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Targeting sirtuin activity with nicotinamide riboside reduces neuroinflammation in a GWI mouse model.

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Abstract:
Gulf War Illness (GWI) affects 30% of veterans from the 1991 Gulf War (GW), who suffer from symptoms that reflect ongoing mitochondria dysfunction. Brain mitochondria bioenergetics dysfunction in GWI animal models corresponds with astroglia activation and neuroinflammation. In a pilot study of GW veterans (n = 43), we observed that blood nicotinamide adenine dinucleotide (NAD) and sirtuin 1 (Sirt1) protein levels were decreased in the blood of veterans with GWI compared to healthy GW veterans. Since nicotinamide riboside (NR)-mediated targeting of Sirt1 is shown to improve mitochondria function, we tested whether NR can restore brain bioenergetics and reduce neuroinflammation in a GWI mouse model. We administered a mouse diet supplemented with NR at 100µg/kg daily for 2-months to GWI and control mice (n = 27). During treatment, mice were assessed for fatigue-type behavior using the Forced Swim Test (FST), followed by euthanasia for biochemistry and immunohistochemistry analyses. Fatigue-type behavior was elevated in GWI mice compared to control mice and lower in GWI mice treated with NR compared to untreated GWI mice. Levels of plasma NAD and brain Sirt1 were low in untreated GWI mice, while GWI mice treated with NR had higher levels, similar to those of control mice. Deacetylation of the nuclear-factor κB (NFκB) p65 subunit and peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α) was an increase in the brains of NR-treated GWI mice. This corresponded with a decrease in pro-inflammatory cytokines and lipid peroxidation and an increase in markers of mitochondrial bioenergetics in the brains of GWI mice. These findings suggest that targeting NR mediated Sirt1 activation restores brain bioenergetics and reduces inflammation in GWI mice. Further evaluation of NR in GWI is warranted to determine its potential efficacy in treating GWI.
Introduction:

Nearly thirty years have elapsed since the 1991 Gulf War (GW) conflict, but 30% of veterans from this conflict continue to suffer from Gulf War Illness (GWI) and experience debilitating symptoms such as pain, fatigue and memory problems\textsuperscript{1,2}. Both clinical and animal studies suggest that disturbances in bioenergetics may be part of the underlying pathological features associated with the heterogeneous symptoms experienced by veterans with GWI\textsuperscript{3–6}. Several imaging studies have shown abnormal levels of bioenergetics markers in the brains of veterans with GWI\textsuperscript{7,8}. Therefore, therapies that are aimed at improving mitochondrial bioenergetics in the brain may help with improving the general health and well-being of veterans with GWI.

Energy disturbances in veterans with GWI are characterized by increases in lipid peroxidation products in plasma, changes in mitochondrial plasma lipids, inability to recover phosphocreatine levels in muscle after exercise, and damage to mitochondrial DNA corresponding with reduced activity of mitochondrial enzymes involved in electron transport chain (ETC) and anti-oxidant defense\textsuperscript{3,5,8,10}. Evidence for central nervous system (CNS) bioenergetics disturbances is suggested by a study showing increased lactate utilization in the prefrontal cortex following an exercise challenge in a subset of veterans with GWI and glucose hypometabolism in the hippocampus of GW veterans with GWI\textsuperscript{6}. In a GWI mouse model, neurobehavioral deficits corresponded with decreases in the Krebs cycle intermediary compounds and downregulation of pathways related to the conversion of pyruvate to lactate and gluconeogenesis\textsuperscript{3}. Lipid analysis of this mouse model showed alterations in mitochondria-specific lipids, such as cardiolipin (CL) and acylcarnitine levels in the brains of GWI mice\textsuperscript{3}. A decrease in several ETC enzymes was also observed in the brains of GW chemical-exposed mice\textsuperscript{11}. A rat model of GW chemical exposure also showed chronic oxidative stress, inflammation, and mitochondrial dysfunction along with memory and mood dysfunction in exposed rats\textsuperscript{4,12}. These studies suggest that mitochondria dysfunction may be a contributory factor in bioenergetics deficits in GWI, as it has been observed both in veterans with this condition and in mouse models developed using GW-relevant chemical exposures.

Nicotinamide adenine dinucleotide (NAD+) is an important cofactor of many metabolic processes, including glycolysis, fatty acid β-oxidation and the Krebs cycle, while its reduced form, NADH, serves as an electron-rich source that takes part in oxidative phosphorylation (OXPHOS), ultimately contributing to the generation of adenosine triphosphate (ATP) as energy\textsuperscript{13,14}. During OXPHOS, NAD+/NADH simply shuttle electrons and are not consumed during this process. However, NAD+ is actively consumed by sirtuins during the protein deacetylation process\textsuperscript{15}. It is well known that NAD+ increases the expression and the activity of sirtuins\textsuperscript{16,17}, which corresponds with reduced inflammation via nuclear-factor κB (NFκB) mediated pathways and targets mitochondria biogenesis through activation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α)-dependent pathways\textsuperscript{18–20}. Although the exact mechanisms remain unknown, depletion of NAD+ or its reduced form NADH is considered a hallmark of aging and is also observed in chronic fatigue syndrome (CFS), which has some clinical resemblance to GWI\textsuperscript{21–25}.
Given the importance of NAD+ in cellular bioenergetics, various approaches have been explored for supplementing NAD+. Supplementation with the NAD+ precursor nicotinamide riboside (NR) appears to be a viable option as this form of NAD+ can enter the cell and cross the blood-brain-barrier (BBB). In the cell, NR is converted to NAD+ by an enzyme nicotinamide riboside kinase (NRK). Supplementation with NR in animal models of neurodegeneration reduces inflammation and attenuates neuropathological phenotypes through sirtuin-1 (Sirt1)-mediated deacetylation of the NFκB pathway. Others have shown that NR treatment enhances cognitive functioning and reduces glial activation. Treatment with NR may be a valuable therapeutic approach due to its high bioavailability and minimal toxicity in humans. In a pilot human study, levels of NAD+ rose by 3-fold after a single oral dose of 300mg of NR. We therefore hypothesize that NR treatment may reduce disturbances in mitochondrial bioenergetics, oxidative stress and chronic neuroinflammation. We therefore tested NR in a mouse model of GWI that exhibits neurobehavioral and brain bioenergetics deficits and neuroinflammation that are relevant to the etiology of GWI. The studies described below provide preclinical evidence for potential use of NR in treating the bioenergetic deficits in this GWI mouse model and provides translational relevance of these findings to veterans with GWI.

