5-2013

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Published in final edited form as:
Exp Physiol. 2013 May ; 98(5): 988–998. doi:10.1113/expphysiol.2012.071555.

Sources of Intravascular ATP During Exercise in Humans: Critical Role for Skeletal Muscle Perfusion

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Abstract

Exercise hyperemia is regulated by several factors and one factor known to increase with exercise that evokes powerful vasomotor action is extracellular ATP. The origination of ATP detectable in plasma from exercising muscle of humans is, however, a matter of debate and ATP has been suggested to arise from sympathetic nerves, blood sources (e.g. erythrocytes), endothelial cells, and skeletal myocytes, among others. Therefore, we tested the hypothesis that acute augmentation of sympathetic nervous system activity (SNA) results in elevated plasma ATP draining skeletal muscle, and that SNA superimposition during exercise further increases ATP vs exercise alone. We show that increased SNA via −40mmHg lower body negative pressure (LBNP) at rest does not increase plasma ATP (51±8 vs 58±7 nmol/L with LBNP), nor does it increase [ATP] above levels observed during rhythmic handgrip exercise (79±11 exercise alone vs 71±8 nmol/L with LBNP). Secondly, we tested the hypothesis that active perfusion of skeletal muscle is essential to observe increased plasma ATP during exercise. We identify that complete obstruction of blood flow to contracting muscle abolishes exercise-mediated increases in plasma ATP (90±19 to 49±12 nmol/L), and further, that cessation of blood flow prior to exercise completely inhibits the typical rise in ATP (3 vs 61%; obstructed vs intact perfusion). The lack of ATP change during occlusion occurred in the face of continued muscle work and elevated SNA, indicating the rise of intravascular ATP is not resultant from these extravascular sources. Our collective observations indicate that the elevation in extracellular ATP observed in blood during exercise is unlikely to originate from sympathetic nerves or the contacting muscle itself, but rather is dependent on intact skeletal muscle perfusion. We conclude that an intravascular source for ATP is essential and points toward an important role for blood sources (e.g. red blood cells) in augmenting and maintaining elevated plasma ATP during exercise.

Keywords

adenosine triphosphate; muscle blood flow; contractions

Introduction

The control of blood flow and oxygen delivery to skeletal muscle during exercise is dynamic and highly integrative in nature. Muscle contractions produce an assortment of vasomotor responses resultant from the compressive mechanical forces of muscle contraction, local

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production of metabolic factors, and autonomic nervous system activation (Saltin *et al.*, 1998; Clifford & Hellsten, 2004). A common means by which exercise-induced stimuli can signal a change in flow is through release of and subsequent communication via a signaling molecule. One such molecule implicated in governing blood vessel diameter within active muscle is extracellular ATP. Importantly, extracellular ATP is prevalent in venous plasma draining skeletal muscle from humans and concentrations are known to rise significantly during exercise (Forrester, 1972; Gonzalez-Alonso *et al.*, 2002; Kirby *et al.*, 2012). While recent advances have shed substantial light on the unique vasomotor action of ATP (Kirby *et al.*, 2008; Rosenmeier *et al.*, 2008; Crecelius *et al.*, 2012) and identified the notable presence of this transmitter in the luminal compartment (Gonzalez-Alonso *et al.*, 2002; Mortensen *et al.*, 2011; Kirby *et al.*, 2012), investigations have been limited with regard to characterizing where blood extracellular ATP may originate from during exercise in humans.

A multitude of cell sources could prove to be the origin for intravascular ATP, though with respect to conditions associated with contracting skeletal muscle, a few candidates are likely. Evidence indicates that ATP is released from: (i) sympathetic nerve endings following activation (Burnstock & Kennedy, 1986; Todorov *et al.*, 1996), (ii) endothelial cells and red blood cells during conditions of low oxygen and mechanical stress (Yang *et al.*, 1994; Bodin & Burnstock, 1995; Ellsworth *et al.*, 1995; Sprague *et al.*, 1998), and (iii) skeletal myocytes during contraction (Hellsten *et al.*, 1998; Buvinic *et al.*, 2009; Mortensen *et al.*, 2009). Whether any of these candidates give rise to elevated plasma ATP during exercise in humans is unclear.

The overall aim of the present study was to gain insight into which cell source(s) might act as the site of origination for increased extracellular ATP during moderate intensity exercise in humans. Accordingly, we tested the hypothesis that: (a) sympathetic nervous system activation results in nerve ending release of ATP that is detectable in the intravascular space, and (b) blood flow (i.e. muscle perfusion) during exercise is critical to the elevation in plasma ATP during exercise, suggesting release from an intravascular source such as blood and/or endothelial cells. To do so, we employed a lower body negative pressure system to elicit sympathetic nervous system activation alone and during exercise, and blood flow-occlusion methodology to inhibit erythrocyte delivery and minimize stimulus-induced release from erythrocytes and endothelial cells in the face of continued muscle contractions.

Methods

Subjects

With Institutional Review Board approval and after written informed consent, a total of 18 young healthy adults (16 men, 2 women; age = 24± 1 years; weight = 73± 1.3 kg; height = 176 ± 1 cm; body mass index = 23.6± 0.5 kg/m^2; % body fat = 21.7%; and MVC of 47 ± 2 kg; means±S.E.M) participated in the present study. All were non-smokers, non-obese, normotensive, and not taking any medications. Studies were performed after a 4-hour fast with the subjects in the supine position. All studies were performed according to the Declaration of Helsinki.

Arterial Blood Pressure and Heart Rate

Resting arterial blood pressure was measured in duplicate non-invasively over the brachial artery of the control arm after 15 minutes of supine rest before experimental trials (Cardiocap/5, Datex-Ohmeda, Louisville, CO, USA). Beat-by-beat arterial blood pressure (MAP) was measured at heart level by finger photoplethysmography (Finometer, FMS, Netherlands) on the middle finger of the control hand during each trial. Heart rate was determined using a 3-lead ECG (Cardiocap/5, Datex-Ohmeda, Louisville, CO, USA).

