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Biochimica et Biophysica Acta 1757 (2006) 721–726
BIOCHIMICA ET BIOPHYSICA ACTA
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Review

Mitochondrial NADPH, transhydrogenase and disease

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Received 21 December 2005; received in revised form 21 February 2006; accepted 14 March 2006
Available online 5 April 2006

Abstract

Ever since its discovery in 1953 by N. O. Kaplan and coworkers, the physiological role of the proton-translocating transhydrogenase has generally been assumed to be that of generating mitochondrial NADPH. Mitochondrial NADPH can be used in a number of important reactions/processes, e.g., biosynthesis, maintenance of GSH, apoptosis, aging etc. This assumed role has found some support in bacteria but not in higher eukaryotes, a situation which changed dramatically with two recent but separate findings, both using transhydrogenase knockouts, in the nematode *C. elegans* and the mouse strain C57BL/6J. The latter, which is due to a spontaneous deletion mutation in the *Nnt* gene, was serendipitously found during investigations of the diabetic properties of these mice. The implications of these findings for the overall role of transhydrogenase in cell metabolism and disease are discussed.

Keywords: Transhydrogenase; Mitochondria; NADPH; ROS; Oxidative stress; Diabetes

1. Introduction

The mitochondrion is one of the major cellular sources of reactive oxygen species (ROS), generated mainly in Complexes I and III [1]. Electron leakage during respiration leads to the generation of superoxide anions and oxidative stress, defined as a disturbance in the prooxidant–antioxidant balance in favour of the former [1,2]. Mitochondrial ROS-mediated cell damage has been implicated in aging, apoptosis, necrosis and a number of pathological changes, including neurodegenerative diseases and cancer, mediated by, e.g., Ca^{2+} fluxes, apoptotic proteins, poly (ADP-ribose)polymerase (PARP) and mitochondrial channels [2–8]. The primary defenses against mitochondrially generated ROS are superoxide dismutases, such as mitochondrial MnSOD [9] and glutathione peroxidase [10]. Reduced glutathione (GSH) participates in the defense against ROS and repair of mitochondrial oxidative damage both as a potent antioxidant in itself and as a substrate for mitochondrial glutathione peroxidase and glutathione-dependent phospholipid hydroperoxidase [10,11]. Since ROS may diffuse to the cytosol, cytosolic glutathione peroxidase may also be involved in the conversion to non-toxic metabolites [10, 11]. Mitochondrial and cytoplasmic pools of glutathione are separate [12] and GSH levels in these pools are maintained by their respective NADPH-dependent glutathione reductases [13]. For example, the classical initiation of apoptosis by *tert*-butylhydroperoxide [3] is mediated by oxidation of mitochondrial GSH and NAD(P)H [14].

2. Sources of mitochondrial NADPH

A significant contributor to mitochondrial NADPH is the proton-translocating transhydrogenase that catalyzes the reaction

$$H^+_{out} + NADH + NADP^+ \rightleftharpoons H^+_{in} + NAD^+ + NADPH \qquad (1)$$

(for reviews on the properties of transhydrogenase, see Refs. [15,16]). In the presence of an electrochemical proton gradient, Δp, i.e., under physiological conditions, the reaction is strongly shifted towards NADPH formation and the rate of the reaction from left to right is enhanced 5- to 10-fold. The redox level of mitochondrial NADP is normally more than 95% reduced, about half of which is uncoupler sensitive [17]. Thus, the uncoupler-sensitive part is consistent with transhydrogenase-generated NADPH, even though contributions by other sources, e.g., the

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doi:10.1016/j.bbabio.2006.03.010

NADP-isocitrate dehydrogenase, cannot be excluded. As judged from a flux investigation of redox equivalents in *E. coli* under different conditions, transhydrogenase accounts for up to 45% of the NADPH flow, the remaining contributed mainly by the pentose phosphate pathway, NADP-isocitrate dehydrogenase and decarboxylating malate dehydrogenase (malic enzyme) [18]. In mitochondria, the contribution of NADPH by transhydrogenase may thus be assumed to be at least as high or higher. It should also be stressed that transhydrogenase, at the high $NADPH/NADP^+$ redox ratio prevailing in mitochondria, has been proposed to be largely product inhibited [19], i.e., its maximal activity, approx. 30 nmol/min/mg mitochondrial protein, is only reached upon a consumption of the product NADPH at a rate exceeding that of the steady-state rate of transhydrogenase.

