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Original article

Redox imbalance due to the loss of mitochondrial NAD(P)-transhydrogenase markedly aggravates high fat diet-induced fatty liver disease in mice

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ABSTRACT

The mechanisms by which a high fat diet (HFD) promotes non-alcoholic fatty liver disease (NAFLD) appear to involve liver mitochondrial dysfunctions and redox imbalance. We hypothesized that a HFD would increase mitochondrial reliance on NAD(P)-transhydrogenase (NNT) as the source of NADPH for antioxidant systems that counteract NAFLD development. Therefore, we studied HFD-induced liver mitochondrial dysfunctions and NAFLD in C57Unib.B6 congenic mice with ($Nnt^{+/+}$) or without ($Nnt^{-/-}$) NNT activity; the spontaneously mutated allele ($Nnt^{-/-}$) was inherited from the C57BL/6J mouse substrain. After 20 weeks on a HFD, $Nnt^{-/-}$ mice exhibited a higher prevalence of steatohepatitis and content of liver triglycerides compared to $Nnt^{+/+}$ mice on an identical diet. Under a HFD, the aggravated NAFLD phenotype in the $Nnt^{-/-}$ mice was accompanied by an increased H_2O_2 release rate from mitochondria, decreased aconitase activity (a redox-sensitive mitochondrial enzyme) and higher susceptibility to Ca^{2+}-induced mitochondrial permeability transition. In addition, HFD led to the phosphorylation (inhibition) of pyruvate dehydrogenase (PDH) and markedly reduced the ability of liver mitochondria to remove peroxide in $Nnt^{-/-}$ mice. Bypass or pharmacological reactivation of PDH by dichloroacetate restored the peroxide removal capability of mitochondria from $Nnt^{-/-}$ mice on a HFD. Noteworthy, compared to mice that were chow-fed, the HFD did not impair peroxide removal nor elicit redox imbalance in mitochondria from $Nnt^{+/+}$ mice. Therefore, HFD interacted with *Nnt* mutation to generate PDH inhibition and further suppression of peroxide removal. We conclude that NNT plays a critical role in counteracting mitochondrial redox imbalance, PDH inhibition and advancement of NAFLD in mice fed a HFD. The present study provide seminal experimental evidence that redox imbalance in liver mitochondria potentiates the progression from simple steatosis to steatohepatitis following a HFD.

1. Introduction

The increased consumption of fat-rich and energy-dense foods is a nutritional behavior that is associated with a myriad of metabolic, cardiovascular and hepatic diseases of rising worldwide prevalence [1]. Prominent hepatic abnormalities, such as insulin resistance, non-alcoholic fatty liver disease (NAFLD), and increased rates of gluconeogenesis and glucose output can result from a high fat diet (HFD), and their pathophysiological mechanisms have been extensively studied in experimental rodent models [2–5]. Several studies have implicated mitochondrial dysfunctions in the etiology of metabolic and morphological alterations that occur in the liver in response to a HFD [5–8], but a causal relationship is not always clear [5,7,9]. Higher mitochondrial production of reactive species and impaired redox balance have been indicated as events that are involved in the progression from simple steatosis to nonalcoholic steatohepatitis (NASH) [9,10]. Recently, studies have demonstrated the importance of the inhibition of mitochondrial pyruvate dehydrogenase (PDH) in the context of NAFLD by showing that pharmacological or genetic activation of PDH ameliorated the increased hepatic glucose output and the steatosis induced by HFD in mice [3,11,12]. Pyruvate oxidation is relevant in liver mitochondria [3,4], and its decrease seems to cause pyruvate to be channeled into the gluconeogenesis pathway, impairing glucose homeostasis in mice on a HFD [3]. However, a plausible interplay between impaired redox balance and pyruvate oxidation may occur in liver mitochondria following a HFD; the rationale for this hypothesis is presented below.

