Nicotinamide Riboside supplementation does not alter whole-body or skeletal muscle metabolic responses to a single bout of endurance exercise

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Short Title: Post exercise responses to NR supplementation

Keywords: Skeletal Muscle, NAD^+, Exercise, Metabolism

Abstract

Oral supplementation of the NAD^+ precursor Nicotinamide Riboside (NR) has been reported to increase Sirtuin (SIRT) signalling, mitochondrial biogenesis and endurance capacity in rodent skeletal muscle. However, whether NR supplementation can elicit a similar response in human skeletal muscle is unclear. This study aimed to assess the effect of 7-day NR supplementation on exercise-induced transduction and transcriptional responses in skeletal muscle of young, healthy, recreationally active human volunteers. In a double-blinded, randomised, counter-balanced, crossover design, eight male participants (age: 23 ± 4 years, VO_2peak: 46.5 ± 4.4 $mL \cdot kg^{-1} \cdot min^{-1}$) received one week of NR or cellulose placebo (PLA) supplementation (1000 $mg \cdot d^{-1}$) before performing one hour of cycling at 60% Wmax. Muscle biopsies were collected prior to supplementation and pre-, immediately and three-hours post-exercise from the medial vastus lateralis, whilst venous blood samples were collected throughout the trial. Global acetylation, auto-PARylation of PARP1, acetylation of $p53^{Lys382}$ and $MnSOD^{Lys122}$ were unaffected by NR supplementation or exercise. Exercise led to an increase in $AMPK^{Thr172}$ (1.6-fold), and ACC^{Ser79} (4-fold) phosphorylation, in addition to an increase in PGC-1α (~5-fold) and PDK4 (~10-fold) mRNA expression, however NR had no additional effect on this response. There was also no effect of NR supplementation on substrate utilisation at rest or during exercise or on skeletal muscle mitochondrial respiration. Finally, NR supplementation blunted the exercise induced activation of skeletal muscle NNMT mRNA expression, but had no effect on mRNA expression of NMRK1, NAMPT or NMNAT1, which were not significantly affected by NR supplementation or exercise. In summary, one week of NR supplementation does not augment skeletal muscle signal transduction pathways implicated in mitochondrial adaptation to endurance exercise.

Introduction

Nicotinamide adenine dinucleotide (NAD^+), including its reduced form NADH, is a redox co-enzyme that shuttles hydride ions between processes of fuel oxidation, as well as within biosynthethic pathways [1]. In addition to central roles in these critical metabolic processes, NAD^+ has emerged as a signalling moiety and an obligatory co-substrate for sirtuins (SIRTs), poly ADP-ribose polymerases (PARPs) and cyclic ADP-ribose synthetases [2]. Thus NAD^+ is an important substrate in pathways governing metabolic adaptations, DNA repair and apoptosis [1, 2]. Given the regulatory role of NAD^+ in lifespan extending processes, it is unsurprising that strategies to elevate cellular NAD^+ are considered as promising therapies. For example, elevating cellular NAD^+ *in vivo* leads to positive outcomes in murine models of diabetes [3, 4], ageing [3, 5, 6], obesity [7], vascular dysfunction [5], muscular dystrophy [8] and Alzheimer's disease [9].

The vitamin B3 molecule nicotinamide riboside (NR) has emerged as one dietary strategy to elevate NAD^+ *in vivo*. In rodents, oral NR supplementation increases fat oxidation (at least during the light, inactive phase) [7], promotes metabolic flexibility [10], improves insulin sensitivity and may improve endurance performance [7], although a trend towards impaired endurance performance has also been noted [11]. Mechanistically, NR supplementation increases SIRT1 and SIRT3 activity, deacetylation of peroxisome proliferator-activated receptor-γ coactivator 1-α (PGC-1α) and induces mitochondrial biogenesis [7, 8, 12, 13]. Interestingly, and somewhat unsurprisingly, the effects of NR supplementation are much more pronounced during models of elevated cellular stress [7, 8, 12-14].

Studies investigating NR supplementation in humans are in their infancy [6, 15-22]. Importantly, NR displays excellent safety and oral bioavailability in humans [6, 16, 17, 22], with NR supplementation reported to improve blood pressure [6], liver health [16] and physical function in the elderly [16], although the latter is not a consistent finding [6]. However, despite promising evidence from pre-clinical models [7, 10], several studies have reported no effect of chronic NR supplementation on mitochondrial volume, mitochondrial respiration, insulin sensitivity, body composition, cardiac function, lipolysis, VO_2peak and resting or exercising substrate utilisation [6, 17-21], although one study has found increases in relative fat free mass and sleeping metabolic rate [21]. The effect of NR on mitochondrial biogenic signalling in skeletal muscle following exercise remains unstudied.

Therefore, the purpose of this study was to investigate the effects of oral NR supplementation on whole body substrate utilisation and skeletal muscle mitochondrial biogenic signalling at rest and following acute steady-state exercise in humans. It was hypothesised that NR supplementation would increase whole body fat oxidation during exercise and augment SIRT1, SIRT3 and PGC-1α signalling in the post-exercise period compared to placebo.

Materials and Methods

Participants

Eight recreationally active males (mean ± SD: age, 23 ± 4 years; body mass, 72.4 ± 5.3 kg; peak oxygen uptake (VO_2peak), 46.5 ± 4.4 $mL \cdot kg^{-1} \cdot min^{-1}$; maximal aerobic power (Wmax), 224 ±= 29 W) were recruited to participate. Participants were fully informed of the study procedures and their right to withdraw before providing written consent to participate. The study was pre-approved by the National Health Service Research Ethics Committee, Black Country, West

96 Midlands, UK (17/WM/0321) and was conducted in accordance with the Declaration of
97 Helsinki.
98
99 *Experimental overview.*
100 Participants attended the laboratory on five occasions. Prior to the experimental periods,
101 participants attended the laboratory for a pre-testing visit to determine VO_2peak and Wmax.
102 The experimental period then consisted of two identical experimental blocks in which
103 participants visited the laboratory before and after a seven-day supplementation period.
104 During the supplementation period participants received either 1000 $mg \cdot d^{-1}$ nicotinamide
105 riboside (NR; Niagen, ChromaDex, Irvine CA, USA) or 1000 $mg \cdot d^{-1}$ of a cellulose placebo (PLA)
106 in a double-blinded, randomised, counter-balanced, crossover design. Supplements were
107 consumed twice daily such that participants were instructed to consume 500 mg of
108 supplement at ~9 am and ~9 pm each day. A two-week washout period was employed
109 between experimental blocks. After measuring height (Seca 220, Seca, Birmingham, UK) and
110 body mass (Champ II, OHAUS, Griefensee, Switzerland) participants performed a graded
111 exercise test to exhaustion on a cycle ergometer (Lode Excalibur, Groningen, Netherlands).
112 The test began at 50 W with power increasing by 25 W every three minutes thereafter.
113 Respiratory variables were measured continuously during exercise using a breath-by-breath
114 metabolic cart (Vyntus CPX, Jaeger, CareFusion, Germany), heart rate was monitored
115 throughout (RCX5, Polar Electro Oy, Kempele, Finland) and ratings of perceived exertion (RPE)
116 were determined using a 6-20 Borg scale during the final 15 seconds of each 3- minute stage
117 [23]. VO_2peak was determined as the highest rolling 30-second average of VO_2. Wmax was
118 determined as work rate at the last completed stage plus the fraction of time spent in the
119 final non-completed stage multiplied by the increment in work rate (25W).

