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Inhibition of erythrocyte Ca^{2+}-ATPase by activated oxygen through thiol- and lipid-dependent mechanisms

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We have studied erythrocyte Ca^{2+}-ATPase as a model target for elucidating effects of activated oxygen on the erythrocyte membrane. Either intracellular or extracellular generation of activated oxygen causes parallel decrements in Ca^{2+}-ATPase activity and cytoplasmic GSH, oxidation of membrane protein thiols, and lipid peroxidation. Subsequent incubation with either dithiothreitol or glucose allows only partial recovery of Ca^{2+}-ATPase, indicating both reversible and irreversible components which are modeled herein using diamide and *t*-butyl hydroperoxide. The reversible component reflects thiol oxidation, and its recovery depends upon GSH restoration. The irreversible component is largely due to lipid peroxidation, which appears to act through mechanisms involving neither malondialdehyde nor secondary thiol oxidation. However, some portion of the irreversible component could also reflect oxidation of thiols which are inaccessible for reduction by GSH, since we demonstrate existence of different classes of thiols relevant to Ca^{2+}-ATPase activity. Activated oxygen has an exaggerated effect on Ca^{2+}-ATPase of GSH-depleted cells. Sickle erythrocytes treated with dithiothreitol show a heterogeneous response of Ca^{2+}-ATPase activity. These findings are potentially relevant to oxidant-induced hemolysis. They also may be pertinent to oxidative alteration of functional or structural membrane components in general, since many components share with Ca^{2+}-ATPase both free thiols and close proximity to unsaturated lipid.

Introduction

Exposure of cells to activated oxygen is an inevitable consequence of aerobic existence. For example, the spontaneous generation of superoxide, peroxide and hydroxyl radical is detected even for normal erythrocytes [1] due to the relentless autoxidation of hemoglobin [2]. Since erythrocytes are uniquely vulnerable to autoxidation, the red cell membrane serves as a particularly useful model for studying the pathobiology of autoxidation [3]. For example, sickle erythrocytes spontaneously generate excessive amounts of activated oxygen [1], and their membranes reveal evidence of abnormal lipid peroxidation [4–6] and abnormal oxidation of cytoskeletal protein thiols [7]. The possibility that such defects contribute to disease pathophysiology [8] mandates an attempt to define apparent mechanisms of autoxidative damage to specific red cell membrane components.

The studies reported here were prompted by our earlier observation that the brisk murine he-

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Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; GSH, reduced glutathione; Hb, hemoglobin; H_2O_2, hydrogen peroxide; O_2^-, superoxide; PCMB, *p*-chloromercuribenzoate; PCMBS, *p*-chloromercuribenzene sulfonic acid; PMS, phenazine methosulfate.

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molytic anemia induced by the oxidant drug phenylhydrazine is accompanied by profound disruption of erythrocyte calcium homeostasis [9]. In addition to an accumulation of calcium, we observed a significant decrement in activity of the erythrocyte membrane's magnesium-dependent calcium-ATPase (herein referred to as 'Ca^{2+}-ATPase'). Unlike simple structural proteins, Ca^{2+}-ATPase serves an important homeostatic function by actively removing calcium from the cell interior. Moreover, it is particularly useful as a model target since it is embedded within the membrane (in close juxtaposition to lipid), and it has free thiol groups [10].

We here describe the inhibition of erythrocyte Ca^{2+}-ATPase by activated oxygen generated either inside or outside the cell. Also, we provide data suggesting that this defect is due to combined effects of thiol oxidation and lipid peroxidation, either of which can itself inhibit Ca^{2+}-ATPase.

Materials and Methods

Reagents

Except for saponin (Calbiochem-Behring Corp., La Jolla, CA) and [^{203}Hg]PCMB (Amersham, Arlington Heights, IL), reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Erythrocyte manipulations

Activated oxygen. For exposure to extracellular generation of activated oxygen, fresh normal erythrocytes were washed three times with isotonic NaCl and suspended to hematocrit 20% in Hanks' balanced salt solution containing 1 g/l glucose, 136.9 mmol/l NaCl, 5.4 mmol/l KCl, 0.3 mmol/l Na_2HPO_4, 0.3 mmol/l KH_2PO_4, 0.4 mmol/l $MgSO_4$, 1.3 mmol/l $CaCl_2$, 0.5 mmol/l $MgCl_2$, and 4.2 mmol/l $NaHCO_3$ (pH 7.4). They were then incubated at 37°C for 30 or 45 min with oxygen-radical-generating enzymes. Peroxide was generated with glucose/glucose oxidase, and superoxide was generated with xanthine/xanthine oxidase (1 mmol/l substrate, 1 unit/ml enzyme). For exposure to hydroxyl radical, erythrocytes were incubated with both enzymes and both substrates in addition to 2 mmol/l $FeCl_2$/EDTA. Control red cells were incubated without the enzymes. For exposure to intracellular generation of superoxide, erythrocytes were incubated at hematocrit 20% in Hanks' solution for 60 min at 37°C with 0.1–1.0 mmol/l phenazine methosulfate [11].

