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TERT-BUTYLHYDROPEROXIDE BIOACTIVATION TO METHYL RADICAL IN RAT LIVER MITOCHONDRIA AND SUBMITOCHONDRIAL PARTICLES

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Electron paramagnetic resonance spectroscopy (EPR) coupled to the spin trapping technique was used to detect carbon-centered radicals in rat liver mitochondria and submitochondrial particles exposed to *t*-butyl-hydroperoxide (TBH), using the spin trapping agent 3,5-dibromo-4-nitroso-benzenesulfonic acid (DBNBS). The signal recorded was unambiguously assigned to the methyl radical adduct.

DBNBS was added to isolated rat liver mitochondria energized with succinate, and the methyl radical adduct was observed. The addition of NADH, NADPH, inhibitors of the respiratory chain, and of monoaminoxidase (MAO) inhibitors did not cause any relevant modification in the yield of radical adduct formation. Boiling and the addition of a non-ionic detergent inhibited the formation of the radical adduct, while experiments carried out under hypoxic conditions generated a significant increase in methyl radical formation. Further experiments were carried out on sub-mitochondrial particles (SMP) giving rise to, basically, the same results.

From the above results, we are proposing that haem prosthetic groups are the likely source of TBH bioactivation in mitochondria.

KEY WORDS: Spin trapping; *t*-butyl-hydroperoxide; detergents; mitochondria; submitochondrial particles; carbon-centered radical

INTRODUCTION

tert-Butylhydroperoxide (TBH) is used as a model compound able to generate oxidative stress in biological systems. Its complex metabolic pathway has been extensively investigated[1,2] and it is believed that reactive free radical intermediates, including peroxyl-, alkoxyl- and carbon centered radicals, are mediators of the peroxide-dependent injury.

Peroxide metabolism in mitochondria blocks the oxidative pathway and restricts ATP formation from glycolytic substrates[3]. Peroxides added to isolated mitochondria inhibit the aerobic energy-producing pathway[4], and free radical scavengers prevent the hydroperoxide-induced decrease of mitochondrial transmembrane potential[5].

Both oxygen and carbon centered radicals have been demonstrated to arise during the bioactivation process using Electron Paramagnetic Resonance (EPR) spectroscopy – spin trapping[6,7] however the precise mechanism(s) of degradation, the identity of the species produced, and the relevance of such species in causing the toxic effect is still a matter of debate.

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Although there is abundant experimental evidence supporting the involvement of peroxide-derived free radical species in eliciting mitochondrial injury, only one paper has provided a direct demonstration of the radical species involved[8]. Carbon centered radicals have been demonstrated during the bioactivation of TBH in mitochondria using 5,5-dimethyl-pyrroline N-oxide (DMPO) and 2-methyl-2-nitroso propane (MNP) as spin trap.

In the present paper we first wanted to confirm the results obtained in the previous work, using 3,5-dibromo-4-nitrosobenzenesulfonate (DBNBS) as spin trap, which is more hydrophilic and not as light sensitive as MNP.

The second aim of the work was to differentiate between non-enzymatic and enzymatic bioactivation, and identify the enzymatic system(s) responsible.

From the evidence gathered, it is concluded that the free radical generating system is neither completely dependent on respiratory chain enzymes, nor on some specific enzymes, such as monoaminoxidase (MAO). Haem prosthetic groups, on which TBH compete with oxygen, are likely to catalyze TBH free radical bioactivation.

MATERIALS AND METHODS

Chemicals: 3,5-dibromo-4-nitroso-benzenesulfonic acid (DBNBS), *tert*-Butyl Hydroperoxide (70%, TBH) and polyoxyethylenesorbitan monooleate (Tween 80) were obtained from Aldrich Chemical Company (Germany). NADH, NADPH, KCN, p-trifluoromethoxy-carbonylcyanide-phenylhydrazone (FCCP), rotenone, N-methyl-N-benzyl-2-propynylamine (Pargyline) and N-methyl-N-propargyl-3-(2,4-dichlorophenoxy) propylamine (Clorgyline) were purchased from Sigma Chemical Company (St. Louis, MO, USA) and used as specific inhibitors of monoamine oxidase B and A activities, respectively; antimycin A was from Böhringer (Mannheim, Germany), desferrioxamine was obtained from CIBA Pharmaceutical Company (Summit, NJ, USA).

Mitochondria were isolated from the liver of adult male Wistar rats (180–250 g) fasted overnight and sacrificed by decapitation, as described elsewhere[9].

Isolation of submitochondrial particles (SMP) was performed according to Muscari *et al.*[10] by sonicating isolated mitochondria and centrifuging them in 2 mM EDTA pH 8.5 at 10,000 g for 10 min. The supernatant was then centrifuged twice at 104,000 g for 30 min. Two rats were used for each preparation.

