

Peroxidative Damage of Mitochondrial Respiration is Substrate-Dependent

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Summary

The concentration-dependence of *tert*-butyl hydroperoxide (BHP) inhibitory effect on oxygen consumption in isolated rat liver mitochondria was measured in the presence of various respiratory substrates. Strong inhibitory effect at low concentrations of BHP (15-30 μ M) was found for oxoglutarate and palmitoyl carnitine oxidation. Pyruvate and glutamate oxidation was inhibited at higher concentrations of BHP (100-200 μ M). Succinate oxidation was not affected even at 3.3 mM BHP. Determination of mitochondrial membrane potential has shown that in the presence of NADH-dependent substrates the membrane potential was dissipated by BHP but was completely restored after addition of succinate. Our data thus indicate that beside peroxidative damage of complex I also various mitochondrial NADH-dependent dehydrogenases are inhibited, but to a different extent and with different kinetics. Our data also show that succinate could be an important nutritional substrate protecting hepatocytes during peroxidative damage.

Key words

Liver mitochondria • Mitochondrial enzymes • Peroxidative damage • *Tert*-butyl hydroperoxide

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Introduction

It is now commonly accepted that peroxidative damage of cell components is involved in the action of most hepatotoxic drugs (Jaeschke *et al.* 2002) and that the cell energy provision system localized in mitochondria is not only an important generator of oxygen radicals but also an important target of their action (Boveris and Chance 1973, Turrens 2003).

Therefore for the treatment of damaged liver tissue and for protection of the regeneration process more data on the mechanisms through which the oxidative damage proceeds are necessary. There are many data in the literature describing peroxidative damage of particular enzyme reactions occurring in liver mitochondria (Kennedy *et al.* 1992, Kowaltowski and Vercesi 1999, Nulton-Presson and Szveda 2001, Nieminen *et al.* 1997, Kmoníčková *et al.* 2001, Křiváková *et al.* 2007). However, there are not sufficient data comparing and evaluating the sensitivity of various enzyme reactions participating in the mitochondrial energy transformation process to peroxidative damage.

In our previous communication we presented data showing that there is a different sensitivity of NADH and flavoprotein-dependent substrate to peroxidative damage. Oxidation of palmitoyl carnitine by rat liver mitochondria is more sensitive than oxidation of succinate (Červinková *et al.* 2008). This finding could be explained by high sensitivity of complex I to oxidative stress. To obtain more information we tested in this communication various NADH-dependent substrates and

evaluated in more detail the concentration-dependence of the prooxidant inhibitory effect. As prooxidant we used *tert*-butyl hydroperoxide (BHP) because it is not degraded by mitochondrial catalase as hydrogen peroxide (Chance *et al.* 1979) and thus its concentration-dependence on the activity of particular mitochondrial enzymes can be better evaluated.

Materials and Methods

Chemicals

Tert-butyl hydroperoxide, tetraphenylphosphonium (TPP⁺), substrates of respiratory chain (pyruvate, glutamate, malate, succinate, palmitoyl carnitine and oxoglutarate), inhibitors of respiratory chain (rotenone and carbonylcyanide-*p*-trifluoromethoxy-phenylhydrazone – FCCP) and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). Safranin was purchased from Riedel-deHaën (Germany).

Isolation of rat liver mitochondria

Male Wistar rats (Biotest Konarovice, Czech Republic) with body weight of 220-250 g were used. The animals had free access to standard laboratory diet (DOS 2B Velaz, Czech Republic) and tap water. All animals received care according to the guidelines set out by the Institutional Animal Use and Care Committee of Charles University, Prague, Czech Republic. Liver mitochondria were isolated by differential centrifugation as described by Bustamante *et al.* (1977) in a medium containing 220 mM mannitol, 70 mM sucrose, 2 mM HEPES, 0.5 mg/ml bovine serum albumin, pH 7.2. Tissue homogenate was centrifuged for 10 min at 800 x g, and supernatant was centrifuged 10 min at 8000 x g. Sedimented mitochondria were washed twice by centrifugation for 10 min at 10000 x g in the isolation medium without bovine serum albumin (BSA) and final sediment was also resuspended in the isolation medium without BSA.

