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Clinical Research Article

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Nicotinamide Riboside Enhances In Vitro Beta-adrenergic Brown Adipose Tissue Activity in Humans

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Abbreviations: BAT, brown adipose tissue; CT, computed tomography; HFD, high-fat diet; NAD+, nicotinamide adenine dinucleotide; NE, norepinephrine; NR, nicotinamide riboside; OCR, oxygen consumption rate; PET, positron emission tomography; SIRT, sirtuin; SUV, standardized uptake value; SUVmean, mean standardized uptake value; UCP1, uncoupling protein 1; WAT, white adipose tissue.

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Abstract

Context: Elevating nicotinamide adenine dinucleotide (NAD^+) levels systemically improves metabolic health, which can be accomplished via nicotinamide riboside (NR). Previously, it was demonstrated that NR supplementation in high-fat-diet (HFD)-fed mice decreased weight gain, normalized glucose metabolism, and enhanced cold tolerance.

Objective: Because brown adipose tissue (BAT) is a major source of thermogenesis, we hypothesize that NR stimulates BAT in mice and humans.

Design and intervention. HFD-fed C56BL/6J mice were supplemented with 400 mg/kg/day NR for 4 weeks and subsequently exposed to cold. In vitro primary adipocytes derived from human BAT biopsies were pretreated with 50 μM or 500 μM NR before measuring mitochondrial uncoupling. Human volunteers (45-65 years; body mass index, 27-35 kg/m^2) were supplemented with 1000 mg/day NR for 6 weeks to determine whether BAT activity increased, as measured by [18F]FDG uptake via positron emission tomography-computed tomography (randomized, double blinded, placebo-controlled, crossover study with NR supplementation).

Results: NR supplementation in HFD-fed mice decreased adipocyte cell size in BAT. Cold exposure further decreased adipocyte cell size on top of that achieved by NR

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alone independent of ex vivo lipolysis. In adipocytes derived from human BAT, NR enhanced in vitro norepinephrine-stimulated mitochondrial uncoupling. However, NR supplementation in human volunteers did not alter BAT activity or cold-induced thermogenesis.

Conclusions: NR stimulates in vitro human BAT but not in vivo BAT in humans. Our research demonstrates the need for further translational research to better understand the differences in NAD^+ metabolism in mouse and human.

Key Words: NR; brown adipose tissue; mitochondria

Brown adipose tissue (BAT) has been proposed as a promising target to stimulate energy expenditure in humans with obesity or type 2 diabetes. The presence of uncoupling protein 1 (UCP1) in mitochondria of BAT enables heat production instead of ATP synthesis while using glucose and fatty acids as fuel (1). The interest in human BAT was sparked by the discovery of active BAT in adult humans (2-5), and high BAT activity is associated with healthy whole-body metabolism. Cold exposure is the most effective way to stimulate BAT activity in humans (6). Prolonged cold exposure may, however, not be an attractive treatment option for many people; thus, pharmacological alternatives are actively explored. Numerous potential ways to activate BAT have been investigated (eg, example vitamin A, FGF21, thyroid hormones, bile acids (7)). In this context, we and others have shown that bile acids stimulate BAT activity in mice (8) and healthy humans (9); however, it is not known whether this approach is effective in human metabolic disease. Another approach to activate BAT involves activation of the beta-adrenergic receptor via administration of agonists such as mirabegron. However, beta-adrenergic stimulation at doses high enough to activate BAT also affect cardiac function by increasing heart rate and blood pressure because of beta-adrenergic receptors in the heart (10). Therefore, other more specific and safer pharmacological strategies are warranted that stimulate BAT activity in humans that do not need to rely on the activation of the beta-adrenergic receptor.

Nicotinamide adenine dinucleotide (NAD^+) is an important mediator of cellular metabolism. NAD^+ demand is high to carry out metabolic redox reactions that yield energy like glycolysis and oxidative phosphorylation. However, besides these redox reactions, NAD^+ is also consumed by NAD^+-dependent enzymes like sirtuins, poly-ADP-ribose polymerases, and cyclic ADP-ribose synthases (11). Low NAD^+ is associated with aging and decreased metabolic health (12); therefore, increasing NAD^+ concentrations is an interesting approach to boost metabolism. The vitamin B3 analogue nicotinamide ribose (NR) is a NAD^+ precursor that increases activity of sirtuin (SIRT) 1 and 3 (13). In vitro, NR boosts NAD^+ levels (14), resulting in elevated SIRT1/3 activity in vivo (13). In obesity models, NR protected high-fat-diet (HFD)-fed mice from weight gain and glucose intolerance (13, 15). Furthermore, NR supplementation in various rodent models was able to counteract harmful effects of brain damage (16), Alzheimer's disease (17), autism spectrum disorder (18), age-related ovarian infertility (19), senescence (20), and muscular dystrophy through increased mitochondrial function (21).

