Atovaquone, a Broad Spectrum Antiparasitic Drug, Collapses Mitochondrial Membrane Potential in a Malarial Parasite

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At present, approaches to studying mitochondrial functions in malarial parasites are quite limited because of the technical difficulties in isolating functional mitochondria in sufficient quantity and purity. We have developed a flow cytometric assay as an alternate means to study mitochondrial functions in intact erythrocytes infected with Plasmodium yoelii, a rodent malaria parasite. By using a very low concentration (2 nM) of a lipophilic cationic fluorescent probe, 3,3′-dihexyloxycarbocyanine iodide, we were able to measure mitochondrial membrane potential (∆Ψm) in live intact parasitized erythrocytes through flow cytometry. The accumulation of the probe into parasite mitochondria was dependent on the presence of a membrane potential since inclusion of carbonyl cyanide m-chlorophenylhydrazone, a protonophore, dissipated the membrane potential and abolished the probe accumulation. We tested the effect of standard mitochondrial inhibitors such as myxothiazole, antimycin, cyanide and rotenone. All of them except rotenone collapsed the ΔΨm and inhibited respiration. The assay was validated by comparing the EC50 of these compounds for inhibiting ΔΨm and respiration. This assay was used to investigate the effect of various antimalarial drugs such as chloroquine, tetra-cycline and a broad spectrum antiparasitic drug atovaquone. We observed that only atovaquone collapsed ΔΨm and inhibited parasite respiration within minutes after drug treatment. Furthermore, atovaquone had no effect on mammalian ΔΨm. This suggests that atovaquone, shown to inhibit mitochondrial electron transport, also depolarizes malarial mitochondria with consequent cellular damage and death.

Plasmodium spp. are obligate intracellular parasites, spending a major portion of their life cycle within erythrocytes and converting these relatively inactive cells into metabolically thriving active hosts. At present, our knowledge of mechanisms by which the parasite accomplishes these changes is limited, as is our understanding of metabolic processes associated with parasitism. It is generally believed that glycolysis is the main source of ATP in erythrocyclic stages of malarial parasites with little or no contribution by mitochondria to the cellular ATP pool (1, 2). A lack of tricarboxylic acid cycle enzymes (3–6) and an acetate mitochondrial morphology has led to the suggestion that mitochondria in malaria parasite act mainly to serve as an electron disposal sink for dihydronicotinate dehydrogenase, a critical enzyme in pyrimidine biosynthesis (7–9). It is well established through studies in other systems that, in addition to oxidative phosphorylation, mitochondria are also central to many other physiological activities such as the metabolism of molecules such as amino acids, lipids, and heme, as well as intracellular Ca2+ homeostasis (10). These functions are achieved by the action of gene products encoded by both mitochondrial and nuclear genomes. Because most of the mitochondrial proteins are encoded by the nuclear genome and imported into mitochondria, an active import mechanism is necessary for mitochondrial functions. Both metabolites and protein transport require maintenance of membrane potential across the inner mitochondrial membrane (11, 12). The mitochondrial electron transport chain serves to generate this membrane potential (11). Hence, maintenance of ΔΨm is critical not only for ATP synthesis but also for the maintenance of additional metabolic activities of mitochondria. While it is not established which of the non-ATP synthesis functions are present in malarial parasites, it is safe to assume that these are likely to be critical for parasite physiology.

Approaches for investigating mitochondrial functions in malarial parasite are quite limited at present. Mitochondrial DNAs of various Plasmodium spp. have been sequenced and found to encode at least three components of the electron transport chain, viz. subunits 1 and 3 of cytochrome c oxidase, and apocytochrome b (13–16). In addition, mitochondrial preparations have been shown to contain ubiquinone cytochrome c oxidoreductase (bc1 complex) (17), and cytochrome c oxidase activities (18–20). However, detailed studies of mitochondrial functions and their response to antimalarial drugs have been hampered by the technical difficulties of obtaining workable quantities of functional mitochondria. To circumvent these problems, we have explored the possibility of studying mitochondrial functioning in intact parasitized erythrocytes with a fluorescent activated cell scanner (FACS). We have used the ability of the lipophilic cationic fluorescent probe DiOC6 (3) to partition into energized mitochondria as a measure of ΔΨm. By using this assay, we were able to demonstrate that a new antiparasitic drug, atovaquone, rapidly collapses ΔΨm in erythrocytes infected with a rodent malaria parasite Plasmodium yoelii.

MATERIALS AND METHODS

Mice—An animal colony of BALB/cByJ mice was maintained in our American Association of Laboratory Animal Care accredited animal facility.

