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Phospholipid Membrane-Mediated Hemozoin Formation: The Effects of Physical Properties and Evidence of Membrane Surrounding Hemozoin

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Abstract

Phospholipid membranes are thought to be one of the main inducers of hemozoin formation in Plasmodia and other blood-feeding parasites. The “membrane surrounding hemozoin” has been observed in infected cells but has not been observed in vitro experiments. This study focused on observing the association of phospholipid membranes and synthetic β-hematin, which is chemically identical to hemozoin, and on a further exploration into the mechanism of phospholipid membrane-induced β-hematin formation. Our results showed that β-hematin formation was induced by phospholipids in the fluid phase but not in the gel phase. The ability of phospholipids to induce β-hematin formation was inversely correlated with gel-to-liquid phase transition temperatures, suggesting an essential insertion of heme into the hydrocarbon chains of the phospholipid membrane to form β-hematin. For this study, a cryogenic transmission electron microscope was used to achieve the first direct observation of the formation of a monolayer of phospholipid membrane surrounding β-hematin.

Background

Malaria is one of the most dangerous diseases in tropical countries. Each year, there are an estimated 250 million new malaria infections and almost a million deaths due to malaria world-wide [1]. Spreading resistance to current quinoline antimalarials and artemisinin has made malaria a major global problem [2]. Since a vaccine for malaria is not available, it is essential to study the molecular, biochemical, and immunological aspects of malarial parasites to develop vaccines and new antimalarial drugs.

Host protein digestion has two aspects: to obtain amino acids and to regulate osmotic pressure. This hemoglobin digestion takes place in the parasites’ food vacuoles and is carried out by multiple proteases including four aspartic versions [3]: three cysteine proteases [4] and a zinc metalloprotease [falsilysin] [5]. These proteases digest hemoglobin into small fragments consisting of about 20 different amino acids and free ferrous protoporphyrin IX (Fe(II)PPIX), which is rapidly oxidized to Fe(III)PPIX (heme). Heme is the deep red, oxygen-carrying, non-protein, ferrous component of hemoglobin in which the iron is Fe(II) (ferrous iron) and also called reduced hematin. Iron(III), ferriprotoporphyrin IX, (Fe(III)PPIX) is known to be present in solution as hematin (H2O/OH-Fe(III)PPIX). The free heme is oxidatively active and toxic to both the host cell and the malarial parasite, however free heme is rapidly oxidized to hematin and sequestered into hemozoin (malarial pigment). Due to the absence of heme oxygenase, the parasite is unable to cleave heme into an open-chain tetrapyrrole, which is necessary for cellular excretion [6]. To protect itself, the malarial parasite detoxifies free heme via neutralization with a histidine-rich protein 2 [7,8], degradation with reduced glutathione [9,10,11], or crystallization into hemozoin which is a water-insoluble malarial pigment that is not lethal to biological cells [7,12]. However, at least 95% of free heme in P. falciparum is reportedly converted to hemozoin [13,14]. Hemozoin is known to be structurally and chemically identical to in vitro synthetic β-hematin (BH), which is a crystal of the heme (Fe(III)PPIX) dimer of the hematin Fe(III) PPIX dimer [15,16,17]. It has been used for parasite concentration and detection [18,19,20,21,22]. It is also suggested that the blocking of BH formation is an ideal target for antimalarial screening [23,24,25,26]; thus, it is important to understand the mechanism of BH formation.

Several factors such as histidine-rich protein [7,8], elevated temperature [27], lipids [28,29], pre-formed BH [25], alcohols [30], detergent [31], and malarial heme detoxification protein [32] are reportedly responsible for heme crystallization. Among these factors, lipid droplets and phospholipid membranes are proposed as the main inducers of hemozoin formation in Plasmodia and other blood-feeding parasites including Schistosoma and Rhodius.
The mechanism of BH formation induced by neutral lipid droplets both in vitro and in vivo has been well documented [34,35,36,37,38]. The “membrane surrounding hemozoin” has been found in the in vivo ultrastructure [39,41,42], but it has not been observed in vitro experiments. In the present study, we aimed to observe the association of phospholipid membranes and BH crystals, and to further explore the mechanism of phospholipid membrane-induced BH formation.

