Regulation of human erythrocyte glyceraldehyde-3-phosphate dehydrogenase by ferriprotoporphyrin IX

Fausta Omodeo Salè[a,*], Elisa Vanzulli[a], Simone Caielli[a], Donatella Taramelli[b]

[a] Institute of General Physiology and Biochemistry "G. Esposito", Facoltá di Farmacia, University of Milan, Milan, Italy
[b] Institute of Microbiology, University of Milan, Milan, Italy

Abstract **Erythrocyte glyceraldehyde-3-phosphate dehydrogenase (G3PD) is a glycolytic enzyme containing critical thiol groups and whose activity is reversibly inhibited by binding to the cell membrane. Here, we demonstrate that the insertion of ferriprotoporphyrin IX (FP) into the red cell membranes exerts two opposite effects on membrane bound G3PD. First, the enzyme is partially inactivated through oxidation of critical thiols. Dithiothreitol restores part of the activity, but some critical thiols are irreversibly oxidized or crosslinked to products of FP-induced lipid peroxidation. Second, G3PD binding to the membrane is modified and the enzyme is activated through displacement into the cytosol and/or release from its binding site.**
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Keywords: Glyceraldehyde-3-phosphate dehydrogenase; Heme; Erythrocyte; Ferriprotoporphyrin IX

1. Introduction

Heme (ferric protoporphyrin IX, FP) accumulates inside red blood cells (RBCs) in hemoglobinopathies [1] or patients with G6PDH deficiency [2]. Heme is also found in the plasma of patients with hemolytic diseases [3] as well in malaria, although the actual concentration and disposition have not been well quantified, yet. We and others have previously shown that FP rapidly binds to lipid bilayers of artificial liposomes as well as macrophages or RBCs inducing a time- and dose-dependent membrane lipid peroxidation [4–8]. In RBCs, membrane and cytoskeleton proteins are particularly susceptible to oxidative damage which is thought to reduce whole cell deformability [9,10] and cause microrheologic abnormalities similar to those observed in sickle RBCs [11]. It has been previously reported that intercalation of the porphyrin into the membrane is followed by the oxidation of protein's thiols and lysis of RBCs [8,12]. The latter has been attributed to a colloid-osmotic mechanism and/or peroxidative crosslinking of cytoskeleton proteins [12,13].

On the basis of these results, we reasoned that membrane associated enzymes, in particular those containing critical thiols, might be affected by FP. The aim of this study was to elucidate the influence of FP insertion into the membrane on glyceraldehyde-3-phosphate dehydrogenase (G3PD). G3PD is a glycolytic enzyme containing critical thiols, which plays a key role in RBC metabolism and whose activity is modulated by its dynamic association with the cell membrane [14,15].

2. Materials and methods

2.1. Materials

Human A-positive blood from healthy donors was used within 10 days from withdrawal with CPD (citrate/phosphate/dextrose) as anticoagulant.

All biochemicals were purchased from Sigma (Sigma Italia, Milan, Italy), anti-G3PD monoclonal antibody from Chemicon; nitrocellulose membrane and the enhanced chemiluminescence developing system (ECL) from Amersham Bioscience.

FP stock solutions were prepared daily. A weighed amount of FP was resuspended in 0.02 N NaOH and then diluted to the appropriate concentration in isotonic phosphate buffered saline (PBS). The heme equivalents were quantified by dissolving an aliquot in 1 N NaOH and reading the absorbance at 385 nm (ϵ_{385} hematin = 6.1×10^4 M cm^{-1}).

2.2. Preparation of RBC, free cell extracts and RBC membranes

Aliquots of blood were centrifuged at $1850 \times g$ at 4 °C for 5 min, the buffy coat removed and the erythrocyte pellet washed three times with 10 vol of cold (4 °C) PBS. Cells were gently resuspended with PBS-5 mM glucose and used immediately.

RBCs at 10% hematocrit (Htc) in PBS-glucose were treated with different doses of FP from stock solutions (10–20 μl). Incubation was performed in plastic vessels for different times at 37 °C under shaking. In some experiments, the RBC suspension was pre-incubated with vitamin E (50 μM) and then washed before the addition of FP. RBCs were then pelleted by centrifugation at $1850 \times g$ for 10 min, washed twice with cold PBS to remove the unbound porphyrin and lysed with a proper volume of water just before the assay of G3PD activity. RBC cytosol was prepared by hypotonic shock in 5 mM NaHPO$_4$ buffer pH 8.0 (5P8 buffer) or 3 freeze–thawing cycles in PBS. When the effects of FP on G3PD activity were studied in cell free extracts, RBC were lysed and diluted to 10% Htc with 5P8 buffer and the suspension incubated at 37 °C with FP for 30 min.

