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MINI-REVIEW

Facilitation of Ca^{2+}-induced opening of the mitochondrial permeability transition pore either by nicotinamide nucleotide transhydrogenase deficiency or statins treatment

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Abstract

Mitochondrial redox imbalance and high Ca^{2+} uptake induce the opening of the permeability transition pore (PTP) that leads to disruption of energy-linked mitochondrial functions and triggers cell death in many disease states. In this review, we discuss the major results from our studies investigating the consequences of NAD(P)-transhydrogenase (NNT) deficiency, and of statins treatment for mitochondrial functions and susceptibility to Ca^{2+}-induced PTP. We highlight the aggravation of high fat diet-induced fatty liver disease in the context of NNT deficiency and the role of antioxidants in the prevention of statins toxicity to mitochondria.

Keywords: C57BL/6J mice; mitochondrial permeability transition; nicotinamide nucleotide transhydrogenase; *Nnt* mutation; pravastatin; simvastatin

Introduction

Mitochondrial permeability transition (MPT) is characterized by the opening of a high conductance, nonspecific proteinaceous pore in the inner mitochondrial membrane (IMM) called permeability transition pore (PTP) (Kowaltowski et al., 2001). PTP opening is induced by mitochondrial Ca^{2+} overload and leads to the disruption of energy-linked mitochondrial functions, for example, oxidative phosphorylation and Ca^{2+} transport, which may promote cell death under several pathophysiological conditions, such as ischemia/reperfusion injury, neurodegenerative diseases, muscular dystrophy, dyslipidemias, and metabolic diseases and drug toxicity. In addition, there is compelling evidence that further studies on the control of MPT may contribute to a better understanding of the aging process (Rottenberg and Hoek, 2017). The PTP opening occurs in response to increased levels of Ca^{2+} in the mitochondrial matrix and is facilitated by conditions such as oxidative stress, high concentrations of inorganic phosphate, elevated expression of Bcl-2 family proteins, and toxicity induced by chemical compounds, including therapeutic drugs (Figueira et al., 2013). Here, we review the contributions of our laboratory to the understanding of the mechanisms underlying the mitochondrial redox alterations that favor PTP opening under two conditions: NAD(P)-transhydrogenase (NNT) deficiency and statins toxicity. These two conditions have a common denominator: the underlying biochemical mechanism that ultimately leads to mitochondrial oxidative stress. While NNT deficiency impairs mitochondrial antioxidant capacity, the use of statins to reduce cholesterol synthesis leads to increased mitochondrial production of reactive oxygen. Both conditions increase susceptibility to MPT and cell death and are relevant for human metabolic diseases such as fatty liver diseases and atherosclerosis, as will be discussed in the following topics. We have studied the role of NNT and statin toxicity in mouse models with the C57BL6/J genetic background, which carries a spontaneous mutation

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Abbreviations: CoQ_{10}, Coenzyme Q_{10}; CsA, cyclosporin A; GD, glucocorticoids deficiency; HFD, high fat diet; IMM, inner mitochondrial membrane; IDH2, isocitrate dehydrogenase; MPT, mitochondrial permeability transition; NNT, NAD(P)-transhydrogenase; PTP, permeability transition pore; PDH, pyruvate dehydrogenase

of the NNT gene. This mouse strain was used to obtain congenic lines ($NNT^{-/-}$ and $NNT^{+/+}$) to investigate NNT deficiency and also to generate the hypercholesterolemic mice (LDL receptor knockout mice) employed in statins studies.

Role of NNT deficiency in MPT and in high fat diet (HFD)-induced fatty liver disease

NADP-dependent isocitrate dehydrogenase (IDH2) in the Krebs cycle and NNT are presumed to be the main sources of NAPDH in the mitochondrial matrix. NNT is a transmembrane enzyme assembled in the IMM that catalyzes the transfer of hydrides from NADH to $NADP^+$, in a manner coupled to the H^+ movement from intermembrane space to mitochondrial matrix. This reaction can be displaced to the forward direction until it establishes a 500-fold higher NADPH/$NADP^+$ than NADH/NAD^+ ratio in the matrix, at the expense of the proton-motive force across IMM (Rydstrom, 2006). Therefore, NNT is extremely important to supply reducing equivalents in the form of NADPH to maintain the functioning of the mitochondrial antioxidant defense systems.

