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ORIGINAL ARTICLES

Oxidative Effects of Gemfibrozil on Anion Influx and Metabolism in Normal and Beta-Thalassemic Erythrocytes: Physiological Implications

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Abstract To further clarify some peculiar molecular mechanisms related to the physiology and pathophysiology of erythrocytes with respect to oxygen binding and release, metabolism and senescence, we investigated the oxidative effects of gemfibrozil in normal and beta-thalassemic red blood cells. Our results showed that the oxidative stress promoted by the drug, through a direct interaction with hemoglobin, may lead to activation of caspase 3, which in turn influences the band 3 anion flux and glucose metabolism. In a comparative context, we also evaluated the effect on band 3 and caspase 3 activation of orthovanadate (a phosphatase inhibitor) and *t*-butylhydroperoxide (a known oxidant). The results support the hypothesis that gemfibrozil influences band 3 function through several mechanisms of action, centered on oxidative stress, which induces significant alterations of glucose metabolism.

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Introduction

The recent study of Koch et al. (2008) showed that in patients undergoing cardiac surgery transfusion of red cells that had been stored for more than 2 weeks was associated with a significantly increased risk of postoperative complications as well as reduced short-term and long-term survival. These data confirm that some aspects of metabolism, oxygen transport properties and storage conditions of erythrocytes need to be further investigated, even more because they have significant implications in terms of morbidity and mortality. On this basis, we investigated some properties of normal and beta-thalassemic red blood cells (RBCs) in the presence of gemfibrozil (GFZ, a fibric acid derivative widely used in medicine as an antihyperlipidemia agent), a well-known drug that can act both as an allosteric effector and an anti-gelling agent on human hemoglobin (Perutz et al. 1986).

Fibric acid derivatives are a class of drugs generally used in pharmacotherapy as antihyperlipoproteinemia agents with the main aim to reduce incidence of coronary heart disease (Frick et al. 1987; Manninen et al. 1988). Clinical results, however, have been contradictory, mainly because the mortality index was higher in treated patients with respect to those treated with placebo. In particular, some toxic effects seem to be linked to the so-called peroxisome proliferator–activated receptor subtype alpha (PPAR-α).

At the present, fibrates are considered molecules with intriguing biological activity which can be experimentally utilized to stress some pathophysiological conditions

linked to degenerative diseases. In particular, fibrates (clofibric acid, bezafibrate, GFZ) have shown a number of biological activities:

- inhibition of the mitochondrial respiratory chain associated with significant production of oxygen free radicals (Chance and McIntosh 1995)
- strong allosteric interaction with human hemoglobin (Hb), with a consequent reduction of oxygen affinity (Scatena et al. 1995; Marden et al. 1988; Perutz et al. 1986; Hyde et al. 1984)
- strong modulation of band 3 protein (B3), with a consequent increase in anion flux through the erythrocyte membrane (Tellone et al. 2008)

In this study, we demonstrated that the GFZ–Hb complex induces auto-oxidation of the hemoprotein and a significant interaction with the cytoplasmic domain of band 3 in both humans and thalassemic erythrocytes. From these data some implications on the physiology and physiopathology of RBCs can be inferred.

In fact, it is well known that RBCs may be vulnerable to oxidative stress because, throughout their life cycle, they are continuously exposed to high concentrations of iron and oxygen (O_2), even if this harmful combination is supervised by sophisticated oxide-reduction systems (Nagababu et al. 2003; Meister and Anderson 1983; De Flora et al. 2001). In several pathological conditions, like hemoglobinopathies and erythroenzymopathies, this sensitive defense system can be seriously impaired (Carrel et al. 1975; Kahane et al. 1978). For example, in beta-thalassemia syndromes, the accumulation of unpaired alpha-globins and the large concentration of free iron lead to the production of several toxic species such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-), which often induce massive damage of the cellular membrane (Browne et al. 1998; Cappellini et al. 1999; Droge 2002).

Oxidative stress is a major event also in normal erythrocytes, especially when they reach a given degree of senescence. In fact, it has been demonstrated that human mature RBCs contain a functional procaspase, such as caspase 3, and that its activation by oxidative stress leads to the proteolytic cleavage of the N-terminal cytoplasmic domain of band 3 (cdb3) (Mandal et al. 2002, 2003; Matarrese et al. 2005; Clementi et al. 2007).

