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Peroxyl-oxidized Erythrocyte Membrane Band 3 Protein with Anion Transport Capacity is Degraded by Membrane-bound Proteinase

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Human red blood cells anion exchange protein (band 3) exposed to peroxyl radicals produced by thermolysis of 2,2′-azo-bis(2-amidinopropane) (AAPH) is degraded by proteinases that prevent accumulation of oxidatively damaged proteins. To assess whether this degradation affects anion transport capacity we used the anionic fluorescent probe 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-y) amino] ethanosulfonate (NBD-taurine). A decrease of band 3 function was observed after exposure to peroxyl radicals. In the presence of proteinase inhibitors the decrement of anion transport through band 3 was smaller indicating that removal achieved by proteinases includes oxidized band 3 which still retain transport ability. Proteinases recognize band 3 aggregates produced by peroxyl radicals as was evaluated by immunoblotting. It is concluded that decrease of band 3 transport capacity may result from a direct protein oxidation and from its degradation by proteinases and that band 3 aggregates removal may prevent macrophage recognition of the senescent condition which would lead to cell disposal.

Keywords: Erythrocyte; Band 3; Anion transport; Oxidation; Proteinase

INTRODUCTION

Oxidative stress in erythrocytes has been associated to several pathological abnormalities and also to cellular aging. Multiple functional deficiencies are produced by oxidation of cell constituents and they are related to the type of molecule that is being damaged. Erythrocyte band 3 is a multifunctional membrane protein involved in anion transport, binding to membrane skeleton proteins, cell metabolism modulation through binding of glycolytic enzymes and cell aging recognition.[1–3] Band 3 protein alterations have been described in erythrocytes from different pathological situations and *in vitro* studies in which reactive oxygen species and oxidative compounds are involved. Clustering,[3,4] fragmentation[5] higher proteolytic susceptibility,[6–8] tyrosine phosphorylation, binding of oxidized hemoglobin and Heinz bodies[9,10] are some of the reported band 3 alterations. However, studies relating band 3 protein oxidation and anion transport capacity are scarce. Phenylhydrazine has been reported to diminish anion transport through Heinz bodies formation followed by binding to N-terminal domain of band 3.[11]

AAPH is an azocompound which selectively affects the erythrocyte membrane since it neither oxidizes hemoglobin nor decreases the amount of intracellular glutathion.[6] This fact allows the use of AAPH for the exclusive study of erythrocytes membrane damage and the functional implications to the associated anion transport through membrane band 3 protein.

Erythrocytes have antioxidant defenses to prevent oxidative damage. However, if the oxidative stress is high enough to produce oxidatively damaged cells, they have proteolytic systems that prevents

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the accumulation of oxidized proteins.[5,12–14] Specific cytosolic and membrane-bound proteinases to oxidatively damaged erythrocytes membrane proteins have been characterized.[5,15]

It is known that AAPH-derived peroxyl radicals produce an oxidative damage in erythrocytes which gives rise to membrane proteolysis.[6,7] However, whether this proteolysis involves oxidized membrane band 3 protein producing functional alterations in the membrane has not yet been reported.

In this study we report the relation between band 3 proteolysis, as the result of its oxidation by AAPH-derived peroxyl radicals, and the modification of its anion transport capacity, as evaluated by NBD-taurine anion, a fluorescent substrate of the native anion transport system of human erythrocytes.[16]

MATERIALS AND METHODS

Materials

AAPH, NBD-taurine, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), monoclonal antihuman band 3 and protein A-horseradish peroxidase conjugated were purchased from Sigma, USA. Other chemicals were from standard commercial sources.

Oxidative Treatment of Red Blood Cells

Heparinized fresh human blood was collected from healthy volunteers following informed consent. Erythrocytes were separated by centrifugation and plasma and buffy coat were discarded. They were washed three times with isotonic Krebs-HEPES buffer, pH 7.4, and then resuspended to a 16% hematocrit. For AAPH treatment, one volume of resuspended erythrocytes were incubated with an equal volume of 150 mM AAPH at 37°C.[7] After incubation, cells were centrifuged and washed three times with isotonic NaCl buffer (PBS), (NaCl, 145 mM; sodium phosphate, 10 mM, pH 7.4) for AAPH removal. No cell lysis was observed under this condition. Erythrocytes were resuspended in the same buffer and then used immediately for NBD-taurine flux determination and for electrophoresis. When proteinases inhibitors (PMSF, 0.2 mM; EDTA, 1 mM) were used, they were present in all the described procedures.