**Materials and Methods**

**Materials**

Hemin chloride (heme) was purchased from Sigma. L-α-Phosphatidylcholine dilauroyl (dilauroyl-PC), L-α-phosphatidylcholine dimyristoyl (dimyristoyl-PC), L-α-phosphatidylcholine dipalmitoyl (dipalmitoyl-PC), L-α-phosphatidylcholine distearoyl (distearoyl-PC), L-α-phosphatidylcholine dioleoyl (dioleoyl-PC), L-α-phosphatidylserine dipalmitoyl (dipalmitoyl-PS), L-α-phosphatidylethanolamine dimyristoyl (dimyristoyl-PE), L-α-phosphatidylethanolamine dipalmitoyl (dipalmitoyl-PE), dimethyl sulfoxide, and chloroform were provided by Wako Pure Chemicals (Osaka, Japan). The remaining reagents were also acquired from Wako Pure Chemicals.

**Preparation of Lipid Vesicles**

Phospholipids were dissolved in 1 ml chloroform at a concentration of 2 mM. Then they were sprayed and dried on the walls of 1.5 ml micro-tubes under a nitrogen gas flush to create a thin layer, which was then suspended in 1 ml of distilled water. The lipid suspension (2 mM) was sonicated for 10 s and used for BH formation assay, as previously described [43], and for cryo-TEM observation.

**Assay of BH Formation Initiated by Phospholipids**

Stock heme solution (10 mM) was prepared using hemin chloride in dimethyl sulfoxide as described previously [10]. Heme (100 μM) was incubated with various concentrations of phospholipids in 1 ml of 50 mM acetate buffer at pH 4.8. For quantification of BH, after incubating at 37°C for 16 h, the sample was centrifuged for 5 min at 7,000×g, and the supernatant was discarded. The obtained BH was purified and quantified as previously described [44,45]. Values obtained from triplicate assays were plotted, and the EC20 values (M), along with the concentration of heme into BH (EC20 values) for these phospholipids varied from 30–40% of the heme converted to BH at a slightly higher concentration of 70–80% of the heme into BH, indicating a relatively high efficiency. Dimyristoyl-PC converted a maximum of 30–40% of the heme into BH at a slightly higher concentration of the inducer. The concentration that is required to convert 20% of heme into BH (EC20 values) for these phospholipids varied from 5 to 12 μM (Table 1). We also observed that the maximal yield of BH in the presence of lipids was negatively correlated with the EC20 values for these phospholipids varied from 5 to 12 μM (Table 1).

**Statistical Analysis**

Data analysis was performed using the SPSS Version 14. The Pearson correlation was analyzed to evaluate the relationship between the abilities of phospholipids to induce BH formation and their physical properties. Differences in BH formation induced by lipids were analyzed for statistical significance using the nonparametric Mann–Whitney U test. Values were considered significant at p<0.05.

**Results**

BH Formation Induced by Phospholipids

The abilities of various phospholipids to induce BH formation in vitro at 37°C are shown in Fig. 1. BH formation was induced by phospholipids in a biphasic dose-dependent manner. Two phosphatidylcholines, dilauroyl-PC and dioleoyl-PC, were catalyzed by BH formation at low molar concentrations and converted a maximum of 70–80% of the heme into BH, indicating a relatively high efficiency. Dimyristoyl-PC converted a maximum of 30–40% of the heme into BH at a slightly higher concentration of the inducer. The concentration that is required to convert 20% of heme into BH (EC20 values) for these phospholipids varied from 5 to 12 μM (Table 1). We also observed that the maximal yield of BH in the presence of lipids was negatively correlated with the EC20 values for these phospholipids varied from 5 to 12 μM (Table 1).

**Cryo-transmission Electron Microscopy (cryo-TEM)**

The analysis was performed as described previously [47,48]. Briefly, samples (2.5 μL) were applied to glow-discharged microgrids supported by 3-nm-thick carbon films (JEOL, Tokyo, Japan). After removing excess samples with pre-water-soaked filter paper, the samples were quickly frozen by liquid ethane cooled by liquid Nitrogen (EM CPC, LEICA Microsystems, Vienna). The grid was then transferred into a JEOL cryo-electron microscope (JEM3000SFF) and kept at 4.2 K and observed at 300 kV.

**Figure 1. BH formation induced by various concentrations of phospholipids at 37°C.** The ordinate shows the amount of BH induced by incubation with various concentrations of phospholipids for 16 h, as expressed as the percentage of heme converted to BH. Values represent the mean ± SD (n = 3). The results were reproducible. Circle; dioleoyl-PC, triangle; dilauroyl-PC, square; dimyristoyl-PC. doi:10.1371/journal.pone.0070025.g001
### Table 1. BH-forming ability and the physical properties of phospholipids.

