Eliciting the Mitochondrial Unfolded Protein Response by Nicotinamide Adenine Dinucleotide Repletion Reverses Fatty Liver Disease in Mice

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With no approved pharmacological treatment, nonalcoholic fatty liver disease (NAFLD) is now the most common cause of chronic liver disease in Western countries and its worldwide prevalence continues to increase along with the growing obesity epidemic. Here, we show that a high-fat high-sucrose (HFHS) diet, eliciting chronic hepatosteatosis resembling human fatty liver, lowers hepatic nicotinamide adenine dinucleotide (NAD⁺) levels driving reductions in hepatic mitochondrial content, function, and adenosine triphosphate (ATP) levels, in conjunction with robust increases in hepatic weight, lipid content, and peroxidation in C57BL/6J mice. To assess the effect of NAD⁺ repletion on the development of steatosis in mice, nicotinamide riboside, a precursor of NAD⁺ biosynthesis, was added to the HFHS diet, either as a preventive strategy or as a therapeutic intervention. We demonstrate that NR prevents and reverts NAFLD by inducing a sirtuin (SIRT1)- and SIRT3-dependent mitochondrial unfolded protein response, triggering an adaptive mitohormetic pathway to increase hepatic β-oxidation and mitochondrial complex content and activity. The cell-autonomous beneficial component of NR treatment was revealed in liver-specific Sirt1 knockout mice (Sirt10−/−), whereas apolipoprotein E-deficient mice (Apoe−/−) challenged with a high-fat high-cholesterol diet affirmed the use of NR in other independent models of NAFLD. Conclusion: Our data warrant the future evaluation of NAD⁺ boosting strategies to manage the development or progression of NAFLD. (HEPATOLOGY 2016;63:1190–1204)

Nonalcoholic fatty liver disease (NAFLD) encompasses a disease spectrum that can progress from hepatic steatosis to nonalcoholic steatohepatitis (NASH), fibrosis, and, finally, cirrhosis1 and is projected to become the most common indication leading to liver transplantation in the United States by 2030.2 Unfortunately, there are few existing therapies for NAFLD and NASH3,4 which are also suboptimal, hence the urge to find new preventive and therapeutic strategies to manage these chronic hepatic diseases. Often, alterations in lipid homeostasis, associated with obesity or insulin resistance, result in the increase of lipolysis in fat and delivery of free fatty acids

Abbreviations: Ad-GFP, adenovirus virus expressing green fluorescent protein; ADP, adenosine diphosphate; Alb, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ANOVA, analysis of variance; ATP4, activating transcription factor 4; ATP, adenosine triphosphate; CD, chow diet; COX, cytochrome c oxidase; CS, citrate synthase; dH2O, double-distilled water; ER, endoplasmic reticulum; FA, fatty acid; GEO, Gene Expression Omnibus; GRP78, glucose-regulated protein 78; H&E, hematoxylin and eosin; HFC, high-fat high-cholesterol diet; HFHS, high-fat high-sucrose diet; HNE, 4-hydroxynonenal; KO, knockout; LF, low-fat; LOF, loss of function; LPO, lipid peroxidation; mRNA, messenger RNA; mtDNA, mitochondrial DNA; NAD⁺, nicotinamide adenine dinucleotide; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; nDNA, nuclear DNA; NMN, nicotinamide mononucleotide; NR, nicotinamide riboside; OCR, oxygen consumption rate; OXPHOS, oxidative phosphorylation; PAGE, polyacrylamide gel electrophoresis; PAR, poly(ADP)-ribose; PARPs, poly(ADP-ribose) polymerases; PPAR, peroxisome proliferator-activated receptor; qPCR, quantitative polymerase chain reaction; SDH, succinate dehydrogenase; SEM, standard error of the mean; SIRT, sirtuin; TG, triglyceride; TNF-α, tumor necrosis factor alpha; UPR, ER-specific unfolded protein response; UPRer, mitochondrial unfolded protein response.

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to the liver, favoring hepatic lipogenesis.\(^5\) In addition, incomplete fatty acid (FA) oxidation in the context of mitochondrial dysfunction can also contribute to hepatic FA and lipid overload (reviewed in a previous study\(^6\)). With prolonged existence, these alterations in lipid homeostasis can promote oxidative stress and lipid peroxidation (LPO) in the liver, leading to elevated cytokine production, inflammation, and fibrosis. It is this distinction that divides the appearance of a fatty liver with that of the appearance of NASH and, eventually, hepatocellular carcinoma\(^7,\)\(^8\) Because generation of oxidative stress is one of the key mediators of this disease, the pursuit of treatments to improve the dysfunctional state of liver mitochondria has become a prime goal to alleviate the pathophysiology of NASH.

