject may participate if her/his personal physician gives permission for the subject to discontinue the drug for the duration of the subject's participation in the study. In this case, the subject signs a HIPAA form and the physician signs a permission form (see related uploaded documents). Upon obtaining permission from her/his physician, the subject ceases the physician-prescribed antihypertensive drug for a 14-day washout period prior to participating in the first experiment. During the 14 days, she/he self-checks her/his blood pressure with a blood pressure monitor (supplied by us) every 6-8 hours. During the first week, the study nurse contacts the subject daily to check the subject's progress and discuss concerns the subject may have. During the second week, the study nurse contacts the subject every other day to check the subject's progress and discuss concerns the subject may have. If the subject's blood pressure exceeds 168 mmHg systolic or 96 mmHg diastolic during the washout, the subject resumes taking the physician-prescribed antihypertensive drug, and withdraws from the study. We will consult with Dr. Fragin concerning those potential subjects taking a beta blocker because the length of the washout period could be longer than 2 weeks depending upon the dose of beta blocker the subject is taking. We extend the monitoring-protocol used after the first week of washout for washout durations that are longer than two weeks. We advise the subject to follow up with her/his personal physician.

Experiments:

Blood Sample Experiment: When the subject comes to the lab for one of the first experiments, we obtain a 30 ml (2 Tbsp) blood sample to analyze for substances pertinent to the specific aims of this study (e.g. ACE activity, Ang II, Ang 1-7, oxLDL subfraction particles, ApoB, and assymetrical dimethyl L-arginine (ADMA) concentrations (ELISA), and plasma inflammatory cytokine panel (IL-6, sIL-6α receptors, CRP, TNFα, and gp130)). We conduct some of the analyses at the Noll Lab. We send some of the sample to an outside lab for the other analyses (see letter from Joseph Cannon, Ph.D.).

Microdialysis Experiments: (conducted on separate days)

Microdialysis Overview: Microdialysis (MD) is a procedure in which a thin tube of membrane that mimics the capillary blood vessel is implanted in tissue. As a physiological saline perfuses the membrane, there is bi-directional exchange of molecules between the perfusing saline and the fluid bathing the tissue surrounding the membrane. However, the membrane prohibits the exchange of large molecules such as proteins. Substances of interest may be added to the perfusing saline and thus delivered into the surrounding tissues with no systemic effects.

Preparation for all MD experiments: We give printed and verbal instructions outlining what the subjects need to do before they come to the lab. Subjects do not eat or drink anything containing caffeine (ex. coffee, tea, Coca Cola, chocolate) for 12 hours before the experiment.

We measure HR and BP. Women who are not postmenopausal undergo a urine pregnancy test if they have not had one within 2 weeks. The subject washes the ventral forearm with antimicrobial soap.

Microdialysis probe insertion: We place a tight band around the arm so we can visualize veins. For each MD site, we make pairs of pen-marks on the arm 2.5 cm (1 inch) apart and away from veins. We remove the tight band. The MD tubing enters and exits the skin at the marks. We clean the arm with povidone iodine and alcohol. We place an ice bag on the arm for 5 minutes to numb the skin. Then we insert a thin needle into the skin at each entry mark. The needle's tip travels between the layers of skin for 2.5 cm (1 inch) and leaves the skin at the matching exit mark. We thread the MD tubing through the needle. Next, we withdraw the needle leaving the tubing in the skin. Any redness of the skin subsides in about 60 – 120 minutes.

We place a cuff on the upper arm sans the MD probes to measure blood pressure. We place a local skin heater and laser Doppler probe over each MD site. We measure local temperature, and SkBF throughout the MD experiments. We also measure blood pressure every 5-7 minutes (brachial osculation and/or Cardiocap).

MD Experiment 1: Na2S Dose Response and Local Heating

We prepare 4 MD sites. We clamp the temperatures of the local heaters at 33°C (91.4°F). We perform the Na2S Dose Response and Local Heating procedure concurrently.

Na2S Dose Response	Probe 1. Lactated Ringer's only (control) Probe 2. Lactated Ringer's + LNAME

When the experiment begins, the subject rests for a 20-minute baseline. When skin blood flow readings are stable, we add the test substances to the respective probes as indicated above for 60-90 minutes. We obtain a second baseline for about 20 minutes. When the SkBF is stable, we add the first concentration of Na2S to the perfusate of Probes 1-2. We increase the concentration of Na2S approximately every 5 minutes. The nine concentrations of Na2S range from 10^-6M to 10^-1M. After the perfusion with the last concentration of Na2S, we perfuse the sites with Ringer's + SNP for about 30 minutes. Heating and SNP perfusion causes maximum vasodilation.

Local Heating	Probe 3. Lactated Ringer's + Cysteine Probe 4. Lactated Ringer's + Cysteine + AOAA

We collect baseline data for 20 minutes, then we add the investigative substances to the probes. After a second 20-minute baseline, we increase the temperature of the local heaters 0.5°C/5 sec to a target local skin temperature of 42°C (107.6°F). We clamp the local temperature at 42°C (107.6°F). After the SkBF stabilizes (~40 minutes), we switch perfusates at all MD sites to Ringer's + sodium nitroprusside (SNP) and increase the local skin temperature to 43°C (109.4°F) for about 30 minutes. Heating and SNP perfusion causes maximum vasodilation.

Then the experiment is over, and we remove the MD tubing from the skin and place sterile bandages over the sites. If the subject desires, we can also place a bag of ice on the site for 10 minutes to reduce any bruising that may occur. We measure blood pressure and heart rate before the subject departs.

MD Experiment 2: Acetylcholine (ACh) and Norepinephrine (NE) Dose Responses

We prepare 4 MD sites. We clamp the temperatures of the local heaters at 33°C (91.4°F). We perform the ACh and NE Dose Responses concurrently.

ACh Dose Response Probe 1. Lactated Ringer's only (control)
Probe 2. Lactated Ringer's + LNAME
Probe 3. Lactated Ringer's + AOAA

When the experiment begins, the subject rests for a 20-minute baseline. When skin blood flow readings are stable, we add the test substances to the respective probes as indicated above for 60-90 minutes. We obtain a second baseline for about 20 minutes. When the SkBF is stable, we add the first concentration of ACh to the perfusate at each probe. Each site receives 10 increasing concentrations of ACh (10^-10 to 10^-1) over 70 minutes. As SkBF stabilizes with the addition of each concentration of ACh, we proceed to the next concentration. After the last concentration of ACh, we switch perfusates at all sites to Ringer's + SNP and increase the local skin temperature to 43°C (109.4°F) for about 30 minutes. Heating and SNP perfusion causes maximum vasodilation.

NE Dose Response Probe 4. Lactated Ringer's only

We collect baseline data for about 20 minutes. We add the first concentration of NE. We perform the dose response of NE with serial log concentration doses from 10^-12M to 10^-2M for a total of 11 doses. The participant receives 11 different concentrations of NE. After the perfusion with the last concentration of NE, we perfuse the site with Lactated Ringer's only. After the effects of the final concentration of norepinephrine subsides (about 30 minutes), we switch the perfusate to Ringer's + SNP and increase the local skin temperature to 43°C (109.4°F) for about 30 minutes. Heating and SNP perfusion causes maximum vasodilation.

Then the experiment is over, and we remove the MD tubing from the skin and place sterile bandages over the sites. If the subject desires, we can also place a bag of ice on the site for 10 minutes to reduce any bruising that may occur. We measure blood pressure and heart rate before the subject departs.

Experiment: Flow Mediated Dilation (FMD) and sublingual nitroglycerin

FMD: FMD is an assessment of conduit vessel endothelial function. The researchers place a BP cuff on a forearm, gel on the upper arm just above the elbow, and a Doppler ultrasound probe on the gel. The ultrasound measures vessel size and blood velocity. After a 3-minute "resting" measurement, they inflate the cuff for 5 minutes to occlude forearm BF. After deflating the cuff, they perform a second reading.

Sublingual nitroglycerin: This procedure assesses vascular smooth muscle function. A nurse administers the sublingual nitroglycerine and is present throughout the procedure. The subject is supine on a medical bed or recliner. We apply a blood pressure / heart rate monitor (Cardiocap). We may take manual blood pressures. Also, we perform ultrasonography at the brachial artery at the elbow during the procedure. We place a 0.4 mg nitroglycerin tablet under the subject's tongue. The subject closes the mouth immediately after we insert the tablet. The tablet dissolves in 15-90 seconds. The subject refrains from swallowing until the tablet dissolves. Nitroglycerin causes blood vessels to dilate. The effects last for 5-10 minutes. The subject remains supine for 20 minutes following the nitroglycerin administration. The subject remains in the lab until 20 minutes after administration of the nitroglycerin. If the subject has an exceptional reaction to the nitroglycerin (e.g. drop in blood pressure with longer duration), we monitor the subject for up to 60 minutes following nitroglycerin administration.

Experiment: Biopsy:
We give printed and verbal instructions listing what the subjects need to do before they come to the lab. We take two small pieces of skin from the arm (skin biopsy) using standard techniques. First, the subjects wash the site with soap and warm water. Then the subject sits or lies on a bed. We clean the top of the lidocaine-vial with alcohol. An approved clinician cleans the skin with alcohol and injects lidocaine into the skin at the biopsy sites to numb them. We wait a few minutes after injecting the lidocaine to give the drug time to work. We clean the biopsy site 3 times with povidone iodine and an alcohol pad. If the subject is allergic to iodine, we use only alcohol. We gently touch the site with the tip of a needle to see if the subject can feel anything. The participant may feel the slight pain of the pin-prick or only pressure. If the subject can feel pain, we wait a little longer or the approved clinician adds more lidocaine into the skin. We use a punch-tool that looks like a screwdriver that has a round, hollow tip. The tip is 3mm (0.12 in) in diameter. The hollow tip

acts like a cookie cutter. We place the tip of the punch against the skin at the biopsy site and apply mild pressure. The subject feels the pressure. The tip of the punch goes about 3 mm (0.12 in) into the skin. The punch collects a small piece of skin about 3mm x 2mm (0.12 in x 0.08 in). We may apply pressure with sterile dressing to the biopsy site to stop bleeding, if necessary. We place the piece of skin into a small container. We use the punch to remove the second piece of skin in the same way. The researcher applies a sterile bandage to the site. We give instructions concerning care of the biopsy site.

