Impaired Skeletal Muscle Blood Flow Control With Advancing Age in Humans
Attenuated ATP Release and Local Vasodilation During Erythrocyte Deoxygenation

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Rationale: Skeletal muscle blood flow is coupled with the oxygenation state of hemoglobin in young adults, whereby the erythrocyte functions as an oxygen sensor and releases ATP during deoxygenation to evoke vasodilation. Whether this function is impaired in humans of advanced age is unknown.

Objective: To test the hypothesis that older adults demonstrate impaired muscle blood flow and lower intravascular ATP during conditions of erythrocyte deoxygenation.

Methods and Results: We showed impaired forearm blood flow responses during 2 conditions of erythrocyte deoxygenation (systemic hypoxia and graded handgrip exercise) with age, which was caused by reduced local vasodilation. In young adults, both hypoxia and exercise significantly increased venous [ATP] and ATP effluent (forearm blood flow×[ATP]) draining the skeletal muscle. In contrast, hypoxia and exercise did not increase venous [ATP] in older adults, and both venous [ATP] and ATP effluent were substantially reduced compared with young people despite similar levels of deoxygenation. Next, we demonstrated that this could not be explained by augmented extracellular ATP hydrolysis in whole blood with age. Finally, we found that deoxygenation-mediated ATP release from isolated erythrocytes was essentially nonexistent in older adults.

Conclusions: Skeletal muscle blood flow during conditions of erythrocyte deoxygenation was markedly reduced in aging humans, and reductions in plasma ATP and erythrocyte-mediated ATP release may be a novel mechanism underlying impaired vasodilation and oxygen delivery during hypoxemia with advancing age. Because aging is associated with elevated risk for ischemic cardiovascular disease and exercise intolerance, interventions that target erythrocyte-mediated ATP release may offer therapeutic potential. (Circ Res. 2012;111:220-230.)

Key Words: aging ■ blood flow ■ ATP ■ erythrocytes ■ hypoxia

Evidence indicates a close coupling between skeletal muscle blood flow and the oxygenation state of hemoglobin in young healthy humans.1,2 In this respect, the erythrocyte may function as an oxygen sensor in addition to an oxygen carrier and facilitate local vasodilation in relation to the degree of unoccupied hemoglobin binding sites.2–4 In addition to the production of nitric oxide via deoxyhemoglobin-mediated nitrite reduction5 and the possible formation of S-nitrosohemoglobin5 during red blood cell deoxygenation, ATP is also released from the red cell and likely contributes to local vasodilation observed during mismatches in oxygen supply and demand.2,4,6,7 Specifically, during physiological stimuli such as hypoxia and muscle contractions, ATP is believed to assist in the regulation of blood flow and oxygen delivery to meet the metabolic demands of the tissue via binding to purinergic (P2) receptors on the endothelium.2,7 Importantly, and unique to ATP vasomotor signaling in humans, we and others have demonstrated that exogenous ATP administration not only evokes substantial vasodilation but also blunts sympathetic vasoconstriction in a dose-dependent fashion, similar to what occurs in contracting muscle.8–9 Moreover, because sympathoexcitation typically...
occurs during such conditions, intrusive intravascular ATP is thought to have a critical role in blood flow regulation in humans. Collectively, this schema provides a means of rapidly matching tissue oxygen demand with oxygen delivery via alterations in arteriolar resistance as sensed by the red blood cell oxygenation state.

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Although alterations in skeletal muscle vascular tone during conditions of hemoglobin deoxygenation have been determined in young healthy humans, little information exists in aging humans, a population that demonstrates both endothelial dysfunction and heightened sympathetic activity and is at greater risk for ischemic cardiovascular diseases and exercise intolerance. In this context, whether humans of advanced age have significantly impaired blood flow control during conditions of hemoglobin deoxygenation is unclear. Furthermore, whether this decline in local skeletal muscle vascular control and oxygen delivery with age is related to the loss of circulating vasoactive ATP and, more specifically, the loss of ATP release from red blood cells during hemoglobin deoxygenation is unknown. Therefore, we tested the hypothesis that local control of skeletal muscle blood flow is impaired in aging humans during systemic hypoxia and during muscle contractions (both of which are conditions of erythrocyte deoxygenation) and that plasma ATP of blood draining the skeletal muscle is lower in these conditions in older adults in vivo. Additionally, in an effort to gain mechanistic insight into what underlies the diminished circulating ATP with human aging, we tested the hypothesis that any age-associated impairment may be caused in part by augmented ATP hydrolysis by blood components and impaired release of ATP from the red blood cell during hemoglobin deoxygenation in vitro.

### Methods

A detailed and expanded Methods section is available in the online-only Data Supplement.

### Subjects

With Institutional Review Board approval and after written informed consent was obtained, a total of 37 young and 25 older healthy adults participated in the present investigation. This study was approved by the Human Research Committee of Colorado State University and was performed according to the Declaration of Helsinki.

### Experimental Protocols

#### Protocol 1: Forearm Plasma [ATP] During Intra-Arterial ATP Infusion

Evidence in the lower limb indicates that ATP administered intra-arterially is undetectable in the venous circulation; however, the upper limb is a much smaller mass, and less distance is traveled for a single pass through the microcirculation. To establish that venous plasma [ATP] measures (versus arterial) are more closely reflective of effluent draining the skeletal muscle, we determined whether (1) plasma arterial [ATP] equates to plasma venous [ATP] and (2) ATP is rapidly catalyzed in a single passage of the forearm microcirculation. To do so, we infused saline via a brachial artery catheter for 3 minutes (control) followed by infusion of ATP (8.35 nmol·dL⁻¹·min⁻¹) for 3 minutes in young adults. Blood was sampled from both catheters at the end of saline infusion and from the deep venous catheter during ATP infusion and analyzed for plasma [ATP] via the luciferin-luciferase technique. Arterial [ATP] during infusion was calculated as follows: (8.35 nmol·dL⁻¹·min⁻¹ × forearm volume)/forearm blood flow.

#### Protocol 2: Systemic Hypoxia and Age

An initial rest period of 3 minutes was given to establish baseline values while the subject breathed on the mouthpiece open to room air. As described previously by our laboratory, the level of inspired O₂ was then titrated to achieve an O₂ saturation of ~80%, as established by pulse oximetry on the earlobe (SpO₂), while simultaneously clamping end-tidal CO₂ at baseline values via a self-regulating partial rebreathing system. This transition from baseline to the target level of hypoxia typically occurred in 2 minutes. The subject was then maintained at ~80% O₂ saturation for 5 minutes to establish steady-state levels of SpO₂ and etCO₂, as well as forearm hemodynamics during hypoxia. Forearm blood flow (FBF; Doppler ultrasound) and vascular conductance were calculated, and venous blood was sampled for blood gases and plasma [ATP] at the end of normoxia and the end of hypoxia.

#### Protocol 3: Graded-Intensity Forearm Exercise and Age

After 3 minutes of baseline measurements, subjects performed dynamic rhythmic handgrip exercise in the supine position with the nondominant arm for a total of 15 minutes. This 15-minute trial was graded in exercise intensity, whereby 5-minute segments each of 5%, 15%, and 25% maximum voluntary contraction (MVC) workloads were completed. FBF and forearm vascular conductance were calculated, and venous blood was sampled for blood gases and plasma [ATP] at the end of rest and each exercise intensity level.

