Physiological Research Pre-Press Article

SHORT COMMUNICATION

Inhibition of Palmityl Carnitine Oxidation in Rat Liver Mitochondria by *Tert*-Butyl Hydroperoxide

Z. ČERVINKOVÁ[1], H. RAUCHOVÁ[2], P. KŘIVÁKOVÁ[1], Z. DRAHOTA[1, 3]

[1]Department of Physiology, Faculty of Medicine in Hradec Králové, Charles University in Prague, Czech Republic, [2]Institute of Physiology, Czech Academy of Sciences, Center for Cardiovascular Research, Prague, Czech Republic, [3] Institute of Physiology, Czech Academy of Sciences, Center for Applied Genomics, Prague, Czech Republic.

Received

Accepted

Short title: Peroxidative damage of mitochondria

Key words: Liver mitochondria, Palmityl carnitine oxidation, *tert*-butyl hydroperoxide

Reprint requests

Zuzana Červinková, Department of Physiology, Charles University in Prague, Faculty of Medicine in Hradec Králové, Šimkova 870, 500 38 Hradec Králové, Czech Republic.

E-mail: wolff@lfhk.cuni.cz

Summary

Mitochondria as an energy generating cell device are very sensitive to oxidative damage. Our previous findings obtained on hepatocytes demonstrated that Complex I of the respiratory chain is more sensitive to oxidative damage than the other respiratory chain complexes (Drahota *et al.* 2005). We present additional data on isolated mitochondria showing that at a low (200 μM) *tert*-butyl hydroperoxide (*t*BHP) concentration palmityl carnitine oxidation is strongly depressed, while oxidation of the flavoprotein-dependent substrate-succinate is not affected and neither is ATP synthesis inhibited by *t*BHP. In the presence of *t*BHP, the respiratory control index for palmityl carnitine oxidation is strongly depressed, but when succinate is oxidized the respiratory control index remains unaffected. These findings thus show that for the regeneration process in the necrotic liver damaged by oxidative stress, flavoprotein-dependent substrates could be an important nutritional factor.

Liver cells represent an important factor in the traffic of lipids as a nutritional energy source for animal organisms (Havel 1987). Some lipids, namely short and medium chain fatty acids entering the liver through the portal vein, are oxidized directly in hepatocytes by mitochondria to cover their energy demands.

It is now commonly accepted that mitochondria are not only an important generator of reactive oxygen species (Boveris and Chance 1973, Chance *et al*. 1979), but also an important target of their action (Turrens and Boveris 1980, Kovaltowski and Vercesi 1999, Nulton-Presson and Szweda 2001, Turrens 2003). Liver tissue is especially sensitive to various toxic agents that induce processes in which reactive oxygen species play an important role (Shu *et al*. 1997, Knight *et al*. 2003).

There are many studies describing peroxidative damage of liver mitochondria (Kennedy *et al*. 1992, Kowaltowski and Vercesi 1999, Nulton-Persson and Szweda 2001, Lin *et al*. 2002) and hepatocytes (Masaki *et al.* 1989, Nieminen *et al.* 1997, Kmoníčková *et al*. 2001). However, there are not sufficient comparative data to evaluate the sensitivity of various mitochondrial dehydrogenases to peroxidative damage.

This is why in our previous experiments on isolated hepatocytes we studied the peroxidative damage of mitochondrial enzymes induced by *tert*-butyl hydroperoxide (*t*BHP). In these studies we used *t*BHP as the prooxidant because, unlike hydrogen peroxide, it is not degraded by catalase (Chance *et al.* 1979) and consequently its peroxidative effect can be studied over a longer period of incubation. We found that oxidation of NADH-dependent substrates is extremely sensitive to peroxidative damage and also that oxidation of flavoprotein dependent substrates is partially reduced (Drahota *et al*. 2005, Křiváková *et al*. 2007).

It has not been fully elucidated to what extent the toxic effects of prooxidants are due to the direct action on mitochondrial enzymes and to what extent secondary radical metabolites

formed in cytosol (Kennedy *et al.* 1992) could also be involved in peroxidative damage when isolated hepatocytes are used. Following up on our previous experiments on hepatocytes, therefore, in this study we measured isolated mitochondria and we tested to what extent fatty acid oxidation and succinate oxidation are affected by *t*BHP. In order to obtain additional data, we tested the effect of *t*BHP on palmityl carnitine and succinate oxidation on isolated liver mitochondria under the same experimental conditions. We compared the effect of *t*BHP on palmityl carnitine and succinate oxidation, because oxidation of palmityl carnitine involves two mitochondrial dehydogenases, a NADH- and a flavoprotein-dependent one, whereas succinate is oxidized only by the flavoprotein-dependent dehydrogenase.

