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CALCIUM POTENTIATES THE PEROXIDATION OF ERYTHROCYTE MEMBRANE LIPIDS

SUSHIL K. JAIN and STEPHEN B. SHOHET *

Cancer Research Institute, Division of Hematology, School of Medicine, University of California, San Francisco, CA 94143 (U.S.A.)

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Summary

To explore the possible role of intracellular calcium in membrane lipid peroxidation, we subjected red cells to conditions designed to increase intracellular calcium levels and then measured lipid peroxidation after exposure to a peroxidant threat. Human erythrocytes were pretreated for 3 h with either very high levels of $CaCl_2$, or with low levels in the presence of the ionophore A23187. The erythrocytes were subsequently exposed to a peroxide-generating system consisting of xanthine and xanthine oxidase, or H_2O_2 for 1 h at 37°C. As measured by a malonyldialdehyde assay, the calcium-treated cells showed up to a 2-fold increase in lipid peroxidation in comparison to untreated cells. In experiments with the ionophore, calcium concentration-dependent effects were detected at levels as low as 10 μM and were maximal at 50 μM. A significant loss of phosphatidylserine and phosphatidylethanolamine was observed in calcium- and peroxide-treated erythrocytes. This potentiation of membrane lipid peroxidation and lipid loss could be prevented by either lipid antioxidants or EGTA.

The present study shows that pretreatment of erythrocytes with calcium increases their sensitivity to lipid peroxidation. This suggests that increased calcium concentration may be a factor in the potentiation of membrane lipid peroxidation of erythrocytes known to have increased calcium levels such as sickled and senescent red cells.

* To whom correspondence should be addressed.
Abbreviation: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

The concentration of calcium in the erythrocyte membrane is only 10^{-6} M as compared to 10^{-3} M in circulating plasma. This concentration gradient is maintained by the low permeability of calcium across the membrane and by a calcium pump that utilizes ATP [1,2]. The concentration of calcium in the membrane is increased in sickle cells [3,4] and in aged erythrocytes [5] to as high as 10^{-4} M. However, the consequences of such calcium accumulation in the membrane on the pathophysiology of erythrocytes are not clear. Loading of calcium into normal erythrocytes results in their transformation into echinocytes or the cross-linking of membrane proteins [6—8] and accumulation of 1,2-diacylglycerol in the membrane [9]. This paper reports that the addition of comparatively small amounts of intracellular calcium also potentiates the peroxidation of membrane lipids. This effect is blocked by the lipid antioxidants.

Materials and Methods

Fresh blood was drawn from human volunteers into heparinized tubes and then passed through a cotton-wool column to remove white cells. The blood was then centrifuged at 1500 × *g* for 5 min in a refrigerated centrifuge, and the residual cells were similarly washed three times with cold isotonic saline.

Erythrocyte incubations to pretreat with calcium without ionophore. Erythrocytes were suspended to 4% hematocrit in 10 mM Tris-HCl buffered saline (pH 7.8, 290 mosM). The cells were incubated in Erlenmeyer flasks both with and without $CaCl_2$ (50 mM) at 37°C for 3 h in a shaking water bath. After incubation the cells were washed twice with saline or Tris-HCl buffer and resuspended to 4% hematocrit in the Tris-HCl buffer.

Erythrocyte incubations to pretreat with calcium and ionophore. Our incubation system was similar to that used by Sarkadi et al. [10]. Red blood cells were incubated in a shaking water bath for 3 h at 37°C at 15% hematocrit with a 5 μM concentration of the ionophore A23187 and low concentrations of $CaCl_2$ (10—100 μM). In the experiments with EGTA, 1 mM of the chelator was added to the buffer before addition of the ionophore. At the end of the incubations the cells were washed three times with 0.5% albumin in isotonic saline to remove the ionophore from the cells, and then washed three times with isotonic saline without albumin. The cells were resuspended to 4% hematocrit in the Tris-HCl buffer before treatment with peroxide.

