SUPPLEMENTAL MATERIAL

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

Brett S. Kirby[1] Ph.D., Anne R. Crecelius[1] M.S., Wyatt F. Voyles[2,3] M.D., Frank A. Dinenno[1] Ph.D.

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 described[1]. 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 laboratory[2]. 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:

$$FBF = MBV\ (cm/s) * \pi\ (\text{brachial artery diameter}/2)^2 * 60,$$

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 in response to hypoxia and exercise), forearm vascular conductance (FVC) was calculated as (FBF/MAP) * 100, and expressed as ml/min /100 $mmHg^2$. 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 asceptic 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)[2, 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, 6]. 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, 6]. 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, 6], 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: $VO_2 = FBF \times (ctO_{2arterial} - ctO_{2venous})$ 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 RLUs 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 ± 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] = $(ATP_{blood:diluent} - ATP_{hemolysis})*(1.35+1\text{-}HCT)/(1\text{-}HCT)$ [5].

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 – HCT) * p[Hb]/t[Hb]}*100). A correction formula to account for ATP due to RBC hemolysis was created for both young and older populations as previously outlined[5]. 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 ($R^2 = 0.95$; $y = 4.21x$)[5]. 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[4, 7, 8]. 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[1, 2].

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 (37ºC) and 10 µL of exogenous ATP (100 µM final concentration) was added[9]. 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[9]. 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[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 4ºC 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 CaCl2, 1.2 MgSO4, 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[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 4×10^8 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[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[12, 13].

Erythrocyte Deoxygenation

A 20% red cell suspension was placed in a rotating bulb tonometer and warmed to 37ºC (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_2Hb 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

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

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

	Young	Older
Glucose (mmol/l)	3.9±0.2	4.1±0.3
Insulin (pmol/l)	32.2±6.6	43.8±8.8
HbA1C (Proportion)	0.051±0.001	0.052±0.001
Total Intracellular ATP (mmol/l/RBC)	2.01±0.17	1.87±0.21

Online Table II. ATP Infusion Protocol: forearm and systemic hemodynamics at rest (saline) and during ATP infusion

	FBF (ml/min)	FVC (ml/min/100mmHg)	MAP (mmHg)	HR (beats/min)
Saline	31±4	33±4	93±2	48±3
ATP	101±17*	112±17*	89±3	49±3

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

	pH	PO_2 (mmHg)	PCO_2 (mmHg)	ctO_2 (ml/dl)	$ctCO_2$ (ml/dl)	FO_2Hb (%)	Hct (%)	tHb (g/dl)	Hemolysis (%)
Normoxia									
Young	7.371± 0.009	31.5±2.4	44.2±1.6	11.6±1.0	26.3±0.7	55.0±3.8	43.7±1.2	14.9±0.4	0.0064± 0.0008
Older	7.393± 0.011	32.6±1.4	41.3±1.7	12.3±0.8	25.8±0.9	59.4±2.9	42.8±1.2	14.6±0.4	0.0076± 0.0012
Systemic Hypoxia									
Young	7.371± 0.009	28.1±1.9*	43.5±1.6	9.8±1.0*	25.9±0.8	47.1±4.0*	43.0±0.4	14.6±0.4	0.0096± 0.0024
Older	7.395± 0.011	28.4±1.4*	40.6±1.0	10.4±0.9*	25.6±0.8	51.5±3.3*	42.4±1.3	14.5±0.4	0.0080± 0.0010

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

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

	FBF (ml/min)	FVC (ml/min/ 100mmHg)	MAP (mmHg)	HR (beats/min)	SpO_2 (%)	Minute Vent. (l/min, BTPS)	$ETCO_2$ (mmHg)
Normoxia							
Young	37±6	39±6	94±2	61±2	99.4±0.2	9.8±0.8	37.6±0.8
Older	35±3	36±4	99±3	55±3	96.3±2.0	10.3±0.6	34.6±1.3†
Systemic Hypoxia							
Young	54±10*	58±10*	93±3	83±2*	80.8±0.9*	21.5±2.1*	38.7±0.6
Older	35±3†	34±3†	103±4*†	66±3*†	81.5±0.9*	19.2±3.5*	34.4±1.0†

FBF = Forearm blood flow; FVC = Forearm vascular conductance; MAP = Mean arterial pressure; HR = Heart rate; MVC = Maximum voluntary contraction; Vent. = ventilation; $ETCO_2$ = end-tidal CO_2.
*P<0.05 vs Normoxia; †P<0.05 vs young adults