Liver mitochondria were isolated from male albino Wistar rats (b.w. of 220-230 g) by differential centrifugation as described previously (Ješina *et al.* 2004). Oxygen uptake was measured with the High Resolution Oxygraph2K (OROBOROS, Austria). Measurements were taken at 30^{o} C in 2 ml of incubation medium containing 100 mM KCl, 10 mM Tris HCl, 4 mM K-phosphate, 3 mM $MgCl_2$, 1 mM EDTA, 0.5 mg/ml fatty acid free bovine serum albumin, pH 7.4. The rate of oxygen uptake was expressed as pmol/s/mg protein. Oxygen uptake curves are presented as the first negative derivation of the oxygen tension changes.

Fig. 1 demonstrates the experimental conditions used for evaluation of the *t*BHP effect on the oxidation of palmityl carnitine and succinate performed by rat liver mitochondria. In contrast to other respiratory substrates which are used in high (millimolar) concentrations, palmityl carnitine must be tested at micromolar concentrations, because at higher concentrations it exerts a detergent-like effect on mitochondrial membranes. We used 5 μM palmityl carnitine and 2.5 mM malate as a sparker. After the addition of the ADP, the oxygen uptake was highly activated (five-fold, Fig. 1A, Table 1), indicating that mitochondria are well coupled and that the concentration used of the palmityl carnitine had no detergent-like effect. When palmityl carnitine was oxidized, the respiratory rate decreased and another

portion of palmityl carnitine induced a similar response in oxygen uptake (Fig. 1A). The addition of rotenone, an inhibitor of Complex I, completely inhibited palmityl carnitine oxidation (not shown). The addition of succinate after palmityl carnitine again increased the rate of oxygen uptake to values about 30-50% higher than those obtained with palmityl carnitine and malate (Fig. 1A and Table 1). When tBHP was added after palmityl carnitine was oxidized, then subsequent addition of palmityl carnitine induced a much smaller increase in the respiratory rate (Fig.1B). However, subsequent addition of succinate demonstrated that the rate of succinate oxidation was not depressed by tBHP (Fig. 1B, and Table 1). The same results were obtained when the effect of *t*BHP on the oxidation of succinate was tested in the absence of palmityl carnitine and malate (Fig 1C, D, and Table 1).

From our data it is evident that fatty acid oxidation is more sensitive than oxidation of succinate to peroxidative damage (Fig. 1, and Table 1). We may also conclude based on our experiment that under conditions when fatty acid oxidation is highly depressed by *t*BHP (Fig. 1C, D), the ATP synthase is active, because the respiratory rate induced by ADP is not affected (Table1). Our data thus confirm our previous results obtained on isolated hepatocytes (Drahota *et al.* 2005, Křiváková *et al.* 2007), demonstrating that Complex I is the most sensitive part of the mitochondrial respiratory chain to peroxidative damage. Experiments on isolated mitochondria further indicate that the inhibition of Complex I is not necessarily connected with the collapse of membrane potential if flavoprotein dependent substrates are accessible. These conclusions are also supported by our previous data on isolated hepatocytes (Lábajová *et al.* 2006), demonstrating that the dissipation of the mitochondrial membrane potential by *t*BHP, when pyruvate and malate are used as respiratory substrates, can be fully recovered by succinate.

Our data showing that fatty acid oxidation is highly sensitive to oxidative damage could be important information for the treatment of necrotic liver tissue because most hepatotoxic

agents are known to be reactive oxygen species inducers (Knight et *al*. 2003, Sundari *et al.* 1997). For the activation of the regeneration process, therefore, flavoprotein-dependent substrates could play an important role.

Acknowledgement

This work was supported by the grants MSM 0021620820, GAČR 303/03/H065, GAČR 305/04/0500, and 1M6837805003.

References

BOVERIS A, CHANCE B: The mitochondrial generation of hydrogen peroxide. *Biochem J* **134:** 707-716, 1973.

CHANCE B, SIES H, BOVERIS A: Hydroperoxide metabolism in mammalian organs. *Physiol Rev* **59**: 527-605, 1979.

DRAHOTA Z, KŘIVÁKOVÁ P, ČERVINKOVÁ Z, KMONÍČKOVÁ E, LOTKOVÁ H, KUČERA O, HOUŠTĚK J: *Tert*-Butyl hydroperoxide selectively inhibits respiratory-chain enzymes in isolated rat hepatocytes. *Physiol Res* **54:** 67-72, 2005.

HAVEL RJ: Lipid transport function of lipoproteins in blood plasma. *Am J Physiol* **253**: E1-E5, 1987.

JEŠINA J, KHOLOVÁ D, BOLEHOVSKÁ R, ČERVINKOVÁ Z, DRAHOTA Z, HOUŠTĚK J: Glycerophosphate-dependent hydrogen peroxide production by rat liver mitochondria. *Physiol Res* **53:** 305-310 2004.

