Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 68 (2004) 1903–1910
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Plasmodium berghei: dehydroepiandrosterone sulfate reverses chloroquino-resistance in experimental malaria infection; correlation with glucose 6-phosphate dehydrogenase and glutathione synthesis pathway

Innocent Safeukui[a,*], François Mangou[a], Denis Malvy[a], Philippe Vincendeau[a,b], Djavad Mossalayi[a], Gilbert Haumont[a], Remi Vatan[a], Piero Olliaro[c], Pascal Millet[a,b]

[a]*Unité 3677, Bases thérapeutiques des inflammations et infections, Université Victor Segalen Bordeaux II, 146 rue Leo Saignat, 33076 Bordeaux Cedex, France*
[b]*Laboratoire de Parasitologie, Centre Hospitalier, Universitaire de Saint André, Bordeaux, France*
[c]*UNDP/World Bank/WHO Special Programme on Research & Training in Tropical Diseases (TDR), World Health Organization, Geneva, Switzerland*

Received 4 March 2004; accepted 18 May 2004

Abstract

In *Plasmodium falciparum*-infected cells or in *P. berghei* infected mice, increase of reduced glutathione (GSH) levels confers resistance to chloroquine (CQ). GSH is synthesized within the cells through a complex biochemical pathway composed of several well known enzymes, in which glucose-6-phosphate dehydrogenase (G6PD) plays an important role. The physiological hormone dehydroepiandrosterone sulfate (DHEAS) is a potent inhibitor of G6PD activity, and G6PD deficiency is known to exert antimalaria protection. This study aimed to investigate the ability of DHEAS to enhance the antimalarial activity of CQ, via an inhibition of G6PD activity and GSH synthesis. Two *P. berghei* CQ resistant strains (CQR6 and CQR30) were selected in vivo from the sensitive strain NK65. Drug effects were checked both by monitoring the evolution of parasitaemia and by the survival of infected mice. In addition, intra-parasite levels of GSH and G6PD activity were measured before and after the treatment. Results demonstrate that acquisition of CQ resistance in *P. berghei* is associated with a significant increase in parasite G6PD activity and GSH level. Combination of CQ with DHEAS or buthionin sulfoximin (BSO, a specific inhibitor of GSH synthesis) significantly increased sensitivity of resistant parasites to CQ and increased the survival period of the infected mice. This reduction of parasitaemia and improvement of the survival of infected mice were associated with intra-parasite depletion of GSH and inhibition of G6PD activity due to DHEAS action. This experimental study suggests that DHEAS could be used to potentiate antimalarial action of CQ, particularly on CQ resistant strains.

Keywords: Malaria; Chloroquine resistance; Glucose 6-phosphate dehydrogenase; Reduce glutathione; *Plasmodium berghei*; Drug potentiation; Dehydroepiandrosterone sulphate

1. Introduction

Plasmodium falciparum resistance to chloroquine, the traditional first-line antimalarial, is now widespread, while resistance to second-line drugs continues to expand. Cross-resistance between closely related members of the same chemical classes is also present [1]. While several factors concur to generate resistance, it is clear that a better understanding of the mechanisms of action of CQ and how CQ generates resistance from the parasite can help us identify and develop new effective treatments against CQ-resistant malaria.

Both the mode of action and mechanisms of resistance to chloroquine are complex, only partly known, and controversial [2–4]. In general, they are thought to operate

Abbreviations: BSO, buthionine sulfoximine; CQ, chloroquine; CQS, CQ sensitive; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; G6PD, glucose 6-phosphate dehydrogenase; GSH, reduced glutathione; GSSG, glutathione disulfide; Gst, glutathione s-transferase; NADPH, nicotinamide adenosine diphosphate

* Corresponding author. Tel.: 33 5 57 57 12 43; fax: +33 5 57 57 12 10.
E-mail address: isafeukui_no@etud.u-bordeaux2.fr (I. Safeukui).

0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2004.05.049

through separate mechanisms but the two might converge toward a specific point: it has been postulated that the malaria parasites dispose cytotoxic haem (a by-product of haemoglobin degradation) by either packing it up into an insoluble crystal (haemozoin) in the parasite food vacuole [5,6], or by degrading it via hydrogen peroxide in the food vacuole [7] or via reduced glutathione in the cytoplasm [8–10]. In addition to its direct effect on haem, GSH could play a critical role in protecting the parasite against oxidative stress and lipid peroxidation induced by haem itself [11–13]. The correlation between GSH level and chloroquine resistance is supported by the recently reported higher expression of γ-glutamylcysteine synthetase, the first enzyme in the glutathione biosynthetic pathway [14]. To produce GSH from glutathione disulfide (GSSG), glutathione reductase uses nicotinamide adenosine diphosphate (NADPH), which is obtained via the pentose phosphate shunt. G6PD is the key enzyme of pentose phosphate process. A correlation has been shown in cancer cells between increased GSH levels, resistance to oxidant stress and increased G6PD expression [15]. Moreover, the over-expression of G6PD by transcomplementation in GSH depleted cancer cells restored the level of GSH [15]. From the above description, it becomes apparent that the combination of drugs that can deplete parasites from GSH in vivo, via G6PD inhibition could potentiate the antimalarial action of CQ and thus re-establish it as a first line therapy for malaria, irrespective of the localisation of GSH action.

