ELSEVIER

Biochemical Pharmacology 61 (2001) 999–1009

Biochemical Pharmacology

Prooxidant activity of β-hematin (synthetic malaria pigment) in arachidonic acid micelles and phospholipid large unilamellar vesicles

Fausta Omodeo-Salè[a,*], Diego Monti[b], Piero Olliaro[c], Donatella Taramelli[d]

[a]*Institute of General Physiology and Biochemistry, University of Milan, Via Trentacoste 2, I-20134-Milan, Italy*
[b]*Department of Organic Chemistry, CSSON, CNR, University of Milan, Via Trentacoste 2, I-20134-Milan, Italy*
[c]*UNDP, WorldBank, WHO Special Program for Research & Training in Tropical Diseases (TDR), Genova, Switzerland*
[d]*Institute of Microbiology, University of Milan, Via Trentacoste 2, I-20134 Milan, Italy*

Received 28 April 2000; accepted 16 October 2000

Abstract

Intraerythrocytic malaria parasite has evolved a unique pathway to detoxify hemoglobin-derived heme by forming a crystal of Ferri-protoporphyrin IX dimers, known as hemozoin or "malaria pigment." The prooxidant activity of β-hematin (BH), the synthetic malaria pigment obtained from hematin at acidic pH, was studied in arachidonic acid micelles and phospholipid Large Unilamellar Vesicles (LUVs) and compared to that of α-hematin (AH, Ferri-protoporphyrin IX-hydroxide) and hemin (HE, Ferri-protoporphyrin-chloride). Lipid peroxidation was measured as production of thiobarbituric acid reactive substances (TBARS). The extent of peroxidation induced by either AH or BH was strongly dependent upon the content of pre-existing hydroperoxides and efficiently inhibited by triphenylphosphine, a deoxygenating agent able to reduce hydroperoxides to hydroxides and by lipophilic scavengers. BH prooxidant activity was linearly related to the material, whereas that of AH seemed dependent on the aggregation state of the porphyrin. Maximal activity was observed when AH was present in concentration lower than 2 μM. In this case a shift of spectra in the Soret region, leading to the increase of the O.D. 400/385 nm ratio, suggested a transition toward a less aggregated state. BH prooxidant activity was significantly lower than that of monomeric AH, yet higher than that of AH aggregates. Differently from AH aggregates, BH-induced peroxidation was unaffected by GSH and inhibited rather than enhanced by acidic pH (5.7) and chloroquine. UV/Vis spectroscopy of AH aggregates at acidic pH, low GSH concentrations and chloroquine suggests a shift of AH aggregates toward the less aggregated state, more active as peroxidation catalyst.

Keywords: Liposomes; Hemin; β-hematin; Lipid peroxidation; Chloroquine

1. Introduction

Heme is a by-product of hemoglobin catabolism in blood stage *Plasmodium*. The fate of heme after being released from hemoglobin through the combined action of various proteinases [1] is not completely elucidated. Indeed, a proportion of it undergoes crystallization into a solid material called hemozoin (HZ[f] or "malaria pigment") [1–3] that accumulates in the FV. Such HZ crystal is made of heme dimers, in which the iron atoms are coordinated to the propionic side chains of each other porphyrin, that form chains linked by hydrogen bonds [3]. However, it was recently suggested that significant amounts of heme do not crystallize but rather diffuse into membranes and are degraded by GSH in the parasite cytoplasm [4]. Thus, quinoline-type antimalarials such as CQ, would act by inhibiting both HZ synthesis via the formation of heme-CQ adducts inside the FV [5,6], and GSH-induced heme degradation in the parasite cytoplasm [4].

Pigment, and possibly heme, are also present in host phagocytes as they ingest whole parasitized RBC (or "residual bodies") after schizogony [7].

Heme is potentially toxic for both erythrocytes and *Plas-*

* Corresponding author. Tel.: +39-02-215-0579; fax: +39-02-215-0464.

E-mail address: Fausta.Omodeosale@unimi.it (F. Omodeo-Salè).

Abbreviations: AA, arachidonic acid; AH, (ferriprotoporphyrin IX-hydroxide); HE (ferriprotoporphyrin IX chloride) BH, β-hematin; CQ, chloroquine; CU-OOH, cumene hydroperoxide; DMSO, dimethylsulfoxide; FV, food vacuole; GSH, reduced glutathione; HZ, hemozoin; LOOH, hydroperoxides; LUVs, Large Unilamellar Vesicles; PE, phosphatidylethanolamine; PL, phospholipid; RBC, red blood cells; SOD, superoxide dismutase; TBARS, thiobarbituric reactive substances; TPP, triphenylphosphine.

0006-2952/01/$ – see front matter
PII: S0006-2952(01)00558-5

modium itself [8] and inhibition of either mechanism of disposal leads to parasite death. Heme has been shown to bind to lipid bilayers [9,10] as well as protein structures. Its lytic effect upon these cells [11] suggests that its interaction with phospholipid bilayers may lead to membrane perturbation and subsequent changes in permeability. In addition, heme-containing molecules are known to have catalytic activity during both the initiation and the propagation phases of membrane lipid peroxidation [12,13] and also to play a role in diseases such as malaria and porphyrias [14,15]. Alterations of the redox processes have been observed in pathological conditions or genetic diseases accompanied by increased heme loss and hemolytic anemia, and could play a role in cell lysis processes [16]. Therefore, a possible mechanism of heme toxicity could be related to its catalytic activity on membrane lipid peroxidation.

