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Effect of dietary nitrate levels on nitrate fluxes in rat skeletal muscle and liver

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ABSTRACT

Rodent skeletal muscle has high levels of nitrate ions and this endogenous nitrate reservoir can supply nitrite/nitric oxide (NO) for functional hyperemia and/or for other physiological processes in muscle during exercise. Mice with a NOS1 knockout have markedly reduced muscle nitrate levels, suggesting NO production by NOS and its reaction with oxymyoglobin as a source of nitrate. However, oxygen levels are normally low in most internal organs, which raises the possibility that nitrate-derived NO pathway is physiologically important even at "normoxia", and muscle nitrate reservoir is the main endogenous NO backup when exogeneous (dietary) nitrate intake is low. Using dietary nitrate manipulations, we explore the importance of diet for maintaining and renewal of muscle nitrate reservoir and its levels in other tissues. We found that skeletal muscle nitrate is extensively used when nitrate in diet is low. One week of nitrate starvation leads to dramatic nitrate depletion in skeletal muscle and a substantial decrease in liver. Nitrate depleted from skeletal muscle during starvation is quickly recovered from new dietary sources, with an unexpected significant "overload" compared with animals not subjected to nitrate starvation. Our results suggest the importance of dietary nitrate for nitrate reserves in muscle and in other tissues, when compared with endogenous NOS-derived sources. This requires an active transport mechanism for sequestering nitrate into cells, stimulated by lack of dietary nitrate or other enzymatic changes. These results confirm the hypothesis that muscle is a major storage site for nitrate in mammals.

1. Introduction

Nitric oxide (NO) is important for a large array of basic physiological functions, including the determination of vascular tone, affecting platelet activation, being a volume neurotransmitter and maintaining some of the body immunological functions. NO is a reactive molecule with a half-life in the blood in the order of milliseconds. NO is now known to be the active product of both the L-arginine-citrulline nitric oxide synthase metabolic pathway (oxygen-dependent) and the reductive nitrate-nitrite-NO cycle (oxygen-independent). The nitric oxide synthase (NOS) enzymes are found in endothelium (NOS3 or eNOS); brain, skeletal muscle and other tissues (NOS1 or nNOS); or are induced upon inflammation (NOS2 or iNOS). NO produced by the NOS enzymes is always used directly at the production site with the excess oxidized to nitrate by oxy-heme proteins (oxyhemoglobin, oxymyoglobin or other still unknown heme proteins). At physiological normoxic conditions, nitrate is a relatively inert ion and can be considered as an effective storage molecule for NO production. However, enterobacteria can reduce nitrate to nitrite at low oxygen conditions and nitrate can be slowly reduced by Mo-containing proteins such as xanthine oxidoreductase (XOR) and aldehyde oxidase (AO) back to nitrite and then either further reduced by XOR or AO or deoxy-heme proteins (mainly deoxyhemoglobin (deoxyHb) or deoxymyoglobin (deoxyMb)) to NO [1–7].

Previously, we have shown that nitrate is highly concentrated in skeletal muscle, with a concentration gradient from muscle to blood to internal organs, while nitrite is more homogeneously distributed among internal organs, blood and skeletal muscle in Wistar rats [8]. We proposed that nitrate in skeletal muscle at least in part originates from oxidation of NO produced by NOS1 by oxymyoglobin and that this nitrate is likely one of the main sources of endogenous nitrate in the rat. We also showed that skeletal muscle nitrate is accessible and used for generating nitrite and likely NO during vigorous exercise [9].

Here we present data in rats of nitrate and nitrite organ levels after

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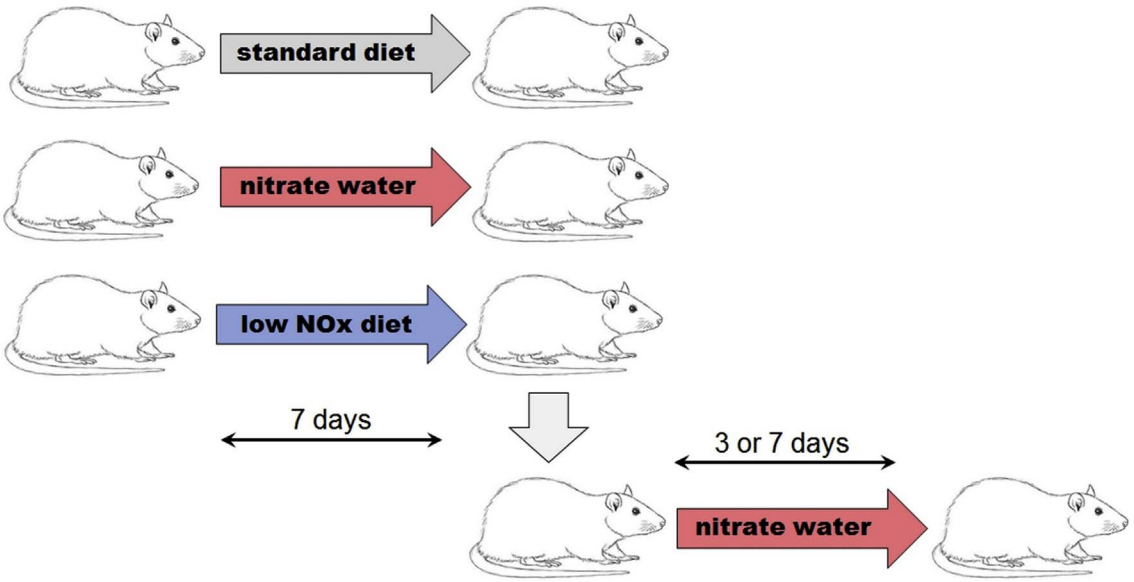

