REVIEW ARTICLE

Copper Homeostasis for the Developmental Progression of Intraerythrocytic Malarial Parasite

Hiroko Asahi[a],*, Fumie Kobayashi[a], Shin-Ichi Inoue[a], Mamoru Niikura[a], Kenji Yagita[b], and Mohammed Essa Marghany Tolba[c,d]

[a]*Division of Tropical Diseases and Parasitology, Department of Infectious Diseases, Kyorin University School of Medicine, Tokyo, Japan;* [b] *Department of Parasitology, The National Institute of Infectious Diseases, Tokyo, Japan;* [c] *Department of Medical Genomics, Graduate School of Frontier Sciences, The University of Tokyo, Tokyo, Japan;* [d] *Department of Parasitology, Faculty of Medicine, The Assiut University, Assiut, Egypt*

Abstract: Malaria is one of the world's most devastating diseases, particularly in the tropics. In humans, *Plasmodium falciparum* lives mainly within red blood cells, and malaria pathogenesis depends on the red blood cells being infected with the parasite. Non-esterified fatty acids (NEFAs), including *cis*-9-octadecenoic acid, and phospholipids have been critical for complete parasite growth in serum-free culture, although the efficacy of NEFAs in sustaining the growth of *P. falciparum* has varied markedly. Hexadecanoic acid and *trans*-9-octadecenoic acid have arrested development of the parasite, in association with down-regulation of genes encoding copper-binding proteins. Selective removal of Cu$^+$ ions has blockaded completely the ring–trophozoite–schizont progression of the parasite. The importance of copper homeostasis for the developmental progression of *P. falciparum* has been confirmed by inhibition of copper-binding proteins that regulate copper physiology and function by associating with copper ions. These data have provided strong evidence for a link between healthy copper homeostasis and successive developmental progression of *P. falciparum*. Perturbation of copper homeostasis may be, thus, instrumental in drug and vaccine development for the malaria medication. We review the importance of copper homeostasis in the asexual growth of *P. falciparum* in relation to NEFAs, copper-binding proteins, apoptosis, mitochondria, and gene expression.

Keywords: Copper-binding protein, Copper homeostasis, Developmental arrest, Gene expression, Non-esterified fatty acids, *Plasmodium falciparum,* Copper ion.

Hiroko Asahi

1. INTRODUCTION

Malaria is one of the world's most devastating diseases particularly in the tropics, with an estimated annual incidence worldwide of 90 million clinical cases. The annual mortality from malaria is estimated to be 584,000 worldwide [1]. This is caused largely by *Plasmodium falciparum*.

The rapid emergence of drug-resistance *Plasmodium* strains has severely reduced the efficacy of conventional drugs employed to treat malaria, and threatens the effectiveness of even combination therapy, which is widely used in the field. A better understanding of the biology of malaria parasites and antimalarial drugs are needed [2– 4].

P. falciparum changes constantly its gene expression to generate a sequence of forms that adapt to different environments: liver and red blood cells (RBCs) in humans; the gut, vascular system and salivary glands in mosquitoes [5]. In humans, *P. falciparum* lives mainly within RBCs and develops through three distinct stages (the ring, trophozoite,

and schizont stages) during its cycle of approximately 48 h [5–7] (Fig. **1**). Pathogenesis depends on the RBCs infected with the parasite, and an impact progressively amplified by repeated 48-h cycles of invasion, intracellular growth, multiplication, egression of merozoites, and re-invasion. However, the mechanisms responsible for the developmental progression are poorly known.

In addition to host RBCs, the development of *P. falciparum* requires human serum [8, 9], a growth-promoting fraction from adult bovine plasma (GFS) [10, 11] or lipid-enriched bovine albumin [12]. In order to identify the factors that control intraerythrocytic development of *P. falciparum*, growth-promoting substances of GFS have been investigated [13]. And Asahi (2009, 2012) [7, 14] has formulated a chemically defined culture medium (CDM) that is suitable for promoting the complete development of intraerythrocytic *P. falciparum*. Non-esterified fatty acids (NEFAs) have been critical for parasite growth in GFS-containing media and the CDM. The efficacy of NEFAs in promoting the growth of *P. falciparum* has varied markedly, depending upon the type, total amount, and various combinations used. Addition of phospholipids with specific structures into culture media containing optimal NEFAs has increased parasite develop-

*Address correspondence to this author at the Division of Tropical Diseases and Parasitology, Department of Infectious Diseases, Kyorin University School of Medicine, Tokyo 181 8611, Japan; Tel: +81-422-47-5512; E-mail: isseix20@ks.kyorin-u.ac.jp

ment to a level similar to that seen with GFS-containing media.
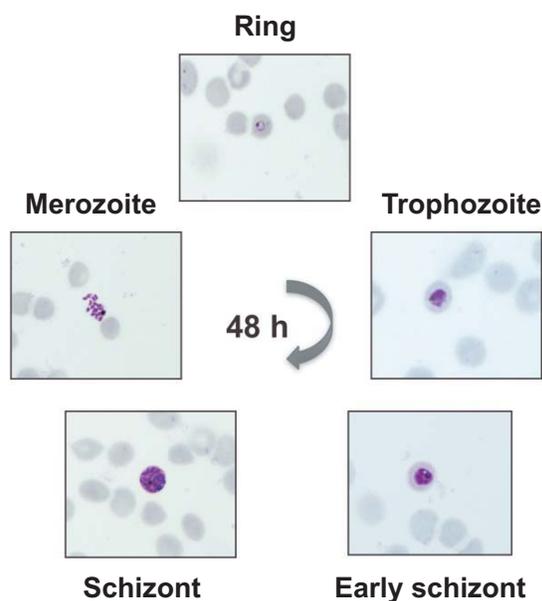
Fig. (1). Different stages of *P. falciparum* cultured synchronously and stained with Giemsa.

The established CDM consists of NEFAs, phospholipids, and specific proteins dissolved in a basal medium of RPMI-1640 [14]. The different NEFAs have played various roles by modifying the developmental stages of *P. falciparum*, ranging from complete development to arrested development at the ring stage [15]. To identify the molecules that regulate development of *P. falciparum* in RBCs, genome-wide transcriptome responses among various stages of *P. falciparum* cultured in diferrent CDMs have been compared [16]. Twenty-six transcripts that are associated with the suppression of schizogony have been predicted, of which 5 transcripts are particularly associated with blockade of trophozoite progression from the ring stage [16]. One of the 5 transcripts has been a putative copper channel. In addition, selective removal of copper ions has inhibited completely the successive ring–trophozoite–schizont progression of the parasite [16, 17]. Inhibition of copper-binding proteins that control copper function by actively associating with copper ions has caused arrested development of *P. falciparum*, implying the importance of copper homeostasis for the developmental progression of *P. falciparum*. Reduced expression of genes encoding copper-binding proteins has been detected, in association with arrested development of the parasite cultured in the medium enriched with specific growth-promoting NEFAs, including hexadecanoic acid (palmitic acid, C16:0) and *trans*-9-octadecenoic acid (C18:1-*trans*-9) [16, 17]. These data have provided intense evidence for a link between healthy copper homeostasis and developmental progression of *P. falciparum*, and may be useful for drug and vaccine development for the malaria eradication.

Here we review the importance of copper homeostasis in the asexual development versus arrest of *P. falciparum* in relation to NEFAs as growth promoting factors, copper-

binding proteins, apoptosis, mitochondria, and gene expression in the growth regulation of the parasite.

2. GROWTH OF *P. FALCIPARUM* IN HUMAN SERUM-FREE CULTURE MEDIUM

2.1. NEFAs as Critical Growth Factors for *P. falciparum*

Continuous *in vitro* culture of intraerythrocytic *P. falciparum* with human serum has facilitated a significant advance in malaria research [9]. The mechanisms that underlie *P. falciparum* development remain largely unknown. Elucidation of the functional components required for the growth of *P. falciparum* is needed to provide important clues to understanding the biology of parasite development in RBCs.

Based on the characterization of the ability of components of GFS to sustain development of *P. falciparum*, a simple total lipid fraction containing NEFAs has been detected as essential factors, which is obtained after lipid extraction of GFS, to sustain parasite development. The importance not only of the simple total lipid fraction, but also of specific proteins, including bovine albumin, in parasite development has been also indicated [13].

The components of the NEFA fraction of the simple total lipids of GFS have contained mainly *cis*-9-octadecenoic acid (C18:1, oleic acid, 43%), C16:0 (21%), octadecanoic acid (C18:0, stearic acid, 14%), *cis,cis*-9,12-octadecadienoic acid (C18:2), *cis*-9-hexadecenoic acid (C16:1), *cis*-5,8,11,14-eicosatetraenoic acid (C20:4), *cis*-5,8,11,14,17-eicosapentaenoic acid, and *cis*-4,7,10,13,16,19-docosahexaenoic acid [13]. Mixtures of NEFAs, but not individual NEFAs, have sustained parasite development only to a small extent [13]. However, parasite development even in the presence of several combinations of NEFAs has been much lower than that with medium containing the simple total lipid fraction, GFS or human serum [13]. These results imply that: i) the NEFAs are functional components of GFS in promoting parasite development, and ii) other factors must also contribute to the high development-promoting activity of GFS.

