Cysteine Proteinase Inhibitors Block Early Steps in Hemoglobin Degradation by Cultured Malaria Parasites

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Erythrocytic malaria parasites degrade hemoglobin as a source of amino acids for parasite protein synthesis. Cysteine proteinase inhibitors have been shown to block the hydrolysis of globin by cultured parasites, indicating that a malarial cysteine proteinase is required for this process. In the present study, we have evaluated the role of parasite proteinases in earlier steps of hemoglobin degradation, namely the dissociation of the hemoglobin tetramer and the separation of heme from globin. Hemoglobin did not spontaneously denature or release heme under the pH and reducing conditions of the malarial food vacuole, suggesting that parasite enzymatic activity is necessary for early steps in hemoglobin degradation. The incubation of cultured parasites with cysteine proteinase inhibitors inhibited the denaturation of hemoglobin and the release of heme from globin. These results suggest that, in addition to its role in globin hydrolysis, a malarial cysteine proteinase participates in the dissociation of the hemoglobin tetramer and the release of heme from globin. Thus, the malarial cysteine proteinase is a promising target for antimalarial chemotherapy.

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MATERIALS AND METHODS

Reagents. E-64, pepstatin, human globin, human hemoglobin, equine microperoxidase, and saponin were from Sigma (St Louis, MO). Benzoylxyacyarbonyl (Z)-Phe-Arg-CH₂F was kindly provided by Prototek (Dublin, CA). Purified human hemoglobin of known native conformation (based on its normal oxygen-carrying capacity) was kindly provided by Dr Mark Scott (Albany Medical College, Albany, NY). Erythrocytes for hemoglobin assay and parasite culture were obtained from healthy volunteers.

Parasite culture. It-strain Plasmodium falciparum parasites were cultured with human erythrocytes at 2% hematocrit in RPMI media and 10% human serum,22 synchronized with sorbitol,22 and assessed microscopically with Giemsa-stained smears. For analysis of the effects of proteinase inhibitors, cultures were incubated in 24-

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well culture plates with proteinase inhibitors added to the cultures from 100% stocks in water (E-64) or dimethylsulfoxide (DMSO; Z-Phe-Arg-CH₂F, pepstatin). Control cultures containing equivalent volumes of water and DMSO were maintained and evaluated in parallel.

Preparation of parasite proteins. Erythrocytes from parasite cultures were lysed with the nonionic detergent saponin, which selectively lysed erythrocytes, but not parasite membranes, as follows. Erythrocytes were collected from parasite cultures and washed in phosphate-buffered saline (PBS). Erythrocyte pellets were mixed well with 5× pellet volumes of 0.1% saponin in PBS and incubated for 10 minutes on ice, 1 mL of ice-cold PBS was added, the samples were centrifuged (13,000 g for 5 minutes at 4°C), and the remaining parasites and erythrocyte ghosts were washed four times in ice-cold PBS (13,000 g for 5 minutes at 4°C) to remove erythrocyte cytoplasmic contents (including erythrocyte hemoglobin). Parasite proteins were mixed with the appropriate sample buffers before electrophoresis. For control erythrocyte lysates, erythrocytes were diluted 1:5 in H₂O and subjected to three freeze-thaw cycles; the resulting lysate was mixed with appropriate sample buffers for electrophoresis. The concentration of purified native hemoglobin used in individual experiments was determined using a Bradford assay, with commercial hemoglobin as a control.

