Biochimica et Biophysica Acta, 1144 (1993) 77–84

BBABIO 43858

Membrane-associated redox cycling of copper mediates hydroperoxide toxicity in *Escherichia coli*

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(Received 21 July 1992)
(Revised manuscript received 15 February 1993)

Key words: Copper; Hydroperoxide toxicity; Respiratory chain injury; (*E. coli* membranes)

We are studying the action of *tert*-butylhydroperoxide (t-BOOH) on *Escherichia coli* as a model system for peroxide toxicity. In our previous report (De la Cruz-Rodriguez, L.C., Farías, R.N. and Massa, E.M. (1990) Biochim. Biophys. Acta 1015, 510–516), the respiratory chain was identified as a major target of t-BOOH. In the present paper, we study further the effect of t-BOOH on the NADH oxidase of the *E. coli* respiratory chain to clarify the mechanism of damage, especially regarding the identity and role of the metal ion involved. The results are: (a) t-BOOH toxicity is mediated by membrane-bound copper ions; (b) a small pool of the membrane-bound copper is reduced from Cu(II) to Cu(I) in the presence of NADH and other respiratory substrates (succinate, D-lactate); (c) this reduction of copper occurs at 37°C but not at 0°C or when the membranes are inactivated by previous heating; (d) the Cu(I) generated by reduction of Cu(II) during membrane preincubation with NADH, is oxidized by t-BOOH with simultaneous inactivation of the NADH oxidase, whereas treatment with only t-BOOH (without NADH) has no effect on the oxidase. It is concluded that the effect of t-BOOH on the respiratory chain is mediated by redox cycling of copper. It is proposed that the damage results from activation of the hydroperoxide through its interaction with Cu(I) in a site-specific Fenton-type reaction.

Introduction

We are studying the action of *t*-butylhydroperoxide (t-BOOH) on *Escherichia coli* as a model system for organic peroxide toxicity. In a previous report [1], we described the toxic effect of t-BOOH on *E. coli* cells and identified the respiratory chain as a major target of the in vivo hydroperoxide action.

In *E. coli*, the components of the respiratory chain are bound to the cytoplasmic membrane [2,3]. It was previously shown [1] that exposure of *E. coli* membranes to t-BOOH in the presence of NADH, led to impairement of the respiratory chain monitored by inactivation of the NADH oxidase. This inactivation was irreversible since it persisted after membrane washing and resuspension in fresh medium without added hydroperoxide. The inactivating process induced by t-BOOH was time-dependent and required the presence of the substrate NADH [1]. The involvement of a metal ion as a mediator of the hydroperoxide action was inferred by the lack of effect of t-BOOH on the NADH oxidase in the presence of the chelator EDTA [1].

In the present paper, we study further the t-BOOH effect on the NADH oxidase of the *E. coli* respiratory chain to clarify the mechanism of damage, especially regarding the identity and role of the implicated metal. It is shown that the toxic effect of t-BOOH is mediated by redox cycling of membrane-bound copper ions. It is demonstrated, for the first time, that *E. coli* membranes have the ability to reduce Cu(II) to Cu(I) in the presence of NADH and other respiratory substrates (succinate, D-lactate), and that this Cu(I) is oxidized by t-BOOH leading to impairement of the respiratory chain. It is proposed that the damage results from t-BOOH activation through its interaction with Cu(I) in a site-specific Fenton-type reaction.

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Abbreviation: t-BOOH, *tert*-butylhydroperoxide.

Materials and Methods

Reagents. The following compounds were from Sigma Chemicals Co. (St. Louis, MO, USA): *t*-butylhydroperoxide (70% aqueous solution); chelating resin (sodium form); β-nicotinamine adenine dinucleotide, reduced form (NADH); 2,2′-biquinoline (cuproine); bathocuproinedisulfonate (disodium salt); 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine, sodium salt (ferrozine; PDT disulfonate). Other chemicals used were of analytical grade.

Bacterial strain and growth conditions. E. coli ML 308225 ($i^- z^- y^+ a^+$), obtained from Dr. H.R. Kaback, was grown aerobically at 37°C in minimal salt medium M9 [4] omitting the $CaCl_2$ (composition per liter of distilled water: KH_2PO_4 3 g; Na_2HPO_4 6 g; NaCl 0.5 g; NH_4Cl 1 g; $MgSO_4 \cdot 7H_2O$ 0.2 g) and containing 0.5% glycerol as carbon and energy source. Bacteria were harvested at the exponential phase of growth by centrifugation (6000 × *g*, 10 min) at room temperature.

