European Review for Medical and Pharmacological Sciences 2012; 16: 143-150

Regulation of blood flow by aspirin following muscle ischemia

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Abstract. – *Background:* **The vascular endothelium secretes a balance of dilator and constrictor substances which regulate vascular tone. During ischemic stress, this balance changes. After a short period of ischemia, a protective mechanism known as reactive hyperemia (RH) contributes to a post-ischemic increase in blood flow. The agents regulating this phenomenon remain controversial.**

Aim: **The purpose of this study was to examine whether aspirin regulates vascular endothelial function following ischemia.**

Methods: **Sixteen healthy volunteers presented for two visits, each serving as their own control, and randomized to receive 500 mg aspirin or placebo. Forearm blood flow (FBF) was measured at baseline and during reactive hyperemia (RH) which was induced by five minutes of arterial occlusion. Blood samples were analyzed for vWF and lipids.**

Results: **After ischemia, RH was attenuated when subjects were pre-medicated with 500 mg aspirin compared to placebo: $AUC_{[aspirin]}$=1450 ± 201 mL/100 mL tissue/min vs. $AUC_{[placebo]}$= 2207 ± 294 mL/100 mL tissue/min; ($p < 0.05$). Separation of the subjects with high HDL or low HDL levels resulted in a similar peak FBF response with placebo, but in the high-HDL group only, aspirin ingestion attenuated peak FBF after ischemia compared to the placebo condition (22.6 ± 1.7 mL/100 mL tissue/min vs. 33.5 ± 3.2 mL/100 mL tissue/min, respectively) ($p < 0.05$).**

Conclusions: **Aspirin partially regulates the RH response following ischemia compared to placebo, and this effect appears to be more profound when adjusting for plasma HDL concentration in healthy individuals. This suggests that the post-ischemic RH response may be partially mediated by arachidonic acid-derived mediators such as the prostaglandins.**

Key Words:

Reactive Hyperemia (RH), High Density Lipoprotein (HDL), Forearm Blood Flow (FBF), Aspirin, Prostaglandins.

Introduction

The vascular endothelium secretes a large number of dilator and constrictor agents which regulate vascular tone, including nitric oxide (NO) and members of the prostaglandin family. Endogenous prostaglandin inhibition was found to impair endothelial-dependent vasodilation in patients with hypertension, but not in normotensive individuals[1]. Treatment of patients with coronary artery disease (CAD) with a prostaglandin analog decreased basal vascular tone whilst improving endothelial-dependent dilation[2]. Interestingly, the beneficial vascular effect of angiotensin-converting enzyme inhibitors is lost in hypertensive patients administered with 1000 mg of aspirin[3]. Taken together, the role of endogenous prostaglandins in regulating vascular tone has important implications in both health and disease, and in the response to therapeutic intervention.

Some studies have shown that pharmacological inhibition of prostaglandin synthesis causes a decrease in blood flow in the arteries of healthy subjects[4,5]. Aspirin inhibits platelet and endothelial cell prostaglandin synthesis by irreversible acetylation and non-specific inhibition of cyclooxygenase (COX) isoforms, COX1 and COX2. Numerous reports have demonstrated that low-dose aspirin therapy protects against adverse cardiovascular and cerebrovascular events, but the protective mechanism is mostly ascribed to platelet inhibition and a diminished propensity to form thrombi rather than to the regulation of blood flow in vascular networks[6,7].

Ischemic challenge in normal arteries is followed by a marked increase in blood flow (reactive hyperemia, RH), which is presumably a protective response[8]. RH in humans was shown to correlate with endothelial cell function, and the

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post-ischemic increase in blood flow is believed to be predominantly NO-mediated[9-11]. However, it has also been suggested in healthy subjects that prostaglandins may be involved in regulating blood flow after ischemia[12], while other reports state that RH is markedly attenuated by inhibitors of prostaglandin synthesis[13,14]. Studies in healthy subjects attributed forearm exercise-mediated hyperemia to NO rather than prostaglandins as a function of age[12,15]. These conflicting findings may be due to differences in plasma mediators that alter signaling properties of the vascular endothelium and agents in the plasma milieu which may alter the way the vasculature responds to prostaglandin inhibition. Based on the conflicting reports regarding the role of prostaglandins in regulating blood flow in the human vasculature in response to ischemia, we designed a study in healthy subjects to assess the role of aspirin in regulating reactive hyperemia following muscle ischemia. Ischemia is also reported to degranulate the vascular endothelium via Weibel Palade body exocytosis[16,17], and so we measured plasma vWF in plasma as a surrogate for endothelial cell integrity.