3. Proposed roles of transhydrogenase

As mentioned above, mitochondrial peroxides are reduced by GSH and glutathione peroxidase, and the GSSG formed is assumed to be reduced primarily by NADPH generated by transhydrogenase. By estimating oxidative damage as release of Ca^{2+}, mitochondria generating NADPH by the addition of a NADH-linked substrate are therefore less sensitive to *tert*-butylhydroperoxide than mitochondria provided with a NADPH-linked substrate [14]. Initially, this seemed confusing but is, in fact, consistent with the properties of the transhydrogenase reaction. Because of the linkage to Δp, the reaction is able to generate a high $NADPH/NADP^+$ ratio at a relatively low rate, in contrast to, e.g., the NADP-isocitrate dehydrogenase reaction which generates a lower $NADPH/NADP^+$ ratio but at a higher maximal rate. The experiment with *tert*-butylhydroperoxide therefore allows the important conclusion that protection against mitochondrial oxidative stress depends on the redox level of NADP(H), i.e., $NADPH/NADP^+$, and thus GSG/GSSG, rather than the capacity for NADPH generation. Replenishment of GSH in brain mitochondria involves transhydrogenase [20], and transhydrogenase also enables *Rhodobacter sphaeroides* to metabolize methanol through a GSH-linked degradation system [21]. As will be described in more detail in the following chapter, transhydrogenase protects *C. elegans* nematodes against ROS through the generation of GSH. Thus, it may be generally concluded that an important function of transhydrogenase is to maintain a high GSH/GSSG ratio.

Other proposed roles include a regulation of NADPH-dependent protein thiols [19], and thereby activities of, e.g., crucial membrane proteins such as the mitochondrial permeability transition pore (MPTP) [22]. By reversing reaction [1], another role of transhydrogenase would be to generate a short burst of a proton motive force (Δp) under anaerobic/ischemic conditions. However, since ΔG^0 for the nucleotide substrates/products of reaction [1] is close to zero, this burst would be very short considering that the total free energy available is in the form of a high product/substrate ratio and equilibration would be expected to be fast. A significant contribution of the reverse reaction to Δp would therefore be insignificant. It was early proposed that mitochondrial NADPH, under conditions of an increased demand for cytosolic NADPH, transhydrogenase-generated NADPH could contribute to the synthesis of isocitrate through the reversible NADP-isocitrate dehydrogenase, followed by transport of isocitrate/citrate to the cytosol by the tricarboxylate carrier and a reversal of the analogues cytosolic NADP-isocitrate dehydrogenase system [19]. Subsequently, Jackson and coworkers [23] suggested that transhydrogenase, together with the NAD- and NADP-isocitrate dehydrogenases, would regulate the Krebs cycle by forming a Δp-consuming futile cycle. Although potentially very attractive, there is no experimental support for this mechanism. On the contrary, a specific inhibitor of the NADP-isocitrate dehydrogenase affects the NADPH level in a manner inconsistent with the proposal [23]. In addition, as pointed out in the proposal, this mechanism of course requires a coexistence of transhydrogenase and NADP-isocitrate dehydrogenase, which seems to be true for mitochondria from different sources [24] but not yeasts which generally do not have transhydrogenase but do have NADP-isocitrate dehydrogenase [25]. Obviously, an interaction between the NADP-isocitrate dehydrogenase and transhydrogenase appears inevitable, but the relevance and extent of this interaction for the regulation of the Krebs cycle in the intact cell remains to be demonstrated.

Transhydrogenase activities of *E. coli* and other enteric bacteria are apparently co-regulated with glutamate dehydrogenase, and can vary several-fold depending on, e.g., the source of ammonia; it was proposed that transhydrogenase is a direct source of NADPH in the ammonia assimilation system [26]. Also, growth on branched amino acids, e.g., leucine, suppresses transhydrogenase [27]. Transhydrogenase (Pnt) knock-outs in *E. coli* show a phenotype with limited growth in the absence of the pentose phosphate pathway, but otherwise no apparent phenotypes [28–30]. *E. coli* has a separate, soluble transhydrogenase (UdhA), co-existing with the membrane-bound enzyme (Pnt), which seems to be working during excess NADPH production [18]. However, the existence of UdhA seems to be restricted to *E. coli* and possibly other bacteria, and has not been found in mitochondria.

