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Peroxynitrite oxidizes erythrocyte membrane band 3 protein and diminishes its anion transport capacity

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Abstract

We describe an altered membrane band 3 protein-mediated anion transport in erythrocytes exposed to peroxynitrite, and relate the loss of anion transport to cell damage and to band 3 oxidative modifications. We found that peroxynitrite down-regulate anion transport in a dose dependent relation (100–300 μmoles/l). Hemoglobin oxidation was found at all peroxynitrite concentrations studied. A dose-dependent band 3 protein crosslinking and tyrosine nitration were also observed. Band 3 protein modifications were concomitant with a decrease in transport activity. (−)-Epicatechin avoids band 3 protein nitration but barely affects its transport capacity, suggesting that both processes are unrelated. *N*-acetyl cysteine partially reverted the loss of band 3 transport capacity. It is concluded that peroxynitrite promotes a decrease in anion transport that is partially due to the reversible oxidation of band 3 cysteine residues. Additionally, band 3 tyrosine nitration seems not to be relevant for the loss of its anion transport capacity.

Keywords: *Erythrocyte, peroxynitrite, anion transport, band 3*

Introduction

Peroxynitrite anion, a product of the reaction of superoxide radicals and nitric oxide, and its protonated form (peroxynitrous acid), are potent oxidants known to be produced under physiological conditions. Both oxidants can directly react with biomolecules such as proteins, carbohydrates, lipids, DNA and low molecular weight thiols with significant second order rate constants. Furthermore, peroxynitrite can react "via" hemolytic cleavage of the protonated form. As consequence of peroxynitrite reactions with proteins there is a direct oxidation of aminoacid residues leading to –SH oxidation and crosslinking, as well as tyrosine and tryptophan groups nitration [1]. Multiple studies of protein disfunction due to exposure to peroxynitrite have been reported. Importantly, several pathologies [1–4] have been associated to the over-production of peroxynitrite anion.

Erythrocytes are exposed to peroxynitrite during its circulation since it can be produced by activated leucocytes [5] and synthesized when $O_2^{\cdot-}$, produced during hemoglobin autoxidation, reacts with NO· derived from other cells. Extracellular generated peroxynitrite gets into erythrocytes by crossing the membrane barrier by two pathways: the anion form through band 3 protein anion exchanger and the protonated form by passive diffusion through the lipid bilayer [6,7]. At physiological pH, 80% of peroxynitrite is present as the anion form and 20% as the

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protonated form, with an overall lifetime of *ca*.1s. Therefore band 3 protein appears as the main route of erythrocyte peroxynitrite uptake.

Anion exchange band 3 protein is the major intrinsic protein of erythrocytes membranes. Anion transport in erythrocytes is important for oxygen delivery to tissues and for the acid–base status of body fluids. Erythrocytes exposed to peroxynitrite *in vitro* show both intracellular and membrane constituents damage: hemoglobin is oxidized [6,8,9], enzyme activities are decreased [10], K^+ transport is altered [11], and band 3 protein is modified. The latter includes crosslinking [12,13], and nitration and phosphorylation of tyrosine residues [14,15].

Erythrocytes exposed to oxidative stress show an altered transport capacity through the anion exchange band 3 protein. Phenylhydrazine decreases anion transport due to band 3 protein damage mediated by hemoglobin oxidation [16]. Peroxyl radicals derived from AAPH, an extracellular free radical generator, decrease erythrocytes anion transport capacity. However, not all oxidized band 3 protein molecules lose its transport capacity since crosslinked band 3 partially retains its anion transport function [17]. Damage to erythrocyte band 3 protein by peroxynitrite is known but, to our knowledge, no studies have been reported regarding its effect on anion transport.

In this report we describe an altered band 3-mediated anion transport in erythrocytes exposed *in vitro* to a bolus of peroxynitrite, and relate the loss of anion transport capacity with band 3 protein oxidative modification assessed from tyrosine nitration and crosslinking.

Materials and methods

Materials

4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) was obtained from Molecular Probes, goat anti mouse IgG peroxidase-conjugated and mouse anti-nitrotyrosine monoclonal antibodies were from Chemicon, monoclonal anti-human band 3 was obtained from Sigma, hydrogen peroxide and sodium nitrite were from Merck, and the super signal west pico chemiluminescence kit was obtained from Pierce. Other chemicals were from standard commercial sources.