Pharmacotherapy Protocol 1: (Hypertensive subjects only)
Note: All medications will be double-checked by two people to ensure correctness. No generic substitutions are allowed

1) Subjects take their assigned pharmacotherapy (ACEi, SH-ACEi, or diuretic).
   a) They take the drug for 16 weeks + the time necessary to repeat the experiments.
   b) Subjects receive enough pills for 4 weeks of therapy at a time.
   c) Women who are not postmenopausal undergo a pregnancy test every 2 weeks during pharmacotherapy.
   Special Note The women have a pregnancy test during the monthly visits to the lab to pick up pills and conduct a pregnancy test at home two weeks after the visit. We supply the pregnancy test for subjects to use in at home.
2) The research nurse contacts subjects weekly to check compliance.
3) After starting the treatment, subjects either (1) come to the lab every 4 weeks to pick up pills or (2) if they are male or post-menopausal female, then pills can be mailed directly to the subjects' residence by the pharmacy.
   a) During the monthly visit, we check blood pressure: either (1) we will directly take their blood pressure or (2) if participants own an automatic blood pressure cuff, they will take their own blood pressure and report to us the results via telephone.
   b) Women who are not postmenopausal, have a urine pregnancy test, and receive test supplies to take home.
4) At week 4 of the treatment, subjects also have a blood draw (about 8.5 ml, 0.6 Tbsp) to check potassium, BUN, and creatinine.
5) At week 8 of the treatment, subjects repeat the 24-hour blood pressure monitoring.
   a) If subjects who are taking HCTZ have not reached the target blood pressure (140/90 mmHg) the dose is Increased to 50 mg/day.
6) At week 12 of treatment (Subjects taking HCTZ only)
   a) If subjects who are taking HCTZ have not reached the target blood pressure (140/90 mmHg), the subjects are given the opportunity to switch to one of the ACEi pharmacotherapies for 16 weeks. The ACEi pharmacotherapy chosen is randomized. The subjects washout for two weeks before starting the ACEi or SH-ACEi pharmacotherapy.
   b) The nonresponders are replaced to achieve the necessary number of participants who respond to HCTZ.
4) At week 16 of the treatment
   a) Subjects repeat the 24-hour blood pressure monitoring.
   b) Subjects repeat all experiments.
   c) We take another blood sample (about 8.5 ml, 0.6 Tbsp) to check potassium, BUN, and creatinine.

Pharmacotherapy Protocol 2: (Hypertensive subjects who stopped physician-prescribed drugs only)
Note: All medications will be double-checked by two people to ensure correctness. No generic substitutions are allowed

1) Subjects take their assigned pharmacotherapy (ACEi, SH-ACEi, or diuretic). A participant who has a history of being a non-responder to one of the research blood pressure drugs before they enter the study will be randomly assigned to one of the other study drugs.
   a) They take the drug for 16 weeks + the time necessary to repeat the experiments.
   b) During the first month, subjects receive enough pills for 2 weeks of therapy at a time.
   c) Women who are not postmenopausal undergo a pregnancy test every 2 weeks during pharmacotherapy.

Special Note The women have a pregnancy test during the monthly visits to the lab to pick up pills and conduct a pregnancy test at home two weeks after the visit. We supply the pregnancy test for subjects to use in at home.

2) The research nurse contacts subjects weekly to check compliance.
3) After starting the treatment, subjects come to the lab every 2 weeks for the first month for blood pressure check and to pick up pills.
   a) If a subject's blood pressure is > 140/90 after 2 weeks of pharmacotherapy, we titrate the dose of the medication upward to reduce blood pressure below 140/90.
   b) Women who are not postmenopausal, have a urine pregnancy test, and receive test supplies to take home.
4) After the first month on the pharmacotherapy, subjects will either (1) come to the lab every 4 weeks thereafter to pick up enough pills for 4 weeks of therapy at a time or (2) if they are a male or a post-menopausal female, pills can be mailed directly to the subjects' residence by the pharmacy.
   a) During the monthly visit, we check blood pressure: either (1) we will directly take their blood pressure or (2) if participants own an automatic blood pressure cuff, they will take their own blood pressure and report to us the results via telephone.
      i) Any subjects whose blood pressure is > 140/90 at 4 or more weeks after starting the pharmacotherapy end participation in the study.
   b) Postmenopausal women who are continuing in the study, have a urine pregnancy test, and receive test supplies to take home.
4) At week 4 of the treatment, subjects also have a blood draw (about 8.5 ml, 0.6 Tbsp) to check potassium, BUN, and creatinine.
5) At week 8 of the treatment, subjects repeat the 24-hour blood pressure monitoring.
4) At week 16 of the treatment
   a) Subjects repeat the 24-hour blood pressure monitoring.
   b) Subjects repeat all experiments.
   c) We take another blood sample (about 8.5 ml, 0.6 Tbsp) to check potassium, BUN, and creatinine.

Equipment:

Laser Doppler Flowmetry: The Laser Doppler Flowmeter (Moor Instruments, Inc.) non-invasively provides a qualitative measure of skin blood flow to a depth of about 1 mm in the skin using a weak laser light. This measure is a dimensionless value called "flux" that reflects the speed and number of blood cells moving through the microvasculature in an area of skin. The flowmeter continuously measures skin blood flow using a fiber optic probes that fit into holders taped to the skin. *approved by the FDA*

Blood Pressure, ECG (Heart rate): The CardioCap 5 (General Electric – GE) critical care monitor measures blood pressure via a cuff inflated every 5-7 minutes on the upper arm and heart rate via 3-lead ECG probes taped to the skin of the chest. *approved by the FDA*

Flow Mediated Dilation (FMD): FMD measures the health of blood vessels. The researchers place a blood pressure cuff around the forearm. They put gel on the upper arm just above the elbow. Then they place a Doppler ultrasound probe on the gel. The ultrasound makes sound waves to measure the size of blood vessels and the speed of the blood. They make a "resting" measurement before they inflate the cuff. Then they inflate the cuff for 5 minutes to stop blood flowing to and from the forearm. After they deflate the cuff, they perform a second reading for 3 minutes. ATL HDI 5000 SonoCT (Ultrasound) *FDA 510 (k) approved by the FDA*

7.3 Duration of Participation

Screening (1 Visit)	less than 1.5 hour
MD Experiments (4 Visits)	5 hours each
FMD/sublingual Nitroglycerin (2 visits)	1.5 hours
Biopsy (2 visits)	less than 1 hour
Obtain pills (3 Visits, Hypertensives only)	about 15 minutes each

Total: 27.25 Hours (7-12 visits; It could take a minimum of 18 weeks to complete the study.)Note: The biopsy may occur on the same day as one of the other visits.

Hypertensive subjects taking physician-prescribed drugs:
In addition to the 27.25 hours described above, these subjects undergo a 2-week washout period during which they monitor their blood pressure prior to the first experiment.

HCTZ non-responders only	
Screening (1 Visit)	less than 1.5 hour
MD Experiments (4 Visits)	5 hours each
FMD/sublingual Nitroglycerin (2 visits)	1.5 hours
Biopsy (2 visits)	less than 1 hour
Obtain pills (6 Visits)	about 15 minutes each

Total: 28 Hours (10-15 visits; It could take a minimum of 32 weeks to complete the study.)

Note: The biopsy may occur on the same day as one of the other visits.

7.4 Test Article(s) (Study Drug(s) and/or Study Device(s))

7.4.1 Description

Devices: See "7.2 Study Procedures"

Used with Microdialysis:

Acetylcholine ACh Form: Powder
Role in Current Study: Local heating combined with the local perfusion of endothelial agonist, acetylcholine, specifically examines the attenuated endothelium-dependent vasodilation (eNOS-derived NO) associated with aging or vascular pathologies.
Concentration: 10^-10 to 10^-1M

Aminooxyacetic acid AOAA Form: Powder
Role in current study: Aminooxyacetic acid is a general inhibitor of pyridoxal phosphate (PLP)-dependent enzymes and an inhibitor of cystathionine Y-lyase (CSE), an enzyme that aids in endogenous production of hydrogen sulfide from the substrate, l-cysteine.
Concentration: 5mM

Vitamin C L-Ascorbate Form: Powder
Role in Current Study: L-ascorbate stabilizes norepinephrine in solution. A small amount will be delivered by MD to a nickel-sized are of skin.
Concentration: 3mM

Cysteine L-Cysteine Form: Powder
Role in Current Study: L-cysteine is a substrate for the enzyme, cystathionine Y-lyase (CSE) that aids in the production of endogenous production of hydrogen sulfide in the body.
Concentration: 20 mM

LNAME N^G-nitro-L-arginine methyl ester Form: Powder
Role in current study: A nitric oxide synthase inhibitor. L-NAME is an analog to the amino acid, L-arginine. L-NAME is a non-specific inhibitor for nitric oxide synthases, thereby inhibiting the production of nitric oxide that causes vasodilation. We deliver small doses of LNAME to a nickel-sized area of the skin.
Concentration: 10mM

Norepinephrine Noradrenaline Form: Powder

Role in Current Study: Norepinephrine acts on both alpha-1 and alpha-2 adrenoreceptors to cause vasoconstriction. A small amount will be delivered by MD to a nickel-sized are of skin.
Concentration: 10^-12 to 10^-1 M

Sodium Nitroprusside SNP Form: Powder
Role in Current Study: SNP, acting an NO donor, dilates blood vessels maximally thereby achieving maximal skin blood flow (SkBF). Maximal SkBF is a reference point for other measures of skin blood flow.
Concentration: 28mM

Sodium sulfide Na_2S Form: Powder
Role in current study: Once in the human body, sodium sulfide breaks down to produce hydrogen sulfide. The hydrogen sulfide causes vasodilation. Sodium sulfide solution is strongly alkaline; therefore the solution is titrated with HCl is added to correct the pH to 7 thereby rendering the solution suitable for perfusion through a MD probe.
Concentration: 10^-6 to 10^-1 M

Lactated Ringer's Ringer's Form: Liquid
Role in current study: Lactated Ringer's acts as the vehicle for the other research substances and as a flush.