In some experiments, membranes were prepared from control or FP exposed RBCs by hypotonic lysis and extensive washing in 20 vol of 5P8 buffer containing 0.1 mM EDTA and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). White ghosts were stored at −80 °C in a small volume of the same buffer containing a protease inhibitor cocktail (Sigma) (5 μl/200 μg proteins).

2.3. G3PD assay

G3PD (E.C. 1.2.1.12) activity was measured according to Beutler with minor modifications [16]; since G3PD is inactive in its membrane-bound state [15,17], the determination of the membrane activity was performed in the presence of 0.02% Triton X-100. Enzyme activity

*Corresponding author.
E-mail address: fausta.omodeosale@unimi.it (F. Omodeo Salè),

Abbreviations: G3PD, glyceraldehyde-3-phosphate dehydrogenase; FP, ferriprotoporphyrin IX; RBC, red blood cells

was referred to the hemoglobin content calculated spectrophotometrically from the absorbance at 412 nm (A_{412}) for intact RBC and RBC cytosol or to the phospholipid phosphorus, determined according to Bartlett [18], for RBC ghosts.

2.4. Gel electrophoresis and Western blot analysis

About 20 µg of membrane proteins were solubilized in 2× loading buffer, incubated for 15 min at 60 °C and separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [19]. Proteins were stained with Coomassie blue R-250 and quantified by densitometric analysis (CAMAG VideoScan) or transferred to nitrocellulose membrane at 100 mA, overnight. Blots were blocked with 10% non-fat dry milk in Tris buffered saline-1% Tween-20 (TBS-T) and incubated for 1 h at room temperature with monoclonal anti-G3PD antibody (1:2000 in 5% no-fat milk in TBS-T). Blots were washed in TBS-T, followed by incubation at room temperature for 1 h with anti-mouse IgG (1:10000 in TBS-T) and finally detected with ECL.

3. Results and discussion

The treatment of RBC with FP results in a time and dose-dependent insertion of the porphyrin into the membrane, followed by oxidation of protein sulfhydryl groups and hemolysis [8]. On this basis, we investigated the effect of FP on the activity of G3PD, a glycolytic enzyme containing critical thiol groups [20]. It is known that G3PD is a tetrameric protein bound through electrostatic interactions to the N-terminal cytoplasmic tail of the anion-transport protein band 3 [14]. The binding site for band 3 is located in the catalytic domain of G3PD and therefore the enzyme is inactive in its bound state [15]. The association is reversed by elevation of the ionic strength [21], free oxygen radicals [22] or S-nitrosylation by NO donors [23]. In a first set of experiments, a pure G3PD solution was incubated with FP for 30 min: a significant and dose-dependent inhibition of activity was observed (Fig. 1A, dotted line). A similar sensitivity to inactivation by FP was seen with G3PD associated to RBC ghosts. FP almost completely abolished the enzyme activity at doses above 40 µM (Fig. 1A) and it was active at very low concentrations (1–5 µM), as well. This suggests that the sulfhydryl group at the active site of G3PD is sensitive to oxidation by FP and that it is only partially protected by the association of the enzyme to the membrane. A similar pattern of enzyme inactivation is observed with H_2O_2.

