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Band 3 tyr-phosphorylation in human erythrocytes from non-pregnant and pregnant women

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Abstract

Pregnancy is associated with changes in circulating red blood cells, mainly involving band 3 protein and membrane lipid peroxidation. Membrane band 3 is a multifunctional protein containing four Tyr-phosphorylatable residues which modulate the physiological status of erythrocytes by regulating glycolysis, cell shape and membrane transport. Erythrocytes from nine pregnant and 12 age-matched non-pregnant healthy women were subjected to oxidative and hyperosmotic stress conditions and the extent of band 3 Tyr-phosphorylation and membrane Syk recruitment as a membrane marker were evaluated. Results indicated that, in pregnancy, red blood cells show a decrease in band 3 Tyr-phosphorylation and a clear-cut rearrangement of band 3 protein within the membrane. In fact, band 3 shows a decrease in high molecular weight aggregates (HMWA), with different subdivision between Triton-soluble and -insoluble compartments, and an increase in proteolytic fragments. In conclusion, it is demonstrated that pregnancy is associated with membrane adjustments which reduce the sensitivity of erythrocytes to both oxidative and osmotic stress. Band 3 Tyr-phosphorylation is proposed as a new parameter in the evaluation of erythrocyte membrane arrangement.

Keywords: Human erythrocytes in pregnancy; Band 3 Tyr-phosphorylation; Oxidative stress; Osmotic stress; Band 3 electrophoretic pattern

1. Introduction

Pregnancy is associated with many physiological changes, some of them involving the maternal hematopoietic system. Circulating red blood cells have been shown to undergo many physiological alterations [1]. For example, during pregnancy, a reduction in size and a decrease in specific gravity are observed [2,3]. Pro-oxidative processes are also intensified in pregnant women, as indicated by the increase in lipid peroxidation products [4,5].

Oxidative treatment of normal human erythrocytes with pervanadate and diamide acting as PTPase inhibitors induces an increase in protein Tyr-phosphorylation [6–8], mostly involving four tyrosine residues of band 3 protein [8,9], in a two-step mechanism. Syk tyrosine kinase, belonging to the Syk family, triggers the so-called "primary phosphorylation" of Tyr 8 and 21 which, once phosphorylated, act as docking sites for Lyn tyrosine kinase, belonging to the Src family, which catalyses the "secondary phosphorylation" of Tyr 359 and 904 [8]. P-Tyr-359 is then recognized by the SH2-domain of SHP-2, a protein tyrosine phosphatase which, when recruited to band 3, dephosphorylates Tyr-residues 8, 21 and 904, and thus contributes to restoration of the normal erythrocyte band 3 Tyr-phosphorylation level [10]. Alteration in P-Tyr-protein content has been demonstrated to affect the cell's physiological status: regulation of glycolysis, through the release of glycolytic enzymes from membrane band 3 following phosphorylation of its cytoplasmic domain [11–13], alteration of red blood cell shape [14] and volume [15], and membrane transport [15,16] are all aspects of cellular life involving band 3 Tyr-phosphorylation.

Moreover, our recent findings assigned to band 3 Tyr-phosphorylation a new important task, as a marker of the physiological status of erythrocyte membranes [17,18]. When treated with several different effectors, including oxidants, erythrocyte response is clearly mediated by membrane reorganization, as indicated by the modulation in their ability to recruit Syk from cytosol to membranes [17]. This was further

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confirmed by the finding that, in pathological conditions, such as the chronic lowering of anti-oxidant defences in the metabolic disorder of Glucose-6-Phosphate Dehydrogenase deficiency (G6PD d), both band 3 Tyr-phosphorylative and membrane Syk recruitment responses are useful in evaluating the impaired erythrocyte response to both oxidative and osmotic stress [18].

In this work, we demonstrate that band 3 is directly involved in the changes induced by both diamide and hyperosmotic stress, and compare these changes with the re-adjustments previously reported in pregnancy [19].

2. Materials and methods

2.1. Materials

Anti-P-Tyr and anti-Syk monoclonal antibodies were purchased from MP Biomedicals (Aurora, Ohio) and Upstate (Lake Placid, NY), respectively. Rabbit-anti SHP-2 (C-18) polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PP2 and Protease inhibitor cocktail were obtained from Calbiochem (Darmstadt, Germany) and Roche, respectively. γ[^{32}P] adenosine 5′-triphosphate (ATP) was purchased from Amersham Pharmacia Biotech (Little Chalfont, UK). Other reagents were purchased from Sigma.

2.2. Methods

2.2.1. Isolation of human erythrocytes

Human erythrocytes were prepared, as previously described [20], from fresh blood collected from 9 healthy pregnant women (PW) at the third trimester and 12 age-matched non-pregnant women. Five of the nine pregnant women received supplements of ferrous sulfate (90 mg/day of elemental iron).

None of them showed oxidative stress, as indicated by analysis of GSH/GSSG content (Table 2) and reticulocyte counts (1.2±0.6 vs. 0.9±0.4 of normal donors, expressed as % RBC). Hemoglobin determination (10.9±1.1 vs. 12.1±0.9 g/dl) (on an automated Sismex X6 2100 analyser, Dasit) showed no significant differences between the two groups.

