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Biphasic Oxidation of Mitochondrial NAD(P)H

Victor V. Lemeshko[1]
School of Physics, Science Faculty, National University of Colombia, Medellin Branch, AA 3840 Medellin, Colombia

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The redox state of mitochondrial pyridine nucleotides is known to be important for structural integrity of mitochondria. In this work, we observed a biphasic oxidation of endogenous NAD(P)H in rat liver mitochondria induced by *tert*-butylhydroperoxide. Nearly 85% of mitochondrial NAD(P)H was rapidly oxidized during the first phase. The second phase of NAD(P)H oxidation was retarded for several minutes, appearing after the inner membrane potential collapse and mitochondria swelling. It was characterized by disturbance of ATP synthesis and dramatic permeabilization of the inner membrane to pyridine nucleotides. The second phase was completely prevented by 0.5 μM cyclosporin A or 0.2 mM EGTA or was significantly delayed by 25 μM butylhydroxytoluene or trifluoperazine. The obtained data suggest that the second phase resulted from oxidation of the remaining NADH via the outer membrane electron transport system of permeabilized mitochondria, leading to further oxidation of the remaining NADPH in a transhydrogenase reaction.

Key Words: **permeability transition; pyridine nucleotides; hydroperoxides; ATP synthesis; antioxidants; free radicals.**

Most of the mechanisms of necrosis and apoptosis appear to include mitochondrial permeability transition (MPT) that leads to the rupture of the outer mitochondrial membrane and the release of various apoptotic factors from the intermembrane space (1–6). Opening of the permeability transition pore (PTP) in the inner mitochondrial membrane causes the uncoupling of oxidative phosphorylation (7). Mitochondria permeabilization depends on Ca^{2+} overload (see 5, 8–10, and references therein) and is stimulated by oxidative stress (1, 2, 5, 11), among many other permeabilization factors (10, 12).

Abbreviations used: MPT, mitochondrial permeability transition; PTP, permeability transition pore; tBH, *tert*-butylhydroperoxide.

[1] Address correspondence and reprint requests to author. Fax: (57) (4) 2604489. E-mail: vvasilie@perseus.unalmed.edu.co.

Mitochondria permeabilization by oxidative stress is widely studied by applying *tert*-butylhydroperoxide (tBH) to isolated mitochondria (5, 3, 14) or intact cells (15–17). A biphasic character of mitochondrial NAD(P)H oxidation after tBH addition to intact hepatocytes was shown recently (16). It was demonstrated that the second phase is observed during reactive oxygen species generation in mitochondria and suggested that MPT is the subsequent event (16, 17). These data awake again an attention to a possible mechanism of the inner membrane permeabilization under oxidation of mitochondrial pyridine nucleotides (11, 12, 15, 18, 19).

Lehninger *et al.* (18) showed that the release of Ca^{2+} from mitochondria is regulated by redox-state of pyridine nucleotides. Haworth and Hunter have demonstrated that NADH, as ADP, is able to inhibit a nonspecific permeability of the inner membrane and acts synergistically with ADP (20). Allosteric modulation of PTP by the matrix NADH was also suggested by Chernyak and Bernardi (21), similar to that demonstrated by Lee *et al.* (22) for modulation of the voltage-dependent anion channel of the outer membrane.

According to other data, the redox-state of NADPH/$NADP^+$ may be more closely linked to MPT (11, 23), since NADPH directly participates in elimination of hydroperoxides through the glutathione peroxidase/glutathione reductase and thioredoxin peroxidase/thioredoxin reductase enzyme systems (11, 13, 19). Thus, both NADH and NADPH oxidations were assumed to be important for MPT modulation. On the other hand, some data suggest that oxidation of mitochondrial NAD(P)H may be a consequence of PTP opening (24, 25). However, it is also not excluded that oxidation of a certain fraction of mitochondrial NAD(P)H is critical for PTP opening, while oxidation of the remaining fraction might be activated by MPT.

In this work, the biphasic character of endogenous NAD(P)H oxidation in rat liver mitochondria was observed in the presence of tBH and rotenone, and its relation to the inner membrane permeability transition and disturbance of ATP synthesis was studied. The obtained data suggest that the first phase of endogenous NAD(P)H oxidation is a causative factor for MPT, while the second phase seems to be caused by

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MPT. The second phase might be explained by the release of the remaining NADH from permeabilized mitochondria and its oxidation via the external pathway of NADH oxidation (26–29), leading to a further oxidation of the remaining NADPH through a reversible transhydrogenase reaction.