The relevance of the functions and regulation of transhydrogenase in bacteria for the mitochondrial enzyme is presently unknown. However, it is conceivable that the role of transhydrogenase varies with the organism in which it is located.

Fig. 1 outlines transhydrogenase with an emphasis on its role as a redox buffer and thus a redox regulator of glutathione, protein thiols and other thiol systems, notably mitochondrial thioredoxin (Trx2) and glutaredoxin (Grx2). Both of the latter are maintained reduced by the mitochondrial Trx-reductase (TrxR); Trx2 and Grx2 have important roles in the regulation and protection of protein thiols [31]. Oxidized mitochondrial phospholipids are reduced by glutathione peroxidase including the mitochondrial phospholipid hydroperoxidase (HGPx) [11].

4. Properties of a *C. elegans* transhydrogenase deletion mutant

Transhydrogenase in *C. elegans*, denoted NNT, is globally expressed [32], with a particularly high expression in the pharyngeal–intestinal valve and certain neurons. In the mouse, high

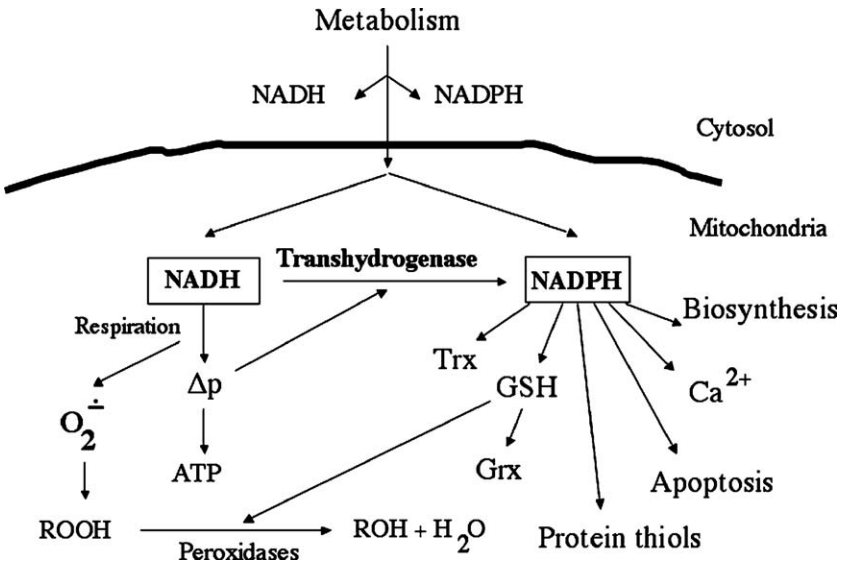

Fig. 1. Role of transhydrogenase in the maintenance of the mitochondrial NADPH and thiol redox homeostasis. Trx and Grx denote mitochondrial thioredoxin (Trx2) and glutaredoxin (Grx2), respectively.

expression was observed in heart, kidney, bladder, lung, ovary and brain [32]. In order to further identify the physiological role of transhydrogenase in *C. elegans*, a deletion mutation, *nnt-1 (sv34)*, was constructed which was lacking essential parts of the transhydrogenase gene, resulting in a phenotype devoid of transhydrogenase protein as indicated by Western blots following SDS-PAGE [33]. These nematodes showed essentially a normal life span and other properties typical of wildtype nematodes living in a normal environment. However, analysis of the cellular glutathione redox level, i.e., the GSH/GSSG ratio, which in wildtype was approximately 60, dropped to 12 in the mutant [33]. Due to the fact that the mitochondrial pool of glutathione is approximately 10–15% of the total pool, this drop in GSH (and increase in GSSG), was too large in order to only reflect a mitochondrial stress. An "overflow" of mitochondrial oxidative stress to the cytoplasm is therefore assumed [33]. Thus, it would be expected that the mutant nematodes would be more sensitive to especially mitochondrial oxidative stress than the wildtype ones. Indeed, when exposed to 0.1–0.4 mM methylviologen (Paraquat), a known redox cycler generating superoxide anions primarily in mitochondria, eggs of the mutant nematode were considerably more sensitive to mitochondrial oxidative stress than wildtype eggs, assayed as growth to adult nematodes. The defense against oxidative stress could be rescued by reintroducing the *nnt-1* gene. Similar effects were seen with eggs treated with siRNA.

