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Overexpression of NRK1 ameliorates diet- and age-induced hepatic steatosis and insulin resistance

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ABSTRACT

NAD$^+$ is a co-enzyme in redox reactions and a substrate required for activity of various enzyme families, including sirtuins and poly(ADP-ribose) polymerases. Dietary supplementation of NAD$^+$ precursors nicotinamide mononucleotide (NMN) or nicotinamide riboside (NR) protects against metabolic disease, neurodegenerative disorders and age-related physiological decline in mammals. Here we sought to identify the roles of nicotinamide riboside kinase 1 (NRK1) plays in regulating hepatic NAD$^+$ biosynthesis and lipid metabolism. Using adenovirus mediated gene transduction to overexpress or knockdown NRK1 in mouse liver, we have demonstrated that NRK1 is critical for maintaining hepatic NAD$^+$ levels and triglyceride content. We have further shown that the hepatic expression of *Nmrk1* mRNA is significantly decreased either in mice treated with high-fat diet or in aged mice. However, adenoviral delivery of NRK1 in these diet- and age-induced mice elevates hepatic NAD$^+$ levels, reduces hepatic steatosis, and improves glucose tolerance and insulin sensitivity. Our results provide important insights in targeting NRK1 for treating hepatic steatosis.

1. Introduction

Fatty liver disease is a reversible condition wherein large vacuoles of triglyceride fat accumulate in liver cells via the process of steatosis [1,2]. The main reasons causing fatty liver disease are due to the popular high-fat/calorie diet or heavy alcohol intake, which lead to nonalcoholic fatty liver disease (NAFLD) or alcoholic fatty liver disease (AFLD) [3]. Abnormal cytoplasmic lipid accumulation in the liver is primarily caused by imbalance of hepatic lipid acquisition and removal, which involves augmented lipid uptake, enhanced de novo lipogenesis, impaired fatty acids β-oxidation, and/or decreased lipid export as the form of very low-density lipoprotein (VLDL) in liver [3]. Interestingly, both NAFLD and AFLD are strongly associated with metabolic syndrome and type 2 diabetes, suggesting that fatty liver, independent of origin, promotes systemic metabolic dysfunction [4]. In clinical practice, fatty liver diseases encompass a continued spectrum of liver damage, which progresses from simple hepatic steatosis to advanced steatohepatitis, and in some cases, even to fibrosis, cirrhosis, and hepatocellular carcinoma [5].

Nicotinamide adenine dinucleotide (NAD$^+$) serves as a coenzyme in cellular redox reactions and is also a substrate for multiple enzymes, including sirtuins and poly(ADP-ribose) polymerases (PARPs) [6,7]. Accumulating studies have demonstrated that NAD$^+$ participates in a variety of biological processes, including the regulation of energy metabolism, DNA repair and transcriptional regulation through the activity of the sirtuins, which are a family of NAD$^+$-dependent histone/protein deacetylase [6,7]. The NAD$^+$ concentration in the organism appears to be variable as it is regulated by time and nutrient availability [8–11]. Importantly, previous evidence in animal models showed that a decrease in the hepatic NAD$^+$ pool is a significant feature of aging and fatty liver related liver injury [12,13].

Mammalian NAD$^+$ biosynthesis is accomplished through either

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the de novo pathway from tryptophan or salvage pathway from three NAD^+ precursor vitamins, nicotinamide (NAM), nicotinic acid (NA) and nicotinamide riboside (NR) [14]. As a natural precursor of NAD^+, NR supplementation increases lifespan in yeast and improves high-fat diet-induced metabolic complications in mice [15–17]. NR is present in milk and its conversion to NAD^+ is initiated by phosphorylation of NR to NMN by NR kinases (NRKs) [18,19]. NRKs are highly conserved enzymes in all eukaryotes [20,21]. In mammals there are two NRK enzymes, NRK1 and NRK2, encoded by genes *Nmrk1* and *Nmrk2*, respectively [22]. *Nmrk1* is ubiquitously expressed in all tissues while the expression of *Nmrk2* is highly restricted to muscle [20,21]. Recent evidence has shown that NRK1 is not only rate-limiting for NR metabolism in mammalian cells, but also necessary for the conversion of extracellular NMN to NR for cellular uptake and NAD^+ synthesis [20]. Liver, the organ mediating many of the metabolic effects of NR, displays high NRK1 levels [20]. However, whether NRK1 indeed mediates the increase in hepatic NAD^+ pool in vivo is still largely unknown. In the present study, we show that adenovirus (Ad)-mediated overexpression of NRK1 upregulates hepatic NAD^+ levels and ameliorates high-fat diet- or age-induced hepatic steatosis.