MATERIALS AND METHODS

Liver mitochondria were isolated from adult, 5–7 months old, rats (starved overnight) by the standard procedure of differential centrifugation. The medium, composed of 210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 5 mM Hepes–Tris, pH 7.4, and 0.3 mg/ml of bovine serum albumin (fraction V, free of fatty acids), was used for tissue homogenization. Mitochondria were washed two times and finely suspended in the medium composed of 210 mM mannitol, 70 mM sucrose, 0.02 mM EGTA, 5 mM Hepes–Tris, pH 7.4. The final concentration of mitochondria in suspension was about 60 mg of mitochondrial protein/ml, determined by the biuret method, using Na^+–cholate, with bovine serum albumin as standard.

NADH was measured fluorometrically (340/450 nm), using the Aminco–Bowman luminescence spectrometer, Series 2 (Spectronics, U.S.A.). To maximally minimize the influence of light scattering on the fluorescence measurements, the cuvette holder was modified: the exciting and emitting light beams were focused only on the 1.5 × 1.5 mm corner of the cuvette. The fluorometer, constructed in our laboratory, was also used for some experiments: it allows the fluorescence measurements from the same wall of the cuvette where the exciting light enters. The biphasic oxidation of mitochondrial NAD(P)H was observed using both fluorometers.

The electrical potential on the inner mitochondrial membrane was monitored by measuring the safranin O fluorescence (30), at 520/580 nm. Light scattering was measured at 520/520 nm, using the Aminco–Bowman luminescence spectrometer. All three parameters, NAD(P)H fluorescence, safranin O fluorescence and light scattering, were measured from the same corner of the cuvette, with a 1.0-s time resolution. The medium, composed of 100 mM mannitol, 100 mM sucrose, 40 mM glucose, 5 mM phosphate–Tris, 5 mM succinate–Tris, and 10 mM Hepes–Tris, pH 7.4, was used. The resulting contamination of this medium was 13–14 μM Ca^{2+}, determined by atomic absorption spectrometry.

Real-time ATP synthesis was determined fluorometrically, registering NADPH accumulation in hexokinase/glucose-6-phosphate dehydrogenase enzyme system (31). The composition of the ATP-registering system is shown in the legend to Fig. 3.

All experiments were performed at 37°C with constant stirring. All reagents were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO).

RESULTS

Rapid oxidation of mitochondria NAD(P)H is known to occur in the presence of the oxidizing factor, tBH (13, 14, 16, 19). Figure 1A (curve b) shows that rat liver mitochondria can almost completely recover their NAD(P)H level after the reduction of 25 μM tBH. Only a partial recovery was observed in the case of 50 μM tBH (Fig. 1A, c); the additional 50 μM tBH caused a more profound oxidation, without any following NAD(P)H recovery.

At 100 μM tBH concentration (or higher), the typical biphasic character of NAD(P)H oxidation was observed

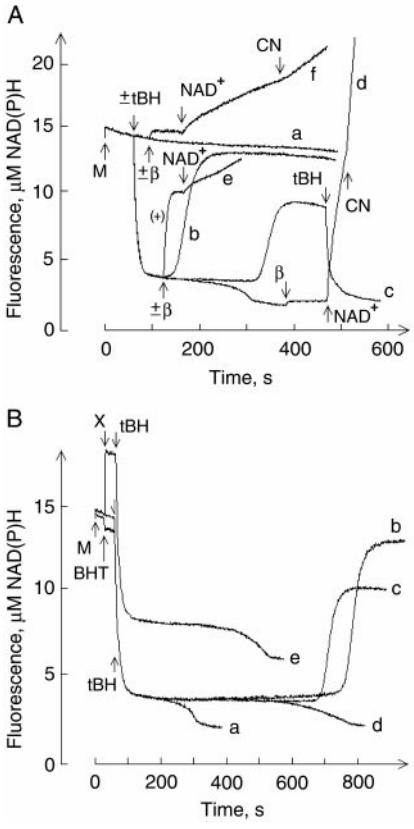

FIG. 1. Influence of *tert*-butylhydroperoxide on oxidation–reduction of endogenous pyridine nucleotides in rat liver mitochondria. M, mitochondria, 0.3 mg mitochondrial protein/ml, and 2.5 μM rotenone addition into the incubation medium composed of 100 mM mannitol, 100 mM sucrose, 40 mM glucose, 5 mM phosphate–Tris, 5 mM succinate–Tris, 10 mM Hepes–Tris, pH 7.4. (A) tBH, 25 μM (b), 50 μM (c) or 100 μM *tert*-butylhydroperoxide (d, e); a, f, no tBH addition; β, 5 mM β-hydroxybutyrate–Tris; NAD^+, 0.5 mM NAD^+; CN, 1 mM KCN. (B) tBH, 100 μM *tert*-butylhydroperoxide; BHT, 25 μM butylhydroxytoluene (d); X, 0.5 μM cyclosporin A (b), 0.2 mM EGTA (c), or 25 μM trifluoperazine (e); a, no BHT or X addition.