120

121 *Experimental trials.*

122 Participants refrained from alcohol for 72 h, caffeine for 24 h and exercise for 48 h prior to
123 each experimental trial. For 72 h prior to each experimental trial participants consumed a
124 replicated diet. For the first 48 h of this period participants consumed a diet that replicated
125 their ad libitum intake recorded via a weighed food diary prior to the first experimental visit.
126 For the final 24 h prior to each experimental visit participants were provided with a
127 standardised fixed energy intake diet (energy: 2271 kcal; macronutrient composition: 63%
128 carbohydrate, 21% fat and 16% protein). For the pre-supplementation visit, participants
129 arrived at the laboratory at ~8:30am following an ~12-hour overnight fast. Upon arrival,
130 participants rested in the supine position for approximately five minutes before a venous
131 blood sample was collected via venepuncture from an antecubital forearm vein. A resting
132 skeletal muscle biopsy was then taken from the medial vastus lateralis. Participants then
133 consumed the first 500 mg dose of their supplement prior to leaving the laboratory. For the
134 post-supplementation visit, participants arrived at the laboratory at ~7:30 am following an
135 ~12-hour overnight fast. Participants rested in the supine position for ten minutes prior to a
136 20-minute measurement of resting metabolic rate under a ventilated hood using the
137 GEMNutrition indirect calorimeter (GEMNutrition, Daresbury, UK). A cannula was then
138 inserted into an antecubital forearm vein and a baseline venous blood sample was collected
139 prior to providing a pre-exercise skeletal muscle biopsy from the medial vastus lateralis.

140

141 Participants then cycled for one-hour at 60% Wmax before a second skeletal muscle biopsy
142 was taken immediately post-exercise (completed within two minutes of exercise cessation)
143 after which they rested in a supine position prior to a third skeletal muscle biopsy obtained

144 three-hours post-exercise. A new incision was made for each biopsy at least 2 cm proximal
145 from the previous site. Venous blood was collected throughout rest periods and during
146 exercise. Respiratory variables were measured pre-exercise and at 15-minute intervals
147 throughout exercise, heart rate was monitored continuously throughout exercise and RPE
148 was determined at 15-minute intervals throughout exercise. Carbohydrate and fat oxidation
149 were calculated from VO_2 and VCO_2 using the moderate-high exercise intensities equation of
150 Jeukendrup and Wallis [24] during exercise and Frayn [25] at rest. Participants were allowed
151 to drink water ad libitum during rest and exercise periods during the visit following the first
152 supplementation period, which was matched during the second experimental trial.
153
154 *Muscle biopsies.*
155 Muscle biopsies were obtained from separate incision sites on the medial vastus lateralis
156 under local anaesthesia (1% lidocaine; B. Braun, Melsungen, Germany) by a Bergström needle
157 adapted with suction. Muscle was rapidly blotted to remove excess blood and was
158 immediately flash frozen in liquid nitrogen. In the case of pre-supplementation and pre
159 exercise biopsies, an ~20mg section was removed prior to freezing and placed in ice-cold
160 BIOPS buffer (2.77 mM CaK2EGTA, 7.23 mM K2EGTA, 5.77 mM Na2ATP, 6.56 mM MgCl2, 20
161 mM taurine, 15 mM Na2Phosphocreatine, 20 mM imidazole, 0.5 mM DTT, and 50 mM MES)
162 for the immediate measurement of mitochondrial respiration. Frozen muscle was powdered
163 using a Cellcrusher tissue pulverizer on dry ice and stored at -80°C prior to analysis.
164
165 *High-resolution respirometry.*
166 Skeletal muscle fibres were mechanically separated under a light microscope and
167 permeabilised by incubation in BIOPS buffer containing 50 $mg \cdot ml^{-1}$ of saponin for 30 minutes

followed by a 15-minute wash in MiR05 buffer (0.5 mM EGTA, 3 mM MgCl2.6H2O, 60 mM K lactobionate, 20 mM taurine, 10 mM KH2PO4, 20 mM HEPES, 110 mM sucrose, and 1 g.L-1 fatty acid-free bovine serum albumin). Samples were then weighed and analysed in duplicate using an Oroboros O2K (Oroboros Instruments, Innsbruck, Austria). When substantial variability was apparent between duplicates a third sample was run. Data was collected at 37°C in hyperoxygenated (200-400 μM) conditions in MiR05 buffer. The substrate-uncoupler inhibitor titration performed was as follows: 5 mM pyruvate, 2 mM malate, and 10 mM glutamate was added to measure leak respiration through complex one (CIL); 5 mM ADP was then added to measure coupled oxidative phosphorylation through complex one (CIP); 10 mM succinate was then added to measure coupled oxidative phosphorylation through complexes one and two (CI+IIP); 10 μM cytochrome-c was added to test outer mitochondrial membrane integrity; titrations of 0.5 μM FCCP until maximal respiration were then added to measure maximal electron transport chain capacity (CI+IIE); 5 μM antimycin A was then added to measure nonmitochondrial respiration. Respiration was normalised to tissue masses and non-mitochondrial respiration was subtracted to give mass-specific mitochondrial respiration. In all samples the increase in respiration following addition of cytochrome-c was less than 10%, indicating preserved mitochondrial membrane integrity.

Immunoblotting.

Tissue was homogenized in a 10-fold mass excess of ice-cold sucrose lysis buffer (50 mM tris, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM Na4P2O7-10H_2O, 270 mM sucrose, 1 M triton-X, 25 mM β-glycerophosphate, 1 μM trichostatin A, 10 mM nicatinamide, 1mM 1,4 dithiothreitol, 1% phosphatase inhibitor Cocktail 2; Sigma, 1% phosphatase inhibitor cocktail 2; Sigma, 4.8% cOmplete mini protease inhibitor cocktail; Roche) using an IKA T10 basic

ULTRA-TURRAX homogeniser (IKA, Oxford, UK) followed by shaking at 4°C for 30 minutes and centrifuging at 4°C and 8000 g for 10 minutes to remove insoluble material. Protein concentrations were determined by the DC protein assay (Bio-Rad, Hercules, California, USA). Samples were prepared in laemmli sample buffer, boiled at 97°C for 5 min (with the exception of an aliquot set aside for determination of electron transport chain protein content which remained unboiled) and an equal volume of protein (18-36 µg) was separated by SDS-PAGE on 8 - 12.5% gels at a constant current of 23 mA per gel. Proteins were transferred on to BioTrace NT nitrocellulose membranes (Pall Life Sciences, Pensacola, Florida, USA) via wet transfer at 100 V for one hour. Membranes were then stained with Ponceau S (Sigma-Aldrich, Gillingham, UK) and imaged to check for even loading. Membranes were blocked in 3% dry-milk in tris-buffered saline with tween (TBST) for one hour before being incubated in primary antibody overnight at 4°C. Membranes were washed in TBST three times prior to incubation in appropriate horse radish peroxidase (HRP)-conjugated secondary antibody at room temperature for one hour. Membranes were then washed in TBST three times prior to antibody detection via enhanced chemiluminescence HRP substrate detection kit (Millipore, Watford, UK). Imaging and band quantification were undertaken using a G:Box Chemi-XR5 (Syngene, Cambridge, UK).

Antibodies.

All primary antibodies were used at a concentration of 1:1000 in TBST unless otherwise stated. Pan-acetylation (ab193), ac-MnSOD^{K122} (ab214675) and OXPHOS cocktail (ab110411) were purchased from abcam; AMP-activated protein kinase alpha (AMPKα; 2603), p-AMPK^{Thr172} (2535), p-ACC^{Ser79} (3661), calmodulin dependent kinase II (CAMKII; 3362), p-CAMKII^{Thr268} (12716), cAMP response element binding protein (CREB; 1°: 1:500; 9197), p-

CREB^{Ser133} (1°: 1:500; 9191), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:5000; 2118), p38 mitogen activated protein kinase (p38 MAPK; 9212), p-p38 MAPK$^{Thr180/Tyr182}$ (4511), poly ADP-ribose polymerase 1 (PARP1; 1°: 1:500; 9542), tumour protein 53 (p53; 2°: 1:2000; 2527) and acp53^{K382} (1°: 1:500 in 3% BSA, 2°: 1:2000; 2570) were purchased from Cell Signaling Technology; acetyl CoA carboxylase (ACC; 05-1098), superoxide dismutase (MnSOD; 1°: 1:2000; 06-984), PGC-1α (ab3242) and poly-ADPribose (PAR; 1°: 1:500; MABE1031) were purchased from Merck Millipore. Secondary antibodies were used at a concentration of 1:10000 in TBST unless otherwise stated. Anti-rabbit (7074) and anti-mouse (7076) antibodies were from Cell Signaling Technology.