The simple total lipid fraction of GFS has contained phospholipids, diacylglycerides, cholesterol, monoglycerides, cholesteryl esters, and NEFAs [13]. Although the efficacy of NEFA mixtures for sustaining parasite development has been much lower than that of the simple total lipid fraction, addition of phospholipids such as dioleoylphosphatidylcholine has amplified the low development-promoting efficacy of NEFAs to an extent similar to that observed with the simple total lipid fraction- and GFS-containing media. Nevertheless, NEFAs have been assumed to be the dominant factors involved in the development promotion, because phospholipids alone have been unable to promote development of the parasite in the absence of NEFAs. And various types, combinations and concentrations of NEFAs that are effective for sustaining development of the parasite have been shown to be no different between culture media added with and without phospholipids, although developmental rates in the presence of phospholipids have been much higher [14, 15].

In contrast to normal mature human RBCs, phospholipid metabolism is present in *P. falciparum*-infected RBCs, and

total lipid content in membrane increases markedly [18, 19]. It has been thought that *P. falciparum* satisfies its own requirements for nutrition and membrane-building using phospholipids [19, 20]. In addition to the *de novo* synthesis of phospholipids, RBCs infected with *P. falciparum* or *Plasmodium knowlesi* have readily taken up intact phospholipids from surrounding culture media [21–25]. Studies in *P. falciparum* have elucidated new metabolic pathways for the synthesis of the parasite phospholipids. Moreover, the importance of the phospholipid metabolic pathway has been highlighted in the development of antimalarial therapies [3]. Further studies are necessary to determine the mechanisms responsible for the actions of phospholipids on the development of the parasite in association with NEFA mixtures.

2.2. Distinct Roles of NEFAs in the Development of *P. falciparum*

The efficacy of NEFAs in sustaining the development of *P. falciparum* has varied notably, depending on the type, total amount, and combinations. The NEFAs involved in the growth promotion of *P. falciparum* have required to be at least in specific pairs (unsaturated and saturated NEFAs); the most effective combination has comprised the two most abundant NEFAs in GFS and human serum, C18:1 and C16:0. On the other hand, the combination of C18:1 and C18:0 has been less effective [13–15, 26].

Various NEFAs added individually or in combination have exerted distinct effects on each growth step of *P. falciparum*, including schizogony, merozoite formation, and re-invasion into RBCs [15]. Four typical effect patterns, including suppressed schizogony (SS), suppressed formation of merozoites (SMF), inhibited merozoite invasion into new RBCs (IMI), and no inhibition (comparable), have been detected. The detrimental effect on any growth step has disrupted the cycles of the parasite and caused reduced parasitemia (Fig. **2A**) [15]. Thus, different NEFAs have played various roles during development of *P. falciparum* in RBCs by promoting development of the parasite at different stages. Carbon-chain length, degree and position of unsaturation, and isomerism of NEFAs have contributed to the development of the different stages of the parasite in RBCs, and to general growth of the parasite. For example, all stages of the parasite cultured in medium supplemented with optimal NEFAs (C18:1 plus C16:0), phospholipids, and albumin (CDRPMI) have been comparable to those grown in the complete GFS-containing medium (GFSRPMI). On the other hand, unsaturated or saturated NEFAs with longer or shorter carbon-chain length than C18:1 or C16:0, higher degrees of unsaturation, and *trans*-forms have resulted in lower parasite growth, mainly through SS and SMF effects. C18:1 plus C14:0 and C18:1 plus C18:0 have sustained moderate development by a partial IMI effect (less inhibited). Culture media exerting IMI or partial IMI effects have produced high numbers of released merozoites at 45 h after inoculation, but the merozoites have been unable to invade new RBCs, because percentage of new ring forms at 45 h (re-invaded) has been low. These results suggest that abnormal merozoites have been formed by IMI effect. The position of unsaturation of NEFAs with 18 carbons and one double bond, such as C18:1, *cis*-6-octadecenoic acid (C18:1-*cis*-6), and *cis*-11-octadecenoic acid (C18:1-*cis*-11), also has affected the de-

velopment of the parasite by partial IMI effect (Fig. **2B**) [15].
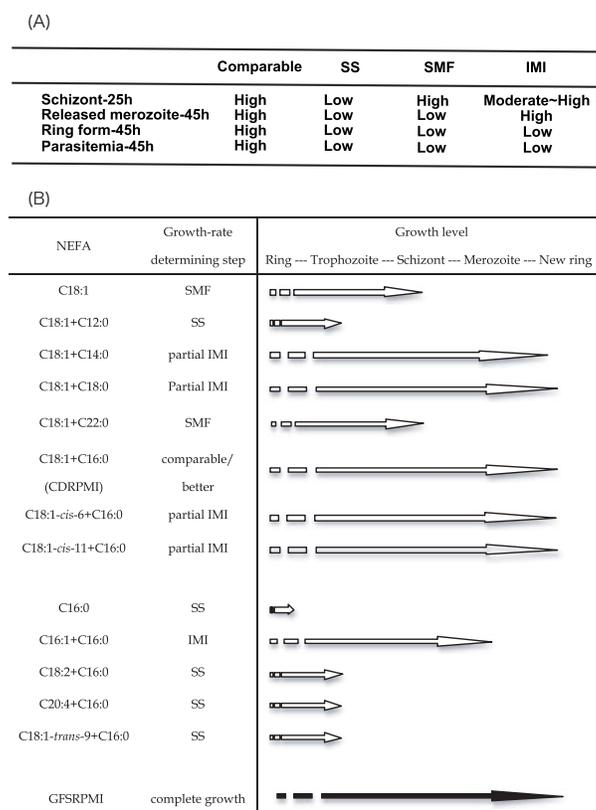
Fig. (2). (A) Representative modification of growth of *P. falciparum* cultured synchronously in the presence of various growth promoters, indicating comparative growth, SS, SMF, and IMI. Each developmental stage was compared with complete growth in GFSRPMI (control): schizonts at 25 h (Schizont-25h), merozoites at 45 h (Released merozoite-45h), ring forms at 45 h (Ring form-45h), and parasitemia at 45 h (Parasitemia-45h). **(B)** Growth-rate-determining step and growth level in development of *P. falciparum* cultured in the presence of various NEFAs. C12:0, dodecanoic acid; C14:0, tetradecanoic acid; C22:0, docosanoic acid; C18:1; C18:1-*cis*-6; C18:1-*cis*-11; C16:1; C18:2; C20:4; C18:1-*trans*-9.

2.3. Developmental Arrest with Saturated and *trans*-form NEFAs in Association with Reduced Expression of a Putative Copper Channel

The SS effect with C16:0 (CDM-C16alone) and C18:1-*trans*-9 can be helpful for the identification of molecular factors that regulate early developmental stages of *P. falciparum*, including its growth progression through the ring–trophozoite–schizont stages. Genome-wide transcriptome responses have been compared. Expressed genes in different developmental stages of *P. falciparum* which is cultured in the presence of different NEFAs have been selected to identify putative key molecules in early developmental arrest [16]. Twenty-six transcripts have been inferred to be associated with the schizogony suppression: these transcripts have been loosely categorized into 6 groups, including (i) 7 erythrocyte-binding or apical complex-associated, (ii) 4 RNA or DNA binding or transcription related, (iii) 1 signal transduc-

tion related, (iv) 2 lipid function related, (v) 3 sexual stage associated, and (vi) 9 others (not classified). Five of these, including merozoite surface protein 2, a putative DEAD/DEAH box RNA helicase, serine repeat antigen 3, a putative copper channel, and palmitoyl acyltransferase, have been assumed to blockade the trophozoite formation from the ring forms because of profound differences in transcript levels between the ring and trophozoite stages [16]. Among the five proteins, the putative copper channel is particularly intriguing since copper ions play an extensive role in living organisms, by regulating the activities of critical copper-binding proteins. The additional involvement of the other 4 proteins is, however, not excluded.

2.4. Putative Mechanisms Responsible for Various Functions of NEFAs

2.4.1. Effects of NEFAs in Relation to Programmed Cell Death (Apoptosis), Mitochondria, and Endoplasmic Reticulum (ER) Stress

There is evidence that NEFAs participate in a large number of biological processes, including activation of protein kinases, and cell proliferation, differentiation, and death, in addition to their basal functions, including acting as important intermediates in lipid biosynthesis. In particular, saturated NEFAs, such as C16:0 and C18:0, have caused apoptosis, induction of Jun-N-terminal kinase, inhibition of insulin signaling in various cells, a significant increase in reactive oxygen species (ROS), DNA damage, and mitochondrial dysfunction [27–38].