(Fig. 1A, d). The rate of the second phase of oxidation was evaluated as 6.9 ± 0.4 nmol NAD(P)H per minute per milligram of protein (n = 8). Addition of β-hydroxybutyrate before the second phase was able to maintain the reduced state of nearly a half of the total pool of mitochondrial NAD(P)H (Fig. 1A, e); only a negligible level was maintained after the second phase (Fig. 1A, d). In contrast, the rate of exogenous NAD^+ reduction by β-hydroxybutyrate was more than 10 times higher after the second phase (Fig. 1A, d) than before it (Fig. 1A, e), or in the control (Fig. 1A, f). The rate of exogenous NAD^+ reduction was more than 2 times higher in the presence of cytochrome *c* oxidase inhibitor, KCN (Fig. 1A, d) that is known to inhibit the external pathway of NADH oxidation.

The second phase of mitochondrial NAD(P)H oxidation was prevented by 0.5 μM cyclosporin A (Fig. 1B, b) or 0.2 mM EGTA (Fig. 1B, c), added 30 s before tBH. The same results were observed when cyclosporin A or

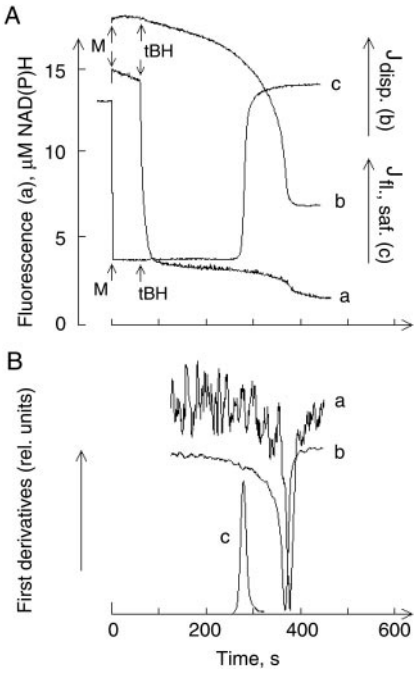

FIG. 2. Influence of *tert*-butylhydroperoxide on endogenous NAD(P)H oxidation, mitochondria light scattering and safranin O fluorescence changes in rat liver mitochondria. M, mitochondria, 0.3 mg mitochondrial protein/ml, and 2.5 μM rotenone addition into the incubation medium (see the legend to Fig. 1). The medium was supplemented with 10 μM safranin O, when the inner membrane potential was monitored. (A) a, NAD(P)H; b, light scattering; c, safranin O fluorescence; tBH, 100 μM *tert*-butylhydroperoxide. (B) a, b, c, first derivatives of curves a, b and c in A, respectively.

EGTA were added 30 s after tBH (data not shown). A significant recovery of endogenous NAD(P)H was observed after finishing the reduction of 100 μM tBH in the presence of these agents (Fig. 1B, b and c). The appearance of the second phase may also be delayed nearly 3 or 2 times by 25 μM butylhydroxytoluene (Fig. 1B, d) or 25 μM trifluoperazine (Fig. 1B, e), respectively. Desferrioxamine, at concentrations 0.5–1.0 mM, was ineffective under our conditions (data not shown).

The obtained data show that the second phase of mitochondrial NAD(P)H oxidation relates to PTP opening. To determine, whether the PTP opening results from the second phase or precedes it, the mitochondria swelling (Fig. 2A, b) and the inner membrane potential (Fig. 2A, c) were also registered (at the same response time, temperature and the magnetic stirring velocity). One can see that the inner membrane potential collapse preceded the mitochondria swelling (Fig. 2A, c and b, respectively), and the mitochondria swelling preceded the second phase (Fig. 2A, b and a, respectively). The peak of the first derivative of the decrease in the light scattering was observed 83 $\pm$ 3 s (n = 4) later than the peak of the first derivative of the inner membrane potential decrease, and 12.5 $\pm$ 2.5 s (n = 4) before than the peak of the first derivative of the second phase of mitochondrial NAD(P)H oxidation (Fig. 2B, b, c, and a, respectively).

