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

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Key Points:

- Acute Nicotinamide riboside (NR) supplementation does not alter substrate metabolism at rest, during or in recovery from endurance exercise.
- NR does not alter NAD⁺-sensitive signaling pathways in human skeletal muscle.
- NR supplementation and acute exercise influence the NAD⁺-metabolome.

Abstract

Oral supplementation of the NAD⁺ precursor Nicotinamide Riboside (NR) has been reported to alter metabolism alongside increasing sirtuin (SIRT) signalling and mitochondrial biogenesis in rodent skeletal muscle. However, whether NR supplementation can elicit a similar response in human skeletal muscle is unclear. This study assessed the effect of 7-day NR supplementation on whole-body metabolism and exercise-induced mitochondrial biogenic signalling in skeletal muscle. Eight male participants (age: 23 ± 4 years, $\text{VO}_{2\text{peak}}$: $46.5 \pm 4.4 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) received one week of NR or cellulose placebo (PLA) supplementation ($1000 \text{ mg}\cdot\text{d}^{-1}$). Muscle biopsies were collected from the medial vastus lateralis prior to supplementation and pre-, immediately post- and three-hours post-exercise (one-hour of $60\% W_{\text{max}}$ cycling) performed following the supplementation period. There was no effect of NR supplementation on substrate utilisation at rest or during exercise or on skeletal muscle mitochondrial respiration. Global acetylation, auto-PARylation of PARP1, acetylation of p53^{Lys382} and MnSOD^{Lys122} were also unaffected by NR supplementation or exercise. NR supplementation did not increase skeletal muscle NAD⁺ concentration, however did increase the concentration of deaminated NAD⁺ precursors (NAR and NAMN) and methylated NAM breakdown products (Me2PY and Me4PY), demonstrating the skeletal muscle bioavailability of NR supplementation. In summary, one week of NR supplementation does not alter whole-body metabolism or skeletal muscle signal transduction pathways implicated in the 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 biosynthetic pathways (Ying, 2008). 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 (Belenky *et al.*, 2007). Thus NAD⁺ is an important substrate in pathways governing metabolic adaptations, DNA repair and apoptosis (Belenky *et al.*, 2007; Ying, 2008). 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 (Ramsey *et al.*, 2008; Yoshino *et al.*, 2011), ageing (Ramsey *et al.*, 2008; de Picciotto *et al.*, 2016; Martens *et al.*, 2018), obesity (Canto *et al.*, 2012), vascular dysfunction (de Picciotto *et al.*, 2016), muscular dystrophy (Ryu *et al.*, 2016) and Alzheimer's disease (Long *et al.*, 2015).

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) (Canto *et al.*, 2012), promotes metabolic flexibility (Shi *et al.*, 2017), improves insulin sensitivity and may improve endurance performance (Canto *et al.*, 2012), although a trend towards impaired endurance performance has also been noted (Kourtzidis *et al.*, 2016). Mechanistically, NR supplementation increases SIRT1 and SIRT3 activity, deacetylation of peroxisome proliferator-activated receptor- γ coactivator 1- α (PGC-1 α) and induces mitochondrial biogenesis (Canto *et al.*, 2012; Cerutti *et al.*, 2014; Khan *et al.*, 2014; Ryu *et al.*, 2016). Interestingly, the effects of NR supplementation are much more pronounced during models of elevated cellular stress (Canto *et al.*, 2012; Cerutti *et al.*, 2014; Khan *et al.*, 2014; Frederick *et al.*, 2015; Ryu *et al.*, 2016). Furthermore,

genetically elevating skeletal muscle NAD^+ levels, via overexpression of nicotinamide phosphoribosyltransferase (NAMPT), augments endurance capacity in voluntary wheel running mice but not their sedentary counterparts (Costford *et al.*, 2018). Thus, elevating NAD^+ levels appears more effective during conditions of cellular NAD^+ stress, such as during exercise (White & Schenk, 2012).

Studies investigating NR supplementation in humans are in their infancy (Trammell *et al.*, 2016; Airhart *et al.*, 2017; Dellinger *et al.*, 2017; Dollerup *et al.*, 2018; Martens *et al.*, 2018; Dollerup *et al.*, 2019; Elhassan *et al.*, 2019; Dollerup *et al.*, 2020; Remie *et al.*, 2020). Importantly though, NR displays excellent safety and oral bioavailability in humans (Trammell *et al.*, 2016; Dellinger *et al.*, 2017; Dollerup *et al.*, 2018; Martens *et al.*, 2018; Elhassan *et al.*, 2019). Clinical trials investigating the efficacy of NR supplementation have reported improvements in blood pressure (Martens *et al.*, 2018), liver health (Dellinger *et al.*, 2017) and physical function in the elderly (Dellinger *et al.*, 2017), although the latter is not a consistent finding (Martens *et al.*, 2018). However, despite promising evidence from pre-clinical models (Canto *et al.*, 2012; Shi *et al.*, 2017), several studies have reported no effect of chronic NR supplementation on substrate utilisation, insulin sensitivity, body composition, cardiac function, lipolysis or VO_2peak (Dollerup *et al.*, 2018; Martens *et al.*, 2018; Dollerup *et al.*, 2019; Elhassan *et al.*, 2019; Dollerup *et al.*, 2020; Remie *et al.*, 2020), although one study has found increases in relative fat free mass and sleeping metabolic rate (Remie *et al.*, 2020). Furthermore, chronic NR supplementation does not augment mitochondrial volume or mitochondrial respiratory capacity in humans (Elhassan *et al.*, 2019; Dollerup *et al.*, 2020; Remie *et al.*, 2020). However, as pre-clinical models of genetic or nutritional NAD^+ elevation are more effective during cellular stress, including exercise (Costford *et al.*, 2018), it is likely that the context of NR supplementation is important. Indeed, combining NR supplementation with exercise may be more efficacious than supplementation in the sedentary condition.

