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Clotrimazole enhances lysis of human erythrocytes induced by t-BHP

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ABSTRACT

Clotrimazole (CLT) is an antifungal and antimalarial agent also effective as a Gardos channel inhibitor. In addition, CLT possesses antitumor properties. Recent data provide evidence that CLT forms a complex with heme (hemin), which produces a more potent lytic effect than heme alone. This study addressed the effect of CLT on the lysis of normal human erythrocytes induced by tert-butyl hydroperoxide (t-BHP). For the first time, it was shown that 10 μM CLT significantly enhanced the lytic effect of t-BHP on erythrocytes in both Ca^{2+}-containing and Ca^{2+}-free media, suggesting that the effect is not related to Gardos channels. CLT did not affect the rate of free radical generation, the kinetics of GSH degradation, methemoglobin formation and TBARS generation; therefore, we concluded that CLT does not cause additional oxidative damage to erythrocytes treated with t-BHP. It is tempted to speculate that CLT enhances t-BHP-induced changes in erythrocyte volume and lysis largely by forming a complex with hemin released during hemoglobin oxidation in erythrocytes: the CLT–hemin complex destabilizes the cell membrane more potently than hemin alone. If so, the effect of CLT on cell membrane damage during free-radical oxidation may be used to increase the efficacy of antitumor therapy.

1. Introduction

High hemoglobin content and continual contact with oxygen unavoidably result in the formation of reactive oxygen species (ROS) in erythrocytes [1,2]. In addition, in the body, erythrocytes are attacked by exogenous ROS, originating from other blood cells (platelets, neutrophils, monocytes, and macrophages) and vessel endothelium [3,4], as well as from certain xenobyotics and medicines [5,6].

Physiological erythrocyte aging and sequestration of senescent cells are thought to be due to oxidative damage accumulated in erythrocytes over their lifespan in the circulation (about 120 days) [7].

In the norm, the endogenous antioxidant defense system provides for the balance between ROS generation and scavenging. In various disease conditions (certain enzymo- and hemoglobinopathies, inflammation, sepsis, ischemia–reperfusion, shock, and burns), the balance of ROS production and degradation is impaired, resulting in the so-called "oxidative stress" [8]. Clinical efficacy of antioxidants and cytotoxic drugs cannot be achieved without a detailed understanding of the mechanisms by which ROS affect cells and subcellular structures. This line of research has been extensively pursued over the last decades.

A model substance often used in the studies of oxidative processes in erythrocytes is tert-butyl hydroperoxide (t-BHP), which, being lipophilic, passes easily across the erythrocyte membrane [9]. In erythrocytes treated with t-BHP (0.1–3 mM) for less than 1 h, a dramatic decline in the reduced glutathione (GSH) content is observed, followed by hemoglobin (Hb) oxidation, changes in cell morphology and in the ion permeability of cell membranes [10–15]. Erythrocytes long exposed to t-BHP at various concentrations may undergo oxidative isotonic lysis [16–18]. As we showed earlier [19], incubation of erythrocytes with 1–3 mM t-BHP in a Ca^{2+}-containing medium for 20–30 min resulted in a dose-dependent cell swelling. If the incubation medium was Ca^{2+}-free, high in K^+, and/or supplemented with 10 μM clotrimazole (CLT), an inhibitor of Ca^{2+}-activated K^+ channels (Gardos channels), the extent of swelling was significantly greater. This result led us to conclude that t-BHP acted by activating Gardos channels of erythrocytes suspended in a Ca^{2+}-containing medium, thereby raising K^+ efflux and moderating cell swelling.

This study addressed the effect of CLT on the swelling and lysis of normal human erythrocytes induced by t-BHP. For the first time, it was shown that 10 μM CLT significantly enhanced the lytic effect of t-BHP on erythrocytes, even in a Ca^{2+}-free medium. To elucidate the mechanism of action of CLT on the erythrocyte membrane, we investigated how CLT affected the rate of ROS generation and

Abbreviations: CLT, clotrimazole; t-BHP, tert-butyl hydroperoxide; ROS, reactive oxygen species; GSH, glutathione; Hb, hemoglobin; TBARS, thiobarbituric acid reactive substances; DCFH-DA, dichlorofluorescin-diacetate; TCA, trichloroacetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoate); MetHb, methemoglobin; MDA, malonyldialdehyde.