#### Protocol 4: ATP Hydrolysis in Whole Blood From Exercising Forearm and Age

Given the observations of reduced extracellular ATP in the blood of older adults during exercise (Results), we sought to determine whether any observed age-associated differences in venous plasma [ATP] were caused by an enhanced capacity to degrade extracellular ATP within the blood of older adults during exercise. To do so, subjects performed exercise as described above in protocol 3, and blood was sampled at the end of rest and each exercise intensity level. Because data indicate that whole blood degrades ATP more rapidly than plasma and that blood cells such as leukocytes are a primary source for nucleotidase activity, we determined the catalysis of 100 μmol/L ATP in whole blood rather than plasma alone. In this respect, intraluminal extracellular ATP is exposed to the mixed media of blood cells, and thus, determination of ATP degradation in whole blood (versus plasma alone) is a more accurate representation of ATP breakdown in vivo.

#### Protocol 5: Erythrocyte ATP Release During Hemoglobin Deoxygenation and Age

Given the collective observations of reduced extracellular ATP in the blood of older adults during 2 conditions of hemoglobin deoxygenation, and given that ATP is catabolized to a similar extent in young and older adults during exercise (Results), we aimed to determine whether the release of ATP from erythrocytes of aging humans during low O₂ exposure was impaired. To do so, erythrocytes were diluted to 20% hematocrit and equilibrated with normoxic gas (16% O₂/6% CO₂) for 15 minutes in a rotating-bulb tonometer. The gas mixture was then changed to a 3% O₂/6% CO₂ mixture for 7 minutes, followed by a secondary drop in O₂ (1.5%/6% CO₂) for 7 more minutes. Our goal was to reduce PO₂ and the fraction of oxyhemoglobin (FO₂Hb) to levels within the range observed in vivo.

### Non-standard Abbreviations and Acronyms

- eCO₂: end-tidal carbon dioxide
- FBF: forearm blood flow
- FO₂Hb: fraction of oxyhemoglobin
- MVC: maximal voluntary contraction
- SpO₂: oxygen saturation pulse oximetry
Red cells were sampled at the end of each gas equilibration period, and ATP was measured as described in the online-only Data Supplement and normalized to red cell count.\textsuperscript{18,19}

**Data Acquisition and Analysis**

Data were collected and stored on a computer at 250 Hz and analyzed off-line. Baseline FBF, heart rate, and mean arterial pressure represent an average of the last 30 seconds of each condition.\textsuperscript{4} To account for changes in FBF and its impact on [ATP] concentration measurements and to quantify the rate of total ATP draining the active muscle, ATP effluent was calculated as FBF × [ATP]/1000, similar to other methods of data quantification when blood flow is altered.\textsuperscript{2,20}

**Statistical Analysis**

All values are reported as mean±SEM. Specific hypothesis testing during hypoxia or exercise studies across age was performed with 2-way repeated-measures ANOVA. In the case of a significant $F$ value, Student-Newman-Keuls post hoc pairwise comparisons were made to identify significant differences. Comparison of the outcome variables at specific time points between conditions were made with unpaired $t$ tests, and the values within each condition were compared with paired $t$ tests. SigmaPlot 11.0 (Systat Software, Inc, San Jose, CA) was used for all analyses, and statistical significance was set a priori at $P<0.05$.

**Results**

**Subject Characteristics**

The mean age difference between the young and older adults was 41 years (23±1 versus 64±1 years). Older adults had a greater body mass index, percentage of body fat, total and low-density lipoprotein cholesterol, and triglycerides; however, all measured variables were within the normal healthy range. All subject characteristics are noted in Online Table IA. Additional characteristics of those subjects in protocol 5 are presented in Online Table IB.

**Protocol 1: Intra-Arterial ATP Infusion in Young Adults**

Exogenous ATP infusion in young adults (n=8) significantly increased arterial plasma [ATP] compared with saline control (38±8 versus 956±148 nmol/L); however, venous plasma [ATP] was unchanged (52±13 versus 53±11 nmol/L; Figure 1). Forearm and systemic hemodynamics are presented in Online Table II. These data demonstrate elevations in arterial plasma [ATP] without subsequent increases in venous plasma [ATP], which supports the concept that extracellular ATP is rapidly degraded once in the circulation.\textsuperscript{2,17,21} Given these findings and the fact that the milieu (PO$_2$, pCO$_2$, pH) of venous blood is more closely representative of the microcirculatory environment and conducive to ATP release, we focused our investigation in aging humans on plasma [ATP] within venous blood draining the skeletal muscle.

**Protocol 2: Systemic Hypoxia and Age**

The degree of systemic hypoxia was not different between young (n=10) and older (n=8) adults (80.8±0.9% SpO$_2$ versus 81.5±0.9% SpO$_2$), and t$_{ET}$CO$_2$ was clamped at baseline (Online Table III). FBF and forearm vascular conductance increased significantly during systemic hypoxia from normoxia in young adults but not in older adults (Figures 2A and 2B; Online Table IIIB). Mean arterial pressure increased in older adults in response to hypoxia, and absolute mean arterial pressure was greater than in young adults (Online Table IIIB). Heart rate increased during systemic hypoxia in both groups; however, the response was lower in older adults (Online Table IIIB).

Venous plasma [ATP] was not different at rest between young and older adults (64±8 versus 47±10 nmol/L; Figure 2C). Young adults had significantly greater [ATP] during hypoxia than during normoxia (106±16 nmol/L; Δ 43±19 nmol/L; Figure 2C). Remarkably, older adults had significantly lower [ATP] than young adults during hypoxia and did not experience a significant increase in [ATP] above rest (48±8 nmol/L; Δ 1±9 nmol/L; Figure 2C). Similar to plasma [ATP], resting ATP effluent was not different with age. During hypoxia, ATP effluent increased in young adults (2.6±0.7 versus 5.3±1 nmol/min; Figure 2D), whereas there was no change in older adults (1.8±0.4 versus 1.7±0.3 nmol/min; Figure 2D). Respiratory variables and venous blood gases are presented in Online Table III.

**Protocol 3: Graded-Intensity Forearm Exercise and Age**

FBF and forearm vascular conductance increased in a graded fashion during progressive handgrip exercise. Older (n=12) compared with young (n=14) adults had significantly lower absolute FBF at 15% and 25% MVC exercise, as well as significantly less forearm vasodilation (forearm vascular conductance) during all exercise workloads (Figures 3A and 3B). Heart rate and mean arterial pressure increased in both young and older adults in response to exercise, and absolute mean arterial pressure was greater in older subjects (Online Table IVB).

Venous plasma [ATP] was not different at rest between young and older adults (62±5 versus 59±9 nmol/L; Figure 3C). Young adults had significantly greater [ATP] during all exercise intensities than during rest conditions (5% MVC, 96±13 nmol/L; 15% MVC, 105±9 nmol/L; and 25% MVC, 126±12 nmol/L; Figure 3C). Older adults did not have a significant increase in [ATP] above rest and had significantly
lower [ATP] than young adults at all exercise intensities (5% MVC, 52 ± 7 nmol/L; 15% MVC, 73 ± 7 nmol/L; and 25% MVC, 83 ± 8 nmol/L; Figure 3C). ATP effluent was not different with age at rest (young, 2.4 ± 0.4 nmol/min versus older, 2.0 ± 0.3 nmol/min; Figure 3D). Although both young and older adults had greater ATP effluent during exercise than during rest, ATP effluent was significantly attenuated (50% to 60%) with age (Figure 3D and Figure 6). Venous blood gases are presented in Online Table V.