Forearm Blood Flow and Vascular Conductance

A 12 MHz linear-array ultrasound probe (Vivid 7, General Electric, Milwaukee, WI, USA) was used to measure brachial artery mean blood velocity (MBV) and brachial artery diameter and was placed in a holder securely fixed to the skin proximal to the catheter site as previously described by our laboratory (Kirby *et al.*, 2012). For blood velocity measurements, the probe insonation angle was maintained at <60° and the frequency used was 5 MHz. The Doppler shift frequency spectrum was analyzed via a Multigon 500M TCD (Multigon Industries, Mt. Vernon, NY) spectral analyzer from which mean velocity was determined as a weighted mean of the spectrum of Doppler shift frequencies. Brachial artery diameter measurements were made in triplicate in duplex mode at end diastole and between contractions during steady-state conditions. Forearm blood flow (FBF) was calculated as: FBF = MBV (cm/s) * π (brachial artery diameter/2)^2 * 60, where the FBF is in ml/min, the MBV is in cm/s, the brachial diameter is in cm, and 60 is used to convert from ml/s to ml/min. As an index of forearm vascular tone (and vasodilation), forearm vascular conductance (FVC) was calculated as (FBF/MAP) * 100, and expressed as ml/min /100 mmHg. Studies were performed in a cool temperature-controlled environment with a fan directed toward the forearm to minimize the contribution of skin blood flow to forearm hemodynamics.

Rhythmic Handgrip Exercise

Maximum voluntary contraction (MVC) was determined for each subject as the average of at least three maximal squeezes of a handgripdynamometer (Stoelting, Chicago, IL, USA) that were within 3 percent of each other. For the exercise trials, weights corresponding to 15% MVC were attached to a pulley system and lifted 4–5 cm over the pulley at a duty cycle of 1 s contraction-2 s relaxation (20 contractions per minute) using audio and visual signals to ensure the correct timing. We have recently demonstrated that handgrip exercise at this intensity increases venous plasma ATP draining the contracting tissue (Kirby *et al.*, 2012).

Sympathetic Nervous System Activation via Lower Body Negative Pressure

Subjects lay supine and weresealed at the level of the iliac crests in a chamber designed for administering lower body negative pressure (LBNP) (Kirby *et al.*, 2005). LBNP was administered at −40 mmHg to unload cardiopulmonary and arterial baroreceptors and evoke reflex increases in muscle sympathetic nerve activity (Davy *et al.*, 1998). This level of LBNP was administered for 2 minutes and was chosen because it evokes reproducible increases in sympathetic vasoconstrictor activity. Previous studies in our laboratory indicate that, when separated by 15 minutes of rest, the forearm vasoconstrictor response to LBNP under resting conditions are repeatable over time (Kirby *et al.*, 2005).

Blood Flow Occlusion via Cuff Inflation

Blood flow was occluded by rapidly inflating a blood pressure cuff to suprasystolic blood pressure (≥ 200mmHg ; D.E. Hokanson, Bellevue, WA, USA). The cuff was placed around the upper arm proximal to the ultrasound probe. Obstruction was confirmed by identification of a complete loss in the blood velocity signal.

Venous Catheterization

A choice of 18 – 22 gauge 5.1 cm catheter, depending on visual assessment of vein size, was inserted at the antecubital crease in retrograde fashion into a vein draining the muscle tissue of the experimental arm for venous blood samples to be used for ATP determination and blood gas analysis via clinical blood gas analyzer (Siemens Rapid Point 405 Series Automatic Blood Gas System, Los Angeles, CA). Saline was continuously infused through this catheter at a rate of approximately 3 ml/minfor the duration of the study to keep it patent

(Kirby *et al.*, 2012). In the present investigation we focused on venous ATP given that (a) we previously demonstrated that graded rhythmic handgrip exercise does not significantly elevate arterial [ATP] and (b) ATP in the arterial circulation is undetectable in venous samples given the rapid (< 1 sec) breakdown by nucleotidases (Pearson *et al.*, 1980; Mortensen *et al.*, 2011; Kirby *et al.*, 2012).

Sampling and Measurement of Plasma ATP

Blood was drawn by syringe, immediately centrifuged, and plasma assayed for ATP as previously described in detail (Kirby *et al.*, 2012). Venous blood was sampled and used for ATP measurement because this blood is post exposure to the microcirculation, and during forearm exercise arterial ATP sampled from the brachial artery changes minimally (Kirby *et al.*, 2012). Briefly, venous blood was drawn through the catheter directly into a pre-heparinized 10cc syringe in which 2 ml of blood was at once gently expelled into a tube containing 2.7 mL of an ATP stabilizing solution to equal a blood:diluent ratio of 1.35 (Gorman *et al.*, 2007; Kirby *et al.*, 2012). The ATP stabilizing solution was used to inhibit degradation of ATP via nucleotidases and additional ATP release post sampling. Blood:diluent samples were immediately centrifuged at 4,000 rpm for 3 minutes at 22°C. Directly following centrifugation, 100 μL of supernatant was taken for plasma ATP determination via luciferin-luciferase assay. An ATP standard curve was created on the day of the experiment prior to all experimental trials and in plasma medium from each subject studied.

Because small amounts of RBC hemolysis can lead to significant increases in ATP, samples were accounted for with a hemolysis-ATP calculation (Kirby *et al.*, 2012). 1 mL of supernatant from the same blood:diluent sample used for plasma [ATP] measurements was analyzed for plasma Hb via spectrophotometry (Molecular Devices SpectraMax) at wavelengths 415, 380, and 450. This plasma [Hb] reading provided an indication of hemolysis (% Hemolysis = {(100 − HCT) * p[Hb]/t[Hb]}*100). Any sample that was more than 2 standard deviations from the mean in % hemolysis was excluded in the analysis and considered technical error.

Experimental Protocols

Protocol 1: Influence of Sympathetic Nervous System Activation on Venous Plasma ATP

The purpose of this protocol was to determine whether sympathetic activation results in significantly elevated plasma ATP or further augments plasma ATP during exercise. While in the supine position, subjects (N = 9) underwent both of the following trials in a randomized order with trials separated by at least 30 minutes. One trial consisted of 2 minutes of quiet rest followed by 2 minutes of −40 mmHg LBNP, where this level of LBNP elicits robust sympathetic nervous system activation (Davy *et al.*, 1998). The other trial involved 2 minutes of quiet rest followed by 6 minutes of continuous rhythmic dynamic handgrip exercise performed at a moderate intensity (15% MVC). LBNP at −40 mmHg was superimposed during forearm exercise for the final 2 minutes of this trial. Blood samples were collected at the end of rest, exercise, or LBNP.

