Nitric Oxide 76 (2018) 29–36

Contents lists available at ScienceDirect

Nitric Oxide

journal homepage: www.elsevier.com/locate/yniox

Impact of cell-free hemoglobin on contracting skeletal muscle microvascular oxygen pressure dynamics

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ARTICLE INFO

Keywords:
Nitric oxide
Exercise
Hemolysis
Haptoglobin
Sickle Cell Disease

ABSTRACT

Free hemoglobin (Hb) associated with hemolysis extravasates into vascular tissue and depletes nitric oxide (NO), which leads to impaired vascular function and could impair skeletal muscle metabolic control during exercise. We tested the hypothesis that: 1) free Hb would extravasate into skeletal muscle tissue, reducing the contracting skeletal muscle O_2 delivery/O_2 utilization ratio (microvascular PO_2, PO_2mv) to a similar extent as that observed following NO synthase (NOS) blockade, and 2) that the Hb scavenging protein haptoglobin (Hp) would prevent Hb extravasation and inhibit these skeletal muscle tissue effects. PO_2mv was measured in eight rats (phosphorescence quenching) at rest and during 180 s of electrically induced (1-Hz) twitch spinotrapezius muscle contractions (experiment 1). A second group of seven rats was also used to investigate the effects of Hb + Hp (experiment 2). For both experiments, measurements were made: 1) during control conditions, 2) following a bolus infusion of either Hb (50 mg/kg) or Hb + Hp (50 mg/kg), and 3) following local superfusion of NG-nitro-*l*-arginine methyl ester (L-NAME; 10 mg/kg). Additional experiments were completed to visualize Hb extravasation into the muscular tissue using Click chemistry techniques. There were no significant differences in the PO_2mv observed at rest for any condition in either experiment ($p > 0.05$ for all). In experiment 1, both Hb and L-NAME reduced the PO_2mv significantly during the steady-state of muscle contractions when compared to control conditions with no differences between Hb and L-NAME (control: 24 ± 1, Hb: 21 ± 1, L-NAME: 20 ± 1 mmHg, $p < 0.05$). In experiment 2, only L-NAME resulted in a significantly lower PO_2mv during the steady-state of muscle contractions (control: 25 ± 1, Hb + Hp: 22 ± 2, L-NAME: 18 ± 1 mmHg, $p < 0.05$). Free Hb lowered the blood-myocyte O_2 driving force to a level not significantly different from L-NAME. However, infusing Hb bound to Hp resulted in no significant differences in steady-state PO_2mv during muscle contractions when compared to control. Surprisingly, we did not observe Hb accumulation in skeletal muscle tissue. Taken together these data suggests that free Hb impairs O_2 delivery/utilization via a NO scavenging effect. Furthermore, the unchanged PO_2mv steady-state observed following Hb + Hp further indicates that vascular compartmentalization of Hb by the scavenger protein haptoglobin may improve skeletal muscle metabolic control and potentially exercise tolerance in those afflicted with hemolytic diseases.

1. Introduction

Muscular exercise requires frequent and rapid transitions in the metabolic demands of the contracting myocytes which dictate equally robust increases in skeletal muscle blood flow ($\dot{Q}m$) to meet the increasing ATP requirements. The rise in $\dot{Q}m$ is accomplished by a coordinated effort of mechanical (i.e., muscle pump) and vasodilatory controllers [1] which serve to match O_2 delivery ($\dot{Q}O_2$) to utilization ($\dot{V}O_2$). Local release of the vasodilator nitric oxide (NO) helps to relax vascular smooth muscle within the arteriolar bed and, in parallel with elevated cardiac output, facilitates the tight matching of $\dot{Q}O_2/\dot{V}O_2$ within the contracting myocytes [2–4]. Per Fick's Law of Diffusion, the O_2 pressure gradient (PO_2) between the capillary blood and that in the intramyocyte space, serves as the principal driving force for O_2 flux into the myocyte such that if microvascular PO_2 (PO_2mv) falls skeletal muscle $\dot{V}O_2$ becomes compromised, thus placing a greater reliance on substrate level phosphorylation (e.g., creatine phosphate) and other anaerobic and finite means of energy production [5].