The discovery in 2005 of a widely used experimental mice substrain (i.e., C57BL/6J) that carry a loss-of-function homozygous mutation in NNT gene (*Nnt*) (Toye et al., 2005) provided an experimental model that we used to evaluate the relationship between mitochondrial redox functions and the process of MPT (Ronchi et al., 2013; Ronchi et al., 2016). We showed that the rate of NADPH-dependent peroxide removal by mitochondria is severely impaired in the absence of NNT activity. Moreover, our data indicated that NNT becomes the unique source of NADPH when Krebs cycle flux, and hence IDH2 flux, are inhibited in an anoxia-like condition (Ronchi et al., 2013, 2016). Considering the redox regulation of MPT, it was demonstrated that only mitochondria from mice deficient in NNT exhibited spontaneous NADPH oxidation, which was accompanied by increased rates of H_2O_2 release and higher susceptibility to Ca^{2+}-induced PTP opening as compared to mitochondria from *Nnt* wild type mice (Ronchi et al., 2013). These results evidenced for the first time that an in vivo condition of redox imbalance (i.e., low NADPH levels due to spontaneous *Nnt* mutation) corroborates a redox-sensitive PTP opening model that was generated by in vitro experimentation, where NADPH was oxidized by oxidants such as tert-butyl hydroperoxide, acetoacetate, or other compounds (Kowaltowski et al., 2001). Therefore, the study of mitochondria devoid of a main NADPH source, that is, NNT, strongly supported the redox nature of the MPT process that was first described in 1978 (Lehninger et al., 1978; Kowaltowski et al., 2001).

Data regarding the effects of the Nnt mutation in humans are scarce. Mutations in *Nnt*, which are predictably dysfunctional, have been found in patients with familial glucocorticoid deficiency (Meimaridou et al., 2012). The relation between the glucocorticoids deficiency (GD) and NNT mutation is attributed to a diminished supply of reducing equivalents for the adrenodoxin oxidoreductase mitochondrial system, involved in the glucocorticoids biosynthesis pathway (Fujisawa et al., 2015). Another mechanism that implicates NNT deficiency in the GD is the cell oxidative stress induced by NNT deficiency leading to adrenal gland cortex dysfunction (Meimaridou et al., 2012). Nonetheless, more studies are needed to clarify the mechanism through which the loss of NNT function generates this endocrine disease in suitable experimental models.

As mentioned above, the most widely used mice substrain in biomedical research, the C57BL/6J, carries mutated *Nnt* alleles (named $Nnt^{C57BL/6J}$). Despite this mutation was discovered in 2005 (Toye et al., 2005), it may have arose nearly 40 years ago in The Jackson Laboratory. Astonishingly, mice carrying the genetic background of C57BL/6J with mutated *Nnt*, including knockout and transgenic models, have been widely used in innumerable studies of drug toxicity and diet induced metabolic diseases. Indeed, after the discovery of NNT mutation, several studies reported major metabolic differences between C57BL6 strains with and without the NNT mutation, including glucose intolerance, susceptibility to insulin resistance, diabetes, and obesity (Fisher-Wellman et al., 2016), showing that NNT deficiency in vivo is highly relevant for susceptibility to metabolic disturbances.