DHEAS and dehydroepiandrosterone (DHEA) are androgen precursors mainly secreted from the adrenal cortex in humans [16]. The amount of DHEAS produced and the serum concentrations of DHEAS are higher than those of any other steroids in the body, and present in adult male plasma at an approximately 6 μM concentration [17,18]. DHEAS was suggested to be a reservoir and a precursor of DHEA, a precursor of sex steroids, which is released by the action of endogenous DHEAS sulfatase, found widely distributed in peripheral tissues [19]. DHEA has a track record of safe clinical use for ageing, cardiovascular illness, and cancer. DHEA and DHEAS are potent inhibitors of G6PD [20]. Reports indicate that inhibition of G6PD activity by DHEA or other analogs induced growth arrest, decreased DNA synthesis, or both in a variety of normal or tumor cell lines and tissues [21–24]. It has been shown that 16alpha-bromoepiandrosterone, an analogue of the human adrenal steroid hormones DHEA and DHEAS was endowed with antimalarial activity against several strains of *P. falciparum* in vitro and against *Plasmodium berghei* in a mouse model [25], possibly mediated by enhanced opsonization and phagocytosis of ring stage parasitized erythrocytes [26,27]. In population-based studies it has been established that inherited deficiency of erythrocyte G6PD confers protection against severe *P. falciparum* malaria [28]. Interestingly, another study [29] has indicated that age-related decreases in the frequency and density of *P. falciparum* parasitaemia were greater during puberty and that the blood level of DHEAS was a significant predictor of resistance. Recently, DHEAS levels has been significantly associated with decreased parasite density and increased haemoglobin level in pubertal girls living in Kenya [30].

In this study, the ability of DHEAS to enhance the antimalarial action of CQ was investigated in *P. berghei* mouse model in vivo. Drug effect was checked both by monitoring the evolution of parasitaemia and the survival of infected mice. We also assessed whether such enhancement could be obtained directly or via inhibition of G6PD activity and GSH level.

2. Materials and methods

2.1. Mice and parasites

Female Swiss mice 6–10 weeks old and 3 *P. berghei* strains were used in this study: a CQ sensitive strain CQS (NK65 from Professor I. Landau, Museum d'Histoire Naturelle, Paris [31]) and two resistant strains obtained from strain NK65 by drug pressure with 6 and 30 mg/kg CQ for at least 30 treatment courses using the methodology described in Peters [32] (herein after referred to the CQR6 and CQR30 lines respectively). The CQR6 and CQR30 strains CQ resistance levels were maintained by CQ pressure during successive passages in naive animals, and it was loosed after about five passages without treatment. All experiments were performed on parasites obtained from infected untreated mice for one passage. In vitro and in vivo sensitivity profile of *P. berghei* CQ sensitive (CQS) and CQ resistant strains (CQR6 and CQR30) are presented in Table 1.

2.2. In vivo study

In order to study the effect of CQ alone or combined with DHEAS or BSO on parasite growth and survival of infected mice, we adapted the test described by Hawking [33].

2.2.1. Effect on parasite growth

Groups of five mice were inoculated with 3×10^7 *P. berghei* CQ30 strain infected erythrocytes. They were then treated with three daily doses (d_0, d_1 and d_2) starting 4 days after infection, with CQ (30 mg/kg/day) alone or combined with either DHEAS (400 mg/kg/day; Sigma) or BSO (1100 mg/kg/day; Sigma). CQ was administrated by intraperitoneal (i.p.) injection, DHEAS and BSO by subcutaneous (s.c.) injection. The effect of BSO was evaluated since it has been shown that it reverses *P. berghei* CQ-resistance [34]. Mice in the control group received NaCl solution (0.9%) s.c., which was also used to dissolve CQ, DHEAS and BSO. Parasitaemia was followed by Giemsa-stained thin blood smears before treatment start (d_0), and

Table 1
Full sensitivity profile of *P. berghei* chloroquine sensitive (CQS) and chloroquine resistant strains (CQR6 and CQR30)

Characteristics	*P. berghei* strains				
	CQS	CQR6[a]	R[a]	CQR30	*R*
In vivo chloroquine sensitivity: ED_{50} (mg/kg)	1.5 ± 0.3	50 ± 7	33.3	>90	>60
In vitro chloroquine sensitivity: IC_{50} (μM)	0.26 ± 0.19	20 ± 1.9	75.8	45 ± 1.8	145.5
In vitro amodiaquine sensitivity: IC_{50} (μM)	0.26 ± 0.03	3.5 ± 0.3	13.4	15.2 ± 2.5	58.0
In vitro quinine sensitivity: IC_{50} (μM)	1.53 ± 0.34	10 ± 0.6	6.5	31 ± 2.7	20.3
In vitro mefloquine sensitivity: IC_{50} (μM)	0.11 ± 0.04	0.2 ± 0.02	1.7	2.5 ± 0.8	21.7

R: resistance index (IC_{50} on each strain/IC_{50} on the CQS line).
[a] Data published by Platel et al. [34].

then 24 h (d_3) and 5 days (d_7) after the last dose of the treatment. Each experiment was performed in triplicate.

2.2.2. Effect on infected mice survival

An identical method was used. The *P. berghei* strain used was NK65. Treatment was either CQ (1 mg/kg/day) alone or combined with DHEAS (400 mg/kg/day), or NaCl solution as control (0.9 %). Mice were followed until death. Each experiment was performed in triplicate.

2.3. Biochemical analysis: effect of DHEAS on intra-parasite G6PD activity and GSH level

Infected mice (20–50% parasitaemia) were divided into two groups, and received for 3 days: DHEAS s.c. (400 mg/kg/day) or NaCl s.c (0.9 %) alone (control). Measurements of G6PD activity and GSH level were done on isolated parasites 24 h after the last dose. All experiments were performed ≥5 times.

