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Oxidative damage of rat liver mitochondria during exposure to t-butyl hydroperoxide. Role of Ca^{2+} ions in oxidative processes

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ABSTRACT

Aims: The present study was designed for further evaluation of the biochemical mechanism of hepatic mitochondrial dysfunction under oxidative damages induced by organic hydroperoxide, tert-butyl hydroperoxide (tBHP), for estimation of the molecular targets impaired during oxidative stress, and for investigation of the role of Ca^{2+} ions in mitochondrial oxidative reactions and of the protective effect of melatonin during mitochondrial peroxidative damage.

Main methods: Mitochondria were isolated by differential centrifugation from the rat liver. The effects of tBHP exposure, EDTA, Ca^{2+} ions and melatonin on mitochondrial respiratory activity, mitochondrial enzyme activities and redox status were measured.

Key findings: The present study provides evidence that tBHP (at low concentrations of 0.02–0.065 mM, in EDTA-free medium) induced uncoupling of the oxidation and phosphorylation processes and decreased the efficiency of the phosphorylation reaction. This effect depended on the respiratory substrate used. The presence of EDTA prevented oxidative impairment of mitochondrial respiration, but Ca^{2+} ions in the medium enhanced oxidant-induced mitochondrial damage considerably. In the presence of 0.5 mM EDTA, tBHP (at high concentrations, 0.5–2 mM) considerably oxidized mitochondrial reduced glutathione, enhanced accumulation of membrane lipid peroxidation products and mixed protein–glutathione disulfides and led to an inhibition of oxoglutarate dehydrogenase and succinate dehydrogenase.

Significance: Direct oxidative modification of enzymatic complexes of the respiratory chain and mitochondrial matrix, mitochondrial reduced glutathione depletion, protein glutathionylation, membrane lipid peroxidation and Ca^{2+} overload are the main events of mitochondrial peroxidative damages. Experiments in vitro demonstrated that melatonin inhibited the mitochondrial peroxidative damage, preventing redox-balance changes and succinate dehydrogenase inactivation.

Introduction

Mitochondria play a central role in cell biology. Disturbances in mitochondrial function will lead to disruption of cellular structure and function and result in apoptotic and necrotic cell death (Duchen, 2004). Mitochondria are major producers of free radical species and first of all of a superoxide anion radical (the primary reactive oxygen species, ROS), possibly also of nitric oxide, and despite the presence of various antioxidant defenses are also the major targets of oxidative damage. It is widely accepted that one of the major sites of radical generation lies at complex III of mitochondria (ubiquinol–cytochrome c reductase) (Turrens, 2003).

Many xenobiotics, toxic agents and oxidants are inhibitors of the mitochondrial respiratory chain. Cytotoxicity resulting from many pathological insults, such as ischemia–reperfusion injury, intoxication, stroke and excitotoxicity, is known, to involve mitochondria-associated processes: mitochondrial oxidative damage, intracellular Ca^{2+} ion level increase and mitochondrial permeability transition (MPT) (Jaeschke et al., 2002; Baranov et al., 2008). The organic hydroperoxide, tert-butyl hydroperoxide (tBHP), a water-soluble analog of lipid hydroperoxides, is a useful model compound to study the mechanisms of oxidative cell injury, and to evaluate the sensitivity of cells and cellular components to oxidative metabolic stress (Nieminen et al., 1997; Krivakova et al., 2007). The effect of tBHP on mitochondrial energy metabolism is very complex and depends on many parameters: the oxidant concentration, the time of its exposure, and the respiration substrates used (Bellomo et al., 1984; Kmonícková et al., 2000; Krivakova et al., 2007). As was shown in the earlier works of Bellomo et al. (1982, 1984), at low concentrations of tBHP, pyridine nucleotides and SH groups are the targets of its action in mitochondria. It

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was further suggested that tBHP modified the iron–sulfur clusters or SH groups of the NADH dehydrogenase enzyme complex in mitochondria (complex I) (Drahota et al., 2005). The metabolism of tBHP (at low concentrations) leads to a release of Ca^{2+} from intracellular stores via oxidation of pyridine nucleotides and reduced glutathione, and subsequent membrane damage is not directly associated with this Ca^{2+} efflux but results from continued cycling of released Ca^{2+} (Bellomo et al., 1982, 1984). The authors suggested that different regulatory mechanisms modulate mitochondrial (NADPH-dependent) and extramitochondrial (thiol-dependent) Ca^{2+} compartmentation and that disturbance of normal Ca^{2+} homeostasis might be critical in peroxide-induced cytotoxicity (Bellomo et al., 1982). Later it was shown that tBHP (0.1 mM) caused an early increase of mitochondrial free Ca^{2+} uptake in hepatocytes, leading to increased matrix Ca^{2+}-induced MPT, mitochondria depolarization, rapid pyridine nucleotide oxidation and reactive oxygen species (ROS) generation, leading to death of hepatocytes (Nieminen et al., 1997; Lemasters et al., 1998; Byrne et al., 1999). Onset of the MPT was preceded by pyridine nucleotide oxidation, mitochondrial generation of ROS, and an increase of mitochondrial free Ca^{2+}.

