Intracellular Ferriprotoporphyrin IX Is a Lytic Agent

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Human erythrocytes were treated with menadione to oxidatively denature hemoglobin and release ferriprotoporphyrin IX (ferriheme, FP) intracellularly. The high affinity of FP for chloroquine was used to detect its release. After incubation for 1 hr at 37°C and pH 7.4 with 0.5 mM menadione, erythrocytes bound 14C-chloroquine with an apparent dissociation constant of 10^-8 M. Untreated erythrocytes did not bind chloroquine with high affinity. At a chloroquine concentration in the medium of 2 μM, for example, menadione-treated erythrocytes bound 70 μmole chloroquine/kg and untreated erythrocytes bound 13.4 μmole/kg. The intracellular location of FP released by menadione was verified by finding that Tween 80 did not prevent chloroquine binding. By contrast, Tween 80 inhibited the binding of chloroquine to erythrocytes treated with extracellular FP. The hemolytic response to menadione was characteristic of the hemolytic response to FP. Thus, 5 μM chloroquine caused hemolysis to increase to 60% from baseline values of 5% in experiments using erythrocytes treated either with 0.5 mM menadione or with 5 μM FP; and, in both cases, the potentiating effect of chloroquine was inhibited by 1 μM mefloquine or 10 μM quinine. Higher concentrations of menadione caused hemolysis in the absence of chloroquine. We conclude that FP released by menadione exists intracellularly in a form that is accessible to bind chloroquine and to express its lytic activity.

ERYTHROCYTES AND MALARIA parasites exposed to extracellular ferriprotoporphyrin IX (ferriheme, FP) swell, become spherical, and subsequently lyse.1,1 Detailed studies of mouse erythrocytes showed that hemin (FP chloride) induces hemolysis by a colloid-osmotic mechanism.2 Although studied less extensively, hematin (FP hydroxide) has similar effects on mouse erythrocytes. The membrane lesion has not been completely described, but FP is known to bind to phospholipids,6 and the hemolytic response can be potentiated or inhibited by diverse amphipathic agents, such as Tween 80 (polyoxyethylenesorbitan mono-oleate) and the antimalarial drugs, chloroquine, quinine, and mefloquine, which also bind to phospholipids.7,8 The lysis of erythrocytes by FP occurs without peroxidation of membrane phospholipids, as detected by the production of malonyldialdehyde, and is not inhibited by various free radical scavengers.7 Consequently, it is probable that hemolysis results from a perturbation of membrane structure induced by the binding of FP to membrane phospholipids.8

Because extracellular FP is a lytic agent, it is important to know whether intracellular FP likewise would lyse erythrocytes. FP is produced intracellularly when Heinz bodies are formed by oxidative denaturation of hemoglobin.9,10 Indeed, oxidizing agents may cause enough hemoglobin breakdown to stain the erythrocyte membrane green.11 We have proposed, therefore, that FP could contribute to excessive hemolysis as well as to protection against malaria in patients with glucose-6-phosphate dehydrogenase deficiency, thalassemia, and other Heinz body hemolytic anemias.1,2,4,5 We now provide support for this proposal by presenting evidence that FP functions as a lytic agent in human erythrocytes exposed to menadione.

Menadione releases FP by oxidatively denaturing hemoglobin in vitro,12 and it can cause hemolytic anemia.13,14 To relate these two phenomena to each other, we designed experiments (A) to detect FP release in erythrocytes during exposure to menadione and (B) to compare the hemolytic responses of erythrocytes to FP and menadione. FP binds chloroquine with high affinity,15 and this property was used to detect it in intact erythrocytes in the presence of high concentrations of hemoglobin. Neither hemoglobin, methemoglobin, nor other normal erythrocyte constituents bind chloroquine with high affinity.15,16 To accomplish the second objective, we took advantage of the fact that amphipathic agents characteristically either potentiate or inhibit the hemolytic response to FP.9

