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Inhibition of enzymes and oxidative damage of red blood cells induced by *t*-butylhydroperoxide-derived radicals

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The effects of *t*-butylhydroperoxide ('BHP), its alkoxyl radical ('BuO·) and its peroxyl radical ('BuOO·) in model systems and on red blood cells were studied. Glyceraldehyde-3-phosphate dehydrogenase was strongly inhibited by 'BHP via a direct reaction of the hydroperoxide with an essential sulfhydryl group in the enzyme molecule. Several other enzymes were unaffected by 'BHP. Alcohol dehydrogenase was strongly inhibited by 'BuO· but was much less sensitive to 'BuOO·. Lysozyme, lactate dehydrogenase and trypsin, on the other hand, were very sensitive to the peroxyl and not, or much less, to the alkoxyl radical, whereas acetylcholinesterase was very sensitive to both radicals. 'BuOO· caused covalent binding of tryptophan, tyrosine, histidine and methionine to serum albumin. The corresponding alkoxyl radical was ineffective in this respect. Conversely, 'BuO· caused peroxidation of linolenic acid, whereas 'BuOO· did not. Incubation of human erythrocytes with 'BHP caused lipid peroxidation and K^+ leakage. Both effects were caused by 'BHP-derived radicals generated in a reaction of the hydroperoxide with hemoglobin. With radical scavengers it was possible to dissociate 'BHP-induced lipid peroxidation and K^+ leakage, demonstrating that these two processes are not causally related. Experimental results indicate that 'BuO· causes lipid peroxidation, whereas 'BuOO· is responsible for K^+ leakage.

Introduction

Hydroperoxides and their catalytic decomposition products are thought to play an important role in metabolism under physiological and pathological conditions [1–5]. Therefore, a correct understanding of the mode of action of these compounds in biological systems is essential. With ESR spin trapping it has been shown that catalytic decomposition of 'BHP by hematin(Fe^{3+}) yielded predominantly the peroxyl radical [4,6], whereas with ferrous bipyridyl as a catalyst the alkoxyl radical was formed [7]. Also, photolytic breakdown of hydroperoxides yielded primarily alkoxyl radicals [8,9]. Apparently, these observations can be used to manipulate the decomposition route of hydroperoxides in biological model systems and thus to discriminate between the effects of hydroperoxide-derived alkoxyl and peroxyl radicals.

'BHP has been used in numerous investigations as a model to establish the effects of hydroperoxides on biological systems [2]. Although these studies clearly indicate that hydroperoxides have a profound influence on many cellular structures and functions, it is in most cases unknown whether these effects should be attributed to the reactivity of the hydroperoxide itself, its alkoxyl radical or its peroxyl radical. Yet to elucidate the actual role of hydroperoxides in cellular metabolism, such a distinction is essential.

It was attempted in the present study to distinguish between the effects of 'BHP, 'BuO· and 'BuOO· on biological model systems and on red blood cells. It will be shown that the reactivities of 'BHP, its alkoxyl radical and its peroxyl radical towards various biologically important molecules are quite different.

Materials and Methods

Glyceraldehyde-3-phosphate dehydrogenase, alcohol dehydrogenase, lactate dehydrogenase, acetyl cholinesterase, lysozyme and trypsin were obtained from Boeh-

Abbreviations: 'BHP, *t*-butylhydroperoxide; 'BuO·, *t*-butylalkoxyl radical; 'BuOO·, *t*-butylperoxyl radical; BHT, butylated hydroxytoluene.

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ringer. ^{14}C-labeled compounds were purchased from Amersham.

Heparinized human blood was centrifuged shortly after collection. The red blood cells were washed three times and resuspended at 10% hematocrit in phosphate-buffered isotonic NaCl solution. Incubations were carried out at room temperature. Resealed, Na^+-containing erythrocyte ghosts, with a residual hemoglobin content of less than 0.01 mg hemoglobin/mg total membrane protein were prepared as described by Girotti and Thomas [10]. Carbonmonooxyhemoglobin-containing red blood cells were prepared as described previously [11]. Conversion of cellular hemoglobin to methemoglobin was brought about as described by Thornalley et al. [12].