2.2.2. Treatment of erythrocytes

Packed cells (50 μl), prepared as described above, were resuspended (at 20% hematocrit) in an isosmotic buffer A (20 mM Tris–HCl, pH 7.5, 125 mM NaCl, 10 mM KCL, 1 mM $MgCl_2$, 50 mM glucose, 1 mM adenosine) or a hyperosmotic buffer (buffer A containing 700 mM sorbitol), centrifuged for 3′ at 3000 rpm, discarding supernatant (to balance the cells osmotically), immediately resuspended in 200 μl of the respective buffers, and incubated for 30 min at 35 °C in the presence or absence of 0.7 mM diamide dissolved in buffer A. When required, 1 μM PP2 inhibitor (or DMSO vector) was added to the incubation mixture together with diamide or hyperosmotic buffer. After incubation, each sample was centrifuged and the packed cells were subjected to hemolysis in 1.5 ml of hypotonic buffer containing 5 mM sodium phosphate, pH 8.0; 0.02% NaN_3, 30 μM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate, and protease inhibitor cocktail.

Membranes were separated from the cytosol by centrifugation (20,000×*g* for 20 min) and washed once in hypotonic buffer. Aliquots of membranes (10 μg, corresponding to about 5 μl of packed erythrocytes) or cytosol (15 μl, corresponding to about 0.5 μl of packed cells) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% gels), transferred to nitrocellulose membranes, and immunostained with the appropriate antibody.

2.2.3. Preparation of band 3 proteolytic fragment (cdb3)

The 40/45 kDa fragment of the cytoplasmic domain of band 3 (called as cdb3) was obtained by α-chymotrypsin-promoted breakdown of inverted membrane vesicles derived from ghosts and isolated by DE 52 chromatography according to [21].

2.2.4. Protein kinase assay

The protein kinase assay was performed according to [18]. Briefly, packed treated erythrocytes were subjected to several freeze/thaw cycles to yield a homogeneous cell lysate. 4 μl of each were assayed for tyrosine kinase activity at 30 °C for 10 min in 30 μl incubation mixture containing 50 mM Tris HCl, pH 7.5, 10 mM $MnCl_2$, 30 μM γ[^{32}P] ATP (specific activity 1000 cpm/pmol), 10 μM sodium orthovanadate, and 3 μg cytoplasmic domain of band 3 (cdb3) as a substrate for tyrosine kinase activity. After incubation, the reaction was stopped by the addition of 1% SDS and 1% β-mercaptoethanol (final concentrations) and heated for 5 min at 100 °C. Solubilized proteins were subjected to SDS-PAGE (10% gels), the gels were stained with Coomassie blue, treated with 2 mM NaOH for 60 min at 55 °C, dried, and autoradiographed for 2 days [22].

2.2.5. Protein phosphatase activity

4 μl of the cell lysate obtained as described above were assayed for tyrosine protein phosphatase activity in 30 μl incubation mixture containing 50 mM Tris–HCl pH 7.5 and 3 μg [^{32}P]cdb3 obtained as described in [10] at 30 °C for 5 min. The reaction was stopped after incubation by the addition of 1% SDS and 1% β-mercaptoethanol (final concentrations), followed by 5 min heating at 100 °C. Solubilized proteins were subjected to SDS-PAGE (10% gels). The gels were stained with Coomassie blue, treated with 2 mM NaOH for 60 min at 55 °C, dried, and autoradiographed for 3 days.

2.2.6. Quantitative determination of total glutathione (GSSG+GSH) and oxidized glutathione (GSSG) content in erythrocytes

Total glutathione was determined according to the method of Tietze [23]. Briefly, 10 μl of cytosol obtained from erythrocytes of normal and pregnant women as described above, was added to the glutathione assay mixture [23] and analysed spectrophotometrically. GSSG content was evaluated in 10 μl cytosol incubated in a glutathione assay mixture in which vinylpyridine, was added in accordance with [24]. The level of GSH was determined by calculating the difference between the two assays.

2.2.7. Quantitative determination of methemoglobin (metHb) content in erythrocytes

The concentration of methemoglobin in lysate samples was determined by the KCN addition method [25]. Samples of treated and untreated erythrocytes of normal and pregnant women were hemolysed in hypotonic buffer. Determinations of hemoglobin/methemoglobin were carried out on diluted cytosol with a UV-VIS spectrophotometer (mod. V-530, Jasco) at room temperature, using 1-cm path length quartz cuvettes.

2.2.8. Quantitative determination of lactate

Supernatants of 20% hematocrit incubation samples (see above), obtained by centrifuging for 3 min at 3000×*g* were assayed for determination of lactate. Measurements were carried out in 0.1 M Tris–HCl, pH 8.5, containing 2 mM NAD^+ and 5 U/ml L-lactate dehydrogenase (Sigma). NADH formation was followed at 340 nm on a V-530 Jasco spectrophotometer according to [26].

2.2.9. Preparation of membrane skeletal and soluble fractions

Membranes, obtained from 200 μl treated and untreated erythrocytes and recovered as described above, were extracted with 2 volumes of buffer C containing 50 mM Tris–HCl pH 7.5, 1% (final) Triton X-100, 1 mM vanadate and protease inhibitor cocktail for 1 h at 4 °C. After removal of an aliquot for Western blot analysis, the remainder was microfuged at 80,000×*g* for 40 min. Both supernatant (corresponding to the Triton-soluble fraction) and pellet (corresponding to the Triton-insoluble fraction) (membrane skeleton) were then collected, and the pellet was resuspended in the same soluble-fraction volume with buffer C. 10 μg of total membrane and the corresponding soluble and cytoskeleton fractions were then subjected to Western blotting and revealed with the appropriate antibodies (or SDS-PAGE followed by Coomassie blue staining for use as sample loading controls).

2.2.10. Statistical analysis

The data reported are expressed as means±standard error of the mean (S.E.M.). Data processing was carried out using Sigma Plot software (Jandel Scientific). The values of each group were compared by analysis of variance

(ANOVA). *P* values less than 0.05 were considered to indicate significance in all cases.