Isolated hepatocytes exposed to tBHP (1 mM) showed a significant time-dependent increase in cytosolic free calcium level, perhaps due to inner mitochondrial membrane permeability changes and mitochondrial Ca^{2+} release (opening of Ca^{2+}-dependent pores) (Kmoníčková et al., 2000). In isolated rat hepatocytes, permeabilized by digitonin, flavoprotein-dependent substrate (succinate) oxidation is much less sensitive to oxidative stress (tBHP exposure) than oxidation of NADH-dependent substrates (glutamate + malate) (Drahota et al., 2005). Similarly, it was shown using isolated rat liver mitochondria that exposure to tBHP (0.2 mM) resulted in considerable damage of complex I (palmitoyl carnitine oxidation) and did not affect succinate oxidation as well as ATP synthesis (Cervinková et al., 2008). In isolated hepatocytes in EDTA-containing medium, only high (3 mM) tBHP concentration (10-min incubation) had an uncoupling effect in the presence of succinate as a substrate, which was manifested in an increase of respiration in the absence of ADP and a decrease of the respiration control index as well as a dissipation of membrane potential (Kriváková et al., 2007).

A large number of studies have shown that melatonin, N-acetyl-5-methoxy-tryptamine, and its metabolites are highly effective free radical scavengers and ubiquitously acting antioxidants, which play an essential role in reducing oxidative stress under a variety of experimental settings (Reiter, 2000; Tan et al., 2007; Gallano et al., 2011). Many of the beneficial effects of melatonin administration may depend on its action on the mitochondria (Jou et al., 2010; Paradies et al., 2010; Dragicevic et al., 2012). High concentrations of melatonin were found in mitochondria (Martín et al., 2000; Tan et al., 2000; Tan et al., 2013). There is evidence that mitochondria may have the capacity to synthesize and metabolize melatonin (Tan et al., 2013). Earlier we demonstrated that melatonin administration in a pharmacological dose affected the mitochondrial function and prevented mitochondrial dysfunction under experimental diabetes and intoxication, demonstrating mitochondria-specific activity of melatonin (Cheshchevik et al., 2011; Zavodnik et al., 2011; Cheshchevik et al., 2012).

Thus, the results of numerous investigations of organic hydroperoxide interactions with mitochondria are controversial and the details of this process are not fully understood. The present study was designed to evaluate the biochemical mechanism of hepatic mitochondrial dysfunction under oxidative damages induced by tBHP and to estimate the molecular targets impaired during oxidative stress, as well as to investigate the role of Ca^{2+} ions in mitochondrial oxidative reactions and the protective effect of melatonin during mitochondrial peroxidative damage which is responsible for development of some pathologic states. Our results confirmed high sensitivity of mitochondria to oxidative stress and involvement of Ca^{2+} in oxidative processes in mitochondria.

Materials and methods

Chemicals

tert-Butyl hydroperoxide (tBHP), 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), calcium chloride dehydrate, thiobarbituric acid (TBA), trichloroacetic acid (TCA), N-acetyl-5-methoxytriptamine (melatonin), succinic acid disodium salt hexahydrate, L-glutamic acid sodium salt, 2-oxoglutarate sodium salt, 2,6-dichlorophenol-indophenol (DCPI) and ADP were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). All other reagents were purchased from POCh (Gliwice, Poland) and Reakhim (Moscow, Russia) and were of analytical grade. All solutions were made with water purified in the Milli-Q system.

Rat liver mitochondria: isolation and respiration measurements

Mitochondria were isolated by the standard procedure of differential centrifugation from the rat liver (Johnson and Lardy, 1967). The mitochondrial pellet was resuspended in the buffer to an approximate protein concentration of 35–40 mg/ml. The protein concentration was determined by the method of Lowry et al. (1951).

The respiration of mitochondria was measured using a laboratory-made oxygen Clark-type electrode and a hermetic polarographic cell (volume 1.25 ml) with constant gentle stirring (Dremza et al., 2006). The incubation medium contained 0.125 M KCl, 0.05 M sucrose, 0.01 M Tris–HCl, 0.0025 M KH_2PO_4, and 0.005 M $MgSO_4$, with 0.0005 M EDTA (EDTA-containing) or without EDTA (EDTA-free medium), pH 7.4, The experiments were performed at 25 °C using 5 mM succinate, oxoglutarate or L-glutamate as respiratory substrates. Mitochondrial protein concentration in the sample was 1.0 mg/ml. The functional state of mitochondria was determined by the acceptor control ratio (ACR), equal to the ratio of the respiratory rates (V_3/V_2) of mitochondria in States 3 and 2, and the coefficient of phosphorylation (ADP/O) (the ratio of the amount of ADP added to the amount of oxygen consumed throughout phosphorylation). State 2 corresponded to the respiration in the presence of substrate (glutamate, oxoglutarate or succinate) added (V_2). The rate of mitochondrial respiration corresponding to State 3 (V_3) was recorded after addition of 180 μM ADP (in the presence of 210 nm ADP in the polarographic cell). To study peroxidative damage of mitochondria, we used such concentrations of tBHP at which the free radicals generated caused damage of mitochondrial functional activity. We used high concentrations of tBHP (up to 2 mM) in the presence of EDTA for producing mitochondrial damage and lower concentrations of tBHP (up to 0.1 mM) in the absence of EDTA or in the presence of calcium ions. Similar concentrations of tBHP were used to study oxidative damage in mitochondria previously (Kriváková et al., 2007; Drahota et al., 2005).

