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Inhibition of the Ca pump of intact red blood cells by *t*-butyl hydroperoxide: importance of glutathione peroxidase

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Incubation of human red blood cells (RBCs) with *t*-butyl hydroperoxide (tBHP) resulted in inhibition of the Ca-pump ATPase. This was demonstrated using an assay of the Ca-pump ATPase activity in intact RBCs. In this assay, activity of the Ca-pump ATPase is expressed as the rate constant of the initial loss of ATP in RBCs exposed to Ca and A23187. Pseudo-first-order rate constants (Ca-pump ATPase rate constants) were lower in the presence of tBHP versus controls. Incubation of RBCs with tBHP resulted in both a time- and concentration-dependent inhibition of the Ca-pump ATPase ($IC_{50} \approx 1$ mM). Incubation of RBCs with tBHP also resulted in decreased oxyhemoglobin, increased methemoglobin and increased thiobarbituric acid reactive substances (TBARS). GSH levels were significantly lower in the presence of tBHP. GSH fell from a control value of 2.2 mmol/l RBC to 0.46 mmol/l RBC after incubation with 0.25 mM tBHP for 15 min. Both butylated hydroxytoluene and stobadine prevented the formation of TBARS and were partially effective in protecting the Ca-pump ATPase from tBHP-induced inhibition. Dithiothreitol was completely effective in preventing the tBHP-induced formation of TBARS as well as inhibition of the Ca-pump ATPase. However, when added after exposure to tBHP, dithiothreitol was unable to restore Ca-pump ATPase activity completely. An activity of dithiothreitol independent of enzymic thiol group reduction was apparent. In the presence of mercaptosuccinate, a potent inhibitor of glutathione peroxidase, the ability of dithiothreitol to protect the Ca-pump ATPase from tBHP-induced inhibition was abolished. Therefore, protection by dithiothreitol may be afforded by its ability to replenish GSH from oxidized glutathione, thus allowing glutathione peroxidase to metabolize tBHP. These results may be interpreted to suggest that inhibition of the Ca-pump ATPase in intact RBCs occurs as a result of tBHP-induced oxidant stress and subsequent lipid peroxidation which can be prevented by certain antioxidants including butylated hydroxytoluene, stobadine, and thiol-containing compounds such as dithiothreitol. These findings provide further insight into the mode of action of hydroperoxides and certain reactive oxygen species that have been implicated in oxidative stress associated with various pathological conditions. The importance of the GSH/glutathione peroxidase system in metabolizing organic hydroperoxides is also demonstrated.

Introduction

There is increasing evidence for the involvement of free radicals in numerous human diseases, including cancer, stroke, ischemia/reperfusion injury, trauma and rheumatoid arthritis [1]. Molecular oxygen is a major source of free radicals in these diseases. Thus, oxygen, although essential for life can also cause considerable damage to aerobic organisms due to its ability to form reactive oxygen species such as hydrogen peroxide and free radicals such as superoxide anion and hydroxyl radical. Free radicals may attack a variety of biological molecules. One target for oxygen-generated free radicals is the lipid bilayer of living cells. The resultant exposure to such free radicals may lead to a number of membrane changes including lipid peroxidation [2], protein cross-linking [3], protein fragmentation [4], and inhibition of ion transporting enzymes including the Na/K-pump ATPase [5] and the Ca-pump ATPase [3,6–8]. We recently reported that iron-generated free radicals cause inhibition of the Ca-pump ATPase, protein cross-linking, and lipid peroxidation [9] in isolated red blood cell (RBC) membranes. The purpose of the current study was to investigate the effects of oxidant stress on the Ca-pump ATPase of intact RBCs. Using tBHP, a membrane permeable hydroperoxide, and an assay of the Ca-pump ATPase in intact RBCs [10], we examined the effects of oxidant stress on the Ca-pump ATPase. This whole cell assay allows for the measurement of the maximal capacity of the Ca-pump ATPase in a small volume of packed RBCs [10] and does not require isolating plasma membranes. In addition, oxidative stress can be assessed, without delay, directly on the intact cell.

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Stobadine, (−)-*cis*-2,8-dimethyl-2,3,4,4a,5,9b-hexahydro-1*H*-pyrido-(4,3*b*)-indole dihydrochloride, is an investigational drug which has been found to decrease ischemic injury and to inhibit lipid peroxidation in rat brain [11,12]. Stobadine, a membrane permeable antioxidant, has also been shown to be a potent scavenger of hydroxyl radicals [13]. In the current work we report that stobadine protected the Ca-pump ATPase from inhibition induced by tBHP. The protective effects of stobadine, were compared with those of the classical antioxidant, butylated hydroxytoluene (BHT), and the classical reducing agent, dithiothreitol (DTT), as well as mercaptosuccinate (MCS), an inhibitor of glutathione peroxidase (GSHpx).

Materials and Methods

DTT and GSH were purchased from Boehringer Mannheim (Indianapolis, IN): BHT, 1,1,3,3-tetraethoxypropane, *p*-chloromercuribenzoate, 5,5′-dithiobis(2-nitrobenzoic acid) (DNTB), tBHP, mercaptosuccinate (MCS), and sodium arsenite were all purchased from Sigma (St. Louis, MO). Luciferin-luciferase (Picozyme F) was purchased from Coral Biomedical (San Diego, CA). GSHpx and A23187 were purchased from Calbiochem. Stobadine was graciously provided by Dr. Olga Ondrejickova of the Institute of Experimental Pharmacology, Bratislava, Slovakia. All other chemicals were analytical reagent grade.

Preincubation

Fresh RBCs were obtained from healthy human subjects by venipuncture. Heparinized blood was washed three times in 10 mM phosphate buffer (pH 7.4), containing 135 mM NaCl (PBS) at 2000 × g for 5 min. 50 μl of packed cells (82–90% hematocrit) were added to reaction vessels, containing PBS with or without tBHP and/or test drugs as specified, to a final volume of 1 ml. When present, test drugs were added before tBHP. Preincubations were carried out in a Dubnoff shaking incubator at 37°C. At the conclusion of the preincubation period, samples were immediately spun down at 2000 × *g* for 5 min. The supernate was aspirated off and cells were rewashed in 2 ml of PBS and spun again. After aspiration of the supernate, the packed RBCs were stored on ice until assayed. For reversibility with DTT, washed samples were resuspended in PBS containing 5 mM DTT at a final volume of 1 ml. Reaction vessels were further incubated for 20 min at 37°C, at which time samples were prepared for assay of the Ca-pump ATPase.

