DISSERTATION

NOVEL ROLE OF ACETYLCHOLINE IN VASCULAR CONTROL IN HUMANS

Submitted by

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ABSTRACT

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The vascular endothelium is remarkably sensitive to the molecule acetylcholine (ACh), which binds to muscarinic receptors to initiate endothelium-dependent vasodilation. Although vasodilatory responsiveness to ACh is considered the gold standard index of endothelial function, an obligatory role for ACh in peripheral blood flow control has been challenging to elucidate. Thus, muscarinic ACh receptors on endothelial cells are widely considered to be evolutionary remnants with no real physiological function in humans. Administration of exogenous ACh amplifies endothelial sensitivity to other vasodilatory stimuli and blunts sympathetic vasoconstrictor signaling; therefore, we sought to determine whether endogenous ACh contributes to these processes in vivo. Accordingly, the overall goal of this dissertation research was to evaluate the role of ACh in modulating sympathetic α-adrenergic vasoconstriction and eliciting vasodilation in healthy, young adults.

The primary findings are that 1) ACh interacts with the endothelium-dependent vasodilator adenosine triphosphate (ATP) to augment vasodilation and limit α_1-adrenergic vasoconstriction in the skeletal muscle resistance vasculature, 2) endogenous ACh blunts sympathetic vasoconstriction within active skeletal muscle and is an obligatory mechanism of functional sympatholysis during exercise at high intensities, and 3) ACh mediates flow-induced vasodilation of conduit arteries in response to sustained and transient increases in shear rate induced by handgrip exercise and reactive hyperemia, respectively. Collectively, these studies reveal a novel, physiological role of ACh in peripheral blood flow regulation in humans.

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DEDICATION

This work is dedicated to the people who influenced my path and supported me along the way. Dr. Christopher Baldi initially sparked my interest in research, and I am grateful to him and Dr. Matthew Brothers for the research experience and mentorship they provided. I would also like to thank my unofficial mentor, Dr. Jordan Patik, for helping me to navigate graduate school and for his continued friendship. I am immensely grateful to my wonderful labmates, whose camaraderie is forever appreciated. Finally, I dedicate this to Josh for his endless support and to my family for their encouragement.

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INTRODUCTION

The vascular endothelium is a single layer of cells that lines entire cardiovascular system and fundamentally contributes to all aspects of cardiovascular function. Endothelial cells constantly monitor chemical and mechanical cues and communicate with underlying vascular smooth muscle cells to regulate vascular tone; thus, the endothelium plays a critical role in regulating blood flow and tissue oxygen delivery. Because of its essential role, a decline in endothelial function has long been recognized as an independent risk factor for cardiovascular disease. Accumulating evidence suggests that the endothelium is a key site for integration of information from the bloodstream, sympathetic nervous system, and surrounding tissues; yet, how the endothelium processes simultaneous stimuli and transduces signals to control arterial diameter is not entirely clear.

Role of acetylcholine in regulating vascular tone

The endothelium is so profoundly sensitive to the molecule acetylcholine (ACh) that administration of ACh is considered the gold standard assessment of endothelial health. However, despite the close association between endothelial dysfunction and cardiovascular disease risk, numerous attempts to elucidate a physiological role of ACh in blood flow regulation in humans have been unsuccessful (Armstrong & Laughlin, 1986; Buckwalter *et al.*, 1997; Shoemaker *et al.*, 1997; Brock *et al.*, 1998). Because ACh is classically regarded as a neurotransmitter, researchers historically investigated the possibility that ACh was released by sympathetic vasodilator nerves or "spilled over" from motor nerve terminals (Corcondilas *et al.*, 1964; Donald *et al.*, 1970; Williams *et al.*, 1985; Joyner *et al.*, 1992; Welsh & Segal, 1997).

More recent evidence from isolated vessels demonstrates that vascular endothelial cells synthesize and secrete ACh in response to shear stress (Wilson *et al.*, 2016), which then binds to muscarinic receptors to elicit vasodilation in an autocrine manner. Evidence of this non-canonical source of ACh released directly to the circulation raises the possibility that local cholinergic mechanisms may regulate vascular tone in humans.

Muscle blood flow regulation and functional sympatholysis

During whole-body exercise, elevated sympathetic nervous system-mediated vasoconstriction limits blood flow to inactive tissues, which facilitates redistribution of blood to metabolically-active skeletal muscle without compromising arterial blood pressure. Although sympathetic nerve activity is also increased to skeletal muscle circulation, post-junctional α-adrenergic vasoconstriction is substantially blunted in the vasculature of contracting muscle (Remensnyder *et al.*, 1962). This ability of working skeletal muscle to modulate sympathetic vasoconstriction, termed 'functional sympatholysis', is essential to ensure adequate blood flow and oxygen delivery to active muscle in the face of elevated sympathetic outflow. Advancing age and many disease states are characterized by a combination of elevated sympathetic nerve activity and an impaired ability of contracting skeletal muscle to limit sympathetic vasoconstriction (Dinenno *et al.*, 2002; Saltin & Mortensen, 2012). The resulting malperfusion of working skeletal muscle limits delivery of oxygen and substrates to metabolically-active tissue, which accelerates fatigue development and diminishes exercise tolerance (Tyni-Lenné *et al.*, 1996; Mortensen *et al.*, 2012). Thus, elucidating the signaling mechanisms that underlie functional sympatholysis in humans is an important biomedical goal with implications for understanding and treating exercise intolerance in aging and clinical populations.

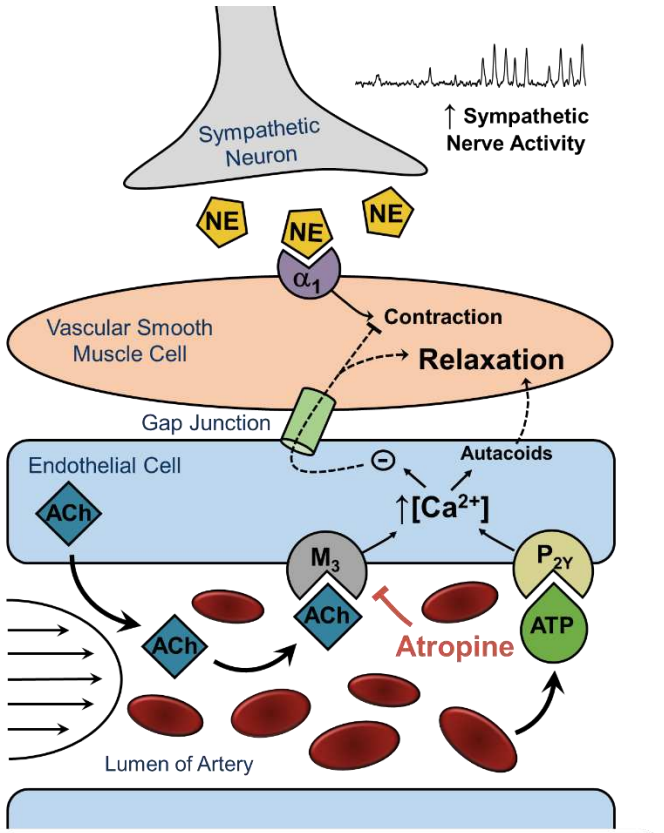


Figure 1.1: Proposed mechanism

Acetylcholine (ACh) is released from endothelial cells in response to shear stress and adenosine triphosphate (ATP) is released from erythrocytes in response to deoxygenation or deformation. ACh binds to muscarinic (M_3) receptors and ATP binds to purinergic (P_{2Y}) receptors on endothelial cells, which increases cytosolic Ca^{2+}. The rise in intracellular Ca^{2+} concentration elicits hyperpolarization ⊖ and production of vasodilatory autacoids. Hyperpolarization spreads to vascular smooth muscle cells through gap junctions, where it attenuates sympathetic vasoconstriction by modulating the post-junctional response to activation of α_1-adrenergic receptors by norepinephrine (NE).

Mechanisms of functional sympatholysis

The mechanisms that modulate α-adrenergic signaling within active skeletal muscle remain poorly understood (Saltin & Mortensen, 2012). However, mounting evidence suggests that the vascular endothelium is a critical site for integration of local vasodilatory and sympathetic vasoconstrictor signaling (Behringer & Segal, 2012*a*; Kerr *et al.*, 2012; Hearon *et al.*, 2016), and evidence from experimental animals suggests that hyperpolarization originating in

the endothelium limits sympathetic vasoconstriction in a variety of vascular beds (Behringer & Segal, 2012*a*). Currently, the only vasoactive molecule known to attenuate α-adrenergic vasoconstriction when administered exogenously to humans under resting conditions is adenosine triphosphate (ATP) (Rosenmeier *et al.*, 2008), which binds to $G_{q/11}$ protein-coupled receptors (purinergic P_{2Y} receptors) on the endothelium (Fig. 1.1). ACh elicits vascular hyperpolarization through a similar $G_{q/11}$ protein-coupled receptor-mediated mechanism (muscarinic M_3 receptors; Fig. 1.1), and application of exogenous ACh to skeletal muscle resistance arterioles blunts sympathetic vasoconstriction in rodents (Kurjiaka & Segal, 1995). We have observed that intra-arterial infusion of exogenous ACh blunts α_1-adrenergic during forearm exercise (Hearon *et al.*, 2016, 2017), which suggests a potential role of ACh in vascular control within contracting muscle. To date, no study has determined whether endogenous ACh contributes to functional sympatholysis in humans.

Flow-mediated vasodilation

The ability of the endothelium to facilitate vasodilation in response to vascular wall shear stress is a critical component of vascular function. Due to its close association with sensitivity to infused ACh, flow-mediated dilation (FMD) of the brachial artery is commonly used as a surrogate measure of endothelial function. Interestingly, muscarinic ACh receptor activation contributes to basal and flow-induced nitric oxide synthesis and vasorelaxation of coronary artery rings superfused with effluent from isolated canine conduit arteries (Martin *et al.*, 1996). Together with recent evidence that ACh released from endothelial cells in response to shear mediates endothelial calcium signaling and nitric oxide production ex vivo (Wilson *et al.*, 2016), these findings suggest that locally-released ACh plays a role in endothelial mechanotransduction and flow-mediated dilation.

Summary and overall goal

The vascular endothelium is highly responsive to ACh, and evidence from isolated arteries indicates that ACh is synthesized and released from endothelial cells in response to shear stress (Martin *et al.*, 1996; Wilson *et al.*, 2016). Interestingly, activation of endothelial cells has been shown to amplify the response to additional vasodilatory stimuli (Sonkusare *et al.*, 2014; Lee *et al.*, 2018; Hearon *et al.*, 2019), and application of ACh to resistance vessels of resting skeletal muscle markedly attenuates sympathetic vasoconstriction in rodents (Kurjiaka & Segal, 1995). We have recently demonstrated that intra-arterial administration of exogenous ACh enhances sympatholysis during mild intensity exercise (Hearon *et al.*, 2016); however, whether endogenous ACh is involved in the integrative control of vascular tone in humans is unknown. Therefore, the overall goal of this dissertation is to determine the physiological role of endogenous ACh in functional sympatholysis and conduit artery flow-mediated dilation in humans.

SPECIFIC AIMS

Experiment 1: To determine whether combined action of the endothelium-dependent agonists ACh and ATP enhances vasodilation and blunts α_1-adrenergic vasoconstriction in the forearm resistance vasculature.

Experiment 2: To determine the role of endogenous ACh in functional sympatholysis in contracting skeletal muscle.

Experiment 3: To determine the contribution of endogenous ACh to flow-mediated vasodilation of the brachial artery in response to sustained and transient shear stimuli.

CHAPTER 2 – MANUSCRIPT 1

Endothelium-dependent agonists ACh and ATP interact to augment vasodilation and limit α_1-adrenergic vasoconstriction in human skeletal muscle

INTRODUCTION

Vascular tone is regulated in a highly specific manner that relies on integration of numerous vasoactive mechanisms. The vascular endothelium constantly monitors physiological cues and processes multiple chemical and mechanical stimuli arising from the bloodstream and surrounding tissues to regulate vessel diameter. Precisely how the endothelium integrates simultaneous sensory inputs to govern an appropriate response is not clear; however, it appears to involve complex interactions and communication between neighboring endothelial cells (ECs) and underlying vascular smooth muscle cells (VSMCs).

Activation of the endothelium by physiological stimuli is typically mediated by fluctuations in cytosolic Ca^{2+}. Vasodilatory molecules such as adenosine triphosphate (ATP) and acetylcholine (ACh) stimulate an increase cytosolic Ca^{2+} when they bind to their respective purinergic (P_{2Y}) and muscarinic (M_3) receptors on the endothelium. Likewise, shear forces exerted by blood flowing along the endothelium initiate cytosolic Ca^{2+} signals. In addition to stimulating the production and release of vasodilatory autacoids such as nitric oxide and prostaglandins, elevated Ca^{2+} within ECs stimulates Ca^{2+}-activated K^+ (K_{Ca}) channels. The resulting efflux of K^+ results in endothelium-derived hyperpolarization, which is conducted to adjacent ECs and spreads to underlying VSMCs to promote relaxation.

Mounting evidence suggests that activation of the endothelium enhances its sensitivity to further stimulation. Using freshly isolated arteries from rats, Lee et al. (2018) recently

determined that muscarinic and purinergic receptors are heterogeneously distributed among ECs such that clusters of ECs detect each agonist and communicate the information with neighboring cells to coordinate an appropriate response. When a combination of ATP and ACh is applied to the endothelium, a synergistic response occurs with distinct Ca^{2+} signals that are not observed when either dilator is applied in isolation, which suggests that ATP and ACh augment sensitivity to each other. Such interactions also appear to affect vasomotor responses. For example, in the cutaneous microcirculation, administration of ATP enhances subsequent cholinergic vasodilation (Fujii et al., 2015). ATP activates K_{IR} channels, which have been shown to amplify electrical signaling in ECs in response to cholinergic vasodilation (Sonkusare et al., 2016); thus, circulating ATP could act on the endothelium to enhance its sensitivity to other stimuli. Indeed, we recently demonstrated that vasodilation to ACh is selectively amplified in contracting skeletal muscle (Hearon et al., 2019) when circulating ATP is elevated (González-Alonso et al., 2002; Kirby et al., 2012). Collectively, these observations highlight the ability of the endothelium to process complex interactions between multiple signals and evoke an appropriate hemodynamic response.

In addition to sensing circulating vasodilators, the endothelium receives input from and communicates with VSMCs to regulate vascular tone. This is particularly important during whole-body exercise, when elevated sympathetic nerve activity stimulates α-adrenergic receptors on VSMCs to elicit vasoconstriction. Although muscle sympathetic nerve activity is increased during exercise, α-adrenergic vasoconstriction is attenuated within the resistance vasculature of contracting skeletal muscle, which facilitates redistribution of blood flow to active muscle. This phenomenon of "functional sympatholysis" is essential to ensure adequate delivery of oxygen to the working muscle despite elevated sympathetic outflow. Although the mechanisms that

attenuate post-junctional α-adrenergic signaling in the microcirculation of exercising muscle are unclear, accumulating evidence suggests that the endothelium plays a central role in limiting sympathetic vasoconstriction (Kerr et al., 2012). In rodents, endothelium-dependent hyperpolarization counteracts sympathetic vasoconstriction (Kurjiaka & Segal, 1995), and in humans, activating the endothelium with either ATP or ACh enhances functional sympatholysis (Hearon et al., 2016). Thus, the endothelium appears to play a key role in limiting sympathetic vasoconstriction; yet it is currently unknown whether endothelium-dependent stimuli interact to modulate sensitivity to sympathetic constriction.

Therefore, in the present study, we aimed to determine whether combined action of the endothelium-dependent agonists ATP and ACh elicits greater vasodilation and further limits α_1-adrenergic vasoconstriction compared to the combined action of either dilator with the endothelium-independent dilator SNP.

METHODS

Participants and ethical approval

All experimental protocols were approved by the Institutional Review Board at Colorado State University (protocol 14-5392H) and performed in accordance with the Declaration of Helsinki except for registration in a database. After providing written, informed consent, 11 healthy volunteers (5 women, 6 men; 24 ± 0.7 y, 26 ± 2% body fat; means ± SE) completed the study. Participants were free from cardiovascular and metabolic disease and were not taking any medications aside from oral contraceptives (3 participants).

Instrumentation

Participants arrived at the laboratory at 7 AM following an overnight fast and lay supine with the non-dominant arm abducted 90 degrees resting on a table slightly above heart level

throughout the study visit. After local anesthesia (1% lidocaine), a 20 g, 7 cm catheter was inserted in the brachial artery using sterile technique. The catheter was continuously flushed with heparinized saline and connected to a 3-way port for infusion of vasoactive drugs. Mean arterial pressure (MAP) was determined using a pressure transducer connected to the catheter and heart rate (HR) was assessed with a three-lead electrocardiogram (Cardiocap/5). Studies were conducted in a temperature-controlled room with a fan directed at the experimental arm to minimize skin blood flow.

Forearm blood flow

Forearm blood flow was determined using Doppler ultrasound. Brachial artery blood velocity and diameter were measured proximal to the catheter using a 12 MHz linear array ultrasound probe (Vivid7, General Electric, Milwaukee, WI). Velocity was measured with a probe insonation angle <60° at a frequency of 5 MHz, and a spectral analyzer (Multigon 500 M, Multigon Industries, Mount Vernon, NY) was used to determine mean blood velocity (MBV) as the weighted mean of the spectrum of Doppler shift frequencies. Brachial artery diameter was measured at end-diastole at 30 s intervals throughout each trial from images recorded on a DVD. Forearm blood flow (FBF) was calculated as $FBF = MBV \times \pi \times (diameter \div 2)^2$ and expressed in ml/min and forearm vascular conductance (FVC) was calculated as $FVC = FBF \div MAP \times 100$ and expressed in ml/min/100 mmHg.

Endothelium-dependent and independent vasodilation

All vasoactive drugs were infused through the arterial catheter at low inflow rates (approximately 2 ml/min) to elicit local effects within the experimental forearm without affecting central hemodynamics. Doses were adjusted according to each participant's forearm volume (FAV) determined by dual X-ray absorptiometry.

Adenosine triphosphate (ATP; A7699, Sigma-Aldrich, St. Louis, MO, USA) and acetylcholine (ACh; Miochol-E, Novartis, Basel, Switzerland) were used to stimulate endothelium-dependent vasodilation and sodium nitroprusside (SNP; Hospira, Lake Forest, IL, USA) was used to stimulate direct vascular smooth muscle relaxation that occurs independently of the endothelium. Each drug was infused at a low dose with the intention of elevating FBF to approximately 3 times baseline FBF. ATP was infused at 1.28 μg/dl FAV/min in all participants, and the doses of ACh and SNP were adjusted to elicit similar steady-state vasodilation to that observed during ATP. Initially, doses of 1.50 μg ACh/dl FAV/min and 0.20 μg SNP/dl FAV/min were used; however, in subsequent participants, the doses were adjusted to better match average steady-state vasodilation (ACh, 1.48 ± 0.01, range: 1.36 – 1.50 μg/dl FAV/min; SNP, 0.28 ± 0.02, range: 0.18 – 0.35 μg/dl FAV/min). Due to time constraints, it was not possible to precisely match the response to each dilator within individual participants. However, the same dose of each dilator was used for the single dilator and combination trials (described below) within each participant.

