นิพนธ์ต้นฉบับ

Mitochondrial function of human malarial parasite Plasmodium falciparum cultivated in vitro : implications for antimalarial drug design

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Several lines of evidence suggest that there are some key differences between malarial parasites and their mammalian hosts in mitochondria and energy metabolism. Mitochondria from human malarial parasite Plasmodium falciparum were then purified by differential centrifugations and Percoll density gradient. The two mitochondrial enzymes, dihydroorotate dehydrogenase and cytochrome c oxidase, were assessed for markers during the organelle purification. By using cytochrome c oxidase, more stable than the other enzyme, 32% yield of the mitochondrial organelles was obtained. The purified mitochondria from P. falciparum in the erythrocytic stages were found to be heterogenous as examined by electron microscopy and rhodamine 123 fluorescence microscopy. A variety of antimitochondrial drugs including quinone analogs, electron transport components inhibitors, and mitochondrial ATP synthetase inhibitors, were found to inhibit the growth of P.falciparum in in vitro cultures. Our results suggest that the erythrocytic malarial parasite has functional mitochondria that contribute significantly to the overall energy metabolism.

Key words: *Mitochondria, P.falciparum, Malaria, Antimalarial drug, Drug design.*

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จากหลักฐานต่าง ๆ แสดงว่าเชื้อมาลาเรียและเจ้าบ้านจะมีความแตกต่างกันในเมตาบอลิสมของพลังงานและไมโตคอนเดรีย ในการศึกษานี้ได้ทำการแยกไมโตคอนเดรียให้บริสุทธิ์จากเชื้อพลาสโมเดียมฟัลซิปารัม ระยะที่อยู่ในเม็ดเลือดแดง โดยวิธีการปั่นด้วยความเร็วต่างกัน และตามด้วยเปอร์คอลที่ทำให้เกิดความแตกต่างของระดับความหนาแน่น ได้ติดตามการแยกให้บริสุทธิ์โดยเอ็นไซม์ของออร์กาเนลนี้ 2 ตัวคือ ไดไฮโดรออโรเทท ดีไฮโดรจีเนส และไซโตโครม ซี ออกซิเดส โดยได้ผลลัพธ์ของการแยกเท่ากับ 32 เปอร์เซ็นต์ การตรวจสอบรูปร่างด้วยกล้องจุลทรรศน์อิเล็คตรอน และตรวจสอบการทำงานโดยสารโรดามีนตามด้วยกล้องจุลทรรศน์เรืองแสง พบว่ามีความหลากหลายของออร์กาเนลนี้ และสารที่ยับยั้งไมโตคอนเดรียชนิดต่าง ๆ จะสามารถยับยั้งการเจริญเติบโตของเชื้อมาลาเรียในจานทดลองได้ดีอีกด้วย ผลงานดังกล่าวให้ข้อเสนอแนะว่าเชื้อมาลาเรียระยะที่อยู่ในเม็ดเลือดแดงมีไมโตคอนเดรียที่ทำหน้าที่เกี่ยวกับเมตาบอลิสมของพลังงาน

Malaria has once more become a global health problem due to the rapid spread of Plasmodium falciparum parasites which are resistant to currently used antimalarial drugs. In order to develop novel chemotherapeutic agents for malaria control, a rational approach to drug design is advocated. This requires a thorough understanding of the biochemical differences between the parasite and its host and the design of effective drugs to interfere selectively with the parasites' biochemical pathways.

Several lines of evidence suggest that there are some key differences between malarial parasites and their analogous mammalian systems in mitochondria metabolism and its linked to pyrimidine biosynthesis. [1-7] The mitochondria of Plasmodium spp. in the asexual erythrocytic stages lack a full complement of Krebs'cycle enzymes,[8] and it is generally believed that the parasites derive most, if not all, of their energy requirements via glycolysis.[9] It has been demonstrated that the mitochondria of the parasites actively maintain an electron transport-dependent transmembrane electrical potential[10] and possess an electron transport chain that operates in energy-consuming processes[6,11] and hat most parasite species tested require and utilize O_2 .[6,8,9] It has been suggested that the exclusive role of the mitochondria in the parasites is to serve as an electron disposal mechanism in de novo pyrimidine biosynthesis. [5,6,9,12]

We report here the purification and characteristics of mitochondria from asexual erythrocytic stages of P.falciparum in vitro. The effect of a range of inhibitors that are relatively specific for various aspects of mitochondrial function is presented. Many of these inhibitors efficiently impair the parasite viability, thereby indicating that the mitochondrial function is important for the growth and survival of the parasite, and it may be chemotherapeutic target for antimalarial drug design.

