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Skeletal muscle as an endogenous nitrate reservoir

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ABSTRACT

The nitric oxide synthase (NOS) family of enzymes form nitric oxide (NO) from arginine in the presence of oxygen. At reduced oxygen availability NO is also generated from nitrate in a two step process by bacterial and mammalian molybdopterin proteins, and also directly from nitrite by a variety of five-coordinated ferrous hemoproteins. The mammalian NO cycle also involves direct oxidation of NO to nitrite, and both NO and nitrite to nitrate by oxy-ferrous hemoproteins. The liver and blood are considered the sites of active mammalian NO metabolism and nitrite and nitrate concentrations in the liver and blood of several mammalian species, including human, have been determined. However, the large tissue mass of skeletal muscle had not been generally considered in the analysis of the NO cycle, in spite of its long-known presence of significant levels of active neuronal NOS (nNOS or NOS1). We hypothesized that skeletal muscle participates in the NO cycle and, due to its NO oxidizing heme protein, oxymyoglobin has high concentrations of nitrate ions. We measured nitrite and nitrate concentrations in rat and mouse leg skeletal muscle and found unusually high concentrations of nitrate but similar levels of nitrite, when compared to the liver. The nitrate reservoir in muscle is easily accessible via the bloodstream and therefore nitrate is available for transport to internal organs where it can be reduced to nitrite and NO. Nitrate levels in skeletal muscle and blood in $nNOS^{-/-}$ mice were dramatically lower when compared with controls, which support further our hypothesis. Although the nitrate reductase activity of xanthine oxidoreductase in muscle is less than that of liver, the residual activity in muscle could be very important in view of its total mass and the high basal level of nitrate. We suggest that skeletal muscle participates in overall NO metabolism, serving as a nitrate reservoir, for direct formation of nitrite and NO, and for determining levels of nitrate in other organs.

1. Introduction

In mammals nitric oxide (NO) is a signaling molecule with a broad array of effects – NO has been implicated in such diverse processes as vascular homeostasis, blood clotting, the immune response and neuronal signaling [1–4]. When oxygen supply in tissue is adequate, NO is produced mostly from enzymatic conversion of arginine to citrulline by the family of nitric oxide synthase (NOS) enzymes. In conditions of reduced oxygen supply, reduction of nitrite either by the 5-coordinated ferrous heme of deoxyhemoglobin or other ferrous hemoproteins and molybdopterin-containing enzymes in several tissues provide additional NO [5–9]. Nitrite is supplied either by oxidation of NO excess in blood or by reduction of nitrate via molybdopterin-containing mammalian nitrate reductases, such as xanthine oxidoreductase (XOR) or aldehyde oxidase (AO) [10–16] or various salivary bacteria nitrate reductases [17,18]. Nitrate is a final end product of either NO or nitrite oxidation by oxyheme proteins, such as oxyhemoglobin or oxymyoglobin, and also enters the body from diverse dietary sources. For details of the current knowledge of the NO cycle, see extensive review by van Faassen et al. [3].

Generally, only blood and the internal organs, especially the heart and liver, have been considered as active sites where the nitric oxide metabolites cycle [7,9,12,19,20]. Smooth muscle was recognized early as a target organ for NO effects, but very little has been presented about the possibility of skeletal muscle playing an important role in the NO cycle or its function. However, Murad et al. found in 1993 that neuronal NOS (nNOS or NOS1) is expressed in skeletal muscle tissue in significant quantities [21]. In fact, it was later shown that skeletal muscle contains two different active nNOS splice variants, nNOSμ – an essential member of the dystrophin-associated protein complex in sarcolemma, and nNOSβ – located in the Golgi complex [22]. The exact roles of both NOS proteins in myocytes are still under debate. Interestingly, progression of dystrophin loss in some types of muscular dystrophies (especially Duchenne and

Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal NOS (NOS1); eNOS, endothelial NOS (NOS3); XOR, xanthine oxidoreductase; AO, aldehyde oxidase.

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Becker) is associated with increasing disruption of dystrophin-associated protein complex and dissociation of nNOSμ into the cytoplasm [23]. It is worth noting that sarcopenia – process of muscle mass loss with aging – has been recently connected with NOS deficiency in mice [24].

Also in recent years it has also been recognized that NO plays an important role in skeletal muscle function. It is produced in muscle tissue even at rest and during contraction its production substantially increases [25]. When NO released from isolated rat muscle into a bathing solution was measured, there was ~60 pmol of NO/mg at resting conditions and the value almost tripled to ~150 pmol of NO/mg during electrical stimulation [25]. When L-NMMA, a general NOS inhibitor was added, NO release was suppressed to about half of resting value and addition of arginine or SNP (an NO donor) doubled the released NO compared to resting conditions. Those values show that NOS activity of skeletal muscle is important, even at the resting conditions, which would likely make the skeletal muscle, as the largest organ in the mammalian body, the main site of production of NO and its metabolites. NO participates both in the regulation of resting and exercise-induced blood flow, possibly contributing to active functional hyperemia, and it is also involved in skeletal muscle metabolism by increasing glucose uptake into cells – for review on NO effects in skeletal muscle see Ref. 26. Presumably, due to its action on mitochondrial respiratory chain complex IV, NO also improves muscle endurance capabilities and muscle repair – for more details on the variety of known NO functions in muscle cells see Refs. 27–29.

With increasing knowledge about NO metabolism pathways, greater attention has been given to sources of NO such as nitrite and, especially recently, nitrate. Both ions had been quantified using chemiluminescence in most of the internal organs [7,9,12,19,20], but we are not aware of any similar measurements done in skeletal muscle.

