Enhancement of Heme-Induced Membrane Damage by the Anti-malarial Clotrimazole: the Role of Colloid-Osmotic Forces

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Two recent studies have demonstrated that clotrimazole, a well-known potential antifungal agent, inhibits the in vitro growth of chloroquine-resistant strains of the malaria parasite, Plasmodium falciparum. In a previous study, we suggested that clotrimazole acts as an anti-malarial agent by inhibiting heme catabolism in the malaria parasite and by enhancing heme-induced membrane damage. In this paper, we examined the mechanism of action by measuring hemolysis as an indicator of membrane damage. Our results showed that clotrimazole does not promote the binding of heme to membranes, and that the enhancement of heme-induced hemolysis by clotrimazole is not caused by lipid peroxidation or by oxidation of thiol groups in membrane proteins. Instead, clotrimazole inhibits glutathione-dependent heme degradation, resulting in an enhancement of heme-induced hemolysis. We also found that clotrimazole increases the susceptibility of erythrocytes to hypotonic lysis in the presence of heme and that sucrose could inhibit hemolysis induced by heme–clotrimazole complexes. Thus, it appears that the enhancement of heme-induced hemolysis by clotrimazole in our experiments is due to a colloid osmotic hemolysis mechanism. The hydrophobicity and the large molecular size of the heme-clotrimazole complex might be key factors for induction of hemolysis.

Key words clotrimazole; heme; hemolysis mechanism; malaria; membrane

Clotrimazole (CLT), an antifungal drug containing an imidazole group (Fig. 1), is effective against a wide range of fungal pathogens.11 Many investigations have shown that the fungistatic mechanism of CLT is associated with inhibition of sterol 14 alpha-demethylase and microsomal P-450 dependent enzyme.2,3) CLT is also reported to be an effective immunosuppressant,4,5) and recent studies have raised the possibility that CLT may be an effective anti-malarial drug.6,7) Although the anti-malarial mechanism of CLT is not well understood, it may be due to its ability to cause major changes in calcium ion fluxes.8—10)

During development and proliferation in human erythrocytes, the malarial parasite degrades hemoglobin for use as a major source of amino acids. This is accompanied by the release of free heme. Because free heme is oxidatively active, it is highly toxic to the cell and malarial membrane and causes parasite death. To protect itself, the malarial parasite detoxifies free heme by neutralization and removal from membrane using Plasmodium falciparum histidine rich protein 2 (PfHRP2),11) polymerization initiated by PfHRP2 in food vacuole,12) or degradation by reduced glutathione (GSH),13—15) a compound that exists at millimolar levels in red blood cells and parasite compartments.16,17) These detoxification processes are initiated by the formation of heme–PfHRP2 and heme–GSH complexes.

Recently, our spectroscopic investigations showed that one heme molecule binds to two CLT molecules with high affinity, and that heme–CLT complex contains a ferric atom in low-spin state (S=1/2) with two nitrogenous ligands derived from the imidazole moieties of CLTs.18,19) In addition, CLT inhibits GSH-dependent degradation of free heme and enhances heme-induced hemolysis. These results indicate that CLT might have another anti-malarial mechanism, namely the disruption of the malarial heme detoxification pathways. However, it is unclear why the heme–CLT complex causes more severe hemolysis than the equivalent concentration of free heme. In this paper, we found that this may be due to a promotion of heme-induced membrane damage.

MATERIALS AND METHODS

Preparation of Heme
At start of each experiment, a stock heme solution was prepared by dissolving hemin chloride (Sigma, U.S.A.) in 20 mM NaOH. After shaking vigorously for 10 min in dark, insoluble heme was removed by centrifugation at 15000 rpm for 10 min. The concentration of the heme solution was estimated from the absorbance at 385 nm with ε_max of 58400 in 100 mM NaOH.20) The heme solution was diluted to 1 mM with 20 mM NaOH, kept on ice in dark as a stock, and used within 24 h.