Peroxynitrite was synthesized in a quenched-flow reactor as described by Beckman [18] with modifications according to Saha [19] and stored at −80°C. Peroxynitrite anion concentration in 1 M NaOH was determined spectrophotometrically at 302 nm ($\epsilon = 1670\,M^{-1}\,cm^{-1}$). Peroxynitrite concentration in the top layer of a partially frozen solution was 180–216 mM. Dilutions in saline phosphate buffer (buffer A) (mmoles/l:sodium phosphate, 80, NaCl, 45, pH 7.4, 0°C) were prepared immediately before use. Decomposed peroxynitrite in buffer A was obtained by incubation for 5 min at 25°C.

Erythrocyte oxidation by peroxynitrite. Heparinized fresh blood was collected from healthy volunteers following informed consent. After centrifugation at 12,000*g* for 30 s, plasma and buffy coat were removed and erythrocytes were washed three times by centrifugation in buffer A [20]. Resuspended erythrocytes in buffer A (1% hematocryt) were exposed for 3 min at 25°C to a bolus (100–300 μmoles/l) of fresh or decomposed peroxynitrite. Alternatively washed erythrocytes were resuspended in saline-phosphate bicarbonate buffer (mmoles/l:sodium phosphate, 80, NaCl, 20, $NaHCO_3$, 25, pH 7.4) (1% hematocrit) and exposed to peroxynitrite under the same conditions used when resuspended in buffer A. Immediately after, erythrocytes suspensions were cooled at 0°C, kept at this temperature for 5 min and washed three times by centrifugation with cold buffer A. Peroxynitrite-treated erythrocytes were used for sulfate transport measurements, membrane protein fragmentation analysis by immunoblotting, evaluation of lysis, and intracellular hemoglobin oxidation. Also, they were employed for membrane isolation according to the method described by Dodge [21].

Prior to peroxynitrite treatment, (−)-epicatechin was added to some erythrocyte suspensions. In other experiments, peroxynitrite treated erythrocytes were incubated with *N*-acetylcysteine (600 μmoles/l) in PBS (mmoles/l:NaCl, 145, sodium phosphate, 5, pH 7.4) at 25°C for 60 min. Red cells were separated by centrifugation and resuspended in a freshly prepared *N*-acetylcysteine solution to perform transport anion evaluation.

Sulfate influx kinetics. Peroxynitrite-treated and untreated erythrocytes (hematocryt 3%) were kept for 1 h at 25°C in PBS. After centrifugation, red blood cells were re-suspended in sulfate buffer (mmoles/l:Na_2SO_4, 70, NaCl, 10, HEPES, 25, pH 7.4) and incubated at 25°C for 2 h. Samples were removed at several time intervals and added to a volume of ice-cold DIDS solution to obtain a final DIDS concentration of 50 μmoles/l, and then centrifuged at 12,000*g* for 90 s. Erythrocytes sediment was resuspended in free sulfate buffer (mmoles/l; NaCl, 90, HEPES, 25, pH 7.4) and then washed three times with free sulfate buffer. Intracellular sulfate concentration was measured after cell lysis in perchloric acid solution and pellet separation was performed by centrifugation. Supernatant sulfate anions were precipitated as $BaSO_4$ and measured at 405 nm according to Teti et al. [22]. Influx constant rate (min^{-1}) were obtained by fitting the data to a first order kinetic equation [23]:

$$C_t = C_\infty(1 - e^{-kt})$$

where C_t and C_∞ represent sulfate concentration at times t and ∞ respectively and k is the influx constant

rate. Only data with correlation coefficients (r) higher than 0.95 were considered.

Sulfate concentrations were normalized by the number of red blood cells used as described by Cabantchik [24]. Values were corrected to take into account the small degree of lysis taking place during sulfate influx measurements.

Hemoglobin oxidation and cell lysis. Hemoglobin oxidation was assessed spectrophotometrycally in erythrocyte lysates of treated and untreated red blood cells, as described by Winterbourn [25]. Cell lysis, assessed according to Celedón [26] was measured immediately after peroxynitrite treatment and during the following 2 h.

