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PLATELET INHIBITION WITH CLOPIDOGREL AUGMENTS CUTANEOUS REACTIVE HYPEREMIA IN HEALTHY, MIDDLE-AGED SKIN

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ABSTRACT

Daily low-dose aspirin (ASA, 81 mg) therapy is the gold standard for primary and secondary prevention of atherothrombotic disease. Clopidogrel (Plavix®, 75 mg) is the second most widely used prescription drug in the world for secondary atherothrombotic disease treatment. ASA and clopidogrel inhibit platelet function through cyclooxygenase- (COX) and ADP-receptor inhibition, respectively. Reflex cutaneous vasodilation (VD) is significantly impaired in humans taking ASA and clopidogrel via independent mechanisms. However, the impact of platelet inhibition on whole blood viscoelastic properties and their influence on shear-induced VD is unknown. We hypothesized that systemic low-dose aspirin and clopidogrel each would attenuate shear-induced cutaneous vasodilation by lowering whole-blood viscosity. Five healthy men and women (55±3 years) were orally-administered aspirin (81mg), clopidogrel (75mg), or placebo for 7 days in a double-blind fashion with 2 weeks of washout between treatments. A reactive hyperemia protocol was performed, utilizing bretylium-tosylate iontophoresis on one site to inhibit sympathetic adrenergic function. Skin blood flow was measured using laser-Doppler (LD) flowmetry. Cutaneous vascular conductance was calculated (CVC=LDF/MAP). Data were normalized to maximal CVC (%CVC_{max}: local heat to 43°C). Clopidogrel significantly increased VD through augmented area under the curve (AUC) measurements ($AUC_{clopidogrel}$ = 4187 ± 376 %CVCmax·s vs. Control AUC = 3559 ± 259 %CVCmax·s) ($p<0.05$), but there was no change with ASA (AUC_{ASA} = 3523 ± 481 %CVCmax·s). Total hyperemic response (THR) values approached significance between clopidogrel and placebo ($p=0.068$). Contrary to our hypothesis, clopidogrel augmented cutaneous reactive hyperemia, indicating that platelet ADP-receptor inhibition may improve endothelial function in the cutaenous microvasculature.

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CHAPTER 1
LITERATURE REVIEW

Skin Blood Flow

Skin blood flow (SkBF) is one of the primary mechanisms by which humans thermoregulate (6). SkBF in humans is controlled by two branches of the sympathetic nervous system: the adrenergic system which causes vasoconstriction and the sympathetic cholinergic system which mediates vasodilation (VD). Additionally, local mediators within the endothelium also contribute to changes in SkBF. The skin is a readily accessible, model circulation to non-invasively examine microvascular function with its changes often preceding changes that occur in the conduit circulation.

Reflex cutaneous VD is attenuated in older adults. This is a result of decreased nitric oxide bioavailability, among other co-released cholinergic vasodilators (4). This attenuated vasodilation puts older humans at a greater risk for heat-related morbidities due to less-efficient dry heat loss mechanisms and increased cardiovascular strain.

Platelets

Platelets cause the formation of hemostatic plugs during vascular injury (1). ADP, released by the platelet, activates $P2Y_{12}$, which leads to signaling cascades that stimulate platelet aggregation and thrombus formation. Another platelet-released factor is TXA_2. This molecule amplifies the platelet's adhesion response and is produced from arachidonic acid through the enzymatic conversion by cyclooxygenase-1 (COX-1). Clots are formed through platelets adhering to, and aggregating at, vessel walls (12). Problems arise when these clots become pathogenic thrombi which are associated with diseases such as peripheral artery disease and acute coronary artery disease. Because dislodged clots leading to heart attack and stroke are

problematic in these diseases, platelet-inhibiting drugs, such as aspirin and clopidogrel, were developed.

Aspirin and Clopidogrel (Plavix®)

Daily low-dose aspirin (ASA, 81 mg) therapy is the gold standard for primary and secondary prevention of atherothrombotic disease. ASA inhibits platelet function through cyclooxygenase-1 (COX-1) acetylation in the liver. COX is inhibited for the life of the platelet (~10 days), while endothelial cells are still able to produce COX-dependent vasoactive products (18). TXA_2 also cannot be formed during this time (1).

Clopidogrel bisulfate (Plavix®, 75 mg) is the second most widely used prescription drug in the world for the secondary prevention of heart attack and stroke. Clopidogrel inhibits platelet function through irreversible ADP-receptor inhibition (22). By inhibiting the release of ADP from granula in platelets, $P2Y_{12}$ receptors are unable to be activated, and therefore, cannot stimulate platelet aggregation. $P2Y_{12}$ receptors have also been shown to be present on the vascular smooth muscle and endothelium of blood vessels. However, because clopidogrel is metabolized in the liver and has a short half-life, ADP $P2Y_{12}$ receptors on the platelet are more likely affected, not the vascular smooth muscle.

Acute administration of clopidogrel improves endothelial dysfunction in conduit circulation of patients with coronary artery disease (21), but no further improvements are seen with chronic administration (13). The effect of clopidogrel in the microvasculature of these patients, however, is unknown.

Although these two medications have been proven effective in the primary and secondary prevention of atherothrombotic disease, both ASA and clopidogrel attenuate reflex cutaneous VD in aged humans (5,7). This attenuation may further worsen the already blunted VD response

in older adults, making it even a greater possibility to develop heat-related illnesses. Our lab has found that chronic systemic low-dose ASA therapy attenuates reflex cutaneous VD in healthy middle-aged humans (5,7). In these studies, SkBF (measured in red blood cell flux by laser-Doppler flowmetry) was significantly attenuated in subjects who took ASA. Additionally, the time it took subjects to increase their body core temperature by one degree Celsius was also significantly shorter than the time taken by a subject not on an ASA regime. Similarly, more dramatic decreases in SkBF were found with clopidogrel. This attenuated SkBF and shorter time to increase body core temperature one degree Celsius in a water-perfused suit model of heat stress raises the questions if there may be functional thermoregulatory consequences through decreases in dry heat loss mechanisms in subjects taking either aspirin or clopidogrel during more traditional modes of heat stress (i.e. exercise heat stress).

Whole Blood Viscosity

Whole blood viscosity is determined by: plasma volume, total plasma protein concentration, red blood cell number, erythrocyte's internal viscosity, and erythrocyte and platelet aggregation tendencies (20). Both ASA and clopidogrel decrease whole blood viscoelastic properties (2, 14-16). It is unclear, however, how altering whole blood viscoelastic properties affects shear-induced cutaneous VD, which may be an important mechanisms for increasing SkBF. Altering whole blood viscosity changes flow-mediated dilation in the conduit circulation (14,15), however this response has not been studied in the skin.

Shear Stress and Cutaneous Reactive Hyperemia

Shear-induced VD, or the result of the force created by flowing blood upon the endothelium, may contribute to the rise in SkBF during hyperthermia. Reactive hyperemia (RH) is a tool used in the clinical setting to assess both macrovascular and microvascular function of

patients diagnosed with hypercholesterolemia, hypertension, and other cardiovascular risk factors (23). RH is the process of increasing SkBF significantly after a brief period of arterial occlusion. In the cutaneous circulation this can be measured using laser-Doppler flowmetry. The total magnitude of the RH-mediated VD is related to the metabolic deficit created by the arterial occlusion because the occluded blood flow creates a build-up of metabolites (17). The total magnitude of the RH response may also be related to the shear rate of the blood upon the vessel. However, there is controversy concerning if RH in skin induces a shear-mediated VD response.

One argument set forth is that although shear-mediated VD is present in the conduit circulation (10), it is not present in the skin because nitric oxide does not directly contribute to the RH response in the skin (23). In addition, there are methodological limitations in measuring cutaneous microvessel diameter and flow to calculate shear rate in human skin (5). On the other hand, there may be differences in the microcirculation where shear-induced VD is not NO-dependent and instead may be mediated by a host of other pathways including endothelium derived hyperpolarization factor (EDHF). EDHF-dependent pathways have been shown to contribute to cutaneous RH (11). Despite this controversy, significant questions remain as to the impact of altering blood viscoelastiticy on the cutaneous RH response and, more importantly, the effects of platelet inhibition on cutaneous microvascular vasodilatory responsiveness.

CHAPTER 2
INTRODUCTION

Daily low-dose aspirin (ASA, 81 mg) therapy is the gold standard for primary and secondary prevention of atherothrombotic disease. ASA inhibits platelet function through cyclooxygenase-1(COX-1) inhibition. COX is inhibited for the life of the platelet because platelets are not nucleated and thus do not have the genetic machinery to synthesize additional COX (~10 days). Endothelial cells, however, are nucleated and can still produce COX-dependent dilators (18). Platelet inhibition with chronic systemic use of low-dose aspirin significantly attenuates reflex cutaneous vasodilation (VD) in middle-aged skin (5,7). In addition to COX-dependent platelet inhibition, clopidogrel, which inhibits platelets via a different mechanism, also significantly attenuates reflex cutaneous VD in healthy middle-aged (58±3 years) human skin (7). The underlying mechanisms mediating this reduction in skin blood flow with whole body heat stress during systemic platelet inhibition are unknown.

Clopidogrel bisulfate (Plavix®, 75 mg) is the second most widely used prescription drug in the world for the prevention of heart attack and stroke. Clopidogrel inhibits platelet function through ADP-receptor inhibition. It has been demonstrated that acute administration of clopidogrel improves endothelial dysfunction in conduit circulation of patients with coronary artery disease (21). However, no further improvements are seen with chronic administration (13). The effect of clopidogrel in microvasculature endothelial function of these patients is unknown.

One potential explanation for our recent findings that platelet inhibition attenuates reflex cutaneous VD is that platelet inhibitors decrease whole blood viscosity, thereby decreasing the shear stimulus on the cutaneous microcirculation. The determinants of the shear stimulus include

the rate of blood flowing across the endothelium, the flow pattern, and the viscoelastic properties of the blood. Whole blood viscoelastic properties are determined by: plasma volume, total plasma protein concentration, red blood cell number, erythrocyte's internal viscosity (i.e. erythrocyte deformability), and erythrocyte and platelet aggregation tendencies (20). Both ASA and clopidogrel decrease whole blood viscoelastic properties within one week of chronic administration (2, 14-16). It is unclear, however, how altering whole blood viscoelastic properties affects shear-induced cutaneous VD. It may be possible that platelet inhibition and anticoagulatory therapy, through the use of ASA and clopidogrel, reduce the shear stress stimulus, thereby impairing shear-induced cutaneous VD.