It could be useful to recall that band 3 (CD233, anion exchanger-1 [AE-1]) is a member of the anion exchanger gene family and consists of two structurally and functionally distinct domains: an amino-terminal 43-kDa cytoplasmic domain and a 55-kDa membrane domain. The amino-terminal domain interacts with the cellular cytoskeleton through ankyrin and binds proteins 4-1 and 4-2, hemoglobin, enzymes of the glycolytic cycle and the protein tyrosine kinase (PTK) p72 SYK. The carboxy-terminal membrane domain spans the membrane 12–14 times and carries a number of blood group antigens; especially the fourth segment is heavily glycosylated. The extreme C-terminal tail ends intracellularly, and amino acids 886–890, LDADD, are the binding site of carbonic anhydrase II (CAII). Tyrosine-904, adjacent to the binding of CAII, is phosphorylated by p72 SYK, which seems to be triggered by a decrease in the cell volume. Interestingly, CD233 interacts with glycophorin A (GPA), which facilitates movement of CD233 toward the cell surface. The interaction of cdb3 seems to modulate both the anion influx, which is oxygen-dependent, and the metabolism, which is characteristically shifted toward the pentose phosphate pathway (PPP) in the high-oxygenation state of RBCs and toward the Embden-Meyerhof-Parnas pathway (EMP) in the low-oxygenation state (De Rosa et al. 2007; Giardina et al. 1995; Galtieri et al. 2002; Russo et al. 2008).

Since GFZ interacts strongly with Hb (Perutz et al. 1986; Abraham et al. 1983) at a different site with respect to cdb3, it is predictable that the macrocomplex Hb–GFZ–cdb3 may differently modulate the anion influx (Tellone et al. 2008). Moreover, the new conformation and functional properties of the binary complex Hb–GFZ, promoting hemoprotein auto-oxidation, could activate caspase 3, inducing new and not negligible effects on B3 function and RBC metabolism.

These premises have led us to determine whether the presence of GFZ could induce or increase oxidative stress in normal and beta-thalassemic erythrocytes. Moreover, we investigated the possibility of drug-induced modifications of the kinetic characteristics of the B3 anion flux and their linkage to the aging process of these cells.

Materials and Methods

Materials

All reagents were from Sigma-Aldrich (St. Louis, MO). Citrate fresh human blood was obtained from informed heterozygous beta-thalassemic and healthy donors aged 30–50 years who had avoided any drug treatment for at least 1 week before sample collection.

Preparation of RBCs

Citrate blood samples were washed three times with an isoosmotic NaCl solution. During washings the white blood cells were discarded from the pellet. After washing, RBCs were resuspended (hematocrit 3%) in the incubation buffer (35 mM Na_2SO_4, 90 mM NaCl, 25 mM *N*-(2-hydroxyethyl)-piperazine-N^1-2-ethanesulfonic acid [HEPES], 1.5 mM $MgCl_2$), adjusted to pH 7.4 or 7.3 and 310 ± 20

mOsmol/kg, measured by an Osmostat OM-6020 apparatus (Daiichikagakuco, Kyoto, Japan).

Methemoglobin (MetHb) levels and hemolysis degree were determined at the end of the incubation time as follows: Hemolysis was determined by measuring spectrophotometrically (DU70 spectrophotometer; Beckman, Palo Alto, CA) the Hb concentration in the supernatants obtained from centrifugation at 2,500*g* for 5 min at 4°C; MetHb levels were determined spectrophotometrically on lysed cells (Zijlstra et al. 1991).

Kinetic Measurements

Cells were incubated in the above incubation buffer at 25°C, under different experimental conditions. At several time intervals, 10 μmol of the stopping medium 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS) were added to each test tube containing the RBC suspension. Cells were then separated from the incubation medium by centrifugation (J2-HS Centrifuge, Beckman) and washed three times at 4°C with a sulfate-free medium to remove the sulfate trapped outside. After the last washing, the packed cells were lysed with perchloric acid (4%) and distilled water. Lysates were centrifuged for 10 min at 4,000*g* (4°C), and membranes were separated from the supernatant. Sulfate ions were precipitated from the supernatant by adding glycerol/distilled water mixture (1:1, V/V), 4 M NaCl, 1 M HCl and 1.23 M $BaCl_2 \cdot 2H_2O$ in order to obtain a homogeneous barium sulfate precipitate. The absorbance of this suspension was measured at 350–425 nm.