Recent evidence demonstrates that nicotinamide adenine dinucleotide (NAD\(^+\)) repletion protects organisms from metabolic diseases, induced by genetic factors, diet, or aging\(^9-\)\(^14\) These findings corroborate the role of sirtuins, many of which rely on NAD\(^+\) as a cosubstrate, in eliciting metabolic improvements in various tissues (reviewed in a previous study\(^15\)). However, despite the rapidly advancing field, the role of NAD\(^+\) in the development and protection from NAFLD is still unspecified. Furthermore, the influence of NAD\(^+\) repletion on mice with preexisting liver hyperlipidemia and aberrant lipid metabolism has yet to be determined.

Several approaches have been recently explored to elevate NAD\(^+\) levels \emph{in vivo}. This includes the use of natural NAD\(^+\) precursors, such as nicotinamide riboside (NR), that are readily converted into bioavailable NAD\(^+\) in metabolic tissues after dietary administration\(^16\). In addition to NR, there are three other molecules described as root substrates for mammalian NAD\(^+\) biosynthetic pathways, including tryptophan, niacin, and nicotinamide\(^15\). Once NR is transported into cells by nucleoside transporters\(^17\), it is phosphorylated by NR kinases 1 and 2\(^15\). Phosphorylation of NR generates nicotinamide mononucleotide (NMN), an intermediate compound of NAD\(^+\) biosynthesis, which can also be intraperitoneally administered to animals to increase tissue NAD\(^+\) levels\(^11\). This metabolite can then be converted to NAD\(^+\) through the action of NMN adenylyltransferase.

In this study, we demonstrate that a Western diet, including high levels of fat and sucrose, could induce dysfunction in hepatic NAD\(^+\) homeostasis and contribute to the development of NAFLD, and that NAD\(^+\) repletion by feeding animals with a diet enriched in NR may

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prevent or reverse NAFLD by inducing the mitochondrial unfolded protein response (UPRmt) to elicit a mito-
hormetic response. We further confirmed that NAD+ repletion can reverse liver mitochondrial dysfunction in
HFC-fed Apoe−/− mice, another well-known model of
NAFLD.(19)

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 21-23°C
and had ad libitum access to water and food throughout
the experiment. From the age of 8 weeks, mice were
separated into four groups of 10 animals. Animal
cohorts were fed either a Western high-fat and high-
sucrose (HFHS) diet with 44.6% of kcal derived from
fat (of which 61% is derived from saturated fatty acids)
and 40.6% of kcal derived from carbohydrates (primarily
sucrose 340 g/kg diet) (TD.08811, 45% kcal Fat Diet;
Harlan Laboratories Inc., Madison, WI)(20) or normal
chow diet (CD). NR-treated animals were fed with pel-
lets containing vehicle (double-distilled water; ddH2O)
or NR (400 mg/kg/day) for 2, 9, or 18 weeks. All ani-
mal experiments were carried according to national
Swiss and European Union ethical guidelines and
approved by the local animal experimentation commit-
tee of the Canton de Vaud under license #2465.

Generation of Sirt1L2/L2 mice has been previously
described.(21) Liver-specific Sirt1 knocko ut mice were
generated by breeding Sirt1L2/L2 mice with mouse albu-
mun (Alb)-Cre mice (AlbcreTg/0), (22) both of which have
been backcrossed to C57BL/6J mice for 10 generations.
These mice lines were then further intercrossed to gen-
erate mutant AlbcreTg0/Sirt1L2/L2 mice, which were termed
Sirt1bpb−/− mice. The following Sirt1bpb−/− and Sirt1L2/L2
mice were fed with HFHS pellets containing vehicle
(ddH2O) or NR (400 mg/kg/day) for 14 weeks.