Treatment with peroxide. 2-ml of 4% red cell suspensions were treated with a peroxide-generating system [11] consisting of 5 mM xanthine and a varying concentration of xanthine oxidase as specified in the figure or tables. The cells were incubated in a shaking water bath together with the peroxide-generating system at 37°C for 1 h. In a few experiments where the direct addition of H_2O_2 (2.5—5 mM) was used, the cells were first incubated with NaN_3 at 37°C for 1 h to vitiate catalase activity.

Addition of antioxidants. Tocopherol acetate was prepared as described by Stocks and Dormandy [12]. Aliquots of butylated hydroxyanisole in ethanol were first dried in the test tubes with N_2 before adding red cell suspensions to the tubes. 2-ml of a 4% suspension were preincubated with either 0.5 mM

tocopherol acetate or 0.5 mM butylated hydroxyanisole for 2 h at 37°C before treatment with peroxide.

Measurement of lipid peroxidation. Lipid peroxidation was assayed by measuring malonyldialdehyde, an end product of peroxidation reactions. Malonyldialdehyde reacts with thiobarbituric acid to form a colored complex, which has its maximum absorbance at 532 nm. In preliminary experiments, we confirmed that the presence of calcium did not interfere with the complex formation. Thiobarbituric acid reactivity of the control and peroxide-treated erythrocytes was carried out by using the method of Stocks and Dormandy [12].

Separation of young and old erythrocytes. Washed erythrocytes were resuspended in phosphate-buffered saline containing potassium and glucose [13] to approx. 30% hematocrit and layered on the top of continuous stractan gradient as described by Clark et al. [13]. This technique separated red cells according to their densities. The topmost layer in the gradient (approx. 2%) containing reticulocytes was discarded. The layer just below the topmost layer was designated as the top layer. The top and bottom layers of the gradient, which each constituted about 5% of the total red cells, were removed with a pasteur pipett. The cells were washed with saline four times before their treatment with calcium and peroxide as described above. Since, as the erythrocyte ages its mean cell hemoglobin concentration is increased [14], the hemoglobin concentrations of the washed erythrocytes from the top and the bottom fractions were measured to confirm their separation as young and old cells. The hemoglobin concentrations of the top and bottom fractions were 33 ± 1 and 38 ± 1 g/100 ml, respectively.

Lipid extraction and quantitation. Lipids of the erythrocytes were extracted according to the method of Rose and Oklander [15] using isopropanol/chloroform. Phospholipid classes in the lipid extract were separated by two-dimensional thin-layer chromatography with silica gel H plates using the solvent system described by Jain and Subrahmanyam [16]. The phospholipid phosphorus in the silica gel spots was quantitated by using the method of Fiske and SubbaRow [17].

TABLE I

THE EFFECT OF DIFFERENT CONCENTRATIONS OF XANTHINE OXIDASE ON THIOBARBITURIC ACID REACTIVITY OF NORMAL AND CALCIUM-TREATED ERYTHROCYTES

Values are mean ± S.D. of four samples. Values marked with an asterisk are statistically significant as compared to respective controls. 2 ml of a 4% erythrocyte suspension in 10 mM Tris-HCl buffered saline, pH 7.8, were incubated with the peroxide-generating system at 37°C for 1 h. The peroxide-generating system consisted of 5 mM xanthine and different concentrations of xanthine oxidase as given in the table. The units of xanthine oxidase are as given by Kellog and Fridovich [11]. Thiobarbituric acid reactivity was measured by using the method of Stocks and Dormandy [12].