Mitochondrial oxygen consumption

Oxygen consumption by rat liver mitochondria was measured at 30 °C with a High Resolution Oxygraph2K (Oroboros, Austria) in 2 ml of incubation medium containing 80 mM KCl, 10 mM Tris-HCl, 5 mM K-phosphate, 3 mM MgCl₂, 1 mM EDTA, pH 7.3. The oxygraphic curves presented are the first negative derivatives of oxygen tension changes in the incubation medium (Gnaiger *et al.* 1995). For calculation of oxygen uptake rates and for presentation of oxygraphic curves

Oroboros software Datlab 4.1 was used. Oxygen uptake is expressed as pmole oxygen/s/mg protein.

Measurement of mitochondrial membrane potential by safranin O accumulation

The mitochondrial membrane potential ($\Delta\psi$) was detected according to Wasilewski *et al.* (2004) using the cationic dye safranin O. Safranin O uptake by mitochondria (Akerman and Wikstrom 1976) was determined from fluorescence quenching monitored at the wavelengths of 495 nm (excitation) and 586 nm (emission) with AMINCO-Bowman Series 2 Luminescence Spectrometer. Measurement was performed in 1 ml of a medium containing 120 mM KCl, 10 mM Tris-HCl, 0.5 mM EGTA and 1 mM K-phosphate.

Determination of mitochondrial membrane potential by TPP⁺-sensitive electrode

Membrane potential of rat liver mitochondria was measured as tetraphenylphosphonium (TPP⁺) concentration changes in the incubation chamber (Labajová *et al.* 2006a). The decrease in TPP⁺ concentration indicated TPP⁺ uptake by the energized mitochondria. The TPP⁺ electrode was connected to a high impedance measurement card PCI-6036E (National Instruments USA) and the Ag/AgCl electrode was used as reference electrode. Signal acquisition processing and display were carried out by MATLAB/Simulink software (The Math Works, Inc., USA). The TPP⁺-sensitive membrane was prepared according to Labajová *et al.* (2006a). Measurements were performed at room temperature in 2 ml of the medium used for oxygen consumption measurements described above. Before each measurement the electrode was calibrated by stepwise addition of TPP⁺ (1-6 μ M). The membrane potential was expressed as logarithm of μ M TPP⁺ concentration (pTPP). The corresponding values of membrane potential ($\Delta\psi$) were corrected for nonspecific TPP⁺ binding as described by Zolkiewska *et al.* (1989).

Determination of proteins

Protein content was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Results

Figure 1 demonstrates experimental conditions for evaluation of the inhibitory effect of BHP on