The effects of ^{t}BHP were studied in solutions containing 1 mM of the hydroperoxide. Generation of ^{t}BuOO$^\cdot$ was accomplished by the addition of 0.5 mM hematin(Fe^{3+}) to the reaction mixture, as described by Kalyanaraman et al. [4], whereas the formation of ^{t}BuO$^\cdot$ was catalyzed by addition of 0.5 mM ferrous bipyridyl [7]. In some experiments, ^{t}BuO$^\cdot$ was generated by irradiation (254 nm) of ^{t}BHP-containing mixtures with a low-pressure mercury lamp (fluence rate: 0.2 J/m^2 per s) [8,9]. In control experiments, it appeared that neither hematin(Fe^{3+}) nor ferrous bipyridyl had any influence on the parameters measured in the present studies when added in the absence in ^{t}BHP.

Linolenic acid solutions were prepared in 1% Tween 20 as described by Tien et al. [13]. The influence of ^{t}BHP and ^{t}BHP-derived radicals on solubilized enzymes was studied by incubation of the enzyme solution (1 mg/ml) in 50 mM phosphate buffer (pH 7.4) at room temperature with ^{t}BHP alone, or in the simultaneous presence of either hematin or ferrous bipyridyl. Subsequently, enzyme activity was measured after appropriate dilution. Glyceraldehyde-3-phosphate dehydrogenase was measured according to Wu and Racker [14], acetylcholinesterase as described by Ellman et al. [15], lactate dehydrogenase by the method of Bergmeyer and Berndt [16], alcohol dehydrogenase as described by Bergmeyer et al. [17], lysozyme according to Shugar [18] and trypsin by the method of Schwert and Takenaka [19]. Labeling of the essential sulfhydryl group of glyceraldehyde-3-phosphate dehydrogenase with iodo-[^{14}C]acetic acid was performed as described by Trentham [20]. Covalent binding of amino acids to serum albumin was measured as described before [21], utilizing ^{14}C-labeled amino acids.

K^+ leakage from red blood cells and Na^+ leakage from ghosts were determined with a flame photometer and expressed as a percentage of total efflux evoked by lysis of the cells in distilled water. Lipid peroxidation was assayed by measuring the generation of thiobarbituric-acid-reactive products [22] and by monitoring the formation of chromolipids [23].

For all measurements, values were within the range ± 8%, with three to six independent experiments for each datum point.

Results

Inhibition of solubilized enzymes

Exposure of glyceraldehyde-3-phosphate dehydrogenase to ^{t}BHP in the absence of a catalyst resulted in fast inactivation of the enzyme. Residual enzyme activities after incubation periods of 5, 10, 15 and 30 min were 23, 12, 7 and less than 2%, respectively, whereas iodo[^{14}C]acetate binding to the enzyme decreased simultaneously to 21, 15, 6 and 3% of the initial value. None of the other enzymes tested was inhibited by ^{t}BHP in the absence of a catalyst.

Alcohol dehydrogenase was strongly inhibited in the simultaneous presence of ^{t}BHP and ferrous bipyridyl, whereas with ^{t}BHP and hematin a much slower inhibition developed (Fig. 1). With lysozyme the results were opposite: in the presence of hematin(Fe^{3+}), ^{t}BHP caused a fast and complete inhibition of the enzyme, whereas in the presence of ferrous bipyridyl and ^{t}BHP only a slight inhibition was observed (Fig. 1). As this suggested that different enzymes may have different susceptibilities towards alkoxyl and peroxyl radicals, three other enzymes were included in these experiments. The results are shown in Table I. Lactate dehydrogenase, acetylcholinesterase and trypsin were all strongly inhibited with hematin(Fe^{3+}) as a catalyst. With ferrous bipyridyl, acetylcholinesterase was inactivated, whereas lactate dehydrogenase and trypsin were much less sensitive.