Exercise regulates the concentrations of NAD⁺ and NADH in an intensity-dependent manner. Submaximal exercise at 50% VO_{2max} decreases total muscle NADH concentrations (Katz & Sahlin, 1987), while continuous exercise at 75% VO_{2max} did not alter NADH levels (Sahlin *et al.*, 1990). Fluctuations in the ratio of NAD⁺/NADH are also affected by exercise intensity, for example, NADH decreased while the cytosolic NAD⁺/NADH ratio was unaffected during exercise at 40% VO_{2max} but at higher intensities, 75% and 100% VO_{2max}, NADH increased above pre-exercise values with no changes in NAD⁺ concentration (Sahlin, 1985; Sahlin *et al.*, 1987). However, the regulation of NAD⁺ concentration is more complex than just NAD⁺/NADH ratios with biosynthetic and salvage pathways contributing to the maintenance of cellular NAD⁺ (Bogan & Brenner, 2008). The effect of exercise on the wider NAD-metabolome remains unstudied.

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-1 α signalling in the post-exercise period compared to placebo.

Methods

Ethical Approval

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 Midlands, UK (17/WM/0321) and was conducted in accordance with the Declaration of Helsinki.

Experimental overview

Eight recreationally active males (mean $\pm$ SD: age, 23 ± 4 years; body mass, 72.4 ± 5.3 kg; peak oxygen uptake ($\text{VO}_{2\text{peak}}$), 46.5 ± 4.4 mL $\cdot$ kg $^{-1}\cdot$ min $^{-1}$; maximal aerobic power (W_{max}), 224 ± 29 W) were recruited to participate. Participants attended the laboratory on five occasions. Prior to the experimental periods, participants attended the laboratory for a pre-testing visit to determine $\text{VO}_{2\text{peak}}$ and W_{max} . The experimental period then consisted of two identical experimental blocks in which participants visited the laboratory before and after a seven-day supplementation period. During the supplementation period participants received either 1000 mg $\cdot$ d $^{-1}$ nicotinamide riboside (NR; Niagen, ChromaDex, Irvine CA, USA) or 1000 mg $\cdot$ d $^{-1}$ of a cellulose placebo (PLA) in a double-blinded, randomised, counter-balanced, crossover design. This dose has been demonstrated to increase plasma NAD^{+} bioavailability for at least 24 hours (Trammell *et al.*, 2016) and augment the skeletal muscle NAD^{+} -metabolome after three weeks (Elhassan *et al.*, 2019). Supplements were consumed twice daily such that participants were instructed to consume 500 mg of supplement at ~ 9 am and ~ 9 pm each day. A two-week washout period was employed between experimental blocks. After measuring height (Seca 220, Seca, Birmingham, UK) and body mass (Champ II, OHAUS, Griefensee, Switzerland) participants performed a graded exercise test to exhaustion on a cycle ergometer (Lode Excalibur, Groningen, Netherlands). The test began at 50 W with power increasing by 25 W every three minutes thereafter. Respiratory variables were measured continuously during exercise using a breath-by-breath metabolic cart (Vyntus CPX, Jaeger, CareFusion, Germany), heart rate was monitored throughout (RCX5, Polar Electro Oy, Kempele, Finland) and ratings of perceived exertion (RPE) were determined using a 6-20 Borg scale during the final 15 seconds of each 3-minute stage (Borg, 1973). $\text{VO}_{2\text{peak}}$ was determined as the highest rolling 30-second average of VO_2 . W_{max}

was determined as work rate at the last completed stage plus the fraction of time spent in the final non-completed stage multiplied by the increment in work rate (25W).