NR supplementation also stimulated mitochondrial biogenesis in muscle and BAT in a mouse model of mitochondrial myopathy (22). Interestingly, in mice NR, safeguarded body temperature following a cold challenge (13), thus implicating the involvement of BAT because of the thermogenic properties of BAT. Combined, these findings generated 2 additional questions: (1) Is beta-adrenergic–stimulated BAT metabolism altered in murine adipocytes and in cultured adipocytes derived from human BAT following NR? (2) Does NR enhance cold-stimulated BAT activity in humans? Therefore, we assessed how NR affected BAT morphology in a in vivo HFD-fed mouse model. Because murine BAT is different from human BAT and the effect of NAD^+ boosting on human BAT remain unclear, we also investigated the effects of NR on human BAT by using in vitro primary cultured adipocytes derived from human BAT biopsies. Finally, we performed a first human clinical trial in which we supplemented human volunteers with 1000 mg/day NR to examine the effects on in vivo BAT activity in humans.

Materials and Methods

Animal Experiments

Male C57BL/6J mice were purchased from Charles River Laboratories (Wilmington, MA) and were housed under a 14-hour light, 10-hour dark cycle at 21°C and had ad libitum access to water and food throughout the experiment. From the age of 8 weeks, mice were split into 4 groups of 10 animals. All animals received an HFD (D12492) for 4 weeks from Research Diets Inc. (New Brunswick, NJ). One-half of animals were fed with pellets containing vehicle (double-distilled water) or NR-supplemented by

providing NR (400 mg/kg/day) for 4 weeks as previously described (23). Mouse body weight was assessed weekly. Cold test was performed as described (24) in 7 animals on HFD alone and in 7 animals receiving HFD and NR. The rest of the animals were maintained at room temperature. In cold-test groups, mice were anesthetized with sodium pentobarbital after 6 hours of cold exposure (IP injection, 50 mg/kg body weight). In the RT group, mice were anesthetized at the same time with sodium pentobarbital (IP injection, 50 mg/kg body weight). BAT was stored in 4% formaldehyde or used for lipolysis experiments. Body composition was determined by Echo-MRI (Echo Medical Systems, Houston, TX). All animal experiments were carried according to national Swiss and European Union ethical guidelines and approved by the local animal experimentation committee of the Canton de Vaud under license #2868.

Lipolysis Assay

Murine BAT was isolated and tissue explants were minced and incubated with lipolysis medium at 37°C and 5% CO_2. After 2 hours, media was collected and incubated for of 5 minutes at 37°C with free glycerol reagent from Sigma-Aldrich (St. Louis, MO). Following absorption was measured at 540 nm as previously described (25). In cultured adipocytes derived from human BAT and white adipose tissue (WAT), glycerol release was determined using the enzyChrom adipolysis assay kit from BioAssay systems (Hayward, CA) according to the manufacturer's instructions.

Histology

Murine BAT samples were fixed overnight in buffered 4% formaldehyde and embedded in paraffin. The 5-µm-thick serial sections were made from paraffin-embedded tissue t were subsequently stained with hematoxylin and eosin.

Culture of Human Primary Adipocytes

The collection of BAT and WAT biopsies in patients was reviewed and approved by the ethics committee of Maastricht University Medical Center (METC 10-3-012, NL31367.068.10, NCT03111719). Isolation of the stromal vascular fraction and differentiation of cultured adipocytes derived from human BAT and WAT have been described previously (9). In short, collected cells from the stromal vascular fraction were grown to confluence. Differentiation was initiated by a cocktail containing biotin (33 µM), pantothenate (17 µM), insulin (100 nM), dexamethasone (100 nM), 3-isobutyl-1-methylxanthine (250 µM), rosiglitazone (5 µM), T3 (2 nM), and transferrin (10 µg/mL). Cells were transferred to maintenance medium consisting of biotin (33 µM), pantothenate (17 µM), insulin (100 nM), dexamethasone (10 nM), T3 (2 nM), and transferrin (10 µg/mL) until lipid-accumulating adipocytes had formed.

Mitochondrial Respiration

Mitochondrial respiration in cultured adipocytes derived from human BAT and WAT has been described before (9). In short, adipocytes derived from human BAT and WAT were differentiated in XF96-well plates. Oxygen consumption rates were measured using the XF96 extracellular flux analyzer from Seahorse Biosciences (North Billerica, MA). Cells were incubated for 1 hour at 37°C in unbuffered DMEM (2 mM GlutaMAX, 1 mM sodium pyruvate, and 25 mM glucose). Basal oxygen consumption was measured followed by injection of 2 µM oligomycin subsequently followed by injection of the compounds of interest (1 µM NE, 0.3 µM FCCP, 1 µM antimycin A + rotenone). When indicated, cells were preincubated with NR for 24 hours before the start of the experiment. Data are plotted as a percentage compared with uncoupled respiration following oligomycin.

RNA Isolation and Gene Expression Analysis

Total RNA was extracted from cultured adipocytes derived from human BAT and WAT using the miRNEasy kit from Qiagen (Hilden, Germany) according to the manufacturer. cDNA was created using the high-capacity RNA-to-cDNA-kit from Applied Biosystems (Foster City, CA). Gene expression data were normalized to TATA box-binding protein and further analyzed using the $2^{-\Delta\Delta Ct}$ method. Primers for SIRT3 (Hs00953477_m1), SOD2 (Hs00167309_m1), and UCP1 (Hs00222453_m1) were from Applied Biosystems. SYBR-green quantitative PCR primers for PGC1A: forward primer 5′-TGCTGAAGAGGGAAAGTGAGCGA TTAGTTGA-3′, reverse primer 5′-AGGTGAAAGTGT AATACTGTTGGTTGA-3′; SIRT1: forward primer 5′-AGAGCCTCACATGCAAGCTCTAG-3′, reverse primer 5′- GCCAATCATAAGATGTTGCTGAAC-3′; TFAM: forward primer 5′- TTCCCAAGACTTCATTTCATTGTC-3′, reverse primer 5′- GATGATTCGGCTCAGGGAAA-3′. Primers for TATA box-binding protein have been described previously (26).