Methods

Human subject:
As described previously, human plasma samples were utilized from 2 different cohorts: (1) Veterans with GWI and healthy GW-deployed control veterans recruited at the Roskamp Institute Clinic and (2) GWI cases and healthy GW-deployed control veterans from the Boston Gulf War Illness Consortium (GWIC). The Roskamp Institute Clinic study was approved by the Western Institutional Review Board (IRB) and the protocol and procedures were carried out in accordance with the Declaration of Helsinki. Samples collected at the Boston GWIC were approved by the Boston University IRB. For both studies, written IRB-approved informed consent was obtained from all study participants. Veterans with GWI were diagnosed using the Kansas GWI criteria, which requires that GW veterans show symptoms in at least 3 out of 6 symptom domains (fatigue/sleep problems, somatic pain, neurological/cognitive/mood symptoms, gastrointestinal symptoms, respiratory symptoms and skin abnormalities). Potential study participants were excluded if they reported being diagnosed with another medical condition that could explain their chronic health symptoms. Gulf War veterans were considered controls if they did not meet the Kansas diagnostic criteria and also did not meet the exclusionary criteria. Veterans who volunteered for the study were interviewed and participated in the study protocol in person. After the interview, blood samples were obtained as described previously. Detailed study participant demographics are provided below in Table 1.

<table>
<thead>
<tr>
<th>Table 1. Demographics of the Gulf War veteran cohort.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (GW veteran)</td>
</tr>
<tr>
<td>N total</td>
</tr>
<tr>
<td>Age (Mean ± SD) Range</td>
</tr>
<tr>
<td>t-test (df= 16) = 16.75, p = 0.43</td>
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</table>
Male (%)

<table>
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<tr>
<th>Ethnicity</th>
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<th>Chi square (p=0.38)</th>
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</thead>
<tbody>
<tr>
<td>Caucasian</td>
<td>10 (83.3%)</td>
<td>24 (77.4%)</td>
</tr>
<tr>
<td>others</td>
<td>2 (16.6%)</td>
<td>7 (22.6%)</td>
</tr>
</tbody>
</table>

Plasma preparation and peripheral blood mononuclear cell (PBMC) Isolation:

Blood was drawn from age- and gender-similar subjects. Plasma was collected as described previously. Plasma aliquots were immediately transferred to a -80°C freezer until further use. Human PBMC were isolated using a density gradient technique. The Ficoll-Paque PLUS (GE Healthcare Life Sciences# 17144002) density gradient was used according to manufacture protocol. Briefly, 5ml of human blood was diluted with 5ml of PBS and the diluted blood was layered on top of a 10ml Ficoll-Paque PLUS. This mixture was centrifuged at 400 x g for 35 minutes at room temperature in a swinging bucket rotor without brakes. The mononuclear cell layer was carefully transferred to a new tube, where the cells were diluted with 10 ml of PBS and centrifuged at 400g for 15 minutes at room temperature to remove Ficoll-Paque. The supernatant was separated, and the pellet was resuspended in the fetal bovine serum with 6% DMSO and stored at -80°C until used for western blotting.

Animal Handling:

All procedures conducted on mice were approved by the Roskamp Institute’s Institutional Animal Care and Use Committee and were conducted in compliance with the Office of Laboratory Animal Welfare and Laboratory Animal Care guidelines as previously described. Male C57BL/6J mice (3 months of age, weight 25 g ± 0.7 SD) were either co-administered 0.7 mg/kg of PB (Thermo Fisher Scientific, Waltham, MA) and 200 mg/kg of PER (Sigma Aldrich, St. Louis, MO) in a single 50μl intraperitoneal injection in dimethyl sulfoxide (DMSO) (Sigma Adrich, St. Louis, MO) or DMSO alone (as control) daily for 10 consecutive days. Mice were divided into 4 groups: control, control + NR, PB+PER and PB + PER + NR. After 6 months post-exposure to GW chemicals, mice were fed with 100μg/kg of NR (MedKoo Biosciences, Research Triangle Park, North Carolina # 329479) supplemented in normal mouse chow (Envigo, Madison, # 2018) that comprised of 58.2% carbohydrate, 10.6% fat and 19% protein by calorie or were fed chow alone as control. Mice were euthanized 2 months after the NR treatment; a timeline of the study procedures is provided as Figure 1.

Forced swim test:

We performed the forced swim test (FST) as described previously. Briefly, mice were acclimated to the behavior testing room at least 30 minutes prior to testing. The FST apparatus comprised of a cylindrical tank (30 cm height x 20 cm diameters) filled with warm tap water (approximately 22 °C) to a depth of 25 cm, preventing the tail and feet from reaching the apparatus. Each animal was placed in the tank for 6 minutes, after which the mouse was removed to a warm and dry environment. Data were recorded and captured using the Ethovision XT software version 7. Latency to stop swimming and time spent being immobile were recorded as the outcome measures. Since our previous work showed that fatigue-type behavior emerges after
a few minutes of swimming during the FST, we split the FST test duration into two halves, first half (2-4min) and second half (4-6min). The difference between the two halves is denoted as delta immobile time ($\Delta$).