Six-week-old male Apoe−/− mice were purchased from
The Jackson Laboratory (Bar Harbor, ME) and randomly
assigned to modified AIN-93M diets (Dyets, Inc., Beth-
elehem, PA) that included low-fat (Apoe−/−/LF), high-fat
and high-cholesterol (Apoe−/−/HFC), and HFC supple-
mented with NR (Apoe−/−/NR-Ther; see Supporting
Table 1). Seven-week-old mice were placed on either LF
(4% fat by weight) or HFC (22% fat, 0.2% cholesterol by
weight) diets for 3 weeks to induce aberrant lipid metabo-
lim. At 10 weeks of age, 500 mg/kg/day (mpk) of NR
was supplemented to the assigned HFC diet group for 7

weeks. All procedures concerning Apoe−/− mice were
approved by the Animal Care and Use Committee at the
University of Connecticut (Storrs, CT).

The procedures used for NR food preparation, along
with in vivo mouse phenotyping and sacrifices, are
described in the Supporting Methods.

PRIMARY HEPATOCYTES

Primary hepatocytes from Sirt1 floxed (Sirt1L2/L2) or
Sirt3 floxed (Sirt3L2/L2) mice (described in previous
studies(21,23), respectively) were isolated with Liberase
Blendzyme (Roche Diagnostics, Indianapolis, IN) perfu-
sion as described previously, with minor modifications.(24)
Isolated hepatocytes were then plated in Dulbecco’s
modified Eagle’s medium (4.5 g/L of glucose; Gibco,
Gaithersburg, MD) with 10% fetal bovine serum and
penicillin and streptomycin and maintained at 37°C in a
5% CO2 atmosphere. Four hours after plating, cells were
transduced with adenovirus expressing green fluorescent
protein (Ad-GFP) or Cre recombinase (Ad-Cre) at a
multiplicities of infection = 5 to generate matched
Sirt1L2/L2 or Sirt3L2/L2 (loss-of-function) and Sirt1L2/L2 or
Sirt3L2/L2 wild-type hepatocytes, respectively. Hepato-
cytes were then incubated overnight before changing
media. Primary hepatocytes were then treated with 1
mM of NR or vehicle (ddH2O) for 24 hours.

HISTOLOGY AND LIVER
FUNCTION

Preparation of histological tissue sections, staining
procedures for hematoxylin and eosin (H&E), Oil Red
O, Picrosirius red, cytochrome c oxidase activity, succi-
nate dehydrogenase activity, CD45, Masson’s tri-
chrome, and blinded scoring for steatosis are described
in the Supporting Methods. Mitochondrial function in
fresh liver tissue was evaluated with high-resolution
respirometry and citrate synthase activity, as also
described in the Supporting Methods.

QUANTIFICATION OF NAD+ AND
ADENOSINE TRIPHOSPHATE
LEVELS

NAD+ was extracted using acidic and alkaline extrac-
tion methods, respectively. Tissue NAD+ was analyzed
with mass spectrometry, as previously described.(25) Total
adenosine triphosphate (ATP) content was measured by
the CellTiter-Glo luminescent cell viability assays (Prom-
ega). Typically, luminescence was recorded with a Victor
X4 plate reader (PerkinElmer, Waltham, MA), and values were normalized by the total protein concentration, determined using a Bradford assay.

**LIVER TRIGLYCERIDE, CHOLESTEROL, AND LPO MEASUREMENTS**

Hepatic lipids were extracted as performed previously. Triacylglyceride (TG) and cholesterol contents in hepatic lipid fractions were quantified using enzymatic assays (Roche). The by-product of LPO and a marker of oxidative stress, 4-hydroxynonenal (HNE), was measured following the manufacturer’s protocol of the OxiSelect HNE-His Adduct enzyme-linked immunosorbent assay Kit (Cell Biolabs Inc., San Diego, CA).

**IDENTIFICATION OF TRANSCRIPT CORRELATIONS IN MICE AND HUMANS**

Liver microarray data (Affymetrix Mouse Gene 1.0 ST) from a BXD mouse genetic reference population were analyzed for transcript expression correlations between NAD\(^+\) regulating and \(\beta\)-oxidation genes using the GeneNetwork program (http://www.genenetwork.org). Raw microarray data are also publicly available on Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo) under the accession number GSE60149. Similarly, human microarray data were retrieved from the GSE48654(27) and GSE9588(28) GEO data sets.

**TRANSCRIPT AND PROTEIN EXPRESSION**

Real-time quantitative polymerase chain reaction (qPCR), mitochondrial DNA (mtDNA) content, western immunoblotting, and blue native polyacrylamide gel electrophoresis (PAGE) techniques are described in the Supporting Methods.

