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Original Contribution

Stress response and cytoskeletal proteins involved in erythrocyte membrane remodeling upon *Plasmodium falciparum* invasion are differentially carbonylated in G6PD A$^-$ deficiency

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ABSTRACT

Multiple glucose-6-phosphate dehydrogenase (G6PD)-deficient alleles have reached polymorphic frequencies because of the protection they confer against malaria infection. A protection mechanism based on enhanced phagocytosis of parasitized G6PD-deficient erythrocytes that are oxidatively damaged is well accepted. Although an association of this phenotype with the impairment of the antioxidant defense in G6PD deficiency has been demonstrated, the dysfunctional pathway leading to membrane damage and modified exposure of the malaria-infected red cell to the host is not known. Thus, in this study, erythrocytes from the common African variant G6PD A$^-$ were used to analyze by redox proteomics the major oxidative changes occurring in the host membrane proteins during the intraerythrocytic development of *Plasmodium falciparum*, the most lethal malaria parasite. Fifteen carbonylated membrane proteins exclusively identified in infected G6PD A$^-$ red blood cells revealed selective oxidation of host proteins upon malarial infection. As a result, three pathways in the host erythrocyte were oxidatively damaged in G6PD A$^-$: (1) traffic/assembly of exported parasite proteins in red cell cytoskeleton and surface, (2) oxidative stress defense proteins, and (3) stress response proteins. Additional identification of hemichromes associated with membrane proteins also supports a role for specific oxidative modifications in protection against malaria by G6PD polymorphisms.

Deficiency of glucose-6-phosphate dehydrogenase (G6PD) is a common X-linked erythroenzymopathy, though most carriers of polymorphic alleles are asymptomatic. A large number of G6PD variants have reached polymorphic frequencies in various parts of the world [1] because of the relative protection they confer against malaria infection [2–5]. In the red blood cell (RBC) the only source of NADPH is the reactions catalyzed by G6PD and 6-phosphogluconate dehydrogenase. NADPH is essential in this cell to maintain a reduced environment, which protect against physiological high levels of reactive oxygen species (ROS) [1]. Consequently, in G6PD deficiency, an increased oxidative damage occurs to the RBC membrane proteins, either directly through oxidation of thiol groups or indirectly by the binding of hemoglobin denaturation by-products (hemichromes and Heinz bodies) [6]. Infection by *Plasmodium falciparum*, independent of the G6PD status, increases oxidative stress within the host RBC as a consequence of the large amounts of ROS generated by the active metabolism of the growing malaria parasite and the degradation of hemoglobin generating free heme [7,8]. As a result, in parasitized erythrocytes, abnormal cross-linking of membrane proteins and changes in membrane fluidity and membrane lipid composition are produced [9,10]. High parasitemias and cellular adhesion of parasitized erythrocytes are the grounds of lethal malaria. In malaria-endemic countries, the selective advantage of polymorphic G6PD-deficient alleles is explained as a consequence of the increased susceptibility to oxidative stress of the infected deficient erythrocyte, resulting in its accelerated phagocytosis by the host [11], which might finally reduce the risk of severe malaria [2–5].

A reasonable step forward in identifying potential oxidant determinants of phenotypic adaptation to malaria in G6PD deficiency is the differential display of posttranslational redox modifications of membrane proteins between parasitized G6PD-deficient and normal erythrocytes. For this rationale, we have attempted to identify differential changes in the carbonylation pattern of the G6PD A$^-$ RBC membrane proteome caused by *P. falciparum* infection.

Materials and methods

Plasmodium falciparum Dd2 culture

Venous peripheral blood samples from blood group 0 G6PD B and hemizygous G6PD A$^-$ healthy male subjects were collected in CPDA

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(citrate phosphate dextrose adenine) vials (Vacuette). All blood donors provided informed consent. Highly synchronous cultures of *P. falciparum Dd2* at 50–60% parasitemia were grown following strictly the protocol previously described [12].

Preparation of membrane protein controls from uninfected RBCs

Peripheral whole blood was collected in CPDA vials and stored without shaking for 72 h at 4 °C to allow maturation of reticulocytes to RBCs. White blood cells were suspended in density gradient medium with Limphoprep (Axis-shield) and removed together with the upper layer containing RBCs. Erythrocytes were washed twice with 10 ml of RPMI 1640:Hepes, pH 7.4, buffer (Sigma). Finally, the packed RBC was diluted in the same buffer and used to obtain membrane proteins according to previously reported procedures [13,14]. RBC membrane ghosts were suspended in ice-cold 5 mM phosphate buffer, pH 8, 1% SDS, and 100 μM 3,5-di-*tert*-4-butylhydroxytoluene (BHT; Sigma) antioxidant and supplemented with one Complete-Mini protease inhibitor cocktail tablet per 10 ml buffer protease inhibitor (Roche). The protein concentration was determined using a modified Bradford method (Bio-Rad).

Preparation of membrane protein from infected RBCs (iRBCs)

Because, 18 h postinvasion, iRBCs undergo osmotic lysis on isotonic solutions of sorbitol [15,16], samples of iRBCs from late rings (18 h) to schizonts (40 h) were suspended in 5 volumes of 280 mM sorbitol in RPMI 1640:Hepes, pH 7.4, and incubated for 10 min at room temperature to lyse the iRBCs selectively. Lysates were centrifuged at 180*g* for 5 min at 4 °C and supernatants containing ghost cells were collected. The pellets were washed two more times in 280 mM sorbitol. For iRBCs at ring stage (12 h), samples were mixed with 0.05% saponin/PBS (w/v) for 5 min at room temperature to lyse erythrocyte membranes [8] and centrifuged at 180*g* for 5 min at 4 °C and the supernatants containing suspended ghosts were collected.