Electrophoresis. For reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), samples were solubilized in standard SDS-PAGE sample buffer with β-mercaptoethanol and boiled for 3 minutes before electrophoresis in 15% acrylamide gels. For nonreducing SDS-PAGE, the procedure was identical except that β-mercaptoethanol was omitted from the sample buffer and samples were not boiled. For nondenaturing PAGE, electrophoresis was performed without SDS using a modification of established methods, as follows. Samples were solubilized in nondenaturing sample buffer (50 mmol/L Tris HCl, 10% glycerol, 3% Tween 20, pH 6.9); β-mercaptoethanol was not added and samples were not boiled. Electrophoresis was performed in 5.6% acrylamide stacking gels (50 mmol/L Tris HCl, pH 6.9) and 8% acrylamide separating gels (150 mmol/L Tris HCl, pH 8.9) using distinct cathode (53 mmol/L Tris HCl, 52 mmol/L glycine) and anode (0.1 mol/L Tris HCl, pH 8.0) buffers. After electrophoresis, proteins in the gels were stained with Coomassie blue or blotted onto nitrocellulose.

Chemiluminescence assays. A chemiluminescence assay was used to detect heme moieties, taking advantage of the inherent peroxidase activity of heme. For these assays, electrophoresed proteins were blotted onto nitrocellulose and peroxidase activity was detected by its oxidation of luminol, which causes the emission of light, using reagents and protocols from the ECL detection system (Arlington Heights, IL). Light emission was detected with XOMAT AR film (Eastman Kodak, Rochester, NY).

Immunoblotting. For immunoblotting, electrophoresed proteins were blotted onto nitrocellulose membranes, and blots were blocked with 1% bovine serum albumin in tris-buffered saline/0.05% Tween 20, incubated with rabbit antihuman hemoglobin antiserum (1:4,000 dilution; DAKO, Carpenteria, CA) for 1 hour, washed extensively in Tris-buffered saline/0.05% Tween 20, incubated with alkaline phosphatase-conjugated goat antirabbit IgG (1:7,500; Promega, Madison, WI) for 30 minutes, washed extensively in tris-buffered saline/1% Tween 20, and developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate following the Promega Protoblott II protocol.

RESULTS

Native hemoglobin was fairly well resolved using nondenaturing PAGE. Critical for our studies was a simple assay that allowed the differentiation of native and partially or fully denatured hemoglobin. We optimized a nondenaturing PAGE system for this purpose. Although resolution was somewhat less sharp than that with SDS-PAGE, nondenaturing PAGE did allow us to easily distinguish native hemoglobin (purified from human erythrocytes or present in erythrocyte lysates) from denatured hemoglobin migrating as α- and β-globin monomers (Fig 1). After boiling, the rapid denaturation of native hemoglobin to globin molecules was readily identified by Coomassie blue staining (Fig 1A) and by a chemiluminescence assay that detects heme-containing proteins (Fig 1B).

Hemoglobin maintained its native conformation and heme-carrying capacity at the acid pH of the malarial food vacuole. A recent study showed that hemoglobin was denatured, as measured by spectral changes, when subjected to acid pH. However, the denaturation of hemoglobin was very slow at a pH greater than 4.0, suggesting that, in the
hemoglobin also retained heme, as shown with chemiluminescence (Fig 2C). The more sensitive chemiluminescence assay did identify a small subset of slower-migrating hemoglobin molecules that appeared to be partially denatured. However, the fact that the most of the hemoglobin retained its native conformation and heme-carrying capacity at pH ≈5.0 suggests that enzymatic activity, and not simply the acidity of the food vacuole, is required for the extensive hemoglobin denaturation and heme release that occurs in erythrocytic parasites.

**Effects of proteinase inhibitors on hemoglobin denaturation and heme release.** To determine if malarial cysteine proteinase activity is involved in initial steps in hemoglobin degradation in addition to its role in globin hydrolysis, parasites were cultured with the cysteine proteinase inhibitor E-64, and parasite proteins were subsequently analyzed using multiple assays (Fig 3). As shown previously, E-64 caused trophozoites to develop swollen, dark-staining food vacuoles and, as determined using SDS-PAGE, to abnormally accumulate large quantities of globin (Fig 3A). When pro-
teins were separated by nonreducing SDS-PAGE, chemiluminescence assays showed that the globin retained by the E-64–treated parasites remained bound to heme (Fig 3B). When proteins were separated by nondenaturing (native) PAGE, Coomassie blue, chemiluminescence, and immunoblot assays showed that E-64–treated parasites had accumulated abundant hemoglobin that migrated, in part, at the position of native hemoglobin and, in part, as a more slowly migrating smear, suggesting partial denaturation (Fig 3A through C). Control parasites contained negligible heme-bound globin or hemoglobin. In summary, when incubated with cultured malaria parasites, the cysteine proteinase inhibitor E-64 partially inhibited the denaturation of the hemoglobin tetramer and blocked the separation of heme from globin.