**STATISTICAL ANALYSIS**

Statistical analysis was performed with Prism Software (version 6.0; GraphPad Software Inc., La Jolla, CA). The significance of differences between two groups was determined by unpaired two-tailed Student t test. For comparison of multiple groups, we applied a one-way analysis of variance (ANOVA) with a post-hoc Bonferroni test. Results are presented as mean ± standard error of the mean (SEM). A P value <0.05 was considered significant.

**Results**

**REGULATION OF NAD\(^+\) HOMEOSTASIS AND LIPID METABOLISM**

Exploring the potential for NAD\(^+\) dysfunction in the development and progression of NAFLD, we first evaluated gene expression patterns related to NAD\(^+\) homeostasis and lipid metabolism in two separate existing liver tissue data sets that include 221(27) and 427(28) normal human samples. These data sets conveyed a positive correlation between custom-designed gene sets for \(\beta\)-oxidation and genes involved in NAD\(^+\) biosynthesis (Fig. 1A,C). Specifically, transcripts for nicotinamide phosphoribosyltransferase, the rate-limiting enzyme for NAD\(^+\) salvage from nicotinamide, and NRK1 correlated positively to \(\beta\)-oxidation genes, suggesting a beneficial effect of NAD\(^+\) synthesis on NAFLD. In contrast, negative correlations were observed when compared to NAD\(^+\) consumers, such as poly adenosine diphosphate (ADP)-ribose (PAR) polymerase 1 (PARP1), and the cyclic ADP-ribose synthase, CD38. These correlations were conserved in livers of genetically diverse strains of mice from the BXD genetic reference population fed either chow or high-fat diets (described in previous works(9,26); Fig. 1B,D). A unique custom-designed gene set was also created for the comparison of matching genes between the human and mouse data sets (Supporting Fig. 1A). To experimentally corroborate this link observed in human and mice, we examined a mouse model of NAFLD.(20) Mice given an HFHS diet displayed a progressive loss of liver NAD\(^+\) levels that was matched by step-wise increases in liver TG levels, linking changes in NAD\(^+\) homeostasis to liver metabolism (Fig. 1E,F). Thus, NAD\(^+\) regulating enzymes, reflecting more NAD\(^+\) availability, appear to correlate with lipid metabolism in both human and mouse liver samples.

**NAD\(^+\) REPLETION, AS A THERAPEUTIC TREATMENT, CAN REVERT INDICATIONS OF NASH IN HFHS-FED MICE**

To evaluate the potential of NAD\(^+\) repletion to protect mice from the development of NAFLD, we compared 7-week-old mice exposed to 18 weeks of HFHS diet to mice given the same diet supplemented with NR (400 mg/kg/day) from the onset of the HFHS diet (prevention mode) or 9 weeks after the start of the diet.
Both preventive and therapeutic approaches improved glucose tolerance (Fig. 2B, C) and insulin sensitivity (Fig. 2D) with concurrent reductions in body weight (Fig. 2E) and fat mass (Fig. 2F), each being frequently measured as secondary outcomes in NASH-related clinical trials. NR boosted whole-liver lysate and mitochondrial NAD⁺ levels in HFHS-fed animals from both cohorts.