Western Blot Analysis

Protein was extracted using lysis buffer (50 mM Tris, 1 mM EDTA, 1% NP40, 5 mM nicotinamide, 1 mM sodium

butyrate, 150 mM KCl, protease inhibitors [pH 7.4]). Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Blocking and antibody incubations were performed in 5% BSA. Proteins were detected and quantified using the Odyssey from LI-COR Biosciences. Antibodies against OXPHOS were purchased from Abcam (Cambridge, UK). Beta-actin (A5316) was detected using an antibody from Sigma-Aldrich.

NAD^+ Determination

NAD^+ concentrations were determined using the enzyChrom NAD^+/NADH assay kit from BioAssay Systems (Hayward, CA) according to the manufacturer's instructions.

ATP Determination

ATP concentrations were determined using the CellTiter-Glo Luminescent cell viability assay from Promega (Madison, WI) according to the manufacturer's instructions.

Clinical Trials in Humans

The ethics committee of Maastricht University Medical Center approved the study protocol (METC 16-30-19, NL58119.068.16, NCT02835664), and all volunteers provided written informed consent. Eight healthy overweight and obese men and postmenopausal women were recruited. Inclusion criteria were 45 to 65 years of age, body mass index 27 to 35 kg/m^2, sedentary lifestyle (<3 hours of exercise per week), nonsmoking for at least 6 months, no alcohol use of >2 servings per day, stable body weight for at least 6 months, and no active diseases. A randomized controlled, double blinded, placebo-controlled, crossover study with NR supplementation was performed. Volunteers underwent 2 times per 6-week period an oral supplementation of either NR 1000 mg daily (NIAGEN, Chromadex) or placebo (capsules identical to NR in external appearance and number). Supplements were consumed with breakfast (500 mg) and lunch (500 mg). Measurements were performed on the last day of each supplementation period. Participants fasted for at least 11 hours when entering the test facilities.

For the determination of cold-induced BAT activity, volunteers were wrapped in a water-perfused suit (ThermaWrap Universal 3166; MTRE Advanced Technologies Ltd., Yavne, Israel). First, volunteers remained at thermoneutral conditions (32°C water) for 30 minutes, during which basal metabolic rate was measured by indirect calorimetry (IDEE, Maastricht Instruments, Maastricht, The Netherlands). Thereafter, an individualized cooling procedure was started to determine nonshivering thermogenesis as described before (27). In brief, volunteers were cooled down in order to maximize nonshivering thermogenesis after which energy expenditure was measured for 30 minutes. Next, 75 MBq of [18F]FDG was injected via the intravenous canula. Cold exposure was continued for 60 minutes while volunteers were instructed to remain lying still. Next, the volunteers were unwrapped and underwent a static [18F]FDG-positron emission tomography (PET)/computed tomography (CT) scan (Gemini TF PET-CT, Philips, The Netherlands). This consisted of a low-dose CT scan (120 kV, 30 mAs) followed by a PET scan. Six to 7 bed positions (5 minutes per bed position) were used, to cover the area from the skull to the iliac crest. Tracer uptake was determined with the PET scan, whereas the CT was used for attenuation correction and anatomical localization of the active BAT.

The scans were analyzed with PMOD software (version 3.0; PMOD Technologies). The regions of interest were manually outlined, whereas a threshold of 1.5 standardized uptake value (SUV) and Hounsfield units between -10 and -180 were used, as described previously by our group (27). Additionally, fixed volumes (10 mm × 10 mm) were placed in the cervical adipose tissue behind the clavicula to measure general uptake values as described before (28). BAT activity was expressed in SUV: [18F]FDG uptake kBq/mL/(injected dose [kBq/patient weight in grams]). The activity was determined as average SUV (SUVmean) and as total SUV (SUVmean × the volume of interest).

Statistics

Two means were compared using Student *t* test, or with the Wilcoxon signed-rank test in case of nonparametric data. Comparison of multiple means was assessed by ANOVA. $P < 0.05$ was considered statistically significant. Data are expressed as mean ± standard error of the mean. Analyses were performed using Graph Pad Prism (San Diego, CA).

Results

NR Combined With Cold Exposure Decreases Adipocyte Cell Size in Murine BAT Without Affecting Lipolysis

NR-supplemented mice were able to maintain a higher body temperature when faced with a cold tolerance test (13), suggesting the involvement of BAT. Therefore, we specifically investigated BAT by examining morphology and adipocyte cell size following NR supplementation and cold exposure (4°C) in HFD-fed mice. Four

weeks of HFD (body weight: 32.9 ± 1.71 g; fat mass: 5.9 ± 1.16 g; average ± SD, n = 12) compared with HFD supplemented with NR (body weight: 32.1 ± 1.42 g; fat mass: 5.2 ± 0.70 g; average ± SD, n = 12) did not change body composition. As expected, acute cold decreased brown adipocyte cell size (Fig. 1A and 1B). Brown adipocyte cell size also decreased following NR supplementation to HFD (Fig. 1A and 1B). NR supplementation and cold combined resulted in the smallest adipocyte cell size (Fig. 1A and 1B), thus potentially hinting at increased lipolysis. To examine whether NR supplementation affected lipolysis in mouse BAT, we performed ex vivo lipolysis experiments in mouse BAT harvested at room temperature and after acute cold exposure. At room temperature, supplementation of NR to a HFD did not alter lipolysis (Fig. 1C). Also, under conditions of acute cold exposure, supplementation of NR did not significantly change lipolysis in murine BAT (Fig. 1C).