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Knowing that reduced NADP is the driving energy source for peroxide removal via the glutathione- and thioredoxin-dependent antioxidant systems [8,13,14] and that its oxidation is associated with the opening of the mitochondrial permeability transition pore (PTP) [15,16], we hypothesized that another consequence of HFD-induced PDH inhibition is redox imbalance and aggravation of NAFLD when the supply of mitochondrial NADPH is not adequately shifted towards sources that are independent of pyruvate oxidation and Krebs cycle flux, such as the enzyme NAD(P)-transhydrogenase (NNT). Previous findings collectively demonstrated that pyruvate oxidation via PDH and downstream Krebs cycle reactions is important for NADPH-dependent peroxide removal because the generated isocitrate sustains NADPH production by NADP-dependent isocitrate dehydrogenase (IDH2) [17,18]. IDH2 is a main source of NADPH, along with NNT in liver mitochondria [17,19,20]. Thus, if pyruvate flux is impaired, concurrent sources of mitochondrial NADPH, particularly NNT, will need to increase their relative contributions to maintain redox balance. NNT is located in the inner mitochondrial membrane and reduces $NADP^+$ at the expense of NADH oxidation and H^+ translocation down the protonmotive force across the inner mitochondrial membrane, thus maintaining NADP in a highly reduced state [13,16]. Interestingly, C57BL/6J mice have a spontaneous mutation of the NNT gene (*Nnt*) [18,21], are apparently more susceptible to HFD-induced metabolic diseases than other substrains [22–24], and are widely used as models of HFD-induced obesity, insulin resistance and NAFLD [2,7,22]. Nonetheless, in addition to *Nnt* mutation, other genetic modifiers could play a role in phenotypic differences between mice substrains [25–31]. As a result of this *Nnt* mutation, liver mitochondria that are devoid of NNT function display a lower peroxide removal capacity as well as other redox abnormalities [17,18]. Therefore, the role of NNT in counteracting HFD-induced redox imbalance linked to PDH inhibition seems a sound and unexplored hypothesis.

We have recently generated congenic C57Unib.B6 mice bearing wild type ($Nnt^{+/+}$) or the mutated ($Nnt^{-/-}$) *Nnt* allele from the C57BL/6J substrain [17]. In the current study, we maintained these congenic mice (C57Unib.B6-$Nnt^{+/+}$, C57Unib.B6-$Nnt^{-/-}$) on a chow diet or a HFD to investigate the role of NNT-dependent mitochondrial redox balance in HFD-induced NAFLD. For comparative purposes, the liver histology of C57BL/6J mice on a HFD was also evaluated because innumerous studies have employed this substrain.

2. Material and methods

2.1. Reagents

The fluorescent probes Calcium Green™-5N and Amplex Red® were purchased from Invitrogen (Carlsbad, California, USA) and dissolved in deionized water and dimethyl sulfoxide (DMSO), respectively. The primary antibodies against PDH-E1α (code # 110330) and serine^{293}-phosphorylated PDH-E1α (code # 177461) were ordered from Abcam (Cambridge, MA, USA). Secondary antibodies conjugated with HRP were purchased from BD Bioscience (rabbit anti-mouse, code # 554002; San Jose, CA, USA) and Cell Signaling (goat anti-rabbit, code # 7074, Beverly, MA, USA). Malic acid, sodium pyruvate, succinic acid, oxaloacetic acid, sodium α-ketoglutarate, glutamic acid, sodium isocitrate, citric acid, palmitoylcarnitine, tert-butyl hydroperoxide (t-BOOH), rotenone, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), oligomycin, oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$), reduced nicotinamide adenine dinucleotide (NADH), adenosine diphosphate (ADP), sodium dichloroacetate, NADP-isocitrate dehydrogenase, peroxidase from horseradish type VIA (HRP), a protease inhibitor cocktail (code P8340) and most other chemicals were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Stock solutions of respiratory substrates, nucleotides and sodium dichloroacetate were prepared in a 20 mM HEPES solution with the pH adjusted to 7.2 using KOH.