**Fig. 1.** NAD⁺ regulating enzymes and NAD⁺ levels correlate with lipid metabolism in both humans and mice. Transcripts from NAD⁺-synthesis genes (shown in blue font), in contrast to NAD⁺-consuming genes (shown in red font), were positively correlated with regulators of β-oxidation using custom-designed data sets derived from (A) two human data sets, including 427(28) and 220(27) human liver samples or (B) two mouse data sets, including 42 BXD strains fed either chow or high-fat diets. As seen on a correlogram, blue correlations are positive (red correlations are negative)—intensity of the colors correlates with level of significance. NRK1 and NAMPT transcripts, in contrast to Parp1, were positively correlated to mitochondrial β-oxidation genes, ACADL, ACADM, and HADH, in data sets from 427(28) and 220(27) human liver samples (C) or 42 BXD strains fed either chow or high-fat diets. (D) Consistent with these findings in mice and humans, we see reductions in (E) hepatic NAD⁺ levels, followed by increases in (F) hepatic TG levels, in mice fed 9-18 weeks with a HFHS diet (n = 5-10). **P < 0.001; ***P < 0.0001 compared to the CD cohort. Data are expressed as mean ± SEM. One-way ANOVA with a post-hoc Bonferroni test was used for all statistical analyses.
with each cohort consuming similar quantities of food (Fig. 3A). As a result, plasma elevations in alanine aminotransferase (ALT) and aspartate aminotransferase (AST), indicators of liver damage, induced by an HFHS diet, were completely attenuated with NR (Fig. 3B). Hepatic TG, cholesterol, and LPO levels were also reduced upon NR administration (Fig. 3B). Remarkably, the 9-week intervention, similar to the 18-week preventive treatment, reversed changes in liver weight that occurred after HFHS diet (Fig. 3C), indicating that even therapy initiated after the onset of disease was beneficial. Furthermore, NR improved liver H&E histology scores for severity and extension of steatosis (Fig. 3C,D). Indications for increased lipid use with an NR-supplemented diet was evident after just 5 weeks of treatment, with reductions in the respiratory exchange ratio during the dark and light cycles (Supporting Fig. 2A-C). The development of a pale white liver color, which occurs with increased lipid content or steatosis, was attenuated by NR and was corroborated by reduced staining with Oil Red O for lipid content (Fig. 3D). NR also reduced hepatic fibrosis, as indicated by less Picrosirius red and Masson’s trichrome staining (Fig. 3D and Supporting Fig. 3), substantiated by reduced transcript expression of Col, Fn1, and Acta2 (Fig. 3E). In line with this, although the observations were not linked to NAD$^+$ metabolism, inhibition of PARP, a major consumer of NAD$^+$, was shown to protect against chemically induced liver fibrosis. In parallel, in NR-treated animals, there was a reduction in the HFHS-induced liver transcripts of the peroxisome proliferator-activated receptor (Ppar)γ, a mediator of lipogenesis (reviewed in a previous work) (31), resulting in the reduction of Srebf1, Acc, Fasn, and Scd1 messenger RNAs (mRNAs; Fig. 3E). NR treatment also lowered the expression of genes involved in lipid uptake (Cd36), TG formation (Dgat1), and packaging for lipoprotein secretion (Cideb, Apob; Fig. 3E). In agreement with the NR-mediated improvements in liver health and lipid content, there was also a reduction in the hepatic expression of inflammatory transcripts Tnfa, Mip, Vam, Il-6, and Il-1b (Fig. 3E). These observations were complimented by the attenuation of plasma tumor necrosis factor alpha (TNF-α) levels (Fig. 3F) and the reduced infiltration of CD45-positive leukocytes into the liver with NR treatment (Supporting Fig. 3).
NR THERAPY REVERSES HFHS-INDUCED MITOCHONDRIAL DYSFUNCTION AND INCREASES TRANSCRIPTS FOR β-OXIDATION

Development of NAFLD, and the ensuing progression of liver cirrhosis, relies mainly on abnormal liver lipid accumulation. Whereas increased lipid uptake and lipogenesis can lead to NAFLD, incomplete FA oxidation in the context of mitochondrial dysfunction also contributes to hepatic FA and lipid overload. Although NR has been demonstrated to prevent diabesity in HFD-fed animals, the ability of NAD+ repletion to reverse liver damage, brought on by mitochondrial dysfunction, has yet to be shown. Here, we demonstrate an improvement in liver mitochondrial function following NR, with increased citrate synthase (CS) activity and oxygen consumption rates (OCRs) driven by complex I (and trending with complex II) in the presence of ADP (Fig. 4A,B). Additionally, cytochrome c oxidase (COX) and succinate dehydrogenase (SDH) activity was enhanced in liver sections after NR (Fig. 4C). Changes...
in citrate cycle activity and oxidative phosphorylation (OXPHOS) with NR led to a recovery in liver ATP concentrations compared to mice fed only an HFHS diet (Fig. 4D). These functional changes are supported by NR-induced increases in transcripts for Nampt, Sirt1, Nrfl1, and Tfam, as regulators of mitochondrial biogenesis (Fig. 4E). mRNA levels of Ppara and Pparα/β, transcriptional activators of genes involved in β-oxidation and which protect against NAFLD, showed trends of reduction with an HFHS diet and...
recovery with NR (Fig. 4E). To analyze how PPARz, the predominant PPAR isoform in hepatocytes, contributes to the beneficial effects of NR on β-oxidation and mitochondria biogenesis, we exposed AML12 hepatoma cells to small interfering RNA for Pparz (Supporting Fig. 4). NR treatment of AML12 cells did not increase transcript levels of Pparz, yet upon knockdown of Pparz, expression of Sir1 showed a tendency towards reduction along with the significant attenuation of the stimulatory effects of NR on β-oxidation genes, such as Msd and Acox1, and the expression of Tfam, a crucial transcription factor for mitochondrial genes. Therefore, NR lost some of its ability to enhance β-oxidation and mitochondrial function when Pparz was absent.