The respiratory control index (RCI) was determined on each mitochondrial preparation, using a Clark oxygen electrode, adding succinate and ADP as substrates. Mitochondria with a RCI higher than 3 were used for the experiments; protein concentration was adjusted to 8 mg/ml.

EPR – spin trapping experiments were carried out on a Bruker 200 D EPR spectrometer; usual settings were microwave power 10 mW; modulation amplitude 0.5 G; scanning width 100 G; cavity temperature 37°C. The mitochondrial (or sub-mitochondrial) pellet was gently resuspended in 10 mM K phosphate buffer pH 7.4, 210 mM mannitol, 70 mM sucrose, 10 mM DBNBS, 400 μM desferrioxamine, 5 mM succinate, and 5 mM TBH.

When indicated NADPH (0.5 mM), NADH (0.5 mM), Tween 80 (2.5 mM), antimycin A (4 μM), KCN (2 μM), FCCP (2 μM), Clorgyline (100 μM), or Pargyline (500 μM) was added to the suspension and incubated at 37°C in a flat EPR cell within the cavity. The spectra were recorded at regular intervals. The signal reached a maximum within 10 minutes.

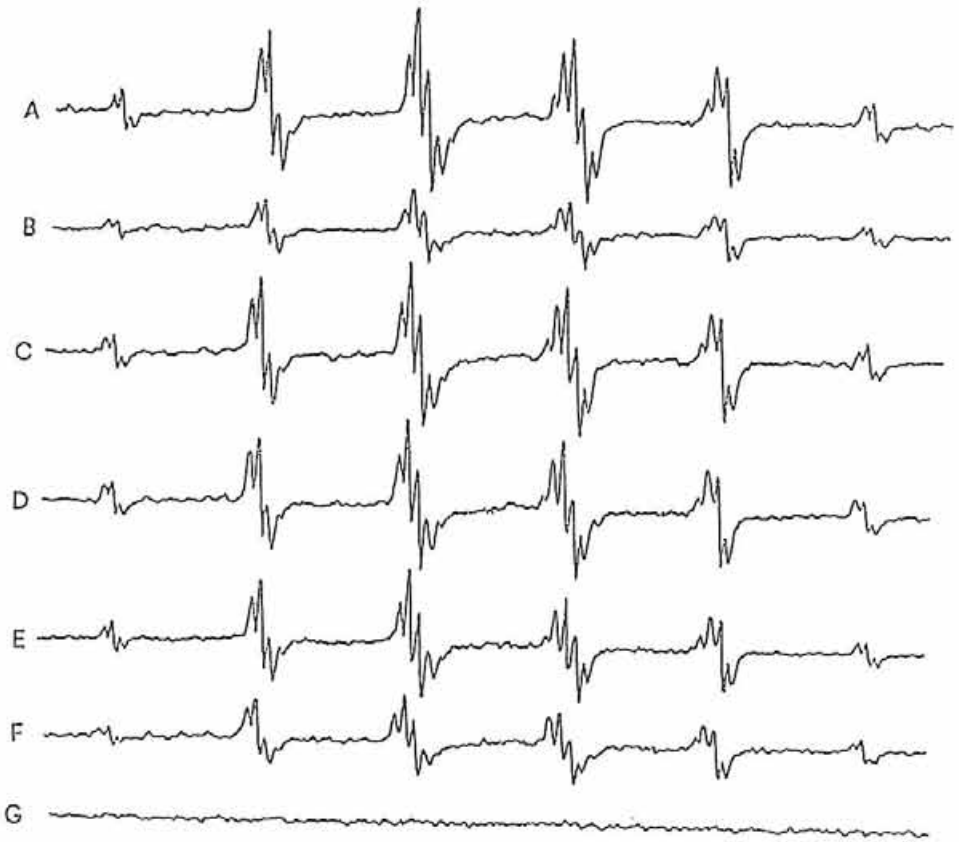

FIGURE 1 Typical EPR spectra observed in rat liver mitochondria. (A) Whole mitochondria incubation at 37°C for 10 min, 8 mg/ml mitochondrial protein, 400 μM Desferal, 5 mM TBH, 10 mM DBNBS, 210 mM mannitol, 70 mM sucrose, 10 mM K phosphate buffer, pH 7.4. (B) same as A except 2.5 mM Tween 80 being added to mitochondria. (C) same as A, +0.5 mM NADPH. (D) same as A, +0.5 mM NADH. (E) same as A, +3 μM antimycin A. (F) boiled mitochondria (100°C for 15 min). (G) mitochondria omitted.