In contrast, HZ has generally been considered a nontoxic storage form of heme. However, recent data from ourselves and other groups indicated that phagocytosis of pigment impairs macrophage activation and functions [17–19]. Further studies allowed to postulate that some of the effects mediated on macrophages by BH, the synthetic crystal derivative of hematin identical to native HZ [2–3,20], could be related to peroxidative stress, to which peritoneal macrophages are eminently susceptible [21].

The mechanisms of HZ or BH-induced peroxidation, as well as the reason of the absence of toxicity of the pigment as long as retained within the *Plasmodium* FV, are largely unknown. On this basis, the present study aimed at investigating the ability of BH to promote lipid peroxidation of two different artificial systems, AA micelles and PL LUVs. The prooxidant activity of BH, AH, and HE were compared in different experimental settings with or without CQ.

2. Materials and methods

2.1. Chemicals

AH, HE, GSH (G4251), PE from bovine brain, AA, CQ, TPP, 1,1,3,3-tetraethoxypropane, CU-OOH, diethyl-enetriaminepentaacetic acid, α-tocopherol, and catalase (1.11.1.6, from bovine liver), were purchased from Sigma and thiobarbituric acid, SOD (1.15.1.1) from bovine erythrocytes from Fluka.

2.2. Hematin and β-hematin preparation

BH was synthesized from a solution of AH precipitated by the addition of acetic acid [22]. Unreacted AH was removed by extracting the precipitate twice for 3 hr in 0.1 M sodium carbonate buffer, pH 9.1. The purity of the final product was routinely controlled by infrared spectroscopy [22]. Batches of BH, which did not meet criteria for purity, were discarded. Fresh AH, HE, or BH stock solutions were prepared daily. A weighed amount of AH, HE, or BH was re-suspended in 0.02 NaOH, DMSO, or methanol, respectively. The heme equivalents present in the different stock solutions were quantitated by dissolving an aliquot in 1 N NaOH and reading the absorbance at 385 nm. (ε_{385} hematin $= 6.1 \times 10^4$ M cm^{-1}). Proper aliquots of the HE stock solution were diluted in the assay buffer to keep the final DMSO concentration lower than 0.2% and added to the incubation mixture just before the experiment. CQ was dissolved to 4 mM in water and porphyrin-CQ mixtures were made in the molar ratio 1:2 (heme equivalents: CQ).

2.3. Preparation of LOOH containing AA and PE and LOOH determination

Unsaturated lipids stored dried tend to autooxidize [23]. Therefore, enrichment of AA or PE with LOOH was obtained by storing at room temperature dried aliquots of the lipid for 10–24 hr. The absolute LOOH content used in the peroxidation experiments was determined by the xylenol orange method [24] using CU-OOH for the calibration curve.

2.4. AA micelles and LUVs of RBC ghost membrane phospholipids

AA in chloroform (either peroxide-free or containing different amounts of oxidized AA) was dried with a stream of nitrogen and further lyophilized for $\geq$3 hr. Micelles were prepared by re-suspending the sample in 10 mM Tris HCl buffer (pH 7.4), 154 mM NaCl, 1 mM EDTA to achieve a final AA concentration of 3.75 M.

White ghosts were prepared from normal RBC by hypotonic lysis and extensive washing in 5 mM Na phosphate, pH 8.0. Membranes were then washed in the same 2.5 mM buffer. PL were extracted from ghosts [25], purified [26], and then quantified as inorganic phosphorus [27]. PL in the amount required for each experiment—with the addition of partially oxidized PE (20% molar) and CU-OOH (5% molar) in some experiments—were dried with a stream of nitrogen, lyophilized overnight and resuspended in 10 mM Tris HCl buffer (pH 7.4), 154 mM NaCl, 1 mM EDTA in order to achieve a final PL concentration of 2.0 M. The suspension was extruded under nitrogen through two stacked 25-mm polycarbonate filters (0.1 μm pore size) (Nucleopore Corp.) using a liposome extruder (Lipex Biomembranes). The vesicles were collected and reextruded through the filter 10 times to ensure uniformity. To make LUVs containing AH, the PL mixture was resuspended in buffer containing AH and extruded as described above. Fresh preparations of LUVs were made the same day of the experiment.

2.5. Lipid peroxidation

Lipid peroxidation was monitored measuring TBARS according to Buege and Aust [28] and quantified using

1,1,3,3-tetraethoxypropane for the calibration curve. The lipid concentration of micelles and liposomes in the reaction mixture was 750 μM and 400 μM, respectively. TPP or α-tocopherol were introduced into liposomes by coevaporation with PL from organic solvent, while hydrophilic antioxidant or enzymes (SOD, catalase) were added to the incubation mixture just before the addition of LUVs. TBARS are expressed as nmol/μmol AA or nmol/μmol PL. The values reported in the Result section are the net values, obtained by subtracting the value of TBARS production in the absence of AH, HE or BH.

2.6. Statistical analysis

Data are reported as mean ± standard deviation of the experiments conducted. Results were compared by using the analysis of variance (ANOVA).