2.4. Preparation of isolated parasites

Infected erythrocytes were obtained from mouse whole blood collected by cardiac puncture into a 3.8% solution of sodium citrate (v/v). Leukocytes and platelets were removed as described in Dubois et al. [35]. Cells were washed three times with 0.9% NaCl. A thin smear was made to check for absence of leukocytes. Parasites were isolated by lysis of infected erythrocytes by saponin (0.2%, v/v), during 2 min at room temperature. After lysis, parasites were washed three times with 0.9% NaCl, counted on malassez cell after coloration with "bleu de Una" and diluted in PBS (pH 7.4), in order to obtain a suspension of 10^9 parasites/ml. The purity of the parasite suspension was assessed by detection for acetylcholinesterase using the method of Ellman et al. [36] to estimate contamination by red blood cells.

2.5. Determination of G6PD activity and GSH level

G6PD activity was determined spectrophotometrically (MRP1 G6PD, 124672, Boehringer) based on Löhr and Waller [37]. The parasite suspension (10^9/ml) was lysed by three times 10 s sonication on ice. After centrifugation of the suspension for 5 min at 12000 rpm at 4 °C, the supernatant was used for the determination of G6PD activity. Results were expressed in international units (i.u)/10^9 parasites.

GSH level was measured as previously described in Dubois et al. [35] using the Brehe and Burch method [38]. The suspension of 10^9 parasites/ml was lysed into 1 volume of distilled water and 1 volume of 5-sulfosalycilic acid (0.12%, w/v). The precipitation was allowed to proceed on ice for 10 min. The tube was span for 5 min at 800 rpm and 4 °C, and the supernatant removed and diluted 1/10 for measurement of total GSH. To measure GSSG, the parasites extract was immediately incubated for 1 h at room temperature following the addition of *N*-ethylmaleimide (NEM stock solution 0.05 M) (4 volumes per 10 volumes of sample) to block the reduction to GSH. NEM was extracted five times with an equal volume of ether. GSSG was measured as for total GSH.

2.6. Statistical analysis

Average of asexual parasitaemia and levels of parasite growth inhibition were considered as quantitative variable. Differences between average of G6PD activity, GSH and GSSG levels and asexual parasitaemia means were evaluated with the non-parametric Mann–Whitney *U* test. The Logrank (Mantel-cox) test was used to compare survival between groups. The differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Parasite GSH content and G6PD activity versus CQ resistance

Intraparasite GSH levels and G6PD activity were compared between the CQS, CQR6 and CQR30 strains. GSH levels ($P < 0.02$ for both CQS/CQR6 and CQS/CQR30) and G6PD activity ($P < 0.02$ for both CQS/CQR6 and CQR6/CQR30, and $P < 0.007$ for CQS/CQR30) increased significantly with level of CQ resistance (Figs. 1 and 2). However, no difference was observed in GSH levels between CQR6 and CQR30 strains ($P > 0.7$) (Fig. 1).

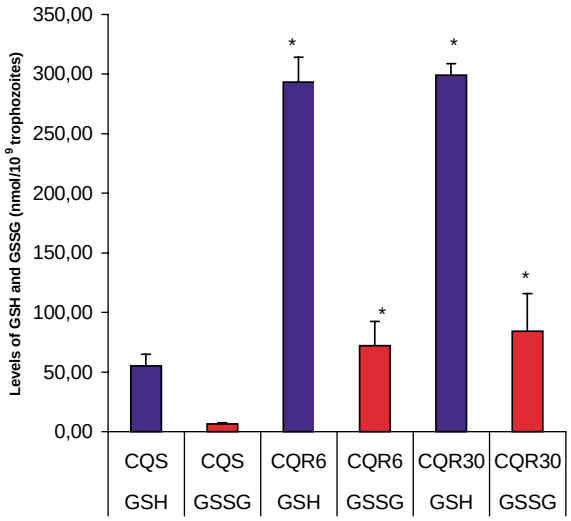

Fig. 1. Levels of GSH and GSSG in trophozoites of *P. berghei* chloroquine sensitive (CQS) and chloroquine resistant (CQR6 and CQR30) strains isolated from infected and untreated mice for one passage. All experiments were done when the parasitaemia reached 20–50%. Columns indicated with symbols (∗) are significantly different in relation to isolated sensitive parasites at the $P < 0.02$. All experiments were performed at least five times.

Likewise intraparasite GSSG levels increased significantly with level of CQ resistance ($P < 0.02$ for both CQS/CQR6 and CQS/CQR30) (Fig. 1). There was no statistically significant difference in the levels of GSSG between CQR6 and CQR30 strains ($P > 0.5$) (Fig. 1).

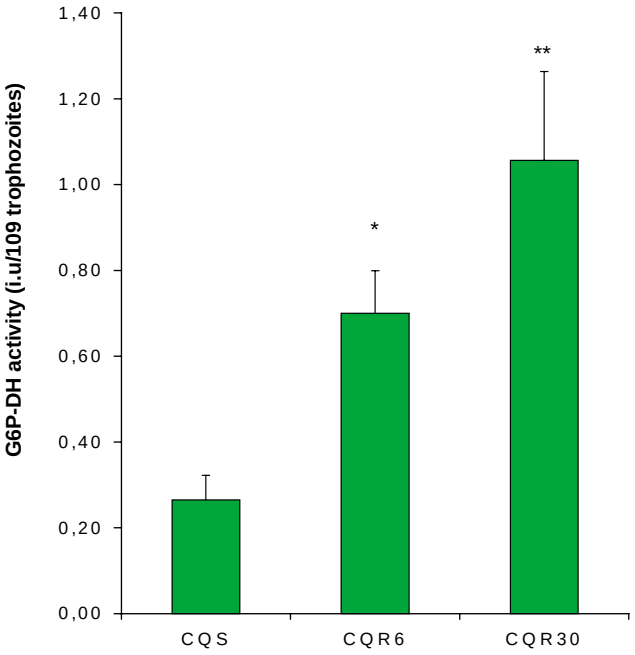

Fig. 2. G6PD activity in trophozoites of *P. berghei* chloroquine sensitive (CQRS) and chloroquine resistant strains (CQR6 and CQR30) isolated from infected and untreated mice for one passage. All experiments were done when the parasitaemia reached 20–50%. Columns indicated with symbols (∗) and (∗∗) are significantly different at the $P < 0.02$ (CQS vs. CQR6) and $P < 0.007$ (CQS vs. CQR30) levels, respectively. All experiments were performed at least five times.