Assay of the Ca-pump ATPase activity in intact RBCs

Determination of the Ca-pump ATPase activity was performed as previously described [10]. Briefly, packed RBCs (20 μl) preincubated under various conditions, were added to 1.0 ml of buffer containing 20 mM Hepes (pH 7.4), 140 mM KCl, 2 mM $MgCl_2$, 1 mM iodoacetic acid (IAA), and 0.1 mM $CaCl_2$. After incubation for 5 min at 37°C, 10 μl of A23187 in ethanol was added to the suspension to a final concentration of 3.8 μM at time zero. This results in a massive influx of Ca and 'short-circuits' the Ca-pump ATPase which rapidly consumes ATP [10]. Incubation at 37°C was continued with removal of 20 μl aliquots every 2 min for 10 min. Aliquots were immediately diluted in 1.0 ml of a lysing solution consisting of 0.5 mM $MgSO_4$ in 10 mM Tris buffer (pH 7.75). Then, 15 μl of the lysed aliquot of RBCs was added to 40 μl of a solution containing luciferin-luciferase (Picozyme F) in 0.25 mM $MgSO_4$ in 5 mM Tris buffer (pH 7.75) in a Packard luminometer. The ATP content was determined in duplicate at each time point. In the presence of A23187, ATP levels in the RBCs declined rapidly with pseudo-first-order behavior. The data were fitted with a first-order equation from which the rate constant of the Ca-pump ATPase was estimated.

Determination of methemoglobin and oxyhemoglobin

Methemoglobin (MetHb) and oxyhemoglobin (OxyHb) content were determined by the spectrophotometric method of Winterbourn [14].

TBARS measurement

Thiobarbituric acid-reactive substances (TBARS) were assayed as previously described by Stocks and Dormandy [15], with some modification. 800 μl of preincubation solution was 'stopped' by the addition of 400 μl of 28% (w/v) of trichloracetic acid (TCA) to which sodium arsenite was added to 0.1 M concentration. Samples were mixed and centrifuged at 1500 × *g* for 5 min. 800 μl of the supernate from each of these samples was then combined with 200 μl of 1% thiobarbituric acid (TBA) (w/v) in 0.05 M NaOH for a final volume of 1.0 ml. Samples were boiled for 15 min in capped microcentrifuge tubes, cooled in ice, and absorbance at 535 nm was determined with quantification based upon a molar extinction coefficient of $1.54 \cdot 10^5$ M/cm obtained from standard curves generated using 1,1,3,3-tetraethoxypropane.

Glutathione measurement

GSH content was assayed according to the colorimetric method of Prins and Loos by titration with DTNB [16].

Results

tBHP and the Ca-pump ATPase

Exposure of fresh normal RBCs (hematocrit 82–90%) to tBHP resulted in inhibition of the Ca-pump ATPase. Fig. 1, inset, shows typical raw data of an

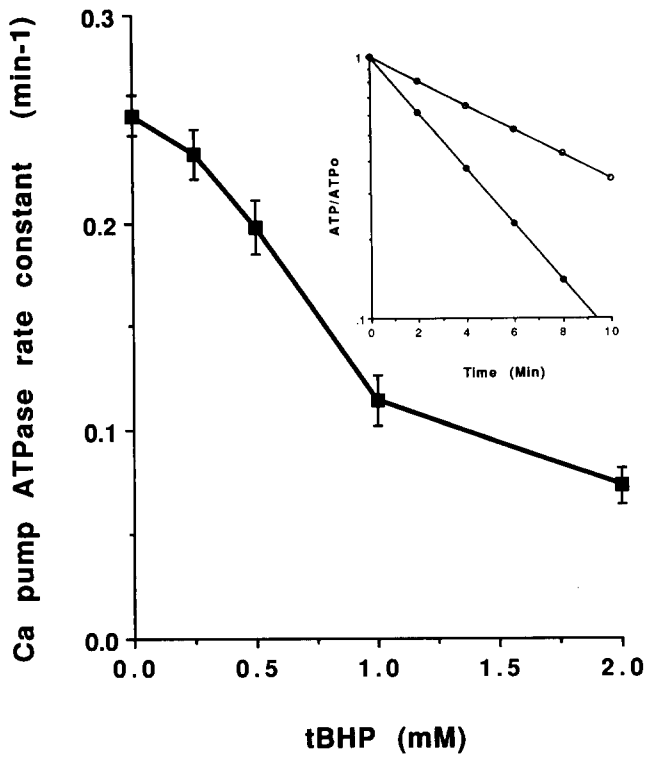

Fig. 1. tBHP-induced inhibition of the Ca-pump ATPase of intact RBCs. Fifty μl of RBCs were incubated with various concentrations of tBHP for 20 min. Following preincubation, Ca-pump ATPase activity was determined. Data ($n = 3$, $\pm$S.E.M.) are expressed as the pseudo-first-order rate constant of the Ca-pump ATPase (Ca-pump rate constant). Inset: typical raw data of the Ca-pump ATPase assay in intact RBCs of control cells (●) and cells treated with 1.0 mM tBHP (○). Data are presented as a semilogarithmic plot of the ATP content, normalized to the ATP content at time zero. In each case the data were fitted with an exponential and the correlation coefficient was > 0.99. The slopes of the lines were taken as the estimated pseudo-first-order rate constants (min^{-1}).

experiment with and without 1.0 mM tBHP. Loss of ATP was pseudo-first-order and the solid lines represent the best-fit equations for the data as previously shown [10]. The disappearance of ATP was slower in RBCs incubated with tBHP. In this example, the rate constant for loss of ATP in cells incubated with tBHP was less than half that of control, decreasing from a value of 0.246 min^{-1} for the control to 0.107 min^{-1} in the presence of 1.0 mM tBHP. Incubation of RBCs with tBHP resulted in a concentration- and time-dependent inhibition of the Ca-pump ATPase activity (Figs. 1 and 2), with maximal inhibition occurring after 20 min and with an apparent IC_{50} of approximately 1 mM tBHP.