Membrane preparation. E. coli membranes were prepared as described by Evans [5]. Basically, the procedure consisted in treatment of the cells with lysozime-EDTA to disrupt the cell wall and obtain the spheroplasts, which were sedimented by centrifugation and then lysed by resuspension in a hypotonic buffer (20 mM Tris-HCl, pH 7.4). The membranes were sedimented, washed twice with the same buffer, and suspended (6–8 mg of protein per ml) in 20 mM Tris-HCl (pH 7.4) containing 1 mM $MgCl_2$. The membrane suspensions were fractioned in small portions which were stored frozen at −30°C until they were used.

Assays of oxidases from the E. coli respiratory chain. NADH oxidase, succinate oxidase and D-lactate oxidase involve, respectively, the components of the electron transport chain between the dehydrogenase specific for each substrate (NADH, succinate or D-lactate) and oxygen as the final electron acceptor [2,3]. The NADH oxidase activity of *E. coli* membranes was assayed as in our previous report [1] recording the absorbance changes at 340 nm in a Gilford spectrophotometer, at 37°C. The reaction was started by addition of 10–20 μl of NADH to 0.5 ml of membrane suspension in 50 mM sodium phosphate (pH 7.5). Succinate and D-lactate oxidases were assayed at 37°C recording the oxygen consumption of membrane suspensions in 2 ml of 50 mM sodium phosphate (pH 7.5), in the presence of 10 mM succinate or 10 mM D-lactate, respectively.

Oxygen uptake. The oxygen consumption was measured at 37°C in a Gilson oxygraph with a Clark electrode.

Determination of Cu(I) and total acid-extractable copper. A method that had been devised for extraction and determination of copper with glacial acetic acid and biquinoline [6], was adapted for estimating the valency state of the copper bound to *E. coli* membranes subjected to different treatments. To 0.25 ml of membrane suspensions (0.4–0.5 mg of protein in 50 mM sodium phosphate, pH 7.5), it was added 10 μl of 5 mM *p*-chloromercuribenzoate (to block protein sulfhydryl groups which could reduce cupric ions to the cuprous state during the extraction process), 20 μl of an ethanolic solution of 2,2′-biquinoline (1 mg/ml), and 0.25 ml of glacial acetic acid. After mixing and allowing to stand 10 min at 37°C, membrane proteins were sedimented in an Eppendorf microfuge and the absorbance of the supernatants was read at 550 nm to detect Cu(I) as a colored complex with biquinoline. Then, a small spatula tipful (5–7 mg) of solid sodium ascorbate was added to reduce the Cu(II) present to cuprous state, and the samples were read again at 550 nm to estimate the total acid-extractable copper. When copper concentrations were too low, greater volumes of samples were processed and the colored biquinoline-Cu(I) complex was quantitatively extracted into 0.5 ml of chloroform, the absorbance of which was read at 550 nm. Blanks without membranes or without biquinoline were used. The amount of copper was calculated from a calibration curve carried out using standard solutions of $CuCl_2$ in 10 mM HCl, and reducing the cupric ions to Cu(I) by addition of solid ascorbate under conditions identical to those of the samples.

It is important to note that before determination of the membrane copper content, substances like NADH or t-BOOH must be eliminated from the samples by centrifugation to avoid interferences. Under the acidic conditions of the assay and in the presence of the chelator biquinoline, NADH acts as a chemical reductant of the Cu(II) present in the sample (just as ascorbate does) and, therefore, only the total copper would be evaluated in the presence of NADH. Although we did not test the effect of the presence of t-BOOH in the copper assay, we routinely eliminated it from the medium to avoid possible reactions of the hydroperoxide during the process of copper extraction and determination.

Transition metal additions. Cu(II), Mn(II), and Fe(III) were added as their chloride salts. The stock solutions were prepared in 0.1 M HCl. A small volume of concentrated salt solution was added to the membrane suspensions so that the pH of the buffered medium was not changed. Fe(III) was also added as an Fe(III)-ADP complex (1 : 10) prepared by diluting the stock solution of ferric chloride with the chelator to the appropriate concentration.