3. Results

Experiments performed on AA micelles proved that the pro-oxidant activity of either BH or AH depend upon the oxidation state of the fatty acid of the micelle. Fig. 1a shows that thiobarbituric acid reactivity increased with increasing amounts of AA-LOOHs. Contrary to AH, BH had a measurable, though low prooxidant activity in peroxide free micelles (1% AA-LOOH); in peroxide-containing micelles, an increase in activity with the AA-LOOH content was detected, although this was significantly lower than that observed with AH. The importance of the presence of LOOHs was confirmed by experiments performed in the presence of TPP, a LOOH scavenger, that significantly reduced BH and AH-induced peroxidation. The BH- and AH-induced TBARS production in micelles containing AA-LOOH (12%) was dose dependent (Fig. 1b). TBARS production in the presence of HE was identical to that of AH (data not shown).

When tested in LUVs, the thiobarbituric acid reactivity induced by BH or AH was low in non-oxidized vesicles but increased significantly in vesicles containing a partially oxidized PE (3% LOOHs) (Fig. 2a). The response of BH was initially linear up to 3 μM to reach a plateau beyond 4 μM. In contrast, a bell-shaped dose-response curve was observed in the presence of AH, as well as HE, reaching a maximum at 2 μM heme equivalents and decreasing at higher concentrations. This effect was not observed when LUVs were extruded in the presence of AH. In these conditions, AH was directly entrapped in liposomes and the pro-oxidant activity of 5 μM AH was approximately 3-fold that of 1 μM AH (data not shown).

In the presence of 2 or 5 μM BH and 2 μM AH the extent of peroxidation was higher in liposomes containing LOOH-PE or CU-OOH than in peroxide-free liposomes and was significantly reduced by TPP (Fig. 2b). However, LOOH-PE and TPP did not affect the extent of peroxidation induced by 5 μM AH.

Alpha-tocopherol and its partially water soluble analogue, trolox C, significantly reduced the extent of BH and AH-induced peroxidation, whereas catalase, SOD or hydrophilic reducing agents (GSH and ascorbate) were not inhibitory (Table 1). Surprisingly, GSH enhanced AH-induced peroxidation.

3.1. Effect of CQ

CQ reduced by 50% the peroxidation induced by 5 μM BH (Fig. 3a). The same effect was observed at 2 μM. TPP further lowered TBARS production. In contrast, CQ increased AH as well as HE-induced peroxidation, particularly at heme concentrations >2 μM (Fig. 3a, b). The CQ-induced enhancement of peroxidation at 5 μM AH was particularly marked when partially oxidized LUVs were used (Fig. 3a); in this latter case such effect was inhibited by TPP.

3.2. Effect of pH

The effect of two different pH (7.4 or 5.7) on BH-, AH-, or HE-induced peroxidation was investigated. Experiments were performed in 10 mM Na-phosphate buffer, 154 mM NaCl, 1 mM EDTA. The extent of BH-induced peroxidation was 50% lower at acidic—than at neutral pH, whereas that of either AH or HE was 30% higher (data not shown).

3.3. Spectra analysis of AH and HE in different experimental conditions

The UV/Vis spectra of AH were analyzed in different experimental conditions. As shown in Fig. 4a, and in agreement with previous reports [29], the U.V./Vis spectra of 5 μM aqueous solution (pH 7.4) of AH was characterized by a spectrum with a maximum of absorbance at 385 nm, leading to a O.D. 400/385 nm ratio of 0.892 ± 0.03. In these conditions, as further developed in Discussion, AH is expected to be present largely as dimers and higher aggregates. In the spectrum of AH at 2 μM a higher O.D. 400/385 nm ratio was seen (1.020 ± 0.020), indicating a higher percentage of monomers. A similar shift at 2 μM was observed with HE, although the initial O.D. 400/385 nm ratio at 5 μM HE was higher (0.905 ± 0.02) than the corresponding values with 5 μM AH. In the presence of CQ (at AH/CQ 1:2 molar ratio) (Fig. 4b), or at acidic pH (Fig. 5a), or in the presence of 100 μM GSH (Fig. 5b), a similar increase in the O.D. 400/385 nm ratio was observed, suggesting a shift toward a less aggregated state. No effect on the AH spectrum was exerted by 100 μM ascorbic acid (Fig. 5c). Comparable shifts of the U.V./Vis spectra were observed with 5 μM HE in the same experimental conditions (data not shown).