Sympathetic α_1-adrenergic vasoconstriction

To assess sensitivity to sympathetic stimulation, the α_1-adrenergic receptor agonist phenylephrine (PE) was infused via the arterial catheter at 0.125 μg/dl FAV/min. The rate of PE was adjusted according to FBF in order to carefully control the sympathetic stimulus, as described previously (Dinenno & Joyner, 2003).

Experimental protocol

Figure 2.1 illustrates the general experimental protocol (A) and timeline for each trial (B). Vasodilation and α_1-adrenergic vasoconstrictor sensitivity were assessed during infusion of endothelium-dependent (ATP and ACh) and endothelium-independent (SNP) dilators alone and

in combination. Initially, the response to each dilator was evaluated separately in randomized order, then the following combinations were tested in randomized order: 1) ATP + ACh, 2) SNP + ACh, and 3) SNP + ATP.

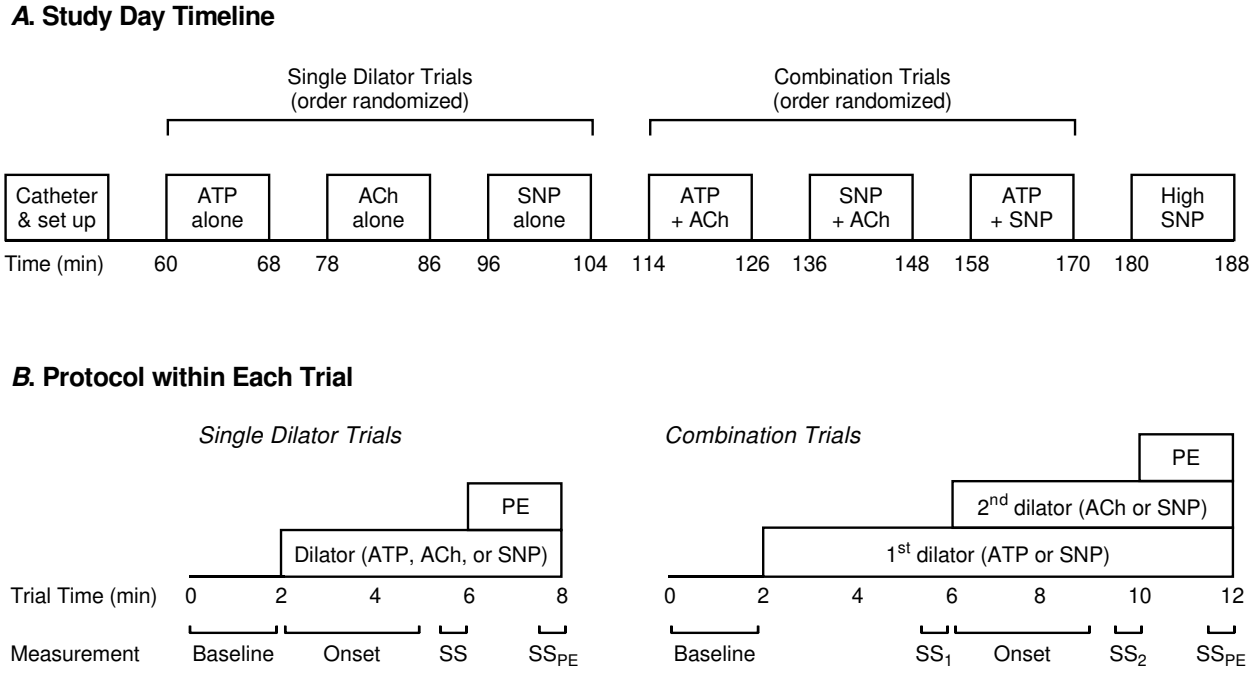


Figure 2.1: Experimental protocol.

A) Following brachial artery catheterization, vasodilation and α_1-adrenergic vasoconstrictor sensitivity were assessed during infusion of endothelium-dependent (ATP, ACh) and endothelium-independent (SNP) dilators alone and in combination in trials separated by ≥ 10 min. *B*) When each dilator was infused separately, baseline values were recorded, then the onset of vasodilation was assessed for 3 min. After steady-state (SS) measurements were obtained, the vasoconstrictor response to PE infusion was assessed with steady-state measurements at 2 min of PE infusion (SS_{PE}). In the dilator combination trials, the first dilator was infused for 3 min to achieve steady-state vasodilation (SS_1), then the second dilator was co-infused. The onset of vasodilation to the second dilator was recorded for 3 min, after which steady-state (SS_2) measurements were obtained and PE was infused for 2 min. ATP, adenosine triphosphate; ACh, acetylcholine, SNP, sodium nitroprusside

Single dilator trials

In each trial, baseline hemodynamics were assessed during 2 min of saline infusion, then the onset of vasodilation was recorded over the initial 3 min of dilator infusion. Steady-state

hemodynamics were assessed from minutes 3-4, then PE was infused for 2 min and hemodynamics were measured in the final 30 s of the trial. The dose of PE was adjusted according to FBF to match the concentration of PE in the blood across trials, as described previously by our laboratory (Kirby et al., 2008, 2011).

Combination trials

Baseline hemodynamics were recorded during 2 min of saline infusion prior to each trial, after which the first dilator was infused for 4 min to obtain steady-state values. The second dilator was then co-infused and the onset of vasodilation to the second dilator was assessed over 3 min. Following steady-state measurements of combined dilator infusion, PE was infused for 2 min and hemodynamics were measured in the final 30 s of the trial.

High SNP trial

Because we hypothesized that vasodilation would be augmented during combination of two endothelium-dependent dilators (ATP + ACh) compared to combination of either dilator with SNP (ATP + SNP and SNP + ACh), an additional trial was performed in which a high dose of SNP (2.52 ± 1.8 μg/dl FAV/min) was used to elicit vasodilation comparable to that observed in the ATP + ACh trial. The purpose of this trial was to serve as a "high flow" control condition to better assess vasoconstrictor sensitivity to PE.

Data acquisition and analysis

Data were collected at 250 Hz and stored on a computer for analysis with signal processing software (Windaq; DATAQ Instruments). Baseline hemodynamics reflect 30 s average values immediately preceding the start of dilator infusions. The onset of vasodilation was assessed in 3 s bins throughout the initial 3 min of infusion, and steady-state hemodynamics

reflect 30 s average values obtained between minutes 3 and 4 of each dilator infusion. Because we were limited in our ability to precisely match the hyperemic response to individual dilators within each participant, steady-state hemodynamics varied within participants prior to infusion of the second dilator. Therefore, vasodilatory responses were expressed as the absolute change in FVC from baseline (single dilator trials) or the first dilator (combination trials). Hemodynamics during PE reflect 30 s average values at the end of 2 min of PE infusion. Vasoconstrictor sensitivity to PE was calculated as $(FVC_{PE} - FVC_{Pre\text{-}PE}) \div FVC_{Pre\text{-}PE} \times 100$ and expressed as the percent change in FVC.

Statistics

All values are presented as means ± SE. Baseline hemodynamics prior to each trial were compared using one-way, repeated measures ANOVA. The onset of vasodilation was compared using two-way, repeated-measures ANOVA (drug × time) of 15 s time-binned averages. Steady-state responses to each dilator were compared using one-way, repeated-measures ANOVA, and hemodynamics before and after PE were compared using two-way, repeated-measures ANOVA (drug × condition: pre vs. post PE). Sensitivity to α_1-adrenergic vasoconstriction was compared with one-way, repeated-measures ANOVA. Tukey's post-hoc testing was performed when appropriate based on significant main effects or interactions between factors. In the event of a non-normal distribution, data were log transformed for analysis. All comparisons were performed using R statistical software, and significance was evaluated as $P < 0.05$.

RESULTS

Onset and steady-state vasodilation

Resting hemodynamics were similar prior to the start of all trials (Table 2.1). The time course of dilation when each dilator was infused individually is presented in Fig. 2.2*A* & *C*.

Infusion of ATP or ACh caused rapid vasodilation that peaked within 60-75 s, then declined until reaching a steady-state plateau. Vasodilation to SNP occurred more slowly and reached a plateau by ~105 s. As intended, steady-state vasodilation was similar with each dilator (Figs. 2.2*A* & *C* and 3*A*).

Table 2.1: FVC at baseline and during the first dilator

	FVC (ml/min/100 mmHg)	
	Baseline	First Dilator
Single Dilator Trials		
ATP	23 ± 2	—
ACh	21 ± 1	—
SNP	23 ± 1	—
Combination Trials		
ATP + ACh	21 ± 2	65 ± 7*
SNP + ACh	23 ± 2	73 ± 10*
ATP + SNP	21 ± 2	64 ± 4*
High SNP	21 ± 2	—

Values are means ± SE. Statistical comparisons were performed using one-way and two-way (drug × time), repeated-measures ANOVA. FVC, forearm vascular conductance. *$P < 0.05$ *vs.* baseline within trial.

In the combination trials, steady-state hemodynamics for were similar during administration of the first dilator (Table 2.1). However, because sensitivity to each dilator varied within individual participants, FVC was not precisely matched during the first dilator within each participant. Therefore, vasodilation was assessed as the change in FVC from the first dilator. The onset of vasodilation when the second dilator was co-infused is presented in Fig. 2.2*B* and *D*. Combination of the two endothelium-dependent dilators (ATP + ACh) caused a steady rise in FVC and resulted in sustained vasodilation that was greater than combination of either dilator with SNP (Figs. 2.2*B* & *D* and 2.3*B*). In the SNP + ACh trial, addition of ACh resulted in a rapid

peak at 45 s; however, vasodilation was temporary and FVC gradually declined toward baseline over the course of 3 min. In the ATP + SNP trial, addition of SNP resulted in a slow onset of vasodilation that reached a plateau by ~2 min.

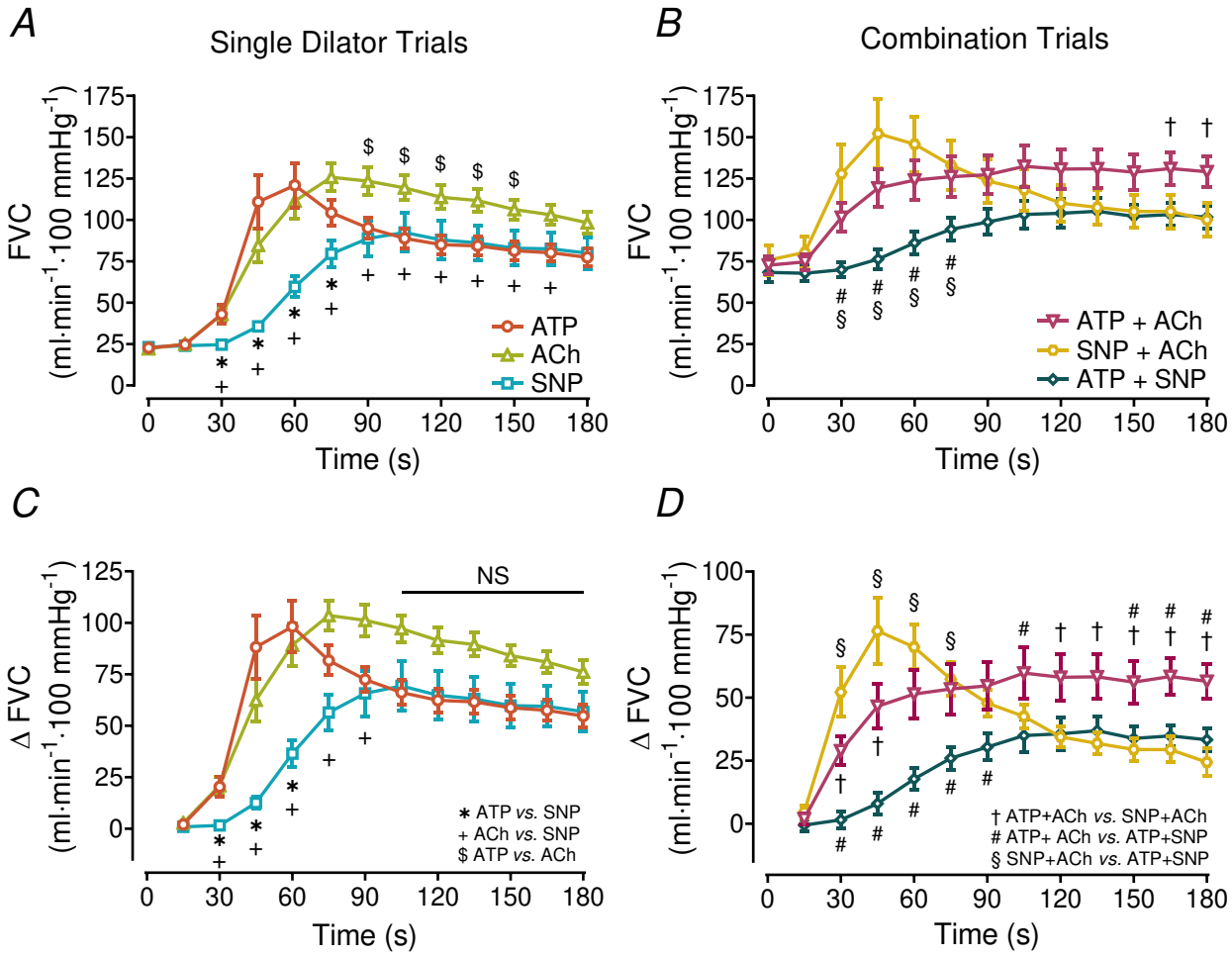


Figure 2.2: Onset of vasodilation.

ATP and ACh each evoked rapid vasodilation when administered separately (*A* & *C*), whereas the onset of vasodilation to SNP occurred more slowly. By 105 s of dilator infusion, Δ FVC was similar between trials (*C*). The onset of vasodilation upon co-infusion of a second dilator is shown in panels *B* & *D*. The combination of two endothelium-dependent dilators (ATP + ACh) caused sustained vasodilation that was greater than combination of either dilator with SNP from 105 s onward. In contrast, combination of SNP + ACh evoked rapid vasodilation that peaked at 45 s, then declined toward baseline, whereas combination of ATP + SNP resulted in a slow onset of vasodilation at a lesser degree than ATP + ACh (*D*). Data represent means ± SE for $n = 11$ participants (5 women, 6 men). FVC, forearm vascular conductance. * $P < 0.05$ ATP *vs.* SNP; $^{+}$ $P < 0.05$ ACh *vs.* SNP; $^{\$}$ $P < 0.05$ ATP *vs.* ATP; † $P < 0.05$ ATP + ACh *vs.* SNP + ACh; $^{\#}$ $P < 0.05$ ATP + ACh *vs.* ATP + SNP; § $P < 0.05$ SNP + ACh *vs.* ATP + SNP

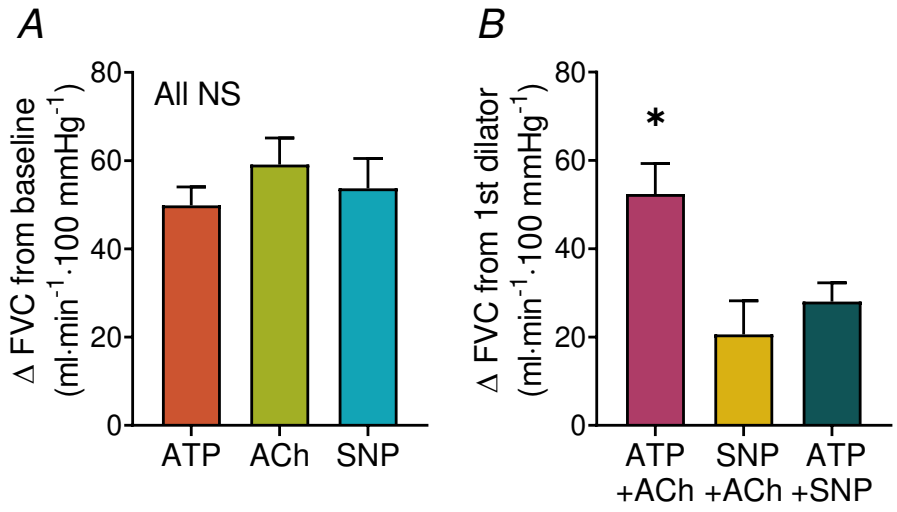


Figure 2.3: Steady-state vasodilation.

Each dilator evoked similar vasodilation when administered separately (*A*). Combination of the endothelium-dependent dilators ATP + ACh resulted in greater vasodilation than combination of either dilator with SNP (*B*). Data represent means ± SE for $n = 11$ participants (5 women, 6 men). * $P < 0.05$ *vs.* other combinations

Sympathetic α_1-adrenergic vasoconstriction

Vasoconstrictor sensitivity to PE during the individual dilator trials is presented in Fig. 2.4*A* & *C*. Steady-state FVC was similar during infusion of each dilator prior to administration of PE (Fig. 2.4*A*). The vasoconstrictor response to PE was greater in the SNP trial compared to ATP or ACh (Fig. 2.4*A* & *C*).

Vasoconstrictor sensitivity to PE during the combination dilator trials is presented in Fig. 2.4*B* & *D*. Prior to administration of PE, steady-state FVC was greater in the ATP + ACh trial compared to other trials. Administration of PE reduced FVC to a similar level during infusion of SNP + ACh, ATP + SNP, and High SNP, whereas vasoconstriction was attenuated when the endothelium-dependent dilators were combined in the ATP + ACh trial (Fig. 2.4*B* & *D*).

