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Tert-Butyl Hydroperoxide Selectively Inhibits Mitochondrial Respiratory-Chain Enzymes in Isolated Rat Hepatocytes

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Summary

Sensitivity of various mitochondrial enzymes to oxidative damage was tested on isolated rat liver hepatocytes permeabilized by digitonin. In permeabilized hepatocytes normal respiratory control values were obtained and mitochondrial membranes remained intact. Respiratory rates of NADH-dependent (glutamate+malate, palmitylcarnitine + malate) and flavoprotein-dependent (succinate) substrates were determined in hepatocytes exposed for 5 min to 0.5-3 mM *tert*-butyl hydroperoxide before addition of digitonin. Our data showed that oxidation of NADH-dependent substrates is much more sensitive to oxidative stress than oxidation of flavoprotein-dependent ones, evidently due to the modification of iron-sulfur clusters or SH groups in the NADH dehydrogenase enzyme complex (Complex I).

Key words

Hepatocytes • Mitochondrial enzymes • *Tert*-butyl hydroperoxide

Introduction

The cellular system of energy provision localized in mitochondria is a target of many hepatotoxic substances and oxidative stress is one of the most important mechanisms through which hepatotoxic factors induce apoptotic and necrotic processes (Kroemer *et al.* 1998, Pedersen 1999, Quian *et al.* 1999, Ferenčíková *et al.* 2003, Uličná *et al.* 2003, Kucharská *et al.* 2004). Effective protection of cellular damage induced by oxidative stress requires more information about reactions involved in this process. Therefore various prooxidants such as hydrogen peroxides (Masaki *et al.* 1989, Nieminen *et al.* 1997) redox cycling xenobiotics (Orrenius *et al.* 1990) and anoxia reoxidation injury (Nazareth *et al.* 1991) were used in these studies. As prooxidant, *tert*-butyl hydroperoxide (*t*-BHP) was widely used and many effects on cell metabolism have been described, e.g. changes in calcium homeostasis (Nicotera *et al.* 1988), increase of lipid peroxidation or decrease of mitochondrial membrane potential (Rubin and Farber 1984, Kmoníčková *et al.* 2001). Two mechanisms for *t*-BHP action were proposed: depletion of cellular stores of GSH and oxidation of functionally important SH groups on mitochondrial enzymes (Masaki *et al.* 1989), and/or changes of mitochondrial membrane integrity induced by peroxidation of membrane lipids (Rubin and Farber 1984, Kmoníčková *et al.* 2001). Recent studies

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have indicated that besides modification of SH groups, oxidative modification of iron-sulfur clusters in various enzyme complexes is also involved (Genova *et al.* 2001).

Experiments on isolated hepatocytes are thus a useful model system for evaluation of the toxic effect of various prooxidants which act directly on mitochondrial enzymes. Hepatocytes permeabilized by digitonin can be used for evaluation of changes in the activity of mitochondrial enzymes better than isolated mitochondria because under controlled conditions the plasma membrane becomes fully permeabilized, but the cytoskeleton and cellular organelles remain intact (Katz and Wals 1985, Adams *et al.* 1988, Chowdhury *et al.* 2000). Mitochondrial interactions with other cell organelles and structures such as lysosomes, endoplasmic reticulum and microtubule or actin filaments (Frey and Mannella 2000, Garesse and Vallejo 2001) might also be essential for correct function of the whole mitochondrial energy-providing system.

The present investigation confirms the observations of Moreadith and Fiskum (1984) that mitochondria *in situ* in digitonin-permeabilized hepatocytes have intact membranes and are tightly coupled. Using these experimental conditions we tested the effect of t-BHP on the activity of various mitochondrial enzymes.

Methods

Materials

Fatty acid free bovine serum albumin, *tert*-butyl hydroperoxide, 2, 4-dinitrophenol, antimycin A, oligomycin, respiratory substrates, ADP and digitonin were purchased from Sigma (St. Louis, MO) and all other chemicals of analytical grade were obtained from Lachema (Czech Republic).

Isolation of rat hepatocytes

Male Wistar rats (Velaz Lysolaje, Czech Republic) with the body mass 230-250 g were used. The rats were housed at 23±1 °C, 55±10 % relative humidity, air exchange 12-14 times/h, and 12-hour light-dark cycle periods (6:00 to 18:00 h). The animals had free access to standard laboratory rat chow (DOS 2B Velaz, Czech Republic) and tap water. All animals received care according to the guidelines set by the institutional Animal Use and Care Committee of the Charles University, Prague, Czech Republic.

Hepatocytes were isolated by collagenase perfusion as described before (Moldeus *et al.* 1978, Farghali *et al.* 1994). Isolated cells were suspended in Krebs-Henseleit medium. The number of cells was counted and their viability was evaluated by trypan blue exclusion. For experiments only preparations with the viability higher than 95 % were used.