Experimental trials

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

Participants then cycled for one-hour at 60% W_{max} before a second skeletal muscle biopsy was taken immediately post-exercise (completed within two minutes of exercise cessation) after which

they rested in a supine position prior to a third skeletal muscle biopsy obtained three-hours post-exercise. A new incision was made for each biopsy at least 2 cm proximal from the previous site. Venous blood was collected throughout rest periods and during exercise. Respiratory variables were measured pre-exercise and at 15-minute intervals throughout exercise, heart rate was monitored continuously throughout exercise and RPE was determined at 15-minute intervals throughout exercise. Carbohydrate and fat oxidation were calculated from VO_2 and VCO_2 using the moderate-high exercise intensities equation of Jeukendrup and Wallis (Jeukendrup & Wallis, 2005) during exercise and Frayn (Frayn, 1983) at rest. Participants were allowed to drink water ad libitum during rest and exercise periods during the visit following the first supplementation period, which was matched during the second experimental trial.

Muscle biopsies

Muscle biopsies were obtained from separate incision sites on the medial vastus lateralis under local anaesthesia (1% lidocaine; B. Braun, Melsungen, Germany) by a Bergström needle adapted with suction. Muscle was rapidly blotted to remove excess blood and was immediately flash frozen in liquid nitrogen. In the case of pre-supplementation and pre-exercise biopsies, an ~20mg section was removed prior to freezing and placed in ice-cold BIOPS buffer (2.77 mM CaK2EGTA, 7.23 mM K2EGTA, 5.77 mM Na2ATP, 6.56 mM MgCl₂, 20 mM taurine, 15 mM Na2Phosphocreatine, 20 mM imidazole, 0.5 mM DTT, and 50 mM MES) for the immediate measurement of mitochondrial respiration. Frozen muscle was powdered using a Cellcrusher tissue pulverizer on dry ice and stored at -80°C prior to analysis.

High-resolution respirometry

High-resolution respirometry was performed on resting skeletal muscle biopsies (i.e. pre-supplementation and pre-exercise following the supplementation). Skeletal muscle fibres were mechanically separated under a light microscope and permeabilised by incubation in BIOPS buffer containing 50 $\mu\text{g}\cdot\text{ml}^{-1}$ of saponin for 30 minutes followed by a 15-minute wash in MiR05 buffer (0.5 mM EGTA, 3 mM $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$, 60 mM K lactobionate, 20 mM taurine, 10 mM KH_2PO_4 , 20 mM HEPES, 110 mM sucrose, and 1 $\text{g}\cdot\text{L}^{-1}$ 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 Na₄P₂O₇-10H₂O, 270 mM sucrose, 1 M triton-X, 25 mM β -glycerophosphate, 1 μ M trichostatin A, 10 mM nicotinamide, 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 μ g) 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 (AMPK α ; 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-1 α (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 μ L 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· μ L⁻¹ and reverse transcribed to cDNA in 20 μ L 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 μ L 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 $2^{-\Delta\Delta Cq}$ method (Livak & Schmittgen, 2001) with the geometric mean of Cq values for ACTB, B2M and GAPDH used as an internal control (Vandesompele *et al.*, 2002). Optimal stability of housekeeper genes was determined using RefFinder (Xie *et al.*, 2012). Statistical analyses were performed on log-transformed $\Delta\Delta Cq$ 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.

Metabolites were extracted from skeletal muscle as previously described (Elhassan *et al.*, 2019). Briefly, ~20-25 mg of skeletal muscle was suspended in 200 μ L of ice-cold HPLC grade MeOH, placed on ice and then 300 μ L of ice-cold HPLC grade water was added. Metabolites were extracted by probe sonication in an acetone/water ice bath (-4°C) followed by shaking at 85°C for 5 min at 1050 rpm. Samples were returned to ice for 5 min and centrifuged at top speed for 10 min at 4°C. The supernatant was transferred to clean tubes and dried in a speed-vac at 30°C. The extract was resuspended in 80 μ L of 10 mM ammonium acetate, centrifuged at top speed for 3 min at 4°C and the supernatant transferred into 2 mL glass HPLC vials with inserts. Vials were stored at -20°C until liquid chromatography-mass spectrometry (LC-MS) analysis. Metabolites were separated on an Amide XBridge HPLC column (Waters, Millford, MA; 2.1 mm x 100 mm, 3.5 μ m) maintained at 40°C, connected to a Shimadzu Nexera LC-30AD liquid chromatography (LC) system (Shimadzu, Kyoto, Japan) with a gradient profile consisting of mobile phase A [95:5 (v:v) water:acetonitrile with 20 mM ammonium acetate and 20 mM ammonium hydroxide; pH 9] and mobile phase B as described in Table 2 with a total run time of 25 min. The column was equilibrated for 20 min before the first injection. 5 μ L of each sample was injected in a randomised order. The LC system was coupled to a QTRAP 6500+ tandem mass spectrometer (AB Sciex, Foster City, CA, USA) operating in both positive and negative ion mode using multiple-reaction monitoring (MRM). Peaks were automatically selected using MultiQuant v. 3.0.3 and reviewed to confirm appropriate peak selection while blinded to condition. A non-blind deconvolution script was developed to resolve overlapping NAAD-NAD, NAMN-NAM and NAR-NR using MATLAB's Optimisation Toolbox. Metabolite concentration/mg of wet tissue was determined using external standard curves and corrected for recovery using controls spiked with the same standards used for the curves.