NR Stimulates Norepinephrine-stimulated Mitochondrial Uncoupling Only in Adipocytes Derived From Human BAT

To investigate if the positive effects of NR on BAT morphology in mouse can be translated to humans, we incubated cultured adipocytes derived from human BAT and WAT with 50 or 500 µM NR to examine effects on norepinephrine-induced mitochondrial uncoupling. Whereas 50 µM NR did not increase NAD^+ levels, 500 µM NR significantly increased NAD^+ levels in adipocytes derived from human BAT and WAT (Fig. 2A and 2B). Both doses of NR were without effect on ATP concentrations in adipocytes derived from human BAT or WAT (Fig. 2C and 2D). To further investigate the effect on mitochondrial uncoupling in adipocytes derived from BAT and WAT, cultured adipocytes were treated with oligomycin to block ATPase and subsequently stimulated with NE. NE enhanced mitochondrial uncoupling in adipocytes derived from BAT, whereas this response was negligible in adipocytes derived from human WAT (Fig. 2E and 2F). Totals of 50 µM and 500 µM NR were unable to potentiate NE-stimulated mitochondrial uncoupling in WAT (Fig. 2F and 2H). In human primary brown adipocytes, however, 500 µM NR increased NE-stimulated mitochondrial uncoupling (Fig. 2E and 2G), as well as maximal FCCP-induced mitochondrial respiration (Fig. 2E and 2G). At 500 µM NR, antimycin A and rotenone resulted in higher oxygen consumption rate (OCR) compared with 0 µM NR and 50 µM NR. In human primary brown adipocytes, basal OCR (86.9 ± 22.22 pmol/minutes, average ± SD, n = 6) and basal OCR following 500 µM NR incubation (73.6 ± 16.39 pmol/minute, average ± SD, n = 6) was comparable.

NR Does Not Affect Lipolysis in Cultured Adipocytes Derived From Human BAT and WAT

To investigate whether lipolysis plays a pivotal role in NR-stimulated mitochondrial uncoupling in human BAT cells, we examined lipolysis following NR treatment in cultured adipocytes derived from human BAT and WAT. Both basal and beta-adrenergic NE-stimulated lipolysis was

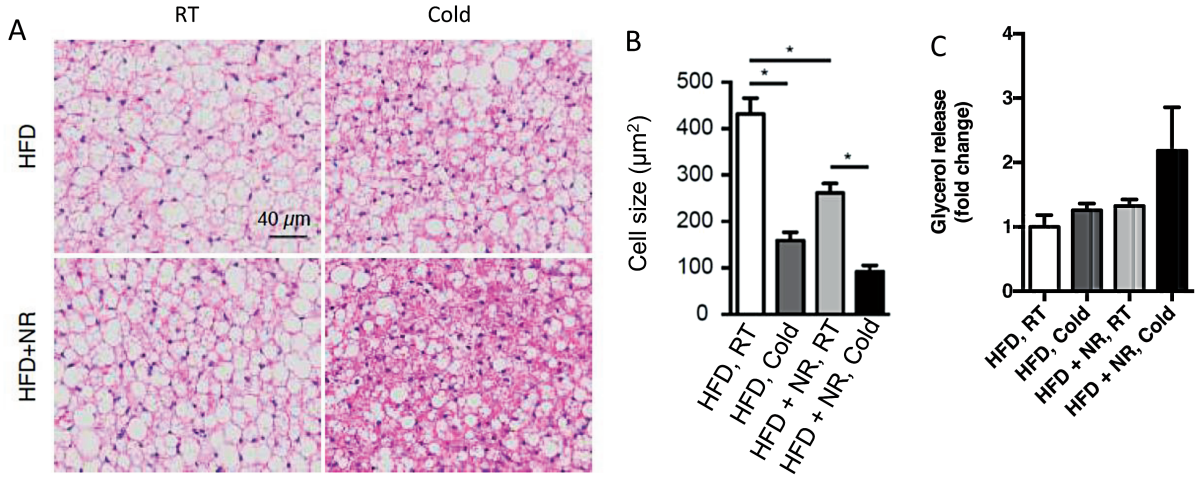

Figure 1. NR supplementation alters adipocyte cell size in mouse BAT following an HFD. Mice were fed an HFD or an HFD supplemented with NR (HFD + NR). Following the 4-week diet, animals were acutely exposed to cold (cold) or remained at room temperature (RT). (A) HE staining of BAT. (B) Cell size quantification of results obtained in panel A. *$P < 0.05$ (n = 5-7). (C) Ex vivo lipolysis was performed on BAT explants (n = 2-3). Data are expressed as mean ± standard error of the mean. Abbreviations: BAT, brown adipose tissue; HE, hematoxylin, and eosin; HDF, high-fat diet; NR, nicotinamide riboside.