Reactive hyperemia (RH) is a method used to assess both macrovascular and microvascular function in a number of clinical populations (23). There is controversy as to whether shear-induced VD occurs in the human cutaneous circulation because shear-rate cannot be measured due to the anatomy of the skin vessels and methodological limitation. Despite this, RH is still the most appropriate method to evaluate shear-mediated cutaneous VD (23). Altering whole blood viscosity through platelet inhibition may affect the cutaneous RH response (shown in Figure 1). Parameters of the RH response include the time for skin blood flow (SkBF) to peak after occlusion release, peak SkBF (as a % CVC_{max}), the decay time from peak SkBF back to baseline (tau), and the area under the curve (AUC, gray in figure 1). The total hyperemic response (THR), or the area under the curve minus the baseline SkBF times the time of the RH response, is another parameter of interest.

The purpose of this study was to examine the role of platelet inhibition upon shear-induced VD through systemic interventions (low-dose aspirin and clopidogrel bisulfate) in middle-aged skin. Our working hypothesis was that both systemic low-dose aspirin and

clopidogrel separately would attenuate shear-induced VD and that whole blood viscoelastic properties would be positively associated with shear-induced VD. More specifically, we hypothesized that platelet inhibition through the use of ASA and clopidogrel would lower whole blood viscoelastic properties and would attenuate the cutaneous RH response. Additionally, peak SkBF after platelet-inhibition would be attenuated, time to peak and tau would be longer in duration, and the AUC and THR would be decreased compared to placebo conditions. Further, because clopidogrel is a more potent platelet inhibitor, there would be greater attenuation in the RH response during the clopidogrel trials compared to the ASA trials. Finally, whole blood viscoelasticity would be positively related with shear-induced cutaneous VD.

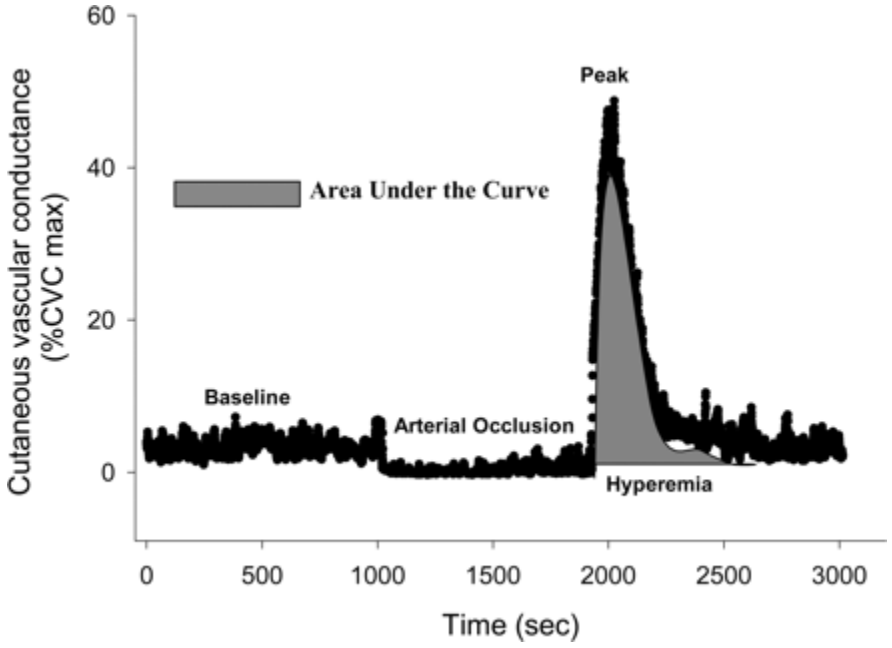

Figure 1: Representation of a typical reactive hyperemia response. Depicted are baseline, 5 min. arterial occlusion, peak SkBF, and SkBF return to baseline. The grey region above represents the area under the curve (AUC) of the RH response. Source: **Wong, B. J., B. W. Wilkins, et al.** (2003). "Nitric oxide synthase inhibition does not alter the reactive hyperemic response in the cutaneous circulation." J Appl Physiol **95**(2): 504-10.

CHAPTER 3
MATERIALS AND METHODS

Subjects

Experimental protocols were approved by the Institutional Review Board at the Pennsylvania State University. All subjects submitted voluntary verbal and written consent prior to participation. Repeated measures with placebo control experiments were performed on 5 healthy subjects (55±3 yr, 2 men, 3 women).

All subjects underwent a complete medical screening which included blood chemistry, lipid profile analysis (Quest Diagnostics Nichol Institute, Chantilly, VA), coagulation study (prothrombin time), resting electrocardiogram, and physical examination. All subjects performed a graded exercise test on a recumbent bicycle to identify VO_{2max} peak to evaluate the existence of underlying cardiovascular disease. No subjects were previously taking low-dose ASA or any other medication including vitamin supplements.

Systemic drug treatments

A randomized double-blinded placebo control study was performed with a two week washout period between trials. Subjects were instructed to consume non-identifiable capsules (81 mg ASA (Bayer)), 75 mg clopidogrel (Plavix® Bristol-Myers Squibb), or placebo (sucrose), compounded by a registered pharmacist (Boalsburg Apothecary), once per day for 7-10 days prior to experimentation. Full platelet aggregation inhibition, through treatment with ASA and clopidogrel, has been shown to occur within 4 days of initiating treatment (15, 19). After at least seven days of drug intervention, RH protocols were performed, followed by a two-week washout period before the next drug intervention. This washout time period has been shown to be effective for full platelet recovery (15). A fourth experimental trial was performed following the

final drug intervention washout in which the subject to ingested 40 mg of furosemide (Lasix®) in order to induce a 3% isoosmotic dehydration, decrease plasma volume, and increase hematocrit (Hct) with the intention of increasing blood viscoelasticity.

Instrumentation and measurements

Protocols were performed in a thermoneutral laboratory with the subject in a semisupine position and the experimental arm at heart level. Bretylium tosylate (BT, Sigma) iontophoresis (Moor Instruments, Iontophoresis Controller MIC2, Devon, UK) was performed at one site on the right ventral forearm arm to block sympathetic adrenergic function and inhibit vasoconstriction. A 200 μA current was administered over a 3-cm^2 area of skin for 20 minutes (9), followed by a 45-60 minute hyperemia period. Previous studies in our laboratory using this same protocol have shown that the BT block remains in effect for at least 7 hours after iontophoresis (9). A non-treated skin site was used as a control.

Cutaneous red blood cell flux was measured with an integrated laser-Doppler flowmeter probe placed in a local heater maintained at 33°C (MoorLAB, Temperature Monitor SH02, Moor Instruments, Devon, UK) on the skin above the BT-treated and control sites as an index of SkBF. All laser-Doppler probes were calibrated using Brownian standard solution. Cutaneous vascular conductance (CVC) was calculated by dividing the red blood cell flux by the mean arterial pressure (MAP).

Mean skin temperature was clamped at 33ºC using a water-perfused suit that covered the entire body, except the hands, feet, head, and experimental arm. Three minutes of vigorous whole-body cooling with 6°C water circulating through the water-perfused suit was implemented to test the integrity of the BT-treated site to prove the efficacy of the total sympathetic adrenergic

blockade (8). If laser-Doppler flux did not decrease to the magnitude of the control site during this cooling period, the BT block was deemed intact.

Blood samples (13mL each sample) were taken at the beginning and conclusion of the experiment to measure blood viscoelastic properties as well as hematocrit, hemoglobin, sodium, potassium, prothrombin time, and international normalized ratio. A third blood sample was taken during the furosemide intervention day in order to determine blood parameters a after a 3% decline in body weight before the start of the experimental protocol.

Experimental protocol

Upon arrival at the laboratory, subjects were required to give a urine sample to confirm euhydration (urine specific gravity < 1.015). If a euhydration state was not present, subjects were required to drink an additional 32-64 oz. of water, until euhydration was confirmed by an additional urine sample. BT iontophoresis was then performed on one site of the right arm for 20 min. A 45 minute hyperemia and instrumentation period followed a blood draw and BT treatment. After hyperemia and instrumentation was complete, a stable baseline flux was determined to compare SkBF values during the whole-body cooling in order to test the integrity of the BT site (test of sympathetic adrenergic inhibition). After a second stable baseline (5 minute reading), the subjects were taken through an RH protocol. Each trial of the protocol consisted of a 5-minute arterial occlusion in which a blood pressure cuff on the right upper arm was inflated to suprasystolic pressure (~220 mmHg). After 5 minutes, the cuff was rapidly deflated and the ensuing RH response was measured continuously. A 20-min recovery period followed the occlusion release to return to stable SkBF values. Each protocol consisted of three RH trials. Upon reaching a final steady state after the third occlusion, local skin temperature was

gradually increased (0.5°C / 5 sec) to 43°C using local heaters to induce maximal cutaneous VD (5). At the completion of the experiment, a second blood draw was taken to measure the same parameters as the first blood draw.

The furosemide-intervention experiment involved an additional blood draw after the initial urine sample, followed by the administration of 40 mg of furosemide. BT iontophoresis then occurred after subjects lost 3% of their body weight via isoosmotic dehydration. After fluid loss, the experiment was conducted as previously described, with two more 13mL blood draws after the BT-treatment and at the end of the experiment.

Blood viscoelasticity was determined using Vilastic-3 Viscoelasticity Analyzer (Vilastic Scientific, Inc., Austin, TX) at a frequency of 2 Hz and a temperature matching the subject's measured internal temperature. Data were analyzed through the department of Bioengineering at the Penn State University.

Data acquisition and analysis

Data were collected using Windaq software and Dataq data-acquisition systems (Austin, TX). The data were collected at 40 Hz, digitized, recorded, and stored on a personal computer for further analysis. CVC data were averaged over 5 min periods at baseline and occlusion and are presented as a percentage of CVC_{max} (%CVC_{max}). Peak SkBF was defined as the highest blood flow value after release of the occlusion. Its value is represented as a % of CVC_{max}. Tau, the time constant of the exponential decay of the response, was calculated by dividing the time it took peak blood flow values during the RH response to return to a steady state flux by three. AUC analysis procedures were taken from the RH protocol performed by Wong, et. al.(22). Finally, the THR was found by subtracting the baseline skin blood flow values prior to occlusion

multiplied by the time it took for the blood flow to return to baseline [i.e. THR = AUC - (baseline SkBF as % CVC_{max} x duration of hyperemic response in s)] (21). Absolute CVC_{max} was calculated as an average of peak stable laser-Doppler flux plateau during 43°C local heating divided by MAP.