MATERIALS AND METHODS

After obtaining informed consent, blood was drawn from healthy men and women, heparinized to prevent clotting, and centrifuged to obtain a pellet of erythrocytes. The erythrocytes were washed twice with an aqueous medium consisting of 141 mM NaCl and 10 mM Tris (hydroxymethyl)aminomethane (pH 7.7, 25°C), and then they were used to prepare membrane ghosts or they were resuspended in the Tris-saline medium for treatment with menadione or FP as described in the legends for the figures. The effects of these treatments on the binding of chloroquine and on hemolysis were measured. Ring-labeled 3-14C-chloroquine (2.36 μCi/μ mole) was purchased from New England Nuclear Corporation, Boston, MA, to permit radiochemical measurement of chloroquine bound to erythrocytes and remaining in the medium after an incubation period of

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sufficient length to ensure that steady-state conditions had been reached. For measurement of radioactivity, the chloroquine was quantitatively extracted under alkaline conditions into heptane. To evaluate hemolysis, intact erythrocytes were sedimented by centrifugation at the end of an incubation period, and the amount of hemoglobin in the supernatant solution was determined by measuring absorbance at 540 nm. Then the erythrocyte pellet was lysed with distilled water, the hemoglobin content was measured, and percent hemolysis was calculated.

To overcome solubility problems, a stock solution of 1 mM FP was prepared in 0.02 N NaOH freshly each day and kept on ice until final dilutions were made with the standard medium. After dilution, the pH of solutions containing FP was 7.7 at 25°C. Menadione was prepared immediately before use in the Tris-saline medium, with the aid of Tween 80 (0.001%-0.02% v/v) and sonication to obtain a homogeneous suspension. Mefloquine was prepared in ethanol and subsequently diluted with the Tris-saline medium. Erythrocytes were never exposed to ethanol concentrations exceeding 0.1% (v/v), and control experiments revealed that this concentration had no detectable effect on the hemolytic responses to menadione and extracellular FP.

Mefloquine hydrochloride was provided by the Walter Reed Army Institute of Research. Hemin, crystalline human hemoglobin, quinine sulfate, menadione, and Tween 80 were purchased from Sigma Chemical Company, St. Louis, MO.

RESULTS

In agreement with earlier studies of mouse erythrocytes, human erythrocytes did not bind chloroquine with high affinity (Fig. 1). After treatment with menadione, however, the erythrocytes did bind chloroquine with high affinity (Fig. 1), as predicted for the formation of a complex with FP. In repetitions of these experiments, the difference between menadione-treated and untreated erythrocytes was reproducible and generally greater than is illustrated in Fig. 1. At a concentration of 2 μM chloroquine in the medium, for example, erythrocytes treated with 0.5 mM menadione bound 70.0 ± 18.1 μmole chloroquine/kg and untreated erythrocytes bound 13.4 ± 3.2 μmole/kg (means ± SD for 4 separate experiments with each preparation). Also, in complementary experiments, menadione had no effect on chloroquine binding by white erythrocyte membrane ghosts. However, when crystalline human hemoglobin was added to the ghosts, menadione readily induced high-affinity chloroquine binding.

The apparent dissociation constant ($K_d$) for the binding of chloroquine to menadione-treated erythrocytes was estimated to be approximately $10^{-6} \text{ M}$ in each of 4 experiments, similar to the one illustrated in the top panel of Fig. 1. A double reciprocal transformation of the data was used to permit estimation of the apparent $K_d$. For comparison, the apparent $K_d$ for the binding of chloroquine to extracellular FP adsorbed to erythrocytes is approximately $10^{-7} \text{ M}$. The present results with FP (Fig. 1) are in agreement with a more extensive earlier study in which erythrocytes were preincubated with FP, and the free FP was removed prior to measuring chloroquine binding. Also, as expected from earlier studies, Tween 80 inhibited chloroquine binding to erythrocytes treated with extracellular FP (middle panel of Fig. 1), but did not inhibit chloroquine binding to erythrocytes treated with menadione (top panel of Fig. 1). Tween 80 had no effect on chloroquine uptake by erythrocytes in the absence of menadione and FP (bottom panel of Fig. 1).