Biochemical measurements

The amount of membrane lipid peroxidation products, TBA-reactive species (TBARS), in rat liver mitochondria after tBHP treatment was measured using the method of Stocks and Dormandy (1971). Mitochondria (15 mg protein/ml) were exposed to tBHP in 150 mM KCl, 20 mM KH_2PO_4, 0.5 mM EDTA, pH 7.4, at 25 °C for 30 min. The concentration of the lipid peroxidation products, TBA complex, was assessed spectrophotometrically, using the extinction coefficient of 156 mM^{-1} cm^{-1} (532 nm). The concentration of GSH in mitochondria was determined spectrophotometrically by the method of Ellman (1959), using the extinction coefficient of 13.6 mM^{-1} cm^{-1} at 412 nm. Mixed disulfides (GSSP) formed by glutathione and accessible sulfhydryl groups of mitochondrial proteins after tBHP treatment were determined by the method described by Rossi et al. (1993). Oxoglutarate dehydrogenase (OGDH) activity was assayed as the rate of NAD reduction that was measured spectrophotometrically at 340 nm upon addition of fractured

mitochondria (by rapid freezing–thawing of mitochondria, three times) to the medium containing 0.1 M potassium phosphate buffer, pH 7.4, 5.0 mM $MgCl_2$, 40.0 μM rotenone, 2.5 mM oxoglutarate, 0.1 mM CoA, 0.2 mM thiamine pyrophosphate, and 1.0 mM NAD at 25 °C (Nulton-Persson and Szweda, 2001). The protein concentration in the reaction mixture was 50 μg/ml. The activity of mitochondrial succinate dehydrogenase (SDH) was spectrophotometrically determined by the rate of 2,6-dichlorophenol–indophenol reduction at 600 nm upon addition of fractured mitochondria (final protein concentration of 50 μg/ml) to the reaction mixture containing 0.1 M potassium phosphate buffer, pH 7.4, 25 mM sodium succinate, 0.5 mM phenazinemetasulfate, 2.5 mM sodium azide and 0.05 mM DCPI (Nulton-Persson and Szweda, 2001).

Statistical analysis

The results were expressed as the means of four to five replicates ± SD and the statistical analysis was conducted using analysis of variance (ANOVA). We used the standard Student's *t*-test for the comparison of the raw and transformed data showing no departures from normality (according to Shapiro–Wilk's test).

Results

In the present work, we studied the mechanism(s) of oxidative damage of isolated liver mitochondria during exposure to organic hydroperoxide tBHP and evaluated the effects of Ca^{2+} ions, EDTA, divalent ions chelator, and antioxidant melatonin during oxidation.

Respiratory parameters of mitochondria: effects of tBHP, EDTA and Ca^{2+} ions

Fig. 1 represents the effect of tBHP on the mitochondrial respiratory parameters in EDTA-free medium. (Mitochondria were exposed to the oxidant for 2 min at 25 °C before starting measurements.) The oxidative damage of mitochondria considerably decreased the ADP-stimulated respiration rate V_3 in the case of glutamate as substrate and increased the substrate-dependent respiration rate V_2 in the presence of both glutamate and succinate (Fig. 1a and c). The acceptor control ratio (ACR) reached 1.0 due to uncoupling of the processes of oxidation and phosphorylation and the phosphorylation coefficient (ADP/O) reached 0 at 0.065–0.1 mM tBHP in the case of glutamate or succinate as respiratory substrates (Fig. 1b and d). The glutamate-dependent respiration was more susceptible to the oxidant than the succinate-dependent one (Fig. 1a–d).

In the EDTA-containing medium, the oxidative effect of tBHP on mitochondria was much lower. In the case of oxoglutarate as a substrate, V_3 and the ACR and ADP/O ratios decreased considerably in the presence of high concentrations of tBHP (above 1 mM), whereas V_2 was not changed at the same oxidant concentration (Fig. 2a and b). The oxidative agent at the concentrations used (0.4–2 mM) did not change mitochondrial respiration when mitochondria used succinate as a substrate in EDTA-containing medium (data not shown). The respiratory parameters of mitochondria were more susceptible to the oxidative agent tBHP, when mitochondria consumed oxoglutarate in comparison with glutamate or succinate. Fig. 3 summarizes the effect of varying EDTA concentrations on the tBHP-induced oxidative impairment of ADP-stimulated mitochondrial respiration in the case of glutamate as a substrate. 1 mM EDTA, divalent ions chelator, completely prevented inhibition of respiration during exposure of mitochondria to tBHP, showing that endogenous Ca^{2+} ions (and ions, released from damaged mitochondria) participate in mitochondrial oxidative impairments.

At the next step of the work, we evaluated the role of exogenous Ca^{2+} ions in the oxidative damage of mitochondria. In the EDTA-free medium, exogenous Ca^{2+} ions at low concentrations of 4–30 μM dose-dependently inhibited the mitochondrial respiration V_3 (Fig. 4a) and decreased the ACR in the case of glutamate-energized mitochondria (Fig. 4b). In the case of succinate as a substrate, the effect of Ca^{2+} ions