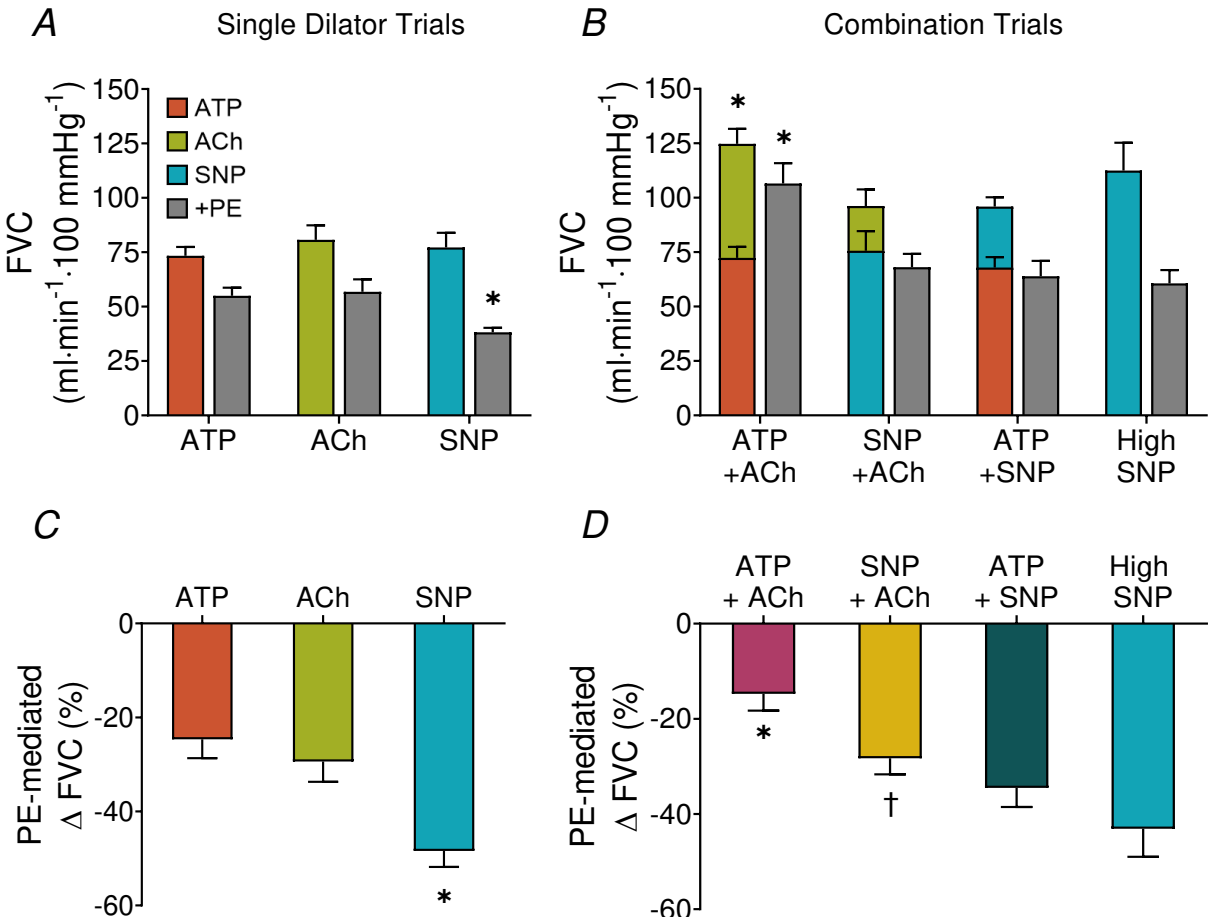


Figure 2.4: Endothelium-dependent dilators combine to attenuate α_1-adrenergic constriction.

When administered separately, ATP and ACh limited vasoconstriction to PE compared to SNP (*A* & *C*). Steady-state FVC was higher during combination of ATP + ACh compared to other trials (*B*) and combination of the endothelium-dependent dilators limited vasoconstriction to PE compared to other combinations or High SNP (*D*). Sensitivity to α_1-adrenergic vasoconstriction was further reduced during combination of ATP + ACh (*D*) than when either dilator was administered alone (*C*). Data represent means ± SE for $n = 11$ participants (5 women, 6 men). * $P < 0.05$ *vs.* all other single dilators or combinations at same time point; † $P < 0.05$ *vs.* High SNP

DISCUSSION

The present study was designed on the premise that the vascular endothelium simultaneously processes numerous stimuli and integrates information in a complex manner to elicit an appropriate vasomotor response. We tested the hypotheses that stimulating the endothelium with a combination of ATP and ACh would: 1) elicit greater vasodilation and 2)

limit α_1-adrenergic vasoconstriction compared to the combined action of either dilator with SNP, which acts directly on vascular smooth muscle. The results demonstrate that endothelium-dependent agonists interact to augment vasodilation and attenuate α_1-adrenergic vasoconstriction, whereas such interactions do not occur when either agonist is paired with an endothelium-independent vasodilator. These findings highlight the unique capacity of the endothelium to synthesize inputs from multiple vasoactive stimuli in human skeletal muscle.

Onset and steady-state vasodilation

Recent *ex vivo* evidence revealed a synergistic effect of ATP and carbachol, an ACh analogue, to increase the magnitude and alter the time course of Ca^{2+} signaling within the endothelium (Lee *et al.*, 2018); yet, it was previously unknown whether such interactions occur or affect vascular tone in human skeletal muscle. Therefore, in the present study, we evaluated whether intra-arterial infusion of ATP enhances vasodilatory sensitivity to ACh. The results demonstrate that combination of ATP + ACh causes sustained vasodilation that is augmented compared to combination of either dilator with SNP (Figs. 2.2*D* & 2.3*B*). Interestingly, although both ATP and ACh initiate rapid vasodilation that peaks within ~60 s when administered separately, the onset of vasodilation to ACh occurred more slowly and lacked the characteristic peak when it was co-infused on top of ATP. In contrast, the rapid peak still occurred when ACh was co-infused on top of SNP. These findings suggest that ATP interacts with ACh to influence the time course and magnitude of vasodilation, which adds to the growing body of evidence that stimulating the endothelium heightens its sensitivity to further activation.

In the combination trials, the response to co-infusion of a second dilator was attenuated such that the vasodilatory response to each dilator was not additive when two dilators were combined. When administered separately, the dose of each dilator increased FVC by

approximately 50-60 ml/min/100 mmHg (Fig. 2.2*A*). In contrast, the same dose increased FVC by only 20-30 ml/min/100 mmHg when it was infused as the second dilator during the SNP + ACh and ATP + SNP trials (Fig. 2.2*B*). Thus, the vasodilatory response to the second dilator was only preserved when two endothelium-dependent dilators were combined during the ATP + ACh trial, where FVC increased by an additional ~50 ml/min/100 mmHg (Fig. 2.2*B*). Although the reasons for this are unclear, we speculate that the elevation in blood flow generated by the first dilator may have diluted the concentration of the second dilator in the arterial circulation. Alternatively, it is possible that the overperfusion of resting tissue relative to its metabolic demand may have caused other mechanisms to restrain vasodilation (Hester *et al.*, 1982; Welsh *et al.*, 1998). Therefore, while vasodilation was clearly enhanced during combination of the two endothelium-dependent dilators compared to other combinations, we are limited by our approach of sequentially adding two dilators.

Sympathetic α_1-adrenergic vasoconstriction

Accumulating evidence suggests that the endothelium merges inputs from vasodilatory stimuli and the sympathetic nervous system to regulate vascular tone (Segal, 2015). In a hamster cremaster preparation, hyperpolarization arising from the endothelium limits sympathetic vasoconstriction (Kurjiaka & Segal, 1995), and in humans, infusion of ATP reduces sensitivity to α-adrenergic agonists (Kirby *et al.*, 2008). These examples of myoendothelial communication have important implications for blood flow distribution when sympathetic nerve activity is elevated. During whole-body exercise, sympathetic outflow increases to muscle and splanchnic circulations; yet, sensitivity to sympathetic vasoconstriction is reduced within the vasculature supplying contracting skeletal muscle (Remensnyder *et al.*, 1962). Although the mechanisms underlying functional sympatholysis are unknown, we previously demonstrated that stimulating

the endothelium with low doses of ATP or ACh enhances sympatholysis in active muscle (Hearon *et al.*, 2016). This reflects a specific ability of the endothelium to modulate α_1-adrenergic vasoconstriction, as similar results are not observed during infusion of SNP or KCl, which act directly on vascular smooth muscle to cause vasodilation.

Therefore, in the present study, we sought to determine whether endothelium-dependent agonists interact to further limit α_1-adrenergic vasoconstriction. When administered separately, ATP and ACh each reduced sensitivity to the α_1-adrenergic receptor agonist PE (Fig. 2.4*C*). Combined infusion of ATP + ACh further limited sympathetic vasoconstriction compared to either dilator alone (Fig. 2.4*C* & *D*; $P = 0.03$ *vs*. ATP alone; $P = 0.02$ *vs.* ACh alone) or in combination with SNP. Indeed, because steady-state vascular conductance was approximately 2-fold higher during ATP + ACh (Fig. 2.4*B*) compared to infusion of either dilator alone (Fig. 2.4*A*) and sensitivity to PE is assessed as a percentage change, this comparison likely underestimates the effects of combined infusion to restrain sympathetic constriction. These findings suggest that combined activation of the endothelium enhances its capacity to regulate vascular tone through specific interactions with post-junctional α_1-adrenergic signaling, which further emphasizes the role of the endothelium in integrating information from a multitude of inputs.

Potential cellular signaling mechanisms

Many chemical and mechanical stimuli that act on the endothelium are transduced as localized cytosolic Ca^{2+} signals which give rise to endothelium-dependent hyperpolarization and stimulate production of vasodilatory autacoids. The results of this study indicate an interaction between ATP and ACh, which each bind to $G_{q/11}$ coupled protein receptors on the endothelium. Activation of these receptors mobilizes Ca^{2+} from both intracellular stores and influx across the

cell membrane, and the resulting rise in cytosolic Ca^{2+} propagates to neighboring ECs, possibly by direct exchange of Ca^{2+} or IP_3 via gap junctions or through the spread of hyperpolarization, which enhances Ca^{2+} entry (Behringer & Segal, 2015). Interestingly, ATP and ACh initiate distinctive Ca^{2+} oscillations which are influenced by the presence of the other agonist such that combined activation of ATP and ACh receptors generates a new, synergistic Ca^{2+} signal (Lee *et al.*, 2018). Ultimately, a rise in cytosolic Ca^{2+} stimulates production of autacoids such as nitric oxide and prostaglandins and opens Ca^{2+}-activated K^+ (K_{Ca}) channels. Release of K^+ through these channels initiates hyperpolarization that spreads through gap junctions to adjacent ECs and VSMCs, where it activates inwardly-rectifying (K_{IR}) channels to further efflux K^+ and boost the electrical signal. In the end, hyperpolarization of vascular smooth muscle inhibits entry of Ca^{2+} through voltage-gated Ca^{2+} channels to promote relaxation and vasodilation.

The cellular signaling mechanisms by which ATP and ACh interact to augment vasodilation and limit α_1-adrenergic vasoconstriction are unclear. Further, it is important to note that the effects on vasodilation and sympathetic constriction may be mediated by different mechanisms. Membrane potential hyperpolarization enhances Ca^{2+} entry in ECs in response to ACh (Behringer & Segal, 2015), and augmented Ca^{2+} signaling like that reported in the rat carotid endothelium during combined application of ATP + ACh could increase vasodilation through several mechanisms, including electrical signaling and release of autacoids. Activation of K_{IR} channels amplifies hyperpolarization, and we and others have observed that K_{IR} channels enhance endothelium-dependent vasodilation in a variety of circumstances (Rivers *et al.*, 2001; Smith *et al.*, 2008; Sonkusare *et al.*, 2016; Hearon *et al.*, 2019). In contrast to the wide array of mediators which could enhance vasodilation, the ability of the endothelium to limit sympathetic vasoconstriction appears to be more specific. Indeed, the sympatholytic properties of ATP

remain intact even during combined inhibition of K_{IR} channels, the Na^+/K^+-ATPase, and synthesis of nitric oxide and prostaglandins (Hearon *et al.*, 2017), which is in line with other observations that these pathways are not required for sympatholysis (Crecelius *et al.*, 2015; Hearon *et al.*, 2016). In rodent vessels, conducted vasodilation in response to endothelial stimulation opposes sympathetic vasoconstriction (Kurjiaka & Segal, 1995), and the conducted vasodilatory responses to ATP and ACh are mediated by activation of small- and intermediate-conductance K_{Ca} channels (Marchenko, 2002; Winter & Dora, 2007). Collectively, augmented Ca^{2+} signaling in response to combination of ATP + ACh coupled with the dense localization of K_{Ca} at myoendothelial projections (Ledoux *et al.*, 2008) points toward involvement of K_{Ca}-mediated electrical signaling in the ability of the endothelium to modulate sympathetic vasoconstriction.

Implications for blood flow regulation

Interactions involving ATP and ACh may have physiological relevance for controlling peripheral vascular tone. Endothelial cells and erythrocytes release ATP in response to deoxygenation and mechanical forces such as shear stress and deformation (Milner *et al.*, 1990; Faris & Spence, 2008; Wang *et al.*, 2015); thus, ATP serves as a vasodilatory signal in areas with low tissue PO_2 to help match O_2 delivery with metabolic demand (Ellsworth *et al.*, 2016). In humans, circulating levels of ATP are elevated during hypoxia and muscle contractions (González-Alonso *et al.*, 2002; Kirby *et al.*, 2012), which may serve to enhance the endothelium's sensitivity to further stimuli. In support of this idea, ATP potentiates flow-mediated vasodilation in isolated rat mesenteric arteries; indeed, the presence of ATP is required for sustained dilation in response to shear (Liu *et al.*, 2004). Likewise, an increase in flow rate enhances vasodilatory sensitivity to ATP. Although it is not clear whether endogenous ACh

plays a physiological role in blood flow regulation in humans (Joyner & Dietz, 2003), studies of cultured endothelial cells and isolated arteries suggest that endothelial cells synthesize and release ACh in response to shear stress to facilitate flow-mediated vasodilation (Milner *et al.*, 1990; Martin *et al.*, 1996; Wilson *et al.*, 2016). We have observed that infusion of exogenous ACh amplifies vasodilation in response to exercise (Hearon *et al.*, 2019), and both ATP and ACh augment functional sympatholysis within contracting muscle in humans (Hearon *et al.*, 2016).

The results of the present study indicate that activating the endothelium with multiple agonists enhances vasodilation and limits sympathetic vasoconstriction, which may have profound implications for blood flow regulation under physiological conditions. Interactions between vasoactive stimuli may enable the endothelium to effectively process several inputs and coordinate an appropriate response to regulate blood flow distribution. Indeed, the results of this study and others suggest that the presence of multiple stimuli may alter the threshold for eliciting an endothelium-dependent vasodilatory response (Liu *et al.*, 2004; Lee *et al.*, 2018; Hearon *et al.*, 2019). This may be particularly important during physiological stressors such as exercise and hypoxia, when a number of stimuli act in concert to regulate skeletal muscle blood flow. Moreover, the observation that endothelium-dependent agonists combine to limit α_1-adrenergic vasoconstriction provides insight regarding the mechanisms of functional sympatholysis, as a number of stimuli act on the microcirculatory endothelium within contracting skeletal muscle that may serve to attenuate sympathetic vasoconstriction. Further experiments will be required to determine whether such interactions occur to influence vascular tone under normal physiological circumstances.

Limitations

Due to time constraints, we were unable to precisely match the response to each dilator within individual participants. Thus, although each dilator caused similar vasodilation when assessed as a group average response, this was not always the case for individual participants. As a result, steady-state FVC varied during infusion of the first dilator in combination trials. Therefore, we assessed vasodilation to the second dilator as the absolute change in FVC to account for baseline differences. Our inability to carefully match vasodilation for each participant is a clear limitation that likely increased the variability of the results; nonetheless, the findings support the conclusion that endothelium-dependent agonists combine to promote greater vasodilation than is observed during combination with a direct smooth muscle dilator.

The present investigation was limited to three vasodilatory substances and one vasoconstrictor. Moreover, vasodilators were always infused in the same order during the combination trials. It is therefore unclear whether the interactions we observed between ATP and ACh also occur with other endothelium-dependent vasodilators, such as bradykinin and substance P, or whether the same interactions would occur if the order of dilators was reversed. Similarly, it is unclear whether combined activation of the endothelium also affects vasoconstriction mediated by other mechanisms, such as angiotensin II, endothelin, or α_2-adrenergic receptors. In rodent preparations, the distribution of receptors and sensitivity to many of these agonists is heterogeneous among neighboring endothelial cells (Tomlinson *et al.*, 1991; Marie & Bény, 2002), which is comparable to the distinct clusters of cells that respond to ATP or ACh (Lee *et al.*, 2018). Thus, we speculate that cellular characteristics such as the distribution of surface receptors and gap junctions within a population of endothelial cells and across different circulatory beds determine how various vasoactive stimuli interact. Ultimately, while we designed the present study to assess the role of endothelium-dependent *vs*.

endothelium-independent vasodilation, our interpretation is limited to interactions between the specific dilators we employed in the human forearm circulation.

Perspectives

Many patient populations at elevated risk for cardiovascular disease display both endothelial dysfunction and elevated sympathetic nervous system activity. Thus, understanding the interactions between vasodilator and vasoconstrictor signaling may lead to strategies to improve tissue blood flow and oxygen delivery in at-risk populations (Saltin & Mortensen, 2012). We have observed impairments in ATP release from erythrocytes of older adults which correspond to reductions in circulating ATP during exercise and systemic hypoxia (Kirby *et al.*, 2012). Therefore, therapeutic approaches to enhance circulating ATP in older adults (Racine & Dinenno, 2019) could potentially improve vasodilatory sensitivity to shear stress or ACh, which decline with age (Celermajer *et al.*, 1994; Gerhard *et al.*, 1996), while also improving the capacity to overcome exaggerated sympathetic activation.

CONCLUSIONS

The present study was designed to investigate potential interactions between endothelium-dependent vasodilators and sympathetic vasoconstriction. The findings demonstrate that the endothelium-dependent agonists ATP and ACh combine to augment vasodilation compared to that observed when either agonist is paired with the endothelium-independent dilator SNP, which suggests that activation of the endothelium enhances its sensitivity to further stimuli. In addition to augmenting vasodilation, the combination of ATP and ACh attenuates α_1-adrenergic vasoconstriction in resting skeletal muscle, which has implications for functional sympatholysis. Collectively, these observations highlight the endothelium as a key site for integrating multiple vasoactive inputs to efficiently regulate tissue blood flow. Yet, how such

interactions occur to regulate vascular tone in humans under physiological conditions remains to be determined.

CHAPTER 3 – MANUSCRIPT 2

Endogenous ACh limits α_1-adrenergic vasoconstriction in contracting skeletal muscle of humans

INTRODUCTION

The hemodynamic response to whole-body exercise entails an increase in sympathetic nervous system activity that serves to increase cardiac output and regulate systemic vascular resistance. Sympathetic vasoconstriction limits blood flow to inactive tissues, which facilitates redistribution of blood to metabolically-active skeletal muscle without compromising arterial blood pressure. Although elevated sympathetic outflow is also directed toward active skeletal muscle, α-adrenergic vasoconstriction is dramatically attenuated within the vasculature of contracting muscle (Remensnyder *et al.*, 1962). This ability of working skeletal muscle to limit sympathetic vasoconstriction, termed 'functional sympatholysis,' is essential to ensure adequate blood flow to the active muscle. In older adults and some patient populations, the ability of working muscle to modulate sympathetic constriction becomes impaired (Koch *et al.*, 2003; Dinenno *et al.*, 2005; Vongpatanasin *et al.*, 2011), which restricts oxygen consumption, increases blood lactate, and elevates arterial pressure (Mortensen *et al.*, 2012). Thus, elucidating the signaling mechanisms that underlie functional sympatholysis is an important goal with implications for exercise intolerance in aging and clinical populations (Saltin & Mortensen, 2012).