Fig. 2 describes the mechanism of protection by transhydrogenase of *C. elegans* against methylviologen-dependent oxidative stress. Through a high GSH/GSSG ratio, transhydrogenase-generated NADPH contributes to a detoxification of peroxides formed from superoxide and lipid peroxidation.

This is the first time that a physiological role of transhydrogenase has been demonstrated in a higher eukaryote. The *C. elegans* system, because of its simplicity, short generation time, and established genome sequence, constitutes a very attractive model system for further studies on, e.g., mechanisms of neuronal damage related to transhydrogenase and oxidative stress. A further advantage with the *nnt-1(sv34)* mutant is that specific mitochondrial mechanisms of ROS toxicity may be studied. In this respect, the *nnt-1(sv34*) mutant resembles the *mev-1* mutant which generates excessive amounts of superoxide due to a mutation in cytochrome b of Complex II [34].

5. Diabetes: a disease potentially inked to a defective transhydrogenase gene

Oxidative stress, especially but not exclusively, mitochondrial oxidative stress, is believed to be involved in type 2 diabetes [35,36]. In the first demonstration ever of a linkage between a disease and a defective transhydrogenase gene, Toye et al. [37] showed that insulin release induced by high glucose

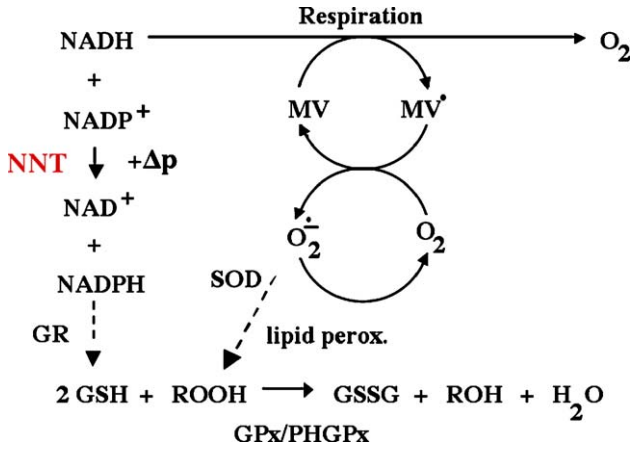

Fig. 2. Generation of toxic organic peroxides by Paraquat in *C. elegans* mitochondria, and the detoxification mechanism involving transhydrogenase. NNT (in red), transhydrogenase; MV, methylviologen or Paraquat; SOD, superoxide dismutase; GPx, glutathione peroxidase; PHGPx, phospholipid hydroperoxidase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was strongly impaired in C57BL/6J mice as compared to other mouse strains, e.g., C3H/HeH, DBA/2 and BALB/c. Thus, the phenotype of the C57BL/6J mice was typical of a type 2 diabetes condition, with an abnormal glucose tolerance. The defective glucose tolerance was linked to a mutated *Nnt* gene in which a multi-exon deletion was found, essentially resulting in a transhydrogenase knock-out. The absence of transhydrogenase attenuated the closure of the K_{ATP} channel in response to glucose metabolism. Consequently, opening of the Ca^{2+}-channel in the β cells was impaired, leading to an inhibition of insulin release. The detailed mechanism leading to decreased insulin release is discussed in more detail below.

In this context, it should be mentioned that Huang et al. [38] discovered that DBA/2J mice containing a mutated Sod2 gene, coding for the mitochondrial MnSOD, lived considerably longer (8 days) as compared to the same mutation in C57BL/6J mice (less than 24 h). As in the work by Toye et al. [37], C57BL/6J mice were shown to essentially lack transhydrogenase protein. The extended lifetime of the MnSOD-deficient DBA/2J mice was therefore concluded to be due to the presence of transhydrogenase. Thus, the absence of MnSOD was compensated by the presence of transhydrogenase.