Aspartic proteinases have been identified in the malarial food vacuole and shown to degrade hemoglobin in vitro.13-15 In our previous studies, the aspartic proteinase inhibitor pepstatin was toxic to cultured parasites, but it did not cause the accumulation of undegraded globin.11,17 Using reducing SDS-PAGE and nondenaturing PAGE, we compared the effects of pepstatin on the initial steps of hemoglobin degradation with those of E-64 and the specific falcipain inhibitor Z-Phe-Arg-CH₂F (Fig 4). As shown above with E-64, Z-Phe-Arg-CH₂F inhibited the denaturation of hemoglobin and heme release. However, in pepstatin-treated parasites, negligible hemoglobin of either native or denatured conformation was detectable by either Coomassie blue staining or the chemiluminescence assay. Our data indicate that, when incubated with cultured malaria parasites, the broadly effective cysteine proteinase inhibitor E-64 and the specific falcipain inhibitor Z-Phe-Arg-CH₂F, but not the aspartic proteinase inhibitor pepstatin, inhibited the processing of hemoglobin. This inhibition in processing included at least partial blocks in hemoglobin denaturation, heme release, and globin hydrolysis.

DISCUSSION

Erythrocytic malaria parasites degrade hemoglobin in an acidic food vacuole to provide amino acids and perhaps iron for parasite metabolic needs. Hemoglobin degradation involves multiple, probably interrelated steps (Fig 5). We have previously shown that cysteine proteinase inhibitors block the hydrolysis of globin to free amino acids (Fig 5, step 4)11 and, thus, that a cysteine proteinase, presumably the food vacuole proteinase falcipain,12 is required for globin hydrolysis. However, in older studies using SDS-PAGE techniques, we were unable to determine whether the cysteine proteinase is also involved in earlier steps in hemoglobin degradation, including dissociation of the hemoglobin tetramer (step 2) and the separation of heme from globin (step 3).

To determine if the acid pH of the malarial food vacuole was itself sufficient to denature hemoglobin and/or allow the separation of heme from globin, we incubated native hemoglobin under acid conditions and evaluated the conformation of the protein using nondenaturing PAGE. In agreement with an earlier study that used spectrophotometric assays,9 we found that hemoglobin was quite rapidly denatured at pH less than 4.5, but that at the milder acid conditions of the malarial food vacuole, which has been shown to have pH of 5.0 to 5.4 by two independent methods,18,19 native hemoglobin did not appear to be significantly altered. Thus, it appeared that a parasite enzymatic activity was required for hemoglobin denaturation and heme release.

To characterize the activity required for early steps in hemoglobin degradation, we used nondenaturing PAGE to determine the conformation of the hemoglobin that accumulated in malarial trophozoites after incubation with proteinase inhibitors. Parasites incubated with the cysteine proteinase inhibitors E-64 and Z-Phe-Arg-CH₂F, but not the aspartic proteinase inhibitor pepstatin, accumulated hemoglobin that...
was partially in the native tetramer conformation. Most of the hemoglobin that accumulated in these parasites appeared to be partially denatured, and so enzymes in addition to falcipain are likely also involved in hemoglobin denaturation. However, the quantity of native hemoglobin that accumulated in cysteine proteinase inhibitor-treated parasites may have been underestimated by our assays, because hemoglobin may have been denatured not only during parasite culture, but also during sample preparation. In any event, even the partial block in hemoglobin denaturation that was clearly shown in our studies likely contributes to the antimalarial effects of cysteine proteinase inhibitors, because the efficient processing of hemoglobin appears to be critical to parasite development.