The precise mechanisms by which a saturated NEFA, C16:0, exerts pleiotropic effects on various cells are not completely understood. In general, cell death that is caused with saturated NEFA (C16:0) is characterized by markers of apoptosis, such as cytochrome c release, caspase activation, and DNA fragmentation. The involvement of the loss of mitochondrial membrane potential and mitochondrial swelling, has been also described for C16:0-induced cell death by using primary cardiomyocyte cultures from embryonic chicks and neonatal rats [29, 39]. These may be initiated through a reduction in the mitochondrial membrane phospholipid, cardiolipin [30, 39], an increase in ceramide synthesis [29], and increased generation of ROS [40].

Several papers have described the relationship between ER stress and cell death in hepatocytes [41–44], pancreatic beta cells [45], Chinese hamster ovary cells, and cardiomyoblasts [46]. Long-chain, saturated NEFAs have induced ER stress and activation of the unfolded protein response (URP). This has been involved in apoptosis and cell death by several mechanisms, such as activation of caspases, interplay of Bcl-2 protein and the ER, extrusion of luminal calcium, and induction of CCAAT/enhancer-binding proteins [44]. On the other hand, Borradaile *et al.* (2006) [46] have demonstrated that, in Chinese hamster ovary cells, the induction of ER stress in the presence of C16:0 is accompanied by incorporation of C16:0 into the ER, leading to compromised ER structure and integrity, and apoptosis. This suggests that impairment of ER function is involved in the cellular responses to C16:0, such as apoptosis, through a more direct mechanism in spite of previous studies indicating that C16:0-induced cellular responses converge on the mitochondrial damage.

Apoptotic markers have been described for *P. falciparum*. This implies that *P. falciparum* may experience programmed cell death, although, the putative machinery for death of the parasite has been different significantly from that in their human host [47]. The programmed cell death pathway in *P. falciparum* has been mediated by clan CA cysteine proteases (a family of papain-family enzymes) [48]. It is unclear whether apoptosis is involved in the developmental arrest of *P. falciparum* with C16:0.

C18:1 has been known to prevent C16:0- or C18:0-induced lipotoxicity in mitochondrial dysfunction and apoptosis [32], cell growth inhibition in human aortic endothelial cells [49], and insulin resistance in L6 myotubes [37]. Listenberger *et al.* (2003) [50] have provided evidence, in Chinese hamster ovary cells, that NEFAs exert the toxicity by promoting triglyceride accumulation in the cells. An addition of C18:1 has led to its accumulation in triglycerides and been well tolerated. On the other hand, excess C16:0 has been poorly incorporated into triglyceride and caused apoptosis. C18:1 rescues C16:0-induced apoptosis by bringing C16:0 away from pathways leading to apoptosis.

Similar to the protection against C16:0-induced apoptosis, C18:1 has protected *P. falciparum* from the development-arresting effect of C16:0, because the parasite cultured in medium containing C18:1 and C16:0 has developed similar to that in complete medium [14, 15]. The mechanisms responsible for the profound developmental arrest with C16:0 and the protective effect of C18:1 in the parasite remain to be investigated in depth.

2.4.2. NEFAs as Signals in Regulating Gene Expression

Based on current understanding, NEFAs are now considered to be intracellular signaling molecules. Precise molecular mechanisms underlying for the action of NEFAs and the relationships between the structure and various cellular activities are, however, not well understood. NEFAs have modified numerous cellular systems and functions, such as i) regulation of gene expression, ii) ion channel functions, and iii) membrane fusion [51–54]. NEFAs have been known to affect particularly gene transcription in various cell types. The effect has been mediated either by direct binding to various nuclear receptors (peroxisome proliferator activated receptors (PPARs), liver X receptor, and hepatic nuclear factor), or indirectly as the result of changes in regulatory transcription factors (sterol regulatory element binding protein-1c) [52–58]. On the other hand, it has been inferred that the mechanism involved in NEFA regulation of gene transcription is not single and several transcription factors other than PPARs are likely candidates. NEFAs have altered transcription via different routes, depending upon the cell-specific context and the target gene [58].

In *P. falciparum*, profound differences in the transcripts of a large number of genes have been detected when the parasites are cultured in media containing different NEFAs presenting different growth-promoting ability. Development of the parasite has been arrested at the ring and (or) trophozoite stage along with profound changes in transcript levels [16]. It is unclear whether NEFAs regulate the genes expres-

sion involved in the developmental arrest of *P. falciparum* in a similar manner to other cell types, because *Plasmodium* species are thought to lack peroxisomes in the presence of classic mitochondria [59–61].

3. COPPER HOMEOSTASIS AND ASEXUAL GROWTH VERSUS ARREST OF *P. FALCIPARUM*

3.1. Extensive Role of Copper Ions

Copper ions are essential trace nutrients for all living organisms only at extremely low concentrations. Copper metabolism is crucial to diverse vital cellular functions such as energy production, antioxidant defense, and metabolism of iron and peptide hormones. Copper ions play an important role in higher plants and animals by regulating the activities of various copper-binding proteins such as cytochrome c oxidase (CCO), Cu/Zn superoxide dismutase (SOD), dopamine β -hydroxylase, prion protein, tyrosinase, X-linked inhibitor of apoptosis protein, lysyl oxidase, metallothionein, ceruloplasmin, and other proteins. Monogenic Wilson's and Menkes diseases are well known to be caused by disturbed copper metabolism [62–66]. Also in mice carrying a null mutation of a high-affinity copper-uptake protein gene (*Ctr1*), early embryonic lethality and deficiency in copper uptake in the brain have been described [67, 68]. Particularly in relation to microbes, copper ions have been critical factors in a variety of mitochondrial functions: respiratory reaction, energy generation, regulation of iron acquisition, oxygen transport, the cellular stress response, antioxidant defense, and various other important processes [62, 64, 69].

However the production of hydroxyl radicals under aerobic conditions has arisen from the redox nature of copper through the Fenton reaction, leading to oxidative damage to proteins, DNA and lipids [70]. The acquisition and insertion of life-supporting copper into proteins of all organisms must be balanced by strict homeostatic mechanisms by a complex series of transporters and carrier proteins to prevent inappropriate interactions of copper with cellular components or generation of ROS.

In the past several decades, the importance of cellular copper homeostasis has been described. This involves the concerted action of various proteins, which regulate copper uptake, transport, distribution and efflux. Despite remarkable insights have been obtained in the field, a comprehensive understanding of copper function is still incomplete in many aspects.

3.2. Role of Copper Ions in *P. falciparum* Development

Experimental findings show: i) selective removal of Cu^+ with 2,9-dimethyl-1,10-phenanthroline, hydrochloride (neocuproine, $C_{14}H_{12}N_4$) has inhibited completely the developmental stages of *P. falciparum* in RBCs (ring forms, trophozoites and schizonts) [16, 17, 71]; ii) a tetrathiomolybdate, which is a specific inhibitor of copper-binding proteins that regulate copper physiology and function by actively associating with copper ions, has arrested development of the parasite [17]; and iii) a specific growth-promoting factor (C16:0) has arrested early development of the parasite in association with profoundly down-regulated expression of genes encoding copper-binding proteins [7, 15, 17]. These

findings suggest the optimal copper homeostasis is critical for the intraerythrocytic developmental succession of *P. falciparum*. Kenthirapalan *et al.* [72] have also described copper-transporting P-type ATPase (CuTP, PBANKA_041650 at PlasmoDB [73]) as a critical factor to murine malaria parasite fertility of both genders of gametocyte.

The understanding of the copper pathway and trafficking, and copper handling in malaria parasites, which are obligate intracellular protozoa, is still in its infancy. Notwithstanding, a novel and unexpected role for copper homeostasis in parasite growth should be clarified.

Although it is not an intracellular parasite, the yeast *Saccharomyces cerevisiae* may be informative to speculate copper transport of *P. falciparum*. In *S. cerevisiae* cells, copper enters the cell in the reduced Cu^+ form, whereas extracellular copper is mostly in the more stable oxidized form, Cu^{2+}. Therefore, reduction of Cu^{2+} to Cu^+ by a metalloreductase in the plasma membrane must be accompanied with the uptake of Cu^{2+} ions by yeast cells. Transport of the Cu^+ ions across the plasma membrane is subsequently carried out primarily with the Ctr copper transporter family (Ctr1 and Ctr3). In the cell, Cu^+ ions are bound to the copper chaperones Atx1, Cox17, and CCS to be carried into the Golgi complex, mitochondria, and Cu/Zn SOD, respectively [62–64, 69]. For example, copper ions are delivered to the mitochondria to be inserted into CCO via the Cox17 protein. This protein has been found in both the cytoplasm and the mitochondrial intermembrane space of the yeast. The Cox17 has been, however, considered to play a role in carrying Cu^+ within the mitochondrial intermembrane space [74, 75]. The chaperone Atx1 is involved in the copper secretory pathway. Atx1 has delivered Cu^+ to a P-type ATPase (Ccc2) located in the *trans*-Golgi network [76].