To investigate the changes of G3PD activity in whole erythrocytes, RBC were incubated with doses of FP ranging between 10 and 80 µM, at 37 °C, for 30–180 min. At the end of treatment, lysed cells were discarded and the enzymatic activity measured in the intact cells recovered by centrifugation,
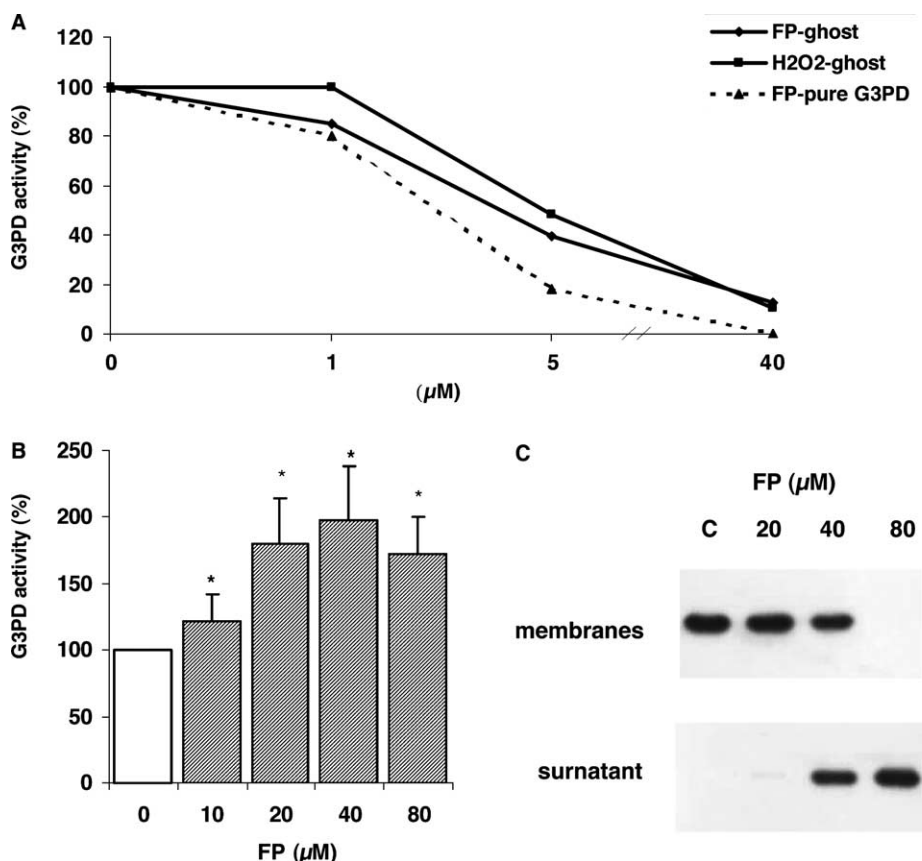

Fig. 1. (A) Effect of different concentrations of FP (30 min, 37 °C) on the activity of pure G3PD and G3PD of RBC ghosts. H_2O_2 effect on G3PD activity of RBC ghosts is also shown. With the exception of samples exposed to 1 µM H_2O_2 all samples treated with FP or H_2O_2 were significantly different from controls ($P < 0.001$). (B) Increased G3PD activity of whole RBC as a function of FP concentrations. RBC at 10% Htc in PBS 5 mM glucose were exposed to FP for 30 min at 37 °C under gentle shaking. The enzyme activity was measured on the erythrocytes recovered intact after the incubation, washed with PBS and lysed immediately before the enzymatic assay. *$P < 0.01$ vs. control. (C) Release of G3PD from RBC ghosts. Ghosts were exposed to different concentrations of FP (30 min, 37 °C). After centrifugation proteins recovered in the soluble fraction and the pellet were subjected to SDS–PAGE and immunoblotting with anti-G3PD antibody.

washed with PBS and lysed just before the assay. As shown in Fig. 1B, a 2-fold increase of total G3PD activity compared to controls was observed, with a plateau at 40 μM FP after 30 min of incubation. No further increase was seen after a longer incubation time (data not shown). This finding led us to hypothesize that G3PD could be activated by displacement from the membrane following the treatment with FP. The FP capability of displacing G3PD was confirmed by incubating control ghosts with increasing amount of FP. After 30 min treatment at 37 °C, the membranes and the surnatants were recovered by centrifugation and subjected to SDS–PAGE, followed by Western blotting with anti-G3PD antibody. The results, shown in Fig. 1C, demonstrate a dose-dependent release of G3PD from the membrane and a parallel recovery of the protein in the supernatant.

To verify the displacement of the enzyme in intact cells, as well, we measured the G3PD activity on the cytosol of FP-treated RBC. Intact RBC recovered after incubation with 40 μM FP, were lysed by three cycles of freezing/thawing or by 2 min mechanical homogenization in a glass potter and the cytosol was separated from the membranes by centrifugation for 30 min at $20000 \times g$. G3PD activity in the cytosol was significantly increased (140–145% compared to controls), although to a lesser extent than in the whole cell (data not shown and Fig. 1B). Taking into account that in the presence of FP, RBC become spherocytic [12], this result is in agreement with the recent observation of Campanella et al. [24] showing by specific antibody and confocal microscopy that G3PD is partly displaced into the cytosol in spherocytic or otherwise abnormal (senescent, dehydrated) cells. Interestingly, no increase of G3PD activity was found when the cytosol was prepared by hypotonic lysis of RBC, compared to mechanical or thermic shock (data not shown). This latter finding suggests that the FP-induced displacement of G3PD into the cytosol is a transient event, requiring a ionic strength of physiological value (0.15 M) and that G3PD can re-associate to the membrane when the cells are lysed in hypotonic buffer.