<u>Drugs not used with microdialysis</u>

Lidocaine (1%) Form: Liquid
Role in Current Study: We use Lidocaine (without epinephrine) to numb the skin for biopsy in an FDA-approved manner.

Nitroglycerin Form: Tablets
Role in Current Study: Sublingual nitroglycerin coupled with ultrasonography of the brachial artery assesses the function of vascular smooth muscle. This is not an on-label, FDA-approved use for nitroglycerin. However, researchers at Noll Lab and elsewhere have successfully used sublingual nitroglycerin in this manner without problem. We included this use of nitroglycerin in the IND application for this study.

<u>Used as Pharmacotherapy</u>:

Enalapril maleate Vasotec Form: Tablets
Role in Current Study: Enalapril is an Angiotensin Converting Enzyme (ACE) inhibitor. Clinically, oral administration of Enalapril is used in the management of hypertension. Vasotec is FDA-approved for the management of hypertension. Titration and doses are performed in accordance with the FDA-approved labeling.

Hydrochlorothiazide Form: Tablets
Role in Current Study: Hydrochlorothiazide USP is a diuretic and antihypertensive. Clinically, oral administration of hydrochlorothiazide is used in the management of hypertension either as the sole therapeutic agent or to enhance the effectiveness of other antihypertensive drugs in the more severe forms of hypertension. Hydrocholothiazide is sold under various brand names (e.g. Aquazide H, HydroDIURIL, Microzide). Titration and doses are performed in accordance with the FDA-approved labeling.

Captopril Form: Tablets
Role in Current Study: Captopril is an Angiotensin Converting Enzyme (ACE) inhibitor. Captopril contains a sulfhydryl moiety, which has been associated with the capacity to scavenge oxygen-free radicals. The antioxidant activity of Captopril has been shown to be present at cardiac and vascular levels both *in vitro* and *in vivo*. Captopril is approved by the FDA for clinical use in the United States. Titration and doses are performed in accordance with FDA-approved labeling.

7.4.2 Treatment Regimen

Investigative Substances used with Microdialysis

The following is a table of the research agents used with intradermal microdialysis. Based upon research literature, we use 14% for calculating maximum delivery of the research agents for all IND applications for our MD studies.

Research Agent	14% delivery (mg)
Acetylcholine (USP)	0.051
Aminooxyacetic acid	0.033
Vitamin C	0.002
Cysteine	0.092
L-NAME	0.100
Norepinephrine	0.005
Sodium Nitroprusside	0.070
Sodium Sulfide	0.067

Drugs not used with microdialysis

Lidocaine (1%): The dose is less than 0.5mg / biopsy site.

Nitroglycerine: 0.4 mg under the tongue during FMD/sublingual nitroglycerin experiment

Used as Pharmacotherapy:

Enalapril: The recommended initial dose is 5 mg orally once/day. Dosage is adjusted according to blood pressure response. The typical maintenance dosage range is 10 to 40 mg per day administered in a single dose or two divided doses. Titration and doses are performed in accordance with the FDA-approved labeling.

Hydrochlorothiazide: The recommended initial dose is 25 mg orally once daily. The maintenance dose can increase to 50 mg orally daily, as a single or 2 divided doses. Titration and doses are performed in accordance with the FDA-approved labeling.

Captopril: Typical initial dose is 25mg orally given 2-3 times daily. Dosage is adjusted according to blood pressure response. Target dose usually achieved within 4 weeks. Maintenance dose range is 25-150mg given 2-3 times daily (usually not necessary to exceed 150mg/day). The typical maintenance dose of Captopril Tablets in hypertension usually does not exceed 50 mg three times per day. The maximum dose of 450mg/day is not to be exceeded. Titration and doses are performed in accordance with the FDA-approved labeling.

Also see “7.2 Study Procedures”

7.4.3 Method for Assigning Subject to Treatment Groups

The hypertensive subjects are randomly assigned to a treatment group. If a hypertensive subject has contraindications for one or two of the treatments, the subject is assigned to one of the remaining treatments.

7.4.4 Subject Compliance Monitoring

We contact subject weekly to ask if they have been compliant during their treatment period.

7.4.5 Blinding of the Test Article

The members of the research team conducting experiments and data analysis are blinded to the pharmacotherapy treatment and do not handle the treatment drugs during the study. The research nurse and research physicians know which treatment the subject receives. Subjects are not blinded to their assigned treatment. We caution the subjects not to reveal the identity of their treatment to the research team conducting experiments and data analysis.

7.4.6 Receiving, Storage, Dispensing and Return

See uploaded Physician Oversight SOP.

7.4.6.1 Receipt of Test Article

Used with Microdialysis: Detailed records are kept in laboratory log books. The containers are dated upon receipt and when opened.

Acetylcholine	source: USP
Aminooxyacetic acid	source: MPBiochemicals, Sigma
Vitamin C	source: Sigma
Cysteine	source: MP Biomedicals
LNAME	source: EMD, Tocris
Norepinephrine	source: USP
Sodium Nitroprusside	source: USP
Sodium sulfide	source: EMD, Sigma
Lactated Ringer's	source: VWR, Owens and Minor, McKesson

From uploaded Physician Oversight SOP:
The investigative agents are purchased by the lab members from reputable sources as indicated in the IRB and the FDA IND applications and in accordance with the standards and guidelines imposed by the FDA.
A license from the CRC physician, the nurse manager, or the overseeing physician is filed with vendors (e.g. VWR , Owens and Minor) requiring the documentation for the purchase of some investigative agents (e.g. Lactated Ringer's) .
The investigative agents are shipped to the Noll Lab or picked up from the pharmacy by lab personnel.
Copies of the prescriptions and orders are maintained in the laboratory's files.

Not used with Microdialysis: Detailed records are kept in laboratory log books and in individual subjects ' charts. The containers are dated upon receipt and when opened.

Lidocaine (1%)	source: University Health Services Pharmacy
Nitroglycerin:	source: University Health Services Pharmacy.

From uploaded Physician Oversight SOP:
Some of the investigative agents are prescription drugs (e.g. lidocaine for skin biopsy). The lab personnel obtain prescriptions from a CRC physician, the research nurse manager, or the overseeing physician for the investigative agents that are prescription drugs and forward the prescriptions to the pharmacy (usually UHS Pharmacy). The investigative agents are shipped to the Noll Lab or picked up by lab personnel.

A license from the CRC physician, the research nurse manager, or the overseeing physician is filed with reputable vendors requiring the documentation for the purchase of some investigative agents.
Copies of the prescriptions and orders are maintained in the laboratory's files.

Used as Pharmacotherapy: Detailed records are kept in laboratory log books and in individual subjects ' charts. The containers are dated upon receipt and when opened. All medications will be double-checked by two people to ensure correctness. No generic substitutions are allowed

Vasotec, Captopril, and HCTZ:	source: Boalsburg Apothecary

From uploaded Physician Oversight SOP:
Some of the investigative agents are prescription drugs (e.g. lidocaine for skin biopsy). The lab personnel obtain prescriptions from a CRC physician, the research nurse manager, or the overseeing physician for the investigative agents that are prescription drugs and forward the prescriptions to the pharmacy (usually UHS Pharmacy). The investigative agents are shipped to the Noll Lab or picked up by lab personnel.

A license from the CRC physician, the research nurse manager, or the overseeing physician is filed with reputable vendors requiring the documentation for the purchase of some investigative agents.

Copies of the prescriptions and orders are maintained in the laboratory's files.

7.4.6.2 Storage

Used with Microdialysis:
The investigational substances are stored under environmental conditions according to manufacturers' instructions in cabinets, refrigerators, or freezers located in Room 228 or 224A Noll Laboratory. The room is locked when unoccupied. Each location has a thermometer. The temperatures are recorded daily during weekdays. The temperature of the room is also monitored by the Central Control System of the Environmental Systems at PSU.

Not used with Microdialysis:
Lidocaine, Nitroglycerin: We store the investigational substances under environmental conditions according to manufacturers' instructions in cabinets, refrigerators, or freezers located in Room 228 or 224A Noll Laboratory. The room is locked when unoccupied. Each location has a thermometer. The temperatures are recorded daily during weekdays. The temperature of the room is also monitored by the Central Control System of the Environmental Systems at PSU.

Used as Pharmacotherapy:
Vasotec, HCTZ, Captopril: We store the drugs in a locked cabinet in Room 228 under environmental conditions according to manufacturers' instructions. The room is locked when unoccupied. The location has a thermometer. The temperatures are recorded daily during weekdays. The temperature of the room is also monitored by the Central Control System of the Environmental Systems at PSU.