Measurement of oxygen uptake by isolated hepatocytes

Oxygen consumption was measured by the High Resolution Oxygraph OROBOROS (Austria). Measurements were performed in 2 ml of incubation medium at 30 °C. Intact isolated hepatocytes were incubated in Krebs-Henseleit medium. Digitonin-permeabilized hepatocytes were incubated in potassium-medium containing 100 mM KCl, 10 mM Tris HCl, 4 mM K-phosphate, 3 mM $MgCl_2$, 1 mM EDTA, and pH 7.4. For evaluation of oxygen uptake OROBOROS software was used. Oxygen uptake curves are presented as the first derivation of oxygen tension changes.

Results

The isolated digitonin-permeabilized cells were used for the evaluation of toxic effects of *tert*-butyl hydroperoxide (*t*-BHP) on function of mitochondrial enzyme complexes. Because we preincubated isolated hepatocytes with digitonin in the oxygraph cuvette in potassium medium, we first tested to what extent the transfer of hepatocytes from the isolation (sodium) medium into the potassium medium can change the rate of their endogenous respiration. Table I demonstrates that the rate of endogenous respiration was the same in both media. Respiration in both media was inhibited by rotenone and rotenone-sensitive portion was about 60 % of the original rate of respiration (Table 1, Fig. 1A). Endogenous respiration was further increased approximately twofold by an uncoupler dinitrophenol (Fig. 1B). These data indicate that mitochondria in intact hepatocytes are well coupled in potassium medium.

As shown in Table 1 and Figure 2, digitonin highly increased the rate of respiration after the addition of succinate and ADP. Because the optimum concentration of the digitonin for cell membrane permeabilization may differ in different cells, we tested optimum concentration of digitonin required for the maximal rate of oxygen uptake in our experimental conditions. We observed maximum activation of the respiratory rate in the concentration range between 10-20 μg digitonin per ml of the medium (Fig. 2B).

Table 1. Comparison of the respiratory rate of intact and digitonin-permeabilized hepatocytes.

	Oxygen uptake
Krebs-Henseleit medium	
Control cells	250.9 ± 19.2
+ Rotenone (2 μM)	108.9 ± 10.3∗
Rotenone sensitive (%)	56.4 %
Rotenone-insensitive (%)	43.6 %
Potassium-medium	
Control cells	208.9 ± 12.2
+ Rotenone (2 μM)	84.1 ± 14.6∗
Rotenone sensitive (%)	59.8 %
Rotenone-insensitive (%)	40.2 %
Digitonin-permeabilized cells	102.8 ± 19.7∗∗
+ Rotenone (2 μM) + succinate (10 mM)	585.3 ± 59.8∗∗
+ ADP (0.5 mM)	1274.8 ± 58.2∗∗
+ Glutamate (10 mM) + malate (3 mM)	146.4 ± 18.2∗
+ ADP (0.5 mM)	809.2 ± 57.5∗∗

Oxygen uptake is given in pmol oxygen/s/10^6 cells). Significant differences were evaluated using Student's t-test and are related to values of control cells (∗ $p \leq 0.05$, ∗∗ $p \leq 0.001$).

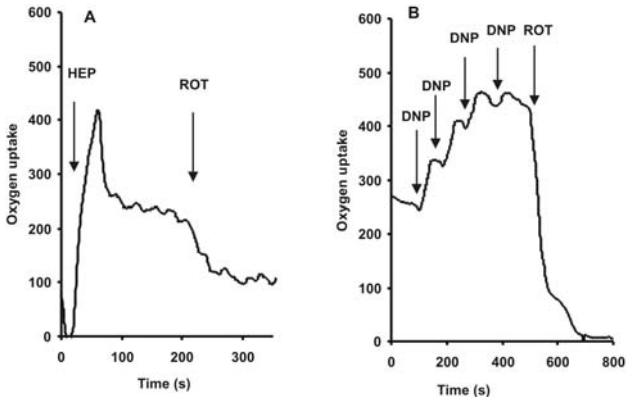

Fig. 1. Respiration of isolated rat liver hepatocytes. **A.** Inhibition of respiration of hepatocytes by rotenone. Hepatocytes (0.5 million cells/ml) were incubated in Krebs-Henseleit medium. Where indicated, rotenone (ROT, final concentration 2 μM) was added. **B.** Hepatocytes were incubated in Krebs-Henseleit medium. Where indicated, 2, 4-dinitrophenol (DNP, final concentration 16.6 μM) or rotenone (ROT, final concentration 2 μM) were added. Oxygen uptake was expressed as pmol oxygen/s/10^6 cells.