Protocol 2: Influence of Obstructing Skeletal Muscle Perfusion During Moderate Intensity Exercise on Venous Plasma ATP

The purpose of this protocol was to determine whether obstructing skeletal muscle perfusion as a means to inhibit erythrocyte delivery and minimize stimulus-induced release of ATP from erythrocytes and endothelial cells during exercise results in reduced plasma ATP or an inability to elevate plasma during exercise. To do so, subjects were instrumented with a blood pressure cuff wrapped around the upper arm proximal to the anitecubital fossa that

was connected to a rapid cuff inflation unit (Hokanson, Bellevue, WA, USA). A total of 3 trials were performed in a randomized order. Two minutes of quiet rest took place prior to each trial. One trial (N = 9) consisted of 6 minutes of continuous moderate intensity handgrip exercise and thus acted as a time control trial to characterize the expected change in plasma ATP during unobstructed flow conditions. A second trial (N = 9) was conducted to determine whether muscle perfusion was necessary to maintain high levels of plasma ATP, and was performed similar to the time control trial, yet the last two minutes of exercise were performed in conjunction with complete obstruction of blood flow to the forearm achieved via inflation of the blood pressure cuff to suprasystolic levels (200 mmHg). A third trial (N = 8) was conducted to determine the capacity to increase plasma ATP during moderate exercise when tissue perfusion is obstructed *prior to* onset of exercise. This trial involved 2 minutes of rest with perfusion followed by two minutes of rest with complete occlusion via blood pressure cuff inflation. Moderate intensity handgrip exercise was then initiated for 2 minutes while occlusion was maintained. All subjects rested for a minimum of 30 minutes between each trial. Blood samples were collected at the end of rest, exercise, or occlusion + exercise.

Data Acquisition and Analysis

Data was collected and stored on computer at 250 Hz and analyzed off-line with signal-processing software (WinDaq, DATAQ Instruments, Akron, OH, USA). Mean arterial pressure (MAP) was determined from the arterial pressure waveform. An average of 30 seconds was calculated for FBF, FVC, HR, and MAP for each trial segment of interest. Sigmaplot (Systat Software) was used for all statistical comparisons.

Statistics

All values are reported as means ± S.E.M. Specific hypothesis testing within each trial was performed using a one-way repeated measures ANOVA, and for the case of a significant F, Student Newman Kuels post hoc analysis was performed. Comparison of ATP values at specific time points were made with unpaired t-tests, and the values within a condition with paired t-tests. Significance was set *a priori* at $P<0.05$.

Results

Protocol 1: Influence of Sympathetic Nervous System Activation on Venous Plasma ATP

Forearm and systemic hemodynamics for Protocol 1 are presented in Table 1 and blood gas parameters are presented in Table 2. LBNP evoked significant reductions in forearm blood flow at rest (−31 ± 4%; $P<0.05$) confirming efficacy of the LBNP system to activate the sympathetic nervous system and evoke vasoconstriction. Despite substantial sympathetic nervous system activation, venous plasma ATP was not greater with addition of LBNP (51±8 vs 58±7 nmol/L; Figure 1A). In the exercise trial, moderate intensity handgrip exercise elicited significant increases in ATP ($P<0.05$) but these values were unaltered by SNA superimposition (47±5, 79±11, and 71±8 nmol/L; Rest, Exercise, and Exercise + LBNP; respectively; Figure 1B). As expected, functional sympatholysis occurred during exercise and thus limited our ability to detect vasoconstriction as an index of SNA (Kirby *et al.*, 2005). Nevertheless, augmentation of SNA was evidenced by a significant increase in heart rate resultant from LBNP (Table 1).

Protocol 2: Influence of Obstructing Skeletal Muscle Perfusion during Moderate Intensity Exercise on Venous Plasma ATP

Forearm and systemic hemodynamics for Protocol 2 are presented in Table 3 and blood gas parameters are presented in Table 4. Forearm handgrip exercise (15% MVC) elicited a

significant elevation in plasma ATP in both the time control trial (53±5 vs 75±11 nmol/L; Figure 2A) and the occlusion trial prior to blood flow obstruction (50±7 vs 90±20 nmol/L; Figure 2B), resulting in an average increase of ~60% from resting baseline. However during the occlusion, plasma ATP was significantly reduced from steady state exercise levels (Figure 3), and was no longer greater than rest (49±12 nmol/L; Figure 2B). In contrast, plasma ATP during continued exercise with freely perfused conditions was unaltered (Δ 3±9%; Figure 3) and maintained above rest (72±8 nmol/L; Figure 2A). In the trial where occlusion occurred at rest prior to exercise, a small but non-significant increase in ATP was observed following cuff inflation at rest (63±5 vs 72±11 nmol/L; P=0.49; Figure 2C), however exercise was unable to significantly augment plasma ATP concentrations (Δ 3±17%) compared to that seen with flow-unobstructed exercise (Δ 61±13%; Figure 4).

As a result of exercise combined with blood flow obstruction, a significant elevation in mean arterial pressure was observed in both trials utilizing this approach ('Exercise then occlusion' and 'Occlusion then Exercise'; Table 3). This observation is consistent with muscle metaboreflex activation often observed when oxygen demand exceeds oxygen delivery (O'Leary *et al.*, 1999), and provides an indication that substantial sympathetic activation had occurred (Fadel *et al.*, 2003). Lastly, all subjects were able to continue handgrip exercise despite blood flow obstruction indicating that muscle work performed was maintained throughout trials. Confirming this, blood gas analysis data are provided in Table 4 and depict the directional changes in parameters expected from increased metabolic activity alone and in combination with reduced oxygen delivery (i.e. ischemia).

Discussion

The present investigation was designed to gain insight into which cell sources contribute to the increase in plasma ATP draining active skeletal muscle during exercise in humans. We demonstrate that sympathetic activation via LBNP does not increase venous plasma ATP draining skeletal muscle at rest nor during moderate intensity forearm exercise, indicating a limited contribution of ATP from sympathetic nerves to intravascular ATP concentrations during exercise. Next, we identify that skeletal muscle perfusion is obligatory to maintain increased levels of intravascular ATP during exercise despite augmentation of ATP releasing stimuli (e.g. hypoxemia), and that plasma ATP cannot increase during exercise without intact muscle perfusion. This phenomenon occurs in spite of continued muscle contractions and endogenous reflex activation of the sympathetic nervous system, arguing against the muscle itself and confirming that sympathetic nerves are unlikely to be significant sources for extracellular ATP found within blood. We conclude that augmentation and maintenance of elevated intravascular ATP during exercise requires intact skeletal muscle perfusion and suggests an important role for blood and/or endothelial cells as an intravascular ATP source.

