Extracellular Heme Enhances the Antimalarial Activity of Artemisinin

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Artemisinin exerts the antimalarial activity through activation by heme. The hemolysis in malaria results in the elevated levels of plasma heme which may affect the activity of artemisinin. We hypothesized that the extracellular heme would potentiate the antimalarial activity of artemisinin. Hemin (ferric heme) at the pathologic concentrations enhanced the activity of artemisinin against Plasmodium falciparum in vitro and increased the levels of the lipid peroxidation products in the presence of artemisinin. The antimalarial activity of artemisinin and potentiation by hemin was decreased by vitamin E. Hemin had no effect on the activity of quinoline drugs (chloroquine, quinine and mefloquine). Furthermore, the oxidative effect of hemin in the presence of artemisinin or quinoline drugs was studied using low-density lipoprotein (LDL) oxidation as a model. Artemisinin enhanced the effects of hemin on lipid peroxidation and a decrease of tryptophan fluorescence in LDL whereas the quinoline drugs inhibited the oxidation by hemin. In conclusion, the extracellular heme enhances the antimalarial activity of artemisinin as a result of the increasing oxidative effect of hemin.

Key words artemisinin; heme; hemin; malaria; vitamin E

MATERIALS AND METHODS

Ethics Statement This study was approved by the Ramathibodi Hospital Ethics Committee. The informed consent was obtained from volunteers in the written form in accordance with the Declaration of Helsinki.

Reagents Hemin (ferric heme chloride, ferrprotoporphyrin IX), artemisinin, chloroquine, quinine, mefloquine, Drabkin’s reagent, vitamin E and all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Hemin was dissolved in 0.1 M NaOH and adjusted to the desired concentration with 10 mM phosphate buffered saline (PBS) (used immediately). The concentration of hemin was checked by using the molar extinction coefficient at 383 nm of 62000 M\(^{-1}\)cm\(^{-1}\). The 0.01 M stock solution of artemisinin, quinine, and mefloquine was prepared in dimethylsulfoxide (DMSO). Chloroquine stock solution (0.01 M) was prepared in RPMI1640.

Measurement of Plasma Heme The plasma heme from six patients with severe malaria classified according to the WHO criteria was measured by the colorimetric method with Drabkin’s reagent.

In Vitro Culture of Plasmodium falciparum The P. falciparum laboratory strain TM267R was used in this study. The parasites were cultured with the washed human erythrocytes (group O) in RPMI1640 media as previously described. The stage synchronization of parasite was done using 5% d-sorbitol and the parasitized erythrocytes were maintained at 2—3% parasitemia.

Drug Sensitivity Test The synchronous ring stage parasites (10—15 h of age) at 3% parasitemia were exposed to different concentrations of hemin, artemisinin, quinoline drugs, or combination of drug and hemin in 24-well plates in triplicates for 24 h. The thin smear was performed and stained in Giemsa’s solution. The mature schizonts with ≥5 nuclei from random 3000 erythrocytes were counted under the light microscope. The % growth inhibition was calculated from the ratio of schizont number in the presence of drugs or hemin

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Separation of LDL. The fasting blood was collected from healthy volunteers in glass tube using ethylenediaminetetraacetic acid (EDTA) (1.5 mg/mL) as anticoagulant. The plasma was separated by centrifugation at 2500 g for 10 min at 4°C and kept at −70°C. LDL was separated by the sequential density gradient ultracentrifugation[2] using a Beckman Optima LE-80K centrifuge and a 90Ti rotor at 4°C. LDL was stored at −70°C for experiments within 1 week.

Oxidation of LDL. The separated LDL was dialyzed in 10 mM PBS (> 50 fold volume excess over LDL) to remove salts from the density solution. The dialyzed LDL (100 µg protein/mL) was incubated with various concentrations of artemisinin, chloroquine, quinine, and mefloquine; both in the absence and presence of 10 µM hemin (freshly prepared prior to use) in 10 mM PBS, pH 7.4 at 37°C for a period up to 8 h. The levels of thiobarbituric acid reactive substances (TBARs), the indicators of lipid peroxidation, were measured as described previously.[3] In separate experiments, the tryptophan fluorescence was recorded at 281 nm excitation and 335 nm emission. The decrease of tryptophan fluorescence corresponds to the decomposition of this amino acid and represents the oxidation of the protein portion (apolipoprotein B) of LDL.[3]

Statistical Analysis. The data were analyzed by GraphPad Prism 5. All data represent means ± standard error of mean (S.E.M.). Comparison was performed by the unpaired t-test or analysis of variance (ANOVA) with the accepted p-value < 0.05.

