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Report

Selective Toxicity of the Antimalarial Primaquine—Evidence for Both Uncoupling and Inhibitory Effects of a Metabolite on the Energetics of Mitochondria and Its ATP Synthase Complex

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A methylene-linked dimeric metabolite of primaquine can exist as an oxidized form (PD^+) or a reduced form (PDH). Using a rat liver mitochondrial preparation, low concentrations ($E_{50\%}$ = 12 μM) of PD^+ were found to act as a classical uncoupler of oxidative phosphorylation leading to a stimulation of mitochondrial respiration. At higher concentrations, PD^+ was found to inhibit a purified F_1-ATPase preparation, while its effects on the complete ATP synthase complex (F_0F_1-ATPase) was bimodal. A working hypothesis relating F_0F_1-ATPase and glucose-6-phosphate dehydrogenase activity to the selective toxicity of primaquine on the malaria parasite and host is presented.

KEY WORDS: F_0F_1-ATPase; glucose-6-phosphate dehydrogenase; parasites; malaria; primaquine metabolism.

INTRODUCTION

Primaquine remains the antimalarial of choice for the treatment of the tissue phase of the *Plasmodium* parasite despite the manifold toxic effects of the drug. Much of the past research on the toxicity of primaquine has focused on the pentose phosphate pathway because of the very early finding that those individuals with a deficiency in glucose-6-phosphate dehydrogenase (G6PD) often develop a severe hemolytic anemia when administered primaquine (1). Studies on the effects of primaquine on *Plasmodium* have also focused on that organism's partial deficiency of G6PD (2). It is proposed here that primaquine is metabolized to a dimeric cation metabolite (PD^+; Fig. 1) of a high lipophilicity that is then accumulated in the mitochondria of the parasite, with the proton gradient of the inner mitochondrial membrane as a primary driving force. The PD^+ species then binds to the F_0F_1-ATPase within the parasite's mitochondria, resulting in inhibition of oxidative phosphorylation (Fig. 2), which ultimately leads to the destruction of the parasite. The host is protected from these toxic effects by the recycling of PD^+ by the pentose phosphate pathway (Fig. 2) to its reduced form (PDH; Fig. 1), which would not be accumulated in the mitochondria of the host.

Because of the primary amino group on its side chain, primaquine has been shown to be selectively concentrated in the liver of the host (3), where it was rapidly converted to a number of metabolites. In fungi (4), rats (5), monkeys (6), and humans (7), the majority of the primaquine undergoes an oxidative deamination in which the terminal group of the side chain is converted from an amine to a carboxylic acid. In rat (3) and monkey (6), it was found that the carboxylic acid metabolite has a very low affinity for tissue compartments, and preliminary findings indicated that the metabolite had a low mammalian toxicity and a low antimalarial activity. In these laboratories it has also been shown that fungi can metabolize primaquine to a methylene dimer with unusual stereochemical properties (8,9). This fungal metabolite had the structure shown for PDH shown in Fig. 1 [R = $CH_3C(=0)NHCH_2CH_2CH_2(CH_3)CH-$], and its solutions are colorless, while the oxidized form of the metabolite (PD^+) displays an intense blue color. Although positively charged, PD^+ was unusually lipophilic, and it was also observed that aqueous solutions of the PD^+ are almost instantly reduced back to PDH when zinc dust and HCl were added.

It has been shown that pyronin-Y (Fig. 1) and the structurally similar dye, rhodamine-123, have a high affinity for mitochondria of different cell types and that these agents have potent effects on oxidative phosphorylation (10,11). Rhodamine-123 has also been shown to be taken up very selectively in the matrix of *Plasmodium yoelii* malaria parasites residing in a mouse erythrocyte host (11). Being a highly lipophilic cation, rhodamine-123 was accumulated within the parasite using the proton gradient produced by the electron transport chain as the primary driving force. In a similar manner, it could be anticipated that PD^+ would be concentrated in the mitochondria of the parasite residing within the host liver cell.