Removal of transition metal ions from the phosphate buffer. In an attempt to limit the trace amounts of adventitious transition metals normally present in most laboratory chemicals and reagents, phosphate buffer used in the assay of NADH oxidase was treated with a chelating ion exchange resin (chelating resin from

Sigma Chemicals Co., comprised of a styrene-divinylbenzene copolymer matrix within which are attached iminodiacetate active groups, similar to the Chelex 100 resin from Bio-Rad Laboratories) which shows high preference for copper, iron and other heavy metals over such cations as sodium, potassium and calcium. 1 liter of 50 mM sodium phosphate (pH 7.5) was passed through a column (15 mm in diameter) loaded with 6.5 g of resin (sodium form), at a flow rate of 1 ml/min. The pH of the eluate was tested until it decreased from an initial value of about 10 to a stable value of 7.5, point at which the eluting buffer began to be recovered. All the glassware used in the experiments with this resin-treated buffer was washed with diluted HCl and rinsed with bidistilled water. Iron concentration (evaluated by atomic absorption spectroscopy) in the 50 mM phosphate buffer was $1.8 \cdot 10^{-6}$ M, and it decreased to $2.5 \cdot 10^{-7}$ M after treatment with the resin. Dosage of copper (by atomic absorption spectroscopy) was performed in 10-fold more concentrated phosphate (500 mM) giving a value of $1.6 \cdot 10^{-6}$ M, which decreased to an undetectable limit (below 10^{-7} M) after resin treatment.

The resin-treated buffer was used in the experiments of Fig. 1 and 3; all the other experiments were performed with untreated buffer.

Protein determination. Membrane proteins were measured by the method of Lowry et al. [7].

Results

Identification of the cation implicated in t-BOOH toxicity

To identify the metal that mediates the effect of t-BOOH on the NADH oxidase from the *E. coli* respiratory chain, two experimental approaches were used.

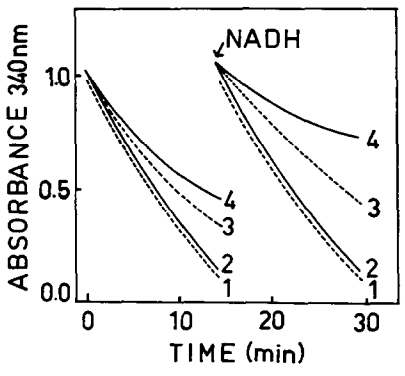


Fig. 1. Time-course of NADH oxidase in the absence and presence of t-BOOH, using resin-treated or untreated phosphate buffer. NADH oxidase was assayed in the untreated 50 mM sodium phosphate, pH 7.5 (samples 2 and 4), and in the same buffer previously treated with the chelating resin as indicated in Materials and Methods (samples 1 and 3). t-BOOH (0.5 mM) was present in samples 3 and 4. More NADH was added to the samples when indicated by the arrow. Membrane protein concentration was 15 μg/ml.

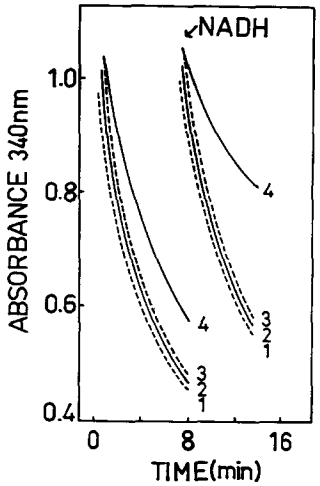


Fig. 2. Blockade of the t-BOOH effect by the chelator bathocuproine disulfonate. NADH oxidase was assayed in the absence (samples 1 and 2) and presence (samples 3 and 4) of 0.5 mM t-BOOH. Bathocuproine disulfonate (35 μM) was present in samples 1 and 3. More NADH was added to the samples when indicated by the arrow. Membrane protein concentration was 23 ug/ml.

One of them was based on the employment of chelators specific for transition metals suspected of being implicated in the process; the other was based on the addition of different cations to the system to detect whether anyone was able to increase the t-BOOH effect.

Traces of metal cations such as iron, copper, lead and others are usually present as impurities in phosphate buffers. Therefore, we tried to eliminate or decrease these metals from the buffer used for the NADH oxidase assay by treatment with a chelating resin (see Materials and Methods). As shown in Fig. 1, the t-BOOH effect on the NADH oxidase was decreased but not eliminated when the resin-treated phosphate buffer was used.