Sympathetic Nerves as a Source for Plasma ATP in Humans

While norepinephrine-mediated vasoconstriction clearly results from sympathetic activation, ATP can also be co-released from sympathetic nerves and lead to vasoconstriction (Burnstock & Kennedy, 1986; Todorov *et al.*, 1996; Kluess *et al.*, 2010). Additionally, stimulation of purinergic 2X receptors appears to provide a tonic vasoconstrictor signal during exercise in which the sympathetic nervous system is activated (Buckwalter *et al.*, 2004), further substantiating such claims of nerve-mediated ATP release and associated vasoconstriction. In humans, ATP increases in the interstitial space during exercise and may in part result from elevated sympathetic activity (Li *et al.*, 2005; Cui *et al.*, 2011). While a recent study aimed to address a similar question to ours through intra-arterial administration of tyramine (to evoke endogenous spillover of norepinephrine) (Mortensen *et al.*, 2011), tyramine does not authentically elevate sympathetic nerve activity and fails to evoke co-

release of neuronal ATP (Muramatsu, 1987; Driessen *et al.*, 1996). Rather, usage of LBNP in humans does appear to elicit nerve-mediated ATP release (Pelleg & Burnstock, 1990; Taddei *et al.*, 1990), and was therefore used as our stimulus to increase sympathetic nervous system activation. We hypothesized that intravascular ATP may result as overflow from nerve release of ATP in humans.

In contrast to our hypothesis, extracellular ATP was not increased in the blood draining skeletal muscle as a result of sympathetic activation despite significant elevations in sympathetic outflow (Figure 1A). Further, while moderate intensity forearm exercise stimulated a significant rise in plasma ATP, this was not augmented following addition of LBNP during this high flow condition (Figure 1B). Consistent with this logic, handgrip exercise of mild-to-moderate intensity does not significantly increase SNA (Victor & Seals, 1989; Seals & Victor, 1991), yet plasma ATP is increased during exercise at these workloads (present study and (Kirby *et al.*, 2012). Collectively, these data suggest that sympathetic nerves are unlikely to be a principal source for extracellular ATP within the blood of humans during exercise.

Impact of Exercise and Altered Skeletal Muscle Perfusion on Venous Plasma ATP in Humans

While exercise hyperemia, and the milieu created by muscle contractions are associated with a rise in plasma ATP *in vivo*, what specifically about exercise is cause for elevated intravascular ATP is unclear. Nevertheless, important roles for hemoglobin conformational state (Gonzalez-Alonso *et al.*, 2002; Farias *et al.*, 2005; Akatsu *et al.*, 2010; Kirby *et al.*, 2012) and the modulation by acidosis (Ellsworth *et al.*, 1995), hypercapnia (Bergfeld & Forrester, 1992), or temperature (Kalsi & Gonzalez-Alonso, 2012), as well as tissue mechanical compression (Crecelius *et al.*, 2010; Mortensen *et al.*, 2011) have all been put forth as potential mediators for increased plasma ATP in various models. All of these stimuli occur with muscle contractions. One obvious aspect of these exercise-mediated stimuli associated with elevated intravascular ATP is the substantial overlap with basic erythrocyte function, and interestingly, erythrocytes are well recognized to release ATP in response to all of the above stated stimuli (Ellsworth *et al.*, 2009). Additionally, endothelial cells may also release ATP in response to low oxygen and increased mechanical stress (Bodin *et al.*, 1991; Bodin & Burnstock, 1995). The latter however has been refuted as a means to increase ATP because pharmacologically-induced increases in blood flow (and concurrent shear stress) do not elevate plasma ATP in humans (Mortensen *et al.*, 2009; Kirby *et al.*, 2012).

Our goal in protocol 2 was to cause complete occlusion of blood flow to the active muscle as a means to: (a) inhibit red blood cell delivery to the stimulus rich environment, and (b) significantly reduce the stimulus-induced release from red cells and/or endothelial cells (e.g. shear stress) to release ATP. Because these intravascular cell sources are associated with ATP release to stimuli known to increase plasma ATP, we hypothesized that elevations in plasma ATP during exercise are dependent on intact muscle perfusion.

The results of Protocol 2 clearly demonstrate that inhibition of blood flow to active skeletal muscle after initiation of exercise abolishes the normal rise in plasma ATP, such that ATP is returned to resting baseline levels (Figure 2B). Moreover, this reduction in ATP seen during complete occlusion clearly contrasts the lack of change in plasma ATP observed when muscle perfusion remains uninterrupted (Figure 3). These observations occur in the face of augmented stimuli for ATP release during occlusion (e.g. hypoxemia) and highlight the crucial contribution of muscle perfusion in detecting elevated intravascular ATP. When we reversed the experimental design and fully obstructed muscle blood flow *prior* to exercise, elevations in intravascular ATP during muscle contractions were completely inhibited (< 5% increase vs ~60% with perfusion intact; Figure 4). It is important to note that these subjects

in Protocol 2 were able to continue forearm contractions without faltering during full occlusion, indicating muscle work was unchanged. Thus, if ATP release were occurring from muscle and interstitial ATP were passing into blood, then a maintained elevation in plasma ATP would have been expected, but this did not occur. Taken together, our findings demonstrate an obligatory role for skeletal muscle perfusion in mediating the rise in plasma ATP with exercise and are consistent with the concept that extracellular ATP within the bloodstream originates from an intravascular source rather than from the extravascular compartment.

Lastly, a noticeable rise in mean arterial blood pressure was observed during ischemic exercise resultant from metaboreflex activation indicating clear sympathetic nervous system engagement (Victor *et al.*, 1987). Yet, plasma ATP was still diminished during occlusion and unable to rise significantly when exercise was initiated (Figure 3 & 4). This observation serves to substantiate our primary findings from Protocol 1. Our collective observations indicate that sympathetic nerves and skeletal muscle that may contribute to extravascular ATP are unlikely to be the dominant sites for exercise-mediated elevations in intravascular ATP of humans. Rather, it is more apparent that something specific to the delivery of blood is obligatory to augment plasma ATP concentrations during moderate intensity exercise; presumably the continuous presence of new red blood cells.