Methods

Subject Selection

This study included healthy males and females aged between 25 and 50 years. An initial telephone screen took place to ensure subjects met our study criteria. The following factors served as exclusion criteria: diabetes, smoking, hypertension, dyslipidemia, body mass index (BMI) >30 kg/m^2, bleeding disorders, gastrointestinal disorders, known intolerance to aspirin or non-steroidal anti-inflammatory medication, or current prescription of vasoactive medications or hormone replacement therapy. All subjects were relative sedentary, and were asked to refrain from changes in usual activity patterns for the duration of the investigation. With the exception of one subject who was taking fluoxetine, none of the remaining subjects were taking prescribed or over-the-counter (OTC) medications. According to the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) criteria, the subjects were free from dyslipidemias[18]. This study was approved by the Institutional Review Boards (IRB) from both Syracuse University and SUNY Upstate Medical University. Each subject signed an informed consent document and had the opportunity to ask questions and to withdraw from the study at any point without penalty.

Study Protocol

Subjects presented for two visits. After twenty minutes of rest, venous blood was drawn and analyzed for plasma vWF concentration as well as for plasma cholesterol and cholesterol sub-fractions. Following resting measurements, the subjects received either 500 mg aspirin or placebo by mouth. This dose of aspirin was selected as it is less than what patients may take to alleviate chronic inflammation but larger than what patients would take for primary prevention (81 mg in the U.S.). Each visit was in a randomized order and the subjects and investigators were blinded to the treatment. Forearm blood flow (FBF), blood pressure, and heart rate measurements taken every ten minutes and before induction of forearm ischemia did not differ from baseline (t=0 minutes) on either visit (Figure 1).

Two days before each visit, subjects were asked to abstain from any physical activity and vasoactive agents, including: nicotine, tobacco, caffeine, and alcohol. No subject had taken aspirin or other non-steroidal anti-inflammatory agents for at least 90 days, and each study visit was separated by at least 14 days to abrogate a treatment pre-conditioning effect. The female subjects were all tested in the latter half of the menstrual cycle. Blood pressure was measured before and during each study day using a manual sphygmomanometer (Welch Allyn, Skaneateles, NY, USA). All studies were carried out in a quiet, temperature-controlled room maintained at 22°C to 24°C to minimize variation in vascular reactivity.

During both visits, forearm blood flow (FBF) was measured in the non-dominant forearm using a mercury-filled Silastic strain-gauge plethysmography (EC-4, D.E. Hokanson, Inc., Bellevue, WA, USA), with the forearm positioned above heart level[17]. The strain gauge was placed around the widest part of the forearm and was connected to a plethysmographic device. One minute prior to and throughout the FBF measurements, hand circulation was arrested with a wrist cuff inflated at a pressure of 50 mm Hg above systolic blood pressure. A blood pressure cuff placed in the upper arm was then inflated to 50 mm Hg for 7 sec in each 15-sec inflation-deflation cycle using a rapid cuff inflator (EC-20, D.E. Hokanson, Inc.). Six