The sulfate concentration was determined using a calibrated standard curve, obtained by measuring the absorbance of suspensions with known sulfate amounts (Romano et al. 1998). Experimental data of sulfate concentration as a function of incubation time were analyzed by best-fitting procedures according to the following equation: $c(t) = c_\infty\ (1 - e^{-kt})$, where $c(t)$ represents sulfate concentration at time t, c_∞ intracellular sulfate concentration at equilibrium and k the rate constant of sulfate influx.

Analysis of Caspase 3 Activity

Caspase 3 activity was measured using a Sigma (St. Louis, MO) assay kit following the manufacturer's instructions. Acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (Ac-DEVD-pNA) was used as a colorimetric substrate.

Human mature erythrocytes were incubated for 2 h at 25°C in HEPES 25 mM, NaH_2PO_4 1 mM, NaCl 110 mM, KCl 5 mM, $MgCl_2$ 2 mM, at pH 7.4 and 290 ± 5 mOsm/kg^{-1} measured by the Osmostat OM-6020 apparatus. Cell suspensions were then separated from the incubation medium by centrifugation (J2-HS Centrifuge) and washed three times at 4°C with HEPES washing buffer. After the last washing, the packed cells were lysed with cycles of freezing and defrosting. Lysate was centrifuged at 10,000*g* for 1 min at 4°C, and supernatant was used for caspase 3 activity. The protein concentration was determined by the BCA assay. Protease activity was determined by spectrophotometer detection at 405 nm of the chromophore pNA after its cleavage by caspase 3 from the labeled caspase-3-specific substrate (DEVD-pNA). Acquired data were fitted to a polynomial 2° grade equation. Before performing spectrophotometer determinations, hemolysates were filtered (50,000 cut-off) in order to eliminate interference by hemoglobin spectra.

SDS-PAGE

Treated and untreated erythrocytes were osmotically lysed in hypotonic solution, and the lysate was incubated overnight with rabbit anti-caspase 3 (Santa Cruz Biotechnology, Santa Cruz, CA) antibody at 4°C. Protein A/G agarose (Santa Cruz Biotechnology) was added and incubated for 1 h at room temperature, and the immunoprecipitate was washed, denatured and separated by SDS-PAGE, as previously described (Mandal et al. 2002). Proteins were electrophoretically transferred to a nitrocellulose membrane and probed with anti-caspase 3 antibody followed by alkaline phosphatase–linked goat anti-rabbit secondary antibody (Santa Cruz Biotechnology) and finally visualized using color developing reagent (Bio-Rad, Hercules, CA). Images of nitrocellulose membranes were acquired (Bio-Rad Gel Doc 2000) and scanned (Bio-Rad GS800) using Bio-Rad Quantity One software.

Statistical Analysis

Differences were analyzed with a two-tailed Student's *t*-test for unpaired data. Upon occurrence, group means were compared by analysis of variance (ANOVA), followed by a multiple comparison of means by Dunnett's test. The results are expressed as means ± SD of at least three different experiments performed in triplicate, unless otherwise specified. $p < 0.05$ was considered significant.

Results

In this study, the effects of GFZ in normal and beta-thalassemic erythrocytes were evaluated. The results reported in Table 1 show that GFZ induced rapid oxidation of the hemoglobin molecule as demonstrated by the increase in the MetHb levels, which paralleled the induction of a significant hemolysis.

Table 1 Effects of GFZ in red blood cell

Conditions	Incubation time (min)	MetHb (%)	Hemolysis (%)
Control	15	0	0
GFZ	15	15 ± 2.0 ($n = 4$)	45 ± 2.9 ($n = 4$)
Control	90	0	0
GFZ	90	20 ± 2.1 ($n = 4$)	55 ± 3.8 ($n = 3$)
Control	Overnight	0	0
GFZ	Overnight	6 ± 1.8 ($n = 4$)	25 ± 3.0 ($n = 4$)

Moreover, as shown in Fig. 1, the presence of GFZ strongly increased anion SO_4^-/Cl^- exchange through both normal and thalassemic RBC membranes (increments of about 340% and 150% for normal and beta-thalassemic RBCs, respectively; $p < 0.001$ for both samples). Intriguingly, in basal conditions a significantly greater flux was evident in beta-thalassemic RBCs with respect to normal RBCs (about +80%, $p < 0.05$) and this in spite of the reported different B3 content between normal and beta-thalassemic RBCs (20.6% ± 0.7 and 14.3% ± 0.9, respectively) (Alekperova et al. 2004).

