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Oxidative Processes Induced by *tert*-Butyl Hydroperoxide in Human Red Blood Cells: Chemiluminescence Studies

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Abstract—The erythrocyte is a good model for investigation of the mechanisms of cell damage induced by oxidizing agents. Oxidative damage to cell components and cellular metabolism results in impaired rheological properties of circulating red blood cells and is involved in the development of some pathologies. The aim of the present study was to elucidate further the oxidative processes induced by *tert*-butyl hydroperoxide (tBOOH) in erythrocytes, identify cellular targets damaged by the oxidant, as well as estimate the energy and stoichiometry of the reactions that occur. The generation of free radicals in the cell was registered using the chemiluminescence technique. The products of oxyhemoglobin (oxyHb) oxidation, changes in intracellular glutathione (GSH) pool, and accumulation of the stable products of membrane lipid peroxidation were concurrently measured. The oxidative processes induced by tBOOH in red blood cells can be described as follows: 1) rapid GSH oxidation (30-60 sec) by glutathione peroxidase; 2) formation of radicals in the reaction between tBOOH and cellular Hb, which are then immediately consumed in lipid peroxidation reactions; 3) generation of chemiluminescence by the radicals formed. Several stages of the oxidative processes can be revealed. The order of the chemiluminescence reaction (n) with respect to oxidant was estimated to be equal to 2.5 at oxidant concentrations less than 0.5 mM and equal to 1.0 at higher oxidant concentrations. The order of the reaction of membrane lipid peroxidation was found to be $n = 2.2$ at 0.25-0.6 mM tBOOH and $n = 0.5$ at higher oxidant concentrations. The apparent activation energy of membrane lipid peroxidation was 55.8 ± 6.4 kJ/mol, and that of oxyHb oxidation was 108 ± 16 kJ/mol. It is shown that the interaction of tBOOH and HOCl in erythrocytes is accompanied by changes in both the total number of radicals generated in the cell and the time corresponding to the maximal rate of radical generation.

Key words: human red blood cells, oxidative stress, organic hydroperoxide, hypochlorous acid, hemoglobin, chemiluminescence

Chemical and biological interactions of free radicals as well as oxidizing agents of non-radical nature with cellular components are constantly of interest to researchers. Numerous damaging effects of oxidizing agents at the molecular level (oxidation of proteins, peroxidation of lipids, DNA damage), cellular level (disruption of cellular signal transduction pathways, affects on gene expression), and tissue level (development of pathologies, aging) are well known. These effects range between subtle regulation of numerous intracellular signal transduction cascades and induction of sudden cell death. Nonspecific oxidative modification of cellular structures is one of the most potentially dangerous processes for the cell [1]. The toxic effect of free radicals on cellular functions has resulted in the formation of a multilevel antioxidant defense system.

In the last 15 years, "oxidative stress" has become a key term for many scientific investigations. According to the classical definition, oxidative stress means "the disturbance of the prooxidant–antioxidant balance in favor of prooxidants, which can cause potential damage" [1].

Generation of radicals in cells is determined by metabolic activity, oxygen concentration, availability of transition metal ions, and the level of reducing compounds [2]. A convenient cell model for studies of oxidation damage mechanism is erythrocytes, which are highly specialized red blood cells without protein synthesis apparatus and therefore lacking the ability to repair damage. They circulate in blood vessels for 120 days at constant high oxygen concentration, and they also contain high concentration of transition metals ions (iron ions in hemoglobin) [3, 4]. Oxidative damage to structural elements in the membrane and in erythrocyte metabolism leads to disruption of the rheological properties of the cir-

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culating blood cells, which plays a crucial role in the development of certain pathologies [5, 6].

The organic hydroperoxide *tert*-butyl hydroperoxide (tBOOH) is a model oxidizing agent widely used in numerous studies [7]. Noticeable damaging effects of tBOOH in various cell types are well investigated [6, 7]. Mechanisms of generation and types of the formed radicals are also known [8, 9]. Among possible mechanisms of tBOOH cytotoxicity we can mention the supposed disruption of intracellular calcium homeostasis, which follows the oxidation of the reduced glutathione and protein thiols in the cell [10, 11], as well as appearance of DNA strand breakage [7], peroxidation of lipids [12], and an increase in intraglobular mobility of membrane proteins [13]. However, the cellular targets that are primarily interacting with the oxidant and the cascade from oxidative modification of primary target to cell death require further investigation.

Chemiluminescence (i.e., light emission accompanying chemical reactions) is widely used in the studies of processes involving free radicals [14]. In this work, we used the luminol-dependent chemiluminescence technique for estimation of parameters of generation of free-radical intermediate products in the reaction between human erythrocytes and organic hydroperoxide tBOOH (a water soluble analog of lipid hydroperoxides that easily penetrates the cell membrane). Earlier we used the chemiluminescence technique for investigation of the interactions between tBOOH and human hemoglobin (Hb) [15].