To evaluate the retention of heme within hemoglobin molecules in proteinase inhibitor-treated parasites, we used a chemiluminescent assay that takes advantage of the inherent peroxidase activity of heme. Under the reducing SDS-PAGE conditions that were used in prior studies with proteinase inhibitors, the globin that accumulated in cysteine proteinase inhibitor-treated parasites did not retain heme. When these proteins were electrophoresed under nonreducing SDS-PAGE conditions, hemoglobin was seen to have denatured not only to globin monomers, but these monomers retained their heme moieties. With nondenaturing PAGE, the native and partially denatured hemoglobin that accumulated in cysteine proteinase inhibitor-treated parasites was seen to retain large quantities of heme. These results suggest that falcipain has an important role not only in hemoglobin denaturation, but also in the separation of heme moieties from hemoglobin.

Our results suggest that the inhibition of falcipain may be toxic for malaria parasites for multiple reasons, all related to a block in hemoglobin degradation. Most clearly, inhibition of falcipain activity may starve the parasite for free amino acids. This loss of available amino acids for protein synthesis is likely principally responsible for the block in parasite development at the trophozoite-schizont stage that is engendered by cysteine proteinase inhibitors. As a second mechanism for toxicity, falcipain inhibitors may limit parasite supplies of iron required for the synthesis of iron-containing proteins such as ribonucleotide reductase and for the synthesis of parasite-derived heme, which appears to be required for parasite protein synthesis. The source of free iron for malaria parasites is uncertain. Potential sources of parasite iron include serum iron (obtained via transferrin receptors or transferrin-independent iron uptake mechanisms), free intraerythrocytic iron, erythrocyte ferritin, and hemoglobin. However, the presence of transferrin receptors on parasitized erythrocytes has been refuted, the lack of an antimalarial effect of iron deficiency argues against parasite use of serum iron or ferritin, and the lack of an antimalarial effect of intraerythrocytic iron chelators suggests that free erythrocytic iron is not used by parasites. Thus, although malarial heme degrading enzymes have not been identified, it remains likely that parasites use a small portion of hemoglobin iron for their metabolic needs. If this is the case, the inhibition of heme-globin dissociation might exert an antimalarial effect.

As a third mechanism for toxicity of falcipain inhibitors, a block in the separation of heme and globin may allow the persistence of potentially toxic heme-globin complexes. The persistence of these complexes would be expected to limit hemozoin formation. Indeed, it was recently shown that the cysteine proteinase inhibitor E-64, but not the aspartic proteinase inhibitor pepstatin or the antimalarials chloroquine or artemisinin, inhibited hemozoin formation by cultured parasites. Free heme is toxic to malaria parasites and heme-chloroquine complexation has been offered as a possible explanation for the antimalarial effects of this drug. We have shown that cysteine proteinase inhibitors cause the persistence of heme-globin complexes, as shown by nondenaturing PAGE and nonreducing SDS-PAGE. It seems plausible that the heme-globin complexes that accumulate under these conditions may be toxic to intraerythrocytic parasites.

Our results suggest that falcipain is a promising target for antimalarial chemotherapy and that cysteine proteinase inhibitors have antimalarial effects. Further studies will be needed to understand the mechanisms involved.
inhibitors have potential as antimalarial drugs. Indeed, specific fluoromethyl ketone inhibitors of falcipain have been shown to block hemoglobin degradation by cultured parasites and to cure malaria in Plasmodium vinckeii-infected mice. The current results show that the inhibitors acted not only against globin hydrolysis but also at the more proximal steps of hemoglobin denaturation and heme release, suggesting that falcipain has a key role in these processes and perhaps explaining the marked potency of cysteine proteinase inhibitors against malaria parasites.

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REFERENCES

5. Fulton JD, Grant PT: The sulphur requirements of the erythrocytic form of Plasmodium knowlesi. Biochem J 63:274, 1956
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