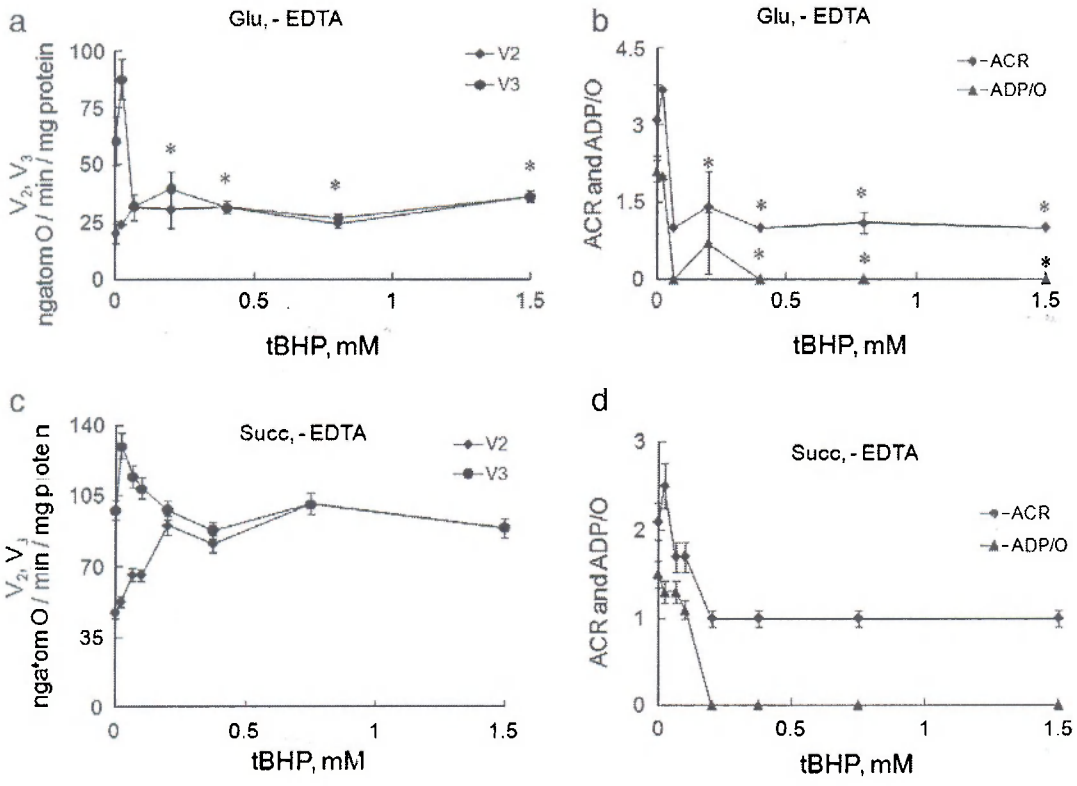

Fig. 1. Effect of organic hydroperoxide tBHP on respiratory parameters of isolated rat liver mitochondria in EDTA-free medium; respiratory substrates: glutamate (a, b) and succinate (c, d); substrate-dependent respiration rate V_2 and ADP-stimulated respiration rate V_3 (a, c) and acceptor control ratio ACR and phosphorylation coefficient ADP/O (b, d). 0.125 M KCl, 0.05 M sucrose, 0.01 M Tris–HCl, 0.0025 M KH_2PO_4, 0.005 M $MgSO_4$, pH 7.4, 25 °C, the time of preliminary exposure of mitochondria to tBHP was 2 min. Mitochondrial protein concentration was 1.0 mg/ml and respiratory substrate concentrations were 5 mM.

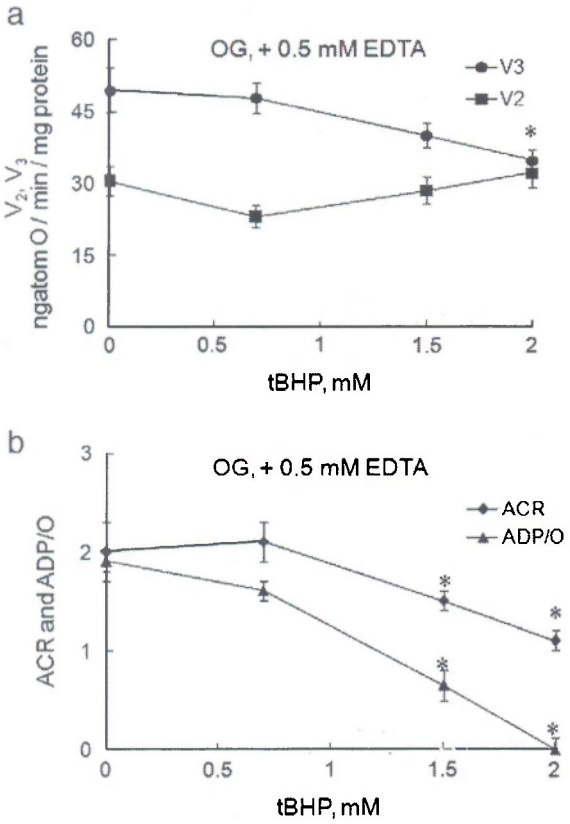

Fig. 2. Effect of organic hydroperoxide tBHP on respiratory parameters of isolated rat liver mitochondria in EDTA-containing medium: oxoglutarate-dependent respiration rate V_2 and ADP-stimulated respiration rate V_3 (a) and acceptor control ratio ACR and phosphorylation coefficient ADP/O (b). 0.125 M KCl, 0.05 M sucrose, 0.01 M Tris–HCl, 0.0025 M KH_2PO_4, 0.005 M $MgSO_4$, 0.0005 M EDTA, pH 7.4, 25 °C, the time of preliminary exposure of mitochondria to tBHP was 2 min. Mitochondrial protein concentration was 1.0 mg/ml.

on the respiratory parameters of mitochondria was lower in comparison with glutamate (data not shown). In the presence of EDTA (0.5 mM), Ca^{2+} ions did not change the respiratory parameters of mitochondria consuming succinate or glutamate (data not shown).