To further study the biological roles of NNT and address specific questions, we have recently generated a congenic mice model carrying the mutated $Nnt^{C57BL/6J}$ allele from the C57BL/6J substrain (Ronchi et al., 2016). These congenic mice were subjected to a standard chow or a HFD for 20 weeks prior the functional analysis of their isolated liver mitochondria and liver histology. The results have indicated that HFD interacts with *Nnt* mutation to elevate mitochondrial redox imbalance (Navarro et al., 2017). In fact, such interaction caused further impairment of mitochondrial peroxide removal because it led to the inhibitory phosphorylation of pyruvate dehydrogenase (PDH). Noteworthy, it is expected that NADP-dependent isocitrate dehydrogenase (part of the Krebs cycle) will be the main source of NADPH in the absence of NNT. Therefore, PDH inhibition may impair the flux through Krebs cycle and the production of NADPH that are dependent on the oxidation of carbon skeletons from pyruvate (Ronchi et al., 2016). Despite the development of fatty liver disease following a HFD in *Nnt*-wild type mice, their mitochondria seemed to cope well with this metabolic challenge, neither exhibiting increased susceptibility to Ca^{2+}-induced MPT nor redox abnormalities in most studied conditions. Nonetheless, in the absence

of functional NNT (i.e. in *Nnt*-mutated mice), the mitochondrial redox imbalance promoted by the HFD was accompanied by higher susceptibility to Ca^{2+}-induced MPT and an aggravated fatty liver disease phenotype. Indeed, *Nnt*-mutated mice on a HFD displayed histological markers of progression from simple steatosis to steatohepatitis. This is an important finding that comprises a proof of the concept that mitochondrial redox imbalance may potentiate the development of aggravated forms of HFD-induced fatty liver disease (Navarro et al., 2017).

MPT and statins toxicity to mitochondria

Statins are safe and efficient cholesterol-lowering drugs used worldwide that reduce the risk of cardiovascular mortality by inhibiting the rate-limiting step in cholesterol synthesis (3-hydroxy-3-methylglutaryl coenzyme A reductase). As a consequence, statins also diminish the production of coenzyme Q_{10} (CoQ_{10}) and other intermediates such as dolichols and prenylated isoprenoids. Besides reducing cholesterol, statins present other lipid-independent protective actions called pleiotropic effects. Literature data suggest that statins contribute to the treatment of diseases associated with oxidative stress such as cardiac diseases as well as sepsis, neurological conditions and even tumors (Tobert, 2003). However, the most well characterized benefit of statins treatment is the protection against the hypercholesterolemia-induced atherosclerosis. The key step in the pathogenesis of atherosclerosis is the oxidation of low-density lipoprotein (LDL) caused by ROS generated by vascular wall cells. However, the exact mechanism of how this process occurs is not completely known. Previous works from our laboratory have shown that the hypercholesterolemic LDL receptor knockout mice ($LDLr^{-/-}$, C57BL6/J background), the best model for familial hypercholesterolemia, exhibit increased mitochondrial ROS production and higher susceptibility to MPT in several tissues (Oliveira et al., 2005; Paim et al., 2008). We also showed in $LDLr^{-/-}$ mice higher liver steroidogenesis and lipid secretion rates, in order to compensate for the lack of LDL-cholesterol uptake. We proposed that the increased steroidogenesis in $LDLr^{-/-}$ mice would deplete the reducing equivalents from NADPH pool generating a mitochondria and cell redox imbalance (Oliveira et al., 2005). Since mice on the C57BL6/J background carry the spontaneous Nnt gene mutation, the tissues from these $LDLr^{-/-}$ mice present two mechanisms to explain their higher susceptibility to mitochondrial oxidative stress and MPT: 1—less generation of NADPH due to NNT deficiency, and 2—higher consumption of NADPH to steroidogenesis due to the LDL receptor gene deletion. Therefore, we hypothesized that the inhibition of cholesterol synthesis by statins would spare NADPH consumption and could improve mitochondrial function. Thus, our group investigated the effects of statins treatment in vivo and in isolated mitochondria from $LDLr^{-/-}$ mice. Unexpectedly, in vitro statins treatments induced MPT in a dose-dependent and class-dependent manner. These effects were associated with an increase in protein thiol groups oxidation (50% decrease in free SH- groups) and were prevented by the reducing agent dithiothreitol. In addition, short-term lovastatin-treated mice also resulted in a higher susceptibility to develop Ca^{2+}-induced MPT in liver and muscle mitochondria (Velho et al., 2006). These data indicated that statins have a prooxidant action on mitochondria shown either in vivo or in vitro leading to MPT, that in turn, can ensue cell death.