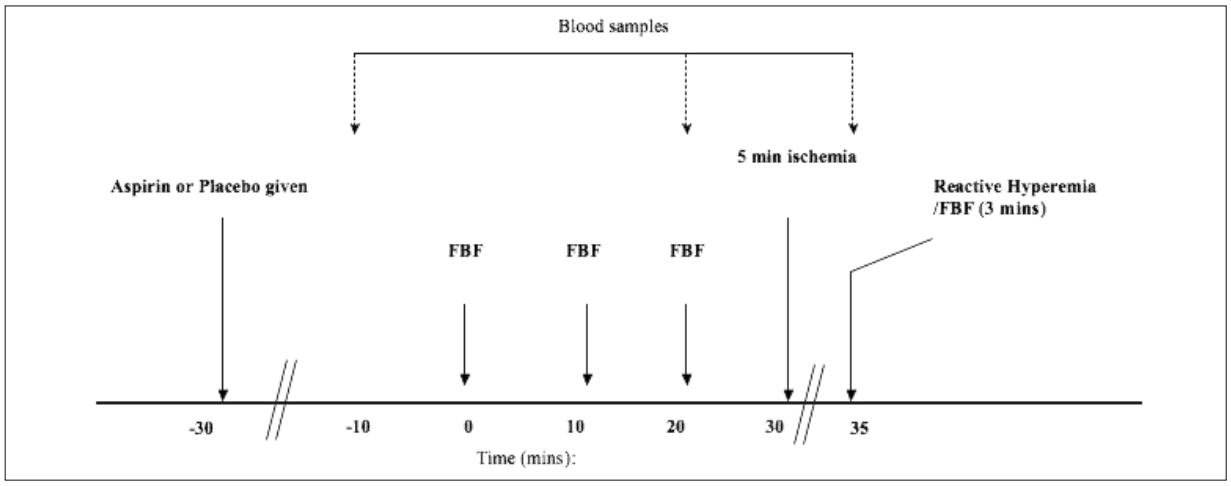

Figure 1. Study protocol.

plethysmographic measurements were averaged to obtain baseline FBF. To induce reactive hyperemia, a blood pressure cuff was inflated around the upper arm to 240 mm Hg for 5 minutes. After release of the forearm ischemic occlusion cuff, FBF was measured for 3 minutes. FBF was expressed as mL/100 mL of tissue/min. The reliability of FBF measurement in our lab was calculated using the resting FBF ($r = 0.90$, $p < 0.05$), AUC of FBF in response to RH ($r = 0.81$, $p < 0.05$) and peak FBF in response to RH ($r = 0.82$, $p < 0.05$).

Blood pressure was monitored in the opposite arm in which FBF was being measured at intervals throughout each study (Figure 1, Table I). Heart rate was also measured at the same time as blood pressure using a Polar heart rate monitor. Prior to all measurements, a 22G antecubital vein cannula was inserted into the non-dominant arm, and used for the withdrawal of blood for analysis.

Blood Cholesterol Measurement

A venous blood sample drawn before either placebo or aspirin administration was used to assess lipoproteins on a point-of-care testing (POCT) device (Cholestech Instruments, Hayward, CA, USA) following the manufacturers guidelines. Assay precision by coefficient of variation (%CV) during the time of sample measurement was as follows: 7.6% for HDL, 7.2% for LDL, 0.37% for TG, and 1.0% for total cholesterol.

Body Composition Analysis

Total body fat was measured using the BodPod according to the manufacturer's guidelines (Life Measurement, Inc., Concord, CA, USA).

Blood vWF Measurement

At three time-points (Figure 1), a venous blood sample was drawn, placed in sodium citrate collection tubes (BD Diagnostic Systems,

Table I. Clinical characteristics of study subjects.

	All subjects (n = 16)	High HDL (n = 8)	Low HDL (n = 8)
Gender (men, women)	12.4	4.4	8
Age (years)	32.6 ± 1.8	31.4 ± 2.5	33.8 ± 2.7
Body mass index (kg/m^2)	24.4 ± 0.6	23.5 ± 1	25.3 ± 0.9
% body fat	19.5 ± 2.3	21.1 ± 0.9	17.9 ± 6.0
Heart rate (b/min)	60 ± 3.8	62 ± 4.2	57 ± 6.4
Mean arterial pressure (mmHg)	84 ± 7	83 ± 3.2	85 ± 1.8
High density lipoproteins (mg/dL)	51 ± 3	61 ± 1.9	42 ± .04*
Low density lipoproteins (mg/dL)	100 ± 5	104 ± 7.8	98 ± 7.1
Triglycerides (mg/dL)	98 ± 16	83 ± 5.3	113 ± 25.1
Total cholesterol (mg/dL)	172 ± 7.5	181 ± 9.5	170 ± 12.7

Mean ± SEM; *$p < 0.001$ low vs. high HDL levels.

Swedesbro, NJ, USA), centrifuged at 1000 × g, and the isolated plasma was stored at -80°C. Plasma vWF concentration was determined in duplicate against prepared recombinant vWF standard solutions using a commercially-available 96-well vWF Enzyme-Linked Immunosorbant Assay (ELISA) kit (IMUBIND® vWF ELISA, American Diagnostica, Stamford, CT, USA). Before analysis, plasma samples were diluted 1:200 in double-distilled water.