The aim of this work was to further clarify the oxidation processes that are induced in human erythrocytes by tBOOH, to determine key elements in the cell being primarily damaged by the oxidant, and to study the energy and stoichiometry of the reactions. Equilibrium concentrations of intermediate free-radical products registered by the chemiluminescence technique were compared with the concentration of final stable products of oxidation reactions associated with tBOOH metabolism in erythrocytes. The oxidant was added into erythrocyte suspension both as a single injection and continuously at a constant rate. Also, we considered the interaction of organic tBOOH hydroperoxide in human erythrocytes with one of the strongest biological oxidants, hypochlorous acid.

MATERIALS AND METHODS

Reagents. For this work we used *tert*-butyl hydroperoxide (tBOOH), sodium hypochlorite (NaOCl), 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman reagent), 2-thiobarbituric acid (TBA), trichloroacetic acid (TCA), glutathione reductase, NADPH, and sodium nitrite from Sigma-Aldrich (Germany); luminol from Serva (Germany); other reagents were of chemically pure grade from Reakhim (Russia).

Erythrocytes. Erythrocytes from healthy donors were received at the Blood Transfusion Station in Grodno. After removing plasma and the leukocyte layer, erythrocytes were washed three times with cold isotonic buffered salt solution (PBS: 0.145 M NaCl, 19 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4) and used as a suspension with 5% hematocrit in PBS. Erythrocytes containing hemoglobin in met-form were obtained by incubating erythrocytes (5% hematocrit) with 4 mM $NaNO_2$ for 30 min at 20°C and then washing three times with cold PBS.

Oxidation damage of erythrocytes. tBOOH was used as a freshly made 100 mM solution in PBS. The required amount of the oxidant was added into the erythrocyte suspension preincubated at the reaction temperature for 15 min. For studying the cooperative effect of tBOOH and hypochlorous acid, the erythrocytes were preincubated with HOCl for 5 min.

The concentrations of stable products of membrane lipid peroxidation reacting with TBA (TBA reactive substances, TBARS) were determined spectrophotometrically using the method of Stocks and Dormandy [16], assuming that the molar absorption coefficient ε_{532} is $1.56 \cdot 10^5$ $M^{-1} \cdot cm^{-1}$. The intracellular concentration of reduced glutathione (GSH) was determined spectrophotometrically by the method of Ellman [17] using molar absorption coefficient $\varepsilon_{412} = 1.36 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$. The total glutathione concentration (GSH + GSSG) was determined enzymatically using cyclic GSH reduction in the glutathione reductase–NADPH–Ellman reagent system by the method of Akerboom and Sies [18] in the acid-soluble fraction of the cell suspension.

The level of mixed glutathione disulfides with erythrocyte proteins was determined according to the method described by Rossi et al. [19]. The concentration of intracellular hemoglobin (Hb) forms was determined spectrophotometrically (0.1 ml of suspension with 5% hematocrit was lysed with 1 ml H_2O) using the equations proposed by Winterbourn et al. [20, 21].

Chemiluminescence measurements. Kinetic curves of free radical generation and scavenging were detected by the technique of luminol-dependent chemiluminescence (CL) using an KhLM1Ts-01 chemiluminometer (USSR). The total volume of the sample containing erythrocyte suspension in PBS (5% hematocrit) and 10 μM luminol (6 mM luminol stock solution in ethanol) was 3 ml. In the course of the reactions, the oxidant was introduced directly into the measuring cuvette both by single injection and continuously with constant rate using a micro dosing system. Kinetic curves of CL were analyzed by the following parameters: maximum intensity, time of reaching maximum CL, total light emission. It should be noted that the CL maximum for luminol (425 nm [14]) is overlaid by the hemoglobin absorption band. CL absorption by hemoglobin and scattering by the cell suspension will change detected light intensity.