Other treatments. Erythrocytes were incubated at hematocrit 10% (30 min at 37°C) in Hanks' solution containing one of the following reagents: 5 mmol/l diamide (diazenedicarboxylic acid bis(*N*,*N*-dimethylamide)); 0.05 mmol/l PCMB, 0.05 mmol/l PCMBS; 1 mmol/l *t*-butyl hydroperoxide; 0.5 mmol/l CDNB. Controls were incubated with Hanks' solution alone. For reaction with malondialdehyde, erythrocytes were incubated at hematocrit 5% (2 hours at 37°C) in Hanks' solution with up to 1 mmol/l malondialdehyde, prepared immediately before use by acid hydrolysis of malonaldehyde bis(dimethyl acetal) [12].

Reversibility of Ca^{2+}-ATPase inhibition

When the above manipulations inhibited Ca^{2+}-ATPase activity, erythrocytes were further examined to determine whether this effect could be reversed with reducing agents. The treated erythrocytes were washed four times with NaCl, suspended to hematocrit 10% in Hanks' balanced salt solution with or without 10 mmol/l dithiothreitol, and incubated at 37°C. A parallel incubation with glucose (1 g/l) but without dithiothreitol assessed the ability of erythrocytes to restore Ca^{2+}-ATPase activity spontaneously. When inhibition of Ca^{2+}-ATPase was in fact reversible, in most cases restoration of activity was complete by 40 min incubation. However, if activity was not fully restored by this 40 min exposure to dithiothreitol or glucose, incubation was carried out as long as 240 min before we concluded that restoration was not possible.

Ca^{2+}-ATPase assay

Erythrocytes were washed five times with NaCl and were immediately assayed for Ca^{2+}-ATPase activity [9]. They were suspended to hematocrit 10% in Tris-HCl (0.172 mol/l (pH 7.4)) and admixed with saponin [13] at a ratio of 10 μg saponin per 10 μl erythrocytes. After 15 min incubation at room temperature, any unlysed red cells were removed by low-speed centrifugation (since they would result in artifactual lowering of apparent

Ca^{2+}-ATPase activity). Red cell lysate (0.1 ml) was admixed with 0.2 ml Tris-HCl buffer and 0.7 ml reaction mixture (30 mmol/l imidazole, 100 mmol/l KCl, 15 mmol/l NaCl, 4.5 mmol/l $MgCl_2$, 300 μmol/l ouabain, and 4.5 mmol/l ATP before dilution). Finally, 0.02 ml of either 1.25 mmol/l $CaCl_2$ or 5 mmol/l EGTA was added, and duplicate samples were incubated for 60 min at 37°C. Following precipitation with 0.1 ml 50% trichloracetic acid, inorganic phosphate (P_i) was measured using the Fiske-SubbaRow method [14]. Ca^{2+}-ATPase activity was calculated by subtracting the P_i content of the EGTA-containing sample (i.e., the Mg^{2+}-ATPase activity) from that of the calcium-containing sample (i.e., the Mg^{2+}-ATPase and Ca^{2+}-ATPase activities). Using this method, we find normal erythrocyte Ca^{2+}-ATPase activity to be 81.15 ± 9.15 μmol P_i/g Hb per h (mean ± S.D.; $n = 22$), with duplicate samples varying by no more than ± 5%.

Since this Ca^{2+}-ATPase assay utilizes the red cell's own cytoplasm as a calmodulin source, control experiments examined admixture of membranes from untreated erythrocytes with cytoplasm from treated erythrocytes. None of the agents used here affected the cytoplasm's ability to serve as a calmodulin source (data not shown). Hence, the method is valid as used in these studies.