To obtain hemoglobin-free infected membrane protein fractions, all supernatants were centrifuged at 9000*g* for 20 min at 4 °C. Next, the supernatants containing hemoglobin were discarded and infected ghost pellets were washed with 5 volumes of ice-cold 5 mM phosphate buffer, pH 8, supplemented with antioxidant BHT and protease inhibitor. Washes were repeated until the supernatants appeared colorless. Thereafter, infected ghosts were centrifuged at 20,000*g* followed by Na_2CO_3 treatment [14], solubilized, and quantified as mentioned above.

Normal and deficient RBC membrane protein fractions (uninfected and infected) were derivatized with 2,4-dinitrophenylhydrazine (DNPH) immediately after being obtained and stored in aliquots at −80 °C until used. Carbonylated proteins were detected using anti-DNPH antibodies by one-dimensional Western blotting analysis (OxyBlots), as previously described [17]. Infected RBC membrane protein fractions (trophozoites, 34 h) used in two-dimensional OxyBlots were derivatized in IPG strips after isoelectric focusing (IEF) as mentioned below.

One-dimensional OxyBlots in normal and G6PD-deficient RBC membrane protein samples

DNPH-derivatized uninfected RBC membrane proteins (10 μg) from two normal and two G6PD-deficient donors were electrophoresed by 10% SDS–PAGE and transferred onto polyvinylidene difluoride (PVDF; Amersham Biosciences) membranes using a Bio-Rad transfer apparatus (1 mA/cm^2 of gel). PVDF membranes were blocked for 1 h at room temperature with 10% nonfat dry milk in phosphate-buffered saline. Blocked membranes were incubated with rabbit polyclonal anti-DNPH antibody at 1:4000 (Sigma) in PBS–Tween 20 0.05%, milk 10%, for 2 h at room temperature with gentle rocking, followed by incubation with peroxidase-linked anti-rabbit IgG antibody at 1:5000 (Amersham Biosciences; 1 h at room temperature). Chemiluminescence signals were developed using SuperSignal West Pico rabbit IgG detection kit from Thermo Scientific. Quantity One 4.0 software (Bio-Rad) was used to compare protein bands.

Carbonylation patterns across intraerythrocytic cycle

To study the oxidative damage caused by *P. falciparum* on membrane proteins of host cells across the intraerythrocytic cycle, triplicate cultures of normal and G6PD-deficient RBCs were harvested at various postinvasion times corresponding to ring (12 h), late ring (18 h), early trophozoite (24 h), trophozoite (34 h), and schizont (40 h) forms (Fig. 1). DNPH-derivatized infected RBC membrane proteins (1 μg) were analyzed using 1D OxyBlots as described above.

Identification of carbonylated proteins by 2D gel electrophoresis and MALDI-TOF-TOF

Infected and uninfected RBC membrane protein fractions were precipitated with Clean Up (GE Healthcare), resuspended in 50 μl of lysis buffer II (7 M urea, 2 M thiourea, 4% Chaps, 2 mM TCEP–HCl, and 1% carrier ampholites, pH 3–10 NL), and solubilized for 1 h at room temperature with agitation.

Two-dimensional electrophoresis

One hundred micrograms of membrane protein fractions was loaded onto gels used to identify proteins by MALDI-TOF-TOF analysis. Samples were mixed with rehydration buffer and electrophoresed following the conditions previously described [18].

SYPRO ruby staining

Gels used to identify proteins were stained with SYPRO ruby protein gel stain (Sigma). The gels were fixed in 10% MeOH/7% acetic acid for 30 min, incubated in SYPRO ruby staining solution overnight, washed in 10% MeOH/7% acetic acid twice for 30 min/each, and finally, washed twice with water for 10 min each.

Two-dimensional OxyBlots

Twenty micrograms of membrane protein fractions were loaded in duplicate onto gels used to detect carbonylated proteins. Samples were diluted in rehydration buffer, applied to IPG strips, and separated by IEF as described [18]. After the first dimension, the strips were derivatized with 10 mM DNPH in 10% trifluoroacetate according to the procedure described by Reinheckel et al. [19]. Next, the strips were equilibrated, reduced with dithiothreitol, alkylated with IA (Iodo Acetamide), and separated in the second dimension as described. Two-dimensional-gel separated proteins were transferred to Immobilon-P (Millipore) membranes using Tris–glycine transfer buffer (2.93 g glycine, 5.81 g Tris, 0.375 g SDS in 1 L with 200 ml of methanol) at 200 mA for 1 h 15 min in a semidry system (Pharmacia Biotech Multiphor II). Transferred membranes were blocked and incubated with anti-DNPH rabbit polyclonal antibody as mentioned. Replicates and several exposure times of the OxyBlots were used to avoid potential errors in spot matching with 2D gels. Only common spots, present in both replicates of each sample, were considered carbonylated.

Image analysis

Gels stained with SYPRO ruby were scanned in a Typhoon 9400 variable mode imager (GE Healthcare) equipped with a 532-nm excitation laser (green) with an emission filter of 610 nm BP 30 nm (SYPRO ruby, ROX, EtBr) and 100-μm resolution. The photomultiplier tube setting was altered to 650 V to optimize sensitivity to background ratios. One- and two-dimensional X-ray films of carbonylated proteins