Real time RT-qPCR.

RNA was extracted from ~20 mg of muscle by homogenising in 1 mL of Tri reagent (Sigma Aldrich, Gillingham, UK) using an IKA T10 basic ULTRATURRAX homogeniser (IKA, Oxford, UK). Phase separation was achieved by addition of 200 µL of chloroform and centrifugation at 12000 g for 15 minutes. The RNA-containing supernatant was removed and mixed with an equal volume of 2-propanol. RNA was purified on Reliaprep spin columns (Promega, Madison, Wisconsin, USA) using the manufacturer's instructions, which includes a DNase treatment step. RNA concentrations were determined using the LVis function of the FLUOstar Omega microplate reader (BMG Labtech, Aylesbury, UK). RNA was diluted to 30 ng·µL^{-1} and reverse transcribed to cDNA in 20 µL volumes using the nanoScript 2 RT kit and oligo(dT) primers (Primerdesign, Southampton, UK) as per the manufacturer's instructions. RT-qPCR analysis of mRNA content was performed in triplicate by using Primerdesign custom designed primers (Table 1) and commercially available ACTB, B2M GAPDH, (Primerdesign) and Precision plus qPCR Mastermix with low ROX and SYBR (Primerdesign) on a QuantStudio3 Real-Time PCR

System (Applied Biosystems, Thermo Fisher, UK). The qPCR reaction was run as per the manufacturer's instructions (Primerdesign) and followed by a melt curve (Applied Biosystems) to ascertain specificity. 2-20 ng of cDNA was added to each well in a 20 µL reaction volume. qPCR results were analysed using Experiment Manager (Thermo Fisher). mRNA expression is expressed relative to the expression in the pre-exercise sample during FED for each individual using the 2-ΔΔCQ method [26] with the geometric mean of Cq values for ACTB, B2M and GAPDH used as an internal control [27]. Optimal stability of housekeeper genes was determined using RefFinder [28]. Statistical analyses were performed on log-transformed ΔΔCQ values.

Blood analyses.

Blood samples were collected into tubes containing ethylenediaminetetraacetic acid (EDTA; BD, Oxford, UK) for the collection of plasma. Samples were placed immediately upon ice prior to centrifugation at 1600 g at 4°C for 10 minutes before collection of plasma from the supernatant. Plasma was frozen at -80°C until further analysis. Plasma samples were subsequently analysed on an autoanalyser (iLAB650, Instrumentation Laboratory, Bedford, MA, USA) for glucose, lactate, non-esterified fatty acid (NEFA) and glycerol (Randox Laboratories, County Antrim, UK) using commercially available kits.

Statistics.

Two-way repeated measures ANOVAs assessed effects of time, treatment and time*treatment interaction effects for all time-course data. Ryan-Holm-Bonferroni multiple comparison corrections were applied post-hoc where applicable. Differences in means for resting and exercising VO_2, VCO_2, respiratory exchange ratio (RER), substrate utilisation, heart

rate and RPE were assessed using repeated-measures t-tests. All statistics were performed using the Statistical Package for the Social Sciences (SPSS) version 22.0. Data are presented as means with 95% confidence intervals. Statistical significance was accepted as $p < 0.05$.

Results

Substrate utilisation and systemic availability.

Seven days of NR supplementation did not influence resting metabolic rate (PLA: 1859 ± 202 vs NR: 1772 ± 211 kcal·d^{-1}; $p = 0.486$). Furthermore, substrate utilisation at rest was similar following supplementation of NR or PLA (carbohydrate oxidation: PLA: 0.09 ± 0.04 vs NR: 0.11 ± 0.03 g·min^{-1}; $p = 0.446$, fat oxidation: PLA: 0.10 ± 0.03 vs NR: 0.09 ± 0.02 g·min^{-1}; $p = 0.395$, RER: PLA: 0.79 ± 0.04 vs NR: 0.80 ± 0.03; $p = 0.563$). Carbohydrate and fat oxidation during exercise were also similar between trials (Table 2). VO_2, VCO_2, RER, heart rate and RPE did not differ between trials during exercise (Table 2). There was no effect of NR on resting or exercising plasma NEFA, glycerol, glucose or lactate (Figure 1). Plasma NEFA concentration initially decreased during the first 15 minutes of exercise before returning to pre-exercise values for the remainder of the exercise bout (main effect of treatment; $p = 0.891$, time; $p < 0.001$, interaction; $p = 0.296$). Following exercise (80 minutes) plasma NEFA concentration increased and remained elevated above pre-exercise values from 120 minutes until the end of the trial (240 minutes). Plasma glycerol concentration increased during exercise and remained elevated above pre-exercise values for the remainder of the trial (main effect of treatment; $p = 0.106$, time; $p < 0.001$, interaction; $p = 0.720$). Plasma glucose was marginally, although significantly, decreased from pre-exercise values at two hours after the cessation of exercise (main effect of treatment; $p = 0.175$, time; $p = 0.010$, interaction; $p = 0.174$). Plasma

lactate increased during exercise and remained elevated for the first 20 minutes of recovery (main effect of treatment; p = 0.192, time; p = 0.001, interaction; p = 0.585).