Identifying cdb3 as one of the main targets through which GFZ exerts its effect (Tellone et al. 2008), we also tested the fibrate influence on caspase 3 (procaspase) activity because its activation leads to the proteolytic cleavage of cdb3 (Clementi et al. 2007; Mandal et al. 2003).

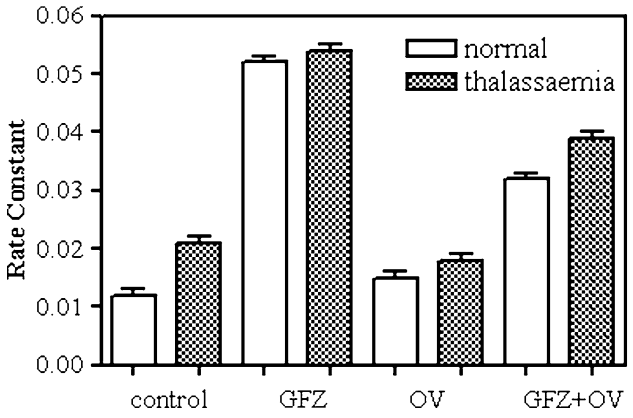

Fig. 1 Effect of GFZ and OV concentrations on the rate of sulfate influx. Results of a series of experiments performed by incubating normal (*white boxes*) and beta-thalassemic (*cross-hatched boxes*) human adult erythrocytes without and with 3 mmol/l of GFZ, 3 mmol/l of OV and 3 mmol/l of GFZ plus 3 mmol/l of OV. See "Materials and Methods" for further experimental details. $Control_n$ vs. GFZ_n $p < 0.001$, $control_n$ vs. OV_n $p < 0.001$, $control_n$ vs. $(GFZ + OV)_n$ $p < 0.001$, $control_\beta$ vs. GFZ_β $p < 0.001$, $control_\beta$ vs. $(GFZ + OV)_\beta$ $p < 0.001$, $control_n$ vs. $control_\beta$ $p < 0.001$, $GFZ_{n/\beta}$ vs. $(GFZ + OV)_{n/\beta}$ $p < 0.001$, $(GFZ + OV)_n$ vs. $(GFZ + OV)_\beta$ $p < 0.001$

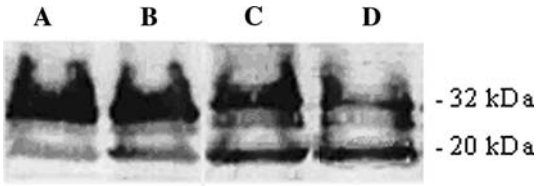

Fig. 2 Caspase 3 activation by GFZ treatment. Normal and beta-thalassemic erythrocytes were treated with 3 mmol/l of GFZ at 25°C for 2 h. Lysates were immunoprecipitated with anti-caspase 3 antibody and analyzed for caspase 3 by Western blotting as described in "Materials and Methods." *Lane A*, untreated normal cells; *lane B*, normal cells treated with 3 mmol/l of GFZ; *lane C*, untreated beta-thalassemic cells; *lane D*, beta-thalassemic cells treated with 3 mmol/l GFZ. The 20- and 32-kDa bands represent, respectively, the activated and inactivated forms of caspase 3

Western blot analysis (Fig. 2) indicated the presence of a higher concentration of the active form of caspase 3 in beta-thalassemic RBCs with respect to normal RBCs; this was significantly increased after incubation with GFZ for 2 h at 25°C. The appearance of the caspase 3 active form is evidenced by the band at 20 kDa.

In light of our previous results (Tellone et al. 2008), we excluded a phosphorylative action of the fibrate in promoting caspase activation. Moreover, since the increase of caspase 3 activity depends on the oxidative stress (Clementi et al. 2007; Mandal et al. 2003), we tried to emphasize the fibrate role as "promoter" or "amplifier" of oxidative stress.

Interestingly, the level of caspase 3 activation was slightly increased in beta-thalassemic RBCs with respect to normal RBCs (about +35%, $p < 0.05$). Most importantly, GFZ strongly increased caspase activation in both normal and thalassemic erythrocytes (increments of about 130% and 70% for normal and beta-thalassemic RBCs, respectively; $p < 0.001$ for both samples), a fact that indirectly confirms the presence of oxidative stress induced by GFZ.