Western blot immunoassays. Proteins of erythrocytes membrane were separated by electrophoresis (5–17%) (SDS-PAGE) under reducing conditions. After transfer to a nitrocellulose paper, blots were washed with Tris buffer saline (TBS) (mmoles/l:NaCl 150, Tris–HCL, 50, pH 7.4) and then blocked by incubation for 1 h at room temperature with 5% bovine serum albumin, 0.3% Tween-20 in TBS. Nitrocellulose membranes were then incubated for 1 h with mouse anti-nitrotyrosine monoclonal antibodies (1:2000, in 5% albumin, 0.6% Tween-20 in TBS) and then washed three times with 2,5% albumin, 0.3% Tween-20 in TBS before incubating for 1 h with goat anti mouse IgG peroxidase-conjugated (1:5000). After extensive washing, presence of nitrotyrosine residues was revealed by chemiluminescence. For band 3 protein evaluation, blocking solution was a 5% non-fat milk, 1% Tween-20 in PBS [17]. When necessary, western blot stripping was performed according to the procedure described by Kaufmann [27]. Positive controls for nitrotyrosines were prepared by exposure of isolated membranes to peroxynitrite (1 mmoles/l) for 3 min. Protein bands were evaluated by a Gel-Pro analyser 4.0 program.

Statistical analysis. Results are expressed as mean ± SEM averaged over the different blood samples. On each sample, duplicate measurements were performed. Statistical significance of the obtained results was assessed by Friedman test followed by Dunn's multiple comparison test.

Results and discussion

Erythrocytes are exposed to peroxynitrite during its lifespan under physiological and pathological conditions. Many *in vitro* studies on the consequences of red cells exposure to peroxynitrite in membrane constituents [11], hemoglobin [6,8,9], intracellular redox state [12,28], cell metabolism [29,30], and cell lysis [31] have been published.

In this study we report the effect of peroxynitrite on erythrocyte anion transport capacity and make an evaluation of the transport function alteration. This change is related to band 3 and cell damage inflicted by the oxidant, measured as cell lysis, hemoglobin oxidation and oxidative damage to band 3 protein.

Cell lysis is clearly observed when erythrocytes are exposed to large peroxynitrite concentrations (Table I). This effect is not observed when previously decomposed peroxynitrite is employed. Lysis extent observed in the present work is smaller than that reported by others [31], a difference that could be due to the higher temperature (37°C) employed by these authors. To evaluate if cell lysis induced by peroxynitrite continues after its consumption, pre-treated cells were incubated for 2 h in buffer at 25°C. A small increment in lysis was observed, 5 ± 1% and 14 ± 2% for peroxynitrite concentrations of 200 and 300 μmoles/l respectively. This lysis is not due to secondary oxidative reactions from products of peroxynitrite reactions with hemoglobin, since no increment in hemoglobin oxidation was observed during this period (data not shown). It can be speculated that cell damage inflicted by peroxynitrite cannot be completely stopped and/or repaired by intracellular antioxidants, due to an irreversible membrane damage that leads to a delayed colloidal osmotic lysis. This effect could be enhanced by a decrease in the antioxidant defenses of the cell. In this regard, it has been reported a decrease in glutathion concentrations in peroxynitrite treated red cells [12,28].

Peroxynitrite permeates erythrocytes and readily reacts with oxyhemoglobin [6]. This process represents the most important route for peroxynitrite consumption due to the high protein intracellular

Table I. Lysis and hemoglobin oxidation in peroxynitrite-treated red cells.

Peroxynitrite (μmole/l)	Lysis (%)	OxyHb (%)	MetHb (%)	$OxyHb_{consumed}/ONOO^-_{molecule}$
0		1.0 ± 0.1	96.4 ± 0.3	0.50 ± 0.2
100	1.2 ± 0.1	78.1 ± 1.0*	18.8 ± 0.8*	0.35 ± 0.01
200	2.0 ± 0.6	44.0 ± 1.0†	56.0 ± 2.0†	0.55 ± 0.01
300	10.0 ± 2.0	21.0 ± 4.0‡	80.0 ± 4.0‡	0.52 ± 0.01
200 (Decomposed)	0.3 ± 0.2	95.7 ± 0.2^{NS}	0.9 ± 0.1^{NS}	–

$n = 3$ for lysis studies and $n = 6$ for hemoglobin oxidation; NS, non significant; $^\star p < 0.05$; $^\dagger p < 0.01$ and; $^\ddagger p < 0.001$ for peroxynitrite as compared to absence of peroxynitrite.

concentration (20 mmoles/l). Under our experimental conditions, peroxynitrite quantitatively oxidizes hemoglobin to methemoglobin. This oxidation process is very efficient, since one hemoglobin molecule is oxidized by two peroxynitrite molecules added to the suspension. This large extent of hemoglobin oxidation is similar to that previously described [6] and implies that at least half peroxynitrite molecules are able to penetrate the red cell during is short lifetime. The reaction mechanism involves a net one-electron oxidation of the ferrous hem, with production of superoxide and hydrogen peroxide [9]. Both oxidants can contribute to the secondary damage of lipids and proteins.