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how GSH, methemoglobin (MetHb), and thiobarbituric acid reactive substances (TBARS) varied with time in human erythrocytes treated with t-BHP. The results tempt us to speculate that t-BHP-induced swelling and eventually hemolysis may be due to membrane destabilization produced by hemin, which is released during oxidation of erythrocytes. Forming a complex with hemin, CLT further enhances the effect of hemin on the membrane.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade. tert-Butyl hydroperoxide, HEPES, glucose, DMSO, clotrimazole, albumin and A 23187 were from Sigma (St. Louis, MO) or Sigma–Aldrich.

2.2. Preparation of washed erythrocytes

Freshly drawn blood (obtained from normal donors after informed consent) was anticoagulated with a citrate solution in a blood-to-citrate ratio of 9:1. Thereupon, erythrocytes were isolated and washed two times in 10 mM HEPES (pH 7.4) containing 5 mM KCl, 0.8 mM $MgSO_4$, 5 mM glucose, and NaCl at a concentration required to achieve isotonic osmolality (U = 300 mOsm/kg) (Buffer1). At each washing step, the buffy coat was removed. Washed erythrocytes were resuspended in the same Buffer1 to a hematocrit (Hct) of 60%. The suspension obtained was stored at 4 °C for no longer than 3 h.

2.3. Experimental design

The initial 60% suspension of washed erythrocytes was diluted to Hct = 5% with Buffer1. In experiments meant to study the role of calcium ions, some samples were prepared using Buffer1 supplemented with 1.5 mM $CaCl_2$ (Buffer2) or 1 mM EGTA (Buffer3). Immediately before measurements, a 100 mM stock solution of CLT in ethanol was diluted 1:100 with Buffer1 and added into erythrocyte suspension to a final concentration of 10 μM. A 100 mM t-BHP stock solution was prepared in Buffer1 from a 70% commercial solution. Erythrocytes were incubated with 1–3 mM t-BHP (final concentrations) with or without CLT at 37 °C. Control cells were incubated with Buffer1, Buffer2, or Buffer3.

In experiments with albumin, erythrocyte suspension was split into 1 ml samples into which we added 2 mM t-BHP or 10 μM CLT + 2 mM t-BHP. The samples were incubated at room temperature for 15 min and then centrifuged to pellet the cells. The supernatant was discarded, and the cells were resuspended in 1 ml of Buffer1 not containing or containing albumin at different concentrations. The suspensions were placed at 37 °C. Hemolysis was read 2 h after addition of t-BHP for samples with CLT and 3.5 h after addition of t-BHP for samples without CLT.

2.4. Osmotic resistance distribution

The osmotic resistance of erythrocytes was determined using our modification of the profile migration method of Lew [20,21]. Briefly, light transmission was measured on a Thermomax microplate reader (Molecular Devices, Sunnyvale, United States) at room temperature. For these measurements, a graded series of buffered lysis media (25, 50, 75, 100, 125, 150, and 300 mOsm/kg) was prepared by mixing the isotonic Buffer1 and the same buffer lacking NaCl (i.e., 25 mOsm/kg in osmolality) at appropriate ratios. The first horizontal row of flat-bottom wells contained distilled water (300 μl/well); the second and consecutive rows, lysis buffer (300 μl/well) in the order of increasing osmolality. A multipipette was used to distribute 6-μl aliquots of erythrocyte samples (5% suspensions) so as to have each vertical row corresponding to one erythrocyte sample. The final Hct in each well was 0.1%.

The microplate was placed on an MS1 minishaker (IKA Werke, Staufen, Germany) for 30 min at room temperature. After incubation, 20% NaCl (20 μl/well) was added into the wells to bring their osmolality from the initial values of 0–150 mOsm/kg to 425–565 mOsm/kg, after which light transmission was immediately read at λ = 650 nm. The intracellular hemoglobin concentration and, correspondingly, the erythrocyte refraction index increase significantly on going into the hypertonic osmolality range but vary only slightly within that range. Therefore, the light transmission is determined largely by the concentration of cells that have escaped lysis. This method makes it possible to obtain osmotic lysis curves simultaneously for 12 erythrocyte samples [21]. The osmolality value at which 50% cells undergo lysis (Mc, mOsm/kg; center of the osmotic resistance distribution of erythrocytes) was chosen as a quantitative index of osmotic resistance of erythrocytes.