Effect of tBHP incubation on intracellular proteins of the RBC

Incubation of RBCs with tBHP resulted in a decrease in OxyHb content and an increase in MetHb formation (Fig. 3). This effect of tBHP was concentration-dependent. It is suggested that, although tBHP was added to the exterior of the RBC, it is able to penetrate the lipid bilayer and oxidize intracellular constituents. Indeed, incubation of RBCs with tBHP resulted in a change of the RBCs from a bright red to brown that was dependent on the concentration of tBHP used in the experiment. As Fig. 3 indicates, apparently not all of the OxyHb was converted to MetHb.

GSH was also sensitive to incubation with tBHP. We found that GSH levels decreased in a dose-dependent manner when RBCs were preincubated with tBHP: GSH fell from 2.2 mmol/l RBC to 0.46 mmol/l RBC after incubation with 0.25 mM tBHP for 15 min (Fig. 4).

tBHP stimulated lipid peroxidation in intact RBCs

The possibility that tBHP may stimulate lipid peroxidation in RBCs was assessed by measuring the formation of TBARS. In intact RBCs tBHP induced a brisk generation of TBARS that was dependent on the concentration of tBHP and time (Figs. 2 and 5). At 1 mM tBHP, the highest concentration used in such an experiment, the amount of TBARS increased 6-fold from a control value of 4.65 $\pm$ 4.67 μmol/l RBC to

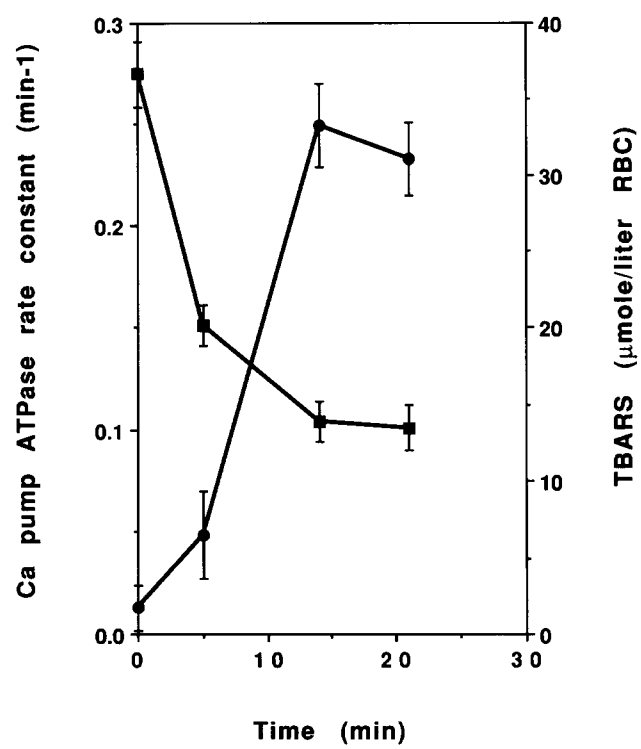

Fig. 2. Time-course of tBHP-induced lipid peroxidation and inhibition of the Ca-pump ATPase. RBCs were preincubated with 1.0 mM tBHP for various times and the Ca-pump ATPase rate constant was assayed as in Fig. 1, and in separate experiments samples were assayed for TBARS. Data are expressed as rate constant (■) ($\pm$S.E.M.) or μmol TBARS per liter of RBC (●) ($\pm$S.D.) plotted as a function of time. For each experiment $n = 3$.

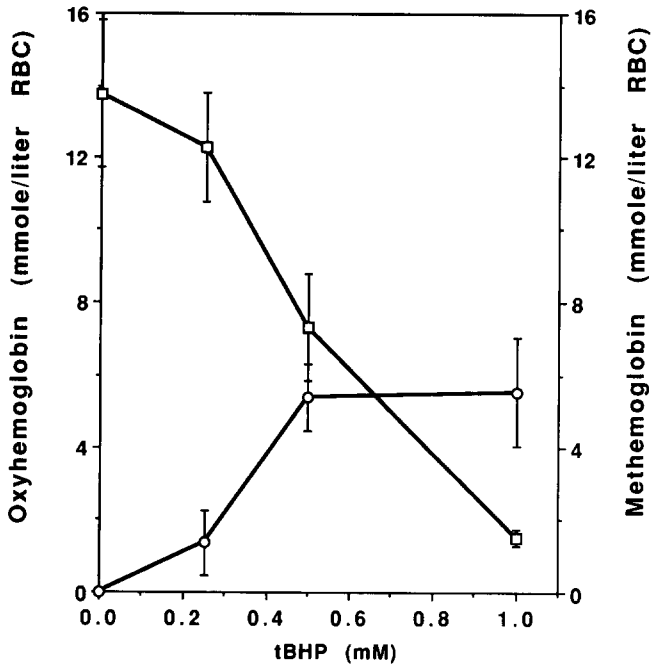

Fig. 3. Oxidation of hemoglobin by tBHP. RBCs were preincubated for 15 min at various concentrations of tBHP. Following preincubation, oxyhemoglobin (□) and methemoglobin (○) content was determined. Data represent the mean of three different experiments (±S.D.).