Glutathione (GSH) assay

After thorough washing with large volumes of NaCl, erythrocyte GSH was determined by titration with 5,5′-dithiobis(2-nitrobenzoic acid) [15]. Using this method, we find GSH levels for normal erythrocytes to be 6.79 ± 1.05 μmol GSH/g Hb (mean ± S.D.; $n = 17$), with duplicate samples varying by no more than ± 5%.

Assay for thiol oxidation and lipid peroxidation

Thiol oxidation resulting from the various treatments was detected using thiol-disulfide exchange chromatography of solubilized erythrocyte membranes exactly as recently described [7], a technique which detects even intramolecular thiol oxidation. Lipid peroxidation due to various red cell treatments was monitored by measuring the thiobarbituric acid reactive peroxidation byproducts ('malondialdehyde') in aliquots of the erythrocyte suspensions after trichloroacetic acid precipitation [16].

Displacement of [^{203}Hg]PCMB by GSH

Erythrocytes were treated with PCMB as above, except that 0.05 mmol/l [^{203}Hg]PCMB (61 mCi/g) was used, and they were washed with NaCl until supernatant radioactivity returned to background. Solubilized membranes were then prepared by admixing equal volumes of 2% sodium dodecyl sulfate and red cell ghosts derived from washing labeled erythrocytes four times with 5 mmol/l sodium phosphate (pH 7.4). The ensure complete precipitation of membranes upon subsequent acidification, 5% albumin was added to aliquots of labeled solubilized membranes, which were then incubated for 30 min at 37°C with an equal volume of buffer with or without 10 mmol/l GSH. After precipitation with one-fifth volume trichloroacetic acid, radioactivity in supernatants was determined. The number of acid-precipitable cpm was determined from samples incubated with buffer alone. The percentage of acid-precipitable cpm released by exposure to reducing agent was determined from samples incubated with GSH.

Statistical analysis

Statistical analysis employed Student's *t*-test.

Results

Activated oxygen and Ca^{2+}-ATPase

Exposure of fresh normal erythrocytes to activated oxygen results in a significant inhibition of Ca^{2+}-ATPase activity (Table I). This is illustrated for exposure to both extracellular oxidant (generated enzymatically) and intracellular oxidant (generated using PMS). The PMS stimulates the generation of superoxide [11] and is thus most analogous to physiologic or pathologic oxidant stress. Results using extracellular generation of superoxide (O_2^-) and/or peroxide (H_2O_2) suggest that hydroxyl radical is a particularly potent inhibitor. This is evident in that fact that Ca^{2+}-ATPase inhibition due to both O_2^- and H_2O_2 is even greater if iron is additionally present.

Further data on perturbation by activated oxygen are shown at the top of Table II. The depicted data were derived using enzymatically-

TABLE I

INHIBITION OF ERYTHROCYTE Ca^{2+}-ATPase BY ACTIVATED OXYGEN

Normal erythrocytes were exposed to activated oxygen for 45 min at 37°C and then assayed immediately for Ca^{2+}-ATPase activity. Extracellular exposure utilized enzymatic generation of superoxide (O_2^-) and/or peroxide (H_2O_2) with and without iron/EDTA. Intracellular exposure was achieved using PMS. Data represent mean ± S.D. for four experiments. Control≡ 100%.

Erythrocyte treatment	Ca^{2+}-ATPase activity	
	% of control	*P*
Extracellular exposure		
O_2^-	83.1 ± 10.1	< 0.02 [a]
H_2O_2	92.0 ± 8.5	n.s.
$O_2^- + H_2O_2$	76.5 ± 10.0	< 0.01 [a]
$O_2^- + H_2O_2$ + 2 mmol/l iron	54.7 ± 11.0	< 0.005 [a], < 0.05 [b]
Intracellular exposure		
PMS, 0.1 mmol/l	55.9 ± 2.6	< 0.001 [a]
PMS, 0.5 mmol/l	35.2 ± 8.9	< 0.001 [a]
PMS, 1.0 mmol/l	24.6 ± 2.4	< 0.001 [a]

[a] Compared to control incubation.
[b] Compared to $O_2^- + H_2O_2$ without iron.

generated oxidant (for 30 min in Table II as compared to 45 min for Table I). However, results derived using PMS (not shown) were qualitatively identical. The decrement in Ca^{2+}-ATPase activity induced by activated oxygen is accompanied by a parallel decrement in cytoplasmic GSH level, an increase in membrane protein thiol oxidation, and a moderately brisk generation of the lipid peroxidation byproduct, malondialdehyde.