2.5. Hemolysis measurements

Samples (5% erythrocyte suspension) were incubated with t-BHP at 37 °C with gentle slow agitating. At various time intervals, 200-μl aliquots were removed and centrifuged at 7000 rpm for 5 min. The supernatant was mixed 2:1 or 5:1 (depending on the extent of hemolysis) with Drabkin's solution, and 300-μl aliquots of the mixture were transferred into wells of a microplate absorbance spectrophotometer (Thermomax, Molecular Devices). The optical density was measured at λ = 490 nm. An aliquot of control suspension was brought to complete hemolysis (25 μl suspension + 975 μl Drabkin's solution); the optical density of its supernatant times a factor of 40 was taken as corresponding to 100% hemolysis.

2.6. ROS assay

The intracellular production of ROS was assayed using the fluorescent probe 2′,7′-dichlorodihydrofluorescin diacetate (DCFH-DA) [22]. Briefly, 10 mM DCFH-DA in DMSO was added to 5% erythrocyte suspension in Buffer1 to a concentration of 100 μM for 60 min at room temperature in the dark. Thereupon, the cells were incubated with t-BHP or t-BHP + CLT, and aliquots were drawn every 5–10 min over a period of 40–65 min to determine the cell fluorescence distribution. Measurements were taken using a Fluorescence Activated Cell Sorter (Becton-Dickinson FACS-Sort Flow Cytometer) in FL1-H. The fluorescence channel geometric mean (G_{mean}) was derived using Win MDI-2.8 software.

2.7. Reduced glutathione assay

GSH was assayed spectrophotometrically, as described in [23], with some modifications. Specifically, we adapted the technique so as to take optical density measurements using a Thermomax microplate reader (Molecular Devices, Sunnyvale, United States). Briefly, 100 μl aliquots of 5% erythrocyte suspension, being incubated with t-BHP or t-BHP + CLT, were taken at different moments and put into 200-μl Eppendorf-type tubes. To each aliquot, 50 μl of 28% trichloroacetic acid (TCA) containing 100 mM sodium arsenite was added. The tubes were vigorously vortexed and then centrifuged at 7000 rpm for 5 min. Without pH adjustment, the TCA supernatant was applied to a 96-well flat-bottom microtiter plate (100 μl per well containing 200 μL Tris buffer). Then 20 μl of 10 mM DTNB (pH 7.0) was added to each well for 5 min, and the optical density was read at λ = 405 nm with a microplate reader. The GSH content was determined from a calibration curve. The cal-

ibration curve was obtained by putting GSH solutions of known concentrations instead of erythrocyte suspensions into tubes with TCA.

2.8. Methemoglobin assay

Methemoglobin and total hemoglobin were determined by a modified Evelyn and Malloy method [24].

2.9. Lipid peroxidation assay

Lipid peroxidation in erythrocytes was assessed by measuring the malonyldialdehyde (MDA) formed by the thiobarbituric acid (TBA) reaction using the spectrophotometric technique [25] with some modifications. To 1 ml of the TCA supernatant prepared as described above for GSH measurements, 250 μl of 1% TBA in 0.05 M NaOH was added. The mixture was placed in a boiling water bath for 15 min and then cooled under running cold water. The MDA production was assessed by measuring the absorbance at $\lambda = 532$ nm against the blank containing all the components except for the suspension being studied. $\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$ [26].

2.10. Statistics

Experimental results are presented as single observations representative of at least three others, or as means ± S.E.M. of *n* parallel observations. Where appropriate, comparisons were made using Student's paired *t*-test.

3. Results

3.1. Modulation of the effect of t-BHP on erythrocyte osmotic resistance by calcium and CLT

As we reported earlier [19], erythrocytes incubated with t-BHP (1–3 mM) for 20–30 min swelled significantly. The effect was most pronounced if Gardos channel activation was prevented. A decrease in the osmotic resistance of t-BHP-treated erythrocytes was taken as an index of their swelling. Fig. 1 depicts the osmotic resistance—t-BHP concentration curves for erythrocytes treated with t-BHP for 30 min in a Ca^{2+}-containing medium (curves 1 and 3) and in a Ca^{2+}-free medium (curves 2 and 4) in the absence (curves 1 and 2) or in the presence (curves 3 and 4) of 10 μM CLT. Obviously, at every t-BHP concentration, osmotic resistance decreased to a greater extent in the presence of CLT, whether in a Ca^{2+}-containing medium or in a Ca^{2+}-free medium.