We measured arterial plasma [ATP] sampled from the brachial artery during exercise in 5 young adults who were instrumented with arterial catheters for other studies in the laboratory. Arterial [ATP] was 34 ± 9 nmol/L at rest and 34 ± 6 nmol/L during 5% MVC exercise. During 15% and 25% MVC exercise, arterial [ATP] was 44 ± 10 and 50 ± 4 nmol/L (P=0.2 versus rest), respectively. Importantly, arterial [ATP] at rest or during any exercise intensity was always lower than the average resting venous [ATP] (≈60 nmol/L; Online Figure 1), which suggests that arterial plasma [ATP] detectable at the brachial artery does not increase in this model of exercise and furthermore does not explain elevations in venous plasma [ATP] that result from exercise or explain the age group differences found in this protocol.

**Protocol 4: ATP Hydrolysis in Whole Blood From Exercising Forearm and Age**

The percentage of ATP remaining of 100 μmol/L ATP in whole blood was significantly lower from the previous time point of measurement, which indicates rapid and progressive ATP degradation over time within whole blood in young (n=8) and older (n=8) adults (P<0.05; Figures 4A through 4E). There were no age group differences in ATP hydrolysis at any exercise intensity or time point. At minute 15, the percentage of ATP remaining was lower at all exercise intensities than at rest for all subjects (P<0.05), which indicates that acute exercise augments ATP catabolism in whole blood.

**Protocol 5: Erythrocyte ATP Release During Hemoglobin Deoxygenation and Age**

Erythrocytes from young (n=8) and older (n=8) adults were similarly deoxygenated when exposed to 3% O2 and 1.5% O2 compared with 16% O2 normoxic gas (Online Table V). Po2, pCO2, and pH were not different between age groups during normoxic and hypoxic gas exposure.

Absolute ATP release from red cells of young adults tended to increase during the first level of hypoxia (P=0.087) and was significantly elevated during the second level of
The relative (%) change in ATP from baseline was graded with the level of deoxygenation. In contrast, red blood cells of older adults did not release ATP at either level of deoxygenation, and a clear age-associated impairment was observed during the second level of hypoxia (205±57% versus 43±24%; Figure 5B).

**Discussion**

To the best of our knowledge, this is the first study to directly investigate alterations in extracellular intravascular ATP in aging humans and the control of blood flow and vascular tone at rest or during hemoglobin-deoxygenating stimuli such as systemic hypoxia and exercise. There are several new find-
ings from the present series of in vivo and in vitro experiments. First, despite an augmented perfusion pressure in older adults, skeletal muscle blood flow during conditions of erythrocyte deoxygenation (systemic hypoxia and exercise) was markedly reduced in aging humans because of impaired local vasodilation. Second, although venous plasma [ATP] increased during systemic hypoxia and during dynamic forearm exercise in young adults, healthy older humans did not

Figure 4. Hydrolysis of ATP in whole blood acquired during graded rhythmic handgrip exercise. Exogenous ATP (100 μmol/L) was progressively catabolized in human whole blood samples over 15 minutes at all exercise intensities. No difference with age at rest or across exercise intensities was observed when percentage of ATP remaining was determined at rest (A), 5% maximum voluntary contraction (MVC; B), 15% MVC (C), or 25% MVC (D). E, ATP hydrolysis (after 15 minutes) was significantly greater than at rest during exercise for all subjects but was not dependent on intensity. *P<0.05 vs prior time point. ‡P<0.05 vs rest.
experience an increase in [ATP] and had significantly lower [ATP] than young adults under these conditions. Third, the rate of ATP effluent from skeletal muscle increased during systemic hypoxia and graded-intensity forearm exercise in young adults, whereas no increase in ATP effluent was observed during hypoxia in older adults, and the increase was substantially blunted during exercise (50%–65% versus young adults; Figure 6). Importantly, the reduced [ATP] and ATP effluent in older adults occurred concomitantly with less vasodilation. Fourth, although extracellular ATP was degraded rapidly within the human circulation and this catabolism was enhanced by exercise, aging did not appear to augment ATP hydrolysis in whole blood at rest or during exercise. Finally, release of ATP from erythrocytes of older adults in response to deoxygenation was markedly impaired, and the present data are the first evidence of such impairment in aging humans. Collectively, reductions in erythrocyte-mediated ATP release and intravascular ATP may be a novel mechanism by which skeletal muscle blood flow and oxygen delivery are impaired during hypoxemia with advancing age in humans.

Aging, Blood Flow, and Intravascular ATP During Conditions of Hemoglobin Deoxygenation (In Vivo Studies)

Evidence indicates a close coupling between skeletal muscle blood flow and the oxygenation state of hemoglobin, and deoxygenation-mediated ATP release from red blood cells is believed to play a role in this coupling by evoking local vasodilation via binding to P2 receptors on the endothelium. Intravascular ATP evokes a robust endothelium-dependent vasodilation and can blunt sympathetically mediated vasoconstriction in young healthy humans, both of which will serve to improve blood flow and oxygen delivery to hypoxic or metabolically active tissue. Because aging is classically associated with endothelial dysfunction, and older adults present with an impaired ability to modulate sympathetic vasoconstriction in contracting muscle (“functional sympatholysis”), we recently directly tested whether these unique properties of ATP were impaired with age. In contrast to our hypotheses, older adults demonstrated preserved ATP-mediated vasodilation and the ability to blunt sympathetic α-adrenergic vasoconstriction. These collective observations suggest that if ATP is involved in the impairment of vascular control during mismatches in oxygen supply and demand in aging humans, older adults are likely to have diminished intravascular ATP during conditions of hemoglobin deoxygenation.
The findings from protocol 2 in the present investigation are the first to demonstrate an age-related impairment in the local vasodilation and muscle blood flow response to systemic isocapnic hypoxia in humans (SpO$_2$ ≈80%). Specifically, although local vascular conductance increased in young adults during systemic hypoxia, this was essentially nonexistent in older adults (Figure 2B). In line with our central hypothesis, plasma [ATP] and ATP effluent increased during systemic hypoxia in young adults; however, we observed no significant change in either index of vasoactive ATP in aged humans (Figures 2C and 2D). This absence of local vasodilation in conjunction with a lack of net ATP release in vivo in older adults is consistent with prior in vitro studies demonstrating that red blood cells are crucial to observe hypoxic vasodilation, and within the present study, they imply the loss of vasoactive ATP as a potential mechanism.

The findings from protocol 3 are consistent with many studies in older adults demonstrating that local vascular conductance (vasodilation) is impaired and thus muscle blood flow during exercise is reduced despite an augmented perfusion with age. However, whether age-associated reductions in exercise hyperemia and oxygen delivery are associated with lower levels of intravascular ATP has never been determined. In young subjects, mild contractions (5% MVC) evoked a significant increase in plasma [ATP], which was maintained during 15% MVC and increased further during 25% MVC exercise. In contrast, despite similar levels at rest, plasma [ATP] did not increase above baseline values in older subjects during any exercise intensity, and these concentrations were significantly lower than in the young. Somewhat similarly, ATP effluent increased progressively with exercise intensity in both young and older adults, but ATP effluent was significantly lower in older adults at every exercise intensity (≈50%–65%). As in protocol 2, we clearly showed that older adults had significantly lower muscle blood flow during exercise, which occurred concomitantly with less plasma [ATP] and ATP effluent. Although the mechanism underlying these impairments in hyperemia and oxygen delivery with age may be somewhat dependent on the stimulus, our collective data imply that blunted vasodilation with age during 2 physiological conditions of erythrocyte deoxygenation may be caused in part by reductions in intravascular ATP. In turn, this could lead to reductions in P$_2$ receptor stimulation and reduced endothelium-dependent signaling for direct vasodilation or the modulation of sympathetic vasoconstriction and thus impaired control of peripheral vascular tone in aged humans.