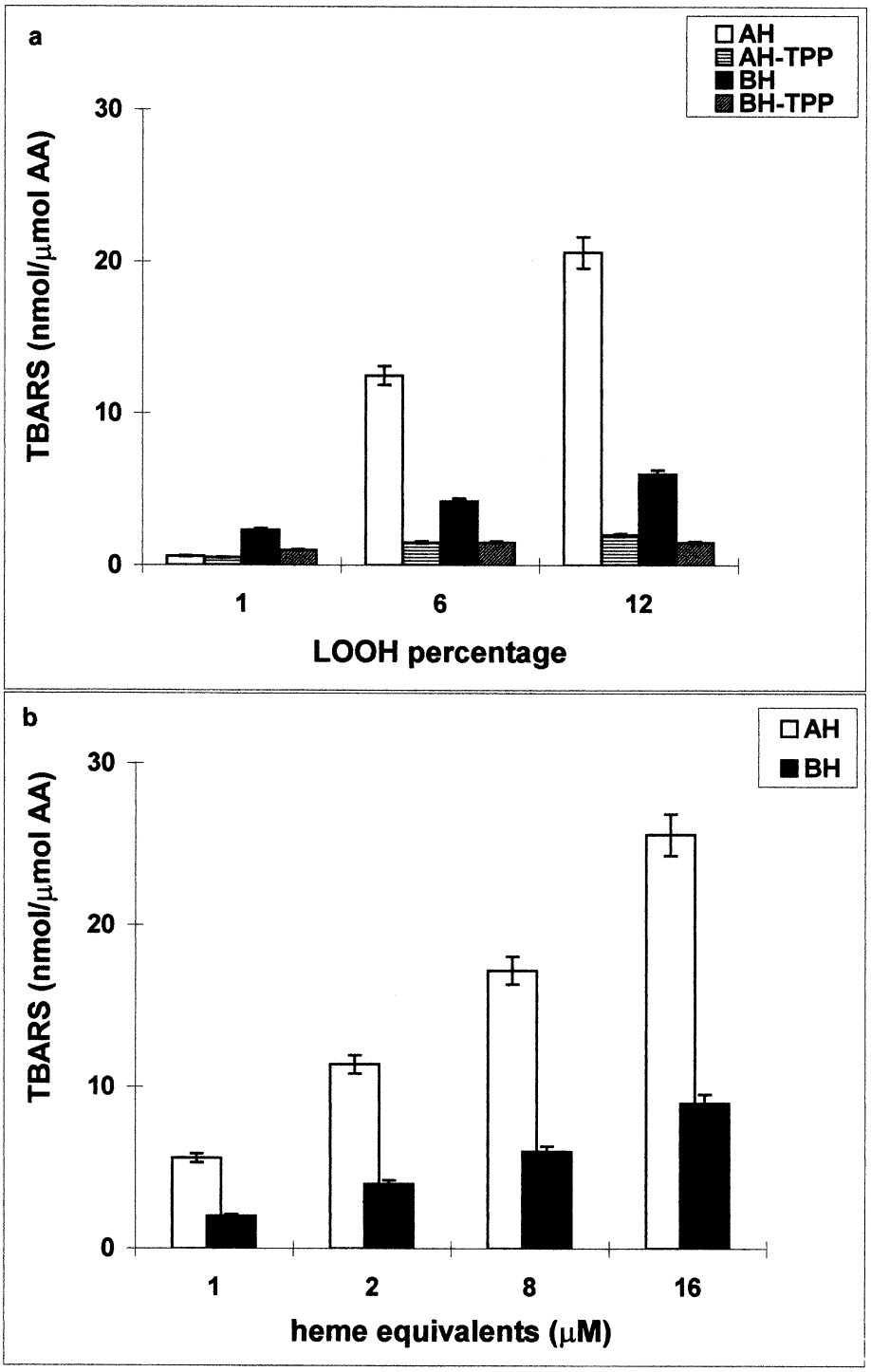

Fig. 1. AH and BH-induced TBARS production in AA micelles. (a) In the presence of 10 μM AH or BH (as heme equivalents) before and after addition of TPP in micelles containing different percentage of AA-LOOH. (b) In the presence of increasing amount of AH or BH. Values are mean ± SD (N = 3).

4. Discussion

4.1. Susceptibility of AA micelles and PL LUVs to BH and AH-induced oxidative stress

The pro-oxidant activity of BH, the synthetic crystal identical to malarial pigment [3], on either cellular or artificial membranes depends on the oxidation status of the membrane. Our experiments with AA micelles and LUVs enriched with LOOHs show that the level of pre-existing hydroperoxides is critical in determining membrane susceptibility to BH-mediated free radical production. Therefore it can be deducted that BH, as well as AH, promotes lipid