It was demonstrated by the addition of cytochrome *c* (Fig. 2A) that the outer mitochondrial membrane remains intact in permeabilized hepatocytes. This is in agreement with our previous finding on cultured fibroblasts (Chowdhury *et al*. 2000).

Table 1 together with Figures 2A and 3A also indicate to what extent the rate of respiration was enhanced by the addition of respiratory substrates and ADP to permeabilized hepatocytes. Succinate-dependent respiration was twofold increased after the addition of ADP and there was a fivefold increase of glutamate + malate-dependent respiration, which thus presented an additional evidence that mitochondria in permeabilized hepatocytes are tightly coupled.

In further experiments we tested the effect of *t*-BHP on the activity of various mitochondrial enzymes. Isolated hepatocytes were incubated for 5 min with *t*-BHP and then permeabilized by digitonin. The rate of the oxygen uptake after addition of various substrates and ADP was determined in control and in *t*-BHP-treated hepatocytes.

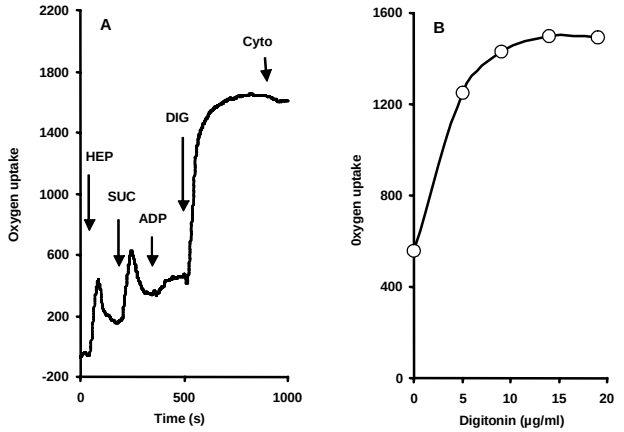

Fig. 2. Activation of respiration of isolated hepatocytes by digitonin. **A.** Hepatocytes were suspended in potassium-medium under conditions described in methods. Where indicated succinate (SUC, final concentration 10 mM) ADP (final concentration 0.5 mM), cytochrome *c* (final concentration 20 μM) and digitonin (0.02 mg/ml) were added. **B.** activation of succinate oxidation by digitonin. Oxygen uptake is expressed as pmol oxygen/s/10^6 cells.

Figure 3B shows that after 5 min preincubation of hepatocytes with 1.5 mM *t*-BHP the respiration dependent on glutamate + malate is drastically depressed. Critical concentration of *t*-BHP used under our experimental conditions was in the range of 0.75-1.5 mM (Fig. 3C). Contrary to glutamate + malate, oxidation of succinate was less sensitive to *t*-BHP action (Fig. 3B). At 1.5 mM *t*-BHP, the glutamate + malate oxidation was decreased by 90 % but succinate oxidation only by 10 %. Very pronounced inhibitory effect of *t*-BHP was also found when palmityl carnitine + malate were used as respiratory substrates (Fig. 4).

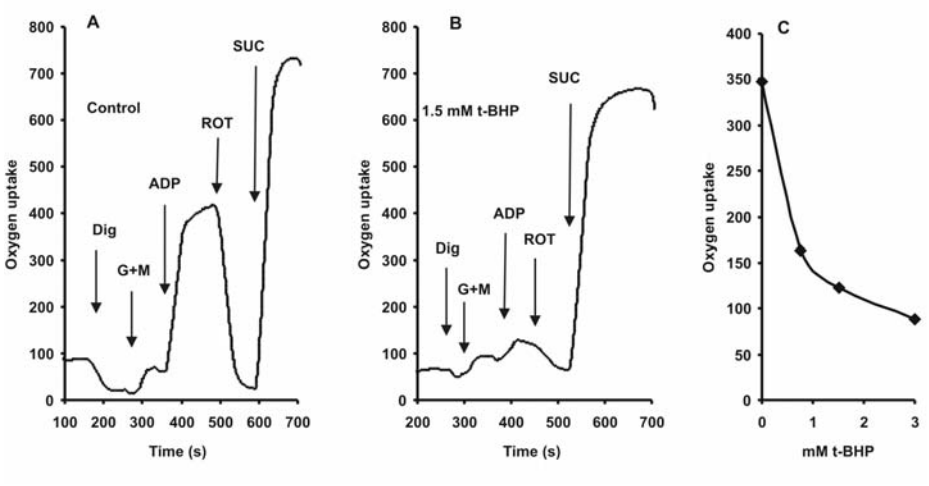

Fig. 3. The effect of *t*-BHP on respiration of glutamate + malate and succinate. Hepatocytes were incubated in K-medium as described in methods. **A.** control hepatocytes (5 x10^5 cells/ml), **B.** hepatocytes preincubated for 5 min with 1.5 mM *t*-BHP, **C.** the effect of various *t*-BHP concentrations on respiration of glutamate + malate. Where indicated, digitonin (DIG, 0.02mg/ml), glutamate (final concentration 10 mM), malate (final concentration 3 mM, G+M), ADP (final concentration 1mM), rotenone (ROT, final concentration 2 μM) and succinate (SUC, final concentration 10 mM) were added.