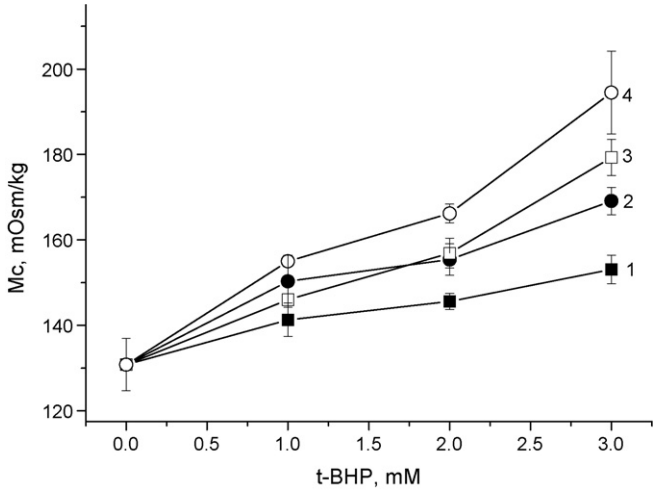

Fig. 1. Osmotic resistance index Mc plotted versus t-BHP concentration: Effects of calcium and CLT. Cells resuspended in calcium-containing Buffer2 (curves 1 and 3) or calcium-free Buffer3 (curves 2 and 4) were incubated with varied concentrations of t-BHP (1 and 2) in the absence or (3 and 4) in the presence of 10 μM CLT for 30 min. Aliquots for recording the osmotic resistance curves were taken from each sample. Each data point (Mc, mOsm/kg) is the mean ± S.E.M. of 6–20 separate experiments.

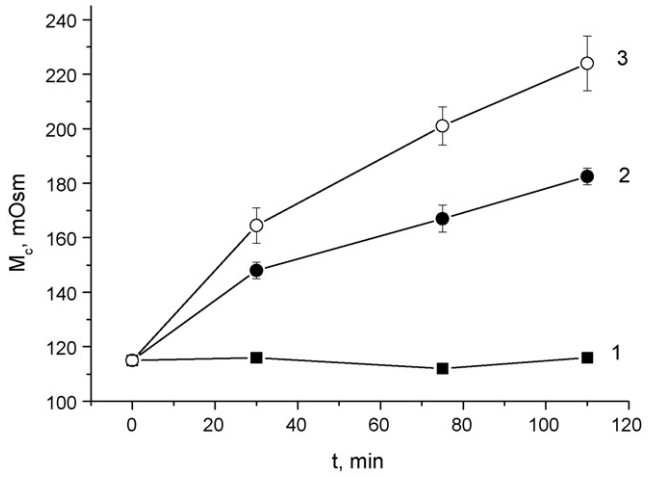

Fig. 2. Effect of CLT on the time course of t-BHP-induced changes in the osmotic resistance index Mc. Cells were resuspended in calcium-free Buffer3 (1) without or (2 and 3) with t-BHP (2 mM) (2) in the absence or (3) in the presence of 10 μM CLT. Aliquots for recording the osmotic resistance curves were taken at 30, 75 and 110 min. Each data point (Mc, mOsm/kg) is the mean ± S.E.M. of three separate experiments.

Apparently, Gardos channel inhibition may contribute to the effect of CLT on the erythrocyte osmotic resistance in a Ca^{2+}-containing medium [21,27]; however, in a Ca^{2+}-free medium, the mechanism of erythrocyte swelling should be different.

3.2. Time course of t-BHP-induced changes in the erythrocyte osmotic resistance: effect of CLT in a Ca^{2+}-free medium

To gain further insight into oxidation-induced erythrocyte volume change, we examined how the cell volume varied with the time of t-BHP treatment.

Erythrocytes were incubated with 2 mM t-BHP in the presence or in the absence of CLT in a Ca^{2+}-free medium. Aliquots for testing the osmotic resistance were taken 30, 75, and 110 min after the start of incubation. The results (Fig. 2) provide evidence that the extent of erythrocyte swelling increases with the incubation time and that CLT nearly doubles the swelling effect.