2.2. Animals

C57BL/6J mice were obtained from the Campinas University Multidisciplinary Center for Biological Research in Laboratory Animals (CEMIB/UNICAMP, Campinas, Brazil), which breeds a mouse colony that is confirmed to be homozygous for mutated *Nnt* alleles (a 17,814-bp deletion in the *Nnt* gene, resulting in the absence of exons 7–11) [17,18]. A congenic mouse model carrying wild-type or the *Nnt* mutated alleles from the C57BL/6J substrain was recently generated on the genetic background of C57BL/6/JUnib mouse substrain in our laboratory, as detailed in Ronchi et al. [17]. These congenic mice were bred in our department's animal facility and the N7 (the seventh backcrossed generation) was used in this study. The full designations of these congenic mice are C57Unib.B6-$Nnt^{+/+}$ and C57Unib.B6-$Nnt^{-/-}$, which will be referred to by their *Nnt* genotype only (i.e., $Nnt^{+/+}$ and $Nnt^{-/-}$). We ordered the "genome scanning service" from The Jackson Laboratory to analyze four independent samples from N7 congenic mice and two independent samples from C57BL/6J mice (purchased from the local provider) using the "C57BL/6 substrain characterization panel" of 150 validated SNPs; the results indicated that C57BL/6J mice from the local provider exhibited all 150 SNPs identical to The Jackson Laboratory reference C57BL/6J substrain; also, the comparison between C57BL/6J and four mice from our congenic model showed a difference of 28% SNPs. Male mice were kept under standard laboratory conditions (at 20–22 °C and a 12 h/12 h light/dark cycle) with free access to tap water and to either a standard diet or a high fat diet (specified below) in the local animal facility, according to Brazilian guidelines and the "Guide for the Care and Use of Laboratory Animals" from the National Academy of Sciences. Male mice were chosen because they are more susceptible to HFD-induced NAFLD than female (which are protected by estrogens) and develop markers of inflammation like humans with NASH [32,33]. The mice were euthanized by cervical dislocation prior to harvesting the liver for further analysis. The use of mice and the experimental protocols were approved by the local Committee for Ethics in Animal Research (CEUA-UNICAMP, approval number 3914-1). The animal procedures comply with national Brazilian guideline number 13 for "Control in Animal Experiments", published on September 13th, 2013 (code 00012013092600005, available at ⟨http://portal.in.gov.br/verificacao-autenticidade⟩).

2.3. High fat diet (HFD) treatment

One-month-old congenic and C57BL/6J male mice were randomly assigned to groups fed either the standard diet (Chow, total energy of 3.9 kcal/g, 5% fat, 12% of total calories from fat) from Nuvital (Nuvilab CR1, Nuvital, Colombo, PR, Brazil) or a HFD from PragSoluções (Hyperlipidic diet, PragSoluções biociências, Jaú, SP, Brazil). The composition of the HFD was as follows: 31% lard fat, 20% casein, 13% dextrinized cornstarch, 12% cornstarch, 10% sucrose, 5% microcrystalline cellulose, 4% soybean oil, 3.5% mineral mix AIN 93 G, 1% vitamin mix AIN 93, 0.3% L-cysteine, 0.25% choline bitartrate, and 0.0028% butylhydroxytoluene (also present as a preservative in the standard chow). The caloric content of this HFD is 5.3 kcal/g, from which ~60% of total energy were derived from fats (90% from lard fat and 10% from vegetable oil). Mice were maintained on a standard diet or HFD for 20 weeks prior to being sacrificed for liver removal and use in histology or mitochondrial isolation.

3. Measurements

3.1. Liver histology

Approximately 3 mm-sided fragments of the two largest liver lobules were cut and placed in 10% formaldehyde for 24 h at room temperature. Then, tissue samples were embedded in paraffin before being sectioned (5 μm thick slices). These liver slices were placed on