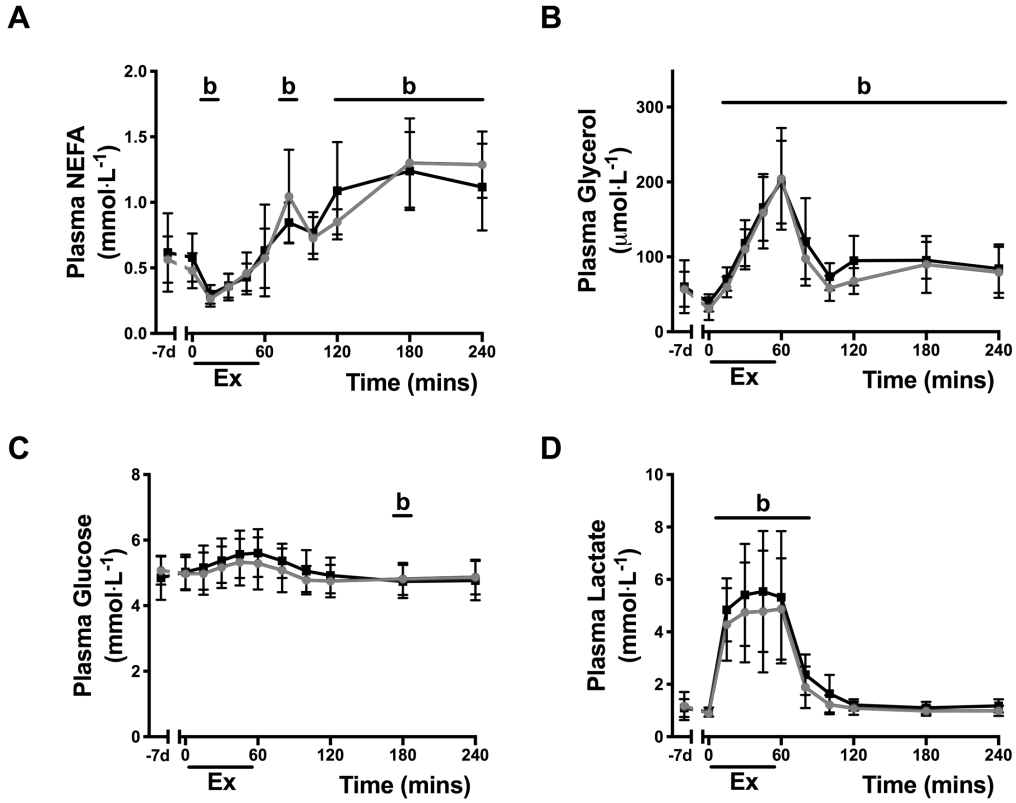
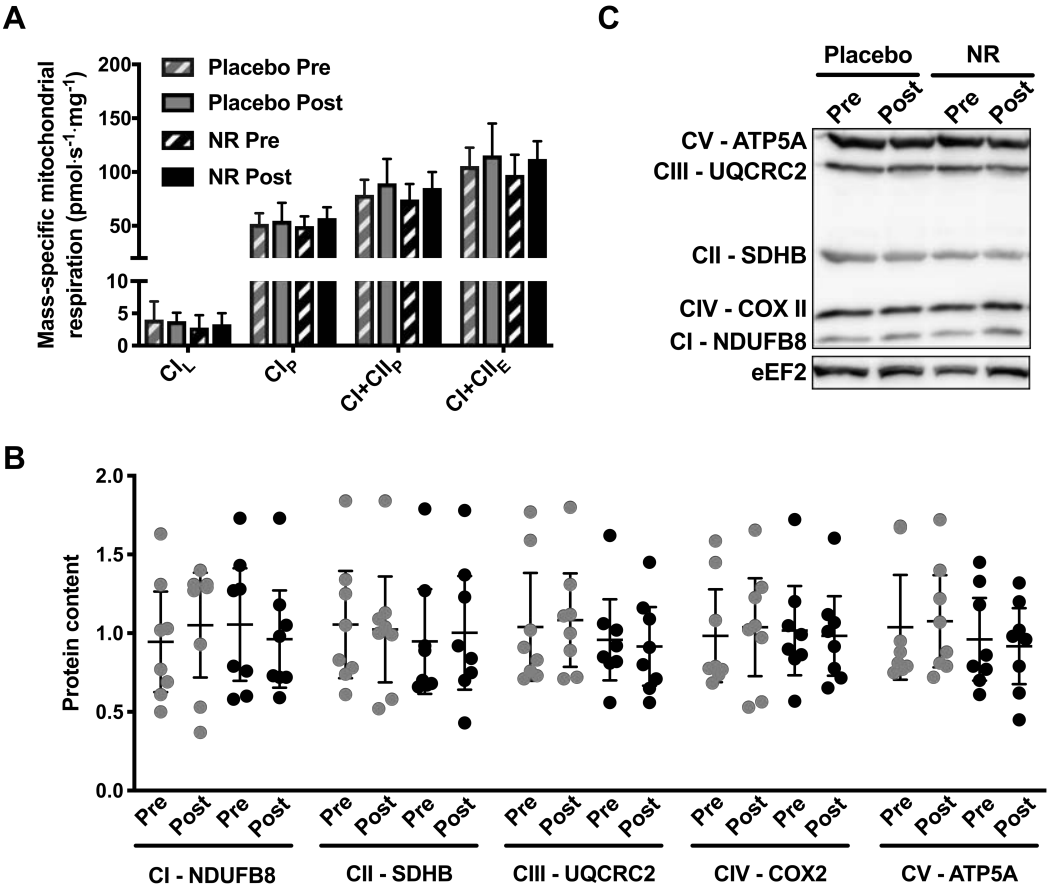


Figure 1. NR supplementation does not alter plasma NEFA, glycerol, glucose or lactate at rest or during exercise. Time-course for plasma NEFA (A.), glycerol (B.), glucose (C.) and lactate (D.) in PLA (black) and NR (grey). b: main effect of time (significantly different to pre-exercise; p ≤ 0.05. Data presented as means ± 95% confidence intervals (n = 8).

Skeletal muscle mitochondrial function and protein content.

Rates of mitochondrial respiration were similar to those previously reported [29, 30]. There were no changes observed in CIL (main effect of treatment; p = 0.319, time; p = 0.833, interaction; p = 0.588), CIP (main effect of treatment; p = 0.979, time; p = 0.388, interaction; p = 0.551), CI+IIP (main effect of treatment; p = 0.612, time; p = 0.216, interaction; p = 0.993) or CI+IIE (main effect of treatment; p = 0.657, time; p = 0.190, interaction; p = 0.621)

301 respiration following supplementation of NR or PLA (Figure 2A). Furthermore, the content of
302 proteins within each of the five electron transport chain complexes were unchanged
303 following NR or PLA supplementation (Figure 2B; $p > 0.05$).



304

305 **Figure 2. Seven days of NR supplementation does not induce mitochondrial biogenesis in**
306 **skeletal muscle.** *A. There were no changes in the mass-specific mitochondrial leak respiration*
307 *through complex I (CIL), coupled respiration through complex I (CIP), coupled respiration*
308 *through complexes I and II (CI+IIP), or maximal electron transport chain capacity (CI+IIE)*
309 *following seven days of NR supplementation ($p > 0.05$). B. Similar content of proteins within*
310 *the five electron transport chain complexes pre- and post-supplementation of PLA (grey) or*
311 *NR (black) ($p > 0.05$). C. Representative immunoblot images. Data presented as means ± 95%*
312 *confidence intervals ($n = 8$).*

313

314 Skeletal muscle signalling.

315 Global acetylation within skeletal muscle was unaffected by NR supplementation or exercise
316 (Figure 3A; main effect of treatment; $p = 0.845$, time; $p = 0.120$, interaction; $p = 0.106$).
317 Furthermore, the acetylation of $p53^{Lys382}$, a SIRT1 deacetylation target [31], and $MnSOD^{K122}$,
318 a SIRT3 deacetylation target [32], were unchanged throughout the intervention (Figure 3C &
319 D; $p53^{Lys382}$: main effect of treatment; $p = 0.723$, time; $p = 0.786$, interaction; $p = 0.354$,
320 $MnSOD^{K122}$: main effect of treatment; $p = 0.324$, time; $p = 0.409$, interaction; $p = 0.332$). The
321 protein content of PARP1 was unaffected by NR supplementation as post-hoc analyses
322 revealed no significant difference despite a significant treatment*time interaction effect
323 (main effect of treatment; $p = 0.498$, time; $p = 0.520$, interaction; $p = 0.040$; Figure 4A). Auto-
324 PARylation of PARP1, a proxy of PARP1 activity [33], was also unchanged by NR or exercise
325 (main effect of treatment; $p = 0.512$, time; $p = 0.255$, interaction; $p = 0.115$; Figure 4B).
326