Parasites—P. yoelii 17XL was maintained in vivo in either male or
female BALB/cByJ mice. Blood was collected in Hank's balanced salt solution (HBSS) containing 10 units of heparin/ml of HBSS at approximately 60% parasitemia. Red blood cells were washed three times with HBSS at low speed centrifugation (800 × g for 10 min each time). Washed erythrocytes were diluted 1:1 in phosphate-buffered saline, pH 7.4, and passed over a microcrystalline cellulose column to remove leukocytes and platelets (21). Red blood cells depleted of leukocytes and platelets were washed and resuspended in phosphate-buffered saline, and infected red blood cells were enriched by centrifugation over a discontinuous Percoll density gradient as described elsewhere (22). Infected erythrocytes within 65% Percoll and 65–75% Percoll interphase were collected and contained predominantly trophozoites and schizonts as determined by Giemsa-stained thin blood smears. These preparations were free of leukocytes and platelets as judged by Giemsa staining. These fractions were pooled, washed twice with RPMI 1640 medium containing 1% fetal calf serum and used in all of the experiments described here.

Inhibitors—All of the mitochondrial respiratory chain inhibitors, e.g., antimycin, myxothiazole, rotenone, and cyanide; uncoupler carbonyl cyanide m-chlorophenyl hydrazone (CCCP); and antimalarials tetracycline and chloroquine, were purchased from Sigma. The antimalarial atovaquone was a gift from Glaxo Wellcome, Research Triangle Park, NC.

Flow Cytometric Assay for ΔΨm—Flow cytometric assays were carried out using a FACScan (Becton Dickinson Cellular Imaging). The flow cytometer was adjusted for forward scattering profile, side scattering profile and fluorescence detection of infected red blood cells at channel 1. Parasitized cells at a concentration of 5 × 10⁶/ml in RPMI 1640 medium containing 1% fetal calf serum were incubated with a 2 nM final concentration of DiOC₆(3) for 20 min at 37°C. At the end of the incubation period the suspension was aliquoted into eight tubes of 250 μl each. Different concentrations of the compound to be tested were added, and the mixture was incubated for an additional 20 min. At the end of the incubation period, each sample was subjected to flow cytometric analysis. For each sample, 10,000 events were counted at the same flow cytometer setting. The results were calculated from the mean fluorescence of 10⁶ cells in a histogram. In each experiment, measurements of fluorescence in infected erythrocytes in the presence of dye (F₁,ₒ) and in absence of dye (Fₒ) were carried out to establish baselines.

Rate of O₂ Consumption by P. yoelii-infected Erythrocytes—Mitochondrial uptake of O₂ was measured in a closed system using a Clark's oxygen electrode and K-IC oxygraph (Gilson Medical Electronics Inc., Middleton, WI) in a reaction volume of 1.5 ml at 37°C following the manufacturer's instructions. Briefly, 1.5 × 10⁸ parasitized cells in 1.5 ml of RPMI 1640 medium containing 1% fetal calf serum were added into the chamber, and the rate of O₂ consumption by infected erythrocytes was followed for 5 min. A desired concentration of the compound to be tested was added into the chamber using a Hamilton syringe. The rate of O₂ consumption by infected erythrocytes was followed for the next 5 min. The rate of O₂ consumption was expressed as nAO/10⁸ infected erythrocytes/min. The difference in the rate of O₂ consumption by infected erythrocytes in the presence versus the absence of the compound was calculated as the measure of respiration inhibition. All the compound concentrations were tested individually in a separate set of experiments.

RESULTS

Determination of Optimal Conditions for DiOC₆(3) Assay of ΔΨm—DOIC₆(3) is a cationic, lipophilic fluorescent compound. When incubated with infected erythrocytes, it diffuses into cells and is concentrated several orders of magnitude into negative-inside mitochondria. A collapsed ΔΨm will result in diffusion of the probe out of the mitochondria resulting in dissipation of the signal. As shown in Fig. 1A, incubation of parasitized erythrocytes with 2 nM DiOC₆(3) led to the accumulation of the probe into parasites, which was reflected by the increase in fluorescence intensity. The fact that the accumulation of the probe within the mitochondria was dependent upon a membrane potential was shown by the dissipation of the signal intensity after incubation of infected erythrocytes with the protonophore CCCP (Fig. 1B). As shown in Fig. 2A, cell-associated DiOC₆(3) fluorescence intensity increased as a function of the probe concentration up to 50 nM. Incubation with CCCP dissipated cell-associated DiOC₆(3) fluorescence. Maximal dissipation of about 70% was obtained at the probe concentration of 2 nM. At higher probe concentrations the cell-associated fluorescence became relatively resistant to CCCP-mediated dissipation. The inhibition of the fluorescence above 50 nM appears to be the result of the inhibition of electron transport by the dye (see below). The decrease in the extent of quenching by CCCP above 2 nM appears to result from the self-quenching of the dye on the interface of the mitochondrial membrane, which thus saturates the signal. Hence, we decided to use 2 nM DiOC₆(3) as the final probe concentration for the ΔΨm assay for malarial parasite. We also measured the kinetics of probe accumulation at 2 nM concentration in infected erythrocytes as shown in Fig. 2B. A rapid accumulation of the probe in infected erythrocytes was observed, reaching maximum levels within 30 min. The slight reduction of fluorescent intensity after 30 min suggested that some self quenching occurred even at 2 nM dye concentration. Continued incubation of infected erythrocytes with the probe for longer than 60 min did not result in any significant change of fluorescence profile of the probe, indicating that partitioning of the probe into mitochondria had reached its equilibrium.