Erythrocytes anion transport was evaluated from sulfate influx. This approach has been used by others and has the advantage of the slow rate of the process. Furthermore, the absence of endogenous sulfate ensures that its intracellular concentration is directly related to anion uptake [32]. Figure 1 shows the time dependent profile of sulfate influx in untreated erythrocytes and after exposure to 200 μM peroxynitrite. Rate of uptake when erythrocyte suspension is treated with previously decomposed peroxynitrite is also shown. Results show that addition of peroxynitrite significantly decreases the rate of the process ($p < 0.01$, $n = 6$). On the other hand, addition of predecomposed peroxynitrite does not significantly modify the rate of sulfate intake.

The decrease in sulfate influx rate elicited by the exposure of red cells to peroxynitrite is concentration dependent. Rate constants for the process are collected in Table II. Data obtained in presence of DIDS, an inhibitor of band 3 anion exchange, are also included. Rate constants obtained in non treated erythrocytes are similar to those previously reported

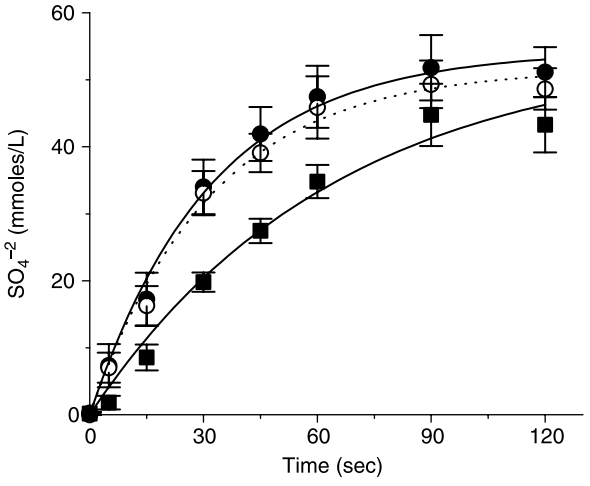

Figure 1. Time course of sulfate influx in erythrocytes. (■) With 200 μmoles/l peroxynitrite, (○) with 200 μmoles/l peroxynitrite previously decomposed, (●) without peroxynitrite. Cells number: 3.6×10^8 cells/ml. Data are representative of six independent experiments.

Table II. Peroxynitrite effect on sulfate influx rate constant (k_{in}) in red cells.

Peroxynitrite (μmoles/l)	k_{in} (min^{-1})
0	0.049 ± 0.006
100	0.042 ± 0.004
200	0.020 ± 0.007
300	0.013 ± 0.001
Peroxynitrite (μmoles/l) + DIDS (50 μmoles/l)	
0	0.002 ± 0.002
200	0.003 ± 0.001
300	0.000

$n = 3$.

[30]. Addition of peroxynitrite (300 μM) reduces (down to 26 ± 1%) the rate of sulfate influx. In presence of DIDS, transport is negligible, both in untreated and peroxynitrite exposed red cells. This indicates that this oxidant does not promote unspecific anion transport due to membrane damage. This is compatible with the low level of lipoperoxidation associated to the process [31].

The efficiency of added peroxynitrite in reducing band 3 transport is compared to that of other oxidants in Table III. Data collected show that the efficiency of peroxynitrite is similar to that of AAPH derived radicals. In fact, in both systems, on average, nearly 3000 oxidants are needed to inactivate a band 3 protein. On the other hand, the efficiency of phenylhydrazine is nearly an order of magnitude smaller. This low efficiency could result from a different action mechanism, since for this later compound it has been proposed that its action takes place mainly via Heinz body formation. However, both peroxyl radicals and peroxynitrite derived radicals could promote protein inactivation by direct reaction with the macromolecule. In any case, the large number of radicals needed (on average) for inactivating one transporter (assuming an all/or none transport situation) reflects both the multiplicity of targets in the red cell and the resistance of the protein to its inactivation. In particular, the rather low damage of band 3 could reflect the large scavenging capacity of the intracellular hemoglobin.