Erythrocyte Preparation
Blood was taken from healthy volunteers, heparinized (1 mg heparin for 1 ml blood) and erythrocytes were collected by centrifugation at 1500×g for 3 min. Next, the erythrocytes were washed 6 times with Tris-buffered saline (TBS, 20 mM Tris–HCl, pH 7.4, containing 0.9% NaCl) at 25°C and used to prepare membrane ghosts or resuspended in TBS for hemolysis experiments.

Preparation of Erythrocyte White Ghost Membrane
Human erythrocyte white ghost membrane was prepared on ice as described previously21) with slight modifications. Washed erythrocytes (2.5 ml) were homogenized in 35 ml of 5 mM phosphate buffer, pH 8. After shaking vigorously, the hemolysate was centrifuged at 35000×g for 20 min to sediment membranes. This step was repeated again, after which the membrane was washed repeatedly with 50 mM Tris–HCl,
pH 7.4 until the pellet became white. At each centrifugation step, the brown pellet that had been accumulated at the bottom of the membrane fraction was carefully removed to avoid contamination with proteinases. Finally, the membrane was resuspended with 9 ml of the wash buffer. The erythrocyte white ghost membrane thus obtained was stored on ice and used within 24 h. The amount of membrane was determined as the membrane protein concentration using a protein assay kit (Bio-Rad, U.S.A.) with bovine serum albumin as the standard.

**Effect of CLT on the Binding of Heme to Erythrocyte White Ghost Membranes**

Suspensions of 20 μg erythrocyte white ghost membranes were incubated for 7 min at room temperature with 5 μM heme or heme–CLT complex (5 μM heme and 10 μM CLT) in 0.6 ml of 0.2 M HEPES, pH 7.4 to form heme-membrane or heme–CLT–membrane complexes. The membranes were washed twice with the same buffer by centrifugation at 15000 rpm for 10 min. Next, the membrane pellet was dissolved in 50 mM Tris–HCl, pH 7.4 containing 2.5% SDS, and the amount of membrane-bound heme was determined spectrophotometrically (Hitachi U-3300, Tokyo, Japan). Concentrations of ferric heme and heme–CLT complex were calculated independently using molar extinction coefficients at 402 nm that had been determined prior to adding to the membrane fraction. The amount of membrane-bound heme was expressed as nmol heme per mg membrane protein.

**Degradation of Membrane-Bound Heme by GSH and Its Inhibition by CLT**

Suspensions of 20 μg erythrocyte white ghost membrane were incubated for 7 min at room temperature with 5 μM heme or heme–CLT complex (5 μM heme and 10 μM CLT) in 0.6 ml of 0.2 M HEPES, pH 7.4 to combine heme or heme–CLT complex with membranes. Next, membranes were sedimented by centrifugation at 15000 rpm for 10 min and washed twice with the same buffer. The heme-loaded white ghost membranes were resuspended in 0.6 ml of the same buffer and then incubated at 37 °C for 2 h with 10 mM GSH or a mixture of 10 mM GSH and 10 μM CLT. The heme–CLT complex-loaded membranes were also incubated with 10 mM GSH under the same conditions. Before and after incubation, 0.3 ml of heme– or heme–CLT complex-loaded membrane suspensions were removed and centrifuged at 15000 rpm for 10 min. The membrane pellets were dissolved in 0.3 ml of 50 mM Tris–HCl, pH 7.4 containing 2.5% SDS (w/v), and the absorbance at 402 nm was measured to determine the amounts of heme and heme–CLT complex. The amount that was undegraded was determined by comparing the absorbance with that for 5 μM heme or heme–CLT complex (5 μM heme and 10 μM CLT) in the same solution.