3. Results

3.1. Oxidative stress in pregnancy

Previous findings report that, during pregnancy, lipid peroxidation products increase in erythrocytes as a signal of enhanced oxidative stress, due to intensified pro-oxidative processes, mainly in the third trimester [4,5,27]. In human erythrocytes, oxidative conditions can trigger membrane protein phosphorylation, mainly involving band 3 protein [6,8,10] through modulation of membrane protein thiol status [7]. It has also been demonstrated that impairment of anti-oxidant cell defences as in pathological conditions (favism) amplifies the phosphotyrosine level of band 3 induced by mild oxidizing treatment of red blood cells, indicating a powerful correlation between these two processes [18].

We examined erythrocytes from 9 pregnant and 12 age-matched non-pregnant healthy women in oxidative conditions by adding to them diamide, a mild oxidant commonly used to trigger band 3 Tyr-phosphorylation in red blood cells [7,8,10].

In unstimulated conditions, no membrane band 3 P-Tyr level was evident in PW erythrocytes (panel A, lane b; panel A′, lane b′) or in normal cells (lanes a; a′), highlighting the fact that enhanced pro-oxidative processes do not involve alteration of the normal P-Tyr status of band 3. Unexpectedly, in the presence of diamide, the extent of band 3 Tyr-phosphorylation was clearly reduced PW versus normal cells (Fig. 1A, compare lanes d and lane c). This was not due to alteration of the phosphorylative sequence, as demonstrated by adding PP2, a Src family kinase inhibitor, to diamide–treated cells (Fig. 1A′). The two-step sequential mechanism also occurred in PW red blood cells since, following PP2-induced Src inhibition, the band 3 Tyr-phosphorylation level declined by about the same proportion in both kinds of cells (Fig. 1A′, lanes c′, d′ compared with panel A, lanes c, d), as expected by inhibition of the Src-dependent band 3 Tyr-phosphorylation of residues 359 and 904. This finding excludes a different mechanism of band 3 Tyr-phosphorylation between the two groups. This was further confirmed by the finding that SHP-2 was recruited to the membranes of both groups (Fig. 1, panels C, C′) but only according to the extent of band 3 P-Tyr (panel C, lanes c, d, compared with respective controls a, b)—as expected, since the recruiting site for phosphatase is band 3 P-

Table 1
Erythrocytes from normal and pregnant women were studied in oxidizing and unstimulated conditions

	Band 3–P-Tyr	% of membrane Syk content	% of membrane SHP-2 content
Normal RBC	n.d.	100	100
PW RBC	n.d.	70.0±10.2	71.5±9.8
Normal diamide	0.80±0.04	170.3±9.5	215.0±12.2
PW diamide	0.45±0.05	130.5±12.3	123.4±9.0
Normal hypert.	0.95±0.05−	99.5±1.2	99.6±0.8
PW hypert.	0.35±0.03−	72.1±3.5	72.3±2.9
Normal-PP2	n.d.	99.5±1.2	99.6±1.5
PW-PP2	n.d.	71.7±3.5	72.0±3.2
Normal diam-PP2	0.50±0.05	167.0±10.2	98.5±2.5
PW diam-PP2	0.27±0.04	123.3±9.5	72.5±5.2
Normal hypert.-PP2	0.57±0.04	99.5±3.5	99.3±2.7
PW hypert.-PP2	0.18±0.05	73.5±2.5	72.8±2.9

Membranes were analysed by Western blotting and immunorevealed by anti-P-Tyr, anti-Syk or anti-SHP-2 antibodies, and panels were counted in a densitometer. For band 3 P-Tyr evaluation, an arbitrary unit was chosen [17]; for enzyme recruitment, amounts of Syk or SHP-2 in normal resting cells were used as 100% value.
Data are expressed as means±S.E.M. (n=5).

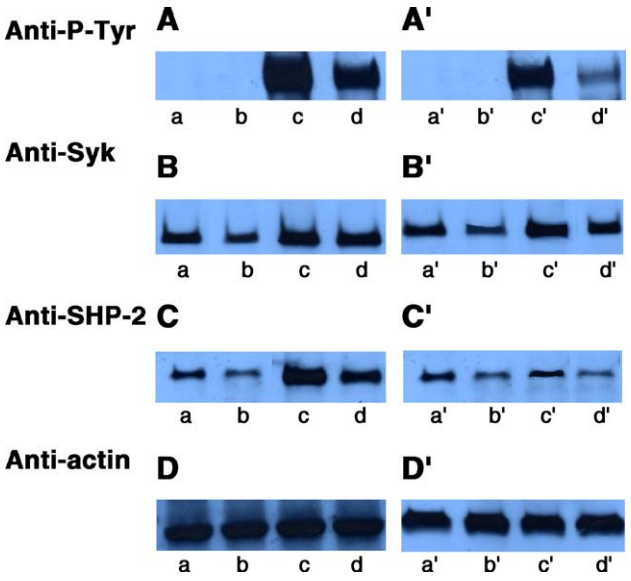


Fig. 1. Oxidative stress in pregnancy: effect on band 3 Tyr-phosphorylation (panels A, A′), Syk (B, B′) and SHP-2 (C, C′) recruitment to membrane. Erythrocytes from normal (lanes a, c, a′, c′) and pregnant women (lanes b, d, b′, d′) were incubated with 0.7 mM diamide (lanes c, d, c′, d′) or without (lanes a, b, a′, b′), in the presence (panels A′, B′, C′, D′) or absence (panels A, B, C, D) of 1 μM PP2. Membranes, recovered as described in Methods, were analysed by Western blotting and immunostained with anti-P-Tyr (panels A, A′), anti-Syk (panels B, B′) anti-SHP-2 (panels C, C′) or anti-actin (panels D, D′) for loading controls. Figure is representative of 9 separate experiments.