RESULTS

Plasma Heme in Malaria and in Vitro Drug Sensitivity Test. The levels of plasma heme were higher in malaria than normal subjects (3.5 ± 0.7, 0.3 ± 0.2 µM, respectively) (Fig. 1A). Artemisinin had antimalarial activity against TM267R strain of P. falciparum with IC₅₀ of 7.0 ± 1.1 nM (Fig. 1B) and showed the maximal growth inhibition of 100%. Hemin did not show significant activity (IC₅₀ = 2.3 ± 1.6 nM, maximal inhibition = 20%); therefore, the isobologram was not constructed. Combination of artemisinin with hemin at 0.1 and 1 µM, which were the concentrations within the in vivo range, increased the activity of artemisinin as observed from the reduction of IC₅₀. In contrast, hemin had no effect on the activity of quinoline drugs (Table 1). Vitamin E could reduce the antimalarial activity of artemisinin. The IC₅₀ of artemisinin in the presence of 20 µM vitamin E and 0.1 µM hemin + 20 µM vitamin E were 81.3 ± 5.1 and 74.3 ± 11.7 nM, respectively (n = 4 each).

To examine whether hemin could enhance the oxidative effect of artemisinin, the TBARs levels were measured in the parasite culture media (20— 30% parasitemia) after 24 h incubation (Fig. 1C). The TBARs levels in media were measured as markers of lipid peroxidation. Artemisinin alone at 10 nM did not change the levels of TBARs in media. In the presence of 1 and 10 µM hemin, 10 nM artemisinin increased the TBARs levels significantly. Moreover, the TBARs levels in parasitized erythrocytes were measured. Ten nM artemisinin in combination with 0.1 µM hemin increased the TBARs levels, and the addition of 20 µM vitamin E blocked the effect (Fig. 1D). These results suggest the potentiation by hemin of
artemisinin-induced oxidative damage.

**Effect of Hemin and Antimalarial Drugs on LDL Oxidation** The lipid peroxidation in LDL was determined by measuring TBARs in LDL solution (100 µg protein/mL). The oxidizing activity of hemin appeared to depend on its concentration. Hemin induced TBARs formation in dose-dependent manner, and the time required to reach the peak maximum was approximately 360 min (6 h) (Fig. 2A). After 6 h, the levels of TBARs remained constant. TBARs reached the maximal levels when the concentration of hemin was 10 µM. Hence, 10 µM hemin was chosen in the following series of experiments. All antimalarial drugs used in this study (200 µM artemisinin, chloroquine, quinine, and mefloquine) did not change the TBARs levels, even when they were incubated with LDL for 8 h. When co-incubated with 10 µM hemin in LDL solution, 200 µM artemisinin enhanced the hemin-induced TBARs formation. In contrast, the quinoline drugs inhibited TBARs formation induced by hemin.

The dependence of pre-incubation of 200 µM artemisinin with 10 µM hemin was assessed. It was found that the maximal levels of TBARs were unchanged upon varying the pre-incubation period up to 1 h, suggesting the stability of the reactive intermediates (data not shown). In addition, the potential effect of dissolving solvent (DMSO) for artemisinin was tested because of the hydroxyl radical scavenging property of DMSO. DMSO, over the range from 8 µM to 140 µM, had no effect on the maximal levels of TBARs generated from the reactions between hemin and artemisinin (data not shown).

The oxidation of apolipoprotein moiety was continuously monitored by the reduction of tryptophan fluorescence. In the absence of hemin, all drugs (200 µM) had no effect on the fluorescent intensity of tryptophan, when compared to controlled LDL. In LDL solution (100 µg protein/mL) containing 10 µM hemin, the two-step kinetics of change in tryptophan fluorescence were observed: lag and propagation steps (Fig. 2B). In the presence of 200 µM artemisinin, the tryptophan fluorescence decreased rapidly to remain about 26% within the first 15 min. The lag phase was not observed, and the kinetics of change appeared to be one propagation step. The tryptophan fluorescence was then gradually decreased to remain about 10% after 150 min of reaction. Again, these data showed that artemisinin could enhance the oxidative effect of hemin. In contrast to artemisinin, quinoline drugs inhibited the hemin-induced reduction of tryptophan fluorescence. In the presence of both hemin and quinoline drugs, the tryptophan fluorescence remained about 85% of the initial values even after 360 min of incubation.