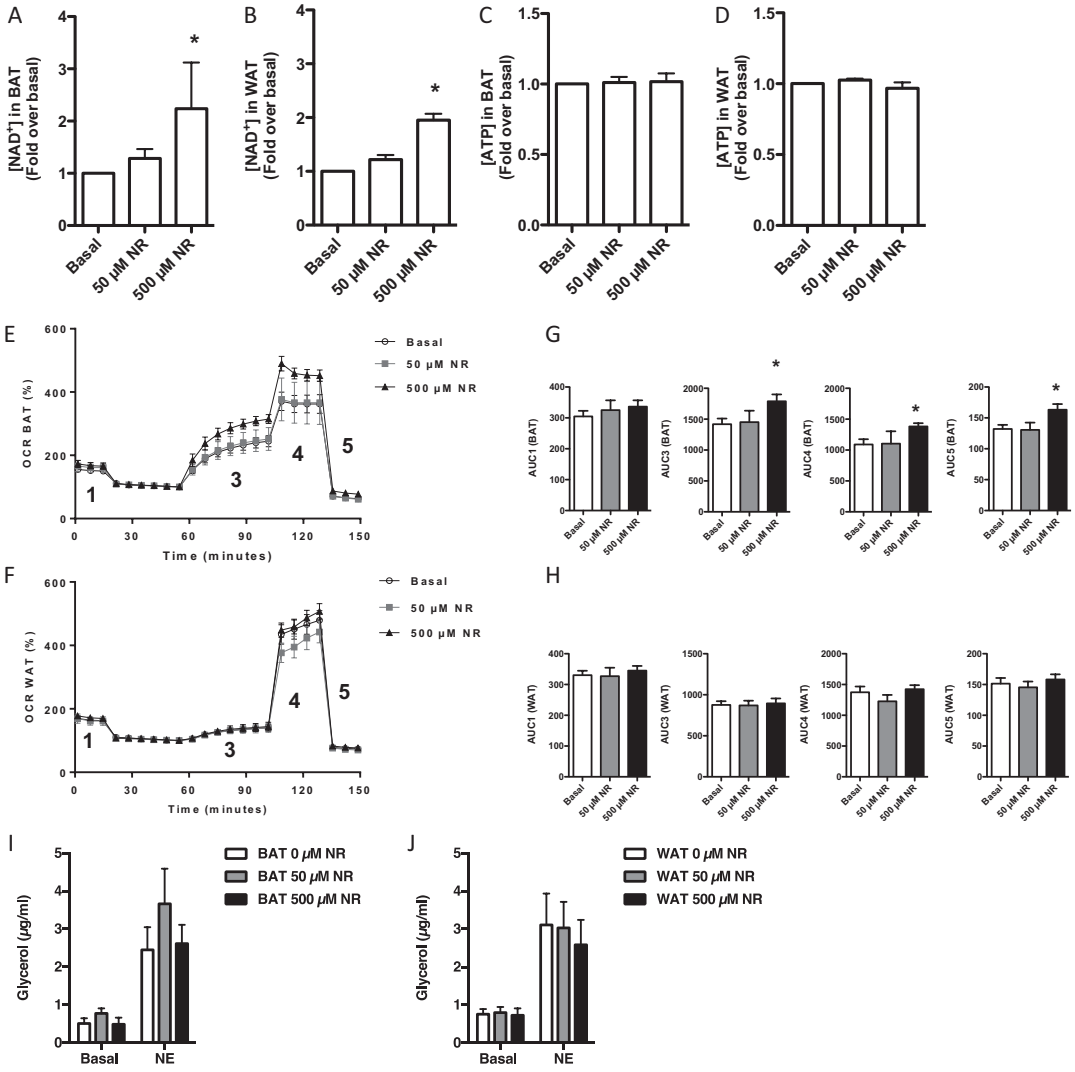

Figure 2. NR stimulates mitochondrial uncoupling in cultured primary adipocytes derived from human BAT. (A, B) NAD^+ and (C, D) ATP levels were determined in cultured adipocytes derived from human BAT or WAT after 24 hours with 0 µM, 50 µM, or 500 µM NR. Data are expressed as a fold increase compared with 0 µM NR. Cellular respiration was measured using the Seahorse bioanalyzer in cultured adipocytes derived from human (E) BAT and (F) WAT following 24-hour incubation with 0 µM, 50 µM, or 500 µM NR. Mitochondrial respiration was measured following injections with oligomycin, NE, FCCP, and antimycin A + rotenone. (G) Quantification of AUC from results in panel E. (H) Quantification of AUC from results in panel F. NR does not alter lipolysis in cultured adipocytes derived from human BAT. Cultured adipocytes derived from human (I) BAT and (J) WAT were incubated for 24 hours with 0 µM, 50 µM, or 500 µM NR. Following adipocytes were stimulated with NE to measure glycerol release as a marker of lipolysis. Data are expressed as mean ± standard error of the mean. $*P < 0.05$ (n = 4-6). Abbreviations: AUC, area under the curve; BAT, brown adipose tissue; WAT, white adipose tissue; NE, norepinephrine; NR, nicotinamide riboside.

unchanged following NR treatment in adipocytes derived from human BAT and WAT (Fig. 2I and 2J).