### Potential Mechanisms for Attenuated Plasma ATP With Age During Conditions of Hemoglobin Deoxygenation (In Vitro Studies)

Evidence indicates that ATP catabolism to downstream metabolites occurs rapidly within whole blood and that this process is enhanced during exercise in healthy donors. In humans who demonstrate endothelial dysfunction, we recently observed under resting conditions that vasodilation evoked via exogenous ATP infusions does not occur via P$_2$ receptors in either young or older adults, which provides indirect evidence that nucleotidase activity is not augmented with age at rest. However, whether ATP catabolism is augmented during exercise with human aging (independent of other comorbidities) and could in part explain the reduced ATP levels we observed in the circulation of older adults during exercise is unknown. Therefore, we directly determined ATP degradation in whole blood at rest and during exercise. We found no evidence of augmented ATP breakdown in the blood of older versus young adults sampled at rest or during exercise. Nevertheless, the present data support the concept that ATP hydrolysis is greater during conditions of exercise (Figure 4E). Similar to previous reports, an [ATP] greater than we observed in plasma was used for the ATP degradation assay and therefore, true in vivo catabolism rates cannot be extrapolated from the present findings. However, in light of our observations, we speculate that the reduced intravascular ATP during hypoxia and exercise in aging humans may result from either augmented breakdown via endothelial cell–bound nucleotidases (which was not accounted for in our catabolism studies) or a loss of ATP production and/or efflux of extracellular ATP into circulation.

Studies in isolated red blood cells have clearly demonstrated that hemoglobin deoxygenation evokes ATP release that is graded with the level of deoxygenation. Therefore, in an effort to reveal a possible mechanism underlying our in vivo observations, we determined whether ATP release from erythrocytes of healthy older adult humans was impaired compared with red cells of healthy young adults. By study design, we focused on deoxygenating erythrocytes to FO$_2$Hb levels similar to our in vivo measures. In support of this hypothesis, we observed that ATP release from red blood cells of young adults increased by 71% from baseline normoxia (FO$_2$Hb=≈95%) during the first level of low-oxygen exposure (FO$_2$Hb=≈71%) and by 205% during the second level (FO$_2$Hb=≈43%). In contrast, red cells from older adults did not significantly release ATP during either low-oxygen condition despite similar total intracellular [ATP], and a clear age impairment was present (Figure 5). Although it is currently unclear why this impairment exists, these data are consistent with observations from red cells of diabetic patients that demonstrated blunted ATP release and subsequent vasodilation compared with red cells of healthy adults. Interestingly, deoxygenation-induced ATP release is significantly impaired from red cells of poor deformability, and reduced erythrocyte deformability occurs with advanced donor age. Therefore, it is plausible to speculate that the “erythrocyte dysfunction” observed in the present study may be secondary to red cell stiffening with advancing age. Nonetheless, it is apparent that red blood cells from humans of advanced age fail to release ATP during hemoglobin deoxygenation, and this is likely one mechanism underlying the age-related reduction in plasma [ATP] and vasodilation during hypoxemia with age. Although not directly established in the present in vivo studies, data obtained in vitro clearly demonstrate that a blunted ATP release from red cells is associated with impaired vasomotor responses, and importantly, that vasodilation can be restored when ATP release from red cells is rescued.
Integrative Perspectives
We used several in vivo and in vitro experimental approaches to gain an understanding of whether intravascular ATP is impaired in aging humans during physiological conditions of hemoglobin deoxygenation and, if so, what potential mechanisms are involved. The concept that the red blood cell can serve as an oxygen carrier and sensor is certainly intriguing; however, this leads to complex physiology and associated measures in vivo, particularly during conditions of high blood flow within contracting skeletal muscle. One important consideration under these experimental conditions relates to the measurement of plasma [ATP] as solely representing the available circulating vasoactive ATP. In the present experiments, there was a clear increase in [ATP] in young adults during 5% MVC exercise that did not increase significantly above this level until 25% MVC (Figure 3A). Because total muscle blood flow (and hence blood volume) increased up to 10-fold during exercise, this means that a significant release of ATP release into circulation must have occurred, otherwise [ATP] would have decreased via a dilution effect by the large volume of blood. This latter point has been demonstrated recently experimentally during high-flow nonexercise conditions (acetylcholine infusions) in humans. Thus, although plasma [ATP] may represent one measure of intravascular ATP, we believe that quantification of ATP effluent ([ATP] × flow) may be important as well to describe the total circulating rate of vasoactive ATP available to evoke vasodilation during a given stimulus.2 Accordingly, we summarized ATP effluent across all in vivo conditions in the present study and demonstrated that although it is normal under resting conditions in older adults, ATP effluent is abolished during systemic hypoxia and is substantially reduced with age during forearm exercise (Figure 6).

A second point relates to the influence of elevated blood flow caused by vasodilation and the number of red blood cells exposed to the low-oxygen environments of systemic hypoxia and exercise, because total released ATP is dependent on the number of red cells.29 By means of the Färnæus and phase separation effects, vasodilation increases red cell supply and microvascular hematocrit.7,30 This is important in that during exercise, PO2 and hemoglobin oxygenation within skeletal muscle decrease significantly at the onset of low-intensity exercise and remain relatively constant as exercise intensity increases2–31 (Online Table IVA). However, given the elevation in total muscle blood flow, redistribution of blood to the active muscle fibers, and elevations in microvascular hematocrit, the total number of red cells exposed to the low-oxygen environment is significantly elevated, which provides a greater stimulus for net ATP release in vivo. Furthermore, the integrative physiological conditions of contracting muscle not only result in hemoglobin deoxygenation but also evoke changes in pH and CO2, as well as mechanical stresses, all of which can induce extracellular ATP release from erythrocytes into circulation.2,6,7,32 Thus, within skeletal muscle, a simple correlation between hemoglobin deoxygenation and [ATP] in vivo does not exist, nor should it be required to exist to support this overall hypothesized regulation of tissue blood flow and oxygen delivery by the red cells. A final consideration regarding the complexity of this physiology is the ability of endothelial cell–bound ectonucleotidases21 and other blood constituents to rapidly degrade ATP in vivo; the latter have been shown to be upregulated during exercise15 (Figure 4E). This very tight regulation between red cell delivery, ATP release, and ATP degradation may aid in explaining why venous plasma [ATP] levels are not substantially greater as exercise intensity and blood flow are increased even in young adults.

Experimental Considerations
There are multiple sources of ATP, including sympathetic nerve terminals and skeletal muscle; however, recent studies have demonstrated that ATP does not cross the endothelium,2,28,33 and thus, intravascular [ATP] from these sources is unlikely. Furthermore, older adults demonstrate greater levels of sympathetic activity both at rest and during conditions of deoxygenation,10,11 and exercise intensity and forearm muscle mass were similar between groups; thus, these cannot explain the reductions in intravascular ATP with age.

To definitively determine the contribution of intravascular ATP to vasomotor control during systemic hypoxia and exercise, the use of a specific P2Y receptor antagonist would be ideal. Unfortunately, agents used in animal preparations in vivo are significantly flawed,34 and no substances are currently approved for human use. Regardless, the loss of ATP release from red cells has been linked repeatedly with impaired vascular function and lends credibility to the present in vivo and in vitro observations.4,5,7,18,35,36 Independent from vascular control, ATP as a signaling molecule has a wide range of effects on cardiovascular function. Profoundly, attenuated intravascular ATP is associated with a prothrombotic environment, leukocyte adhesion, platelet activation, enhanced ischemia-reperfusion injury, and hyperinflammation.37 Therefore, the present observations of diminished intravascular ATP in humans of advanced age have clear implications and therapeutic potential for many cardiovascular complications in addition to oxygen delivery during hypoxemia.