Hypoxia was obtained by incubating the suspension in gas-permeable teflon tubing. The sample was drawn into a gas permeable teflon capillary tube (Zeus Industries, Raritan N.J.) of 0.081 mm inside diameter and 0.0038 ± 0.0013 mm wall thickness. The tube containing the sample was folded in two and inserted in a quartz EPR tube, open at both ends, placed in the cavity Dewar. Gas of desired composition was flowed through the Dewar and around the sample.

RESULTS

Experiments were performed on freshly prepared mitochondria, having a RCI $\geqslant 3$. The incubation carried out at 37°C in the presence of DBNBS and TBH gave rise to a well detectable radical adduct (Figure 1). The signal was characterized by hyperfine splittings from nitrogen ($a_N = 14.3$ G), three equivalent methyl protons ($a_{H^{CH3}} = 13.71$ G), and two meta-protons ($a_{H^{meta}} = 0.76$ G) and assigned to the methyl radical adduct.

TABLE I

	Whole Mitochondria	SMP
Control	23 ± 5.1	21.8 ± 4.5
+NADPH (0.5 mM)	18.7 ± 1.9	22.2 ± 5.8
+NADH (0.5 mM)	16.3 ± 1.4	25.1 ± 4.6
+Clorgyline (0.1 mM)	18.7 ± 6.7	18.7 ± 6.3
+Pargyline (0.5 mM)	20.3 ± 2.9	20.5 ± 1.5
boiling 15 min	14.3 ± 3.1*	15 ± 3.9*
+Tween 80 (2.5 mM)	13.1 ± 2.2**	14.9 ± 4.3*

DBNBS-methyl radical adduct (arbitrary units) observed in mitochondria and SMP exposed to TBH for 30 minutes under normoxic conditions; mean ± s.d, n ⩾ 3; * = $P < 0.05$.; ** = $P < 0.01$, paired data Student's *t* test.

The incubation of all the reactants, but mitochondria, resulted in the formation of a small, however reproducible, methyl radical adduct, whose formation was completely prevented by the addition of desferrioxamine.

Table I describes the effects observed after addition of NADPH and NADH, which did not cause any significant change in the methyl radical adduct yield; equally inefficient was the addition of Clorgyline and Pargyline, inhibitors of MAO A and B activities.

Experiments using SMP gave rise to results similar to those observed in whole mitochondria (Table I).

Boiling the mitochondrial suspension for 15 min or the addition of the non-ionic detergent Tween 80 caused a significant decrease in signal intensity, both in whole mitochondria and SMP (Table I).

Further experiments were carried out, on whole mitochondria, with the addition of highly specific inhibitors of the respiratory chain, such as rotenone, which blocks the electron flow on complex I, antimycin A, which blocks the electron flow on complex II, and KCN, which blocks complex III (Figure 2). The oxidative phosphorylation uncoupling agent FCCP was also tested. The results are reported in Figure 2.

In hypoxic experiments, mitochondria were exposed either to air or to oxygen-free nitrogen for ten minutes before the addition of TBH and the spin trap. The results obtained, as shown in Table II, demonstrated a highly significant increase of the radical adduct. The same results were observed in SMP (not shown).

DISCUSSION

Methyl radical, as demonstrated by EPR – spin trapping technique, is apparently the only type of free radical adduct which has been observed in mitochondria during bioactivation of TBH using DBNBS as spin trap. Reactive free radical intermediates, including peroxyl-, alkoxyl- and carbon centered radicals have been demonstrated during TBH bioactivation[6,7,11], however oxygen centered radicals have only been demonstrated in either purified enzymatic systems or in isolated microsomes. The carbon centered radical observed is presumably due to the rapid decomposition of *tert*-butoxy radicals; in fact *tert*-butoxy radicals decompose through β-scission to form methyl radicals and acetone much faster in water than in organic solvents[12]; concurrently the alkoxy-DBNBS radical adduct, if formed,

