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Original Contribution

A spontaneous mutation in the nicotinamide nucleotide transhydrogenase gene of C57BL/6J mice results in mitochondrial redox abnormalities

Juliana A. Ronchi [a], Tiago R. Figueira [a,**], Felipe G. Ravagnani [a], Helena C.F. Oliveira [b], Anibal E. Vercesi [a], Roger F. Castilho [a,*]

[a] Department of Clinical Pathology, Faculty of Medical Sciences, State University of Campinas, Campinas, SP 13083-887, Brazil
[b] Department of Structural and Functional Biology, Biology Institute, State University of Campinas, Campinas, Brazil

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ABSTRACT

NADPH is the reducing agent for mitochondrial H_2O_2 detoxification systems. Nicotinamide nucleotide transhydrogenase (NNT), an integral protein located in the inner mitochondrial membrane, contributes to an elevated mitochondrial NADPH/$NADP^+$ ratio. This enzyme catalyzes the reduction of $NADP^+$ at the expense of NADH oxidation and H^+ reentry to the mitochondrial matrix. A spontaneous *Nnt* mutation in C57BL/6J (B6J-Nnt^{MUT}) mice arose nearly 3 decades ago but was only discovered in 2005. Here, we characterize the consequences of the *Nnt* mutation on the mitochondrial redox functions of B6J-Nnt^{MUT} mice. Liver mitochondria were isolated both from an *Nnt* wild-type C57BL/6 substrain (B6JUnib-Nnt^{W}) and from B6J-Nnt^{MUT} mice. The functional evaluation of respiring mitochondria revealed major redox alterations in B6J-Nnt^{MUT} mice, including an absence of transhydrogenation between NAD and NADP, higher rates of H_2O_2 release, the spontaneous oxidation of NADPH, the poor ability to metabolize organic peroxide, and a higher susceptibility to undergo Ca^{2+}-induced mitochondrial permeability transition. In addition, the mitochondria of B6J-Nnt^{MUT} mice exhibited increased oxidized/reduced glutathione ratios as compared to B6JUnib-Nnt^{W} mice. Nonetheless, the maximal activity of NADP-dependent isocitrate dehydrogenase, which is a coexisting source of mitochondrial NADPH, was similar between both groups. Altogether, our data suggest that NNT functions as a high-capacity source of mitochondrial NADPH and that its functional loss due to the *Nnt* mutation results in mitochondrial redox abnormalities, most notably a poor ability to sustain NADP and glutathione in their reduced states. In light of these alterations, the potential drawbacks of using B6J-Nnt^{MUT} mice in biomedical research should not be overlooked.

Introduction

The mitochondrial electron transport chain is a quantitatively relevant source of superoxide ($O_2^{\bullet -}$) in the majority of cell types. Physiological levels of $O_2^{\bullet -}$ and its derivatives (reactive oxygen species; ROS) are important signaling intermediates in a variety of cellular processes, while the excessive accumulation of mitochondrial ROS is implicated in the pathophysiology of many diseases [1]. To handle ROS, mitochondria possess an antioxidant system to metabolize $O_2^{\bullet -}$ into hydrogen peroxide (H_2O_2) via mitochondrial superoxide dismutase (SOD2); in turn, H_2O_2 is mainly transformed into H_2O through the action of specific peroxidases and at the expense of reduced glutathione and thioredoxin [1,2]. The ultimate reducing power for this H_2O_2-detoxifying system is NADPH, which is oxidized by specific reductases that catalyze the rereduction of the oxidized forms of glutathione and thioredoxin [2]. Therefore, the redox status of NADP is critical for the enzymatic removal of mitochondrial H_2O_2 and, in turn, for redox homeostasis [2–4].

Abbreviations: 3-OHB, 3-hydroxybutyrate; 3-OHBDH, 3-hydroxybutyrate dehydrogenase; AA, antimycin A; AcAc, acetoacetate; APAD, 3-acetylpyridine adenine dinucleotide; B6JUnib-Nnt^{W} mice, C57BL/6/JUnib mice carrying wild-type *Nnt* alleles; B6J-Nnt^{MUT} mice, C57BL/6J mice carrying mutated *Nnt* alleles; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulfide; IDH2, mitochondrial NADP-dependent isocitrate dehydrogenase; IDH3, mitochondrial NAD-dependent isocitrate dehydrogenase; Isoc, isocitrate; ME3, mitochondrial NADP-dependent malic enzyme; MPT, mitochondrial permeability transition; NAD, β-nicotinamide adenine dinucleotide; NADH, reduced form of NAD; NAD^+, oxidized form of NAD; NADP, β-nicotinamide adenine dinucleotide phosphate; NADPH, reduced form of NADP; $NADP^+$, oxidized form of NADP; NNT, nicotinamide nucleotide transhydrogenase; PCoA, palmitoyl coenzyme A; ROS, reactive oxygen species; SOD2, mitochondrial superoxide dismutase; t-BOOH, *tert*-butyl hydroperoxide; t-BOH, *tert*-butyl alcohol; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride..

