Changes in the Ultrastructure of Human Erythrocytes and in Their Content of Free Fatty Acids during Incubation with Hydroperoxide and Calcium Ions *In Vitro*

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It is shown that incubation of a suspension of human erythrocytes with H_2O_2 and Ca^{2+} mainly results in echinocytic transformation and hemolysis; incubation with H_2O_2 in the absence of Ca^{2+} is attended by polymorphous changes in erythrocytes: discocyte swelling, formation of stomatocytes and echinocytes, and their hemolysis. The level of free fatty acids in human erythrocytes increases for incubation with Ca^{2+} and calcimycin under anaerobic conditions and drops for H_2O_2-induced activation of lipid peroxidation.

Key Words: *erythrocyte ultrastructure; lipid peroxidation*

During *in vitro* and *in vivo* aging, erythrocytes undergo structural transformations in the order normocyte - echinocytes I-IV - spherocyte, and the membrane material pinches off in the form of vesicles (microvesicles) some 0.2 μ in diameter [4,6,7]. This has been shown to be paralleled by an increase in the levels of diacylglycerides [3,5] and lysolecithin [9], indicating activation of phospholipases C and A [9]. The contribution of lipid peroxidation (LPO) to erythrocyte aging is of interest. For example, it has been demonstrated that the effect of H_2O_2 on erythrocytes manifests itself in an increase of the concentration of fluorescing LPO products and of the amount of spectrin-hemoglobin associates, as well as of the degree of echinocytosis; the experiments were performed in a Ca^{2+}-free medium [8,10]. Our study, aimed at determining phospholipase A activity by measuring the free fatty acid (FFA) content for incubation of erythrocytes with calcium ions and calcimycin, demonstrated that the erythrocyte level of FFA does not always increase, although a Ca-activated accumulation of FFA in "ghosts" obtained from the same erythrocytes always occurs. This could be attributed to the activated peroxidation of FFA liberated during the hydrolysis of plasmalemma phospholipids.

The objective of the present study was to investigate changes in the ultrastructure of erythrocytes, in the levels of FFA and malonic dialdehyde (MDA), and in the degree of hemolysis in the case of simultaneous induction of LPO and phospholipase hydrolysis of lipids. LPO was activated by the classical method of H_2O_2 application, and phospholipases were activated by combined application of calcium ions and calcimycin (A23187).

MATERIALS AND METHODS

An erythrocyte suspension was obtained from heparinized fresh blood by thriple centrifugation in isotonic phosphate buffered saline. The incubation medium (37°C) contained 145 mM NaCl and 7 mM sodium phosphate, pH 7.4. Erythrocytes were added to the incubation medium to attain a 5%

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hematocrit. H_2O_2 in a concentration of 10 mM was added to the suspension of erythrocytes preincubated with sodium azide (1 mM). In other cases $CaCl_2$ (2 mM), calcimycin (A23187) (5 mM), and EDTA (2 mM) were added. Anaerobic conditions were created by adding 10 mM glucose and 25 IU/ml glucose oxidase to the suspension. In the course of the experiment samples were taken to determine the degree of hemolysis and the FFA and MDA content. The degree of hemolysis was calculated as the ratio between the optical densities (540 nm) of supernatants (3000 g, 10 min) obtained in the test erythrocyte suspension and in the complete saponin-induced hemolysis. The FFA content was determined by Anderson's method in the modification of Nikitina *et al.* [1], and the MDA content was measured by the reaction with thiobarbituric acid; the values were calculated per 100 mg hemoglobin. Morphological investigations were performed in a separate series of experiments performed under similar conditions. Erythrocytes for scanning electron microscopy (SEM) were fixed in 2.5% glutaraldehyde during 1-4 h, washed in distilled water by centrifugation, dehydrated in ascending grades of ethanol, precipitated by centrifugation, placed on cover slips, air-dried, and then shadowed with gold on a Giko apparatus (Japan). The preparations were examined in a Hitachi electron microscope (Japan).