The studies by Cox and coworkers of the role of transhydrogenase in the regulation of insulin secretion have recently been extended with, e.g., siRNA directed towards transhydrogenase in an insulin-secreting cell line. Glucose-induced uptake of Ca^{2+} was markedly lowered by siRNA directed towards either of the NAD(H)-binding or the NADP(H)-binding domains of transhydrogenase, leading to a close to 100% lack of transhydrogenase protein in the cells [39]. Endogenously generated ROS in β cells isolated from wild-type and homozygous Nnt G745D mutant mice was several-fold higher in the mutant mice as compared to wild-type [39]. The G745D transhydrogenase mutant is predicted to be inactive since glycine 745 is located in the membrane domain of the enzyme (J. Rydström, unpublished). Together, these results strongly suggest that the diabetic characteristics of C57BL/6J mice are primarily due to an impaired transhydrogenase activity. Indeed, these results also strongly support the proposal of Arkblad et al. [33] that an important role of transhydrogenase is to control ROS. In this context, it should be pointed out that transhydrogenase is strongly inhibited by fatty acyl CoA, especially long chain acyl CoA [40], which may further suppress the transhydrogenase activity in the presence of a high cellular content of fatty acids, typical of a pre-diabetic condition [35].

The work by Cox and coworkers [37,39] is remarkable and no doubt a breakthrough in transhydrogenase research. Three important questions may be raised by these results: (i) Is the C57BL/6j mice a model for human type 2 diabetes? (ii) What is the mechanism of the decreased insulin release in C57BL/6J mice? (iii) What other states/diseases may be visualized to be caused by the lack of transhydrogenase?

The first question is really an epidemiological problem which is presently investigated by others, but is beyond the scope of this review.

The second question obviously involves one or more steps in the mechanism of insulin release in β-cells. As described above, the K_{ATP} channels are normally closed by a high cellular ATP/ADP ratio generated by the metabolism of glucose and the resulting synthesis of ATP. Closing of the K_{ATP} channel results in a depolarization of the plasma membrane, activation of Ca^{2+}-channels, an increased cytosolic concentration of Ca^{2+}, fusion of insulin vesicles with the plasma membrane and release of free insulin [37,41]. The key factor seems to be an increased level of oxidative stress and a resulting decreased ATP/ADP ratio, indirectly caused by the lack of transhydrogenase. An attractive mechanism proposed by Cox and coworkers [39], involves UCP2, a homologue of the H^+-conducting uncoupler protein UCP1 responsible for thermogenesis [42]. UCP2, and possible also UCP3, is regulated by products of oxidative stress and lipid