Bathocuproine disulfonate is a water-soluble chelator of Cu(I) [8] with no appreciable capacity for complexing iron [9]. When present in the assay medium at 35 μM, this chelator blocked completely the t-BOOH effect on the NADH oxidase (Fig. 2).

The water soluble chelator ferrozine forms a stable and intensely colored complex with Fe(II) [10,11]. Although it also forms a colored complex with Cu(I) [12], its efficiency as a copper chelator is much lower than that of bathocuproine disulfonate, fact evidenced by competition tests for Cu(I) bound to the chelator 2,2'-biquinoline. Spectroscopic studies (not shown) indicated that about 80% of the Cu(I) binding was transferred from 2,2'-biquinoline to bathocuproine disulfonate at identical concentrations of these chelators; meanwhile, no appreciable displacement of the Cu(I) from its complex with 2,2'-biquinoline was observed with a 10-fold higher concentration of ferrozine. In an experiment similar to that of Fig. 2, ferrozine was

unable to avoid the effect of t-BOOH on the NADH oxidase, at concentrations up to 250 μM.

The effect of bathocuproine disulfonate and ferrozine in our system correlates with their relative abilities for complexing Cu(I), strongly indicating that the metal implicated is copper.

Addition of $CuCl_2$ to the assay system at concentrations from 0.2 to 1 μM accelerated the inactivation of NADH oxidase by t-BOOH (Fig. 3). At these concentrations, $CuCl_2$ had no effect on the oxidase in the absence of the hydroperoxide (Fig. 3), although at levels higher than 1 μM copper alone began to be inhibitory. The concentration range in which $CuCl_2$ accelerates the t-BOOH effect but does not by itself inhibit the NADH oxidase, varies in parallel with the membrane concentration (see below, Fig. 4).

Neither $FeCl_3$ nor $MnCl_2$, added at concentrations between 0.2 and 10 μM in an experiment similar to that of Fig. 3, had any influence on the effect of t-BOOH on the NADH oxidase. Not either iron added as an Fe(III)-ADP complex at concentrations up to 0.2 mM Fe(III)/2 mM ADP could accelerate the effect of t-BOOH on the NADH oxidase, even though at that concentration the iron-ADP complex was able to stimulate the NADH-dependent lipid peroxidation in bovine heart mitochondria [13,14].

These results indicate that copper ions mediate the effect of t-BOOH on the NADH oxidase.

Copper ions involved in t-BOOH toxicity bind to the membranes

The data shown above made us suspect that *E. coli* membranes contain copper capable of mediating the t-BOOH effect, and that that copper pool could be increased by binding of cupric ions from the medium. To test that possibility, membranes were preincubated in phosphate buffer with or without addition of $CuCl_2$ and then sedimented by centrifugation, rinsed and resuspended in phosphate buffer to determine the variations in membrane copper content, NADH oxidase activity and t-BOOH effect. In order to evaluate the valency state of the membrane-bound copper, a method based on the formation of a colored complex with biquinoline (see below) was used for estimating the variations in membrane copper content. As shown in Fig. 4A, membrane exposure to $CuCl_2$ led to increased levels of membrane-bound Cu(I) and total copper [Cu(I) plus Cu(II)]. The NADH oxidase activity remained unchanged up to about 20 μM $CuCl_2$ and thereon began to decrease (Fig. 4A), indicating that high levels of copper are by themselves (in the absence of t-BOOH) toxic to the NADH oxidase. The effect of t-BOOH was faster in membranes preincubated with $CuCl_2$ and increased in parallel with the copper concentration (Fig. 4B), in the range in which copper alone had no effect on the NADH oxidase. That was a 'functional' way of visualizing the binding of cupric ions to a membrane copper pool involved in t-BOOH toxicity. Note that for detection of the membrane-bound copper, the experiment in Fig. 4 was performed with a membrane concentration about 20-fold higher than that of the experiment in Fig. 3. Consequently, the concentration range in which $CuCl_2$ accelerated the t-BOOH effect but did not by itself inhibit the

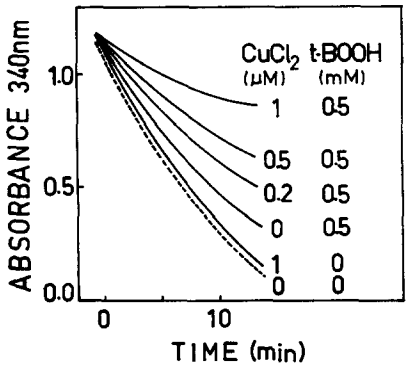


Fig. 3. Time-course of NADH oxidase in the absence and presence of t-BOOH, at different concentrations of added $CuCl_2$. NADH oxidase was assayed in the resin-treated phosphate buffer, in the presence of the $CuCl_2$ and t-BOOH concentrations indicated for each sample. Membrane protein concentration was 20 μg/ml.