To investigate whether it was changes in β-oxidation or lipogenesis that initially led to the NR-mediated reduction of liver steatosis, we compared the activation of genes associated with β-oxidation or lipogenesis in NR-treated mice fed an HFHS diet for a short 2-week period, to avoid the confounding changes in mouse body weight observed after long-term HFHS diets. After 2 weeks of diet, NR treatment accentuated the elevation in transcripts for β-oxidation genes after 24 hours of fasting (Fig. 4F). In contrast, no difference in transcripts of lipogenesis genes were found in refed NR-treated animals. Consequently, in our long-term studies, NR-mediated reductions in lipogenesis transcripts (Fig. 3E) were not caused by the active repression of lipogenesis genes. Therefore, functional and molecular improvements in β-oxidation and OXPHOS upon NR treatment, instead of reduced lipogenesis, are responsible for the attenuation of NAFLD in HFHS animals and support the hypothesis that NAD⁺ repletion protects and enhances liver mitochondria during periods of FA overload.

**NAD⁺ REPLETION PROMPTS MITONUCLEAR PROTEIN IMBALANCE, INDUCING THE UPRMT MARKERS CLPP AND HSP10 IN VIVO, WHILE REDUCING ENDOPLASMIC RETICULUM STRESS**

Next, we examined whether the severe hepatic lipid accumulation and respiratory defects that were improved by NR administration were the result of changes in mitochondrial content, complex formation, and UPR⁶° signaling. Recently, sirtuin-dependent mitochondrial changes were shown to rely, in part, on induction of UPR⁶°, triggered by an imbalance in mitochondrial-versus nuclear-encoded mitochondrial proteins. This mitonuclear imbalance activates a retrograde signal that induces a mitohormetic and adaptive nuclear response, ultimately repairing and improving mitochondrial function. These mitohormetic signals can attenuate the harmful repercussions of aging, inherited mitochondrial diseases, or a high-fat diet on whole body metabolism. In Caenorhabditis elegans and muscle with NAD⁺ boosting strategies. Mitonuclear protein imbalance has previously been shown to trigger the UPR⁶° (35). In line with this premise, protein and transcript levels of CLPP and HSP10 (Hspt1), two UPR⁶° biomarkers, were elevated in mice treated with NR (Fig. 5D). In contrast to NR-induced UPR⁶°, NR reduces endoplasmic reticulum (ER) stress. In fact, an ER-specific unfolded protein response (UPR⁺ER) is activated during liver steatosis and occurs in NAFLD, viral hepatitis, and alcohol-induced liver injury (reviewed in (37)). Persistent activation of UPR⁺ER can cause ER stress-dependent alterations in lipid homeostasis and is suggested to contribute to steatosis. The activation of UPR⁺ER was confirmed in HFHS-fed animals by qPCR, demonstrating increased expression of the transcription factor, activating transcription factor 4 (ATF4), which transactivates the promoter of the binding immunoglobulin protein/glucose-regulated protein 78 (GRP78) and the CCAAT/enhancer-binding protein homologous protein, a proapoptotic transcription factor (Fig. 5E). Also, at the protein level, ATF4 and GRP78 were induced (Fig. 5E). These transcript and protein markers of UPR⁺ER activity were all reduced after both NR treatment regimens (Fig. 5E).