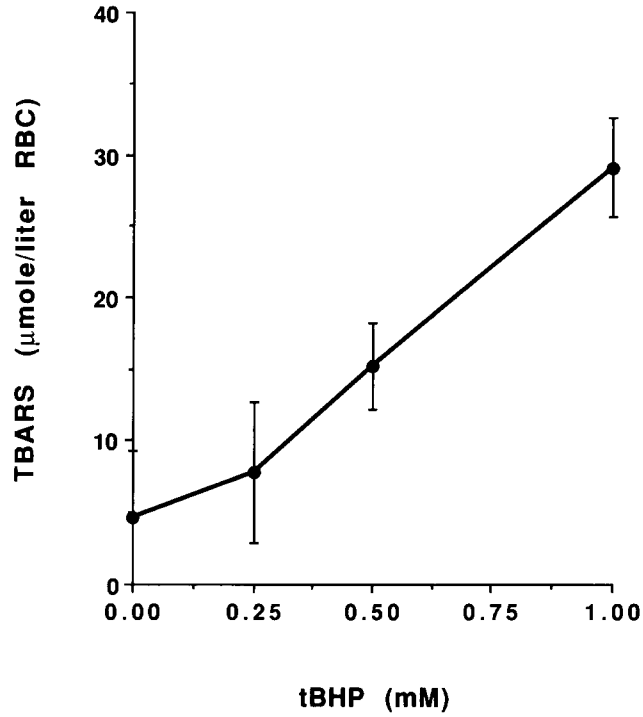

Fig. 5. Lipid peroxidation induced by tBHP. RBCs were preincubated with varying concentrations of tBHP for 15 min then assayed for TBARS. Data represent the mean of three separate experiments (±S.D.).

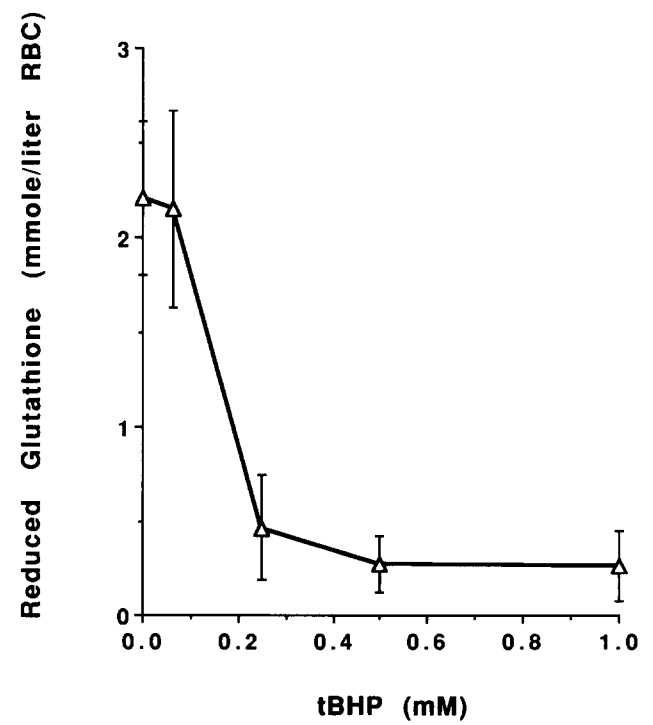

Fig. 4. Loss of reduced glutathione induced by tBHP. RBCs were preincubated for 15 min at various concentrations of tBHP. Following preincubation, reduced glutathione levels within RBCs were determined. Data represent the mean of three different experiments (±S.D.).

29.1 ± 3.49 μmol/l RBC at 1 mM tBHP when incubated with RBCs for 15 min (Fig. 5). TBARS also increased in a time-dependent manner when RBCs were preincubated with tBHP. There was a notable lag phase in the production of TBARS that reached a plateau after approx. 15 min (Fig. 2). It is noteworthy that 45% inhibition of the Ca-pump ATPase occurred within the first 5 min of preincubation with tBHP. During the same time period, TBARS were only 21% of the total TBARS formation (Fig. 2).

Time-course of tBHP-induced inhibition of the Ca-pump ATPase, loss GSH, and oxidation of hemoglobin

Fig. 6 depicts time-courses for several parameters when RBCs were preincubated with tBHP. GSH levels fell immediately upon addition of tBHP. Even when RBCs were sampled immediately after addition of tBHP (nominal time zero point), the amount of GSH had already decreased to levels comparable to all additional time points (approx. 0.20 mmol/l RBC). In contrast, when tBHP was omitted from the preincubation reaction, GSH levels were approx. 2 mmol/l RBC (dashed line, Fig. 6).

Incubation of RBCs with tBHP resulted in a decrease in OxyHb in a time-dependent fashion that closely resembled the inhibition of the Ca-pump ATPase (compare Figs. 2 and 6). The production of MetHb also increased over time, reaching a plateau after 14

min (Fig. 6). It appeared that, upon incubation of RBCs with tBHP, GSH levels fell first, followed by the loss of OxyHb and inhibition of the Ca-pump ATPase. MetHb increased gradually, while the formation of TBARS occurred more slowly, with significant production occurring only after 14 min (compare Figs. 2 and 6).

Effect of several antioxidants on tBHP-induced lipid peroxidation and inhibition of Ca-pump ATPase activity

BHT and stobadine were tested for their ability to prevent lipid peroxidation and to prevent inhibition of the Ca-pump ATPase caused by tBHP. Both BHT and stobadine were partially effective in preventing tBHP-induced inhibition of the Ca-pump ATPase. Ca-pump ATPase activities were 35% higher in the presence of 300 μM stobadine and 1.0 mM tBHP than in the presence of tBHP alone (Fig. 7). BHT was also partially effective in preventing tBHP-induced inhibition of the Ca-pump ATPase and was more potent than stobadine. Ca-pump ATPase activity was approx. 41% greater in the presence of 50 μM BHT and 1.0 mM tBHP than with tBHP alone (Fig. 7). At the concentrations tested, neither stobadine nor BHT was completely effective in preventing tBHP-induced inhibition.

Although both BHT and stobadine gave only partial protection from tBHP-induced inhibition of the Ca-pump ATPase, both drugs were effective in preventing the formation of TBARS. In the presence of tBHP and either 50 μM BHT or 300 μM stobadine, the formation of TBARS was 71% less than that in the presence of tBHP alone (table I). Interestingly, neither BHT nor stobadine prevented the oxidation of OxyHb and the loss of GSH caused by tBHP (table I).