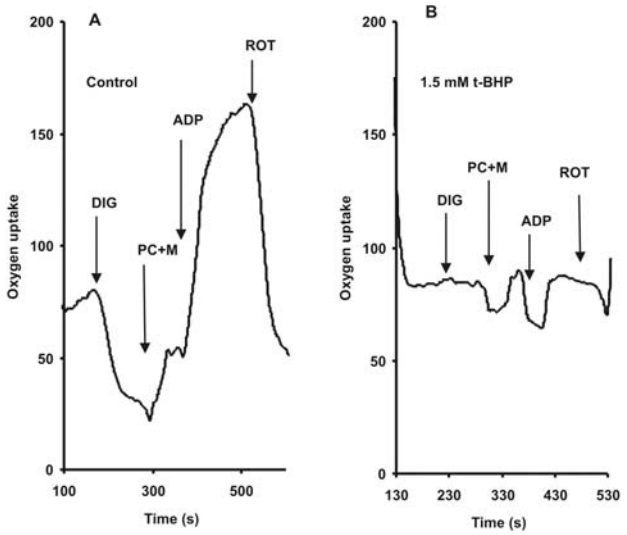

Fig. 4. Inhibition of palmityl carnitine oxidation in permeabilized rat liver hepatocytes. Hepatocytes were incubated in K-medium as described in methods. **A.** control hepatocytes (5 x10^5 cells/ml), **B.** hepatocytes preincubated for 5 min with 1.5 mM *t*-BHP. Where indicated, 0.02mg/ml digitonin, palmitylcarnitine (final concentration 0.5 mM), malate (final concentration 3 mM, PC+M), ADP (final concentration 1 mM) and rotenone (ROT, final oncentration 2 μM) were added. Oxygen uptake is expressed as pmol oxygen/s/10^6 cells.

Discussion

Oxidative stress induced by various factors can lead to necrotic or apoptotic processes (Quian *et al.* 1999, Yang *et al.* 2000). However, neither the mechanism responsible for production of oxygen reactive species (ROS) nor their specific action on various cellular components are fully understood. The results of the present study extend our information about the sensitivity of mitochondrial enzyme complexes to the ROS inhibitory action. We used isolated rat hepatocytes permeabilized by digitonin as a model system. The advantage of permeabilized hepatocytes in comparison with isolated liver mitochondria is that they better reflect situation *in vivo*. Data in literature indicate that outer mitochondrial membrane is partially damaged during homogenization (Nedergaard and Cannon 1979). Furthermore, mitochondrial interaction with other cytosolic structures is impaired as well as the inter-mitochondrial contacts. Both, the interaction of mitochondria with the cytoskeleton network (Garesse and Vallejo 2001) and their integration into functional clusters and filaments (Skulachev 2001) are factors that are important for the maintenance of mitochondrial functional activity.

Figure 3C shows the concentration range of digitonin that enable substrates and ADP to enter mitochondria without damage of the mitochondrial membranes. Under these experimental conditions the effect of *t*-BHP on activity of various mitochondrial enzymes can be evaluated. We found that Complex I activity is much more sensitive to the peroxidative action of *t*-BHP than the activity of Complex II. These data can be explained on the basis of recent findings indicating extremely high sensitivity of aconitase to ROS produced inside the cell (Powel and Jackson 2003). The high sensitivity was explained by modification of the iron-sulfur components required for the aconitase function. It is well known that Complex I also has iron-sulfur clusters as an integral component of the enzyme complex, which are required for the transfer of reducing equivalents from NADH to coenzyme Q. Therefore similar inhibitory effect of ROS was also proposed for Complex I inhibition (Wallace 1999, Genova *et al.* 2001, Powell and Jackson 2003). Complex I was mostly studied as one of the sites

of the respiratory chain, where ROS are produced (Turrens and Boveris 1980), but the possibility that its function can be inhibited by ROS was neglected.

Increased ROS production induced by various toxic factors (Ferenčíková *et al.* 2003) or pathological states (Ješina *et al.* 2004) is often accompanied with activation of uncoupling proteins as a compensatory mechanism decreasing mitochondrial membrane potential (Yang *et al.* 2000). Together with other findings that propose participation of reversible inhibition of Krebs cycle enzymes by ROS in regulation of cell energy metabolism (Nulton-Persson and Szweda 2001), we also have to accept the possibility, that ROS can act as regulatory factor of cell metabolism besides its participation in necrotic and apoptotic processes.

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