Subsequent incubation with either dithiothreitol or glucose results in full restoration of GSH levels. Despite this, neither glucose nor dithiothreitol accomplishes full restoration of Ca^{2+}-ATPase activity, although both agents allow significant improvement in its activity. Thus, Ca^{2+}-ATPase inhibition by activated oxygen has both a reversible and an irreversible component.

The significance of these results was elucidated by comparing the effect of activated oxygen to the effect of various membrane perturbants. As discussed below, the resulting data suggest that the reversible component of Ca^{2+}-ATPase inhibition by activated oxygen is related to thiol oxidation and is reversed as GSH recovers, while the irreversible component reflects the effect of lipid peroxidation.

Classical thiol reactants (Table II)

Diamide. Diamide, a reversible thiol oxidant [17], induces significant and parallel reduction in Ca^{2+}-ATPase activity and GSH levels. There is a general increase in membrane thiol oxidation, but no significant lipid peroxidation. Ca^{2+}-ATPase activity and GSH levels are both fully restored by subsequent exposure to dithiothreitol or glucose. However, while full recovery in response to dithiothreitol required only 40 min, full spontaneous recovery (i.e., in response to glucose) required 240 min. This is consistent with the known slow recovery of GSH after exposure to such high concentrations of diamide [17]. The smaller decrement in Ca^{2+}-ATPase activity induced by lower diamide concentrations is fully reversed during the standard 40 min glucose incubation (these data are included in Fig. 1, discussed below).

PCMBS. PCMBS, which reacts only with external membrane thiols under the conditions employed here [18], affects neither Ca^{2+}-ATPase nor GSH of intact erythrocytes. However, not shown in the table is the fact that this same concentration of PCMBS does profoundly reduce the Ca^{2+}-ATPase activity of open red cell ghosts to 15 ± 21% ($n = 4$) of control values.

PCMB. PCMB, which is similar to PCMBS but which penetrates erythrocytes under the conditions employed here [18], significantly inhibits Ca^{2+}-ATPase without altering GSH levels. Despite full restoration of Ca^{2+}-ATPase by subsequent incubation with dithiothreitol, PCMB-treated erythrocytes fail to show any spontaneous (i.e., GSH-mediated) Ca^{2+}-ATPase recovery whatsoever. This failure of GSH to restore the Ca^{2+}-ATPase of PCMB-treated erythrocytes is not due to a chemical inability of GSH to disrupt the PCMB metallo-thiol bond, since GSH displaces 88 ± 11% ($n = 4$) of the radioactivity from SDS-solubilized membranes prepared from erythrocytes treated with [^{203}Hg]PCMB (as described in Materials and Methods). Thus, PCMB inhibits Ca^{2+}-ATPase by affecting membrane thiols which are sterically inaccessible for reduction by GSH.

TABLE II

EFFECT OF VARIOUS MEMBRANE PERTURBANTS ON ERYTHROCYTE Ca^{2+}-ATPase

Normal erythrocytes were incubated for 30 min at 37°C with the indicated agent. The first four columns show the effect of the initial treatment on cytoplasmic GSH level, Ca^{2+}-ATPase activity, formation of malondialdehyde (MDA), and percent of membrane proteins having oxidized thiols. The last three columns show effect on GSH and Ca^{2+}-ATPase of a subsequent incubation with either 10 mmol/l dithiothreitol (DTT) or 10 mmol/l glucose. This second incubation examines the ability of reducing agent to reverse the Ca^{2+}-ATPase defect induced by the initial incubation. Results are shown as mean ± S.D. for 3–5 experiments. n.d., not determined.

Treatment	Effect of initial treatment				Reversibility		
	GSH (% of control)	Ca^{2+}-ATPase (% of control)	Lipid peroxidation (nmol MDA/ml RBC)	Thiol oxidation [b] (%)	GSH using DTT of glucose [d]	Ca^{2+}-ATPase using DTT	Ca^{2+}-ATPase using glucose
Control incubation	≡100	≡100	0.98 ± 0.76	14.1 ± 1.4	≡100	≡100	≡100
Activated oxygen	62.6 ± 10.1 [a]	76.3 ± 8.6 [a]	7.50 ± 1.25 [a]	22.9 ± 1.3 [a]	104.4 ± 12.0	86.9 ± 4.7 [a]	86.0 ± 3.7 [a]
Diamide	16.2 ± 5.7 [a]	48.8 ± 7.3 [a]	1.60 ± 1.21	27.8 ± 1.0 [a]	98.5 ± 4.8	103.5 ± 8.6	99.4 ± 7.5
PCMBS	99.4 ± 3.3	102.9 ± 4.2	0.36 ± 0.62	n.d.	n.d.	n.d.	n.d.
PCMB	98.8 ± 5.0	55.8 ± 1.9 [a]	0.10 ± 0.09	18.3 ± 1.1 [a]	n.d.	101.3 ± 5.7	59.6 ± 2.5 [a]
t-Butyl hydroperoxide	83.0 ± 3.6 [a]	46.6 ± 23.0 [a]	16.79 ± 8.39 [a]	14 [c]	102.0 ± 14.9	39.5 ± 6.5 [a]	42.6 ± 17.0 [a]