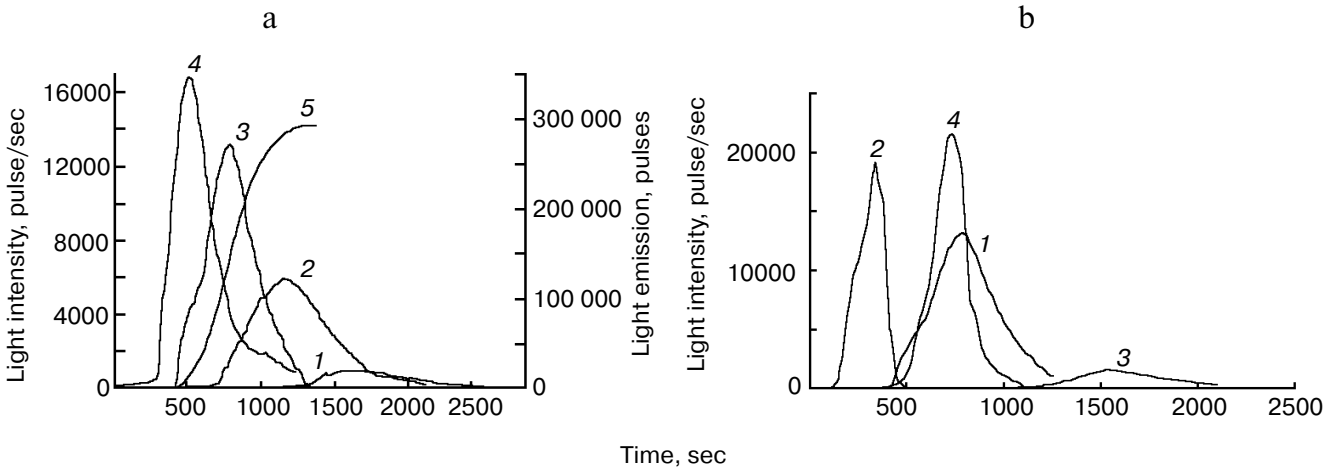

Fig. 1. Kinetic curves for chemiluminescence induced in human erythrocyte suspension by single injection (a) and continuous supply (b) of tBOOH (5% hematocrit, PBS, pH 7.4, 37°C, 10 μM of luminol). a) CL at different tBOOH concentrations (mM): *1*) 0.32; *2*) 0.63; *3*) 1.25; *4*) 1.87; *5*) integral curve of CL corresponding to differential curve *3* (1.25 mM tBOOH concentration). b) CL at the rates of tBOOH injection equal to 2.5 and 10 μmol/ml of suspension per hour (curves *3* and *4*, respectively); *1*) CL registered on single-injection supply of the same tBOOH total amount (1.25 mM) added during the whole observation time at 2.5 μmol/ml of suspension per hour flow rate; *2*) CL registered on single-injection supply of the same tBOOH total amount (5 mM) injected during the whole observation time at 10 μmol/ml of suspension per hour flow rate.

Statistical analysis. All results are presented as the mean values of 4-6 measurements ± standard deviation; significance of differences was evaluated using Student's *t*-test.

RESULTS

Chemiluminescence of erythrocyte suspensions. The sensitivity of human erythrocytes towards oxidative stress induced by tBOOH was estimated by the parameters of CL signal, level of membrane lipid peroxidation products, changes in intracellular glutathione pool, and ratio of erythrocyte hemoglobin forms. Figure 1a shows kinetic curves for luminol-dependent CL induced in human erythrocytes containing oxyhemoglobin (oxyHb) by different tBOOH concentrations added as a single injection. Figure 1b shows kinetic curves for CL induced in erythrocytes by tBOOH that was injected into the suspension continuously at different rates. For comparison, there are also presented curves for CL induced in erythrocytes by the single injection of the same amounts of tBOOH that was added via constant supply during the whole observation time. Under constant supply of tBOOH into the erythrocyte suspension, the CL generation starts at oxidant amount of 17.5 ± 2.5 μmol/ml packed cells (at oxidant injection rate of 2.5 μmol/ml of cell suspension per hour). The threshold amount of hydroperoxide required for CL generation increased as the oxidant supply rate increased (Fig. 1b). At low rates of oxidant supply (2.5 μmol/ml of suspension per hour) total light emission of CL was significantly lower than total light emission of CL generated from a single injection of the same amount of oxidant as was injected into the suspension continuously during the observation.

Kinetic curves of the CL induced in human erythrocytes containing oxyHb and methemoglobin (metHb) and in hemolysates of erythrocytes are presented in Fig. 2. (Erythrocytes initially oxidized by sodium nitrate con-

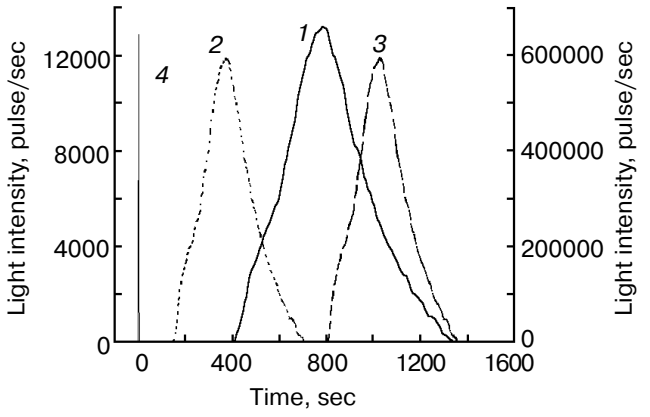

Fig. 2. Kinetic curves for chemiluminescence induced by tBOOH (1.25 mM) in human erythrocyte suspension (*1*, *2*) and in hemolysate of the corresponding cell amount (*3*) (left axis): *1*) erythrocytes containing oxyHb; *2*) erythrocytes containing metHb (preliminarily treated with $NaNO_2$); *3*) hemolysate (5% hematocrit, PBS, pH 7.4, 37°C, 1.25 mM tBOOH, 10 μM of luminol; CL of hemolysate was registered in 0.05 M phosphate buffer, pH 7.4); *4*) kinetic curve of chemiluminescence induced in the system luminol + HOCl (right axis); concentration of luminol, 10 μM; HOCl, 1 mM; PBS, pH 7.4, 37°C.