Peroxynitrite is known to oxidize erythrocyte membrane proteins. In isolated membranes of pretreated cells, it has been reported band 3 protein aggregation, membrane sulfhydryls group decreased [12,13], and band 3 tyrosine residues nitration [14,15]. We confirm band 3 crosslinking and tyrosine nitration due to peroxynitrite oxidative damage to band 3 protein aminoacid residues under the present experimental conditions (Figures 2, 3 and 4). We also visualized band 3 protein aggregates in whole erythrocytes:they were submitted to electrophoresis after their direct digestion in the corresponding buffer. Separated proteins were analyzed by western blots with anti-band 3 antibodies under reducing conditions. A peroxynitrite dose dependent increase of band 3

Table III. A comparison of anion transport capacity of erythrocytes treated with oxidants.

Oxidants (mmoles/l)	Decrement (%)	Oxidant/erythrocyte (molecules/cell) $\times 10^{-8}$	Oxidant/band 3 (molecules/inactivated molecule)	Reference
Phenylhydrazine				
2.0	40	200	50,000	[16]*
Peroxynitrite				
0.1	14	5.5	3900	This report
0.2	61	11.0	1800	
0.3	74	16.5	2200	
AAPH-derived peroxyl radicals				
0.5	15	3.4	2300	[17]

* Calculated from reported data in [16].

aggregates due to non sulfhydryls bonds were observed together with a decrease in band 3 monomer (Figure 2A). These results are in accordance with studies that detected band 3 protein patches in red blood cells treated with peroxynitrite, together with a decrease of band 3 protein levels, as evaluated by immunofluorescence analysis and flow cytometry [28].

In order to detect crosslinking due to sulfhydryl oxidations, immunoblots of isolated membranes of erythrocytes pretreated with peroxynitrite were performed without pre-reduction of disulfide bridges (Figure 2B). An increase of band 3 aggregates was observed as compared to reducing conditions, evidencing a reversible oxidation of band 3 cysteine residues. Although band 3 protein aggregation (*ca.* 50%, 200 μM peroxynitrite) is similar to the reduction in band 3 transport (50–60%), both effects could not be directly related since we have found that crosslinked band 3 in erythrocytes treated with peroxyl radicals retain anion transport capacity [17].

Peroxynitrite nitrates free or protein tyrosines giving rise to 3-nitrotyrosine. It has been suggested that, in absence of a catalyst, nitration takes place through ·OH and $\cdot NO_2$ radicals. Tyrosine reacts first with ·OH yielding tyrosil radical, which recombines with $\cdot NO_2$ to produce 3-nitro-tyrosine. 3,3′-Dityrosine is also formed from tyrosine derived radicals dimerization [33]. Figure 3(c) shows a protein band of nitrotyrosine that co-migrates with band 3 protein in electrophoresis assays employing membranes isolated from peroxynitrite treated erythrocytes. This band is not present in a control without peroxynitrite (Figure 3a) or if peroxynitrite is decomposed previous to its addition to the cells (Figure 3b). In these assays, actin is employed as internal standard.

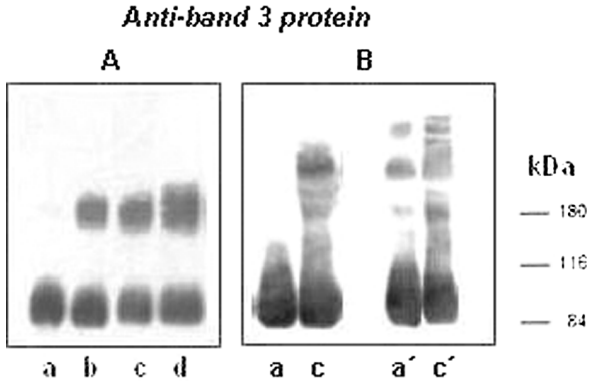

Figure 2. Crosslinking of band 3 protein in erythrocytes. (A) SDS-PAGE electrophoresis of pretreated erythrocytes under reducing conditions and subsequent immunoblotting using mouse anti human band 3. (B) SDS-PAGE electrophoresis under reducing conditions (a, c) and under non reducing conditions (a′, c′), of isolated membranes of pretreated erythrocytes. Peroxynitrite concentrations were (μmoles/l): 0 for a and a′, 100 for b; 200 for c and c′ and 300 for d. Data are representative of three independent experiments.