[a] Values so indicated differ from controls by at least $P < 0.01$.

[b] Percent of membrane protein having oxidized thiols as measured by thiol-disulfide exchange chromatography [7].

[c] This value is a best estimate for actual membrane proteins, taking into account the required correction for the great amount of membrane-associated hemoglobin having oxidized thiols in this preparation. This problem with *t*-butyl hydroperoxide is discussed in detail elsewhere [31]. The normality of membrane proteins from *t*-butyl hydroperoxide treated cells was proven in these studies using polyacrylamide gel electrophoresis as previously described [7].

[d] Values for glucose and dithiothreitol (DTT) were nearly identical in each case; specific numbers shown are those derived from the glucose incubation.

Effect of lipid peroxidation (Table II)

The effect of lipid peroxidation upon Ca^{2+}-ATPase was assessed by exposing erythrocytes to a concentration of *t*-butyl hydroperoxide which induces brisk generation of malondialdehyde but which leaves GSH levels and membrane thiols relatively intact. In response to peroxidation induced by this agent, Ca^{2+}-ATPase activity is markedly diminished. In striking contrast to the demonstrated reversibility of thiol-dependent Ca^{2+}-ATPase inhibition, there is no recovery whatsoever upon subsequent incubation with dithiothreitol or glucose. Indeed, despite full restoration of GSH, continued incubation with glucose results only in continued malondialdehyde formation and further deterioration of Ca^{2+}-ATPase activity (data not shown).

Since peroxidation of lipid can secondarily affect thiols [19], additional experiments examined the possibility that reversible thiol blockage might protect erythrocytes from peroxidation-induced Ca^{2+}-ATPase inhibition. Since the effect of PCMB on Ca^{2+}-ATPase is fully reversible (Table II), erythrocytes were treated with this agent prior to exposure to *t*-butyl hydroperoxide. After exposure to the hydroperoxide, the PCMB was removed (and its effect reversed) with dithiothreitol. The Ca^{2+}-ATPase of such cells was no less deficient than that of cells exposed to *t*-butyl hydroperoxide without benefit of reversible thiol blockage (data not shown), suggesting that lipid peroxidation does not inhibit Ca^{2+}-ATPase through the PCMB-sensitive thiol. Likewise, similar experiments using diamide to protect thiols yielded the same result. Thus, the data suggest that lipid peroxidation inhibits Ca^{2+}-ATPase through a non-thiol mechanism.

Exposure of erythrocytes to as much as 1

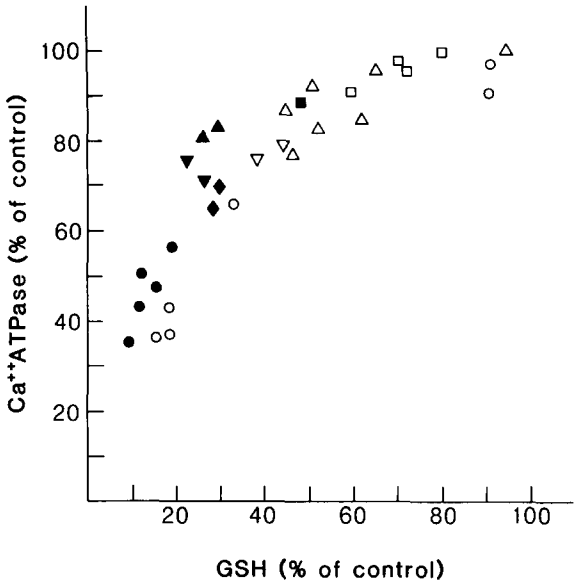

Fig. 1. Ca^{2+}-ATPase of diamide-treated erythrocytes recovers only coincident with GSH. Red cells were incubated for 30 min with diamide (■, 1 mmol/l; ▲, 2.5 mmol/l; ▼, 3.5 mol/l; ◆, 4 mmol/l; ●, 5 mmol/l). They were then incubated with 1 g/l glucose for 40–240 min (open symbols, coded to diamide concentrations as above). Ca^{2+}-ATPase activity and GSH level are expressed as percent of those of control cells incubated in parallel but without diamide exposure.

mmol/l reagent malondialdehyde had no effect on Ca^{2+}-ATPase activity (data not shown), so peroxidation effects apparently are not due to modification by this peroxidation byproduct per se.