**Sample preparation:**

Mice were exsanguinated via cardiac puncture, using an 18-gauge wide-bore needle to prevent hemolysis of red blood cells (RBC) during blood collection. Blood samples were collected into 1.5ml Eppendorf tubes containing 10 units of Ethylenediaminetetraacetic acid (EDTA) to a final concentration of 1x. Samples were immediately centrifuged at 3000 x g for 5 min, and the plasma was transferred to a new 1.5 ml Eppendorf tube and snap frozen in liquid nitrogen. Plasma were stored at -80°C until further biochemical studies were performed. Subsequently, all animals were perfused with PBS, and the right brain hemispheres were immediately frozen in liquid nitrogen and transferred to a -80°C freezer until further use. Using a dounce homogenizer, brains were homogenized in chilled lysis buffer containing a protease inhibitor cocktail (Roche, Indianapolis, IN) and phosphatase inhibitor (Pierce, Grand Island, NY) cocktails. The total protein content of each sample was determined by the bicinchoninic acid assay (BCA assay, Pierce, Grand Island, NY).

**Western Blot:**

Western blotting was performed as described previously. List of primary antibodies used is provided in the supplementary table. Briefly, protein concentrations in the brain homogenates were quantified using bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). Approximately 30µg of protein from each brain homogenate was separated by 4–15% Mini-PROTEAN™ TGX Stain-Free™ Protein Gels (BioRad, Hercules, CA, USA) and transferred into polyvinylidene fluoride (PVDF) membranes (BioRad, Hercules, CA, USA) (100 mA constant current for 2 hour). The membrane was blocked with 6% skim milk (BioRad, Hercules, CA, USA) for 1 hour at room temperature. The primary antibody diluted with milk was incubated overnight at 4°C. Each membrane was incubated with the recommended dilution (1:5000) of corresponding horseradish peroxidase-conjugated secondary antibody (Cell Signaling, Danvers, MA) in blocking buffer at room temperature for 1hr. Protein bands were visualized using enhanced chemiluminescence detection reagents (Thermo Fisher Scientific, MA USA). Band intensities were analyzed using the ChemiDoc imaging system (BioRad, Hercules, CA, USA). Results were calculated using Image Lab software (BioRad, Hercules, CA, USA).

**Immunoprecipitation of Sirt1, PGC-1α and p65:**

Sirtuin 1 is an NAD+ dependent deacetylase that regulates metabolism and inflammation. To determine if Sirt1 interacts and deacetylates p65 (NFkB subunit), we performed pull down experiments. Briefly, using a dounce homogenizer, the left hemisphere was homogenized in 1ml of mammalian protein extraction reagent (Thermo Fisher Scientific, Waltham, MA) containing a protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA). About 200µl of PBS was added in 100µl brain homogenate and then centrifuged at 2000 x g, 4°C for 15 min. Supernatant was collected and combined with 500µl methanol and again centrifuged at 2000 x g 4°C for 15 min. Approximately 200µl Triethylammonium bicarbonate (TEAB) buffer was added to the pellet (cytosolic fraction). Primary antibody (p65 or Sirt1) was added at 1:100 to the cytosolic fraction and incubated with gentle rocking overnight at 4°C. This antibody complex was incubated for 20 min at room temperature with pre-washed magnetic bead pellet (Cell Signaling, Danvers, MA). Beads were pelleted with a magnetic separation rack (Cell Signaling, Danvers,
MA). The pellet was washed several times before adding 30μl of 3X sodium dodecyl sulfate (SDS) sample buffer. Samples were boiled for 5 minutes, beads were pelleted, and the collected supernatant underwent SDS-PAGE and western blot analysis as mentioned above. The primary antibodies were against Sirt1(1:1000), acetylated p65 (K310) (1:1000), p65 (NFκB subunit) (1:1000), acetylated-lysine (1:500) and PGC-1 alpha (1:1000). All antibodies, including primary and secondary antibodies, were purchased from Cell Signaling (Cell Signaling, Danvers, MA). HRP-conjugate secondary antibody was used to detect the protein of interest. Band intensities were analyzed using the ChemiDoc imaging system (BioRad, Hercules, CA, USA). Results were calculated using the Image Lab software as mentioned above (BioRad, Hercules, CA, USA).

Enzyme-Linked Immunosorbtent Assay:
To assess the impact of NR diet on inflammatory pathways, we also examined C-C chemokine receptor type 2 (CCR2) and its ligand, CCL2, as well as fractalkine receptor (CX3CR1). Mouse CX3CR1, CCL2 and CCR2 enzyme linked immunosorbent assay (ELISA) kits (Lifespan Biosciences, Seattle, WA) were used for the detection of these proteins as per manufacturer’s instructions. This procedure was followed for all CX3CR1, CCL2 and CCR2 ELISAs. Intra-Assay: CV<10.6% Inter-Assay: CV<12.6% for all the ELISA. There was no reported cross reactivity with other proteins for the primary antibody used in these kits.

Multiplex Cytokine assay
Cytokine levels in the plasma and brain were analyzed using Meso Scale Discovery (MSD) 96-Well MULTI-SPOT®Ultra-Sensitive V-PLEX Proinflammatory Panel 1 Mouse Kit, using electrochemiluminescence detection on an MSD Sector Imager™ 6000 with Discovery Workbench software (version 3.0.18) (MSD®, Gaithersburg). Cytokines were measured using the TH1/TH2 8-plex kit, which included 8 markers: Interferon gamma (IFN-γ), Interleukin-1β (IL-1β), IL-2, IL-4, IL-5, IL-10, IL-13 and Tumor necrosis factor-α (TNF-α). IL-4 and IL-13 were not detected in mouse brain and plasma. All assays were performed according to manufacturer’s instructions in duplicates. Plasma samples were diluted 1:2 and added to the plate, which contained the capture antibody immobilized on a working electrode. This procedure was followed as per manufacturer’s instructions. Results are expressed as the percentage to control.

Thiobarbituric Acid Reactive Substance assay:
Malondialdehyde (MDA) results from lipid peroxidation of polyunsaturated fatty acids. Since it is unstable, it is generally measured indirectly using a Thiobarbituric Acid Reactive Substance (TBARS) formed during the reaction of MDA with thiobarbituric acid. Levels of TBAR in brain homogenates were measured using a TBARS Assay Kit (Cayman Chemical, Ann Arbor #10009055) as per the manufacturer’s protocol.