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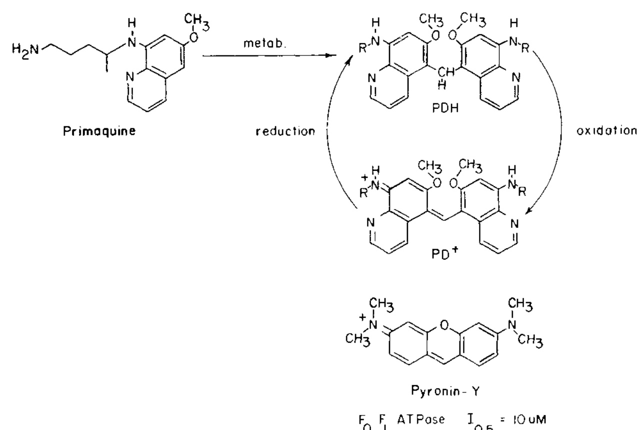

Fig. 1. Metabolic conversion of primaquine to a reduced methylene dimer (PHD) and to an oxidized dimer (PD^+) having a structure similar to the F_0F_1-ATPase inhibitor pyronin-Y.

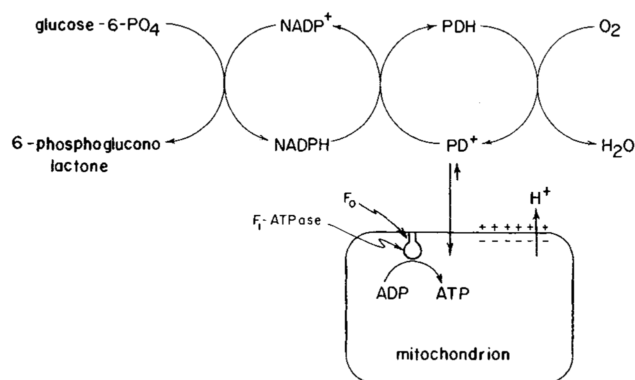

Fig. 2. Proposed mechanism for the transport of PD^+ into parasite mitochondria and the mechanism of detoxification of PD^+ by the host.

Although a number of other compounds structurally related to rhodamine-123 and pyronin-Y were accumulated in mitochondria, not all are cytotoxic. The more potent compounds such as pyronin-Y were found to be atypical uncouplers of oxidative phosphorylation, presumably through interaction with the membrane-bound oligomycin-sensitive ATP synthetase (F_0F_1-ATPase) (10). Using rat liver mitochondria, the effects of the agents on the F_0F_1-ATPase system was detectable at concentrations as low as 1×10^{-8} *M*. It was also concluded that the binding site of pyronin-Y is probably physically close to the oligomycin binding site on the F_0 protein subunit (10). Because of the marked similarity of the placement of the two nitrogens of pyronin-Y and the two nitrogens of PD^+ (Fig. 1), it might be predicted also that PD^+ would bind to the same receptor site. An effect of PD^+ on the F_0F_1-ATPase (or at least the inner mitochondrial membrane) is further suggested by studies on the effects of primaquine on mitochondrial ultrastructure of the exo-erythrocytic schizonts of *Plasmodium yoelii* (13). After the rats were infected with the parasite, then treated with primaquine, liver biopsy revealed that the mitochondrial membranes of the parasite are extensively damaged, while no changes were observed in the structure of the mitochondrial membranes of the host hepatocytes.

The primary objective of the present study was to determine if PD^+ had an effect on oxidative phosphorylation. If so, this effect of PD^+ could account for the toxic effect of primaquine on the host and parasite, while the differential concentration of PD^+ and PDH might account for the selectivity of the toxic effect on the parasite.