Covalent binding of amino acids to serum albumin

^{14}C-labeled tryptophan, tyrosine, methionine and histidine were incubated with serum albumin and ^{t}BHP

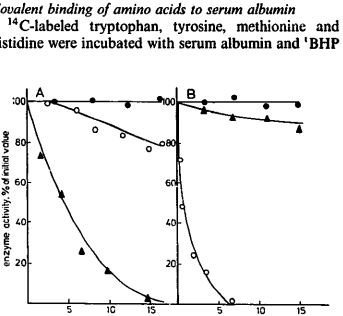

Fig. 1. Inhibition of alcohol dehydrogenase (A) and lysozyme (B) during incubation with ^{t}BHP (●), ^{t}BHP plus hematin (○) and ^{t}BHP plus ferrous bipyridyl (▲).

TABLE I

Inhibition of solubilized enzymes by 'BHP, 'BuO· and 'BuOO·

Inhibition (mean of three to five experiments) was measured after an incubation period of 15 min with 'BHP, 'BHP plus ferrous bipyridyl or 'BHP plus hematin.

	Inhibition (%)		
	'BHP	'BHP + Fe^{2+}-bipyridyl	'BHP + Fe^{3+}-hematin
Glyceraldehyde-3-phosphate dehydrogenase	93	–	–
Alcohol dehydrogenase	0	98	10
Lysozyme	0	8	100
Lactate dehydroganase	0	< 4	85
Acetylcholinesterase	0	85	92
Trypsin	0	12	72

alone, or in the simultaneous presence of either hematin(Fe^{3+}) or ferrous bipyridyl. As covalent binding always reached a constant level after an incubation period of 20 min, binding of the amino acids to serum albumin was measured after an incubation period of 30 min. The results showed that covalent binding occurred only in the simultaneous presence of 'BHP and hematin, indicating that the 'BuOO· radical is involved in these processes (Table II).

Lipid peroxidation

In the absence of a catalyst, 'BHP did not cause peroxidation of solubilized linolenic acid or erythrocyte membrane lipids. Also, addition of hematin(Fe^{3+}) to the reaction mixture did not lead to lipid peroxidation. Only in the simultaneous presence of 'BHP and ferrous bipyridyl did lipid peroxidation occur, as indicated by the generation of thiobarbituric-acid-reactive products and the formation of chromolipids (Fig. 2).

Effects on intact red blood cells

'BHP induced lipid peroxidation and K^+ leakage when added to a suspension of intact red blood cells. Pretreatment of the cells with CO slightly stimulated both processes, whereas conversion of cellular hemoglobin to methemoglobin strongly inhibited both K^+

TABLE II

Binding of ^{14}C-labeled amino acids to serum albumin after incubation of the mixtures with 'BHP, 'BHP plus ferrous bipyridyl or 'BHP plus hematin

	Binding (nmol amino acid/mg albumin)		
	'BHP	'BHP + Fe^{2+}-bipyridyl	'BHP + Fe^{3+}-hematin
Tryptophan	0	0.02	1.60
Tyrosine	0	0.01	23.81
Methionine	0	0.08	1.42
Histidine	0	0.04	4.25

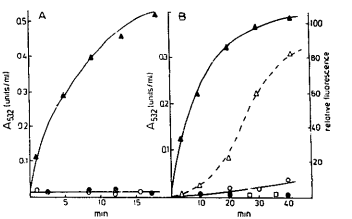

Fig. 2. Lipid peroxidation of linolenic acid (A) and of erythrocyte membrane lipids (B) as measured by the generation of thiobarbituric acid-reactive products (A_{532}) (○, ● and ▲) and of chromolipids (relative fluorescence) (□ and △). ○, 'BHP; ●, 'BHP plus hematin; ▲, 'BHP plus ferrous bipyridyl; □, 'BHP and hematin; △, 'BHP and ferrous bipyridyl.

loss and lipid peroxidation (Fig. 3). The effects of a number of radical scavengers are shown in Table III. BHT and promethazine strongly inhibited both lipid peroxidation and K^+ leakage. Most interesting are the effects of thiourea and diphenylamine. Thiourea inhibited 'BHP-induced K^+ loss, without affecting lipid peroxidation. Diphenylamine, contrarily, inhibited 'BHP-induced lipid peroxidation, but augmented K^+ leakage.