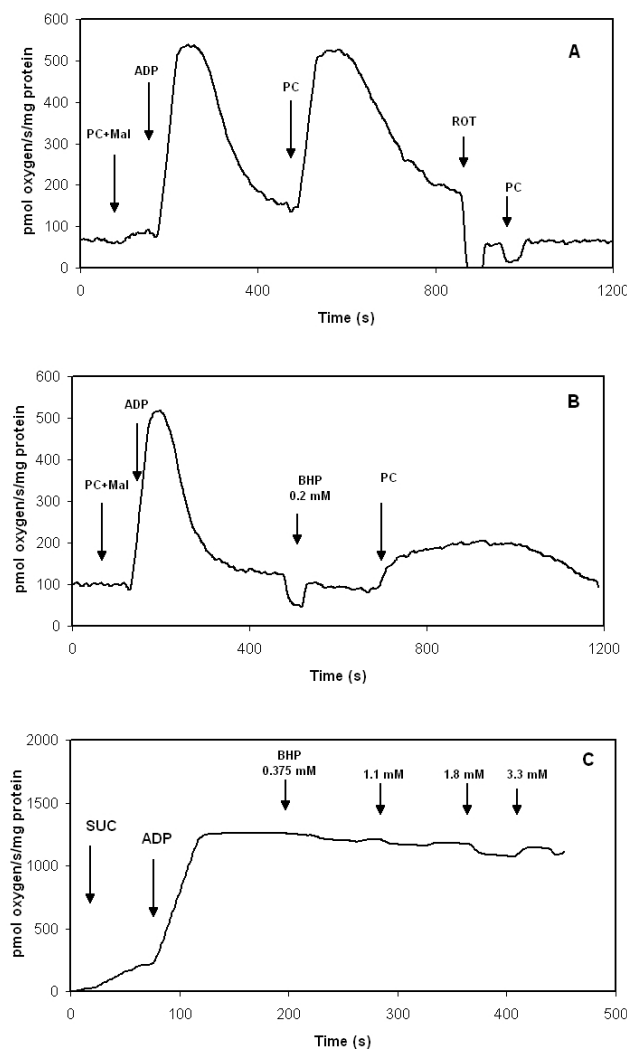


Fig.1. Experimental conditions for detection of the inhibitory effect of BHP. **A:** Oxidation of palmitoyl carnitine by rat liver mitochondria. **B:** Inhibition of palmitoyl carnitine oxidation by BHP. **C:** Inhibition of succinate oxidation by BHP. Rat liver mitochondria (0.2 mg protein/ml) were incubated in 2 ml of KCl medium. Where indicated 1.5 mM malate (MAL), 12.5 μ M palmitoyl carnitine (PC), 1.5 mM ADP, 10 mM succinate (SUC), 2 μ M rotenone (ROT) and BHP were added. The experiment was performed five times with different mitochondrial preparations with the same results.

oxidation of various substrates by rat liver mitochondria. Because higher concentrations of palmitoyl carnitine may act as detergents on mitochondrial membranes, we used low, micromolar concentrations of palmitoyl carnitine that already activate respiration. Under these conditions the added palmitoyl carnitine is quickly oxidized and another addition of palmitoyl carnitine induces the same response (Fig. 1A). The advantage of this arrangement is that after the first addition of palmitoyl carnitine various concentrations of BHP can be added and the maximum rate of palmitoyl carnitine oxidation as the total amount of oxygen consumed before and after addition of the

Table 1. Reference values for respiration rates of various substrates by rat liver mitochondria.

pmol oxygen/s/mg protein	
<i>Palmitoyl carnitine</i> (10 μ M)	
+ malate (3 mM)	118.9 \pm 20.5
+ ADP (1.5 mM)	773.7 \pm 64.9
RCI	6.5 \pm 1.3
<i>Succinate</i> (10 mM)	
+ ADP (1.5 mM)	1543.2 \pm 387.1
RCI	6.3 \pm 1.6
<i>Pyruvate</i> (10 mM)	
+ malate (1.5 mM)	89.2 \pm 16.8
+ ADP (1.5 mM)	1371.3 \pm 98.1
RCI	15.4 \pm 2.8
<i>Glutamate</i> (10 mM)	
+ malate (1.5 mM)	108.4 \pm 42.8
+ ADP (1.5 mM)	1300.2 \pm 324.6
RCI	12.0 \pm 3.6
<i>Oxoglutarate</i> (10 mM)	
+ malate (1.5 mM)	80.2 \pm 19.3
+ ADP (1.5 mM)	770.3 \pm 139.8
RCI	9.6 \pm 1.6

Data are mean \pm SEM from 8 preparations of rat liver mitochondria (0.2 mg protein/ml) for palmitoyl carnitine and succinate oxidation and from 4 preparations for pyruvate + malate, glutamate + malate and oxoglutarate + malate, respectively. RCI means respiratory control index.

inhibitor may be calculated (Fig. 1B).

The effect of BHP on oxidation of other respiratory substrates (succinate, pyruvate with malate, oxoglutarate with malate and glutamate with malate) was determined as shown for succinate (Fig. 1C). The data in Figures 1B and 1C confirmed our previous findings (Červinková *et al.* 2008) that succinate oxidation is not affected by the concentration of BHP that strongly inhibits oxidation of palmitoyl carnitine. It is evident (Fig. 1B) that the rate of palmitoyl carnitine oxidation was highly depressed (62 % inhibition). However, the total amount of oxygen consumed during palmitoyl carnitine oxidation decreased in the presence of BHP only by 27 %, from 44.5 μ mole oxygen per added palmitoyl carnitine to 37.2 μ mole, respectively. These data indicate that almost all added palmitoyl carnitine was oxidized, but at a lower rate. The time required for this oxidation increased approximately twice (Fig. 1B).