MATERIALS AND METHODS

The oxidized, acetylated methylene dimer of primaquine [Fig. 1; PD^+ where R = $CH_3C(=O)NHCH_2CH_2CH_2(CH_3)CH-$] was obtained in a chloride salt form as a gift from Charles D. Hufford, School of Pharmacy, University of Mississippi.

The isolation of rat liver mitochondria and the measurement of respiration using an oxygen electrode were essentially the same as in a previously published procedure (14). The mitochondria and test compound were incubated in a medium consisting of 5 m*M* succinate, 0.15 mg/ml bovine serum albumin, 1.5 m*M* $MgCl_2$, 1 mg/ml mitochondrial protein, 220 m*M* mannitol, 70 m*M* sucrose, 2.5 m*M* KH_2PO_4, 2 m*M* HEPES, and 0.5 m*M* EDTA at a final pH of 7.4.

Measurement of membrane-bound F_0F_1-ATPase activity was carried out using inverted inner membrane vesicles of rat liver mitochondria (13). The vesicles (5 mg/ml) and the test compound were incubated at 4°C for 20 min, then a 5-μl portion of the mixture was removed for the ATPase assay using the spectrophotometric procedure (15).

F_1-ATPase was purified as previously described (15). The effect of the test compound on the purified F_1-ATPase was measured in a manner similar to the above procedure. Varying concentrations of PD^+ were incubated for either 20 min, 1.0 hr, or 3.0 hr at room temperature with 67 μg of F_1-ATPase in 100 μl of pH 7.6, 50 m*M* Tris–HCl buffer. At the end of the incubation, 2 μl of the mixture was removed for the ATPase assay.

RESULTS

When the rat liver mitochondrial preparation respiring at a state IV rate of 18 nEq oxygen/min/mg was treated with 150 μ*M* ADP (Table I), the rate of respiration showed the ex-

Table I. Effect of PD^+ on Rat Liver Mitochondrial Respiration

	Respiration rate (nEq of oxygen/min/mg)
Expt 1	
Mitochondria	18
+ 150 μ*M* ADP	94
Expt 2	
Mitochondria	18
+ 200 μ*M* PD^+	
Initial	67
After 3 min	28
After 10 min	28
Expt 3	
Mitochondria	20
+ 200 μ*M* PD^+	
Initial	57
After 3 min	20
+ 50 μ*M* dinitrophenol	21

pected increase to a state III rate as a feedback response of the oxidative phosphorylation system to convert ADP to ATP. The respiration rate then returned to the very slow state IV rate (data not shown). When mitochondria respiring in state IV were treated with PD^+, the respiration rate increased, which is a response typical of the classical uncouplers such as 2,4-dinitrophenol (16). While the increase in respiration produced by dinitrophenol lasts for a moderately long time, the increase in respiration produced initially by PD^+ was followed by a depression in respiration (Expt 2, Table I). It was also found that dinitrophenol could no longer evoke an increase in the respiration once the mitochondria had been treated with PD^+. If the concentration of PD^+ was kept fairly low, the predominant effect on the state IV mitochondria was a stimulation of respiration. This effect was found to be half-maximal at a PD^+ concentration of 12 μM (Fig. 3).

Using the inverted membrane vesicle F_0F_1-ATPase model system, low concentrations of PD^+ were found to stimulate the ATPase activity (Fig. 4), but at higher concentrations the stimulated activity was inhibited. This observations suggests that low concentrations of PD^+, similar to 2,4-dinitrophenol, exhibit an uncoupling effect resulting in a stimulation of ATPase activity. However, at higher concentrations, PD^+ inhibits the F_0F_1-ATPase complex. Significantly, additional studies showed that this inhibitory effect was exerted at the level of the F_1 moiety. Thus, with the purified F_1-ATPase model system that was free of the F_0 subsystem and the membrane, PD^+ was found to inhibit the enzyme at moderately low concentrations (Fig. 5). The extent of inhibition increased with longer periods of incubation with PD^+, but the concentration producing a half-maximal effect was in the 100–200 μM range for all three experiments.