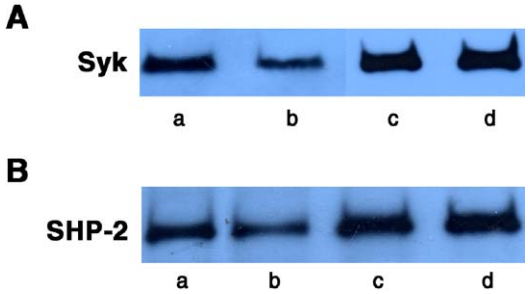


Fig. 2. Determination of Syk (panel A) and SHP-2 (panel B) between membranes (lanes a, b) and cytosol (lanes c, d) in erythrocytes from normal (lanes a, c) and pregnant (lanes b, d) women. Erythrocyte membranes (10 μg) and cytosol (15 μl), obtained as described in Methods, were analysed by Western blotting and revealed with anti-Syk (panel A) or anti-SHP-2 (panel B) antibodies. Figure is representative of 8 separate experiments.

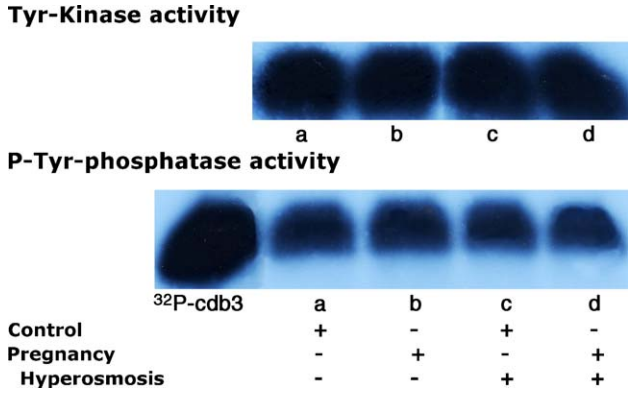


Fig. 3. Tyr-kinase and Tyr–phosphatase activities by total cell lysate. Erythrocytes from normal (lanes a, c) and pregnant women (lanes b, d) were incubated in isotonic (lanes a, b) or hyperosmotic (lanes c, d) conditions. Packed erythrocytes recovered by centrifugation were subjected to several freeze/thaw cycles and 4 μl of total cell lysate were incubated with cdb3 and [^{32}γ-P]ATP for tyrosine kinase or [^{32}P]-cdb3 for tyrosine phosphatase activity, all as described in Methods. Samples analysed by SDS-PAGE and gels, subjected to NaOH treatment, were autoradiographed for 2 days. Figure is representative of 3 separate experiments.

Tyr 359 [10]. Once inhibited by PP2, this site does not accomplish its task, and SHP-2 is no longer recruited (panel C′, lanes c′, d′). However, Syk distribution clearly differed between the two kinds of cells, being higher in controls (panel B, lane a) than in PW (lane b) in unstimulated conditions. We have previously reported that diamide induces Syk recruitment to erythrocyte membranes in a SH2-P-Tyr independent manner, but probably following diamide-induced membrane alteration [17] (lane c compared with lane a). After diamide treatment, Syk content in the membranes of both normal (lane c) and pregnant (lane d) cells increased, and was not affected by the addition of PP2 (panel B′, lanes c′, d′) (Table 1).

In order to determine whether this discrepancy was due to loss of enzyme content during transformation of PW red blood cells, we analysed Syk (Fig. 2, panel A) and SHP-2 (panel B) contents in the two groups, from both membrane

Table 2
Total glutathione and GSSG contents in RBC from controls and pregnant women subjected to various treatments

Effectors	Normal RBC		PW RBC	
	Glu_{tot} (mmol/l)	GS-SG	Glu_{tot} (mmol/l)	GS-SG
Basal	2.04±0.06	0.25±0.02	2.00±0.06	0.25±0.02
0.7 mM diamide	1.33±0.04	0.33±0.02	1.35±0.06	0.29±0.03
Hyperosmosis	2.00±0.06	0.24±0.04	2.01±0.04	0.28±0.02

Erythrocytes, incubated as indicated, were hemolysed, cytosol was recovered and total glutathione (GSSG+GSH) mM and oxidized glutathione (GSSG) mM determined, all as described in Methods.
Means±S.E.M. of six experiments.
n.d.: not detected.

