EFFECT OF DIHYDROARTEMISININ ON THE ANTIOXIDANT CAPACITY OF *P. FALCIPARUM*-INFECTED ERYTHROCYTES

Wanida Ittarat, Apichai Sreepian, Apapan Srisarin and Kritsana Pathepchotivong

Department of Clinical Microscopy, Faculty of Medical Technology, Mahidol University, Bangkok, Thailand

Abstract. Many lines of evidence reveal that artemisinin, an antimalarial containing endoperoxide, generates free radicals to kill malaria parasites. The present study re-evaluated the antioxidants of *P. falciparum*-infected erythrocytes in the absence and presence of 0.25, 0.5 and 1.0 ng/ml of dihydroartemisinin (DHA), the active metabolite of artemisinin. The ratio of reduced to oxidized glutathione (GSH/GSSG) and activities of superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) were determined. The data indicated that malaria infection induced oxidative stress in erythrocytes that resulted in a significant lower GSH in parasitized cells compared to the non-parasitized. DHA showed no effect on the antioxidant levels of non-parasitized erythrocytes treated under similar conditions as *P. falciparum*-infected erythrocytes. However, significantly lower GSH as well as catalase and GPx activities in parasitized cells were seen at drug concentrations of 0.5 and 1.0 ng/ml (p < 0.05). GSH is the most sensitive indicator of oxidative stress in malaria-infected erythrocytes both in the absence and in the presence of DHA. Parasite GPx might play a more important role than catalase in the elimination of peroxide. Parasite viabilities in the presence of DHA were analyzed simultaneously and were affected to a greater extent than the antioxidant levels. The present observation showed that although DHA killed malaria parasites by generating free radicals from the endoperoxide bridge causing the reduction of antioxidants, but the depletion of parasite antioxidants is not a prerequisite for the parasite death.

INTRODUCTION

Artemisinin, a sesquiterpene endoperoxide derived from a traditional Chinese herbal remedy, is a new promising antimalarial, useful against multidrug resistant *P. falciparum* (Meshnick, 1998; Olliaro *et al*, 2001). The endoperoxide can generate carbon-centered free radicals that may kill the parasite by alkylating one or more essential malarial proteins (Asawamahasakda *et al*, 1994). It is also possible that artemisinin derivatives may exert their effect by inhibition of the parasite heme polymerization (Berman and Adams, 1997). Despite widespread clinical use, the mode of artemisinin action is not completely understood but it is clear that the drug is a pro-oxidant (Olliaro *et al*, 2001). We investigated whether its antimalarial properties could aggravate oxidative stress in malaria-infected erythrocytes by interfering with the parasite antioxidant defence. We measured the activities of total superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) as well as the ratio of reduced to oxidized glutathione (GSH/GSSG) of erythrocytes harboring the trophozoite of *P.falciparum* in the presence and absence of dihydroartemisinin (DHA), an active metabolite of artemisinin.

MATERIALS AND METHODS

Chemicals

Unless specified, all chemicals used in the present study were purchased from Sigma (St Louis, MO, USA). All solutions used for enzymatic assays and GSH/GSSG determination were prepared using deionized water.

Malaria cultivation

The laboratory strain of *P. falciparum* (K1) was maintained in continuous culture according to the method of Trager and Jensen (1976). The parasites were grown in RPMI 1640 medium (GIBCO Labs, Green Island) supplemented with

Correspondence: Dr Wanida Ittarat, Department of Clinical Microscopy, Faculty of Medical Technology, Siriraj Hospital, Mahidol university. Bangkok 10700, Thailand.
Tel: 66 (0) 2419 7169; Fax: 66 (0) 2412 4110
E-mail: mtwit@mahidol.ac.th

10% heat-inactivated human O serum. Erythrocytes were washed 3 times with isotonic phosphate buffer. Centrifugations were performed at 1,400g at 4ºC. Parasite culture was synchronized by sorbitol lysis as described previously (Lambros and Vanderberg, 1979).