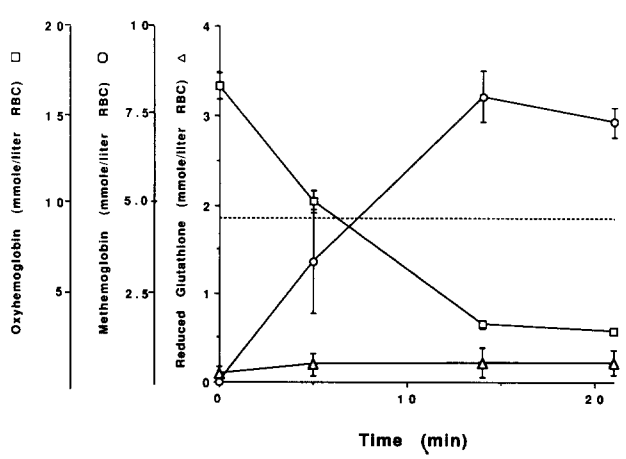

Fig. 6. Time-course of tBHP-induced loss of reduced glutathione, and oxidation of hemoglobin. GSH (△), OxyHb (□), and MetHb (○) levels as a function of time of preincubation with 1 mM tBHP. The dashed line represents the reduced glutathione level with no additions. Data represent three separate experiments (±S.D.).

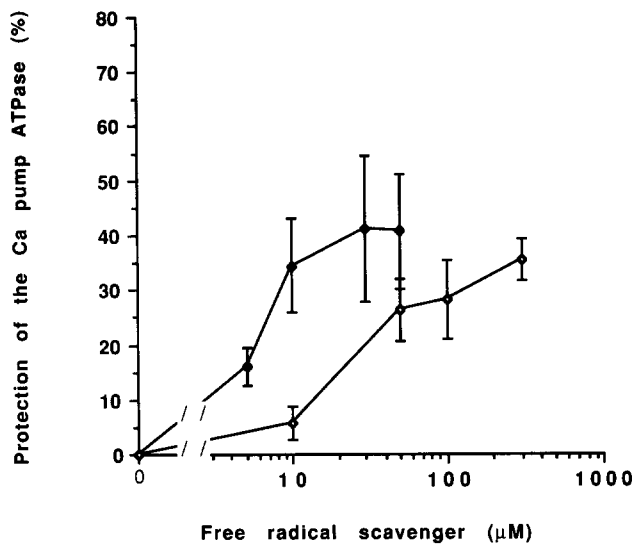

Fig. 7. Free radical scavenger protection from tBHP-induced inhibition of the Ca-pump ATPase. RBCs were preincubated for 15 min in the presence of 1.0 mM tBHP and varying concentrations of BHT (♦) or stobadine (◇). Following preincubation, the Ca-pump ATPase activity was determined. Data (±S.E.M.) are expressed as percent protection of ATPase activity determined in the absence of tBHP. Activity determined in the presence of 1 mM tBHP alone was taken as 0% protection ($n = 3$).

Effect of DTT on tBHP-induced lipid peroxidation and inhibition of the Ca-pump ATPase

Because inhibitors of lipid peroxidation were only partially effective in preventing tBHP-induced inhibition, we investigated thiol oxidation in the inhibition of the Ca-pump ATPase. RBCs were incubated with tBHP and either DTT alone or DTT plus BHT. Compared to tBHP incubation alone, where ATPase activity was inhibited to less than 50% of the control, complete protection of the Ca-pump ATPase was obtained using either BHT plus DTT or DTT alone (Fig. 8).

DTT was also very effective in preventing the formation of TBARS. In the presence of DTT and tBHP, the formation of TBARS was strongly inhibited with absorbance values near controls (Table I). Thus, DTT inhibited lipid peroxidation in RBCs caused by tBHP. MetHb formation was also prevented in the presence of DTT and tBHP. It is noteworthy that, of the agents used, only DTT gave complete protection of all parameters tested; DTT not only prevented lipid peroxidation and inhibition of the Ca-pump ATPase, but it also prevented the loss of OxyHb and GSH (Table I).

Reversibility of Ca-pump ATPase inhibition

Because of the ability of DTT to provide complete protection of ATPase activity when preincubated in the presence of tBHP, we further examined the possibility that inhibition of the Ca-pump ATPase of intact RBCs, as caused by tBHP, may involve oxidation of sulfhydryl groups on the Ca-pump ATPase. Therefore, we tested

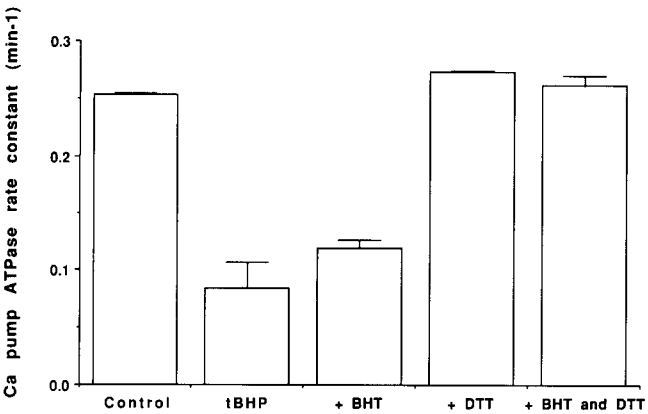

Fig. 8. Protection by DTT from tBHP-induced inhibition of the Ca-pump ATPase. Ca-pump ATPase activities were determined under different conditions, as labeled (where + BHT = tBHP + BHT; + DTT = tBHP + DTT; + BHT and DTT = tBHP + BHT + DTT). RBCs were preincubated with tBHP and BHT (50 μM) and/or DTT (1 mM) for 15 min then assayed for ATPase activity. Data represent three separate experiments ($\pm$S.E.).