Power calculation

The current study was powered to detect differences in exercising fat oxidation seen during fed versus fasted exercise of a similar intensity (De Bock *et al.*, 2005). After 60 mins of exercise De Bock *et al.* reported fat oxidation rates (mean $\pm$ SD) of 0.52 ± 0.24 and 0.77 ± 0.15 . This equates to a mean difference of 0.25, a pooled SD of 0.20 and, thus, a cohen's d of 1.25. An a priori sample size calculation was performed for repeated-measures t-tests in the G*Power 3.1 software (Faul *et al.*, 2009). At an α -level of 0.05 and a power (1- β) of 0.80, a sample size of 8 is required to detect a significant effect with an effect size of 1.25.

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 $p \leq 0.05$.

Results

Substrate utilisation and systemic metabolite availability.

To investigate the effect of NR supplementation on whole-body metabolism, substrate utilisation and systemic metabolite availability were measured at rest and during steady-state exercise (one

hour at 60% $W_{\max}$). 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 3). VO_2 , VCO_2 , RER, heart rate and RPE did not differ between trials during exercise (Table 3). 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; $p = 0.585$).

Skeletal muscle mitochondrial function and protein content.

Given NR supplementation increases mitochondrial volume, protein content and enzyme activity in rodents (Canto *et al.*, 2012; Cerutti *et al.*, 2014; Khan *et al.*, 2014; Ryu *et al.*, 2016), albeit not in humans (Elhassan *et al.*, 2019; Døllerup *et al.*, 2020; Remie *et al.*, 2020), we were interested in whether seven days of NR supplementation influenced mitochondrial respiration and protein content in human skeletal muscle. 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) respiration following supplementation of NR or PLA (Figure 2A). Furthermore, the content of proteins within each of the five electron transport chain complexes were unchanged following NR or PLA supplementation (Figure 2B; p > 0.05).

Skeletal muscle signalling.

Both NR treatment and exercise activate the deacetylases sirtuin 1 (SIRT1) and 3 (SIRT3) in rodents (Philp *et al.*, 2011; Canto *et al.*, 2012; Cerutti *et al.*, 2014; Khan *et al.*, 2014), thus we studied the influence of NR supplementation and exercise on acetylation in human skeletal muscle. Global acetylation within skeletal muscle was unaffected by NR supplementation or exercise (Figure 3A; main effect of treatment; p = 0.845, time; p = 0.120, interaction; p = 0.106). Furthermore, the acetylation of p53^{Lys382}, a SIRT1 deacetylation target (Vaziri *et al.*, 2001), and MnSOD^{K122}, a SIRT3 deacetylation target (Tao *et al.*, 2010), were unchanged throughout the intervention (Figure 3C & D; p53^{Lys382}: main effect of treatment; p = 0.723, time; p = 0.786, interaction; p = 0.354, MnSOD^{K122}: main effect of treatment; p = 0.324, time; p = 0.409, interaction; p = 0.332). The protein content of PARP1 was unaffected by NR supplementation as post-hoc analyses revealed no significant difference despite a significant treatment*time interaction effect (main effect of treatment; p = 0.498, time; p = 0.520, interaction; p = 0.040; Figure 4A). Auto-PARylation of PARP1, a proxy of PARP1 activity (Tao *et al.*, 2009), was also unchanged by NR or exercise (main effect of treatment; p = 0.512, time; p = 0.255, interaction; p = 0.115; Figure 4B).

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; $p < 0.001$, treatment; $p = 0.697$, interaction; $p = 0.695$). 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).

PPARGC1A & PDK4 mRNA response

As NR treatment and exercise both activate PGC-1 α (Egan *et al.*, 2010; Canto *et al.*, 2012; Stocks *et al.*, 2019) and activation of PGC-1 α can coordinate mitochondrial biogenic processes (Kang & Li Ji, 2012), we studied the resting and post-exercise induction of PPARGC1A mRNA expression. 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 ($p = 0.029$ vs pre-exercise). mRNA expression of PDK4 was similar between PLA and NR trials (main effect of treatment; $p = 0.827$, interaction; $p = 0.521$).