The slopes of the viscoelastic plots were determined between 10 and 1000 inverse seconds for each blood sample.

Statistical analyses

Separate two-way repeated measures ANOVA were performed to examine the effect of oral treatment period (placebo, low-dose aspirin, clopidogrel, or furosemide) on the parameters of the RH response (time to peak, peak, AUC, THR, and tau), on absolute CVC max (flux/MAP), and on blood viscoelastic properties. Significance was set at $\alpha=0.05$. Planned post hoc comparisons were performed when appropriate with Bonferroni correction.

CHAPTER 4
RESULTS

Subject characteristics are presented in Table 1. All subjects were healthy, free from cardiovascular disease, and had never been on a low-dose ASA regime or had taken clopidogrel. No subjects were currently taking or had taken systemic anti-inflammatory drugs for at least one month.

Table 1. Subject Characteristics

Sex (M,F)	2, 3
Age, years	55±2
BMI, kg/m^2	24.0±4.1
Total Cholesterol, mg/dl	160.0±34.0
HDL, mg/dl	63.4±14.6
LDL, mg/dl	81.0±22.8
HbA1c, %	5.4±0.2
MAP, mmHg	83.7±4.9
PT, sec	11.7±0.8
INR	0.9±0.01

Table 1: Values are means ±SEM; BMI, body mass index; HDL, high density lipoprotein; LDL, low density lipoprotein; HbA1c, glycated hemoglobin; MAP, mean arterial pressure; PT, protrombin time; INR, international normalized ratio.

Table 2 displays hemoglobin, hematocrit, prothrombin time, and international normalized ratio (INR) averages for each drug intervention trial. Furosemide Hct was significantly higher than placebo ($p<0.05$). All other parameters were not significantly different.

Table 2. Blood Characteristics.

	Placebo	Low-dose ASA	Clopidogrel	Furosemide
Hb, gm/dl	14.42±0.38	14.32±0.34	14.38±0.57	15.50±1.03
Hct, %	41.58±0.65	41.92±0.72	41.50±1.08	44.67±1.82*
PT, sec	12.74±0.42	12.32±0.70	12.76±0.75	12.15±1.08
INR	1.08±0.04	1.02±0.06	1.08±0.06	1.00±0.08

Table 2: Values are means ± SEM; Hb, hemoglobin; Hct, hematocrit; PT, prothrombin time; INR, international normalized ration. Furosemide had significantly larger Hct values compared to placebo (*$p<0.05$).

During the furosemide drug intervention experiment, subjects lost 2.99% ± 0.06% body weight in two to two-and-a-half hours.

There was no difference between drug intervention trials (clopidogrel, ASA, and placebo) in the viscosity and elasticity measurements. However, there was a significant difference in whole blood viscosity with furosemide compared to placebo trial days (Table 3). A representative tracing of viscosity measurements is shown in Figure 2.

Table 3. Average Slopes of the Whole Blood Viscosity Graphs for Each Drug Intervention

Drug Intervention	Slope ($*10^{-4}$)
Placebo	-1.07±0.34
ASA	-1.40±0.52
Clopidogrel	-1.48±0.99
Furosemide	-1.75±0.46*

Table 3: Average slopes (±SD) of the whole blood viscosity graphs for each drug intervention. * $p<0.05$ significant difference compared to placebo

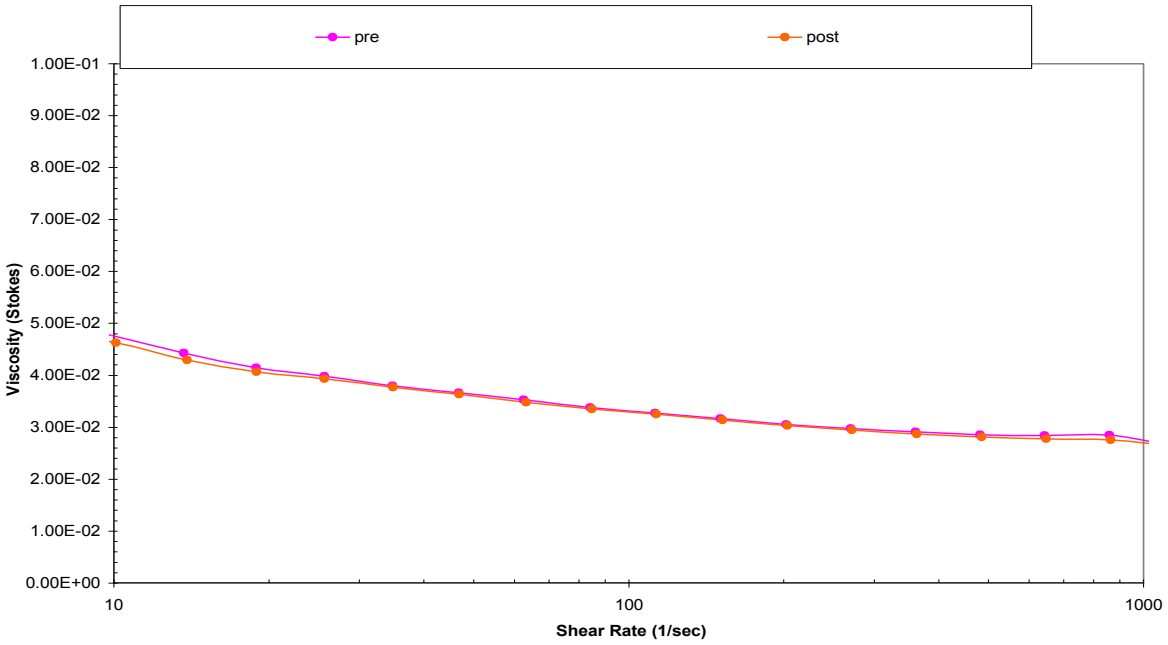

Figure 2: A representative whole blood viscosity curve. There was little variability in whole blood viscosity and elasticity measurements between drug interventions and between subjects except during the furosemide trials.

Average THR is presented in Figure 3 for all four drug-intervention trials.THR in the clopidogrel trial neared significance compared to placebo (p=0.0676). There was also a significant difference between control and BT-treated sites during the clopidogrel intervention (2280 ± 358 %CVC_{max} vs. 1367 ± 188 %CVC_{max}; $p<0.05$). Clopidogrel had the greatest THR value among the control sites, while ASA had the greatest THR value among the BT-treated sites (1855 ± 238 %CVC_{max}).

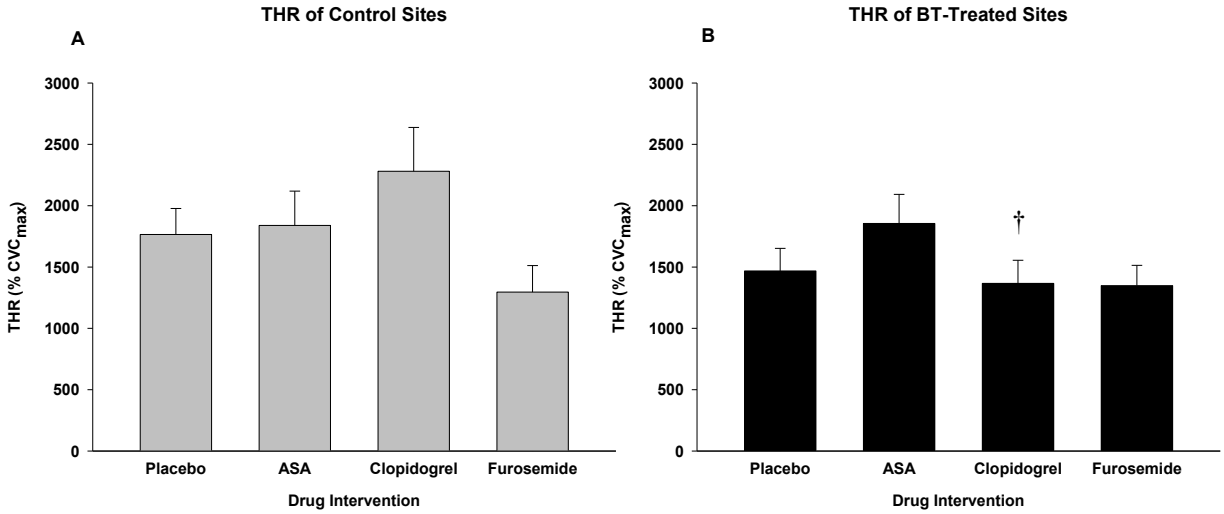

Figure 3: Total Hyperemic Response (THR). (A) Clopidogrel increased and furosemide decreased the THR compared to the placebo trial. **(B)** In bretylium treated sites, systemic aspirin increased the THR compared to the placebo trial. BT significantly decreased the THR in clopidogrel trial compared to the control sites. † $p<0.05$ significant difference from control site

Average AUC is presented in Figure 4 for all four drug-intervention trials. Clopidogrel AUC values were significantly greater than ASA, placebo, and furosemide (all $p<0.05$). Furosemide AUC was lower than placebo control site AUC (2945 ± 494%CVC_{max} vs. 3559 ± 259%CVC_{max}; $p<0.05$). AUC in the control sites for the clopidogrel and placebo drug interventions were higher than their BT-treated counterparts ($p<0.05$). The control site during the clopidogrel drug intervention had the greatest AUC compared to all other systemic drug interventions. In the BT site the ASA intervention had the greatest AUC which was significantly

greater than the BT sites during the clopidogrel, placebo, and furosemide drug-interventions ($P<0.05$).