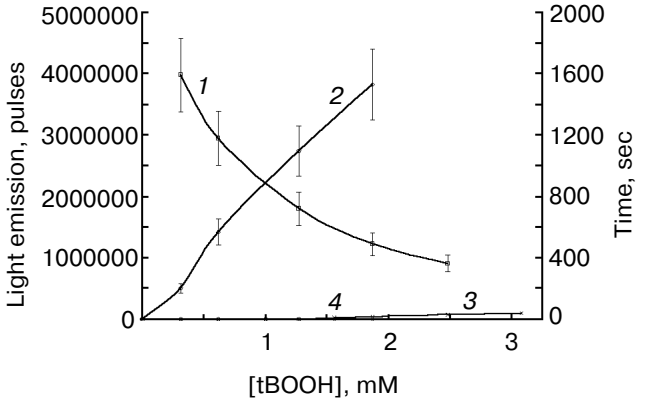

Fig. 3. Dependence of time corresponding to maximum intensity of tBOOH-induced chemiluminescence in erythrocyte suspension CL (curve *1*) and integral light emission value (*2*, *3*) on oxidant concentration in the presence (*2*) of luminol and in the absence of luminol (*3*) (5% hematocrit, PBS, pH 7.4, 37°C, 10 μM of luminol); *4*) CL registered in the course of interaction between tBOOH and luminol (PBS, pH 7.4, 37°C, 10 μM of luminol).

tained ~90% of intracellular Hb in met-form and 100% of intracellular reduced glutathione, as determined in preliminary experiments.)

It should be noted that chemiluminescence curves are individual characteristics of the blood samples and vary (by maximum intensity, total light emission of CL) from one sample to another and during storage. Kinetic curves of CL generation have complex shape, which reflects a number of stages in the process of generation and deactivation of the free radical products in the system; lag-phase, intensive generation phase, and termination phase (Fig. 1) can be distinguished. Obviously, the concentration of radical products in the system is determined by a number of competing processes. In the case of suspension of erythrocytes containing metHb, the shape of CL kinetic curves sufficiently differs: the area under the curve and maximum CL intensity decreases, and the curves are shifted along the time axis towards the origin (Fig. 2, curve *2*). Preliminary hemolysis of erythrocytes in hypotonic medium decreased the total light emission of CL and increased the time corresponding to the maximum light intensity (Fig. 2, curve *3*).

Kinetic curves of CL (Fig. 1) describe a differential form of the process, each point on the curve characterizing the rate of accumulation of free radicals in the system (registered by the CL technique) at a corresponding moment in time, the intensity of the curve maximum corresponds to the maximal reaction rate, and area under the CL curve is proportional to the total number of generated radical products. By integrating the corresponding differential curves, we obtain a number of integral curves for tBOOH-induced CL, where each point represents the total number of quanta emitted from the start of the reaction (Fig. 1a, curve *5*).

Under our experimental conditions, we did not register CL in the absence of luminol, and direct interaction of luminol and tBOOH was not accompanied by CL (Fig. 3, curves *3* and *4*). As the tBOOH concentration increases the integral light (total light emission of CL) increases (until tBOOH concentration of 2.5 mM after single injection), while the time of CL maximum is sharply decreased (Figs. 1 and 3). In the oxidant concentration range of 0.5-2.5 mM a linear dependence between the total light emission of CL and oxidant concentration can be observed (Fig. 3).

Oxidation processes in human erythrocytes induced by tBOOH. Besides tBOOH-induced CL in human erythrocytes, we could also detect such processes as oxidation of intracellular oxyHb and reduced glutathione and peroxidation of membrane lipids. Kinetic curves of these processes are presented in Fig. 4. tBOOH-induced oxidation of erythrocytes leads to the formation of metHb and also the ferryl form of Hb and hemichrome. By registering spectra of erythrocyte hemolysates exposed to tBOOH and using the algorithms proposed by Winterbourn et al. [20, 21], we calculated the content of different Hb forms in the cells (Fig. 4, curves *2-4*).

Figure 5 presents the changes in concentration of different forms of intracellular glutathione (GSH) in the erythrocytes exposed to various tBOOH concentrations. Oxidation of GSH into its disulfide form occurs in less than 60 sec (Fig. 4, curve *1*) and is not accompanied by a significant change in concentration of total (GSH + GSSH) intracellular glutathione (Fig. 5, curve *2*). When oxidant concentrations exceed 0.5 mM, we observe the formation of mixed glutathione disulfides with erythrocyte proteins (GSSP) (Fig. 5, curve *3*). Human erythrocytes (according to our measurements) initially contain

