Original Article

MOLECULAR METABOLISM

Nicotinamide riboside kinases display redundancy in mediating nicotinamide mononucleotide and nicotinamide riboside metabolism in skeletal muscle cells

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ABSTRACT

Objective: Augmenting nicotinamide adenine dinucleotide (NAD^+) availability may protect skeletal muscle from age-related metabolic decline. Dietary supplementation of NAD^+ precursors nicotinamide mononucleotide (NMN) and nicotinamide riboside (NR) appear efficacious in elevating muscle NAD^+. Here we sought to identify the pathways skeletal muscle cells utilize to synthesize NAD^+ from NMN and NR and provide insight into mechanisms of muscle metabolic homeostasis.

Methods: We exploited expression profiling of muscle NAD^+ biosynthetic pathways, single and double nicotinamide riboside kinase 1/2 (NRK1/2) loss-of-function mice, and pharmacological inhibition of muscle NAD^+ recycling to evaluate NMN and NR utilization.

Results: Skeletal muscle cells primarily rely on nicotinamide phosphoribosyltransferase (NAMPT), NRK1, and NRK2 for salvage biosynthesis of NAD^+. NAMPT inhibition depletes muscle NAD^+ availability and can be rescued by NR and NMN as the preferred precursors for elevating muscle cell NAD^+ in a pathway that depends on NRK1 and NRK2. Nrk2 knockout mice develop normally and show subtle alterations to their NAD+ metabolome and expression of related genes. NRK1, NRK2, and double KO myotubes revealed redundancy in the NRK dependent metabolism of NR to NAD^+. Significantly, these models revealed that NMN supplementation is also dependent upon NRK activity to enhance NAD^+ availability.

Conclusions: These results identify skeletal muscle cells as requiring NAMPT to maintain NAD^+ availability and reveal that NRK1 and 2 display overlapping function in salvage of exogenous NR and NMN to augment intracellular NAD^+ availability.

Keywords Skeletal muscle; NAD^+; Energy metabolism; Nicotinamide riboside

1. INTRODUCTION

Nicotinamide adenine dinucleotide (NAD^+) was first described as a vital cofactor in cellular redox reactions important to cellular energy metabolism [1,2]. NAD^+ also serves as a consumed substrate for enzymes such as sirtuins that post translationally modify proteins by deacetylation, yielding nicotinamide (NAM) and 2′-and 3-O-acetyl-ADP ribose in the process [3]. Sirtuins have been characterized as regulatory sensors that coordinate metabolic and transcriptional adaptations to cellular and tissue energy requirements [4,5].

Skeletal muscle requires a high turnover of ATP to sustain contraction, facilitated by glycolysis and oxidative phosphorylation, which depend on the redox functions of NAD^+ [6]. Because of the activity of NAD^+ consuming enzymes, replenishment of NAD^+ through biosynthesis and salvage pathways is vital [7,8]. NAD^+ can be synthesized de novo from tryptophan and by salvage of nicotinic acid (NA), a form of vitamin B3, via the Preiss-Handler pathway [9,10]. Along with NA, nicotinamide (NAM) is also called vitamin B3 (collectively termed niacin) and as a nutrient or by recycling following NAD^+ consumption, is metabolized by nicotinamide phosphoribosyltransferase (NAMPT) to nicotinamide mononucleotide (NMN), which is converted to NAD^+ via NMN adenylyltransferases (NMNAT) [11]. A final route to NAD^+ is the salvage and phosphorylation of the recently discovered form of vitamin B3 nicotinamide riboside (NR) to NMN, through the nicotinamide riboside

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kinase 1 and 2 (NRK1 and 2) pathway [2,12,13]. Despite mammalian cells demonstrating pathway diversity for maintaining NAD^+ levels in various tissues, the relative contribution of these pathways to NAD^+ biosynthesis in skeletal muscle remained unclear. The metabolic benefits of augmenting muscle NAD^+ availability is being realized through the application of NAD^+ precursor supplementation strategies. Historically, NA and NAM supplementation has been used to treat hypercholesterolemia and pellagra [14]. However, undesirable side effects in humans have limited their utility. NA causes flushing through activation of the GPR109A receptor in a large proportion of patients, leading to poor compliance [15]. Large doses of NAM are required to increase NAD^+ leading to adverse effects and NAM mediated inhibition of sirtuin activity [16,17]. The NA derivative Acipimox has shown the potential of using NAD^+ precursors to improve and enhance mitochondrial function and ATP content of skeletal muscle in the context of diabetes [15].

NMN and NR have emerged as NAD^+ precursors with the potential to circumvent the adverse side effects associated with high dose niacin and augment NAD^+ synthesis and sirtuin activity [18]. NMN, being an intermediate of NAD^+ biosynthesis, has been used to successfully ameliorate a number of pathological scenarios, including normalization of glucose tolerance in diet induced diabetes [19], and is able to restore mitochondrial function in aged muscle [20,21]. Dietary supplementation of NR in mice can negate the metabolic consequences of high fat diet and increase oxidative performance [22], delay disease progression in mice with mitochondrial myopathy, inducing sirtuin dependent mitochondrial biogenesis and the mitochondrial unfolded protein response [23]. In addition, NR improves glycemic control and opposes development of diabetic neuropathy in mice and allows rats to resist development of chemotherapeutic neuropathy [24,25]. Importantly, NR has been shown to safely elevate human NAD^+ metabolism [26].