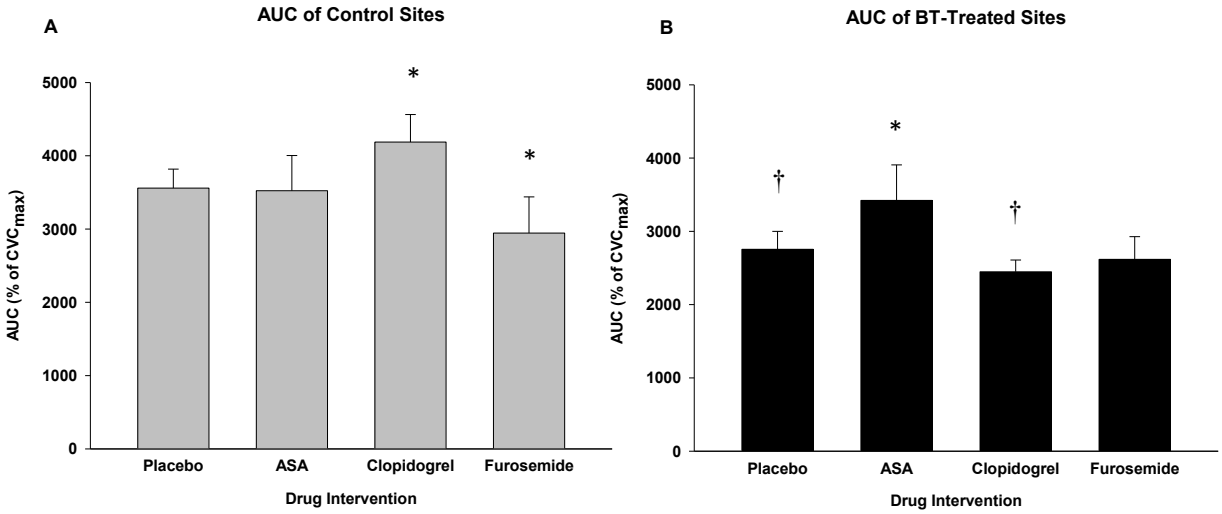

Figure 4: Area Under the Curve (AUC). **(A)** Clopidogrel increased and furosemide decreased the AUC compared to the placebo trial. **(B)** Aspirin significantly increased the AUC response among the BT-treated sites compared to placebo. BT decreased the AUC in both placebo and clopidogrel compared to control sites. * $p<0.05$ significant difference from placebo trial; † $p<0.05$ significant difference from control site

There were no significant differences in decay time (tau), time to peak SkBF, and peak SkBF between intervention trials and between control and BT-treated sites (Figures 5-7).

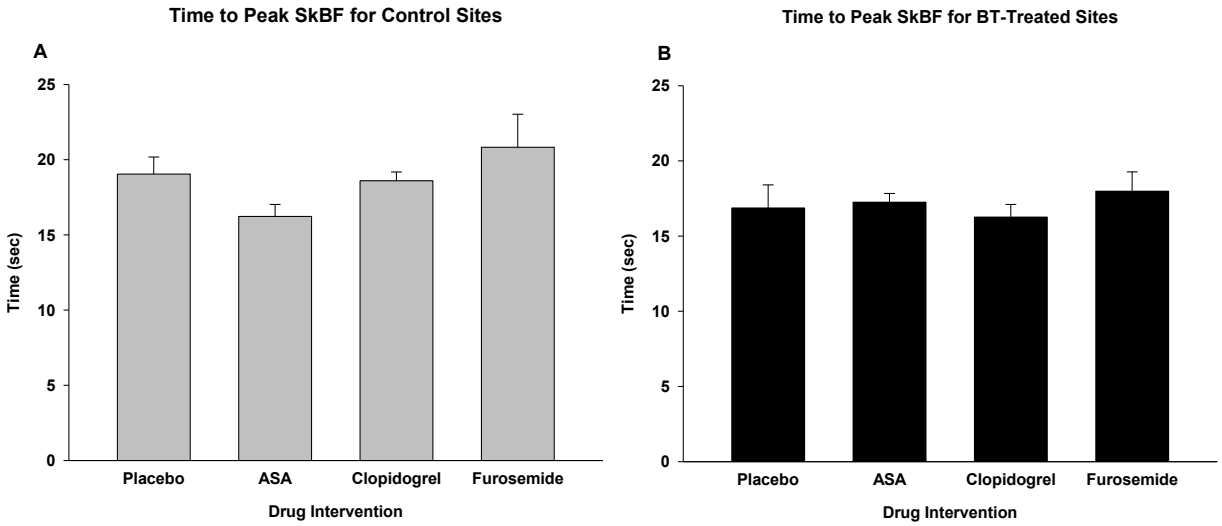

Figure 5: Time to reach peak skin blood flow. There were no differences between trials and treatment sites.

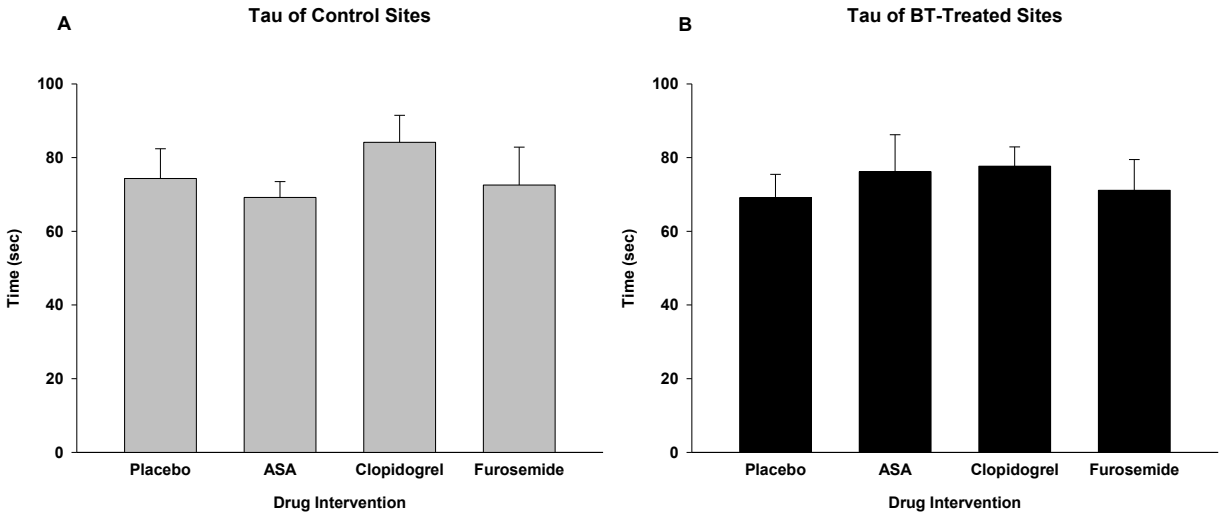

Figure 6: Tau. There were no significant differences in decay values (seconds) between drug interventions or treatment sites. Tau was calculated by dividing the time it took peak skin blood flow to return to steady-state blood flow responses by three.

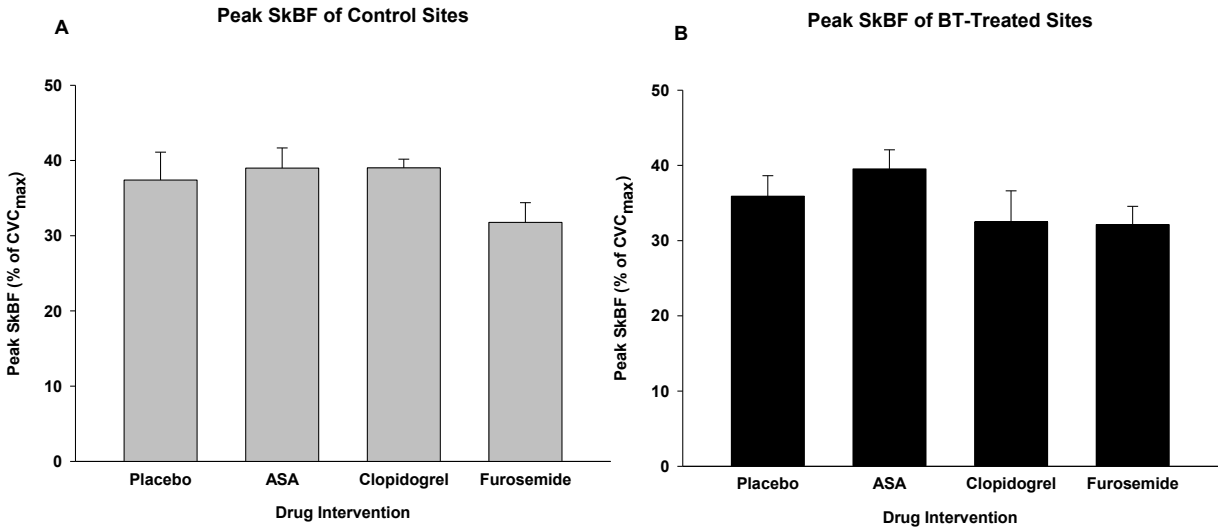

Figure 7: Peak skin blood flow. There were no significant differences in peak skin blood flow values between drug interventions and treatment sites.

Table 4 displays maximum absolute CVCs for each measurement site and for each drug treatment. There were no differences in CVC_{max} in the control site with the systemic drug interventions, however there was a difference in the ASA trial for the BT treated site ($p<0.05$).

Table 4. Absolute CVC_{max}

	Control	Bretylium-Treated
Placebo	1.86 ± 0.23	2.02 ± 0.24
ASA	1.40±0.61	2.36±1.07*
Clopidogrel	1.47±0.19	2.43±0.15
Furosemide	1.74±0.19	1.70±0.39

Table 4: Absolute CVC_{max} data. There was a significant difference in values between ASA control and BT-treated sites (* $p<0.05$).

CHAPTER 6
DISCUSSION

The primary findings of this study were that systemic platelet inhibition with a $P2Y_{12}$ receptor antagonist significantly augmented cutaneous reactive hyperemia (RH) in middle-aged adults. This is demonstrated by the increase in the area under the curve (AUC) and the total hyperemic response (THR) during the clopidogrel trials. Furthermore, there was a decrease in the AUC during systemic isoosmotic dehydration induced by furosemide. There were no differences in blood visoelastic properties with the platelet inhibitors compared to the placebo conditions, but there was a significant increase in these properties through isoosmotic dehydration.

While these results contradict our original hypothesis, they suggest that platelet inhibition does not alter blood viscoelasticity and does not attenuate cutaenous reactive hyperemia. In relation to our previous data with whole body heating, these results indicate that platelet inhibition is not affecting shear-induced vasodilation and instead platelets may be releasing some additional VD factor that is causing the cutaneous VD during whole body heat stress. Finally our results are in agreement with the augmentation in flow-mediated VD in the conduit arteries associated with systemic clopidogrel treatment (3), further supporting that the skin is a useful surrogate circulation for assessing microvascular function.

There is the possibility that clopidogrel could be affecting not only platelets, but the vascular smooth muscle (VSM) surrounding blood vessels. Wihlborg, et. al. (22) found that $P2Y_{12}$ receptors had the second highest mRNA expression in internal mammary artery segments. They also demonstrated that these receptors mediated contractile effects within the VSM. The augmented RH response during our experiments could be a result of clopidogrel inhibiting ADP

receptors, not only on platelets, but on the VSM. This inhibition could prevent vascular vasospasms and decrease vasoconstrictor tone upon the endothelium.