The mechanisms that modulate post-junctional α-adrenergic signaling within active skeletal muscle remain poorly understood (Saltin & Mortensen, 2012). A variety of substances including nitric oxide, prostaglandins, and potassium have been proposed as potential mediators

of functional sympatholysis (Rosenmeier *et al.*, 2003; Dinenno & Joyner, 2004; Keller *et al.*, 2004; Crecelius *et al.*, 2015); yet the signaling pathways capable of blunting sympathetic constriction in humans have been challenging to elucidate. To date, no study employing pharmacological inhibition to investigate potential mechanisms in humans has clearly impacted the ability of contracting muscle to blunt sympathetic vasoconstriction (Rosenmeier *et al.*, 2003; Dinenno & Joyner, 2004; Keller *et al.*, 2004; Crecelius *et al.*, 2015). Indeed, functional sympatholysis remains intact even during combined inhibition of nitric oxide, vasodilatory prostaglandins, inwardly-rectifying potassium channels, and the sodium/potassium pump (Crecelius *et al.*, 2015).

Thus, our current understanding relies on studies which have employed exogenous administration of vasoactive molecules to mimic functional sympatholysis. Mounting evidence from these studies suggests that the vascular endothelium is a critical site for integration of local vasodilatory and sympathetic vasoconstrictor signaling (Behringer & Segal, 2012*b*; Kerr *et al.*, 2012; Hearon *et al.*, 2016; Terwoord *et al.*, 2017), and evidence from experimental animals suggests that hyperpolarization originating in the endothelium limits sympathetic vasoconstriction in a variety of vascular beds (Behringer & Segal, 2012*b*). In rodents, application of the endothelium-dependent vasodilator acetylcholine (ACh) elicits vascular hyperpolarization and blunts sympathetic vasoconstriction in skeletal muscle resistance arterioles (Kurjiaka & Segal, 1995), and in humans, intra-arterial infusion of ACh enhances functional sympatholysis during mild intensity exercise (Hearon *et al.*, 2016).

Collectively, these observations suggest a potential role of ACh in vascular control within contracting muscle. Therefore, in the present study, we tested the hypothesis that endogenous ACh contributes to functional sympatholysis in humans. The results indicate that muscarinic

ACh receptors modulate α_1-adrenergic vasoconstriction during heavy intensity exercise, demonstrating a novel, functional role of ACh receptors in the peripheral vasculature in humans.

METHODS

Participants and ethical approval

All experimental procedures were approved by the Institutional Review Board at Colorado State University (protocol 14-5392H) and performed in accordance with The Declaration of Helsinki except for registration in a database. Sixteen healthy volunteers (8 women, 8 men; 26 ± 1 y; 25 ± 2% body fat; means ± SE) completed the study after providing written, informed consent. Participants were free from cardiovascular disease and reported the following medications: levothyroxine and a selective serotonin reuptake inhibitor (1 participant) and oral contraceptives (4 participants).

Instrumentation

Participants arrived at the laboratory in the morning following an overnight fast. Studies were performed in a cool (19-20°C), temperature-controlled laboratory where participants lay supine with the experimental (non-dominant) arm abducted 90° and supported slightly above heart level. Following local anesthesia with 2% lidocaine, a 20 g, 7 cm catheter was introduced to the brachial artery using sterile technique. The catheter was continuously flushed with heparinized saline and connected to a 3-way port for local drug administration. Heart rate (HR) was monitored with a 3-lead ECG (Cardiocap/5) and arterial pressure was continuously recorded with a transducer connected to the catheter. A fan was directed at the experimental forearm throughout the study to minimize skin blood flow.

Forearm blood flow and vascular conductance

Brachial artery diameter and blood velocity were measured with a 12 MHz linear array Doppler ultrasound probe (Vivid7, General Electric, Milwaukee, WI) proximal to the catheter to measure forearm blood flow (FBF). Beat-by-beat velocity was measured at a frequency of 5 MHz using an insonation angle of 60° and mean blood velocity (MBV) was determined as the weighted mean of the spectrum of Doppler shift frequencies (Multigon 500 M, Multigon Industries, Mount Vernon, NY). Diameter was measured in triplicate at end-diastole and FBF was calculated as $FBF = \pi \times (diameter \div 2)^2 \times MBV$ and expressed in ml/min. Forearm vascular conductance (FVC) was evaluated as an index of vascular tone. FVC was calculated as $FVC = FBF \div MAP \times 100$ and expressed in ml/min/100 mmHg.

Rhythmic handgrip exercise

Participants performed dynamic handgrip contractions to lift a weight connected to a pulley system. The weight corresponded to 15 or 25% of the participant's maximal voluntary contraction force (MVC; see experimental design below) and was lifted 4-5 cm at a rate of 20 contractions per minute (1 s contraction followed by 2 s relaxation).

Vasoactive drug administration

All drugs were infused through the arterial catheter at low flow rates (approximately 2 ml/min) to cause local effects within the forearm without altering systemic hemodynamics. Each participant's forearm volume (FAV) was determined prior to the study using dual X-ray absorptiometry for normalization of drug doses.

Control vasodilator

In the resting trial, adenosine or sodium nitroprusside was infused as a control vasodilator intended to match steady-state hyperemia to that observed during exercise at 15% MVC (see Experimental Protocol). Adenosine and sodium nitroprusside were selected because they do not exhibit sympatholytic properties in our laboratory (Kirby *et al.*, 2008; Crecelius *et al.*, 2015; Hearon *et al.*, 2016). Based on drug availability, six participants (4F, 2M) received adenosine with a final dose of 19 ± 7 µg/dl FAV/min and five participants (2F, 3M) received sodium nitroprusside with a final dose of 4.8 ± 0.3 µg/dl FAV/min.

Sympathetic α_1-adrenergic vasoconstriction

To evaluate sympathetic vasoconstrictor sensitivity, phenylephrine (PE, α_1 agonist) was infused at a dose of 0.125 µg/dl FAV/min. In an effort to normalize the concentration of PE in the arterial inflow, the rate of PE was adjusted according to forearm blood flow, as described previously (Dinenno & Joyner, 2003).

ACh dose-response

The response to infusion of exogenous ACh was assessed under control conditions and following inhibition of muscarinic ACh receptors to determine blockade efficacy. ACh was infused at doses of 4, 8, and 16 µg/dl FAV/min for 3 min per dose.

Muscarinic ACh receptor blockade

Following control trials, atropine (muscarinic ACh receptor antagonist) was infused to inhibit ACh receptors. Initially, a total of 0.2 mg was administered over the course of 3 min as a loading dose (Shoemaker *et al.*, 1997; Brock *et al.*, 1998). Prior to subsequent trials, an additional 0.07 mg was infused over 1 min as a maintenance dose. Infusion of atropine always

occurred as a bolus dose approximately 2 min before the start of each trial; atropine was not continuously infused during exercise or vasodilator administration.

Experimental protocol

An overview of the experimental protocol is shown in Figure 3.1. To determine the role of ACh in functional sympatholysis, we evaluated sensitivity to α_1-adrenergic vasoconstriction at rest and during rhythmic handgrip exercise under control conditions and following local inhibition of muscarinic ACh receptors.

Because sympatholysis is graded with exercise intensity, we assessed sympathetic vasoconstriction during both moderate (15% MVC) and heavy (25% MVC) intensity handgrip exercise. In the resting trial, we infused a vasodilator (adenosine or sodium nitroprusside) to match forearm blood flow to that observed during 15% MVC exercise; this served as a "high flow" control condition to compare sympathetic vasoconstriction from a similar starting level of hyperemia. At the beginning of each trial, baseline data were collected for 2 min, then the hyperemic condition (dilator infusion or forearm exercise) began (Fig. 3.1*A*). Steady-state hemodynamics were assessed at 4 min of dilator infusion or exercise, then PE was infused for 2 min. Data reflect 30 s of steady-state measurements at the end of each time point (baseline, pre-PE, and PE).

Following control trials, muscarinic ACh receptors were blocked with intra-arterial atropine and trials were repeated. The response to graded doses of exogenous ACh was evaluated under control conditions and following atropine to determine blockade efficacy. All trials were separated by ≥ 10 min and the order of trials was counterbalanced across participants.

Data acquisition and analysis

Data were recorded at 250 Hz and stored on a computer for later analysis with signal processing software (Windaq; DATAQ Instruments). Hemodynamic values reflect 30 s of steady-state measurements as illustrated in Fig. 3.1. Sensitivity to α_1-adrenergic vasoconstriction was calculated as the PE-mediated change in FVC and expressed as a percentage of FVC prior to PE infusion: $\Delta\ FVC_{PE}\ (\%) = (FVC_{PE} - FVC_{SS}) \div FVC_{SS} \times 100$.

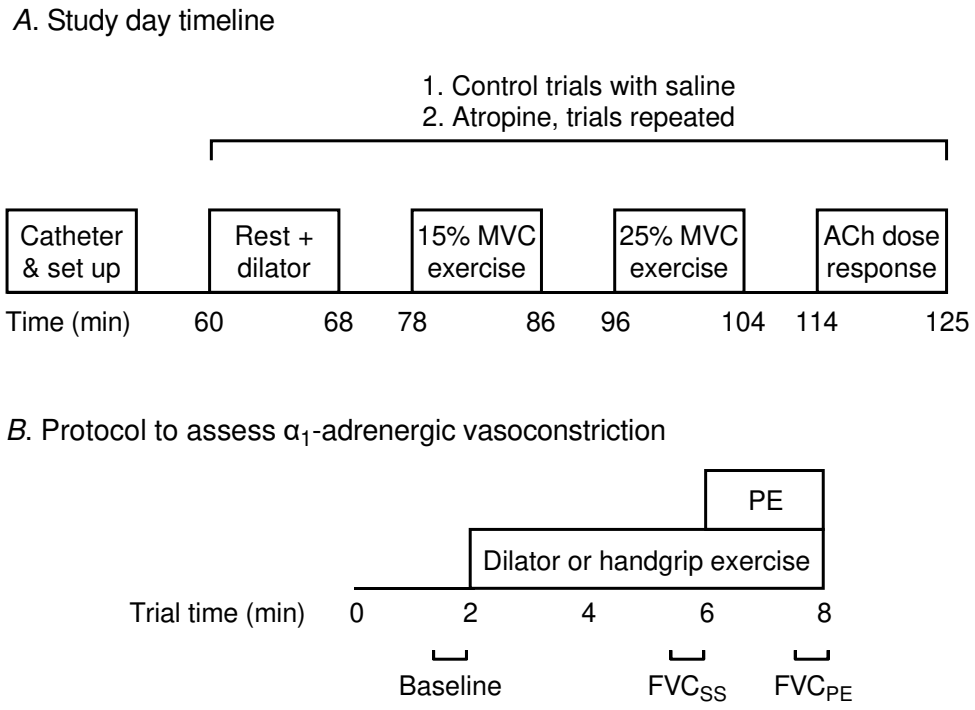


Figure 3.1: Experimental protocol.

An overview of the study timeline is shown in *A*. Sympathetic vasoconstrictor sensitivity was assessed during separate trials at rest and during handgrip exercise at 15 and 25% MVC; the timeline for each trial is illustrated in *B*. Participants performed rhythmic handgrip exercise for 4 min to assess steady-state hemodynamics, then PE (α_1-adrenergic agonist) was infused and hemodynamics were assessed again after 2 min. In the resting trial, a control vasodilator was infused to match steady-state forearm blood flow to that observed during exercise at 15% MVC prior to PE infusion. Sensitivity to infusion of exogenous ACh was evaluated in a separate trial. Following control trials, a bolus dose of atropine was infused to block muscarinic acetylcholine receptors and trials were repeated. Trials were separated by ≥10 min rest and the order of trials within control and atropine conditions was counterbalanced across participants. FVC, forearm vascular conductance; MVC, maximal voluntary contraction; PE, phenylephrine; SS, steady-state

Statistics

All values are means ± SE. Many comparisons are possible with this experimental design; therefore, we have focused on the most relevant statistical analyses to address our hypotheses. All comparisons were performed using R statistical programming. Due to the large increase in blood flow as a result of exercise or control dilator infusion, baseline values prior to each trial (Table 3.1 & Fig. 3.2) were analyzed separately and compared using two-way (drug × trial), repeated-measures ANOVA. Steady-state values during the hyperemic trials (Table 3.1 & Fig. 3.2) were compared with three-way (drug × trial × time), repeated-measures ANOVA, and vasoconstriction to PE (Fig. 3.3) was assessed with two-way (drug × trial), repeated-measures ANOVA. The vasodilatory response to ACh infusion (Fig. 3.4) was compared with two-way (drug × ACh dose), repeated-measures ANOVA. Data were log transformed prior to analysis when appropriate as a result of non-normal distributions. Statistical significance was determined as $P < 0.05$, and Tukey's post hoc tests were performed in the event of significant main or interaction effects.

RESULTS

Sympathetic α_1-adrenergic vasoconstriction

Baseline hemodynamics were similar prior to each trial (Table 3.1 & Fig. 3.2). As intended, dilator infusion in the rest trial elevated steady-state FBF and FVC to a level comparable to that observed during exercise at 15% MVC (Table 3.1 & Fig. 3.2). Under control conditions, infusion of PE reduced FVC to a greater extent at rest compared to during exercise at 15% or 25% MVC (Fig. 3.2). Sympathetic vasoconstrictor sensitivity was limited in parallel with exercise intensity (Fig. 3.3*B*), indicating functional sympatholysis. Atropine had no effect on baseline or steady-state hemodynamics prior to PE in the rest trial or during exercise (Table 3.1

& Fig. 3.2). Atropine did not alter sympathetic vasoconstrictor sensitivity at rest or during exercise at 15% MVC; however, atropine attenuated functional sympatholysis during exercise at 25% MVC such that the vasoconstrictor response to PE increased approximately two-fold (Fig. 3.3).

Blockade efficacy

Under control conditions, infusion of exogenous ACh caused robust vasodilation (Fig. 3.4). Atropine reduced vasodilation to progressive doses of ACh by 66 ± 4, 58 ± 6, and 46 ± 5%.

Table 3.1: Forearm and systemic hemodynamics

	Control			Atropine		
	Baseline	Pre-PE	PE	Baseline	Pre-PE	PE
Forearm blood flow (*ml/min*)						
Rest + dilator	27 ± 2	155 ± 18	103 ± 18†‡	29 ± 2	162 ± 19	108 ± 17†‡
15% MVC	28 ± 3	179 ± 18	146 ± 13†‡	27 ± 2	190 ± 20	153 ± 16†‡
25% MVC	29 ± 3	320 ± 38‡	295 ± 30‡	32 ± 3	333 ± 36‡	272 ± 28*†‡
Mean arterial pressure (*mmHg*)						
Rest + dilator	87 ± 2	81 ± 2‡	84 ± 2†‡	88 ± 2	82 ± 2‡	85 ± 2†‡
15% MVC	88 ± 1	89 ± 2	93 ± 2†‡	89 ± 2	89 ± 1‡	93 ± 2†‡
25% MVC	87 ± 1	94 ± 2	100 ± 3†‡	90 ± 2	95 ± 2‡	101 ± 3†‡
Heart rate (*beats/min*)						
Rest + dilator	51 ± 2	55 ± 3	54 ± 3	48 ± 2	59 ± 3	53 ± 3
15% MVC	52 ± 2	57 ± 2	54 ± 2	50 ± 2	54 ± 3	55 ± 3
25% MVC	50 ± 2	57 ± 2	55 ± 2	53 ± 3	59 ± 3	59 ± 3

Values are means ± SE. Data were not obtained for each trial in all participants, thus $n = 11$ for rest + dilator, $n = 16$ for 15% MVC, and $n = 15$ for 25% MVC. Abbreviations: MVC, maximal voluntary contraction; PE, phenylephrine. * $P < 0.05$ *vs.* control at same time point; † $P < 0.05$ *vs.* pre-PE within trial; ‡ $P < 0.05$ *vs.* all other trials at same time point (within drug).

DISCUSSION

The primary observation from the present study is that local inhibition of muscarinic ACh receptors with atropine reduces the ability of contracting skeletal muscle to modulate α_1-adrenergic vasoconstriction, which indicates a key role of ACh as a mechanism of functional

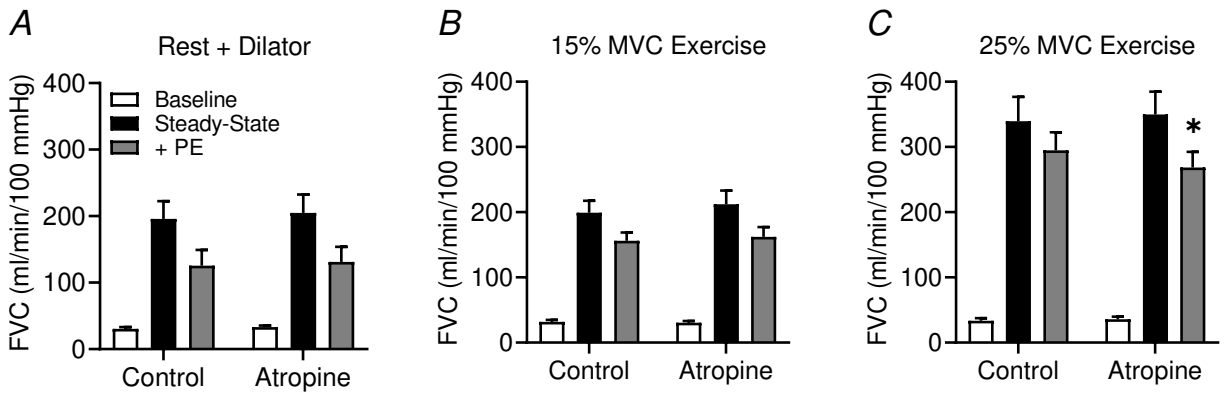


Figure 3.2: Forearm vascular conductance responses throughout each trial.

FVC is presented at baseline, steady-state hyperemia, and during infusion of phenylephrine (PE; α_1 agonist) for each trial. In the resting trial (*A*), a control vasodilator was infused to match steady-state FVC to that observed during 15% MVC exercise (*B*) prior to administration of PE. Atropine (muscarinic ACh receptor antagonist) did not affect FVC throughout the rest trial (*A*) or 15% MVC exercise trial (*B*). However, during 25% MVC exercise (*C*), FVC was significantly lower during infusion of PE under atropine compared to control conditions. FVC, forearm vascular conductance; MVC, maximal voluntary contraction. * $P < 0.05$ *vs.* control at same time point

sympatholysis. The results demonstrate that ACh receptors limit sympathetic vasoconstriction within active skeletal muscle during exercise at high workloads. This is the first study to reveal an essential mechanism of functional sympatholysis, which has important implications for understanding the pathophysiology of exercise intolerance in clinical populations. Moreover, the results demonstrate a novel, physiological role for ACh in blood flow regulation in humans.