Data Analysis

Forearm blood flow, heart rate, blood pressure, cholesterol, and vWF data were reported as mean ± SEM unless otherwise specified. Reactive hyperemia during vasodilation after ischemia has two components: peak FBF and time taken for blood flow to return to baseline; the latter can be examined using a recovery time constant, (τ_{rec}). To assess the kinetics of the three-minute RH response, data were analyzed as follows: (1) as the area under the curve (AUC) by integrative calculus to approximate the duration of RH using Microcal Origin (OriginLab Corporation, Northampton, MA, USA); (2) by fitting the recovery phase FBF after post-ischemic dilation during RH with a mono-exponential function $FBF(t) = FBF(0)\ (1\text{-}exp^{-t/\tau rec})$. FBF(t) is the FBF at time t, FBF(0) is the initial pre-recovery FBF at maximum vasodilatory capacity, and τ_{rec} is the mono-exponential recovery time constant which is greater in magnitude with prolonged vasodilation; (3) by measuring the peak FBF observed with vasodilation following ischemia when the upper arm occlusion cuff is released. A paired *t*-test was employed to compare FBF measures between the aspirin and placebo condition. A Student's unpaired *t*-test was used to compare the descriptive characteristics between the high and low HDL groups, while a 2 (low vs. high HDL groups) × 2 (aspirin vs. placebo) mixed model ANOVA with repeated measures was employed for the blood flow comparisons between these groups. Statistical analysis was conducted using SPSS statistical package (version 16.0). A *p* value of 0.05 was considered appropriate for rejecting the null hypothesis.

Results

Table I shows the demographic profile of the subject population. After telephone screening, sixteen subjects were ultimately enrolled, consisting of four females and twelve males with a mean age of 32.6 ± 7.3 years.

Effect of Aspirin Treatment on Reactive Hyperemia

Baseline FBF was indistinguishable between treatment groups (3.8 ± 0.50 mL/100 mL tissue/min for placebo treatment and 3.7 ± 0.47 mL/100mL tissue/min for aspirin treatment, p = NS). Peak FBF increased by approximately 10-fold following vascular occlusion, with gradual recovery to baseline over the course of three minutes (Figure 2A). Pre-medication with 500 mg aspirin did not significantly change peak FBF (placebo: 30.4 ± 3.0 mL/100 mL tissue/min vs. aspirin: 24.0 ± 1.8 mL/100 mL tissue/min, p = 0.07, Figure 2A) or the percentage increase in FBF from baseline (placebo 877 ± 134%, aspirin 620%, p=NS). The recovery time constant to baseline FBF after ischemia was faster for aspirin compared to the placebo condition (Aspirin τ_{rec} = 40.2 ± 2.4 sec vs. Placebo τ_{rec} = 62.3 ± 4.2 sec, $p < 0.05$, Figure 2A). RH following muscle ischemia as assessed by the area under the curve (AUC) was attenuated in the aspirin condition compared to the placebo condition: $AUC_{[aspirin]}$ = 1450 ± 201 vs. $AUC_{[placebo]}$ = 2207 ± 294 mL/100 mL tissue/min , ($p < 0.05$, Figure 2B).

Plasma HDL and Reactive Hyperemia

NCEP ATP III suggests evidence-based cut-off values for low HDL concentration at 40 mg/dL and high HDL concentration at 60 mg/dL[18]. An HDL concentration of > 60 mg/dL is considered a negative, independent risk factor for the purposes of risk stratifying cardiovascular diseases[18]. Therefore, we took 50 mg/dL as a mean cut-point for separating those patients with higher HDL from those with lower HDL. The sub-group of subjects with lower plasma HDL (42 ± 8.0 mg/dL) was compared to another sub-group with higher plasma HDL (61 ± 5 mg/dL). We compared each HDL sub-group of healthy volunteers for differences in other cholesterol sub-fractions, body fat, heart rate, blood pressure, age, and body mass index and found there to be no differences other than HDL concentration (Table I). Baseline blood flow was similar in the sub-group with high HDL (3.96 ± 0.52 mL/100 mL tissue/min) compared to the sub-group with low HDL (3.76 ± 0.52 mL/100 mL tissue/min, p = 0.06). In the placebo condition, there was no sig-