NAD^+ precursor utilization pathways in muscle require further definition. Naturally available NR is phosphorylated to NMN by the NR kinases (NRKs encoded by *Nmrk1* and *2*) [12,27], highly conserved enzymes, but little is known of their roles in skeletal muscle [12]. It is unclear whether NMN is truly available to muscle as an intermediate of NAD^+ biosynthesis or is dependent on NRKs as a consequence of extracellular metabolism to NR prior to cellular uptake and incorporation into the cellular NAD^+ pool [28]. Here we investigate NRK expression in skeletal muscle and define the influence of NRKs on NR and NMN metabolism to NAD^+ in loss-of-function muscle cells derived from *Nmrk*1 and 2 knockout mice (NRK1KO, NRK2KO). We show that the NRKs have overlapping and redundant activity in muscle cells critical to the conversion of exogenous NR and NMN to NAD^+.

2. MATERIALS AND METHODS

Unless otherwise specified all materials and reagents were acquired from Sigma–Aldrich, UK.

2.1. Animal care

Mice were group housed in a standard temperature (22 °C) and humidity-controlled environment with 12:12- hour light:dark cycle. Nesting material was provided and mice had ad libitum access to water and standard chow. Mice were sacrificed using schedule one cervical dislocation and tissues were immediately. All experiments were in groups of up to 6 and conducted within the UK Home office regulations.

2.2. Generation of NRK loss of function mice

NRK2KO mice were acquired from the Jackson Laboratory. The *Nmrk2* KO mutant allele was generated on a C57BL/6NTac background through the Knockout Mouse Phenotyping Program (KOMP2). A ZEN-UB1 Velocigene cassette (beta-galactosidase coding sequence from E. coli lacZ gene; polyadenylation signal; loxP site; promoter from the human ubiquitin C gene; neomycin phosphotransferase; polyadenylation signal; loxP site) was inserted through homologous recombination into the gene in place of all coding exons inhibiting transcription. Deletion of *Nmrk2* was validated by qPCR and immunoblotting.

NRK1KO mice generated on a C57BL/6NTac background have been previously described [28].

2.3. Exercise and fibre-typing

As a preliminary experiment, mice ($n = 3$) were acclimatized to the treadmill environment and exercised 3 times a week for 1 h at 0.25 M/Sec, at a 10° incline for 6 weeks.

Muscle sections were fiber typed using immunofluorescence following an established protocol [29–31]. Briefly, 10 μm sections were cut using a cryostat and mounted onto slides. Primary antibodies detecting different myosin heavy chain subunits (BA-F8 – MHC1 (1:50), BF-F3 – MHC IIb (1:100), SC-71 (1:600) – MHC IIa, 6H1 – MHC IIx (1:50)) (Developmental Studies Hybridoma Bank, University of Iowa) were added, followed by fluorescent secondary antibodies (IgG AF 647– Blue (1:500), IgM AF 555 – Red (1:500), IgG AF 488 – Green (1:500)). Sections were formalin fixed and mounted and then analyzed using a Zeiss Axio Observer inverted microscope (Carl Zeiss, Germany). Fibers were manually counted across the entire section using Image J (Fiji) software and recorded as positive for each fiber expressing the relevant visible color.

2.4. RNA extraction and qPCR

RNA was extracted from tissue and cells using TRI-reagent (Invitrogen). RNA quality was determined by visualization on a 1.5% agarose gel and quantified using a nanodrop. Reverse transcription was conducted using 500 ng RNA that was incubated with 250 μM random hexamers, 5.5 mM $MgCl_2$, 500 μM dNTPs, 20 units RNase inhibitor 63 units multiscribe reverse transcriptase, and 1× reaction buffer. Reverse transcription was performed using a thermocycler set at the following conditions: 25 °C for 10 min and 37 °C for 120 min before the reaction was terminated by heating to 85 °C for 5 min qPCR was performed in a 384-well plate in single-plex format. Primers and probes were purchased as Assay on Demand (FAM) products (Applied Biosystems). Total reaction volumes used were 10 μl containing Taqman Universal PCR mix (Applied Biosystems). All Ct values were normalized to 18s rRNA (VIC) (Applied Biosystems). The real-time PCR reaction was performed under the following protocol: 95 °C for 10 min, then 40 cycles of 95 °C for 15 s, and 60 °C for 1 min using an ABI7500 system. Data were collected as Ct values and used to obtain deltaCt (dCt) values.

2.5. Western blotting

Protein lysates were extracted from tissues in RIPA buffer (50 mmol/l Tris pH 7.4, 1% NP40, 0.25% sodium deoxycholate, 150 mmol/l NaCl, 1 mmol/l EDTA) and protease/phosphatase inhibitor cocktail (Roche, Lewes, U.K.). Total protein concentration was quantified by Bio-Rad assay. Total proteins (25 μg) were resolved on a 12% SDS-PAGE gel and transferred onto a nitrocellulose membrane. Primary antibodies specific for NRK1/2 were generated and affinity purified by BioGenes (GmbH) Berlin, Germany and used at a 1:2000 dilution. Primary antibodies including NAMPT (Abcam, USA), β-Actin (Cell Signaling, USA, #12262) and α-Tubulin (Santa Cruz, USA, SC-5286) were all commercially available and used at a 1:1000 dilution.