* Corresponding author. Fax: +55 19 3521 9434.
** Also to be corresponded.
E-mail addresses: figueirat@yahoo.com.br (T.R. Figueira), roger@fcm.unicamp.br (R.F. Castilho).

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Indeed, NADPH oxidation promptly triggers mitochondrial permeability transition (MPT) in Ca^{2+}-loaded mitochondria, a process that has been implicated in mitochondrial dysfunction and cell death [5–8].

Mitochondria possess three enzymatic sources of NAPDH: NADP-dependent isocitrate dehydrogenase (IDH2), nicotinamide nucleotide transhydrogenase (NNT), and NADP-dependent malic enzyme (ME3) [3,9–11]. If pyruvate supply to mitochondria is not compromised, the last source may be negligible because ME3 displays a low affinity for malate, low maximal activity, and product inhibition by pyruvate [9]. The relative contributions of the first two sources have been proposed to be nearly 50% each [10]. Nonetheless, the functional importance of the IDH2- and NNT-mediated $NADP^+$ reduction in mammalian mitochondria under controlled conditions remains to be determined. While IDH2 catalyzes a simple dehydrogenation reaction, the NNT reaction is more complex. NNT is assembled at the inner mitochondrial membrane and couples its transhydrogenase activity to H^+ movement between the mitochondrial intermembrane space and the matrix [10,12–15], as illustrated in Fig. 1.

The electrochemical gradient across the inner mitochondrial membrane shifts the NNT equilibrium toward NADP reduction, at the expense of NAD oxidation and H^+ reentry from the intermembrane space back to the mitochondrial matrix [13,16,17]. If the electrochemical gradient is experimentally dissipated and NAD is oxidized, NNT is able to catalyze the reverse reaction [6,13,14]. In fully functional mitochondria, NNT should comprise a high-capacity source of NADPH because it may unite the capacities of several mitochondrial dehydrogenases that yield NADH. Nonetheless, the physiological roles of NNT are still gradually being revealed [3,18].

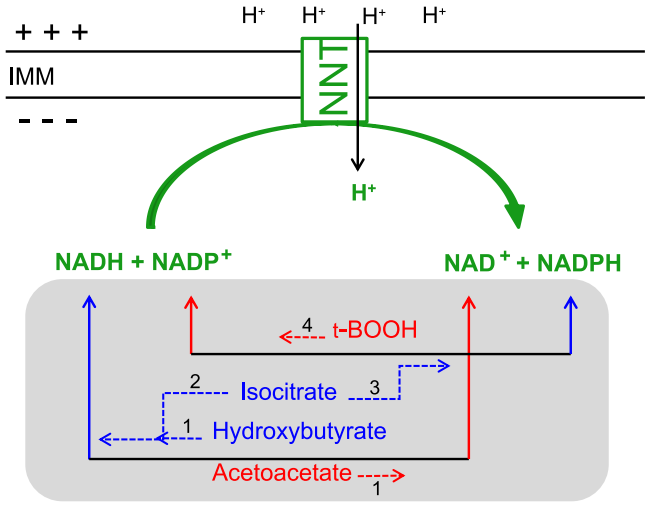

Fig. 1. Schematic of the reaction catalyzed by mitochondrial nicotinamide nucleotide transhydrogenase (NNT) and other NAD(P)-redox reactions. NNT is assembled across the inner mitochondrial membrane (IMM) and translocates H^+ to the mitochondrial matrix as $NADP^+$ is reduced at the expense of NADH on the matrix side; the electrochemical gradient across the IMM shifts the NNT equilibrium to the right. The gray box is not part of NNT reaction and is shown to depict mitochondrial NAD(P)-redox reactions (numbers **1** to **4** next to dashed arrows) that were used as a source or sink of each nucleotide involved in the NNT reaction. The reactions that had their equilibrium displaced by the addition of exogenous substrates are: (**1**) *acetoacetate+NADH+H^+ ⇌ 3-hydroxybutyrate+NAD^+*, through 3-OHBDH; (**2**) *isocitrate+NAD^+ ⇌ α-ketoglutarate+NADH+CO_2+H^+* through IDH3; (**3**) *isocitrate+$NADP^+$ ⇌ α-ketoglutarate+NADPH+CO_2+H^+*; through IDH2; (**4**) *t-BOOH+NADPH+H^+ → t-BOH+H_2O +$NADP^+$*, the overall reaction through glutathione reductase and peroxidase. In this cartoon, the blue color represents the reductants and the reduction of $NAD(P)^+$, while the red color represents the oxidants and the oxidation of NAD(P)H. NADH and NADPH, but not their respective oxidized forms, exhibit strong fluorescence signals at 366 nm excitation and 450 nm emission, which allow them to be spectrofluorometrically monitored in suspensions of isolated mitochondria.