Relationship between Ca^{2+}-ATPase and GSH

As discussed above, the Ca^{2+}-ATPase inhibition induced by classical, penetrating thiol-reactive agents is promptly and fully reversed with the reducing agent dithiothreitol (Table II). Yet, the spontaneous (GSH-mediated) Ca^{2+}-ATPase recovery of erythrocytes treated with diamide is delayed, providing an opportunity to examine the relationship between GSH levels and Ca^{2+}-ATPase activity. Indeed, after diamide exposure, Ca^{2+}-ATPase activity only recovers coincident with spontaneous GSH regeneration (Fig. 1).

In this regard, Ca^{2+}-ATPase inhibition is not simply an effect of lowered GSH concentration per se, as evidenced by experiments using CDNB, which non-oxidatively but irreversibly depletes erythrocyte GSH [20]. Despite profound GSH depletion, Ca^{2+}-ATPase remains near-normal in CDNB-treated erythrocytes (line 3 in Table III).

The important restorative role of GSH in terms of reversing thiol-dependent Ca^{2+}-ATPase inhibition is further illustrated by comparison of the effect of activated oxygen upon GSH-replete and GSH-depleted cells (Table III). As compared to GSH-replete erythrocytes exposed to activated oxygen, GSH-depleted (CDNB-treated) red cells which are subsequently exposed to activated oxygen manifest a greater decrement in Ca^{2+}-

TABLE III

ENHANCED SUSCEPTIBILITY OF GSH-DEPLETED ERYTHROCYTES TO INHIBITION OF Ca^{2+}-ATPase BY ACTIVATED OXYGEN

Erythrocytes were prepared without (GSH-replete) or with (GSH-depleted) CDNB preincubation (0.5 mmol/l at 37°C for 30 min). Each type was then incubated without or with exposure to enzymatically generated activated oxygen. Data shown as mean ± S.D. for four experiments.

Erythrocyte type		GSH (% of control)	Ca^{2+}-ATPase (% of control)	Malondialdehyde (nmol/ml RBC)
GSH-replete erythrocytes	control incubation	≡100	≡100	1.02 ± 0.73
	activated oxygen	62.6 ± 10.1	76.3 ± 8.6	7.50 ± 1.25
GSH-depleted erythrocytes	control incubation	15.7 ± 1.5	94.4 ± 3.4	0.47 ± 0.90
	activated oxygen	10.8 ± 7.4	53.8 ± 8.5	16.23 ± 9.34

TABLE IV

EFFECT OF DITHIOTHREITOL ON NORMAL AND SICKLE ERYTHROCYTE Ca^{2+}-ATPase ACTIVITY

Erythrocytes were incubated for 30 min at 37°C in Hanks' balanced salt solution with or without 10 mmol/l dithiothreitol before determination of Ca^{2+}-ATPase activity.

Erythrocyte type	Ca^{2+}-ATPase activity (μg P_i/g Hb per h)	
	without dithiothreitol	with dithiothreitol
Normal 1	85.64	85.23
Normal 2	83.54	84.19
Normal 3	81.36	81.50
Sickle 1	86.09	86.62
Sickle 2	85.67	92.06
Sickle 3	77.13	83.45
Sickle 4	75.35	75.63
Sickle 5	53.92	79.01
Sickle 6	52.45	49.62

ATPase activity and much greater malondialdehyde formation. In addition, since the GSH deficiency of CDNB-treated erythrocytes is irreversible [20], subsequent incubation of these cells with glucose produces no Ca^{2+}-ATPase recovery whatsoever (data not shown).