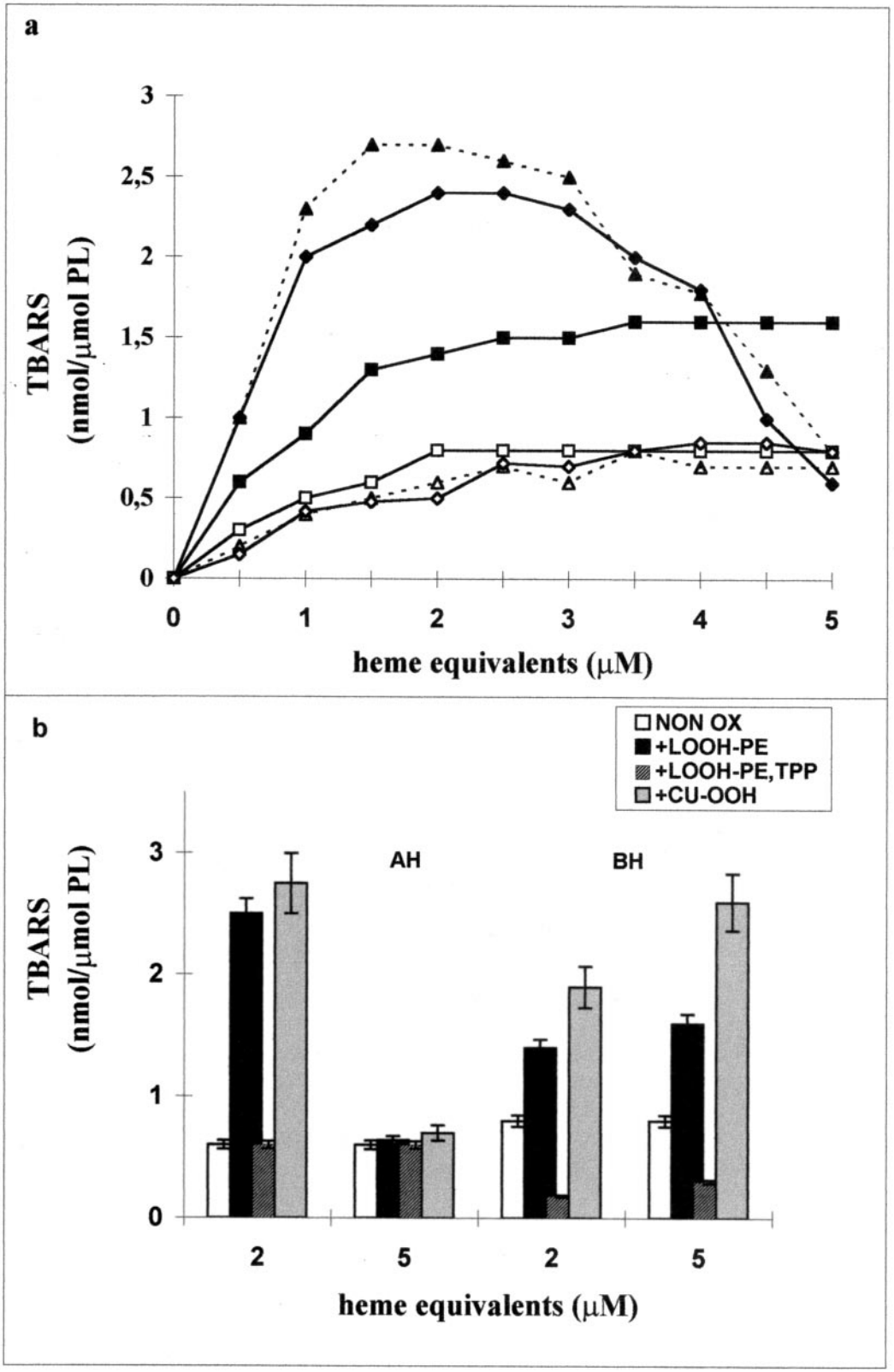

Fig. 2. AH, HE and BH-induced TBARS production in phospholipid LUVs. (a) At different concentrations of heme equivalents. Open symbols: non oxidized LUVs (AH = ◇-◇, HE = Δ-Δ, BH = □-□); Closed symbols: LUVs containing LOOH-PE (3% of total PL, AH = ◆-◆, HE = ▲-▲, BH = ■-■). (b) AH and BH-induced TBARS production in phospholipid LUVs at two different concentrations of heme equivalents in non oxidized LUVs (non ox), in LUVs containing LOOH-PE (LOOH-PE, 3% of total PL) ± TPP or in LUVs containing cumene hydroperoxide (CU-OOH, 5% of total PL). In non oxidized and LOOH-PE containing LUVs the total amount of PE was the same. Values are mean ± SD (N = 3).

Table 1
Effect of different scavengers and antioxidants on AH and BH-induced peroxidation of LUVs.

	Rate of peroxidation (%)	
Addition	AH	BH
None	100	100
Catalase (400 U/mL)	91 ± 7	90 ± 6
SOD (300 U/mL)	93 ± 5	82 ± 7
Ascorbate (100 μM)	80 ± 5	95 ± 5
GSH (100 μM)	135 ± 10	100 ± 6
α-Tocopherol (150 μM)	5 ± 2	10 ± 2
Trolox C (150 μM)	10 ± 5	10 ± 6

The assays were performed in partially oxidized LUVs and in the presence of 2 μM AH or 5 μM BH. The reaction mixture and incubation conditions were as reported in section 2.

peroxidation through the decomposition of lipid peroxyl species and derived hydroperoxides, which, in the presence of polyunsaturated fatty acids, can propagate new chain reactions [12,30,31]. This conclusion is substantiated by the results with TPP, a deoxygenating agent that reduces LOOH to hydroxides (LOH) whilst being oxidized to triphenylphosphine oxide [32]. The role of LOOH in the radical chain reactions promoted by BH supports in-vitro data on the antimalarial activity of polyunsaturated fatty acids, that was ascribed to their oxidized forms [33]. It is worthwhile to be noted that, in the absence of LOOH, the pro-oxidant activity of BH in both AA micelles and LUVs is slightly higher than that of AH/HE (Fig. 1a, Fig. 2a); the possibility that the crystalline structure of the polymer may act as an oxygen "trap," favoring peroxidation, is under investigation.

4.2. Correlation between aggregation state and pro-oxidant activity of heme

Our data indicate that the aggregation state of heme influences the degree of lipid peroxidation. When the experiment is performed on AA micelles, the LOOH-dependent increase in pro-oxidant activity is linearly related to the concentration tested of BH, AH or HE (Fig. 1b). In contrast, when LUVs are used as the peroxidative substrate, BH proves to be sensitive to LOOH and to the scavenging effect of TPP at both concentrations (2 and 5 μM heme equivalents) while AH does so only at the lower concentration (Fig. 2b).

The aggregation state of heme in aqueous solutions is known to be concentration-dependent: monomeric at low concentrations (<1 μM), dimeric [29] or more aggregated state at the higher concentrations [6,34]. Noteworthy, when hematin is dissolved in basic solutions, oxo-bridge dimers form which can also further aggregate [35]. This was identified as a possible pitfall of the method. To minimize this problem, experiments were performed in parallel using as control stock solutions of HE prepared in DMSO. In such solvent hemin is eminently monomeric [36]. No significant difference was observed between the two heme preparations, ruling out the possibility that some of the above findings (such as the shift of the U.V./Vis spectra, heme concentration dependence of TBARS production, or the effects of pH) could be due to pre-existing oxo-bridge dimers.