How Might Perfusion Be Crucial to Elevated Intravascular ATP?

Observations from human studies suggest that enhanced shear stress is unlikely the cause for increased ATP from exercise (Mortensen *et al.*, 2009; Kirby *et al.*, 2012), and may indicate a relatively weak role for endothelial cell derivation as the principal plasma ATP source. The present study completely inhibited the replenishment of fresh erythrocytes to the stimuli rich environment. In this manner, ATP in plasma resultant from red cells is likely to be substantially reduced without addition of fresh red cells because no new cells are delivered and ATP release from already present cells does not largely increase as a result of increased exposure time to a stimulus (Sprague *et al.*, 2009; Kalsi & Gonzalez-Alonso, 2012). Therefore, it is presumable that increases in erythrocyte delivery and the renewal of fresh red cells to active muscle are of utmost importance in augmenting plasma ATP during exercise.

Along these lines, another point of thought relates to the detailed interaction between exercise intensity and the associated magnitude of hyperemia and ATP releasing stimuli. We notice in protocol 1, LBNP evoked ~30% reduction in blood flow and a concurrent rise in stimuli for ATP release (e.g. deoxygenation), but plasma ATP was not significantly altered (up nor down). Observations such as these may suggest an acute regulatory balance between blood flow and ATP releasing stimuli that works in unison to optimize ATP release and its impact on vessel tone, rather than an all-or-none phenomenon. Hence, our current working hypothesis is that factors associated with muscle contraction not only create stimulus for immediate ATP release but may also in effect facilitate the augmentation of blood flow and additional red cell delivery for sustained balance. This interaction between exercise hyperemia and stimulus may also be modulated by ectoenzymes to provide the net plasma ATP concentrations, thus controlling the transmitter for purinergic receptor activation and the ensuing vasomotor response. A detailed schematic of this working hypothesis is presented as Figure 5.

Experimental Considerations

Occlusion completely abrogated exercise hyperemia yet ATP was still detected in the plasma suggesting release from certain sources occurs to some extent independent of active perfusion. Whether this is associated with reductions in ectoenzyme activity (from loss of

blood flow/shear stress (Yegutkin *et al.*, 2000)) or a general basal ATP release from an unidentified source is indiscernible, but further study is warranted. Alternatively, because we completely occluded arterial inflow and venous outflow by way of a blood pressure cuff, blood within the 'enclosed' space was effectively stagnant and thus endogenous ATP concentrations could also have been impacted by local ectoenzymes and/or the ADP suppression effect on red cell ATP release (Pearson *et al.*, 1980; Wang *et al.*, 2005). Regardless, our data clearly demonstrate a crucial need for continuous intact skeletal muscle perfusion in order to elevate and effectively maintain high levels of extracellular ATP within the vessel lumen during exercise in humans.

A separate consideration is that if ATP were able to bypass the endothelium from muscle to plasma, muscle ATP release could have been reduced during ischemia by way of limiting oxygen delivery as a substrate for 'aerobic' metabolism. This is unlikely to explain our observations because muscle glycogen stores are plentiful and do not deplete within minutes and are sufficient to sustain ATP production (Bertocci *et al.*, 1992). Further, low oxygen may evoke, if anything, an increase in ATP release from muscle rather than a decrease (Clemens & Forrester, 1981).

Conclusions

The results from the present investigation demonstrate that sympathetic nervous system activation does not result in elevated intravascular ATP at rest nor augments exercise-mediated increases in plasma ATP. Alternatively, elevations in plasma ATP as a result of moderate intensity exercise were abolished by completely limiting perfusion to the active skeletal muscle and ATP did not elevate when blood flow was impeded prior to the onset of exercise, despite increased stimuli for release. Given that muscle contractions persisted in the face of blood flow occlusion and plasma ATP was unaffected, it can be concluded that extracellular ATP is unlikely to originate from the active muscle itself. Taken together, our observations suggest increased delivery of blood to the working muscle resultant from exercise is requisite for exercise-induced augmentation and maintenance of intravascular ATP in humans.

Acknowledgments

We would like to thank Leora Garcia for her assistance with the project and the subjects who volunteered to participate. This research was supported by National Institutes of Health awards HL095573 and HL102720 (F.A. D).

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New Findings

- What is the central question of this study?
- Plasma ATP increases during exercise in humans, but whether ATP predominantly originates from extravascular (nerves and muscle) or intravascular (blood and endothelial cells) sources is unclear.
- What is the main finding and its importance?
- The collective observations indicate that neither sympathetic nerves nor active skeletal muscle are likely to be origin for intravascular ATP during dynamic muscle contractions in humans. Further, elevations in skeletal muscle perfusion are requisite to increase and maintain high plasma ATP during exercise, suggesting ATP release from an intravascular cell source.