Sickle erythrocytes

Since sickle erythrocytes undergo excessive autoxidation [1] and their membranes reveal evidence of both abnormal thiol oxidation [7] and lipid peroxidation [4–6], we determined Ca^{2+}-ATPase activity of sickle and normal red cells before and after treatment with dithiothreitol. As shown in Table IV, dithiothreitol does not change the Ca^{2+}-ATPase activity of normal erythrocytes. However, for some of the sickle patients, Ca^{2+}-ATPase activity improved noticeably upon exposure to dithiothreitol.

Discussion

We have demonstrated the inhibition of erythrocyte membrane Ca^{2+}-ATPase by activated oxygen. This occurs with both extracellular and intracellular generation of activated oxygen (Table I), and it is accompanied by peroxidation of lipid and oxidation of membrane thiols (Table II).

Whether or not this perturbation of calcium homeostasis is physiologically relevant is discussed below. However, Ca^{2+}-ATPase was investigated due to its suitability as a model target for studying the adverse effects of oxidative phenomena on erythrocyte membranes. That is, erythrocyte Ca^{2+}-ATPase is potentially vulnerable to autoxidative modification because it has free thiol groups and resides in close proximity to unsaturated membrane lipids [10]. Consequently, the general conclusions supported by these studies may be pertinent to a variety of membrane proteins having this environment in common with Ca^{2+}-ATPase. Examples include the $(Na^+ + K^+)$-ATPase, the transmembrane protein band 3, and the cytoskeletal protein ankyrin.

When erythrocytes with Ca^{2+}-ATPase inhibited by exposure to activated oxygen are subsequently incubated with either dithiothreitol or glucose, Ca^{2+}-ATPase activity improves significantly; but in either case restoration is only partial. Thus, the inhibition of Ca^{2+}-ATPase by activated oxygen appears to have both reversible and irreversible components (Table II). The significance of these findings is elucidated by the studies examining the effect of various thiol and lipid perturbants on Ca^{2+}-ATPase activity. These suggest that the reversible component of Ca^{2+}-ATPase inhibition reflects thiol oxidation and recovers as cytoplasmic GSH recovers. In contrast, the irreversible component appears to reflect lipid peroxidation.

Thiol oxidation

Inhibition of erythrocyte Ca^{2+}-ATPase by thiol-reactive agents is a well-known phenomenon [21–24], but the present data illustrate several important aspects of the thiol-dependence of Ca^{2+}-ATPase activity.

First, these studies establish a close linkage between maintenance of cytoplasmic GSH levels and preservation of Ca^{2+}-ATPase activity. For example, after diamide exposure erythrocyte Ca^{2+}-ATPase recovers only coincident with GSH recovery (Fig. 1). Experiments utilizing CDNB (Table III) reveal that GSH concentration per se does not affect Ca^{2+}-ATPase. Rather, GSH protects the Ca^{2+}-ATPase from oxidative inhibition. Thus, exposure of GSH-depleted (CDNB-treated)

erythrocyte to activated oxygen results in greater Ca^{2+}-ATPase inhibition than does exposure of GSH-replete erythrocytes to activated oxygen. Furthermore, the diminished Ca^{2+}-ATPase activity of CDNB/oxidant treated erythrocytes shows no spontaneous recovery whatsoever, since the GSH within these cells cannot be restored metabolically [20].

Thus, these studies are consistent with the hypothesis that maintenance of optimal Ca^{2+}-ATPase activity depends, at least in part, upon preservation of membrane thiols in their reduced state. Indeed, the erythrocyte Ca^{2+}-ATPase is a reasonably thiol-rich protein [10]. Hence, one mechanism by which activated oxygen inhibits Ca^{2+}-ATPase appears to be formation of disulfide bond(s), since reducing agents significantly (albeit only partially) improve Ca^{2+}-ATPase after oxidant exposure. In this regard, our data indicate that there are different classes of membrane thiols relevant to Ca^{2+}-ATPase activity, something previously noted regarding the $(Na^+ + K^+)$-ATPase [18]. For example, blockage/oxidation of both PCMB-sensitive and diamide-sensitive thiols is fully reversed with dithiothreitol. However, the latter thiol is also reduced by cytoplasmic GSH while the former is not.

One way to explain inaccessibility to GSH is for the thiol to be buried within the lipid bilayer. This, in fact, is probably the case for some of the enzyme thiols of sarcoplasmic reticulum Ca^{2+}-ATPase [25]. If this is also true for erythrocyte Ca^{2+}-ATPase thiols, the thiol-dependent component of oxidant-induced Ca^{2+}-ATPase inhibition might be irreversible to the extent that it involves thiols which do not protrude into the cytoplasm. That this may, in fact, be the case is suggested by studies on sickle erythrocytes. These cells spontaneously generate abnormal amounts of activated oxygen [1] and their membranes have undergone abnormal oxidation of protein thiols [7] despite the fact that their GSH levels are only minimally depressed [26]. Treatment of sickle erythrocytes with dithiothreitol increases Ca^{2+}-ATPase activity for some patients (Table IV).