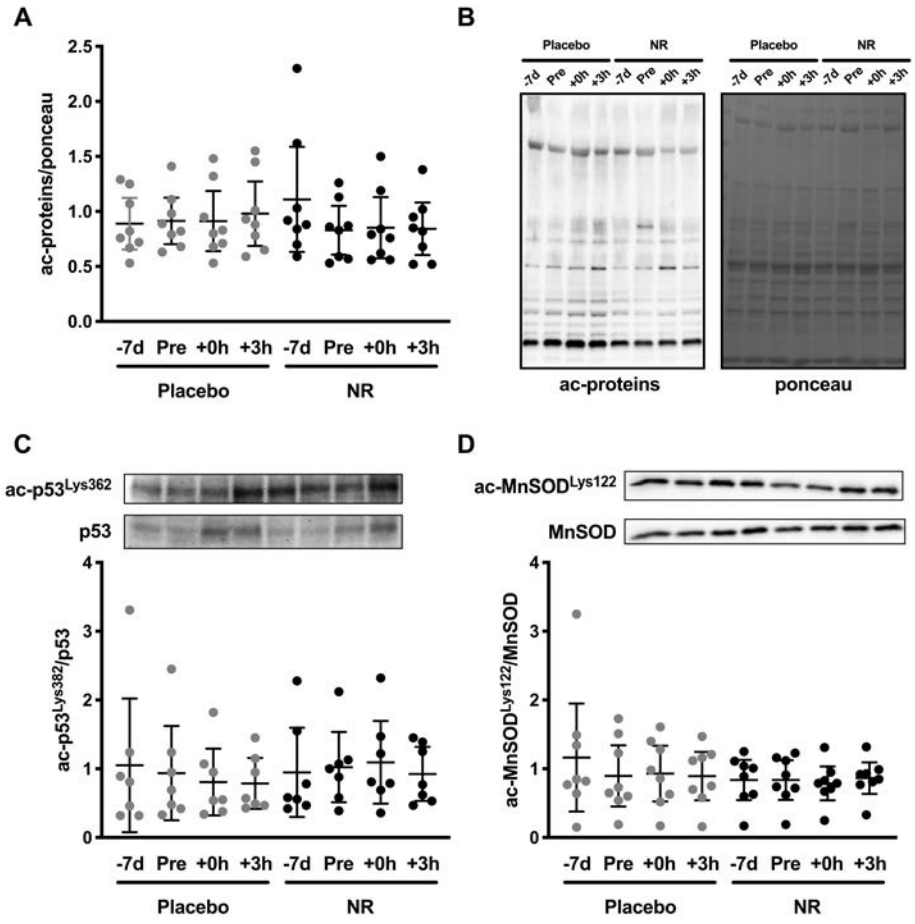


Figure 3. Seven days NR supplementation does not influence Sirtuin deacetylase activity at rest or following endurance exercise. *A. Global acetylation within skeletal muscle is unaffected by NR supplementation or exercise (n = 8; p > 0.05). B. Representative immunoblot images of global acetylation and Ponceau S stain. C. Acetylation of p53^{Lys382}, a SIRT1 deacetylation site, is unchanged by NR supplementation at rest or following endurance exercise (n = 7; p > 0.05). D. Acetylation of MnSOD^{Lys122}, a SIRT3 deacetylation site, is unchanged by NR supplementation at rest or following endurance exercise (n = 8; p > 0.05). -7d: pre-supplementation; Pre: pre-exercise (post-supplementation); +0h: immediately post-exercise; +3h: three hours post-exercise. All values are presented relative to the group mean for all pre-supplementation samples. Data presented as means ± 95% confidence intervals.*

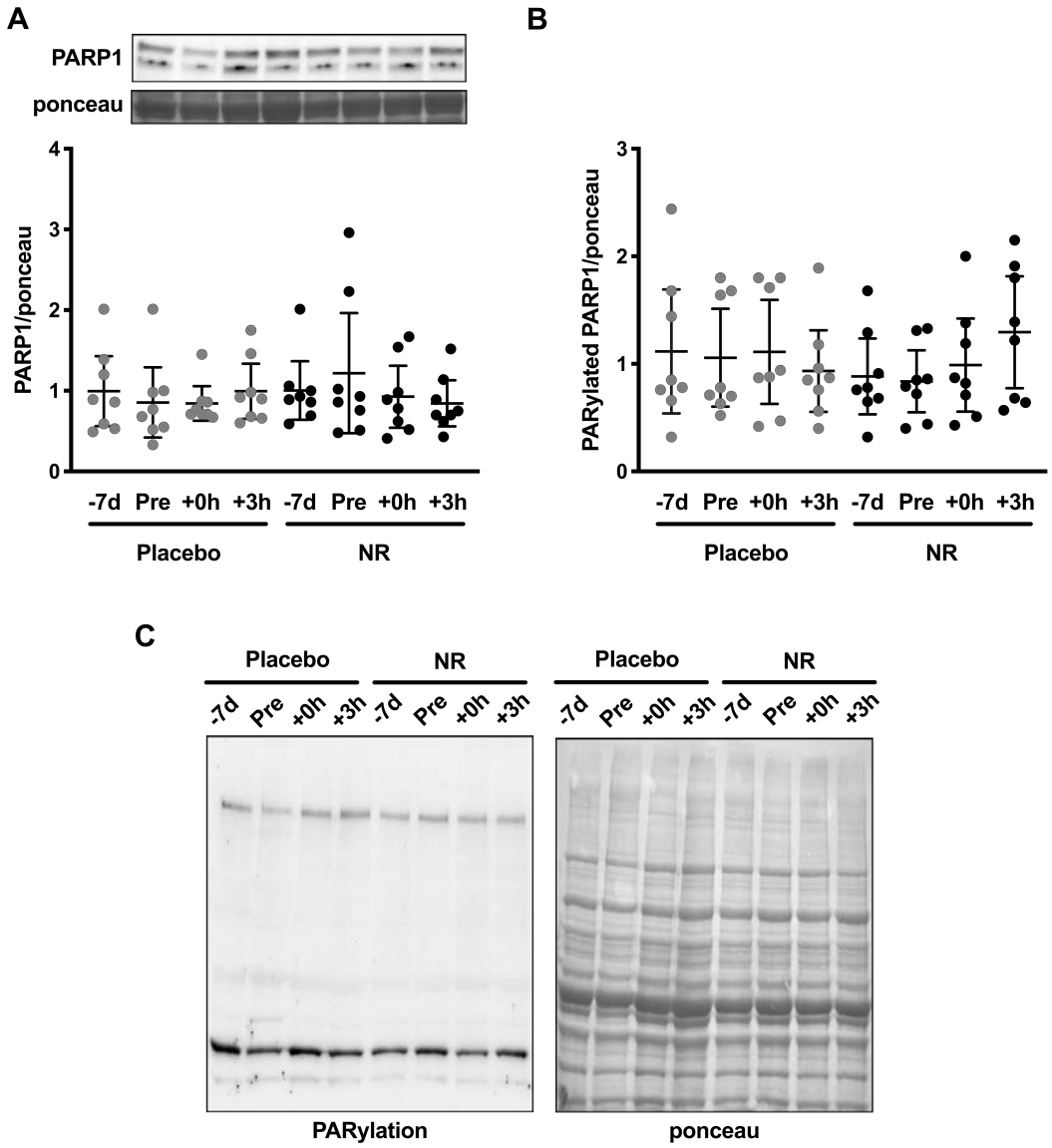


Figure 4. Seven days of NR supplementation does not influence PARP1 protein content or PARylated PARP1 protein content. *PARP1 protein content (A.) and auto-PARylation of PARP1 (B.) are unaffected by NR supplementation or exercise ($p < 0.05$). C. Representative immunoblot images of PARylation and Ponceau S stain. -7d: pre-supplementation; Pre: preexercise; +0h: immediately post-exercise; +3h: three hours post-exercise. All values are presented relative to the group mean for all pre-supplementation samples. Data presented as means ± 95% confidence intervals (n = 8).*