<table>
<thead>
<tr>
<th>Phospholipids</th>
<th>Acyl groups</th>
<th>$T_m$ (°C)</th>
<th>MWa</th>
<th>Total net charge</th>
<th>No. of anion charge</th>
<th>logP</th>
<th>logD’</th>
<th>Hydrogen bond acceptors</th>
<th>Hydrogen bond donors</th>
<th>Freely rotating bonds</th>
<th>Polar surface area (Å²)</th>
<th>Polarizability’ ($\times 10^{-24}$)</th>
<th>BH induction (EC20 values, mM)</th>
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<tr>
<td>dilauroyl-PC</td>
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<td>0</td>
<td>621.8</td>
<td>0</td>
<td>1</td>
<td>0.72</td>
<td>9</td>
<td>6.62</td>
<td>9</td>
<td>1</td>
<td>32</td>
<td>118.17</td>
<td>1</td>
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<tr>
<td>dimyristoyl-PC</td>
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<td>23</td>
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<td>1</td>
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<td>9</td>
<td>8.65</td>
<td>9</td>
<td>1</td>
<td>36</td>
<td>121</td>
<td>1</td>
</tr>
<tr>
<td>dipalmitoyl-PC</td>
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<td>42</td>
<td>734.0</td>
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<td>1</td>
<td>10.09</td>
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<td>9</td>
<td>1</td>
<td>40</td>
<td>121</td>
<td>1</td>
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<td>distearoyl-PC</td>
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<td>55</td>
<td>790.1</td>
<td>0</td>
<td>1</td>
<td>12.12</td>
<td>9</td>
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<td>1</td>
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<tr>
<td>dimyristoyl-PE</td>
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<td>48</td>
<td>635.8</td>
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<td>1</td>
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<td>9</td>
<td>8.51</td>
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<td>36</td>
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<td>41</td>
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*Values were retrieved from ChemSpider (www.chemspider.com), predicted by Advanced Chemistry Development (ACD/Laboratories) software.

*Performed by this study.

BH formation was correlated with their membrane fluidity. BH was positively correlated with the reaction temperature. These phospholipids (Table 1, $T_m$) could induce BH formation. Phospholipids such as dioleoyl-PC showed morphologies that were almost identical (Fig. 4). The dioleoyl-PC liposome was not observed around the BH, but a monolayer membrane-like structure that surrounded the BH was observed, and is indicated by arrows in Figs. 4B and 4D. With longer irradiation from an electron beam, this membrane-like monolayer membrane-like structure was not observed around the BH, but a monolayer membrane-like structure was observed as variable-sized crystals with long thin shapes. These morphological characteristics were similar to those seen in previous reports of BH both in vivo and in vitro [50,51]. As shown in previous reports, BH was observed to have two distinct structures: the BH core and the BH shell. The BH core was spherical vesicles with a bilayer membrane (Fig. 3A), while a few spherical vesicles was related to structure, observations by cryo-TEM were mostly observed as multilamellar vesicles with a bilayer membrane (Fig. 3B). The formation of BH catalyzed by dioleoyl-PC liposome was also observed morphologies that were almost identical (Fig. 3A). The BH formation spontaneous occurs with no inducer at high temperature (37°C), catalyzed by dioleoyl-PC liposome was also observed as multilamellar vesicles. However, dilauroyl-PC liposomes were observed as spherical vesicles with a bilayer membrane (Fig. 3A). The BH formation spontaneous occurs with no inducer at high temperature (37°C). Our results showed that the ability of phospholipids to induce BH formation was inversely correlated with the gel-to-fluid phase transition temperature ($T_m$) of phospholipids. A lower $T_m$ was more effective in inducing BH formation than a higher $T_m$. In contrast, other physical properties of phospholipids such as dioleoyl-PC with a lower $T_m$ were more effective in inducing BH formation than those with a higher $T_m$. Our results showed that the ability of phospholipids to induce BH formation was inversely correlated with the gel-to-fluid phase transition temperature ($T_m$) of phospholipids. To further understand the mechanism of phospholipid-mediated BH formation, cryo-TEM images of BH were observed using cryo-TEM. The cryo-TEM images of BH showed morphologies that were almost identical (Fig. 4). The BH formation was correlated with their membrane fluidity. BH formation was correlated with their membrane fluidity. BH formation was correlated with their membrane fluidity. BH formation was correlated with their membrane fluidity. BH formation was correlated with their membrane fluidity. BH formation was correlated with their membrane fluidity.
structure was burned and developed a white color, as observed under cryo-TEM. Given the fact that only the dioleoyl-PC in the mixture had the potential to form a monolayer structure, this observation strongly suggests that the membrane-like structure was formed by a phospholipid.