Protein concentration was determined by Lowry's method (Lowry *et al*, 1951) with bovine serum albumin as standard.

Effect of dihydroartemisinin on antioxidants of uninfected erythrocytes

The erythrocytes from the same donor and kept as those used in parasite culture were diluted to 1.5% hematocrit and treated with dihydroartemisinin to final concentrations of 0.25, 0.5 and 1.0 ng/ml. Controls were untreated erythrocytes and erythrocytes treated with 1% DMSO. The treated cell suspension was kept in a candle jar at 37ºC for 18-20 hours and collected for estimation of antioxidants.

Effect of dihydroartemisinin on antioxidants of parasitized erythrocytes

The highly synchronized parasitized erythrocytes (PRBC) at the ring stage, 5% parasitemia and 1.5% hematocrit, were treated with dihydroartemisinin (DHA) to investigate the effect of drug on parasite antioxidants. DHA was dissolved in 1% DMSO and added to the cell suspension to final concentrations of 0.25, 0.5 and 1.0 ng/ml. The treated cell suspension was incubated in a candle jar at 37ºC and harvested after incubation for 18-20 hours. The treated red cells were subjected to antioxidant analysis. Controls were untreated PRBC and PRBC treated with 1% DMSO since DHA was dissolved in DMSO. The controls were kept under similar conditions as tested samples.

Determination of the antioxidants

Total glutathione (GSH + GSSG) in different erythrocyte lysate was measured based on DTNB-glutathione reductase recycling assay as described previously (Griffith, 1980). The oxidized glutathione (GSSG) was reduced to GSH by glutathione reductase in the presence of NADPH. Then total GSH was determined by 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB). The formation rate of thionitrobenzene (TNB) was measured at 412 nm every 15 seconds for 2.5 min-utes. The DTNB-glutathione reductase recycling assay was also used to determine the level of GSSG after removing the endogenous GSH by 2-vinylpyridine. The ratio of GSH/GSSG was then calculated.

Glutathione peroxidase was quantitated according to the method described by Lawrence and Burk (1976). The reaction mixture contained NADPH, GSH and H_2O_2. The reaction was initiated by the addition of glutathione reductase and the erythrocyte lysate. The conversion of NADPH to NADP was evaluated at 340 nm.

Catalase activity was estimated according to the method described by Aebi (1984) using hydrogen peroxide as a substrate. The degradation rate of H_2O_2 was monitored continuously at 240 nm.

Total superoxide dismutase (SOD) was determined by a nitroblue tetrazolium (NBT) based assay as described previously by Spitz and Oberley (1989). Xanthine-xanthine oxidase was used to generate superoxide radicals. The activity of SOD was then measured by the degree of inhibition by NBT. One unit of SOD was defined as the amount of enzyme causing half the maximum inhibition of NBT reduction.

Determination of parasite viability after treated with dihydroartemisinin

To investigate the parasite death after DHA treatment, the parasite viability in the presence of the same concentrations of drug was estimated by tritiated hypoxanthine incorporation as described previously (Desjardins *et al*, 1979).

The infected erythrocytes, 5% parasitemia, 1.5% hematocrit and synchronized at the ring stage were incubated with DHA at 0.25, 0.5 and 1.0 ng/ml in a 96-well plate. ^{3}H-hypoxanthine, 0.25 μCi in RPMI 1640, was added to each well. After incubation at 37ºC in a candle jar for 18-20 hours, the parasites were harvested and the radioactivity was determined in a liquid scintillation.