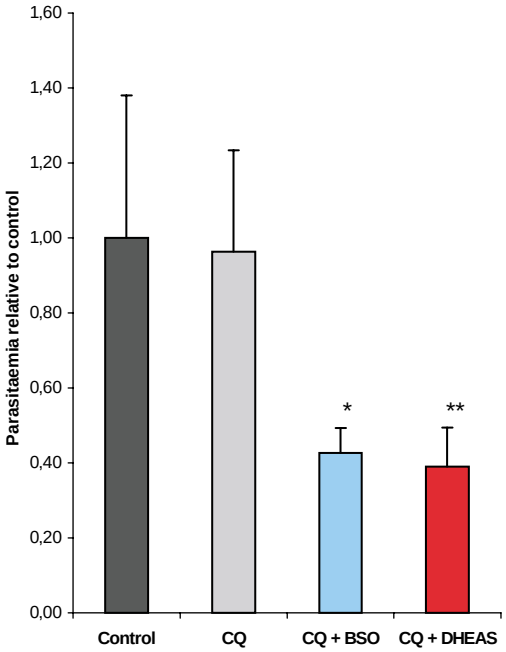

Fig. 3. Study of the reversion of chloroquine resistance by BSO (CQ + BSO) and DHEAS (CQ + DHEAS), as assessed from the degree of parasitaemia relative to untreated mice infected with *P. berghei* CQR30 strain. Results show means of 15 mice ± S.D. in each group. Columns indicated with symbols (∗) and (∗∗) are significantly different at the $P < 0.0001$ and $P < 0.001$ levels, respectively (comparing parasitaemia at day 3 vs. day 0).

3.2. Partial reversion of CQ resistance induced by DHEAS

P. berghei, CQR30 strain, infected mice were treated with CQ plus DHEAS (a specific inhibitor of G6PD activity) or BSO (a specific inhibitor of GSH synthesis). DHEAS and BSO partially increased CQ activity against the highly CQ-resistant strain CQR30 (Fig. 3). Parasitaemia decreased significantly 24 h after administration of the last dose in mice treated with CQ/DHEAS ($d_3 < d_0$ parasitaemia: $P < 0.001$) and those treated with CQ/BSO ($d_3 < d_0$ parasitaemia: $P < 0.0001$). No significant difference was seen between mice treated with CQ/DHEAS (60.96% inhibition) or with CQ/BSO (57.38% inhibition) ($P > 0.2$). In contrast, parasitaemia increased significantly in the same period in CQ-treated and untreated controls ($d_3 > d_0$ parasitaemia: $P < 0.0001$ in both CQ-treated mice and untreated control). In both combination treatment groups, this initial reduction was followed by a significant increase 5 days after the last dose of the treatment ($d_3 < d_7$ parasitaemia: $P < 0.0001$ in both groups of mice treated with CQ/DHEAS and CQ/BSO) (data not shown).

3.3. Effect of DHEAS on mice survival

P. berghei, CQ-sensitive strain, infected mice treated 3 days with CQ alone or associated with DHEAS were followed until death. Infected mice treated with CQ/

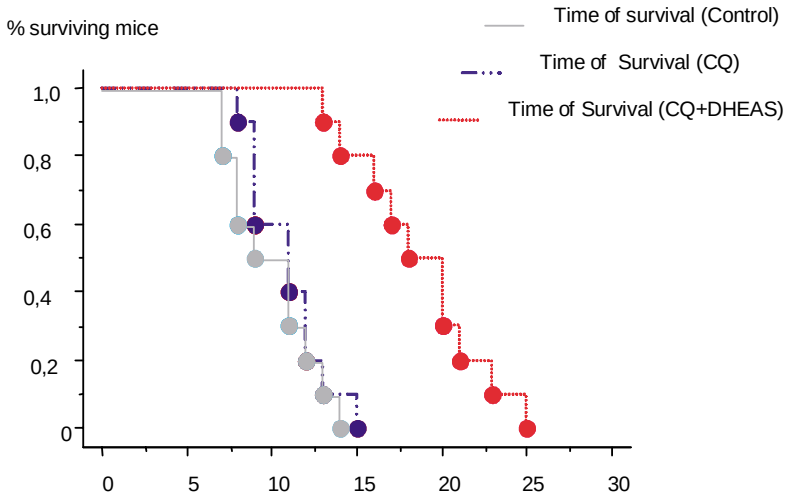

Fig. 4. Times of survival of *P. berghei* NK65 strain infected mice untreated (Control) and after 3 days of treatment with chloroquine (CQ) or drug association chloroquine/DHEAS (CQ + DHEAS). Results obtained from 15 mice of each group.

DHEAS survived longer (1.64 and 2.0 times) than those treated with CQ only or untreated, respectively ($P < 0.0001$). There was no difference between groups treated with CQ only or untreated ($P > 0.2$) (Fig. 4).