In *Plasmodium* species, the proteins, which are inferred to be involved in copper pathways and trafficking, have been identified, although the mechanism of copper metabolism and function remains to be investigated. Malaria parasites, including *Plasmodium berghei*, *Plasmodium chabaudi*, *Plasmodium cynomolgi*, *P. falciparum*, *Plasmodium gallinaceum*, *P. knowlesi*, *Plasmodium reichenowi*, *Plasmodium vivax*, and *Plasmodium yoelii*, harbor >100 genes that are predicted to relate to copper, including a putative copper channel, a copper transporter, CuTP, a putative CCO copper chaperone (COX17), a CCO subunit 3, a putative CCO assembly protein COX11, and a putative mitochondrial ribosomal protein S22 precursor. Thirteen and 11 genes are for *P. falciparum* 3D7 and IT strains, respectively. *P. falciparum* parasites harbor at least three genes, which are predicted to be involved in copper transport to maintain the healthy copper homeostasis in the parasite: two proteins harboring Ctr copper transporter domain, a putative copper channel (PF3D7_1421900, XP_001348385.1 at PlasmoDB and NCBI [the National Center for Biotechnology Information] and a copper transporter [PF3D7_1439000, XP_001348543.1], and CuTP (PF3D7_0904900, XP_001351923.1) [71, 72, 77]. Four proteins, which are predicted to relate to mitochondrial copper, have been described. These include a putative COX17 (PF3D7_1025600, XP_001347536.1, [78]), COX11 (PF3D7_1475300, XP_001348895.1), COX3 (mal_mito_1 at PlasmoDB), and a

putative mitochondrial ribosomal protein S22 precursor (PF3D7_1027200, XP_001347551.1).

3.2.1. A Putative Copper Channel of P. falciparum

A putative copper channel of *P. falciparum* (PF3D7_1421900) consists of 160 amino acids, and is a component of the apicoplast membrane. This protein is also referred to as "a putative Ctr copper transporter domain containing protein" (NCBI), and contains an $M^{131}XXXM^{135}$ motif in the second transmembrane domain, which is important for copper uptake, and four transmembrane regions at locations 139–159, 113–136, 63–86, and 6–26. The putative copper channel gene of *P. falciparum* has orthologs of the copper channel in *Plasmodium* species, including *P. reichenowi, P. vivax, P. knowlesi, P. yoelii, P. berghei,* and *P. chabaudi,* with the exception of avian malaria parasite, *P. gallinaceum.* Orthologs of this gene appear to be absent from apicomplexan species such as *Babesia bovis, Theileria annulata, Theileria parva* and *Theileria lestoquardi, Cryptosporidium muris, Cryptosporidium parvum,* and *Cryptosporidium hominis*, with the exception of *Toxoplasma gondii.*

3.2.2. Copper Transporter of P. falciparum

Copper transporter (PF3D7_1439000) consists of 235 amino acids, and is a component of the plasma membrane [77]. This protein contains an $M^{181}XXXM^{185}$ motif in the second transmembrane domain, which is important for copper uptake, and two transmembrane regions at locations 181–204 and 113–136. The gene of *P. falciparum* has orthologs of the copper transporter in the aforementioned *Plasmodium* species, and also an ortholog in *P. gallinaceum* in contrast to the above-mentioned copper channel. The aforementioned apicomplexan species each possess orthologs or homologs of this copper transporter, and also an ortholog in *T. gondii* in contrast to the above-mentioned copper channel.

3.2.3. CuTP of P. falciparum

CuTP (PF3D7_1439000) consists of 2568 amino acids, and is also referred to as "Cu^{2+}-transporting ATPase" (NCBI). This protein contains seven predicted transmembrane regions and distinctive features of copper P-ATPases, including one metal-binding motif, $M^{403}XCXXC^{408}$ at the N-terminal region preceding the first transmembrane domain. Also cation-translocation motifs, $C^{1897}PC^{1899}$ and $M^{2235}XXSS^{2239}$, which are believed to function in heavy-metal translocation, are present in membranes and contribute to the Cu^{+} ion translocation [71, 72, 79, 80].

Copper transporting P-type ATPases, including CuTP of *P. falciparum*, are ubiquitous heavy metal pumps, which are driven with ATP and transport heavy metal ions such as Cu^{+}, Cu^{2+}, Zn^{2+}, Co^{2+} and Pb^{2+} across membranes. The molecules are evolutionarily highly conserved in most organisms as well as *Plasmodium* species [79–81]. The CuTP is a component of the plasma membrane of the parasite and is also detected in the plasma membrane of host RBCs (PlasmoDB, [71]). In *P. falciparum*, this protein has been suggested to mediate copper ion efflux or export to minimize toxic effects of copper ions excess [71]. On the other hand, in a murine malaria model, *P. berghi* CuTP (PbCuTP) has been suggested to have a role in intracellular Cu^{+} redistribution and storage for cuproenzyme biosynthesis [72]. PbCuTP has been important only for male and female fertility, but not for blood stage development of the parasite [72]. It has been found to localize in vesicular bodies, which are predicted as copper storage organelles [72]. Function of copper-transporting P-type ATPases in *Plasmodium* species needs to be further elucidated.

3.2.4. A Putative COX17 of P. falciparum

A putative COX17 (PF3D7_1439000, [78]) consists of 67 amino acids, and contains no transmembrane region. In yeast as well as other cells, copper delivery to the mitochondria is markedly complex, but is essential for functioning of the CCO complex. Copper chaperon such as Cox17 is critical for the assembly of functional CCO and for delivery of copper ions to the mitochondria [82].

Mitochondrial CCO has been identified in *P. falciparum* [59]. Similar function of the putative COX17 of *P. falciparum* to the Cox17 has been considered. The putative COX17 contains two cysteine residues, $C^{27}CVC^{30}$, as a Cu^{+} binding site. It has been noted in the cytosol of the parasite through the course of asexual development [78].

3.3. Mode of action of copper chelators and a copper inhibitor

3.3.1. A Tetrathiomolybdate Elicits Severe Developmental Arrest of P. falciparum

Tetrathiomolybdates such as TTM (ammonium salt; $(NH_4)_2MoS_4$) and ATN-224 (choline salt) are potent anticopper agents. Although knowledge of the reaction chemistry and structures of tetrathiomolybdate complexes with various proteins is limited, the surprising stability of sulfur-bridged copper-molybdenum clusters and of a TTM complex with a copper-binding protein has been observed using crystallographic method [83]. TTM has been, thus, considered to abrogate copper-binding proteins controlling copper ion functions by formation of a sulfur-bridged copper–molybdenum cluster, rather than by direct chelation of copper ions [83]. Tetrathiomolybdates have efficacy as antiangiogenic and anti-tumor agents against several types of cancer, possibly inhibiting the activities of a variety of cuproenzymes. Also, the inhibition of nuclear factor κB is suggested for the anti-cancer mechanism of TTM [84–86].

TTM has caused developmental arrest of *P. falciparum* at early stages, with IC_{50} (the concentration required to inhibit the growth of the parasite by 50%) of 12.3 ± 0.1 μM (Fig. **3A**). An approach wherein *P. falciparum*-infected and normal RBCs are treated separately and co-cultured after washing has shown that development of *P. falciparum* pretreated with TTM has been profoundly arrested, even after a short period of treatment 0.5 h (Fig. **4A**). On the other hand, treatment of uninfected RBCs has caused developmental arrest to a much lesser extent. These results indicate that the target molecules for TTM that are involved in the developmental arrest of *P. falciparum* can occur predominantly in the parasite, but not in host RBCs. Further TTM has reacted irreversibly with the copper-binding proteins of the parasite [17].

IC_{50} of TTM for *P. falciparum* has been relatively high [17] in comparison with IC_{50} for several oxidases (1–5 µM) [87] and IC_{50} of ATN-224 for myeloma cells (~5 µM) and SOD (0.3 µM) [85]. It is suggested that the relatively high IC_{50} may be attributed to intracellular parasite, *P. falciparum* and RBCs (host cells) that contain high concentrations of copper-binding protein such as SOD [88]. TTM may interact with copper-binding proteins in RBCs prior to binding with *P. falciparum* proteins. Molecules contributing to the developmental arrest and the target molecules of TTM in the parasite remain to be determined.