3.3. Effects of calcium and CLT on t-BHP-induced lysis of erythrocytes

Expectedly, more prolonged incubation with the oxidant resulted in a gradual increase in hemolysis. In Fig. 3A, hemolysis is presented as a function of time for a t-BHP concentration of 2 mM. In Fig. 3B, hemolysis is given as a function of t-BHP concentration for an incubation time of 3 h. As can be seen from the figures, hemolysis was not affected by the presence of Ca^{2+} in the medium, whereas CLT considerably and significantly enhanced hemolysis at all t-BHP concentrations in both Ca^{2+}-containing and Ca^{2+}-free media.

3.4. Effects of albumin on t-BHP-induced lysis of erythrocytes

If cells treated with 2 mM t-BHP (with or without CLT) for 15 min were placed in a new medium containing albumin at different concentrations, we observed a concentration-dependent decrease in the extent of hemolysis (Fig. 4).

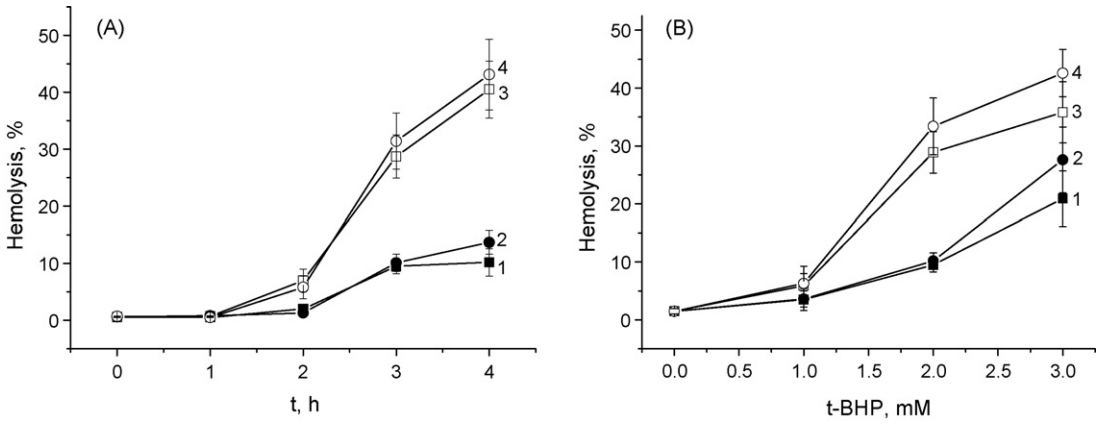

Fig. 3. Effects of calcium and CLT on t-BHP-induced hemolysis. (A) Hemolysis as a function of time (t-BHP concentration, 2 mM); (B) hemolysis as a function of t-BHP concentration (incubation time, 3 h) curve 1, Buffer3, no CLT; curve 2, Buffer2, no CLT; curve3, Buffer3, 10 μM CLT; curve 4, Buffer2, 10 μM CLT. Each data point is the mean ± S.E.M. of 10 separate experiments.

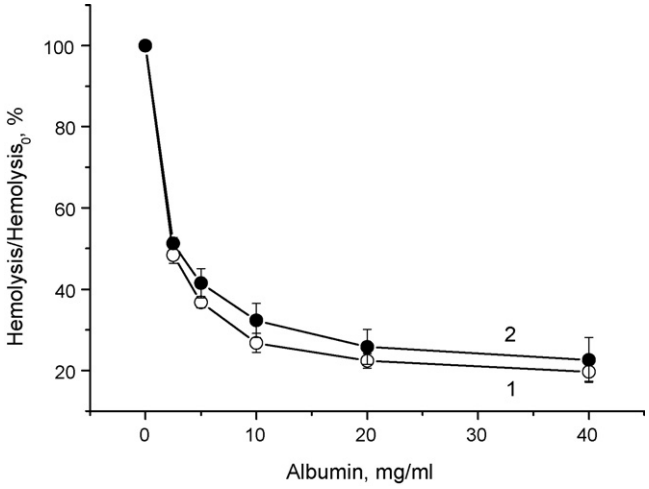

Fig. 4. Effect of albumin on the extent of lysis caused by t-BHP in erythrocyte suspension. Suspensions of human erythrocytes (5%) incubated for 15 min at room temperature with 2 mM t-BHP or 10 M M CLT + 2 mM t-BHP were centrifuged and the supernatant was replaced with Buffer1 without albumin (control) or Buffer1 containing albumin at different concentrations. The resuspended cells were kept at 37 °C. The extent of hemolysis was determined 2 h after addition of t-BHP for samples with CLT (curve 1) or 3.5 h for sample without CLT (curve 2). The extent of hemolysis is given as percentage of the respective control taken as 100%. Each data point is the mean ± S.E.M. of three separate experiments.