**IN VIVO UPRMT-DEPENDENT ACTIVATION OF MITOCHONDRIAL BIOGENESIS REQUIRES SIRT1**

Furthermore, we set out to determine whether NAD⁺ repletion in hepatocytes triggers mitochondrial improvements through a SIRT1- and/or SIRT3-dependent activation of UPR⁶°, as reported
Treatment of primary hepatocytes with NR induced an elevation in NAD$^+$ levels and mtDNA abundance, similar to the effect observed in vivo (Fig. 6A). The SIRT1-dependency of NR-induced mitochondrial biogenesis was first demonstrated in AML12 cells using the SIRT1 inhibitor, EX527 (Fig S5A). Moreover, primary hepatocytes from Sirt1L2/L2 mice, infected with an Ad-GFP (control) or Ad-Cre to induce a Sirt1 loss of function (LOF), showed a clear SIRT1-dependent reduction in the ratio of the nuclear-encoded SDHB and mitochondrial-encoded MTCO1 proteins (SDHB/MTCO1) resulting in a mitonuclear imbalance, which together with the elevation of HSP10, HSP60, and CLPP (data not shown), is indicative of the UPRmt (Fig. 6B). In addition to the Sirt1 LOF, which completely attenuated the UPRmt induction, we observed an analogous, but less robust, effect on primary hepatocytes with a Sirt3 LOF (Supporting Fig. 5B). Thus, the mitonuclear imbalance and activation of UPRmt, observed after boosting NAD$^+$ levels in liver cells, are reliant on the presence of either SIRT1 or SIRT3. We next evaluated the possible cell-autonomous contribution of SIRT1 in mediating the effects of NR using a liver-specific Sirt1-deficient mouse line (Sirt1$^{bp-/}$). In these Sirt1$^{bp-/}$ mice, the effect of NR on body and liver weights after 14 weeks on an HFHS diet were non-significant (Fig. 6C). This was matched by the reversal previously.$^{21,38}$
of the NR-mediated benefits in liver TGs and lipids in Sirt1<sup>bp/2</sup> mice (Fig. 6D,E). These results are further corroborated by clear reductions in NR's efficacy to induce expression of mitochondrial- and β-oxidation-associated genes (Fig. 6F). These data demonstrate that although NR may have some systemic effects that ameliorate the impact of an HFHS diet, SIRT1 in hepatocytes is essential for NR-mediated liver benefits.
To confirm the beneficial effects of NR, we repeated several phenotyping experiments on HFC-fed Apoe²/² mice. Apoe²/² mice were challenged with 3 weeks of HFC diet, then treated (in a therapeutic mode) with an HFC diet supplemented with NR (Apoe²/²/NR-Ther) for another 7 weeks (NR: 500 mg/kg/day). Mice were sacrificed after overnight fasting. NR elevated hepatic NAD⁺ levels resulting in reduced body weight. Elevated NAD⁺ levels reduced circulating ALT, an indicator for liver damage (n = 8-10), and improved hepatic cholesterol and TG accumulation. NR led to less steatosis, as observed by H&E-stained liver sections (n = 8-10), with similar food intake between HFC-fed animals (n = 8-10). NR showed a trend to reduce liver and epididymal fat pad weights. This resulted in a trend to increase mtDNA abundance and in a significant elevation of the relative expression of genes associated with mitochondrial biogenesis and β-oxidation after NR (n = 8-10). *P < 0.05; **P < 0.01; ***P < 0.001 compared to the HFHS cohort. Data are expressed as mean ± SEM. One-way ANOVA with a post-hoc Bonferroni test was used for all statistical analyses. Male mice were used for these experiments.

REVERSAL OF TISSUE STEATOSIS IN THE APOE²⁻/-- MOUSE MODEL OF NAFLD IS NAD⁺-DEPENDENT

To confirm the beneficial effects of NR, we repeated several phenotyping experiments on HFC-fed Apoe²/² mice. Apoe²/² mice were challenged with 3 weeks of HFC diet, then treated (in a therapeutic mode) with an HFC diet supplemented with NR for another 7 weeks (Apoe²/²/NR-Ther). Similar to HFHS-fed C57BL/6J mice, HFC-fed Apoe²/² mice treated therapeutically with NR exhibited increased hepatic NAD⁺ levels and reductions in body weight (Fig. 7A,B), circulating ALT, and total hepatic cholesterol and TG levels (Fig. 7C). NR also reduced hepatosteatosis in this model, as observed with H&E staining, which was not dependent on food intake (Fig. 7D; HFC cohorts). However, given that liver weight and epididymal fat mass did not increase in Apoe²/² mice fed an HFC diet, there was no strong reduction in these parameters with NR (Fig. 7E). Yet, there was a trending increase in mtDNA abundance with NR treatment, which is consistent with increases in Sirt1, Tfam, and β-oxidation gene expression (Fig. 7F). Thus, NR used as a therapeutic
treatment consistently reverses indications of liver damage and mitochondrial dysfunction between two well-known models of NAFLD in mice.