Used systemically in experiment:
Nitroglycerin: We store the drug in a locked cabinet in Room 228 under environmental conditions according to manufacturers' instructions. The room is locked when unoccupied. The location has a thermometer. The temperatures are recorded daily during weekdays. The temperature of the room is also monitored by the Central Control System of the Environmental Systems at PSU.

7.4.6.3 Preparation and Dispensing

Used with Microdialysis:
None of the microdialysis drugs are dispensed to the participant; rather we use them in the experiment, as outlined in the Standard Operating Procedures (Phys Ovrsght SOP). The physician providing oversight approves the laboratory standard operating procedure for the obtainment, preparation, and administration of all investigational agents for MD and other procedures in experiments.

The following procedures have been examined and approved by the FDA.
Trained lab personnel prepare the perfusates in accordance with the procedure described in the IRB and FDA IND applications. When mixing the perfusates, the experienced technician washes the hands, wears protection (e.g. gloves, lab coat), and uses glassware that has been washed with cleaner (designed for use with healthcare instruments , pharmaceutical process equipment , tissue culture apparatus , etc.), and rinsed multiple times in tap and then doubly- distilled water and air-dried. Most of the investigational agents are obtained in ultra-pure solid form. The solid investigational agents are weighed on a microbalance and then mixed with sterile pharmaceutical-grade Lactated Ringer's solution to the desired concentration. The solution is drawn into the syringe through a 0.2 µm filter. Prior to injecting a solution into or withdrawing solution from a sterile container through a sterile hypodermic needle, the stopper of the sterile container is cleaned thoroughly with alcohol. Most perfusates are used within minutes or hours after preparation. Stock solutions are made for some investigational agents. The stock solutions are drawn into sterile 1-cc syringes through 0.2 µm filters for storage. The sterile stock solutions are labeled accordingly (e.g. content, date), and protected from light. Stock solutions are not used beyond 1 week (refrigerated) and 6 months (frozen) after mixing. Stock solutions are diluted with sterile Lactated Ringer's when preparing the perfusate. The final dilutions of the perfusates are drawn into sterile syringes through 0.2 µm filters within minutes or hours of their use in the experiment.
The protocol that includes the administration of the investigative substances has been examined and approved by the FDA.

The administration of the investigative substances is performed by the trained and approved lab personnel as described in the protocol included in the IRB and FDA IND applications. The overseeing physician has observed and approved the microdialysis technique as performed by Dr. Lacy Alexander. The lab personnel performing microdialysis have been trained and approved to perform the technique by Dr. Lacy Alexander, approved by the overseeing physician, and approved by the IRB. Letters of approval from the overseeing physician for each member of the lab who performs microdialysis in the research project have been submitted to the IRB.

Drugs not used with microdialysis
Lidocaine: The research or CRC nurse administers the drug under the authority and according to the instructions provided by the supervising physician. Detailed records are kept in laboratory log books and in individual subjects ' charts.

Nitroglycerin: The research or CRC nurse administers the sublingual nitroglycerin during the experiment. Detailed records are kept in laboratory log books and in individual subjects ' charts.

Used as Pharmacotherapy:
Vasotec, Captopril, and HCTZ: The Boalsburg Apothecary places the pills into bottles labeled with the subject ID number, strength (mg), dosing instructions, date, etc. Each bottle contains the number of pills as prescribed by the CRC clinicians who are working with the subjects. All medications will be double-checked by two people to ensure correctness. No generic substitutions are allowed. The research nurse gives the bottle of pills to the subject along with handouts that contain detailed information and dosing instructions pertinent to their assigned drug. Detailed records are kept in laboratory log books and in individual subjects ' charts.

Also see uploaded Physician Oversight SOP.

7.4.6.4 Return or Destruction of the Test Article

Subjects are required to return any unused pills to the investigators. The pills are disposed of in accordance with the policies of the Penn State's Environmental Health and Safety Department at University Park.

7.4.6.5 Prior and Concomitant Therapy

Subjects are screened out if they are currently taking chronic medications that may affect vascular function or be contraindicated for the pharmacotherapy. Medications that are not specifically mentioned in exclusion criteria may be excluded on a case by case basis upon review by the research clinicians or PI.

8.0 Data and Specimen Banking For Future Undetermined Research

NA

9.0 Statistical Plan

9.1 Sample size determination

Based on our previously published data with pharmacological intervention studies, we calculate that 10 subjects will be necessary to detect an effect size of 15% difference in microdialysis treatment sites (1 sample t-test (pre vs. post intervention), power=0.08, α=0.05). We calculate that 14 subjects per group to detect a meaningful physiological differences between groups and microdialysis treatment sites to measure an effect size of at least 15%; (SA2, 3 group ANOVA, power=0.80, α=0.05). Conservatively, we recommend increasing to 19 subjects per group to account for potential dropout along the course of the 16 week intervention and possibility that not all subjects will respond to the antihypertensive treatments. In our previous interventional studies we have been able to maintain 100% compliance. We plan to include about 10 time-control normotensive subjects.

9.2 Statistical methods

We will use a within-person experimental design and associated repeated measures ANOVA tests with planned contrasts. For all experiments, significance will be accepted at $p<0.05$. P-values and 95% confidence

intervals for the mean difference estimates will be adjusted using Tukey's multiple comparison procedure to account for post-hoc multiple comparison testing and ensure a family-wise type I error rate less than 5%. Unless indicated otherwise, data will be analyzed using SAS PROC MIXED, a flexible analytic tool that accommodates a wide variety of ANOVA and regression models and nested and incomplete data structures.
Microdialysis experiments. For these protocols, the primary outcome variable will be CVC. Separate 3-way repeated measures analysis of variance (ANOVA) will be performed to examine systemic treatment difference (Captopril, Enalopril, or Hydrochlorothiazide), and localized microdialysis treatment differences across the phases or doses of pharmacological or physiological stimuli. Appropriate post-hoc analyses with corrections for multiple comparisons will be performed when main effects are identified.
Pharmacological curve modeling. Prior to analysis, H_2S, ACh, NE dose concentrations will be log-transformed. CVC will be normalized to absolute and as a percentage of maximum or baseline, respectively. Vascular responsiveness will be modeled using a sigmoidal dose-response curve with variable slope, as previously described by Wenner et al. and will be used to obtain parameters describing potency ($logEC_{50}$), and cooperativity (Hill slope). Additional growth curve analysis (nonlinear modeling) will be used to determine maximal vasoactive responses (E_{max}) between groups and treatment conditions.
Biopsy experiments. Repeated measures ANOVA will be used to examine group differences, and changes that accrue with systemic treatment. Densitometry analysis will be conducted to examine differences between the groups in enzyme activity, and the protein concentrations obtained from Western blots.

10.0 Confidentiality, Privacy and Data Management

10.1 Confidentiality.

The computerized data files are password protected, and automatically and manually backed up. Only Anna Stanhewicz, and the research personnel directly involved in these protocols will have access to these passwords. The computerized spreadsheets that are being used are also constructed to ensure the validity and confidentiality of the subject's identity (no names, only subject #), as well as the accuracy of the data itself (i.e., automated check for allowable response range). Once data are entered and completeness is verified, analyses proceed with summary results and statistical analyses. The data collected are used exclusively for research purposes.

The investigators maintain data in the laboratory in locked cabinets and on password-protected computers maintained in locked rooms. Most of the data are coded and do not contain personal identifying information. Documents allowing identification of participants do not leave the investigator's labs and are only available to authorized persons. Coded data shared with unauthorized persons cannot be traced to individuals. The investigators store any list linking the code to participants' identity in locked cabinets and on password-protected computers maintained in a locked room. Potential participants may submit pre-screening questionnaires via the investigator's website / Qualtrics (commercial survey website). All web traffic to and from the Qualtrics application is done via a Secure Socket Layer (SSL) that encrypts the data in transmission. Data is deleted from the Qualtric's servers within 24 hours after the user deletes it from the website. Hard copy data forms containing identifiable information are shredded when no longer needed (within 5 years after publication of results). Screening data from subjects who are not accepted into the study are shredded when the project ends. Subjects may give permission to have their contact information retained in the investigator's secured files if they wish to be considered for participation in future studies. After the investigators complete the study, they remove all identifiers from the study's data and store the data indefinitely. Individual data may be used without identifying the subject to illustrate representative responses.

10.1.1 Identifiers associated with data and/or specimens

All of the data and specimens collected in the laboratory are coded according to subject numbers. These subject numbers are randomly assigned by the CRC personnel when the subject screens for the study. The investigators maintain data in the laboratory in locked cabinets and on password-protected computers maintained in locked rooms. Coded data shared with unauthorized persons cannot be traced to individuals.

10.1.1.1 Use of Codes, Master List

Documents allowing identification of participants do not leave the investigator's labs and are only available to authorized persons. The investigators store any list linking the code to participants' identity in locked cabinets and on password-protected computers maintained in a locked room. Only authorized persons have access to the code. The code is destroyed within 5 years of publication of the data.

10.1.2 Storage of Data and/or Specimens

The investigators maintain data in the laboratory in locked cabinets and on password-protected computers maintained in locked rooms. Most of the data are coded and do not contain personal identifying information. Documents allowing identification of participants do not leave the investigator's labs and are only available to authorized persons. Coded data shared with unauthorized persons cannot be traced to individuals. The investigators store any list linking the code to participants' identity in locked cabinets and on password-protected computers maintained in a locked room. Potential participants may submit pre-screening questionnaires via the investigator's website / Qualtrics (commercial survey website). All web traffic to and from the Qualtrics application is done via a Secure Socket Layer (SSL) that encrypts the data in transmission. Data is deleted from the Qualtric's servers within 24 hours after the user deletes it from the website. Hard copy data forms containing identifiable information are shredded when no longer needed (within 5 years after publication of results). Screening data from subjects who are not accepted into the study are shredded when the project ends. Subjects may give permission to have their contact information retained in the investigator's secured files if they wish to be considered for participation in future studies. After the investigators complete the study, they remove all identifiers from the study's data and store the data indefinitely. Individual data may be used without identifying the subject to illustrate representative responses.