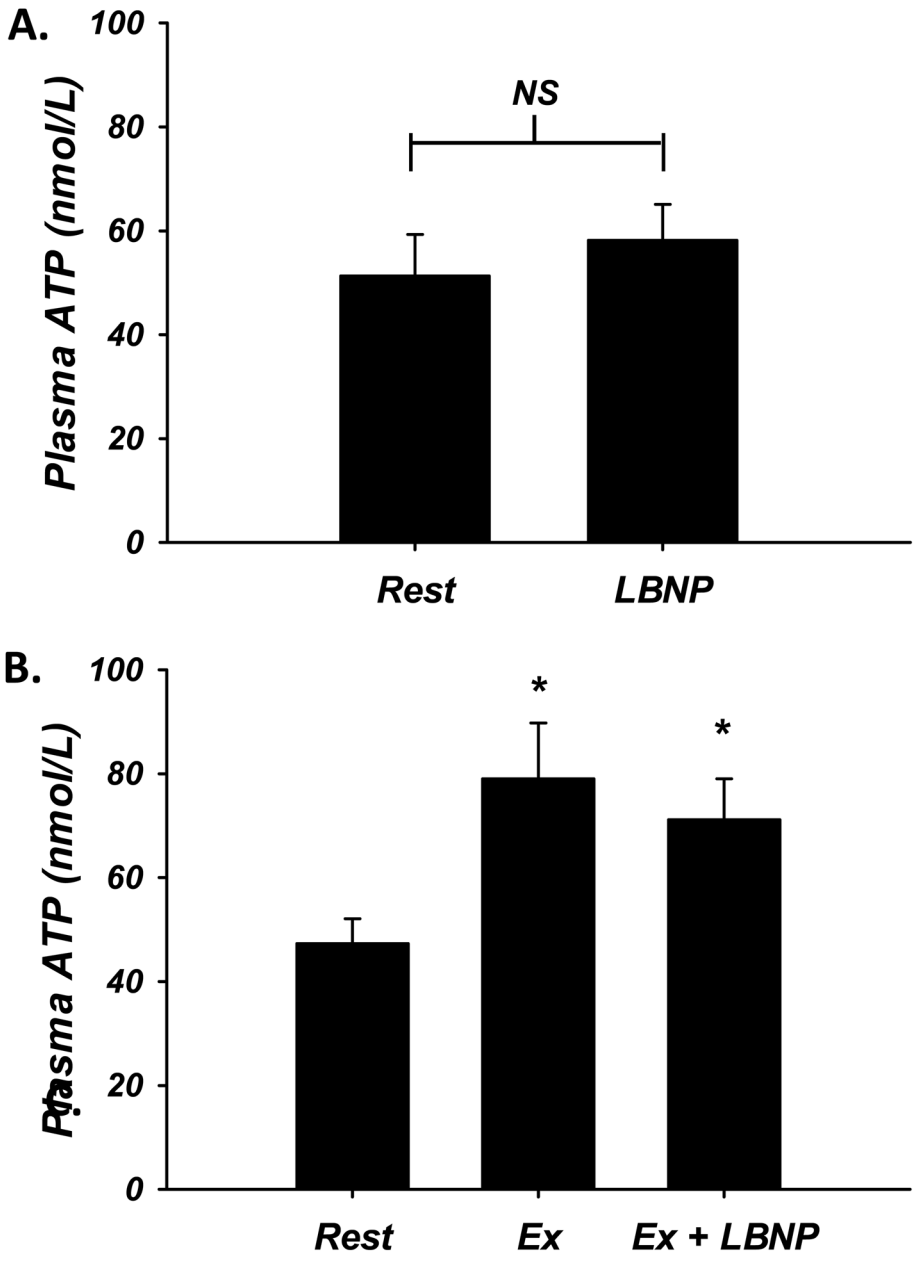

Figure 1. A–B: Protocol 1 – Impact of Sympathetic Nervous System Activation on Venous Plasma ATP at Rest and During Exercise

Lower body negative pressure (LBNP) at −40 mmHg was utilized to augment sympathetic nervous system activity (SNA). SNA had no independent effect on venous plasma ATP when superimposed at rest (A) or during moderate intensity foreram handgrip exercise (B). * P <0.05 vs rest alone.

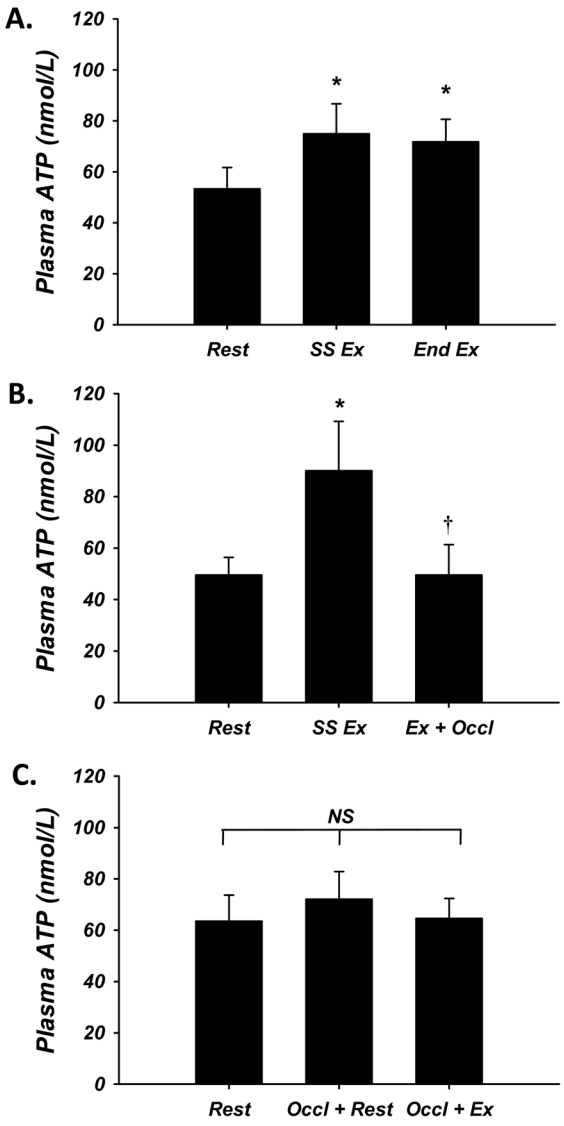

Figure 2. A–C: Protocol 2 – Impact of Occluding Skeletal Muscle Blood Flow during Exercise on Venous Plasma ATP

Moderate intensity forearm handgrip exercise evoked significant elevations in venous plasma ATP during both steady state (SS; 4 minutes) and at end exercise (6 minutes) time points (A). When skeletal muscle perfusion ceased via complete blood flow occlusion (Occl), plasma ATP was significantly reduced from SS exercise and was no longer greater than rest (B). Application of a cuff to completely occlude forearm blood flow prior to exercise abolished any significant increase in plasma ATP resultant from exercise (C). ***** P <0.05 vs rest; † P <0.05 vs SS Ex.