Potential sources of ACh

Muscarinic receptors are ubiquitous throughout the peripheral vasculature; indeed, the vascular endothelium is so profoundly sensitive to ACh that the vasodilatory response to ACh infusion is the gold standard assessment of endothelial function. Yet, despite the close association between endothelial dysfunction and cardiovascular disease risk, numerous attempts to elucidate a physiological role of ACh in blood flow regulation have been unsuccessful

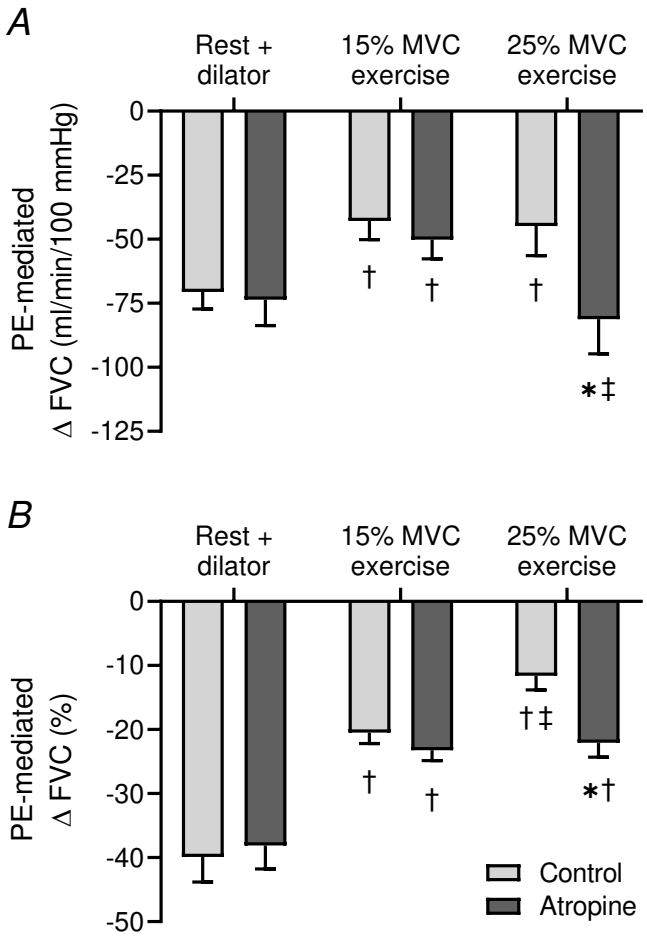


Figure 3.3: Atropine attenuates functional sympatholysis at higher exercise intensities.

The absolute and percent change in FVC in response to PE (α_1 agonist) are presented in *A* and *B*. Under control conditions, handgrip exercise progressively reduced α_1-adrenergic vasoconstriction (*B*), indicating functional sympatholysis. Atropine (muscarinic ACh receptor antagonist) did not affect sensitivity to PE at rest or during exercise at 15% MVC. However, in the 25% MVC exercise trial, PE elicited approximately 2-fold greater vasoconstriction during atropine compared to control conditions. MVC, maximal voluntary contraction; PE, phenylephrine. * $P < 0.05$ *vs.* control; † $P < 0.05$ *vs*. rest; ‡ $P < 0.05$ *vs*. 15% MVC

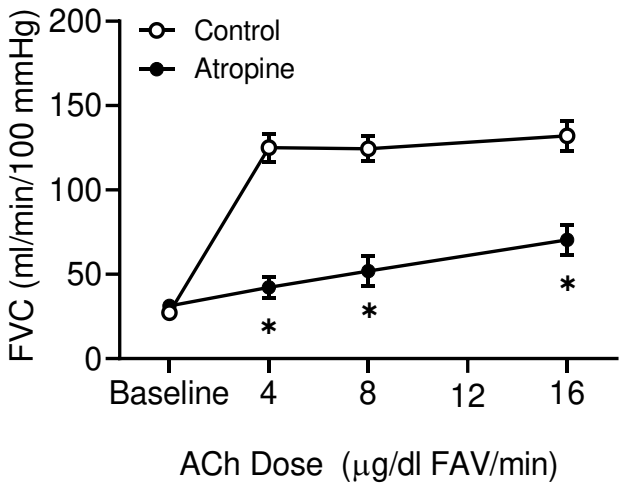


Figure 3.4: Atropine blocked vasodilation to exogenous ACh.

Intra-arterial infusion of atropine (muscarinic ACh receptor antagonist) successfully inhibited the forearm vasodilator response to graded infusions of ACh, as intended. ACh; acetylcholine, FAV; forearm volume; FVC; forearm vascular conductance. * $P < 0.05$ *vs.* Control

(Armstrong & Laughlin, 1986; Buckwalter *et al.*, 1997; Shoemaker *et al.*, 1997; Brock *et al.*, 1998). Historically, researchers investigated the possibility that ACh was released by sympathetic vasodilator nerves (Joyner & Halliwill, 2000; Joyner & Dietz, 2003), as the skeletal muscle circulation of many mammalian species is innervated by sympathetic cholinergic fibers that serve to increase muscle blood flow when sympathetic outflow is elevated (Bülbring & Burn, 1935; Bolme *et al.*, 1970). Initial studies supported this idea by demonstrating atropine-sensitive forearm vasodilation in response to mental or emotional stress (Blair *et al.*, 1959; Dietz *et al.*, 1994); yet, histochemical examination revealed no evidence of sympathetic cholinergic nerves in skeletal muscle of humans or other primates (Bolme & Fuxe, 1970; Bolme *et al.*, 1970). Later, the idea of sympathetic vasodilation was brought into further question when sympathetic nerve traffic recordings and stellate ganglion nerve blockade studies revealed that

such vasodilatory responses could occur in the absence of muscle sympathetic nerve activity (Lindqvist *et al.*, 1996; Halliwill *et al.*, 1997; Reed *et al.*, 2000). Although this is still a topic of some debate (Ishii *et al.*, 2013), these studies cast serious doubt on functional innervation of human skeletal muscle by sympathetic cholinergic nerves (Joyner & Dietz, 2003).

Motor neurons have also been investigated as a potential source of ACh that could act on the skeletal muscle vasculature. ACh released from motor nerve terminals binds to nicotinic receptors at the neuromuscular junction to initiate muscle contractions; thus, spillover of ACh to nearby resistance vessels could facilitate concomitant vasodilation in parallel with the degree of muscle activation (Fuglevand & Segal, 1997; Welsh & Segal, 1997). Kurjiaka & Segal (1995) demonstrated that application of ACh to resistance vessels in the hamster cremaster muscle limits vasoconstriction to sympathetic nerve stimulation; thus, they speculated that ACh released at the motor end plate could be a mechanism of functional sympatholysis. In a later study, they demonstrated that stimulation of motor nerves causes atropine-sensitive vasodilation within the cremaster muscle microcirculation even when muscle contractions were prevented via nicotinic receptor blockade (Welsh & Segal, 1997). However, Dyke et. al (Dyke *et al.*, 1998) employed a similar approach in humans in a subsequent study and reported only minor changes in forearm blood flow in response to attempted handgrip contractions following neuromuscular blockade. Hellsten and colleagues (2009) expanded on this in a study that employed partial neuromuscular blockade such that greater activation of motor neurons (and thus greater release of ACh) was required to perform knee extensor exercise at a given workload. Although femoral blood flow was enhanced during exercise with the partially paralyzed muscle, inhibition of ACh receptors did not alter the response. Furthermore, local inhibition of muscarinic receptors with intra-arterial atropine does not impact rapid vasodilation following a single muscle contraction in the

human forearm (Brock *et al.*, 1998); therefore, the idea that ACh released from motor neurons elicits skeletal muscle vasodilation has been largely disregarded.

The lack of clear evidence for neurogenic vasodilation led many researchers to conclude that local cholinergic mechanisms may account for atropine-sensitive changes in blood flow observed in human muscle during stressful situations. Cultured endothelial cells release ACh in response to shear (Milner *et al.*, 1990), and flow-induced nitric oxide production in isolated canine femoral arteries is mediated by ACh (Martin *et al.*, 1996). Recent studies of isolated rat carotid and mesenteric arteries demonstrate that endothelial cells synthesize and release ACh in response to fluid shear stress and that ACh acts as an autocrine signaling mechanism to stimulate nitric oxide production (Wilson *et al.*, 2016).

Taken together, there is a large body of work investigating sympathetic cholinergic nerves, motor nerves, and endothelial cells as potential sources of ACh that could regulate muscle blood flow. The experimental approach of the present study does not provide insight to the source of ACh; however, because there is no clear evidence of sympathetic vasodilator innervation of human skeletal muscle and muscle sympathetic nerve activity does not change during rhythmic handgrip exercise (Victor *et al.*, 1987), we do not expect that sympathetic cholinergic nerves contributed to our findings. Rather, it is likely that ACh released from endothelial cells mediates functional sympatholysis. Additionally, we cannot rule out motor nerves as a potential source of ACh, as our observation that sympatholysis is inhibited by atropine despite preserved exercise hyperemia suggests that modulation of sympathetic vasoconstriction by ACh can occur independent of vasodilation. The specific effect of atropine on sympatholysis rather than total blood flow is further discussed in a later section. Ultimately, it raises the possibility that ACh released at the neuromuscular junction could limit sympathetic

vasoconstriction despite the minor effect of attempted contractions on muscle blood flow following muscle paralysis (Dyke *et al.*, 1998).

Role of ACh in exercise hyperemia

The results of this study and others indicate that inhibition of muscarinic ACh receptors via atropine has little to no effect on muscle blood flow during exercise (Armstrong & Laughlin, 1986; Buckwalter *et al.*, 1997; Shoemaker *et al.*, 1997; Brock *et al.*, 1998). It is important to note that redundant vasodilatory signaling may mask a contribution of endogenous ACh to exercise hyperemia, particularly under circumstances that do not elicit an increase in sympathetic outflow (Joyner & Wilkins, 2007; Hellsten *et al.*, 2012). In this context, rhythmic handgrip exercise is not associated with increased muscle sympathetic nerve activity (Victor *et al.*, 1987). Therefore, although the results of the present study demonstrate that endogenous ACh modulates sensitivity to sympathetic vasoconstriction in contracting muscle, an obligatory role of endogenous ACh in exercise hyperemia may not be revealed by exercise modalities that do not engage the sympathetic nervous system. Along these lines, systemic inhibition of ACh receptors partially mitigates the fall in peripheral vascular resistance during cycling exercise and completely prevents the reduction in systemic vascular resistance during isometric handgrip exercise (Vianna *et al.*, 2015), which suggests that ACh contributes to vasodilation during exercise that elevates sympathetic outflow.

In addition, we have observed that not all local vasodilators have sympatholytic properties (Hearon *et al.*, 2016), which raises the possibility that ACh is more involved in fine-tuning the distribution of blood flow within the muscle microcirculation rather than regulating bulk blood flow to the tissue (Van Teeffelen & Segal, 2003). Within a contracting muscle, sympathetic α-adrenergic vasoconstriction normally restrains blood flow to inactive regions;

thus, blood flow is specifically increased to the recruited muscle fibers as a result of functional sympatholysis in the most active regions (Heinonen *et al.*, 2012, 2013). Functional sympatholysis becomes impaired with advancing age (Dinenno *et al.*, 2002), and the within-muscle heterogeneity of blood flow distribution is reduced in older men independent of bulk tissue blood flow (Rudroff *et al.*, 2014). Maldistribution of blood flow and oxygen delivery within the muscle has metabolic consequences: impaired sympatholysis in sedentary older men limits tissue oxygen uptake and results in greater lactate release at a given workload compared to active older men with preserved sympatholysis despite similar bulk blood flow between groups (Mortensen *et al.*, 2012). In support of the idea that ACh may regulate blood flow distribution within active muscle, Ishii et al. (Ishii *et al.*, 2013) reported an atropine-induced reduction in oxygenated hemoglobin of the vastus lateralis muscle during one-legged cycling exercise. Collectively, these studies suggest that the role of ACh in functional sympatholysis may be essential for appropriate distribution of blood flow within the exercising muscle independent of total exercise hyperemia.

Mechanisms of functional sympatholysis

Numerous attempts to pharmacologically inhibit functional sympatholysis in the same experimental model have failed to reveal obligatory signaling mechanisms involved in the response. Separate inhibition of nitric oxide or prostaglandin synthesis does not alter sympathetic vasoconstriction in contracting muscle (Dinenno & Joyner, 2003, 2004), and although combined inhibition of these pathways augments α_1-adrenergic vasoconstriction in contracting muscle, it also augments constriction in quiescent muscle which indicates that the effects are not specific to exercise (Dinenno & Joyner, 2004). Similar, non-specific effects are observed during systemic inhibition of ATP-sensitive K^+ (K_{ATP}) channels (Keller *et al.*, 2004). We have observed that

inwardly-rectifying K^+ (K_{IR}) channels and the Na^+/K^+-ATPase contribute substantially to exercise hyperemia in humans (Crecelius *et al.*, 2014); yet, functional sympatholysis is preserved when these vasodilatory pathways are blocked (Crecelius *et al.*, 2015). Indeed, contracting skeletal muscle maintains the ability to blunt α_1-adrenergic vasoconstriction even during simultaneous inhibition of nitric oxide, prostaglandins, K_{IR} channels, and the Na^+/K^+-ATPase (Crecelius *et al.*, 2015). The challenge of elucidating mechanisms involved in functional sympatholysis highlights the significance of the observation that atropine increases sympathetic vasoconstriction exclusively in contracting skeletal muscle in the present study.

Potential signaling pathways

Evidence from animal models suggests that hyperpolarization originating in the endothelium limits sympathetic vasoconstriction (Kurjiaka & Segal, 1995; Behringer & Segal, 2012*b*), and we have observed that endothelium-dependent agonists capable of eliciting hyperpolarization enhance functional sympatholysis in humans (Hearon *et al.*, 2016). ACh binds to $G_{q/11}$-coupled receptors on the endothelium, which activates phospholipase C and results in Ca^{2+} release from the endoplasmic reticulum as well as Ca^{2+} entry across the plasma membrane. The rise in cytosolic Ca^{2+} opens Ca^{2+}-activated K^+ (K_{Ca}) channels, and the resulting efflux of K^+ hyperpolarizes membrane potential. Ultimately, hyperpolarization spreads from endothelial cells to underlying vascular smooth muscle cells, where it inhibits Ca^{2+} entry via voltage-gated Ca^{2+} channels and thus promotes relaxation.

In addition to stimulating K^+ efflux through K_{Ca} channels, ACh initiates downstream signaling pathways including enzymatic production of vasodilatory autacoids as well as activation of K_{IR} channels and the Na^+/K^+-ATPase. In this regard, we have observed that pharmacological inhibition of nitric oxide and prostaglandin synthesis actually enhances the

ability of ACh to blunt α_1-adrenergic vasoconstriction (Hearon *et al.*, 2016). Thus, ACh-stimulated production of vasodilatory autacoids does not appear to contribute to its effects on sympathetic vasoconstriction; rather, inhibition of these pathways may cause greater reliance on hyperpolarizing mechanisms that modulate sympathetic signaling. We have also demonstrated that the sympatholytic effect of exogenous ATP, which binds to similar $G_{q/11}$-coupled receptors on the endothelium, is intact even during combined inhibition of nitric oxide synthesis, prostaglandin synthesis, K_{IR} channels, and the Na^+/K^+-ATPase (Hearon *et al.*, 2017). Taken together with observations that functional sympatholysis is preserved during exercise when these pathways are blocked (Crecelius *et al.*, 2015), we speculate that hyperpolarization arising from activation of K_{Ca} channels is central to the ability of ACh to blunt sympathetic vasoconstriction.

In a previous study, we demonstrated that ACh interacts with ATP to limit α_1-adrenergic vasoconstriction in resting muscle such that co-infusion of ACh with ATP mimics the functional sympatholysis observed during exercise. We have also observed that the ability of ACh to blunt sympathetic vasoconstriction is greatly enhanced by muscle contractions. Indeed, a moderate dose (4 ± 2 µg/dl FAV/min) of ACh completely abolishes vasoconstriction to PE during mild intensity handgrip exercise, whereas substantial constriction still occurs when a much higher dose (12 ± 4 µg/dl FAV/min) of ACh is infused at rest in the same participants (Hearon *et al.*, 2016). These findings raise the possibility that ACh is permissive to other mechanisms involved in limiting sympathetic vasoconstriction. In this regard, several lines of evidence suggest that ATP may play a key role in functional sympatholysis. ATP is released from erythrocytes and endothelial cells in response to deoxygenation and cellular deformation and circulating ATP increases with graded exercise intensity. Although we are unable to block ATP receptors to confirm a role of ATP in functional sympatholysis, infusion of exogenous ATP blunts

α_1-adrenergic vasoconstriction in a dose-dependent manner. In isolated vessels, ACh and ATP have a synergistic effect to amplify Ca^{2+} signaling within endothelial cells, which could enhance endothelium-dependent hyperpolarization and thus blunt sympathetic vasoconstriction. Therefore, we speculate that ACh interacts with other factors associated with muscle contractions to facilitate functional sympatholysis.

Effect of exercise intensity

The effect of atropine on functional sympatholysis was intensity-dependent such that atropine doubled the vasoconstrictor response to PE during high intensity (25% MVC) exercise, whereas it did not significantly affect vasoconstriction during exercise at a lower intensity (15% MVC). The reasons for this are not entirely clear; however, it is likely that more ACh is released during higher workloads as a result of greater shear-mediated release from endothelial cells and/or greater spillover from motor nerves. Interestingly, circulating pyruvate levels influence the synthesis and release of ACh by endothelial cells (Wilson *et al.*, 2016); thus, greater pyruvate release with graded exercise intensity (Henderson *et al.*, 2004) may also contribute to greater ACh release. Considering the aforementioned effect of contractions and intra-arterial ATP to enhance the sympatholytic effect of exogenous ACh (Hearon *et al.*, 2016), it is also possible that ACh interacts with another factor associated with contraction intensity to limit sympathetic vasoconstriction.

Experimental considerations and limitations

The site of action of drugs infused intra-arterially is unclear and deserves consideration. Bolus infusion of atropine in the present study resulted in a substantial but incomplete blockade of the vasodilatory response to intra-arterial ACh. However, although atropine is lipophilic and able to cross the blood-brain barrier, its diffusion across the vascular wall appears to be limited

(Lew *et al.*, 1989); thus, intraluminal infusion of atropine may not inhibit muscarinic receptors at other sites where ACh could act, such as the adventitial surface of endothelial cells. This is particularly relevant when considering potential sources of ACh that would initially encounter the interstitial surface, such as release from nerve terminals or release from the adventitial surface of endothelial cells.