Effect of exercise and NR on the skeletal muscle NAD⁺-metabolome

In order to confirm the bioavailability of NR in skeletal muscle following seven days of supplementation and to study the influence of NR and exercise on NAD metabolism, we measured the NAD-metabolome within skeletal muscle. Skeletal muscle NR concentration did not change following NR treatment or exercise (main effect of treatment; $p = 0.0624$, time; $p = 0.221$, interaction; $p = 0.639$; Figure 7A). Furthermore, neither NR supplementation nor exercise altered NAD⁺ (main effect of treatment; $p = 0.093$, time; $p = 0.440$, interaction; $p = 0.280$; Figure 7B), NAD phosphate (NADP; main effect of treatment; $p = 0.388$, time; $p = 0.153$, interaction; $p = 0.365$; Figure 7C), nicotinamide (NAM; main effect of treatment; $p = 0.265$, time; $p = 0.114$, interaction; $p = 0.382$; Figure 7D) or nicotinamide mononucleotide (NMN; main effect of treatment; $p = 0.603$, time; $p = 0.816$, interaction; $p = 0.712$; Figure 7E) concentrations within skeletal muscle. However, NR supplementation did increase nicotinic acid mononucleotide (NAMN; main effect of treatment; $p = 0.003$, time; $p = 0.05$, interaction; $p = 0.024$; Figure 7F) and nicotinic acid riboside (NAR; main effect of treatment; $p = 0.002$, time; $p = 0.001$, interaction; $p = 0.001$; Figure 7G), as well as the methylated NAM products N-methyl-2-pyridone-5-carboxamide (Me2PY; main effect of treatment; $p = 0.002$, time; $p < 0.001$, interaction; $p < 0.001$; Figure 7I) and N-methyl-4-pyridone-5-carboxamide (main effect of treatment; $p = 0.002$, time; $p = 0.001$, interaction; $p = 0.001$; Figure 7J). Exercise displayed a trend towards increased skeletal muscle concentration of methylated NAM (MeNAM) at 3h post-exercise (vs pre-exercise; $p = 0.063$, main effect of time; $p = 0.007$, Figure 7G), although there was no effect of NR on MeNAM (main effect of treatment; $p = 0.108$, interaction; $p = 0.332$). Exercise also increased skeletal muscle NAR concentration immediately post-exercise (vs pre-exercise; $p = 0.004$, main effect of time; $p = 0.001$), which was apparent in both NR and PLA trials (PLA 0h post-exercise vs PLA pre-exercise: $p = 0.075$, NR 0h post-exercise vs NR pre-exercise: $p = 0.091$; Figure 7G). Neither

NR nor exercise altered the concentration of the NAD breakdown product ADPr (main effect of treatment; $p = 0.232$, time; $p = 0.953$, interaction; $p = 0.861$; Figure 7K).

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

Given that NR and exercise altered the NAD-metabolome, NR and exercise may influence the expression NAD-biosynthesis and salvage enzymes. NR supplementation did not alter the mRNA expression of nicotinamide riboside kinase 1 (NMRK1; main effect of treatment; $p = 0.432$) within skeletal muscle (Figure 8A), however NMRK1 mRNA expression did show a tendency to decrease following exercise (main effect of time; $p = 0.071$). There was no treatment*time interaction effect for NMRK1 mRNA ($p = 0.203$). mRNA expression of NAMPT, the rate limiting enzyme in NAD⁺-salvage (Frederick *et al.*, 2015; Fletcher *et al.*, 2017; Costford *et al.*, 2018), was unaffected by NR supplementation or exercise (Figure 8B; main effect of treatment; $p = 0.303$, time; $p = 0.305$, interaction; $p = 0.442$). Nicotinamide mononucleotide acetyl transferase 1 (NMNAT1) mRNA expression was not influenced by NR supplementation (Figure 8C), however it showed a trend to decrease three hours post-exercise ($p = 0.065$ vs pre-exercise, main effect of time: $p = 0.046$). There was no effect of treatment ($p = 0.482$) nor a treatment*time interaction effect ($p = 0.168$) on NMNAT1 mRNA expression. Nicotinamide N-methyltransferase (NNMT; Figure 8D) mRNA expression displayed a significant treatment*time interaction effect ($p = 0.029$). Post-hoc analyses identified that NNMT mRNA expression only significantly increased post-exercise in the PLA trial (PLA 3h post-exercise vs PLA pre-exercise: $p = 0.010$). There was a trend for an increase in NNMT mRNA expression three hours post-exercise in the NR trial ($p = 0.118$), however there was also a trend towards a difference between NR and PLA three hours post-exercise ($p = 0.116$). This points towards a reduced or delayed exercise-induced NNMT mRNA expression following NR supplementation.

Discussion

Contrary to our hypothesis, seven days of NR supplementation ($1000 \text{ mg}\cdot\text{d}^{-1}$) did not alter whole-body metabolism or substrate utilisation at rest or during exercise. The activity of the NAD^{+} -dependent deacetylases SIRT1 and SIRT3 and the mRNA expression of PPARGC1A were also unaffected by NR supplementation, both at rest and in the post-exercise recovery period. However, NR did augment the skeletal muscle NAD-metabolome, increasing the concentration of NAR and NAMN as well as the methylated products Me2PY and Me4PY, although skeletal muscle NAD^{+} concentration remained unchanged. This lends further support to the tissue bioavailability of oral NR supplementation in humans although NAD^{+} concentrations remain under tight homeostatic regulation.