	Thiobarbituric acid-reactivity (A_{352nm})		
Xanthine oxidase:	0	0.2 U	0.4 U
Untreated	0.008 ± 0.001	0.017 ± 0.001	0.026 ± 0.001
Ca (50 mM)	0.01 ± 0.002	0.033 ± 0.001 *	0.049 ± 0.002 *
Ca (25 μM) + A23187 (5 μM)	0.009 ± 0.001	0.031 ± 0.001 *	0.042 ± 0.002 *

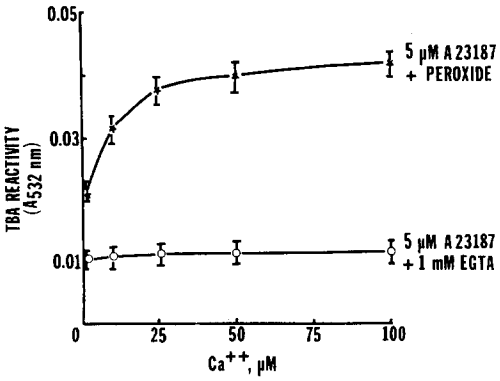

Fig. 1. The effect of different concentrations of calcium on peroxide-induced lipid peroxidation. Conditions of incubation and the method for malonyldialdehyde assay are as given in Table I. 0.2 U of xanthine oxidase were used to generate peroxide. The error bars represent standard deviations of four samples. TBA, thiobarbituric acid.

Results

Table I shows the thiobarbituric acid reactivity of calcium-pretreated cells after their exposure to different concentrations of the peroxide-generating system. These treatments resulted in lipid peroxidation as measured by thiobarbituric acid reactivity, and the extent of reactivity was proportional to the concentration of xanthine oxidase used. When red cells were incubated with a high concentration of calcium (50 mM) alone or a low concentration of calcium (25 μM) with ionophore (5 μM) prior to their exposure to peroxide-generating systems, thiobarbituric acid reactivity was significantly increased ($P < 0.01$).

The effect of different concentrations of calcium on peroxide-induced lipid peroxidation in ionophore-treated cells is shown in Fig. 1. Calcium concentrations as low as 10 μM showed some potentiation of membrane lipid peroxidation. This effect was dependent on calcium concentrations up to approx.

TABLE II

THE EFFECT OF EGTA AND ANTIOXIDANTS ON CALCIUM- AND PEROXIDE-INDUCED PEROXIDATION OF LIPIDS

Values are mean ± S.D. of four samples. All values except those marked with an asterisk were statistically significant. ($P < 0.01$) when compared to respective controls. Conditions of incubation and the method for malonyldialdehyde assay are as given in Table I. Concentrations used are: A23187, 5 μM; Ca, 25 μM; EGTA, 1 mM; Tocopherol, 0.5 mM; butylated hydroxyanisole, 0.5 mM.

Treatment	Increase in thiobarbituric acid reactivity (ΔA_{532nm})	
	2.5 mM H_2O_2	5.0 mM H_2O_2
Untreated	0.024 ± 0.003	0.055 ± 0.002
A23187 + Ca	0.045 ± 0.001	0.091 ± 0.001
EGTA + A23187 + Ca	0.026 ± 0.002 *	0.052 ± 0.003 *
Tocopherol + A23187	0.021 ± 0.001 *	0.042 ± 0.001
Butylated hydroxyanisole + A23187	0.012 ± 0.002	0.029 ± 0.001

TABLE III

THE EFFECT OF CALCIUM AND PEROXIDE TREATMENT ON YOUNG AND OLD ERYTHROCYTES

Values are mean ± S.D. of three samples. Values marked with an asterisk were statistically significant when compared to respective controls or to each other ($P < 0.01$). Populations of young and old erythrocytes were separated from normal blood using a stractan gradient [13]. Conditions of incubation and the method for malonyldialdehyde assay are as given in Table I. Concentrations used are: A23187, 5 μM; Ca, 25 μM; H_2O_2, 2.5 mM.

Treatment	Thiobarbituric acid reactivity (A_{532nm})	
	Young erythrocytes	Old erythrocytes
Untreated	0.011 ± 0.001	0.014 ± 0.002
A23187 + Ca	0.014 ± 0.002	0.015 ± 0.002
H_2O_2	0.041 ± 0.001 *	0.050 ± 0.004 *
A23187 + Ca + H_2O_2	0.065 ± 0.007 *	0.087 ± 0.006 *

50 μM after which saturation occurred. At a concentration of 10 μM, red cells showed a 1-fold increase in thiobarbituric acid reactivity as compared to cells incubated in the absence of calcium.