**Oxidant Effects of Heme and Heme–CLT Complex on Membrane Protein**

Suspensions of 0.2 mg erythrocyte white ghost membranes were incubated at 37 °C for 2 h in 0.2 M HEPES, pH 7.4 in the presence of heme (5 μM) or heme–CLT complex (5 μM heme and 10 μM CLT containing 0.2% DMSO). In the control experiment, membrane was incubated with 0.2 M HEPES, pH 7.4 containing 0.2% DMSO. The membrane was sedimented by centrifugation at 15000 rpm for 10 min and lysed with 0.6 ml of 0.3 M phosphate buffer, pH 7.4 containing 0.8 M urea and 2% SDS. The membrane thiol content was determined by measuring the difference in absorption at 412 nm before and 10 min after the addition of 12 μg of dithionitrobenzoic acid in 0.6 ml of 0.3 M phosphate buffer, pH 7.4 containing 0.8 M urea and 2% SDS.  

**Lipid Peroxidation by Heme and Heme–CLT Complex**

The extents of lipid peroxidation induced by heme, heme–CLT complex and Cu²⁺ were determined by measuring the malonyldialdehyde (MDA) formed by the thiobarbituric acid (TBA) reaction as previously described. Suspension of 20 μg erythrocyte white ghost membranes in 0.3 ml of TBS were incubated with 5 μM heme, heme–CLT complex (5 μM heme–10 μM CLT) or 5 μM CuSO₄. After incubation at 37 °C for 2 h, the samples were mixed with 0.3 ml of 0.375% TBA (v/v) solution containing 0.25 M HCl and 1% Triton X-100 (v/v). These mixtures were heated for 30 min in a boiling bath, immediately cooled on ice, and centrifuged at 15000 rpm for 10 min. The MDA production in the supernatant was determined by measuring the absorbance at 534 nm. Samples lacking membranes but containing either heme or heme–CLT complex were used as blanks. Performing the assay using 1,1,3,3-tetraethoxypropane, a precursor of MDA, confirmed that heme and heme–CLT complex did not affect the reaction between TBA and lipid peroxide.

**Effect of Heme and Heme–CLT Complex on the Osmotic Fragility of Erythrocytes**

Aliquots of a 1% suspension of erythrocytes in TBS were incubated for 2 h with heme or heme–CLT complex as described in the legend for Fig. 2. Non-hemolyzed erythrocytes were collected by centrifugation, washed three times by gentle resuspension in TBS, and then incubated for 30 min at 37 °C in 20 mM Tris, pH 7.4 containing various concentrations (42—140 mM) of NaCl. At the end of the incubation, the percentage of hemolysis was evaluated as described in our previous report.(18) Briefly, the erythrocyte suspension was centrifuged at 1500 × g for 3 min to collect non-hemolyzed erythrocytes, and the amount of hemoglobin in the supernatant was determined by absorbance at 578 nm. The hemoglobin content in the sedimented non-hemolyzed erythrocytes was measured following lysis in distilled water. The percent hemolysis was calculated from the ratio of hemoglobin content of hemolyzed erythrocytes to that of the total erythrocytes.

Because sucrose is reported to be an inhibitor of the colloid-osmotic hemolysis, we also used it to evaluate the osmotic effects of heme–CLT complex on the hemolytic response. Aliquots of a 1% suspension of erythrocytes in TBS were incubated at 37 °C with heme or heme–CLT complex in the presence or absence of 100 mM sucrose. After 2 h, these samples were centrifuged to measure the extent of hemolysis as described above.

**RESULTS**

**Effect of CLT on the Heme Binding by Erythrocyte White Ghost Membranes**

To begin to investigate the mechanism by which CLT enhances heme-induced hemolysis, we first examined the effect of CLT on heme binding by erythrocyte white ghost membranes. After incubation of membranes with heme or heme–CLT complex, the amount of bound heme was determined spectrophotometrically (Table 1). The amount of membrane-bound heme–CLT complex was not significantly different than for heme alone or with...
Table 1. Effects of CLT on Binding of Heme to White Ghost Membranes

<table>
<thead>
<tr>
<th>Additives</th>
<th>Heme bound to membrane (nmol heme/mg membrane protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heme (5 μM)</td>
<td>42.4±3.0</td>
</tr>
<tr>
<td>Heme–CLT (5—10 μM)</td>
<td>44.0±1.8</td>
</tr>
<tr>
<td>Heme (5 μM)+DMSO (0.2%)</td>
<td>42.6±4.2</td>
</tr>
</tbody>
</table>

Data shown are calculated from 3 experiments.