NR supplementation for one week did not alter circulating substrate availability or whole-body substrate utilisation either at rest or during 60% W_{max} cycling in healthy recreationally active males. This is in accordance with recent reports where NR supplementation of $1000 \text{ mg}\cdot\text{d}^{-1}$ for six weeks or $2000 \text{ mg}\cdot\text{d}^{-1}$ for 12 weeks had no effect on resting energy expenditure, substrate utilisation or fasting concentrations of glucose or NEFA (Dollerup *et al.*, 2018; Martens *et al.*, 2018; Elhassan *et al.*, 2019; Remie *et al.*, 2020). Furthermore, six weeks of NR supplementation does not alter RER during an incremental exercise test in elderly males (Martens *et al.*, 2018). However, these data are in contrast to rodent studies, which have demonstrated that NR supplementation can increase metabolic flexibility (Shi *et al.*, 2017) and fat oxidation during the inactive phase (Canto *et al.*, 2012), which occurs alongside induced mitochondrial biogenesis (Canto *et al.*, 2012). Changes in substrate utilisation with NR supplementation may therefore be a physiological outcome of mitochondrial biogenesis. 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. Indeed, 6 and 12 weeks of NR supplementation also failed

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to increase skeletal muscle mitochondrial volume or respiratory capacity in humans (Elhassan *et al.*, 2019; Dollerup *et al.*, 2020; Remie *et al.*, 2020). Thus, it is clear that oral NR supplementation in humans, at least with current dosing strategies, does not alter whole-body metabolism or skeletal muscle mitochondrial respiration.

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 (Canto *et al.*, 2012; Cerutti *et al.*, 2014; Khan *et al.*, 2014). 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. The discrepancy between rodent and human studies likely arises from the inability of NR supplementation to alter NAD⁺ concentration in human skeletal muscle (Fig. 7B) (Elhassan *et al.*, 2019; Dollerup *et al.*, 2020; Remie *et al.*, 2020), whereas effects of NR supplementation are more pronounced in rodents (~10% increase in NAD⁺) (Canto *et al.*, 2012). NAD⁺-dependent signalling does appear to be more tightly regulated in human compared to rodent skeletal muscle. For example, endurance exercise increases SIRT1 activity in mice (assessed via p53 deacetylation) (Philp *et al.*, 2011) but does not produce the same response in humans (Figure 3). Swimming exercise increases NAD⁺ in mouse skeletal muscle (Cantó *et al.*, 2010), whereas, here, no change in skeletal muscle NAD⁺ was apparent after cycling at 60% W_{max}, nor is NAD⁺ concentration altered in skeletal muscle of humans exercising across a range of intensities (Sahlin, 1985; Henriksson *et al.*, 1986; Sahlin *et al.*, 1987). Collectively, therefore, NAD⁺ metabolism in human skeletal muscle seems to be more tightly regulated and resistant to exercise and precursor supplementation than in rodents.

Despite not altering skeletal muscle NAD^+ -content, oral NR supplementation does alter the NAD^+ -metabolome within human skeletal muscle. Seven days of NR supplementation increased NAR and NAMN concentration, as well as the methylated products of NAM breakdown, Me2PY and Me4PY. The increase in skeletal muscle NAR and NAMN concentration following oral NR supplementation is consistent with elevated deaminated NAD^+ metabolites in plasma and tissues following NR supplementation (Trammell *et al.*, 2016; Elhassan *et al.*, 2019). Furthermore, elevated NAR and NAMN is indicative of microbiome-mediated conversion of NR into deaminated NAD^+ precursors via the bacterial nicotinamidase (PnCA) prior to systemic absorption (Shats *et al.*, 2020). Indeed, the microbiome is essential for the NAD^+ -boosting effects of oral NR treatment in mice (Shats *et al.*, 2020). The increase in methylated products of NAM breakdown, Me2PY and Me4PY, are a well described product of NR supplementation (Trammell *et al.*, 2016; Elhassan *et al.*, 2019) and probably reflects the excretion of excess NAD^+ to maintain NAD^+ homeostasis. This likely prevents the NAD^+ -boosting properties of NR in conditions of NAD^+ sufficiency. Despite this, studies of NR supplementation in human conditions of NAD^+ deficiency, where NR-derived additional NAD^+ may be retained, are warranted.

Endurance exercise increased skeletal muscle NAR in both NR and PLA trials, which could reflect either an increased uptake of NAR during exercise, as skeletal muscle blood flow increases (Saltin *et al.*, 1998), or a decrease in the conversion of NAR to NAMN. Partial support for the latter is our observation that mRNA expression of NMRK1, an isoform of the enzyme responsible to the conversion of NAR to NAMN, showed a trend to decrease post-exercise. Exercise also increased skeletal muscle MeNAM concentrations, however this effect was not altered by NR supplementation. Increased plasma MeNAM following exercise has previously been reported in mice (Chlopicki *et al.*, 2012). Elevated MeNAM concentrations occurred alongside increased mRNA expression of NNMT, a methyltransferase of NAM that produces MeNAM and, thus, prevents NAD^+ -

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salvage (Aksoy *et al.*, 1994). The increase in expression of NNMT mRNA was delayed in the NR trial, possibly reflecting a negative feedback loop due to elevated methylated NAM products (Me2PY and Me4PY) during NR supplementation. The functional significance of elevated MeNAM following exercise remains unclear, however systemic MeNAM has been reported to induce adipose tissue lipolysis (Ström *et al.*, 2018). It may also reflect increased NAD⁺-consumption during exercise leading to NAM excretion, although evidence of increased NAD⁺-consuming processes, e.g. sirtuin and PARP activity, were not apparent in this study.