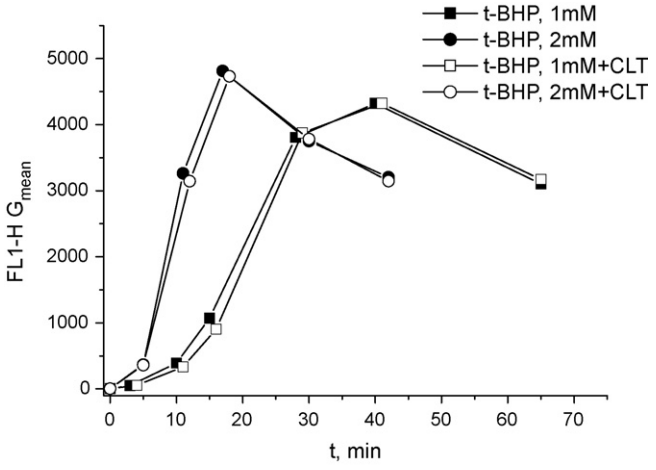

Fig. 5. ROS formation in human erythrocytes treated with t-BHP in the presence and in the absence of 10 M CLT. DCFH-DAwere incubated with t-BHP at a concentration of 1 mM or 2 mM in the presence (open symbols) or in the absence (filled symbols) of 10 M CLT. The data are representative of three separate experiments.

In an attempt to understand the mechanism of action of CLT on the erythrocyte membrane, we studied whether the rate of free radical generation and the kinetics of MetHb formation, GSH oxidation, and TBARS production in human erythrocytes treated with 1–3 mM t-BHP were affected by CLT.

3.5. Effects of CLT on t-BHP-induced ROS formation

As follows from Fig. 5, CLT produced little effect, if any, on the ROS formation in the incubation mixture.

3.6. Effect of CLT on t-BHP-induced oxidation of Hb and GSH and TBARS formation

In our experiments, the kinetics of t-BHP-induced GSH oxidation and recovery (Fig. 6) and the rate of Hb oxidation (Fig. 7) did not depend on the presence of CLT (10 μM). The formation of TBARS during incubation of erythrocytes with t-BHP (2–3 mM) was also not affected by the presence of CLT (Fig. 8).

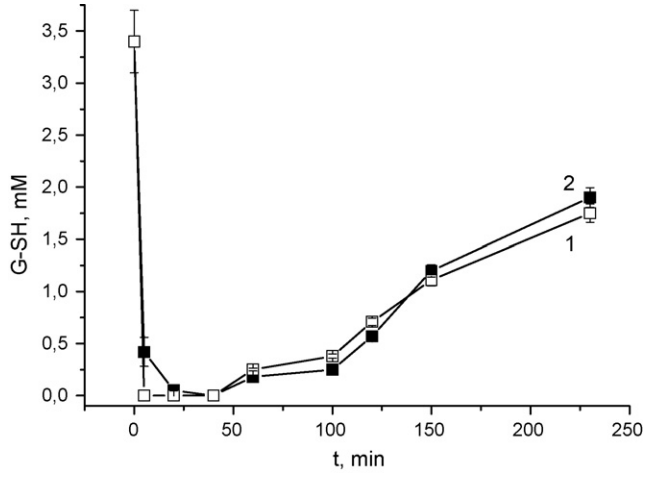

Fig. 6. Effect of CLT on GSH oxidation in erythrocytes treated with t-BHP. Erythrocytes were incubated with 2 mM t-BHP (1) in the presence or (2) in the absence of 10 M CLT. Each data point is the mean ± S.E.M. of four separate experiments. Reduced glutathione concentration was determined as described in Section 2.