In the present in vivo aging experiments, we did not sample for arterial plasma [ATP]; however, studies in young subjects demonstrated that arterial infusion of ATP was not detectable in the venous blood of the same limb (Figure 1) and that arterial [ATP] was lower than venous values and did not increase during handgrip exercise (Online Figure I). Finally, a wealth of data exist that demonstrate no significant elevation in arterial plasma [ATP] during systemic hypoxia or in response to exercise (eg, Dufour et al38). Thus, these data are consistent with our observations that arterial plasma [ATP] does not explain venous plasma [ATP] or the observed impairments with age.

The regulation of blood flow to contracting muscle is complex and involves many factors in addition to intravascular ATP, such as K+ efflux from myocytes, mechanical factors, and a variety of endothelial and metabolically derived substances.39 In this context, we have shown a robust association between plasma ATP and FBF (Figure 3E) that is left-shifted in older adults and likely depicts a compensation of lowered plasma [ATP] by other vasoinductive signals rather than enhanced ATP receptor responsiveness.23,24 Thus, de-
spite clear impairments in intravascular ATP and hyperemic responses during exercise in older subjects, blood flow still increases during exercise, and this would be expected given the redundant control of blood flow to active muscle.

Conclusions
In young healthy humans, oxygen delivery is effectively matched to oxygen demand during hypoxia and exercise via increases in skeletal muscle blood flow; however, older adults demonstrate an altered control of the vasculature (impaired vasodilation and functional sympatholysis) that may predispose this population to increased risk for ischemia-related cardiovascular disease and exercise intolerance.12,13 The collective findings from the present investigation indicate that skeletal muscle blood flow during conditions of erythrocyte deoxygenation is markedly reduced in aging humans because of impaired local vasodilatation associated with reductions in plasma ATP. Furthermore, we suggest that loss of erythrocyte-mediated ATP release may be a novel mechanism underlying impaired oxygen delivery during hypoxemia with advancing age and that rectification of this dysfunction could prove beneficial for older healthy and diseased humans.

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Disclosures
None.

References


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### Novelty and Significance

**What Is Known?**

- Human aging is a predominant risk factor for ischemia-related cardiovascular events.
- Muscle blood flow is closely coupled to the oxygenation state of hemoglobin in young healthy humans.
- Extracellular ATP within blood vessels has potent vasoactive properties and is released from erythrocytes.

**What New Information Does This Article Contribute?**

- Skeletal muscle blood flow is markedly reduced in humans of advanced age during conditions of erythrocyte deoxygenation because of impaired local vasodilation.
- Unlike young adults, intravascular plasma [ATP] does not increase during conditions of erythrocyte deoxygenation in older adults.
- Although ATP hydrolysis by blood is augmented during exercise, aging does not alter this capacity.
- Red blood cells from humans of advanced age are significantly impaired in their ability to release ATP during hemoglobin deoxygenation.

Aging is associated with endothelial dysfunction and heightened sympathetic activity, and it increases the risk for ischemic cardiovascular diseases and exercise intolerance. Nevertheless, it is unclear whether a decline in local skeletal muscle vascular control and oxygen delivery with age is related to the loss of circulating vasoactive ATP, and more specifically, the loss of ATP release from red blood cells during hemoglobin deoxygenation. Here, we present evidence supporting the notion that skeletal muscle blood flow, local vasodilation, intravascular plasma ATP, and erythrocyte-mediated ATP release during conditions of erythrocyte deoxygenation are markedly impaired in aged humans. This “erythrocyte dysfunction” may be a novel mechanism underlying impaired vasodilation and oxygen delivery during hypoxemia with advancing age. Because aging is associated with elevated risk for ischemic cardiovascular disease and exercise intolerance, and because ATP has notable effects on vascular function, interventions to improve erythrocyte-mediated ATP release may be of therapeutic value.
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SUPPLEMENTAL MATERIAL

Impaired skeletal muscle blood flow control with advancing age in humans: attenuated ATP release and local vasodilation during erythrocyte deoxygenation

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SUPPLEMENTAL METHODS

Subjects
With Institutional Review Board approval and after written informed consent, a total of 37 young and 25 older healthy adults participated in the present investigation. Of those, eleven young and nine older subjects participated in multiple protocols. This study was approved by the Human Research Committee of Colorado State University and was performed according to the Declaration of Helsinki. All subjects were free from overt cardiovascular disease as assessed from a medical history, were sedentary to moderately active, free of medications, and considered healthy as previously described¹. Older subjects were further evaluated for clinical evidence of cardiopulmonary disease with a physical examination and resting and maximal exercise electrocardiograms. All subjects fasted for at least 4 hr prior to experimentation. Experiments described below for each protocol were performed on different study days with subjects in the supine position.

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 30 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 hypoxia and the graded exercise experiments. Heart rate was determined using a 3-lead ECG (Cardiocap/5, Datex-Ohmeda, Louisville, CO, USA).

Body Composition and Forearm Fat Volume
Body composition, forearm volume, and forearm fat-free mass was determined by dual-energy X-ray absorptiometry (DEXA; Hologic, Inc; Bedford, MA, USA)¹. Body mass index (BMI) was calculated as bodyweight (kg) divided by height (meters) squared.

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 laboratory². 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:

\[ \text{FBF} = \text{MBV (cm/s)} \times \pi \left( \text{brachial artery diameter}/2 \right)^2 \times 60, \]

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 in response to hypoxia and exercise), forearm vascular conductance (FVC) was calculated as (FBF/MAP) * 100, and expressed as ml/min /100 mmHg². FBF and FVC were normalized to 100g of fat free mass (FFM). 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.
Arterial and Venous Catheterization

A 20 gauge, 7.6 cm catheter was placed in the brachial artery of the non-dominant arm at the antecubital crease facing upstream under aseptic conditions after local anesthesia (2% lidocaine) for local administration of study drugs and blood sampling. The catheter was connected to a 3-port connector as well as a pressure transducer for mean arterial pressure (MAP) measurement and continuously flushed at 3 ml/hr with heparinized saline. One side port was used for drug infusion (Protocol 1) and the other for blood sampling (exercise subgroup without drug infusion; Protocol 1)\(^3\). In addition, an 18 gauge, 5.1 cm catheter was inserted at the antecubital crease in retrograde fashion (facing downstream) into a vein draining the muscle tissue of the experimental arm for venous blood samples\(^4\) to be used for systemic electrolyte monitoring 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/min for the duration of the study to keep it patent.

Drug Administration

Saline or ATP was administered through the brachial artery catheter to create a local effect in the forearm. Saline was infused for 3 minutes, followed by ATP (P\(_2\) receptor agonist; Sigma A7699) at a dose of 8.35 nmol/dL forearm volume/min for 3 minutes in 8 young adults. This dose of ATP elevates forearm blood flow to levels typically observed during mild-moderate intensity handgrip exercise\(^3\). ATP was prepared in saline and confirmed sterile and free of fungus/endotoxin and particulate matter with a standard microbiology report (JCB-Analytical Research Labs, Wichita, KS) prior to use. Forearm volume used for normalization of ATP dose across subjects was determined from regional analysis of whole-body dual-energy X-ray absorptiometry scans (QDR series software, Hologic, Inc, Bedford, MA).