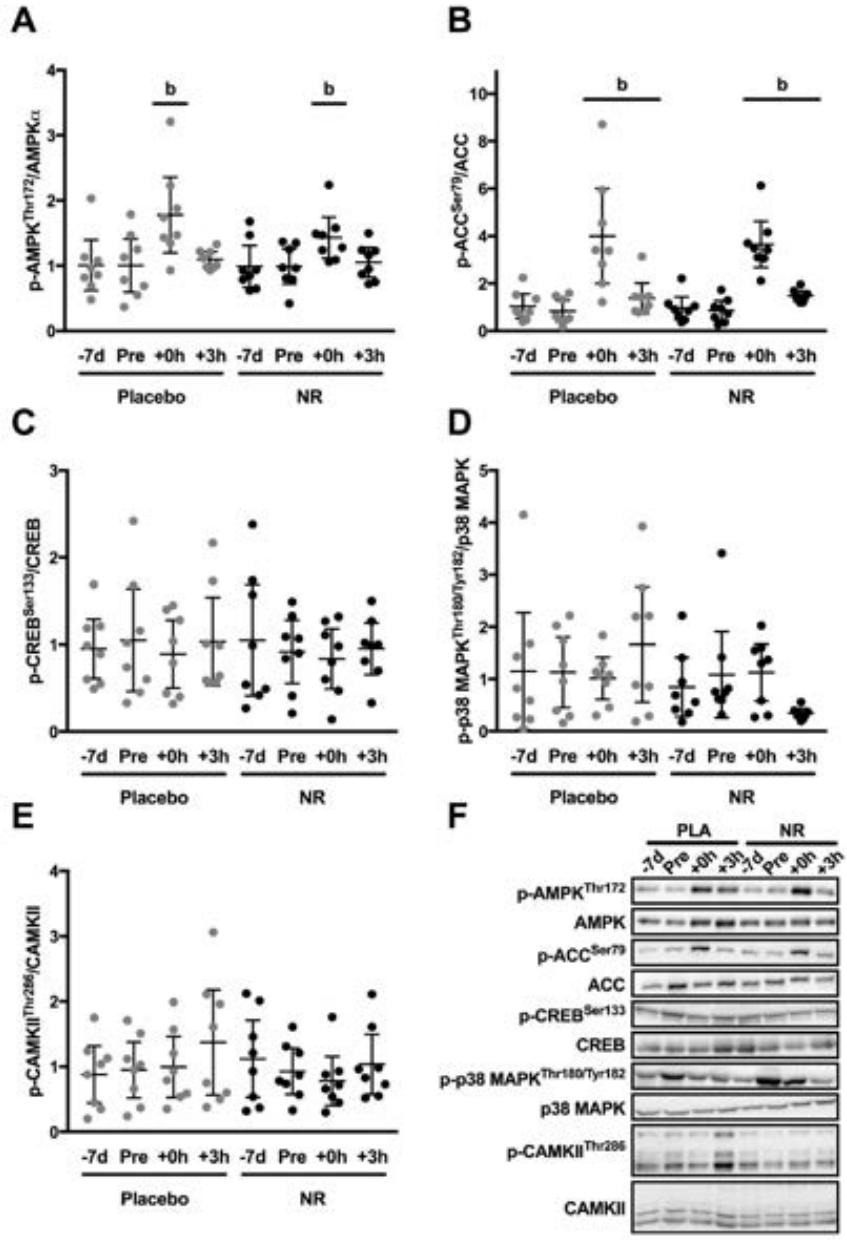


Figure 5. Activation of exercise-sensitive signalling pathways following NR supplementation and endurance exercise. *A. Phosphorylation of AMPK^{Thr172} is increased immediately post exercise in each trial. B. Phosphorylation of ACC^{Ser79} is increased immediately postexercise and remains elevated three hours post-exercise in each trial. C. CREB^{Ser133}, D. p38 MAPK$^{Thr180/Tyr182}$ and E. CAMKII^{Thr286} remain unchanged throughout the intervention. F. Representative immunoblot images. -7d: presupplementation; Pre: pre-exercise (post supplementation); +0h: immediately post-exercise; +3h: three hours post-exercise. b: main effect of time (significantly*

different to pre-exercise; p ≤ 0.05). All values are presented relative to the group mean for all pre-supplementation samples. Data presented as means ± 95% confidence intervals (n = 8).

Exercise increased the phosphorylation of AMPK^{Thr172} (Figure 5A, main effect of time; p = 0.002) by ~1.6-fold immediately post-exercise (p = 0.031 vs pre-exercise). There was no effect of treatment (p = 0.216) or a treatment*time interaction effect (p = 0.472). Phosphorylation of ACC^{Ser79} (Figure 5B) increased ~4-fold immediately post-exercise (p < 0.001 vs pre-exercise) and remained ~1.4-fold elevated 3-h post-exercise (p = 0.013 vs pre-exercise, main effect of time; p < 0.001). CREB^{Ser133} phosphorylation was unaffected by exercise or NR (main effect of treatment; p = 0.651, time; p = 0.462, interaction; p = 0.810; Figure 5C). p38 MAPK$^{Thr180/Tyr182}$ phosphorylation was not significantly affected by exercise or NR (Figure 5D), as post-hoc analyses revealed no significant differences despite a treatment*time interaction effect (main effect of treatment; p = 0.124, time; p = 0.942, interaction; p = 0.034). CAMKII^{Thr286} phosphorylation was not altered by exercise or NR (main effect of treatment; p = 0.574, time; p = 0.177, interaction; p = 0.236; Figure 5E).

<u>Metabolic mRNA response.</u>

Seven days of NR supplementation did not alter resting PPARGC1A mRNA expression in skeletal muscle (Figure 6A). PPARGC1A mRNA increased ~5-fold three hours post-exercise (p = 0.025 vs pre-exercise, main effect of time; p = 0.003). Post-exercise PPARGC1A mRNA expression was similar in PLA and NR trials (main effect of treatment; p = 0.257, interaction; p = 0.591). Expression of pyruvate dehydrogenase kinase 4 (PDK4; Figure 6B) increased post-exercise (main effect of time; p = 0.001) and was ~10-fold elevated three hours post-exercise

380 (p = 0.029 vs pre-exercise). mRNA expression of PDK4 was similar between PLA and NR trials

381 (main effect of treatment; p = 0.827, interaction; p = 0.521).

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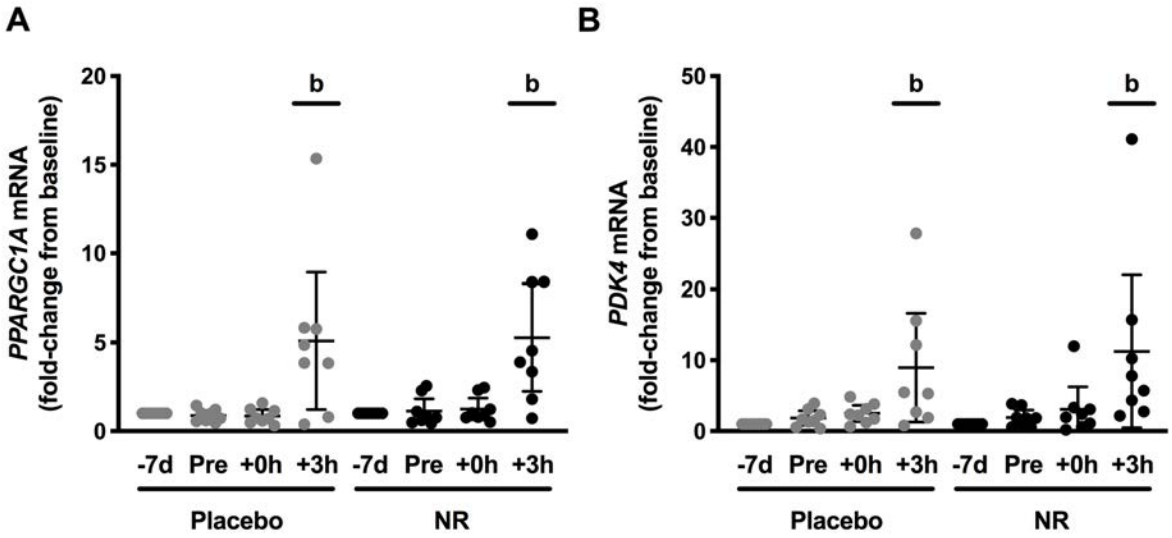

383

384 **Figure 6. Seven days of NR supplementation does not alter resting or exercise-induced PGC-**

385 **1α or PDK4 mRNA expression.** *A. Resting and exercise induced PGC-1α mRNA expression is*

386 *similar between NR and PLA trials. B. Resting and exercise induced PDK4 mRNA expression is*

387 *similar between NR and PLA trials. -7d: pre-supplementation; Pre: pre-exercise (post-*

388 *supplementation); +0h: immediately post-exercise; +3h: three hours post-exercise. b: main*

389 *effect of time (significantly different to pre exercise; p ≤ 0.05). All values are presented relative*

390 *to individual pre supplementation values for each trial and reported as means ± 95%*

391 *confidence intervals (n = 8).*

392

393 mRNA expression of enzymes within the NAD^+ synthesis and salvage pathways.