The data in Table II show the potentiation of peroxidation of membrane lipids by calcium when the cells were treated with peroxide alone. This effect was blocked by both antioxidants and EGTA. The extent of lipid peroxidation in calcium (25 μM) and peroxide (5 mM) treated erythrocytes was nearly 5% of the total peroxidizable lipid, as calculated from the highest thiobarbituric acid reactivity obtained after exposing the erythrocytes to varying concentrations of H_2O_2 (1—300 mM) under similar conditions.

Table III illustrates that older erythrocytes, separated from normal blood using a density gradient, are more susceptible to membrane lipid peroxidation than younger erythrocytes when exposed to the same amount of peroxidant threat. Further calcium preatreatment of these cells potentiated peroxide-induced membrane lipid peroxidation by 102 ± 8% in older erythrocytes and by 66 ± 10% in younger erythrocytes.

The phospholipid composition of calcium- and peroxide-treated erythrocytes is presented in Table IV. The treatment of red cells with peroxide resulted in a significant loss ($P < 0.05$) of phosphatidylserine and phosphatidylethanolamine in the membrane. This loss of phosphatidylserine and phosphatidylethanolamine was significantly greater ($P < 0.01$) when the cells were pretreated with calcium before their exposure to peroxide. The composition of phosphatidylserine and phosphatidylethanolamine was 10.66 ± 0.24 and 26.27 ± 0.93%, respectively, of the total phospholipid in calcium- and peroxide-treated cells as compared to 13.54 ± 1.05 and 30.28 ± 0.21%, respectively, in untreated cells. This loss of phospholipids was blocked in the presence of tocopherol and butylated hydroxyanisole.

Discussion

Lipids are major components of the erythrocyte membrane and are vital for maintaining its structural and functional integrity [18,19]. A contribu-

TABLE IV

EFFECT OF CALCIUM AND H_2O_2 TREATMENT ON HUMAN ERYTHROCYTES PHOSPHOLIPIDS

Values are mean ± S.D. of three observations and are expressed as % of total phospholipid. Differences between a and b ($P < 0.05$), b and c ($P < 0.05$) and a and c ($P < 0.01$) were found to be statistically significant using the Student's *t*-test. Conditions of incubations are as given in Table II. Concentrations used are: A23187, 5 μM; EGTA, 1 mM; Ca, 25 μM; H_2O_2, 5 mM; tocopherol, 0.5 mM; butylated hydroxyanisole (BHA), 0.5 mM.

	Untreated	H_2O_2	A23187 + EGTA + Ca	A23187 + Ca + H_2O_2	A23187 + Ca + tocopherol + H_2O_2	A23187 + Ca + BHA + H_2O_2
Sphingomyelin	22.15 ± 0.55	24.23 ± 1.43	22.13 ± 0.81	23.64 ± 1.62	21.89 ± 0.59	22.41 ± 1.42
Phosphatidylcholine	32.06 ± 1.55	32.55 ± 1.83	32.22 ± 1.11	34.59 ± 1.83	31.73 ± 0.93	32.23 ± 1.42
Phosphatidylinositol	1.06 ± 0.13	0.87 ± 0.16	0.81 ± 0.55	1.20 ± 0.18	1.43 ± 0.50	1.02 ± 0.25
Phosphatidylserine	13.54 ± 1.05 [a]	11.12 ± 0.75 [b]	13.05 ± 0.38	10.66 ± 0.24 [c]	14.34 ± 1.60	13.67 ± 1.19
Phosphatidylethanolamine	30.28 ± 0.21 [a]	28.04 ± 0.76 [b]	30.25 ± 1.01	26.27 ± 0.93 [c]	29.41 ± 1.50	28.56 ± 0.93
Phosphatidic acid	1.91 ± 0.50	2.55 ± 0.48	1.51 ± 0.52	2.59 ± 0.42	1.97 ± 0.38	2.06 ± 0.18

tion of erythrocyte membrane lipid peroxidation to the pathophysiology of certain hemolytic anemias has been suggested [20—28].

