Deficiency of the Mitochondrial NAD Kinase Causes Stress-induced hepatic steatosis in mice

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Abstract

Background & Aims—The mitochondrial nicotinamide adenine dinucleotide (NAD) kinase (NADK2, also called MNADK) catalyzes phosphorylation of NAD to yield NADP. Little is known about the functions of mitochondrial NADP and MNADK in liver physiology and pathology. We investigated the effects of reduced mitochondrial NADP by deleting MNADK in mice.

Methods—We generated MNADK-knockout (KO) mice on a C57BL/6NTac background; mice with a wild-type *Mnadk* gene were used as controls. Some mice were placed on an atherogenic high-fat diet (16% fat, 41% carbohydrate and 1.25% cholesterol supplemented with 0.5% sodium cholate) or given methotrexate intraperitoneally. We measured rates of fatty acid oxidation in

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primary hepatocytes using radiolabeled palmitate and in mice using indirect calorimetry. We measured levels of reactive oxygen species in mouse livers and primary hepatocytes. Metabolomic analyses were used to quantify serum metabolites, such as amino acids and acylcarnitines.

Results—The KO mice had metabolic features of MNADK-deficient patients, such as increased serum concentrations of lysine and C10:2 carnitine. When placed on the atherogenic high-fat diet, the KO mice developed features of non-alcoholic fatty liver disease and had increased levels of reactive oxygen species in livers and primary hepatocytes, compared with control mice. During fasting, the KO mice had a defect in fatty acid oxidation. MNADK deficiency reduced the activation of cAMP-responsive element binding protein-hepatocyte specific and peroxisome proliferator-activated receptor alpha, which are transcriptional activators that mediate the fasting response. The activity of mitochondrial sirtuins was reduced in livers of the KO mice. Methotrexate inhibited the catalytic activity of MNADK in hepatocytes and in livers in mice with methotrexate injection. In mice given injections of methotrexate, supplementation of a diet with nicotinamide riboside, a NAD precursor, replenished hepatic NADP and protected the mice from hepatotoxicity, based on markers such as increased level of serum alanine aminotransferase.

Conclusion—MNADK facilitates fatty acid oxidation, counteracts oxidative damage, maintains mitochondrial sirtuin activity, and prevents metabolic stress-induced nonalcoholic fatty liver disease in mice.

Keywords

CREBH; PPARα; liver injury; mouse model; steatohepatitis

Introduction

Mitochondrial NADP, the phosphorylated form of nicotinamide adenine dinucleotide (NAD), plays essential roles in liver biology.[1] NADPH is a cofactor for many mitochondrial enzymes, such as 2,4 dienoyl-coa reductase (DECR), a crucial enzyme for polyunsaturated fatty acid oxidation (FAO) and α-aminoadipic acid semialdehyde synthase (AASS), which controls the first step in lysine degradation.[2, 3] Mitochondria represent a major source of oxidative stress, because the majority of reactive oxygen species (ROS) are generated from the mitochondrial respiratory chain.[4] NADPH, the reduced form of NADP, regenerates cellular oxidative defense systems via glutathione reductase to counteract oxidative damage.[4, 5] Therefore, as a universal electron carrier in cellular electron transfer reactions, NADP(H) is required by mitochondria in a multitude of metabolic processes.[5]

NAD has been studied for decades as the prototypical cellular electron donor and acceptor. Recently the interest in NAD has increased largely because of research on sirtuins (SIRT), NAD-dependent nutrient-sensing deacetylases whose levels and activity are increased by caloric restriction.[6] Reduced SIRT1 activity or expression level results in hepatic steatosis,[7] a hallmark of nonalcoholic fatty liver disease (NAFLD), which is caused by an imbalance among lipid synthesis, storage, oxidation and secretion.[8] In contrast to the wealth of research examining NAD, the *in vivo* roles of mitochondrial NADP in liver physiology and pathology, such as NAFLD, are largely unknown.

The NAD kinase (NADK) is the sole NADP biosynthetic enzyme.[9] Because NADP is membrane-impermeable, eukaryotes need compartment-specific NADKs for different organelles. Consistently, in both yeast and plants, three compartment-specific NADKs have been identified.[10] In contrast, even though human cytosolic NADK was identified in 2001,[11] the identity of a hypothesized mitochondrial NADK remained elusive[9], until the recent discovery that the uncharacterized human gene C5ORF33 encodes a mitochondrion-localized NADK, referred to as MNADK or NADK2.[10, 12, 13] Two MNADK-deficient patients were then identified with symptoms characteristic of mitochondrial disease.[3, 14] Metabolically, both patients displayed elevated plasma C10:2 carnitine and lysine,[3] due to the reduced activity of the NADPH-dependent DECR and AASS, respectively.[3] Biochemically, mitochondria isolated from patient fibroblasts has reduced NADP(H) levels.[3] Therefore, in humans, MNADK is the mitochondrial NAD kinase that generates mitochondrial NADP(H).[3]

In this study, we addressed the functional involvement of MNADK in liver biology and the development of NAFLD. We examined the consequences of reduced mitochondrial NADP by deleting MNADK in mice. Metabolically the MNADK KO mice phenocopy MNADK-deficient patients, by having elevated plasma concentrations of lysine and C10:2 carnitine, and exhibit hepatic steatosis on an atherogenic high-fat (AHF) diet, in part, due to increased ROS and reduced activities of the key hepatic regulators in FAO and lipolysis, including CREBH (cAMP-responsive element binding protein, hepatic) and PPARα (peroxisome proliferator-activated receptor α). Furthermore, methotrexate (MTX), a widely prescribed drug for treating cancers and autoimmune diseases, is notoriously known for its hepatotoxicity. We found that MTX is a potent inhibitor of MNADK, providing a mechanistic explanation of MTX's hepatotoxicity. These results provide significant insights into the roles of mitochondrial NADP and MNADK in liver physiology as well as metabolic disease.

Materials and methods

Materials

Synthetic oligonucleotides were purchased from Integrated DNA Technologies, Inc. Mouse monoclonal anti-PPARα antibody was purchased from Millipore Corp. Polyclonal anti-CREBH antibody was developed in our lab as previously described.[15] Kits for measuring TG and FA were from BioAssay System.

Mice

MNADK KO mice on the C57BL/6NTac background Nadk2^{tm1a}(EUCOMM)Wtsi were obtained from the European Mouse Mutant Archive, as part of the IMPC. Mouse genotyping was determined by PCR with the primers: 5′-TCGTGGTATCGTTATGCGCC-3′; 5′-AGGTGCTGTTTGCCTTCTGT-3′; 5′-AAAGCCCACAGGTCCCTACT-3′. WT and targeted alleles resulted in PCR bands of 195 and 120 bp, respectively. Correct gene targeting was confirmed by Western blotting with MNADK antibody (Sigma). For the diet study, the AHF diet (16% fat, 41% carbohydrate, supplied with 0.5% sodium cholate by weight) was from Harlan Laboratories. To treat mice with PPARα agonist,[16] mice were

orally treated by gavage with 10 mg/kg Wy-14,643 (Sigma) dissolved in 2% methylcellulose vehicle (Sigma) daily for 7 days and control mice received methylcellulose only. To make NR diet, the powder food (Research Diets, New Brunswick, NJ) was mixed with water (vehicle) or with NR (High Performance Nutrition, Newport Beach CA), corresponding to NR intake of 400 mg/kg/day based on 3 g food intake/mouse (30g)/day. Mice were fed with chow or NR diet for 8 weeks. At beginning of the 8th week on the NR diet, some groups of mice were *i.p.* injected with MTX (20 mg/kg body weight) daily for 7 days.

Mouse CLAMS assays

Indirect calorimetry was used to determine the volume of oxygen consumed (VO_2), carbon dioxide produced (VCO_2), respiratory exchange ratio (RER), energy production and locomotive activity. VO_2 and VCO_2 were recorded using a Comprehensive Laboratory Animal Monitoring System (CLAMS, Columbus Instruments) at the Nutrition Obesity Research Center, University of Michigan. VO_2 and VCO_2 were scaled to lean mass determined using an NMR-based analyzer (Minispec LF90II, Bruker Optics, Billerica, MA). Whole-body carbohydrate and fat oxidation were estimated from nonprotein RQ. All animal experiments were performed in accordance with the protocols approved by the Animal Care and Use Committees of both Wayne State University and University of Michigan.

Mouse primary hepatocytes

Isolation of mouse primary hepatocytes is as previously described.[17] Briefly, livers from WT and MNADK KO mice were perfused by HBSS supplemented with 8 mM HEPES (pH 7.35) and 1mM sodium pyruvate followed by collagenase digestion. Hepatocytes were collected by centrifugation at 50g, washed with DMEM medium, and seeded in the collagen-coated plates with Williams E medium. For a full description of materials and methods, refer to Supplemental Materials and Methods.

Results

MNADK KO mice phenocopy the MNADK-deficient patient, exhibiting elevated plasma levels of lysine and C10:2 carnitine

MNADK KO mice carrying the MNADK knockout-first allele (*Nadk2*[tm1a(EUCOMM)Wtsi]) were generated on a C57BL/6NTac background by the International Mouse Phenotyping Consortium.[18] The MNADK knockout-first allele contains an artificial splicing acceptor followed by an IRES:lacZ trapping cassette inserted into the intron following *MNADK* exon 5. The artificial splicing acceptor results in expression of LacZ and a frameshift mutation in *MNADK* exons following exon 5 (Figure 1A). The MNADK gene targeting in mice was confirmed by PCR (Figure 1B) and Western blotting analyses (Figure 1C). Indeed, Western blotting analysis showed that the MNADK protein was deleted using this strategy. The MNADK KO mice were born at the expected Mendelian ratio and displayed no gross developmental defects. On the chow diet, levels of free fatty acids (FFA), triglycerides (TG), ketone bodies, glycerol, and insulin were comparable between WT and KO mice (Supplemental Figure 1).

Because the MNADK-deficient patient exhibited characteristic metabolic abnormalities, *i.e.*, elevated plasma lysine and C10:2 carnitine, we examined these metabolites in MNADK KO mice. Indeed, compared to WT mice, serum levels of lysine were more than tripled (P < 0.01) and those of C10:2 carnitine were doubled (P < 0.01) in the KO mice (Figure 1D and E). Therefore, MNADK KO mice phenocopied the MNADK patient by having elevated serum levels of lysine and C10:2 carnitine. Furthermore, serum levels of D- and L-enantiomers of lysine (DL-lysine) exhibited a 7-fold increase (Figure 1F). L-lysine is an essential amino acid that cannot be synthesized in mice and humans and is degraded in the liver, and we therefore examined lysine levels in the liver. Levels of lysine and N-α-acetyllysine in the livers of MNADK KO mice were increased for about 4- and 7-fold, respectively, compared to that in the WT livers (Figure 1G and H). In addition to C10:2 carnitine, the KO mice had increased levels of other acylcarnitine species, including C4-OH, C14-OH, C16:1 and C18:2 acylcarnitines (Figure 1I-L; Supplemental Table 1).

MNADK KO mice develop hepatic steatosis and hypertriglyceridemia on an atherogenic high-fat diet

Because MNADK is highly enriched in the liver, we investigated the liver phenotypes of MNADK KO mice. We placed the mice on an atherogenic high-fat (AHF) diet, which consists of 16% fat, 41% carbohydrate and 1.25% cholesterol supplemented with 0.5% sodium cholate.[19] After 3 weeks on the AHF diet, the MNADK KO mice displayed enlarged, pale livers (Figure 2A), while exhibiting comparable body weights and fat composition (data not shown), compared to the WT mice. Liver TG levels were significantly increased in the KO mice (Figure 2B). Oil-red O staining of hepatic lipids consistently revealed that the KO mice exhibited massive TG accumulation in the livers (Figure 2E). Moreover, the levels of TG and FFA in the serum of the AHF diet-fed KO mice were significantly higher than those in the control mice (Figure 2C and D). Histological analyses based on H&E staining and Sirius-red staining for hepatic collagen deposition suggested that the MNADK-null livers had a tendency for elevated hepatocyte ballooning and fibrosis (Figure 2F and G), as quantified by NAFLD grading (Figure 2H). Taken together, these lines of evidence suggest that MNADK deletion results in a profound liver phenotype under the metabolic stress, characterized by severe hepatic steatosis and hypertriglyceridemia.

MNADK deficiency reduces mitochondrial NADPH

MNADK was previously shown to localize in the mitochondria of HepG2 cells.[12] To verify the subcellular localization of MNADK in the liver, we isolated mitochondrial and cytosolic protein fractions from WT mouse livers. Western blotting analysis confirmed that MNADK was highly enriched in the mitochondrial fraction of mouse livers (Figure 3A). Because MNADK is a mitochondrial NAD kinase, we tested whether MNADK deficiency altered cellular levels of NADP(H) in mouse livers. Examination of the levels of NADP(H) in mitochondrial and cytosolic fractions indicated that the levels of mitochondrial NADP(H) in the livers of the MNADK KO mice were significantly reduced, compared to those in WT mice, while the levels of cytosolic NADP(H) were comparable (Figure 3B and C). This result indicates that MNADK is critical in generating mitochondrial NADP(H) *in vivo*.

MNADK deficiency leads to increased hepatic levels of reactive oxygen species

NADP(H) is essential in neutralizing ROS and in regenerating cellular oxidative defense systems via glutathione reductase to counteract oxidative damages.[10] Indeed, GSSG:GSH ratio (oxidized *vs.* reduced glutathione), a marker for oxidative stress, was elevated in KO livers (Figure 3D), consistent with the NADPH dependency of glutathione reductase. We next examined the alteration in ROS levels in both livers and primary hepatocytes in the absence of MNADK. Dihydroethidium (DHE), a superoxide indicator, was used to examine the superoxide amount in mouse liver sections. The amount of superoxide staining signal was comparable in the livers of the MNADK KO and WT control mice on the normal control diet (NCD); on the AHF diet, however, the KO mice had a dramatic increase in superoxide levels in the liver (Figure 3E and G).

To further evaluate the involvement of MNADK in hepatic ROS formation, we isolated primary hepatocytes from MNADK KO and WT mice, and treated the cells with BSA-complexed palmitic acid (PA) (20μg/ml) or PBS vehicle for 6 hours. The cell permeable reagent 2′,7′-dichlorofluorescin diacetate (DCFDA) can be deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into the highly fluorescent compound 2′, 7′-dichlorofluorescein (DCF). We utilized DCFDA to quantify the amount of ROS in MNADK KO and WT hepatocytes treated with PA or PBS. In PBS treated cells, MNADK-null cells had a mild, yet significant, increase in ROS. In PA-treated cells, both WT and MNADK KO cells had increased ROS; the latter, however, showed approximately 3-fold higher ROS levels (Figure 3F and H). Taken together, MNADK deficiency reduces mitochondrial NADP(H) and increases ROS accumulation in livers and primary hepatocytes.

MNADK deficiency impairs fasting-induced fat oxidation

Reduced FAO has a causal role in hepatic steatosis.[20] We therefore examined the FAO rate in primary hepatocytes by using ^{3}H labeled palmitate as the radioactive substrate. In WT hepatocytes, glucagon treatment significantly increased the FAO rate, but MNADK deficiency totally abolished glucagon-induced increase in FAO (Figure 4A). We next examined FAO in mice through indirect calorimetry using CLAMS. Mice were monitored for two days and fasting started on day 2. Once fasting started, WT mice showed an increase in FAO, which reached a maximum level about 6 hours following food withdrawal. With *ad libitum* food, MNADK KO mice had a comparable FAO compared to WT mice; however, during fasting, the KO mice had a dramatically reduced level of FAO (Figure 4B). The respiratory exchange ratio (RER), the ratio between the amount of carbon dioxide and oxygen, indicates fuel selection between fat and carbohydrate. In WT mice, the RER is about 0.8 throughout light and dark cycles, but after fasting started, it dropped to around 0.67, indicating that fat is the predominant fuel source. MNADK KO mice showed a significant increase in RER (P<0.01), one hour after fasting started (Figure 4C). This result suggests that MNADK KO mice had defects in utilizing fat, and had increased reliance on carbohydrate for energy production during fasting (Figure 4D). During fasting, MNADK had a modest decrease in energy expenditure (Figure 4E), with significant difference in locomotor activity (Figure 4F). Together, these results suggest that MNADK deficiency resulted in the defective hepatic FAO upon fasting.

MNADK deficiency impairs the activation of the transcriptional regulators CREBH and PPARα

CREBH is a fasting-induced, liver-enriched transcription factor that mediates the transcription of genes involved in FAO and lipolysis.[15] We previously showed that CREBH interacts with PPARα to regulate expression of the metabolic hormone FGF21.[21] In response to metabolic challenges, CREBH is activated by cleavage, releasing an amino-terminal fragment that transits to the nucleus to activate gene transcription.[15, 17] We previously showed that CREBH is activated in mice on the AHF diet, and that CREBH KO mice developed severe NAFLD and hyperlipidemia on this diet.[17] We therefore tested the hypothesis that the AHF diet-induced hepatic steatosis in MNADK KO mice may be partially attributable to defects in CREBH activation.

Indeed, the AHF diet strongly stimulated CREBH activation in WT mice. In the AHF diet-fed KO mice, however, CREBH activation was abolished (Figure 5A). It has been previously shown that CREBH interacts PPARα and PGC1α, to regulate the transcription of genes involved in FAO.[21, 22] We determined the levels of PPARα and PGC1α in the livers of MNADK KO and WT mice. Similar to the levels of cleaved/activated CREBH protein, the levels of PPARα and PGC1α in the livers of MNADK KO mice were significantly decreased (Figure 5A). Further, we examined the interaction between CREBH and PPARα in mouse livers, and WT mice displayed an increase in CREBH-PPARα interaction on the AHF diet, while this interaction was abolished by MNADK deficiency (Figure 5B). Consistently, expression of common target genes of CREBH and PPARα involved in FAO, including *Fgf21*, *Acaa2*, *LCAT*, and *Acot2*, was reduced in the KO mice on the AHF diet (Figure 5C). These results suggest that the reduced activity of the CREBH-PPARα regulatory axis may be responsible, at least in part, for the reduced FAO in MNADK KO mice during fasting.

Increased ROS represses CREBH and PPARα expression and activation in MNADK KO livers

Increased ROS levels alter the transcription of downstream genes partially through modulating sirtuin activity.[23] We tested whether increased ROS in MNADK KO mice contributed to the reduced expression levels of CREBH and PPARα, two stress-inducible, sirtuin-regulated metabolic regulators of FAO.[15, 17] Mitochondrial superoxide dismutase 2 (Mn-SOD) plays a critical role in limiting mitochondrial ROS release during oxidative stress.[24] Rac1 is a signaling G protein associated with NADPH oxidase, which represents a significant source of ROS.[25] Mouse primary hepatocytes were infected with adenoviruses expressing Mn-SOD, N17Rac1 (a dominant negative form of Rac1), or GFP, and then treated with either BSA-complexed PA (20μg/ml) or vehicle. Indeed, in WT primary hepatocytes, Mn-SOD and N17Rac1 suppressed PA-induced ROS production (Supplemental Figure 2A). Consistent with our previous study,[17] treatment of PA induced CREBH cleavage. In MNADK KO primary hepatocytes upon PBS or PA treatment, over-expression of Mn-SOD and/or N17Rac1 increased activated CREBH, PPARα, and PGClα proteins (Supplemental Figure 2B), and decreased PA-induced TG formation (Supplemental Figure 2C and D). N-acetyl-L-cysteine (NAC), a ROS inhibitor, consistently suppressed ROS levels, increased CREBH protein, increased expression of *Fgf21*, a CREBH transcriptional

target,[22] and decreased PA-induced TG formation (Supplemental Figure 2E-H) in MNADK KO primary hepatocytes. These results suggest that elevated ROS accumulation due to MNADK deficiency may be responsible for the reduced levels of activated CREBH and PPARα in AHF diet-fed MNADK KO mouse livers.

Sirtuin activity is reduced in the mitochondria of MNADK KO mice

Sirtuins are a family of NAD-dependent nutrient-sensing deacetylases that are critical in regulating metabolism. Deficiency of SIRT1[7] and SIRT3[26], the nuclear and mitochondrial sirtuins, respectively, results in hepatic steatosis and impaired FAO. Sirtuins can be sensitive to ROS, e.g., SIRT1 is post-translationally modified by oxidants, leading to its degradation via the proteasome.[23] On the AHF diet, the sirtuin activity in the mitochondria, but not nucleus or cytosol, of MNADK KO mice, was significantly reduced, compared to the activity in WT mice (Figure 5D and E). Consistently, hepatic activity of SIRT3 was reduced in the KO mice (Figure 5F). Indeed, MNADK KO mice and SIRT3 KO mice displayed considerable phenotypic similarities, such as hepatic lipid accumulation and reduced FAO.[26] Hepatic NAD levels in mice on the AHF diet were unexpectedly decreased (Figure 5G), which likely contributed to the reduced SIRT activities. These results suggest that MNADK plays a role in maintaining SIRT activity in mitochondria.

MNADK deficiency exaggerates fasting-induced hepatic steatosis

Prolonged fasting can induce fatty livers,[27] and because MNADK deficiency led to reduced FAO during fasting, we examined whether fasting could induce a prominent fatty liver phenotype in MNADK KO mice. In fed mice, hepatic lipid accumulation was virtually not detectable by Oil-Red O staining in liver sections of both MNADK KO and WT control mice. After a 24-hour fasting, hepatic lipid droplet accumulation was detected in both MNADK KO and WT mice, however, the levels of hepatic TG accumulated in the KO livers were significantly higher than those in the WT livers (Figure 6A and B). Consistent with the impairment in fasting-induced FAO, serum FFA levels were significantly increased in the KO mice (Figure 6C).

CREBH activation is strongly induced by fasting, and we therefore examined CREBH activation in MNADK KO mice in response to fasting. In both WT and MNADK KO mice, levels of CREBH precursors were comparable under the fed or fasted state. In WT mice, 24-hour fasting significantly increased the levels of activated CREBH. In contrast, in MNADK KO mice, the fasting-induced CREBH activation was abolished (Figure 6D). Consistently, treatment of glucagon, a fasting-induced hormone that activates CREBH through the cAMP pathway,[28] significantly increased the levels of cleaved CREBH in WT mice, but not in MNADK KO mice (Figure 6F). Because the activity of the CREBH-PPARα axis was downregulated in the KO mice, we examined the potential of phenotypic rescue with the PPARα agonist, Wy-14643. Mice were treated with Wy-14643 for 7 days and indeed, Wy-14643 significantly ameliorated fasting-induced hepatic steatosis, shown by Oil Red O staining of liver sections (Figure 6G) and quantification of hepatic TG (Figure 6H). Consistently, genes having reduced hepatic expression in the KO mice, such as *Fgf21*, were significantly up-regulated in Wy-14643-treated mice (Figure 6I). These results suggest that

MNADK deficiency exaggerates fasting-induced hepatic steatosis, at least, in part, through down-regulating the CREBH-PPARα axis.

Methotrexate (MTX) is an MNADK inhibitor

MTX is a first-line anti-rheumatoid arthritis drug and is also widely used as an anti-cancer chemotherapy drug[29], but its use has been significantly limited by its hepatotoxicity.[30] While MTX was thought to potently inhibit NAD kinase activity about 5 decades ago,[31] the mechanism of inhibition was unclear.[31] Because mammals have a mitochondrial NADK and a cytosolic NADK, we examined the degree by which MTX affects NADK activities in both liver fractions of mice. Mice were treated with MTX (20 mg/kg weight, *i.p.* injection daily for 7 days), and NADK activities were dramatically reduced in both mitochondrial and cytosolic liver fractions (Figure 7A and B).

The 3-dimentional structure of the NADK complexed to NAD has been solved (PDB: 1SUW). Since NADKs across species share highly conserved residues and structures of the catalytic site[10], we examined NADK-MTX docking. Strikingly, SwissDock analysis[32] revealed that MTX and NAD share the same binding pocket (Figure 7C and D), and that MTX can bind to the catalytic site of NADK (Figure 7E and F). We then examined the impact of MTX on the catalytic activity of recombinant MNADK.[12] Approximately 75% of the MNADK activity was suppressed by MTX at a concentration as low as 0.1 μM (Supplemental Figure 3A). In primary hepatocytes, MTX consistently inhibited NADK activities (Supplemental Figure 3B and C), and reduced NADP(H) levels in both mitochondria and cytosol (Supplemental Figure 3D), while MNADK overexpression through adenovirus specifically increased mitochondrial NADP(H) (Figure 7G). Consistently, MTX treatment resulted in TG accumulation in primary hepatocytes, which was rescued by MNADK over-expression, revealed by Oil Red O staining (Figure 7H) and TG quantification (Figure 7I).

Because MTX inhibits MNADK and reduces hepatocyte NADP levels, we examined the degree by which nicotinamide riboside (NR), a NAD$^+$ precursor and vitamin B3,[33, 34] rescues the MTX-induced hepatotoxicity. Mice were fed with either chow or NR-supplemented diet (400 mg/kg/day) for 2 months, with or without MTX treatment (20 mg/kg weight, *i.p.* injection daily for 7 days starting from the 8th week of NR treatment). MTX treatment reduced mitochondrial and cytosolic NADP(H) levels (Figure 7J), increased GSSG:GSH ratio in livers (Figure 7K), and increased serum levels of alanine aminotransferase (ALT) (Figure 7L) and aspartate aminotransferase (AST) (Figure 7M), while NR supplementation replenished hepatic NADP, and effectively protected mice from MTX-induced hepatic hepatotoxicity, such as increased GSSG:GSH ratio, and elevated levels of serum ALT and AST (Figure 7J-M). Taken together, these results suggest that MTX is an MNADK inhibitor, that MTX-induced hepatotoxicity may be partially due to its inhibition of MNADK and that NR supplementation is a potential therapeutic approach to alleviate MTX-induced hepatic hepatotoxicity.

Discussion

Mitochondria, the intracellular compartment crucial for FAO and ROS production, play important roles in the pathogenesis of NAFLD. Defects in FAO lead to an accumulation of FFA, which, in turn, becomes esterified into TG, and increased ROS triggers lipid peroxidation and cell death, all of which contribute to the progression of NAFLD.[35] MNADK deficiency results in reduced FAO and elevated ROS accumulation, both of which should contribute to the severe hepatic steatosis of the KO mice. Mechanistically, elevated ROS accumulation is likely due to reduced mitochondrial NADP(H), which is required to regenerate oxidative defense systems through glutathione reductase, as consistently reflected by the increased GSSG:GSH ratio in the MNADK KO livers. The reduced FAO in the KO mice is likely attributable to multiple mechanisms, including the reduced activation of CREBH and PPARα, reduced activity of the NADP(H)-dependent enzymes, such as DECR[36], as well as decreased mitochondrial SIRT activities.

CREBH and PPARα, as well as their interaction, are critical in mediating fasting response, e.g, fasting fails to induce FGF21 in either CREBH or PPARα-null mice.[21] CREBH is strongly activated by the fasting hormone glucagon, and this glucagon-dependent activation is largely abolished in the MNADK KO mice (Figure 6F). To further delineate the mechanism, we examined cellular ATP and cAMP levels, which were indeed significantly reduced in MNADK KO livers (Supplemental Figure 4A and B). Sirt3 has been shown to be critical in maintaining hepatic ATP levels,[37] and therefore reduced hepatic Sirt3 activity (Figure 5F) may partially explain the reduced ATP, consequently cAMP, levels, blocking the glucagon pathway in CREBH activation in the MNADK KO mice.

MTX, a folic acid antagonist, has been widely used in the treatment of cancers and autoimmune diseases.[38] MTX inhibits dihydrofolate reductase (DHFR), which catalyzes the reduction of dihydrofolate to tetrahydrofolate, a required cofactor involved in DNA biosynthesis, thereby blocking cell proliferation. NADK has been proposed to be a therapeutic target in cancer, because the demand for NADP(H) is particularly high in proliferating cancer cells.[39, 40] Therefore, a potential secondary mechanism for MTX's anticancer activity is through its MNADK inhibition to reduce NADP(H) and to increase ROS accumulation in mitochondria. MTX is also the first-line treatment for chronic inflammatory disorders, such as rheumatoid arthritis, psoriasis, psoriatic arthritis and inflammatory bowel disease.[38] Despite MTX's clinical efficacy, its use has been significantly limited by hepatotoxicity.[41, 42] A clear relationship between hepatic steatohepatitis and cumulative doses of MTX has been established,[43] and MTX is believed to disrupt mitochondrial function and increase oxidative stress,[44, 45] the mechanisms, however, remain poorly understood. Our results that MTX is an MNADK inhibitor and that MTX reduces intracellular NADP provide a direct mechanism for MTX-induced hepatotoxicity. The results that in MTX-treated mice, NR supplementation replenished hepatic NADP, suppressed oxidative stress reflected by decreased GSSG:GSH ratio, and normalized elevated serum levels of ALT and AST, strongly suggest that NR supplementation is a promising therapeutic approach in alleviating MTX-induced hepatotoxicity. Of note, the clinical trial has demonstrated that NR safely boosts NAD metabolism in humans,[34] and therefore the NR strategy is practical. Here, for the first time, we suggest that NR is a

therapeutic agent to treat diseases resulting from drug-induced NADP depletion. It is noteworthy that the two reported MNADK-deficient patients, while having abnormalities such as neurologic dysfunction, seem to lack liver symptoms.[3, 14] Our results show that the detrimental consequences of MNADK deficiency are pronounced under metabolic stress, such as fasting, and therefore our study suggests that MNADK-deficient patients and those undergoing MTX treatment should avoid metabolic stress conditions.

In summary, MNADK plays important roles in generating mitochondrial NADP, facilitating hepatic FAO, protecting hepatocytes from oxidative stress, maintaining mitochondrial sirtuin activities, and maintaining the activity of the CREBH-PPARα axis in the liver. MTX inhibited MNADK activity and suppressed NADP levels in primary hepatocytes and livers, and NR supplementation replenished MTX-induced hepatic NADP reduction, and largely protected mice from MTX's hepatotoxicity. These results suggest that patients deficient for MNADK, those undergoing MTX treatment, and patients whose symptoms result from drug-induced NADP depletion, may benefit from restoring their cellular NADP(H) levels by NR supplementation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AASS	α-aminoadipic acid semialdehyde synthase
AHF	atherogenic high-fat
ALT	alanine aminotransferase
AST	aspartate aminotransferase
CLAMS	the comprehensive lab animal monitoring system
CREBH	hepatocyte-specific cAMP-responsive element binding protein
DCFDA	2′,7′-dichlorofluorescin diacetate
DECR	2,4 dienoyl-coA reductase
DHE	dihydroethidium
DHFR	dihydrofolate reductase
FAO	fatty acid oxidation
GSH	reduced glutathione
GSSG	oxidized glutathione
KO	knockout
LC-MS/MS	liquid chromatography coupled with mass spectrometry
MNADK	mitochondrial NAD kinase, MTX, methotrexate
Mn-SOD	mitochondrial superoxide dismutase 2
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NAFLD	nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
NAC	N-acetyl-L-cysteine
NCD	normal chow diet
NEFA	non-esterified fatty acid
NR	nicotinamide riboside
PPARα	proliferator-activated receptor α
RER	respiratory exchange ratio
ROS	reactive oxygen species

SIRT sirtuins
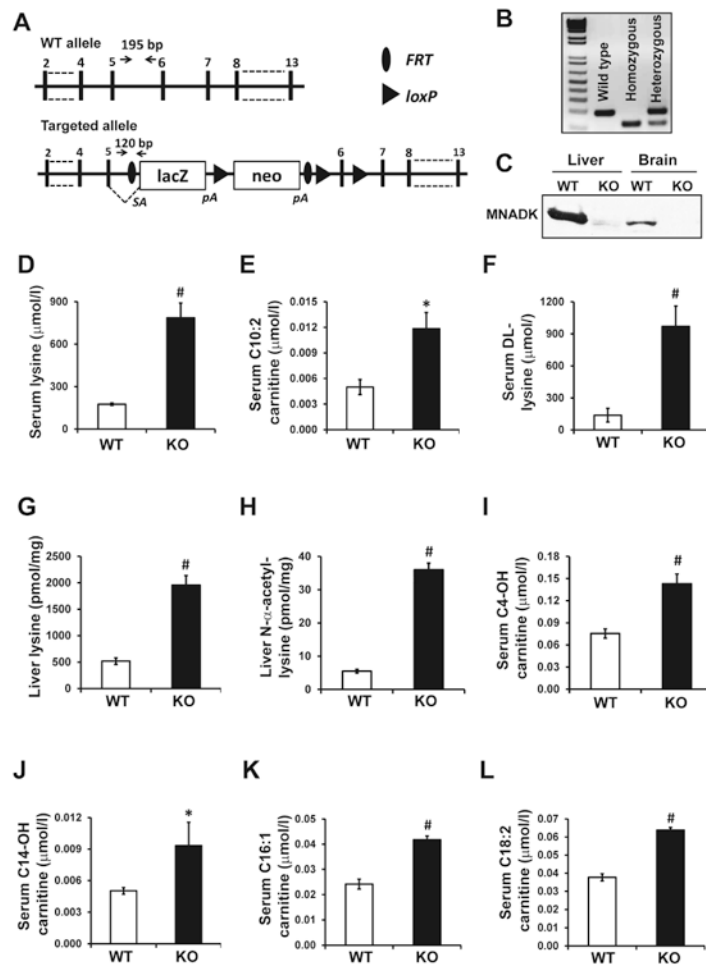
Figure 1. MNADK KO mice phenocopy the MNADK-deficient patient by having elevated serum lysine and C10:2 carnitine

(*A*) Scheme of the *MNADK* WT allele and targeted allele following homologous recombination. Locations of genotyping primers are indicated. (*B*) Mouse genotypes were determined by PCR, and the amplified fragments for WT and homozygous targeted allele were 195 bp and 120 bp, respectively. (*C*) Western blot analysis of homogenates from liver and brain of WT and KO mice. Serum levels of (*D*) lysine (WT, N = 10; KO, N = 7) and (*E*) C10:2 carnitine (WT, N = 7; KO, N = 4). Levels of (*F*) serum DL-lysine, (*G*) liver lysine, and (*H*) N-α-acetyllysine (WT, N = 4; KO, N = 3). Serum levels of (*I*) C4-OH carnitine, (*J*) C14-OH carnitine, (*K*) C16:1 carnitine, and (*L*) C18:2 carnitine (WT, N = 7; KO, N = 4). Level of lysine, lysine degradation products and carnitines were measured using tandem mass spectrometry. All mice were male, 10-week old. Data is presented as mean ± SEM. *, $P<0.05$; #, $P<0.01$.
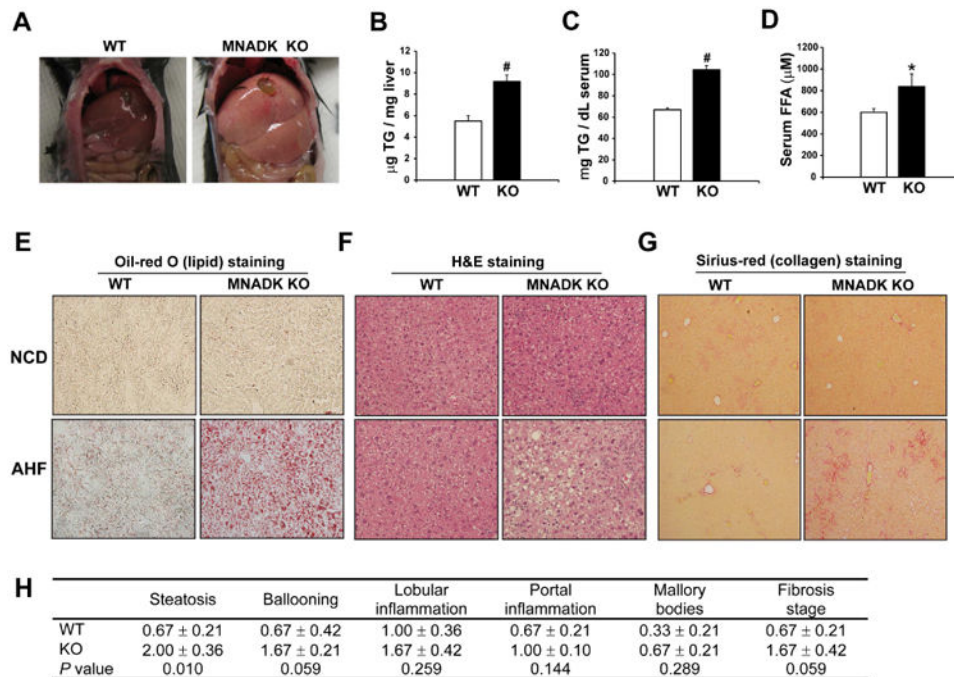
Figure 2. MNADK KO mice develop hepatic steatosis on an atherogenic high-fat diet
(*A*) Gross view of representative livers of WT and MNADK KO mice. (*B*) Serum and (*C*) liver levels of TG, as well as (*D*) serum levels of FFA in WT and MNADK KO mice. (*E*) Oil-red O staining, (*F*) H&E staining and (*G*) Sirius-red staining of liver sections of KO and WT mice. (*H*) Histological scoring for NASH activities in the livers of WT and KO mice after on the AHF diet for 3 weeks. Grade scores were calculated based on the scores of steatosis, hepatocyte ballooning, lobular and portal inflammation, and Mallory bodies. Stage scores were based on the liver fibrosis. P values were calculated by the Mann-Whitney U test. Male 10-week old MNADK KO mice and WT littermates were placed on an AHF diet for 3 wks. WT, N = 5; KO, N = 4. Data is presented as mean ± SEM. *, P<0.05; #, P<0.01.
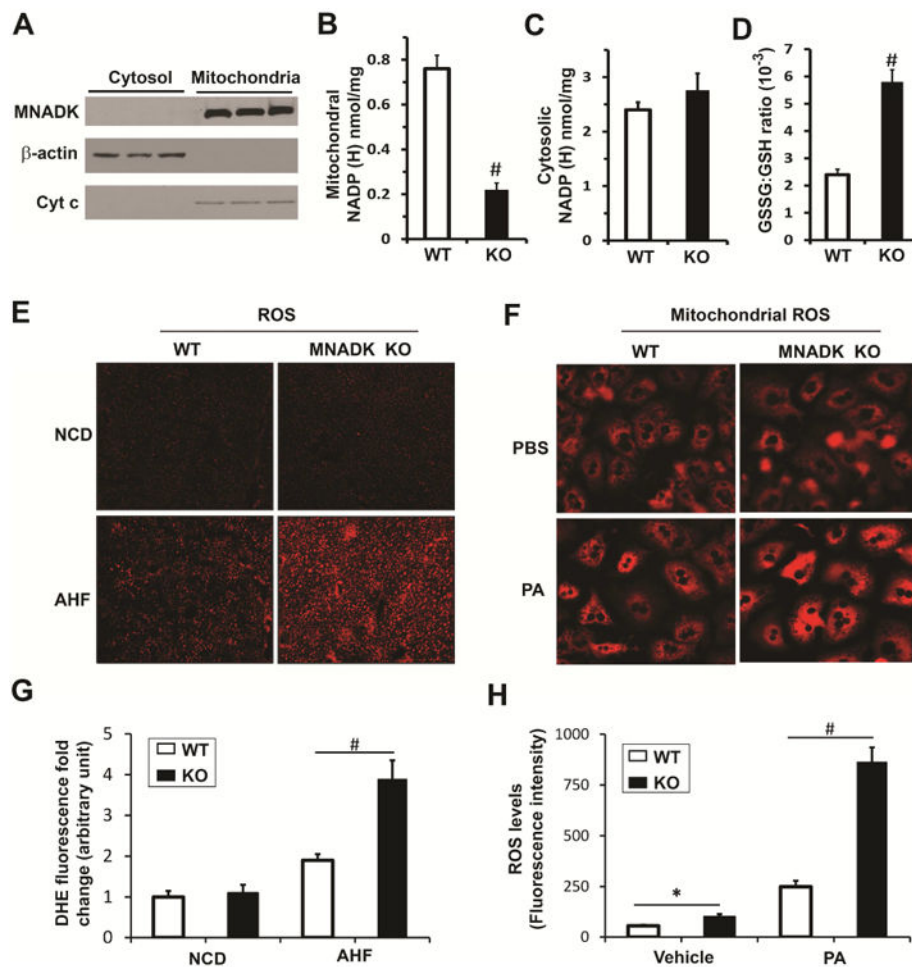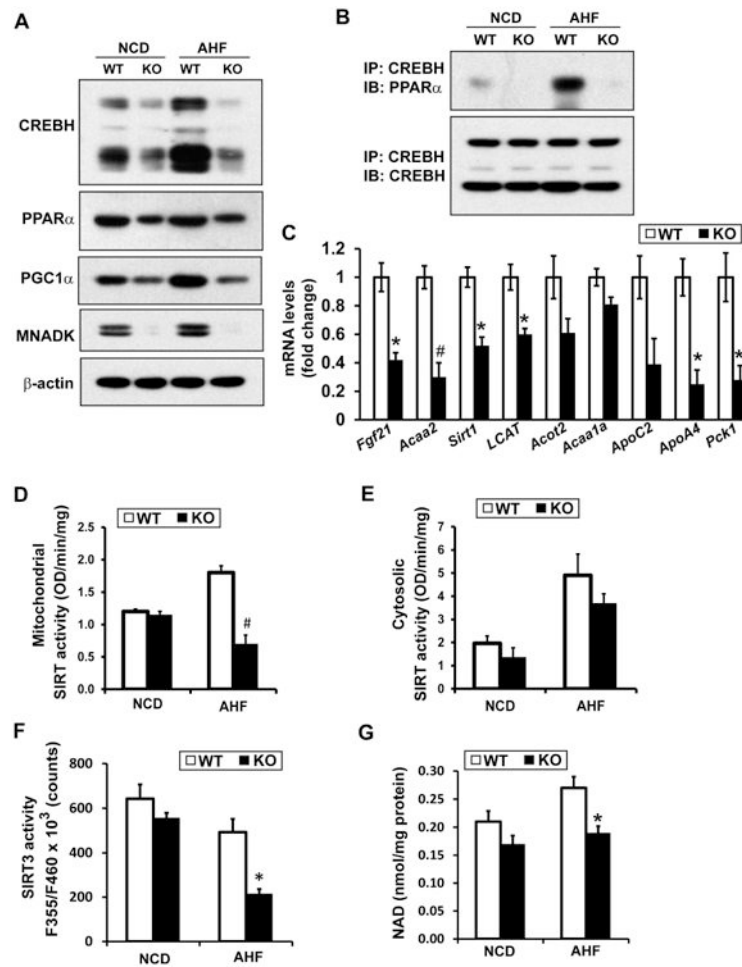
Figure 3. MNADK deficiency reduces mitochondrial NADPH and increases ROS levels
(*A*) Western blotting analysis of mitochondrial and cytosolic fractions prepared from 60 mg of livers of 3 WT male mice. Mitochondria were isolated using Qproteome mitochondria isolation kit (Qiagen). Levels of NADP(H) in (*B*) mitochondrial and (*C*) cytosolic fractions in livers, and (*D*) GSSG:GSH ratio in livers of KO and WT mice (N = 3). NADP (H) levels were quantified with the NADPH quantitation colorimetric kit (Biovision), and GSSG:GSH ratio was determined by GSH/GSSG ratio detection assay kit (Abcam). (*E*) Representative ROS staining in liver sections of WT and MNADK KO mice on either NCD or AHF diets. (*F*) Representative mitochondrial ROS staining in WT primary hepatocytes treated with PBS or palmitic acid (PA). (*G*) Quantification of ROS levels in liver sections of WT and KO mice on either NCD or AHF diets. Dihydroethidium was used to detect superoxide in liver tissue sections. (*H*) ROS levels in WT primary hepatocytes treated with PBS or PA, quantified with ROS quantification kit (Abcam). Primary hepatocytes were treated with 20μg/ml BSA-complexed PA or PBS vehicle for 6 hours. ROS quantification of primary hepatocytes represents three independent experiments using samples in triplicates. All mice were male, 15-16 week old. Data is presented as mean ± SEM. *, P<0.05; #, P<0.01.
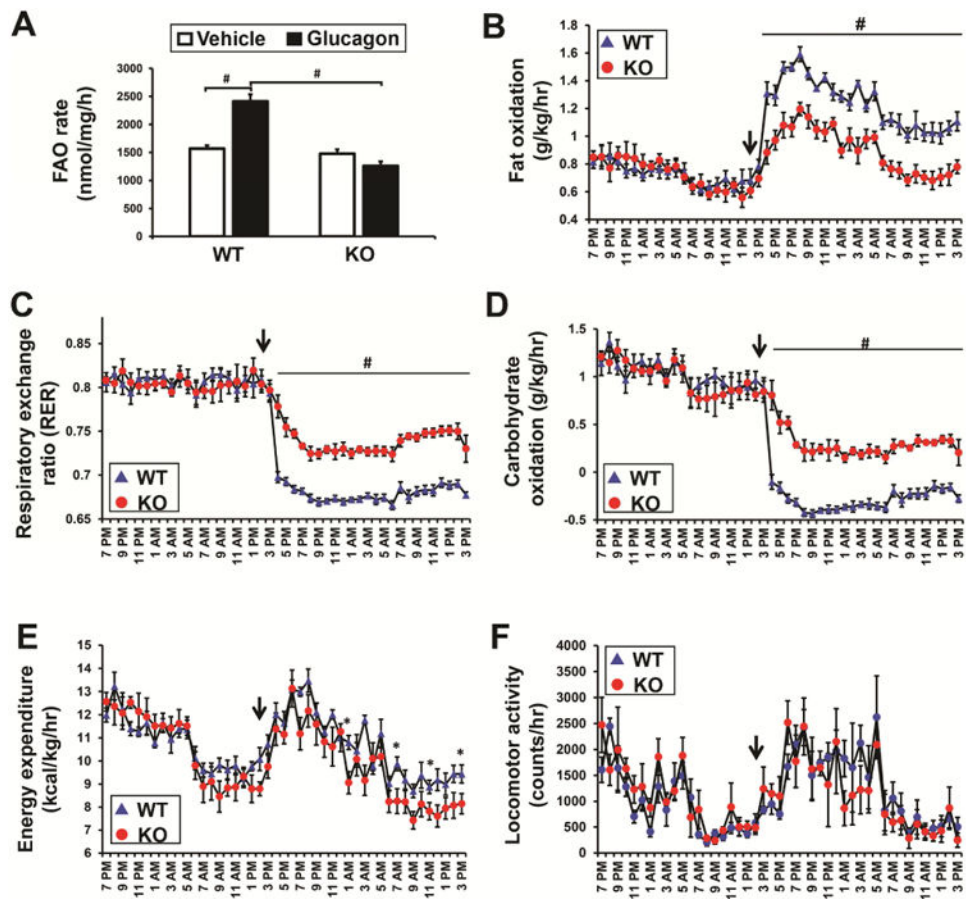
Figure 4. MNADK KO mice have defects in fasting-induced fatty acid oxidation

(*A*) The rate of FAO in primary hepatocytes. The FAO rate of 9,10-^{3}H palmitate by primary hepatocytes was determined with or without glucagon treatment for 12 hrs before measurement. (*B*) fat oxidation, (*C*) respiratory exchange ratio (RER), (*D*) carbohydrate oxidation, (*E*) energy expenditure, and (*F*) locomotive activity in WT and MNADK KO mice determined through indirect calorimetry by using the comprehensive lab animal monitoring system (CLAMS). WT, N = 6; KO, N = 4; all mice were male, 11-week old. Data is presented as mean ± SEM. *, P<0.05; #, P<0.01.

Figure 5. MNADK deficiency impairs AHF diet-induced CREBH activation and reduces mitochondrial SIRT activity

(*A*) Representative Western blotting analysis of full-length and activated CREBH and PPARα in WT and MNADK KO mice on the NCD or AHF diet. (*B*) Reduced interactions between CREBH and PPARα in MNADK KO mice. The interaction between CREBH and PPARα was determined by immunoprecipitation (IP)-Western blot assay. Male MNADK KO and WT mice were placed on the AHF diet for 3 weeks. N = 4-6 per group. (*C*) Reduced hepatic expression in genes involved in FA oxidation in MNADK KO mice on the AHF diet. qPCR analysis of the expression of CREBH transcription targets. Sirtuin activities in (*D*) mitochondrial and (*E*) cytosolic fractions of livers, and (*F*) Sirtuin 3 activity in livers from mice on NCD or the AHF diet. (G) Reduced NAD levels in livers of mice on the AHF diet. Male MNADK KO and WT mice were placed on the AHF diet for 3 weeks. N = 4-6 per group; all mice were male, 13-week old. *, P<0.05; #, P<0.01.
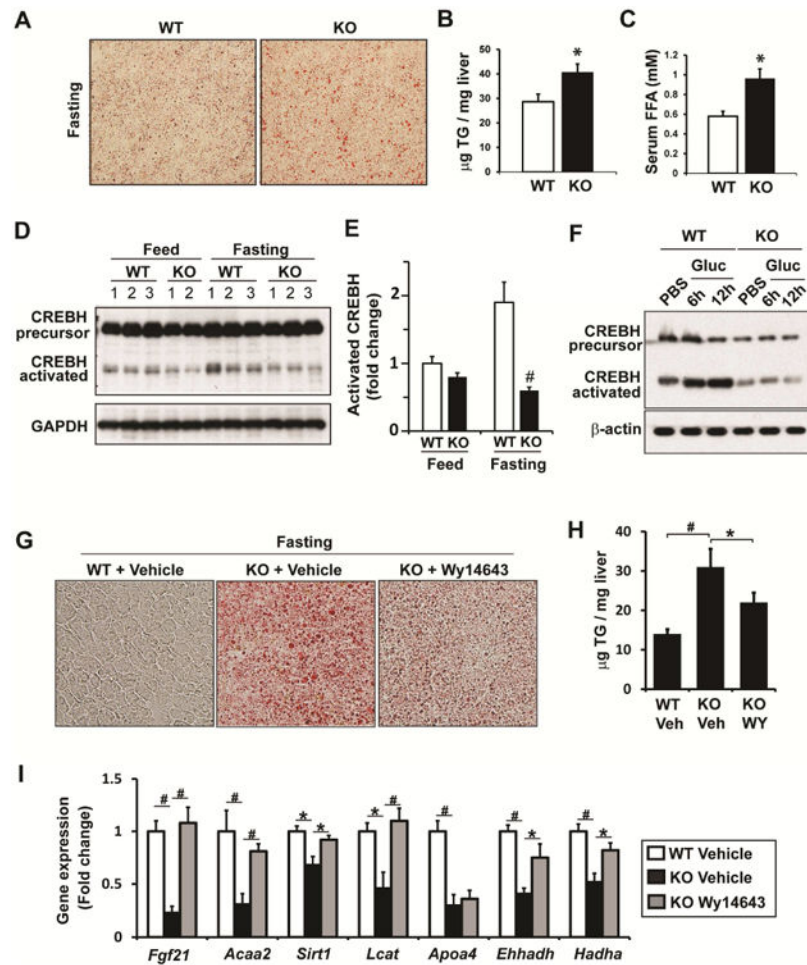
Figure 6. MNADK deficiency exacerbates fasting-induced hepatic steatosis and impairs fasting-induced CREBH activation

(*A*) Oil Red O staining of livers of 24-hr fasted MNADK KO and WT control mice. Levels of (*B*) liver TG and (*C*) serum FFA of WT and KO mice. Fed, N = 3; Fasted, N = 3. (*D*) Representative western blotting analysis for full-length and activated CREBH in WT and MNADK KO mice with or without 24-hr fasting, and (*E*) band intensity was quantified for activated CREBH normalized to GAPDH. Fed: WT, N = 3; KO, N = 2. Fast: WT, N = 3; KO, N = 3. (*F*) Representative Western blotting analysis for full-length and activated CREBH in WT and MNADK KO mice treated with glucagon or PBS were shown. N = 3 per group. (*G*) Oil Red O staining of livers of 24-hr fasted MNADK KO mice, WT mice and KO mice treated with PPARα agonist Wy-14643 by gavage daily for 7 days (10 mg/kg body weight, dissolved in 2% methylcellulose vehicle) and control mice received methylcellulose only. (*H*) Levels of liver TG in WT and KO mice. (*I*) qPCR analysis for the hepatic expression of CREBH-PPARα downstream genes. Gene expression was normalized to β-actin. N = 3 per group, male 8-week old mice. Data is presented as mean ± SEM. *, P<0.05; #, P<0.01.
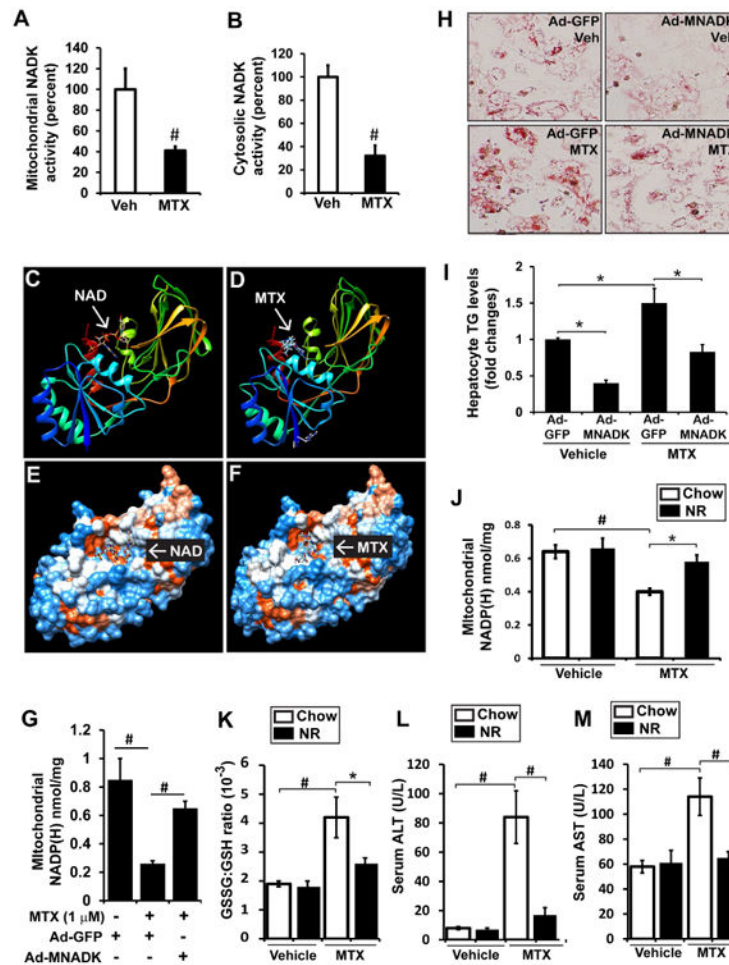
Figure 7. MTX is an MNADK inhibitor

MTX inhibited NADK activity in the (*A*) mitochondrial and (*B*) cytosolic fractions of mouse livers. Ten 8-wk old male C57BL6 (5 per group) were *i.p.* injected with MTX (20mg/kg body weight, dissolved in DMSO, and then further diluted into PBS) or vehicle control for 7 days. Cytosolic and mitochondrial fractions were prepared from liver homogenates, and then were used to assess NADK activity as previously described.[12] (*C*) A structure of NADK complexed to NAD (PDB: 1SUW, chain A. (*D*) Molecular modeling (SwissDock) revealed that MTX binds to the catalytic pocket of NADK. Detailed view of the binding pocket of NAD (*E*) and MTX (*F*) shown as hydrophobicity surface. (*G*) MTX reduced mitochondrial NADP(H) levels that were rescued by MNADK overexpression in primary hepatocytes. NADP(H) levels in the mitochondrial fraction of primary hepatocytes treated with 1 μM MTX for 24 hrs. In primary hepatocytes MTX resulted in TG accumulation that was rescued by MNADK expression. (*H*) Representative images of Oil red O staining and (*I*) TG contents in hepatocytes infected with adenoviruses expressing GFP or MNADK, with or without 1 μM MTX treatment. Primary hepatocytes were infected with adenoviruses expressing either GFP or MNADK at an MOI of 150 when the cells were about 60% confluent, and 36 hours following the virus infection, hepatocytes were incubated with 1 μM MTX for 24 hrs. TG levels were normalized to protein amount. Liver (*J*) mitochondrial NADP(H) levels, and (*K*) GSSG:GSH ratio, and serum (*L*) ALT and (*M*) AST levels in mice

on diets with or without NR-supplementation. 8-week old C57B6 mice were fed with chow diet mixed with water (vehicle) or NR (400 mg/kg/day) for 8 weeks, and MTX treatment started on day 1 of the 8th week (20mg/kg body weight, dissolved in DMSO, and then further diluted into PBS; *i.p.* injected daily for 7 days). N=5-6 per group. *, P<0.05; #, P<0.01.