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https://doi.org/10.1016/j.niox.2018.03.005
Received 3 January 2018; Received in revised form 28 February 2018; Accepted 7 March 2018
Available online 08 March 2018
1089-8603/

Patients with hemolysis stemming from complications with malaria, sickle cell disease (SCD), extracorporeal circulation, or following transfusion of stored blood or heme-based oxygen carriers suffer from vascular abnormalities resulting from severe reductions in NO bioavailability [6]. When released from the red blood cell, hemoglobin (Hb) rapidly scavenges NO which quickly reduces NO stores and promotes pulmonary and systemic vascular dysfunction [7–10]. The ensuing cardiopulmonary abnormalities have been well characterized and include systemic and pulmonary arterial hypertension and left ventricular systolic and diastolic dysfunction, each of which has been associated with severe exercise intolerance and reduced quality of life [11–13].

Central (e.g., cardiopulmonary) abnormalities certainly play a role in the exercise intolerance observed in those afflicted with chronic hemolysis such as SCD. However, the impacts of free Hb on peripheral skeletal muscle vascular and metabolic control have yet to be investigated and likely contribute to the impaired functional capacity of those burdened with hemolytic conditions. Furthermore, recent evidence suggests that the use of Hb scavengers such as the plasma protein haptoglobin (Hp) restores NO signaling [14]. Given the profound effects of free Hb on NO bioavailability discussed above and the well-characterized impacts of NO signaling on contracting skeletal muscle vascular and metabolic control [15,16], it seems logical to postulate that Hb reduces skeletal muscle PO_2mv via reductions in NO bioavailability, which would impair oxidative metabolism and increase the reliance on finite energy sources.

Therefore, the purpose of this investigation was twofold: 1) examine the potentially detrimental role of free Hb in the regulation of skeletal muscle PO_2mv at rest and during contractions under normal conditions and following the blockade of NO synthase (NOS) via the topical superfusion of NG-nitro-*l*-arginine methyl ester (L-NAME), and 2) in a separate experiment, determine the ability of the Hb binding protein Hp to prevent/minimize the loss of NO signaling (and detrimental effects on PO_2mv) within the skeletal muscle following the simultaneous infusion of Hb. Specifically, we tested the hypothesis that 1) acute infusion with free Hb would impair the contracting skeletal muscle PO_2mv to a similar extent as that observed following L-NAME induced NOS blockade, and 2) infusion of the Hb binding protein Hp in combination with Hb (Hb + Hp) would result in a PO_2mv profile not significantly different from control conditions. Using a click-chemistry technique for defining the presence of Hb in tissue, we also sought to determine if the impacts of Hb on the PO_2mv were associated with the translocation of Hb out of the intravascular space and into the surrounding vascular and/or skeletal muscle tissue spaces and if Hb + Hp would prevent this from occurring.

2. Methods

2.1. Animal selection and care

Fifteen adult male Sprague-Dawley rats (~3–4 months of age, Charles Rivers Laboratories, Wilmington, MA, USA, average body mass 398 ± 9 g) were used to investigate the impacts of Hb (experiment 1, n = 8)) and Hb + Hp (experiment 2, n = 7) on skeletal muscle microvascular oxygenation. An additional five rats were used in supplementary experiments to determine if 1) free Hb extravasates out of the systemic circulation and into the skeletal muscle and 2) Hb bound to Hp prevents this extravasation (bringing the total number of animals to n = 20). All rats were maintained on a 12:12-h light-dark cycle and received food and water *ad libitum*. All procedures described herein were approved by the University of Colorado Anschutz Medical Center's Institutional Animal Care and Use Committee and conducted according to the guidelines recommended by the National Institutes of Health.