KENNEDY CH, CHURCH DF, WINSTON GW, PRYOR WA: *Tert*-butyl hydropreoxide-induced radical production in rat liver mitochondria. *Free Radic Biol Med* **12:** 381-387, 1992.

KMONÍČKOVÁ E, DRAHOTA Z, KAMENÍKOVÁ L, ČERVINKOVÁ, Z, MAŠEK K, FARGHALI H: Modulatory effect of cyclosporin A on tert-butyl hydroperoxide-induced oxidative damage in hepatocytes. *Immunopharmacol Immunotoxicol* **23:** 43-54, 2001.

KNIGHT TR, FARISS MW, FARHOOD A, JAESCHKE H: Role of lipid peroxidation as a mechanism of liver injury after acetaminophen overdose in mice. *Toxicol Science* **76**: 229-236, 2003.

KŘIVÁKOVÁ P, LÁBAJOVÁ A, ČERVINKOVÁ Z, DRAHOTA Z: Inhibitory effect of *t*-butyl hydroperoxide on mitochondrial oxidative phosphorylation in isolated hepatocytes. *Physiol Res* **56:** 00-00, 2007. In press

KOVALTOWSKI AJ, VERCESI AE: Mitochondial damage induced by conditions of oxidative stress. *Free Radic Biol Med* **26:** 463-471, 1999.

LIN K-L, HUGHES G, MURATOWSKA A, BLAIKIE FH, BROOKES PS, DARLEY-USMAR V, SMITH RAJ, MURPHY MP: Specific modification of mitochondrial protein thiols in response to oxidative stress. *J Biol Chem* **277:** 17048-17056, 2002.

LÁBAJOVÁ A, KOFRÁNEK J, KŘIVÁKOVÁ P, ČERVINKOVÁ Z, DRAHOTA Z: TTP^{+}-selective electrode as a tool for evaluation of mitochondrial membrane permeability pore function in isolated rat hepatocytes. *Gen Physiol Biophys* **25:** 325-331, 2006.

MASAKI N, KYLE ME, SERRONI A, FARBER JL: Mitochondrial damage as a mechanism of cell injury in the killing of cultured hepatocytes by tert-butyl hydroperoxide. *Arch Biochem Biophys* **270:** 672-680, 1989.

NIEMINEN A-L, BYRNE AM, HERMAN B, LEMASTERS JJ: Mitochondrial permeability transition in hepatocytes induced by t-BuOOH: NAD(P)H and reactive oxygen species. *Am J Physiol* **272:** C1286-C1294, 1997.

NULTON-PRESSON AC, SZWEDA LI: Modulation of mitochondrial function by hydrogen peroxide . *J Biol Chem,* **276:** 23357-23361, 2001.

SHU Z, JUNG M, BEGER HG, MARZINZIG M, HAN F, BUTZER U, BRUCKNER UB, NUSSLER AK: pH-dependent changes of nitric oxide, peroxynitrite, and reactive oxygen species in hepatocellular damage. *Am J Physiol* **273**: G1118-G1126, 1997.

SUNDARI PN, WILFRED G, RAMAKRISHNA B: Does oxidative protein damage play a role in the pathogenesis of carbon tetrachloride-induced liver injury in rat, *Biochim Biophys Acta* **1362:** 169-172, 1997.

TURRENS JF: Mitochondrial formation of reactive oxygen species. *J Physiol* **552**: 335-344, 2003.

TURRENS JF, BOVERIS A: Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. *Biochem J* **191**: 421-427, 1980.