Incubation of human erythrocytes with a 1-mM suspension of menadione caused 30% or greater hemolysis (experiments not shown), as has been previously reported for rat erythrocytes. Incubation with smaller amounts of menadione caused little or no hemolysis, unless chloroquine was included in the incubation medium (top panel of Fig. 2). A similar potentiating effect of chloroquine on hemolysis by extracellular FP chloride (hemin) is shown in the bottom panel of Fig. 2. The explanation for the potentiation by chloroquine remains to be determined, but it may involve the formation of a complex with FP. Chloroquine penetrates the erythrocyte membrane rapidly and, thus, would be able to reach intracellular FP to form such a complex. The effect of chloroquine on the hemolytic responses to menadione and extracel-
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**Fig. 2.** Lysis of erythrocytes treated with menadione or FP. Suspensions of erythrocytes (0.5%) were incubated at 37°C for 2 hr in room air and pH 7.4 with shaking at a rate of 2.3 Hz. At the end of incubation, the hemoglobin contents of the media and erythrocyte pellets were measured for calculation of percent hemolysis.14 The means and ranges of the results of triplicate incubations are shown. (Upper panel) The basal medium contained 0.5 mM menadione suspended with the aid of 0.008% Tween 80. In control experiments, this concentration of Tween 80 was observed with concentrations as low as 0.001%.

Additions to the basal medium were either none (A) or 5 mM chloroquine (B), 5 mM chloroquine plus 1 mM mefloquine (C), or 5 mM chloroquine plus 10 mM quinine (D). (Bottom panel) The basal medium contained 5 mM FP chloride (hemin) and no Tween 80. Additions to the basal medium were as listed for the upper panel. For comparison with the upper panel, the effect of adding 5 mM chloroquine plus Tween 80 is shown in E. The inhibitory effect of Tween 80 was observed with concentrations as low as 0.001%, which was the concentration used to obtain the results represented in E.

Menadione toxicity caused by two other antimalarial drugs, mefloquine and quinine, which can penetrate the erythrocyte membrane. In addition, in agreement with the results shown in Fig. 1, Tween 80 inhibited the potentiating effect of chloroquine on extracellular FP, but did not inhibit the potentiating effect of chloroquine on the hemolytic response to menadione (Fig. 2).

**DISCUSSION**

From the results of the present experiments, we conclude that menadione releases FP intracellularly in a form accessible to bind chloroquine and to express its lytic activity. The release of FP and its accessibility is demonstrated by high-affinity binding of chloroquine to menadione-treated erythrocytes.15 The intracellular location of the FP explains why Tween 80 did not inhibit chloroquine binding to menadione-treated erythrocytes and may explain the relatively high value for the apparent $K_d$, since high intracellular concentrations of hemoglobin and other proteins would influence the interaction between chloroquine and FP. The hemolytic response to menadione is characteristic of the hemolytic response to FP, i.e., in both cases, chloroquine potentiates the response and the effect of chloroquine is inhibited by mefloquine or quinine. And finally, in agreement with our conclusion, previous work has demonstrated that menadione and FP both cause massive potassium efflux, increase osmotic fragility, and lyse erythrocytes in the absence of lipid peroxidation, as detected by the production of malonyldialdehyde.2,19

Menadione toxicity causes hemolytic anemia in experimental animals and in susceptible human subjects, including premature infants and patients with glucose-6-phosphate dehydrogenase deficiency.13,14 Low concentrations of menadione also are toxic for *Plasmodium falciparum* parasites growing intracellularly in erythrocytes from human subjects with glucose-6-phosphate dehydrogenase deficiency or $\beta$-thalassemia.20 Our results indicate that FP is a mediator of menadione toxicity and strongly implicated FP in the pathogenesis of various oxidant-induced hemolytic anemias and in the protection against malaria provided by oxidant-sensitive erythrocytes.

**REFERENCES**

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