University of Research Integrity and Compliance Office

Protocol Title:	Mechanisms of Adaptation to Exercise in Health & COPD: Oxidative stress links aging, activity and mobility
Protocol:	# IRB_00032404
Approval date:	10/30/17
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METHODS

Subjects and General Procedures. 42 young (25 ± 1 yrs) and 45 older (71 ± 1 yrs) healthy volunteers participated in the current study. All subjects were non-smokers, normotensive (<140/90 mmHg), and free of overt cardiovascular disease as determined by health history questionnaire and physical examination. Exclusion criteria included subjects with diagnosed cardiovascular disease, diabetes, hypercholesterolemia, hypertension, and women who were pregnant. Subjects were not taking any prescription medication, and were asked to abstain from vitamin supplements for 10 days prior to enrollment and during the course of the study. Subjects arrived to the laboratory at 0800 in a fasted state, having abstained from exercise and caffeine for 12 hours prior, in accordance with recently published guidelines [24]. Premenopausal females were studied within the first five days of their menstrual cycle, as FMD measurements of the brachial artery have been documented to fluctuate with menstrual phase. Protocol approval and written informed consent were obtained according to the University of Utah and Veteran Affairs Institutional Review Board requirements. All data collection took place with subjects supine, in a thermoneutral environment.

Antioxidant Supplementation. All subjects reported to the laboratory twice within one week (>48 hrs apart) and received the antioxidant (AO) cocktail or placebo (PL) in a balanced, double-blind, crossover design. Supplements were taken in two doses, separated by 30 minutes to improve absorption, consumed 90 and 60 minutes prior to the FMD protocol. The first dose consisted of 300 mg of alpha-lipoic acid, 500 mg Vitamin C, and 200 I.U. Vitamin E, and the second dose was 300 mg alpha-lipoic acid, 500 mg Vitamin C, and 400 I.U. Vitamin E. Placebo microcrystalline cellulose capsules of similar taste, color, and appearance were likewise

consumed in two doses within the same time frame. We have previously reported the efficacy of this antioxidant cocktail to reduce carbon- and O_2-centered free radical levels, as measured by electron paramagnetic resonance (EPR) spectroscopy, in both young [25] and older [26] subject populations.

Measurements. Details of the FMD procedure have been described previously [27], and were performed in accordance with current recommendations [24]. Briefly, a blood pressure cuff was placed on the right arm proximal to the elbow and distal to the placement of the ultrasound Doppler probe on the brachial artery (BA). The BA was insonated approximately midway between the antecubital and axillary regions, and measurements of BA diameter and blood velocity (V_{mean}) measurements were obtained continuously at rest and for 2 minutes following cuff deflation (Logiq 7, GE Medical Systems, Milwaukee, WI).

Analyses. V_{mean} was automatically calculated using commercially available software (Logiq 7). End-diastolic, ECG R-wave gated images were collected via video output from the Logiq 7 for off-line analysis of BA vasodilation using automated edge-detection software (Medical Imaging Applications, Coralville, Iowa). Flow-mediated vasodilation (FMD) was quantified as the maximal percent change in BA diameter following cuff release. Shear rate was calculated as: $Shear\ Rate\ (s^{-1}) = V_{mean} \bullet 8/\ vessel\ diameter$. Blood flow was calculated as: $Blood\ flow\ (ml \bullet min) = (\ V_{mean} \bullet \pi \bullet [Vessel\ Diameter/2]^2 \bullet 60)$. For both shear rate and blood flow, cumulative area-under-curve (AUC) values were integrated with the trapezoidal rule and calculated as $\sum\{y_i[x_{(i+1)} - x_i] + (1/2)[y_{(i+1)} - y_i][x_{(i+1)} - x_i]\}$. Reactive hyperemia was quantified as cumulative BA blood

flow for two minutes (AUC) following cuff occlusion. Normalized FMD was calculated by dividing FMD (%) by the cumulative shear rate AUC at the time of peak BA vasodilation.

Assays. In a subset of subjects (n = 18 young, n = 30 older), blood samples were obtained from the antecubital vein immediately prior to FMD testing on both PL and AO visits. Total AO capacity was assessed by the ferric reducing ability of plasma (FRAP) assay, and endogenous AO activity was assessed by determining superoxide dismutase (SOD, Cayman Chemical Company, Ann Arbor, MI). Plasma ascorbate concentration was also determined (Cosmo Bio, Carlsbad, CA). Quantitative determination of thiobarbituric acid reactive substances (TBARS) was performed to assess lipid peroxidation, a marker of oxidative stress (Bioassay Systems, Hayward, CA). Total nitrate and nitrite were measured using a standard colorimetric nitrate reductase/Greiss reaction assay (Cayman Chemical, Ann Arbor, MI). Measurement of nitrotyrosine (3-NT) was performed using competitive ELISA (Cell Biolabs, San Diego, CA). A lipid panel was obtained for all subjects by standard techniques.

Statistics. Statistics were performed with the use of commercially available software (SigmaPlot 11, Systat Software Inc., Point Richmond, California, USA). Repeated measures two-way analysis of variance (ANOVA) was used to identify significant changes in measured variables between conditions and groups, with the Bonferroni test used for post-hoc analysis when a significant main effect was found. All group data are expressed as mean ± standard error. Significance was established at $P<0.05$.