This study adds to the expanding literature investigating NR supplementation in humans. However, whilst this study was powered to detect meaningful differences in exercising fat oxidation it may be underpowered to detect more subtle differences and those of other parameters. Furthermore, this study investigated the effect of 1000 mg·d⁻¹ of NR and thus cannot rule out effective supplementation with higher doses. Indeed, the lack of translation of rodent findings to human physiology could, in theory, be a result of incomparable dosing. Based on mouse-human dose conversion factors (Reagan-Shaw *et al.*, 2008) the typical dose used in mouse studies (400 mg·kg⁻¹·d⁻¹) is comparable to approximately twice the 1000 mg·d⁻¹ dose used in this and other human studies (Trammell *et al.*, 2016; Elhassan *et al.*, 2019). However, a dose of 2000 mg·d⁻¹ has been supplemented to middle-aged overweight males with no metabolic benefit (Dollerup *et al.*, 2018; Dollerup *et al.*, 2019; Dollerup *et al.*, 2020), whilst the safety of even higher doses in humans remains to be demonstrated.

Conclusions

NR supplementation (1000 mg·d⁻¹) for seven days did not alter total NAD⁺ content, substrate metabolism, mitochondrial respiration or mitochondrial biogenic signalling in resting or exercised

human skeletal muscle. The NAD⁺ metabolome was found to be tightly regulated in skeletal muscle, with NAR and MeNAM the only metabolites shown to be altered in response to exercise. NR supplementation increased the concentration of deaminated NAD⁺ precursors (NAR and NAMN) and methylated NAM breakdown products (Me2PY and Me4PY), demonstrating skeletal muscle bioavailability of NR supplementation in young, healthy male participants. Collectively, our data suggests that NAD⁺ metabolism is tightly regulated in human skeletal muscle and is not perturbed by a single bout of moderate intensity exercise. Our data adds to a growing list of studies suggesting that NR supplementation does not alter NAD⁺ metabolism in healthy human skeletal muscle.

Declaration of interest

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.

Author contributions

Ben Stocks: Conceptualization, Methodology, Formal analysis, Investigation, Data Curation, Writing – Original draft, Writing - Review & Editing, Visualization, Project administration, Funding acquisition.

Stephen P Ashcroft: Methodology, Investigation, Writing - Review & Editing. **Sophie Joannis:** Methodology, Investigation, Writing - Review & Editing. **Linda C. Dansereau:** Investigation, Writing - Review & Editing. **Yen Chin Koay:** Investigation, Writing - Review & Editing. **Yasir S. Elhassan:** Investigation, Writing - Review & Editing. **Gareth G. Lavery:** Resources, Writing - Review & Editing. **Lake-Ee Quek:** Investigation, Writing - Review & Editing. **John F. O'Sullivan:** Investigation, Writing - Review & Editing. **Ashleigh M. Philp:** Investigation, Writing - Review & Editing. **Gareth A. Wallis:**

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Investigation, Writing - Review & Editing, Supervision. **Andrew Philp**: Conceptualization, Methodology, Writing – Original draft, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

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Data Availability Statement

The NAD⁺ metabolome data presented in Figure 7 is openly available on the Cloudstor (www.aarnet.edu.au) platform (<https://cloudstor.aarnet.edu.au/plus/s/NnyXbbc12c1Mk9d>), study reference number (NRAex_Processed_Data).

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Table 1. Gene Accession numbers and corresponding primer sequences.

Gene Name	Accession number	Forward primer (5'-3')	Reverse primer (3'-5')
NAMPT	NM_005746	TTCCCACTACTCCAGCCTAAG	TTTGTGTAAAGGGCAGGTTAA TAAA
NMNAT1	NM_022787	AGTCCTTTGCTGTTCCCAATT	AGCACATCCGATTCATAGATA AAC
NMRK1	NM_017881.2	GCCAGAGTCTGAGATAGAGAC AG	TCCTGGTCTGTTGATACCACAG
NNMT	NM_006169.2	TGCTGTTAGCCTGAGACTCAG	GAGGTGAAGCCTGATTCCATT ATG

Table 2. NAD⁺-metabolome HPLC gradient

Step	Cumulative time (min)	Flow rate (uL/min)	%A	%B
0	0:00	200	15	85
1	2:00	200	15	85
2	10:00	200	30	70
3	13:00	200	85	15
4	17:00	200	85	15
5	17:50	200	15	85
6	19:00	200	15	85
7	22:00	400	15	85
8	24:00	400	15	85
9	24.50	250	15	85
10	25:00	Stop		

Table 3. Effect of NR supplementation on cardio-respiratory changes and substrate utilisation during exercise.