Bell shaped curves for lipid peroxidation induced by hemin or heme-containing proteins had been reported by other authors too [37–39] and interpreted as being due to lower peroxidative efficiency of dimers and higher aggregates. The decline in the monomer fraction in the 5 μM AH is reflected in the U.V./Vis spectra: AH at 5 μM shows a lower O.D. 400/385 nm ratio compared to 2 μM. Taken together, these data indicate a transition toward a more aggregated state, less effective in catalysing peroxidation (Fig. 4a). Such lower reactivity of both AH and HE at 5 μM (Fig. 2a) would suggest that heme aggregates are poorly accessible to LUVs, and that monomeric heme is more apt at initiating new radical chain reactions inside the bilayer through interaction with LOOH. In support of this views is the finding that, when AH (or HE) aggregates (5 μM) are partially incorporated into the LUVs hydrophobic bilayer, they indeed become highly prooxidant. Since hemin incorporated into liposomes was shown to be monomeric or dimeric [40], one can speculate that the recovery of the oxidant capability by AH aggregates in LUVs is due a partial shift to a less aggregated state. A similar shift of the spectrum is observed when 5 μM AH or HE are incubated with AA micelles (data not shown). In this case, the extent of AA peroxidation is linearly related to the concentration AH or HE used in the experiment (Fig. 1b).

However, it is also true that, based of Brown's constant [29], the absolute monomer concentration at 5 μM heme is higher than at 2 μM, despite a lower percentage. Therefore, other factors besides heme dimerization, still related to the concentration of heme, may be responsible of the concentration-dependent pro-oxidant activity of AH or HE and need to be further investigated.

The ranking order of oxidant potential in our experiments is: monomers >BH >AH, HE at concentrations above 2 μM (Fig. 2b). The different structural organization of BH and heme aggregates reflects in a different reactivity of heme iron with the LUVs LOOHs.

Recent data suggest that the amount of non-crystallized heme accumulating in the parasite from mid- to late-stage trophozoite exceeds the amount of HE detoxified as HZ [4]. If this is the case, in addition to HZ, both HE monomers and HE aggregates would be present, representing a dynamic reservoir of highly toxic molecules. In our experimental model, we investigated different conditions that could affect the prooxidant activity of BH and HE aggregates.

4.3. Effect of pH

Lowering the pH from 7.4 to 5.7 (the approximate pH of the FV) significantly decreases BH-induced TBARS pro-

