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Dietary nitrate markedly improves voluntary running in mice

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HIGHLIGHTS

- Voluntary in-cage wheel running is increased in mice supplemented with nitrate.
- When dietary nitrate is removed, running decreases to the control level.
- Dietary nitrate improves submaximal muscle force.
- Dietary nitrate does not enhance endurance or mitochondrial function in sedentary animals.
- Mice given dietary nitrate get a higher oxidative capacity and become more fatigue resistant because they run more.

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ABSTRACT

Nitrate supplementation is shown to increase submaximal force in human and mouse skeletal muscles. In this study, we test the hypothesis that the increased submaximal force induced by nitrate supplementation reduces the effort of submaximal voluntary running, resulting in increased running speed and distance.

C57Bl/6N male mice were fed nitrate in the drinking water and housed with or without access to an in-cage running wheel.

Nitrate supplementation in sedentary mice had no effect on endurance in a treadmill test, nor did it enhance mitochondrial function. However, after three weeks with in-cage running wheel, mice fed nitrate ran on average 20% faster and 30% further than controls ($p < 0.01$). Compared to running controls, this resulted in ~13% improved endurance on a subsequent treadmill test ($p < 0.05$) and increased mitochondrial oxidative capacity, as judged from a mean increase in citrate synthase activity of 14% ($p < 0.05$). After six weeks with nitrate, the mice were running 58% longer distances per night. When nitrate supplementation was removed from the diet, the running distance and speed decreased to the control level, despite the improved endurance achieved during nitrate supplementation.

In conclusion, low-frequency force improvement due to nitrate supplementation facilitates submaximal exercise such as voluntary running.

1. Introduction

Amongst athletes searching for ultimate performance, dietary supplements are very popular. Dietary supplementation with inorganic nitrate reduces oxygen cost of exercise and increases exercise performance in various types of sports [1]. Inorganic nitrate is an oxidation product from endogenous nitric oxide (NO) production. Nitrate can be reprocessed back to NO, thereby creating a large pool of potential NO bioactivity. This pool can be supplemented by a diet consisting of green leafy vegetables such as spinach and beetroot, which are especially rich in inorganic nitrate [2]. Supplemented nitrate is shown to improve the performance of trained cyclists by ~10% during a 10 km time trial [3]. The beneficial effect of nitrate can be partly due to effects on mitochondrial respiration, where it increases mitochondrial efficiency in human subjects [4]. However, nitrate does not seem to have any beneficial effect on mitochondrial function in mice [5].

Recently, it was shown that nitrate supplementation enhances sarcoplasmic reticulum (SR) Ca^{2+} release in fast-twitch mouse muscle [6]. Increased SR Ca^{2+} release results in increased force predominantly at low stimulation frequencies, where the sigmoidal relationship between force and the free cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_i$) is steep. Improved

Abbreviations: $[Ca^{2+}]_i$, free cytosolic $[Ca^{2+}]$; CSQ1, calsequestrin 1; COX1, cytochrome *c* oxidase subunit 1; CS, citrate synthase; Ctrl, control; DHPR, dihydropyridine receptor; EDL, extensor digitorum longus; Nit, nitrate; P/O, oxidative phosphorylation efficiency; RCR, respiratory control ratio; RyR1, ryanodine receptor 1; Run, runner; Sed, sedentary; SR, sarcoplasmic reticulum.

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force production at low stimulation frequency with dietary nitrate supplementation was subsequently confirmed in humans [7].

Voluntary muscle activation is controlled by the drive of higher motor areas on α-motor neurons, and force production consequently depends both on the neural activation and the peripheral response to this activation. The latter is not linear with a steep force-frequency relationship at low stimulation frequencies (~10–30 Hz in human muscle) that reaches saturation at higher frequencies (>50 Hz) [8,9]. Physical activities generally require low to moderate forces and the firing frequencies of α-motor neurons are therefore mostly set to be on the steep part of force-frequency curve [10,11]. Thus, small changes in neural activity significantly affect SR Ca^{2+} release, which will have a large impact on submaximal force. This will have a large impact especially when the neuromuscular system is put under stress, e.g. during fatiguing exercise [12,13]. Accordingly, classical experiments by Angelo Mosso showed that contractile function decreased more rapidly during fatiguing exercise when subjects were mentally exhausted [14], and similar results have subsequently been presented in numerous studies [15,16]. Also, in recent experiments, Flanagan et al. showed that nitrate improves neuromuscular efficiency during resistance training [17].

A recent study showed that running wheels placed in nature were frequently used by wild mice, also when no extrinsic (i.e. food) reward was provided [18]. Moreover, running wheel behaviour of the wild mice was similar to that of captive laboratory mice. Thus, mouse wheel running provides a good opportunity to study the effect of muscle contractile function on voluntary running performance. The present study builds upon the finding that that nitrate supplementation increases SR Ca^{2+} release, which shifts the force-frequency relationship towards lower frequencies [6]. We hypothesize that due to the nitrate-induced increase in submaximal force, the effort of voluntary running will decline and hence running speed and distance will increase. This in turn will induce a greater endurance training effect with a larger improvement in mitochondrial capacity. To test this hypothesis, we fed mice inorganic nitrate and gave some access to an in-cage running wheel, which allowed voluntary running at self-determined speed and duration.