Materials and Methods

Cultivation of P.falciparum in vitro

P.falciparum (a mutant of T9/94) was cultivated in vitro in human red cell type 'O' as previously described ,[13] the RPMI 1640 medium supplemented with 25 mM HEPES, 32 mM $NaHCO_3$ and 10% human serum was used. The culture medium was changed twice daily until 30% parasitemia was obtained at the day of harvest.

Preparation of host cell-free parasite

The parasites from in vitro cultures were isolated from the infected red cells by incubating in 0.15% saponin as described.[4] The isolated intact parasites were washed and then used as starting materials for purification of mitochondria.

Purification of mitochondria from asexual intraerythrocytic parasites.

The host cell-free intact parasites were homogenized in a modified medium of Fry and Beesley. [3] To obtain mitochondria, the parasite homogenates were processed for differential centrifugations and 22% Percoll (Pharmacia LKB) density gradient separation as described.[6] The organelles were assessed by fluorescence and electron microscopic examinations and for the mitochondrial enzyme markers assays.

Enzyme and protein assay

Two mitochondrial enzymes,dihydroorotate dehydrogenase (DHODase) and cytochrome c oxidase, were used as the organelle markers. DHODase and cytochrome c oxidase were essentially determined by established methods.[5,6] Protein concentrations were determined by the method of Bradford .[14]

Microscopy

Fluorescence microscopy was examined by a Nikon microscope. Electron microscopy (EM)

of the mitochondrial samples was performed with a JEOL-100SX transmission electron microscope (TEM). The mitochondrial preparations and their functions were probed by rhodamine123 (Rh123) accumulation and then assessed by fluorescence microscopy as described essentially by Divo et al .[10] They were also examined by TEM by the established technique of Fry and Beesley. [3]

In vitro antimalarial test

Antimalarial activity against P.falciparum in vitro was monitored by examining % parasitemia at 48 hr and 96 hr in the presence of various concentrations of the tested compounds.[15]

Results and Discussion

Purification and characterization of mitochondria from P.falciparum

It has been recognized that the intraerythrocytic malarial parasites in mammals synthesize ATP by anaerobic glycolysis and do not possess a functional Krebs'cycle and also oxidative phosphorylation.[8,9] Nevertheless, O_2 is utilized at a limited requirement and is probably not linked to the ATP synthesis.[9] As proposed by Gutteridge et al, [12] mitochondrial function in the parasites would be coupled to the de novo pyrimidine biosynthesis via DHODase enzyme. Identification of these mitochondrial functions was carried out as follows: (1) isolation and characterization of mitochondria; (2) mitochondrial inhibitiors as antimalarial agents. Figure 1 shows the ultrastructure of P.falciparum-infected red cell examined by TEM. The mature parasites contained at least 2 mitochondrial-like organelles (Fig.1A). The mitochondria were double-membrane organelles with a few tubular-like cristate structure or acristate appearance (Fig.1A&B).

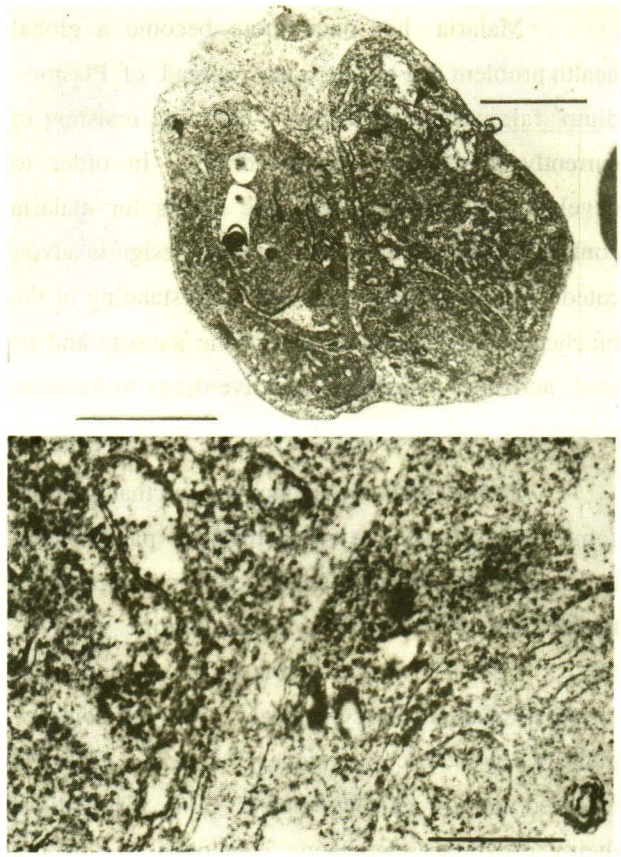

Figure 1. A. Transmission electron micrograph of P.falciparum-infected red cells. Two mature forms of intraerythrocytic parasites with mitochondrial organelles (arrow heads). The bar represents 1.5 μM.