Platelets may also be releasing certain VD factors that could be affecting the VSM such as ATP, ADP, and 5HT. While platelet inhibition of COX-1 or ADP receptors, using ASA and clopidogrel respectively, may inhibit some factors that contribute to the RH VD response, other factors may still be released by the platelet that contribute to this response. Contrary to results from our previous whole body heating studies in which platelet inhibition reduced SkBF, ASA and clopidogrel augmented the RH response. These opposing findings suggest that shear-induced VD does not contribute to reflex VD in skin. This augmented response may further indicate the beneficial effects of platelet-inhibiting drugs in improving vascular endothelium function.

Our previous studies have shown that the time required to increase core temperature by 1.0ºC, using a water-perfuse suit model, took significantly and consistently less time in patients taking platelet-inhibiting drugs (ASA and clopidogrel) (5). A future focus using findings from this and our current study would be to examine the functional thermoregulatory consequences of performing exercise in the heat in middle-aged humans taking platelet-inhibitors due to decreases in dry heat loss mechanisms. This could provide a warning for individuals on platelet-inhibitors if dry heat loss mechanisms are indeed compromised. Other future experiments could determine the thermoregulatory effects of platelet inhibitors in clinical populations in both resting and exercise situations. These studies may give better insight into how much more at risk clinical populations on platelet-inhibitors are for thermoregulatory intolerance. However, it could also be possible that taking these medications could improve cardiovascular health in such a way that it normalizes SkBF responses by enhancing vascular function in diseased populations with damaged vascular endothelium.

Limitations

The sample size for the current experiment was small. Our initial power analysis suggest that a sample size of eight was needed to attain 80% power, however, we analyzed the data with only five subjects completing all of the trials. As described in the results, many of the THR values approached significance at a subject count of n=5.

Throughout the course of the experiments, many of the BT blocks failed in achieving adequate blockade of the sympathetic vasoconstrictor system, as cutaneous vasoconstriction persisted during a vigorous cold stress. However, removing these data points from the group did not alter the mean BT results. We did not anticipate finding differences between the BT and the control sites for several reasons. First, the subjects were supine and therefore there should have been no effect from unloading the cardiopulmonary baroreceptors even during the dehydration protocol. Secondly, local skin temperature was clamped at 33°C. For these reasons we did not anticipate any involvement/activation of the adrenergic vasoconstrictor system. BT has many documented non-specific effects (8) which may mask and/or convolute the interpretation of our results due to any differences from our systemic treatments.

Finally, this study was performed on healthy middle-aged subjects. Therefore, these results may not be applicable to clinical populations for which clopidogrel is routinely prescribed. However, they are still important because they help with determining microvascular mechanisms in healthy humans.

Conclusion

In summary, platelet inhibition, achieved through clopidogrel, but not ASA, significantly augmented shear-mediated VD through an increased AUC during RH in healthy middle-aged

skin. Platelet inhibition did not significantly alter blood viscosity properties, but 3% isoosmotic dehydration increased whole blood viscosity and attenuated the cutaneous RH response. These results suggest that the significant attenuation in reflex cutaneous vasodilation observed with systemic platelet inhibitors are not due to alterations whole blood viscoelastic influencing shear-induced vasodilation but may instead be due to VD factors such as ATP and 5-HT released from platelets. Further research should examine the functional thermoregulatory and cardiovascular consequences of platelet inhibition on limiting dry heat loss mechanisms in middle- and advanced-aged humans.

APPENDIX A

INFORMED CONSENT

INFORMED CONSENT FORM FOR CLINICAL RESEARCH STUDY
The Pennsylvania State University

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DO NOT REMOVE OR MODIFY

IRB#33403 Doc. #1001

The Pennsylvania State University

Title of Project: **Low-dose Aspirin and Human Skin Blood Flow**

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This is to certify that I, _____ have been given the following information with respect to my participation as a volunteer in a program of investigation.

1. Purpose of the study: When you are exposed to heat, nerves in your skin make natural chemicals that cause the skin's blood vessels to get bigger. This increases blood flow through those vessels, helping to cool your body. If something prevents the blood flow increase, you could become prone to heat illness. The researchers observed that taking daily low-dose aspirin or Plavix ® (a similar drug) reduced the increase in skin blood flow (SkBF) in people exposed to whole-body heating. Regular, small doses of aspirin or Plavix ® have "anti-clotting" effects that help to prevent strokes, blood clots, and heart attacks. The positive effects of aspirin and Plavix ® treatment are clear; their effects on how the body responds to heat stress are not. By exploring the actions of aspirin and Plavix ®, the researchers can learn about the effects of these drugs on SkBF. Also, they can explore whether platelets (substances in blood that cause blood to clot) have a role in widening blood vessels to cool your body. The

researchers look at the effect of aspirin and Plavix ® on the blood flow in your skin under several conditions.

2. <u>Some of the procedures used in this study:</u> As many as 30 people may screen for this study.

- This study involves blood draws for the screening and experiments.
- There are two types of experiments: resting and exercise. You repeat each type 3-4 times. You do not have to be in both types. The resting experiment that has two parts: Local Heating (LH) and Reactive Hyperemia (RH). The researchers call the resting experiment "LH/RH."
- You will take aspirin, Plavix ®, or a placebo (fake drug) as pre-treatments for experiments. The order of the drugs is random and unknown to you and the researcher.
- You take Furosemide (Lasix ®) on one day to increase urine output for one LH/RH experiment.
- LH/RH uses a technique called "Microdialysis" (MD). MD involves placing very thin plastic tubing between the layers of your skin. The largest part of the tubing is about 6x the diameter of a human hair. The tubing acts like small blood vessels. The tubing allows substances to pass between the fluid in the tubing and the fluids in your skin. The researchers add test-substances to the fluid that are like some of the natural chemicals found in your body. The substances only reach a nickel-sized area of skin at each tube. The test substances are:
  1. L-NAME (*N^G-nitro-L-arginine methyl ester*) –can prevent your blood vessels from dilating.
  2. SNP (*sodium nitroprusside*) - causes your blood vessels to get as large as they can.
- In another part of the LH/RH experiment, the researchers restrict blood flow to one of your arms for a short time.
- The Exercise experiment involves biking in a warm room. The researchers measure your body's inner temperature with a probe that well-trained personnel place in your esophagus or "food tube."

3. <u>Procedures:</u> *You may request personnel of the same gender to perform procedures.*

Note: Do not exercise hard for 24 hours before a scheduled blood draw (i.e. Screening Day 1 and Experiments).

<u>Note on tests, procedures, and experiments:</u> A test, procedure (e.g. blood draws), or experiment may have to be repeated for various reasons. Examples of these reasons include problems with a blood sample, uncertain test-results, or power outage during an experiment. In such cases, the researchers will ask you to repeat the test, procedure, or experiment. You may decline.

_____ initial <u>Screening Day 1:</u> Do not eat or drink 10 hours before your exam. Report to Noll Lab for your appointment. Well-trained personnel draw 18 ml (4 tsp) of blood from a vein in your arm. The researchers analyze the blood for clotting ability, blood cells, fats in the blood, and blood chemistry. If you take thyroid hormone, well-trained personnel draw an extra 4 ml (1 tsp) to check the level of thyroid hormone. You have a physical exam including blood pressure, height, weight, and 12-lead ECG. Women of childbearing-age submit urine samples for pregnancy tests. The researchers may measure thicknesses of skin-folds on your body to determine your percent body fat.

_____ initial <u>Screening Day 2</u>: Report to Noll Lab for a medical history and graded exercise test (GXT). Bring clothes in which you can exercise. For the GXT, you pedal a bike to measure your fitness level. You wear a nose clip and breathe into a tube to measure exhaled oxygen and carbon dioxide. Well-trained personnel measure blood pressure and monitor ECG. The bike becomes a little harder to

pedal every 2 minutes. You rate how hard you work (RPE) by using a numbered scale matched to short phrases. The test is most accurate if you pedal as long as you can. However, you may stop at any time. The test is 10-20 minutes long. After a 20-30 minute break, you pedal 5-10 minutes while the researchers collect your expired air. They record the bike's workload-setting that matches 60%-70% of your greatest effort in the GXT.

Visits 3 – 9: Experiments

Schedule Overview (Treatments are low-dose aspirin, Plavix ®, or placebo.):

a. Pre-treatment 1 – 7-10 days (in your own home)
b. LH/RH and Exercise Experiments (These occur on two separate days that are at least one day apart and sometime during days 7 - 10 of pretreatment 1)
c. 2-week washout period (at least)
d. Pre-treatment 2 – 7-10 days (in your own home)
e. LH/RH and Exercise Experiments (These occur on two separate days that are at least one day apart and sometime during days 7 - 10 of pretreatment 2)
f. 2-week washout period (at least)
g. Pre-treatment 3 – 7-10 days (in your own home)
h. LH/RH and Exercise Experiments (These occur on two separate days that are at least one day apart and sometime during days 7 - 10 of pretreatment 3)
i. 2-week washout period (at least)
j. Furosemide (Lasix ®) Treatment and LH/RH Experiment (may be at beginning of schedule instead)

    You arrive at Noll Lab around 7 AM. You take 40mg – 80 mg of Furosemide in pill-form. The drug increases urine output for several hours. You lose about 3% of your body's weight in water. About 2 hours later, the researchers collect urine to track weight loss. You ingest sports drink to maintain weight loss at 3%. The researchers measure blood pressure every 15 minutes. Five hours after furosemide, well-trained staff draws blood (about 13 ml, 3 tsp). Then LH/RH starts. You do not drink during LH/RH. After LH/RH, you may have more sports drink.

_____ initial Preparation For All Experiments: You complete the scheduled pre-treatment and/or washout periods.

One day before experiment: Please drink at least 6 glasses of fluid (water, juice, milk, etc.). Do not drink fluids that contain caffeine (i.e. coffee, tea, Coca Cola, etc.) for 12 hours before the experiment.

Day of experiment: Eat your typical breakfast. Bring shorts and short-sleeved shirt. For the exercise experiment, bring comfortable shoes, too.

When you arrive: The researchers measure heart rate, blood pressure, and weight. You wash your forearms and pat them dry. You drink 10 ml of sports drink for every kg of body-weight (or 2 tsp / 2 lbs). For example, someone weighing 72 kg (160 lbs) drinks 720 ml (3 cups). You give a urine sample. Well-trained personnel draw a baseline blood sample (16 ml or 3 tsp).