Biological specimens will be stored at University Park and Quest Lab, Chantilly, VA, Johns Hopkins, Baltimore, MD, and Georgia Health Sciences University, Augusta, GA. At University Park the specimens will be stored in a -80ºC freezer in Noll first floor hallway. All specimens not exhausted upon analysis are maintained no longer than 5 years after publication.

This study will be issued a Certificate of Confidentiality. Researchers will not disclose or provide any identifiable information without the subject's prior consent or where permitted according to NIH's Policy on Issuing Certificates of Confidentiality

10.1.3 Access to Data and/or Specimens

Only IRB approved laboratory personnel will have access to the data. Non-PSU study team members will be made aware they are also subject to the disclosure restrictions according to NIH's Policy on Issuing Certificates of Confidentiality.

10.1.4 Transferring Data and/or Specimens

Some specimens may be transferred to Quest Diagnostics (Chantilly, VA), or the laboratories of Dan Berkowitz, MD, at Johns Hopkins, Baltimore, MD or that of Dr. Joseph Cannon at the Medical College of Georgia. Specimens are labeled with subject numbers only, and the recipients are not given access to the code matching the subject number to information that could identify the research participant. All specimens not exhausted upon analysis are maintained no longer than 5 years after publication.

The researchers do not plan to release identifiable information collected in the study. However, if researchers consider releasing identifiable information in the future – the individual or institution receiving the identifiable information will be made aware they are also subject to the requirements of subsection 301(d) of the Public Health Service Act.

10.2 Privacy

We tell participants that they may decline to answer questions and decline to participate in the study. The researchers obtain only info given to them by the participant. We perform all procedures and experiments in a private room. Only authorized personnel are present during screening and experiments. On occasion (e.g. educational visit, visiting colleague, site visit) participants may give permission for visitors to observe a procedure or experiment. All laboratory personnel are aware and trained to protect and respect participants' confidentiality and privacy.

11.0 Data and Safety Monitoring Plan

11.1 Periodic evaluation of data

Dr. Alexander discusses the study with the group at weekly lab meetings. If necessary, she conducts additional special meetings with relevant personnel if there is an immediate, specific issue must be addressed (e.g. untoward events).

11.2 Data that are reviewed

Relevant data safety and management procedures, interim data evaluation, untoward events (rare in this research), new developments in related research, and quality control issues are discussed.

11.3 Method of collection of safety information

Lab members keep lab notebooks into which they note relevant safety issues and data that they identify for discussion. In the case of untoward events, those lab members present during the event are included in a debriefing and notes of the meeting are taken. The participant involved in an untoward event is interviewed in person or via telephone concerning the event and the responses are included into the notes for that event.

11.4 Frequency of data collection

Data collection (taking notes) concerning safety is ongoing and begins with the beginning of the project.

11.5 Individual's reviewing the data

The PI and lab group review the data.

11.6 Frequency of review of cumulative data

NA

11.7 Statistical tests

NA

11.8 Suspension of research

In the case of an adverse event, research is suspended while evaluation, reporting, and rectification proceeds. Research is restored when the IRB and PI decide that a rectification of the problem is satisfactory.

12.0 Risks

General note: The research group's members are trained and competent in their duties. The group, led by Dr. Alexander, evaluates the effectiveness and safety of protocols and procedures in an ongoing fashion. They discuss the protocol with candidates, invite questions, and offer tours of the laboratory. Prior to medical screening, candidates read and sign informed consent forms detailing protocols, procedures, risks, sensations, compensation, etc. The researchers give candidates witnessed copies of the signed consent forms. After accepting participants into the study, the researchers discuss and review the procedures and protocols with them generally and at each step throughout the project. They frequently remind participants of the option to withdraw from the study at any time. Restricting access to experiments, data, and coding to authorized personnel maintains confidentiality. The Noll Lab's electronics technician certifies the electrical devices for human use. Lists of emergency numbers remain by lab telephones. At least one cell phone is present at each experiment. A hospital and emergency medical services are within 1-2 miles of the lab. An AED hangs nearby in the hallway.

Microdialysis: The researchers insert a 25 g needle horizontally into and then out of the layers of the skin of the ventral forearm. The needle's entry and exit are about 2.5 cm apart. They thread the microdialysis "probe," comprising a tube of membrane (320 um OD) with tubing attached at both ends (650 um OD), through the needle. Then they withdraw the needle leaving the membrane under the skin. They perfuse the probe with sterile saline using a syringe pump.

Cutaneous microdialysis commonly causes some pain and bruising similar to that experienced during venipuncture. There is usually no pain after the probe is in place. The participant may experience mild pain while the researchers remove probe. Minor bleeding may occur. As with routine venipuncture, a participant who is nervous about needles could have increased heart rate and blood pressure, become lightheaded or nauseated, or could faint.

As with venipuncture or any event that breaks the skin, infection is possible. However, no participants in any of the researchers' experiments been reported infection. The researchers place a sterile bandage on the site after the experiment. Although rare, if the membrane should break in half during removal, they remove the remaining half by gently pulling the attached tubing. This presents no additional risk to the participant. In the unlikely event in which the membrane breaks during removal leaving an isolated piece of membrane under the skin, they treat the piece of membrane in a manner similar to that for a splinter in the skin. In this case, they have to make a superficial incision for removal. Such an event has not occurred in projects that have used MD in this lab (over 1,000 MD probes have been placed by Dr. Alexander alone).

Perfusate: Lactated Ringer's solution flows through the microdialysis probes. An allergic reaction to this physiological saline solution is highly unlikely.

All substances (ACh, AOAA, ascorbate, cysteine, LNAME, norepinephrine, propranolol, sodium sulfide, SNP, yohimbine) added to the fluid perfusing the microdialysis probes have been used previously in clinically and/or research in humans:

Microdialysis delivers small amounts of the substances to a nickel-sized area of the skin. The small quantities used and the extremely localized administration during microdialysis does not produce systemic effects. To the researchers' knowledge, there are no reports of short- or long-term side effects of these substances administered through microdialysis. The chance of adverse reactions to these substances is extremely small given the minute amount delivered to the a very small area of skin, the lack of adverse reactions to similar amounts delivered via microdialysis in many other studies, and the lack of adverse effects in human cell cultures. There is a slight chance of allergic reaction to these substances that could produce redness, itching, rash, and/or swelling. A severe reaction (anaphylactic shock) could also cause fever, difficulty in breathing, changes in pulse, convulsions, and/or loss of consciousness.

Pharmacologic Intervention: Subject screening will help to identify potential subjects who have known contraindications. Subjects who have a contraindication (e.g. a condition, medication with a known interaction, known allergy) to one or two of the three pharmacotherapy drugs, may be assigned one of the pharmocotherapy drugs that is not contraindicated. If contraindications do not allow them to be assigned any of the drugs, the subjects are excluded. Subjects receive a handout containing information (e.g. side effects, drug interactions, etc.) and instructions relevant to the drug. The physician associated with the study can decide if the occurrence of a side effect warrants medical treatment and/or removal from the study. The subject is contacted weekly to check on compliance. This also provides an easy weekly opportunity for the subject to ask new questions or express new concerns. Subjects come to the lab monthly for pill-pickup and BP measurement. During the eighth week of therapy the BP measurement comprises 24-hour blood pressure monitoring. Women with normal cycles undergo a urine pregnancy test every two weeks during pharmacotherapy. The subject may choose to stop taking the drug and remove her/himself from the study at any time.

Enalopril: Subjects could have an allergic or anaphylactic reaction to the drug. Liver and kidney problems, history of heart disease/failure, history of blood clots/stroke, diabetes, and angioedema are contraindicative. Titration and doses are performed in accordance with the FDA-approved labeling. See handout for more information.

Possible common mild side effects include:

feeling tired or weak	lightheadedness	cough
blurred vision	confusion	sweating

More severe side effects that include:

feeling like you will faint	pounding heart beats	fluttering-feeling in your chest
slow heart rate	weak pulse	muscle weakness
tingly-feeling	ill feeling	fever
chills	painful mouth sores	painful swallowing
skin sores	flu-like symptoms	trouble breathing

reduced or no urine output	painful urination	swelling of feet or ankles
feeling tired	shortness of breath	

Less common side effects include:

chest pain	cough producing mucus	vomiting
runny stool	fainting	nausea
sneezing	sore throat	tightness in the chest

Hydrochlorothiazide: Subjects could have an allergic or anaphylactic reaction to the drug. Liver and kidney problems, glaucoma, asthma, gout, sensitivity to sulfonamide-derived drugs, and diabetes are contraindicative. Titration and doses are performed in accordance with the FDA-approved labeling. See handout for more information.

Possible milder side effects include:

runny stools	mild stomach pain	blurred vision	constipation

More severe side effects include

eye pain	vision problems	dry mouth/thirst.
Nausea	vomiting	rash.
feeling weak	drowsiness	restlessness
light-headedness	fast/uneven heartbeat	muscle pain or weakness
numbness	tingly feeling.	