The lower increase of activity in the cytosol compared to the whole cell, suggests that the displacement of the protein into the cytosol cannot completely account for the FP-induced activation of G3PD and that most of the enzyme might be activated in the membrane-bound state. In fact, FP insertion into the membrane might interfere with the interaction between band 3 and the bound subunit of G3PD, leading to a greater accessibility of the substrate to the catalytic site and/or relieving the allosteric inhibition on the other three G3PD subunits [15]. This hypothesis is supported by the finding of Muronetz et al. [25] showing that G3PD is capable of functioning in a membrane-bound state, as well. Phosphofructokinase too, whose activity is modulated by the association to band 3, can be activated without changes in the amount of enzyme bound to the membrane [26,27].

In subsequent experiments, control RBC were lysed and the total RBC lysate or the cytosol alone (total lysate minus the membrane fraction) were exposed to FP (Fig. 2). In agreement with the results reported above, no increase of G3PD activity, but rather a 30% inhibition, was observed after treatment of the RBC cytosol with FP, due to a partial inactivation of the enzyme. The effect observed after treatment of the total lysate with FP was in between that observed in the whole cell and in the cytosol alone, being the resultant of two opposing effects:
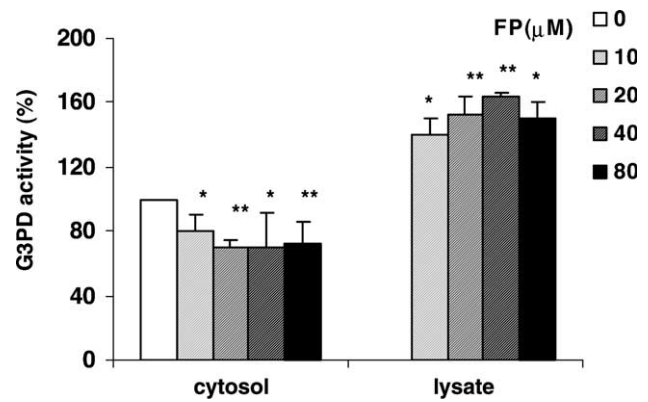

Fig. 2. G3PD activity (expressed as percentage of the control) of the cytosol and total RBC lysate after treatment with different doses of FP. $^*P < 0.05$, $^{**}P < 0.01$ vs. control.

enzyme activation, due to the modified G3PD binding or release from the membrane, and enzyme partial inactivation due to direct exposure to FP.

The association of G3PD to the membrane was investigated in membranes isolated from RBCs incubated with FP (40 μM, 60 min 37 °C). The analysis was performed on membranes of RBC recovered intact after FP treatment as well as membranes of FP-lysed RBC. Membrane of FP-intact RBCs were isolated after freezing–thawing of the cells, the proteins were separated by SDS–PAGE and the electrophoretic pattern subjected to quantitative densitometric analysis using actin as "reference standard" (Fig. 3A and C). G3PD corresponds to band 6, a 37 kDa protein, as confirmed by the immunoblotting analysis using anti-G3PD antibodies (Fig. 3B). Densitometric analyses demonstrate that G3PD decreases only 10% in the membranes of erythrocytes recovered intact after treatment with increasing amounts of FP (Fig. 3C). Similar results were obtained after longer times of exposure (data not shown) confirming that after FP treatment most of G3PD is still bound to the membrane of intact cells. However, considering that in resting RBC the bulk of G3PD (75–80%) is associated with the membrane [14], even this small decrease could account for the 40–45% increase of activity shown in the cytosol.

Differently from intact RBC, G3PD is almost completely lost from the membrane of RBC that undergo lysis during exposure to FP (Fig. 3A and C). Such phenomenon is not observed when RBC are lysed by hypotonic buffer (Fig. 3A, control). In addition, in FP-lysed erythrocytes, we observed a significant decreased of α and β chain of spectrin (240 and 210 kDa proteins) (7% of the total proteins vs. 32% of control) (Fig. 3A). Spectrin is a major structural component of the RBC cytoskeleton, sensitive to sulfhydryl reagent and specific target for oxidative damage of RBC [28–30]. Therefore, based on the present and previous findings [8], disappearance of G3PD is strictly dependent on the FP-induced destabilization of the cytoskeletal network.