In Table 1 reference values for oxidation rates of palmitoyl carnitine, succinate, pyruvate with malate, and

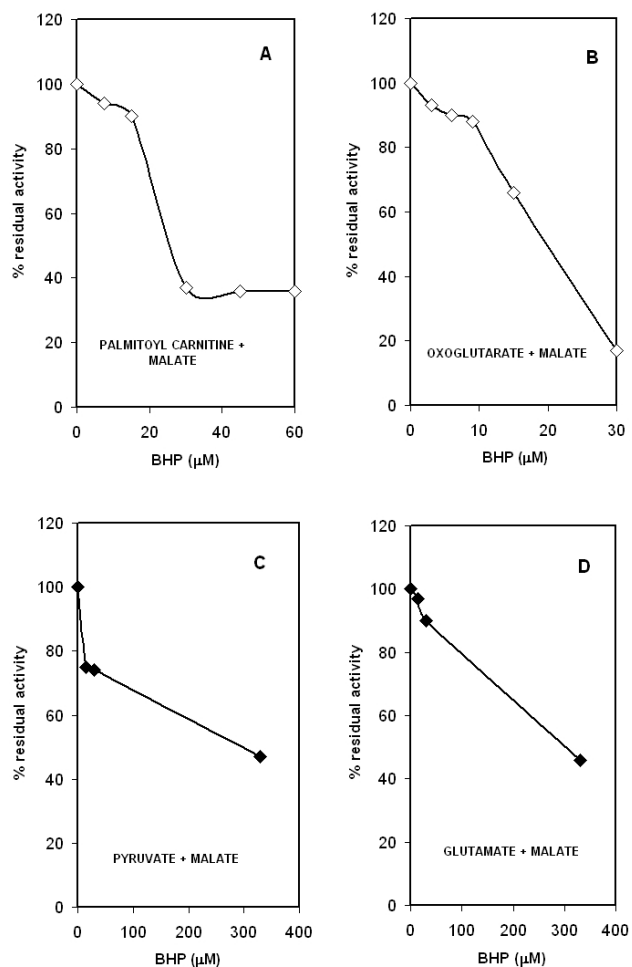


Fig. 2. Concentration dependence of the inhibitory effect of BHP on palmitoyl carnitine + malate (A), oxoglutarate + malate (B), pyruvate + malate (C) and glutamate + malate (D) oxidation by rat liver mitochondria (0.2 mg protein/ml). Inhibition of palmitoyl carnitine oxidation by BHP was measured as described in Fig. 1A and B. The residual activity after addition of increased BHP concentration is expressed in % of the rate obtained before addition of BHP. Inhibitory effect of BHP on oxoglutarate (10 mM) + malate (1.5 mM), pyruvate (10 mM) + malate (1.5 mM) and glutamate (10mM) + malate (1.5 mM) oxidation was detected under the conditions described for succinate oxidation in Fig. 1C.

oxoglutarate with malate and glutamate with malate by rat liver mitochondria are presented. Figure 2 shows the concentration-dependence of the BHP inhibitory effect for various substrates. Oxidation of palmitoyl carnitine (Fig. 2A) and oxoglutarate (Fig. 2B) was strongly inhibited in the concentration range between 15 and 30 μM BHP. Pyruvate- and glutamate-dependent oxidation was inhibited by about 3-fold higher concentration of BHP and also the kinetics of the decrease of the oxidation rate was slower (Figs 2C and 2D). Succinate oxidation was not affected even at 3.3 mM concentration of BHP (Fig. 1C).