NBD-taurine Efflux

NBD-taurine efflux was assessed by the continuous monitoring of NBD-taurine transport by a fluorescence method, as previously described[17] under symmetric conditions ($Cl_{in}^{-1}/Cl_{out}^{-1}$). NBD-taurine was incorporated to erythrocytes by incubating a cell suspension in PBS at 8% hematocrit with NBD-taurine (0.8 mM) at 37°C during 60 min in the dark with gentle shaking. No cell hemolysis was observed during the incubation. Erythrocytes resuspended in PBS were incubated at 37°C for 120 min previous to NBD-taurine incorporation for studies of band 3 degradation effects on anion transport. Extracellular NBD-taurine was removed by three washes with cold PBS by centrifugation at 4°C. Sediments were resuspended in the same buffer to an hematocrit of 4%. An aliquot of 40 μl was added to 3 ml PBS in a cuvette and, as time passes, the efflux of NBD-taurine leads to a time-dependent increase in fluorescence intensity which was continuously recorded at 37°C in a Fluorolog photon-counting spectrofluorometer (Spex). Fluorescence intensities were evaluated at 540 nm, with an excitation wavelength at 478 nm. Fluorescence records correspond to NBD-taurine present in the extracellular medium since intracellular NBD-taurine fluorescence is quenched by hemoglobin. NBD-taurine efflux was ended when cells were lysed with Triton X-100, which released the remaining intracellular probe. The fluorescence intensity at the time of addition of cells and after addition of detergent are denoted as F_0 and F_∞, respectively. Intermediate values of fluorescence intensities at time t are given as $F(t)$.

Results are obtained from the fluorescence/time tracings and analyzed in terms of a first order NBD-taurine efflux. Efflux was also evaluated in the presence of DIDS (1 mM) to inhibit band 3 protein transport and assess anion transport through other pathways present in erythrocyte membrane.

Band 3 Protein Susceptibility to Degradation

As oxidized proteins are degraded by proteinases present in erythrocytes, band 3 protein structure modification was assessed by Western blot analysis of band 3 from AAPH-peroxyl radicals oxidized erythrocytes. After AAPH treatment, erythrocytes were incubated for 180 min at 37°C in the absence of the radical source, to allow proteinases activity on oxidized band 3. When proteinase inhibitors were included, they were present (0.2 mM PMSF and 1.0 mM EDTA) in all procedures employed, starting from erytrocyte washing, AAPH incubation, AAPH removal, incubation in buffer and NBD-taurine incorporation.

Western Blot Analysis of Band 3

Immunoblotting detection of band 3 was carried out according to the method described by Beppu *et al.*[5] A volume of packed erythrocytes (*ca.* 6.6×10^6 cells) were submitted to gel electrophoresis (SDS-PAGE) in the discontinuous buffer system of Laemmli under

reducing conditions using a concentration gradient gel (5–15%) and a 3.5% stacking gel. Cells number was estimated from absorbance at 410 nm of an erythrocyte suspension.[17] Protein bands on SDS-PAGE gel were transferred to a Western blotting membrane. Transferred proteins were incubated with monoclonal anti-human band 3 for 14 h at room temperature and then with protein A-horseradish peroxidase conjugated. Peroxidase activity was detected with hydrogen peroxide and diaminobenzidine. Positive staining reveals band 3 and newly formed band 3-derived fragments. Gel-Pro program was used to analyze immunoblot densitometric profiles.

Statistical Analysis

Wilcoxon test was applied to the obtained data. Results are expressed as mean value ± standard error.

RESULTS AND DISCUSSIONS

Band 3 protein anion transport of erythrocytes exposed to AAPH-derived radicals was evaluated by the continuous monitoring of NBD-taurine fluorescence. Peroxyl radicals were generated by pyrolysis of AAPH at 37°C. It is known that under the used experimental conditions (AAPH concentration, hematocrit) the onset of erythrocytes lysis occurs after 180 min of exposure to the azocompound.[4,6–8,18] In prehemolytic conditions we found a decrease in NBD-taurine efflux when erythrocytes are exposed to AAPH for 90 min (Fig. 1, curve 2). Curve 1 represents NBD-taurine efflux in erythrocytes incubated for 90 min in the absence of AAPH.

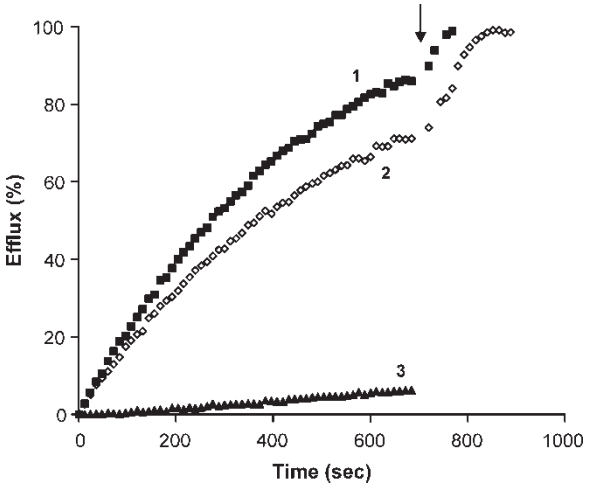

FIGURE 1 Time-dependent NBD taurine efflux of erythrocytes incubated for 90 min in: curve 1, absence of AAPH; curve 2, presence of AAPH (75 mM); curve 3, presence of DIDS (1 mM). Arrow denotes addition of Triton X-100.