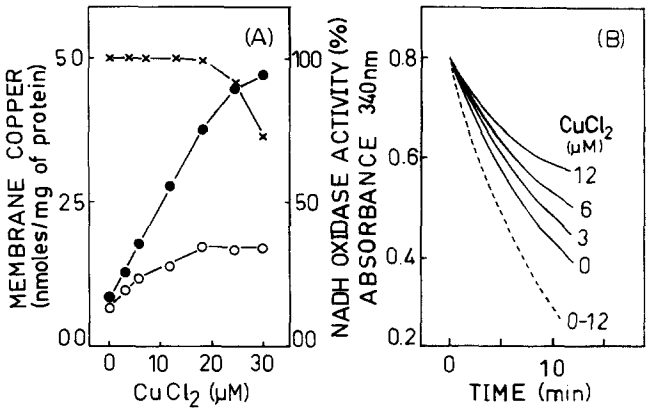


Fig. 4. Variations in membrane copper content, NADH oxidase activity and t-BOOH effect after membrane preincubations with $CuCl_2$. Seven identical samples of membranes (0.51 mg of protein in 1.2 ml of 50 mM sodium phosphate, pH 7.5) were preincubated 10 min at 37°C in the presence of 0, 3, 6, 12, 18, 24, and 30 μM $CuCl_2$, respectively. Then, the membranes were sedimented by centrifugation, rinsed and resuspended in 0.24 ml of the same buffer. From each one of these samples, two 20 μl aliquots were withdrawn for the assay of NADH oxidase in the absence and presence of 0.5 mM t-BOOH, and the remaining 200 μl were used for determining the membrane content of Cu(I) and total copper (see Materials and Methods). Panel A shows the variations in membrane Cu(I) (open circles), total copper (closed circles) and NADH oxidase activity (crosses) as a function of the $CuCl_2$ concentration added to the medium. Each value is the mean of three experiments. Panel B shows the time-course of the NADH oxidase activity in the absence (dashed line) and presence (solid lines) of 0.5 mM t-BOOH, from membranes exposed to the indicated $CuCl_2$ concentrations.

NADH oxidase, was proportionally shifted to higher levels.

The following experiments were performed with membranes preincubated in the presence of $CuCl_2$ within the concentration range in which copper did not by itself inhibit the NADH oxidase.

Redox cycling of copper

Studies have suggested that redox-active transition metals act as catalysts for the formation of free radicals during genesis of oxidative injury [15–17]. A commonly proposed mechanism of damage is associated with site-specific metal-mediated Fenton chemistry, in which a reduced transition metal ($M_e^{(n-1)+}$) reacts with a hydroperoxide (ROOH), leading to its breakdown and free-radical generation [18–21]:

$$M_e^{(n-1)+} + ROOH \rightarrow M_e^{n+} + RO^{\circ} + OH^-$$

Since the effect of t-BOOH on the NADH oxidase depended on the presence of the reductant NADH [1] and was mediated by copper, we hypothesized that the damage resulted from activation of t-BOOH through its interaction with Cu(I) in a Fenton-type reaction. That process would be dependent on reduction equivalents (NADH) to generate Cu(I) from Cu(II) bound to the membranes. Therefore, we tested whether *E. coli* membranes had the ability to reduce Cu(II) to Cu(I) with NADH as electron donor. First, we had to find out a proper method to detect Cu(I) in our system. We adapted a method that had been devised for extraction and estimation of copper with glacial acetic acid and biquinoline [6]. Since the biquinoline forms a colored complex only with cuprous copper, the method is useful for estimating the valency state of the copper in the native material by carrying out the determination in the presence and in the absence of a reducing agent.