Table 2. Oxidation of Membrane Thiols by Heme and Heme–CLT Complex

<table>
<thead>
<tr>
<th>Additives</th>
<th>Thiol groups (nmol/mg membrane protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>55.6±0.9</td>
</tr>
<tr>
<td>Heme (5 μM)</td>
<td>24.6±1.4</td>
</tr>
<tr>
<td>Heme–CLT (5—10 μM)</td>
<td>26.3±0.9</td>
</tr>
<tr>
<td>Heme (5 μM)+DMSO (0.2%)</td>
<td>24.2±0.6</td>
</tr>
</tbody>
</table>

Data shown are calculated from 3 experiments.

Table 3. Effects of Heme–CLT Complex on Membrane Lipid Peroxidation and Erythrocyte Hemolysis

<table>
<thead>
<tr>
<th>Additives</th>
<th>MDA (A432)</th>
<th>Hemolysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.0056±0.0017</td>
<td>1</td>
</tr>
<tr>
<td>Heme (5 μM)</td>
<td>0.0106±0.0015</td>
<td>8</td>
</tr>
<tr>
<td>Heme–CLT (5—10 μM)</td>
<td>0.0202±0.0012</td>
<td>85</td>
</tr>
<tr>
<td>CuSO4 (5 μM)</td>
<td>0.0995±0.0123</td>
<td>2</td>
</tr>
</tbody>
</table>

Data shown are calculated from 3 experiments.

control solution containing only the heme solvent, 0.2% DMSO. Because heme–CLT complex and heme bound to the membrane equally, it appears that CLT does not enhance heme-dependent hemolysis by increasing the binding of heme to membranes.

Degradation of Membrane-Bound Heme by GSH and Its Inhibition by CLT We next examined GSH-dependent degradation of membrane-bound heme in the presence of CLT. Heme-loaded membrane or heme–CLT complex-loaded membrane was incubated with GSH in the presence or absence of CLT, and then centrifuged to separate membrane and unbound heme. Because absorption of heme decreases when decomposed by GSH,13,18) we measured the absorption at 402 nm after membrane lysis in 2.5% SDS to determine the amounts of heme or heme–CLT complex that had not been degraded by GSH in the membrane. Figure 2 shows that approximately 66% of the heme that had been loaded in membrane was degraded following a 2 h incubation with 10 mM GSH in absence of CLT (Fig. 2, Heme+GSH). In contrast, CLT inhibited the degradation of heme by GSH (Fig. 2, Heme+GSH+CLT). In addition, membrane-bound heme–CLT complex was only slightly decomposed by GSH (Fig. 2, Heme–CLT+GSH). These results indicate that CLT protects membrane-bound heme from GSH-dependent degradation.

Influence of CLT on Heme Oxidant Activity The oxidative action of heme on membrane protein is thought to be a mechanism of heme-induced membrane damage.23) Therefore, we investigated the influence of CLT on heme-induced oxidation of membrane protein. As shown in Table 2, the thiol content in erythrocyte white ghost membrane was 55.6 nmol/mg membrane protein when incubated with buffer only. Oxidation by heme reduced the thiol content by approximately 44%. This oxidation was not significantly modified by the presence of CLT or DMSO.