For better comparison of those inhibitory effects

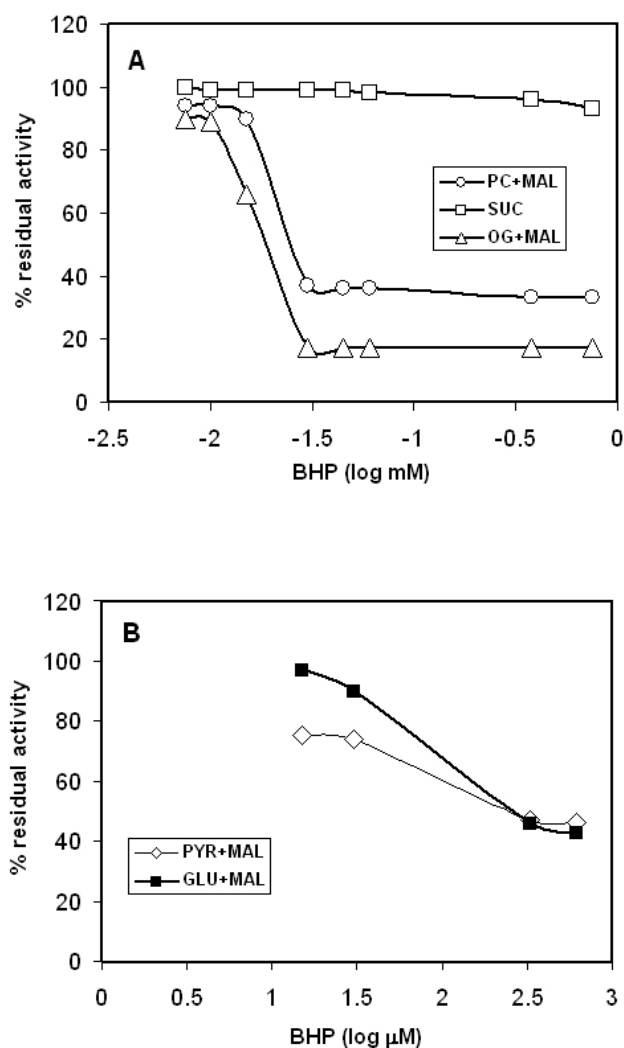


Fig. 3. Concentration-dependence of the inhibitory effect of BHP on oxoglutarate (OG) + malate (MAL), palmitoyl carnitine (PC) + malate and succinate (SUC) oxidation (A) and pyruvate (PYR) + malate and glutamate (GLU) + malate oxidation (B) by rat liver mitochondria (0.2 mg protein/ml). Data from Fig. 1 and 2 are compared and the concentration of BHP is expressed as log μM concentration.

we expressed the BHP concentration in logarithmic scale (Fig. 3). It is evident that all NADH-dependent substrates tested are more sensitive to peroxidative damage than succinate and that pyruvate and glutamate oxidation is inhibited at higher BHP concentration than oxidation of palmitoyl carnitine and oxoglutarate.

In further experiments we confirmed our findings by determination of mitochondrial membrane potential changes induced by BHP in the presence of various substrates. We used two independent methods: a) fluorometric determination of mitochondrial membrane potential by safranin O, and b) TPP⁺-sensitive electrode. We found good correlation between changes of respiration and changes of membrane potential induced by BHP. Figure 4 shows rapid discharge of the