We hypothesized that skeletal muscle is an important factor in NO homeostasis, due to co-localization of the functional nNOS isoforms and oxymyoglobin in the same compartment. The existence of active nNOS suggests the possibility of *in situ* formation of significant quantities of NO, while close proximity of myoglobin, mostly in its oxymyoglobin form, would lead to quick deactivation of excess NO and formation of nitrate. The presence of xanthine oxidoreductase, with its known nitrate reductase activity, may further enhance the importance of skeletal muscle as one of the possible major players in NO homeostasis. In this study we have found that rodent leg skeletal muscle contains unusually high concentrations of nitrate. We also measured the nitrate reductase activity of rat muscle xanthine oxidoreductase and found nitrate reduction, albeit at low levels, even at pH 7.4. Taken together, these findings support the hypothesis that skeletal muscle is an active and important compartment in the NO cycle.

2. Materials and methods

Adult Wistar male rats (n = 6, weight 250 ± 50 g, Charles River Laboratories, Wilmington, MA), adult $nNOS^{-/-}$ mice (n = 5, background C57BL/6, The Jackson Laboratory, USA) and adult wild type (n = 10, C57BL/6J, NCI Frederick, USA) 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-third of total blood volume for an animal of this size. Approximately 1 ml of blood was collected from the mouse. Heparin was used as an anticoagulant in nitrite and nitrate determinations. Immediately after its draw, blood was mixed with "stop" solution containing potassium ferricyanide, NEM and detergent in final ratio 2:1 as described in Ref. 30 in order to conserve nitrite from degradation by hemoglobin. Samples from liver and skeletal muscle from hind leg were collected shortly after the blood was draw and placed into 250 μl of stop solution for chemiluminescence analysis or flash frozen on dry ice for the Western blot and nitrate or nitrite reductase assays. All samples were stored at –80 °C until analysis. Animals were housed in a 12-hour light/dark cycle environment with access to food and drinking water ad libitum. All animal procedures were carried out according to the recommendations in the Guide for the Care and Use of Laboratory Animals of NIH under NIDDK Animal Care and Use Committee approved protocol.

Standard chemiluminescence nitrite and nitrate assays in mouse and rat tissues were carried out according to previously published procedures [30,31]. Tissue samples were weighed, mixed with additional stop solution and homogenized using GentleMacs (Miltenyi Biotec Inc, Auburn, CA). Proteins were precipitated using methanol (dilution 1:3 sample:methanol) and samples were centrifuged at 11,000 *g* for 5 min at 4 °C to separate most of the protein. Supernatants were collected and used to determine nitrite/nitrate content by chemiluminescence (Sievers 280i Nitric Oxide Analyzer, GE Analytical Instruments, USA).

Nitrate reductase assays in rat liver and skeletal muscle tissues were performed according to the recently published procedures [14]. Due to insufficient amount of tissue samples available from mouse, we were restricted to rat tissues in these experiments. Briefly, tissue was homogenized using GentleMacs tissue dissociator (Miltenyi Biotech, Auburn, CA, USA), total protein in homogenate was determined using the bicinchoninic acid (BCA) assay kit (Pierce Rockford, IL) and adjusted as necessary to 7 mg/ml. Then either 100 μM, 300 μM or 500 μM nitrate together with cofactor mix for nitrate reductases (AO/XOR) was added and aliquots were taken at 0 min, 30 min, 1 h, 2 h, 3 h, 4 h and 24 h and analyzed by chemiluminescence for nitrite content. Cofactor mix consisted of 1 mM NADPH (Fluka), 2 mM UDP glucuronic acid (UDPGA), 0.5 mM glutathione (GSH), 0.5 mM NAD+ and NADH (all from Sigma) in 100 mM phosphate buffer pH 7.4 [14]. Experiments were performed at 37 °C and samples were kept at 2% oxygen. Functional hyperemia in skeletal muscle occurs during intense exercise when oxygen availability in muscle tissue is decreased. Resulting reduced oxygen concentration depends on exercise intensity and duration. We choose 2% oxygen to perform the in vitro experiment, because we hypothesized that it could be close to the reduced oxygen tensions muscle may be subjected to during exercise. The experiment in liver homogenate was performed at the same oxygen levels for comparable conditions for XOR and AO.

To investigate if the observed nitrate reduction in rat liver is a result of action of two known nitrate reductases, xanthine oxidoreductase (XOR) and aldehyde oxidase (AO), we used oxypurinol and raloxifene, inhibitors of XOR and AO, respectively. Inhibitors were added at time 0 to the tissue homogenate together with nitrate at a concentration of 300 μM and the nitrate reduction assay proceeded as described earlier. We used 100 μM oxypurinol and 50 nM raloxifene.

Rat liver and skeletal muscle homogenate were prepared by GentleMacs tissue dissociator with RIPA buffer (Sigma, Cat.# R0278) containing protease inhibitor cocktail (Sigma, Cat.# S8830), and then protein concentration was determined by BCA assay. Denatured samples (50 μg) were run on SDS–PAGE and then transferred to nitrocellulose membrane. The membrane was incubated with primary antibodies (Anti-XOR: Santa Cruz Biotechnology, sc-20991; Anti-AO: Santa Cruz Biotechnology, sc-98500; Anti-GAPDH:Sigma-Aldrich, G9545) overnight at 4 °C, then immunoblotted with anti-rabbit fluorescent antibody (Licor Biosciences, 926-32211) for 1 hour at 4 °C. The blots were imaged using the Odyssey imaging system (Licor Biosciences).

Statistical significance of results was tested using one way ANOVA test.

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