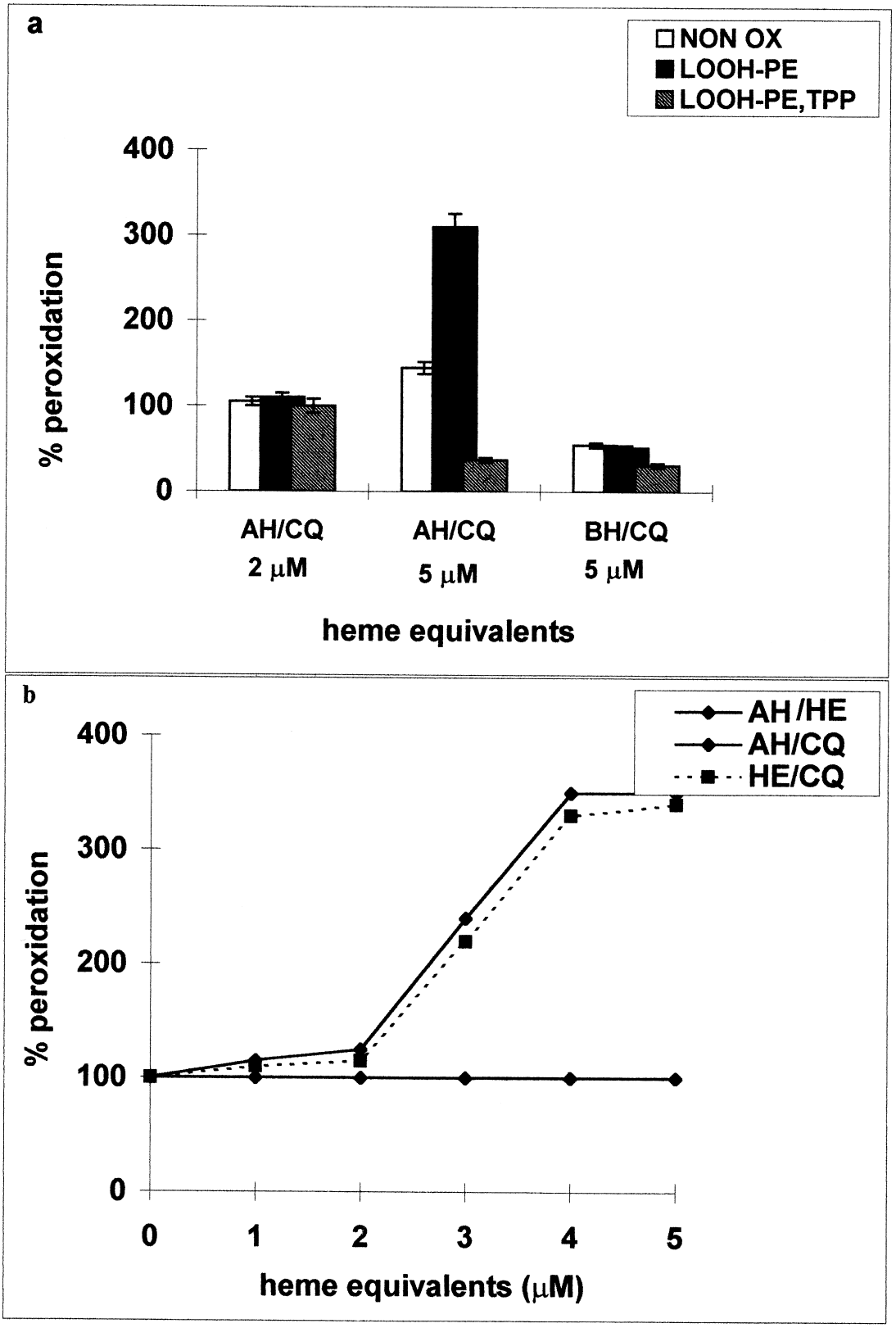

Fig. 3. Effect of chloroquine (CQ) on AH, HE, and BH-induced peroxidation of LUVs (a) At two concentrations of AH or BH heme equivalents (2 and 5 μM) in non-oxidized LUVs (non ox) and in LUVs containing LOOH-PE (3% of total lipids) ± TPP. (b) AH and HE at different concentrations of heme equivalents. Values are mean ± SD (N = 5).

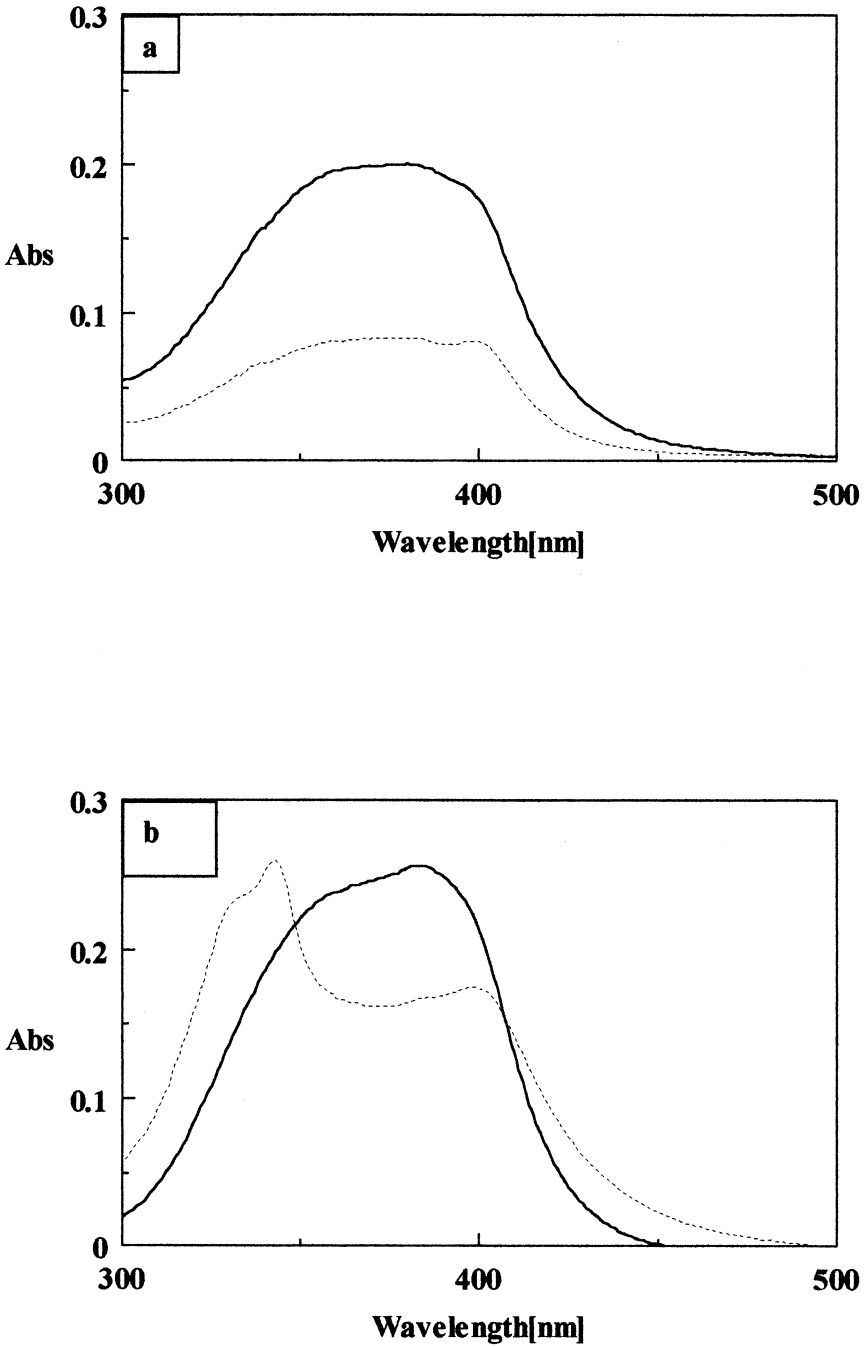

Fig. 4. Effect of heme concentration and CQ on the U.V./Vis spectra (Abs = absorbance) of aqueous solutions of AH (Tris HCl 10 mM, NaCl 154 mM, 1 mM EDTA pH 7.4). (a) Solid line, AH 5 μM; dashed line, AH 2 μM. (b) Solid line, AH 5 μM; dashed line, 5 μM AH + CQ (1:2 molar ratio).

duction. This result can be explained on the basis of the crystal structure of BH, made of chains of hydrogen-bonded dimers [3]. The decrease of inter-chain hydrogen bonds at lower pH might reduce the aggregation state of BH and therefore its oxidative capability. This effect could be particularly relevant for the integrity of the FV where the pigment is synthesized and sequestered.