RESULTS

Antioxidants of parasitized erythrocytes

The levels of antioxidants were measured in synchronized cultures of the parasite with trophozoite-infected erythrocytes (PRBC) compared to the non-parasitized erythrocytes with which they

had been cultured (URBC). As shown in Table 1, the level of reduced glutathione (GSH) showed a significant decrease from 7.45±2.40 to 4.99±3.06 µmol/gram protein during parasite maturation (p < 0.05). Glutathione peroxidase (GPx) exhibited a downward trend but not statistically significant (16.2±4.63 to 14.43±6.15 units/ gram protein). There were no significant differences in the levels of oxidized glutathione (GSSG), catalase and superoxide dismutase during the growth of *P. falciparum.*

Effect of dihydroartemisinin on antioxidants of nonparasitized erythrocytes

The status of antioxidant defense in the presence and absence of DHA was investigated in non-parasitized erythrocytes (URBC, N=30) compared to that treated with 1% DMSO or the untreated. The URBC were not fresh erythrocytes but were erythrocytes kept in a refrigerator and used to culture malaria parasites within 1 month after collection. The treated URBC were incu-

bated under similar condition as those of parasitized erythrocytes. As shown in Table 2, the GSH and GSSG levels in non-parasitized erythrocytes were 7.45±2.40 and 0.64±0.18 µmol/gram protein, respectively. The glutathione ratio (GSH/GSSG) was then 12.05±4.18. The levels of antioxidant enzymes, GPx, catalase and SOD were 16.2±4.63, (18.15±5.31)x10^4 and 3091±948 units/ gram protein, respectively. There was no change of all tested antioxidants in URBC treated with either 1% DMSO or various concentrations of DHA compared to the untreated URBC (p > 0.05).

Effect of dihydroartemisinin on antioxidants of parasitized erythrocytes

Antioxidants in the presence and absence of DHA were estimated in 5% synchronized parasitized erythrocytes carrying the ring stage (PRBC, N=30) compared to that treated with 1% DMSO and the untreated sample. All samples were incubated in a candle jar at 37ºC for 18-20 hours until the parasites were trophozoites before

Table 1

Levels of glutathione and antioxidant enzymes in non-parasitized erythrocytes (URBC) compared to that of parasitized erythrocytes (PRBC) at 5% parasitemia.

(N=30)	URBC	PRBC
GSH (µmol/ gram protein)	7.45 ± 2.40	4.99 ± 3.06
GSSG (µmol/gram protein)	0.64 ± 0.18	0.56 ± 0.23
GSH/GSSG	12.05 ± 4.18	9.69 ± 5.28
GPx (unit/gram protein)	16.2 ± 4.63	14.43 ± 6.15
Catalase (10^{4}unit/ gram protein)	18.15 ± 5.31	19.32 ± 4.51
SOD (unit/gram protein)	3,091 ± 948	3,173 ± 666

Table 2

Antioxidants of non-parasitized erythrocytes (URBC) treated with various concentrations of dihydroartemisinin (DHA) compared to those in the absence of drug and in the presence of 1% DMSO.

(N=30)	Untreated URBC	1% DMSO	DHA 0.25 ng/ml	DHA 0.5 ng/ml	DHA 1.0 ng/ml
GSH	7.45 ± 2.40	6.97 ± 2.73	6.68 ± 3.10	6.30 ± 2.85	7.07 ± 3.82
GSSG	0.64 ± 0.18	0.58 ± 0.17	0.58 ± 0.18	0.59 ± 0.17	0.58 ± 0.13
GSH/GSSG	12.05 ± 4.18	13.15 ± 6.58	12.18 ± 6.06	11.59 ± 6.63	12.11 ± 5.68
GPx	16.2 ± 4.63	15.32 ± 4.47	15.6 ± 5.36	16.27 ± 6.05	15.98 ± 6.25
Catalase	18.15 ± 5.31	17.89 ± 7.1	18.83 ± 8.76	18.66 ± 6.59	18.67 ± 8.88
SOD	3,091 ± 948	3,057 ± 1,174	3,013 ± 879	3,129 ± 898	2,953 ± 735

Values are mean ± standard deviation. SOD and GPx are expressed in (unit/gram protein), catalase in (10^{4}unit/gram protein) and glutathione (GSH, GSSG) in µmol/ gram protein.

being subjected to analysis. As shown in Table 3, the levels of all antioxidants tested in the presence of 1% DMSO were not different from the untreated PRBC (p > 0.05). Treatment of PRBC with DHA at 0.25 ng/ml did not significantly reduced the levels of both antioxidant enzymes and glutathione (p > 0.05) although GPx activity and GSH level showed an almost significant difference (p=0.069 and 0.057, respectively).