Discussion

Although NAFLD is thought to arise from an imbalance between lipid uptake/synthesis and lipid oxidation/export in hepatocytes, relatively little is known about the role of mitochondria in this process. NAD$^+$-mediated changes in sirtuin deacetylase activity have been previously associated with induction of expression of genes and activation of proteins linked to improved mitochondrial function (reviewed in a previous work). Supporting the importance of NAD$^+$ in liver metabolism, we discovered a striking negative correlation between transcripts of NAD$^+$-consuming genes, such as Cd38 and Parp1, and those involved in β-oxidation in liver biopsies from two large human cohorts and in livers from the mouse BXD genetic reference population. In contrast, NAD$^+$ salvage enzyme transcripts, such as Nrk1 and Nampt, were positively correlated to matching gene sets for β-oxidation in mice and humans. Correspondingly, mice that were fed a HFHS diet exhibited a progressive decline in liver NAD$^+$ levels that were matched by an increased liver TG content. Overall, these findings demonstrate a conserved link in humans and mice between expression of NAD$^+$ consumption/biosynthesis genes and genes that regulate β-oxidation in the liver.

From these data, we postulated that by boosting NAD$^+$ levels in long-term HFHS-fed animals, we could increase sirtuin-mediated UPR$^{mt}$ activation and mitochondrial biogenesis, while also reducing UPR$^{er}$ stress response. In agreement, NR attenuated the severe mitochondrial dysfunction, typified by reduced mitochondrial content and function, present in fatty livers of mice fed a long-term HFHS diet. These robust effects of NR on mitochondrial function were reliant on the NAD$^+$-mediated SIRT1 and SIRT3 induction of mitonuclear protein imbalance, triggering the UPR$^{mt}$. Similar to previous reports linking the beneficial effects of NR to the UPR$^{mt}$ activation in livers of these NR-treated animals maintained optimal mitochondrial function despite a chronic FA overload. Interestingly, NAD$^+$ metabolism also appears to play a central role in alcohol-mediated liver damage, leading us to postulate that a proper NAD$^+$ balance is essential for maintaining liver homeostasis.

Boosting NAD$^+$ levels, by using NR as a preventive strategy, proved to avert the onset of NAFLD, indicating that this vitamin B3 analog has potential as a nutraceutical or food supplement to protect liver function. More relevant for a therapeutic setting was our observation that NR administration was able to attenuate mitochondrial dysfunction and liver damage in mice with established NAFLD, as supported by the improvement of primary and secondary indicators of NAFLD. The therapeutic effect of NR was further confirmed in our study of Apoe$^{-/-}$ mice challenged with an HFC diet, as an independent model of NAFLD that is less dependent on changes in body mass. Importantly, using Sirt1$^b6p^{-/-}$ mice, we demonstrate that the beneficial effect of NR on NAFLD is reliant on a nonsystemic component driven by activation of SIRT1 specifically in hepatocytes.

Therefore, our results indicate that NAD$^+$ repletion prevented or reversed the phenotypes associated with NAFLD, such as liver lipid accumulation, liver fibrosis, and the NAFLD molecular signature typified by increased PPARγ mRNA levels and its downstream transcripts linked to lipogenesis. However, the reduction in the activation of lipogenesis genes was shown to be secondary to mitochondrial activation and enhanced β-oxidation and OXPHOS. In parallel to the reversal of lipid accumulation and fibrosis, there were also significant reductions in liver and plasma inflammatory markers associated with NAFLD. Future work should examine how chronic lipid overload and the ensuing inflammation, a process associated with PARP activation (reviewed in a previous work), are linked to the reduction in liver NAD$^+$ levels.

In combination, our data show that the replenishment of depleted liver NAD$^+$ stores in mouse models of NAFLD, by administration of NR supplements, recovers liver NAD$^+$-dependent SIRT1 and SIRT3 signaling to counteract the development of NAFLD. These beneficial effects of NR are largely mediated by the cell-autonomous effect of SIRT1 activation in the liver. Furthermore, our bioinformatics data suggest that reduced NAD$^+$ stores may also be a hallmark of human NAFLD and NASH, warranting further exploration of NAD$^+$ replenishing strategies to treat these pervasive liver diseases.

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