_____ initial A. Local Heating / Reactive Hyperemia Experiment (LH/RH)

This experiment has two parts: "Local Heating" (LH) and "Reactive Hyperemia" (RH). The researchers conduct the two parts of this experiment at the same time, but on different arms. LH usually uses the left arm, and RH uses the right. The researchers tell you the times during the experiment when you may take a break.

_____ initial a. Local Heating: The researchers place a tight band around your upper arm so they can see your veins. The researchers mark four MD sites on your forearm devoid of veins. The researchers connect a battery-powered controller to a small chamber (9.5 mm, 0.4 inch diameter) that contains bretylium tosylate and to an electrode. At two sites, the researchers attach a chamber and electrode nearby. The researchers apply a weak current (200 μamp) for 20 minutes. This moves bretylium into skin without needles (iontophoresis). Bretylium does not allow your skin's blood vessels to constrict (get smaller) when your skin is cold. The researchers remove the chambers and electrodes.

You dress in shorts. Women wear a sports bra, also. You don a suit lined with tubing. You lie on a bed. Water (34-36°C, 93–97°F) flows through the suit's tubing. The researchers tape laser probes and their holders onto your skin at the bretylium-sites. About one hour after iontophoresis, the researchers pump ice water through the suit's tubing for 3 minutes. Then they switch to warm water. If the bretylium works, the experiment proceeds. If the block does not work, you will not do the local heating experiment this day. You may come back another day to try again.

Inserting Microdialysis (MD) Tubing: For each MD site, the researchers make pairs of pen-marks on your arm 2.5 cm (1 inch) apart. MD tubing enters and exits your skin at the marks. The researchers clean your arm with an iodine-based fluid and then alcohol. An ice-bag on your arm for 5 minutes numbs your skin. They insert a thin needle into your skin near an entry mark. The needle's tip travels between the layers of skin for 2.5 cm (1 inch) and leaves your skin near the matching exit mark. The researchers thread the tubing through the needle. They withdraw the needle leaving the tubing in your skin. They insert 4 MD probes. Any redness of your skin subsides in 60 – 120 minutes. They tape a laser probe and holder over each MD site.

Experimental procedure: When the redness from inserting the MD tubing is gone, the experiment begins. The researchers start Lactated Ringer's flowing through the MD tubing. The holders start at about 33°C (91.4°F). After the SkBF is stable for at least 10 minutes, the researchers add L-NAME to the fluid flowing through two sets of MD tubing for the rest of the experiment:

MD Tubing 1: Lactated Ringer's

MD Tubing 2: Lactated Ringer's + L-NAME

MD Tubing 3: Lactated Ringer's (Bretylium pre-treated site)

MD Tubing 4: Lactated Ringer's + L-NAME (Bretylium pre-treated site)

The researchers obtain a second baseline for 20 minutes. They increase the temperatures at all sites to about 42.0°C (107.6°F). After SkBF is stable (about 40 minutes), Lactated Ringer's + L-NAME flows through all MD tubing. The SkBF becomes stable again (about 40 minutes). The researchers increase the temperature to about 43°C (109.4°F) for about 30 minutes. At the same time, they switch the fluid to Lactated Ringer's + SNP. This increases SkBF at all sites to the highest level possible. The experiment ends. The researchers clean where the tubing enters and exits your skin with alcohol. They pull the tubing from your skin and place a sterile bandage over the sites. They may place a bag of ice on your arm for 10 minutes to reduce any bruising.

_____ initial b. Reactive Hyperemia Experiment: The researchers tape two laser probes and their holders on your other forearm. They place a cuff on your upper arm. They inflate the cuff for 5 minutes. During this time, blood does not enter or exit your arm. The researchers deflate the cuff so that blood flow returns to your arm. They wait at least 20 minutes and then repeat 2 more times. Then they increase the temperature to about 43°C (109.4°F) for about 30 minutes. This increases the SkBF at the sites to the highest level possible. Then the experiment ends.

LH/RH Measurements: Throughout both experiments, the researchers measure SkBF with the laser probes. They also measure skin temperatures with the probe-holders. They measure heart rate and blood pressure about every 5-7 minutes with cuffs on your upper arm and/or finger.

LH/RH Experiment Ends: The researchers re-test the Bretylium site (see above). Well-trained personnel draw a blood sample (about 13 ml, 3 tsp). The researchers measure your blood pressure and heart rate before you leave.

_____ initial B. Exercise Experiment

Well-trained personnel place a tube into a vein in your arm so that they can draw blood. ***If the tube should stop working, the well-trained personnel remove it. You may give permission to insert a new tube, stop the experiment and repeat it another day, or decline to be in the experiment. If the tube stops working near the end of the experiment, you may choose to have the last couple of blood draws performed with a regular needle-stick for each draw. The well-trained personnel use normal saline to clear blood from the tube after each blood draw. You may choose to have numbing cream (LMX) applied to the site at which the well-trained personnel will insert the tube. After the personnel apply cream, they wait about 30 minutes prior to inserting the tube in your vein. The cream cannot be used as effectively for replacement tubes inserted into veins once the experiment begins.***

Body temperature: The researchers seal a temperature probe in a tube that looks like a strand of spaghetti. Well-trained personnel coat the tube with Lidocaine gel to make the tube slippery and numb tissue touching the tube. They can use water instead of gel. They insert the tube through your nose and into your esophagus or "food tube." You drink water or sports drink through a straw while the well-trained personnel guide the probe into place. The inserted length of tube is equal to ¼ of your height. The probe in the end of the tube rests in your esophagus at heart level. When the tube is in place, they anchor the free end into a headband. Also, they use tape on your face to keep the tube still. You do not drink or eat, but you can talk with the probe in place. The temperature-reading is more stable if you limit swallowing.

You enter a warm room (about 30°C/86°F and 40% relative humidity). You sit on a stationary, semi-recumbent bike. The researchers tape six wires to your skin (back, calf, thigh, abdomen, chest, and arm) to measure temperature. They tape 2 laser probes and their holders on the skin of your right forearm to measure SkBF. They strap a Polar heart rate monitor around your chest.

Plethysmography: Plethysmography measures the blood flowing into your forearm. The researchers place a cuff around your left wrist and upper arm. They place a strain gauge that looks like a rubber band around your left forearm between the cuffs. During the experiment, the researchers perform a series of 4 readings every 10 minutes. Each series lasts about 1 minute. For each series, the wrist-cuff inflates to stop blood flow to your hand. The upper arm-cuff inflates allowing blood flow into your arm, but blocking flow out. This causes a slight increase in the size of your forearm detected by the gauge. For each series, the wrist cuff remains inflated while the upper arm cuff switches 4 times between inflation (10 seconds) and deflation (5 seconds). Then the researchers deflate both cuffs.

Experimental procedure: You rest for 15 minutes during baseline readings. When the readings are stable, you begin to pedal the bike, and well-trained personnel draw a second blood sample (about 9 ml, 1 tsp). You pedal for one to two hours. The researchers measure your heart rate, blood pressure (every 5-7 minutes), SkBF, forearm blood flow, skin temperature, and your body's temperature throughout exercise. You use charts to rate how hard you are working (RPE) and how warm you feel. Numbers on the charts refer to descriptions of effort or warmth. Qualified personnel draw blood (about 9 ml, 1 tsp) when your body's temperature rises about ½ °C (about 1°F) above your baseline temperature. They draw another blood sample (about 9 ml, 1 tsp) when your temperature rises 1°C (about 2°F) above baseline. When your temperature reaches 39°C (102 °F) or you have pedaled for 2 hours (whichever comes first), they draw a blood sample (about 9 ml, 1 tsp). Then exercise ends. However, you may stop pedaling sooner if you wish. The researchers cool the room to 25°C (77°F) and reduce the humidity to 30%. You rest on the bike for 45 minutes. As you rest, the researchers heat the laser probe-holders to 43°C (109.4°F) for about 30 minutes. This increases the SkBF at all sites to the highest level possible. Well-trained personnel draw a last blood sample (about 8.5 ml, 0.7 Tbsp), remove the tube, and apply a bandage. Well-trained personnel remove the probe from your throat. The researchers measure your weight and take a urine sample. Then the experiment ends.

4. Discomforts and risks: Note: Before the researchers perform any procedure, they verbally inform you of the training and/or experience of the researcher performing the procedure. You may stop a procedure at any time. During screenings and experiments, medical assistance is readily available. If a problem arises that requires medical attention during an experiment, a researcher stays with you. Another researcher summons assistance from the emergency medical system by calling 911 and meets the responders at the door of Noll Lab. All of the researchers have current basic life support training. In addition, some of the researchers have current first aid training. If a problem arises while you are at the Clinical Research Center, the problem is handled according to their standard procedures.

Microdialysis (MD): The risks are less than that for a blood draw because microdialysis uses only a small, local area of skin. In contrast, a blood draw involves large blood vessels and blood also. MD can cause some pain and bruising like that from a blood draw. However, the researchers use ice to numb your arm and small needles to reduce pain when they insert the tubing. You are not likely to feel pain from MD tubing once it is in place. You may feel a little pain when the researchers remove the tubing. You may be nervous about needles. If so, your blood pressure and heart rate may increase for a little while. You may also feel lightheaded, sick to your stomach, or may faint. If the tubing breaks during removal, they remove the tubing by pulling on the other end. This presents no extra risk to you. The tubing could break leaving a small piece under your skin. This has not occurred in any of our studies. In this case, they may have to cut the thin layer of skin over the tubing to remove the tubing. Mild pressure with sterile gauze stops any slight bleeding. Infection is possible, but has never occurred in our or any other lab of which the researchers know. Sterile techniques and supplies keep the risk minimal. The researchers apply a sterile bandage afterward. They tell you how to care for the sites.

Fluid flowing through the tubing: The substances flowing through the tubing only go to a 2.5 cm^2 (0.4 $inch^2$) area of skin at each tubing-site. The amount entering the skin is very small. There is a remote chance of your having a bad reaction to the substances. This reaction could produce redness, itching, rash, and/or swelling. A worse reaction could also cause fever, breathing problems, changes in pulse, convulsions, blood pressure change and/or fainting.

Lactated Ringer's Solution: This fluid is similar to the natural fluids in your skin. This fluid contains salt, potassium, lactate, and chloride. The acid content is like that of your body's fluids. A bad reaction to this fluid is highly unlikely.