The real-time ATP synthesis was dramatically decreased after the second phase (Fig. 3, b). After the first phase, but before the second one, the rate of ATP synthesis (Fig. 3, c) was still close to the control (Fig. 3, a). The ATP synthesis was almost completely blocked by 1 μM oligomycin (data not shown), or 0.5 μM trifluoromethoxy carbonyl cyanide phenylhydrazone (Fig. 3, d), confirming that the method, used in these experiments, indeed allows registering the mitochondrial oxidative phosphorylation. A very high rate of $NADP^+$ reduction was observed after 0.5 mM ATP addition (Fig. 3, b and d), showing that the rate capacity of the used ATP-registering system is not a limiting factor for determining the rate of ATP production by mitochondria.

DISCUSSION

The obtained data show that tBH, at concentration 100 μM or higher, causes a biphasic oxidation of endogenous NAD(P)H in rat liver mitochondria. The glutathione peroxidase/glutathione reductase and thioredoxin peroxidase/thioredoxin reductase enzyme systems, coupled to transhydrogenase, are known to metabolize hydroperoxides in the mitochondrial matrix (11, 13, 19). The inner membrane proton-motive force potentiates steady-state reduction of $NADP^+$ by NADH, which in turn may be recovered in various NAD^+-dependent dehydrogenase reactions in the matrix. As it was observed, after the second phase, mitochondria failed to reduce the oxidized pool of pyridine nucleotides by β-hydroxybutyrate (Fig. 1A, d). If the second phase results from MPT, the endogenous pyridine nucleo-

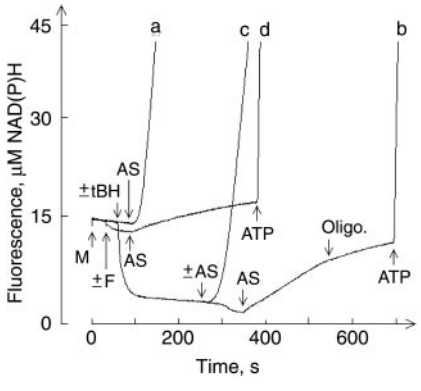

FIG. 3. Influence of *tert*-butylhydroperoxide on the real-time ATP synthesis by rat liver mitochondria. M, mitochondria, 0.3 mg mitochondrial protein/ml, and 2.5 μM rotenone addition into the incubation medium (see the legend to Fig. 1). tBH, 100 μM *tert*-butylhydroperoxide (b, c); a, d, no tBH addition; F, 0.5 μM trifluoromethoxy carbonyl cyanide phenylhydrazone (d); Oligo., 1 μM oligomycin; AS, ATP-registering system addition: 2 U hexokinase, 2 U glucose-6-phosphatase dehydrogenase, 0.35 mM AMP, 0.5 mM $NADP^+$, 2 mM $MgCl_2$; ATP, 0.5 mM ATP.

tides may be released from mitochondria through activated PTP and be diluted in the external medium, thus lowering the endogenous NAD^+ concentration in the matrix far less than K_m of β-hydroxybutyrate dehydrogenase for NAD^+. Under these conditions, exogenous 0.5 mM NAD^+ was rapidly reduced (Fig. 1A, d), as if it was entering into the mitochondrial matrix through open PTP. After the first phase, but before the second one, almost half of the total pool of endogenous pyridine nucleotides was rapidly reduced by β-hydroxybutyrate, while the rate of exogenous NAD^+ reduction was relatively small (Fig. 1A, e).

Our results confirm that the second phase of endogenous NAD(P)H oxidation is interrelated with MPT that was previously observed by other authors in experiments with intact hepatocytes (6, 16, 17). The second (late) phase was interpreted by these authors as a causative factor for MPT, and MPT as a causative factor for the inner membrane potential decrease (6, 16, 17). The latter seems to be still uncertain, because the biphasic oxidation of mitochondrial NAD(P)H in hepatocytes was studied with time resolution in the order of minutes. In addition, the interference of many other factors may take place at the cellular level. In our work with isolated mitochondria, the 1.0-s time resolution data demonstrate that the collapse of the inner membrane potential and the mitochondria swelling preceded the second phase of endogenous NAD(P)H oxidation (Fig. 2).