Sampling for Plasma [ATP] and Blood Gases

An ATP ‘stop solution’ containing EDTA, NaCl, KCl, tricine buffer, nitrobenzyl thioinosine, forskolin, and isobutylmethylxanthine was used to inhibit degradation of ATP via nucleotidases and ATP production from other blood sources (such as platelets)\(^5\). Venous blood samples were drawn through an 18-gauge catheter directly into a pre-heparinized 10cc syringe in which 2 ml of blood was gently and at once expelled into a tube containing 2.7mL of an ATP ‘stop solution’ to equal a blood:diluent ratio of 1.35 as described by Gorman and colleagues\(^5\). The blood:diluent volume provided sufficient volume for plasma ATP measurements in triplicate and a plasma [Hb] measurement per sample. Blood:diluent samples were immediately centrifuged at 4,000 rpm for 3 minutes at 22°C. This ATP ‘stop solution’ has been shown to maintain stable ATP values for up to ~30 minutes\(^5\), and pilot studies in our lab confirmed stability of samples for at least 15 minutes (< 5% change over time). Regardless, to minimize any potential alteration of plasma [ATP], samples were analyzed for plasma [ATP] and plasma [Hb] immediately following centrifugation. In addition, a 2mL blood sample was drawn into a pre-heparinized 3cc syringe for co-oximetry blood gas parameters measured via blood gas analyzer (Siemens Rapid Point 405 Series Automatic Blood Gas System, Los Angeles, CA). Forearm oxygen consumption (VO\(_2\)) was calculated from the venous blood gas sample and assuming an arterial oxygen content of 20 ml/dl across all conditions using the Fick equation: VO\(_2\) = FBF x (ctO\(_2\)arterial – ctO\(_2\)venous) and expressed as ml/100g FFM/min.

Measurement of Plasma [ATP] and [Hb]

Directly following centrifugation of blood:diluent samples, 100 µL of supernatant was used for subsequent plasma [ATP] determination via the luciferin-luciferase technique similar to previously described\(^5\). First, 25 µL of Mg\(^{2+}\) solution (44.25 mmol/L, 40 mmol/L tricine buffer, pH 7.75) was automatically injected into a 100 µL plasma sample followed by 100 µL of luciferase (ATP Bioluminescence Assay Kit CLS II: Roche Diagnostics) two seconds later via an automated dual injector single tube luminometer in which relative light units (RLU) were collected (Turner BioSystems 20/20n, Sunnyvale, CA, USA). After three seconds, cumulative light output in RLU\(_s\) was measured for ten seconds and averaged. 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. Specifically, a baseline blood:diluent sample was obtained and 90 µL plasma samples were spiked with 10 µL of varying concentrations of ATP standard (equating to final concentration of 165.0, 82.6, 41.3, 20.6 nmol/L). The average standard curve $r^2$ value was ~0.996 and the coefficient of variation within a plasma sample reading was $4.0 \pm 0.7\%$. Any ATP standard or plasma sample that provided >10% variation in RLUs was discarded and reanalyzed. After accounting for background RLUs from an unspiked plasma sample, RLU’s were plotted vs ATP and a least squares linear regression line was fit to the data. Plasma [ATP] was calculated as: Final venous plasma [ATP] = $\frac{\text{ATP}_{\text{blood:diluent}} - \text{ATP}_{\text{hemolysis}}}{(1.35+1-HCT)/(1-HCT)}$.

To account for venous concentrations of ATP induced from hemolysis, 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). Plasma [Hb] was calculated from absorbance output 415, 380, and 450 wavelengths. This plasma [Hb] reading provided an indication of hemolysis (% Hemolysis = $\{(100 – \text{HCT}) \times \text{p[Hb]/t[Hb]}\} \times 100$). A correction formula to account for ATP due to RBC hemolysis was created for both young and older populations as previously outlined. ATP concentration from RBC pellets was plotted vs plasma [Hb] (range 0-25 mg/L) and data was fit with a linear regression line in 6 young ($R^2 = 0.92; y = 3.20x$) and 5 older subjects ($R^2 = 0.95; y = 4.21x$). Because small amounts of RBC hemolysis can significantly increase ATP, samples were accounted for with the above hemolysis-ATP calculation. Any sample that was more than 2 standard deviations from the mean in % hemolysis was excluded in the analysis and considered technical error.

Systemic Isocapnic Hypoxia

We employed the use of a self-regulating partial rebreathe system developed by Banzett and colleagues and previously used in our laboratory to isolate the effects of hypoxia. This system allows for constant alveolar fresh air ventilation independent of changes in breathing frequency or tidal volume. Additionally, this system allows for clamping of end-tidal carbon dioxide (ETCO$_2$) levels despite large changes in minute ventilation in response to hypoxia. Oxygen (O$_2$) levels were manipulated by mixing nitrogen with air via a medical gas blender. The level of O$_2$ was titrated down to achieve a steady arterial O$_2$ saturation (SpO$_2$) of ~80% as assessed by pulse oximetry of the earlobe. Subjects breathed through a scuba mouthpiece with a nose-clip to prevent any nasal breathing. Gas concentrations were monitored at the mouthpiece by an anaesthesia monitor (Cardiocap/5, Datex-Ohmeda, Louisville, CO, USA) that was also used to determine heart rate (HR; 3-lead ECG). Ventilation was measured via a pneumotachograph (model VMM-2a, Interface Associates, Laguna Niguel, CA, USA).

Rhythmic Handgrip Exercise

Maximum voluntary contraction (MVC) was determined for each subject as the average of at least three maximal squeezes of a handgrip dynamometer (Stoelting, Chicago, IL, USA) that were within 3 percent of each other. For dynamic handgrip exercise, weights corresponding to 5, 15, or 25% 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.

Measurement of ATP Catabolism in Blood

Blood was sampled and collected into 6 mL preheparinised tubes. 990 µL of whole blood was immediately placed into a water bath (37°C) and 10 µL of exogenous ATP (100 µM final concentration) was added. ATP was determined in whole blood samples immediately, 5, 10, and 15 minutes following the ATP addition. To do so, whole blood aliquots were diluted 500x with saline so that interference with the ATP assay did not occur and that further ATP degradation was minimized to allow for duplicate measurements. An ATP standard curve was determined for all experiments and was performed similar to that described above in plasma. Exogenous ATP in whole blood from young and older adults at rest and each exercise intensity was calculated as the percent remaining after 5, 10, and 15 minutes of incubation time post initial measurement. Because ATP is so rapidly degraded in whole blood, it was impossible for us to determine ATP catabolism rates with the luciferase assay technique at a physiological
concentration observed in the present study (~100 nM). Therefore, we utilized exogenous [ATP] of 100 
µM previously described in the literature, and of which could be observed in various pathological states\textsuperscript{9-11}.

**Erythrocyte Isolation and Extracellular ATP Measurement**

Blood was obtained by venipuncture into a syringe containing 500 units heparin. Erythrocytes were isolated by centrifugation (500g at 4°C for 10 min) with plasma and buffy coat removed. Packed red blood cells were resuspended and washed 3 times in PSS (4.7 KCl, 2.0 CaCl\(_2\), 1.2 MgSO\(_4\), 140.5 NaCl, 21.0 Tris-base, and 5.5 dextrose with 0.5% BSA, pH adjusted to 7.4. This method of isolation yields a red blood cell suspension devoid of platelets and less than one leukocyte per 50 high-power fields. Studies on isolated red cells were performed the morning of collection\textsuperscript{12,13}.

ATP was measured via luciferin-luciferase technique with light emission during the reaction detected by luminometer. A sample of 20% HCT was diluted 500 fold and a 200 µL red cell suspension (0.04%) was injected into a cuvette containing 100uL of 10mg/mL crude firefly tail extract (Sigma) and 100 µL of 0.5 mg/mL D-luciferin (RPI). Extracellular ATP was normalized to a cell count of 4 x 10\(^8\) cells/mL. For intracellular ATP measurements, a 50 µL sample of erythrocytes (20% HCT) was obtained and diluted 8000 fold, and analyzed for ATP. Intracellular ATP was normalized to ATP concentration per erythrocyte as determined by direct cell counting. A standard curve for ATP (Calbiochem) was obtained for each individual experiment\textsuperscript{12,13}. To confirm that ATP release was not due to hemolysis, red cell suspensions acquired for ATP analysis were analyzed for free hemoglobin similar to previous reports\textsuperscript{12,13}.