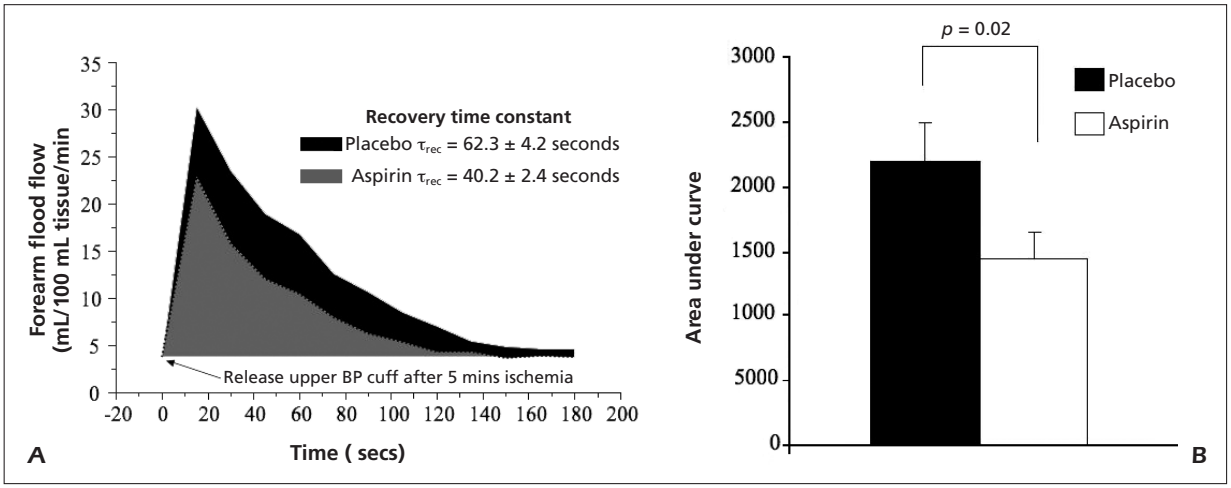

Figure 2. Reactive hyperemic response to aspirin and placebo trials. ***A,*** Forearm flood flow for five minutes following the ischemic condition and recovery time constant, τ_{rec}. ***B,*** Area under the curve (AUC) following muscle ischemia. Values are means ± SEM, n = 16.

nificant difference in peak FBF after ischemia between groups (high HDL sub-group: 33.5 ± 3.2 mL/100 mL tissue/min vs. low HDL sub-group group: 26.7 ± 1.9 mL/100 mL tissue/min, p = NS; (Figure 3). In the high HDL sub-group, aspirin treatment resulted in an attenuation of peak FBF after ischemia (aspirin: 22.6 ± 1.7 mL/100 mL tissue/min vs. placebo: 33.5 ± 3.2 mL/100 mL tissue/min, $p < 0.05$; Figure 3). Aspirin administration did not significantly change peak FBF in the low HDL sub-group.

Using plasma vWF as a surrogate marker protein in the blood for acute vascular endothelial cell degranulation, we observed there was no effect of ischemia on vWF secretion or with aspirin pre-treatment (data not shown).

Discussion

Prostaglandins have been implicated in the regulation of blood flow in response to ischemia in healthy individuals. The findings of the present study demonstrated that aspirin treatment reduced FBF (AUC) following ischemia by 33% compared to placebo. Furthermore, aspirin administration decreased the peak reactive hyperemic response (peak FBF) only in individuals with high HDL levels. Based on the pharmacodynamic profile of aspirin, these data suggest a role for prostaglandins in the regulation of RH in healthy subjects. The dose of aspirin we used (500 mg) is smaller than what patients would take for chronic inflammation but larger than the 81 mg or 350 mg dose that patients may take for primary and secondary prevention of cardiovascular disease, respectively. As such, these finding have important implications in patients with cardiovascular disease in whom preservation of protective, prostaglandin-mediated mechanisms like RH is beneficial.

Aspirin and Reactive Hyperemia

Aspirin appears to attenuate the absolute increase in blood flow (the maintenance of increased blood flow—shown as AUC is Figure 2B) but not peak blood flow following five minutes of forearm ischemia (Figure 2A). Based on these findings, RH may not be entirely NO-mediated as has been suggested in other studies[10]. Dif-