3.4. Effect of DHEAS in parasite G6PD activity and GSH and GSSG content

After 3 days of treatment of *P. berghei* CQR strains infected mice with DHEAS, we observed a significant reduction of G6PD activity (Fig. 5) and of GSH level (Fig. 6) in parasites, 24 h after the last treatment. G6PD activity decreased significantly in both CQR6 ($P < 0.02$) and CQR30 ($P < 0.003$) (inhibition level = 37.5 and 70.31% in CQR6 and CQR30, respectively). GSH levels too decreased significantly ($P < 0.004$ and <0.005 for CQR6 and CQR30, respectively; inhibition level = 64.72 and 69% in CQR6 and CQR30, respectively). In contrast, GSSG content increased significantly 24 h after 3 days of treatment of CQR strains of *P. berghei* infected mice with DHEAS (Fig. 6) ($P < 0.004$ and <0.005 for CQR6 and CQR30, respectively).

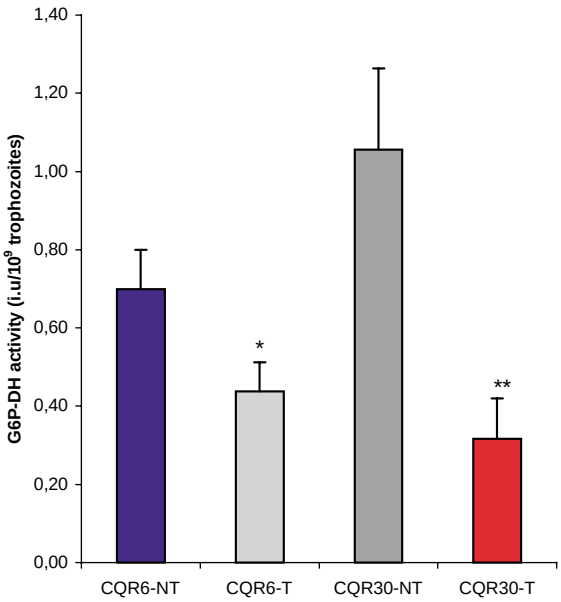

Fig. 5. G6PD activity in trophozoites of *P. berghei* chloroquine resistant strains (CQR6 and CQR30) isolated from infected mice untreated (NT) and after 3 days of DHEAS treatment (T). All experiments were done 24 h after the last treatment. *P*-value < 0.02 (∗) and <0.003 (∗∗) comparing level of G6PD activity between treated and untreated parasites for CQR6 and CQR30, respectively. All experiments were performed at least five times.

4. Discussion

Our group has previously reported that the acquisition of CQ resistance in *P. berghei* was associated with an increase in GSH [10,35]. This high level of CQ resistance can partially be reversed by treatment with butionin sulfoximin (BSO), a specific inhibitor of GSH synthesis [34]. Concomitantly, the resistance reversal effect of verapamil is lost [34]. We further hypothesised that inhibition of G6PD activity in CQ-resistant *P. berghei* could reduce GSH production and consequently increase parasite sensitivity to CQ. The present study demonstrates that selection of high CQ resistant strains of *P. berghei* by CQ pressure correlates with a significant increase of the parasite G6PD activity, thus resulting in increased GSH levels. We further confirm that CQ-resistant *P. berghei* produces significantly more GSH than sensitive strains, though no correlation with the degree of resistance was apparent.

It is generally accepted that CQ forms adducts with haem [11,12]. The cascade of events leading to parasite death is not clearly identified. There are indications that crystallisation does not account for all haem disappearance and the alternative pathways of disposal might exist,

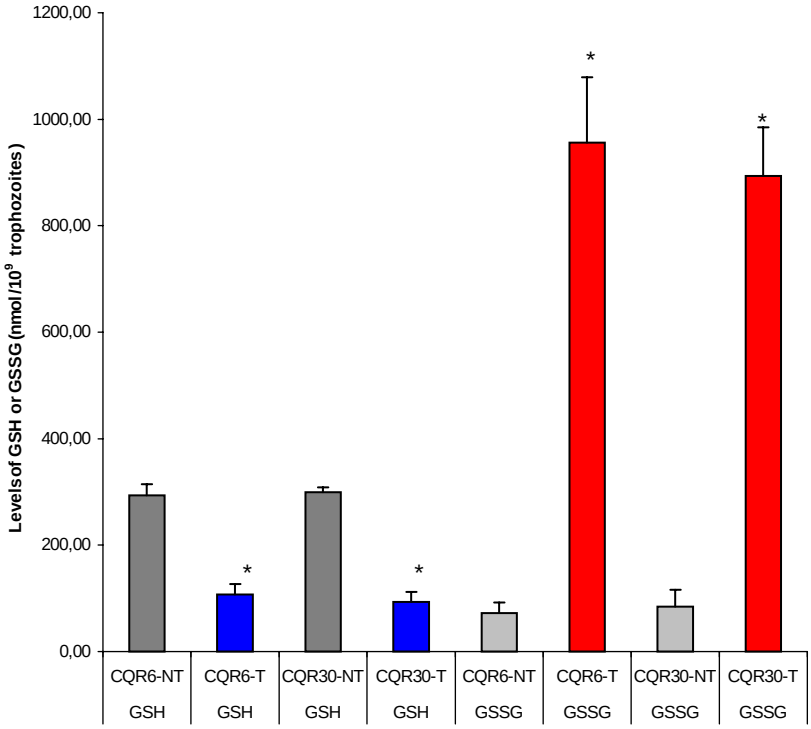

Fig. 6. Levels of GSH and GSSG in trophozoites of *P. berghei* chloroquine resistant strains (CQR6 and CQR30) isolated from infected mice untreated (NT) and after 3 days of DHEAS treatment (T). All experiments were done 24 h after the last treatment. Columns indicated with symbols (∗) are significantly different between treated and untreated isolated parasites at the $P < 0.005$. All experiments were performed at least five times.

