Journal of Traditional and Complementary Medicine 6 (2016) 17–22

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Contents lists available at ScienceDirect

Journal of Traditional and Complementary Medicine

journal homepage: http://www.elsevier.com/locate/jtcme

Original article

Nitrate-containing beetroot enhances myocyte metabolism and mitochondrial content

Roger A. Vaughan [a, b, c, d, *], Nicholas P. Gannon [b], Colin R. Carriker [b, e]

[a] Department of Nutritional Sciences, Texas Tech University, PO Box 41270, Lubbock 79409, TX, USA
[b] Department of Health, Exercise and Sports Science, University of New Mexico, Albuquerque 87131, NM, USA
[c] Department of Biochemistry and Molecular Biology, University of New Mexico Health Sciences Center, School of Medicine, Albuquerque 87131, NM, USA
[d] Department of Individual, Family, and Community Education: Nutrition, University of New Mexico, Albuquerque 87131, NM, USA
[e] Department of Kinesiology, Recreation, and Sport, Indiana State University, Terre Haute 47809, IN, USA

ARTICLE INFO

Article history:
Received 12 October 2014
Received in revised form
26 October 2014
Accepted 11 November 2014
Available online 8 January 2015

Keywords:
dietary supplement
ergogenic
mitochondrial uncoupling protein 3 (UCP3)
nitrate
mitochondrial efficiency
peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α)

ABSTRACT

Beetroot (甜菜 tián cài) juice consumption is of current interest for improving aerobic performance by acting as a vasodilator and possibly through alterations in skeletal muscle metabolism and physiology. This work explored the effects of a commercially available beetroot supplement on metabolism, gene expression, and mitochondrial content in cultured myocytes. C2C12 myocytes were treated with various concentrations of the beetroot supplement for various durations. Glycolytic metabolism and oxidative metabolism were quantified via measurement of extracellular acidification and oxygen consumption, respectively. Metabolic gene expression was measured using quantitative reverse transcription–polymerase chain reaction, and mitochondrial content was assessed with flow cytometry and confocal microscopy. Cells treated with beetroot exhibited significantly increased oxidative metabolism, concurrently with elevated metabolic gene expression including peroxisome proliferator-activated receptor gamma coactivator-1 alpha, nuclear respiratory factor 1, mitochondrial transcription factor A, and glucose transporter 4, leading to increased mitochondrial biogenesis. Our data show that treatment with a beetroot supplement increases basal oxidative metabolism. Our observations are also among the first to demonstrate that beetroot extract is an inducer of metabolic gene expression and mitochondrial biogenesis. These observations support the need for further investigation into the therapeutic and pharmacological effects of nitrate-containing supplements for health and athletic benefits.

1. Introduction

Dietary sources of nitrate are found in a variety of foods including roots and green leafy vegetables, and more recently, some cured/processed meats. Dietary sources of nitrate have become of interest for ergogenic and metabolic purposes because of the ability of dietary nitrate to increase nitric oxide (NO) biosynthesis.[1] Nitrate consumption results in heighted plasma nitrite, which has been previously recognized as an *in vivo* biomarker of NO production.[2,3] Consumption of nitrate-rich foods prior to endurance events including cycling, rowing, and running results in improved performance,[4–6] resulting in increasing popularity of nitrate-containing foods.[7–13] As a result, commercially available dietary products high in nitrate content have emerged as ergogenic aids purported to increase athletic performance.

NO is a potent cellular stimulus capable of increasing intracellular Ca^{2+} and cyclic guanosine monophosphate (cGMP) leading to activation of Ca^{2+}/calmodulin-dependent protein kinase and 5′-adenosine monophosphate-activated protein kinase (AMPK). Dietary NO precursors including arginine and arginine-like metabolites increase NO, stimulating phosphorylation and activation of AMPK, which later acts to increase the expression and phosphorylation of peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α).[14,15] PGC-1α acts as a master metabolic regulator of energy metabolism and mitochondrial biogenesis by controlling the expression of nuclear respiratory factor (NRF) and its downstream target, mitochondrial transcription factor A (TFAM).[16–19] In adipocytes, arginine was shown to increase

* Corresponding author. Department of Nutritional Sciences, Texas Tech University, PO Box 41270, Lubbock, TX 79409, USA.
E-mail address: roger.vaughan@ttu.edu (R.A. Vaughan).
Peer review under responsibility of The Center for Food and Biomolecules, National Taiwan University.

http://dx.doi.org/10.1016/j.jtcme.2014.11.033

mitochondrial content under ambient conditions, and intensify cold-induced adipocyte browning.[20]

Despite the heightened interest in nitrate consumption, findings about the affects of beetroot (甜菜 tián cài; BR) supplementation have been inconsistent.[10,21–23] The primary aim of this work is to characterize the effects a commercially available BR supplement beverage on metabolism and related metabolic gene expression in skeletal muscle.

2. Materials and methods

2.1. Cell culture

Murine myocytes (C2C12) were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium containing 4500 mg/L glucose and supplemented with 10% heat-inactivated fetal bovine serum and 100 U/mL penicillin/streptomycin, in a humidified 5% CO_2 atmosphere at 37 °C (standard conditions). Following overnight seeding, cells were treated with various concentrations of the nitrate-containing commercially available BEET IT Pro-Elite Shot from James White Drinks Ltd (Ipswich, UK) for 24 hours (ingredients are displayed in Table 1).