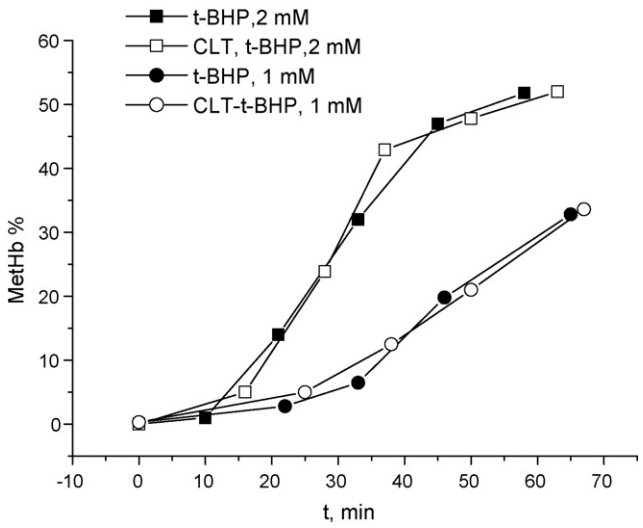

Fig. 7. Effect of CLT on of hemoglobin oxidation induced by t-BHP. Erythrocytes were incubated with t-BHP at a concentration of 1 mM or 2 mM in the presence (open symbols) or in the absence (filled symbols) of 10 M CLT. The data are representative of three separate experiments.

4. Discussion

Interaction with the organic hydroperoxide t-BHP offers a useful model for studying oxidative damage to erythrocytes. Over decades, the cytotoxic effect of t-BHP and the mechanisms involved have been a subject of extensive studies [9–16,28–31]. When t-BHP is added to an erythrocyte suspension, intracellular GSH and oxyHb oxidation and membrane lipid peroxidation are observed [10,11,28,30], which result in oxidative damage to erythrocytes that includes impaired ability of the cells to maintain cation gradients [15,25,32], a decrease in the protein thiol content, rearrangement in the cytoskeleton structure, morphological changes, and eventually hemolysis [9,18,33].

Hb oxidation leads to the formation of denatured hemoglobin monomers (irreversible hemichromes), which bind to the cell membrane and release hydrophobic heme (hemin) [14,30,34]. Chiu [35] reported the results of direct measurements of the rate of hemin release from hemoglobin oxidized with H_2O_2. There are studies in which free hemin content was determined in the membranes of erythrocytes treated with phenylhydrazine [34] and menadione [36,37]. Exogenous hemin (Ferriprotoporphyrin IX) is known to readily incorporate into the membrane, causing its destabilization and hemolysis [36,38–40].

CLT is an imidazole derivative efficacious as an antifungal agent. CLT is also known to be a Gardos channel inhibitor, which can be used perorally in therapy for severe sickle-cell anemia and thalassemia [41,42]. CLT inhibits in vitro growth of *Plasmodium falciparum* and may prove efficacious for treating malaria [43]. Moreover, CLT also possesses antitumor properties related to its ability to inhibit Ca^{2+}-ATPase of the sarcoplasmic reticulum [44]. Recent data provide evidence that CLT forms a complex with heme (hemin) that produces a more potent lytic effect than heme alone [45].

As we showed earlier [19], a 20- to 30-min incubation with 2 mM t-BHP resulted in erythrocyte swelling, which was less in a Ca^{2+}-containing medium. Given that this effect was abolished in the presence of the Gardos channel inhibitor CLT, as well as in media high in K^+, we suggested the contribution from the Gardos effect, that is, partial cell dehydration because of elevated intracellular Ca^{2+}. The results of the present study confirmed this suggestion. However, CLT appeared to enhance t-BHP-induced erythrocyte swelling not only in a Ca^{2+}-containing medium, but also in a Ca^{2+}-free medium (Fig. 1), which means that, besides Gardos channel inhibition, there should be other, Ca^{2+}-independent, mechanisms behind this effect.

A longer incubation resulted in larger swelling (Fig. 2) and, at 2 h, lysis began to occur. By 3 h, hemolysis reached 30–40% at a t-BHP concentration of 2 mM. Whatever the t-BHP concentration, the extent of hemolysis did not depend on the presence of Ca^{2+} in the medium (Fig. 3, panels A and B). One can see that CLT considerably and significantly enhanced hemolysis at all t-BHP concentrations in both Ca^{2+}-containing and Ca^{2+}-free media.