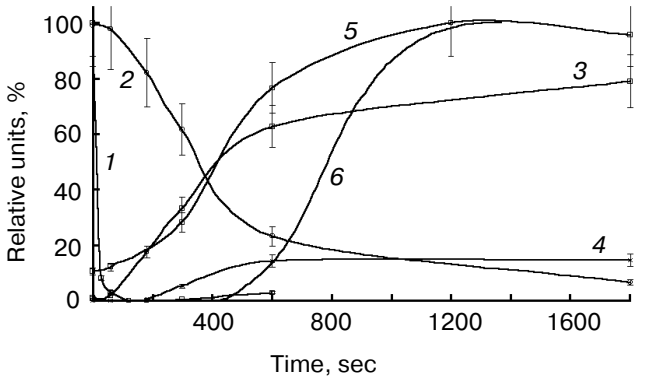

Fig. 4. Oxidative processes induced by the organic hydroperoxide tBOOH in human erythrocytes: *1*) reduced glutathione oxidation; *2*) oxyHb oxidation; *3*) metHb accumulation; *4*) ferryl form of Hb accumulation; *5*) peroxidation of membrane lipids (formation of TBARS); *6*) chemiluminescence integral curve (10 μM of luminol) (5% hematocrit, 1.25 mM tBOOH, PBS, pH 7.4, 37°C). All measured parameters are normalized to their maximum values.

109 ± 17 nmol of mixed glutathione disulfides with proteins per ml of packed cells, mainly with erythrocyte Hb. As illustrated in Fig. 4, oxidation processes of erythrocyte oxyHb and lipid peroxidation proceed in parallel and start after the completion of intracellular GSH oxidation. Generation of free radical products in the tBOOH reaction with erythrocytes is registered by the CL technique only after the completion of these processes.

Preliminary oxidation of intracellular oxyHb into met-form inhibits the peroxidation of erythrocyte membrane lipids; at the same time, lipid peroxidation occurs with the same efficiency in both whole cells and in erythrocytes hemolysate (data not presented).

Interaction of tBOOH and hypochlorous acid in reaction with human erythrocytes. Different oxidants generate different types of radical products in the course of interaction with cellular components. Hypochlorous acid (one of the strongest biological oxidants) reacting with luminol induces a short (few seconds) intense CL flash (Fig. 2, curve *4*). Injection of tBOOH into such system (HOCl–luminol) at the moment of complete quenching of the HOCl-induced light emission results in a second but less intense flash.

Preliminary injection of hypochlorous acid into the erythrocyte–luminol–tBOOH system significantly changes the parameters of generation of radical products induced by tBOOH. tBOOH was introduced into the erythrocyte suspension 5 min after HOCl addition. By this time, CL caused by the interaction between HOCl and luminol is completely quenched. As HOCl concentration increased we observed increase in the total number of CL quanta (at HOCl concentrations lower than 200 μM) with subsequent decrease; the time corresponding to maximum CL intensity decreases with increasing HOCl concentration (Fig. 6).

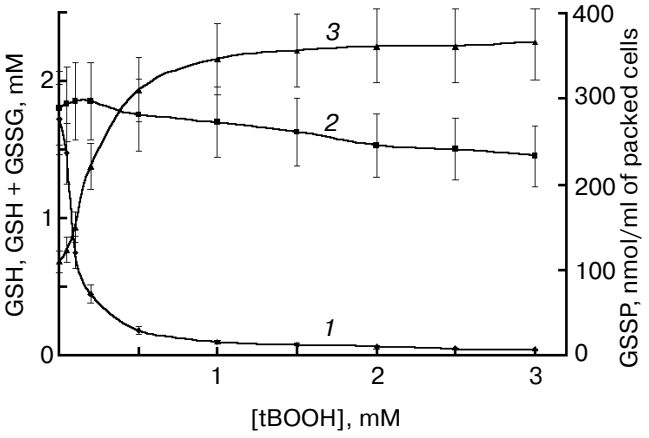

Fig. 5. Oxidative processes induced by tBOOH in human erythrocytes. Dependence on oxidant concentration of reduced glutathione (GSH) content (*1*), total glutathione (GSH + GSSG) content (*2*), and content of mixed glutathione disulfides with cell proteins (*3*) (5% hematocrit, PBS, pH 7.4, 37°C; erythrocyte exposure time, 30 min).

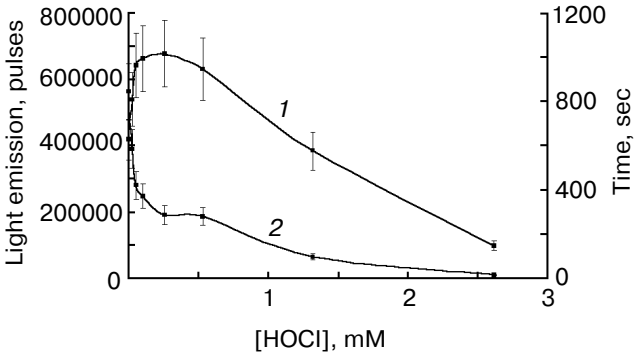

Fig. 6. Influence of hypochlorous acid on integral light emission (*1*) and time (*2*) corresponding to maximum intensity of CL induced by tBOOH in erythrocyte suspension. CL light emission was determined as the area under the CL curve (5% hematocrit, PBS, pH 7.4, 37°C, 1.25 mM tBOOH, 10 μM of luminol; HOCl was introduced in the medium 5 min before tBOOH injection).