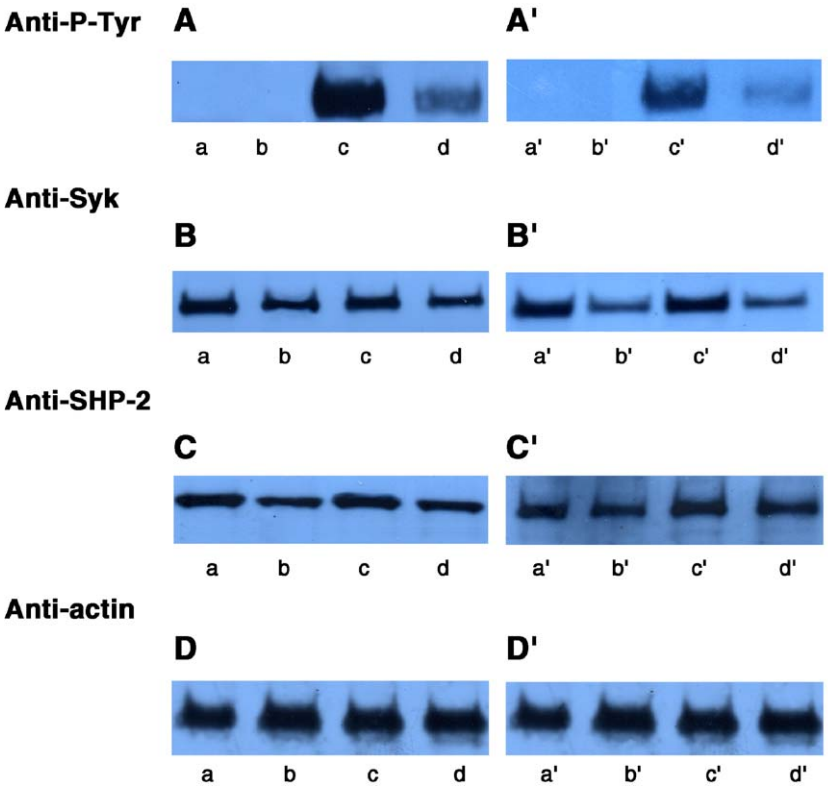


Fig. 4. Osmotic stress in pregnancy: effect on band 3 Tyr-phosphorylation (panels A, A′), Syk (B, B′) and SHP-2 (C, C′) recruitment to membrane. Erythrocytes from normal (lanes a, c, a′, c′) and pregnant women (lanes b, d, b′, d′) were incubated in hypertonic (1.1 Osm) (lanes c, d, c′, d′) or isotonic (lanes a, b, a′, b′) buffer, in presence (panels A′, B′, C′, D′) or absence (panels A, B, C, D) of 1 μM PP2. Membranes, recovered as described in Methods, were analysed by Western blotting and immunostained with anti-P-Tyr (panels A, A′), anti-Syk (panels B, B′), anti-SHP-2 (panels C, C′) or anti-actin (panels D, D′) for loading controls. Figure is representative of 9 separate experiments.

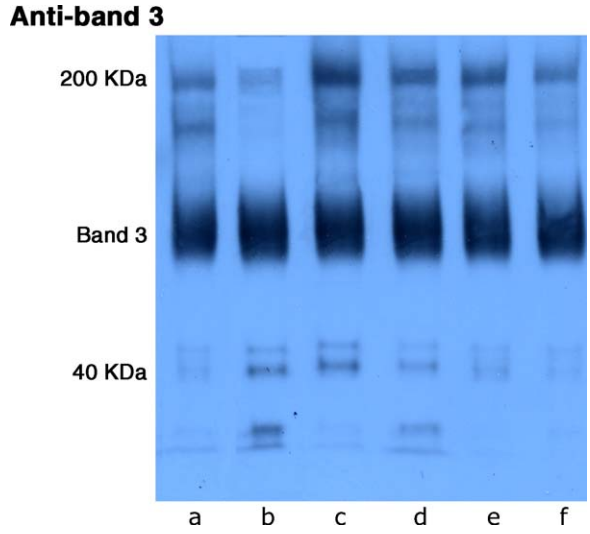


Fig. 5. Electrophoretic pattern of band 3 in normal and PW red blood cells. Erythrocytes from normal (lanes a, c, e) and pregnant (lanes, b, d, f) women were treated with 0.7 mM diamide (lanes c, d), 1.1 Osm buffer (lanes e, f), or untreated (lanes a, b). Membranes (10 μg), recovered as described in Methods, were analysed by SDS/8% PAGE in non-reducing conditions and immunorevealed with anti-band 3 antibodies. Figure is representative of at least 8 separate experiments.

and cytosol. Western blotting analysis with anti-Lyn antibodies revealed no difference in Lyn concentration between the two groups (Fig. 6F).

As indicated in Fig. 2, no difference in total enzyme content or activity (Fig. 3) but altered enzyme distribution was involved for both enzymes, with lower membrane retention for both Syk and SHP-2 in PW erythrocyte membranes (Table 1).

We also analysed both kinds of cells for their GSH–GSSG content and found no difference between them, even after diamide treatment (Table 2). Comparison of Met(Hb) content between two the groups evidenced no particular alteration even after diamide treatment, but ranged from 0.4±0.2 for normal erythrocytes to 0.6±0.2 (% of total hemoglobin) for cells from PW in both unstimulated and stimulated conditions.

Data on the varying degrees of diamide-induced band 3 P-Tyr and enzyme contents in PW membranes in both unstimulated and stimulated conditions, together with the lack of any increase in oxidizing mechanism or impairment in pro-oxidative/anti-oxidative processes, suggest that the membrane structure of red blood cells is altered.

3.2. Osmotic stress in pregnancy

Taken all together, our data suggest that certain properties in the RBC membranes are modified during pregnancy. To assess this hypothesis, we subjected erythrocytes to hyperosmosis, a condition which has been demonstrated to induce band 3 Tyr-phosphorylation through a morphological change to a flattened, slightly crenated shape [18,28]. The pattern of the Tyr-phosphorylation of membrane band 3 (Fig. 4) shows that this discrepancy, found in mild oxidative stress conditions, is even higher in hyperosmotic conditions in pregnancy.

The normal response to hyperosmosis is shown in Fig. 4 (panel A lane c), with substantial band 3 Tyr-phosphorylation. It follows the same sequential Syk/Lyn-catalysed phosphorylation, well characterized for the erythrocyte response to oxidative stress [8,10], as indicated by the reduction of about 50% in band 3 P-Tyr following PP2 addition (Fig. 4, panel A′, lane c′ vs. lane c). Once again, the condition of pregnancy revealed the lower membrane Tyr-phosphorylation of hypertonic-treated red blood cells (Fig. 4A, lane d; panel A′, lane d′ vs. control lanes c and c′), although the same ratio between Syk/Src dependent band 3 Tyr-phosphorylation was respected, as indicated by a definite PP2-induced decrease.