Mai and Allison (10) had reported that pyronin-Y and other compounds similar in structure to PD^+ stimulated the

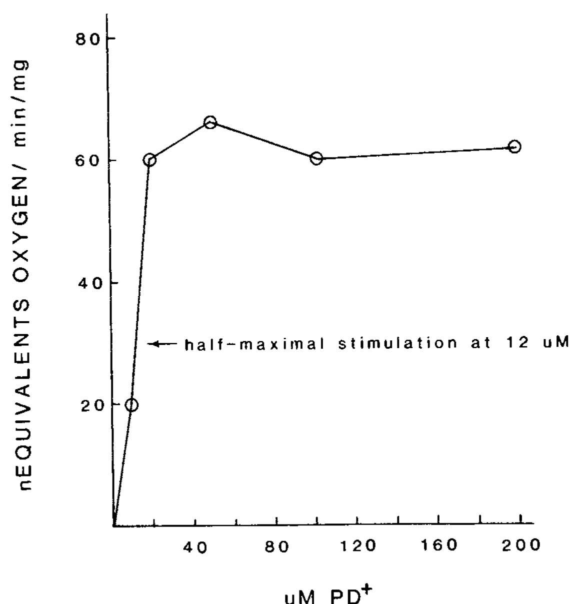

Fig. 3. Effect of PD^+ on the state IV respiration of rat liver mitochondria. Rate measurements were made immediately after the addition of PD^+.

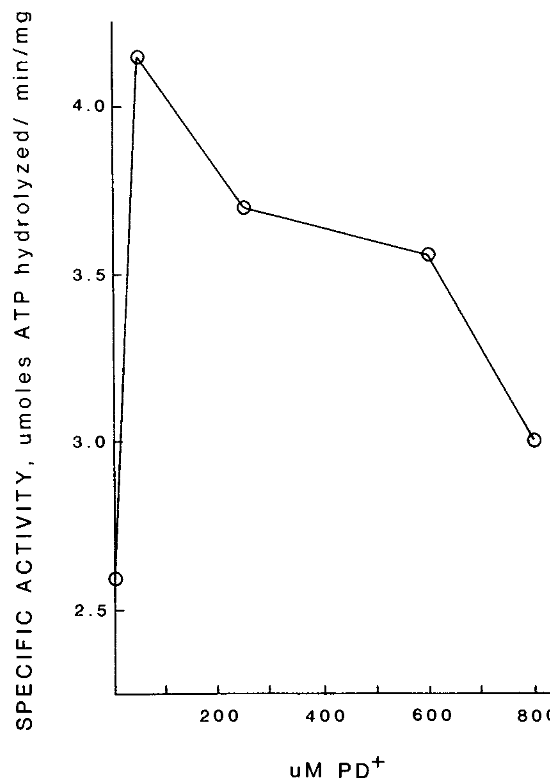

Fig. 4. Effect of PD^+ on the membrane-associated F_0F_1-ATPase.

respiration of state IV rat liver mitochondria when the agent was used at low concentrations, while higher concentrations of pyronin-Y produced a decrease in respiration. They also found that oligomycin, which interacts selectively with the F_0 subsystem, (10,16), could block the increase in mitochondrial respiration produced by these cationic dyes. In contrast, oligomycin could not block the effect of PD^+ when added either before or after PD^+ (Table II).