DISCUSSION

Toxic water-soluble tBOOH fairly quickly penetrates the plasma membrane of mammalian cells [22] and is primarily metabolized by intracellular glutathione peroxidase and glutathione S-transferase [22, 23]:

$$ROOH + 2GSH \rightarrow ROH + GSSG + H_2O.$$

The exposure of mammalian cells to the organic hydroperoxide tBOOH induces a cascade of cytotoxic reactions: noticeable inhibition of cellular proliferation, decreased ability of cells to form colonies [11], morphologic transformation of the cells [6], disruption of permeability of mitochondrial membrane, and necrotic death of the cells [24].

In the case of erythrocytes, the oxidative damage induced by tBOOH is associated with the peroxidation of membrane lipids and degradation of cytoskeleton proteins (horizontal junctions of the cytoskeleton is a specific target) [6], efficient inhibition of Ca-ATPase of cell membranes [23], and sharp increase in the passive cation permeability of the membrane [25]. The GSH–glutathione peroxidase system plays a crucial role in the metabolism of organic hydroperoxide and in antioxidant protection of erythrocytes [23]. Already in the early work of Ataullakhanov et al., it was shown that the introduction of tBOOH into human erythrocytes with rate exceeding the rate of the pentose-phosphate pathway results in the rapid and complete oxidation of reduced glutathione and release of K^+ and hemoglobin [26]. According to this work, the increase in the membrane

permeability for Na^+ and K^+ correlates with peroxidation of lipids [26].

As we demonstrated earlier, the exposure of human erythrocytes to tBOOH resulted in the following sequence of oxidative processes: GSH oxidation → oxyHb oxidation → peroxidation of membrane lipids. This was accompanied by morphologic transformations of the cell: vesiculation, aggregation, formation of echino- and stomatocytes, complex changes in the structure of the plasma membrane and its hyperpolarization [27-30]. Hence, the organic hydroperoxide initiates a cascade of complex reactions in erythrocytes, the chemistry and biochemistry of these being fairly well known; the main stage of this cascade is an interaction of erythrocyte oxyHb with tBOOH.

Currently, there are a number of proposed schemes describing the interaction between heme-containing proteins and enzymes with organic hydroperoxides, including a peroxidase mechanism and homolytic cleavage of hydroperoxide [8, 9, 31]. In the case of interaction between Fe^{3+}-hematin and tBOOH, it has been suggested that alkoxyl radical formed as a result of the homolytic cleavage of the O–O bond in hydroperoxide is a primary radical [9]:

$$\text{porphyrin–Fe(III)} + \text{tBOOH} \rightarrow$$

$$\rightarrow \text{porphyrin–Fe(IV)=O} + \text{tBO}^{\bullet},$$

$$\text{porphyrin–Fe(IV)=O} + \text{tBOOH} \rightarrow$$

$$\rightarrow \text{porphyrin–Fe(III)} + \text{tBOO}^{\bullet}.$$

The peroxidase mechanism includes formation of ferryl form of heme protein (Fe(IV)=O) and peroxyl radical $tBOO^{\bullet}$ (heterolytic cleavage of hydroperoxide) [8, 9]:

$$\text{porphyrin–Fe(III)} + \text{tBOOH} \rightarrow$$

$$\rightarrow \text{porphyrin}^{+\bullet}\text{–Fe(IV)=O} + \text{tBOH},$$

$$\text{porphyrin}^{+\bullet}\text{–Fe(IV)=O} + \text{tBOOH} \rightarrow$$

$$\rightarrow \text{porphyrin–Fe(IV)=O} + \text{tBOO}^{\bullet}.$$

In this case, hydroperoxide undergo double-electron reduction into a corresponding alcohol. Ferryl-hemoglobin is formed as an apoprotein radical, which explains the transfer of the second oxidation equivalent (the radical is localized on an aromatic amino acid residue).

Another possible mechanism is a reduction of tBOOH into alkoxyl radical in the Fenton reaction:

$$\text{porphyrin–Fe(II)} + \text{tBOOH} + H^+ \rightarrow$$

$$\rightarrow \text{porphyrin–Fe(III)} + \text{tBO}^{\bullet} + H_2O.$$

Another tBOOH molecule will be oxidized into peroxyl radical by one electron transfer with concomitant reduction of ferri-heme [9]:

$$\text{porphyrin–Fe(III)} + \text{tBOOH} \rightarrow$$

$$\rightarrow \text{porphyrin–Fe(II)} + \text{tBOO}^{\bullet}.$$

Barr et al. demonstrated the formation of peroxyl, alkoxyl, and methyl radicals in cytochrome P450–cumene hydroperoxide, suggesting, however, that the primary radical is alkoxyl radical [8].