Discussion

Numerous studies have suggested that neutral lipid droplets are the main templates for hemozoin formation [34,35,36,37,38]. For many years, phospholipid membranes have also been proposed as the site of hemozoin formation in malaria [39,40]. Recently, Kapishnikov et al. used cryogenic soft X-ray tomography to demonstrate the hemozoin formation templates on the inner layer of a digestive vacuole [41]. However, very few studies have investigated BH formation induced by phospholipid membranes in an in vitro experiment [40,52]. As far as could be ascertained, the present study is the first to observe a membrane surrounding BH that was induced by phospholipid vesicles (Fig. 4), which was a structure that was similar to that observed in vivo by Hempelmann et al. [39]. The advantage of cryo-TEM was that it allowed the direct observation of phospholipid membranes and BH formation with no fixation or dehydration of the sample on the grid. In addition, cryo-TEM was suitable for the detection of phospholipid membranes because the phosphorus groups have a low permeability to the electron beam. Moreover, the association of polar lipids with malarial hemozoin observed by the thin layer chromatography also supports our results [53].

The differences in morphology between dioleoyl-PC and dipalmitoyl-PC vesicles, as observed by cryo-TEM, suggested that a smooth regular shape of vesicles is required for the site of a BH template. The differences in morphology between dioleoyl-PC and dipalmitoyl-PC vesicles were probably due to their different physical properties, in particular the different states – fluid or gel – caused by the differences in \( T_{m} \) values. Moreover, since the hydrophobic interactions between their inducers and heme have been proposed as an important force in the creation of a precursor heme dimer [38,45,54,55], it was interesting to explore the correlation between the physical properties of phospholipids and their ability to induce BH. Our results demonstrated that phospholipids could induce BH formation only in the fluid phase, and could not do so in the gel phase. Recently, Hoang et al. showed that a blending of five neutral lipids lower the melting temperature of lipid droplets compared to the homogeneous samples and the blending of five neutral lipids produced more hemozoin compared to that of homogeneous lipids, further supporting the role of lipid fluidity in the hemozoin formation.

Evidence showed that an increase in membrane fluidity results in the membrane insertion of heme [56], which is positively correlated with an increase in the BH induction of phospholipids (Fig. 2). These observations suggest that the mechanism of BH formation involves the acyl chains of the phospholipid membranes.
Furthermore, free heme can be quickly and easily inserted into phospholipid vesicles as monomeric heme at a ratio of 1 heme per 4–5 phospholipid molecules in the fluid phase [57,58]. Taken together, these observations suggest that the free heme inserts its vinyl groups deeply into the hydrophobic acyl chains of phospholipids while the charged propionate groups are exposed to the aqueous solution. The hydrophobic environment of acyl chains helps to form monomeric heme, which favors the formation of a BH dimer. However, other mechanism cannot be excluded, and further studies are needed to clarify this issue.

A biphasic dose-dependent manner of BH formation induced by phospholipids was also observed in the assay induced by a detergent [31], probably due to the over dilution of the heme molecules in the high number of vesicles. Further studies are required to clarify this mechanism.

Conclusions
Our results showed that the abilities of phospholipids to induce BH is inversely correlated with the phase transition temperatures, suggesting a required insertion of heme into the hydrophobic acyl chains of a phospholipid membrane in order to form BH. Finally, a monolayer of membrane surrounding BH was observed using cryo-TEM.

Author Contributions
Conceived and designed the experiments: NTH YS A. Maeda TTM A. Miyazawa KK. Performed the experiments: NTH YS A. Maeda TTM AH. Analyzed the data: NTH YS A. Maeda TTM KH AH A. Miyazawa KK. Contributed reagents/materials/analysis tools: NTH A. Miyazawa KK. Wrote the paper: NTH YS A. Maeda TTM KH AH A. Miyazawa KK.

Figure 3. Cryo-TEM images of lipid liposomes prepared in water at 37°C. Liposome of dioleoyl-PC (A and B) or dipalmitoyl-PC (C and D) prepared in water by ultrasonication was observed by cryo-TEM. Scale bars are 50 nm in Figs. A and B and 100 nm in Figs. C and D. doi:10.1371/journal.pone.0070025.g003
References


Figure 4. Cryo-TEM images of BH catalyzed by dioleoyl-PC liposome. Heme (100 μM) was incubated with dioleoyl-PC liposome in 1 ml of acetate buffer at pH 4.8. After incubation for 16 h at 37 °C, the samples were observed by cryo-TEM as described in the Methods section. Figs. B and D are magnified images of the squared area in Figs. A and C, respectively. Black arrows in Figs. B and D indicate the monolayers of the phospholipids. Scale bars are 100 nm. doi:10.1371/journal.pone.0070025.g004