However, in this case, both Syk (panels B, B′) and SHP-2 (panels C, C′) were not recruited to membranes, indicating that membrane/morphology alterations induced by hyperosmosis occurred without involving kinase or phosphatase location, but probably reorganization of membrane protein, thus allowing Tyr-phosphorylation to take place.

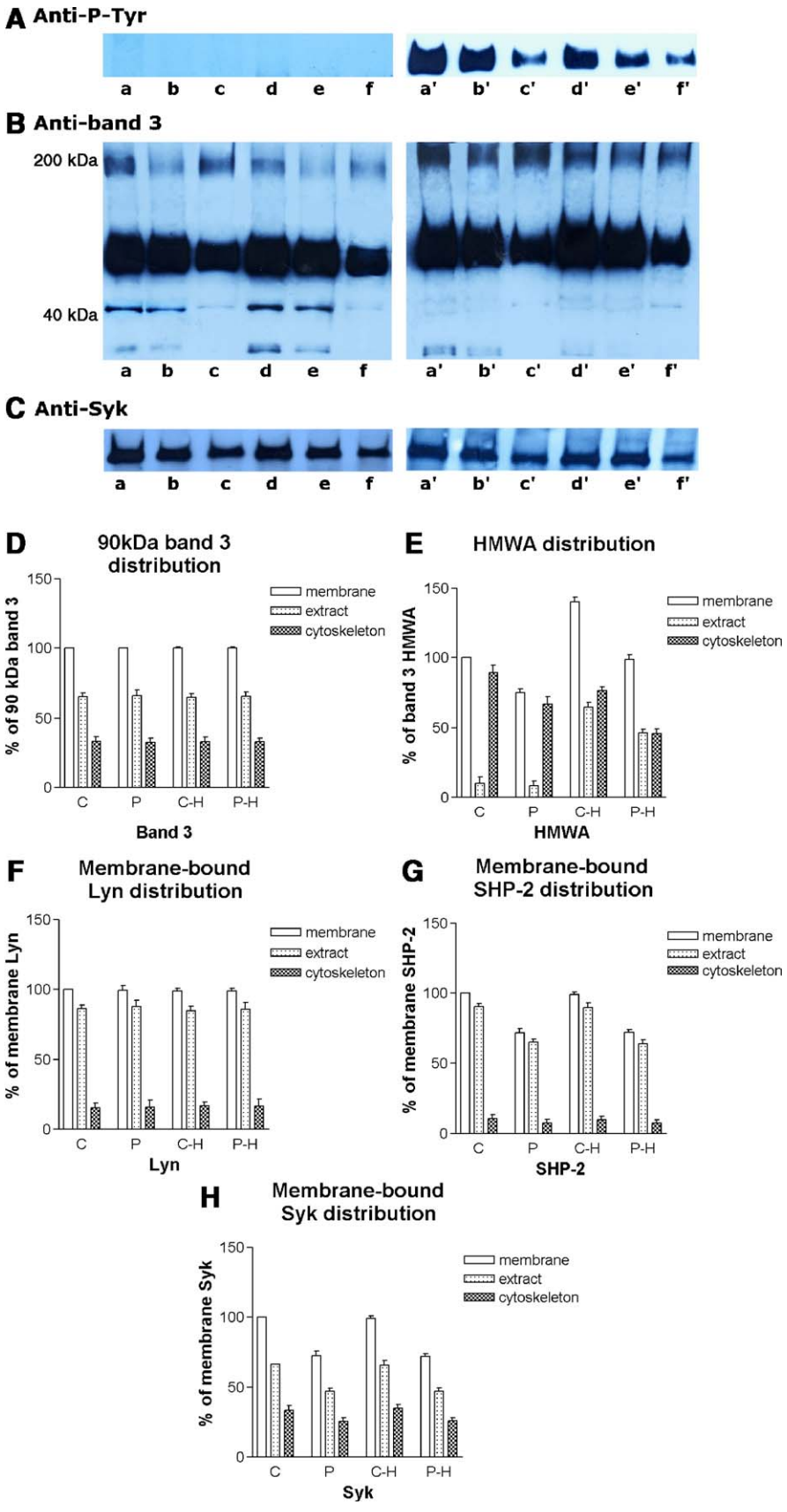
3.3. Electrophoretic pattern of membrane band 3

Previous studies has identified band 3 as a possible marker of pregnancy, since this protein is involved in the erythrocyte membrane modification occurring in the last trimester, showing a reduction in high molecular weight band 3 aggregates and an increase in low molecular weight fragments [19].

We analysed membranes from both kinds of cells (normal and from pregnant women) in normal, oxidative and hyperosmotic conditions, with anti-band 3 antibodies, and evaluated bands by densitometry (1D Analysis software, Kodak).

As previously described, normal and pregnancy–derived erythrocytes differed in band 3 aggregate and proteolytic fragment distribution: in normal red cells, only a slight proteolytic pattern was evident, involving four distinct bands ranging from 20 kDa to 40–45 kDa, and a band at 200 kDa corresponding to high molecular weight aggregates (HMWA). Different patterns were revealed in PW erythrocytes which were reduced by about 30% of their content of band 3 aggregates (compared with normal cells) (Fig. 5) and showed the considerable presence of band 3 proteolytic fragments (lane b compared with lane a).