Yoshida et al. suggest the formation of *tert*-butoxyl and *tert*-butylperoxyl radicals and also ferryl hemoglobin (HbFe(IV)=O; $Hb^{\bullet+}Fe(IV)=O$) accompanying the reactions of tBOOH in erythrocyte hemolysates [31]:

$$\text{HbFe(II)} \xrightarrow{\text{ROOH}} \text{HbFe(III)},$$

$$\text{Fe(III)} + \text{ROOH} \rightarrow \text{HbFe(III)}(^{-}\text{OOR}) + H^+,$$

$$\text{HbFe(III)}(^{-}\text{OOR}) \rightarrow \text{HbFe(IV)=O} + \text{RO}^{\bullet} \rightarrow$$

$$\rightarrow Hb^{\bullet+}\text{Fe(IV)=O} + \text{RO}^{-},$$

$$\text{HbFe(IV)=O} + \text{ROOH} \rightarrow \text{HbFe(III)} + \text{ROO}^{\bullet} + HO^{-}.$$

The formed ferryl groups of heme proteins are exceptionally strong oxidants involved in lipid peroxidation reactions [31].

Hydroperoxide transformation in cells probably involves several mechanisms simultaneously, and the presented mechanisms do not exhaust all hydroperoxide reactions in human erythrocytes. It should be mentioned that interactions between heme proteins and hydroperoxides are characterized by high cytotoxicity. Hydroperoxide cleavage in erythrocytes is one of a few oxygen activation mechanisms triggered by hemoglobin. Redox reactions of hemoglobin in erythrocytes initiate a cascade of oxidation reactions serving as a source of reactive forms of oxygen (and nitrogen), which can induce oxidative stress and participate in the regulation of important physiological processes.

Generated in erythrocytes exposed to tBOOH, the radicals are able to oxidize luminol and induce CL. It is well known that luminol quickly penetrates through the cell membrane and is able to react with different reactive forms of oxygen generated both intracellularly and extracellularly [32]. The CL technique is widely employed for quantitative analysis of free radical generation in erythrocytes [33, 34]. Early investigations of kinetics of CL induced by bivalent iron ions in nuclear membrane suspension allowed identifying four stages of the CL genera-

tion process, which correlate with TBARS accumulation stages in the membranes: "rapid flash" of CL, "latent period", "slow flash", and "stationary light emission" [35]. Long lag-phase of CL generation observed in our experiments on tBOOH injection into the cell suspension with constant rate exceeding (according to Ataullakhanov et al.) the rate of the pentose-phosphate pathway in erythrocytes [26] can be explained by the tBOOH metabolism by the erythrocyte antioxidant system.

Disruption of the erythrocyte plasma membrane by osmotic hemolysis decreases the total light emission of CL and increases the time for reaching maximal reaction rate of radical generation by changing the local balance of Hb/tBOOH concentrations (Fig. 2, curve *3*). It is worth mentioning that lysis of erythrocytes will decrease CL light scattering observed in the cell suspension.

Preliminary oxidation of erythrocyte oxyHb into metHb by sodium nitrate significantly decreases the total number of radical products formed (light emission of CL) and the time required for radical generation to start. We did not observe tBOOH-induced peroxidation of membrane lipids in erythrocytes containing metHb (data not presented), which indicates a different role of radicals generated by HbFe(II) and HbFe(III) in further oxidation processes in the cell. Earlier, it was revealed that alkoxyl radicals play the main role in the induction of the lipid peroxidation reaction [36]. Perhaps the reason for almost complete inhibition of TBARS accumulation in metHb containing erythrocytes is a dependence of lipid peroxidation processes on oxygen content in the cell, which significantly drops after conversion of intracellular oxyHb into met-form.

Parameters for free radical generation in erythrocytes depend on oxidant concentration: the time of the process decreases; generation rate and amount of radicals generated increase (Figs. 1 and 3).

By presenting the dependence of maximal radical generation rate (proportional to maximal CL intensity) on tBOOH concentration (after single injection) as the following equation:

$$\ln I_{max} = n \ln[\text{tBOOH}],$$

we estimated order of reaction as $n = 2.5$ at tBOOH concentrations lower than 0.5 mM (under these conditions the process is described by the dependence similar to the quadratic one) and $n = 1.0$ at higher oxidant concentrations (the process is described by the linear dependence) (Fig. 7, curve *1*). We also estimated the order of CL generation reaction in the conditions of continuous oxidant supply, using the equation:

$$\ln I_{max} = n \ln V_{tBOOH},$$

where V_{tBOOH} is the rate of tBOOH injection into the erythrocyte suspension. In this case, the order of CL generation reaction is $n = 2.5$ at oxidant injection rate 2-5 μmol/ml of suspension per hour and $n = 0.5$ at higher rates of tBOOH injection (Fig. 7, curve *3*).