**Erythrocyte Deoxygenation**

A 20% red cell suspension was placed in a rotating bulb tonometer and warmed to 37°C (Eschweiler GmbH & Co. KG, Germany). Normoxic and hypoxic gases were blended via gas blender (MCQ Gas Blender Series 100, Italy), humidified, and introduced into the enclosed tonometer. Normoxia consisted of 15 minutes of 16% O\(_2\), 6% CO\(_2\), and N\(_2\) balanced gases. Red cell deoxygenation was facilitated by 7 minutes of 3%O\(_2\), 6% CO\(_2\), and N\(_2\) balanced exposure for the first level, followed by 1.5%O\(_2\), 6% CO\(_2\), and N\(_2\) balanced for the second level of deoxygenation. Our goal was to reduce PO\(_2\) and FO\(_2\)Hb to levels within the range observed in vivo. Blood gases were confirmed with blood gas analysis (Siemens Rapid Point 405 Series Automatic Blood Gas System, Los Angeles, CA) and are presented in Table V.
SUPPLEMENTAL TABLES

Online Table IA. Subject characteristics for all protocols

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Older</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male:Female</td>
<td>33:5</td>
<td>24:2</td>
</tr>
<tr>
<td>Age (years)</td>
<td>23±1.0</td>
<td>64±1*</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>23.7±0.3</td>
<td>26.5±0.7*</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>17.9±1.0</td>
<td>27.0±1.1*</td>
</tr>
<tr>
<td>Forearm volume (ml)</td>
<td>1055±35</td>
<td>1121±53</td>
</tr>
<tr>
<td>Forearm fat-free mass (g)</td>
<td>895±38</td>
<td>871±64</td>
</tr>
<tr>
<td>MVC (kg)</td>
<td>47.5±1.5</td>
<td>44.5±2.7</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>3.6±0.1</td>
<td>4.8±0.2*</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>3.3±0.1</td>
<td>4.3±0.1*</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.1±0.1</td>
<td>1.0±0.1</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.8±0.1</td>
<td>1.1±0.1*</td>
</tr>
</tbody>
</table>

For forearm volume and MVC, n=30 for young and n=18 for older. For blood lipid measures n=22 for young and n=23 for older. MVC = Maximum voluntary contraction; LDL=Low density lipoprotein, HDL=High density lipoprotein. *P<0.05 vs young adults

Online Table IB. Additional subject characteristics for isolated erythrocyte study participants

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Older</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/l)</td>
<td>3.9±0.2</td>
<td>4.1±0.3</td>
</tr>
<tr>
<td>Insulin (pmol/l)</td>
<td>32.2±6.6</td>
<td>43.8±8.8</td>
</tr>
<tr>
<td>HbA1C (Proportion)</td>
<td>0.051±0.001</td>
<td>0.052±0.001</td>
</tr>
<tr>
<td>Total Intracellular ATP (mmol/l/RBC)</td>
<td>2.01±0.17</td>
<td>1.87±0.21</td>
</tr>
</tbody>
</table>
Online Table II. ATP Infusion Protocol: forearm and systemic hemodynamics at rest (saline) and during ATP infusion

<table>
<thead>
<tr>
<th></th>
<th>FBF (ml/min)</th>
<th>FVC (ml/min/100mmHg)</th>
<th>MAP (mmHg)</th>
<th>HR (beats/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>31±4</td>
<td>33±4</td>
<td>93±2</td>
<td>48±3</td>
</tr>
<tr>
<td>ATP</td>
<td>101±17*</td>
<td>112±17*</td>
<td>89±3</td>
<td>49±3</td>
</tr>
</tbody>
</table>

FBF = Forearm blood flow, FVC = Forearm vascular conductance, MAP = Mean arterial pressure, HR = Heart rate *P<0.05 vs saline
n = 8 young
Online Table IIIA. Hypoxia Protocol: venous blood gases and plasma hemolysis

<table>
<thead>
<tr>
<th></th>
<th>pH (mmHg)</th>
<th>PO₂ (mmHg)</th>
<th>PCO₂ (mmHg)</th>
<th>ctO₂ (ml/dl)</th>
<th>ctCO₂ (ml/dl)</th>
<th>FO₂Hb (%)</th>
<th>Hct (%)</th>
<th>tHb (g/dl)</th>
<th>Hemolysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>7.37±0.01</td>
<td>31.5±2.4</td>
<td>44.2±1.6</td>
<td>11.6±1.0</td>
<td>26.3±0.7</td>
<td>55.0±3.8</td>
<td>43.7±1.2</td>
<td>14.9±0.4</td>
<td>0.0064±0.0008</td>
</tr>
<tr>
<td>Older</td>
<td>7.39±0.02</td>
<td>32.6±1.4</td>
<td>41.3±1.7</td>
<td>12.3±0.8</td>
<td>25.8±0.9</td>
<td>59.4±2.9</td>
<td>42.8±1.2</td>
<td>14.6±0.4</td>
<td>0.0076±0.0012</td>
</tr>
<tr>
<td>Systemic Hypoxia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>7.37±0.01</td>
<td>28.1±1.9*</td>
<td>43.5±1.6</td>
<td>9.8±1.0*</td>
<td>25.9±0.8</td>
<td>47.1±4.0*</td>
<td>43.0±0.4</td>
<td>14.6±0.4</td>
<td>0.0096±0.0024</td>
</tr>
<tr>
<td>Older</td>
<td>7.39±0.02</td>
<td>28.4±1.4*</td>
<td>40.6±1.0</td>
<td>10.4±0.9*</td>
<td>25.6±0.8</td>
<td>51.5±3.3*</td>
<td>42.4±1.3</td>
<td>14.5±0.4</td>
<td>0.0080±0.0010</td>
</tr>
</tbody>
</table>

All blood gas values are venous blood. *P<0.05 vs Normoxia

Online Table IIIB. Hypoxia Protocol: forearm and systemic hemodynamics and ventilatory responses

<table>
<thead>
<tr>
<th></th>
<th>FBF (ml/min)</th>
<th>FVC (ml/min/100mmHg)</th>
<th>MAP (mmHg)</th>
<th>HR (beats/min)</th>
<th>SpO₂ (%)</th>
<th>Minute Vent. (l/min, BTPS)</th>
<th>ETCO₂ (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>37±6</td>
<td>39±6</td>
<td>94±2</td>
<td>61±2</td>
<td>99.4±0.2</td>
<td>9.8±0.8</td>
<td>37.6±0.8</td>
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<tr>
<td>Older</td>
<td>35±3</td>
<td>36±4</td>
<td>99±3</td>
<td>55±3</td>
<td>96.3±2.0</td>
<td>10.3±0.6</td>
<td>34.6±1.3†</td>
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<tr>
<td>Systemic Hypoxia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>54±10*</td>
<td>58±10*</td>
<td>93±3</td>
<td>83±2*</td>
<td>80.8±0.9*</td>
<td>21.5±2.1*</td>
<td>38.7±0.6</td>
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<tr>
<td>Older</td>
<td>35±3†</td>
<td>34±3†</td>
<td>103±4*†</td>
<td>66±3*†</td>
<td>81.5±0.9*</td>
<td>19.2±3.5*</td>
<td>34.4±1.0†</td>
</tr>
</tbody>
</table>