In the presence of low concentrations of tBHP (0.025 mM), the effect of Ca^{2+} ions on the mitochondrial respiration significantly enhanced (Fig. 5a and b). Low calcium concentrations (4–8 μM) decreased the respiration rate V_3 (Fig. 5a) and the coefficients ACR and ADP/O (the respiration rate V_2 did not change), when added to mitochondria in the presence of the oxidant in EDTA-free medium, reflecting uncoupling of the oxidation and phosphorylation in mitochondria and a decrease of the efficacy of the electron-transport chain (Fig. 5b). The susceptibility of mitochondrial respiratory parameters to the combined action of the oxidant and Ca^{2+} ions was higher when mitochondria consumed glutamate in comparison with succinate.

Membrane lipid peroxidation reduced glutathione level and enzyme activities in mitochondria: effects of tBHP, Ca^{2+} ions, EDTA and melatonin

tBHP (at high concentrations of 0.5–2 mM) induced considerable oxidation of mitochondrial reduced glutathione (Fig. 6a) and mixed glutathione–protein disulfide formation (Fig. 6b), mitochondrial membrane lipid peroxidation (Fig. 6c), SDH (complex II) and OGDH inactivations (Fig. 6d) during mitochondria exposure to the oxidant for 30 min at 25 °C in the presence of EDTA. We found that the inhibition of SDH correlated with the levels of membrane lipid peroxidation products ($r^2 = 0.72$, $p < 0.0005$) (Fig. 7a) and not with GSH depletion, and the OGDH inactivation during tBHP treatment correlated with mitochondrial GSH oxidation ($r^2 = 0.85$, $p < 0.0001$) (Fig. 7b).

Melatonin prevented mitochondrial oxidative damage, inhibiting membrane lipid peroxidation, GSH oxidation and GSSP formation, as well as complex II inactivation (Fig. 6a–d). The effect of melatonin depended on the melatonin/oxidative agent ratio. At the oxidant

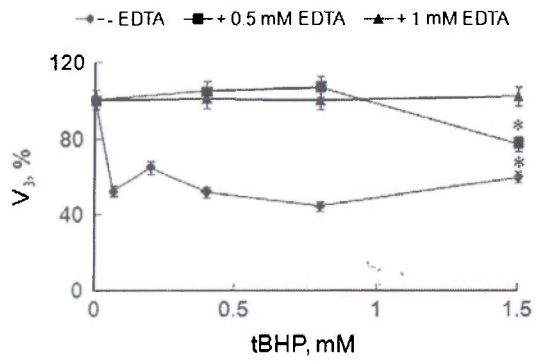

Fig. 3. Effect of organic hydroperoxide tBHP on ADP-stimulated respiration rate V_3 of rat liver mitochondria in the absence or in the presence of varying EDTA concentrations: 0, 0.5 mM and 1.0 mM. 0.125 M KCl, 0.05 M sucrose, 0.01 M Tris–HCl, 0.0025 M KH_2PO_4, 0.005 M $MgSO_4$, pH 7.4, 25 °C, the time of preliminary exposure of mitochondria to tBHP was 2 min. Mitochondrial protein concentration was 1.0 mg/ml and respiratory substrate (glutamate) concentration was 5 mM.

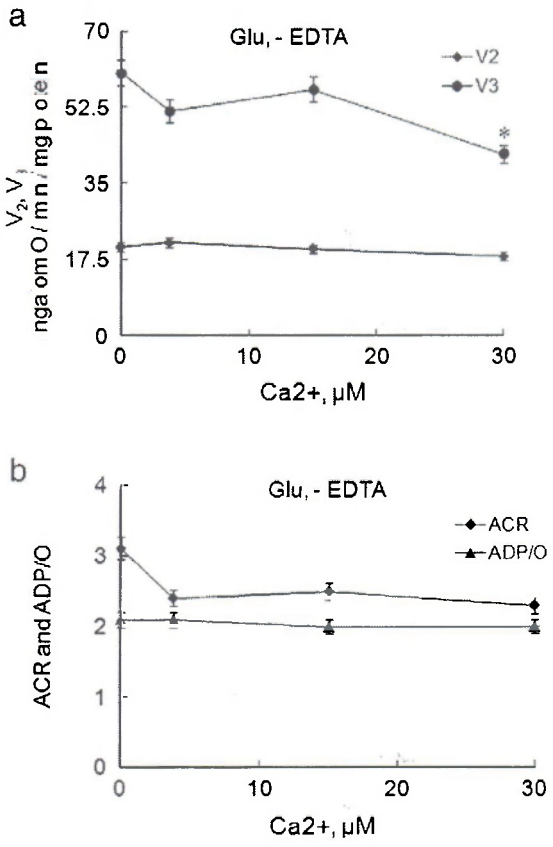

Fig. 4. Effect of Ca^{2+} ions on respiratory parameters of rat liver mitochondria in EDTA-free medium. Substrate-dependent respiration rate V_2 and ADP-stimulated respiration rate V_3 (a), and acceptor control ratio ACR and phosphorylation coefficient ADP/O (b). 0.125 M KCl, 0.05 M sucrose, 0.01 M Tris–HCl, 0.0025 M KH_2PO_4, 0.005 M $MgSO_4$, pH 7.4, 25 °C. Mitochondrial protein concentration was 1.0 mg/ml and respiratory substrate (glutamate) concentration was 5 mM.