In the experiment of Fig. 5, membranes were preincubated in the presence and absence of NADH and then exposed to t-BOOH to analyze the variations in the redox state of copper and in the NADH oxidase activity. As can be seen, membrane preincubation with NADH at 37°C led to a significant increase in the level of membrane Cu(I) while the amount of total copper remained unchanged, demonstrating reduction of Cu(II) to Cu(I) under the given conditions. Copper reduction did not occur neither when the incubation with NADH was carried out at 0°C (condition under which t-BOOH had no effect on the NADH oxidase), nor when the membranes were inactivated by heating at 80°C for 5 min before the incubation with NADH at 37°C (not shown). Thus, copper reduction with NADH as electron donor was mediated by membrane proteins. Incubations with NADH for periods longer than 10 min did not result in enhancement of the membrane Cu(I) content, indicating that that was the maximal

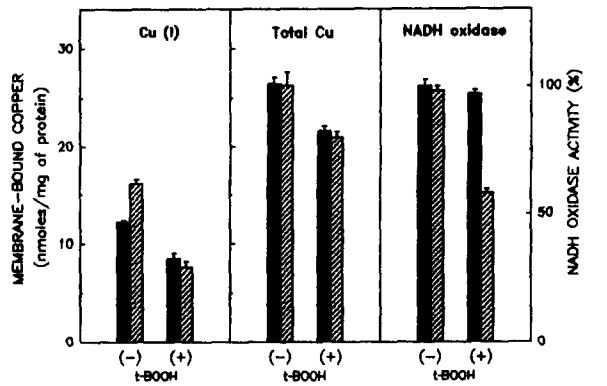


Fig. 5. Interaction between t-BOOH and the membrane-bound copper, and its effect on the NADH oxidase activity. Four membrane samples (0.32 mg of protein in 1 ml of 50 mM sodium phosphate, pH 7.5) were subjected to three sequential preincubations of 10 min at 37°C under the following conditions (after each preincubation the membranes were spun-down, rinsed and resuspended in the same buffer): 1st, 9 μM $CuCl_2$ present in all the samples; 2nd, two samples in the absence (black bars) and the other two in the presence (dashed bars) of NADH (its consumption was followed at 340 nm to avoid depletion by repeated additions of 10 μl of 15 mM NADH); 3rd, 0.5 mM t-BOOH present in one sample from each pair. Finally, aliquots were taken from the samples to determine the Cu(I), the total copper, and the NADH oxidase activity. This activity was expressed as a percentage of the control preincubated without NADH and t-BOOH. Data are the mean ± standard deviation of six experiments.

amount of copper reducible by NADH under the given conditions. Maximal reduction of copper was achieved in less than 10 min, but the time-course of copper reduction was not accurately established because no suitable way for stopping the redox reaction before membrane centrifugation (which took about 5 min) was available.

As shown in Fig. 5, exposure to t-BOOH resulted in significant inactivation of the NADH oxidase from membranes preincubated with NADH, whereas membrane exposure to only t-BOOH (preincubation without NADH) had little effect on the NADH oxidase activity. This is consistent with the view that NADH-supported copper reduction mediates the effect of t-BOOH on the NADH oxidase. As can be seen (Fig. 5), t-BOOH interacted not only with the Cu(I) generated by reduction of Cu(II) during the preincubation with NADH, but also with a fraction of the Cu(I) already present in the membranes preincubated in the absence of NADH. Note that the amount of Cu(I) remaining after the t-BOOH treatment was the same for membranes preincubated with and without NADH; this Cu(I) seemed to be unable to interact with t-BOOH since longer incubations with the hydroperoxide did not result in a greater decrease of the Cu(I) content.

The experiment in Table I was designed to study further the interaction between membrane Cu(I) and t-BOOH. As it can be seen, exposure of the membranes to t-BOOH led to a decrease in the Cu(I)

TABLE I

Redox cycling of copper

5 identical membrane samples (0.45 mg of protein in 1 ml of 50 mM sodium phosphate (pH 7.5)) were subjected to five sequential preincubations of 10 min at 37°C under the following conditions (after each preincubation, the membranes were sedimented by centrifugation, rinsed and resuspended in 1 ml of the same buffer): during the first preincubation 6 μM $CuCl_2$ was present in all the samples; in the second preincubation 0.5 mM t-BOOH was present in all the samples except 1; in the third preincubation NADH was added only to samples 3, 4 and 5 (the amount of added NADH was controlled as indicated in the legend of Fig. 5); in the fourth preincubation 0.5 mM t-BOOH was present only in samples 4 and 5; in the fifth preincubation NADH was added to sample 5. Finally, the membranes were resuspended in 0.25 ml of the phosphate buffer to determine their Cu(I) and total copper contents. Data are expressed as the mean ± S.D. of six experiments.