Fig. 1. Experimental setup. Rats were fed either standard diet (control rats) or had nitrate added into drinking water (high NOx diet) or fed low NOx diet for 7 days. After 7 days on low NOx diet, group of animals was switched to high NOx diet for 3 or 7 days.

Table 1
Corresponding doses in mg/kg/day and μmol/kg/day used in experimental setup described in Fig. 1. Calculations are done for 250 g rat drinking 15 ml of water and eating 25 g of food per day. These doses are comparable to those reported in several human studies [17].

Diet	Nitrate		Nitrite	
	(mg/kg/day)	(μmol/kg/day)	(mg/kg/day)	(μmol/kg/day)
lowNOx	0.2	2.3	0.04	0.5
Standard	2.9	34.1	0.05	0.7
High nitrate	60.2	708.3	0	0

nitrate deprivation and following nitrate intake reintroduction. Following dietary nitrate deprivation, endogenous nitrate in skeletal muscle decreases greatly, possibly serving as nitrite and NO source. In contrast, when an excess of dietary nitrate is consumed, skeletal muscle nitrate levels are increased above levels found in animals consuming standard diet and nitrate values in the liver are much higher than previously measured. Surprisingly, when excess nitrate is reintroduced after a period of nitrate starvation, nitrate uptake into skeletal muscle exceeds greatly the value observed after simple elevation of dietary nitrate.

We believe that this high nitrate "overload" suggests that nitrate is an essential ion and that the array of metabolic pathways in which the nitrate/nitrite/NO pathway is involved is much wider than previously suspected. Our results also raise the possibility of influence of enzyme reduction and/or active transport mechanisms in organs, especially muscle during dietary nitrate alterations. In summary, our findings suggest that high nitrate diet is very important for achieving proper physiological levels of nitric oxide, regardless of physical activity and/or oxygen availability. Based on the results we can also hypothesize that the question about what is the optimal healthy level of nitrate and nitrite in mammalian or human body is still unanswered. Based on the body of available literature, it is certain that even small changes in nitrite and nitrate content in the diet do have large physiological effects and health consequences. Our findings about nitrate and nitrite fluxes in rats, already confirmed in humans [10], may lead to understanding of factors that determine the availability of the nitrate/nitrite/NO reductase pathways in human physiology and lead to simple and effective preventive and supportive therapies for an array of cardiovascular as well as muscular diseases.

2. Materials and methods

2.1. Dietary interventions

All experiments were done under NIDDK animal protocol K049-MMB-15 and in agreement with NIH animal care policies. Young Wistar rats (n = 15, 250 ± 50 g) were divided into 3 groups (n = 5 in each group) according to diet type. Control group rats were fed NIH standard rat diet (NIH07, nitrate 340.3 ± 13.5 nmol/g, nitrite 7.4 ± 0.1 nmol/g); high nitrate diet groups consumed water containing 1 g/l of sodium nitrate (Sigma, St. Louis, MO) and nitrate deprived groups were fed a low nitrate/nitrite diet (TD99366, Harlan Teklad, South Easton, MA, nitrate 23.4 ± 0.7 nmol/g, nitrite 5.3 ± 0.7 nmol/g). Low and high nitrate diets were maintained for 7 days. A separate group of rats was first given low NOx diet for 7 days, after which they were switched to high nitrate diet for another 3 or 7 days to determine if we could "rescue" nitrate levels after depletion. Fig. 1 shows a flow chart of those experiments and nitrate and nitrite doses are in Table 1.

2.2. Sample collection

Rats were enclosed in an anesthesia box and anesthetized using 5% isoflurane mixture with air. Anesthetized animals were placed on a pad in supine position and anesthesia continued through a nose cone. The thoracic cavity was opened and ~9–10 ml of blood collected by cardiac puncture; representing about two-thirds of total blood volume for a rat of this size. Heparin was used as an anticoagulant in nitrite and nitrate determinations. Immediately, blood was mixed with a solution containing potassium ferricyanide, NEM and detergent in final ratio 2:1 as described in Ref. [11] to conserve nitrite from oxidation by hemoglobin. Animals were then perfused using heparin-containing saline to flush the remaining blood out of the tissues. Perfusion continued until no blood was detected in outgoing saline and liver and kidneys were significantly discolored. Samples from liver and skeletal muscle from the hind legs were then collected and placed into 250 μl of nitrite preserving solution containing potassium ferricyanide for chemiluminescence analysis. All samples were stored at −80 °C until analysis. Animals were housed in a 12 h light/dark cycle environment with access to food and drinking water *ad libitum*.