Text to Fig. 1

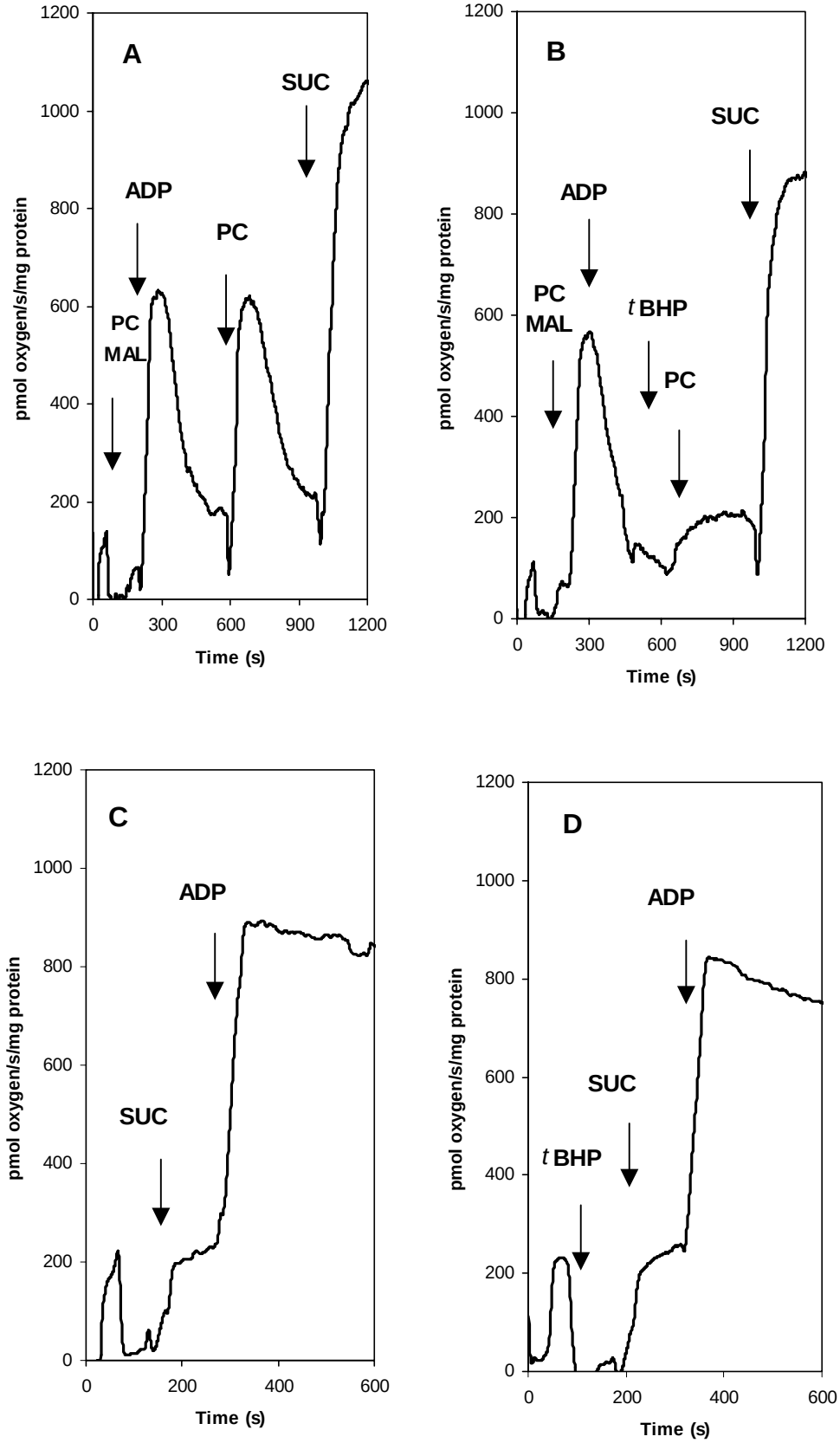
Fig. 1. Respiration of rat liver mitochondria in the presence of palmityl carnitine and succinate: Mitochondria (0.2 mg protein/ml) were incubated in a KCl medium with 0.5 mg of bovine serum albumine. Where indicated, 2.5 mM malate (MAL), 5 μM palmityl carnitine (PC), 1.5 mM ADP, 10 mM succinate (SUC) and 200 μM t-butyl hydroperoxide (tBHP) were added. The respiratory control index (RCI) in part A was 5.2 and 5.0; in B 4.8 and 1.8; in C 4.3; and in part D, 3.9.

Table 1. Inhibition of palmityl carnitine and succinate oxidation by tBHP.

Additions	**pmole oxygen/s/mg protein**	**+tBHP/-tBHP**
Palmityl carnitine + malate	117.0 ± 14.0	
Palmityl carnitine + malate + ADP	601.5 ± 27.6	
Palmityl carnitine + malate + ADP + tBHP	245.7 ± 27.4	0.40 ($p \leq 0.001$)
Succinate + ADP	907.6 ± 53.0	
Succinate + ADP + tBHP	883.9 ± 29.9	0.87 (n.s.)

Mitochondria were incubated as described in the legend to Fig. 1. The palmityl carnitine used was 5 μM, malate 2.5 mM, ADP 1.5 mM, succinate 10 mM and *tert*-butyl hydroperoxide 200 μM. The data presented depict the average± SEM from six liver mitochondrial preparations isolated from six adult male rats. The statistical significance was analysed using t-test.

Fig. 1


A
SUC
ADP
PC
PC
MAL
pmol oxygen/s/mg protein
Time (s)
B
SUC
ADP
PC
MAL
tBHP
PC
pmol oxygen/s/mg protein
Time (s)
C
ADP
SUC
pmol oxygen/s/mg protein
Time (s)
D
ADP
SUC
tBHP
pmol oxygen/s/mg protein
Time (s)