394 NR supplementation did not alter the mRNA expression of nicotinamide riboside kinase 1
395 (NMRK1; main effect of treatment; p = 0.432) within skeletal muscle (Figure 7A). NMRK1
396 mRNA expression did show a tendency for a main effect of time (p = 0.071). There was no
397 treatment*time interaction effect for NMRK1 mRNA (p = 0.203). mRNA expression of NAMPT,
398 the rate limiting enzyme in NAD^+-salvage (8, 12, 14), was unaffected by NR supplementation
399 or exercise (Figure 7B; main effect of treatment; p = 0.303, time; p = 0.305, interaction; p =
400 0.442). Nicotinamide mononucleotide acetyl transferase 1 (NMNAT1) mRNA expression was
401 not influenced by NR supplementation (Figure 7C), however showed a trend to decrease
402 three hours post-exercise (p = 0.065 vs preexercise, main effect of time: p = 0.046). There was
403 no effect of treatment (p = 0.482) nor a treatment*time interaction effect (p = 0.168).
404 Nicotinamide N-methyltransferase (NNMT) increased in expression three-hours post-exercise
405 (Figure 7D; p = 0.010 vs pre-exercise, main effect of time: p = 0.001). However, the post-
406 exercise mRNA expression of NNMT was suppressed following NR supplementation
407 (treatment*time interaction: p = 0.029), such that the exercise-induced NNMT mRNA
408 expression was only increased in the PLA trial (PLA 3h post-exercise vs PLA pre-exercise: p =
409 0.010), while there was also a trend towards a difference between NR and PLA three hours
410 post-exercise (p = 0.116). There was no main effect of treatment (p = 0.148).
411

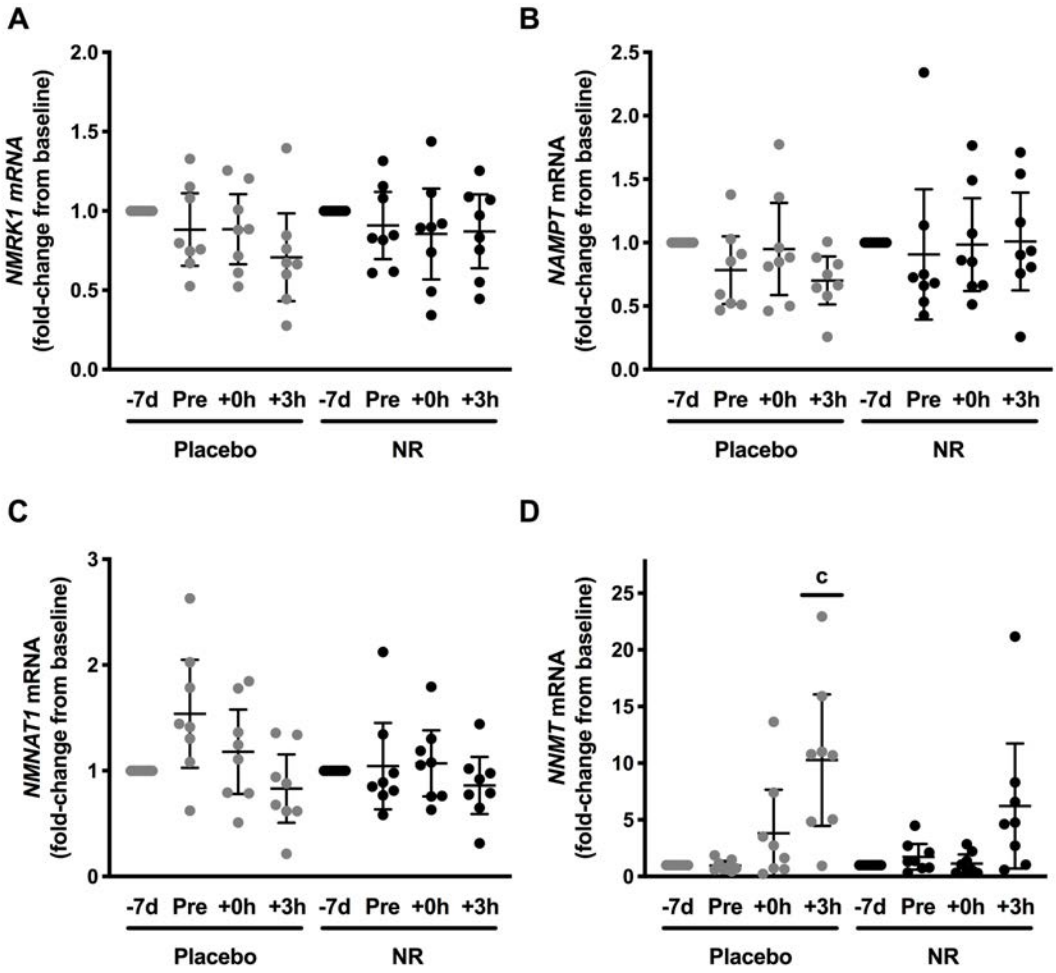

Figure 7. mRNA expression of enzymes in the NAD+ synthesis and salvage pathways within skeletal muscle following NR supplementation and endurance exercise. *mRNA expression of A. NMRK1 and B. NAMPT were unaffected by NR supplementation or endurance exercise. C. NMNAT1 mRNA expression displayed a tendency to decrease three hours post-exercise (p = 0.065). D. mRNA expression of NNMT increased three hours post-exercise in PLA but this was impaired following NR supplementation. -7d: pre-supplementation; Pre: pre-exercise (post supplementation); +0h: immediately post-exercise; +3h: three hours post-exercise. c: interaction effect (different to pre-exercise within Nutrition and post-exercise energy-sensing in skeletal muscle treatment; p ≤ 0.05). All values are presented relative to individual pre supplementation values for each trial and reported as means ± 95% confidence intervals (n = 8).*

Discussion

Contrary to our hypothesis, the activity of the NAD^+-dependent deacetylases SIRT1 and SIRT3 and the mRNA expression of PPARGC1A were unaffected by NR supplementation, both at rest and in the post-exercise recovery period. Furthermore, seven days of NR supplementation (1000 $mg \cdot d^{-1}$) did not alter whole-body metabolism or substrate utilisation. Finally, and somewhat surprisingly, NR impaired the exercise-induced increase of NNMT mRNA expression, an enzyme putatively involved in regulating whole-body fatty acid metabolism [34].

Previous work in rodents supplemented with NR for periods of 4-16 weeks have reported an increase in skeletal muscle NAD^+ content in parallel to increased SIRT1 and SIRT3 activity and mitochondrial biogenesis [7, 8, 12, 13]. However, in the current study, NR supplementation did not alter sirtuin activity or PPARGC1A mRNA expression in human skeletal muscle at rest or following endurance exercise. NAD^+-dependent signalling does appear to be more tightly regulated in human compared to rodent skeletal muscle [16]. For example, endurance exercise increases SIRT1 activity in mice (assessed via p53 deacetylation) [35] but does not appear to produce the same response in humans (Figure 3), possibly indicative of slower NAD^+ turnover in human skeletal muscle or additional levels of regulation [16]. In addition, the protein content and activity of the NAD^+-dependent protein PARP1 was also unchanged by exercise or NR supplementation in the current study. However, this does support rodent data showing that NR supplementation does not alter basal PARylation within skeletal muscle [7, 8]. Therefore, NR supplementation appears less efficient in altering skeletal muscle NAD^+-content in healthy human skeletal muscle [18, 20, 21] when compared to rodent skeletal muscle, where effects of supplementation are more pronounced (~10% increase in NAD^+) [7]

and even more so when NR is provided during metabolic stress (~30% increase in NAD^+) [7]. We have previously shown that 21 days NR supplementation increases MeNAM and NAAD content in old human skeletal muscle indicative of altered NAD^+ flux [20, 21], however did not observe changes in NAD^+ content or mitochondrial respiration [20, 21]. Collectively, therefore, NAD^+ metabolism in human skeletal muscle seems to be more tightly regulated and resistant to precursor supplementation than in rodents.