RESULTS

Our findings on the FFA and MDA levels and the degree of hemolysis under different conditions of incubation of erythrocytes are presented in Fig.

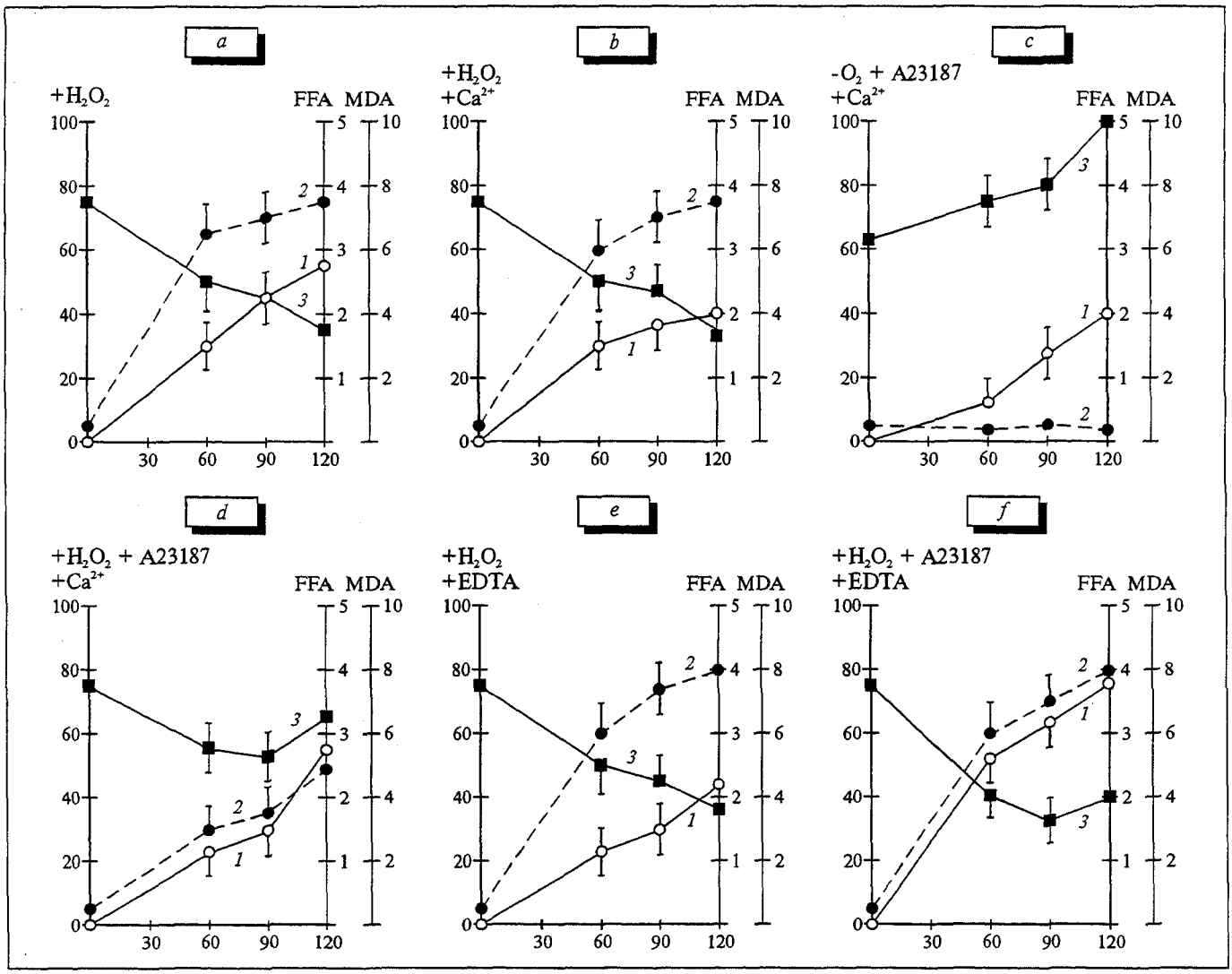

Fig. 1. FFA and MDA content and degree of hemolysis of human erythrocytes during incubation with hydroperoxide and calcium ions *in vitro*. Incubation conditions are shown on each plot by the following designations: $+H_2O_2$: 10 mM H_2O_2; $+Ca^{2+}$: 2 mM $CaCl_2$; +A23187: 5 mM calcimycin; +EDTA: 2 mM EDTA; $-O_2$: anaerobic conditions. Ordinate on each plot at right: MDA and FFA content (nmol per 100 mg hemoglobin). The mean values from 8 experiments are shown. Ordinate (left): Degree of hemolysis, % ; abscissa: Time, min. *1*) degree of hemolysis; *2*) MDA content; *3*) FFA content.