NR Does Not Alter SIRT-associated Genes or OXPHOS Protein Expression in Adipocytes Derived From Human BAT

SIRTs are NAD^+-dependent deacetylases, and NR stimulates SIRT activity by increasing the level of its obligatory cosubstrate NAD^+ (29). Next, we assessed whether NR was able to alter gene expression of SIRT-related pathways. Adipocytes derived from human BAT and WAT were incubated with vehicle, 50 µM NR or 500 µM NR. In cultured adipocytes derived from human WAT, 50 µM NR decreased PGC1A gene expression (Fig. 3E) and 500 µM NR increased transcript levels of TFAM (Fig. 3F) compared with vehicle. In cultured adipocytes derived from human BAT, NR did not significantly change the transcript

levels of UCP1, SIRT3, SOD2, PGC1A, or TFAM (Fig. 3A-F). We also determined protein expression of OXPHOS by western blot analysis examining the individual complexes in adipocytes derived from human BAT and WAT. The highest concentration of 500 µM NR decreased the amount of complex I (Fig. 3G) in adipocytes derived from human BAT, whereas 50 µM NR increased protein abundance of complex I in adipocytes derived from human WAT (Fig. 3H).

NR Does Not Affect BAT Activity or Energy Expenditure in Human Volunteers

Our results show that NR can enhance BAT activity in human primary brown adipocytes. These promising results urged us to examine the true translational potential of NR on human BAT; therefore, we performed a first double-blind, placebo-controlled crossover design clinical trial in a small cohort of volunteers with obesity. During 6 weeks, 1000 mg/day NR or placebo was supplemented. Cold-stimulated BAT activity was determined via uptake of [18F]FDG using PET-CT and whole-body energy expenditure was also measured. However, NR supplementation for 6 weeks had no effect on [18F]FDG-mediated glucose uptake accessed as SUVmean (Fig. 4A) and maximum SUV (Fig. 4B) in fixed volumes. Cold exposure was able to significantly increase energy expenditure in human volunteers (Fig. 4C and D). However, the increase in energy expenditure following cold was similar in human volunteers receiving placebo or human volunteers receiving NR (Fig. 4C and 4D). Also, nonshivering thermogenesis was unchanged following NR supplementation (Fig. 4E). The respiratory