Nicotinamide adenine dinucleotide assay:
Nicotinamide adenine dinucleotide (NAD) is a co-enzyme and is involved in redox reactions. As a result of electron transfer, NAD+ is reduced to NADH and NADH is oxidized to NAD+ forms. Levels of NAD were measured using a commercially available competitive ELISA kit (Cloud Clone, Houston# CEG409Ge). Briefly, NAD were extracted by homogenizing Brain in the PBS and brain NAD levels were normalized against total protein content determined by BCA. The assay used competitive inhibition enzyme immunoassay technique to measure total NAD in the
sample. The detection range of the kit is between 123.5-10,000 ng/mL. This assay reports no significant cross-reactivity with other NAD analogues. The kit is high sensitivity and specificity for detection of NAD.

**Acylcarnitine analyses:**

Acylcarnitine profiling is performed to biochemically screen for disorders of fatty acid oxidation (FAO) in some metabolic disorders. Acylcarnitines were quantified using a liquid-chromatography/mass spectrometry (LC/MS) assay as previously described with some modification. Briefly, 5 μl of labeled internal standard mix (Nsk-b-1, Cambridge isotope laboratories, MA, USA) were spiked into 100 μl of brain homogenate followed by an addition of 1000 μl of 25% methanol in acetonitrile (ACN) and then centrifuged at 20,000 x g for 20 min at 4 °C. Extracts were dried and resuspended in 100 μl of mobile phase A and centrifuged for 5 min at 10,000 x g. Supernatants were analyzed using a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA) coupled with a Shimadzu 20A HPLC System (Shimadzu scientific instrument, Columbia, Maryland), using a 2.1mm ID x 50mm Kinetex HILIC column containing 1.7 μm particles (Phenomenex, Torrance, CA, USA). A gradient was run from 95% solvent A (ACN: water:100mM ammonium formate, 90:5:5 v/v) in 5% solvent B (ACN: water:100mM ammonium formate, 50:45:5 v/v) to 70% B in 10 min with a 10 min hold at the final conditions. The flow rate was 250 μl/min with the column temperature at 40°C. Data were acquired by full scan MS in positive and negative modes with a mass range of 140-600 m/z (13 μscans/sec, spray voltage: 1500V, resolution: 17,500, max inject time: 200msec). List of target species along with retention time and precursor and product ions are provided in supplementary table 2. The Tracefinder™ software (Thermo Fisher Scientific, Waltham, MA) was used for peak identification and integration of acylcarnitine species in each run. Target compound lists of expected analytes for each chosen acylcarnitine species were used to find peaks of interest with ion windows of 5 ppm mass accuracy for each expected ion.

**Statistical analyses:**

For the human study, the Student’s t-test was used to examine group differences between GWI cases and controls. Neuropathological and protein marker data were analyzed using ANOVA to determine statistical significance. Normally distributed data were analyzed using parametric tests; otherwise non-parametric tests were used. For multiple groups where we examined the effects of GW chemicals and NR treatment, we generated a single variable incorporating GWI and NR status as different codes (control+ vehicle = 0, control + NR = 1, GWI+vehicle = 2, and GWI+NR = 3) and one-way analysis of variance (ANOVA) was conducted followed by the Tukey post-hoc test to examine the independent effect of each exposure. These analyses were performed using the GraphPad Prism7 software (San Diego, CA, USA), and data were expressed as means ± standard deviation. All graphs were generated using GraphPad Prism7 (San Diego, CA). For the acylcarnitine study, after data analysis using MLM, Fisher’s least significant difference (LSD) followed by Benjamini–Hochberg (B-H) procedure test was used for performing multiple-testing correction and to control for the false discovery rate (FDR) for all species between all groups. Acylcarnitine data were analyzed using SPSS version 22.0.0 (IBM Corporation, Armonk, NY). Since human NAD and Sirt1 data were not normally distributed, Kolmogorov-Smirnov test (K-S test) were performed to check for significance. All statistical analyses were performed as two-tailed test and two-tailed p < 0.05 was considered significant.
**Result**

**GWI is associated with low NAD and Sirt1 levels in the plasma**

We examined plasma NAD and Sirt1 protein levels in PBMC in a pilot cross-sectional study of age- and gender-similar healthy GW veterans and those with GWI (see demographics in Table 1). These studies showed that NAD (t-test\(\text{df}=12\) = 8.51, \(p = 0.002\), Figure 2A) and Sirt1 (t-test \(\text{df}=16\) = 22.75, \(p = 0.083\), Figure 2B) levels were decreased in veterans with GWI compared to controls. There was a 2-fold decrease in NAD and 1.6-fold decrease in Sirt1 level in GWI compared to healthy controls.

**Nicotinamide riboside improves fatigue-like behavior in GWI mice**

To determine whether NR treatment reduced fatigue-like behavior in GWI mice, we performed the FST at 2-months post-treatment with NR (8-months post-exposure). There was no difference between the groups for a change in total immobile time (F\(\text{3,26}\) = 1.32, \(p = 0.28\), Figure 3A). Also, the mean velocity did not differ between the exposure and the treatment groups (Figure 3B). There were significant differences between the groups when we examined ∆ immobile time between the first and the second half of the FST (F\(\text{3,22}\) = 4.2, \(p = 0.01\); Figure 3C). *Post-hoc* analysis showed a significant difference between GWI and control mice as well as GWI mice treated with NR compared to GWI mice on control diet (\(p = 0.05\)).

**Nicotinamide riboside treatment helps recover normal NAD levels in GWI mice**

Since NR is shown to increase the bioavailability of NAD, we examined NAD levels and found that there was a significant effect of the GWI status and NR treatment on NAD levels in the brain (F\(\text{3,12}\) = 7.33, \(p = 0.004\), Figure 3D) and plasma (F\(\text{3,12}\) = 12.33, \(p < 0.001\), Figure 3E). In the brain, *post-hoc* analyses showed that although there were no group differences in NAD levels between untreated GWI and control mice (\(p > 0.05\)), both GWI and control mice treated with NR had increased NAD levels (\(p < 0.05\)). In plasma, *post-hoc* analysis showed that NAD levels were decreased in GWI compared to control mice (\(p < 0.05\)) and NR treatment increased NAD levels in GWI mice (\(p < 0.01\); Figure 4B).