By that time, a growing interest of statins use in cancer therapy emerged, especially regarding prostate cancer (Tobert, 2003). However, the exact antitumor mechanisms of these drugs were incompletely characterized. In our studies, apoptosis and necrosis were observed in prostate cancer PC3 cells under low (10 μM) and high doses (60 μM) of simvastatin. The partial prevention of necrosis by MPT inhibitors indicated PTP opening in the cancer cell death mechanism (Oliveira et al., 2008). These effects of simvastatin on PC3 cells were later proven to be sensitive to piracetam (membrane stabilizer) and L-carnitine (an antioxidant) (Costa et al., 2013). Although statins are being considered as adjuvants in the treatment of cancer, more studies are needed to better clarify this matter.

It is noteworthy that about 0.5–10% of the statin treated patients present dose-dependent and class-dependent adverse effects, mainly in skeletal muscle. In light of our previous studies, we further investigated the effects of a widely prescribed statin (i.e., simvastatin) on mitochondrial functions of skeletal muscle biopsies from rats. We observed that simvastatin (1 μM) significantly decreased the content of CoQ_{10} (reduced form), inhibited mitochondrial respiration and increased H_2O_2 production in soleus muscle. Interestingly, L-carnitine or CoQ_{10} co-incubation with simvastatin, both of which can act as free radical scavengers, protected muscle mitochondria against respiratory inhibition (La Guardia et al., 2013). Data from this and other studies suggested that statins causes mitochondrial respiration inhibition at the level of complex I and II (La Guardia et al., 2013; Busanello et al., 2017) and complex III (Schirris et al., 2015). Inhibition of respiration by statins stimulates superoxide generation, which in turn attacks their own 4Fe-4S clusters, amplifying superoxide production and then inducing thiol oxidation, protein aggregation and pore formation (MPT). There are propositions, using in silico docking simulation, that statins interact directly with some sites of the respiratory complexes (Schirris et al., 2015). However, direct proof was not obtained yet.

Interestingly, the scavenger ability of CoQ_{10} seems to be more important than its electron transport activity for