2.2. Surgical preparation

Rats were anesthetized using a ketamine/xylazine mixture (75 mg/kg and 6 mg/kg, respectively; given i.m. to effect) with the level monitored continuously via the toe-pinch and blink reflexes and anesthesia supplemented as necessary. Rats were then placed on a heating pad to maintain constant body temperature (~38 °C) throughout the entirety of the experimental protocol. The medial aspect of the left leg was then shaved, and a 2-cm incision was made to afford isolation and cannulation of the left femoral artery and vein via blunt dissection. A polyethylene (PE-50) catheter was then introduced into the left femoral artery for measurement of mean arterial pressure (MAP) and heart rate (HR), infusion of the phosphorescent probe (see below), and blood sampling. A second catheter was placed in the femoral vein for the infusion of Hb or Hb + Hp (see experimental protocols 1 and 2 below) and the incisions were then closed.

Following instrumentation, the skin and overlying fascia of the mid-dorsal region of the rat were reflected carefully to expose the right spinotrapezius muscle in a manner which insured the integrity of the neural and vascular supply to the muscle [17]. Silver wire electrodes were then sutured (6-0 silk) to the rostral (cathode) and caudal (anode) regions of the muscle. Once exposed, the spinotrapezius muscle was superfused continuously with a warm (38 °C) Krebs-Henseleit bicarbonate buffered solution equilibrated with 5% CO_2–95% N_2 and surrounding exposed tissue was covered with Saran Wrap (Dow Brands, Indianapolis, IN, USA). The spinotrapezius muscle originates in the lower thoracic and upper lumbar region, inserts on the scapula, and is closely associated with the vertebral column. It was selected specifically based on its mixed muscle fiber type composition and citrate synthase activity, which is similar to that found in human quadriceps muscle [18,19].

2.3. Experimental protocol

Three separate bouts of contractions were performed for each experiment. Contractions for experiment 1 occurred under control superfusion (5-ml of Krebs Henseleit), Hb infusion (50 mg given as a bolus i.v. infusion via the femoral vein catheter, total volume 0.25 ml), and L-NAME superfusion (non-isoform-specific NO synthase inhibitor; 5 ml of a 1.5 mM solution) conditions. Experiment 2 consisted of control superfusion (5-ml of Krebs Henseleit), an equimolar dose (relative to the Hb condition) of Hb + Hp infusion (50 mg infused i.v., total volume 2.5 ml) and L-NAME (5 ml of a 1.5 mM solution) conditions. Hb + Hp was infused via the femoral vein catheter at a rate of 0.16 ml/min for 15 min using a Chemyx infusion pump (model Fusion 200, Stafford, TX, USA). L-NAME was purchased from Sigma-Aldrich (St. Louis, MO, USA) and concentrations were chosen based on previous studies [16,20,21]. The phosphorescent probe meso-tetra (4 carboxyphenyl)tetrabenzoporphorphyrin-dendrimer (G2: 1–5 mg/kg dissolved in 0.4 ml of saline) was infused via the femoral artery catheter. Following a brief stabilization period of ~10 min, the common end of the light guide of a frequency domain phosphorimeter (PMOD 5000, Oxygen Enterprises, Philadelphia, PA) was positioned ~2–4 mm superficial to the dorsal surface of the right spinotrapezius muscle over a randomly selected field absent of any large vessels thus ensuring that the region contained principally capillary blood. PO_2mv was measured using phosphorescence quenching (see below) and reported at 2 s intervals throughout the 180-s contraction protocol (1 Hz, ~6 V, 2 ms pulse duration) elicited via a Harvard Apparatus Stimulator (model 6002, Holliston, MA, USA). Upon completion of the study, each animal was euthanized using a pentobarbital sodium overdose (≥50 mg/kg administered into the femoral artery catheter).

2.4. PO_2mv measurement and curve-fitting

The Stern-Volmer relationship allows the calculation of PO_2mv through the direct measurement of a phosphorescence lifetime via the following equation [22]: