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Vitamin B5 and N-acetylcysteine in nonalcoholic steatohepatitis: a pre-clinical study in a dietary mouse model

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Abstract

Background—Nonalcoholic fatty liver disease (NAFLD) is the number one cause of chronic liver disease and second indication for liver transplantation in the Western world. Effective therapy is still not available. Previously we showed a critical role for caspase-2 in the pathogenesis of nonalcoholic steatohepatitis (NASH), the potentially progressive form of NAFLD. An imbalance between free Coenzyme A (CoA) and acyl-CoA ratio is known to induce caspase-2 activation.

Objectives—We aimed to evaluate CoA metabolism and the effects of supplementation with CoA precursors, pantothenate and cysteine, in mouse models of NASH.

Methods—CoA metabolism was evaluated in methionine-choline deficient (MCD) and Western diet mouse models of NASH. MCD-diet fed mice were treated with pantothenate and N-acetylcysteine or placebo to determine effects on NASH.

Results—Liver free CoA content was reduced, pantothenate kinase (PANK), the rate-limiting enzyme in the CoA biosynthesis pathway, was down-regulated, and CoA degrading enzymes were increased in mice with NASH. Decreased hepatic free CoA content was associated with increased caspase-2 activity, and correlated with worse liver cell apoptosis, inflammation and fibrosis. Treatment with pantothenate and N-acetylcysteine did not inhibit caspase-2 activation, improve NASH, normalize PANK expression, or restore free CoA levels in MCD diet-fed mice.

Conclusion—In mice with NASH, hepatic CoA metabolism is impaired, leading to decreased free CoA content, activation of caspase-2, and increased liver cell apoptosis. Dietary supplementation with CoA precursors did not restore CoA levels or improve NASH, suggesting that alternative approaches are necessary to normalize free CoA during NASH.

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Keywords

nonalcoholic steatohepatitis; coenzyme A; caspase-2; pantothenate; N-acetylcysteine

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the obesity-related liver disease. It is estimated that worldwide, one billion people are affected by NAFLD (1). Most patients have non-progressive simple steatosis, but up to one fifth develop nonalcoholic steatohepatitis (NASH), the type of NAFLD that can trigger progressive liver fibrosis (2). Liver outcomes in NASH are dictated by the severity of fibrosis (3, 4). NASH-related cirrhosis is already the second liver disease among adults awaiting liver transplantation in the US (5). An effective therapy for NASH is still not available. Recent large, randomized, placebo-controlled trials identified two agents that seem to improve histology in patients with NASH: vitamin E and obeticholic acid (6, 7). Vitamin E improved hepatocellular ballooning and inflammation, but it failed to improve fibrosis (6). Also, safety issues with vitamin E preclude its widespread use. Obeticholic acid seems to improve liver histology, including fibrosis, however it worsened the metabolic profile in patients with NASH (6, 7). Hence, it is imperative to search for better NASH therapies.

NASH is characterized by increased hepatocyte apoptosis. Caspase-2 induces apoptosis when lipids accumulate in the context of starvation (8). Our group showed that caspase-2 activation is also a pivotal player in NASH-related lipotoxicity (9). We demonstrated increased caspase-2 expression and correlated this with fibrosis stage, in human NASH and several NASH animal models. Moreover, caspase-2 deficient mice were protected from diet-induced NASH, developing less hepatocyte cell death and fibrosis than wild type mice that were fed the same diets (9). Caspase-2 activation is regulated by free coenzyme-A (CoA) content and free CoA/acyl CoA ratio. Decreases in free CoA/acyl CoA ratio occur during hepatic lipogenesis. This may activate caspase-2 during NASH by inhibiting the calcium/calmodulin-regulated protein kinase II (CAMK II)-dependent phosphorylation of caspase-2 at serine-135, which inactivates the caspase (10). Interestingly, either fasting or high-fat diet and diabetes have been shown to decrease the free CoA/acyl-CoA ratio in hepatocytes (11). This may explain why both starvation and energy-surplus promote caspase-2 activation and suggests that approaches to replenish free CoA might reduce caspase-2 activation in these conditions.

CoA is a cofactor of 4% of all known enzymes and is required for intermediary metabolism (12). CoA can be synthesized from pantothenate, cysteine, and ATP (13). Pantothenate (from the Greek “pantos” meaning “everywhere”) is the water-soluble vitamin B5 (14). Because mammalian cells cannot synthesize pantothenate, they need vitamin B5 to be provided by the diet or generated by the intestinal microbiome in order to synthesize CoA (15). Pantothenic acid deficiency rarely occurs because most foods are rich in vitamin B5 (16). When dietary pantothenate is depleted in animals, however, liver levels of CoA decline, and serum levels of triglyceride and free fatty acids increase, suggesting that hepatic lipid metabolism has become de-regulated (17). Consistent with this concept, inhibiting the

first enzyme in the synthesis of CoA from pantothenate also causes hypertriglyceridemia. Further, blocking the synthesis of CoA from pantothenate leads to mitochondrial dysmorphology similar to human NASH, impairs hepatic β -oxidation of fatty acids, induces hepatic steatosis, and increases hepatic gluconeogenesis (18-20). These observations suggested to us that the generation of CoA from pantothenate might be impaired in NASH, reducing hepatic stores of free CoA and thereby promoting excessive activation of caspase-2 and increased hepatocyte apoptosis. Therefore, we performed a pre-clinical study to evaluate the effect of treatment with vitamin B5 (pantothenate) and a cysteine precursor (N-acetylcysteine), substrates for CoA biosynthesis, in animal models of NASH.

METHODS

Animal Studies

Male wild-type (WT) mice C57Bl/6 were obtained from Jackson Laboratory and fed either chow diet (Picolab[®] Rodent diet 20, #5053), methionine-choline deficient (MCD) diet (MP Biomedicals, #960439) for 8 weeks or Western diet (45% high-fat diet, enriched in saturated fat, supplemented with 0.2% cholesterol and high-corn syrup equivalent in the drinking water, TD.120330 22% HVO + 0.2% cholesterol diet, Teklad Research) for 16 weeks. Diet specifications are summarized in **Supplemental Tables 1 and 2**. Two separate experiments were done, the first compared chow-fed diet (n=6), MCD diet-fed (n=8) and Western diet-fed (n=8) mice; the second experiment studied 30 mice divided into 3 groups: chow diet, MCD diet and MCD diet treated with Pantothenate and N-acetylcysteine. Pantothenate (Sigma C8731) was administered IP in a solution in PBS, at a dose of 250 mg/kg three times per week. The control group was injected with same volume of PBS. N-acetylcysteine (Sigma, A9165) was administered in the drinking water, resulting in an estimated consumption of 250 mg N-acetylcysteine /kg body weight per day.

Animal care and procedures were approved by the Duke University Institutional Animal Care and fulfilled National Institutes for Health and Duke University IACUC requirements for humane animal care.

Histopathological analysis

Formalin-fixed, paraffin-embedded liver biopsies were cut into 5 μ m serial sections, were stained with H&E and evaluated for severity of NAFLD.

For immunohistochemistry, sections were deparafinized with xylene and rehydrated. Slides were incubated in 3% hydrogen peroxide/methanol. Antigen retrieval was performed by heating in 10 mM sodium citrate (pH 6.0) or pepsin digestion, according to the primary antibody. Horseradish peroxidase-conjugated IgG secondary antibody was used. Antigens were demonstrated by diaminobenzidine (K3466, Dako). Tissue sections were counterstained with Mayer's Hematoxylin (S3309, Dako). Morphometric analysis was done with Metamorph Software (Molecular Devices Corporation, Downingtown, PA), in randomly chosen sections (20x magnification, 20 fields/sample). Immunohistochemistry antibodies are specified in **Supplemental Table 3**.

Serum and tissue analysis

Liver enzymes, alanine and aspartate aminotransferases (ALT and AST) were determined colorimetrically (Biotrom Diagnostics Inc, #398917 and #50726, respectively). Free CoA was quantified in flash frozen liver samples with Coenzyme-A detection kit (Abcam, ab102504) according to manufacturer protocol. Triglycerides were measured in the liver with Triglyceride Colorimetric Assay kit (Cayman Chemical Company: #10010303). Liver hydroxyproline content was colorimetrically quantified in flash frozen liver samples, as previously described (21). Caspase-3 activity was evaluated in 200 ug of whole liver cytosolic extracts, with Caspase-3 Colorimetric Activity Assay kit (EMD Millipore, APT 165), according to manufacturer protocol. Thiobarbituric acid reactive substances (TBARS) liver content was quantified with TBARS Assay Kit (Cayman Chemical Company, Catalog # 10009055).

Molecular Studies

mRNA quantification by Real-time Reverse Transcription-PCR (RT-PCR)—

Total RNA was extracted from livers using TRIzol (Invitrogen). RNA was reverse transcribed to cDNA templates using random primer and Super Script RNase H-Reverse Transcriptase (Invitrogen) and amplified. Semiquantitative qRT-PCR was performed using iQ-SYBR Green Supermix (Bio-Rad) and StepOne Plus Real-Time PCR Platform (ABI/Life Technologies), as previously described (22). For primers, see **Supplemental Table 4**.

Western Blotting—Total proteins were extracted from whole liver using RIPA buffer (Sigma). Proteins were separated by electrophoresis on 4%-20% Criterion gels (BioRad), transblotted into polyvinylidene-difluoride membranes, and incubated with the following primary antibodies: mouse anti-caspase-2 (Cell Signaling, #2224) and rabbit anti-PANK1 (Abcam, ab96173).

Statistics

Results were expressed as mean±SEM. Significance was established using Mann-Whitney tests, with significance $p<0.05$. Correlations evaluated with Spearman coefficient.

RESULTS

CoA metabolism is perturbed in mouse models of NASH

Our group previously showed that caspase-2 is up-regulated in the livers of patients with NASH and correlates with the severity of fibrosis (9). Also, animal models of NASH suggest a crucial role for caspase-2 in the pathogenesis of NASH, particularly in regulating cell death and fibrogenesis (9). Because it has been reported that decreased free CoA promotes caspase-2 activation (10), we evaluated free CoA content and metabolism in a mouse model of NASH induced by feeding methionine-choline deficient (MCD)-diet. As compared to mice fed chow diet, mice fed with MCD diet for 8 weeks, developed a marked decrease in free CoA liver content (**Figure 1.A**). Decreased free CoA content might reflect a shift in the free CoA/acyl CoA ratio that accompanies hepatic accumulation of free fatty acids in this model (23). A decrease in CoA synthesis could also be involved. Therefore, we used qRT-PCR (**Figure 1.C**) and Western-blot (**Figure 1.D**) to evaluate the liver expression

of enzymes in the CoA synthetic pathway, including PANK1, the rate-limiting enzyme in this process. A marked decrease in PANK expression was observed. This finding was intriguing because a feedback inhibition of PANK expression by acyl-CoA has been described (24-26). Lastly, we evaluated the expression of enzymes in the CoA degradation pathway, vanin-1 and vanin-3. qRT-PCR demonstrated a significant up-regulation of these genes (**Figure 1.C**). We found similar changes in CoA metabolism in a different model of NASH caused by feeding Western diet for 16 weeks (**Supplemental Figure 1**), which also associates with increased caspase-2 expression/activation (27). Thus, in two mouse models of NASH, we found a profound decrease in liver free CoA content. Two potential mechanisms for this were also demonstrated, i.e., decreased synthesis of free CoA and increased CoA degradation. To assess whether or not CoA metabolism might be similarly altered in human NASH, we examined our previous microarray analysis of NASH patients(28) and found CpG hypermethylation and decreased PANK-1 gene expression in patients with advanced fibrosis (i.e. fibrosis grade 3-4, n=32), as compared to those with mild fibrosis (i.e. fibrosis grade 0-1, n=40). Thus, the human data support the findings in pre-clinical models: both suggest that free CoA stores can decline during NASH.

Hepatic free CoA content negatively correlates with apoptosis and liver injury

In mice fed either chow diet or MCD diet, we found strong negative correlations between hepatic levels of free CoA and evidence of cell death by apoptosis as assessed by ALT serum levels, TUNEL immunohistochemistry and caspase-3 activity (**Figure 2.A**). Consistent with the concept that free CoA inhibits caspase-2 activity and protein stability, we found an inverse correlation with free CoA and expression of activated caspase-2 protein on Western blot (**Figure 2.A**). We also found strong negative correlations between free CoA content and the fibroductular reaction (**Figure 2.B**), proinflammatory cytokine TNF- α , and macrophage markers F4/80 and YM-1 (**Figure 2.D**). A much weak correlation was found between hepatic triglycerides and free CoA content (**Figure 2.C**).

Treatment with pantothenate and N-acetylcysteine does not protect from liver injury in the MCD model

Because we found decreased levels of free CoA and evidence for decreased CoA synthesis in mouse models of NASH, we performed a second experiment to evaluate the effect of treatment with the CoA precursors, pantothenate (P) and N-acetylcysteine (NAC) (15), on liver injury. Treatment with CoA precursors did not prevent weight loss in the MCD model and caused a mild increase in liver-to-body ratio (**Figure 3.A**). Such hepatomegaly might reflect the tendency for increased liver triglyceride accumulation in P+NAC-treated mice (**Figure 3.D**). Also, treated and untreated mice submitted to MCD diet showed similar liver injury as assessed by aminotransferases serum levels (**Figure 3.B**) and evaluation of H&E stained liver sections (**Figure 3.C**). Treatment also did not improve inflammation as assessed by qRT-PCR analysis of pro-inflammatory genes (TNF- α , F4/80 and YM-1) in whole liver. In fact, those transcript levels were higher in MCD diet-fed mice treated with P +NAC than untreated MCD diet-fed mice. We found similar results in mice fed with MCD diet and treated with pantothenate or NAC alone (**Supplemental Figure 2**).

Treatment with pantothenate and N-acetylcysteine does not protect from fibroductular response in the MCD model

In human NASH, the level of injury does not independently dictate liver-related morbidity or mortality. Rather, fibrosis is the most important factor that predicts worse liver outcomes (4). Hence, it is the repair response to lipotoxicity that dictates prognosis (29). Taking that into consideration, we evaluated the effect of P+NAC on the ductular reaction (i.e., accumulation of ductular-appearing progenitors and myofibroblasts) because fibrosis severity in NASH patients and animal models of NASH strongly correlates with the intensity of the ductular reaction (30). Compared to untreated mice, treated mice were not protected from liver fibrosis, as assessed by hydroxyproline content (**Figure 4.A**) or qRT-PCR/immunohistochemistry analysis of the fibrogenic markers α -smooth muscle actin (α -SMA), collagen-1a and desmin (**Figure 4.B and C**). The progenitor reaction of treated and untreated mice was also similar, i.e., P+NAC did not diminish MCD diet-related induction of the progenitor markers, K7 and K19, as evaluated by qRT-PCR and immunohistochemistry (**Figure 4B and C**).

Treatment with Pantothenate and N-acetylcysteine does not reverse caspase-2 up-regulation or impairment in CoA metabolism, in the MCD model

Since we gave precursors of CoA, which promotes caspase-2 inactivation (10), and caspase-2 seems to have a crucial role in NASH pathogenesis (9), we evaluated caspase-2 induction (by qRT-PCR) and activation (by Western-blot analysis for cleaved caspase-2) in whole liver extracts. Treatment with P+NAC did not protect mice from MCD diet-related induction or activation of caspase-2. In retrospect, this finding was not unanticipated because we found that treatment with CoA precursors did not increase the hepatic content of free CoA, or reverse the MCD diet-related down-regulation of rate-limiting enzymes in the CoA synthesis pathway in our model (**Figure 5**).

Treatment with Pantothenate and N-acetylcysteine does not decrease MCD-induced oxidative stress

Oxidative stress is believed to play a crucial role in the pathogenesis of human NASH (31, 32). Antioxidant enzymes, such as superoxide dismutase 1 and 2 (SOD-1 and -2) neutralize toxic hydroxyl radicals, nitric oxide radicals, and superoxide anions, converting them into H_2O_2 , which is further detoxified by glutathione peroxidase (GPx) and catalase. Thus, oxidative stress typically triggers compensatory induction of SOD and GPx. N-acetylcysteine provides a source of cysteine to replenish stores of reduced glutathione and thus, NAC has antioxidant properties that reduce the stimulus for antioxidant enzyme induction (33). NAC has also been reported to reduce hepatic steatosis and inflammation in a high-fat diet rat model of NAFLD (34).

In mice submitted to MCD diet, however, treatment with NAC did not improve mRNA expression of anti-oxidant enzymes (**Supplemental Figure 3**). Also, it did not improve oxidative stress as assessed by gene expression of heme oxygenase [known to be up-regulated in response to oxidative stress (35)], by immunohistochemistry for 4-hydroxynonenal (4-HNE), a byproduct of lipid peroxidation and by determination of

TBARS. These findings suggest that supplementing NAC was not sufficient to overcome the negative impacts of CoA depletion on hepatic redox balance in MCD diet-fed mice.

DISCUSSION

Our study showed that the hepatic content of free CoA is significantly decreased in two animal models of nonalcoholic steatohepatitis (NASH). We also discovered that hepatic levels of free CoA strongly and inversely correlate with liver caspase-2 activation, apoptosis, inflammation, the intensity of fibrogenic repair responses such as the ductular reaction, and liver fibrosis severity. In addition, we identified two potential mechanisms that may contribute to the reduced free CoA content in mice with NASH, namely down-regulation of key enzymes in the biosynthetic pathway of CoA from pantothenate, and up-regulation of enzymes in the CoA degradation pathway. Reciprocal changes in free CoA synthesis and degradation could confound the increased demand for free CoA to generate the acyl-CoA required for fatty acid metabolism. Preliminary evidence for decreased expression of the first enzyme in CoA biosynthesis was also documented in NASH patients with severe fibrosis, relative to those with mild fibrosis suggesting that free CoA synthesis may also be impaired in human NASH. Decreases in the free CoA/acyl-CoA ratio has critical consequences for lipid and glucose metabolism, since this regulates key metabolic reactions catalyzed by acyl-CoA synthetase, pyruvate dehydrogenase, and α -ketoglutarate dehydrogenase (13). Importantly, decreases in free CoA are also known to activate caspase-2 (10), a pivotal pro-apoptotic protein in the pathogenesis of NASH (9). In an attempt to restore free CoA levels during NASH, we treated MCD diet-fed mice with pantothenate and N-acetylcysteine, precursors of CoA. However, this approach did not reduce liver injury or improve fibrosis. Treatment failure might have reflected the fact that our protocol was unable to normalize hepatic free CoA content in this model. Consistent with its failure to improve free CoA levels, treatment did not blunt the induction of caspase-2, a key driving force for liver apoptosis in NASH pathogenesis. We cannot exclude the possibility that the treatment failed because of the specificities of the protocol. However, in an experimental model of NASH induced by high fat diet in mice with perturbed ketogenesis, *ex vivo* liver perfusion with pantothenate and L-cysteine restored CoA levels (36). We used a high dose of pantothenate, which has been shown by others to be effective in improving insulin resistance, dyslipidemia and hepatic triglyceride content in a hypothalamic mouse model of obesity (37), but that is three times lower the toxic dose (38). The beneficial metabolic effect seen in the obesity mouse model seems to be a consequence of decreased food intake in treated animals. This may reflect an increase in hypothalamic malonyl-CoA concentration, given that hypothalamic malonyl-CoA is known to have anorexigenic properties (37). Furthermore, we used a protocol for N-acetylcysteine that has resulted in improvement of oxidative stress in other diseases mouse models (39).