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

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

Figure Headings.

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 and NR. b: main effect of time (significantly different to pre-exercise; $p \leq 0.05$). Data are presented as means $\pm$ 95% confidence intervals ($n = 8$).

Figure 2. Seven days of NR supplementation does not alter mitochondrial respiration or protein content in skeletal muscle. A. High-resolution respirometry was performed pre- and post-supplementation in resting skeletal muscle ($p > 0.05$). B. Protein content of electron transport chain subunits were measured via immunoblotting ($p > 0.05$). C. Representative immunoblot images. Data are presented as means $\pm$ 95% confidence intervals ($n = 8$).

Figure 3. Seven days of NR supplementation does not influence sirtuin deacetylase activity at rest or following endurance exercise. Immunoblotting assessed total acetylation of skeletal muscle (A. & B.; $n = 8$) and site-specific acetylation of $p53^{\text{Lys382}}$ (C. $n = 7$) and $MnSOD^{\text{K122}}$ (D. $n = 8$). 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 are presented as means $\pm$ 95% confidence intervals.

Figure 4. Seven days of NR supplementation does not influence PARP1 protein content or PARylated PARP1 protein content. Skeletal muscle PARP1 protein content (A.) and auto-PARylation of PARP1 (B.) were assessed by immunoblotting. C. Representative immunoblot images of PARylation and Ponceau S stain. -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 are presented as means $\pm$ 95% confidence intervals ($n = 8$).

Figure 5. Activation of exercise-sensitive signalling pathways following NR supplementation and endurance exercise. The phosphorylation of AMPK^{Thr172} (A.), ACC^{Ser79} (B.), CREB^{Ser133} (C.), p38 MAPK^{Thr180/Tyr182} (D.) and CAMKII^{Thr286} (E.) were assessed throughout the intervention using immunoblotting. F. Representative immunoblot images. -7d: pre-supplementation; Pre: pre-exercise (post-supplementation); +0h: immediately post-exercise; +3h: three hours post-exercise. b: main effect of time (significantly different to pre-exercise; $p \leq 0.05$). All values are presented relative to the group mean for all pre-supplementation samples. Data are presented as means $\pm$ 95% confidence intervals ($n = 8$).

Figure 6. Seven days of NR supplementation does not alter resting or exercise-induced PGC-1 α or PDK4 mRNA expression. PPARGC1A (A.) and PDK4 (B.) mRNA expression were assessed using RT-qPCR. -7d: pre-supplementation; Pre: pre-exercise (post-supplementation); +0h: immediately post-exercise; +3h: three hours post-exercise. b: main effect of time (significantly different to pre-exercise; $p \leq 0.05$). All values are presented relative to individual pre-supplementation values for each trial. Data are presented as means $\pm$ 95% confidence intervals ($n = 8$).

Figure 7. NR supplementation and exercise influence the NAD⁺-metabolome. The concentration of NAD⁺ metabolites within skeletal muscle (A. – K.) were assessed by mass spectrometry. L. Schematic of the NAD⁺ synthesis and salvage pathways influenced by NR. -7d: pre-supplementation; Pre: pre-exercise (post-supplementation); +0h: immediately post-exercise; +3h: three hours post-exercise. c: interaction effect (significantly different between treatments within time point; $p \leq 0.05$). Data are presented as means $\pm$ 95% confidence intervals ($n = 8$).

Figure 8. mRNA expression of enzymes in the NAD⁺ synthesis and salvage pathways within skeletal muscle following NR supplementation and endurance exercise. mRNA expression of *NMRK1* (A.), *NAMPT* (B.), *NMNAT1* (C.) and *NNMT* (D.) were assessed using RT-qPCR. -7d: pre-supplementation; Pre: pre-exercise (post supplementation); +0h: immediately post-exercise; +3h: three hours post-exercise. d: interaction effect (significantly different to pre-exercise within treatment; $p \leq 0.05$). All values are presented relative to individual pre-supplementation values for each trial. Data are presented as means $\pm$ 95% confidence intervals (n = 8).

Author Profile.

This work formed a part of Ben Stocks' PhD thesis performed within the School of Sport, Exercise and Rehabilitation Sciences at the University of Birmingham. Ben has since moved on, becoming a postdoctoral researcher at the Center for Basic Metabolic Research at the University of Copenhagen, where he predominantly implements proteomics in the study of metabolism and disease. Whilst much of his current research focuses on the circadian control of metabolism, Ben retains a strong interest in Exercise Physiology and many of his current and future research avenues are/will be aimed at uncovering novel mechanisms behind the metabolic benefits of exercise.