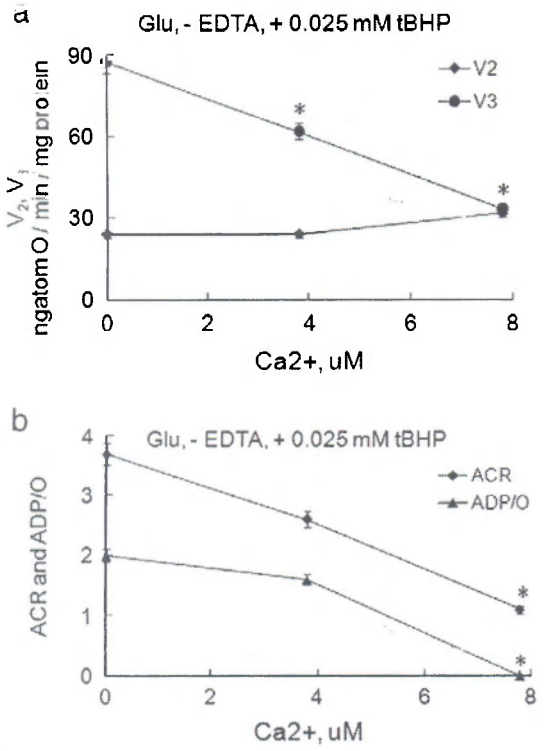

Fig. 5. Effect of Ca^{2+} ions on respiratory parameters of rat liver mitochondria in the presence of organic hydroperoxide tBHP (0.025 mM), EDTA-free medium: glutamate-dependent respiration rate V_2 and ADP-stimulated respiration rate V_3 (a) and acceptor control ratio ACR and phosphorylation coefficient ADP/O (b) 0.125 M KCl, 0.05 M sucrose, 0.01 M Tris–HCl, 0.0025 M KH_2PO_4, 0.005 M $MgSO_4$, pH 7.4, 25 °C, the time of preliminary exposure of mitochondria to tBHP was 2 min. Mitochondrial protein concentration was 1.0 mg/ml. Glutamate concentration was 5 mM.

concentration higher than that of melatonin no prevention of GSH oxidation or GSSP formation was observed.

Ca^{2+} ions (30–150 μM) alone neither induced oxidation of mitochondrial constituents (GSH or membrane lipids) nor influenced on the tBHP-induced oxidation of GSH and membrane lipids. At the same time 0.5–1 mM EDTA slightly prevented oxidative damage of mitochondria induced by tBHP. Addition of respiratory substrate (5 mM succinate) similarly diminished tBHP-induced oxidative damage of mitochondria (data not shown).

Discussion

The cellular bioenergetic machinery localized in mitochondria is a target of oxidative stress induced in cells and tissue under many pathological conditions (Duchen, 2004; Kroemer et al., 1998). Exposure of rat liver mitochondria to tBHP results in production of methyl-, tert butoxyl- and tert butylperoxyl-radicals due to direct reduction of chemical oxidant by either cytochrome c1, cytochrome c, or both of these of the respiratory chain components in mitochondria (Kennedy et al., 1992). It was shown in mitochondria that the tBHP-induced reaction was initiated by organic hydroperoxide homolytic cleavage by the complexes of cytochrome c/cardiolipin (Belikova et al., 2009). The same complexes of cytochrome c/cardiolipin catalyze the important reaction of heterolytic reduction of fatty acid hydroperoxides in mitochondria (Belikova et al., 2009). On the other hand, the reductive metabolism of peroxide by glutathione peroxidase/reductase induces an oxidative shift in redox state of mitochondria (Greco and Fiskum, 2010).

In the present work, we showed that tBHP considerably impaired respiratory and phosphorylation functions of mitochondria due to uncoupling of phosphorylation and respiration processes and inhibition of the OGDH and the complexes of electron-transport chain in the presence of all the substrates used (oxoglutarate, glutamate, succinate). The susceptibility of mitochondrial function to the oxidant during consumption of different substrates increased in the following order: succinate < oxoglutarate < glutamate. The divalent ions chelator EDTA (0.5–1.0 mM) considerably prevented oxidative impairment of mitochondrial respiration, and Ca^{2+} ions in the medium appreciably enhanced oxidant-induced mitochondrial damage. At high concentrations of 0.5–2 mM, tBHP oxidized mitochondrial reduced glutathione and induced membrane lipid peroxidation and mixed protein–glutathione disulfide formations (mitochondrial protein glutathionylation) in the presence of EDTA. It should be noted that in the presence of 0.5 mM EDTA and at high concentrations of tBHP (2 mM), about 40% of SDH activity were blocked, unaffecting the respiration of mitochondria consuming succinate but 35% of OGDH activity reduction (at concentrations of tBHP, 2 mM) were accompanied by a considerable inhibition of mitochondrial respiration (by 30%) and a decline of the phosphorylation coefficient ADP/O to zero in the case of oxoglutarate as a substrate. At 1 mM tBHP, we observed the decrease of OGDH activity by 15% without alterations in the mitochondrial respiration, but further 20% activity reduction (at 2 mM tBHP) led to 30% decrease of respiration (ADP/O decreased by 100%), pointing to a strong dependence of respiration on the OGDH activity launched after the 15% activity threshold. Similarly, the respiration of oxoglutarate-oxidizing mitochondria also strongly depended on NADH dehydrogenase activity (complex I) (Drahota et al, 2005). The OGDH inhibition correlated with the disturbed mitochondrial redox balance (reduced level of intramitochondrial GSH level and increased level of protein glutathionylation). The OGDH activity has already been demonstrated to be modulated through enzymatic glutathionylation and deglutathionylation and this inactivation does not appear to involve oxygen radicals formed upon metal-catalyzed oxidation (Nulton-Persson et al., 2003). Recently it has been shown that redox-triggered mitochondrial protein glutathionylation is the main event of cell redox-signaling (Chen et al., 2007). One of the possible reasons of SDH inactivation might be a disturbance of protein–lipid interactions of transmembrane hydrophobic domain of SDH and therefore destroying compact protein structure as a result of enhanced level of membrane lipid peroxidation. We observed that the respiratory substrates inhibited oxidative processes in mitochondria. At the same time the addition of respiratory substrates results in a considerable increase of methyl-radical production during mitochondria exposure to tBHP (Kennedy et al., 1992).