We did not measure ACh in the present study and therefore do not know if circulating ACh increased in an exercise-intensity dependent manner. ACh is rapidly degraded and rarely detected in plasma, although circulating ACh has been demonstrated through the use of an acetylcholinesterase inhibitor (Kawashima *et al.*, 1987). Studies of isolated vessels clearly show that ACh released from endothelial cells activates muscarinic receptors (Wilson *et al.*, 2016); therefore, we speculate that the role of muscarinic receptors in the present investigation is due to activation by ACh. However, it is also possible that mechanical activation of the receptors by shear stress or muscle contractions contributes to functional sympatholysis, as many G_q-coupled protein receptors exhibit ligand-independent mechanosensitivity (Mederos y Schnitzler *et al.*, 2008). Furthermore, sensitivity of some G_q-coupled protein receptors to ligand activation is enhanced when combined with mechanical stimuli (Mederos y Schnitzler *et al.*, 2008); therefore, it is possible that ACh and mechanical stimuli combine to activate muscarinic receptors during exercise.

Due to time constraints, we did not include a second resting control trial where forearm blood flow was elevated to match that observed during exercise at 25% MVC. Sensitivity to PE was not affected by atropine at rest when forearm blood flow was matched to that observed during 15% MVC exercise; yet, it is unknown whether atropine might have affected vasoconstrictor sensitivity if flow was further elevated. Although this consideration is relevant

because shear stress stimulates ACh release from endothelial cells, infusing a control vasodilator at rest to match blood flow at the brachial artery results in different shear patterns throughout the vascular tree compared to the shear forces evoked by muscle contractions. Therefore, it is not possible to precisely match shear stimuli between resting and exercise conditions. We speculate that atropine would not affect α-adrenergic vasoconstriction at rest if forearm blood flow were matched to 25% MVC exercise; however, we acknowledge this as a limitation of our study design.

Perspectives

The results of this study advance our understanding of the basic mechanisms of blood flow regulation in humans and lay the foundation for future strategies to improve exercise tolerance in aging and clinical populations. Many people at elevated risk for cardiovascular complications display both endothelial dysfunction (i.e. reduced sensitivity to ACh) and elevated sympathetic nervous system activity. Thus, understanding the interactions between endothelium-dependent signaling and sympathetic vasoconstriction may lead to ideas to improve tissue blood flow and oxygen delivery in at-risk populations. We recently demonstrated that infusion of ACh restores functional sympatholysis in older adults (Hearon Jr. *et al.*, 2020), which suggests that the downstream signaling pathways by which ACh modulates α_1-adrenergic constriction remain intact in older adults. Therefore, therapeutic approaches that enhance ACh release or sensitivity have potential to improve peripheral blood flow regulation and oxygen delivery by enhancing functional sympatholysis.

CONCLUSIONS

Sympathetic vasoconstriction is dramatically attenuated within the vasculature of contracting skeletal muscle. This phenomenon of functional sympatholysis is imperative for

redistribution of blood flow to active muscle during exercise. The present study demonstrates that muscarinic ACh receptors powerfully modulate post-junctional α_1-adrenergic signaling within contracting skeletal muscle in humans, thereby revealing endogenous ACh as a mechanism of functional sympatholysis. This is the first study to ascertain a signaling pathway that modulates sympathetic vasoconstriction in contracting muscle in humans and, importantly, the findings establish a novel, physiological role for ACh in the regulation of muscle blood flow during exercise.

CHAPTER 4 – MANUSCRIPT 3

Endogenous ACh facilitates brachial artery flow-mediated vasodilation in humans

INTRODUCTION

The vascular endothelium regulates vessel diameter in response to chemical and mechanical cues to ensure tissues receive an adequate supply of blood flow and oxygen. Endothelial cells detect changes in intraluminal shear stress, which stimulates endothelium-dependent vasodilation that is largely attributed to nitric oxide synthesis and initiation of endothelium-dependent hyperpolarization (Joannides *et al.*, 1995; Lieberman *et al.*, 1996; Bellien *et al.*, 2006). Because flow-induced vasodilation is a critical function of the endothelium, flow-mediated dilation (FMD) of the brachial artery is widely evaluated in clinical research as an index of vascular health. Endothelial dysfunction precedes and independently predicts development of cardiovascular disease (Gokce *et al.*, 2002; Broxterman *et al.*, 2019); thus, FMD is often assessed as a primary outcome measure in studies designed to detect or treat vascular disease. However, despite the physiological and clinical relevance, the mechanisms by which shear stress stimulates nitric oxide production and vasodilation are unclear.

Flow-mediated dilation corresponds closely with sensitivity to the endothelium-dependent vasodilator acetylcholine (ACh). Interestingly, activation of muscarinic ACh receptors contributes to basal and flow-induced nitric oxide synthesis and vasorelaxation of coronary artery rings superfused with effluent from isolated canine conduit arteries (Martin *et al.*, 1996). Together with recent evidence that ACh released from endothelial cells mediates calcium signaling and nitric oxide production in response to shear in isolated rat carotid arteries (Wilson

et al., 2016), these findings suggest that locally-released ACh plays a role in endothelial mechanotransduction and flow-mediated dilation.

Accordingly, the purpose of the present study was to investigate whether endogenous ACh facilitates flow-mediated vasodilation in humans. Prior studies indicate that different mechanisms underlie conduit artery dilation in response to transient and sustained shear stimuli (Mullen *et al.*, 2001; Bellien *et al.*, 2006); therefore, we tested whether intra-arterial infusion of atropine to inhibit muscarinic ACh receptors reduces vasodilation of the brachial artery in response to a transient or sustained shear stimulus. The results suggest a novel, physiological role of ACh in blood flow regulation in humans and provide insight regarding the basic mechanisms of flow-mediated vasodilation.

METHODS

Participants and ethical approval

All procedures were approved by the Institutional Review Board at Colorado State University and performed according to the standards of the Declaration of Helsinki except for registration in a database. After providing written, informed consent, 11 healthy participants volunteered for this study (5 women, 6 men; 23 ± 2 y; 26 ± 2% body fat; means ± SE). All participants were non-smokers who were free of apparent cardiovascular or metabolic disease.

Instrumentation and arterial catheterization

Participants arrived at the laboratory in the morning following an overnight fast and lay supine with the experimental arm abducted ~90° and supported slightly above heart level. After local anesthesia (2% lidocaine), a physician placed a 20 g, 12 cm catheter in the brachial artery using sterile technique. The catheter was inserted at the antecubital fossa and threaded proximally such that the tip of the catheter rested midway up the participant's upper arm (Fig.

4.1*A*). The catheter was connected to a three-way port for intra-arterial drug infusions and a continuous drip of heparinized saline. Heart rate was monitored throughout the study with a three-lead electrocardiogram (ECG) and arterial blood pressure was monitored with a pressure transducer attached to the catheter.

Brachial artery diameter and blood velocity

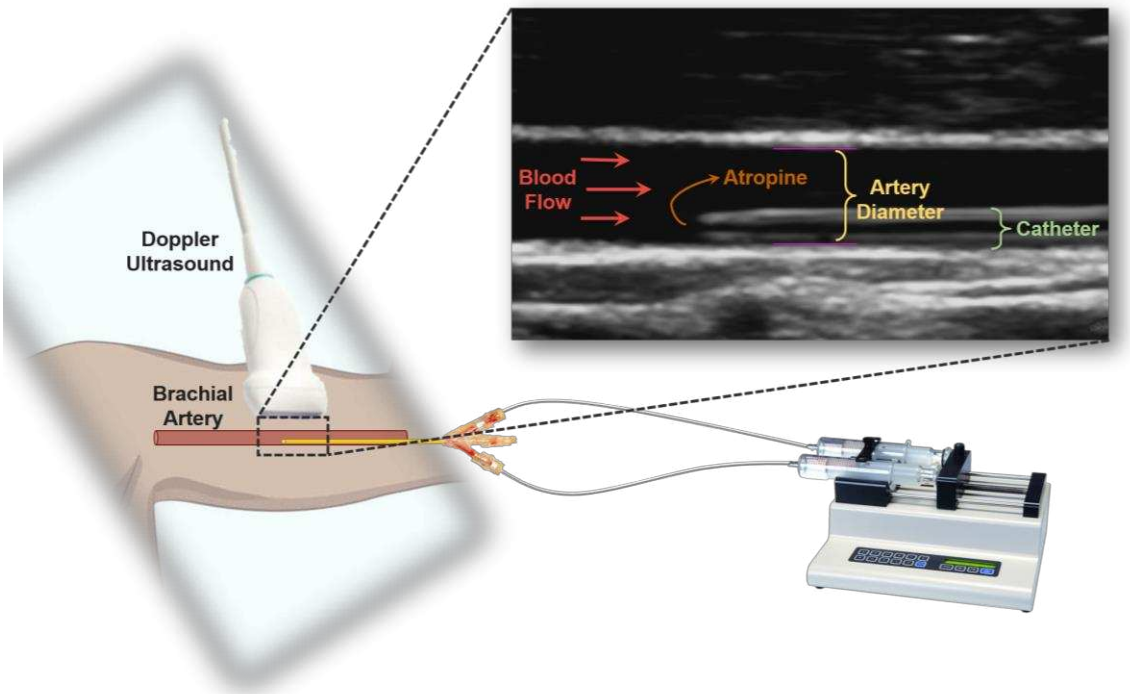
Brachial artery diameter and blood velocity were measured with Doppler ultrasound (Vivid7, General Electric, Milwaukee, WI). The catheter was visualized within the artery using a 12 MHz probe (Fig. 4.1*A*) and diameter measurements were obtained distal to the tip of the catheter (i.e. downstream of intra-arterial drug administration). Images were recorded at end-diastole for each cardiac cycle using the R wave of the ECG as a trigger for image capture (Vascular Imager, Medical Imaging Applications, Coralville, IA) for offline analysis with automatic wall tracking software (Brachial Analyzer, Medical Imaging Applications, Coralville, IA). During the exercise FMD trial and ACh dose-response, steady-state diameter images were recorded in B-mode. During the reactive hyperemia FMD trial, diameter images were recorded in duplex mode to allow simultaneous measurement of blood velocity. Velocity was measured proximal to the tip of the catheter using a 5 MHz frequency and 60° insonation angle. Mean blood velocity (MBV) is reported as the weighted mean of Doppler shift frequencies determined via a spectral analyzer (Multigon 500 M, Multigon Industries, Mount Vernon, NY).

Experimental protocol

An overview of the experimental timeline is presented in Fig. 4.1*B*. Following catheterization and set-up, graded handgrip exercise and reactive hyperemia were used in separate trials to assess FMD, then the vasodilatory response to ACh was determined. All trials were completed under control conditions, then atropine was infused and trials were repeated to

evaluate the contribution of ACh. Trials were always performed in the same order with 10-15 min breaks between trials.

A. Experimental set-up



B. Study day timeline

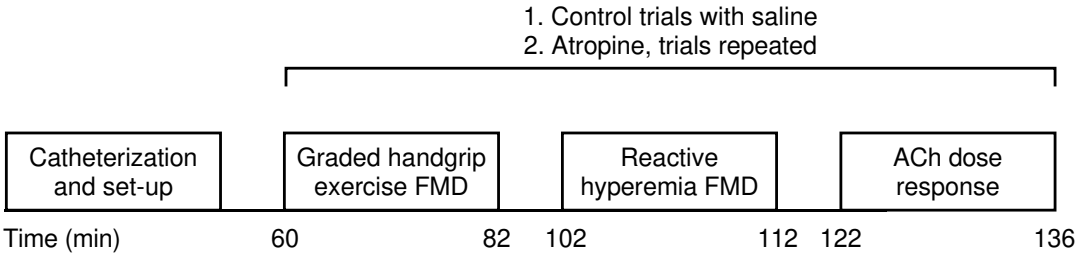


Figure 4.1: Experimental protocol.

The experimental set-up is illustrated in *A*. Doppler ultrasound was used to measure brachial artery diameter and blood velocity. Diameter was measured downstream of the arterial catheter to ensure the site of measurement was exposed to atropine. The study day timeline is shown in *B*. Following catheterization and set-up, participants completed a graded handgrip exercise protocol to assess flow-mediated dilation (FMD) of the brachial artery in response to a sustained shear stimulus. After a ≥20 min break, FMD was assessed in response to reactive hyperemia, which produces a robust, transient shear stimulus. This trial was followed by a ≥10 min break, then the vasodilatory response to infusion of exogenous ACh was evaluated. When control trials were completed, atropine was infused, then all trials were repeated in the same order.

Rhythmic handgrip exercise

A graded handgrip exercise protocol was employed to assess FMD of the brachial artery in response to a steady-state shear stimulus. Participants performed dynamic handgrip contractions to lift a weight 4-5 cm over a pulley system at a rate of 20 contractions per min (1 s contraction, 2 s relaxation). The weights corresponded to 10, 15, 20, and 25% of maximal voluntary contraction (MVC) force, which was determined prior to the study visit using a Stoelting handgrip dynamometer. Two min of baseline data were collected, then participants performed handgrip exercise at 10% MVC for 4-5 min to attain steady-state values. To ensure clear images without movement artifact, participants briefly stopped contractions at the end of each exercise intensity for diameter recordings before beginning the next workload.

Reactive hyperemia

A reactive hyperemia protocol was utilized to assess FMD of the brachial artery in response to a transient shear stimulus. Following 2 min of baseline recordings, a cuff distal to the elbow was inflated to suprasystolic pressure (≥200 mmHg) to occlude forearm blood flow for 5 min, which results in vasodilation of downstream resistance vessels and produces a reactive hyperemic response upon cuff release. Beat-by-beat brachial diameter and blood velocity were recorded for 3 min following cuff release to determine FMD and calculate the shear stimulus. The ultrasound probe position was maintained with a probe holder to minimize movement and the position of the cuff was maintained across control and atropine trials. Data from one participant were excluded from analysis for the reactive hyperemia trial because the image quality was inadequate for accurate diameter measurements with the wall tracking software.

ACh dose-response

The vasodilatory response to ACh infusion was evaluated to assess sensitivity to ACh and efficacy of the blockade with atropine. Forearm volume (FAV) was determined using dual X-ray absorptiometry prior to the study visit for normalization of drug doses. Following 2 min of baseline measurements, ACh was infused at 0.5, 1.5, 4.5, and 15 μg/dl FAV/min for 3 min per dose with an infusion rate of approximately 2 ml/min. The ACh dose-response trial was not performed in one participant due to time restrictions.

Atropine administration

Following control trials, atropine was administered through the arterial catheter to inhibit muscarinic ACh receptors. A bolus infusion of atropine was administered prior to each trial. Initially, a total of 0.2 mg was infused over the course of 3 min as a loading dose, then maintenance doses of 0.07 mg were infused over the course of 1 min prior to subsequent trials. Due to the length of the exercise trial (~20 min), additional maintenance doses of 0.01 mg were infused over 10 s between each exercise intensity.

Data analysis and calculations

Data were collected at 250 Hz for later analysis with data processing software (Windaq; DATAQ Instruments). Steady-state variables in the exercise trial reflect average values over 30 s (MBV, MAP, HR) or 10 cardiac cycles (brachial diameter). The slopes and intercepts for diameter *vs.* shear rate and the change in diameter *vs.* the change in shear rate from rest were calculated for each participant. In the reactive hyperemia trial, variables were assessed for each cardiac cycle following cuff release. Diameter measurements were smoothed using a rolling average of three cardiac cycle bins and the rolling average was used to identify peak diameter. Shear rate was calculated as $8 \times \text{MBV} \div \text{diameter}$ and expressed in s^{-1}. The change in shear rate

from either baseline or occlusion was used to calculate shear rate area under the curve (SR_{AUC}), which is reported from cuff release to peak diameter and to 2 min post-cuff release. Forearm blood flow (FBF) was calculated as $\pi \times (\text{diameter} \div 2)^2 \times \text{MBV} \times 60$ and expressed in ml/min. Forearm vascular conductance (FVC) was calculated as FBF ÷ MAP × 100 and expressed in ml/min/100 mmHg.

Statistics

All data are presented as means ± SE. Steady-state variables in the exercise trial were evaluated using two-way (drug × time), repeated-measures ANOVA followed by Tukey's post-hoc analysis to compare atropine to control conditions. In the event of non-normal distributions, data were log transformed when appropriate. The effect of atropine on relationship between diameter and shear rate (slope and intercept) for each participant was evaluated with paired *t* tests. In the reactive hyperemia trial, the effect of atropine on brachial artery diameter at baseline,

Table 4.1: Forearm and systemic hemodynamics during graded handgrip exercise

	Rest	10% MVC	15% MVC	20% MVC	25% MVC
Forearm blood flow (ml/min) †					
Control	19 ± 3	92 ± 10	145 ± 14	209 ± 17	277 ± 20
Atropine	17 ± 2	90 ± 11	149 ± 17	206 ± 21	270 ± 21
Mean arterial pressure (mmHg) †					
Control	91 ± 2	94 ± 2	96 ± 2	97 ± 2	100 ± 3
Atropine	92 ± 1	93 ± 1	95 ± 1	97 ± 2	101 ± 3
Forearm vascular conductance (ml/min) †					
Control	21 ± 4	98 ± 12	151 ± 14	214 ± 16	280 ± 21
Atropine	18 ± 2	96 ± 11	156 ± 16	211 ± 18	267 ± 18
Heart rate (beats/min) †					
Control	63 ± 2	65 ± 2	65 ± 2	67 ± 2	69 ± 2
Atropine	57 ± 2*	62 ± 3	66 ± 3	70 ± 4	73 ± 3

Values are means ± SE for $n = 11$ participants. * $P < 0.05$ *vs.* control; † $P < 0.05$ main effect of time

occlusion, and peak was assessed using two-way (drug × time), repeated-measures ANOVA followed by Tukey's post-hoc comparisons. The effect of atropine on all other variables in the reactive hyperemia trial was evaluated with paired *t* tests.