In further experiments, methemoglobin-containing cells were exposed to 'BHP in the presence of 100 mM isopropanol and illuminated with ultraviolet light of 254 nm. Isopropanol was added to scavenge the hydroxyl radicals generated during the photolytic decomposition of 'BHP [8,9]. As shown in Fig. 3, ultraviolet illumination caused increased lipid peroxidation. Initially, K^+ leakage was not affected, but after prolonged incubation periods, a small but significant and reproducible increase of K^+ loss was observed. In control experiments it appeared that in the absence of 'BHP, ultra-

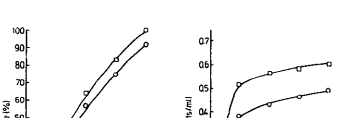

Fig. 3. K^+ leakage and lipid peroxidation in erythrocytes exposed to 'BHP. ○, oxyhemoglobin-containing cells; □, carbon monooxyhemoglobin-containing cells; △, methemoglobin-containing cells; ●, methemoglobin-containing cells exposed to ultraviolet light.

TABLE III

Effect of free-radical scavengers on K^+ leakage and lipid peroxidation in red blood cells exposed to 'BHP

A 10% suspension of oxyhemoglobin-containing cells was incubated with 1 mM 'BHP for 60 min. Initial A_{532} and K^+ leakage = 0. Figures in parentheses are percent of control without scavengers.

Addition	Concentration (mM)	K^+ leakage (%)	Lipid peroxidation (A_{532} units/ml suspension)
None		75 (100)	0.583 (100)
BHT	0.1	12 (16)	0.050 (8)
Promethazine	0.1	5 (7)	0.056 (10)
Thiourea	10.0	20 (27)	0.545 (94)
Diphenylamine	0.1	90 (120)	0.053 (9)

violet illumination did not cause lipid peroxidation or K^+-loss. Further, without ultraviolet illumination, isopropanol did not affect 'BHP-induced K^+ leakage and lipid peroxidation in methemoglobin-containing cells.

The experiments on intact red blood cells were also conducted with cumene hydroperoxide instead of 'BHP. Under all experimental conditions, the results were quite similar.

Effects on resealed ghosts

Addition of 'BHP or cumene hydroperoxide to resealed, low-hemoglobin ghosts did not cause lipid peroxidation or Na^+ leakage above the control. If ferrous bipyridyl was simultaneously added, lipid peroxidation occurred readily (fig. 2B), whereas Na^+ leakage increased only slightly (Fig. 4). With hematin(Fe^{3+}) as a catalyst, the effects were reversed. No lipid peroxidation could be detected with the thiobarbituric acid method or the chromolipid assay, whereas Na^+ loss was strongly enhanced (Fig. 4). With intact, methemoglobin-containing red blood cells similar results were obtained: ferrous bipyridyl augmented 'BHP-induced lipid peroxidation without affecting K^+ loss, whereas hematin(Fe^{3+}) augmented 'BHP-induced K^+ leakage, without affecting lipid peroxidation (data not shown).

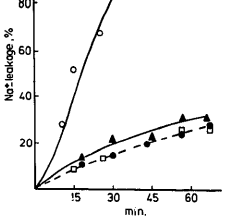

Fig. 4. 'BHP-induced Na^+ leakage from resealed ghosts. □, control; ●, 'BHP; ▲, 'BHP plus ferrous bipyridyl; ○, 'BHP plus hematin.