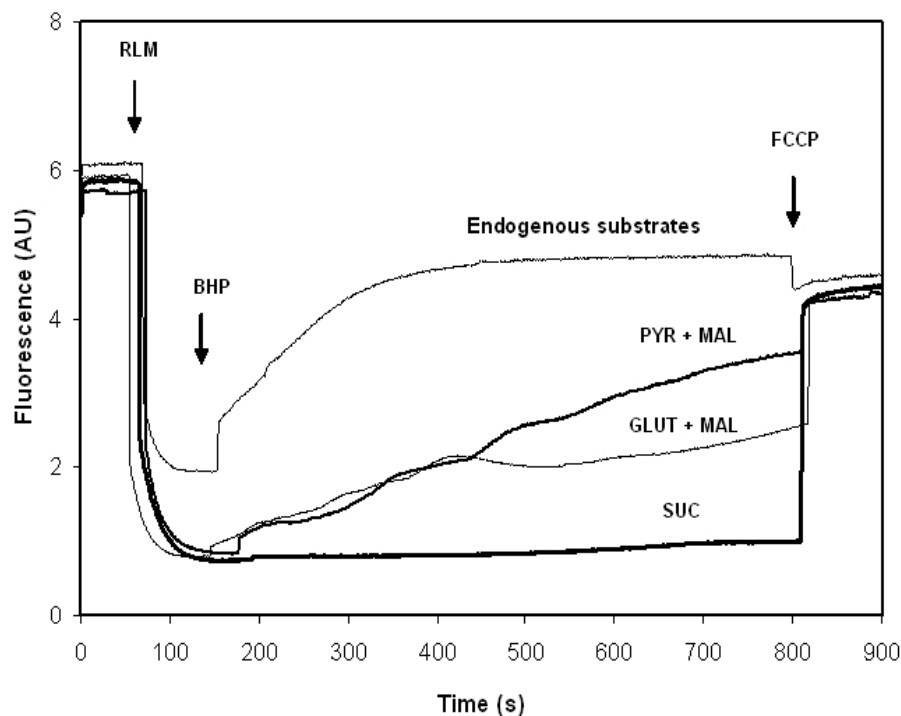


Fig. 4. Changes of mitochondrial membrane potential detected from fluorescence quenching of safranine O. A decrease of the fluorescence corresponds to an increase of mitochondrial membrane potential. The medium contained safranine O (10 μ M) only or safranine O and various mitochondrial substrates – 10 mM succinate (SUC), 10 mM glutamate + 1.5 mM malate (GLUT+MAL) or 10 mM pyruvate + 1.5 mM malate (PYR+MAL). The arrows indicate addition of 0.1 mg/ml of isolated rat liver mitochondria (RLM), BHP (1.5 mM) and 1 μ M FCCP. The experiment was performed three times with different mitochondrial preparations yielding the same results.

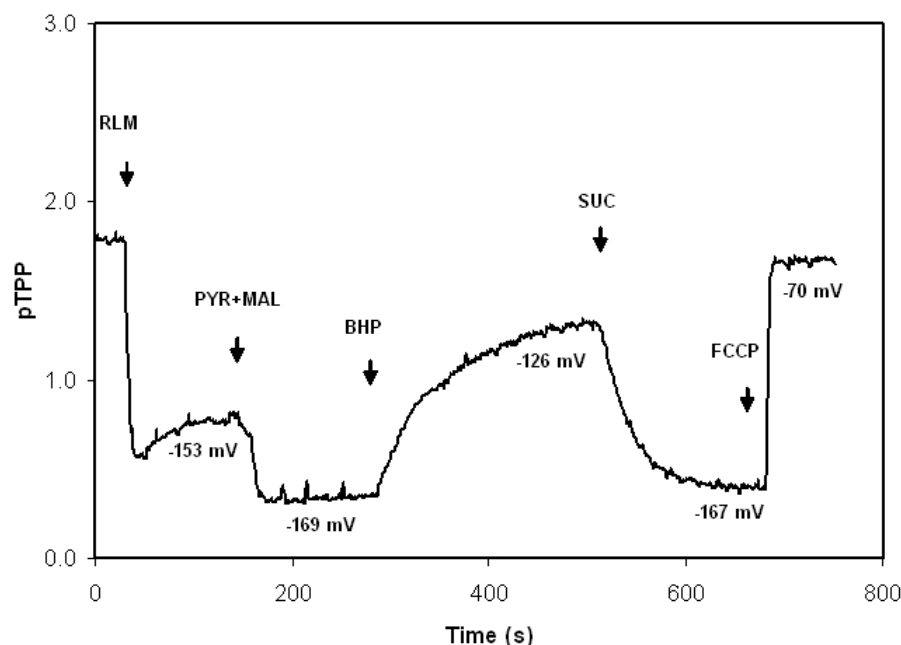


Fig 5. Changes of mitochondrial membrane potential detected as TPP⁺ concentration changes by the TPP⁺-sensitive electrode. Changes of the mitochondrial membrane potential are expressed as logarithm of TPP⁺ μ M concentration (pTPP) induced by addition of various respiratory substrates and BHP to mitochondrial suspension. The arrows indicate addition of 0.5 mg/ml of isolated rat liver mitochondria (RLM), 10 mM pyruvate + 1.5 mM malate (PYR+MAL), 1.5 mM BHP, 10 mM succinate (SUC), and 1 μ M FCCP. The corresponding values of mitochondrial membrane potential after particular additions are shown below the curve. The experiment was performed three times with different mitochondrial preparations yielding the same results.

mitochondrial membrane potential induced by BHP when mitochondria utilize endogenous substrates or pyruvate with malate or glutamate with malate. In contrast, when succinate was used as respiratory substrate, BHP even at a higher concentration (3.3 mM) had no effect on mitochondrial membrane potential and its value was maintained during the whole period tested. Similarly when a TPP⁺-sensitive electrode was used for determination of mitochondrial membrane potential in the presence of pyruvate and malate, membrane potential was

discharged by BHP, but after addition of succinate it was again fully restored (Fig. 5).