Captopril: Subjects could have an allergic or anaphylactic reaction to the drug. Known hypersensitivity to captopril or another ACE inhibitor is contraindicative. Titration and doses are performed in accordance with the FDA-approved labeling. See handout for more information

Possible common side effects include:

light-headedness	flushing (warmth, redness, or tingly feeling	loss of taste sensation
mild itching/rash	numbness, tingling, or burning pain in hands/feet	cough

More severe side effects include:
dizziness
reduced or increase urinating
chest pain or pressure, pounding heartbeats, fluttering in chest
shortness of breath (even with mild exertion), swelling, rapid weight gain
high potassium (slow heart rate, weak pulse, muscle weakness, tingly feeling)
sudden weakness or ill feeling, fever, chills, sore throat, painful mouth sores, pain when swallowing, skin sores, cold or flu symptoms

Captopril can cause injury or death to the unborn baby if the medicine is taken during the second or third trimester of pregnancy. Women who are not post-menopausal should use reliable birth control while taking Captopril. If a woman becomes pregnant, she will stop taking Captopril, notify the her personal physician and the researcher, and exit the study.

Sublingual Nitroglycerine: Subjects may experience some of the following reactions to the nitroglycerine

headache	lightheadedness	dry mouth	flushing
irregular heart beat	weakness	nausea	vomiting
5-10 minute drop in blood pressure	fainting	dizziness	sweating

Subjects may notice a sweet taste and/or tingling sensation in the mouth while the tablet dissolves. All these effects are usually short-lived. We minimized the effects by having subjects remain supine for 20 minutes after receiving the tablet. In subjects who experience a drop in blood pressure, values usually return to within 10 mmHg of baseline levels upon resolution of the testing. We monitor subjects for up to an hour after they receive the nitroglycerin if they have a strong or bad reaction. Subjects could have a mild or severe allergic response to

the drug. This response could include rash, itching, difficulty breathing, and swelling of the face, lips, tongue, or throat. In the event that blood pressure does not return to baseline, coupled with related symptoms, we refer subjects for medical follow-up. In the event of a severe reaction (e.g. anaphylaxis) we call 911.

The effects of nitroglycerin on pregnant or nursing women are unknown. We exclude subjects who are pregnant or nursing. The use of nitroglycerin for artery measurements is not an FDA-approved use of this drug. However, nitroglycerin has been used in this way in many research studies nation-wide without problem. Also, sublingual nitroglycerin is often prescribed for and self-administered by heart patients who have, or are at risk for, angina (heart pain).

Laser Doppler Flowmetry: The probe attaches to the skin with double-sided tape and measures skin blood flow in a 1mm3 volume of skin. Weak lasers can hurt the eye if one should stare into the light for a long time. The red light seen on the surface of the skin is harmless. The researchers have used this technique in their lab with IRB approval for many years without incident. The researchers do not turn on the laser until they tape the probes to a surface. They remove the tape carefully afterward.

Local Heating and Local Skin Temperature: The local heating control unit (Moor Instruments) precisely controls and monitors the temperature of the heating-rings and heated probe holders used with the Laser Doppler Flowmeter. To determine the maximal skin blood flow, the researchers increase the temperature of the heating units slowly (about 0.1°C every 1 second). The skin feels very warm but not painful. Local heating causes temporary redness of the skin that subsides within several hours. This technique is very unlikely to produce long-term ill effects. The local heating controllers (Moor Instruments) precisely control and monitor the temperature of the heating-rings and heated probe holders. The system has programmed maximum temperature limits. This technique is very unlikely to produce long-term ill effects.

Blood pressure (manual; CardioCap 5): The cuff inflates on the upper arm. The cuff slowly deflates while the researchers listen to pulse-sounds at the inside of the elbow with a stethoscope, or the CardioCap 5 takes a measurement. The inflated cuff may make the arm (manual and CardioCap 5) feel tingly and numb, and the cuff may temporarily bruise the arm. Efficient and competent measurement technique minimizes the duration of cuff inflation.

Blood draw: Blood draws can cause anxiety (with increased heart rate and blood pressure), mild pain, swelling, nausea, lightheadedness, fainting, or bleeding. There is a slight chance of infection. Subjects may have pain or bruising at the site where the catheter is placed in the arm, and there is a small risk of infection. Fainting sometimes occurs during or shortly after blood is drawn. A competent nurse performs blood draws using standard venipuncture-procedure and techniques that minimize the chance of infection. Participants may recline for the procedure. To minimize the risk of infection associated with blood collection, only sterile, single use catheters are used.

Povidone Iodine: Researchers and hospitals use this orange-colored fluid to clean the skin. If the subject informs us of a known allergic to iodine, we use only alcohol. An allergic reaction could cause redness, itching, rash, and/or swelling. A worse reaction could also cause fever, breathing problems, changes in pulse, convulsions, and/or fainting.

Tape and adhesive disks: Participants could be sensitive to the adhesive of the tape and double-sided adhesive disks used in the study causing redness, rash, tenderness, and/or itching. The researchers remove the tape and adhesive disks carefully. Ointment is available, if needed.

Medical Screening: The screening includes blood sample, height, waist circumference, weight, heart rate, blood pressure, resting ECG, pregnancy test, and history performed by the competent research staff. Participants may be uncomfortable giving medical information or being measured. The participants may decline to answer questions or participate in measurements. The researchers conduct screenings professionally and privately.

Initial Screening form: The researchers use the form to initially determine a candidate's suitability for the study. The initial interview gathers minimally invasive personal data that is kept confidential. They used similar

interviews in the past without problem. Candidates may complete the form at the lab group's web site via the Qualtrics commercial survey website. The participant may decline to answer questions. The researchers conduct phone interviews professionally and privately. Only authorized personnel may access completed forms.

Local Heating: The local heating control unit (Moor Instruments) precisely controls and monitors the temperature of the heated probe holders used with the Laser Doppler Flowmeter. To determine the maximal SkBF, the researchers increase the temperature of the heating units slowly (about 0.1°C every 1 second). The skin feels very warm but not painful. Local heating causes temporary redness of the skin that subsides within several hours. This technique is very unlikely to produce long-term ill effects. The local heating controllers (Moor Instruments) precisely control and monitor the temperature of the heated probe holders. The system has programmed maximum temperature limits. This technique is unlikely to cause long-term ill effects.

Skin Biopsy: Skin biopsy could cause pain, lightheadedness, nausea, and/or fainting. Subjects who are nervous about needles could experience temporary increased heart rate and blood pressure. There is a chance of infection. Subjects may stop the procedure at any time. Trained staff performs the biopsy. Subjects may lie on a bed or sit in a Trendelenburg chair. The researcher insures subjects are informed and ready. Trained staff uses proper techniques and sterile supplies to keep risk of infection minimal. The stopper of the vial of lidocaine is cleaned with alcohol before needle insertion. The skin of the biopsy sites is cleaned with alcohol. The research nurse infiltrates the sites with 1% Lidocaine w/o epinephrine intradermally to numb the sites so the subject feels little or no pain. Pressure with sterile dressing stops bleeding. A sterile bandage is placed on sites. Scarring can be reduced through proper care. The researcher instructs subjects concerning care of biopsy sites. Subjects may take acetaminophen should they experience minor pain when the lidocaine wears off. People with a history of bleeding problems, excessive scarring, or keloid formation do not participate in the biopsy.

Lidocaine: Subject's may feel brief pain from the needle and a mild burning during the infiltration. Although unlikely, a bad reaction to lidocaine cause fever, breathing problems, changes in pulse and/or blood pressure, convulsions, shock, and/or loss of consciousness. We ask subjects if they are allergic to lidocaine. If so, saline infiltration or ice can be used to reduce pain instead. In the case of a severe reaction, we call 911.

Thermoregulation Lab Website: Potential subjects may enter data into the screening form (see above) via the Qualtrics website. Qualtrics is a secure website and survey application designed to support data capture for research studies. All web traffic to and from the Qualtrics application website is done via a Secure Socket Layer (SSL) that encrypts the data in transmission. The questionnaire contains statements advising of the limitations of technology and that there is no confidentiality guarantee. Data is deleted from the Qualtric's servers within 24 hours after the user deletes it from the website. Participants may choose a personal interview instead.

Flow Mediated Vasodilation (FMD) Test / Doppler Ultrasound: There is a small chance the probe could irritate the skin. Placing the probe on the arm's skin may cause temporary minor redness. The inflated cuffs may cause the participant's arms and feet to feel numb or tingly, and the skin's color to change slightly. The cuffs could cause mild bruising. The gel is the same as that used with medical ultrasound tests. The gel may feel cool or cold on the skin. A bad reaction to the gel is highly unlikely. The cuffs inflate for a minimal amount of time. The temporary redness from the probe is unlikely to have lasting ill effects. The participant may decline the test.

Relevant only to people who stop taking their doctor-prescribed drugs to lower their blood pressure:

Stopping doctor-prescribed blood pressure drugs: Deleterious effects from abruptly stopping blood pressure medications are uncommon[1]. However, withdrawal effects can happen with stopping any blood pressure drug Most effects occur when stopping drugs that affect the central nervous system (e.g. propranolol, verapamil, clonidine)[1]. Symptoms could include agitation, headache, sweating and nausea. Less commonly, blood pressure could suddenly spike upwards[1,2]. A person who has heart disease who abruptly stops taking beta blockers could be at increased risk for chest pain, heart attack, or sudden death. Any subjects whose blood pressure is > 140/90 at 4 or more weeks after starting the pharmacotherapy ends participation in the study. The risk of symptoms is greater when a subject suddenly stops more than one blood pressure drug at the same time[1]. Therefore, we do not include potential subjects the study if they are taking more than one drug prescribed by doctors to control blood pressure. We will exclude patients who are taking clonidine. We will exclude African Americans who are taking a calcium

channel blocker (Norvasc). Also, we will consult with Dr. Fragin, the cardiologist providing medical oversight for this study concerning those potential subjects taking a beta blocker because the length of the washout period could be longer than 2 weeks depending upon the dose of beta blocker the subject is taking.