Online Table IVA. Exercise Protocol: venous blood gases and plasma hemolysis

	pH	PO_2 (mmHg)	PCO_2 (mmHg)	ctO_2 (ml/dl)	$ctCO_2$ (ml/dl)	FO_2Hb (%)	VO_2 (ml/100g FFM/min)	Hct (%)	tHb (g/dl)	Hemolysis (%)
Rest										
Young	7.366± 0.005	29.2± 1.5	46.7± 1.1	10.9± 0.9	27.6± 0.5	50.9± 3.5	0.33± 0.03	44.4± 1.0	15.0± 0.3	0.0058± 0.0008
Older	7.376± 0.008	29.6± 1.0	44.0± 0.8	10.6± 0.5	26.4± 0.6	52.1± 2.1	0.39± 0.06	42.8± 1.1	14.5± 0.4	0.0060± 0.0006
5% MVC										
Young	7.354± 0.004*	21.2± 0.9*	50.0± 0.7*	6.3± 0.4*	28.8± 0.5*	30.3± 1.6*	1.48± 0.09	43.5± 1.0	15.4± 0.7	0.0081± 0.0009*
Older	7.360± 0.008*	21.4± 1.1*	48.3± 1.2*	6.2± 0.4*	28.2± 0.9*	30.3± 1.6*	1.37± 0.16	41.7± 1.0	14.2± 0.3	0.0085± 0.0008*
15% MVC										
Young	7.315± 0.006*	22.6± 1.0*	55.5± 1.1*	6.7± 0.5*	29.3± 0.5*	31.7± 1.8*	3.23± 0.20	43.9± 1.0	14.9± 0.3	0.0084± 0.0007*
Older	7.319± 0.006*	21.5± 0.8*	52.8± 1.5*	6.0± 0.4*	28.2± 0.8*	29.9± 1.8*	2.89± 0.19	42.1± 1.0	14.3± 0.3	0.0093± 0.0009*
25% MVC										
Young	7.287± 0.008*	24.7± 1.0*	56.1± 2.6*	7.3± 0.5*	29.1± 0.6*	34.0± 1.9*	5.60± 0.38	44.3± 1.0	15.1± 0.3	0.0084± 0.0007*
Older	7.291± 0.010*	24.1± 0.6*	58.5± 2.2*	6.9± 0.3*	29.2± 0.7*	34.0± 1.5*	4.63± 0.38	42.8± 1.0	14.6± 0.3	0.0100± 0.0009*

*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

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

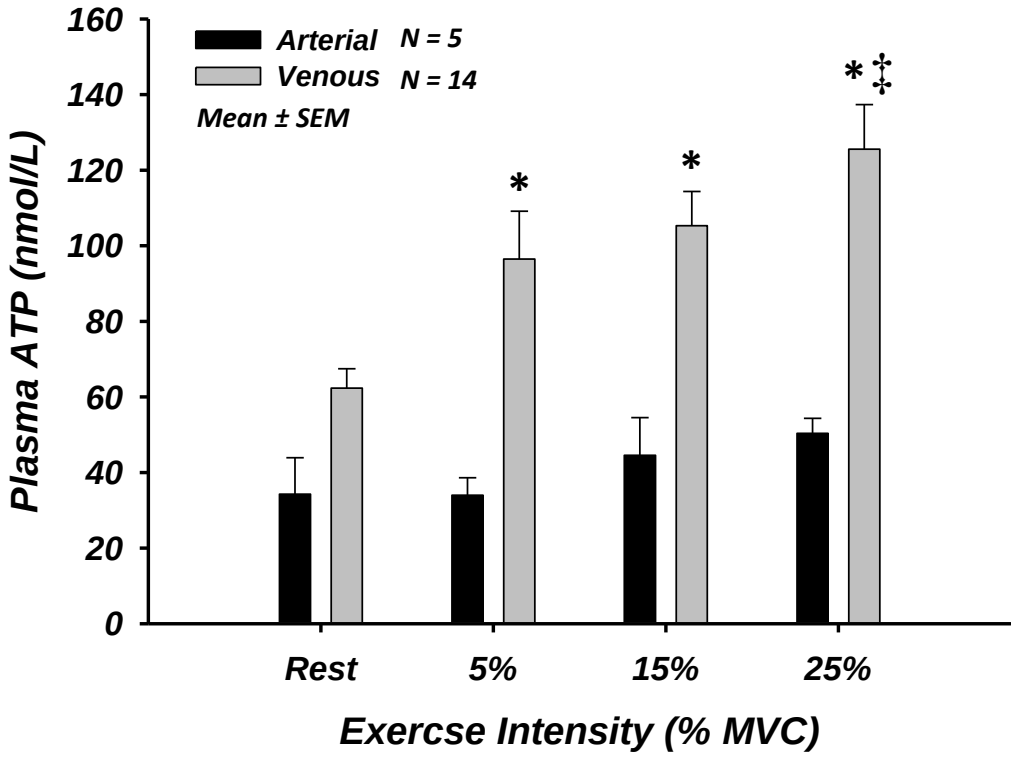
FBF = Forearm blood flow, FVC = Forearm vascular conductance, MAP = Mean arterial pressure, HR = Heart rate, MVC = Maximum voluntary contraction. *P<0.05 vs rest (within group); †P<0.05 vs young adults

Online Table V. Isolated Erythrocyte Protocol: red cell solution blood gases

	pH	PO_2 (mmHg)	PCO_2 (mmHg)	FO_2Hb (%)
16% O_2				
Young	7.276±0.005	109.9±2.3	39.8±1.1	94.9±0.2
Older	7.283±0.010	110.0±2.9	39.1±0.9	95.2±0.2
3% O_2				
Young	7.280±0.007	47.0±1.2*	41.0±0.9	71.6±1.4*
Older	7.289±0.009	45.1±1.0*	40.1±1.0	71.1±1.1*
1.5% O_2				
Young	7.291±0.006	29.2±0.8*	40.6±1.1	42.4±2.1*
Older	7.296±0.009	28.2±1.1*	39.8±0.6	43.1±2.5*

*P<0.05 vs 16% O_2 condition (within group)

SUPPLEMENTAL FIGURES



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.

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