GPx and catalase activities, as well as GSH levels in treated PRBCs were significantly lower than those of untreated samples when the concentration of DHA was increased to 0.5 ng/ml (p=0.023, 0.017 and < 0.001, respectively). The lower GSH level in treated PRBC resulted in a significantly lower GSH/GSSG ratio than of the untreated PRBC (p=0.019). As expected, a greater significant reduction of GPx, catalase and GSH levels was found when the DHA concentration was increased to 1.0 ng/ml (Table 3).

It should be noted that SOD activities as well as GSSG levels of PRBCs in the presence of DHA at all concentrations tested were not different from those of untreated PRBCs (Table 3). Higher DHA concentration resulted in lower SOD activity. Treatment with DHA at 1.0 ng/ml (p=0.065) showed a greater difference than at 0.5 ng/ml (p=0.071) when compared to the control without drugs. Unlike SOD, the effect of increased drug concentration was not seen with the GSSG levels.

Parasite viability after dihydroartemisinin treatment

To investigate whether the reduction of antioxidant capacity in DHA-treated PRBC was secondary to the parasite death, the parasite viability in the presence of the same concentrations of drug was measured (N=5). The data showed that percentages of the surviving parasites after being treated with DHA at 0.25, 0.5 and 1.0 ng/ml were 86.5±5.72%, 45±5.69% and 23±10.14%, respectively (Table 4). DHA at 0.25 ng/ml reduced the level of the glutathione ratio to a greater extent than the parasite death. When the percentage of parasite viability after DHA treatment was compared to the percentages of the remaining antioxidant enzyme activities or the GSH/GSSG ratio at higher drug concentrations, it was found that DHA had a stronger effect on parasite death than on antioxidant capacity (Table 4). The re-

sults indicated that primary effect of higher drug concentrations was parasite death which then secondary leading to the low level of antioxidants.

DISCUSSION

Attempts have been made to elucidate the status of antioxidant defense in malaria-infected erythrocytes (PRBC) in the presence of artemisinin. Although the free radical production by artemisinin is well accepted, the mode of artemisinin action remains uncertain (Meshnick, 1998). It has been postulated that it may involve the alkylation of some essential malarial proteins (Asawamahasakda *et al*, 1994; Bhisutthibhan *et al*, 1998), the selective accumulation of drug in parasitized red cells (Kamchonwongpaisan *et al*, 1994) or the inhibition of heme polymerase (Berman and Adams, 1997). The present study investigated the effects of artemisinin on parasite antioxidants, catalase, glutathione peroxidase (GPx), total superoxide dismutase (SOD) and a reduced glutathione : oxidized glutathione ratio (GSH/GSSG).

It should be noted that the levels of antioxidants shown in Table 1 were not the regular normal values reported for fresh normal erythrocytes since the non-parasitized erythrocytes in the present study were erythrocytes collected from blood donors, kept in a refrigerator and used to culture malaria within 1 month. Moreover, they were incubated in a candle jar at 37ºC for 18-20 hours after the addition of dihydroartemisinin. However, the levels of antioxidants in Table 1 were still in the ranges of previous reports for GSH and SOD (Bilman *et al*, 2001), GPx and catalase (Viroonudomphol *et al*, 2000) and for GSSG (Jain and McVie, 1999).