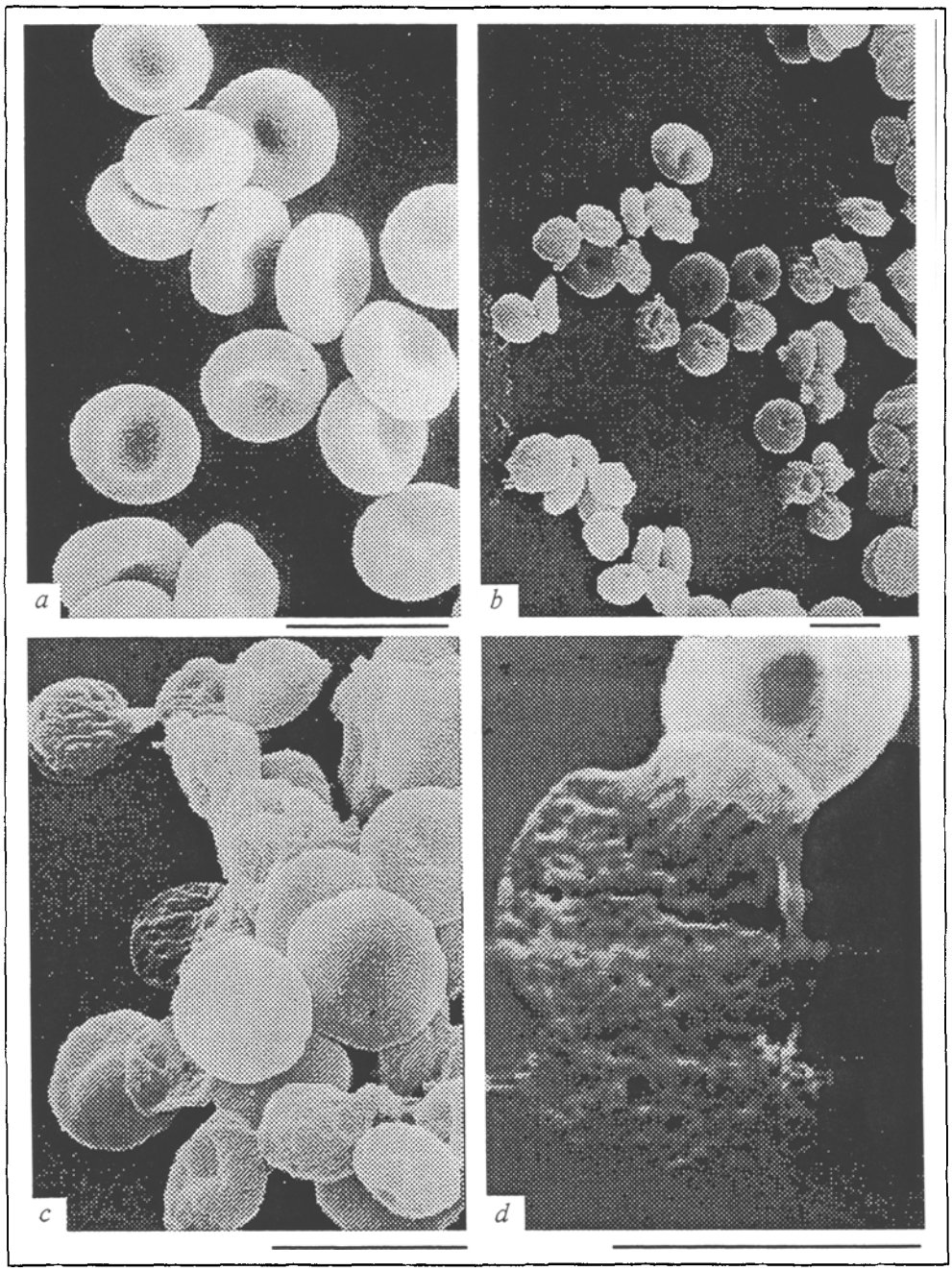

Fig. 2. Ultrastructure of human erythrocytes for incubation with H_2O_2 *in vitro*. *a*) initial erythrocytes – discocytes. *b*) 120 – min incubation with H_2O_2; echinocytes and stomatocytes I – IV, spheroechinocytes, vesiculation, swollen discocytes, release of hemoglobin. *c*) the same as in *b*; erythrocytes at different stages of hemolysis, including ghosts. *d*) erythrocyte ghost with residual hemoglobin. (SEM. Scale 5 μ.)

1. As was expected, an increased MDA concentration and a pronounced hemolysis were observed in all cases (in the presence or absence of Ca^{2+}, or even in the presence of EDTA), except for the variant where erythrocytes were incubated under anaerobic conditions. It should just be noted that the rate of MDA accumulation and hemolysis was much lower (twofold) in the presence of Ca^{2+} and calcimycin in the incubation medium. In the same cases the FFA level dropped; only when erythrocytes were incubated under anaerobic conditions in the presence of Ca^{2+} and calcimycin was a marked elevation of the FFA level observed.

A comparison of the data in Fig. 1, *c* and *d* yields very interesting results. The rates of hemolysis in these experiments were equal, being minimal as compared to the other variants. Presumably, the major cause of hemolysis in both cases was the accumulation of lysophospholipids due to Ca^{2+}-induced activation of phospholipase A, since lysophospholipids are far more effective hemolytic agents than FFA. In the case shown in Fig. 1, *c* this is self-evident; to explain the results depicted in Fig. 1, *d* we may speculate that intensive peroxidation of phospholipid unsaturated fatty acids, leading to lysophospholipid accumula-