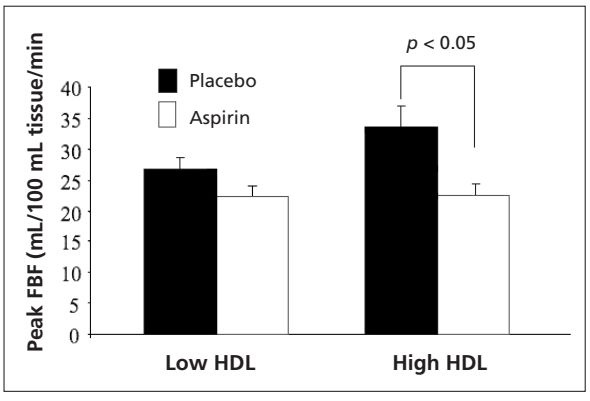

Figure 3. HDL effect on the post-ischemic response to aspirin. Values are means ± SEM, n = 8 for each group.

ferences in our findings from those of Joannides *et al*. may be due to differences in methodology since Johannides *et al*. used a two-fold higher concentration of aspirin, a shorter duration of ischemia (three minutes), and they employed Doppler technology rather than plethysmography. Johannides *et al*. studied blood flow more distally in the radial artery compared to our study of brachial artery ischemia[10]. Using indomethacin, Kilbom *et al*, observed an attenuation of RH with an additional effect on peak blood flow. Kilbom *et al*, used the same experimental design as our study and, based on the mechanism of drug action for indomethacin, prostaglandins may indeed be involved in RH[14]. Furthermore, Doshi *et al*. showed that RH was only partially relieved by pharmacologic inhibition of endogenous NO production and raised the possibility that some other endogenous mediator or mechanical property exerted by the vasculature under acute changes in blood flow (the so-called myogenic response) may contribute to post-ischemic changes in FBF[19]. Lastly, it is very clear that situations in which subjects have enhanced sympathetic tone (hypertension, heart failure, and obesity hypoventilation syndrome) may cause alterations in RH, though our selection criteria was designed as such to eliminate these confounding variables[20-23]. This suggests that the intrinsic subject characteristic, the duration of ischemia, the drug chosen for prostaglandin inhibition, and the technique used to assess FBF may contribute to conflicting reports in the literature regarding the role of prostaglandins in RH.

Kinetics of Reactive Hyperemia

Aspirin did not change peak FBF after ischemia, but diminished the maintenance phase of RH (33% reduction in AUC between conditions), with a more rapid return to baseline blood flow (smaller recovery time constant between conditions). Carlsson and Wennmalm[13] concluded that drugs which inhibit prostaglandin synthesis have the capacity to decrease post-occlusive reactive hyperemia, and they theorized that activation and local release of arachidonic acid and vasodilaor prostaglandins is one of the main factors behind the vascular smooth muscle relaxation response following arterial occlusion.

Separation of our subjects into high and low HDL sub-groups revealed that peak FBF was reduced by ~30% in the high HDL sub-group following aspirin administration. Consistent with previous studies, our findings suggest that individuals with high HDL have greater vasodilator capacity and may explain why aspirin treatment appears only to have greater functional consequence in people with high HDL levels. This is consistent with a previous study in which HDL was an independent predictor of FBF in type 2 diabetics[24]. Additionally, HDL isolated from pre-menopausal versus post-menopausal women differs in its ability to activate eNOS[25]. Antoniades *et al*.[26] reported that elevated lipid levels (total cholesterol greater than 200 mg/dL) decreases forearm vasodilatory response in RH, independent of LDL, HDL, and triglycerides. Higher HDL in subjects may lead to greater prostaglandin production, increased blood flow, and this effect may be pronounced at the time of ischemia[27,28]. Further research is needed to explore if HDL can also regulate abluminal prostaglandin secretion.

Endothelial Function and vWF

Since acute vWF secretion from endothelial cells during ischemia was reported, we measured plasma vWF concentration throughout our protocol[29-32]. We found that aspirin did not alter plasma vWF concentration, which remained physiologic at approximately 900 mU/mL throughout our protocol[33]. Based on these data, endothelial cell integrity was likely preserved during ischemia.

In conclusion, aspirin inhibits the duration of vasodilation but not peak blood flow seen in RH following ischemia. Individuals with high plasma HDL levels demonstrate attenuated peak FBF in response to ischemia after aspirin treatment. Further investigation is needed regarding the dose of aspirin which affects the RH response and its relationship in individuals with differing HDL levels.

Acknowledgements

Sincere thanks to Rose Kingbury, RN, NP for the placement of the hep locks. This project was partially funded by NIH grant R21DK063179 to JAK. SJC was partly funded by a research grant from the Department of Medicine, SUNY Upstate Medical University

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