FBF = Forearm blood flow; FVC = Forearm vascular conductance; MAP = Mean arterial pressure; HR = Heart rate; MVC = Maximum voluntary contraction; Vent. = ventilation; ETCO₂ = end-tidal CO₂.
*P<0.05 vs Normoxia; †P<0.05 vs young adults
Online Table IVA. Exercise Protocol: venous blood gases and plasma hemolysis

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>PO₂ (mmHg)</th>
<th>PCO₂ (mmHg)</th>
<th>ctO₂ (ml/dl)</th>
<th>ctCO₂ (ml/dl)</th>
<th>FO₂Hb (%)</th>
<th>VO₂ (ml/100g FFM/min)</th>
<th>Hct (%)</th>
<th>tHb (g/dl)</th>
<th>Hemolysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rest</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Young</td>
<td>7.366±</td>
<td>29.2±</td>
<td>46.7±</td>
<td>10.9±</td>
<td>27.6±</td>
<td>50.9±</td>
<td>0.33±</td>
<td>44.4±</td>
<td>15.0±</td>
<td>0.0058±</td>
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<tr>
<td>Older</td>
<td>7.376±</td>
<td>29.6±</td>
<td>44.0±</td>
<td>10.6±</td>
<td>26.4±</td>
<td>52.1±</td>
<td>0.39±</td>
<td>42.8±</td>
<td>14.5±</td>
<td>0.0060±</td>
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<tr>
<td>5% MVC</td>
<td></td>
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<tr>
<td>Young</td>
<td>7.354±</td>
<td>21.2±</td>
<td>50.0±</td>
<td>6.3±</td>
<td>28.8±</td>
<td>30.3±</td>
<td>1.48±</td>
<td>43.5±</td>
<td>15.4±</td>
<td>0.0081±</td>
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<tr>
<td>Older</td>
<td>7.360±</td>
<td>21.4±</td>
<td>48.3±</td>
<td>6.2±</td>
<td>28.2±</td>
<td>30.3±</td>
<td>1.37±</td>
<td>41.7±</td>
<td>14.2±</td>
<td>0.0085±</td>
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<td>15% MVC</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>7.315±</td>
<td>22.6±</td>
<td>55.5±</td>
<td>6.7±</td>
<td>29.3±</td>
<td>31.7±</td>
<td>3.23±</td>
<td>43.9±</td>
<td>14.9±</td>
<td>0.0084±</td>
</tr>
<tr>
<td>Older</td>
<td>7.319±</td>
<td>21.5±</td>
<td>52.8±</td>
<td>6.0±</td>
<td>28.2±</td>
<td>29.9±</td>
<td>2.89±</td>
<td>42.1±</td>
<td>14.2±</td>
<td>0.0093±</td>
</tr>
<tr>
<td>25% MVC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>7.287±</td>
<td>24.7±</td>
<td>56.1±</td>
<td>7.3±</td>
<td>29.1±</td>
<td>34.0±</td>
<td>5.60±</td>
<td>44.3±</td>
<td>15.1±</td>
<td>0.0084±</td>
</tr>
<tr>
<td>Older</td>
<td>7.291±</td>
<td>24.1±</td>
<td>58.5±</td>
<td>6.9±</td>
<td>29.2±</td>
<td>34.0±</td>
<td>4.63±</td>
<td>42.8±</td>
<td>14.6±</td>
<td>0.0100±</td>
</tr>
</tbody>
</table>

*P<0.05 vs rest (within group); MVC = Maximum voluntary contraction

Online Table IVB. Exercise Protocol: local and systemic hemodynamics at rest and during exercise

<table>
<thead>
<tr>
<th></th>
<th>FBF (ml/min)</th>
<th>FVC (ml/min/100mmHg)</th>
<th>MAP (mmHg)</th>
<th>HR (beats/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rest</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>36±5</td>
<td>38±5</td>
<td>93±2</td>
<td>58±1</td>
</tr>
<tr>
<td>Older</td>
<td>33±3</td>
<td>34±3</td>
<td>99±2†</td>
<td>58±3</td>
</tr>
<tr>
<td>5% MVC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>98±9*</td>
<td>102±8*</td>
<td>96±3*</td>
<td>60±2</td>
</tr>
<tr>
<td>Older</td>
<td>82±7*</td>
<td>77±7*†</td>
<td>103±3*†</td>
<td>62±3*</td>
</tr>
<tr>
<td>15% MVC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>220±16*</td>
<td>228±13*</td>
<td>96±2*</td>
<td>62±2*</td>
</tr>
<tr>
<td>Older</td>
<td>175±14*†</td>
<td>161±13*†</td>
<td>110±3*†</td>
<td>64±3*</td>
</tr>
<tr>
<td>25% MVC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>382±30*</td>
<td>366±27*</td>
<td>104±3*</td>
<td>68±2*</td>
</tr>
<tr>
<td>Older</td>
<td>299±25*†</td>
<td>260±23*†</td>
<td>116±3*†</td>
<td>67±3*</td>
</tr>
</tbody>
</table>

FBF = Forearm blood flow, FVC = Forearm vascular conductance, MAP = Mean arterial pressure, HR = Heart rate, MVC = Maximum voluntary contraction. *P<0.05 vs rest (within group); †P<0.05 vs young adults
Online Table V. Isolated Erythrocyte Protocol: red cell solution blood gases

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>( \text{PO}_2 ) (mmHg)</th>
<th>( \text{PCO}_2 ) (mmHg)</th>
<th>FO(_2)Hb (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>16% ( \text{O}_2 )</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>7.276±0.005</td>
<td>109.9±2.3</td>
<td>39.8±1.1</td>
<td>94.9±0.2</td>
</tr>
<tr>
<td>Older</td>
<td>7.283±0.010</td>
<td>110.0±2.9</td>
<td>39.1±0.9</td>
<td>95.2±0.2</td>
</tr>
<tr>
<td><strong>3% ( \text{O}_2 )</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>7.280±0.007</td>
<td>47.0±1.2*</td>
<td>41.0±0.9</td>
<td>71.6±1.4*</td>
</tr>
<tr>
<td>Older</td>
<td>7.289±0.009</td>
<td>45.1±1.0*</td>
<td>40.1±1.0</td>
<td>71.1±1.1*</td>
</tr>
<tr>
<td><strong>1.5% ( \text{O}_2 )</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>7.291±0.006</td>
<td>29.2±0.8*</td>
<td>40.6±1.1</td>
<td>42.4±2.1*</td>
</tr>
<tr>
<td>Older</td>
<td>7.296±0.009</td>
<td>28.2±1.1*</td>
<td>39.8±0.6</td>
<td>43.1±2.5*</td>
</tr>
</tbody>
</table>

\*P<0.05 vs 16% \( \text{O}_2 \) condition (within group)
Online Figure I. Arterial and Venous Plasma [ATP] during Forearm Exercise in Young Adults.
Arterial plasma [ATP] was measured during exercise in 5 young adults who were instrumented with arterial catheters for other studies in the laboratory and compared to venous plasma [ATP] from 14 different young adults. Note that the arterial plasma [ATP] was always lower at rest and during all exercise intensities compared with the average venous [ATP] at rest, suggesting that arterial plasma [ATP] detectable at the brachial artery does not increase in this model of exercise and further, is not explanatory for elevations in venous plasma [ATP] resultant from exercise nor the age-group differences observed in Protocol 3. MVC = maximum voluntary contraction. *P<0.05 vs resting venous; ‡ P<0.05 vs 5% and 15% MVC venous.