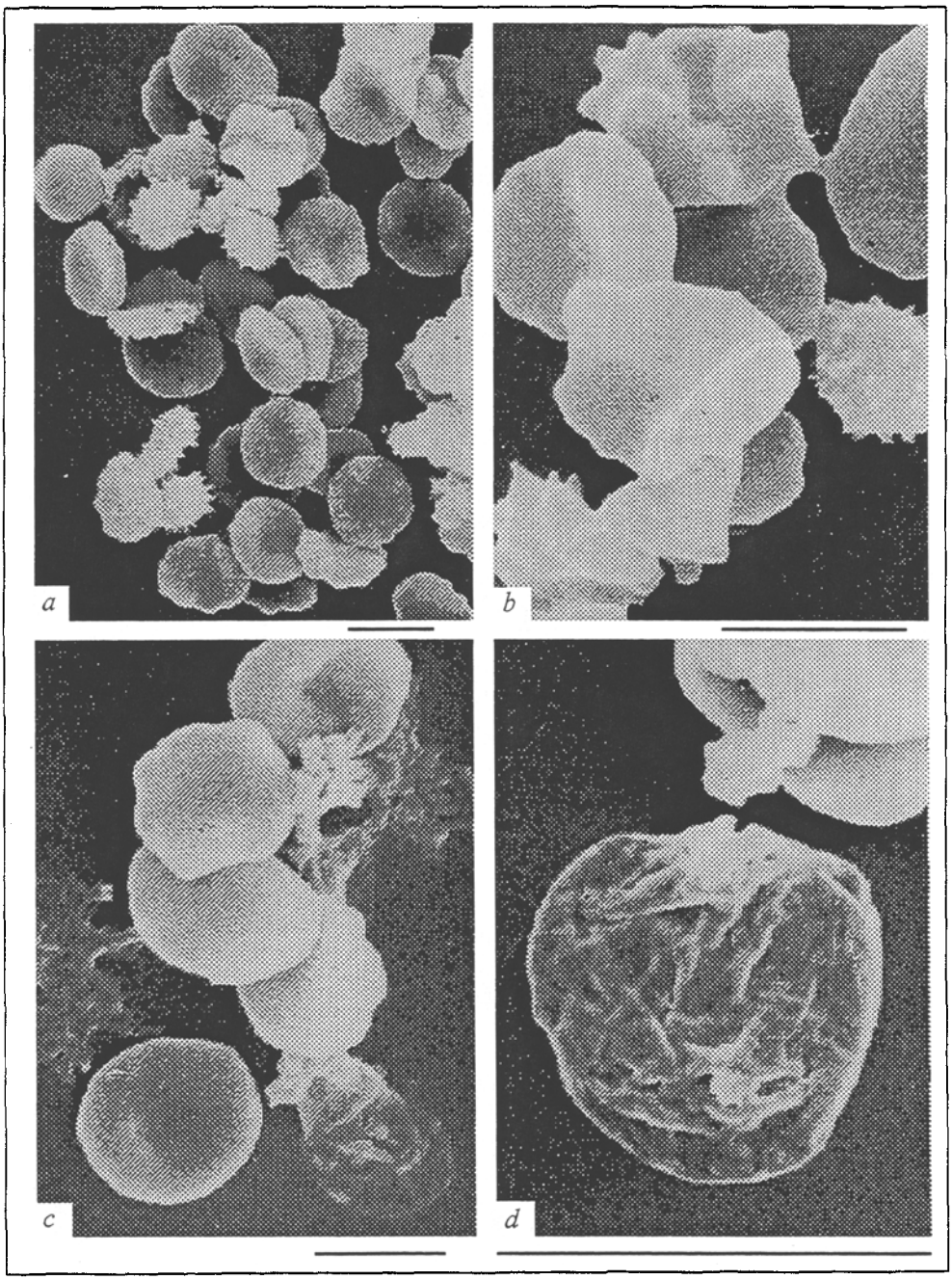

Fig. 3. Human erythrocytes during incubation in the presence of H_2O_2 and calcium ions *in vitro*. *a*) incubation with H_2O_2, Ca^{2+}, and calcimycin for 15 min; pronounced echinocytosis. *b*) the same as in *a*, for 120 min. *c*) incubation with H_2O_2 and Ca^{2+} for 120 min; echinocytes, a swollen discocyte, hemoglobin release, erythrocyte ghost. *d*) erythrocyte ghost. (SEM. Scale 5 μ.)

tion and, respectively, to hemolysis, is markedly lower in the presence of millimolar concentrations of Ca^{2+} than in its absence. In other experiments the addition of calcium ions also lowered the rate of peroxide-induced hemolysis (Fig. 1, *b*), and vice versa, the addition of EDTA (especially together with calcimycin) increased the rate of peroxide-induced hemolysis (Fig. 1, *e* and *f*). On the other hand, since FFA of biomembranes are capable of forming clusters in the presence of Ca^{2+} [2], we may assume that the degree of peroxidation of unsaturated FFA in these clusters will be high due to a high local concentration of double bonds. The latter is very important from the methodological standpoint, since when enzyme lipolysis is judged from an increase in FFA, this may lead to artifacts, which was evidently what was observed in our previous experiments.