**DISCUSSION**

Our study demonstrates that hemin which appears at higher levels in the plasma of malaria patients increases the antimalarial potency of artemisinin because the IC50 of artemisinin is lower in the presence of hemin. This effect of hemin could be due to the increasing oxidative effect of hemin in the presence of artemisinin; which is observed from the model of LDL oxidation. In addition, artemisinin potentiates the effect of hemin on lipid peroxidation in malarial culture. Because the levels of plasma heme are elevated in malaria as a result of hemolysis and dyserythropoiesis, it is possible that the plasma heme could increase artemisinin activity. In contrast to artemisinin, the quinoline drugs show no change in activity in the presence of hemin, but decrease the effect of hemin on LDL oxidation.

Artemisinin is believed to increase the oxidative stress in parasitized erythrocytes by production of hydroxyl radical. Further studies are required to determine the mechanism by which hemin enhances the antimalarial activity of artemisinin.

### Table 1. Antimalarial Activity of Artemisinin and Quinoline Drugs in the Absence and Presence of Hemin

<table>
<thead>
<tr>
<th>Drugs</th>
<th>IC50 (µM) of drug</th>
<th>IC50 (µM) of drug in the presence of 0.1 µM hemin</th>
<th>IC50 (µM) of drug in the presence of 1 µM hemin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artemisinin</td>
<td>7.0±1.1</td>
<td>3.1±0.3*</td>
<td>1.9±0.5*</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>129.9±5.7</td>
<td>160.7±16.3</td>
<td>157.1±5.2</td>
</tr>
<tr>
<td>Quinine</td>
<td>730.8±107.6</td>
<td>751.1±87.4</td>
<td>755.3±109.4</td>
</tr>
<tr>
<td>Mefloquine</td>
<td>35.7±3.5</td>
<td>40.2±1.4</td>
<td>54.9±7.0</td>
</tr>
</tbody>
</table>

Data are means ± S.E.M. (n=3). *p<0.05 vs. IC50 of drug without hemin (ANOVA with Tukey’s multiple comparison test).
and the drug potency is reduced by antioxidants such as N-acetylcysteine. Antioxidants such as vitamin E promote the growth of malaria parasites, and vitamin E deficiency increases the antimalarial activity of artemisinin in vivo. Thus, our results are in agreement with the reports on oxidative stress as an essential process of artemisinin action.

The antimalarial mode of action of artemisinin involves the reductive activation of its endoperoxide bridge, probably by free heme or iron produced during the digestion of hemoglobin in the food vacuole. Ferrous heme reacts with the endoperoxide bond of artemisinin faster than inorganic ferrous ion and ferric heme (hemin). Ferric heme is the major heme species present in plasma during hemolysis. Ferric heme would be reduced to ferrous heme by plasma reductants or artemisinin. The resultant ferrous heme is believed to contribute to the antimalarial action. The reactions of artemisinin and ferrous heme result in the formation of the carbon-centered radicals and intermediates. These intermediates interfere with the heme detoxification process and form adducts with the multiple parasitic proteins such as the P. falciparum translationally controlled tumor protein (PITC) and sarco/endo-plasmic reticulum Ca2+-ATPase (SERCA). The source of heme for activation of artemisinin is controversial. During hemoglobin digestion process in food vacuole, the ferrous heme could be generated transiently and react with artemisinin intracellularly before it is oxidized to ferric heme. Because both ferrous and ferric forms of heme are toxic to the parasite, the parasite detoxifies heme by polymerization into the hemozoin (malarial pigment). Although ferrous heme has been shown in vitro to react with artemisinin faster than hemin, heme also reacts with artemisinin, probably after its reduction to ferrous heme. Additionally, the oxidative effect of hemin in producing membrane damage is enhanced by artemisinin.

Here, we show that the extracellular heme could be also a source for artemisinin activity. Pre-incubation of artemisinin with hemin for 6h decreases the antimalarial activity of artemisinin because of the instability of active intermediates. But in our study, we added hemin and artemisinin into the culture media simultaneously; thus, allowing the short-lived intermediates to act at the parasitic targets. This is relevant to the rapid onset of artemisinin in vivo when the drug is administered and reaches the systemic circulation where it reacts with heme in plasma. However, further study is required to clarify the in vivo significance of plasma heme on artemisinin activity. In conclusion, the extracellular heme could enhance the antimalarial activity of artemisinin as a result of increasing oxidative effect of hemin.

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Conflict of Interest Statement None declared.

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