3.3.2. Effect of Cu+ Chelation on Development of P. falciparum

Neocuproine has caused developmental arrest of intraerythrocytic of *P. falciparum* during the ring–trophozoite–schizont progression in a concentration-dependent manner, with IC_{50} of 0.13 ± 0.06 µM [17] and 0.10 ± 0.01 µM [16]. Trophozoite progression from the ring stage has been blockaded at higher concentrations (Fig. **3B**) [17]. On the other hand, *bis*(cyclohexanone)oxaldihydrazone (cuprizone, $C_{14}H_{22}N_4O_2$) and 2,9-dimethyl-4,7-diphenyl-1,10-phenanthrolinedisulfonic acid, disodium salt (BCS, $C_{26}H_{18}N_2Na_2O_6S_2$) have been noted to exert no visible effect on the growth of the parasite. Neocuproine chelates reduced copper ion (Cu^+) selectively by bidentate ligation, and cuprizone chelates the oxidized form (Cu^{2+}). The both chelators can diffuse through the cell membrane. On the other hand, BCS, which chelates both forms of copper ions, Cu^+ and Cu^{2+}, cannot cross the membrane [89]. From these results, depletion of Cu^+, but not Cu^{2+}, has contributed to the developmental arrest of *P. falciparum*.
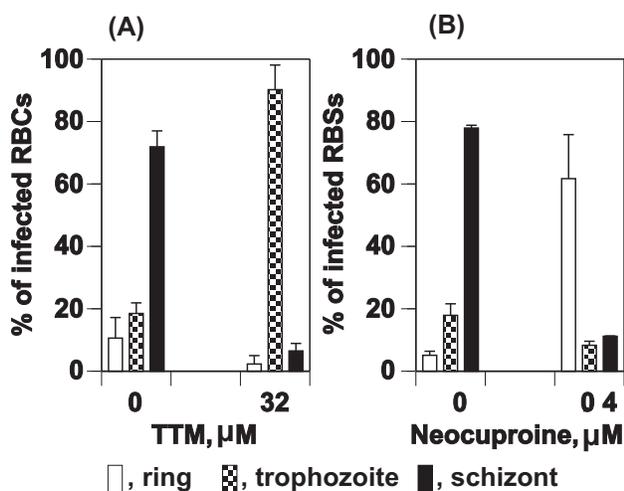
Fig. (3). Effect of TTM (A) and neocuproine (B) on growth of synchronized *P. falciparum* parasite. Synchronized parasites at the ring stage are cultured in the complete medium for 28 h. Each developmental stage is counted after Giemsa staining.

An approach, wherein *P. falciparum*-infected and normal RBCs are treated separately and co-cultured after washing, has shown that development of *P. falciparum* pretreated with neocuproine has inhibited only to a small extent even for a longer time 2.5 h (Fig. **4B**) [17]. This is a marked difference from the results for *P. falciparum* that developed in the presence of neocuproine throughout the culture period. These results imply that neocuproine has chelated Cu^+ ions of the parasite reversibly. Otherwise copper ions may be replenished by host RBCs [17]. Levels as high as a mean 18 µM of copper ions are contained in RBCs, although most of the copper ions present in RBCs is bound to the enzyme SOD [71, 88]. Comprehensive identification of the copper-binding proteins and clarification of the mechanisms responsible for copper homeostasis are necessary to understand anti-developmental mechanisms of the copper chelators, as well as the anti-copper agents (TTM and ATN-224) in *P. falciparum*.
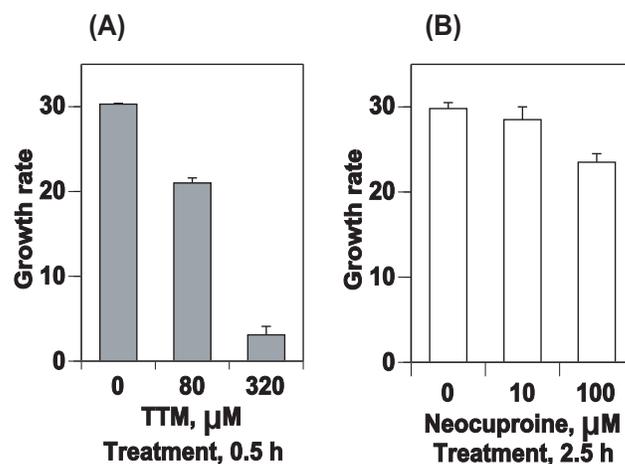
Fig. (4). Growth of *P. falciparum* co-cultured with infected RBCs and uninfected RBCs that are pretreated separately with TTM **(A)** and neocuproine **(B)**. The parasite are cultured in the complete medium for 95 h. The growth rate is estimated by dividing the parasitemia of the test sample after 95 h incubation by the initial parasitemia.

3.4. A Link Between a Healthy Copper Homeostasis and Developmental Progression of *P. falciparum*

A medium containing C16:0 alone as a growth-promoting NEFA (CDM-C16 alone) has caused developmental arrest of *P. falciparum* at the early stage, similar to that with complete media (CDRPMI and GFSRPMI) containing neocuproine or TTM, which cause perturbation of copper homeostasis [17]. A putative copper channel has been severely down-regulated in association with the blockade of trophozoite progression from the ring stage in the parasite [16]. Thus, expression of the genes of the aforementioned copper-binding proteins of *P. falciparum*, including a putative copper channel, a copper transporter, a CuTP, and a putative COX17 has been investigated further by quantitative real-time PCR on cultures grown in CDM-C16alone. Levels of transcripts of the putative copper channel that is detected by genome-wide transcriptome profiling, and the copper transporter have decreased profoundly [17]. These results imply that down-regulation of the two proteins has affected copper pathways and trafficking, and eventually caused the perturbation of copper homeostasis and developmental arrest of the parasite. These results suggest copper ions and copper-binding proteins are critically important for the developmental succession of *P. falciparum*, particularly at the early stages. This implies also that the monounsaturated NEFA, C18:1, has prevented the down-regulation of gene expression

and the developmental arrest of *P. falciparum* observed with C16:0.

3.5. Roles of Copper Ions in Mitochondrial Function, Apoptosis, and Gene Expression

Mitochondrial CCO has a critical requirement for copper ions. Mitochondrion-associated proteins include Cox17, Sco1 and Sco2. These are conserved from yeast to humans, and involved in the delivery of copper ions to CCO. Compromised transport of copper ions to mitochondria affect seriously mitochondrial functions, including the respiratory reaction and energy generation, although the molecular mechanisms responsible for import and mobilization of copper ions are not yet clear, [62, 64].

SODs normally protect cells by dismutating the potentially toxic superoxide anion. Cu/Zn SOD (Sod1 in *S. cerevisiae*) also has a requirement for copper ions. In *S. cerevisiae*, Ccs delivers copper ions to the Sod1 in a series of reactions. Cellular susceptibility to oxidative damage has increased under the condition of copper deficiency. Also, the capability of cells to produce SODs has decreased by copper ion depletion, thus increasing their propensity to oxidative damage involving apoptosis [64, 70]. Oxidative DNA damage has been described in copper deficiency in Jurkat T lymphocytes [90].

In *S. cerevisiae*, various genes such as *CUP1, CRS5, SOD1, CTR11, CTR3* and *FRE1* are transcriptionally activated or suppressed by copper metalloregulatory transcription factors (Ace1 and Mac1) [64, 91]. Several studies have suggested that the capability of copper ions to activate transcription through metal- and oxidative-stress- mediated mechanisms and to induce activation of the mitogen-activated protein kinase signaling pathway through ROS-mediated mechanism [92]. However, the molecular mechanisms involved in copper-induced gene expression remain to be clarified.

In *P. falciparum*, down-regulation of genes encoding copper-binding proteins (a putative copper channel and copper transporter) has been detected in culture with C16:0 [16, 17]. This can be linked to reduced copper ion concentration in parasite organelles, including mitochondria [59, 60], although the copper pathway and trafficking in *P. falciparum* remain to be clarified. Possible direct effects of copper ions on the aforementioned various activities of NEFAs remain also to be elucidated.

4. FUTURE DIRECTIONS

P. falciparum is single-cell eukaryote, and needs to reside in RBCs or liver cells while in a human body. Thus, the parasite requires a more complicated process than that of *S. cerevisiae* to deliver and allocate copper ions to metalloproteins located within organelles, including mitochondria, apicoplasts, and secretory compartments. Much remains to be learned about the cellular copper balance of *P. falciparum*: i) what is a series of copper-binding proteins of *P. falciparum*?; ii) how do the compartments of the copper homeostasis machinery interact?; iii) how does copper make its way from the host RBCs and from the site of import?; iv) what is the factor that reduces Cu^{2+} to Cu^+ in *P. falciparum*? The clarifi-

cation of these obscurities may make us to find a new important principle of copper metabolism in the parasite. The parasitic factors that interact at the molecular level with development-promoting NEFAs should help to reveal the mechanisms underlying the developmental succession versus arrest of *P. falciparum* in relation to copper homeostasis.