**Nicotinamide riboside treatment elevates Sirt1 and Sirt3 expression**

We examined Sirt1 and Sirt3 levels as these proteins use NAD+ for deacetylation reactions. Since sirtuins are primary targets of NR, we examined their protein levels and observed a significant effect of NR and GWI status on Sirt1 protein levels across all groups (F\(\text{3,20}\) = 37.48, \(p = 0.001\); Figure 4A). *Post-hoc* analyses showed lower Sirt1 levels in GWI compared to control mice (\(p < 0.05\)), whereas Sirt1 levels were higher in GWI mice treated with NR (\(p < 0.05\); Figure 5A). We also observed group differences in Sirt3 levels (F\(\text{3,12}\) = 5.48, \(p = 0.001\); Figure 4B) with *post-hoc* analyses showing that there were no differences between GWI and control mice for Sirt3 levels on normal diet (\(p > 0.05\)). However, relative to control diet, NR treatment increased Sirt3 levels by 4-fold in both control (\(p < 0.001\)) and GWI mice (\(p < 0.001\); Figure 4B).

**Nicotinamide riboside increases markers of mitochondria biogenesis in control and GWI mice**

We further investigated the levels of PGC-1α protein associated with mitochondrial biogenesis and UCP-2, a mitochondrial membrane protein that is transcriptionally regulated by
PGC-1α and is involved in thermogenesis and ROS production. Levels of PGC-1α were elevated in the brain following NR treatment ($F_{(3,9)} = 0.18$, $p = 0.03$; Figure 5B). While post-hoc analysis showed no differences in PGC-1α expression between GWI and control mice ($p > 0.05$), PGC-1α protein levels were increased by NR treatment in both control and GWI mice ($p < 0.01$; Figure 5A). We examined acetylated lysine residues of PGC-1α immunoprecipitated from isolated mitochondria as a surrogate for measuring Sirt3 activity. We observed a significant effect of NR and GWI status on acetylated PGC-1α levels across all groups ($F_{(3,9)} = 7.14$, $p = 0.001$; Figure 6B). The post-hoc analyses showed acetylated PGC-1α protein levels to be significantly increased in GWI compared to control mice ($p < 0.05$) and significantly lower in GWI mice treated with NR ($p < 0.05$, Figure 5C). There was a significant difference between the study groups for UCP-2 protein levels ($F_{(3,8)} = 10.33$, $p = 0.03$; Figure 5C). While post-hoc analyses showed no change in UCP-2 expression between untreated control and GWI mice ($p > 0.05$), NR treatment increased UCP-2 expression in both control ($p < 0.05$) and GWI mice ($p < 0.05$; Figure 5C).

**Brain acylcarnitine alterations in GWI mice were normalized by NR treatment which corresponded with reduced oxidative stress and bioenergetics related markers**

Acylcarnitines are mitochondria-specific lipids that transport fatty acid for β-oxidation in mitochondria and shuttle short- and medium-chain fatty acids between peroxisomes and mitochondria. Short-chain acylcarnitine (SCAC) species were affected across all groups ($F_{(3,8)} = 22.068$, $p = 0.01$; Figure 6A). Post-hoc analyses showed that SCAC were significantly upregulated in the brains of GWI mice compared to controls ($p = 0.01$; Figure 6A). This effect in GWI mice was normalized to similar levels in control mice after 2-months of NR treatment in GWI mice ($p = 0.03$). However, there were no significant differences between the study groups for medium-chain acylcarnitine (MCAC; 6–14 carbons) species ($F_{(2,11)} = 4.14$, $p = 0.61$; Figure 6A) or long-chain acylcarnitine (LCAC; 14–20 carbons) species ($F_{(4,12)} = 2.84$, $p = 0.041$; Figure 6A). Very long chain acylcarnitine (VLAC; >22 carbons) species differed across all four experimental groups ($F_{(3,12)} = 10.29$, $p = 0.002$, Figure 6A). Post-hoc analysis showed a significant increase in these species in the brains of GWI mice compared to control mice ($p = 0.009$) and these levels were reduced to control levels in NR treated GWI mice ($p = 0.03$). Figure 6B shows a heatmap of individual species along with their fold changes compared to control, which demonstates that several acylcarnitine species were partially or fully normalized in NR treated GWI mice. Levels of TBARS, indicative of lipid peroxidation, were altered in the brains of GWI and NR-treated mice ($F_{(3,8)} = 65.068$, $p = 0.0001$; Figure 6C). Post-hoc analyses showed high TBARS levels in brains of GWI mice compared to control mice ($p < 0.05$) and these levels were lower in the brains of NR treated GWI compared to GWI mice on control diet ($p < 0.05$). When examining several markers of mitochondrial bioenergetics, an increase in pyruvate precursor, phosphophenol pyruvate, and a decrease in pyruvate itself in GWI mice (supplementary Figure 4). The pyruvate dehydrogenase ultimately regulates conversion of pyruvate to acetyl-CoA for its entry in the Krebs cycle. We then examined pyruvate dehydrogenase level and found its levels to be lower in the brains of GWI mice but increased after NR treatment (supplementary Figure 5).

**Sirt1 directly interacts with p65 and deacetylated p65**

To determine the effects of Sirt1 on NFκB acetylation, we examined Sirt1 immunoprecipitate for NFκB p65 subunit protein and acetylation levels. We found that Sirt1
associated p65 protein levels were significantly altered in GWI and NR treatment groups ($F_{(3,12)} = 8.068, p = 0.0026$; Figure 7A). Post-hoc analyses showed that this interaction was decreased in GWI mice as compared to control mice ($p < 0.05$). This interaction was partially restored in NR treated GWI mice compared to GWI mice on normal diet ($p < 0.05$). We examined lysine at residue 310 of p65 to determine deacetylation of p65 by Sirt1 and this showed a group difference ($F_{(3,12)} = 5.068, p = 0.01$; Figure 7B) with post-hoc analyses showing that GWI mice had an increase in acetylated p65 compared to control mice ($p < 0.05$). On the contrary, NR treated GWI mice had significantly reduced p65 acetylation compared to GWI mice on control diet ($p < 0.05$) and in NR treated control mice compared to those on normal diet ($p < 0.05$). We next examined phosphorylation of the p65 subunit and found it to differ significantly between GWI and NR treatment groups ($F_{(3,8)} = 8.68, p = 0.001$; Figure 7C). Post-hoc analyses showing higher phosphorylated p65 to total p65 ratios in GWI compared to control mice ($p < 0.05$), whereas NR-treated GWI mice had lower ratios of phosphorylated p65 to total p65 protein levels compared to GWI mice on normal diet ($p < 0.05$; Figure 9).