To further confirm the "oxidant" role of the fibrate in caspase activation, we analyzed the *t*-butylhydroperoxide (*t*-BHP) effect on caspase 3 activity in normal and beta-thalassemic RBCs. The results (Fig. 3) showed that *t*-BHP increased caspase 3 activity in both cell types, similarly to what was observed with GFZ in the same experimental conditions (increments of about 106% and 87% for normal and beta-thalassemic RBCs, respectively; $p < 0.001$ for both samples). All this suggests a role of GFZ in caspase 3 activation very likely linked to the drug-induced oxidative stress on Hb.

To better understand the GFZ molecular mechanism, additional experiments were performed in the presence of orthovanadate (OV), which is known to affect protein phosphorylation, including cdb3 (Brunati et al. 2000; Bordin et al. 1995). In term of anion flux, the results showed that OV induced a significant increase of the

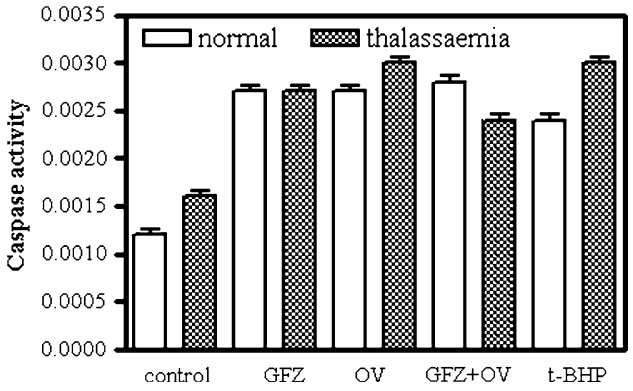

Fig. 3 Caspase 3 activity by GFZ, OV and *t*-BHP treatment. Results of a series of experiments performed by incubating normal (*white boxes*) and beta-thalassemic (*cross-hatched boxes*) adult human erythrocytes without (control) and with 3 mmol/l of GFZ, 3 mmol/l of OV, 3 mmol/l of GFZ plus 3 mmol/l of OV and 3 mmol/l of *t*-BHP. See "Materials and Methods" for further experimental details. Control_n vs. GFZ_n or OV_n or $(\text{GFZ} + \text{OV})_n$ or t-BHP_n $p < 0.001$, control_β vs. GFZ_β or OV_β or $(\text{GFZ} + \text{OV})_\beta$ or t-BHP_β $p < 0.001$, control_n vs. control_β $p < 0.05$, $(\text{GFZ} + \text{OV})_n$ vs. $(\text{GFZ} + \text{OV})_\beta$ $p < 0.001$

sulfate flux in normal, but not in thalassemic, erythrocytes. Moreover, OV partially antagonized the strong stimulation of anion flux induced by GFZ both in normal and in thalassemic samples.

Hence, OV induces caspase activation in both normal and thalassemic erythrocytes almost similarly to what was observed in the presence of GFZ (increases of about 130% and 87% for normal and beta-thalassemic RBCs, respectively; $p < 0.001$ for both samples). Interestingly, the combination of GFZ plus OV does not show a cumulative increase of caspase activity. These findings support the hypothesis that GFZ acts on B3 influx through a different pathway with respect to OV and it does not alter the phosphorylation–dephosphorylation balance of RBCs (Tellone et al. 2008).

Discussion

From a functional point of view, these results should be interpreted in light of the "global allostery model" (Yonetani et al. 2002), according to which the effectors can bind to both the T-state (Perutz et al. 1986) and the R-state (Shibayama et al. 2002; Laberge et al. 2005) of Hb, inducing direct tertiary conformational changes. These tertiary effects could lead to significant changes in oxygen association and hemoglobin functionality (Coletta et al. 1999; Tsuneshige et al. 2002; Perutz et al. 1986; Tellone et al. 2008).

In this context, the increase of erythrocyte anion flux due to GFZ may be linked to the binding of GFZ to hemoglobin (Perutz and Poyart 1983; Abraham et al. 1983) and the subsequent interactions with cdb3, which could give rise to a Hb–GFZ–cdb3 macrocomplex (Tellone et al. 2008).

In this study, normal and beta-thalassemic RBCs following GFZ treatment showed different anion flux values. It is known that beta-thalassemic RBCs represent an intriguing in vitro model to study, through the significant presence of uncoupled alpha chains, binding of GFZ and assembly of "atypical" structural and functional complexes. On this basis, it is hypothesized that the different anion flux values observed could be due to changes in the structure of the macrocomplexes assembled in the two cell types (i.e., Hb–GFZ–cdb3, Hb_β–GFZ–cdb3) (Tellone et al. 2008).