References:

1. *SB, Weber MA, Priest RT, Brewer DD, Hubbell FA. The abrupt discontinuation of antihypertensive treatment. J Clin Pharmacol. 1979 Aug-Sep;19(8-9 Pt 1):476-86.*

2. *Reidenberg, MM. Drug discontinuation effects are part of the pharmacology of a drug. J Pharmacology Exp Ther. 2011 Nov;339(2):324-8.*

13.0 Potential Benefits to Subjects and Others

13.1 Potential Benefits to Subjects

The subjects receive medical screenings providing information about their blood pressure status and physical well-being. We have had occasions when the screening for our projects enabled us to alert a subject to a developing medical condition whereupon the subject obtained prompt professional care that prevented the condition from progressing into a more serious problem.

13.2 Potential Benefits to Others

Hypertension is one of the most important preventable contributors to cardiovascular disease (CVD) and death. It is estimated that 33% of adults are hypertensive. Abundant evidence from clinical trials shows the benefit of antihypertensive treatment for improving clinical outcomes and reducing overall morbidity and mortality. New JNC8 guidelines strongly recommended the use of ACE inhibitors alone or in combination with a thiazide-type diuretic to achieve blood pressure treatment goals. While it is increasingly recognized that the secondary peripheral vascular effects of ACEi significantly contribute to reduced mortality in hypertension associated-disease[83], the precise mechanisms remain unclear.

The present proposal has the potential to advance scientific knowledge of the peripheral vascular effects of antihypertensive treatment. The proposed work addresses key questions and research topics identified by JNC8 and the American Heart Association:

Which classes of antihypertensive agent should be used for the optimal treatment of hypertension?
Does target-based treatment of hypertension lead to superior outcomes?
Identify and characterize new therapeutic targets for the treatment of hypertension.

In addition, this project will provide additional training and learning opportunities for post docs and students of the Pennsylvania State University.

14.0 Sharing Results with Subjects

The researchers give participants copies of their individual lab results. When the researchers complete data analysis, participants may attend an optional presentation of the study's general findings at Noll Lab.

15.0 Economic Burden to Subjects

15.1 Costs

None

15.2 Compensation for research-related injury

It is the policy of the institution to provide neither financial compensation nor free medical treatment for research-related injury. In the event of injury resulting from this research, medical treatment is available but will be provided at the usual charge. Costs for the treatment of research-related injuries will be charged to subjects or their insurance carriers.

16.0 Number of Subjects

Total number of subjects: 70 (about 60 subjects with hypertension, about 10 normal subjects as time-controls)

17.0 Resources Available

17.1 Facilities and locations

The Noll laboratory is a 4-floor (35,000 sq. ft.) free-standing building devoted to basic, clinical, and applied physiological research. Biochemistry laboratories are available for sample processing and analyses. Available equipment includes centrifuges, osmometer, assay plate reader, shaker, washer, NA/K analyzer, spectrophotometer, i-Stat analyzer, refractometer, hemocue, hematocrit system, fraction collector, hydrogen sulphide analyzer, sonicator, microbalances, laser doppler flowmeters, full-field laser perfusion imager, mini-syringe pumps/ controllers, sweat rate monitoring systems, hospital beds, medical infusion chair, ECG analysis system, finipres, plethysmograph, refrigerators, pharmaceutical refrigerator, -20 C and -80 C freezers, heated/refrigerated circulators, water-perfused suits, local skin cooling/ heating systems, critical care monitors, blood pressure monitors, metabolic cart, gas sterilization equipment, and autoclave facilities. In addition to ample office and laboratory laptop or desktop computers for research and student use, Noll Laboratory has two available servers and a trunk line connection to the University's mainframe computer system. Internal, office and laboratory computers are linked. The laboratory has four PC-based data acquisition systems equipped with Dataq hardware driven by Windaq software, two of which are located in computer controlled environmental chambers equipped with treadmills and bikes. A Clinical Research Center is contiguous to the Noll Laboratory in the 14,000 sq. ft. Elmore wing, and available for the proposed studies. Up to 12 subject beds for overnight (or longer) stays, a metabolic kitchen, medical examination rooms, and several specialized procedure rooms are available. Because the CRC is contiguous with existing laboratory space, it is readily available for research support, e.g., pre-test subject screening. Noll Laboratory has exceptional in-house support facilities including machine and electronics shops.

17.2 Feasibility of recruiting the required number of subjects

We are using recruiting methods that will reach a large percent of the general population. We have employed these methods successfully for earlier projects that targeted the same groups.

17.3 PI Time devoted to conducting the research

The researchers have been conducting similar experiments for many years and are familiar with the duration of the procedures/protocols/projects and the amount of staffing required to complete the project while maintaining their other obligations.

17.4 Availability of medical or psychological resources

The CRC is located within the same building as the Noll Lab. A hospital and emergency medical services are within 1-2 miles of the lab. An AED hangs near the labs in the hallway. A CPR mask hangs in each lab. The faculty, staff, grad students, and post docs in the lab group have current BLS training.

17.5 Process for informing Study Team

The PI and senior lab members train new members in the necessary procedures. The researchers conduct weekly lab meetings. They review any applicable changes or issues.

18.0 Other Approvals

None

19.0 Subject Stipend and/or Travel Reimbursements

MD experiments: ($15.00 for each MD probe + $40 for completing an MD experiment)

Na2S Dose Response /Local Heating	$200.00	($100.00 x 2, pre and post-treatment)
ACh/Ne Dose Response	$200.00	($100.00 x 2, pre and post-treatment)
	$400.00	total

Biopsy:	$200.00	$50.00 each (2 pre + 2 post-treatment)
FMD/Sublingual Nitroglycerin:	$100.00	$50.00 each (1 pre + 1 post-treatment)

Total = $700.00

HCTZ non-responders:	Receive $60.00 for the 12 weeks on HCTZ
Switched to an ACEi	$760.00 total = $700.00 (pre and post experiments) + $60.00
Not switched to an ACEi	$410.00 total = $350.00 (pre experiments only) + $60.00

For incomplete experiments, the researchers pay an amount of money equal to the part completed. For instance, if a subject completes half of an MD experiment, the subject receives $15.00 for each MD probe inserted + $20 ($20.00 is one-half of $40.00). The researchers may ask subjects to repeat a trial. If subjects agree to repeat a trial, they receive payment for the repeated trial as stated above. They are reimbursed for gasoline if they live more than 20 miles from Noll Lab.

20.0 Multi-Site Research

NA

21.0 Adverse Event Reporting

21.1 Adverse Event Definitions

For drug studies, incorporate the following definitions into the below responses, as written:	
Adverse event	Any untoward medical occurrence associated with the use of the drug in humans, whether or not considered drug related
Adverse reaction	Any adverse event caused by a drug
Suspected adverse reaction	Any adverse event for which there is a reasonable possibility that the drug caused the adverse event. Suspected adverse reaction implies a lesser degree of certainty about causality than "adverse reaction". • *Reasonable possibility.* For the purpose of IND safety reporting, "reasonable possibility" means there is evidence to suggest a causal relationship between the drug and the adverse event.
Serious adverse event or Serious suspected adverse reaction	Serious adverse event or Serious suspected adverse reaction: An adverse event or suspected adverse reaction that in the view of either the investigator or sponsor, it results in any of the following outcomes: Death, a life-threatening adverse event, inpatient hospitalization or prolongation of existing hospitalization, a persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions, or a congenital anomaly/birth defect. Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered serious when, based upon appropriate medical judgment, they may jeopardize the patient or subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition.
Life-threatening adverse event or life-threatening suspected adverse reaction	An adverse event or suspected adverse reaction is considered "life-threatening" if, in the view of either the Investigator (i.e., the study site principal investigator) or Sponsor, its occurrence places the patient or research subject at immediate risk of death. It does not include an adverse event or suspected adverse reaction that had it occurred in a more severe form, might have caused death.
Unexpected adverse event or Unexpected suspected adverse reaction.	An adverse event or suspected adverse reaction is considered "unexpected" if it is not listed in the investigator brochure, general investigational plan, clinical protocol, or elsewhere in the current IND application; or is not listed at the specificity or severity that has been previously observed and/or specified.

For device studies, incorporate the following definitions into the below responses, as written:	
Unanticipated adverse device	Any serious adverse effect on health or safety or any life-threatening problem or death caused by, or associated with, a device, if that effect, problem, or death

effect	was not previously identified in nature, severity, or degree of incidence in the investigational plan or IDE application (including a supplementary plan or application), or any other unanticipated serious problem associated with a device that relates to the rights, safety, or welfare of subjects.

21.2 Recording of Adverse Events

In the case of untoward events, those lab members present during the event are included in a debriefing and notes of the meeting are taken. The participant involved in an untoward event is interviewed in person or via telephone concerning the event and the responses are included into the notes for that event.

All adverse events (serious or non-serious) and abnormal test findings observed or reported to study team believed to be associated with the study drug(s) or device(s) will be followed until the event (or its sequelae) or the abnormal test finding resolves or stabilizes at a level acceptable to the investigator.

An abnormal test finding will be classified as an *adverse event* if one or more of the following criteria are met:

- The test finding is accompanied by clinical symptoms
- The test finding necessitates additional diagnostic evaluation(s) or medical/surgical intervention; including significant additional concomitant drug treatment or other therapy
  Note: Simply repeating a test finding, in the absence of any of the other listed criteria, does not constitute an adverse event.
- The test finding leads to a change in study drug dosing or discontinuation of subject participation in the clinical research study
- The test finding is considered an adverse event by the investigator.