**Nicotinamide riboside treatment reduces chronic brain inflammation in GWI mice.**

To assess the impact of NR on inflammation, we examined both pro- and anti-inflammatory cytokine levels. Pro-inflammatory cytokines, especially IL-1β ($F_{(4,16)} = 8.68, p = 0.001$; Figure 7E), IFN-γ ($F_{(3,12)} = 5.16, p = 0.003$; Figure 7E) and IL-6 ($F_{(2,8)} = 7.86, p = 0.03$; Figure 7E), were significantly different across all four groups. Post-hoc analyses showed increased expression of several pro-inflammatory cytokines in the brains of GWI mice compared to controls ($p < 0.05$), whereas the brains of NR-treated GWI mice had significantly lower levels compared to GWI mice on normal diet ($p < 0.05$; Figure 7E). Similarly, these cytokines were also upregulated in plasma of GWI mice (Supplementary Figure 6). Chronic NR intervention normalized these pro-inflammatory cytokines in the plasma of GWI mice (Supplementary Figure 6). Since chronic upregulation of IL-6 phosphorylates STAT3 (p-STAT3), we examined its levels in the brains of GWI and control mice, with or without NR treatment. Significant changes in p-STAT3 with respect to GWI and NR treatment ($F_{(3,8)} = 19.068, p = 0.01$; Figure 7D) were detected. The levels of p-STAT3 were elevated in the brains of GWI mice and normalized to control levels in NR-treated GWI mice ($p = 0.034$). There was a significant effect of NR and GWI status on the brain CCL2 ($F_{(1,4)} = 7.46, p = 0.041$, Figure 7F) and CCR2 ($F_{(3,9)} = 7.068, p = 0.01$, Figure 7F) levels, with post-hoc analyses showing increased levels in the brains of GWI mice compared to control and NR-treated GWI mice ($p < 0.05$; Figure 7F).

**Nicotinamide riboside reduced astroglia activation in GWI mice**

We examined GFAP to assess astroglia activation and Iba1 to assess microglia changes after NR treatment. We observed differences in GFAP staining in GWI and NR-treated mice within the dentate gyrus (DG) ($F_{(3,12)} = 15.29.48, p = 0.002$, Supplementary Figure 7A) and post-hoc analysis showed a significant increase in GFAP staining within the DG of GWI mice compared to control mice ($p < 0.05$). Brain GFAP staining was reduced in NR treated GWI mice as compared to GWI mice on a normal diet ($p < 0.05$, Supplementary Figure 7B). The level of GFAP staining in the cortex also showed significant changes across all groups ($F_{(3,12)} = 4.19.48, p = 0.42$; Supplementary Figure 7B) and post-hoc analysis showed a significant difference between GWI and control mice, as well as between GWI mice treated with NR compared to those on control diet ($p < 0.05$). There were no differences across any of the experimental groups.
for microglia staining with Iba1 in the DG (F(3,8) = 6.98, p = 1.72; Supplementary Figure 8A) or cortex (F(3,10) = 4.19, p = 3.42; Supplementary Figure 8B).

**Discussion**

Dysregulated mitochondria function in GWI is characterized by altered bioenergetics molecules, increased oxidative stress, and abnormal levels of mitochondrial metabolites in mouse models and in veterans with GWI\(^3\text{-}^5,^9\). We show significant decreases in NAD and Sirt1 protein levels in veterans with GWI, which were also seen in a mouse model of GWI. Since NR is shown to specifically target NAD recovery\(^32,^40\), we examined its effects in a GWI mouse model and showed that NR treatment increased NAD levels and sirtuin protein and activity in GWI mice. This corresponded with increased expression of proteins associated with mitochondria biogenesis and normalized mitochondria-specific lipids. Increases in sirtuin activity after NR also reduced neuroinflammation and fatigue-like behavior after NR treatment in GWI mice. These clinical studies collectively suggest that NR has high oral bioavailability, is well-tolerated and increases NAD levels. Potential mechanisms of these effects discussed below may be useful in further examining the biological mechanism to explore NR as a GWI treatment.

While there are several redundant pathways for maintaining cellular NAD, their levels diminish during metabolic stress, aging and in certain neurodegenerative conditions\(^41\). Our observation of reduced plasma NAD in veterans with GWI compared to control GW veterans suggests that this bioenergetics system is associated with GWI pathophysiology. These findings are also consistent with previous studies showing reduced mitochondria metabolites in the muscles and blood of veterans with GWI\(^3,^6\). These findings are also consistent with reports of reduced NADPH/NADH in Chronic Fatigue Syndrome, a condition with similar clinical presentation to GWI. Our cross-sectional study showed decreases in plasma NAD levels in ill GW veterans compared to healthy GW veterans. An examination of NAD in our mouse model showed a trend for a decrease in the brain and a significant decrease in plasma NAD of GWI mice. Furthermore, our observations of reduced NAD in plasma of GWI mice and recovery of NAD in plasma after NR treatment suggest potential benefits of NR treatment in GWI for restoring NAD levels.