Nonetheless, perturbation of copper homeostasis disrupts the early-stage behavior of the parasite, leading to complete arrest of its general growth. The importance of copper homeostasis has been confirmed and this may provide critical clues for drug and vaccine development aimed at eradicating malaria.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

REFERENCES

[1]   World Health Organization (WHO): World Malaria Report 2014 http://www.who.int/malaria/publications/world_malaria_report_2014/en/

[2]   Ridley, R.G. Medical need, scientific opportunity and the drive for antimalarial drugs. *Nature,* **2002**, *415*(6872), 686-693.

[3]   Ben Mamoun, C.; Prigge, S.T.; Vial, H. Targeting the Lipid Metabolic Pathways for the Treatment of Malaria. *Drug Dev. Res.,* **2010**, *71*(1), 44-55.

[4]   Miotto, O.; Almagro-Garcia, J.; Manske, M.; Macinnis, B.; Campino, S.; Rockett, K.A.; Amaratunga, C.; Lim, P.; Suon, S.; Sreng, S.; Anderson, J.M.; Duong, S.; Nguon, C.; Chuor, C.M.; Saunders, D.; Se, Y.; Lon, C.; Fukuda, M.M.; Amenga-Etego, L.; Hodgson, A.V.; Asoala, V.; Imwong, M.; Takala-Harrison, S.; Nosten, F.; Su, X.Z.; Ringwald, P.; Ariey, F.; Dolecek, C.; Hien, T.T.; Boni, M.F.; Thai, C.Q.; Amambua-Ngwa, A.; Conway, D.J.; Djimde, A.A.; Doumbo, O.K.; Zongo, I.; Ouedraogo, J.B.; Alcock, D.; Drury, E.; Auburn, S.;Koch, O.; Sanders, M.; Hubbart, C.; Maslen, G.; Ruano-Rubio, V.; Jyothi, D.; Miles, A.; O'Brien, J.; Gamble, C.; Oyola, S.O.; Rayner, J.C.; Newbold, C.I.; Berriman, M.; Spencer, C.C.; McVean, G.; Day, N.P.; White, N.J.; Bethell, D.; Dondorp, A.M.; Plowe, C.V.; Fairhurst, R.M.; Kwiatkowski, D.P. Multiple populations of artemisinin-resistant *Plasmodium falciparum* in Cambodia. *Nat. Genet.,* **2013**, *45*(6), 648-655.

[5]   Bannister, L.; Mitchell, G. The ins, outs and roundabouts of malaria. *Trends Parastol.,* **2003**, *19*(5), 209-213.

[6]   Bannister, L.H.; Hopkins, J.M.; Fowler, R.E.; Krishna, S.; Mitchell, G.H. A brief illustrated guide to the ultrastructure of *Plasmodium falciparum* asexual blood stages. *Parasitol. Today,* **2000**, *16*(10), 427-433.

[7]   Asahi, H. Intraerythrocytic *Plasmodium falciparum* Growth in Serum-Free Medium with an Emphasis on Growth-Promoting Factors. In: *Malaria Parasites*; Omolade O. Okwa Ed.; InTech, Croatia, **2012**; pp. 73-90. ISBN 978-953-51-0326-4.

[8]   Jensen, J.B. Some aspects of serum requirements for continuous cultivation of *Plasmodium falciparum. Bull. World Health Organ.,* **1979**, *57* Suppl 1, 27-31.

[9]   Trager, W.; Jensen, J.B. Continuous culture of *Plasmodium falciparum*: its impact on malaria research. *Internat. J. Parasitol.,* **1997**, *27*(9), 989-1006.

[10] Asahi, H.; Kanazawa, T. Continuous cultivation of intraerythrocytic *Plasmodium falciparum* in a serum-free medium with the use of a growth-promoting factor. *Parasitology.* **1994**, *109*, 397-401.

[11] Asahi, H.; Kanazawa, T.; Kajihara, Y.; Takahashi, K.; Takahashi, T. Hypoxanthine: a low molecular weight factor essential for growth of erythrocytic *Plasmodium falciparum* in a serum-free medium. *Parasitology,* **1996**, *113* (Pt 1), 19-23.

[12]   Cranmer, S.L.; Magowan, C.; Liang, J.; Coppel, R.L.; Cooke, B.M. An alternative to serum for cultivation of *Plasmodium falciparum in vitro. Trans. R. Soc. Trop. Med. Hyg.,* **1997**, *91*(3), 363-365.

[13]   Asahi, H.; Kanazawa, T.; Hirayama, N.; Kajihara, Y. Investigating serum factors promoting erythrocytic growth of *Plasmodium falciparum. Exp. Parasitol.,* **2005**, *109*, 7-15.

[14]   Asahi, H. *Plasmodium falciparum*: Chemically defined medium for continuous intraerythrocytic growth using lipids and recombinant albumin. *Exp. Parasitol.,* **2009**, 121, 22-28.

[15]   Asahi, H.; Izumiyama, S.; Tolba, M.E.; Kwansa-Bentum, B. *Plasmodium falciparum*: Differing effects of non-esterified fatty acids and phospholipids on intraerythrocytic growth in serum-free medium. *Exp. Parasitol.,* **2011**, *127*, 708-713.

[16]   Asahi, H.; Tolba, M.E.M.; Tanabe, M.; Ohmae, H. Molecular factors that are associated with early developmental arrest of intraerythrocytic *Plasmodium falciparum. Can. J. Microbiol.,* **2013**, *59*, 485-493.

[17]   Asahi, H.; Tolba, M.E.; Tanabe, M.; Sugano, S.; Abe, K.; Kawamoto, F. Perturbation of copper homeostasis is instrumental in early developmental arrest of intraerythrocytic *Plasmodium falciparum. BMC Microbiol.,* **2014**, *14*, 167.

[18]   Holz, G.G., Jr. Lipids and the malarial parasite. *Bull. World Health Organ.,* **1977**, *55*(2-3), 237-248.

[19]   Vial, H.J.; Ancelin, M.L. Malarial lipids. In: *Malaria: Parasite Biology, Pathogenesis, and Protection;* Sherman I.W. Ed,; AMS Press, Washington DC, USA, **1998**; pp. 159-175.

[20]   Maguire, P.A.; Sherman, I.W. Phospholipid composition, cholesterol content and cholesterol exchange in *Plasmodium falciparum*-infected red cells. *Mol. Biochem. Parasitol.,* **1990**, *38*(1), 105-112.

[21]   Moll, G.N.; Vial, H.J.; Ancelin, M.L.; Op den Kamp, J.A.; Roelofsen, B.; van Deenen, L.L. Phospholipid uptake by *Plasmodium knowlesi* infected erythrocytes. *FEBS Lett.,* **1988**, *232*(2), 341-346.

[22]   Grellier, P.; Rigomier, D.; Clavey, V.; Fruchart, J.C.; Schrevel, J. Lipid traffic between high density lipoproteins and *Plasmodium falciparum*-infected red blood cells. *J. Cell Biol.,* **1991**, *112*(2), 267-277.

[23]   Haldar, K. Lipid transport in *Plasmodium. Infect. Agents Dis.,* **1992**, *1*(5), 254-262.

[24]   Simoes, A.P.; Moll, G.N.; Slotboom, A.J.; Roelofsen, B.; Op den Kamp, J.A.F. Selective internalization of choline-phospholipids in *Plasmodium falciparum* parasitized human erythrocytes. *Biochim. Biophys. Acta,* **1991**, 1063(1), 45-50.

[25]   Simoes, A.P.; Roelofsen, B.; Op den Kamp, J.A.F. Lipid compartmentalization in erythrocytes parasitized by *Plasmodium* spp. *Parasitol. Today.* **1992**, *8*, 18-20.

[26]   Mitamura, T.; Hanada, K.; Ko-Mitamura, E.P.; Nishijima, M.; Horii, T. Serum factors governing intraerythrocytic development and cell cycle progression of *Plasmodium falciparum. Parasitol. Internat.,* **2000**, *49*(3), 219-229.

[27]   de Vries, J.E.; Vork, M.M.; Roemen, T.H.; de Jong, Y.F.; Cleutjens, J.P.; van der Vusse, G.J.; van Bilsen, M. Saturated but not mono-unsaturated fatty acids induce apoptotic cell death in neonatal rat ventricular myocytes. *J. Lipid Res.,* **1997**, *38*(7), 1384-1394.

[28]   Hardy, S.; Langelier, Y.; Prentki, M. Oleate activates phosphatidylinositol 3-kinase and promotes proliferation and reduces apoptosis of MDA-MB-231 breast cancer cells, whereas palmitate has opposite effects. *Cancer Res.,* **2000**, *60*(22), 6353-6358.

[29]   Kong, J.Y.; Rabkin, S.W. Palmitate-induced apoptosis in cardiomyocytes is mediated through alterations in mitochondria: prevention by cyclosporin A. *Biochim. Biophys. Acta.,* **2000**, *1485*(1), 45-55.