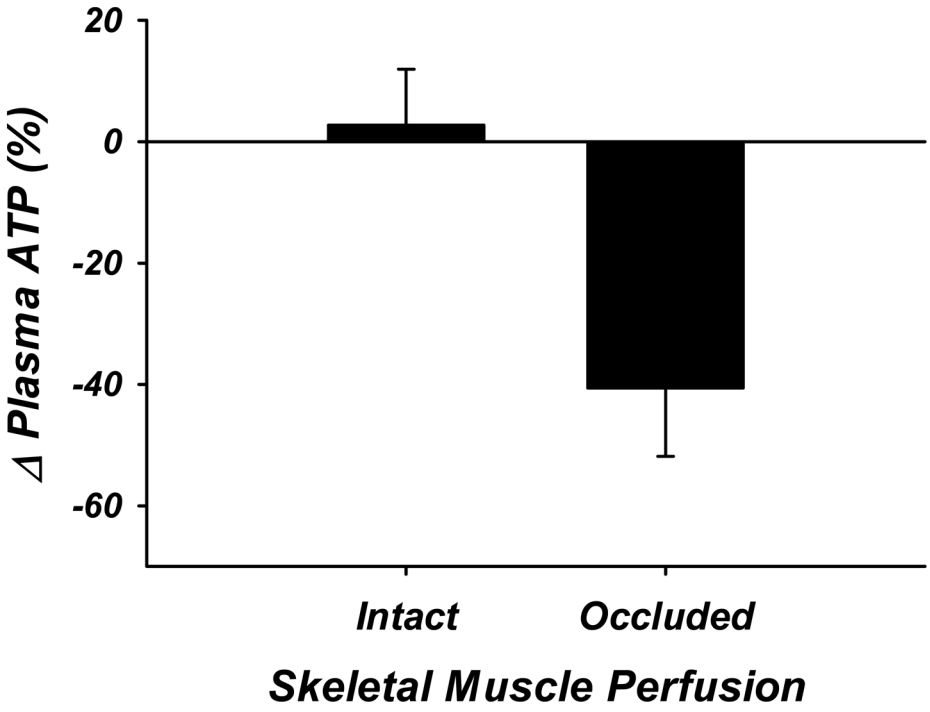

Figure 3. Protocol 2 – Effects of Obstructing Skeletal Muscle Blood Flow During Forearm Exercise on Steady-State Plasma ATP

During forearm exercise, steady-state plasma ATP concentrations are unaffected when muscle perfusion is uninterrupted (intact condition). In contrast, a significant reduction in plasma ATP is observed during blood flow occlusion. ***** $P<0.05$ vs intact perfusion.

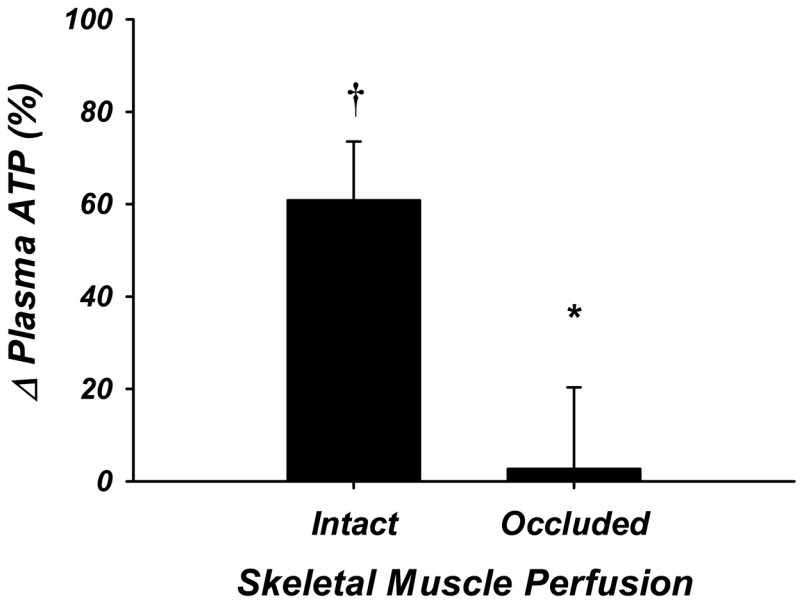

Figure 4. Protocol 2 – Change in Venous Plasma ATP from Rest to Exercise with Intact and Occluded Skeletal Muscle Blood Flow

Moderate intensity forearm exercise evokes a significant increase in venous plasma ATP that is not observed when skeletal muscle perfusion is completely obstructed prior to initiation of muscle contractions. * $P<0.05$ vs intact perfusion; † $P<0.05$ vs zero.