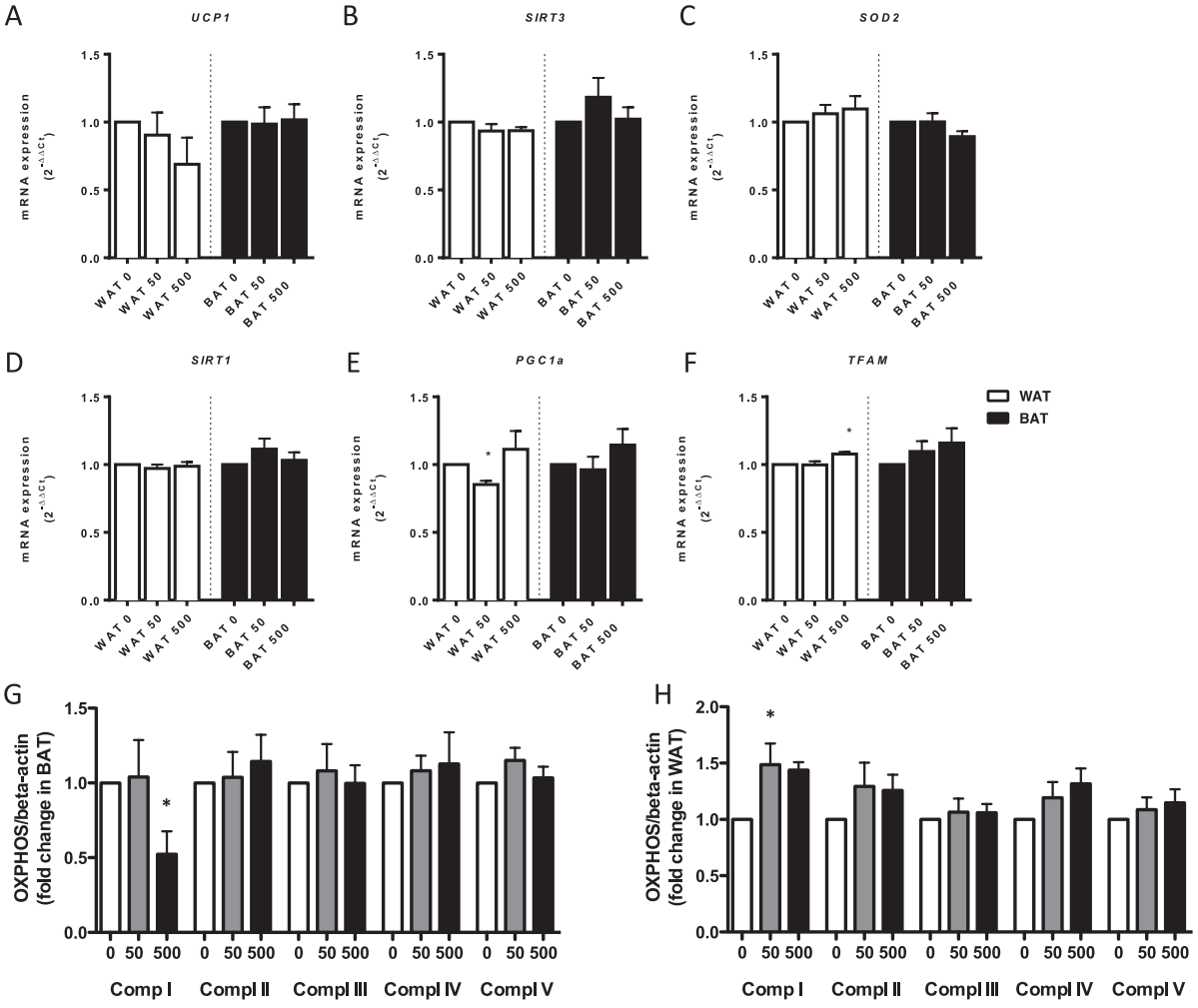

Figure 3. NR does not alter expression of SIRT targets in cultured primary adipocytes derived from human BAT. Cultured adipocytes derived from human BAT (black bars) and WAT (white bars) were stimulated for 24 hours with 0 µM (0), 50 µM (50), or 500 µM (500) NR. Gene expression of (A) UCP1, (B) SIRT3, (C) SOD2, (D) SIRT1, (E) PGC1A, and (F) TFAM were determined using qPCR techniques. Cultured adipocytes derived from human BAT (G) and WAT (H) were stimulated for 24 hours with 0 µM (0), 50 µM (50), or 500 µM (500) NR. OXPHOS complexes were analyzed by Western blot. Data are expressed as mean ± standard error of the mean. *$P < 0.05$ compared with matching control (n = 4). Abbreviations: BAT, brown adipose tissue; NR, nicotinamide riboside; qPCR, quantitative polymerase chain reaction; SIRT, sirtuin; UCP1, uncoupling protein 1; WAT, white adipose tissue.

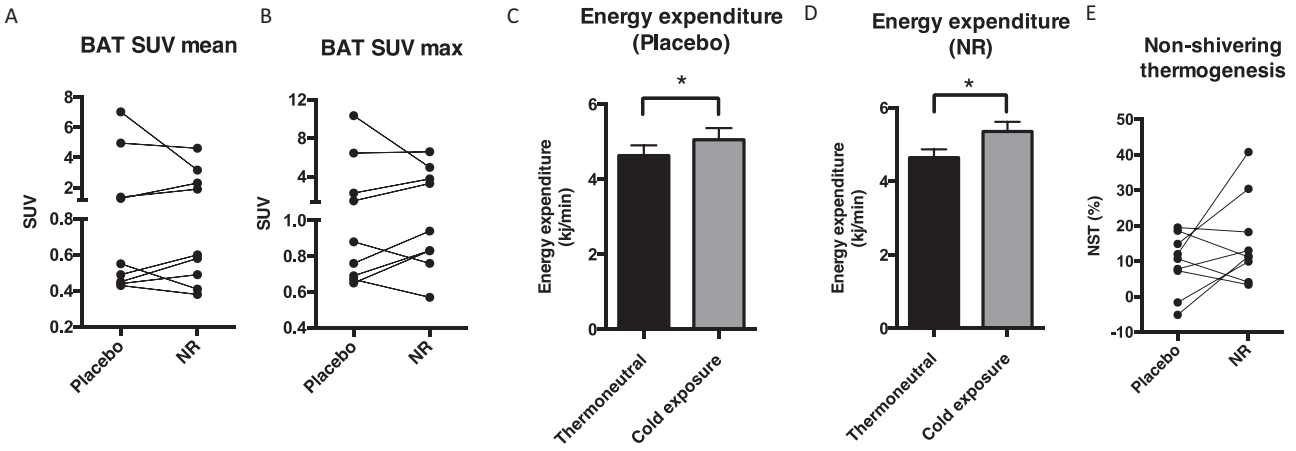

Figure 4. NR does not stimulate energy expenditure or BAT activity in humans. Human volunteers were supplemented with 1000 mg/day NR for 6 weeks in a placebo-controlled crossover design. (A) BAT SUVmean and (B) BAT SUVmax in fixed volumes following cold exposure. Energy expenditure at thermoneutrality and after cold exposure following (C) placebo or (D) NR. NST following NR supplementation. Data are expressed as mean ± standard error of the mean. $*P < 0.05$ (n = 9). Abbreviations: BAT, brown adipose tissue; NR, nicotinamide riboside; NST, nonshivering thermogenesis; SUVmax, maximum standardized uptake value; SUVmean, mean standardized uptake value.

quotient, reflecting substrate oxidation did lower during cold exposure, both in the placebo (from 0.81 to 0.79) and NR (from 0.79 to 0.78) supplementation periods. However, no significant difference was observed in cold exposure–stimulated delta RQ between placebo and NR supplementation.