We also examined the effect of CLT on heme-catalyzed peroxidation of unsaturated lipid by measuring MDA production by the TBA reaction. As shown in Table 3, we found that a 2 h incubation with both heme and heme–CLT complex induced the formation of MDA in erythrocyte white ghost membranes. Compared to controls, heme and heme–CLT enhanced MDA formation 1.9-fold and 3.6-fold, respectively. This indicates that CLT slightly enhanced lipid peroxidation by heme. Under these conditions, the amounts of heme and heme–CLT complex bound to the membranes were nearly identical. With the same concentration of heme–CLT complex used in this experiment, 85% of erythrocytes were hemolyzed. In contrast, only 8% of erythrocytes were lysed by heme alone. In addition, CuSO4, a typical oxidant, caused little hemolysis even though there was much higher lipid peroxidation as determined by MDA formation. Thus, our results suggest that the enhancement of heme-induced hemolysis by CLT is not caused by membrane lipid peroxidation even though CLT can slightly enhance heme-mediated erythrocyte membrane lipid peroxidation.

Effect of Heme and Heme–CLT Complex on the Osmotic Fragility of Erythrocytes A colloid-osmotic mechanism has been proposed as a mechanism for heme-induced hemolysis.23) We therefore assessed the effect of heme and heme–CLT complex on the osmotic fragility of erythrocytes. We were unable to detect an increased susceptibility to hypotonic lysis at a low concentration of heme (3 μM), but at higher concentrations (10, 30 μM), heme enhanced osmotic fragility (Fig. 3). The concentration dependence of the erythrocyte susceptibility to osmotic fragility agrees with previous studies.27) However, in that work,25) as low as 0.5 μM heme could increase the osmotic fragility, suggesting that mice erythrocytes are more susceptible to osmotic fragility than human erythrocytes. This could be due to differences in membrane composition known to exist between various species.
The amount of hemolysis was determined. The results are representative of three independent experiments.

To further investigate the colloid-osmotic mechanism of heme–CLT enhanced hemolysis, sucrose was added to the suspension of erythrocytes in the presence of heme or heme–CLT complex. Figure 4 shows that 100 mM sucrose protects about 50% of erythrocytes from heme-induced or heme–CLT-induced hemolysis. This result suggests that colloid-osmotic lysis contributes to heme–CLT complex-induced hemolysis.

DISCUSSION

In normal red blood cells, hemoglobin constitutes 99% of the non-membrane protein and, although the concentration is as high as 5 mM, it is rarely denatured. However, in malaria-infected erythrocytes, 75% of the hemoglobin is ingested by the parasite, releasing free heme. The free heme readily inserts into both phosphatidylcholine and phosphatidylserine liposomes at ratio of 1 heme per 4—5 phospholipid molecules. This causes a disruption of both erythrocyte and malarial membranes. The malarial parasite possesses a specific pathway for detoxifying free heme through neutralization and removal from membrane using PfHRP2, polymerization to non-toxic insoluble hemozoin initiated by PfHRP2 or degradation by conjugation with GSH.

Recent studies have demonstrated that CLT, a well-known potential antifungal agent, can inhibit the in vitro growth of chloroquine-resistant strains of P. falciparum. The imidazole-lacking CLT metabolite, 2-chlorophenyl-bis-phenyl methanol, has weaker anti-malarial activity than CLT (IC50's of approximately 11 µM and 1 µM, respectively). This indicates that the imidazole group is not essential, but that it is important for the anti-malarial activity of CLT. Several reports suggest that the anti-malarial mechanism of 2-chlorophenyl-bis-phenyl methanol is associated with inhibition of a Ca2+ pump and Ca2+ channel.