the ability of DTT to restore Ca-pump ATPase activity in RBCs subsequent to incubation with tBHP. The results are depicted in Fig. 9 with the data from two separate experiments. In the first experiment, typical inhibition of Ca-pump ATPase activity (to less than 50% of control) was observed when RBCs were preincubated with tBHP. RBCs were then further examined to determine whether this inhibition by tBHP could be reversed using 5 mM DTT (see methods). Little or no restoration of ATPase activity was seen under these conditions: with subsequent incubation with DTT, the Ca-pump ATPase was still inhibited by 45% (Fig. 9a). Even when 10 mM DTT was used for 40 min, no recovery of ATPase activity was seen (data not shown). However, these results were apparently not related to an inability of DTT to reverse thiol oxidation in our

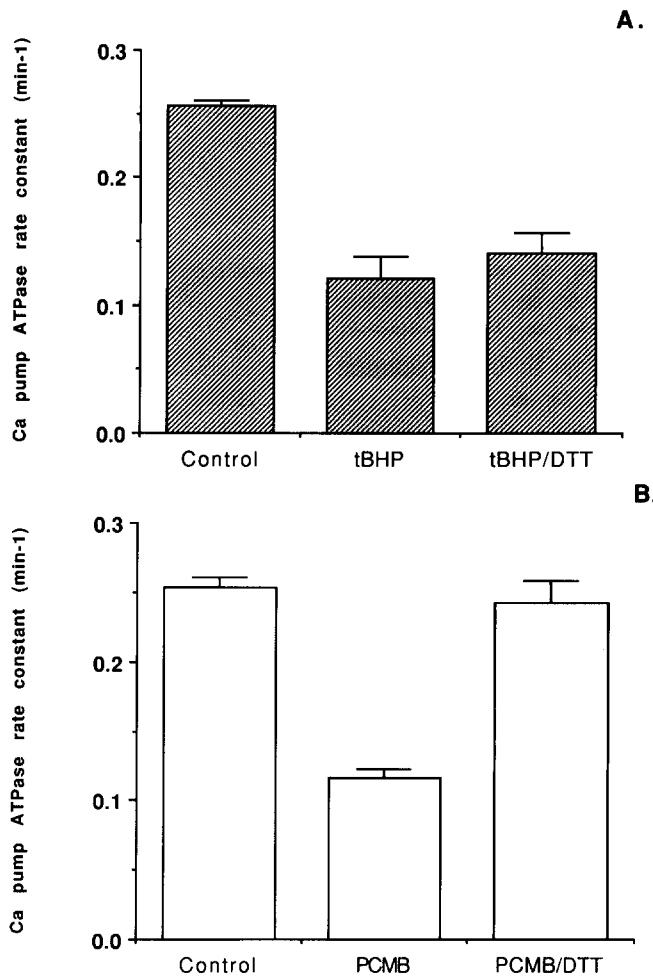

Fig. 9. Reversal by DTT of inhibition of the Ca-pump ATPase due to PCMB but not tBHP. In two separate sets of experiments, RBCs were preincubated with either 1 mM tBHP, (panel A), or 400 μM PCMB (panel B) for 15 min. At the conclusion of the preincubation, washed RBCs were then resuspended in 5 mM DTT and further incubated for 20 min at which time samples were washed and stored on ice until assayed for Ca-pump ATPase activity. For each set of experiments $n = 3$, ($\pm$S.E.).

TABLE I

Effect of various reagents on tBHP-induced oxidation in the intact RBC

Normal erythrocytes were preincubated for 15 min at 37°C with the indicated agents and subsequently oxyhemoglobin, methemoglobin, and glutathione and TBARS were determined. Results are shown as mean $\pm$ S.D. of three experiments.

Treatment	OxyHB (mmol/l RBC)	MetHB (mmol/l RBC)	Glutathione (mmol/l RBC)	TBARS (μmol/l RBC)
Control preincubation	13.3 $\pm$2.17	0	1.58$\pm$0.27	4.38$\pm$1.40
tBHP (1 mM)	2.45$\pm$0.20	6.20$\pm$1.10	0.10$\pm$0.05	33.9 $\pm$4.12
tBHP and DTT (1 mM)	14.9 $\pm$0.35	0	1.54$\pm$0.14	6.27$\pm$1.53
tBHP and BHT (50 μM)	3.04$\pm$0.74	6.86$\pm$1.10	0.12$\pm$0.07	9.57$\pm$1.20
tBHP and Stobadine (300 μM)	2.58$\pm$0.44	7.45$\pm$0.78	0.11$\pm$0.15	9.81$\pm$0.85

TABLE II

Protection by DTT from tBHP-induced inhibition of the Ca-pump ATPase in the presence of mercaptosuccinate (MCS)

Normal erythrocytes were preincubated for 15 min at 37°C with the indicated agents and subsequently, the Ca-pump ATPase rate constants were determined. Data represent the mean of three separate experiments ±S.E.

Treatment	Ca-pump ATPase rate constants (min^{-1})
Control preincubation	0.313±0.004
MCS (1.5 mM)	0.309±0.003
tBHP (1.0 mM)	0.151±0.005
tBHP and DTT (1 mM)	0.316±0.006
tBHP, DTT, and MCS	0.092±0.032

model system. As Fig. 9b shows, 5 mM DTT was fully capable of restoring Ca-pump ATPase activity inhibited by the classical thiol oxidant *p*-chloromercuribenzoate (PCMB).

Role of GSH and GSHpx in tBHP-induced inhibition of the Ca-pump ATPase

Preincubation of intact RBCs with DTT prior to the addition of tBHP prevented the loss of GSH (Table I). Following preincubation with DTT, RBC GSH values were similar to controls. DTT reacts with DNTB like GSH and interferes with the GSH assay if added directly (data not shown). When used in the preincubation, no interference was noted. Apparently, DTT was effectively removed during washing before GSH measurements were performed. The results indicated that the loss of GSH, as caused by tBHP, may be one of the earliest steps in the series of events ultimately leading to inhibition of the Ca-pump ATPase. Therefore, we hypothesized that the mechanism of action of DTT may be that of replenishing GSH from GSSG. To test this hypothesis, we preincubated RBCs with or without tBHP, DTT, and MCS. MCS is a potent inhibitor of GSHpx [17]. Table II presents Ca-pump ATPase rate constants determined after preincubation of RBCs under different assay conditions. As shown, MCS by itself did not inhibit the Ca-pump ATPase. In the presence of tBHP alone, there was approx. 50% inhibition of the Ca-pump ATPase. This inhibition was completely prevented when DTT was also included. However, in the presence of tBHP, DTT, and MCS, not only was protection by DTT abolished, but Ca-pump ATPase rate constants were approx. 70% lower as compared to controls (Table II).