[30]   Hardy, S.; El-Assaad, W.; Przybytkowski, E.; Joly, E.; Prentki, M.; Langelier, Y. Saturated fatty acid-induced apoptosis in MDA-MB-231 breast cancer cells. A role for cardiolipin. *J. Biol. Chem.,* **2003**, *278*(34), 31861-31870.

[31]   Grishko, V.; Rachek, L.; Musiyenko, S.; Ledoux, S.P.; Wilson, G.L. Involvement of mtDNA damage in free fatty acid-induced apoptosis. *Free Radic. Biol. Med.,* **2005**, *38*(6), 755-762.

[32]   Miller, T.A.; LeBrasseur, N.K.; Cote, G.M.; Trucillo, M.P.; Pimentel, D.R.; Ido, Y.; Ruderman, N.B.; Sawyer, D.B. Oleate prevents palmitate-induced cytotoxic stress in cardiac myocytes. *Biochem. Biophys. Res. Commun.,* **2005**, *336*(1), 309-315.

[33]   Karaskov, E.; Scott, C.; Zhang, L.; Teodoro, T.; Ravazzola, M.; Volchuk, A. Chronic palmitate but not oleate exposure induces endoplasmic reticulum stress, which may contribute to INS-1 pancreatic beta-cell apoptosis. *Endocrinology,* **2006**, *147*(7), 3398-3407.

[34]   Pagliassotti, M.J.; Wei, Y.; Wang, D. Insulin protects liver cells from saturated fatty acid-induced apoptosis via inhibition of c-Jun NH2 terminal kinase activity. *Endocrinology,* **2007**, 148(7), 3338-3345.

[35]   Leroy, C.; Tricot, S.; Lacour, B.; Grynberg, A. Protective effect of eicosapentaenoic acid on palmitate-induced apoptosis in neonatal cardiomyocytes. *Biochim. Biophys. Acta,* **2008**, *1781*(11-12), 685-693.

[36]   Ricchi, M.; Odoardi, M.R.; Carulli, L.; Anzivino, C.; Ballestri, S.; Pinetti, A.; Fantoni, L.I.; Marra, F.; Bertolotti, M.; Banni, S.; Lonardo, A.; Carulli, N.; Loria, P. Differential effect of oleic and palmitic acid on lipid accumulation and apoptosis in cultured hepatocytes. *J. Gastroenterol. Hepatol.,* **2009**, *24*(5), 830-840.

[37]   Gao, D.; Griffiths, H.R.; Bailey, C.J. Oleate protects against palmitate-induced insulin resistance in L6 myotubes. *Br. J. Nutr.,* **2009**, *102*(11), 1557-1563.

[38]   Yuzefovych, L.; Wilson, G.; Rachek, L. Different effects of oleate vs. palmitate on mitochondrial function, apoptosis, and insulin signaling in L6 skeletal muscle cells: role of oxidative stress. *Am. J. Physiol. Endocrinol. Metab.,* **2010**, 299(6), E1096-1105.

[39]   Ostrander, D.B.; Sparagna, G.C.; Amoscato, A.A.; McMillin, J.B.; Dowhan, W. Decreased cardiolipin synthesis corresponds with cytochrome c release in palmitate-induced cardiomyocyte apoptosis. *J. Biol. Chem.,* **2001**, *276*(41), 38061-38067.

[40]   Listenberger, L.L.; Ory, D.S.; Schaffer, J.E. Palmitate-induced apoptosis can occur through a ceramide-independent pathway. *J. Biol. Chem.,* **2001**, *276*(18), 14890-14895.

[41]   Wang, D.; Wei, Y.; Pagliassotti, M.J. Saturated fatty acids promote endoplasmic reticulum stress and liver injury in rats with hepatic steatosis. *Endocrinology,* **2006**, *147*(2), 943-951.

[42]   Wei, Y.; Wang, D.; Topczewski, F.; Pagliassotti, M.J. Saturated fatty acids induce endoplasmic reticulum stress and apoptosis independently of ceramide in liver cells. *Am. J. Physiol. Endocrinol. Metab.,* **2006**, *291*(2), E275-281.

[43]   Wei, Y.; Wang, D.; Gentile, C.L.; Pagliassotti, M.J. Reduced endoplasmic reticulum luminal calcium links saturated fatty acid-mediated endoplasmic reticulum stress and cell death in liver cells. *Mol. Cell. Biochem.,* **2009**, *331*(1-2), 31-40.

[44]   Pfaffenbach, K.T.; Gentile, C.L.; Nivala, A.M.; Wang, D.; Wei, Y.; Pagliassotti, M.J. Linking endoplasmic reticulum stress to cell death in hepatocytes: roles of C/EBP homologous protein and chemical chaperones in palmitate-mediated cell death. *Am. J. Physiol. Endocrinol. Metab.,* **2010**, *298*(5), E1027-1035.

[45]   Gwiazda, K.S.; Yang, T.L.; Lin, Y.; Johnson, J.D. Effects of palmitate on ER and cytosolic Ca^{2+} homeostasis in beta-cells. *Am. J. Physiol. Endocrinol. Metab.,* **2009**, *296*(4), E690-701.

[46]   Borradaile, N.M.; Han, X.; Harp, J.D.; Gale, S.E.; Ory, D.S.; Schaffer, J.E. Disruption of endoplasmic reticulum structure and integrity in lipotoxic cell death. *J. Lipid Res.,* **2006**, *47*(12), 2726-2737.

[47]   Deponte, M.; Becker, K. *Plasmodium falciparum*--do killers commit suicide? *Trends Parasitol.,* **2004**, *20*(4), 165-169.

[48]   Ch'ng, J.H.; Kotturi, S.R.; Chong, A.G.; Lear, M.J.; Tan, K.S. A programmed cell death pathway in the malaria parasite *Plasmodium falciparum* has general features of mammalian apoptosis but is mediated by clan CA cysteine proteases. *Cell Death Dis.,* **2010**, *1*, e26.

[49]   Harvey, K.A.; Walker, C.L.; Xu, Z.; Whitley, P.; Pavlina, T.M.; Hise, M.; Zaloga, G.P.; Siddiqui, R.A. Oleic acid inhibits stearic acid-induced inhibition of cell growth and pro-inflammatory responses in human aortic endothelial cells. *J. Lipid Res.,* **2010**, *51*(12), 3470-3480.

[50]   Listenberger, L.L.; Han, X.; Lewis, S.E.; Cases, S.; Farese, R.V., Jr.; Ory, D.S.; Schaffer, J.E. Triglyceride accumulation protects against fatty acid-induced lipotoxicity. *Proc. Natl. Acad. Sci. U. S. A.,* **2003**, *100*(6), 3077-3082.

[51]   Faergeman, N.J.; Knudsen, J. Role of long-chain fatty acyl-CoA esters in the regulation of metabolism and in cell signalling. *Biochem. J.,* **1997**, *323*, 1-12.

[52]   Brandt, J.M.; Djouadi, F.; Kelly, D.P. Fatty acids activate transcription of the muscle carnitine palmitoyltransferase I gene in cardiac myocytes via the peroxisome proliferator-activated receptor alpha. *J. Biol. Chem.,* **1998**, *273*(37), 23786-23792.

[53]   Louet, J.F.; Chatelain, F.; Decaux, J.F.; Park, E.A.; Kohl, C.; Pineau, T.; Girard, J.; Pegorier, J.P. Long-chain fatty acids regulate liver carnitine palmitoyltransferase I gene (L-CPT I) expression

through a peroxisome-proliferator-activated receptor alpha (PPARalpha)-independent pathway. *Biochem. J.,* **2001**, *354*(Pt. 1), 189-197.

[54] Pegorier, J.P.; Le May, C.; Girard, J. Control of gene expression by fatty acids. *J. Nutr.,* **2004**, *134*(9), 2444S-2449S.

[55] Xu, H.E.; Lambert, M.H.; Montana, V.G.; Parks, D.J.; Blanchard, S.G.; Brown, P.J.; Sternbach, D.D.; Lehmann, J.M.; Wisely, G.B.; Willson, T.M.; Kliewer, S.A.; Milburn, M.V. Molecular recognition of fatty acids by peroxisome proliferator-activated receptors. *Mol. Cell,* **1999**, *3*(3), 397-403.

[56] Ou, J.; Tu, H.; Shan, B.; Luk, A.; DeBose-Boyd, R.A.; Bashmakov, Y.; Goldstein, J.L.; Brown, M.S. Unsaturated fatty acids inhibit transcription of the sterol regulatory element-binding protein-1c (SREBP-1c) gene by antagonizing ligand-dependent activation of the LXR. *Proc. Natl. Acad. Sci. U. S. A.,* **2001**, *98*(11), 6027-6032.