Oxidation of membrane protein thiol groups and lipid peroxidation have been reported to be the initial step in heme-induced membrane damage. In addition, chloroquine, a representative anti-malarial drug, enhances not only heme-dependent hemolysis but also heme-stimulated peroxidation of unsaturated lipids. For these reasons, we compared the effects of heme and heme–CLT complex on the membrane protein thiol group and membrane lipid oxidation. We found that the membrane protein thiol contents decreased by 44% following a 2 h incubation with heme. Protein thiol contents were oxidized to the same extent in the presence of heme–CLT complex. Also, CLT slightly enhanced heme-induced lipid peroxidation. Although CuSO4, a typical oxidant, caused a high level of erythrocyte membrane lipid peroxidation, it did not cause hemolysis. These results indicate that the enhancement of heme-induced hemolysis by CLT is not due to the promotion of membrane-protein oxidation or lipid peroxidation.

Erythrocytes are susceptible to hypotonic hemolysis, and heme-induced hemolysis is inhibited by sucrose, suggesting that colloid osmotic lysis is a possible mechanism for heme-induced hemolysis. Indeed, we found that heme–CLT complex-induced hemolysis is inhibited by sucrose and that the heme–CLT complex enhances the susceptibility of erythrocytes to hypotonic hemolysis. These results support the idea that colloid-osmotic lysis at least contributes to heme-induced hemolysis.

Despite the fact that heme is an amphipathic molecule binds with high affinity for membrane, GSH caused the same level of degradation for membrane-bound heme as for free heme in solution. In contrast, GSH was unable to degrade membrane-bound heme in the presence of CLT.
the undegraded heme–CLT complex bound to the membrane. It is thought that formation of the heme–GSH complex initiates heme degradation, but our results suggest that GSH does not bind to heme when CLT is present. This may be due to the fact that heme has a higher affinity for CLT \((K_A = 6.54 \times 10^5 \text{ M}^{-1})\) than for GSH \((K_A = 2.8 \times 10^3 \text{ M}^{-1})\). Furthermore, even an excess of GSH (10 mM) was unable to degrade heme in the presence of 10 μM CLT (Fig. 2), and the absorption spectrum of heme–CLT complex did not change upon the addition of GSH. Because an excess of GSH was used, CLT suppression of GSH-dependent heme degradation was not caused by the direct interaction of CLT and GSH.

Free heme can bind PfHRP2, a protein that initiates the polymerization of heme into non-toxic hemozoin. Through axial ligand exchange, CLT could be able to exchange with PfHRP2 to form heme–CLT complex. A previous study examined this possibility using ESR to determine the effect of adding low levels of CLT to complexes of heme and a 27 amino acid PfHRP2 model peptide. The ESR spectrum revealed that two histidine residues bound to heme at the axial position, and indicated that heme, even when bound to PfHRP2 or GSH-bound can easily exchange with CLT to form a heme–CLT complex. These results indicate that the formation of a heme–CLT complex may interfere with both the GSH- and polymerization-mediated heme detoxification pathways in malaria parasite.

In conclusion, our results suggest that the enhancement of heme-induced hemolysis by CLT is not due to the promotion of membrane heme binding nor to membrane damage caused by membrane lipid peroxidation or membrane protein oxidation. Instead, the anti-malarial activity of heme appears to be due, at least in part, to disruption of GSH-induced degradation. In addition, the heme–CLT complex may enhance colloid-osmotic hemolysis because there was much more membrane damage caused by the heme–CLT complex than by heme alone. Given the molecular structure of CLT, the heme–CLT complex is predicted to contain two CLT molecules binding to the axial positions of heme, making it very bulky and hydrophobic, and thus enhancing colloid-osmotic hemolysis. Furthermore, aggregated heme appears to form larger holes and induce a greater degree of membrane permeability than monomeric heme. Together, this information supports the idea that the molecular size of the heme–CLT complex might participate in the enhancement of heme-induced membrane damage.

A single 1g oral dose of CLT is known to be well absorbed, with plasma levels reaching 2 μM within 2—4 h of administration. The high partitioning of CLT in erythrocytes, and the proven clinical safety after oral administration raise the possibility that CLT can be used as an anti-malarial therapeutic. Therefore, further studies should be performed to continue to elucidate the effects of CLT on the malarial defense system and to better understand its effects in vivo.

REFERENCES