namely via GSH [8–10] and/or H_2O_2 [7]. Although it is not fully explained how GSH would degrade haem, GSH content is higher in CQ resistant parasites than in CQS ones. So it is possible that, resistance may be associated with a shift from crystallisation to GSH- and Gst-mediated disposal of haem in our model [10]. Three observations are in agreement with this hypothesis: (i) the reduced haemozoin synthesis and increased GSH level and Gst activity in *P. berghei* infecting reticulocytes (richer in GSH than mature erythrocytes) [10], (ii) the apparent disappearance of the malaria pigment observed by microscopy (data not shown), and (iii) this malaria pigment reappears when these CQ-resistant strains (CQR6 and CQR30) loosed their CQ resistance (data not shown). In addition, how such event can justify decreased parasite response to CQ remains unclear. One must assume that CQ is less effective in competing with GSH [10] than in inhibiting crystallisation. Alternatively, because of its weak-base properties, it might just be less concentrated in the parasite cytoplasm, or the CQ/haem complex could be degraded by GSH. On the other hand, various mechanisms could be postulated that are not directly related to haem degradation: the higher content in GSH could just provide the parasite with extra anti-oxidant defence against toxic chloroquine-haem adducts, although peroxidation has recently been questioned as a mechanism of action for CQ [39]. Obviously, resistance to CQ is known to be multigenic and multifactorial [40,41].

We also observed that DHEAS (a specific inhibitor of G6PD activity) and BSO (a specific inhibitor of GSH synthesis) enhanced the antimalarial action of CQ on *P. berghei* CQ-resistant strains, by inhibiting parasite growth. Moreover, the survival of mice infected with CQ-sensitive strains was significantly prolonged when treated with otherwise ineffective CQ regimen combined with DHEAS. These results are in agreement with recent findings which shown that some inhibitors of GSH synthesis such as BSO [10], acetaminophen, indomethacin and disulfiram [42], enhanced the antimalarial action of CQ in vivo. Both DHEAS and BSO deplete the parasite in GSH. According to our results, DHEAS action resulted into an accumulation of GSSG within the parasite. This accumulation of GSSG indirectly translates the reduction of NADPH, which is necessary for the reduction of GSSG to GSH. This phenomenon would deprive *Plasmodium* from GSH that normally protects it against toxicity of free and complexed heam. This altered levels of GSH is not the only mechanism, since it has been shown that G6PD deficiency is known to exert antimalarial protection, via enhanced opsonization and phagocytosis of rings, the early parasite stage within the erythrocytes [26,27]. These mechanisms could partially help explain resistance against malaria observed frequently in malaria endemic areas, in people presenting with hereditary deficit in G6PD [28,43,44].

The effect of DHEAS was partial and transient. This could be related to the drug regimen chosen in our study, or

to alternative pathways for NADPH reduction, notably those involving isocitrate dehydrogenase and glutamate dehydrogenase [45,46]. Other mechanisms would be implicated in this phenomenon because our CQR6 and CQR30 strains are also resistant to amodiaquine, mefloquine and quinine. Finally, the parasite obviously possesses other means, such as mutations in the Pgh1 drug pump [40] and in the membrane protein pfcrt [41].

DHEA has a track record of safe clinical use for ageing, cardiovascular illness, and cancer. The effects of DHEA, an androgen hormonal precursor produced by the surrenal glands, are complex. In addition to its inhibitory effect on G6PD activity, DHEAS has a potent immunomodulatory effects and may be in the causal pathway that allowed the induction of protective immune responses. These immunomodulatory effects include activation of T cell function and increased antibody response [47,48]; production of nitric oxide (NO), a vasodilator involved in the control of inflammatory reactions and infection [49,50], such as *falciparum* malaria [51]; increased cytotoxicity of natural killer cells (NK) by insulin like growth factor I (IGF-I) activation [52]. Moreover, a DHEA analogue (16alpha-bromoepiandrosterone) was shown to inhibit in vitro growth of *P. falciparum* and *P. berghei* [25]. Kurtis et al. [29] have shown that postpubertal levels of DHEAS in plasma were significant predictors of resistance against malaria, with respect to lower frequency and density of parasitaemia in multiple comparisons. Recently, it has also been found that DHEAS level was an independent predictor of malaria parasite density in the parasitemic schoolgirls from Kenya who had entered puberty [30]. These authors found that DHEAS levels were significantly associated with decreased parasite density and increased haemoglobin level in pubertal girls [30]. Thus, the effects of DHEAS observed in this study may also relate to enhanced protective immune response against malaria parasite.

In conclusion, *P. berghei* acquisition of CQ resistance is associated with the increase of GSH level and G6PD activity. This study underlines that DHEAS can be used to potentiate the antimalarial action of CQ in vivo, particularly on CQ resistant strains. DHEAS acts on malaria parasite through intra-parasite depletion of GSH by inhibition of G6PD activity or, through induction of protective immune response, which has also been shown to play a critical role in the clearance of resistant *P. falciparum* infections [53]. It suggests that combination of CQ with this drug can serve to treat patients of *P. falciparum* malaria that are infected with CQ-resistant strains, and thus limit the spreading of resistant parasite. However, more data are needed to assess the potential of combining classical antimalarials with drugs affecting GSH. Additional evidence that such mechanisms occur in human malaria infection should be provided to support the rationale for the use of such association, knowing that DHEAS is widely used in many countries as anti-oxidant supplement in humans.

Acknowledgments

This study was financed by the AUPELF-UREF and the French Ministry of Foreign affairs (FSP 97008500). Innocent Safeukui-Noubissi was supported by a fellowship from the AUPELF-UREF.