Endlicher et al. (2009) have also shown that tBHP-induced oxidative damage of rat liver mitochondria is respiratory substrate-dependent. tBHP, when incubated with mitochondria, completely inhibited oxoglutarate dehydrogenase and induced nonenzymatic oxidation of oxoglutarate to succinate (Fedotcheva et al., 2006). At the same time the authors showed that the strong oxidant tBHP did not impair mitochondria respiration when glutamate and malate were used as substrates, because upon tBHP-induced oxidative stress nonenzymatic decarboxylation and transamination of oxoglutarate switch tricarboxylic acid cycle to the formation and oxidation of succinate (Fedotcheva et al., 2006).

In our experiments, exogenous Ca^{2+} alone in EDTA-free medium (but not in the presence of EDTA) impaired glutamate-dependent respiration of mitochondria in state 3, neither affecting the coefficient ADP/O nor inducing significant mitochondrial membrane lipid peroxidation. It is well-known that Ca^{2+} ion overload significantly affects mitochondrial physiological state (Lemasters et al., 2009). In the case of rat brain mitochondria, it was shown the Ca^{2+}-dependent induction of MPT was due to oxidative stress induced by the interaction of Ca^{2+} with the mitochondrial membrane, leading to production of hydrogen peroxide and, subsequently, oxidation of sulfhydryl groups, in particular those of two critical cysteines, most probably located on

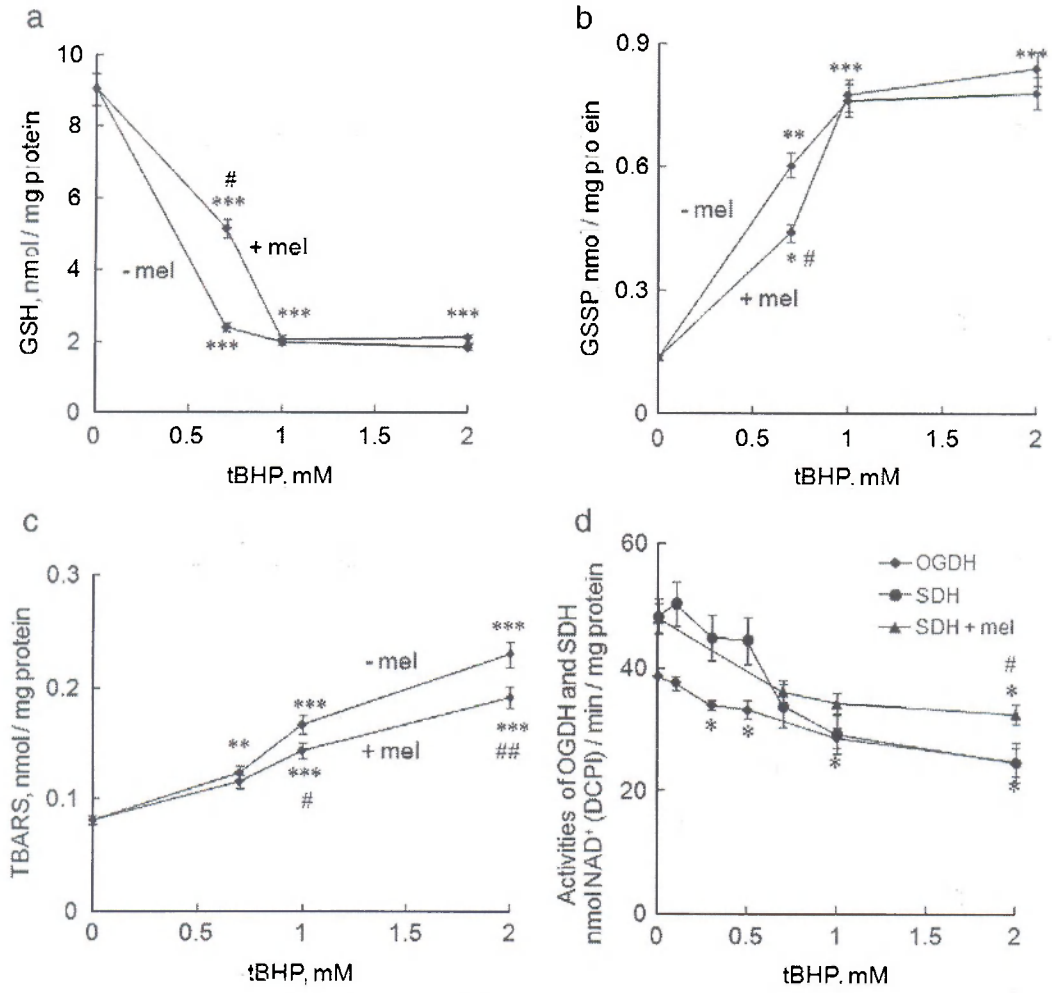

Fig. 6. Oxidative processes in isolated rat liver mitochondria during exposure to tBHP and protection by melatonin (1 mM): a) oxidation of reduced glutathione; b) mixed protein–glutathione disulfide formation; c) formation of membrane lipid peroxidation products (TBARS); d) OGDH and SDH inactivation. Mitochondria were incubated with tBHP for 30 min and preincubated with melatonin for 20 min (0.15 M KCl, 0.02 M KH_2PO_4, 0.0005 M EDTA, pH 7.4, 25 °C).