Because incubation with lipophilic compounds can also affect parasite physiology, we determined the effect of DiOC₆(3) on mitochondrial functioning by measuring the rate of respiration in the presence and absence of DiOC₆(3). As shown in Fig. 3, there was no significant effect of the compound on the rate of O₂ consumption. However, at the higher concentrations inhibition of respiration became apparent, reaching up to 50% at 150 nM DiOC₆(3) concentration. Hence, for the rest of the study, DiOC₆(3) was used at the final concentration of 2 nM.

Effect of Mitochondrial Inhibitors on ΔΨm and Respiration—Having established a way to assay ΔΨm in intact parasitized erythrocytes, we tested the effect of various known mitochon-
Mitochondrial inhibitors on $\Delta \Psi_m$. Inhibitors used were a protonophore (CCCP), a site I inhibitor (rotenone), site II inhibitors (antimycin and myxothiazole), and a site III inhibitor (cyanide). Fig. 4 shows representative histograms showing effects of these inhibitors (at the highest concentration tested) on mitochondrial accumulation of DiOC$_6$(3) in infected erythrocytes. Fig. 5A shows the concentration-dependent dissipation of the fluorescence for each of these inhibitors. Rotenone did not have any significant effect on $\Delta \Psi_m$, which is consistent with the earlier findings regarding the lack of NADH-ubiquinone oxidoreductase (site I) (17) in malarial parasites. Myxothiazole and antimycin collapsed $\Delta \Psi_m$ with an EC$_{50}$ of $2 \times 10^{-7}$ M and $6 \times 10^{-7}$ M, respectively, consistent with the presence of the bc$_1$ complex (site II) in malarial parasites. A much higher concentration of cyanide (6 mM) was required to completely collapse the $\Delta \Psi_m$ and dissipate the DiOC$_6$(3) accumulation with an EC$_{50}$ of $3 \times 10^{-4}$ M.

We also tested the effect of these inhibitors on rate of respiration by infected erythrocytes as shown in Fig. 5B. Both antimycin and myxothiazole inhibited respiration with EC$_{50}$ of $2.5 \times 10^{-7}$ M and $9 \times 10^{-8}$ M, respectively. At higher concentrations, i.e., $1 \times 10^{-6}$ M and above, both these drugs almost completely inhibited respiration. Rotenone had no apparent effect upon the rate of O$_2$ consumption in the range of concentrations tested in this study. Surprisingly, CCCP did not have any appreciable effect on the respiration rate of infected red blood cells. This observation is counter to the classical effect of CCCP where a collapsed membrane potential leads to a release from respiration control over electron transport, resulting in an increased rate of O$_2$ consumption.

Effect of Antimalarial Drugs on $\Delta \Psi_m$ and Respiration of Infected Erythrocytes—Having demonstrated a good correlation between the DiOC$_6$(3) assay of $\Delta \Psi_m$ and respiration by intact infected erythrocytes, we decided to assess the effect of various antimalarial drugs on mitochondrial physiology. Three
Mitochondrial Membrane Potential in Malarial Parasites

**Fig. 5.** The concentration-dependent effect of mitochondrial inhibitors on $\Delta \Psi_m$ and respiration. A, concentration-dependent effect of mitochondrial inhibitors on $\Delta \Psi_m$. Fluorescence intensity was quantitated by FACS analysis in the presence of various concentrations of CCCP ( ), antimycin ( ), myxothiazole ( ), rotenone ( ), and cyanide ( ). B, concentration-dependent effect of mitochondrial inhibitors on parasite respiration. Respiration rate by *P. yoelii*-infected erythrocytes was measured in the presence of various concentrations of antimycin ( ), myxothiazole ( ), cyanide ( ), and CCCP ( ) and is plotted as percent inhibition.

**Fig. 6.** Effect of atovaquone and other antimalarials on $\Delta \Psi_m$. This shows representative histograms showing the effect of high concentrations of atovaquone on $\Delta \Psi_m$ as measured by FACS analysis. The effect of atovaquone on $\Delta \Psi_m$ of mouse lymphocytes is also shown. Control stands for the fluorescence intensity of parasites in the presence of 2 nM DiOC$_6$(3) without any added inhibitors.