Sample	Sequential treatments					Membrane copper (nmol/mg of protein)	
	1st	2nd	3rd	4th	5th	Cu(I)	Total
1	$CuCl_2$					10.73 ± 0.74^{b}	18.70 ± 1.26^{g}
2	$CuCl_2$	t-BOOH				5.95 ± 0.36^{c}	16.10 ± 1.47^{h}
3	$CuCl_2$	t-BOOH	NADH			8.19 ± 0.18^{f}	16.46 ± 1.51^{h}
4	$CuCl_2$	t-BOOH	NADH	t-BOOH		6.10 ± 0.20^{c}	15.99 ± 1.31^{h}
5	$CuCl_2$	t-BOOH	NADH	t-BOOH	NADH	7.96 ± 0.19^{f}	16.36 ± 1.56^{h}

[b,c,f,g,h] Means within each column with a different superscript differ significantly as evaluated by the Tukey-Keuls's test ($P < 0.01$).

content together with a small loss of total copper (compare sample 2 with 1), as already shown in Fig. 5. Note that after exposure to only t-BOOH the membranes retained their full NADH oxidase activity as well as their full potential for being affected by t-BOOH in the presence of NADH (see Fig. 6) and, therefore, the small fraction of total copper eliminated from the membranes by t-BOOH treatment is not implicated in the effect of the hydroperoxide on the NADH oxidase. As shown in Table I, the subsequent incubation of the membranes with NADH resulted in reduction of Cu(II) to Cu(I) without change in the total copper content (compare samples 2 and 3). That Cu(I) was completely oxidized during the subsequent exposure to t-BOOH (compare samples 3 and 4), and reduced again by incubation with NADH (compare samples 4 and 5). Note that the total copper content remained unchanged from the second to the fifth preincubations, while the redox status of a small pool of membrane copper (about 2 nmol/mg protein) cycled between the mono and divalent states. Therefore, data of Table I show that a small pool of the membrane-bound copper can be reduced in the presence of NADH and oxidized by exposure to t-BOOH, in a cyclic fashion. Hence, that pool of membrane copper can catalyze the membrane-mediated electron transfer from NADH to t-BOOH and so mediate the t-BOOH effect on the respiratory chain. The membrane copper pool reducible by NADH increased by exposition of the membranes to $CuCl_2$, up to an apparent saturation value of about 5 nmoles of copper per mg of protein (not shown) which probably represents the maximal size of the copper pool involved in t-BOOH toxicity. That saturation point was reached at the highest $CuCl_2$ concentration in which copper accelerated the t-BOOH effect but did not by itself inhibit the NADH oxidase.

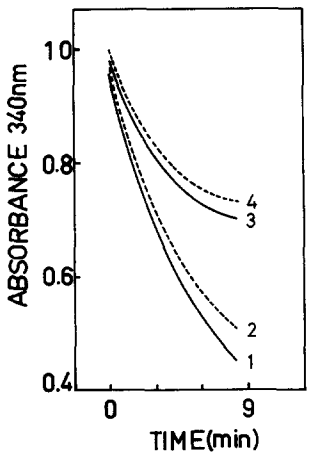


Fig. 6. Membrane preincubation with t-BOOH has no appreciable effect on the NADH oxidase. Four identical membrane samples (0.15 mg of protein) were preincubated 10 min at 37°C with 6 μM $CuCl_2$ in 0.5 ml of 50 mM sodium phosphate (pH 7.5). The membranes were then sedimented and resuspended in 0.5 ml of the same buffer. Two of these samples were preincubated in the absence (solid lines) and the other two in the presence (dashed lines) of 0.5 mM t-BOOH for 10 min at 37°C. After centrifugation and membrane resuspension in the buffer, the NADH oxidase activity of the samples was assayed in the presence (samples 3 and 4) and the absence (samples 1 and 2) of 0.5 mM t-BOOH, starting by NADH addition at zero time on the abscissa.