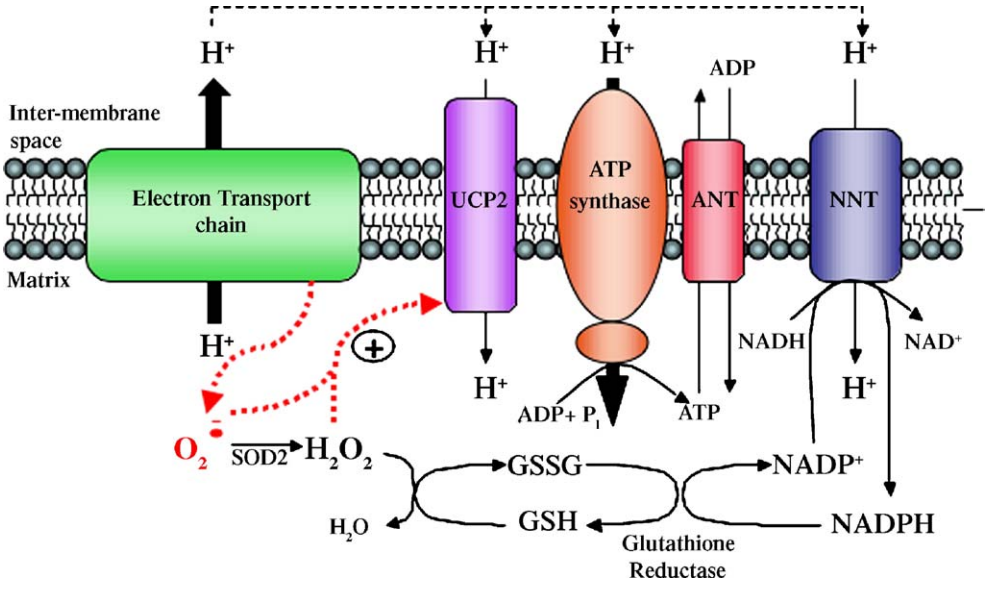

Fig. 3. Proposed role of Nnt in ROS detoxification in β-cells. From Freeman et al. [39] and reproduced with permission. UCP2, uncoupler protein 2; ANT, adenine nucleotide translocator; NNT, transhydrogenase; SOD2, mitochondrial (Mn) superoxide dismutase.

peroxidation such as peroxides, 4-hydroxynonenal and other alkenals [41]. These products bind to UCP2/UCP3, signalling oxidative stress. Oxidative stress depends initially on one-electron redox centers in the respiratory chain, primarily the flavin in Complex I and semiquinone in Complex III, which generate superoxide anions at rates determined by the redox levels of the former centers [3]. The linkage between the respiratory chain and UCP2/UCP3 is that a slight uncoupling will increase electron flow sufficiently for lowering the generation of superoxide anions. Thus, in C57BL/6J mice, the absence of transhydrogenase causes an increased basic level of oxidative stress, a permanent partial uncoupling through UCP2/UCP3, a decreased ATP/ADP ratio and permanently open K_{ATP} channels, regardless of the presence of glucose, and therefore a lack of insulin secretion. Being a proton-utilizing protein itself, a relevant question is how transhydrogenase would affect the delicate balance between ROS generation and proton flow across the inner mitochondrial membrane. However, because of the low ΔG^0 value for the nucleotides, and despite consuming one proton per catalytic turnover, the transhydrogenase reaction is relative insensitive to changes in Δp. This is demonstrated by a lower effect of a decreased Δp on reaction [1] under conditions when oxidative phosphorylation is inhibited [43]. The relationship between the two Δp-requiring reactions, where oxidative phosphorylation may require up to 4 protons/ATP formed, can also be expressed as a relatively small effect of reaction [1] on oxidative phosphorylation. An overview of the mechanism proposed by Freeman et al. [39] is shown in Fig. 3.

The third question may be answered by looking at the large number of investigations that have been carried out and published using C57BL/6J mice. However, this is not a straight forward question since it is not known, of course, when the mutation took place. Searching in PubMed for studies in which C57BL/6J mice were used gives about 7000 hits, the majority of which involves results from disease/toxicology-related investigations. In the light of the present knowledge about the lack of transhydrogenase in this strain, and the resulting increased level of oxidative stress, it is likely that the results of some of these studies have to be reinterpreted. However, inbred strains of C57BL/6J mice show varying levels of transhydrogenase (N. B. Pestov, personal communication), indicating different types of genetic alterations in the transhydrogenase gene, and possibly also in the promoter region.

6. Other diseases/conditions potentially involving transhydrogenase

Apart from type 2 diabetes described above, and possibly also type 1 diabetes, transhydrogenase may well have a role in other diseases/conditions with a mitochondrial etiology [44–47]. Aging, e.g., longevity of *C. elegans* nematodes, is strongly correlated with mitochondrial functions [48], and is apparently also influenced by transhydrogenase in mice [38].

More specifically, there is a large body of evidence suggesting that C57BL/6J mice have a much higher sensitivity to neurotoxic agents, e.g., 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) which causes a Parkinson-like condition in mice probably due to oxidative stress [49–51]. The effect of MPTP was concluded to be related to a putative locus on chromosome 13, where the transhydrogenase gene is located [52]. Unexpectedly, C57BL/6J mice show a *lower* cancer frequency than, e.g., C3H/He and B6C3F1 mice, which was concluded to be due to a lower growth rate of, e.g., liver tissue, rather than a lower rate of apoptosis [53]. It is possible that the latter may be triggered by a lower mitochondrial redox potential of NADP(H) and/or a higher mitochondrial oxidative stress level. Finally, there is an interesting potential linkage between alcohol-related disease in the liver [54,55] heart involving mitochondrial glutathione [56] and transhydrogenase.

Acknowledgement

Supported by the Swedish Research Council.

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