Given that CLT produces little, if any, effect on the rate of free radical generation (Fig. 5), on the kinetics of GSH degradation (Fig. 6), methemoglobin formation (Fig. 7), and TBARS generation (Fig. 8), it is reasonable to conclude that 10 μM CLT does not cause additional oxidative damage to erythrocytes treated with t-BHP. Therefore, our results tempt us to speculate that CLT enhances t-BHP-induced changes in erythrocyte volume and lysis largely by forming a complex with hemin released during hemoglobin oxidation in erythrocytes, and thereby destabilizes the cell membrane more potently [45,46].

Indirect evidence in support of this hypothesis was obtained in experiments where we resuspended t-BHP treated erythrocytes in a buffer containing albumin at different concentrations (Fig. 4). Albumin is known as an active chelator of free heme, contributing, along with hemopexin, into clearing free heme from cell mem-

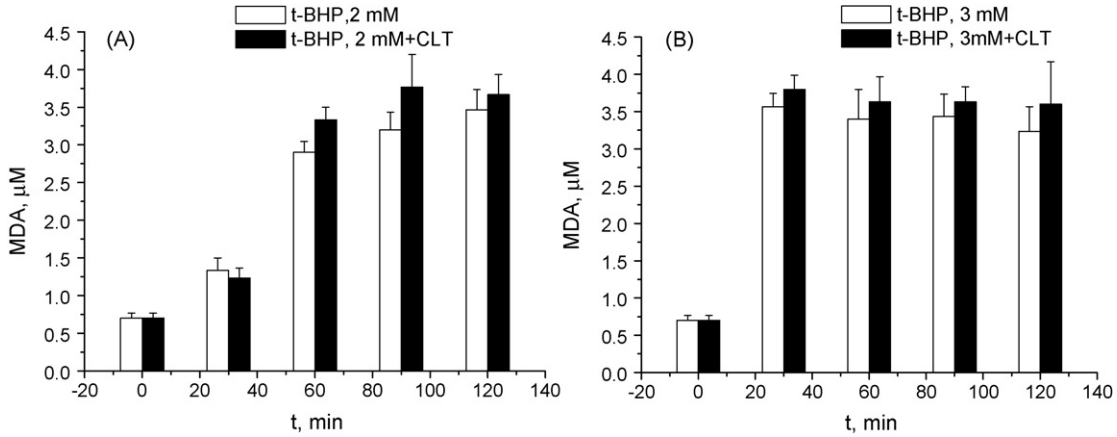

Fig. 8. Effect of CLT on TBARS formation in erythrocytes treated with t-BHP Erythrocytes were incubated with t-BHP at a concentration of (A) 2 or (B) 3 mM in the presence (filled bars) or in the absence (open bars) of CLT (10 μM). The data are representative of 3 separate experiments.

branes [40,47,48]. As can be seen in Fig. 4, the extent of hemolysis decreased with increasing albumin concentration. A possible explanation for these results is that albumin might remove hemin and its complex with CLT from the cell membranes.

Heme/hemin is a functional group of various heme proteins, such as hemoglobin, myoglobin, cytochromes, catalase, and peroxidase. Heme release can occur in, for example, thalassemia, sickle cell anemia, glucose 6-phosphate dehydrogenase deficiency, hemorrhage, and muscle injury. In some pathological cases, heme is present in vivo at high micromolar concentrations [40]. Being a lipophilic molecule, heme is capable of intercalating into the cell membrane, causing cell injury. The heme-degrading enzyme heme oxygenase (HO) plays a significant role in protecting cells from toxic effects of free heme [40,49].

This study is the first to show that CLT at a concentration of 10 μM significantly increases erythrocyte swelling and nearly doubles lysis induced by the model oxidant t-BHP, which indicates that the membrane is damaged to a much greater extent in the presence of CLT. Therefore, it is tempting to speculate that CLT may act as a cytotoxic agent in pathologies associated with oxidative stress and increased hemoglobin release (e.g., intravascular hemolysis).

The consequences of its clinical use may be positive and negative. For example, a concern arises that the use of CLT during painful crisis in sickle cell anemia [42] may have adverse effects on erythrocytes, thereby exacerbating the course of anemia.

On the other hand, we assume that the effect of CLT on cell membrane damage during free radical oxidation may be employed to increase the efficacy of antitumor therapy, even more so that there are data demonstrating inhibition of the heme-degrading enzyme heme oxygenase (HO-1) in the presence of the drug [50].

Conflict of interest

The authors declare that there are no conflicts of interest.

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