It has become apparent that mitochondria are quite heterogeneous with regard to the various functions they serve in different cell types; while ATP synthesis may be a common critical function for mitochondria in most eukaryotic cells, additional crucial functions may be determined by the differentiation status of the cell or the organism in which these organelles reside. Our knowledge of mitochondrial physiology, however, is based largely upon classical studies done with easily accessible systems derived from tissues such as the liver and the heart (30, 31). To broaden our view it is necessary to study mitochondrial physiology in various cell types and organisms.

In this report we describe a flow cytometric assay employing a cationic lipophilic fluorescent probe to monitor $\Delta \Psi_m$ in a rodent malarial parasite. Fluorescence-based demonstration of mitochondrial membrane potential has been used for many years with fluorochromes such as rhodamine 123 (32–35). Most of these studies, however, involved fluorescence microscopy and employed micromolar concentration of the probe. Because the rhodamine dyes have adverse effects on mitochondrial respiration (36, 37) and do not appear to be strictly dependent on $\Delta \Psi_m$ for intramitochondrial accumulation, their use in careful studies on mitochondrial physiology is problematic. A cationic lipophilic fluorochrome, DiOC$_6$(3) originally used at high concentrations to monitor intracellular membranes (39, 40), appears to be a better alternative to assay $\Delta \Psi_m$ when used at low concentrations. We empirically determined the optimal concentration of the probe for $\Delta \Psi_m$ assay in intact malarial parasites as the concentration at which probe accumulation was essentially dependent on membrane potential and maximally dissi-

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Mitochondrial Membrane Potential in Malarial Parasites

Figure 7. The concentration-dependent effect of atovaquone and other antimalarials on $\Delta\Psi_m$ and respiration. A, concentration dependence of the effect of atovaquone and other antimalarials on $\Delta\Psi_m$. Fluorescence intensity of P. yoelii-parasitized erythrocytes is shown in the presence of various concentrations of atovaquone ( ), chloroquine (■), and tetracycline (▲). The effect of atovaquone on mouse lymphocytes is also depicted ( ). B, concentration-dependent effect of atovaquone on respiration rate by P. yoelii-infected erythrocytes.

Atovaquone, a hydroxynaphthoquinone, is a drug initially developed as an antimalarial but now known to be also effective against several other eukaryotic microbial parasites such as toxoplasma and pneumocystis (42). Using cholate-lysed mitochondria from Plasmodium falciparum and P. yoelii, and externally provided heterologous (horse) cytochrome c at 100 micromolar concentration, Fry and Pudney (24) observed that atovaquone inhibited cytochrome c reductase activity. Based upon these results, it was concluded that atovaquone acts at the cytochrome bc1 complex of the malarial respiratory chain. Unique structural features of the parasite cytochrome b were speculated as being responsible for the therapeutic value of some of the hydroxynaphthoquinones as antimalarials (16). Because malarial mitochondria do not seem to contribute much to the ATP pool, it has long been suggested that the main purpose for these organelles was to dispose of electrons generated by dihydroorototate dehydrogenase (7–9), an essential enzyme in pyrimidine biosynthesis. Since the parasites are unable to salvage pyrimidines, inhibition of dihydroorototate dehydrogenase has been suggested as the reason for antimalarial activity of compounds such as atovaquone (43). Our observation that atovaquone rapidly dissipates $\Delta\Psi_m$ provides another possible mechanism for the antimalarial activity of this drug. It is not entirely clear whether the collapse of $\Delta\Psi_m$ is a direct result of respiration inhibition by atovaquone.

Recent studies on programmed cell death (PCD) pathways in metazoan organisms have implicated mitochondria to be involved at earlier stages of the PCD cascade (41, 44–49). PCD has also been observed in unicellular organisms such as Trypanosoma (50, 51), Dictyostelium (52), and Tetrahymena (53) although underlying mechanisms are not known at present. We suggest that the inhibition of mitochondrial electron transport chain in malarial parasites also acts to initiate a cascade of events similar to PCD. It would be important to investigate the possibility that the PCD-like response is elicited by collapsed $\Delta\Psi_m$ in malarial parasites. Nevertheless, maintenance of $\Delta\Psi_m$ is likely to be critical for all stages of the malaria parasite’s life cycle, and drugs that affect $\Delta\Psi_m$ are likely to act on all such stages. In accordance with this proposal atovaquone has been shown also to act against liver (54, 55) and insect stages of malarial parasite (38). Further investigations on parasite mitochondrial physiology will be illuminating in our attempts to understand mechanisms of drug action and the development of newer strategies for chemotherapy of malaria.

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