Discussion

Preclinical data have demonstrated that increasing NAD^+ levels can counteract harmful effects of metabolic disease, and NR supplementation is a powerful intervention to do so. Here, we specifically assessed the effect of NR on mouse BAT morphology and on adipocytes derived from human BAT and WAT in vitro and in vivo in human volunteers. In mice, NR supplementation combined with beta-adrenergic cold-exposure decreased brown adipocyte cell size (Fig. 1). Similarly, in cultured adipocytes derived from human BAT, elevating NAD^+ levels via NR enhanced beta-adrenergic NE-mediated mitochondrial uncoupling (Fig. 2). This stimulatory effect was specific for adipocytes derived from human BAT, which is in line with earlier observation in animals where NAD^+ supplementation increased NAD^+ concentrations in BAT and stimulated BAT metabolism following a cold challenge (13). However, 6 weeks of supplementation with 1000 mg/day NR did not increase cold-stimulated BAT activity in humans. These observations together show that NR and beta-adrenergic signaling can work together to enhance BAT activity; however, the current dose of NR was unable to increase BAT activity in humans.

We examined lipolysis ex vivo in murine BAT and in vitro in human BAT in the context of NR stimulation. There are obvious differences when comparing murine adipose tissue explants with in vitro differentiated human adipocytes. However, in vitro lipolysis in adipocytes derived from human BAT was unaltered following NR (Fig. 2I) and also ex vivo lipolysis in mouse BAT following NR exposure was unaffected (Fig. 1C). This indicates that the direct mode of action of NR is most likely independent of lipolysis in mice and humans; however, further measures of intracellular lipolysis should be explored. A strong link has been suggested between BAT and WAT because BAT volumes correlate with whole-body lipolysis (30), which is mostly mediated by WAT. In our study, we were unable to assess WAT metabolism in human volunteers; however, this could provide explanations in the future why we were unable to detect an increase in BAT activity in human volunteers. Previously, it has been reported that NR increases NAD^+ content in cells and mitochondria (13) and mitochondria are pivotal players in providing energy for lipolysis. In line with unaltered lipolysis in human and murine BAT, we also observed no change in OXPHOS protein, ATP levels, or gene expression related to mitochondrial biogenesis in cultured adipocytes derived from human BAT.

mRNA abundance of the classical mitochondrial marker for BAT, UCP1, was unchanged following NR treatment in cultured adipocytes derived from human BAT. However, when it comes to UCP1 expression and NR, different results have been obtained depending on the experimental setup. In young lean mice on a chow diet supplemented for 5 weeks with NR, UCP1 protein content was increased in BAT; however, in this study, there was no mention of total BAT mass (31). In another study, NR was administered directly after birth in mice, which was subsequently followed by an HFD challenge

for 10 weeks. UCP1 mRNA expression was unaffected in animals on a HFD in BAT, but interestingly the animals on a control diet showed decreased expression of UCP1 mRNA expression following NR in BAT (32). These findings together indicate that gene and/or protein expression alone might not be the best indicator for BAT metabolic activity; therefore, we purposely included metabolic readouts for BAT activity in vitro and in vivo. Next to UCP1-dependent pathways, several UCP1-independent pathways have been described. The first UCP1-independent pathway revolves around insulin. Beta-adrenergic cold stimulation triggers insulin release, resulting in lipolysis to refuel the activated BAT in mice (33); however, whether this mechanism is present in humans warrants further investigation. The second UCP1-independent pathway is based on calcium cycling; specifically, in beige adipocytes, calcium cycling is regulated through SERCA2B and RYR2 (34) resulting in fuel for thermogenesis. The third UCP1-independent driven pathway is creatine-dependent ADP/ATP substrate cycling (35) resulting in increased thermogenesis. These UCP1-independent pathways can be of importance when examining human NR-stimulated (in vitro) BAT activity, especially because human BAT resembles murine beige adipose tissue (26, 36).

Stimulating BAT activity by other means than cold in humans could have beneficial effects on whole-body metabolism. Human BAT activity shows a negative correlation with obesity (4, 37), and stimulation of BAT activity through cold acclimatization increases energy expenditure that potentially could lead to loss of fat mass (38). Furthermore, cold-induced BAT activity is related to increased insulin sensitivity in humans (39, 40). Cold exposure is the most effective method to stimulate BAT activity; however, not the most practical one. Therefore, alternative routes to stimulate BAT in humans are warranted. In the current study, NR was able to stimulate BAT activity in human primary brown adipocytes. However, we could not confirm these results in a clinical trial in humans, which is in line with other clinical trials using NR (41-43). The reason NR had beneficial effects in human primary brown adipocytes but not in vivo in humans, cannot be deduced from the current study. Based on our research, 6 weeks of NR supplementation in healthy volunteers with obesity did not increase NAD or mitochondrial function in skeletal muscle; however, the NAD^+ metabolites nicotinic acid adenine dinucleotide and methylnicotinamide were increased, demonstrating enhanced NAD^+ metabolism following NR (44). SIRTs are targets for NR/NAD^+; however, metabolic disease can alter SIRT expression. For example, NAFLD is associated with lower SIRT expression in human liver (45) and obesity is associated with lower SIRT expression in human WAT (46). This could potentially mean that higher concentrations or longer duration of NR supplementation are needed when examining a human cohort with obesity to see beneficial effects on human BAT. In the future, translational research will be crucial when employing other NAD^+-boosting strategies (eg, nicotinamide mononucleotide (47), dihydronicotinamide riboside (48)) to increase BAT activity. Therefore, further translational research is needed to better understand the differences in NAD^+ metabolism between mouse and man and in vitro and in vivo.

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