References

[1] Olliaro P, Cattani J, Wirth D. Malaria, the submerged disease. J Am Med Assoc 1996;275:230–3.
[2] Ginsburg H, Krugliak M. Quinoline containing antimalarials: mode of action, drug resistance and its reversal. An uptake with unresolved puzzles. Biochem Pharmacol 1992;43:63–70.
[3] Ginsburg H, Famin O, Zhang J, Krugliack M. Inhibition of glutathion-dependent degradation of heme by chloroquine and amodiaquine as a possible basis for their antimalarial mode of action. Biochem Pharmacol 1998;56:1305–13.
[4] Olliaro P. Mode of action and mechanisms of resistance for antimalarial drugs. Pharmacol Ther 2001;2:207–19.
[5] Slater AF, Cerami A. Inhibition by chloroquine of a novel haem polymerase enzyme activity in malaria trophozoites. Nature 1992;355:167–9.
[6] Pagola S, Stephens PW, Bohle DS, Kosar AD, Madsen SK. The structure of malaria pigment beta-haematin. Nature 2000;404:307–10.
[7] Loria P, Miller S, Foley M, Tilley L. Inhibition of peroxydative degradation of haem as the basis of action of chloroquine and other quinoline antimalarials. Biochem J 1999;339:363–70.
[8] Atamna H, Ginsburg H. Heme degradation in the presence of glutathione. A proposed mechanism to account for the high levels of non-heme iron found in the membranes of haemoglobinopathic red blood cells. J Biol Chem 1995;270:24876–83.
[9] Ginsburg H, Ward SA, Bray PG. An integrated model of chloroquine action. Parasitol Today 1999;15:357–60.
[10] Platel DFN, Mangou F, Tribouley-Duret J. Role of glutathione in the detoxification of ferriprotoporphyrin IX in chloroquine resistance *Plasmodium berghei*. Mol Biochem Parasitol 1999;98:215–23.
[11] Vander-Jagt DL, Hunsaker LA, Campos NM. Characterisation of a haemoglobin-degradation, low molecular weight protease from *Plasmodium falciparum*. Mol Biochem Parasitol 1986;18:389–400.
[12] Sugioka Y, Suzuki M. The chemical basis for FPIX-CQ complex induced lipid peroxidation. Biochem Biophys Acta 1991;1074:19–24.
[13] Omodeo-Sale F, Monti D, Olliaro P, Taramelli D. Prooxidant activity of beta-hematin (synthetic malaria pigment) in arachidonic acid micelles and phospholipid large unilamellar vesicles. Biochem Pharmacol 2001;8:999–1009.
[14] Perez-Rosado J, Gervais GW, Ferrer-Rodriguez I, Peters W, Serrano AE. *Plasmodium berghei*: analysis of the gamma-glutamylcysteine synthetase gene in drug-resistant lines. Exp Parasitol 2002;101:175–82.
[15] Salvemini F, Franze A, Iervolino A, Filosa S, Salzano S, Ursini MV. Enhanced glutathion levels and oxydoresistance mediated by increased glucose-6-phosphate dehydrogenase expression. J Biol Chem 1999;274:2750–7.
[16] Nieschlag E, Loriaux DL, Ruder HJ, Zucker IR, Kirschner MA, Lipsett MB. The secretion of dehydroepiandrosterone and dehydroepiandrosterone sulphate in man. J Endocrinol 1973;57:123–34.
[17] Meikle AW, Daynes RA, Araneo BA. Adrenal androgen secretion and biologic effects. Endocrinol Metab Clin N Am 1991;20:381–400.
[18] Nafziger AN, Bowlin SJ, Jenkins PL, Pearson TA. Longitudinal changes in dehydroepiandrosterone concentrations in men and women. J Lab Clin Med 1998;131:316–23.
[19] Miki Y, Nakata T, Suzuki T, Darnel AD, Moriya T, Kaneko C, et al. Systemic distribution of steroid sulfatase and estrogen sulfotransferase in human adult and fetal tissues. J Clin Endocrinol Metab 2002;87:5760–8.