adenine nucleotide translocase, and also oxidation of pyridine nucleotides, resulting in transition pore opening and consequent bioenergetic collapse (Battaglia et al., 2010). Typically the effect of many chemicals and oxidants that promote MPT is to decrease the threshold amount of Ca^{2+} needed to cause MPT pore opening (Lemasters et al., 2009; Bernardi et al., 2006).

In our experiments in vitro, we demonstrated that melatonin inhibited peroxidative damage of mitochondria, preventing GSH oxidation, membrane lipid peroxidation, protein glutathionylation and SDH inactivation. As was shown, micromolar concentrations of melatonin inhibited tBHP-induced peroxidation of cardiolipin, associated with an increased sensitivity of mitochondria to Ca^{2+}-induced MPT and with the release of cytochrome c from the mitochondria (Petrosillo et al., 2009).

In our work, Ca^{2+} ions highly potentiate impairment of mitochondrial respiration during tBHP-induced oxidative stress. We suggest that Ca^{2+} efflux from damaged mitochondria into the mitochondrial suspension (or into the cell) during oxidative stress significantly influenced on the course of oxidative impairments of the rest of mitochondria. The synergistic effect of Ca^{2+} ions and the oxidant allows to suggest that Ca^{2+} is a mediator of mitochondria oxidative injury. We interpret our data to indicate that Ca^{2+} ions promote tBHP-induced mitochondrial damage due to the known Ca^{2+}-dependent events: uncoupling, collapse of the membrane potential, mitochondria swelling (Lapidus and Sokolove, 1994) rather than due to mitochondrial GSH or membrane lipid oxidation.

Conclusions

tBHP considerably impaired the respiratory and phosphorylation functions of mitochondria and this effect depended on the respiratory substrate used. The presence of EDTA prevented oxidative impairment of mitochondrial respiration, and Ca^{2+} ions in the medium enhanced considerably oxidant-induced mitochondrial damage. Ca^{2+} alone (4–30 μM) in the EDTA-free medium impaired the mitochondrial respiratory parameters: the oxygen consumption rate V_3 and ACR but unaffected the coefficient ADP/O. The present study provides evidence that tBHP induced uncoupling of the oxidation and phosphorylation processes and decreased the efficiency of the phosphorylation reaction, and led to a direct oxidative modification of enzymatic complexes of the respiratory chain and mitochondrial matrix as a result of the following events: oxidation of mitochondrial reduced glutathione, protein glutathionylation, enhanced accumulation of products of membranous lipid peroxidation, and Ca^{2+} ion overload. In our experiments in vitro, we demonstrated that melatonin inhibited the peroxidative damage of mitochondria, preventing mitochondrial redox-balance changes and succinate dehydrogenase inactivation.

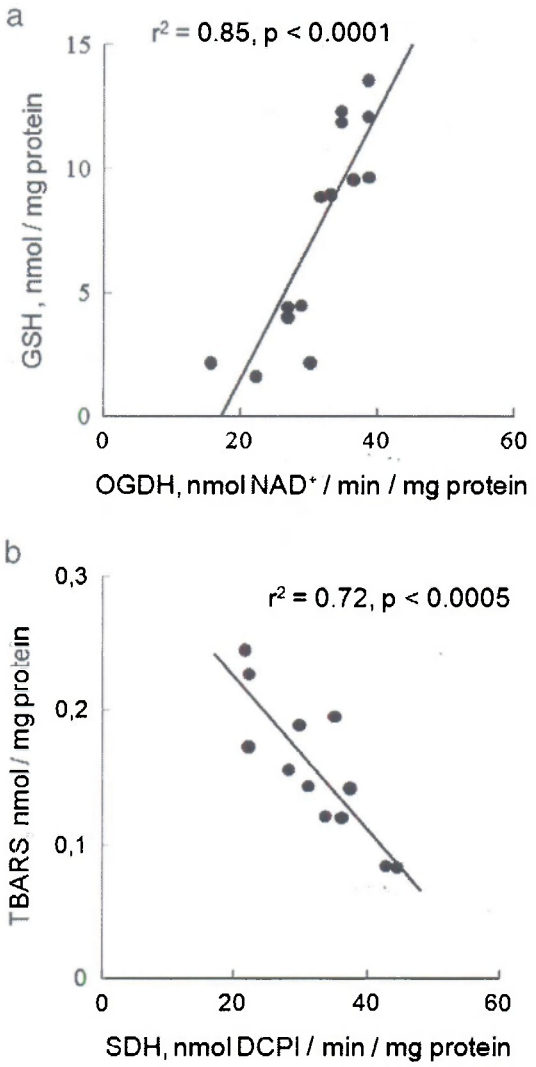

Fig. 7. Relationships between mitochondrial GSH level and OGDH activity (a) and between accumulation of mitochondrial membrane lipid peroxidation products and SDH activity (b).

Conflict of interest statement

The authors declare that there is no conflict of interest.

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