Discussion

'BHP is rather stable in aqueous solution. When a suitable catalyst is added, however, two types of radical can be generated. In the presence of hematin(Fe^{3+}) peroxyl radicals ar formed according to the reaction:

$$\text{hematin}(Fe^{3+}) + \text{ROOH} \rightarrow \text{hematin}(Fe^{2+}) + \text{ROO}^{\cdot} \quad (1)\ [4,6]$$

With ferrous bipyridyl, on the other hand, and during ultraviolet irradiation, alkoxyl radicals are generated according to the reactions:

$$Fe^{2+} + \text{ROOH} \rightarrow Fe^{3+} + OH^- + \text{RO}^{\cdot} \quad (2)\ [7]$$

$$\text{ROOH} \xrightarrow{UV} \text{OH}^{\cdot} + \text{RO}^{\cdot} \quad (3)\ [8]$$

Further, it has been shown before that reaction of hydroperoxides with heme-containing proteins yields both alkoxyl and peroxyl radicals [4,12,25,26]. It should be emphasized that generation of a particular type of radical, according to one of these reactions, may lead to generation of the other radical in secondary reactions, for example:

$$\text{RO}^{\cdot} + \text{ROOH} \rightarrow \text{ROO}^{\cdot} + \text{ROH} \quad (4)\ [8]$$

$$2\text{ROO}^{\cdot} \rightarrow 2\text{RO}^{\cdot} + O_2 \quad (5)\ [4]$$

The velocities of these secondary reactions will obviously depend on factors such as the concentrations of the primarily generated radical and the original hydroperoxide and on the velocity of the reaction of the primarily generated radical with target molecules. Despite this complicating factor, the present studies demonstrate that it is possible to discriminate between the effects of 'BHP itself and its peroxyl and alkoxyl radicals (Fig. 5).

Glyceraldehyde-3-phosphate dehydrogenase was inhibited by 'BHP in the absence of a catalyst. Enzyme activity and iodo[^{14}C]acetate binding decreased to the same extent. As iodoacetate reacts, under the present experimental conditions, exclusively with the essential thiol group in the active center of the enzyme [20], this indicates that enzyme inhibition is caused by reaction of 'BHP with this thiol group, in accordance with earlier observations [11]. With the other enzymes, 'BHP was effective only in the simultaneous presence of a catalyst. The results summarized in Table I demonstrate the different reactivities of 'BuO$^{\cdot}$ and 'BuOO$^{\cdot}$ towards various enzymes. It is impossible, as yet, to correlate the

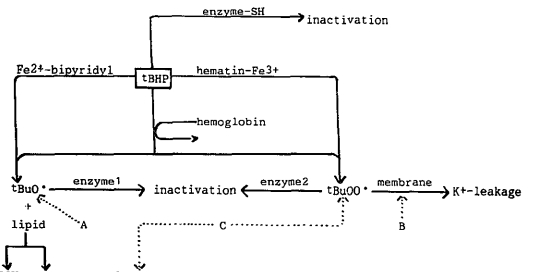

Fig. 5. Scheme of the postulated mechanism of tBHP-induced enzyme inhibition and lipid peroxidation and K^+ leakage in human erythrocytes. L·, lipid radical; LOO·, lipid peroxyl radical; →, scavenging reaction; A, diphenylamine; B, thiourea; C, BHT or promethazine. Enzyme 1, alcohol dehydrogenase, acetylcholinesterase; Enzyme 2, lysozyme, lactate dehydrogenase, acetylcholinesterase, trypsin.

different susceptibilities of these enzymes with molecular structure or amino-acid composition. Further studies will be needed to elucidate the inactivation mechanisms involved. Further, covalent binding of several amino acids to serum albumin was induced by 'BuOO·, but not by 'BuO·. Conversely, lipid peroxidation was initiated by the alkoxyl, but not by the peroxyl radical (Fig. 2). demonstrating again the different reactivities of these radicals.