Morphological changes of human erythrocytes were studied using SEM in three series of experiments: 1) in the presence of H_2O_2 in the incubation medium; 2) in the presence of H_2O_2 + Ca^{2+} + calcimycin; 3) in the presence of H_2O_2 + Ca^{2+}. It was demonstrated that as soon as after 15 min, incubation of erythrocytes with H_2O_2 resulted in swelling of discocytes (15-30%) and their transfor-

mation into stomatocytes (15-20%) and echinocytes I-II (10-15%), in which hemoglobin release was sometimes observed (not illustrated). After 30, 60, and 120 min of incubation we noted a slight increase in the number of stomatocytes and echinocytes, their transformation into echinocytes III-IV along with vesicle detachment, an increase in the number of hemolysed erythrocytes, and ghost formation, as is shown in Fig. 2.

In series 2 and 3 ultrastructural changes of erythrocytes in the course of the experiments were much alike (Fig. 3) and very different from those described above, consisting mainly of erythrocyte transformation in accordance with the echinocytic pattern of aging. Such a transformation is typical of the experiments in which ultrastructural changes of erythrocytes were studied under conditions of ATP deficiency and Ca^{2+} loading [3-7].

In experimental series 2 echinocytic transformation developed faster than in series 3. For instance, after a 15-min incubation of erythrocytes with H_2O_2 + Ca^{2+} + calcimycin, over 70-80% of the cell population was represented by echinocytes, whereas in the same medium but in the absence of the ionophore calcimycin similar changes of erythrocytes were not observed until after 60 min of incubation. Evidently, this delay was due to a slower penetration of Ca^{2+} into the cytosol of erythrocytes and was determined by the time necessary for the barrier function of the plasmalemma to be destroyed and the energy of the erythrocytes to be lost.

In these series of experiments echinocytic transformation was not complete, and up to 10-20% of the cell pool was represented by swollen discocytes and stomatocytes. In these experiments echinocytes did not exhibit an ideal shape, i.e., they differed from those observed for incubation of erythrocytes with Ca^{2+} and calcimycin in the absence of H_2O_2. These peculiarities may be attributed to the effect of hydroperoxide.

Evidently, an obligatory and crucial event in *in vivo* erythrocyte aging is a rise of the Ca^{2+} level in the cytosol to a concentration at which metabolism gives way to catabolism; the critical calcium concentration is probably within the range 10^{-7}-10^{-6} M. Erythrocyte de-energization is a prerequisite, while destruction of the barrier function of the plasmalemma due to external (with respect to erythrocyte) factors (the presence of xenobiotics, mechanical damage, products of pathological processes, including LPO, etc.) is a nonobligatory, though favorable, condition. The most important component of aging is the activation of lipolysis, which results first in a disturbance of the barrier function and then in destabilization of the erythrocyte plasmalemma and a loss of membrane material by vesiculation. Echinocytic transformation of erythrocytes is a morphological manifestation of these processes.

What is the role of lipid peroxidation in erythrocyte aging? Our experiments as well as the findings of other researchers suggest that, in contrast to echinocytic transformation of erythrocytes for the activation of enzymatic lipolysis of the plasmalemma by calcium ions in the presence of calcimycin [4-7], structural changes of erythrocytes under conditions promoting LPO and in the absence of factors activating enzymatic lipolysis (the presence of H_2O_2 and the absence of Ca^{2+}) are polymorphous [10]. In our experiments, under conditions simultaneously promoting LPO and enzyme lipolysis, up to 20% of erythrocytes did not become transformed into echinocytes, and many echinocytes did not appear to be entirely typical; such an effect may be attributed to the formation of hemoglobin-spectrin bonds [10].

Another point worth noting is that when H_2O_2 is present in the incubation medium, it is the FFA which undergo peroxidation rather than the fatty acids which are components of phospholipids; this explanation is not a direct one, but it does not refute the facts.

We, as well as other researchers [6], think that *in vitro* and *in vivo* peroxidation of membrane lipids under routine conditions is not a key component of erythrocyte aging, but when activated by certain factors, it may markedly modify the aging process.

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