B. Higher magnification of mitochondrion in P.falciparum-infected red cells. Arrow heads indicate double-membrane and acristate organelle. The bar represents 0.5 μM.

The organelles were subsequently purified by using differential centrifugations (4,500xg and 24,000xg) and 22% Percoll density gradient separation (Figure 2). The mitochondrial samples from each step of purification were assayed for two mitochondrial enzymes, DHODase[5,16] and cyto-

chrome c oxidase[6], as the organelle markers (Table 1). It was found that the interphase layer of Percoll density gradient contained pure mitochondria with 32 % yield based on the cytochrome c oxidase marker. The lower % yield obtained from the DHODase marker was due to the lability of the enzyme as reported in the subsequent paper. The purity of the mitochondria was determined by TEM (Figure 3). The EM characteristics suggest that the intraerythrocytic parasites have heterogeneity of mitochondria.

Table 1. Mitochondrial marker enzymes during the organelle isolation from a 2.0 ml of host red cell-free P.falciparum.

Steps	DHODase (Units)[1]	Cytochrome c oxidase (Units)
1. Parasite homogenate	24.0	60.4
2. 4,500xg supernate	8.8	44.5
3. 24,000xg pellet	4.2	28.2
4. Percoll density gradient (22%)		
- Interphase layer	2.6(10.8%)[2]	19.4(32.1%)
- Bottom layer	<0.1	<0.4

[1] Unit of the enzyme activity is expressed as nmol/min.

[2] Numbers in parentheses are % yield of mitochondria purified over parasite homogenate of P.falciparum which are fred from the host red cells

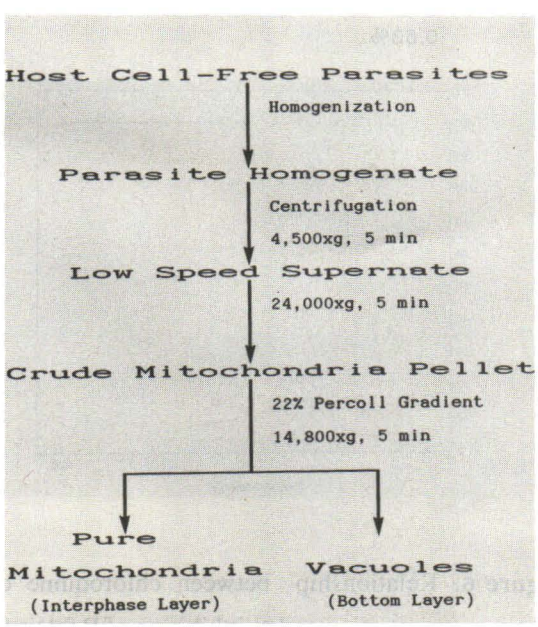

Figure 2. Diagram showing the preparation of P.falciparum homogenate and purification of mitochondria.

Figure 3. Transmission electron micrograph of purified mitochondria by Percoll density gradient. The arrow heads indicate numbers of tubular structures (one arrow head) and Rudzinska and Trager's structure or membrane whorls (two arrow heads). The bar represents 0.2 μM.

Examination of functional status of intact mitochondria purified from the parasite by Rh123 accumulation and retention was performed by fluorescence microscopy (Figure 4). Our results suggest that the mitochondria show their functions on transmembrane electrical potential and also electron transfer.

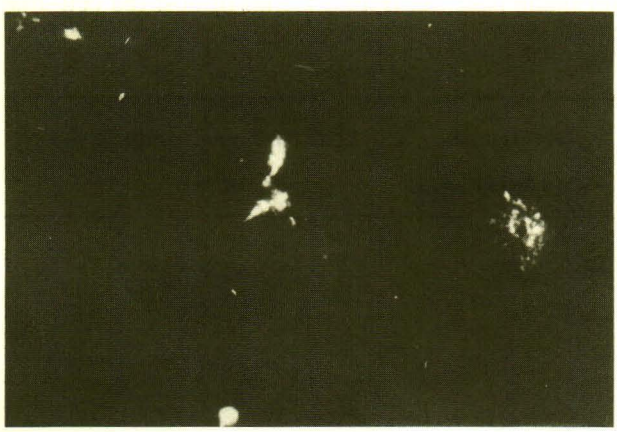

Figure 4. Fluorescent micrograph of purified mitochondria of P. falciparum. At least 5 mitochondrial organelles were stained with rhodamine123 on a wet mount preparation. The photomicrograph magnified x1500.