When subjected to diamide treatment, membranes from both kinds of cells increased their contents of high molecular weight bands but, in PW cells, these aggregates maintained their reduction of about 30% compared with normal cells, with a fall in proteolytic fragments instead of an increase, as found in controls (Fig. 5, lane d compared with lane c, and Fig. 6). This was even more evident when membranes from hypertonic-treated PW cells were analysed (lane f). The bands at 20 kDa had almost completely disappeared, and those at 40 kDa were reduced to 15% of the amount shown in their untreated counterpart (lane b). However, this rearrangement of the electrophoretic pattern of membrane band 3 was characteristic of PW erythrocytes, since normal cells did not undergo such changes, but only a modulation in aggregate composition, with a small difference in proteolytic fragment formation (compare lanes c and e with control, lane a).


A Anti-P-Tyr
a b c d e f a' b' c' d' e' f'
B Anti-band 3
200 kDa
40 kDa
a b c d e f a' b' c' d' e' f'
C Anti-Syk
a b c d e f a' b' c' d' e' f'
D
90kDa band 3 distribution
% of 90 kDa band 3
membrane
extract
cytoskeleton
C P C-H P-H
Band 3
E
HMWA distribution
% of band 3 HMWA
membrane
extract
cytoskeleton
C P C-H P-H
HMWA
F
Membrane-bound Lyn distribution
% of membrane Lyn
membrane
extract
cytoskeleton
C P C-H P-H
Lyn
G
Membrane-bound SHP-2 distribution
% of membrane SHP-2
membrane
extract
cytoskeleton
C P C-H P-H
SHP-2
H
Membrane-bound Syk distribution
% of membrane Syk
membrane
extract
cytoskeleton
C P C-H P-H
Syk

3.4. Characterization of triton-soluble composition of normal versus PW membranes

These findings raised the hypothesis that the different patterns of band 3 protein represented different protein status in membrane organization. To assess this hypothesis, we extracted RBC membranes from both normal and pregnant women with 1% Triton X-100 (see Methods) and centrifuged them to separate membrane skeletal fractions (Fig. 6, lanes c, f, c′, f′) from detergent-soluble fractions (lanes b, e, b′, e′). When immunorevealed with anti-P-Tyr antibodies (panel A), membranes from hypertonic-treated erythrocytes (lanes a′–f′) showed that P-Tyr residues of band 3 were distributed between the soluble (lanes b′, e′) and cytoskeletal (lanes c′, f′) fractions in percentages of 67±3 vs. 33±3%, respectively. When analysed by anti-band 3 antibody (panel B), the same proportion was also evidenced for the band 3 distribution between the two compartments (Fig. 6D, [17]), thus indicating that Tyr-phosphorylation involves uniformly the 90-kDa bands of cell band 3, without distinction. Differences between normal and PW were limited only to the extent of band 3 P-Tyr (lanes a′–c′ and d′–f′) as previously discussed (Fig. 4), not to a different extraction property. When analysed with anti-Syk (Fig. 6C, D), distribution of the enzyme followed the 90 kDa band 3 extraction proportion, the cytoskeleton retaining one third of the total membrane enzyme amount — proportion which did not change in the two kinds of cells in either untreated or treated conditions. Further analysis with anti-SHP-2 and anti-Lyn antibodies (Fig. 6′) confirmed the above results, except that the cytoskeleton retained only about 10% and 15% of membrane phosphatase and kinase, respectively.

The HMWA band (Fig. 6B, E) clearly shows that hyperosmosis affected band 3 profiles mainly in aggregate distribution, since membranes from normal cells (lanes a′–c′) showed that some band 3 aggregates at 200 kDa had shifted to the soluble fraction of the membrane (lane b′), thus increasing soluble band 3 aggregate up to 40–45%. In membranes of PW cells, this was even more evident, since aggregates were equally distributed between the soluble and insoluble fractions (lanes e′, f′).

Diamide did not induce any alterations in the proportion of band 3 HMWA extraction (data not shown).

4. Discussion

Since the first report, as early as in 1857 [29], that pregnancy is associated with specific hematological changes, many researchers have focused attention on the various parameters involved. One of the most important changes in pregnancy occurs in the maternal hematopoietic system, which has to meet the demands of both mother and developing fetus. Thus, hypervolemia protects the mother from hypotension, sequestration of blood in the lower extremities, and hemorrhage at the time of delivery [2,3].

Red blood cells have to undertake additional work in order to meet not only increasing demands for oxygen transport but also to respond to an increase in inflammatory conditions, probably due to activation of neutrophils [30], with the consequent production and release of reactive oxygen species. Pro-oxidative processes are intensified in pregnant women [27] and lipid peroxide levels, due to membrane peroxidation, increase mainly during the last trimester [31]. In human erythrocytes, oxidative stress is a triggering motif for band 3 Tyr-phosphorylation [7,8,10]. We have previously demonstrated that, in Glucose-6-Phosphate Dehydrogenase deficiency (G6PD d), which induces chronic lowering of anti-oxidant defences, the erythrocyte response to diamide, a mild oxidant, is higher than that in normal cells, as evidenced by the increased band 3 phospho-tyrosine level reached in patient's cells as an expression of the patho-physiological disorder [18].