Besides, our results show that beta-thalassemic RBCs with respect to normal ones have a higher concentration of the active form of caspase 3. This finding suggests that caspase 3-mediated cleavage of cdb3 occurs mainly in beta-thalassemic RBCs with respect to normal ones, thus contributing to the decrease in cdb3 binding sites necessary for the formation of Hb–GFZ–cdb3 macrocomplexes.

Nevertheless, it is interesting to note that our studies show very small and not significant differences in the anion exchange between beta-thalassemic and normal RBCs treated with GFZ. In other words, in beta-thalassemic RBCs the drug is able to influence B3 (difference of 150% in sulfate transport between beta-thalassemic RBCs treated and untreated with GFZ) even if in conditions not "favorable" for its modulation.

Moreover, the different modulation of the anionic flux in response to GFZ and OV allowed us to exclude a GFZ involvement in the phosphorylation state of the RBC membrane (Tellone et al. 2008). In fact, the lower anion flux modulation caused by OV with respect to GFZ indicates a different mechanism of action of the two molecules.

OV could affect anionic flux essentially by activating caspase 3; this, once triggered, could perform the cleavage of cdb3 (Mandal et al. 2003), thereby depriving the RBCs of the control mechanisms of both anion exchange and metabolism (Giardina et al. 1995).

In particular, caspase 3 activation by OV would be due to the loss of activity of phosphatase, which may lead in turn to destabilization of the phosphorylation–dephosphorylation balance present at the membrane level on B3. The phosphorylation of tyrosine would cause an increase of oxidative stress (Zipser et al. 1997), followed by caspase 3 activation (Mandal et al. 2002, 2003). The increased production of reactive oxygen species (ROS) in RBCs treated with OV could also be caused by a derangement of glucose metabolism (EMP increases and PPP slows down) due to the displacement of glycolytic enzymes from cdb3 (Low et al. 1997).

This finds further support in the insensibility toward OV of beta-thalassemic RBCs, which are already destabilized as far as the phosphorylation–dephosphorylation balance is concerned (Terra et al. 1998) (Fig. 1).

In summary, OV seems to act by stimulating the kinetic flux on a single front, starting a kind of chain reaction triggered by phosphorylation. The dynamics of the observed anion flux modulation can be better explained through several mechanisms of GFZ on RBCs. This could justify the high increase of anion exchange measured in the presence of GFZ in normal RBCs and persistent in beta-thalassemic RBCs (Fig. 4). In fact, GFZ seems to act on the anion flux not only "indirectly" through caspase 3 activation induced by oxidative stress (Mandal et al. 2002) but also through a "direct" interaction with cdb3.

Interestingly, the increase of oxidative stress measured in the presence of GFZ demonstrates that the oxidative action of fibrate could be due to the strong interaction between this drug and Hb (Perutz et al. 1986; Coletta et al. 1999; Tsuneshige et al. 2002; Tellone et al. 2008).

Thus, it has been shown that the binding of GFZ to Hb induces a shift of the oxygen dissociation curve toward lower O_2 affinity values (Scatena et al. 1995), which in turn results in a greater "availability" of oxygen and derived ROS, potentially dangerous for the cell. This potential toxicity is strengthened by the conversion of Hb to MetHb, which can stimulate the production of superoxide anion and hydrogen peroxide, typical substrates for the Fenton and Haber Waiss reactions.

Hence, our data seem to confirm recent studies demonstrating a strong correlation between oxidative stress and caspase 3 activation (Suzuki et al. 2007; Clementi et al. 2007; Matarrese et al. 2005; Mandal et al. 2002, 2003). Moreover, binding of GFZ to Hb may facilitate the spontaneous auto-oxidation of Hb, leading to caspase 3 activation, proteolytic cleavage of cdb3 and increase in anion flux.

The cellular damage originated by this intriguing pathogenetic mechanism is further exacerbated by the loss of metabolic control due to the displacement of glycolytic enzymes from their inhibitor sites on cdb3, with consequent loss of the fine modulation of the glucose metabolism of the erythrocyte. This deranged metabolic control induces a slowing down of PPP, which impairs the synthesis of NADPH and worsens the oxidative stress, which in turn would cause caspase 3 activation in a sort of "vicious cycle."