We observed a decrease in fatigue-like behavior in GWI mice after NR treatment. Chronic fatigue is one of the primary symptoms of GWI\(^42\); among veterans deployed to the 1991 GW, 23% continue to suffer from fatigue\(^42,^43\). Fatigue in humans is associated with significantly altered brain structure, specifically frontotemporal cortical thinning and reduction in hippocampal volume\(^44\). Changes in these brain structures were also observed in ill GWI veterans\(^45,^46\). Post exertional malaise resulting after a physiological stressor is a major complaint of ill GWI veterans\(^8\). Our mouse study also shows a similar phenotype, with mice freely swimming in the first half of the FST but increasing in immobility in the second half\(^10\). This exertional fatigue type phenotype was lower in GWI mice treated with NR compared to GWI mice on normal diet. While the mechanism behind fatigue and subsequent improvement after NR treatment remains to be fully investigated, our findings do suggest that increasing the bioavailability of NAD may reduce fatigue symptoms in ill GW veterans, possibly by increasing ATP availability.

Bioavailability of NAD\(^+\) is critical for maintaining normal bioenergetics since it serves as a co-factor for many enzymes involved in metabolic processes that support cellular bioenergetics. Among these, sirtuins are NAD\(^+\)-dependent deacetylases that support energy production by promoting lipid metabolism and increasing bioenergetics\(^47,^48\). Our studies
suggested that Sirt1 expression was lower in the blood of veterans with GWI and in the brains of GWI mice. Studies also show that NAD not only affects sirtuin activity, but also increases the protein expression of sirtuin\textsuperscript{27}. In that regard, we observed that NR treatment increased the expression of Sirt1 and Sirt3 in both GWI and control mice. Interestingly, total PGC-1\(\alpha\) levels increased only after NR treatment, but can be a direct consequence of Sirt1’s influence on PGC-1\(\alpha\) gene transcription\textsuperscript{19}. Uncoupling protein-2 is one of the PGC-1\(\alpha\) target genes, which promotes mitochondria biogenesis and mitochondria uncoupling to reduce oxidative stress without compromising ATP production\textsuperscript{30,31}. As UCP-2 levels were increased following NR treatment in both control and GWI mice, it is possible that some of the beneficial effects of NR-induced sirtuin activation may be mediated through PGC-1\(\alpha\) pathways which warrants further investigation.

Additional benefits of NR treatment included decreases in the long chain fatty acid containing acylcarnitine species which accumulated in the brains of untreated GWI mice once treated with NR, suggesting improvements in mitochondrial \(\beta\)-oxidation as these acylcarnitines are preferentially metabolized in mitochondria. This is further supported by decreases in lipid peroxidation after NR treatment in GWI mice, which is also consistent with action by sirtuins on restoring redox balance following NR treatment\textsuperscript{32}. Collectively, these results suggest that PGC-1\(\alpha\) deacetylation by sirtuins leads to increased PGC-1\(\alpha\) protein levels which may contribute to mitochondria biogenesis which requires further investigation. Additional evidence of bioenergetic deficits come from our observations of high phosphoenolpyruvate but low pyruvate and decreases in pyruvate dehydrogenase in the brains of GWI mice. Sirt3 deacetylates pyruvate dehydrogenase and regulates activity of pyruvate dehydrogenase\textsuperscript{33}. Chronic NR treatment for 2 months corrected these metabolite levels and pyruvate dehydrogenase deficiency. Since pyruvate fuels the Krebs’s cycle, this may lead to increases in ATP production, thereby improving bioenergetic in the brains of GWI mice.

Sirtuins also influence inflammatory processes via deacetylation of p65 in an NAD dependent manner, resulting in NF\(\kappa\)B inhibition and decreased pro-inflammatory cytokines\textsuperscript{54–56}. We observed low Sirt1 p65 levels as well as low p65 acetylation in the brains of GWI mice compared to control and GWI mice. However, NR treatment was able to reverse these effects, thereby restoring p65 acetylation in NR-treated GWI mice. As expected, this corresponded with reduced levels of pro-inflammatory cytokines (IL-1\(\beta\) and IFN-\(\gamma\)) in NR-treated GWI mice. In addition, NR treatment also decreased levels of the chemokine CCL2 and chemokine receptors (CX3CR1 and CCR2) as well as STAT3 phosphorylation in the brains of GWI mice. Furthermore, these anti-inflammatory effects of NR corresponded with reduced astroglia in the cortex and the hippocampus of treated GWI mice. This is also consistent with other studies showing a noticeable decrease in reactive astrocytes and astrogial proliferation following NR treatment in Alzheimer’s Disease\textsuperscript{19}. These results suggest that the observed anti-inflammatory effects of NR are likely mediated through increased activity of Sirt1 and may be useful in therapeutic targeting of inflammation associated with GWI.

Despite the small sample size, we detected decreases in NAD levels and Sirt1 protein levels in the blood of veterans with GWI in our pilot study, which clearly warrant further investigation in a larger cohort. Our preclinical studies showed that NR can target several aspects of GWI, including fatigue, lipid peroxidation and inflammation\textsuperscript{10}. Once confirmed using larger studies, we propose that strategies to increase NAD levels via NR intervention will likely be therapeutically beneficial in GWI.
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**Conflict of statement:**
The author(s) declares no competing interests. The contents do not represent the views of the Department of Veterans Affairs or the United States Government.

**Availability of data and materials**
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Reference:**


Legends

Figure 1: Timeline of NR study design

Figure 2: Plasma NAD and Sirt1 levels in veterans with GWI compared to GW control veterans. Mean ± SD. (A) NAD and (B) Sirt1 (n = 6 for controls and n = 12 for GWI) are altered in veterans with GWI compared to controls. T-test *p ≤ 0.05 **<0.001.

Figure 3: Forced swim test data showed NR treatment reduces fatigue-like behavior in GWI mice. Mean ± SD of control (n = 8), GWI mice (n = 8), control + NR (n = 7) and GWI+NR (n = 8). (A) Two months after NR treatment (7-months post-exposure to GW chemicals), immobile time had an increasing trend in GWI mice compared to controls and was reduced in NR treated GWI. (B) There was no change in mean velocity among all 4 groups. (C) Δ immobile time representing difference between two halves across all groups where GWI had elevated Δ immobile time than control mice and lower in GWI treated with NR diet compared to GWI mice. ANOVA with post-hoc Tukey * p ≤ 0.05. (C-D) Brain NAD levels did not differ between control and GWI mice but plasma NAD levels were lower in GWI compared to control mice. Intervention with NR increases brain and blood NAD level in GWI and control mice. ANOVA with post-hoc Tukey’s test * p ≤ 0.05.