The discovery of a spontaneous loss-of-function *Nnt* mutation in C57BL/6J mice in 2005 [19] was puzzling for at least two reasons: (i) these mice would serve as an experimental model to clarify the roles of NNT in mammalian biology; (ii) many published studies may need reevaluation, particularly those that used knockout or transgenic mice engineered on the C57BL/6J genetic background. To illustrate the latter issue, while the genetic deletion of mitochondrial SOD on C57BL/6J mice leads to death at approximately Day 15 of gestation, DBA/2 J mice (*Nnt* wild-type) exhibit an average life span of 8 days [20]. Compared to *Nnt* wild-type strains, C57BL/6J mice also present abnormal metabolic responses [21,22], including impaired glucose tolerance, which results from lowered glucose-induced insulin secretion [19,22,23]. In humans, *Nnt* mutations have been recently described as a primary cause of familial glucocorticoid deficiency. Interestingly, the C57BL/6J mice also display this phenotype [24].

Arguably, the use of C57BL/6J mice in mitochondrial studies would be especially concerning, given the likelihood of the direct impact of NNT's loss of function on the mitochondrial NADP status and the overall redox balance [3]. In light of these issues, the scientific drawbacks of using the C57BL/6J background in biomedical research have been overlooked due to insufficient comprehension about the role of NNT in mitochondrial biochemistry.

Here, we aimed to investigate the consequences of the *Nnt* mutation on the mitochondrial redox state. When compared to an *Nnt* wild-type C57BL/6 substrain (C57BL/6/JUnib), we found that isolated liver mitochondria from C57BL/6J mice displayed major redox abnormalities, including an increase in oxidized/reduced glutathione ratio, higher rates of H_2O_2 release, the spontaneous oxidation of NADPH in the absence of exogenous Krebs cycle substrates, a poor ability to metabolize organic peroxide, and a higher susceptibility to undergo Ca^{2+}-induced MPT.

Materials and methods

Reagents

The majority of the chemicals used, including malic acid, pyruvic acid sodium salt, succinic acid, rotenone, antimycin A, APAD, PCoA, acetoacetic acid lithium salt, *tert*-butyl hydroperoxide, 3-hydroxybutyric acid, isocitric acid trisodium salt, FCCP, TMPD, and NADPH, were obtained from Sigma-Aldrich (St. Louis, MO, USA). Both peroxidase from horseradish type VIA (HRP) and glutathione reductase from baker´s yeast were also obtained from Sigma-Aldrich. Malate, pyruvate, succinate, acetoacetate, 3-hydroxybutyrate, isocitrate, $NADP^+$, and NADPH stock solutions were prepared in 20 mM Hepes-KOH (pH 7.2). The fluorescent probes Calcium Green-5 N hexapotassium salt and Amplex Red were purchased from Invitrogen (Carlsbad, CA, USA).

Animals

C57BL/6J and C57BL/6/JUnib female mice were provided by the Campinas University Multidisciplinary Center for Biological Research in Laboratory Animals (CEMIB/Unicamp, Campinas, Brazil). The C57BL/6J colony was established from founders purchased from The Jackson Laboratory (Bar Harbor, ME, USA) in 2009, while the C57BL/6/JUnib colony was established in 1987 from founders supplied by the Zentralinstitut für Versuchstierzucht (ZfV) (Hannover, Germany). Based on the historic origin of these two mice colonies, C57BL/6J and C57BL/6/JUnib mice were expected to be homozygous for, respectively, mutated and wild-type *Nnt* alleles. This mutation in C57BL/6J comprises a homozygous 17,814-bp deletion in the *Nnt* gene that arose spontaneously and was named $Nnt^{C57BL/6J}$ in the MGI data bank. After genotyping as described