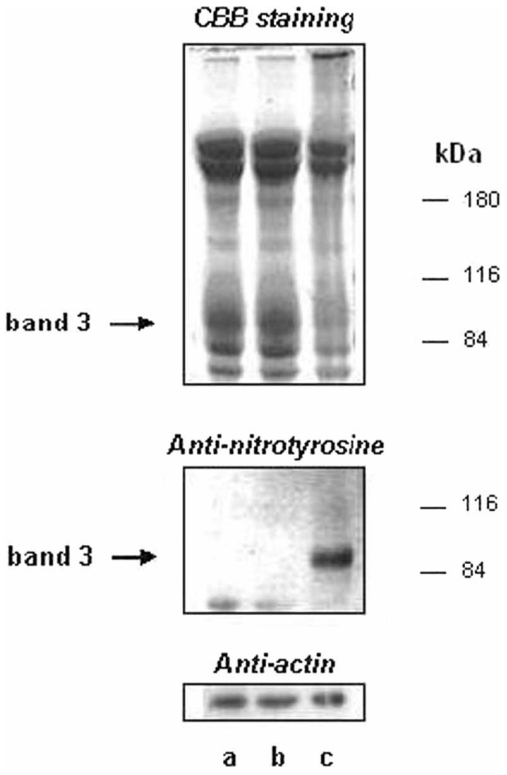

Figure 3. Nitration of band 3 tyrosine. SDS-PAGE electrophoresis under reducing conditions of membrane proteins (higher than 60 kDa) of treated erythrocytes and stained with Coommassie Brilliant Blue (CBB) (upper panel). Immunoblotting using mouse anti-nitrotyrosine (medium pannel) or mouse anti-actin (lower pannel) of isolated membranes of erythrocytes treated with peroxynitrite (μmoles/l): (0) a, (200) previously decomposed b, (200) c. Data are representative of six independent experiments.

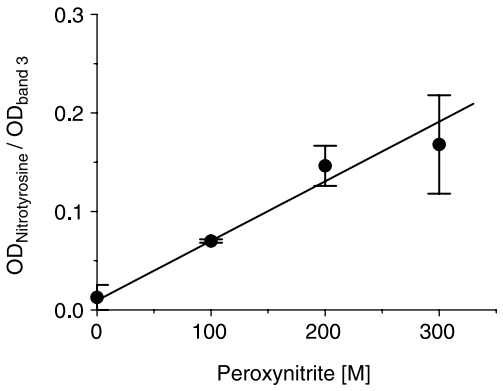

Figure 4. Peroxynitrite concentration dependence of band 3 protein tyrosines nitration. Nitration of band 3 tyrosines are normalized to the amount of band 3 present in each electrophoresis lane. Ratio $OD_{nitrotyrosine}/OD_{band3}$ was obtained from densitometric analysis of immunoblots of membrane proteins separated by SDS-PAGE electrophoresis and then using mouse anti-nitrotyrosine followed by stripping of the nitrocellulose membrane and subsequently mouse anti-human band 3. Data are representative of three independent experiments.

Nitration of band 3 tyrosines is dose dependent (Figure 4) in the same peroxynitrite range where the lost of band-3 activity increases with the oxidant dose (Table II). In order to evaluate if the lost of activity is directly related to band 3 nitration, experiments were performed in presence of (−)-epicatechin, a flavonoid that selectively protects against peroxynitrite reactions. This flavonoid avoids nitration reactions at concentrations two order of magnitude lower than the one needed to protect from other oxidation reactions. Therefore, it gives the possibility to evaluate a putative primary role of nitration reactions [34]. (−)-Epicatechin strongly decreases (*ca*. 90%) nitration of band 3 tyrosine residues (Figure 5). On the other hand, it only moderately decreases (*ca*. 30%) the effect of peroxynitrite in sulfate transport