Malonyldialdehyde, an end product of lipid peroxidation, can react with aminophospholipids to form fluorescent chromolipid products [29]. The presence of these fluorescent compounds in the circulating erythrocytes of patients and of animals treated with oxidant drugs has been documented [30,31]. An affect of these fluorescent compounds on decreased erythrocyte survival has been suggested [30,31].

The present study shows that treatment of erythrocytes with calcium can accentuate lipid peroxidation. However, treatment of erythrocytes with calcium and ionophore resulted in significant echinocyte formation, whereas exposure to calcium without ionophore induced less than 2% echinocyte formation. Therefore, the greatest susceptibility of calcium-treated cells to oxidant stress does not seem to be closely related to morphological differences in the erythrocyte membrane. The greater susceptibility to lipid peroxidation of sickle cells and older erythrocytes which also contain elevated calcium levels has also been previously described [32—34]. The present observation suggests that increased calcium content may be an important factor in potentiating membrane lipid peroxidation in these cells.

The present study further shows that the phosphatidylserine and phosphatidylethanolamine content of the erythrocyte membrane is significantly decreased in cells treated with calcium and peroxide. A similar decrease in phospholipids in erythrocytes treated with peroxide alone has been shown by other investigators [21,27]. A greater decrease in these lipids in calcium-pretreated erythrocytes, compared to untreated erythrocytes after their exposure to peroxide, further shows that calcium potentiates peroxidation of erythrocyte membrane phospholipids. This specific loss of phosphatidylserine and phosphatidylethanolamine during peroxidation is presumably due to the presence of increased amounts of unsaturated fatty acids in these phospholipids [18,19]. It may also be due to the instability of fluorescent complexes [29] formed by the cross-linking of malonyldialdehyde with aminophospholipids [35].

Palek et al. [7] and Siefring and Lorand [8] have observed that sickle cells or calcium-loaded normal cells exhibit aggregation of membrane proteins which these authors have suggested is due to calcium-activated transglutaminase in the membrane. Similar cross-linking of membrane proteins after authentic malonyldialdehyde treatment [36] of erythrocytes has also been reported. Although the intracellular concentration of calcium must be quite high to activate this process, the current studies suggest a mechanism by which calcium-induced membrane protein aggregation might also, at least in part, be due to cross-linking of membrane proteins by malonyldialdehyde. In fact, inhibition of calcium-induced protein aggregation by amines as reported by Palek et al. [7] and Siefring and Lorand [8] could be due to the primary amino group of these amines acting as alternate substrates for malonyldialdehyde [37]. It is of interest to mention here that the appearance of an aggregated protein band, a decrease in spectrin and the accumulation of fluorescent products of lipid peroxidation similar to those in malonyldialdehyde-treated cells have all been observed in older erythrocytes isolated from normal blood [36]. However, it is

not known whether calcium-induced protein cross-linking is blocked in the presence of antioxidants. It would certainly be of interest to see if ^{14}C-labelled malonyldialdehyde could be recovered in high molecular weight protein bands derived from calcium-treated erythrocytes.

Calcium-induced erythrocyte membrane changes could be a consequence of spectrin aggregation occurring in the cytoplasmic surface of the membrane [7]. In addition, an asymmetric distribution of 1,2-diacylglycerol in the inner layer of the erythrocyte membrane could have some role [9]. In the latter regard, we have preliminary data to show that treatment of normal cells (calcium-untreated) with peroxide can result in the formation of 1,2-diacylglycerol similarly to that reported by Allan and Michell [9] in calcium-treated erythrocytes. The mechanism of this phenomenon and its relation to the calcium-induced morphological changes in erythrocytes are currently under investigation.

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