Short communication

Implication of intracellular glutathione and its related enzymes on resistance of malaria parasites to the antimalarial drug arteether

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A B S T R A C T
The control of malaria has been complicated by the increasing resistance of malarial parasites to multiple drugs. However, artemisinin-based drugs offer hope in the fight against drug-resistant parasites. The mode of action of these drugs remains unclear, but evidence suggests a role for free radicals in their mechanism of action. In this study, we examined the relationship between the intracellular levels of glutathione (GSH) and antioxidant enzymes and resistance to the artemisinin-based drug arteether in experimentally selected arteether-resistant Plasmodium vinckei. GSH plays a critical role in the detoxification and protection of cells against oxidative stress. Our comparative studies showed a significant (2.9-fold) increase in the GSH level in arteether-resistant parasites as compared to arteether-sensitive parasites. Simultaneously, significantly increased activities of glutathione reductase, glutathione-S transferase and glucose-6-phosphate dehydrogenase and decreased activity of superoxide dismutase were recorded in resistant parasites; the activity of glutathione peroxidase was comparable in arteether-sensitive and -resistant parasites. Artemisinin derivatives act by generating free radicals and our results indicate that glutathione's antioxidant effects may counteract that drug effect and thereby contribute to the parasites’ resistance to arteether and other artemisinin-based antimalarials.

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1. Introduction

Malaria continues to be the most devastating disease in tropical countries, despite the availability of many classes of antimalarial drugs [1]. The most critical issue limiting the success of malaria treatment is the rapid spread of resistance to most of these antimalarials [2]. The World Health Organization (WHO) has recommended switching to artemisinin-based combination drug therapy in areas where the parasite no longer responds to conventional treatments [3]. Artemisinin derivatives are recognized as the most potent and rapidly acting antimalarial drugs and to date, no cases of clinical resistance to these drugs have been reported. However, variations in sensitivity to two of these drugs artemether and artesunate have been reported in Plasmodium falciparum isolates from French Guinea and Brazil respectively [4,5]. Recently, it was reported that in Western Cambodia, P. falciparum has shown reduced in vivo susceptibility to artesunate, as evidenced by slow parasite clearance [6], suggesting that the parasite may eventually develop resistance to this class of compounds as well. Studies in experimental rodent malaria models have indicated that P. yoelli shows decreased sensitivity to artemisinin [7,8] and stable resistance to artemisinin and artesunate has also been documented in P. chabaudi [9]. Single point mutations have been found to occur in the PfATP6 gene in artemisinin-resistant parasites [4]; however, Afonso et al. [9] saw no alterations in the sequence or copy number of any specific genes in artemisinin-resistant P. chabaudi. Thus, although these resistant parasites have been employed to explore the genetic basis of resistance [9–11], conclusive evidence linking any particular mutation to artemisinin resistance is still lacking [12]. Recent compelling evidence [13] has indicated that artemisinin-based drugs act by reversibly inhibiting a Ca ++-dependent ATPase, the ortholog of mammalian sarco-endoplasmatic reticulum ATPase (SERCA) in the parasite. Eckstein-Ludwig et al. [14] also reported that artemisinin inhibits SERCA in P. falciparum and is involved in the negative regulation of a cationic pump in P. yoelli [15]. Touyz [16] noted that increased levels of reactive oxygen species (ROS) inhibit SERCA activity.

We previously reported the selection of a Plasmodium vinckei strain showing stable resistance to arteether [17]. The present study was aimed at understanding the mechanism by which malarial parasites become resistant to arteether. Since ROS appear to be involved in the mode of action of the artemisinin class of antimalarials and GSH and antioxidant enzymes are directly involved in protecting cells against oxidative stress, we have compared the levels of GSH and the activity of antioxidant enzymes, glutathione reductase (GR), glutathione peroxidase (GPx), glutathione-S-transferase (GST), superoxide dismutase
were measured by spectrophotometer methods as reported earlier [21]. The specific activities of SOD (E.C.1.15.1.1), GR (E.C.1.1.1.4), GPx (E.C. 1.11.1.9) and GST (E.C.2.5.1.18) were measured by the Brøhe and Burch methods [21]. The specific activities of SOD (E.C.1.15.1.1), GR (E.C. 1.1.1.4), GPx (E.C. 1.11.1.9) and GST (E.C.2.5.1.18) were measured by spectrophotometer methods as reported earlier [22–26]. The samples for the SOD assay were partially purified as described by Kakkar et al. [27]. Students’ tests were used to analyze the significance of difference between values for PvAR and PvAS parasites.

2. Materials and methods

Swiss albino mice of between 24 and 26 g body weight were obtained from the Division of Laboratory Animals, Central Drug Research Institute, Lucknow, India. Mice were housed in the animal house at the institute and care was provided as per the guidelines laid down by the ethics committee. Infections were initiated in mice by the intraperitoneal inoculation of 1×10⁶ P. vinckei-infected erythrocytes. Parasitemia was monitored by microscopic examination of Giemsa-stained thin blood smears. The PvAR strains were maintained by arteether pressure during successive passages in naive animals. All experiments were performed on parasites obtained from infected and untreated mice for one passage.