Exposure of intact red blood cells to 'BHP caused lipid peroxidation and K^+ leakage. This K^+ loss can not be attributed to a possible inhibition of the membrane cation pump. The maximal pump flux of human erythrocytes is about 1.6 μM/ml cells per h [24], whereas K^+ leakage, as observed in the present experiments, reached velocities of about 120 μM/ml cells per h. Thus, it should be concluded that 'BHP causes a strongly increased passive permeability of the membrane for K^+. Conversion of cellular hemoglobin into carbonmonooxyhemoglobin stimulated the reactions, whereas conversion of hemoglobin into methemoglobin inhibited both lipid peroxidation and K^+ leakage (Fig. 3). This demonstrates that hemoglobin plays an intermediary role in both processes.

The dissociations of 'BHP-induced K^+ leakage and lipid peroxidation by thiourea and diphenylamine indicate that these two processes are not causally related. Diphenylamine is a powerful scavenger of alkoxyl, but not of peroxyl radicals [8,27]. As diphenylamine strongly inhibited lipid peroxidation (Table III), it is clear that 'BuO·, generated in the reaction between 'BHP and hemoglobin, initiated lipid peroxidation, as proposed earlier by Trotta et al. [25]. On the other hand, diphenylamine did not inhibit K^+ loss. This indicates that neither 'BuO· nor radicals, generated during lipid peroxidation, are implicated in 'BHP-induced K^+ leakage. Actually, diphenylamine augmented hydroperoxide-induced K^+ leakage. This may be caused by diphenylamino radicals generated in the reaction between diphenylamine and 'BuO·.

It is well-documented that peroxyl radical scavengers such as BHT [27] and promethazine [28] inhibit lipid peroxidation by reacting with lipid peroxyl radicals, thus interrupting the radical chain reaction. These scavengers also strongly inhibited K^+ leakage (Table III), indicating that peroxyl radicals are involved in the induction of K^+ leakage. As the effect of diphenylamine indicates that lipid peroxidation (and thus lipid peroxyl radicals) is not implicated in this process, it should be assumed that K^+ leakage is presumably initiated by 'BuOO· generated in the reaction between 'BHP and hemoglobin. Apparently, these peroxyl radicals initiate K^+ leakage by reacting with an as yet unknown membrane target (Fig. 5). The effect of thiourea strongly inhibiting 'BHP-induced K^+ leakage without affecting lipid peroxidation (Table III) corroborates the separate pathways of these two processes. Thiourea is a radical scavenger with, as yet, ill-defined specificity [5,29]. Apparently it reacts with an intermediate in the pathway leading to K^+ leakage, but not with the intermediate, involved in lipid peroxidation (Fig. 5).

The proposed mechanism implies that exposure of erythrocytes to 'BuO· in the absence of 'BuOO· should lead to lipid peroxidation but not to K^+-leakage. This prediction was tested experimentally by ultraviolet irradiation of erythrocytes, incubated with 'BHP, yielding primarily alkoxyl radicals (Eqn. 3). In these experiments, cellular hemoglobin was converted into methemoglobin to minimize radical generation in the reaction between 'BHP and hemoglobin. Further, isopropanol

was added to scavenge the generated hydroxyl radicals. As expected, lipid peroxidation increased during ultraviolet irradiation, whereas K^+ leakage was, initially, not affected (Fig. 3).

Radical formation during exposure of resealed ghosts to ^tBHP is negligible, due to the very low concentration of residual hemoglobin. Addition of hematin(Fe^{3+}) to the incubation mixture caused a fast increase in Na^+ loss from the ghosts, but no lipid peroxidation. Conversely, ferrous bipyridyl-induced generation of $^tBuO^\cdot$ caused lipid peroxidation, without affecting Na^+ leakage (Figs. 2B and 4), again in accordance with the proposed mechanism (Fig. 5).

These observations may have important implications for investigations on the effects of hydroperoxides in vivo. Alkoxyl or peroxyl, singly, or both radicals may be generated in vivo, depending on the availability of suitable catalysts, the enzymatic activation of the hydroperoxide, and the presence of antioxidants and radical scavengers. As alkoxyl and peroxyl radicals may, apparently, have quite different reactivities, elucidation of the various mechanisms is crucial for the better understanding of the role of hydroperoxides under physiological and pathological conditions.

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