When erythrocytes were infected with malaria parasites, reactive oxygen species are produced in the parasite food vacuole during hemoglobin digestion and are able to egress from the parasite to the host cell cytosol (Atamma and Ginsburg, 1994). The local antioxidant depletion may contribute to erythrocyte loss in severe malaria (Griffiths *et al*, 2001). As shown in Table 1, malaria infection, only at 5% parasitemia, can reveal a significantly decreased GSH and GSH/GSSG ratio (p < 0.05). This finding was consistent with the observation in erythrocytes of patients with acute *P. falciparum* malaria (Das and

Table 3
Antioxidants of parasitized erythrocytes (PRBC) treated with various concentrations of dihydroartemisinin (DHA) compared to the untreated PRBC and PRBC treated with 1% DMSO. Significant difference between DHA-treated PRBC and untreated PRBC is indicated in parentheses.

(N=30)	Untreated PRBC	1% DMSO	DHA 0.25 ng/ml	DHA 0.5 ng/ml	DHA 1.0 ng/ml
GSH	4.99 ± 3.06	5.00 ± 3.30	3.84 ± 2.51 (p=0.057)	3.29 ± 2.11 (p=0.017)	2.88 ± 2.05 (p=0.003)
GSSG	0.56 ± 0.23	0.55 ± 0.18	0.5 ± 0.23	0.53 ± 0.17	0.52 ± 0.25
GSH/GSSG	9.69 ± 5.28	8.51 ± 5.52	8.04 ± 4.92	6.43 ± 4.63 (p=0.019)	5.49 ± 4.22 (p=0.001)
GPx	14.43 ± 6.15	14.4 ± 5.81	12.93 ± 5.58 (p=0.069)	10.91 ± 5.96 (p < 0.001)	11.85 ± 4.99 (p=0.008)
Catalase	19.32 ± 4.51	19.3 ± 4.1	18.83 ± 4.74	17.99 ± 4.2 (p=0.023)	17.22 ± 4.33 (p=0.001)
SOD	3,173 ± 666	3,112 ± 812	2,997 ± 961	2,901 ± 859 (p=0.071)	2,849 ± 975 (p=0.065)

Values are mean ± standard deviation. SOD and GPx are expressed in (unit/gram protein), catalase in (10^4 unit/gram protein) and glutathione (GSH, GSSG) in μmol/gram protein.

Table 4
Comparison of parasite viability after treated with various concentration of DHA to the remained antioxidant. Values are mean ± standard deviation.

DHA (ng/ml)	% Survived parasite (N=5)	% Antioxidants remained after DHA treatment (N=30)			
		SOD	CAT	GPx	GSH/GSSG
0	100	100	100	100	100
0.25	86.5±5.72	89.74±22.45	93.38±16.18	89.56±22.28	70.00±31.6
0.5	45±5.69	86.02±19.93	89.31±10.94	78.27±24.88	60.13±31.2
1.0	23±10.14	82.28±21.38	86.97±13.81	79.70±20.02	52.17±36.8

Nanda, 1999). The result indicated that GSH was the most sensitive indicator of oxidative stress in PRBC and might be an endogenous antioxidant which plays a key role in the defense against oxidative stress as generally found in other tissues (Kehrer and Lund, 1994).

Glutathione peroxidase (GPx), a key enzyme involved in the detoxification of peroxides, showed a downward trend in PRBC compared to URBC (Table 1). The result was consistent with previous findings (Mohan *et al*, 1992; Das and Nanda, 1999). It was shown that *P. falciparum* contains an endogenous selenium-dependent GPx with higher enzyme activities was detected only when parasite was cultured in media supplemented with selenium (Gamain *et al*, 1996). However, selenium-independent GPx was also reported in *P. falciparum* (Fritsch *et al*, 1987).

Catalase was found to be significantly decreased both *in vitro* (Mohan *et al*, 1992) and *in vivo* (Das and Nanda, 1999) infection with *P. falciparum*. Nevertheless, Table 1 showed no significant difference of catalase in PRBC compared to URBC. The inconsistent result might be due to the the fact that the 5% parasitemia that we used in the present study was too low to detect the difference. Since the Km (Michaelis constant) value for GPx is lower than that for catalase, it might be possible that malarial GPx plays a more important role than catalase in the elimination of H_2O_2 in physiologic conditions as found in other systems.