2. Methods

2.1. Ethical approval

All experiments complied with the Swedish Animal Welfare Act, the Swedish Welfare Ordinance, and applicable regulations and recommendations from Swedish authorities. The study was approved by the Stockholm North Ethical Committee on Animal Experiments. A total of 68 adult male C57Bl/6N (Harlan, UK) mice were used in this study. Mice were housed in room temperature with a 12/12 h day/night light cycle. The mice were killed by rapid neck disarticulation.

2.2. Experimental design

One week prior to experiments, all mice were given standard chow (R34, Lantmannen, Sweden) checked for low nitrate content with reductive chemiluminescence analysis. Thereafter, one group of mice was provided ad libitum 1 mM $NaNO_3$ dissolved in distilled water, which results in an estimated daily nitrate intake of ~120 μmol kg per kg bodyweight. For comparison, this is equivalent to a human (70 kg) eating 150 g of spinach or drinking 100 ml concentrated beetroot juice (~100 μmol kg^{-1} day^{-1}), a nitrate-source often used in human trials [1,19]. Controls were provided distilled water without nitrate. Some of the nitrate-fed and control mice were housed individually with an in-cage wireless low profile running wheel (ENV-044 Med-Associates, St. Albans City, VT). Running distance and speed was continuously measured for up to 12 weeks. Thus, there were four groups: sedentary control, sedentary nitrate-fed, running control, and running nitrate-fed.

2.3. Treadmill

An exhaustion test was conducted after three weeks on mice from all four groups. Endurance was measured using an animal treadmill (Exer 3/6, Columbus instruments, Columbus, OH). The mice were habituated to the treadmill for four days prior to the exhaustion test. Habituation was performed for 1 h a day with various speeds in order to ensure that the mice were fully familiarised with running on the treadmill. During the test mice ran at 25° uphill. An initial 10 min warm-up (10 $m \cdot min^{-1}$) was followed by gradual increases of 2 $m \cdot min^{-1}$ every 2 min. Exhaustion was determined as the time when the mouse had withstood three mild electric shocks (0.1 mA, 2 Hz) without attempts to continue running. This method is based on Kemi et al. [20], who showed a relation between VO_{2max} and final running speeds.

2.4. Muscle isolation and force measurement

Extensor digitorum longus (EDL) muscles were isolated from mice after 3 weeks with or without nitrate treatment. The muscles were then mounted at optimal length between a force transducer and an adjustable hook in a chamber filled with Tyrode solution (mM): NaCl, 121; KCl, 5.0; $CaCl_2$, 1.8; $MgCl_2$, 0.5; NaH_2PO_4, 0.4, $NaHCO_3$, 24.0; EDTA, 0.1; glucose, 5.5 and fetal calf serum (0.2%). The solution was bubbled with 95% O_2, 5% CO_2 and kept at a temperature of 30 °C. The force-frequency relationship was established by producing 300 ms contractions at 1–100 Hz stimulation frequencies at 1 min interval.

2.5. Western blot

EDL muscles were dissected out from mice sacrificed after 3 weeks of intervention. Muscles were homogenised with a ground glass homogenizer in ice-cold homogenisation buffer at pH 7.4 (20 μl per mg wet weight) consisting of (mM): Hepes, 20; NaCl, 150; EDTA, 5; KF, 25; Na_3VO_4, 1; and 20% glycerol, 0.5% Triton X-100, and protease inhibitor cocktail (Roche, Basel, Switzerland) 1 tablet/50 ml. The homogenate was centrifuged at 700 × *g* for 10 min at 4 °C. Protein content of the supernatant was determined using the Bradford assay (#500-0006, Bio-Rad, Hercules, CA, USA). Samples were diluted 1:1 in Laemmli buffer (Bio-Rad) with 5% 2-mercaptoethanol and heated to 95 °C for 5 min.

10 μg protein was run on a 4–12% precast Bis–Tris gel (NP0336PK2, NuPAGE, Invitrogen Carlsbad, CA, USA) and transferred onto polyvinylidine fluoride membranes (Immobilon FL, Millipore, Billerica, MA, USA). Membranes were then blocked with Li-Cor Blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) followed by incubation overnight at 4 °C with the following antibodies diluted in blocking buffer; mouse anti-dihydropyridine receptor (DHPR; ab2864, Abcam, Cambridge, UK), mouse anti-calsequestrin 1 (CSQ1; ab2824, Abcam), mouse anti-ryanodine receptor 1(RyR1; ab2868, Abcam), mouse anti-oxphos complex IV (cytochrome *c* oxidase), subunit 1 (COX1; #459,600, Invitrogen). Membranes were then washed and incubated with secondary antibody IRDye 680-conjugated donkey anti-mouse IgG and IRDye 800-conjugated donkey anti-rabbit IgG (926–68,072, 926–32,213, LI-COR). Immunoreactive bands were visualized using infrared fluorescence (IR-Odyssey scanner, LI-COR Biosciences). Band density was established using Image Studio v 2.0.38 (LI-COR Biosciences). Equal protein loading was verified with subsequent Coomassie protein staining of the same membranes (#161–0436, Bio-Rad).

2.6. Enzyme assay

Frozen EDL muscles were homogenised in ice-cold homogenisation buffer at pH 7.5 (50 μl per mg wet weight) consisting of (mM): KH_2PO_4, 50; EDTA, 1; and 0.05% TritonX. The homogenate was centrifuged at 1400 × *g* for 1 min at 4 °C. The supernatant was used for analyses of citrate synthase (CS) activity using standard spectrophotometric techniques [21]. The activities were measured at room temperature