L-NAME and SNP: Only minute amounts of these substances enter the nickel-sized area of skin around the MD tubing. The researchers and/or other researchers have used these substances in human skin. There have been no reports of bad reactions.

Low-dose Aspirin (81 mg daily): When taking aspirin you may notice that it takes longer to stop bleeding. You may also bleed or bruise more easily. Minor side effects are upset stomach; heartburn; tiredness; or headache. Other minor side effects are diarrhea or constipation, mild nausea, vomiting, and stomach gas. Serious side effects are black, bloody, or tarry stools; coughing up blood or vomit that looks like coffee grounds; severe nausea, vomiting, or stomach pain; and tinnitus (ringing or other noise in your ears). You could have confusion, general ill feeling or flu-like symptoms, pain on swallowing, redness, blistering, peeling or loosening of the skin, including inside the mouth or nose, trouble passing urine or change in the amount of urine, feeling unusually weak or tired, or yellowing of the eyes or skin. You could have an allergic reaction. This could include a rash, itching, and difficulty breathing, tightness in the chest, and/or swelling of the mouth, face, lips, or tongue. You will receive instructions and more information for aspirin on a separate paper.

Clopidogril (Plavix ®, 25 mg daily): When taking Plavix ® you may notice that it takes longer to stop bleeding. You may also bleed or bruise more easily. Serious side effects are severe allergic reactions (rash; itching; difficulty breathing; tightness in the chest; swelling of the mouth, face, lips, or tongue); bleeding in the eye; change in vision; change in the amount of urine; chest pain; dark or bloody urine; black, tarry stools, unusual or severe bleeding (e.g., increased menstrual bleeding, unexplained vaginal bleeding, unusual bleeding from the gums when brushing); loss of appetite; pale skin; seizures; severe, persistent headache; sore throat or fever; speech problems; unusual bruising; weakness; unexplained weight loss; yellowing of skin or eyes. A rare but serious condition called Thrombotic thrombocytopenic purpura (TTP) may develop while taking Plavix ®. This condition requires urgent treatment and could be fatal. TTP includes kidney problems, broken red blood cells, anemia, problems with the nervous system, fever, and reduced blood platelets. Symptoms of TTP include fever, weakness, fatigue, pallor, shortness of breath on exertion , heart rate over 100 beats per minute, purplish spots in the skin produced by small bleeding vessels near the surface of the skin (purpura), bleeding into the skin or mucus membranes, headache, confusion, speech changes, changes in consciousness, yellowish color to the skin. The FDA warns that some people being treated for cardiovascular disease with Plavix cannot break down the Plavix. This is because they have a low level of a certain enzyme in the liver. The drug does not work very well in these people. Also, Plavix increases their risk of heart attack and stroke when they suddenly stop taking the drug. This lack of ability to break down Plavix occurs in 2% of whites, 4% of blacks, and 14% of Chinese (3% of the population as a whole). A costly genetic test can tell you if you are one of those people at risk; however, the researchers will not be performing this test. These side effects also occurs in people taking certain drugs that reduce stomach acid such as Prilosec, Zegerid (omeprazole), Prevacid (lansoprazole), Protonix (pantoprazole), Aciphex (rabeprazole), and Nexium. These drugs reduce the same enzyme in the liver. You will not be in the study if you are taking these drugs. You will not start taking these drugs while you are in the study. You will receive instructions and more information for Plavix ® on a separate paper.

Furosemide (Lasix ®, 40 mg): Furosemide increases urine output for several hours. Doctors use Furosemide to treat fluid-buildup in people with congestive heart failure, hypertension, liver disease, or some kidney disorders. The researchers use Furosemide to decrease your body's water on one day of LH/RH experiments. This alters your blood's resistance to flow. You are likely to feel thirsty after taking Furosemide. The researchers give you water or sports drink when the experiment ends. You take 40 – 80 mg in pill-form that one day. Most side effects result from long-term use of Furosemide. Mild side effects of Furosemide include diarrhea, constipation, or stomach pain; headache; numbness, burning, pain, or tingly feeling; dizziness; or blurred vision. More serious side effects are dry mouth, thirst, nausea, vomiting; feeling weak, drowsy, restless, or light-headed; fast or uneven heartbeat; muscle pain or weakness; urinating less than usual or not at all; easy bruising or bleeding, unusual weakness; a red, blistering, peeling skin rash; hearing loss; or nausea, stomach pain, low fever, loss of appetite, dark urine, clay-colored stools, jaundice (yellowing of the skin or eyes). An allergic reaction can include hives; difficulty breathing; and/or swelling of the face, lips, tongue, or throat.

Blood Draw: Blood draws often cause mild pain, bruising, swelling, or bleeding. There is also a slight chance of infection or a small clot. You may be nervous about needles. If so, your blood pressure and heart rate may increase for a little while. You may also feel lightheaded, sick to your stomach, or may faint. To keep the chance of infection minimal, the staff uses the same supplies and techniques used in hospitals. Well-trained personnel use a needle to withdraw blood for the screening and the LH/RH experiment. They take out the needle as soon as they obtain the blood sample. Well-trained personnel insert a catheter (a small plastic tube) into a vein for blood samples in the Exercise experiment. They remove the catheter when they withdraw the last blood sample of the Exercise experiment.

ECG: This machine measures the electrical activity of your heart. You have 3-12 wires from the machine taped to spots on your torso. There have been no adverse effects. The tape may irritate your skin.

Medical Screening: You may feel shy about giving health information. The researchers collect information in a private and professional manner. You may feel shy about being measured. You may request someone of the same sex to conduct the screening.

Phone screening form: The form helps us to decide whether you are a good candidate for the study. You may feel shy about answering questions. The researchers collect information in a private and professional manner. Some people fill out forms at our secured website. The website encrypts submitted forms. The researchers keep completed forms confidential and secure.

Laser Doppler Flowmetry: You can hurt your eye if you stare into a weak laser for a long time. The researchers do not turn on the laser until the probes are taped to a surface. The tape may irritate your skin.

Local Heating: The researchers measure the temperature of your skin under the holders. During heating, the skin feels very warm but does not hurt. Heating reddens the skin under the holders like when you take a hot bath. The redness does not last more than several hours. Some people may be more sensitive to heating than others. If your arm feels too warm, tell the researchers, and they reduce or stop heating.

Skin temperatures: The researchers tape wires to 6 sites on your skin. The tape may irritate your skin.

Skin Fold Measurements: The researchers measure your percent body fat using a tool that looks like tongs. The tongs gently measure the thickness of skin folds at several places on your body. There are

no risks to this measure, but you may feel shy. The researchers make the measures privately and professionally.

Blood Pressure (manual and/or Cardiocap 5): The researchers measure blood pressure using the method common in a doctor's office or with a machine. A cuff inflates on your upper arm. As the cuff slowly deflates, the researchers listen with a stethoscope at the bend in your elbow or the machine takes a reading. During the short time the researchers inflate the cuff, your arm may feel numb or tingly. The cuff could cause mild bruising.

Blood Pressure (Finapres): The small cuff on your finger pulses with your blood pressure. In time, your finger may feel numb or tingly. The researchers can move the cuff to another finger or stop the reading to rest your finger.

Metabolic Measurements: On Screening Day 2, well-trained personnel collect your expired air to measure O_2, CO_2, and volume. There are no risks to the collection of expired air.

Ratings of Perceived Exertion (RPE) and Thermal Sensation Scales: The only correct answers are those that truly describe what you are feeling.

Graded Exercise Test (GXT): You will likely have tiredness, sweating, and breathlessness. You will also have increased heart rate and muscle fatigue. You may also have lightheadedness, fainting, nausea, or muscle cramp, but these occur less frequently. More severe reactions include irregular heartbeat, heart attack (< 0.05%), and death (< 0.02%). Severe reactions are rare. The researchers watch you closely.

Forearm Blood Flow: Your arm and/or wrist may feel numb or tingly when the researchers inflate the cuffs. The cuffs may cause mild bruising. The tape may irritate. The mild pressure of the strain gauge may leave a mark on your skin that remains for a short while after the researchers remove the gauge. The technique is unlikely to produce lasting ill effects.

Exercise in the heat: You will feel very warm and will sweat. You will likely have an increase in blood pressure, heart rate, and breathing rate. Exercise in the heat can cause fatigue, cramps, quick shallow breathing, an unsteady breathing pattern, and/or lightheadedness. Although unlikely, you could faint or have nausea. Severe problems like unsteady heart rate, chest pain or heart attack are rare. The researchers watch you closely.

Stationary Bike: It is possible for you to stumble or fall getting on or off the bike leading to cuts, scrapes, dislocations, broken bones, head injury, or abnormal heart rhythms. The researchers assist you on and off the bike. The researchers will tell you the safe use of the bike and watch you closely while you are on the bike.

Core Temperature (esophageal probe): Inserting the probe may tickle your nose. You might sneeze. Although rare, the probe could irritate or scratch soft tissue in your nose and throat. A thin coat of Lidocaine gel on the probe helps to prevent this. You may have us use water instead of gel. You drink as well-trained personnel insert the probe. While drinking, you could choke or get fluid in your windpipe. You may gag. Gagging or coughing when the tip of the probe is at the back of your upper throat could push the probe toward your mouth. In this case, they remove the probe by gently pulling it back through your nose. They reinsert the probe when you are ready. When in place, the probe may feel like a vitamin pill that is stuck in the back of your throat. This feeling is common, usually fades in time, but normally does not go away while the probe is in place. Likely, you will notice the feeling more when you swallow, talk, or turn your head. Although rare, vomiting and nausea can occur. The probe's wires are fragile. Once the probe is in place, the researchers make sure it still works correctly. If not, the well-trained

personnel remove the probe and request to insert another. You may decline and not be in the exercise experiment. Although unlikely, it is possible to place the probe into your windpipe. The researchers have never misplaced a probe into the windpipe in any of our many projects. Placing the probe in the windpipe could cause coughing, difficulty in speaking, and/or could irritate or damage the vocal cords. If the probe enters the windpipe, the well-trained personnel gently remove it.

Lidocaine gel: The gel tastes unpleasant to some people. Therefore, the well-trained personnel put very little gel on the probe. If the taste is strong, it could make you gag. There is a very small chance of an allergic reaction that could include rash, swelling, itching, redness, difficulty in swallowing, and/or heart or breathing trouble.