[57] Wolfrum, C.; Borrmann, C.M.; Borchers, T.; Spener, F. Fatty acids and hypolipidemic drugs regulate peroxisome proliferator-activated receptors alpha - and gamma-mediated gene expression via liver fatty acid binding protein: a signaling path to the nucleus. *Proc. Natl. Acad. Sci. U. S. A.,* **2001**, *98*(5), 2323-2328.

[58] Duplus, E.; Forest, C. Is there a single mechanism for fatty acid regulation of gene transcription? *Biochem. Pharmacol.,* **2002**, *64*(5-6), 893-901.

[59] Krungkrai, J.; Krungkrai, S.R.; Suraveratum, N.; Prapunwattana, P. Mitochondrial ubiquinol-cytochrome c reductase and cytochrome c oxidase: chemotherapeutic targets in malarial parasites. *Biochem. Mol. Biol. Int.,* **1997**, *42*(5), 1007-1014.

[60] Krungkrai, J. The multiple roles of the mitochondrion of the malarial parasite. *Parasitology,* **2004**, *129*(Pt 5), 511-524.

[61] Gabaldon, T. Peroxisome diversity and evolution. *Philos. Trans. R. Soc. Lond. B Biol. Sci.,* **2010**, *365*(1541), 765-773.

[62] Turski, M.L.; Thiele, D.J. New roles for copper metabolism in cell proliferation, signaling, and disease. *J. Biol. Chem.,* **2009**, *284*(2), 717-721.

[63] Markossian, K.A.; Kurganov, B.I. Copper chaperones, intracellular copper trafficking proteins. Function, structure, and mechanism of action. *Biochemistry (Mosc),* **2003**, *68*(8), 827-837.

[64] Festa, R.A.; Thiele, D.J. Copper: an essential metal in biology. *Curr. Biol.,* **2011**, *21*(21), R877-883.

[65] Wang, Y.; Hodgkinson, V.; Zhu, S.; Weisman, G.A.; Petris, M.J. Advances in the understanding of mammalian copper transporters. *Adv. Nutr.,* **2011**, 2(2), 129-137.

[66] Palumaa, P. Copper chaperones. The concept of conformational control in the metabolism of copper. *FEBS Lett.,* **2013**, *587*(13), 1902-1910.

[67] Kuo, Y.M.; Zhou, B.; Cosco, D.; Gitschier, J. The copper transporter CTR1 provides an essential function in mammalian embryonic development. *Proc. Natl. Acad. Sci. U. S. A.,* **2001**, *98*(12), 6836-6841.

[68] Lee, J.; Prohaska, J.R.; Thiele, D.J. Essential role for mammalian copper transporter Ctr1 in copper homeostasis and embryonic development. *Proc. Natl. Acad. Sci. U. S. A.,* **2001**, *98*(12), 6842-6847.

[69] Bertini, I.; Cavallaro, G. Metals in the "omics" world: copper homeostasis and cytochrome c oxidase assembly in a new light. *J. Biol. Chem.,* **2008**, *13*(1), 3-14.

[70] Jomova, K.; Valko, M. Advances in metal-induced oxidative stress and human disease. *Toxicology.* **2011**, *283*(2-3), 65-87.

[71] Rasoloson, D.; Shi, L.; Chong, C.R.; Kafsack, B.F.; Sullivan, D.J. Copper pathways in *Plasmodium falciparum* infected erythrocytes indicate an efflux role for the copper P-ATPase. *Biochem. J.,* **2004**, *381*(Pt. 3), 803-811.

[72] Kenthirapalan, S.; Waters, A.P.; Matuschewski, K.; Kooij, T.W. Copper-transporting ATPase is important for malaria parasite fertility. *Mol. Microbiol.,* **2014**, *91*(2), 315-325.

[73] PlasmoDB (*Plasmodium falciparum* genomic database) [http://www.plasmodb.org]

[74] Horng, Y.C.; Cobine, P.A.; Maxfield, A.B.; Carr, H.S.; Winge, D.R. Specific copper transfer from the Cox17 metallochaperone to both Sco1 and Cox11 in the assembly of yeast cytochrome C oxidase. *J. Biol. Chem.,* **2004**, *279*(34), 35334-35340.

[75] Maxfield, A.B.; Heaton, D.N.; Winge, D.R. Cox17 is functional when tethered to the mitochondrial inner membrane. *J. Biol. Chem.,* **2004**, *279*(7), 5072-5080.

[76] Banci, L.; Bertini, I.; Ciofi-Baffoni, S.; Huffman, D.L.; O'Halloran, T.V. Solution structure of the yeast copper transporter domain Ccc2a in the apo and Cu(I)-loaded states. *J. Biol. Chem.,* **2001**, *276*(11), 8415-8426.

[77] Choveaux, D.L.;Przyborski, J.M.; Goldring, J.P. A *Plasmodium falciparum* copper-binding membrane protein with copper transport motifs. *Malar. J.,* **2012**, *11*, 397.

[78] Choveaux, D.L.; Krause, R.G.; Przyborski, J.M.; Goldring, J.P. Identification and initial characterisation of a *Plasmodium falciparum* Cox17 copper metallochaperone. *Exp. Parasitol.,* **2015**, *148*, 30-39.

[79] Arguello, J.M.; Eren, E.; Gonzalez-Guerrero, M. The structure and function of heavy metal transport P1B-ATPases. *Biometals : Int. J. Role Metal Ions Biol., Biochem., Med.,* **2007**, *20*(3-4), 233-248.

[80] Hodgkinson, V.; Petris, M.J. Copper homeostasis at the host-pathogen interface. *J. Biol. Chem.,* **2012**, *287*(17), 13549-13555.

[81] Palmgren, M.G.; Nissen, P. P-type ATPases. *Annu. Rev. Biophys.,* **2011**, *40*, 243-266.

[82] Cobine, P.A.; Pierrel, F.; Winge, D.R. Copper trafficking to the mitochondrion and assembly of copper metalloenzymes. *Biochim. Biophys. Acta,* **2006**, *1763*(7), 759-772.

[83] Alvarez, H.M.; Xue, Y.; Robinson, C.D.; Canalizo-Hernandez, M.A.; Marvin, R.G.; Kelly, R.A.; Mondragon, A.; Penner-Hahn, J.E.; O'Halloran, T.V. Tetrathiomolybdate inhibits copper trafficking proteins through metal cluster formation. *Science,* **2010**; *327*(5963), 331-334.

[84] Brewer, G.J. Copper lowering therapy with tetrathiomolybdate as an antiangiogenic strategy in cancer. *Curr. Cancer Drug Targets,* **2005**, *5*(3), 195-202.

[85] Juarez, J.C.; Betancourt, O., Jr.; Pirie-Shepherd, S.R.; Guan, X.; Price, M.L.; Shaw, D.E.; Mazar, A.P.; Donate, F. Copper binding by tetrathiomolybdate attenuates angiogenesis and tumor cell proliferation through the inhibition of superoxide dismutase 1. *Clin. Cancer Res.,* **2006**, *12*(16), 4974-4982.

[86] Lee, K.; Briehl, M.M.; Mazar, A.P.; Batinic-Haberle, I.; Reboucas, J.S.; Glinsmann-Gibson, B.; Rimsza, L.M.; Tome, M.E. The copper chelator ATN-224 induces peroxynitrite-dependent cell death in hematological malignancies. *Free Radic. Biol. Med.,* **2013**, *60*, 157-167.

[87] Chidambaram, M.V.; Barnes, G.; Frieden, E. Inhibition of ceruloplasmin and other copper oxidases by thiomolybdate. *J. Inorg. Biochem.,* **1984**, *22*(4), 231-240.

[88] Alexander Bralley, J.; Load, R.S. Minerals. In: *Laboratory evaluations in molecular medicine: nutrients, toxicants, and cell regulators, chapter three*; The Institute for Advances in Molecular Medicine Ed.; Georgia, USA, **2001**; pp. 35-73. ISBN 0967394910. 2001.

[89] Ding, X.; Xie, H.; Kang, Y.J. The significance of copper chelators in clinical and experimental application. *J. Nutr. Biochem.,* **2011**, *22*(4), 301-310.

[90] Pan, Y.; Loo, G. Effect of copper deficiency on oxidative DNA damage in Jurkat T-lymphocytes. *Free Radic. Biol. Med.,* **2000**, *28*(5), 824-830.

[91] Brown, K.R.; Keller, G.L.; Pickering, I.J.; Harris, H.H.; George, G.N.; Winge, D.R. Structures of the cuprous-thiolate clusters of the Mac1 and Ace1 transcriptional activators. *Biochemistry,* **2002**, *41*(20), 6469-6476.

[92] Mattie, M.D.; McElwee, M.K.; Freedman, J.H. Mechanism of copper-activated transcription: activation of AP-1, and the JNK/SAPK and p38 signal transduction pathways. *J. Mol. Biol.,* **2008**, *383*(5), 1008-1018.