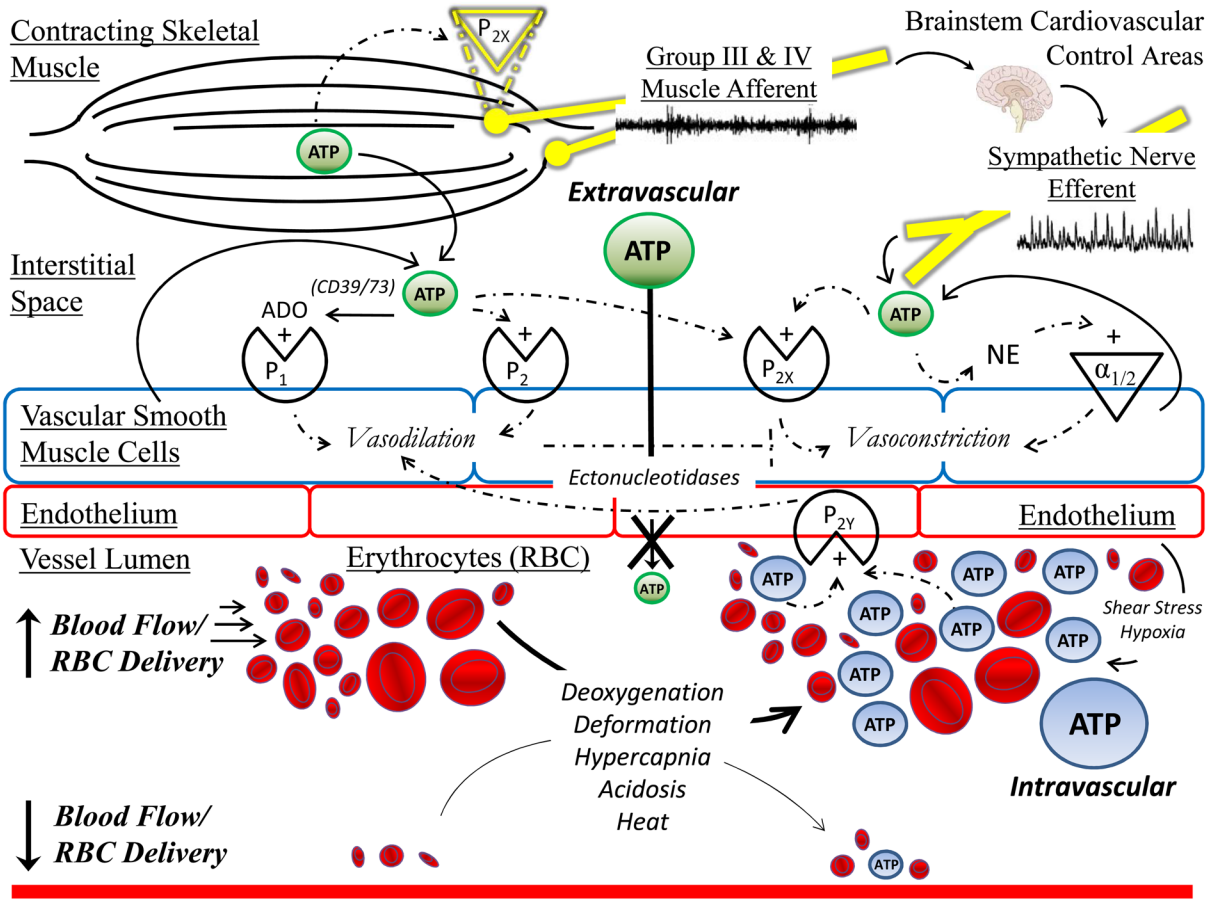

Figure 5. Schematic Depicting Potential Sources of Intravascular ATP

ATP may be released from skeletal muscle and sympathetic nerves to accumulate in the interstitial space during muscle contractions (i.e. extravascular ATP), however this ATP is unlikely to bypass the vascular wall due to endothelial ectonucleotidases. ATP is also released from intravascular cell sources, erythrocytes and endothelial cells in response to specific stimuli concurrent with muscle contractions. Both blood flow/red blood cell delivery and ATP releasing stimuli act in tandem with ATP consuming processes to regulate the absolute plasma ATP concentration (i.e. intravascular ATP). ATP can elicit vasoconstriction, vasodilation, or blunt sympathetic vasoconstriction depending on the location/subtype of purinergic receptor activation. P, Purinergic receptor; ATP, adenosine triphosphate; ADO, adenosine; RBC, red blood cell; NE, norepinephrine.

Table 1

Forearm and Systemic Hemodynamics in Protocol 1

Trial	Condition	Forearm Blood Flow (ml/min)	Mean Arterial Pressure (mmHg)	Forearm Vascular Conductance (ml/min/100 mmHg)	Heart Rate (beats/min)
LBNP during Rest	**Rest**	27.5±2.9	93±2	29.9±3.2	56±3
	+LBNP	18.8±2.0*	92±3	20.5±2.1*	63±2*
LBNP during Exercise	**Rest**	27.1±2.1	93±3	29.5±2.8	57±3
	15% Ex	206.8±12.5*	97±3	214.4±15.2*	63±3*
	+LBNP	199.5±13.7*	97±3	206.4±14.8*	71±2*†

*$P<0.05$ *vs* Rest;

†$P<0.05$ *vs* 15% Ex

Table 2

Venous Blood Gases in Protocol 1

Trial	Condition	pH	pO_2 (mmHg)	pCO_2 (mmHg)	FO_2Hb (%)
LBNP at Rest	**Rest**	7.36±0.01	29.8±2.0	48.8±1.9	50.5±3.9
	+LBNP	7.35±0.01	25.3±1.3 *	49.2±1.9	41.0±3.3 *
LBNP during Exercise	**Rest**	7.35±0.01	28.2±2.1	49.9±1.8	46.8±4.9
	15% Ex	7.30±0.01 *	22.7±0.5 *	60.0±1.5 *	31.3±1.3 *
	+ LBNP	7.30±0.01 *	19.4±0.6 *†	61.1±2.6 *	24.8±1.3 *†

* $P < 0.05$ *vs* Rest;

† $P < 0.05$ *vs* 15% Ex

Table 3

Forearm and Systemic Hemodynamics in Protocol 2

Trial	Condition	Forearm Blood Flow (ml/min)	Mean Arterial Pressure (mmHg)	Forearm Vascular Conductance (ml/min/ 100 mmHg)	Heart Rate (beats/min)
Time Control Exercise	**Rest**	29.3±2.9	87±1	33.7±3.1	51±3
	15% Ex Min 4	222.1±22.2 *	93±2 *	237.1±22.4 *	56±3 *
	15% Ex Min 6	227.7±22.8 *	94±2 *	239.1±21.3 *	56±3 *
Exercise then Occlusion	**Rest**	34.7±3.0	86±2	40.4±3.5	54±2
	15% Ex	228.8±12.1 *	91±2 *	250.1±11.0 *	58±2
	15% Ex + Occlusion	0.0±0.0 *†	109±3 *†	0.0±0.0 *†	67±4 *†
Occlusion then Exercise	**Rest**	30.0±3.3	87±2	34.2±3.4	52±3
	Occlusion	0.0±0.0 *	87±1	0.0±0.0 *	54±3
	Occlusion + 15% Ex	0.0±0.0 *	102±2 *†	0.0±0.0 *	61±4 *†

* $P < 0.05$ *vs* Rest;

† $P < 0.05$ *vs* prior timepoint

Table 4

Venous Blood Gases in Protocol 2

Trial	Condition	pH	pO_2 (mmHg)	pCO_2 (mmHg)	FO_2Hb (%)
Time Control Exercise	**Rest**	7.36±0.01	30.9±2.3	46.0±1.4	54.5±4.7
	15% Ex Min 4	7.29±0.01 *	22.9±0.7 *	59.1±2.1 *	32.2±1.6 *
	15% Ex Min 6	7.29±0.01 *	22.4±0.7 *	59.0±2.0 *	31.2±1.3 *
Exercise then Occlusion	**Rest**	7.37±0.01	26.1±1.2	45.0±1.6	45.0±3.0
	15% Ex	7.30±0.01 *	22.9±0.7 *	57.0±1.7 *	32.0±1.3 *
	15% Ex + Occlusion	7.27±0.02 *	18.9±1.3 *†	57.5±3.6 *	22.4±2.7 *†
Occlusion then Exercise	**Rest**	7.36±0.01	28.5±2.2	47.4±1.6	48.7±5.0
	Occlusion	7.36±0.01	27.4±1.0	41.3±2.2 *	46.0±2.8
	Occlusion+ 15% Ex	7.35±0.01	24.2±0.9 *†	40.6±2.3 *	37.7±1.8 *†

* $P < 0.05$ *vs* Rest;

† $P < 0.05$ *vs* prior timepoint