RESULTS

Graded handgrip exercise FMD

Under control conditions, forearm blood flow increased in parallel with exercise intensity (Table 4.1), which resulted in a progressive increase in shear rate at the brachial artery that

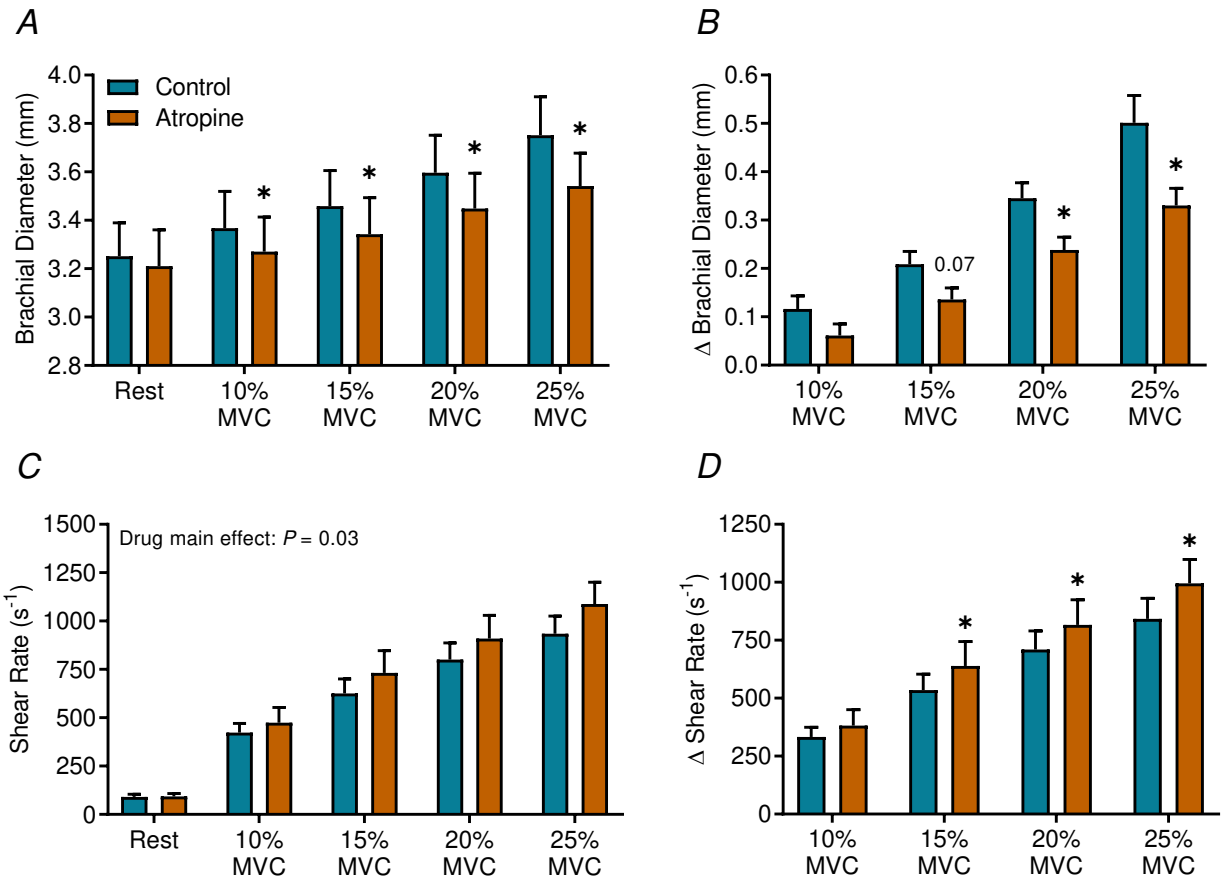


Figure 4.2: Diameter and shear rate during graded handgrip exercise.

Atropine did not affect brachial artery diameter at rest but reduced diameter throughout exercise (*A*) and attenuated the change in diameter from rest at higher exercise intensities (*B*). There was a main effect of atropine to increase shear rate (*C*) and atropine increased the change in shear rate from baseline at the higher exercise intensities (*D*). Values are means ± SE for $n = 11$ participants. MVC, maximal voluntary contraction. *$P < 0.05$ *vs.* control

evoked flow-mediated dilation (Figs 4.2 & 4.3). Inhibition of muscarinic ACh receptors with atropine had no effect on brachial diameter or forearm hemodynamics at rest; however, resting heart rate was slightly lower in the atropine trial (Fig. 4.2 & Table 4.1). Although atropine did not impact forearm blood flow during exercise, it attenuated vasodilation of the brachial artery such that diameter was reduced compared to control exercise (Table 4.1 & Fig. 4.2). There was a main effect of atropine to increase shear rate and the exercise-induced increase in shear rate was greater during the 15, 20, and 25% MVC workloads compared to control (Fig. 4.2). Thus, brachial artery diameter and vasodilation was reduced for a given shear stimulus when ACh receptors were inhibited by atropine (Fig. 4.3 & Table 4.2). Atropine reduced the slope of the relationship between vasodilation and the shear stimulus by 38 ± 8% (Fig. 4.3 & Table 4.2).

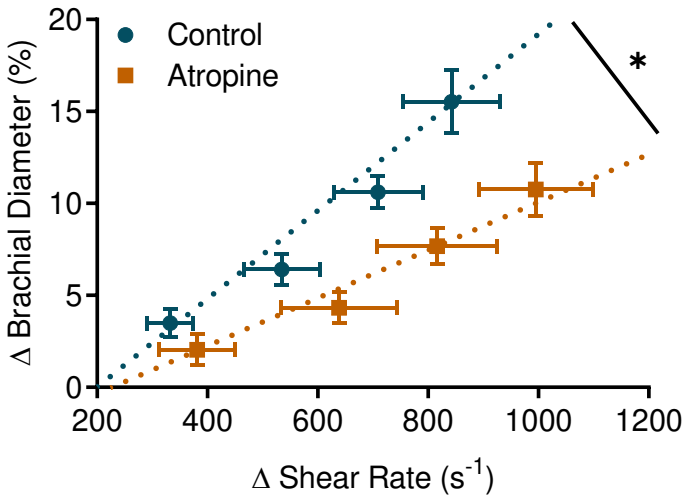


Figure 4.3: Flow-mediated dilation during graded handgrip exercise.

The percent change in brachial artery diameter from rest is plotted against the change in shear rate for each exercise intensity. Dotted lines illustrate the average relationship between the change in diameter and change in shear for n = 11 participants; the slope and intercept are reported in Table 4.2. Atropine reduced the slope describing the relationship between brachial artery vasodilation and the change in shear rate by 38 ± 8%. * $P < 0.05$ control *vs.* atropine slope

Table 4.2: Slopes and intercepts during exercise

	Control	Atropine
Diameter *vs*. Shear Rate		
Slope (mm/s × 10^{-4})	6.3 ± 1.0	3.4 ± 0.4*
Intercept (mm)	3.2 ± 0.1	3.2 ± 0.2
Δ Diameter *vs.* Δ Shear Rate		
Slope (mm/s × 10^{-2})	8.0 ± 1.4	4.1 ± 0.5*
Intercept (mm)	-0.2 ± 0.1	-0.1 ± 0.0
Δ Diameter (%) *vs.* Δ Shear Rate		
Slope (% Δ/s × 10^{-2})	2.4 ± 0.4	1.3 ± 0.1*
Intercept (% Δ)	-4.8 ± 1.6	-3.0 ± 1.3

Values are means ± SE for n = 11 participants.
* $P < 0.05$ *vs.* control

Reactive hyperemia FMD

In the control trial, 5 min of occlusion to produce reactive hyperemia evoked a rapid increase in shear rate at the brachial artery which was followed by brachial vasodilation that peaked by 36 ± 5 s after cuff release (Figs 4.4 & 4.5 and Table 4.3). Atropine did not affect baseline diameter; however, constriction of the brachial artery was observed during cuff occlusion in the atropine trial which was not observed during control conditions (Table 4.3 and Fig. 4.4*A*). Therefore, in addition to traditional analysis of flow-mediated dilation from baseline diameter, we also assessed vasodilation from the occlusion time point. Atropine reduced peak diameter and slowed the time course of vasodilation such that peak diameter did not occur until 63 ± 11 s after cuff release (Table 4.3). When dilation and the shear stimulus were calculated from baseline, atropine attenuated flow-mediated dilation despite an augmented shear rate such that the percent change in brachial artery diameter was reduced by 35 ± 16% when normalized to SR_{AUC} (Fig. 4.5 panels *A*, *C*, & *E*). When calculated from occlusion, atropine did not affect the percent change in brachial diameter (Fig. 4.5*B*). However, atropine resulted in a higher shear

stimulus (Fig. 4.5*D*) such that vasodilation from occlusion still tended to be reduced when normalized to SR_{AUC} (Fig. 4.5*F*; $P = 0.07$ *vs.* control). Similar results were observed when comparing the absolute change in diameter rather than the percent change (data not shown).

Table 4.3: Reactive hyperemia FMD trial

	Control	Atropine
Brachial artery diameter (mm)		
Baseline	3.26 ± 0.17	3.26 ± 0.19
Occlusion	3.26 ± 0.18	3.19 ± 0.18*
Peak	3.48 ± 0.20	3.42 ± 0.22*
Low flow-mediated constriction (%)		
	-0.1 ± 0.7	-2.1 ± 0.8*
Time to peak diameter (s)		
	36 ± 5	63 ± 11*
Shear rate AUC to 2 min (arbitrary units)		
From BL	25,407 ± 3,734	33,477 ± 3,354*
From occl.	29,870 ± 4,472	39,215 ± 3,785*

Values are means ± SE for $n = 10$ participants. AUC, area under the curve; BL, baseline; FMD, flow-mediated dilation. * $P < 0.05$ *vs.* control

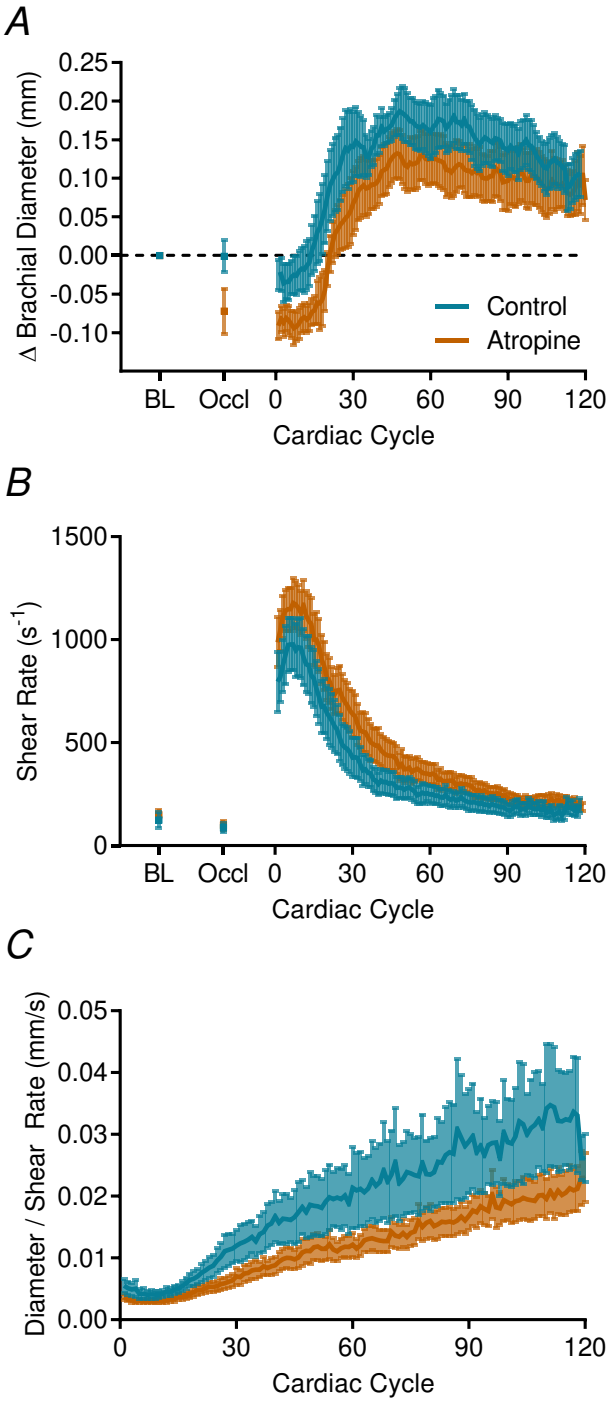


Figure 4.4: Time course of flow-mediated dilation in response to reactive hyperemia.

The time course of the change in brachial artery diameter from baseline (*A*), shear rate (*B*), and diameter normalized to shear rate (*C*) is shown for $n = 10$ participants. BL, baseline; occl, occlusion

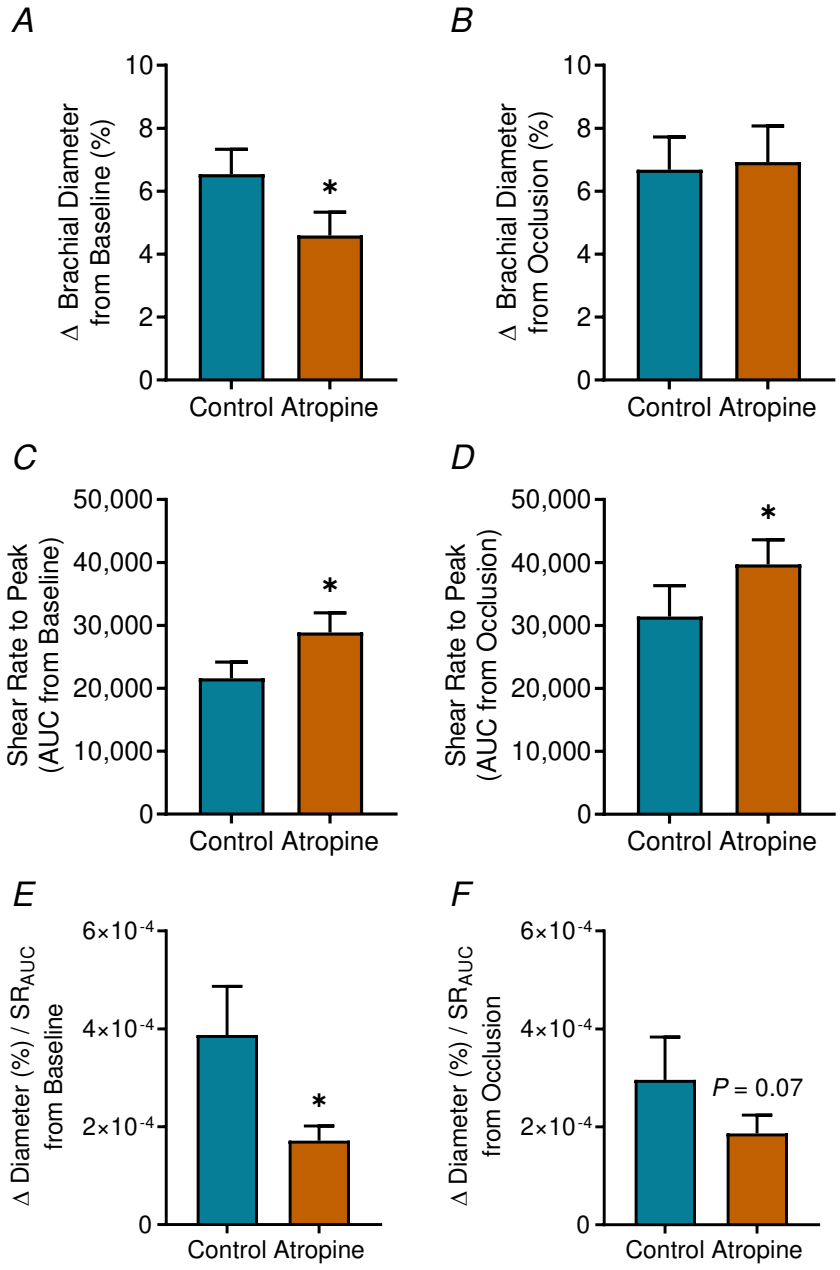


Figure 4.5: Flow-mediated dilation in response to reactive hyperemia.

Traditional analysis of flow-mediated dilation from baseline is shown in panels *A*, *C*, & *E*. Atropine reduced peak vasodilation of the brachial artery following cuff release (*A*) and increased the shear stimulus (*C*) such that the percent change in brachial diameter was diminished by 35 ± 16% when normalized to SR_{AUC} (*E*). An additional analysis of flow-mediated dilation from occlusion is shown in panels *B*, *D*, & *F*. Atropine did not affect the peak change in brachial artery diameter from occlusion (*B*); however, it increased the shear stimulus (*D*) such that vasodilation from occlusion tended to be reduced when normalized to SR_{AUC} (*F*). Values are means ± SE for $n = 10$ participants. AUC, area under the curve; SR, shear rate. * $P < 0.05$ *vs.* control

ACh dose-response

Under control conditions, infusion of ACh caused substantial vasodilation when assessed as the percent change in forearm vascular conductance or brachial artery diameter (Fig. 4.6). Although the lowest dose of ACh (0.5 µg/dl FAV/min) caused only a mild increase in forearm blood flow (~50 ml/min), it resulted in marked vasodilation of the brachial artery that surpassed that observed during the highest exercise intensity (Fig. 4.6*B vs.* Fig. 4.3). Atropine reduced total vasodilation (Δ FVC %) during ACh infusion by 83 ± 3% (averaged across all doses) and reduced brachial artery vasodilation by 74 ± 7% (averaged across all doses).

DISCUSSION

The present study was designed to determine whether endogenous ACh facilitates flow-mediated vasodilation of conduit arteries in humans. We utilized intra-arterial infusion of atropine to inhibit muscarinic receptors at the brachial artery and assessed flow-mediated vasodilation in response to both steady-state and transient shear stimuli. The key findings are as follows: 1) Atropine reduced brachial artery vasodilation in response to graded, steady-state elevations in shear rate produced by handgrip exercise by 38 ± 8%. 2) Atropine also reduced brachial artery vasodilation in response to a robust, transient increase in shear rate produced by the reactive hyperemic response to 5 min of forearm occlusion by 35 ± 16%. These results demonstrate that endogenous ACh elicits conduit artery vasodilation in response to increased flow and reveal a novel, physiological role of ACh in peripheral blood flow regulation in humans.

Flow-mediated dilation is a fundamental homeostatic function by which arteries accommodate changes in blood flow. Because it is mediated by the endothelium, FMD of

conduit arteries is commonly assessed in clinical research as an index of endothelial function. This test has considerable prognostic value: impaired FMD of the brachial or radial artery in response to reactive hyperemia precedes development of cardiovascular disease and is an independent predictor of future cardiovascular events (Celermajer *et al.*, 1992; Gokce *et al.*, 2002; Inaba *et al.*, 2010). Because of the physiological and clinical relevance, there has been

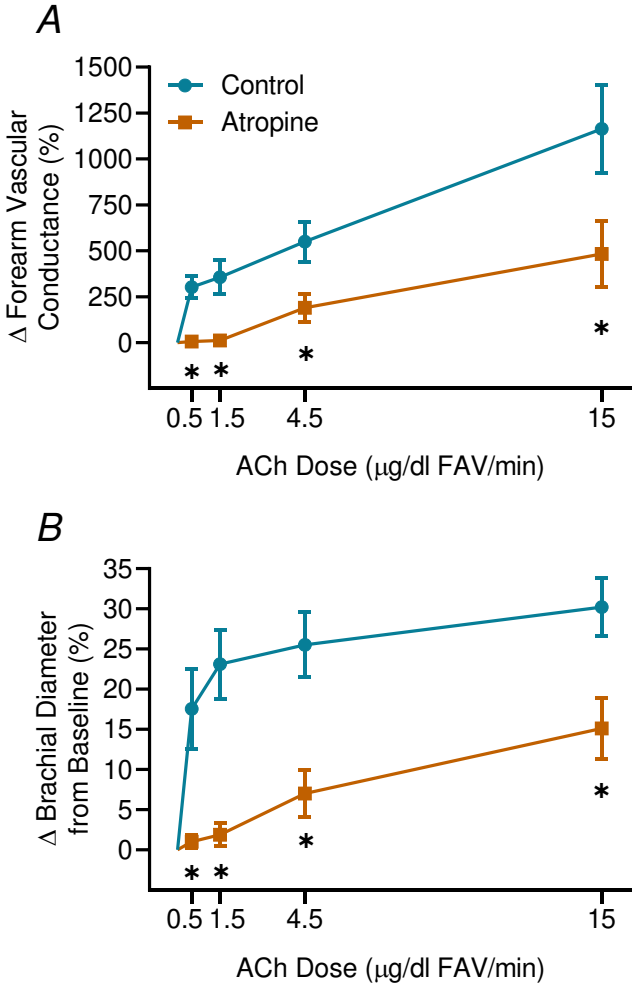


Figure 4.6: Vasodilatory response to acetylcholine infusion.