21.3 Causality and Severity Assessments

The investigator will promptly review documented adverse events and abnormal test findings to determine 1) if the abnormal test finding should be classified as an adverse event; 2) if there is a reasonable possibility that the adverse event was caused by the study drug(s) or device(s); and 3) if the adverse event meets the criteria for a *serious adverse event*.

If the investigator's final determination of causality is "unknown and of questionable relationship to the study drug(s) or device(s)", the adverse event will be classified as *associated with the use of the study drug(s) or device(s)* for reporting purposes. If the investigator's final determination of causality is "unknown but not related to the study drug(s) or device(s)", this determination and the rationale for the determination will be documented in the respective subject's case history.

21.4 Reporting of Adverse Reactions and Unanticipated Problems to the FDA

21.4.1 Written IND Safety Reports

NA

21.4.2 Telephoned IND Safety Reports – Fatal or Life-threatening Suspected Adverse Reactions

NA

21.5 Reporting Adverse Reactions and Unanticipated Problems to the Responsible IRB

Problems Not Requiring Prompt Reporting: The investigators log internal events/problems that are not problems that require prompt reporting to the IRB (i.e., expected and related events) in an accumulative tracking log that is reported to the IRB with the yearly Continuing Progress Report (CPR) and at the close of the study.

Problems Requiring Prompt Reporting: Internal problems that require prompt reporting and are fatal or life-threatening are reported to the IRB in a Problem Report within one weekday of the principal investigator becoming aware of the problem. All other internal problems that require prompt reporting are reported within five weekdays of the principal investigator becoming aware of the event or problem. External problems that require prompt reporting are reported within 30 days of their receipt by the principal investigator. The investigators issue problem reports to the IRB in the case of adverse events that are: (1) unexpected and (2)

related/likely related to the research as determined by the Penn State University (PSU). These events include: specific protocol-defined events that require prompt reporting to the sponsor, breach of confidentiality, incarceration of a participant in a protocol not approved to enroll prisoners, an accidental or unintentional deviation to the IRB-approved protocol that involved risks, an emergency protocol deviation take without prior IRB review to eliminate an apparent immediate hazard to a research participant, a complaint of a participant that indicates an unanticipated risk or any complaint that cannot be resolved by the research staff, information that indicates a change to the risks or potential benefits of the research, change in FDA labeling or withdrawal from marketing of the study drug, device or biologic used in this research protocol, or sponsor-imposed suspension for risk. For each problem, the investigator adds the event to an accumulative problem report log included with the Problem Report submitted to the IRB. The investigator submits the log to the IRB with the yearly Continuing Progress Report (CPR) and at the close of the study.

In accordance with applicable policies of The Pennsylvania State University Institutional Review Board (IRB), the investigator will report, to the IRB, any observed or reported harm (adverse event) experienced by a subject or other individual, which in the opinion of the investigator is determined to be (1) unexpected; and (2) probably related to the research procedures. Harms (adverse events) will be submitted to the IRB in accordance with the IRB policies and procedures.

21.6 Unblinding Procedures

Participants are not blinded. The research team members conducting the experiments will be unblinded to a particular subject's treatment if the subject is having a reaction that could be associated with the treatment. The research nurse and physicians are not blinded. If this is associated with an adverse event, the event is reported.

21.7 Stopping Rules

Although such events are extremely unlikely to occur, we are prepared to immediately stop experiments and seek medical assistance if the subjects should experience the more serious reactions described in the informed consents, and IRB applications such as signs and symptoms of an allergic reaction, anaphylactic shock, chest pain, dizziness, and fainting. As always, we remind our subjects throughout the protocol that they may stop the experiment at any time. Also, the investigators exercise the discretion to end a subject's participation if the subject should engage in behavior that could jeopardize his/her own health and well-being or that of others. The investigators ends the experiments if the subject's systolic or diastolic blood pressures exceed 180 or 110 mmHg, respectively, heart rate exceeds 85% of the age-predicted maximum, or the body's core temperature reaches 39°C (102 °F) or decreases 1°C (1.8 °F) from baseline. Human subjects undergoing the pharmacotherapy end treatment and cease participation in the study if the research physicians determine that the therapy produces undesirable reactions.

22.0 Study Monitoring, Auditing and Inspecting

22.1 Study Monitoring Plan

22.1.1 Quality Assurance and Quality Control

Lacy Alexander, PhD has ultimate responsibility for this study, with oversight from Jason Fragin, D.O. Dr. Alexander meets regularly with the study team to discuss the project, procedures, and data. Dr. Alexander is responsible for the conduct of the study being in compliance with this protocol, with institutional and IRB policies, with Good Clinical Practice guidelines and any other applicable regulatory requirements. The monitoring is ongoing.

Only trained, experienced persons perform the procedures of this project. The licensed research nurse (RN) performs blood draws. If the research nurse is unavailable, a CRC nurse or clinician draws blood. Trained, experienced, and IRB-approved research team members perform the MD insertions and skin biopsies. The CFRC clinician reviews the medical history during screening. Dr. Fragin is the consulting physician overseeing the project. The CRC clinician oversees participants during pharmacotherapy. The members of the research lab (grad students, staff, and select undergrads) have current basic life support training through CentreLife Link in State College, PA.

22.1.2 Safety Monitoring

All adverse events are classified in accordance with the then current policy of the IRB at Pennsylvania State University (Adverse Events, Definitions and Reporting Requirements). A detailed description of each procedure posing risk and the assessment of that risk is included in the relevant sections of the application.

Reporting: The PI evaluates subject eligibility, adherence to protocols and reporting of adverse events at six-month intervals. All adverse events are reported by the PI to the IRB, according to the then current policy of the IRB at Pennsylvania State University (Adverse Events, Definitions and Reporting Requirements) and copied to the Research Safety Advocate. In addition, all changes in protocol procedures will be submitted for review and approval of the IRB and, when approved, will be promptly implemented. The project receives an extensive annual review by the IRB. The PI submits reportable-changes in protocol and annual reports to the FDA.

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Pyrogen References

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   The effect of varying the pH and ionic strength on endotoxin removal (depyrogenation) from Water for Injections (WFI) was investigated. Studies using submicron filters showed that endotoxin aggregation and filter retention increased with increasing molarity and decreasing pH. Using a Sartorius 0.01 micron filter, greater than 98% endotoxin retention could be achieved with 10 endotoxin units (EU)/ml bulk solution, and greater than 97% endotoxin retention with the 500 EU/ml bulk solution. Depyrogenation of active and placebo solutions of the radiopaque, Iohexol (350 mgI/ml), using ultrafilters of varying nominal molecular weight limit (NMWL 10,000- 300,000) and a Pall Posidyne 0.2 micron filter was also investigated. Results with the ultrafilters showed that it was possible to increase the molecular weight cut-off of an ultrafilter from 10,000 to 100,000, without affecting the efficiency of endotoxin removal, thereb increasing flow rate and reducing filtration time. The Posidyne filter was able to depyrogenate Iohexol active and placebo product. The use of submicron filtration in place of ultrafiltration would provide significant cost benefits in terms of filtration time and equipment costs, and they have been shown to be capable of efficient depyrogenation of these pharmaceutical products.
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3. Orwin P, Leung D, Tripp T, Bohach G, Earhart C, Ohlendorf D, Schlievert P. 2002. Characterization of a novel staphylococcal enterotoxin-like superantigen, a member of the group V subfamily of pyrogenic toxins. Biochemistry 41:14033-14040, 2002.
   Staphylococcus aureus is an important human pathogen, causing a variety of diseases. Major virulence factors of this organism include staphylococcal enterotoxins (SEs) that cause food poisoning and toxic shock syndrome. Our study identified a novel enterotoxin-like protein that is a member of the new subfamily (group V) of pyrogenic toxin superantigens (PTSAgs) and examined its biochemical and immunobiological properties. The gene encoding the SE-like protein is directly 5‘ of another recently identified PTSAg, SEK. The SE-like protein had a molecular weight of 26000 and an experimentally determined isoelectric point between 7.5 and 8.0. We demonstrated that the PTSAg had many of the biological activities associated with SEs, including superantigenicity, pyrogenicity, and ability to enhance endotoxin shock, but lacked both lethality in rabbits when administered in subcutaneous miniosmotic pumps and emetic activity in monkeys. Recombinant protein stimulated human CD4 and CD8 T cells in a T cell receptor variable region, β chain (TCRVβ) specific manner. T cells bearing TCRVβ2, 5.1, and 21.3 were significantly stimulated.
4. Orwin P, Fitzgerald JR, Leung D, Gutierrez J, Bohach G, Schlievert P, “Characterization of Staphylococcus aureus Entertoxin L, “Infection and Immunity, Vol. 71, No. 5p.: 2916-2919, 2003.
5. Weber C, Linsberger I, Rafiee-Tehrani M, Falkenhagen D: Permeability and adsorption capacity of dialysis membranes to lipid A. Int J Artif Organs 20: 144-152, 1997.
   Hemodialysis membranes were tested in vitro for possible penetration by low molecular weight endotoxins containing lipid A. Using lipid A from Escherichia coli as a model substance for this kind of pyrogen, different dialyzers (F4, E3. Acepal 1300, Altraflux, F 40, Polyflux 110, Filtral 12, F 60) were challenged by tangential filtration in aqueous medium. All membranes exhibited impermability to lipid A (as well as to LPS from Pseudomonas aeruginosa), which was proved

by additional experiments using culture filtrates of Pseudomonas aeruginosa in bicarbonate dialysis fluid, as well as by employing miniaturized dialyzers with synthetic lipid A as a contaminant. Furthermore, the highest adsorption capacities were found for polysulfone and polyamide membranes.

24.0 Appendix