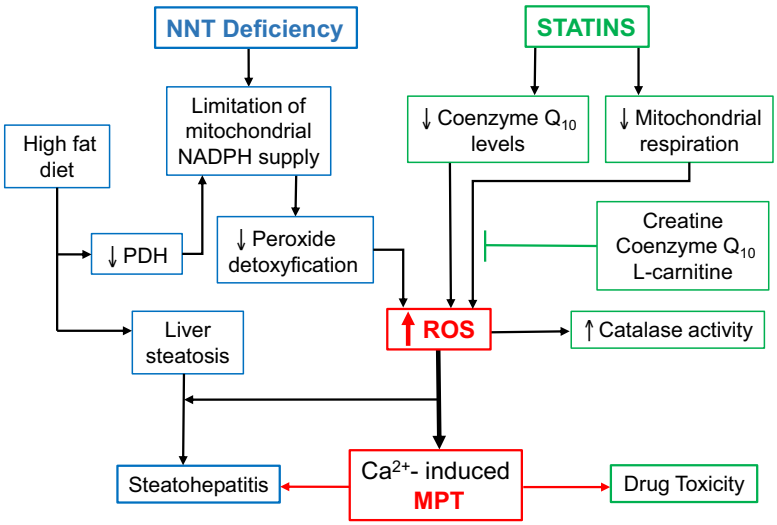

Figure 1 Scheme of main events linking NAD(P)-transhydrogenase (NNT) deficiency or statins treatment to increased levels of reactive oxygen species (ROS) and Ca^{2+}-induced mitochondrial permeability transition (MPT). Arrows indicate the sequence of events described below that converge on redox imbalance (increased ROS generation) and facilitation of Ca^{2+}-induced MPT. NNT deficiency: it occurs due to *Nnt* mutation (present in C57BL/6J mice and in several experimental models) and results in i) impaired mitochondrial supply of NADPH, ii) decreased peroxide detoxification, iii) increased ROS levels and iv) facilitation of Ca^{2+}-induced MPT. High fat diet (HFD) alone promotes liver steatosis, but its interaction with NNT deficiency promotes the disease progression from liver steatosis to steatohepatitis; the role of PDH phosphorylation (inhibition) is also depicted, which leads to further impairment of NADPH supply and peroxide detoxification upon the interaction between HFD and NNT deficiency. Statins: the treatment with these cholesterol-lowering drugs leads to i) depletion of coenzyme Q_{10} levels and ii) inhibition of mitochondrial respiration; both of these alterations increase ROS levels, which, in turn, facilitates Ca^{2+}-induced MPT resulting in drug toxicity/adverse effects. The role of selected antioxidants (creatine, coenzyme Q_{10} or L-carnitine) in counteracting the statins effects is also depicted. The mild oxidative stress induced by statins signals to increased catalase activity as a response mechanism of cytoprotection.

resolving the increased ROS production, since the antioxidant L-carnitine does not replenish CoQ_{10} depletion in muscle biopsies and protects against ROS production and MPT (La Guardia et al., 2013). The same is true for creatine supplementation (Busanello et al., 2017).

To further investigate the mechanisms underlying mitochondrial dysfunction and MPT in skeletal muscle, we changed to a more relevant in vivo context, namely the hypercholesterolemic *LDLr*$^{-/-}$ mice. We studied a chronic treatment (3 months) with moderate therapeutic doses of a less toxic and hydrophilic statin, the pravastatin (Busanello et al., 2017). In this recent study, we observed that respiratory rates were inhibited in the presence of free Ca^{2+} in permeabilized plantaris muscle from *LDLr*$^{-/-}$ mice under pravastatin treatment. Such a decrease in respiratory rates was abolished in the presence of the Ca^{2+} chelator EGTA, the MPT inhibitor cyclosporin A (CsA), the mitochondrial Ca^{2+} uptake inhibitor ruthenium red, or the antioxidant CoQ_{10}. Thus, these results indicate that in vivo pravastatin treatment provoked inhibition of mitochondrial respiration and induced PTP opening in plantaris muscle. Along with the respiratory dysfunction, it was observed that in vivo pravastatin treatment also caused lipids oxidative damage and increased catalase activity. These findings suggest that the oxidative stress provoked by pravastatin signals for the upregulation of cellular antioxidant systems (e.g., catalase), possibly explaining the claimed statins antioxidant properties (Busanello et al., 2017). When such adaptive responses are not sufficient to counteract the oxidative stress, PTP opens and cell death may occur. In addition, we checked whether an in vivo co-treatment with an antioxidant would attenuate the effects of pravastatin. Indeed, muscle from *LDLr*$^{-/-}$ mice that were fed with a creatine-supplemented diet did not present the pravastatin sensitization to Ca^{2+}-induced MPT.

Taken together, the data presented here supports the proposition that statins induce oxidative stress primarily due to inhibition of mitochondrial respiration, increasing superoxide production, inducing thiol oxidation and lipoperoxidation and pore formation (MPT). Several antioxidants may prevent these statin effects, such as L-carnitine, CoQ_{10} and creatine.

Conclusions

We have highlighted the main findings from our studies that aimed at investigating the consequences of NNT deficiency or of statins treatment for mitochondrial (dys)functions. The data collectively indicated that both conditions result in impaired mitochondrial redox balance favoring Ca^{2+}-induced PTP opening (Figure 1). In turn, these mitochondrial

alterations may explain the aggravation of HFD-induced fatty liver disease in NNT deficient mice and statins toxicity to liver and skeletal muscle and the death of tumor cells.

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