The fact that administering pantothenate did not correct hepatic free CoA levels in our mice suggests that CoA levels are tightly regulated. This concept is supported by evidence that high concentrations of pantothenate were able to increase CoA content in a cultured hepatocyte cell line that was depleted of pantothenate (40, 41). Further, early studies showed that adult rodents maintain stable CoA levels when the dietary supply of pantothenic acid is drastically reduced or increased (24). This suggests that pantothenate levels *per se* may not

be rate-limiting factor for CoA biosynthesis, or that tissue levels of pantothenate typically exceed those that are required for CoA synthesis. Indeed, tissue pantothenate levels are maintained by compensatory changes in urinary excretion of vitamin B5 when dietary ingestion of pantothenate is altered (42). CoA synthesis itself is highly regulated at the level of the first enzyme of the pathway, pantothenate kinase (PANK). There is a strong feedback inhibition of PANK expression and activity by acetyl-CoA and Acyl-CoA (13, 24-26). Our results suggest that supplemental pantothenate might be unable to fuel CoA synthesis during NASH because PANK expression is severely suppressed during NASH. Further, newly synthesized CoA from pantothenate would be rapidly esterified with fatty acids, further inhibiting PANK. Consistent with this hypothesis, treatment with pantothenate did not correct the down-regulation of PANK expression in MCD diet-fed mice. Interestingly, pantothenate supplementation has also been shown to improve pantothenate kinase-associated neurodegeneration, a disease associated with impaired function of PANK in the brain. Differences between pantothenate's positive neural effects and its apparent lack of benefit in the liver may reside in the fact that the brain mostly expresses PANK2, whereas the liver expresses PANK1. Unlike cytosolic PANK1, PANK2 localizes to mitochondria and thus, is more sensitive to small increases of palmitoylcarnitine that antagonize the effect of CoA thioesters-mediated PANK inhibition (43, 44). As such, administering CoA precursors might be more effective in restoring CoA levels in the brain than in the liver. On the other hand, CoA levels in NASH are hampered by increased degradation as suggested by increase expression of vanin enzymes. The regulation of vanins expression is complex. PPAR- α is a strong inducer (45, 46). As we previously showed (27) hepatic PPAR- α expression is increased in the Western diet model, but not in the MCD model. Thus, diet-related differences in PPAR- α may explain why vanins expression is more upregulated in the Western diet model than in the MCD diet model. However, vanin expression is also up-regulated by oxidative stress (47) and by direct effect of Sox-9 on vanin transcription (48). Both oxidative stress and up-regulation of Sox-9 are important features induced in the MCD diet model (27), and may explain increased degradation of CoA and hence absence of beneficial effects of supplementation with CoA precursors.

Despite the present dismal results, a modified approach for supplementing pantothenate might have some merit in NASH. Concomitant administration of L-carnitine and pantothenate was reported to overcome the feedback inhibition of PANK (13, 49). This success might reflect the fact that L-carnitine can accept acyl groups from acyl-CoA, thereby importing acyl groups into mitochondria to facilitate fatty acid β -oxidation and liberating CoA (50). Further research is needed to evaluate the effects of supplemental pantothenate + carnitine in NASH. Enthusiasm for this approach should be tempered by the present evidence that treatment with pantothenate and N-acetylcysteine exacerbated MCD diet-related accumulation of mRNAs encoding tumor necrosis factor (TNF)- α (a major pro-inflammatory cytokine) and macrophage activation markers, F4/80 (a marker of classical activation) and YM-1 (a marker of alternative activation). This finding was unexpected, but might be explained by the fact that pantothenate is essential for the growth of pathogenic microorganisms and thus, may have altered the gut microbiome (14). Also, treated mice showed relative hepatomegaly compared to untreated mice that were fed MCD diet. The hepatomegaly does not seem to result from better regenerative ability or decreased liver

injury, since aminotransferases levels are similar. Rather, it may reflect greater fat accumulation, as suggested by a trend to higher hepatic triglyceride content in the treated group. Another surprising result was the fact that we failed to detect improvements in hepatic oxidative stress in mice that were treated with N-acetylcysteine (33), which has known anti-oxidant properties. This fact may further explain the lack of protection to hepatic inflammation with this therapeutic approach.

Finally, a administration of pantethine, rather than pantothenate, might be another strategy to increase CoA. Unlike pantothenate, pantothine may stimulate CoA biosynthesis via PANK-independent mechanisms (51). In addition, pantethine may be able to modulate inflammation and oxidative stress independently of its effects on CoA synthesis (52).