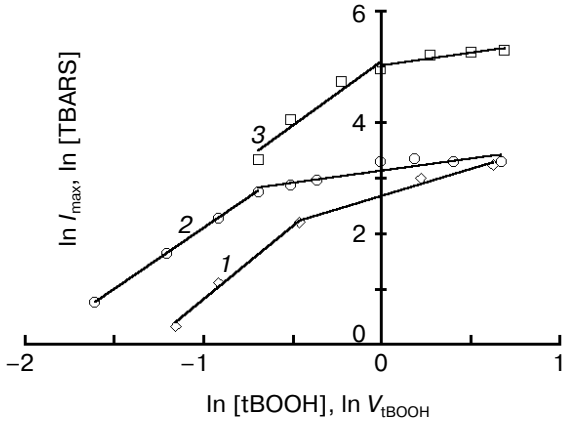

Fig. 7. Dependence of the rate of tBOOH induced oxidation processes in human erythrocytes on oxidant concentration (*1*, *2*) and oxidant injection rate (*3*): *1*, *3*) rate of generation of free radicals registered via the CL technique (in relative units); *2*) rate of accumulation of lipid peroxidation products (TBARS) (5% hematocrit, PBS, pH 7.4, 37°C; TBARS content was measured after erythrocyte incubation for 30 min; oxidant injection rate is presented in relative units).

TBARS accumulation rate expressed as

$$\ln V_{TBARS} = n \ln[\text{tBOOH}],$$

allowed us to estimate the order of the lipid peroxidation reaction (Fig. 7, curve *2*). The peroxidation process also has complex character and includes several stages: at low concentrations (lower than 0.25 mM) oxidant is consumed in inactivation of cell protection systems; at oxidant concentrations of 0.25-0.60 mM the reaction order is $n = 2.2$ (two tBOOH molecules are consumed for formation of one oxidation product molecule); at high tBOOH concentrations the reaction becomes autocatalytic and $n = 0.5$. We estimated the apparent activation energy of peroxidation of erythrocyte membrane lipids by determining the apparent peroxidation rate at different temperatures from linear regions of TBARS accumulation kinetic curves and using the Arrhenius equation; it was 55.8 ± 6.4 kJ/mol.

The main product of tBOOH-induced oxyHb oxidation in erythrocytes (according to hemolysate absorption spectra) is metHb, but hemichrome and ferryl form of Hb are also formed. From the dependence of the apparent rate of metHb formation on temperature, we estimated the apparent activation energy of oxyHb oxidation to be 108 ± 16 kJ/mol. This value is similar to the activation energy of oxyHb auto-oxidation determined by us (120 ± 15 kJ/mol). The fact that the activation energy of the intermediate stage (oxyHb oxidation) is significantly higher than the activation energy of the final stage

(TBARS formation) can be explained assuming that oxyHb oxidation is not a limiting stage of erythrocyte oxidation by tBOOH.

Exposure of erythrocytes to tBOOH significantly changes the content of different forms of intracellular glutathione: reduced glutathione is transformed into the oxidized form, and part of the GSSG is consumed for the formation of mixed disulfides of glutathione with cell proteins (Fig. 5). It is supposed that the reversible S-thiolation of cell proteins can serve as a regulatory mechanism for cellular functions and antioxidant protection during oxidative stress [19].

Kinetic curves of oxidation reactions in human erythrocytes indicate that oxyHb oxidation and lipid peroxidation start only after complete depletion of intra-erythrocyte glutathione. CL, which is generated by radicals formed in the cells, is registered only after the completion of lipid peroxidation (Fig. 4). Perhaps radical concentration in the system is not sufficient for the oxidation of the chemiluminescence probe (luminol), while lipid acceptors of radicals are available. The rate of the interactions of $tBO^{\cdot}$ and $tBOO^{\cdot}$ with membrane lipids is probably higher than for the reaction with luminol.

Hypochlorous acid is a strong two-electron oxidant synthesized by the activated polymorphonuclear leucocytes. It was shown that luminol reacts with ClO^-, which was accompanied by the intensive flash of CL, whose intensity greatly increases in the presence of even small amounts of H_2O_2 [14]. Singlet oxygen formation was assumed in this reaction [14]:

$$H_2O_2 + ClO^- \rightarrow H_2O + Cl^- + {}^1O_2.$$

As was shown for a model system, hypochlorous acid oxidizes tBOOH into *tert*-butyl peroxyl radical [37]. Free radicals formed by organic hydroperoxides in the reaction with HOCl initiate new oxidation processes in the cell [37-39]. The same conclusion can also be made from the dramatic change in parameters of free radical generation induced in human erythrocytes by tBOOH in the presence of hypochlorous acid (Fig. 6).

Using the CL technique we have investigated free radical reactions induced in human erythrocytes by such oxidants as tBOOH and hypochlorous acid. These processes occur according to several mechanisms or in several steps. Sequence of events induced by tBOOH in human erythrocytes can be described as follows: 1) fast (in 30-60 sec) oxidation of GSH in glutathione peroxidase reaction; 2) formation of radical products in the reaction of erythrocyte Hb and tBOOH, which are immediately consumed in the process of lipid peroxidation; 3) CL generation by formed radicals. We have revealed the direct interaction between tBOOH and hypochlorous acid in human erythrocytes accompanied by dramatic changes in parameters of CL initiated by hydroperoxide in the cell.

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