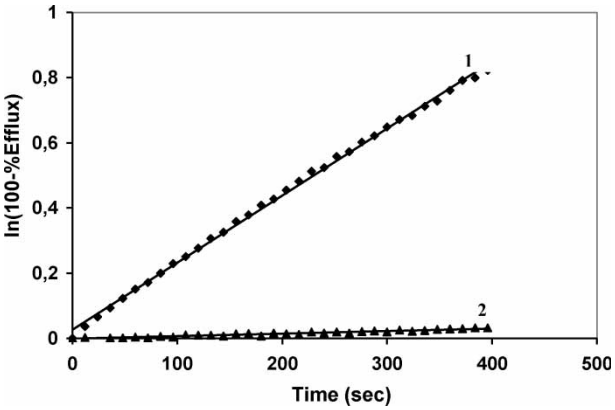

FIGURE 2 NBD taurine efflux as a first order kinetics. Erythrocytes incubated for 90 min in absence of DIDS (curve 1) and in presence of 1 mM DIDS (curve 2).

Most of the transport is band 3 protein-mediated (exit is dramatically reduced in the presence of DIDS, a specific band 3 anion transport inhibitor, curve 3). From data in Fig. 1, the specific rate constant for NBD-taurine efflux (k_{efflux}) can be obtained if it is considered as a first order process. This can be done by taking the slope of the process at a given time, and/or by fitting the data to a first order kinetic law. This last procedure is shown in Fig. 2. Data show a good linear fit up to *ca*. 50% efflux, allowing evaluation of k_{efflux} over this efflux range. k_{efflux} corresponds to the average of k_{inst}, defined by

$$k_{inst}(t) = \frac{1}{F_\infty - F(t)} \frac{dF(t)}{dt}$$

over the efflux range considered.

Measured k_{efflux} takes into account transport mediated by band 3 protein (k_3) and which is not specific (k_{ns}), measured in presence of DIDS. If both processes are considered to follow a first order kinetics, then

$$k_{efflux} = k_3 + k_{ns}.$$

From this consideration, k_{inst} value obtained by subtraction of k_{inst} value, at a particular experimental condition, in the presence of DIDS, from k_{inst} value at that experimental condition in the absence of DIDS, represents that due solely to band 3 protein. From evaluated k_{efflux} and k_{ns}, it is then possible to obtain the value of k_3. Values of k_{ns} and k_3 obtained by this procedure are given in Table I. k_{inst} of NBD-taurine efflux at zero time in the absence of AAPH was $1.82 \pm 0.24 \times 10^{-3}\,s^{-1}$ ($n = 10$); values of k_{inst} are within the range reported by others.[11,17] In the presence of DIDS, k_{inst} decreases to $7.09 \pm 1.62 \times 10^{-5}\,s^{-1}$ ($n = 10$).

From Table I it can be seen that NBD-taurine transport through band 3 decreases with AAPH incubation time, that is, with the increase in the production of peroxyl radicals. This can be

TABLE I NBD-taurine transport in erythrocytes exposed to AAPH-derived radicals. Dependence on proteinase inhibitors

A. Band 3 pathway		
Incubation time (min)		
0	1.003 ± 0.014	($n = 10$)
90	0.850 ± 0.017	($n = 10$)*
90 + (PMSF + EDTA)	0.942 ± 0.006	($n = 6$)*#
B. Other pathways		
Incubation time (min)		
0	1.00 ± 0.010	($n = 10$)
90	1.83 ± 0.070	($n = 10$)**
90 + (PMFS + EDTA)	1.82 ± 0.080	($n = 6$)**

Efflux through band 3 pathway (A) is given as the ratio of k_3 in presence of AAPH to k_3 in absence of AAPH. Efflux through other pathways (B) is given as the ratio of k_{ns} in presence of AAPH to k_{ns} in absence of AAPH. Proteinases inhibitors concentration were 0,2 mM PMSF and 1 mM EDTA. *$p < 0.004$ vs. time zero; **$p < 0.001$ vs. time zero; #$p < 0,003$ vs. AAPH 90 min.

interpreted as the result of band 3 damage by radicals, rendering a non-functional protein. The opposite effect is observed for NBD-taurine transport in the presence of DIDS. The increase in k_{efflux} must be due to the participation of a NBD-taurine transport through membrane defects produced by peroxyl radicals which is different from that of band 3 transport system.