In conclusion, free CoA is reduced in NASH. This may contribute both to metabolic impairment and caspase-2 activation. Hence, strategies to restore free-CoA levels may be beneficial in NASH. Administration of CoA precursors, pantothenate (vitamin B5) and N-acetylcysteine, was unable to correct the reduced hepatic free CoA content in the MCD diet mouse model of NASH. We believe that the lack of efficacy in our study is explained by the NASH-related down-regulation of the rate-limiting enzyme for CoA biosynthesis that cannot be overcome simply by increasing substrate levels alone. Further studies are needed to determine if adding carnitine supplements to pantothenate can overcome this obstacle and thereby restore levels of free CoA, reduce caspase-2 activation, and prevent NASH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations in the order of appearance

NAFLD	nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
CoA	coenzyme A
MCD	methionine-choline deficient
PANK	pantothenate kinase
WT	wild-type
ALT	alanine aminotransferase
AST	aspartate aminotransferase
TBARS	thiobarbituric acid reactive substances
α-SMA	alpha-smooth muscle actin

SOD	superoxide dismutase
GPX	glutathione peroxidase
4-HNE	4-hydroxynonenal
TNF-α	tumor necrosis factor alpha

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Figure 1. Mice with diet-induced NASH show perturbations of CoA metabolism

A. Free CoA liver content in WT mice fed chow (n=6) or methionine-choline deficient (MCD) diet (n=8). B. Summary representation of CoA synthetic pathway. C. qRT-PCR analysis of whole liver mRNA for genes in the CoA synthetic pathway and CoA degradation pathway, in the same mice as A. D. Liver PANK-1 expression analyzed by Western blot. Results were graphed as mean±SEM and normalized to expression in chow-diet fed mice. * P<0.05, ** P<0.01 chow *versus* MCD diet.

Figure 2. Hepatic free CoA content negatively correlates with apoptosis and liver injury

In mice fed either chow diet or MCD diet, correlations between free CoA liver content and apoptosis markers in whole liver (TUNEL, densitometry for caspase-2 assessed by western blot, caspase-3 activity) and ALT serum levels (A), markers of fibroductular reaction (hydroxyproline content, Sirius red morphometry, collagen 1a and K19 expression by qRT-PCR analysis) (B), liver triglycerides content (C), and inflammatory markers TNF- α , F4/80 and YM-1 expression by qRT-PCR analysis (D). Correlations evaluated with Spearman Coefficient.

Figure 3. Treatment with Pantothenate and N-acetylcysteine does not protect from liver injury in the MCD model

WT mice (n=30) were divided in 3 groups: chow diet and MCD diet with or without treatment with pantothenate and N-acetylcysteine. A. At the end of treatment, weight loss and liver to body weight ratio were recorded. B. Aminotransferase serum levels. C. Representative liver sections stained with H&E (200 fold field). D. Right panel: Quantification of liver triglycerides content. Left panel: qRT-PCR analysis of whole liver mRNA for inflammatory genes, normalized for chow diet-fed mice. Results were graphed as mean $\pm$ SEM. * P<0.05, ** P<0.01 versus chow diet. # P<0.05, ## P<0.01 versus MCD diet.

Figure 4. Treatment with Pantothenate and N-acetylcysteine does not protect from fibroductular response in the MCD model

A. Hydroxyproline liver content determination in the same mice as figure 2. B. qRT-PCR analysis of whole liver mRNA for genes associated with fibrogenesis (left panel) and progenitor markers (right panel). C. Liver sections from representative mice stained for markers of the fibroductular reaction (α -SMA, collagen-1 α , desmin, K19) (left panels) and respective morphometry (right panels). Results were normalized for chow-diet mice and graphed as mean $\pm$ SEM. * $P<0.05$, ** $P<0.01$ versus chow diet. ## $P<0.01$ versus MCD diet.

Figure 5. Treatment with Pantothenate and N-acetylcysteine does not reverse caspase-2 up-regulation or impairment in CoA metabolism, in the MCD model

A. Caspase-2 expression, in whole liver, by qRT-PCR and western-blot, in the same mice as figures 2 and 3. B. Free-CoA liver content. C. qRT-PCR analysis of whole liver for genes in the CoA synthetic and degradation pathways. Results were normalized for chow-diet mice and graphed as mean±SEM. * P<0.05, ** P<0.01 *versus* chow diet.