In summary, our results suggest that GFZ increases oxidative stress in RBCs in three different ways:

1. GFZ interacts with Hb, inducing formation of MetHb and other damaging molecules.
2. GFZ activates red cell caspase 3, which is responsible for cdb3 cleavage; this event abolishes cdb3 binding sites for Hb and glycolytic enzymes, thus abolishing

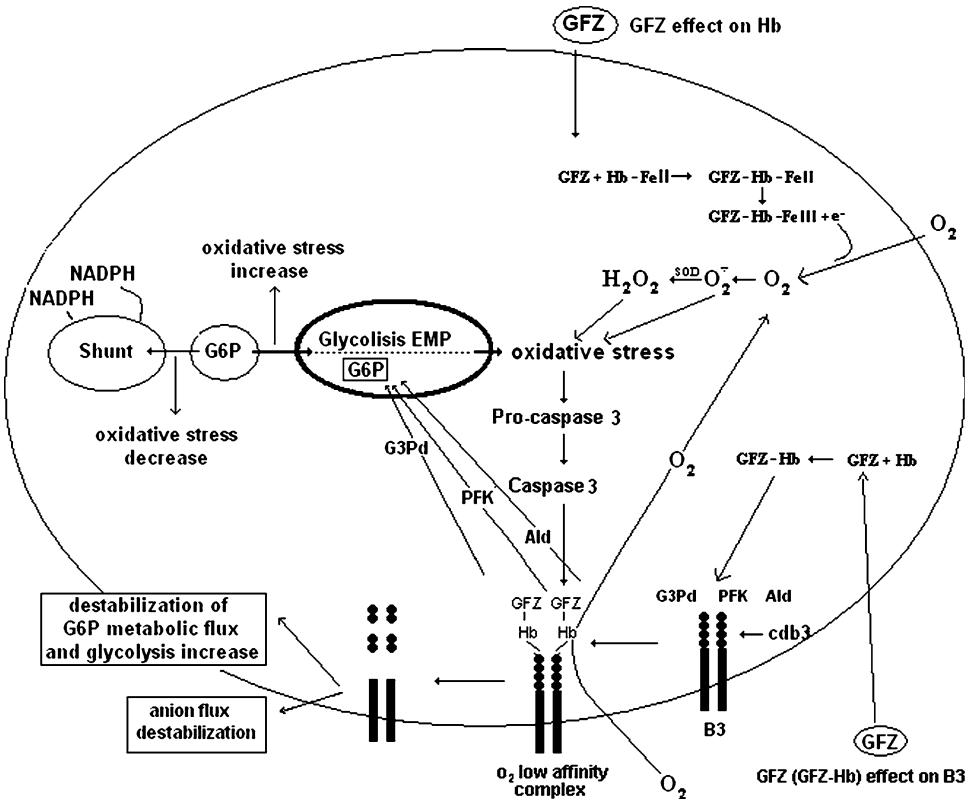

Fig. 4 GFZ effects on RBCs

the oxygen-dependent modulation of erythrocyte metabolism (Giardina et al. 1995).

3. The GFZ–Hb complex is able to bind cdb3 even in the high-oxygenation state. This macrocomplex displaces glycolytic enzymes from their cdb3 binding sites. This finding implies that, particularly under high oxygen pressure, i.e., at the level of the lungs, GFZ-treated erythrocytes have a higher risk of oxidative damage of proteins and lipids because they have lost the ability to increase PPP flux, which is indispensable to maintaining an adequate reducing power and resistance.

In conclusion, these data confirm and deepen previous studies relating Hb oxygenation state with band 3 function and erythrocyte metabolism (Giardina et al. 1995; De Rosa et al. 2008), in an attempt to better clarify the molecular mechanisms governing the life of erythrocytes. Specifically, these data seem to confirm the role of Hb as a modulator of erythrocyte metabolism and erythrocyte senescence. Moreover, in terms of erythrocyte physiology, these results push us to investigate some neglected aspects:

- The molecular mechanisms that link Hb oxygenation state, band 3 anion channel, carbonic anhydrase (through chloride, hydrogen ions and 2,3-biphosphoglyceric acid) may determine at the membrane level a kind of "supercomplex" with peculiar and still not fully understood functional properties.
- The role of the metabolic interrelationships between deoxyHb, cdb3 and glycolysis to sustain the erythrocyte reservoir/storage function of spleen.

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