The percent change in forearm vascular conductance (*A*) and brachial artery diameter (*B*) in response to graded infusions of exogenous ACh are shown for $n = 10$ participants. Atropine diminished total vasodilation (*A*) by 83 ± 3% and reduced brachial artery vasodilation (*B*) by 74 ± 7%. ACh, acetylcholine; FAV, forearm volume. * $P < 0.05$ *vs.* control

considerable interest in identifying the mechanisms that underlie flow-mediated dilation. Prior studies suggest that distinctive pathways contribute to vasodilation in response to sustained and transient increases in shear stress (Mullen *et al.*, 2001) and these responses are differentially affected in clinical populations (Mullen *et al.*, 2001; Bellien *et al.*, 2006; Findlay *et al.*, 2013; Lorthioir *et al.*, 2015). Thus, determining the mechanisms that underlie FMD in response to sustained and transient shear stimuli may provide distinctive insight to vascular function.

FMD in response to sustained shear during handgrip exercise

In the present study, local inhibition of muscarinic ACh receptors did not alter basal brachial artery diameter distal to the site of atropine infusion; however, it reduced brachial artery vasodilation in response to graded bouts of handgrip exercise. Despite the smaller diameter, exercise hyperemia was preserved by a reciprocal increase in blood velocity; therefore, atropine augmented the shear stimulus at a given exercise intensity. In combination, these results indicate that atropine reduces the slope of the relationship between brachial artery diameter and shear rate such that less vasodilation is observed for a given shear stimulus. Thus, endogenous ACh is in part responsible for conduit artery vasodilation in response to a sustained increase in shear stress.

Although atropine diminished flow-mediated dilation during exercise, it had no impact on forearm blood flow. This is in agreement with prior studies which have shown no effect of atropine on exercise hyperemia (Buckwalter *et al.*, 1997; Shoemaker *et al.*, 1997). Given the physiological and clinical importance of flow-mediated dilation, it is noteworthy that preventing a large portion of conduit artery dilation does not alter muscle blood flow. Casey & Joyner (Casey & Joyner, 2009) examined the impact of brachial artery obstruction on exercise hyperemia and observed that inflation of a balloon within the artery caused only a temporary

reduction in forearm blood flow – compensatory vasodilation of the downstream resistance vasculature largely restored perfusion.

FMD in response to reactive hyperemia following cuff occlusion

As in the exercise trial, atropine had no effect on baseline diameter but attenuated brachial artery vasodilation in response to reactive hyperemia. The reduction in FMD occurred despite a greater shear stimulus compared to control conditions. Interestingly, the effect of atropine was partly due to vasoconstriction of the brachial artery that occurred when brachial blood flow was reduced by cuff occlusion, indicating an additional role of ACh in mitigating low-flow mediated constriction (L-FMC). Compared to flow-mediated dilation, much less is known about vasoconstriction of conduit arteries during periods of low shear stress. L-FMC of the radial artery has been proposed to be a marker of vascular function that may provide insight regarding basal flow-induced vasodilation (Gori *et al.*, 2008; Dawson *et al.*, 2012). In contrast to the radial artery, the prevalence and magnitude of L-FMC is reduced in the brachial artery.

Our results are in line with studies that have shown that constriction of the brachial artery during the occlusion period corresponds with a blunted and delayed vasodilatory response (Harrison *et al.*, 2011; Irace *et al.*, 2016; Harbin *et al.*, 2018). As both a reduced magnitude of FMD and a latent response are associated with cardiovascular risk (Irace *et al.*, 2014), constriction of the brachial artery during occlusion may actually be reflective of endothelial dysfunction. In support of this, L-FMC of the brachial artery is augmented in patients with unstable atherosclerosis and lessened in parallel with improvements in FMD in patients recovering from myocardial infarction (Spiro *et al.*, 2011). Collectively, these studies suggest that the degree of constriction during occlusion is an essential contributing factor that reduces FMD following reactive hyperemia. The results of the present study demonstrate that ACh

normally counteracts L-FMC in healthy individuals. To our knowledge, this is the first study to identify a mechanism that regulates L-FMC in the brachial artery.

Atropine did not affect baseline diameter, whereas it enhanced L-FMC and blunted FMD. These observations are somewhat surprising given evidence that ACh is released from endothelial cells in response to shear stress. Many G_q-coupled protein receptors, including M_5 muscarinic receptors, exhibit mechanosensitivity such that deformation of the cell membrane enhances sensitivity to ligand binding (Mederos y Schnitzler *et al.*, 2008), which may explain the lack of effect of atropine on baseline diameter compared to when shear rate is elevated. However, because shear rate was higher at baseline compared to during occlusion, a greater role of ACh would be expected on baseline diameter whereas we only observed an effect during occlusion. The reasons for this discrepancy are not clear. It is possible that ACh is not normally involved in maintaining baseline diameter or that other mechanisms are able to compensate for the loss of ACh under resting conditions. During occlusion, a reduction in shear and thus diminished basal flow-mediated dilation could unmask sympathetic and myogenic vasoconstrictor tone. We have observed that ACh alters post-junctional sensitivity to α-adrenergic receptor activation; therefore, it is possible that ACh is obligatory for preventing sympathetic vasoconstriction during a low-flow condition. Interestingly, intra-arterial atropine in combination with sympathetic α and β receptor blockade by phentolamine and propranolol did not affect radial artery FMD in response to sustained hand warming (Mullen *et al.*, 2001), which raises the possibility that the effect of atropine in the present study may involve an interaction with sympathetic signaling. However, the dose of atropine administered by Mullen et al. was approximately 200 times lower than the dose administered in the present study and they did not

report the blockade efficacy; thus, the lack of effect of atropine on radial artery dilation due to hand warming may be due to insufficient inhibition of muscarinic receptors.

Mechanisms of flow-mediated vasodilation

Prior investigations have explored several potential pathways as mechanisms of flow-mediated dilation. Initial studies showed that inhibition of nitric oxide synthesis abolished FMD in response to reactive hyperemia (Lieberman *et al.*, 1996; Mullen *et al.*, 2001); indeed, vasodilation was converted to constriction in some studies (Joannides *et al.*, 1995; Doshi *et al.*, 2001). These findings led to the prevalent view that FMD serves as an index of nitric oxide bioavailability. Subsequent studies have indicated a lesser role for nitric oxide or observed preserved FMD during inhibition of nitric oxide synthesis (Pyke *et al.*, 2010; Parker *et al.*, 2011; Wray *et al.*, 2013), which suggests that nitric oxide is not always obligatory for FMD in response to reactive hyperemia. Along these lines, the reliance on nitric oxide varies among different protocols employed to induce FMD. Nitric oxide dependence is reduced when longer durations of cuff occlusion are utilized to provoke reactive hyperemia (Mullen *et al.*, 2001) or when the cuff is placed proximal to the site of measurement (Doshi *et al.*, 2001). Compared to the transient shear stimulus associated with reactive hyperemia protocols, a lesser contribution of nitric oxide is observed during sustained increases in shear rate as a result of hand warming or distal vasodilator infusion (Mullen *et al.*, 2001; Bellien *et al.*, 2006), whereas the vasodilatory response to graded handgrip exercise is largely nitric oxide dependent (Wray *et al.*, 2011).

Endothelium-derived hyperpolarization has also been investigated as a mechanism of conduit artery FMD. An increase in cytosolic Ca^{2+} within endothelial cells causes hyperpolarization via K^+ efflux through Ca^{2+}-activated K^+ (K_{Ca}) channels and enhances synthesis of diffusible hyperpolarizing factors such as epoxyeicosatrienoic acids (EETs).

Hyperpolarization of endothelial cells spreads through gap junctions to underlying vascular smooth muscle cells and EETs diffuse to the vascular smooth muscle where they initiate hyperpolarization via K_{Ca} channels. Ultimately, hyperpolarization causes relaxation of vascular smooth muscle and thus vasodilation. Inhibition of K_{Ca} channels reduces FMD in response to hand warming, and this effect is magnified when combined with inhibition of nitric oxide synthesis (Bellien *et al.*, 2006). Similarly, inhibition of a cytochrome P450 epoxygenase to reduce synthesis of EETs attenuates FMD in response to both hand warming and reactive hyperemia, and this effect is also enhanced during combined blockade of nitric oxide production (Bellien *et al.*, 2006; Fischer *et al.*, 2007). These findings support an important contribution of endothelium-derived hyperpolarization to flow-mediated dilation and suggest an interaction between hyperpolarization and nitric oxide signaling.

Taken together, previous studies indicate that nitric oxide and endothelium-derived hyperpolarization contribute to flow-mediated dilation of conduit arteries in humans, although differences in the shear stimulus and crosstalk between pathways obscure definitive conclusions regarding the relative importance of each mechanism. A key aspect of the present study is that ACh is capable of stimulating both nitric oxide synthesis and endothelium-derived hyperpolarization; thus, ACh may serve as an upstream mechanism by which shear stress is transduced to vasodilation through recruitment of these pathways. The idea that ACh is a common precursor to nitric oxide synthesis and hyperpolarization in response to increased flow could also explain the heterogeneous mediators of flow-induced vasodilation, as a reduction in one pathway could shift the response toward reliance on the other pathway. We observed a similar atropine-sensitive component of FMD in response to both sustained and transient shear stimuli, whereas prior studies have identified differential contributions of nitric oxide and

hyperpolarizing pathways depending on the nature of the shear stimulus. This raises the possibility that the contribution of ACh is comparable between trials, yet the effects are shifted toward different downstream mediators with prolonged exposure.

Although atropine substantially blunted FMD, residual vasodilation of the brachial artery was still observed in response to both handgrip exercise and reactive hyperemia. There is considerable redundancy in the signaling mechanisms that regulate blood flow such that upregulation of one pathway compensates for loss of another; therefore, we speculate that additional mechanisms are responsible for the remaining vasodilation. However, it is also possible that incomplete inhibition of ACh receptors with atropine could explain a portion of the residual FMD responses observed.

Muscarinic receptor distribution

Muscarinic receptors are present on both endothelial and vascular smooth muscle cells, and all muscarinic receptor subtypes have been identified in the mammalian vasculature. In small arteries isolated from mouse skeletal muscle, mRNA expression of the M_3 subtype is markedly greater than other subtypes and knockout of the M_3 receptor abolishes vasodilation to ACh, whereas knockout of M_1 or M_5 subtypes has no effect (Gericke *et al.*, 2011). The M_3 subtype is also responsible for femoral artery relaxation to ACh in mice, where the effects are mediated entirely by nitric oxide synthesis (Bény *et al.*, 2008). Although atropine is a nonselective blocker of muscarinic receptors, studies in the human forearm circulation also support a predominant role of the M_3 receptor in the vasodilatory response to ACh (Bruning *et al.*, 1994; Attinà *et al.*, 2008).

In contrast to muscarinic receptors on endothelial cells, activation of M_3 receptors on vascular smooth muscle cells results in contraction. Removal of the endothelium or endothelial dysfunction unmasks a vasoconstrictor response to ACh (Furchgott & Zawadski, 1980; Lüscher

& Vanhoutte, 1986; Treasure *et al.*, 1992). Interestingly, the vasodilatory response to flow has also been reported to revert to vasoconstriction when nitric oxide synthesis is pharmacologically inhibited (Joannides *et al.*, 1995) and in clinical populations at high risk for cardiovascular events (Nguyen *et al.*, 2014); thus, it is possible that ACh release in response to shear activates muscarinic receptors on both endothelial and vascular smooth muscle cells and that the net vasodilatory response shifts to constriction in disease states.

It is likely that the contribution of ACh to flow-induced vasodilation varies among vascular beds and at different levels of the vascular tree. The downstream signaling pathways involved in the response to ACh differ depending on the properties of the vascular bed and size of the artery. For instance, vasodilation of large conduit arteries in response to ACh is primarily attributed to nitric oxide, whereas dilation of arterioles in response to ACh involves substantial activation of hyperpolarizing pathways. In addition to differences in the cellular response to ACh, muscarinic receptor expression and ACh release and degradation may differ throughout the vasculature. Thus, the extent to which ACh contributes to flow-mediated dilation in other arteries is unclear.

Limitations

Several limitations of the present study warrant discussion. First, we did not measure circulating ACh and cannot confirm that it increases with elevated shear rate. Although ACh has been detected in plasma with the use of acetylcholinesterase inhibitors (Kawashima *et al.*, 1987), ACh is rapidly degraded in circulation and thus challenging to measure. Of note, many G_q-coupled receptors exhibit mechanosensitivity and can initiate ligand-independent responses (Mederos y Schnitzler *et al.*, 2008); therefore, it is possible that muscarinic receptors *per se* mediate the response in the absence of ACh. However, treatment with acetylcholinesterase

inhibitors to limit ACh degradation enhances flow-induced endothelial Ca^{2+} signaling and vasorelaxation in ex vivo preparations (Martin *et al.*, 1996; Wilson *et al.*, 2016), which suggests that ACh is released in response to shear.

Another limitation of the current study is that the drug and trial order was maintained across all participants; therefore, we cannot exclude a general effect of time and repeated exposure to shear stimuli on the responses observed. Because the long half-life of atropine precludes randomization of drug order, control trials were always completed first. In a separate study in our laboratory, brachial artery FMD was preserved during repeated, steady-state bouts of handgrip exercise (slope of percent change in diameter per change in shear: 2.4 ± 0.5 *vs.* 2.3 ± 0.4 %/s × 10^{-2}, $P = 0.44$). Thus, the effect of atropine to reduce the slope from 2.4 ± 0.4 to 1.3 ± 0.1 %/s × 10^{-2} in the present study is unlikely to be due to repeated bouts of exercise. Although we have not assessed the effects of repeated measurements on FMD in response to reactive hyperemia in our laboratory, Pyke and colleagues (Pyke *et al.*, 2010; Pyke & Jazuli, 2011) have demonstrated that exposure to several bouts of reactive hyperemia or handgrip exercise does not impact flow-mediated vasodilation. It is also possible that the exercise trial affected the reactive hyperemia trial in the present study, as the order of these trials was held constant for all participants. Llewellyn et al. (Llewellyn *et al.*, 2012) reported a reduction in reactive hyperemia FMD immediately following treadmill running; however, normalization to shear rate abolished the effect of exercise. To mitigate potential carryover effects, we included a 20 min break between exercise and reactive hyperemia trials. Ultimately, any potential impact of exercise on subsequent reactive hyperemia trials should have been comparable between control and atropine conditions.

We measured brachial artery diameter downstream of the arterial catheter to ensure the site of measurement was exposed to atropine. However, because the catheter interfered with the Doppler signal, we were unable to measure blood velocity within the same segment of the vessel as diameter measurements were obtained. Therefore, velocity was assessed proximal to the tip of the catheter with the Doppler gate placed as close as possible while avoiding interference. If atropine did not inhibit muscarinic receptors at this location, it is possible that flow-mediated dilation remained intact. If this were the case, it is likely that our measurements of velocity and therefore shear rate underestimate the actual shear stimulus at the site of diameter measurement during the atropine condition.

Perspectives

Vascular sensitivity to ACh is reduced in aging and clinical populations, and a reduced vasodilatory response to ACh is an independent predictor of cardiovascular disease risk. Due to its close association with sensitivity to ACh, FMD of the brachial artery is commonly used as a surrogate measure of endothelial function. The results of this study clarify the correlation between the vasodilatory response to ACh and flow-mediated dilation through evidence that ACh itself partially mediates FMD. Understanding the signaling pathways that underlie flow-mediated dilation lays the foundation for future approaches to improve endothelial health, which has broad implications for preventing and treating cardiovascular disease.

CONCLUSIONS

The present study provides evidence that endogenous ACh facilitates flow-mediated vasodilation of the brachial artery in humans. The contribution of ACh to flow-mediated dilation is comparable during sustained and transient increases in shear rate induced by handgrip exercise and reactive hyperemia, respectively. These results demonstrate that ACh regulates conduit

artery diameter in response to changes in shear rate and reveal a novel, physiological role of ACh in peripheral blood flow regulation in humans.

CHAPTER 5 – PERSPECTIVES & CONCLUSIONS

This collection of studies provides meaningful insight into the basic physiology underlying blood flow regulation in humans and reveal a novel, physiological role of endogenous ACh as a regulator of peripheral vascular tone. In Experiment 1, we show that ACh interacts with ATP such that combined activation of the endothelium with both agonists amplifies vasodilation, which suggests that activation of the endothelium enhances its sensitivity to other stimuli. In addition to augmenting vasodilation, we show that endothelium-dependent agonists interact to modulate sensitivity to sympathetic vasoconstriction. These observations highlight the endothelium as a key site for integrating multiple vasoactive inputs to efficiently regulate tissue blood flow. In Experiment 2, we demonstrate that endogenous ACh overrides sympathetic vasoconstriction within contracting skeletal muscle. To the best of our knowledge, this is the first study to elucidate an essential mechanism of functional sympatholysis, which is critical to ensure adequate delivery of oxygen and nutrients to metabolically active tissue. Finally, in Experiment 3, we establish that endogenous ACh mediates a substantial portion of brachial artery vasodilation in response to shear stimuli. We also observe that ACh counteracts vasoconstriction of the brachial artery during periods of low blood flow; thus, we conclude that ACh regulates conduit artery diameter in response to changes in shear rate when blood flow is elevated or reduced. Taken together, these findings indicate that endogenous ACh plays a key role in regulating peripheral vascular tone at the level of the resistance vasculature and conduit arteries in humans.

Vascular sensitivity to ACh (i.e. endothelial function) is reduced with age and in clinical populations, and reduced sensitivity to ACh is an independent risk factor that often precedes development of cardiovascular disease. Aging and many disease states are also characterized by

elevated sympathetic nervous system activity, and the ability of contracting skeletal muscle to override sympathetic vasoconstriction is impaired in older adults and clinical populations. The combination of endothelial dysfunction, elevated sympathetic outflow, and an inability to blunt sympathetic vasoconstriction during exercise results in malperfusion of active skeletal muscle. This impairment in blood flow and oxygen delivery leads to accumulation of metabolites, which further increases sympathetic activity, elevates blood pressure, and accelerates development of fatigue. Taken together, our findings implicate endogenous ACh in vasodilation within the skeletal muscle resistance vasculature as well as conduit arteries and demonstrate that ACh modulates sympathetic signaling within contracting muscle. These conclusions have considerable implications for understanding exercise intolerance in clinical populations and lay the foundation for future strategies to prevent and treat cardiovascular disease.

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