Discussion

It is becoming increasingly evident that free radicals, and lipid peroxidation are involved in tissue injury associated with a variety of clinical conditions. Reactive forms of oxygen and oxygen-based free radicals (reactive oxygen species, ROS) have been considered to attack the major classes of cellular components such as nucleic acids, proteins, and lipids [18]. This may cause changes in the structure and function of membranes, particularly the maintenance of ion gradients. One such potential target for these ROS is the plasma membrane Ca-pump ATPase located in essentially all eukaryotic cells [19,20]. Intracellular free calcium is normally maintained at very low levels by a number of mechanisms including a Ca-pump ATPase [21] and an Na/Ca exchanger coupled indirectly to the Na/K-ATPase [22]. It has been proposed that inhibition of the Na/K pump ATPase and the Ca-pump ATPase by ROS leads to the accumulation of intracellular free Ca to toxic levels [9]. In addition, previous work has shown that ROS can inhibit the Ca-pump ATPase in RBCs [3,6,8,23].

One objective of the present research was to confirm and expand these results using an assay recently developed in our laboratory that allows measurement of the Ca-pump ATPase of intact human RBCs. We have shown that the rate constant for ATP loss in intact RBCs can be taken as a measure of the Ca-pump ATPase activity [10]. We chose this assay to examine the effects of ROS on the Ca-pump ATPase for two reasons. First, the intact assay can be carried out on very small volume of RBCs (10 μl) and allows for minimal handling of the RBCs. Second, the assay may be a better physiological model than assays using isolated membranes or RBC ghosts because the integrity of the RBC as well as its antioxidant defenses remains intact.

Incubation of RBCs with tBHP resulted in a lower rate constant for the Ca-pump ATPase than controls. Thus, tBHP inhibited the Ca-pump ATPase in intact RBCs. These results are in agreement with those obtained by Moore et al., who demonstrated nearly 50% inhibition of the CaM-activated Ca-pump ATPase using tBHP and RBC ghosts or isolated membranes [3,23]. Similar results were obtained by Hebbel et al., who showed that incubation of RBCs with 1 mM tBHP resulted in 54% inhibition of the Ca-pump ATPase in RBC lysates [6]. Our results confirm and extend those of Hebbel et al. In our hands, incubation of intact RBCs with tBHP resulted in both a concentration and time-dependent inhibition of the Ca-pump ATPase with 50% inhibition occurring at 1 mM tBHP.

Incubation of RBCs with tBHP also resulted in a decrease in OxyHb content and an increase in MetHb. Van Den Berg et al. obtained similar results using cumene hydroperoxide and intact RBCs. They demonstrated oxidation of hemoglobin (Hb) with a concomitant increase in MetHb levels [24,25]. Our results showing oxidation of Hb by tBHP would suggest that, although tBHP is added to the exterior of the RBC, it is

able to penetrate the lipid bilayer and oxidize intracellular constituents. Interaction of tBHP with Hb may be a critical step in the eventual inhibition of the Ca-pump ATPase by tBHP. According to the model of Van den Berg et al., organic hydroperoxides like tBHP permeate the lipid bilayer and react with Hb near the plasma membrane yielding alkoxyl radicals [26]. These alkoxyl radicals can cause an oxidative stress on the RBC membrane which may lead to alteration and inhibition of membrane proteins such as the Ca-pump ATPase. Our results suggest that tBHP interacts with hemoglobin, oxidizing it to MetHb. According to the Van den Berg model, OxyHb may act as a Fenton reagent as it is converted to MetHb. The product of this Fenton reaction between OxyHb and tBHP, most likely an alkoxyl radical, may then proceed to initiate lipid peroxidation. Data from our experiments support this interpretation in that the production of MetHb preceded any significant formation of TBARS. However, we were surprised at the significant inhibition of the Ca-pump ATPase that occurred within the first 5 min of preincubation with tBHP; a time at which the production of TBARS was minimal. This result can be interpreted in several ways. First, the production of TBARS, indicative of lipid peroxidation, may have nothing to do with inhibition of the Ca-pump ATPase by tBHP. On the other hand, it could be suggested that the inhibitory effect of lipid peroxidation is not 'linear' and that a small amount of TBARS results in substantial inhibition of the Ca-pump ATPase. In support of the latter interpretation were results from Moore et al., who reported roughly 50% inhibition of the CaM-activated Ca-pump ATPase in RBC ghosts after 10 min in the presence of tBHP when the production of TBARS was minimal [23]. We realize that there are pitfalls associated with the TBARS assay; however, from a qualitative perspective the formation of TBARS does indicate whether gross changes have occurred within the lipid bilayer of the RBC.

Incubation of RBCs with tBHP resulted in a loss in GSH. Reduced glutathione is a major RBC defense against oxidant stress. This tripeptide, in concert with its reductant NADPH and GSHpx, can reduce hydrogen peroxide, lipid peroxides, disulfides, ascorbate, and free radicals [2]. Thus, one secondary manifestation of cellular oxidant stress is depletion of reduced intracellular glutathione and/or NADPH. We found a significant decrease in GSH in the presence of as little as 0.25 mM tBHP. The decrease in GSH occurred very rapidly, suggesting that the GSH/GSHpx system provided the first line defense during the oxidative insult. Taken together, these data can be interpreted to suggest that exposure of intact RBCs to tBHP results in significant oxidant stress leading to oxidation of both plasma membrane and cytosolic proteins and to inhibition of the plasma membrane Ca-pump ATPase.