Blood from 5 to 10 mice either infected or uninfected was pooled in acid citrate dextrose (ACD) and centrifuged at 2000×g for 10 min at 4 °C. The plasma anduffy coat were removed and the erythrocyte pellet was suspended in phosphate-buffered saline (PBS) and passed through a CF-11 column (Sigma; Cat # C6288) to remove leukocytes [19]. All enzymatic studies and measurement of total GSH were done through a CF-11 column (Sigma; Cat # C6288) to remove leukocytes [21]. The specific activities of SOD (E.C.1.15.1.1), GR (E.C. 1.1.1.4), GPx (E.C. 1.11.1.9) and GST (E.C.2.5.1.18) were measured by spectrophotometer methods as reported earlier [22–26]. The samples for the SOD assay were partially purified as described by Kakkar et al. [27]. Students’ tests were used to analyze the significance of difference between values for PvAR and PvAS parasites.

3. Results

The sensitivity of the parasites to arteether was tested before and during the exposure to the drug. Sensitivity to arteether was tested at the 135th sequential passage administered to a group of PvAR infected mice at dose 60 mg/kg for four consecutive days from day 0 to 3 and mice became patent on day 16 (Table 1). The result shows that sensitivity of arteether in PvAR strain is ~24-fold higher than the PvAS strain. To confirm the stability of the resistance, the parasites were maintained without drug pressure (i.e., in the absence of drug) for a period of ~300 days from the 82nd passage. Sensitivity to arteether was monitored at 50, 100, 150, 200 and 300 days after the drug treatment was stopped (Fig. 1). The results showed that the sensitivity was not significantly altered during the first 200 days after treatment at 10 or 20 mg/kg/day for four days and the mice became patent within 10 days. However, a marginal loss of sensitivity was recorded after 300 days, since patent infection developed only in the mice treated at 5 mg/kg for 4 days (Table 2).

Pilot studies estimating the glutathione content in blood from mice infected with arteether-sensitive and -resistant parasites demonstrated a sequential increase in GSH levels in PvAR samples, directly proportional to the rise in parasitemia. The levels increased from 276.6±94.36 nmol/10⁹ cells at 5% parasitemia to 492.3±33.07 nmol/10⁹ cells at 20% parasitemia, which is 16% and 53% higher than uninfected blood samples (230.7±41.65 nmol/10⁹ cells) respectively (Fig. 2). The total

![Table 1](image)

<table>
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<th>Number of mice positive/number inoculated on day</th>
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<td>6</td>
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![Table 2](image)

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<th>Number of mice positive/number inoculated on day</th>
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</table>

![Fig. 1](image)

Fig. 1. Assessment of the response to arteether in drug-free parasites (passage in the absence of drug) at different intervals.
GSH level in PvAS infected blood is $213.3 \pm 21.33$, $167.1 \pm 12.82$ and $218.0 \pm 16.71$ nmol/10^9 cells at 5, 10 and 20% parasitemia. Thus results showed that there was no statistically significant difference of the GSH level between PvAS infected and uninfected blood samples (p > 0.5). Analysis of the total GSH content in free parasite preparations showed $260.0 \pm 26.21$ nmol/10^9 cells in PvAR parasites and $87.91 \pm 4.46$ nmol/10^9 cells in PvAS parasites. An almost three-fold higher level in the PvAR parasites: p < 0.0001; Fig. 3). These results indicated that resistance to arteether might be associated with GSH. Our assays of antioxidant enzymes indicated a significant increase in the activity of GST (three-fold; p < 0.001), GR (2.2-fold; p < 0.001) and G6PDH (2.1-fold; p < 0.001) and a marked decrease (2.4-fold; p < 0.001) of the SOD activity in the resistant parasites (Table 3). The activity of Gpx was comparable in the arteether-sensitive and -resistant parasite preparations (p < 0.5).

4. Discussion

The present study has demonstrated that the selection of arteether-resistant strains of P. vinckei by means of drug pressure is correlated with a significant increase in the parasite’s antioxidant enzymatic activity, leading to increased total GSH levels. GSH plays a significant role in protecting cells against ROS-related oxidative stress by detoxifying cytosolic organic peroxides; and the major mechanism by which artemisinin derivatives kill malarial parasites is the production of ROS, which are responsible for reducing antioxidants and glutathione [28]. As mentioned earlier, free radical intermediates of artemisinin-based drugs appear to be involved in the drugs’ antimalarial activity and consistent with this drug effect. Wang and Wu [29] have observed that artemisinin treatment involves the formation of a novel adduct between C-centered free radicals and S-centered free radicals in the presence of catalytic amounts of ferrous sulfate.

Increased levels of GSH and changes in antioxidant enzymatic activities have also been reported in chloroquine-resistant strains of P. falciparum [30], P. berghei [31] and P. chabaudi [32]. The relatively high concentration of GSH that has been observed in the infected RBC might play a role in protection of the parasites from free heme during hemoglobin digestion [33,34]. We also observed that PvAR strains showed enhanced GST activity when compared to PvAS parasites. GST is a detoxification enzyme that catalyzes the conjugation of glutathione (GSH) to a wide range of electrophilic substrates [35]. GST activity, together with increased GSH levels, plays an important role in the development of resistance toward chemotherapeutic agents in malaria [36,37]. Furthermore, the high GR activity and insignificant change in Gpx in the PvAR parasites may help to maintain their reducing environment. The reason for the reduction in SOD activity in PvAR parasites may be a lower level of production of superoxide ions during hemoglobin digestion.

Taken together, the enzymatic changes and increased GSH levels that we identified in resistant P. vinckei would produce a more favorable environment for the survival of the resistant parasites in the presence of lethal concentrations of arteether. Thus, our present work offers additional insights to further explain the complex antioxidant-related mechanism behind the malarial parasite’s resistance to artemisinin derivatives.

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References

[3] WHO: the use of ACT in order to ‘provide effective treatment against malaria and to slow the spread of drug resistance’ in a statement released on 20 February 2002.