Figure 4: Sirtuin 1 and 3 (targets of NR) changes in GWI mice. Mean ± SD (arbitrary value n = 6 per group) (A-B) Western blot analysis demonstrates decline in Sirt1 in GWI and no change in Sirt3 levels between control and GWI mice. The expression of Sirt1 and Sirt3 was upregulated by NR treatment in both control and GWI mice. ANOVA with post-hoc Tukey’s test * p ≤ 0.001.

Figure 5: Brain UCP 2 and PGC-1α levels increase after NR treatment. Mean ± SD (shown as arbitrary units, n = 6 per group). (A) Treatment with NR increased UCP-2 levels in both GWI and control mice compared to those on regular diet within respective groups. (B) Levels of PGC-1α were elevated in NR-treated GWI and control mice compared to their respective groups on normal diet. (C) PGC-1α immunoprecipitation showed increased acetylated PGC-1α in GWI mice compared to control mice and NR treatment increased deacetylated PGC-1α in GWI mice compared to untreated GWI mice. (D) A graphical depiction of how NR treatment decreases PGC-1α acetylation by activating Sirt3. ANOVA with post-hoc Tukey’s test * p ≤ 0.05

Figure 6: NR treatment normalizes increased acylcarnitine in the brain of GWI mice. Mean ± SD (n = 4 per group). (A) Several short-, medium- and long-chain FA-containing acylcarnitine species were elevated in the brains of untreated GWI mice and were reduced in GWI mice treated with NR. (B) Heatmap showing fold change to control for individual acylcarnitine species. ANOVA with post-hoc Tukey * p ≤ 0.05. (C) Lipid peroxidation measured by TBARS showed that GWI mice have higher TBARS level compared to all other groups. ANOVA with post-hoc Tukey’s test * p ≤ 0.05.

Figure 7: Sirt1 deacetylates p65, dephosphorylates NFkB and normalizes inflammation in GWI mice brain. Mean ± SD (shown as arbitrary units n = 6 per group). (A) Analysis of p65 using immunoblotting on Sirt1 immunoprecipitated demonstrates that NR treatment increases the interaction of Sirt1 with p65. (B) Acetylation assays were performed on p65 immunoprecipitated which showed increased acetylated lysine in GWI mice. Deacetylation of p65 at k 310 was higher in NR treated GWI mice. ANOVA with post-hoc Tukey * p ≤ 0.05. (C-D) The ratio of p-p65/p65 as well as p-STAT3/STAT3 was significantly increased in GWI mice. NR treatment normalizes both p65 and STAT3 phosphorylation in GWI mice. ANOVA with post-hoc Tukey * p ≤ 0.05. (E) Cytokine assay showed significant increases in the brain’s IFN-γ, IL-6, and IL-1β level in untreated GWI mice but were lower in NR treated GWI mice. (F) Similarly, CCR2 and CX3CR2 chemokine levels were elevated in GWI mice compared to control mice, but these levels were reduced in GWI mice treated with NR. ANOVA with post-hoc Tukey’s test * p ≤ 0.05.
Figure 2

A

NAD

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Controls | GWI cases

B

Control | GWI case | Positive control

SirT1 120KD

Actin 45KD

SirT1 human

Control | GWI case

0.0 | 0.2 | 0.4 | 0.6

Control | GWI case

0.0 | 0.1 | 0.2 | 0.3 | 0.4 | 0.5
Figure 3

A) Immobile frequency

B) FST Speed

C) Δ immobile time ± SD

D) Brain NAD

E) Plasma NAD
Figure 4

**A**

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**B**

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Sirt1/Actin

Sirt3/Actin
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**Figure 5**

- **A**
  - PGC-1α/Actin
  - Fold change ± SD

- **B**
  - UCP 2/Actin
  - Fold change ± SD

- Control
- GWI
- Control + NR
- GWI + NR
Figure 5

C

IB: Acetylated lysine
IP: PGC-1α

GWI  Control  GWI + NR  Control + NR
Ac:PGC-1α

D

Low energy status

NAD+ → Sir T3

NR treatment

High energy status

PGC-1α alpha

Active

Inactive

Acetylated PGC-1α/PGC-1α

Fold change ± SD

Control  Control + NR  GWI  GWI + NR

* *
Figure 6

A) Bar chart showing the percent change in carnitine levels for different groups. The groups are labeled as Control (DMSO), Control+NR, GWI (PB+PER), and GWI+NR. The y-axis represents the percent change, and the x-axis shows different carnitine levels (C2:0 to C24:0).

B) Heatmap showing the fold change to control for different carnitine levels under different conditions. The conditions are Control+NR, GWI, and GWI+NR. The x-axis represents different carnitine levels (C2:0 to C24:0), and the y-axis represents the percent change. The color bar on the right indicates the fold change range from -1 to 2.

C) Bar chart showing the TBARS levels in different groups. The groups are labeled as SCAC, MCAC, LCAC, and VLCAC. The y-axis represents the TBARS levels, and the x-axis shows different conditions.
Figure 7

A) IP: SirT1, IB: p65

GWI | Control | Control + NR | GWI + NR | mock cells

IP: SirT1
IB: p65

Fold change ± SD

B) IP: p65, IB: Acetylated lysine

GWI | Control | GWI + NR | Control + NR | mock cells

IP: p65
IB: Acetylated lysine

Fold change ± SD

Acetylated Lysine K 310
p65 (Input) 65KD

Fold change ± SD
Figure 7

C

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Fold change. ± SD

D

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Fold change. ± SD
Figure 7

E

Brain cytokine

F

Chemokine

Percent control ± SD

Control

Control+NR

GWI

GWI+NR

IFN-γ
IL-10
IL-1β
IL-2
IL-5
IL-6
TNF-α

CCL2
CCR2
CX3CR1

* indicates statistical significance.