2.2. Determination of BEET-IT nitrate and nitrite content

BEET IT Pro-Elite's nitrate/nitrite content was quantified using the nitrate/nitrite quantification assay kit from Cayman Chemical (Ann Arbor, MI, USA). Nitrate and nitrite were quantified according to the manufacturer's protocol. Briefly, nitrate was quantified by diluting samples in an assay buffer, followed by 1 hour of incubation at room temperature with nitrate reductase and vitamin cofactors. Griess reagents were then added in equivocal volume to each well and incubated for 10 min at room temperature, and then sample absorbance was measured at 540 nm. The beverage nitrate concentrations were 5.312 ± 0.306μM, with undetectable nitrite. All treatments were standardized to determined nitrate concentrations of 20, 10, and 5μM, which correspond with 0.5%, 0.25%, and 0.125% BR, respectively, by volume (determined during preliminary cell viability and mitochondrial staining studies).

2.3. Metabolic assay

Cells were seeded overnight in 24-well culture plate from SeaHorse Bioscience (Billerica, MA, USA) at a density of 5×10^5 cells/well, and treated with either control medium or media containing BR in dilutions corresponding with 0.5%, 0.25%, and 0.125% BR by volume for 24 hours. Following treatment, culture media was removed and replaced with XF Assay Media from SeaHorse Bioscience containing 4500 mg/L glucose free of CO_2 and briefly incubated at 37 °C. As per the manufacturer's protocol, SeaHorse injection ports were loaded with oligomycin, an inhibitor of ATP synthase that induces maximal glycolytic metabolism and reveals endogenous proton leak (mitochondrial uncoupling) at a final concentration of 1.0 μM. Oligomycin addition was followed by the addition of carbonyl cyanide *p*-[trifluoromethoxy]-phenyl-hydrazone, an uncoupler of electron transport that induces peak oxygen consumption (an indirect indicator of peak oxidative metabolism) at final concentration of 1.25μM. Rotenone was then added in 1.0μM final concentration to reveal nonmitochondrial respiration and end the metabolic reactions.[24,25] Extracellular acidification, an indirect measure of glycolytic capacity, and oxygen consumption, a measure of oxidative metabolism, was measured using the SeaHorse XF24 Extracellular Analyzer from SeaHorse Bioscience. SeaHorse XF24 Extracellular Analyzer was run using 8-minute cyclic protocol commands (mix for 3 minutes, let stand for 2 minutes, and measure for 3 minutes) in triplicate as previously described.[26]

Table 1
Food fact panel of *BEET IT* Pro-Elite Shot from James White Drinks Ltd (Ipswich, UK).

Serving Size	**70ml/2.3fl oz**
Servings per container	**1**
Energy/Calories	**306kj/72kcal**
Protein	2.5 g
Total Carbohydrates	16 g
Sugars	16 g
Total fat	<0.1 g
Fiber	<0.5 g
Sodium	<0.1 g
Dietary Nitrate	0.4 g
Cholesterol	0 g
Vitamin A	0%*
Vitamin C	7.20%*
Calcium	1.20%*
Iron	2.80%*

* % daily values based on a 2000 calorie diet.

2.4. Quantitative real-time polymerase chain reaction

Following a 24-hour treatment with either the control medium or media containing BR in dilutions corresponding with 0.5%, 0.25%, and 0.125% BR by volume for various durations up to 24 hours, total RNA was extracted using the RNeasy Kit from Qiagen (Valencia, CA, USA) and cDNA was synthesized using the Retroscript RT kit from Ambion (Austin, TX, USA) according to manufacturer's instructions. Polymerase chain reaction (PCR) primers were designed using the Primer Express software from Invitrogen (Carlsbad, CA, USA) and synthesized by Integrated DNA Technologies (Coralville, IA, USA). Amplifications of PGC-1α, NRF1, TFAM, glucose transporter 4 (GLUT4), and mitochondrial uncoupling protein 3 (UCP3) were normalized to the housekeeping gene, TATA binding protein (*TBP*). Table 2 summarizes the forward and reverse primers of each gene. Quantitative reverse transcription- polymerase chain reactions were performed in triplicate using the LightCycler 480 real-time PCR system from Roche Applied Science (Indianapolis, IN, USA). SYBR Green based PCR was performed in triplicate with final primer concentrations at 10μM in a total volume of 30 μL. The following cycling parameters were used: 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Relative expression levels were determined using the $\Delta\Delta C_p$ method and compared to the lowest expressing group as previously described.[27]

2.5. Flow cytometry

Cells were seeded in six-well plates at a density of 1.0×10^6 cells/well and treated with either control medium or media containing BR in dilutions corresponding with 0.5%, 0.25%, and 0.125% BR by volume for 24 hours. Following incubation, the medium was removed and the cells were resuspended in prewarmed media with 200nM Mitotracker Green from Life Technologies (Carlsbad, CA, USA) and incubated for 45 minutes in a humidified 5% CO_2 atmosphere at 37°C. The cells were pelleted, the media with Mitotracker was removed, and the cells were suspended in prewarmed media. Group mean fluorescence was measured using Facscalibur filtering 488 nm.

2.6. Immunofluorescence and confocal microscopy

To investigate the protein expression of metabolic proteins PGC-1α, cytochrome *C* (Cyt C), GLUT4, and UCP3, cells were seeded in chamber slides obtained from BD Bioscience (Sparks, MD, USA)