To explain the observed biphasic oxidation of mitochondrial NAD(P)H, induced by tBH, the following working hypothesis may be proposed. After a high amplitude PTP opening, the mitochondria swelling leads to the outer membrane rupture. As a result, the remaining NADH may be released from the matrix through the opened PTP. Then, it may be oxidized (even if rotenone is present) through the external pathway of NADH oxidation (Fig. 4). This pathway is known to include the outer membrane electron transport system and cytochrome *c* shuttling between the outer and inner membranes (26–29). The remaining endogenous NADPH may also be oxidized during the second phase, reducing NAD^+ through the reversible transhydrogenase reaction.

The scheme in Fig. 4 explains how the second phase may be realized. It includes only the most important factors related to very complicated mechanisms of mitochondria permeabilization (1, 5, 6, 10–12, 21, 25). The scheme demonstrates that, in addition to Ca^{2+} and GSSG, free radicals may directly participate in PTP activation (5, 7, 12, 14), taking also into account that the free radical scavenger, butylhydroxytoluene, known to delay MPT, significantly delayed the second phase of endogenous NAD(P)H oxidation induced by tBH (Fig. 1B, d). A similar protection was obtained with trifluoperazine (Fig. 1B, e). Although trifluoperazine may react with free radicals, other properties of this phenothiazinic drug may also be responsible for the observed protection (see 33 and references therein). In any case, it is probable that the process of thiol cross-linking is realized through free radical intermediates at the level of PTP (5, 12, 14, 34) and other inner membrane proteins (11, 33, 35). Free radical intermediates of a permeabilization mechanism may be directly modulated by antioxidants, because even the phenylarsine oxide-induced MPT was essentially prevented by butylhydroxytoluene (36). The respiratory chain is the main source of free radicals in the cells (1, 2) influencing a probability of MPT, which is known to synergistically depend on many other permeabilization factors.

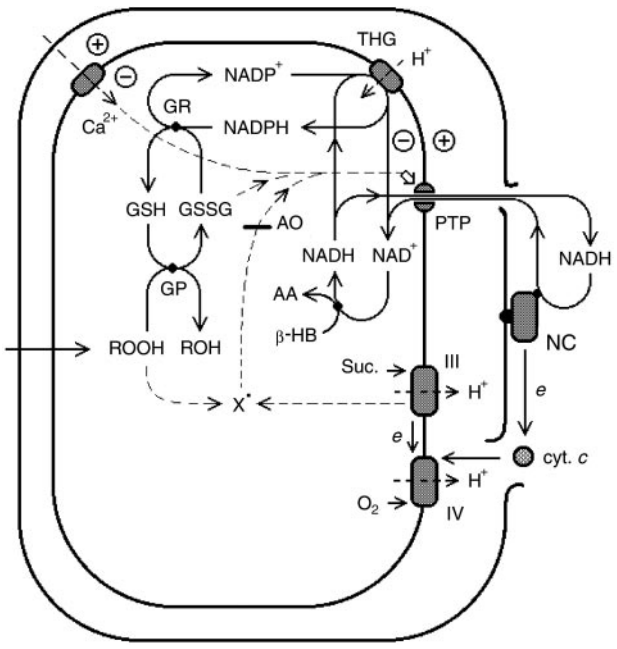

FIG. 4. Simplified scheme demonstrating the influence of hydroperoxides, Ca^{2+}, and free radicals on the mitochondrial permeability transition and possible pathways of NAD(P)H oxidation in mitochondria. THD, transhydrogenase; GR, glutathione reductase; GP, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; X^*, free radicals; AO, antioxidants; PTP, permeability transition pore; NC, NADH–cytochrome *c* reductase system of the outer mitochondrial membrane; AA, acetoacetate; β-HB, β-hydroxybutyrate; Suc., succinate; III, IV, complexes of the respiratory chain.

In conclusion, our data show that the relatively prolonged maintenance of mitochondrial pyridine nucleotides in essentially oxidized state may cause a free radical-dependent MPT resulted in disturbance of ATP synthesis. Such intracellular events are known to be a critical step in mechanisms of apoptosis and necrosis. With respect to the MPT induction by *tert*-butylhydroperoxide, it may be suggested that the first phase of mitochondrial NAD(P)H oxidation is a causative factor for MPT, while the second phase seems to be a consequence of a high amplitude PTP opening. This inter-

pretation is in accordance with the recent data of Zago *et al.* (37) showing that NADPH, but not NADH oxidation in mitochondria is the main causative factor for MPT. Our data suggest that the time interval between the first phase of mitochondrial NAD(P)H oxidation and the PTP opening depends on free radicals generation and antioxidant exhaustion in mitochondria.

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