The level of SOD in PRBC showed no significant difference from those of URBC (Table 1). The result was again consistent with the previous findings both in malaria culture (Areekul *et al*, 1988) and in patients infected with *P.*

falciparum (Das and Nanda, 1999).

The present observations indicate an enhanced oxidative stress in malaria-infected erythrocytes and support the need for further investigation of alternative antimalarial targets. The data also indicate the intraerythrocytic depletion of antioxidants that might play a role in hemolysis and anemia in patients infected by malaria parasites.

Artemisinin and its derivatives can generate free radicals (Butler *et al*, 1998; Meshnick, 1998). We elucidated whether a mode of its action could be explained by an ability to enhance antioxidant depletion in parasitized erythrocytes. As shown in Table 2, treatment of URBC with dihydroartemisinin (DHA), an active metabolite of artemisinin, showed no effect on erythrocyte antioxidants (p > 0.05). The data indicated that DHA was not activated to generate free radicals in URBC.

In the presence of DHA, the levels of antioxidants in PRBC were decreased when compared to the untreated PRBC (Table 3). This finding confirmed that artemisinin is a pro-oxidant and is activated to generate free radicals. The drug activation in PRBC might be initiated during parasite consumption of host hemoglobin (Pandey *et al*, 1999).

As seen in the untreated PRBC (Table 1), the GSH level was the most sensitive index of oxidative stress in the presence of DHA (Table 3). The GSH was almost significantly reduced when DHA was only 0.25 ng/ml (p=0.057). More and more significant reductions were seen when drug concentrations were increased to 0.5 ng/ml (p=0.017) and 1.0 ng/ml (p=0.003). These observations were consistent with the recent report showing that artemether/hemin depleted intracellular glutathione levels in the neuroblastoma cell line NB2a (Smith *et al*, 2001). Although malaria parasites can synthesize GSH *de novo* (Atamma and Ginsburg, 1997) the *de novo* synthesis of GSH might not be efficient to detoxify all the radicals generated from DHA activation. However, the GSSG level in the presence of DHA was not correspondingly increased with the decreased GSH (Table 3). If the turnover of GSH increases; then GSSG should accumulate (Srivastava and Beutler, 1989). This non-correspondence might be that either GSSG is extruded (Atamma and Ginsburg, 1997) or GSH-artemisinin adducts are formed (Mukanganyama

et al, 2001).

The similar effect of DHA on GSH was also observed with the activity of GPx (Table 3). However, the other two enzymes, catalase and SOD showed no difference in the presence of DHA. These findings confirmed that GPx might play a more important role in the elimination of peroxide in erythrocytes infected with *P. falciparum* in the presence of DHA. The activities of catalase and SOD showed a downward trend with the increments of drug concentration (Table 3).

The decreased antioxidants in the presence of DHA might be a primary effect of the drug on parasite antioxidants or might be a secondary effect due to the parasite death. The parasite viability in the presence of similar concentrations of DHA was determined (Table 4). It should be noted that only at 0.25 ng/ml that DHA had a greater effect on the parasite glutathione ratio than on parasite viability. Around 86% of the parasite survived while 70% of the ratio remained after treatment. At higher concentrations of DHA, the parasite viability was affected to a greater extent than the levels of antioxidants (Table 4). The data indicated that reduction of antioxidants at high drug concentrations might be secondary to the parasite death. These observations indicate that the generated free radicals from low concentrations of DHA could primarily reduce the GSH of the parasitized erythrocytes but the reduction was not strong enough to cause the parasite death.

The present findings provide further evidence that artemisinin can generated free radicals upon activation in parasitized erythrocytes and that reduced glutathione is an important determinant related to oxidative stress both in the absence and in the presence of artemisinin derivatives. The data also suggest that the depletion of intraerythocytic antioxidants is not a prerequisite for parasite death. Further investigations for the artemisinin mode of action are still needed.

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