Mitochondrial inhibitors as antimalarial agents: implications for drug design

By using in vitro culture of P.falciparum, antimalarial activity of a range of inhibitors can be experimentally done. Figure 5 shows the effect of antimalarial drug chloroquine of P.falciparum in vitro as examined on 48 hr and 96 hr after incubation at 37°C .[15] The chloroquine concentrations were plotted against % inhibition of the parasite growth (Figure 6). The 90% inhibitory concentration (IC_{90}) was then calculated. A variety of antimitochondrial drugs were found to be toxic by inhibiting P.falciparum in vitro growth. Table 2 shows various quinone analogs having antimalarial activities, except lapachol and lawsone showing little activities. Chloronaphthoquinone has been described as a mechanism-based inhibitor of the mitochondrial DHODase enzyme[7] and atovaquone (Wellcome company), a new antimalarial drug, affects on the electron transport system .[17]

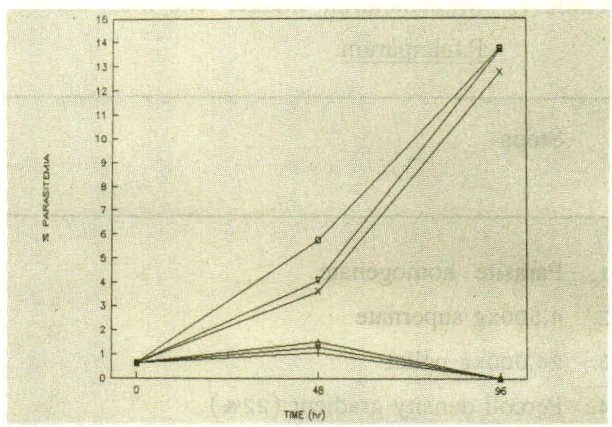

Figure 5. Effect of chloroquine at various drug concentrations on P.falciparum in vitro growth at 48 hr and 96 hr treatment. The symbols used are: +, 1000 nM; ◇, 100 nM; △, 10 nM; x, 1 nM; ▽, 0.1nM; □, drug-free control. The parasitemia used at o hr was 0.63%.

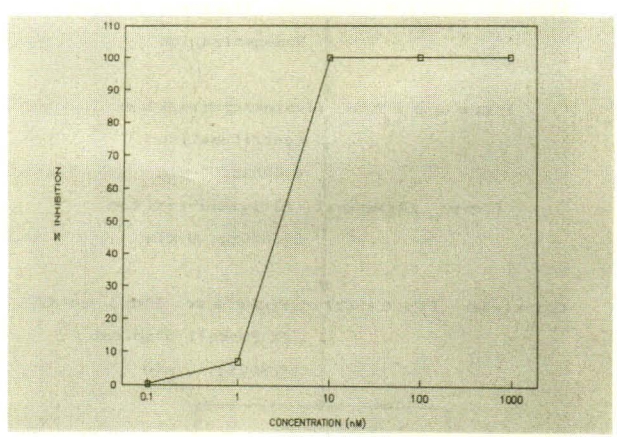

Figure 6. Relationship between chloroquine concentration and % inhibition of P.falciparum growth.

Table 2. Antimalarial activities of some quinone analogs on P.falciparum in vitro growth.

Compounds	IC_{90} (M)
CoQ_0	$4.3x10^{-5}$
Decylubiquinone	$5.8x10^{-5}$
Chloronaphthoquinone	$1.0x10^{-5}$
Menadione	$2.6x10^{-6}$
Lapachol	$2.4x10^{-4}$
Lawsone	$1.9x10^{-4}$
Atovaquone	$5.0x10^{-10}$

Inhibitors of electron transferring complexes and ATP synthetase complex were found to contain antimalarial activities (Table 3). Based on these results, we conclude that the mitochondria in P.falciparum has a functional role in energy metabolism, electron transport and pyrimidine biosynthesis de novo. Our results also point to fundamental and novel approach to antimalarial drug design based on mitochondrial target. At present, the progress towards this target is being done. [7,15-18]

Table 3. Antimalarial activities of antimalarial drugs and some mitochondrial electron transport inhibitors on P.falciparum in vitro growth.

Compounds	IC_{90} (M)
Known antimalarial drugs	
Pyrimethamine	$1.0x10^{-6}$
Chloroquine	$5.0x10^{-8}$
Complex III inhibitors	
Myxothiazole	$1.0x10^{-6}$
Antimycin A	$1.0x10^{-5}$
Complex IV inhibitors	
KCN	$6.0x10^{-5}$
ATP synthetase inhibitors	
Rhodamine123	$1.9x10^{-6}$
Oligomycin	$2.2x10^{-5}$

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