A second aim of the present studies was to investigate the ability of stobadine to prevent lipid peroxidation and inhibition of the Ca-pump ATPase by tBHP. In our system, stobadine prevented the formation of TBARS and gave partial protection of the Ca-pump ATPase from tBHP-induced inhibition. BHT, the classical antioxidant, gave similar results but was more potent. Neither stobadine nor BHT prevented the loss of OxyHb and GSH. It may be that, because BHT and stobadine are relatively lipid soluble, cytosolic proteins such as hemoglobin and GSH would not be afforded much protection from tBHP.

As previously stated, stobadine and BHT were each only partially effective in preventing tBHP-induced inhibition of the Ca-pump ATPase. Moore et al. came to a similar conclusion using tBHP and BHT with isolated membranes or RBC ghosts [23]. They found that BHT prevented enzyme inhibition for only 10 min, after which the activity declined. This may suggest that some additional or other mechanism than lipid peroxidation causes the loss of activity. Perhaps, the alkoxyl radicals produced by the interaction of tBHP with OxyHb not only initiate lipid peroxidation but also inhibit the Ca-pump ATPase by a direct attack on the cytosolic portions of the pump, resulting in the oxidation of critical thiol groups on the enzyme. Inhibition of the Ca-pump ATPase by thiol-reactive agents is well documented [27–30]. We tested the ability of a thiol-reducing agent, DTT, to prevent inhibition of the ATPase by tBHP. When RBCs were preincubated in the presence of both DTT and tBHP, no inhibition of the enzyme was observed. This result was consistent with the interpretation that oxidant stress in RBCs by tBHP caused inhibition of the Ca-pump ATPase at least in part by oxidation of thiol groups on the enzyme. However, when DTT was added after tBHP incubation with RBCs, no recovery of Ca-pump ATPase activity was seen. This result is in agreement with results obtained by Hebbel et al. who incubated RBCs with tBHP. They were able to demonstrate only partial recovery of Ca-pump ATPase activity with DTT after RBCs were preincubated with tBHP [6]. In addition, using the method of thiol-disulfide exchange chromatography [6] Hebbel et al. demonstrated that thiol oxidation does not occur in RBCs incubated with tBHP. Their results and ours suggest that lipid peroxidation may inhibit the Ca-pump ATPase through a thiol-independent mechanism. Interestingly, we were able to demonstrate full restoration of ATPase activity with DTT when a classical thiol-oxidant such as PMCB was used in our model system. Thus, only when DTT was added prior to but not after the incubation of RBCs with tBHP was protection of the Ca-pump ATPase observed.

One possible mechanism by which DTT afforded protection, was by replenishing GSH from GSSG. The majority of oxidation of GSH to GSSG occurs by

GSHpx. GSHpx catalyses the oxidation of GSH to GSSG at the expense of hydrogen peroxide [31]. The enzymatic system of GSH/GSHpx is also capable of metabolizing lipid hydroperoxides to alcohols and represents the sole enzymatic system capable of preventing further propagation of a radical chain reaction that leads to lipid peroxidation [32]. The organic hydroperoxide used in our study, tBHP, is a substrate suitable for GSHpx. We chose to use tBHP because its spontaneous reaction with GSH is lower than that with H_2O_2, and because tBHP is not metabolized by catalase [32]. Previous studies have shown that GSH/GSHpx is an important part of the defense system against ROS that attack cells during an oxidative insult. Jones et al. demonstrated a role for GSHpx in detoxifying H_2O_2 and organic hydroperoxides using isolated hepatocytes [33]. In addition, the GSH/GSHpx system was shown to protect mammalian spermatozoa from loss of motility caused by lipid peroxidation and lipid hydroperoxides [34]. The ability of intracellular GSHpx to metabolize organic hydroperoxides such as tBHP is reliant upon a steady pool of GSH. We used a relatively high concentration of tBHP (1.0 mM), and we suggest that loss of function of GSHpx occurred because of essentially complete conversion of endogenous GSH to GSSG. Under these conditions, no GSH was available as an electron donor substrate to the peroxidase. Thus, GSHpx, having depleted available GSH presumably became 'dormant' leaving a substantial amount of tBHP available to react with Hb and inhibit the Ca-pump ATPase. However, if GSH could be replenished quickly by an exogenous reducing agent, GSHpx would then be capable of metabolizing substantially more tBHP. Our results are compatible with the interpretation that DTT may act as this reducing agent. In the presence of MCS, a potent inhibitor of GSHpx [17], DTT no longer protected the Ca-pump ATPase from tBHP-induced inhibition. These data strongly support a role for DTT in the GSH/GSHpx cycle and demonstrate the crucial coupling between GSH and GSHpx. If either GSH levels were depleted or if GSHpx was inhibited, then the enzymatic system was rendered nonfunctional.

A role for GSHpx metabolizing tBHP in our system was demonstrated by the fact that, in the presence of MCS and tBHP, Ca-pump ATPase rate constants were 40% less than in the presence of tBHP alone. This occurred even though DTT was included in the preincubation medium. These data suggest that inhibition of GSHpx by MCS allows a greater proportion of tBHP to react with OxyHb, producing larger quantities of ROS, and greater inhibition of the Ca-pump ATPase.

In conclusion, we have shown that tBHP inhibits the Ca-pump ATPase when measured in intact RBCs. Incubation of intact RBCs with tBHP caused the loss of OxyHb and formation of MetHb as well as a decrease in GSH within the RBC. All of these changes could be prevented if DTT was added prior to, but not after, the addition of tBHP. Our data support the interpretation that the action of DTT is coupled to GSH/GSHpx, an important enzymatic system in detoxifying organic hydroperoxides. In addition, the feasibility that reducing agents, like DTT, may be therapeutically efficacious under conditions of an oxidative insult has been demonstrated. Finally, stobadine and BHT partially protected the Ca-pump ATPase from tBHP-induced inhibition. Presumably they did so by acting as alkoxyl radical scavengers. Thus, these and other agents, like DTT, may spare cells from damage by protecting the Ca-pump ATPase from inhibition. An uninhibited Ca-pump helps to insure extrusion of Ca from cells which might otherwise become overloaded. Cellular Ca overload has been associated with many disease states including stroke, reperfusion injury, and trauma [1,35].

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