[20] Gordon G, Mackow MC, Levy HR. On the mechanism of interaction of steroids with human glucose 6-phosphate dehydrogenase. Arch Biochem Biophys 1995;318:25–9.
[21] Gordon GB, Shantz LM, Talalay P. Modulation of growth, differentiation and carcinogenesis by dehydroepiandrosterone. Adv Enzyme Regul 1987;26:355–82.
[22] Henderson E, Schwartz A, Pashko L, Abou-Gharbia M, Swern D. Dehydroepiandrosterone and 16 alpha-bromo-epiandrosterone: inhibitors of Epstein-Barr virus-induced transformation of human lymphocytes. Carcinogenesis 1981;2:683–6.
[23] Rais B, Comin B, Puigjaner J, Brandes JL, Creppy E, Saboureau D, et al. Oxythiamine and dehydroepiandrosterone induce a G1 phase cycle arrest in Ehrlich's tumor cells through inhibition of the pentose cycle. FEBS Lett 1999;456:113–8.
[24] Svec F, Porter JR. The actions of exogenous dehydroepiandrosterone in experimental animals and humans. Proc Soc Exp Biol Med 1998; 218:174–91.
[25] Freilich D, Ferris S, Wallace M, Leach L, Kallen-Frincke J, Ahlem C, et al. 16alpha-bromoepiandrosterone, a dehydroepiandrosteron (DHEA) analogue, inhibits *Plasmodium falciparum* and *Plasmodium berghei* growth. Am J Trop Med Hyg 2000;63:280–3.
[26] Cappadoro M, Giribaldi G, O'Brien E, Turrini F, Mannu F, Ulliers D, et al. Early phagocytosis of glucose-6-phosphate dehydrogenase (G6PD)-deficient erythrocytes parasitized by *Plasmodium falciparum* may explain malaria protection in G6PD deficiency. Blood 1998; 92:2527–34.
[27] Ayi K, Giribaldi G, Skorokhod A, Schwarzer E, Prendergast PT, Arese P. 16alpha-bromoepiandrosterone, an antimalarial analogue of the hormone dehydroepiandrosterone, enhances phagocytosis of ring stage parasitized erythrocytes: a novel mechanism for antimalarial activity. Antimicrob Agents Chemother 2002;46:3180–4.
[28] Greene LS. G6PD deficiency as protection against falciparum malaria: an epidemiologic critique of population and experimental studies. Yearb Phys Anthropol 1993;36:153–78.
[29] Kurtis JD, Mtalib R, Onyango FK, Duffy PE. Human resistance to *Plasmodium falciparum* increases during puberty and is predicted by dehydroepiandrosterone sulfate levels. Infect Immun 2001;69:123–8.
[30] Leenstra T, ter Kuile FO, Kariuki SK, Nixon CP, Oloo AJ, Kager PA, et al. Dehydroepiandrosterone sulfate levels associated with decreased malaria parasite density and increased hemoglobin concentration in pubertal girls from western Kenya. J Infect Dis 2003;188:297–304.
[31] Landau I, Chabaud AG. Latency of *Plasmodium* merozoites and drug-resistance. Parasite 1994;1:105–14.
[32] Peters W. The chemotherapy of rodent malaria. II. Host–parasite relationship. Part 2. The relationship between chloroquine sensitivity and the age of the host cell. Ann Trop Med Parasitol 1968;62:246–51.
[33] Hawking F, Worms MJ, Gammage K, Goddard PA. The biological purpose of the blood cycle of the malaria parasite *Plasmodium cynomolgi*. Lancet 1966;2:422–4.
[34] Platel DF, Mangou F, Tribouley-Duret J. High-level chloroquine resistance of *P. berghei* is associated with multiple drug resistance and loss of reversal by calcium antagonist. Int J Parasitol 1998;28: 641–51.
[35] Dubois VL, Platel DFN, Pauly G, Tribouley-Duret J. *Plasmodium berghei*: implication of intracellular glutathion and its related enzyme in chloroquine resistance in vivo. Exp Parasitol 1995;81:117–24.
[36] Ellman GL, Courtney KD, Andrews V, Feath-Erstone RM. A new rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88–95.
[37] Löhr GW, Waller MD. Glucose-6-phosphate dehydrogenase. In: Bergmeyer HV, editor. Methods of enzymatic analysis. New York: Academic Press; 1974. p. 636.
[38] Brehe JE, Burch HB. Enzymatic assay for glutathione. Anal Biochem 1976;74:189–97.
[39] Monti D, Basilico N, Parapini S, Pasini E, Olliaro P, Taramelli D. Does chloroquine really act through oxidative stress? FEBS Lett 2002; 522:3–5.
[40] Reed MB, Saliba KJ, Caruana SR, Kirk K, Cowman AF. Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. Nature 2000;403:906–9.
[41] Sidhu AB, Verdier-Pinard D, Fidock DA. Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by pfcrt mutations. Science 2002;298:210–3.
[42] Deharo E, Barkan D, Krugliak M, Golenser J, Ginsburg H. Potentiation of the antimalarial action of chloroquine in rodent malaria by drugs known to reduce cellular glutathione levels. Biochem Pharmacol 2003;66:809–17.
[43] Friedman MJ. Oxydant damage mediates variant red cell resistance to malaria. Nature 1979;280:245–7.
[44] Miller J, Golenser J, Spira DT, Kosower N. *Plasmodium falciparum*: thiol status and growth in normal and G6PDH deficient human erythrocytes. Exp Parasitol 1984;57:239–47.
[45] Langer BW, Phisphumvidhi P, Jiamparmpoon D. Malarial parasite metabolism: the glutamic acid dehydrogenase of *Plasmodium berghei*. Exp Parasitol 1970;28:298–303.
[46] Golenser J, Marva E, Chevion M. The survival of *Plasmodium* under oxidant stress. Parasitol Today 1991;7:142–6.
[47] Evans TG, Judd ME, Dowell T, Poe S, Daynes RA, Areneo BA. The use of oral dehydroepiandrosterone sulfate as an adjuvant in tetanus and influenza vaccination of the elderly. Vaccine 1996;16:1531–7.
[48] Somekh E, Dagan R, Hanukoglu A. The effect of endogenous dehydroepiandrosterone sulfate on antibody response to hepatitis B vaccine in neonates. Isr Med Assoc J 2000;3:200–2.
[49] Taylor-Robinson AW, Looker M. Sensitivity of malaria parasites to nitric oxide at low oxygen tensions. Lancet 1998;351:1630.
[50] Manabe A, Hata T, Yanagihara T, Hashimoto M, Yamada Y, Irikomo S, et al. Nitric oxide synthesis is increased after dehydroepiandrosterone sulfate administration in term human pregnancy. Hum Reprod 1999;8:2116–9.
[51] Anstey NM, Weinberg JB, Hassanali MY, Mwaikambo ED, Manyenga D, Misukonis MA, et al. Nitric oxide in Tanzanian children with malaria: inverse relationship between malaria severity and nitric oxide production/nitric oxide synthase type 2 expression. J Exp Med 1996; 184:557–67.
[52] Solerte SB, Fioravanti M, Vignati G, Giustina A, Cravello L, Ferrari E. Dehydroepiandrosterone sulfate enhances natural killer cell cytotoxicity in humans via locally generated immunoreactive insuline-like growth factor I. J Clin Endocrinol Metab 1999;9:3260–7.
[53] Djimde AA, Doumbo OK, Traore O, Guindo AB, Kayentao K, Diourte Y, et al. Clearance of drug-resistant parasites as a model for protective immunity in *Plasmodium falciparum* malaria. Am J Trop Med Hyg 2003;69:558–63.