Lipid peroxidation

Unlike classical thiol reactants, activated oxygen induces moderately brisk generation of malondialdehyde. Hence, the failure of reducing agents to restore Ca^{2+}-ATPase after oxidant exposure suggests that lipid peroxidation may also be an important inhibitor. Indeed, *t*-butyl hydroperoxide induces marked malondialdehyde formation and inhibits Ca^{2+}-ATPase, while affecting GSH minimally (Table II). Notably, this effect is not even partially reversed with either dithiothreitol or spontaneous recovery of GSH.

The specific mechanism of peroxidation-induced Ca^{2+}-ATPase deficiency does not appear to involve cross-linking of membrane amino groups by malondialdehyde, since deliberate exposure of erythrocytes to reagent malondialdehyde has no effect on Ca^{2+}-ATPase. We cannot exclude the possibility that lipid peroxidation secondarily induces some type of thiol oxidation [19] which is unaffected by reducing agents such as GSH or dithiothreitol. However, the failure of (reversible) thiol blockage using PCMB or diamide to protect the Ca^{2+}-ATPase from inhibition due to organic hydroperoxide-induced peroxidation at least suggests that it is not the PCMB- or diamide-sensitive thiols which are secondarily modified.

Physiologic significance

Although it is impossible to state with absolute certainty that the present findings are physiologically relevant, certain data suggest that they are. It is true that amounts of oxidant generated extracellularly in these in vitro studies exceed those spontaneously generated by erythrocytes over the same time period [1]. However, the amount of resulting malondialdehyde formation and thiol oxidation (per Table II) is exactly comparable to that found in unmanipulated sickle erythrocytes [6,7]. Moreover, the amounts of PMS used here to inhibit Ca^{2+}-ATPase activity result in the same amount of intracellular superoxide [11] we previously found to be spontaneously generated from sickle erythrocytes [1].

At the least, therefore, the present findings are potentially relevant to the harmful effect of oxidant drugs. For example, murine hemolytic anemia induced by administration of phenylhydrazine is accompanied by a profound decrement in erythrocyte Ca^{2+}-ATPase activity [9]. Likewise, although thiol-perturbants have a great variety of effects upon the membrane, interference with thiols cru-

cial to Ca^{2+}-ATPase activity could be one contributing factor in the attenuated red cell survival induced by *N*-ethylmaleimide [27], PCMB [27] and diamide [28].

Likewise, these data may be relevant to the preferential destruction of glucose-6-phosphate-dehydrogenase-deficient human erythrocytes by oxidant drugs. Our earlier studies demonstrated that diamide induces a markedly greater decrement in Ca^{2+}-ATPase activity in glucose-6-phosphate-dehydrogenase-deficient than in normal erythrocytes, despite the fact that the Ca^{2+}-ATPase activity of glucose-6-phosphate-dehydrogenase-deficient red cells is normal in the absence of oxidant challenge [29]. The explanation for this is evident in the present studies which graphically illustrate the enhanced risk to erythrocyte Ca^{2+}-ATPase when ability to utilize GSH is impaired (Table III).

In summary, these studies suggest that erythrocyte Ca^{2+}-ATPase activity is modulated by cellular 'redox' status (degree of thiol oxidation and lipid peroxidation). It is questionable, however, whether inhibition of Ca^{2+}-ATPase by oxidant would by itself be of physiologic relevance. Due to the extraordinary efficiency of erythrocyte Ca^{2+}-ATPase, it would require a profound inhibition of enzyme activity for this, as an isolated defect, to adversely affect erythrocytes. On the other hand, oxidative damage due to either endogenous or exogenous oxidant is likely to be indiscriminate, resulting in a multitude of defects. This is supported by observation of widespread oxidation of membrane thiols in fresh sickle [7] or glucose-6-phosphate-dehydrogenase-deficient [30] erythrocyte membranes. Consequently, further studies are warranted to ascertain whether the present results are, in fact, relevant to the great variety of red blood cell membrane components having free thiols and close proximity to membrane lipid in common with Ca^{2+}-ATPase.

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