However, when subjected to diamide, PW red blood cells show a clear reduction in band 3 Tyr-phosphorylation compared with controls. There are no alterations in the total amounts of kinase or phosphatase and no reduction of activity, thus invalidating the hypothesis that direct alterations in Tyr-kinase or phosphatase contents occur during pregnancy. Quantification of lactate showed no major metabolic differences between either kind of cells, in both untreated and treated conditions (not shown). This means that alterations depending on control by glycolysis products, such as ATP or glycolysis intermediates, are to be excluded, and point to a separate underlying adjustment. Previous data show that, in pregnancy, the observed increase in membrane peroxidation products is associated to a net increase in anti-oxidative protection, thus leading to a positive response by maternal erythrocytes to increasing oxidative stress. This may be expressed as an index of oxidative stress, as the ratio of pro-oxidative processes (lipid peroxidation) and antioxidant protection (sum of superoxide dismutase, catalase and glutathione peroxidase activities), which indicates the sufficient antioxidative protection in relation to physiological status [31]. Our findings confirm that PW erythrocytes are less sensitive to oxidative stress, as shown by the diamide-induced lower level of band 3 Tyr-phosphorylation. They also exclude any possibilities of imbalance between pro-oxidative/antioxidizing processes due to insufficient antioxidant protection.

The reduced ability of the membrane to retain kinase and phosphatase in resting cells is related to membrane structure

Fig. 6. Distribution and electrophoretic patterns of band 3 in membrane from normal and PW red blood cells in hypertonic conditions. Erythrocytes from normal (lanes a, b, c, a′, b′, c′) and pregnant (lanes d, e, f, d′, e′, f′) women were treated in hyperosmotic conditions (lanes a′–f′), or untreated (lanes a–f). Membranes (10 μg) were extracted with Triton X-100. Membranes (lanes a, d, a′, d′) and extracted (soluble) (lanes b, e, b′, e′) or cytoskeletal (insoluble) (lanes c, f, c′, f′) fractions, collected by ultracentrifugation (see Methods), were analysed by SDS/8% PAGE in non-reducing conditions and immunorevealed with anti-P-Tyr (panel A) anti-band 3 (panel B) or anti-Syk (panel C) antibodies. Figure is representative of 5 separate experiments. Densitometric analysis. Bands corresponding to HMWA, 90 kDa band 3, Syk, SHP-2 and Lyn were counted in a densitometer. Amounts of HMWA, 90 kDa band 3, Syk, SHP-2 and Lyn in unstimulated normal erythrocytes were chosen as 100% value. $P<0.03$.

reorganization, as further confirmed by differences in band 3 Tyr-phosphorylation in response to hyperosmotic stress. Hypertonicity and its ability to induce band 3 Tyr-phosphorylation [28] is another parameter of membrane status. It has been demonstrated in favism that impairment of cellular defences is enhanced in hypertonic conditions, which induce a higher band 3 level of phospho-tyrosine [18]. This is not the case in pregnancy, because erythrocytes show a net decrease in band 3 Tyr-phosphorylation in hypertonic conditions. This implicates a pre-existing alteration in membrane structure, probably provoked by the increasing lipid peroxidation characterizing pregnancy.

Previous findings have shown changes in band 3 electrophoretic profiles, increasing throughout the first, second and third trimesters, with an increase in band 3 proteolytic fragments and a decrease in band 3 HMWA [19]. We find that this anomaly is also evident when the profile patterns of band 3 are analysed on membranes from diamide- or hypertonic-treated erythrocytes: in normal cells, band 3 undergoes a clear defined rearrangement, but in PW cells the changes are slighter. Belo et al. [19] hypothesized that the fewer HMWA in cells in pregnancy reveal a younger erythrocyte population, probably induced by erythropoietin [32]. Recent studies show that, when divided into differing age populations in hypertonic conditions the oldest RBC display higher Tyr-phosphorylation levels of band 3 than the youngest ones [33].

However, the values of band 3 HMWA should not be chosen as an index of erythrocyte aging, because: (i) modifications such as diamide-induced disulfide bond formation are well known to cause the formation of band 3 aggregates [34] in the same cellular population without changing aged or younger fractions; (ii) hyperosmosis, which is used only as a mechanical effector of shape change, can also induce an increase in HMWA. Taking these points together, the presence of HMWA correlates better with membrane alteration. This is confirmed by our finding of Triton X-100 solubilization of membranes. The band 3 protein of the erythrocyte membrane subdivides differently into a soluble (lipid bilayer)/insoluble (cytoskeletal) fraction, about 70% being in the lipid bilayer and 30% anchored to the cytoskeletal counterpart of the whole membrane (Fig. 6B, 6′) [17,35]. Hyperosmosis can alter band 3 interaction/binding properties, as indicated by the different solubility of band 3 HMWA in Triton X-100 (Fig. 5B). In normal and PW cells in resting conditions, band 3 HMWA present in the Triton-soluble (lipid bilayer)/-insoluble (cytoskeletal) fraction is in a 10:90 ratio (Fig. 6B, E). When erythrocytes are treated with hypertonic medium, the ratios of band 3 HMWA in the membrane soluble/cytoskeletal insoluble fraction change to 40:60 and 50:50, respectively in normal and PW cells. This is indicative of inner membrane alterations directly involving protein–protein interactions of band 3 and other membrane proteins making up the cytoskeletal fraction, which become more marked in pregnancy. In this line of evidence, hypertonicity may induce Tyr-phosphorylation of band 3 by rearranging the steric structure of the protein (substrate), thus facilitating the action of kinases more than the counteracting phosphatase dephosphorylation and without inducing activation or relocation of kinases, whose activities remain unchanged (Fig. 3).

In conclusion, we believe that pregnancy alters the normal membrane arrangement of circulating erythrocytes. This is highlighted by membrane band 3 Tyr-phosphorylation processes, changes in which are typical of those occurring in red cells throughout the three trimesters, involving band 3 rearrangement both within the lipid bilayer and in other membrane protein/lipid components.

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