Other respiratory substrates also support the reduction of membrane copper and lead to impairment of the respiratory chain by t-BOOH

Copper reduction might be mediated by the electron flow through the respiratory chain. The membrane copper pool involved in t-BOOH toxicity might be associated to components of the electron transport chain in such a way that copper could be reduced by electrons coming from NADH and other respiratory substrates. Consistent with this view is the fact that

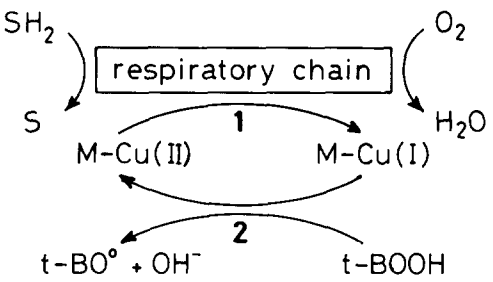
succinate and D-lactate (which in *E. coli* are oxidized by specific membrane-bound dehydrogenases linked to the respiratory chain) also acted as electron donors for the reduction of membrane-bound copper (not shown). Moreover, membrane preincubation with t-BOOH in the presence of anyone of the substrates NADH, succinate or D-lactate led to impairment of the respiratory chain, monitored by irreversible inactivation of the three oxidases (NADH, succinate and D-lactate oxidases); whereas membrane preincubation with only t-BOOH had no significant effect on any of these oxidases (not shown). In every instance, the process of inactivation was accelerated by addition of $CuCl_2$ and avoided by the presence of the chelators EDTA or bathocuproine disulfonate. Taken as a whole, these findings indicate that damage of the respiratory chain by t-BOOH is dependent on the presence of substrates that support an electron flow through the respiratory chain and lead to reduction of membrane-bound copper. Questions about what are the respiratory chain components involved in copper binding and/or reduction and what are the targets inactivated by t-BOOH are under further investigation.

Discussion

This research clearly demonstrates a role of copper as a mediator of t-BOOH toxicity in *E. coli*. The implicated copper is bound to the *E. coli* membranes, and this pool of membrane-bound copper increases when the copper concentration of the medium is raised, up to an apparent saturation value of about 5 nmol of copper per mg of membrane proteins. Copper binding to these sites is strong, since it is not rapidly released after centrifugation and resuspension of the membranes in a medium of much lower copper content. However, copper involved in t-BOOH toxicity is sequestered by chelators such as EDTA and bathocuproine disulfonate, leading to blockade of the hydroperoxide effect. Therefore, the copper content of the membranes (and thus their sensitivity toward t-BOOH) is strongly dependent on their previous treatment and the medium conditions regarding metal concentration and the presence of chelators. Evidence for a catalytic role of copper in oxidative injury has precedents [22–27].

Our previous study [1] suggested the involvement of a metal ion-catalyzed redox reaction in the process of respiratory injury induced by t-BOOH. This is confirmed by the results of the present paper. We demonstrate for the first time the ability of *E. coli* membranes to reduce Cu(II) to Cu(I) in the presence of NADH and other respiratory substrates (succinate, D-lactate), and show that this Cu(I) is oxidized by t-BOOH in a cyclic fashion. Though in our system there is Cu(I) which seems to be quite stable in the presence of oxygen, it can not be ruled out that a fraction of the Cu(I) was oxidized or disproportionated during the previous steps (membrane centrifugation and washing) or during the assay. If that were the case we would have been underestimating the amount of reduced copper

We propose as a working hypothesis that damage results from activation of the hydroperoxide through its interaction with Cu(I) in a site-specific Fenton-type reaction (Eqn. 2) as shown below, where M-Cu represents the membrane-bound copper whose reduction is mediated by membrane proteins and depends on electron flow through the respiratory chain (Eqn. 1), and t-BO° represents the t-butylalkoxy radical generated by reductive cleavage of t-BOOH.



It should be noted that the breakdown of hydroperoxides by transition metals results in free radical formation [28–31]. Since copper is very potent in catalysis of Fenton reactions [17, 32], even a very small fraction of copper in a redox-active form could be a significant mediator of free radical formation. Studies of Davies [33] demonstrated that reductive cleavage of t-BOOH by rat liver microsomes can occur in the presence of NADH or NADPH, generating alkoxyl and carbon-centred radicals detected by electron spin resonance spectroscopy. Therefore, the t-BOOH-induced damage may be a consequence of a free radical attack. The precise mechanism of this attack and the identity of the respiratory chain components that result affected need to be further investigated.

Acknowledgements

We thank María Teresa Foá de Mockevich for her help in the correction of the manuscript. This work was supported in part by Grant PID 3-013800/89 from the Consejo Nacional de Investigaciones Científicas y Técnicas, by the Consejo de Ciencia y Técnica of the Universidad Nacional de Tucumán, and by Fundación Antorchas.

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