The presence of proteinase inhibitors PMSF and EDTA in the incubation medium, should inactivate proteinases responsible of scavenging oxidized proteins by peroxyl radicals, which are produced during AAPH incubation.[5–8] From Table I it can be observed that k_{inst} is slightly but significantly modified in this condition ($p < 0.003$). Therefore proteinase inhibitors avoided the removal of oxidatively modified band 3, which is still functionally active in NBD-taurine transport. Since no change in k_{inst} was observed in the presence of DIDS, the effect of proteinase inhibitors is non-operative in this condition (Table I). This result can be interpreted as an additional AAPH effect on the erythrocyte membrane, different from an oxidative damage to proteins, which may give rise to distinct NBD-taurine transport pathways, such as those through oxidized membrane lipids. It is known that AAPH-derived peroxyl radicals, under conditions used in this study, produce a moderate lipid oxidation.[4,7,8,18].

Band 3 protein structural damage was analyzed by immunoblotting using antiband 3 monoclonal antibodies. Peroxyl radicals give rise to formation of band 3 aggregates, probably band 3 dimers, in a larger extent than in controls (Fig. 3, lane b vs. lane a). Immunoblot bands corresponds to those described by others[5] including monomers (95–100 kD) and 60, 40 and 20 kD fragments. It has been reported that AAPH-exposed erythrocytes undergo lipid oxidation and band 3 aggregation which may result in membrane pore formation and subsequent cellular lysis.[4]

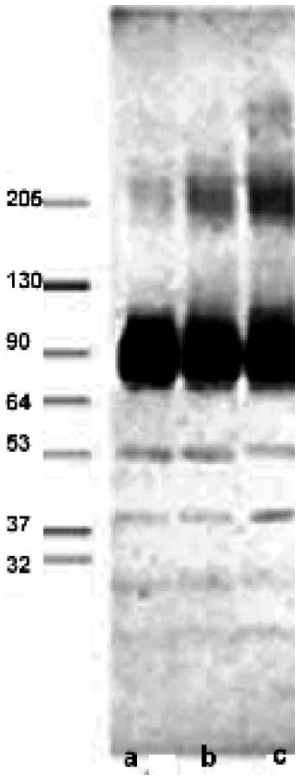

FIGURE 3 Band 3 protein immunoblot of erythrocytes incubated for 90 min in absence of AAPH (lane a); in the presence of 75 mM AAPH (lane b); in the presence of 75 mM AAPH and EDTA (1 mM), PMSF (0.2 mM) (lane c). Numbers denote band molecular weight value expressed in kD.

In this study we do not detect a significant increase in fragments of band 3 monomers. This is probably due to the formation of small fragments, which are not detected under the present electrophoresis conditions. In previous works we have described formation of free amino groups by AAPH-derived peroxyl radicals under similar experimental conditions, and this was explained as due to extensive band 3 proteolysis.[6]

The addition of proteinase inhibitors during the process of oxidation by peroxyl radicals showed the formation of band 3 aggregates and of 40 kD fragments (Fig. 3, lane c), indicating that these structures are efficiently removed by proteinases. Previous studies have demonstrated that erythrocytes have a membrane-bound proteinase activity that prevents accumulation of oxidatively damaged proteins.[5] This proteinase is inhibited by PMSF and EDTA and consequently, our results reflect the action of this proteinase which is selective to oxidized proteins. Our results are also in agreement with Fujino *et al.*[14] who found that this proteinase recognizes band 3 aggregates.

It is known that protein oxidation may give rise to aggregates linked through oxidized aminoacids, like disulfide bridges. However, band 3 aggregates, shown in Fig. 3, are not linked by disulfide bridges

since electrophoresis conditions include a reducing agent. Hence they may result from the linkage through residues (such as tyrosines) unaffected by the reducing conditions present during electrophoresis.

Oxidized band 3 proteolysis is a cellular defense mechanism to scavenge damaged molecules. However, the results of this study shows that this mechanism is destroying oxidized molecules that still retain their ability for anion transport (Table I). It is known that senescent erythrocytes show an increase in band 3 aggregates and this constitutes the antigen recognized by macrophages to eliminate these cells.[14] Presumably, the function of these proteinases as oxidized proteins scavengers is to avoid recognition by macrophages of the senescent antigen, to minimize the destruction of moderately damaged cells at the expense of a diminished anion transport. This is reinforced by the fact that proteinases are not effective on highly oxidized proteins.[5]

In conclusion, band 3 protein anion transport is diminished by peroxyl radicals generated in the external medium. This decrease may rise from a direct protein oxidation and from its degradation by active red blood cell proteinases to oxidized proteins which may protect red blood cells from premature recognition as senescent cells.

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