Bretylium Iontophoresis: Iontophoresis lasts 20 minutes. The controller does not allow the current to run longer than one hour. This technique can cause chemical burns if allowed to run for well beyond an hour. You may feel a slight itchy or tingly feeling while the current is on. Your skin may redden slightly. The redness ends before the experiment begins. The sticky disks may irritate your skin. The bretylium affects the nerves only at the site of iontophoresis (0.64 cm2, 0.1 in^2) and has no whole-body effects. The researchers and other researchers have used iontophoresis of bretylium in humans for many years. There have been no adverse effects of bretylium used in this fashion. Although unlikely, an allergic reaction could produce redness, itching, rash, and/or swelling. A severe allergic reaction could cause fever, difficulty in breathing, changes in pulse, convulsions, and/or fainting.

Cold Stress: The researchers pump ice water through the suit for 3 minutes, but you may have us re-warm you at any time. You may shiver when you skin grows cold. You may find the cold unpleasant. Your heart rate and blood pressure may temporarily increase a little. There are no lasting bad effects.

Povidone Iodine: Hospitals and researchers use this orange-colored fluid to clean and sterilize the skin. You could have a bad reaction if you are allergic to iodine. A bad reaction could cause redness, itching, rash, and/or swelling. A worse reaction could also cause fever, breathing problems, changes in pulse, convulsions, and/or blood pressure change and/or fainting. The researchers can use alcohol instead.

Latex: Some gloves and medical materials are made of latex rubber. If you are allergic to latex, you will not participate in the study.

LMX Cream: LMX (lidocaine) is a numbing cream often used for anal or rectal problems. The researchers will not use LMX if you are allergic to lidocaine. The most common side effects are irritation, numbness, and redness. Other side effects are headache, feeling lightheaded, feeling nervous, and twitching. You could also become confused, vomit, have blurred vision, or feel chest pain. You could become dizzy, feel drowsy, and have an abnormal heart rhythm. Severe allergic reactions include rash, hives, itching, problems breathing, and tightness in the chest, swelling of the mouth, face, lips, or tongue. LMX could cause the skin to become more sensitive. The numbing effects of the LMX are likely to have stopped by the time you leave the lab; nonetheless there is a chance that you could have an accidental injury to the area due to the loss of feeling. Most of the side effects are related to someone being exposed to a large amount of lidocaine. The researchers apply very little LMX to the small area of skin at the site of the tube-insertion. In the case of severe reactions to the cream, the researchers call 911. They can handle minor problems (e.g. skin-rash) at the lab.

5. a. Benefits to you: You will receive a medical screening that could inform you about your health. You could gain some knowledge about how your body works.

b. Potential benefits to society: Heart disease is the number one cause of death of men and women in America. The benefit of daily low-dose aspirin as one way to help prevent heart disease has been well established. The study explores a new side effect of daily aspirin that could put people at greater risk of heat illness. This could prompt a change in the way doctors use aspirin in their patients some day thereby improving the safety and well-being of people taking low-dose aspirin. Also, this study explores the possible means by which aspirin exerts its effects on blood vessels in the skin. This could further our knowledge of how the body controls SkBF during heat stress.

6. Time duration of the procedures and study: You will need to visit the Noll Lab for the following:

_____ initial Visit 1	Screening	1/2 hour.
_____ initial Visit 2	Screening	1 hour.
_____ initial Visits 3-9	Experiment Days:	LH/RH Experiment (4): 6 hours each
		Exercise Experiment (3): 5 hours each

Total: 40.5 hours

7. Alternative Procedures: The researchers could measure your temperature with a probe inserted into your rectum, under your tongue, or in your ear. You could also swallow a pill that measures temperature. These techniques are less accurate for the purposes of this project, and are not used in this study. The other techniques in the study are used in research worldwide. They are the best means by which to meet the goals of this study with minimal discomfort and risk to you.

8. Statement of confidentiality: The researchers code volunteers by an identification number for statistical analyses. They keep all records in a secure location. All records associated with your participation in the study are subject to the usual confidentiality standards applicable to medical records (e.g., such as records maintained by physicians, hospitals, etc.). In the event of any publication resulting from the research, the researchers do not disclose personally identifiable information. The Office of Human Research Protections in the U.S. Department of Health and Human Services, The U.S. Food and Drug Administration (FDA), The Penn State University Office for Research Protections (ORP) and The Penn State University Institutional Review Board may review records related to this project.

9. Right to ask questions: Please contact Lacy Holowatz (W: 814-867-1781, M: 814-880-9217), Rebecca Bruning (W: 814-863-2948, M: 989-351-9080), Susan Slimak (W: 814-863-8556, H: 814-237-4618), or Jane Pierzga (W: 814-865-1236, H: 814-692-4720) with questions, complaints, or concerns about the research. You can also call this number if you feel this study has harmed you. If you have any questions, concerns, problems about your rights as a research participant or would like to offer input, please contact The Pennsylvania State University's Office for Research Protections (ORP) at (814) 865-1775. The ORP cannot answer questions about research procedures. Questions about research procedures can be answered by the research team.

10. Payment for Participation:

LH/RH Experiment: You receive $15.00 for each MD tube inserted in your arm and $40.00 more for each completed experiment. ($100.00 for each experiment x 4 experiments = $400.00 total for RH/LH experiments)

For each incomplete experiment, the researchers pay you an amount equal to the part of the experiment that you complete. For instance, if you complete only half of an LH/RH experiment, the researchers pay

you for each probe that the researchers inserted plus $20.00 for that trial. This is because $20.00 is one half of $40.00.

Exercise Experiment: You receive $100.00 for each completed experiment. ($100.00 for each experiment x 3 experiments = $300.00 total for Exercise experiments).
For incomplete experiments, the researchers pay you a portion of the $100.00 equivalent to the percent of the experiment that you completed.

Total for the project: $700.00 In addition, you may have a lab T-shirt, bag, or sport bottle.

The researchers may ask you to repeat a trial. If you agree to repeat a trial, they pay you for the repeated trial as stated above.

Total payments within one calendar year that exceed $600 will require the University to annually report these payments to the IRS. This may require you to claim the compensation that you receive for participation in this study as taxable income.

11. Injury Clause: In the unlikely event you become injured as a result of your participation in this study, medical care is available. Please call Lacy Holowatz (W: 814-867-1781, M: 814-880-9217), Rebecca Bruning (W: 814-863-2948, M: 989-351-9080), Susan Slimak (W: 814-863-8556, H: 814-237-4618), or Jane Pierzga (W: 814-865-1236, H: 814-692-4720). It is the policy of this institution to provide neither financial compensation nor free medical treatment for research-related injury. By signing this document, you are not waiving any rights that you have against The Pennsylvania State University for injury resulting from negligence of the University or its investigators.

12. Voluntary participation: Your being in this study is voluntary. You may stop at any time. If you decide to withdraw, you will not have a penalty or loss of benefits you would receive otherwise. You may decline to answer certain questions. You may decide not to comply with certain procedures. However, your being in the study may be contingent upon answering these questions or complying with the procedures. The researcher may end your role in the study without your consent if the researcher deems that your health or behavior adversely affects the study or increases risks to you beyond those approved by the Institutional Review Board and agreed upon by you in this document.

14. Abnormal Test Results: In the event that the researchers obtain abnormal test results, they will apprise you of the results immediately and advise you to contact a health care provider for follow-up.

You must be 18 years of age or older to take part in this research study. If you agree to take part in this research study and the information outlined above, please sign your name and indicate the date below.

The researchers will give you a copy of this signed and dated consent form for your records.

_____

Volunteer **Date**

I, the undersigned, have defined and explained the studies involved to the above volunteer.

_____

Investigator **Date**

APPENDIX B
AVERAGE SKIN BLOOD FLOW RESPONSES TO DRUG TREATMENT

Placebo

	Control					BT				
	Peak	Tau	AUC	Time to Peak	THR	Peak	Tau	AUC	Time to Peak	THR
Mean	37.40	74.34	3559.00	19.04	1765.06	35.90	69.13	2755.20	16.87	1467.61
sd	11.73	25.54	819.00	3.62	670.99	8.62	19.98	770.87	4.85	583.12
SE	3.71	8.07	258.99	1.14	212.19	2.73	6.32	243.77	1.53	184.40

Low-Dose Aspirin

	Control					BT				
	Peak	Tau	AUC	Time to Peak	THR	Peak	Tau	AUC	Time to Peak	THR
Mean	39.00	69.19	3522.84	16.23	1839.07	39.54	76.17	3421.32	17.25	1854.66
sd	9.26	14.92	1664.75	2.75	966.89	8.79	34.70	1678.67	2.02	824.24
SE	2.67	4.31	480.57	0.80	279.12	2.54	10.02	484.59	0.58	237.94

Clopidogrel

	Control					BT				
	Peak	Tau	AUC	Time to Peak	THR	Peak	Tau	AUC	Time to Peak	THR
Mean	39.01	84.14	4186.57	18.60	2280.20	32.49	77.68	2447.76	16.26	1366.58
sd	4.04	25.40	1303.09	2.04	1239.08	14.32	18.07	557.65	2.92	651.18
SE	1.17	7.33	376.17	0.59	357.69	4.13	5.22	160.98	0.84	187.98

Furosemide

	Control					BT				
	Peak	Tau	AUC	Time to Peak	THR	Peak	Tau	AUC	Time to Peak	THR
Mean	31.76	72.54	2945.20	20.83	1295.95	32.13	71.11	2617.83	17.98	1348.05
sd	7.43	29.13	1396.40	6.22	611.10	6.84	23.66	878.66	3.65	469.21
SE	2.63	10.30	493.70	2.20	216.05	2.42	8.36	310.65	1.29	165.89

APPENDIX C
AVERAGE BLOOD RESPONSES TO DRUG TREATMENT

Placebo

	PT	INR	Hctr	Hb
Mean	12.74	1.08	41.58	14.42
sd	0.93	0.08	1.45	0.84
SE	0.42	0.04	0.65	0.38

Low-dose Aspirin

	PT	INR	Hct	Hb
Mean	12.32	1.02	41.92	14.32
sd	1.55	0.13	1.62	0.77
SE	0.70	0.06	0.72	0.34

Clopidogrel

	PT	INR	Hct	Hb
Mean	12.76	1.08	41.50	14.38
sd	1.68	0.13	2.40	1.28
SE	0.75	0.06	1.08	0.57

Furosemide

	PT	INR	Hct	Hb
Mean	12.06	1.00	45.18	15.62
sd	0.96	0.07	1.94	0.93
SE	0.43	0.03	0.87	0.42

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