DISCUSSION

At concentrations in the 12 μM range, PD^+ uncouples oxidative phosphorylation in rat liver mitochondria. It is suggested that this uncoupling occurs because the membrane potential (from + to −) is used to take up PD^+ rather than phosphorylate ADP. Once PD^+ is selectively concentrated within the membrane, it may bind at or near the normal un-

Table II. Effect of Oligomycin on PD^+-Stimulated Respiration of Rat Liver Mitochondria

	Respiration rate (nEq of oxygen/min/mg)
Expt 1	
Mitochondria	19
+200 μM PD^+	58
+1 μg/ml oligomycin, then PD^+	58
Expt 2	
Mitochondria	20
+200 μM PD^+	57
+6 μg/ml oligomycin, then PD^+	58

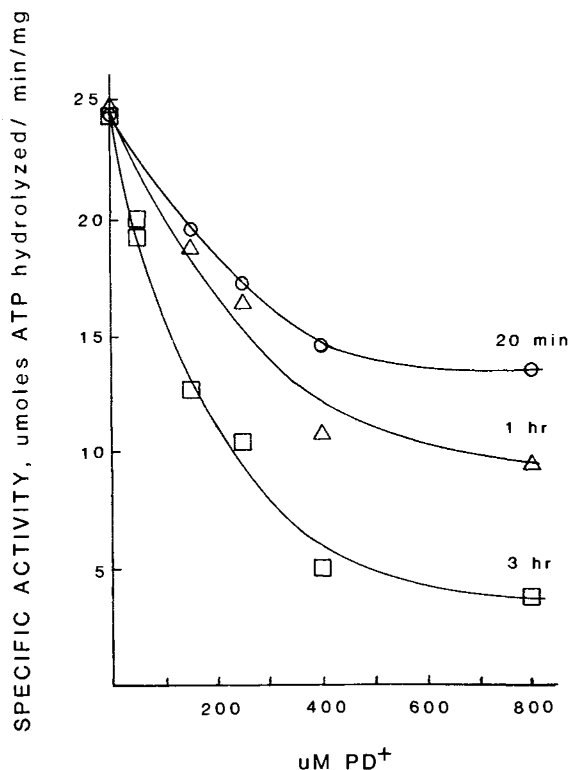

Fig. 5. Effect of PD^+ on the purified F_1-ATPase.

coupler binding site for dinitrophenol, thus preventing further stimulation of respiration by the latter agent. A second, less likely interpretation of the blocking of the dinitrophenol effect is that PD^+ has a direct effect on the electron transport reactions in addition to its inhibition of the F_1 unit.

While there were similarities between the effect of PD^+ and the effect of the structurally similar pyronin-Y on the respiration of the intact rat liver mitochondria, there were also marked differences in the effects of the two compounds. Previous studies (10) have shown that the F_0-selective agent oligomycin can block the action of the pyronin-Y series of compounds, while oligomycin did not block the action of PD^+ in the present study. Mai and Allison (10) also reported that the pyronin-Y series of compounds had no effect on the purified F_1-ATPase, while PD^+ was found in this study to be very effective at inhibiting the purified F_1-ATPase.

SPECULATIONS ON THE SELECTIVE TOXICITY

From these observations, one could propose a model for the toxic action of the antimalarial where primaquine is first transformed to PD^+. Because of its positive charge and highly lipophilic nature, PD^+ would accumulate within the mitochondria, and in the process of doing so it would uncouple oxidative phosphorylation (Fig. 2). Following the concentration of PD^+ within the mitochondria, the high concentrations of PD^+ would interact with the F_1 system, producing an inhibition of the F_0F_1-ATPase and the production of ATP.

On first inspection, one might have expected that the PD^+ species would be accumulated within the mitochondria of both the parasite and the host and inhibit the F_0F_1-ATPase of both. For the present working hypothesis, it is assumed that the pentose phosphate pathway operating within the host liver cell (Fig. 2) converts most of the PD^+ to PDH. Since the PD^+ (but not PDH) would be pumped into the mitochondria, the host would be protected from the toxic effects of primaquine. The parasite with a partially deficient G6PD system (2) would not be able to convert all of the PD^+ to PDH, which would lead to a selective accumulation of PD^+ in the mitochondria of the parasite. In a similar manner, individuals with a genetic deficiency in G6PD would also be subject to the effects of PD^+, which could account for the primaquine-induced hemolytic anemia observed in those individuals.

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