2. Materials and methods

2.1. Animal studies

Male C57BL/6J mice were purchased from Nanjing Biomedical Research Institute of Nanjing University and maintained in an environmentally-controlled room and fed a rodent chow with free access to water. High fat diet (60% calories from fat) was purchased from Research Diets Inc. Adenoviruses carrying NRK1, GFP, Nmrk1-shRNA, and GFP-shRNA were injected into mice at a dose of 1×10^9 pfu via tail vein. Glucose tolerance test (GTT) and insulin tolerance tests (ITT) were performed in mice fasted for 16 h or 4 h before injection of glucose (i.p. 2g/kg) or insulin (i.p. 0.75U/kg), respectively. All animal procedures were performed in accordance with Henan Province Laboratory Animal Care Guidelines for the use of animals in research and were approved by the Institutional Animal Use and Care Committee Xinxiang Medical University.

2.2. Real-time RT PCR

Total RNAs were isolated from tissues or cells using TRIzol reagent (Takara) by the following manufacturer's instructions and converted into cDNA using a cDNA synthesis kit (Vazyme). Real-time PCR analysis was performed using SYBR Green Master Mix (Vazyme) in ABI StepOnePlus Real-Time PCR system.

2.3. Western blot analysis

Protein extracts from cells were made in RIPA buffer (0.5% NP-40, 0.1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-Cl, pH 7.5 and freshly added 1mM PMSF and an additional protease cocktail tablet from Roche at one tablet/10mL final buffer volume). Protein extracts were resolved on an SDS-PAGE gel and transferred to nitrocellulose membrane. The membrane was incubated with HA tag antibody (Proteintech) for overnight at 4 °C. Detection of proteins was carried out by incubations with the HRP conjugated secondary antibody for 1 h at RT, followed by the ECL detection reagents.

2.4. Histological analysis

Mouse liver tissues were dissected and immediately fixed in 4% paraformaldehyde. Tissues were then routinely processed for paraffin embedding, and 5 μm sections were cut and mounted on glass slides. Sections were stained with hematoxylin-eosin (H&E) according to the standard protocol.

2.5. Adenovirus preparation

Adenoviruses carrying NRK1 or GFP were generated using the pAdEasy system (Agilent). Adenoviruses carrying shRNA sequences against *Nmrk1*, or GFP were generated using the BLOCK-iT system (Invitrogen). Adenoviruses were amplified in HEK293A cells and purified by CsCl gradient centrifugation. The viruses were titered using a QuickTiter adenovirus titer immunoassay kit (Cell Biolabs) according to the manufacturer's manual.

2.6. Cell culture

The HEK293A and NIH3T3 cells were cultured in DMEM containing 100 units/ml penicillin, 100 μg/ml streptomycin, 4.5 g/liter glucose, and 10% FBS. Mouse primary hepatocytes were isolated and cultured as described previously [23].

2.7. NAD^+ determination

NAD^+ levels in cells and tissue samples were determined with an enzymatic cycling assay as previously described [23].

2.8. Serum and liver triglyceride analysis

Blood samples were collected from overnight-fasted mice. Hepatic lipids were extracted as previously described [24]. Total triglyceride was analyzed using assay kits from Nanjing Jiancheng Bioengineering Institute.

2.9. Statistical analysis

All data are presented as the mean ± S.E.M. Analysis was performed using 2-tailed unpaired Student's *t*-test, and $p < 0.05$ was considered as significant.

3. Results

3.1. NRK1 is rate-limiting for NR-driven NAD^+ synthesis

In order to efficiently overexpress NRK1 in mammalian cells, adenovirus expressing HA-tagged murine NRK1 (Ad-NRK1) or control adenovirus expressing GFP alone (Ad-GFP) were generated. To determine the efficiency of adenoviral-mediated overexpression, primary hepatocytes were infected with Ad-NRK1 or Ad-GFP for 48hrs. Western blot using HA antibody showed significant expression of NRK1 and control GFP in adenovirus infected cells (Fig. 1A). Since there is no good commercial NRK1 antibody available, mRNA levels of *Nmrk1* were assessed 48hrs after infection. As shown in Fig. 1A, exposure to Ad-NRK1 resulted in ~10-fold increase over endogenous levels of *Nmrk1* mRNA. Once it enters the cell, NR is metabolized into NMN by a phosphorylation step catalyzed by the NRKs. So, NRKs are rate-limiting for NR mediated NAD^+ production. NIH3T3 cells have been shown to have very low endogenous NRK1 and NRK2 levels [20], therefore, cells treated with NR for 24hrs did not display marked differences in NAD^+ levels (Fig. 1B). However, NR treatment elevated NAD^+ levels in cells endogenously express NRK1, such as HEK293 cells (Fig. 1C), and primary hepatocytes (Fig. 1D). Moreover, adenovirus-mediated overexpression of NRK1 is sufficient to boost NR-driven NAD^+ synthesis in all the cells tested (Fig. 1B–D), suggesting a rate-limiting role for NRK1 in NAD^+ production using NR as a precursor.