In spite of the similar amount of membrane-bound enzyme, ghosts prepared from RBC pretreated for 60 min with increasing concentration of FP showed a lower G3PD activity (Fig. 4, white bars). Incubation of the membranes for 30 min with 1 mM dithiothreitol (DTT) reversed the inhibition, but was unable to completely restore the enzymatic activity (Fig. 4,
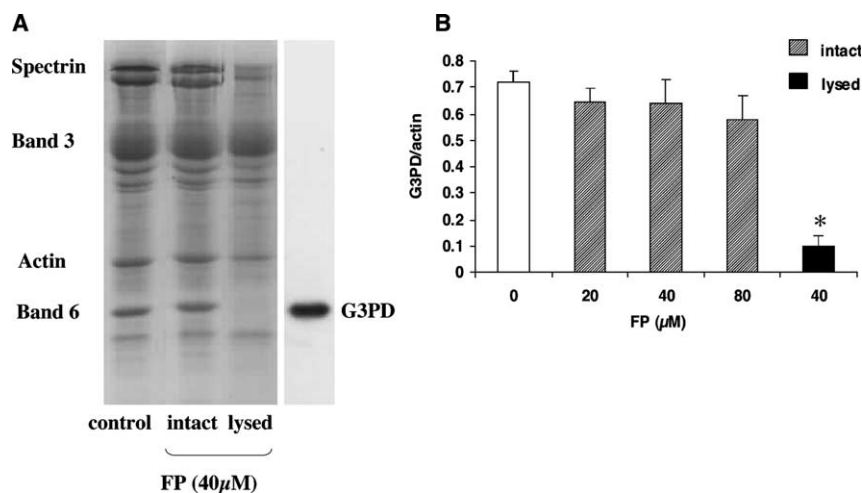
Fig. 3. (A) 10% SDS–PAGE visualized by Coomassie blue staining of membrane proteins prepared from control and FP-treated RBC (60 min, 37 °C). RBC recovered intact or lysed after FP exposure were analysed separately. (B) Identification of band 6 as G3PD using anti-G3PD antibody. (C) Densitometric quantification of Coomassie blue stained gels of membranes prepared from RBC control or exposed to different concentrations of FP (60 min, 37 °C). $^*P < 0.001$ vs. control.
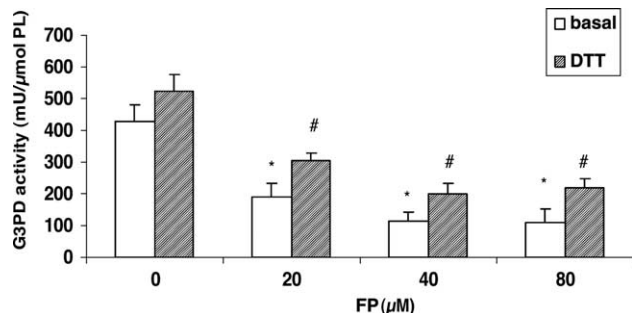

Fig. 4. Decreased G3PD activity of ghosts prepared from RBC treated with different amount of FP and partial recovery of the activity after 30 min treatment of the membranes with 1 mM DTT. $^*P < 0.005$ vs. control, $^\#P < 0.05$ vs. the correspondent sample without DTT.

grey bars). The same result was obtained when the membranes were incubated for 30 min with 2 mM GSH (data not shown).

On the contrary, the membrane-bound activity was completely preserved in RBC pre-incubated for 30 min with 50 μM α-tocopherol, a known scavenger of thiyl radicals and of 4-hydroxy-2-nonenal (HNE) production [31,32] prior to the exposure to FP (data not shown). These findings indicate that FP-induced inactivation of G3PD is only partly due to the reversible oxidation of the thiol group at the catalytic site of the enzyme. It is likely that in the presence of FP, thiyl radicals and HNE, originating from the lipid peroxidation process, are generated [31,33,34] and irreversibly inactivate G3PD by either oxidation of the thiol at the catalytic site to sulfonic acid (RSO_3H) or by alkylation.

In conclusion, these results demonstrate that insertion of FP into the membrane, besides the effects exerted on the structural properties of the erythrocyte, may have important functional implications with regard to the energy metabolism of the cell. FP in fact, exerts two opposite effects on the membrane bound G3PD. On the one hand, it causes a partial inactivation of the enzyme through reversible or irreversible oxidation of critical thiols or crosslinking to products of FP-induced lipid peroxidation. On the other hand, it modifies the binding properties of the enzyme to the cytoplasmic tail of band 3, thereby activating G3PD through displacement into the cytosol and/or release from its original binding site. Since most of the enzyme is concentrated at the membrane surface in a inactive state [14,15], the FP-induced modulation of the binding affinity of even a small number of molecules might play a significant influence in the erythrocyte metabolic control. Further experiments are in progress to investigate whether other membrane-bound glycolytic enzymes [27] are similarly affected by the porphyrin and to understand the mechanisms underlying such effect.

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