Discussion

In our previous communication we found that oxidation of palmitoyl carnitine and glutamate with malate by digitonin-permeabilized hepatocytes is more sensitive to peroxidative damage by BHP than oxidation of succinate (Drahota *et al.* 2005). At higher

concentrations of BHP also succinate oxidation was partially inhibited and mitochondrial potential gradually decreased, but, contrary to palmitoyl carnitine-dependent respiration, at least 50 % of oxidative and phosphorylative capacity remained active (Křiváková *et al.* 2007) and mitochondrial membrane potential supported by NADH-dependent substrates could be recovered by addition of succinate (Lábajová *et al.* 2006b). Because the cellular system of energy provision is a target of many toxic substances and oxidative stress is one of the most important mechanisms through which toxic factors induce apoptotic and necrotic processes (Kroemer *et al.* 1998), it was necessary to obtain more information to what extent the oxidation of various respiratory substrates is resistant to peroxidative damage. Especially in liver cells more information is required for effective protection against peroxidative damage and for optimum supply of nutritional substrates during the regeneration process because liver cells are highly exposed to oxidative stress even in a nonpathological state as a site of biotransformation of various toxic compounds continuously delivered by the portal vein.

We have therefore studied the inhibitory effect of peroxidative damage on the oxidation of palmitoyl carnitine and succinate using isolated rat liver mitochondria (Červinková *et al.* 2008) and we confirmed our previous data obtained on isolated hepatocytes (Drahota *et al.* 2005). This finding also showed that oxidation of palmitoyl carnitine is more sensitive to peroxidative damage than that of succinate. They also indicated that besides inhibition of complex I also inhibition of various mitochondrial dehydrogenases could be involved. Therefore, in this communication we studied this problem in more detail. We tested the concentration dependence of the inhibitory effect BHP on oxidation of various NADH-dependent substrates. Our data showed that, in spite of the fact, that oxidation of all NADH-dependent substrates tested was more sensitive to peroxidative damage than that of succinate, big differences in the sensitivity to peroxidative damage between various NADH-dependent substrates were found (Figs. 2 and 3). We can thus extend our previous proposal that complex I is responsible for inhibition of oxidation of NADH-dependent substrates. Our data presented in this communication showed that besides complex I also the activity of particular dehydrogenases participates in the inhibitory effect.

We found two groups of NADH-dependent

substrates: (a) oxoglutarate and palmitoyl carnitine with high sensitivity and (b) pyruvate and glutamate with lower sensitivity to peroxidative damage (Fig. 3). Differences were detected not only in the sensitivity to BHP but also in the kinetics of the inhibitory effect. Whereas for oxoglutarate and palmitoyl carnitine-dependent oxidation, the maximum inhibitory effect was detected in a relatively narrow concentration range, the inhibition of pyruvate- and glutamate-dependent oxidation occurred in a wider concentration range (Fig. 3).

Measurements of mitochondrial membrane potential have shown that in the absence of Ca^{2+} ions (in the presence of EDTA) the nonspecific mitochondrial membrane permeability transition pore (MPTP) remains closed even at relatively high concentrations of BHP (1.5-3 mM). This also indicates that dissipation of the membrane potential induced by these high concentrations of BHP in the presence of NADH-dependent substrates is due to inhibition of their oxidation (Fig. 4).

Measurements of the membrane potential are in agreement with findings obtained by oxygraphic measurements. Our results showed that membrane potential induced by NADH-dependent substrates, when dissipated by BHP, can be completely recovered after addition of succinate (Fig. 5).

High resistance of succinate oxidation to peroxidative damage and its ability to recover mitochondrial membrane potential dissipated by peroxidative damage when NADH-dependent substrates are oxidized could explain the data appearing in literature that describe positive effect of succinate application *in vivo* in some pathological situations (Chen *et al.* 2003). This effect could be explained by the findings of Fedotcheva *et al.* (2006) and Brooks *et al.* (2006) who observed that upon inhibition of oxoglutarate dehydrogenase by BHP oxoglutarate could be nonenzymatically converted to succinate. By this mechanism the operation of Krebs cycle can be maintained even under conditions of oxidative stress when oxoglutarate dehydrogenase is inhibited.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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