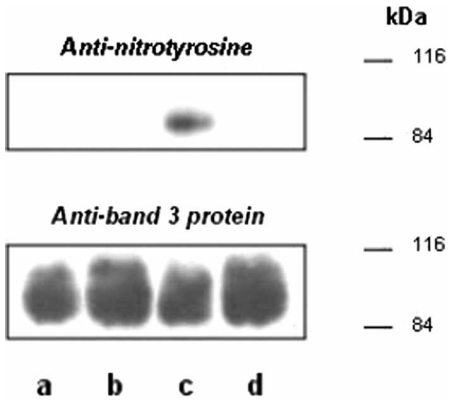

Figure 5. (−)-Epicatechin prevents band 3 protein tyrosines nitration of erythrocytes treated with peroxynitrite (μmoles/l). (0), a, (200) previously decomposed, b, (200), c, and (200) + (−)-epicatechin, d. Data are representative of three independent experiments.

(Table IV). Furthermore, it did not affect intracellular hemoglobin oxidation, confirming a more specific effect on nitration reactions than in preventing other oxidation reactions (Table IV). These results strongly suggest that protein nitration is not a pre-requisite for the lost of anion transport capacity. Furthermore, crosslinking of band 3 protein under reducing conditions was only slightly affected in the presence of this flavonoid (*ca*. 8%) implying that croslinking through oxidized aminoacid residues, most probably dityrosines [35], is not a pre-requisite for the lost of transport capacity.

When erythrocytes were exposed to peroxynitrite in bicarbonate buffer to get close to a biological condition, no changes were observed in anion transport, hemoglobin oxidation or band 3 tyrosine nitration (results not shown). This can be explained considering the reduction of peroxynitrite lifetime which occurs in the presence of bicarbonate [1], and also the incapacity of the adduct nitrosoperoxocarboxylate, formed by reaction between peroxynitrite and bicarbonate, and its decomposition product, the carbonate radical anion [36] to interact with the red cell under our experimental conditions.

To study if the decrement in band 3 anion transport capacity due to treatment with peroxynitrite is reversible, erythrocytes were treated with peroxynitrite and then incubated with *N*-acetyl cysteine before sulfate efflux evaluation. Our results show that *N*-acetylcysteine partially reverted (46%) the anion transport decrement observed in erythrocytes pretreated with peroxynitrite (Table IV). These results imply that a significant part of the activity loss can be reverted by reducing S–S bridges, either intra or interproteins. However, the fact that under the experimental conditions (200 μM peroxynitrite) only around 5% of band 3 proteins are forming reversible dimmers, would indicate that most of the lost of activity is due to the presence of intramolecular S–S bridges. These bridges can correspond to disulfides between protein cysteines and/or mixed disulfides between protein cysteines and glutathione (*S*-glutathiolation). Our results agree with Teti et al. [30] who reported that erythrocytes thiols redox state is important for anion translocation since, in red cells treated with sulfhydryl-blocking or thiol-oxidizing agents, a decrease of anion transport through band 3 was observed.

It is concluded that peroxynitrite promotes a decrease in anion transport through band 3 protein that is partially (46%) due to the reversible oxidation of cysteine residues. This inactivation is concentration dependent and, on average, *ca*. 3000 peroxynitrite molecules are needed to inactivate one band 3 protein molecule of the erythrocyte membrane. Additionally, band 3 protein tyrosine nitration seems not to be relevant for the loss of its anion transport capacity.

Table IV. Effect of (−)-epicatechin and *N*-acetylcysteine (NAC) in peroxynitrite-treated erythrocytes.

Peroxynitrite (μmoles/l)	Sulfate k influx (min^{-1})	MetHb (%)	Nitration
0	0.034 ± 0.005	1.5 ± 0.4	−
200	0.016 ± 0.001	47.5 ± 2.8	+
200 + (−)-Epicatechin (8 μmoles/l)*	0.021 ± 0.001	48.7 ± 2.1	−
0	0.039 ± 0.005	2.0 ± 0.9	−
200	0.014 ± 0.003	47.3 ± 3.8	+
200 + NAC (600 μmoles/l)†	0.029 ± 0.006	47.9 ± 4.2	+

k value (min^{-1}) for sulfate influx with NAC and no peroxynitrite = 0.044 ± 0.007. $n = 3$; *-(−) Epicatechin added before peroxynitrite treatment; †NAC added after peroxynitrite treatment.

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