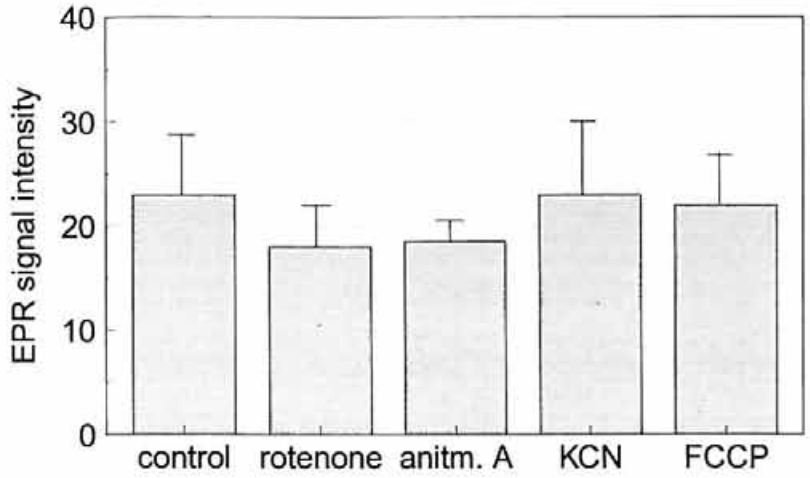

FIGURE 2 Effect of respiratory chain inhibitors and uncoupling agent on methyl radical production observed in rat liver mitochondria incubated in the presence of TBH.

will rapidly decompose, since it is known that DBNBS-oxygen-centered adducts are not stable[13,14].

In this work we have chosen to assess the eligibility of the nitroso spin trap DBNBS in trapping TBH-derived radicals in this model system. This spin trap has recently been under consideration for application in biological model systems[13,14], and its potential application has not yet been fully appreciated. There is a clear advantage in the use of nitroso spin traps, since the radical adduct, in the α position to the nitroxide center, gives detailed information on the structure of the radical trapped.

In the search for an enzymatic system responsible for the TBH activation giving rise to free radical intermediates, experiments were conceived in order to demonstrate a possible role of the respiratory chain. Rotenone, antimycin A and KCN, which inhibit the electron flow on the respiratory chain in a highly specific way, and FCCP, an uncoupler of the oxidative phosphorylation, did not elicit any overt change in the methyl radical adduct formation; hence no active electron transport chain is necessary for the bioactivation of TBH. The possibility of a free-iron dependent hydroperoxide break-down, a well known mechanism verified on several occasions[15], should be taken into consideration. Desferrioxamine chelates iron and prevents it from taking part in the redox reactions. It also prevented radical adduct formation observed in a blank experiment, carried out in the presence of the complete reaction mixture, without mitochondria.

A metal ion complex, not chelatable by desferrioxarmine might be involved in the TBH bioactivation. This is likely to be a haem prosthtetic group but not necessarily a specific group. This view is further supported by experiments in which boiling for 15 min, or treatment with detergent, was shown to significantly inhibit, but not abolish, the adduct formation (Table I).

DBNBS is hydrophilic and with the sulfonic acid moiety mostly dissociated at pH = 7.4, is unlikely to penetrate the outer membrane. Hence the hypothesis

TABLE II

	normoxia	hypoxia
Control	28.3 ± 4.6‡	51.5 ± 8.8
+NADPH (0.5 mM)	25.6 ± 4.5‡	70.0 ± 9.4
+NADH (0.5 mM)	23.0 ± 1.4‡	64.3 ± 6.5
+Tween 80 (2.5 mM)	17.6 ± 2.7**‡	26.9 ± 6.5**

DBNBS-methyl radical adduct (arbitrary units) observed in mitochondria exposed to TBH for 30 minutes; mean ± s.d, n ≥ 3; ** = $P < 0.01$, compared to the control value, ‡ = $P < 0.01$ compared to the related hypoxic value; paired data Student's *t* test.

that the radical species trapped was formed on or on the outside of the external membrane of the mitochondrion.

A possible candidate for TBH bioactivation is the NADPH-dependent mono aminooxidase, placed on the outer mitochondrial membrane[16]. The use of highly specific inhibitors of MAO A and B, Pargyline and Clorgyline, and the addition of NADPH did not elicit any significant modification of the radical adduct yield, ruling out a role for such enzyme(s).

It is known that hypoxia augments TBH toxicity[17]. However, as reported in the same paper, the amount of TBH-derived free radicals trapped in isolated hepatocytes using PBN as spin trap, did not vary under hypoxic conditions. In our experimental model system, a significant increase in the radical adduct yield was observed (Table II). However this yield was not influenced by the addition of reducing equivalents.

These results suggest that the TBH competes with oxygen for access to the same activation site. However the scavenging of the methyl radical by oxygen has also to be taken into consideration.

Taking into account the high concentration of the spin trap, as compared to the concentration of oxygen under normoxic conditions, the results indicate that, of the two possibly concurrent reactions, the first is the more significant, strongly suggesting a haem prosthetic group as site of TBH bioactivation.

In conclusion, we suggest that a metal ion complex, most likely a haem prosthetic group, however not a specific one, is involved in the TBH bioactivation. It seems likely therefore that the free radical generating process in mitochondria is a haem mediated process which might explain the reported[18] inhibition of the oxidative pathway, decrease of ATP concentration and decrease of mitochondrial trans-membrane potential.

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