NR supplementation for one week did not alter circulating substrate availability or whole-body substrate utilisation either at rest or during 60% Wmax cycling in healthy recreationally active males. This is in accordance with recent reports where NR supplementation of 1000 $mg \cdot d^{-1}$ for six weeks or 2000 $mg \cdot d^{-1}$ for 12 weeks had no effect on resting energy expenditure, substrate utilisation or fasting concentrations of glucose or NEFA [6, 17, 20, 21]. Furthermore, six weeks of NR supplementation does not alter RER during an incremental exercise test in elderly males [6]. However, these data are in contrast to rodent studies, which have demonstrated that NR supplementation can increase metabolic flexibility [10] and fat oxidation during the inactive phase [7], which occurs alongside induced mitochondrial biogenesis [7]. Changes in substrate utilisation with NR supplementation may therefore be a physiological outcome of mitochondrial biogenesis. Indeed, in the current study, no changes in skeletal muscle mitochondrial respiration or content of electron transport chain proteins were apparent, which perhaps is unsurprising given the relatively short supplementation period. However, 6 and 12 weeks of NR supplementation also failed to increase skeletal muscle mitochondrial volume or respiratory capacity in humans [18, 20, 21]. In contrast, two weeks of acipimox administration (750 $mg \cdot d^{-1}$), a nicotinic acid-derivative, increased skeletal muscle mitochondrial respiratory capacity in type II diabetes [36], whilst four months of

nicotinic acid supplementation increased mitochondrial mass and cytochrome c oxidase activity in healthy participants and patients with mitochondrial myopathies [37]. It remains unclear why nicotinic acid may be more potent than NR in stimulating mitochondrial biogenesis in human skeletal muscle.

Our data indicates that exercise and NR alter the NAD^+-consumption/salvage machinery within skeletal muscle. The mRNA expression of NNMT, a methyltransferase of nicotinamide (NAM) that produces methylated NAM (MeNAM) and prevents NAD^+-salvage [38], is increased following endurance exercise although this response was impaired by NR supplementation. Previous studies have also shown an increase in skeletal muscle NNMT mRNA and/or protein expression following endurance exercise training in rats [39] and four days of energy restriction in humans [34]. Ström et al [34] went on to demonstrate elevated skeletal muscle NNMT mRNA expression coincided with an increase in circulating MeNAM. In addition, plasma MeNAM concentrations are increased following a single bout of endurance exercise in mice, an effect that could only be partially explained by increased NNMT activity in the liver [40]. MeNAM can be secreted from human primary myotubes and can induce lipolysis in rat primary adipocytes [34]. However, despite elevations in systemic and skeletal muscle MeNAM during NR supplementation in humans [20-22], whole-body fatty acid availability at rest, during exercise and during the post-exercise recovery period are unaffected by NR supplementation. The reduction in exercise-induced skeletal muscle NNMT mRNA expression following NR supplementation is a particularly surprising finding given elevated plasma and skeletal muscle MeNAM concentrations during NR supplementation [20-22]. However, this potentially represents a negative feedback loop preventing additional activation of NNMT in skeletal muscle when MeNAM concentrations are high. The chronic

496 effects of NR on skeletal muscle NNMT content and activity, and whole-body and skeletal
497 muscle fatty acid metabolism, warrant further investigation.
498
499 **Conclusions**
500 NR supplementation (1000 $mg \cdot d^{-1}$) for seven days did not alter substrate metabolism or
501 mitochondrial biogenic signalling in resting or exercised human skeletal muscle. In contrast,
502 NR supplementation did reduce the exercise-induced expression of NNMT mRNA in skeletal
503 muscle, an enzyme proposed to play a putative role in whole-body fatty acid metabolism.
504 Collectively our data would therefore suggest that NAD^+ metabolism is tightly regulated in
505 human skeletal muscle, with short-term NAD^+ precursor supplementation unable to
506 modulate this response at rest or during and in recovery from endurance exercise. Our data
507 therefore adds to a growing list of studies suggesting that NR supplementation does not alter
508 mitochondrial biogenic signalling in healthy human skeletal muscle.
509

Conflict of interests

ChromaDex provided nicotinamide riboside and placebo supplements free of charge under a material transfer agreement with the University of Birmingham. The University of Birmingham did not receive any financial support from ChromaDex for the completion of this trial. The authors declare no other conflicts of interest.

Acknowledgements

The authors would like to thank the participants for their efforts, their time and their tissue. The authors would also like to acknowledge the support of Lauren Homer and Nathan Hodson in the data collection of this study.

Funding

This publication was supported in part through a BBSRC Midlands Integrative Biosciences Training Programme (MIBTP) studentship (BB/J014532/1) to BS and BBSRC New Investigator Award (BB/L023547/1) to AP.

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671

672

673 **Table 1. Gene Accession numbers and corresponding primer sequences.**

674

Gene Name	Accession number	Forward primer (5'-3')	Reverse primer (3'-5')
NAMPT	NM_005746	TTCCCACTACTCCAGCCTAAG	TTTGTGTAAAGGGCAGGTTAATAAA
NMNAT1	NM_022787	AGTCCTTTGCTGTTCCCAATT	AGCACATCCGATTCATAGATAAAC
NMRK1	NM_017881.2	GCCAGAGTCTGAGATAGAGACAG	TCCTGGTCTGTTGATACCACAG
NNMT	NM_006169.2	TGCTGTTAGCCTGAGACTCAG	GAGGTGAAGCCTGATTCCATTATG

675

Table 2. Effect of NR supplementation on cardio-respiratory changes and substrate utilisation during exercise.

		Time (minutes into exercise)					
		15	**30**	**45**	**60**	**Mean**	***p***
Gas exchange							
VO_2 ($L \cdot min^{-1}$)	PLA	2.26 ± 0.25	2.28 ± 0.27	2.35 ± 0.27	2.38 ± 0.28	2.31 ± 0.68	
	NR	2.23 ± 0.24	2.27 ± 0.24	2.28 ± 0.26	2.38 ± 0.23	2.29 ± 0.23	0.702
VCO_2 ($L \cdot min^{-1}$)	PLA	2.11 ± 0.20	2.10 ± 0.24	2.12 ± 0.23	2.13 ± 0.24	2.11 ± 0.22	
	NR	2.09 ± 0.23	2.10 ± 0.23	2.08 ± 0.24	2.17 ± 0.23	2.11 ± 0.23	0.945
Oxidation rates							
Carbohydrate ($g \cdot min^{-1}$)	PLA	2.22 ± 0.26	2.09± 0.36	1.96 ± 0.30	1.93 ± 0.26	2.05 ± 0.28	
	NR	2.20 ± 0.27	2.12 ± 0.28	2.00 ± 0.25	2.08 ± 0.34	2.10 ± 0.22	0.720
Fat ($g \cdot min^{-1}$)	PLA	0.25 ± 0.12	0.30 ± 0.14	0.38 ± 0.14	0.41 ± 0.12	0.33 ± 0.13	
	NR	0.22 ± 0.08	0.27 ± 0.07	0.33 ± 0.07	0.35 ± 0.10	0.29 ± 0.06	0.356
Intensity							
Heart rate ($beats \cdot min^{-1}$)	PLA	153 ± 7	160 ± 7	165 ± 8	169 ± 9	162 ± 7	
	NR	154 ± 10	163 ± 9	168 ± 9	172 ± 9	164 ± 9	0.179
RPE	PLA	11 ± 1	13 ± 2	14 ± 2	15 ± 2	13 ± 1	
	NR	11 ± 1	13 ± 1	14 ± 1	15 ± 2	13 ± 1	0.952

Data presented as meand ± 95% confidence intervals (n = 8). Mean values represent the mean of the recorded values during exercise. *p* values represent reeated-measures t-test comparisons between exercising means for PLA and NR.