On the contrary, AH and HE aggregates are more effective in inducing lipid peroxidation at acidic than at neutral pH. This correlates with a shift in the Soret peak and an increase of the O.D. 400/385 nm ratio, suggesting that, in these conditions, the equilibrium is shifted toward the more toxic monomeric-dimeric form (Fig. 5a). Kinetic studies on hematin μ-oxo oligomers showed a pH dependence of the dimerization constant [35]. In the parasite, this means that, because uncrystallized heme would be more toxic in the acidic environment of the FV, it has to be exported to the cytoplasm. Hence our data would support, though indirectly, these recent data on the fate of free heme [4].

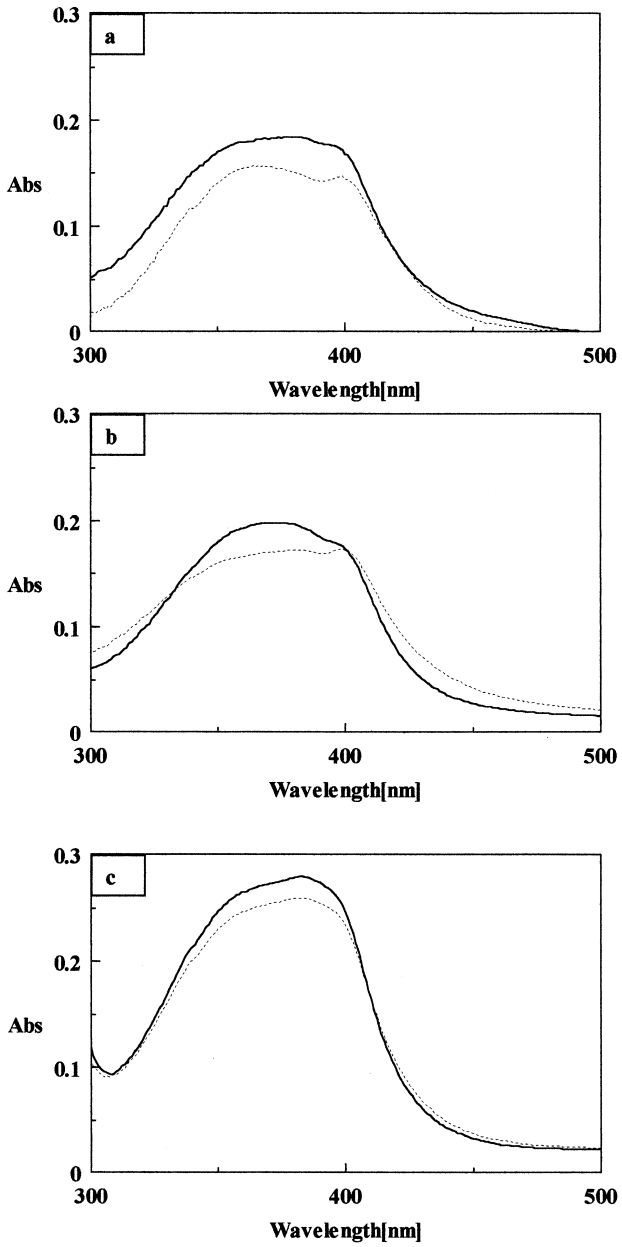

Fig. 5. Effect of pH, GSH, and ascorbic acid on the U.V./Vis spectra (Abs = absorbance) of 5 μM AH. (a) solid line, 10 mM sodium phosphate buffer, 154 mM NaCl, 1 mM EDTA, pH 7.4. (b) Dashed line, 10 mM sodium phosphate buffer, 154 mM NaCl, 1 mM EDTA, pH 5.7. (b) Before (—) and after (– – –) 60 min incubation with 100 μM GSH. (c) Before (—) and after (– – –) 60 min incubation with 100 μM ascorbic acid.

4.4. Effect of free radical scavengers and glutathione

The free radical reaction chain promoted by BH as well HE occurs within the hydrophobic compartment since TBARS production is abolished by lipophilic scavengers (TPP, a-tocopherol) and by trolox, the water-soluble analogue of vitamin E, that can partially partition into the lipid phase of LUVs [41]. By including EDTA in the incubation buffer, we excluded any aspecific involvement of free iron in the peroxidative reactions. Similar results were obtained using diethylenetriaminepentaacetic acid as iron chelating agent (data not shown). SOD and catalase only marginally affected the AH and BH peroxidative activity (thus excluding a significant role of superoxide or hydrogen peroxide, respectively). These findings confirm our previous data on BH-treated macrophages and microglia cell lines [21].

The peroxidative activity of BH is unaffected by low GSH concentrations, whereas that of AH aggregates increases. This could be due to GSH reducing Fe(III)PPIX to Fe(II)PPIX, which would lead to a Fe(II)/Fe(III) ratio that is more favorable to LOOH-mediated peroxidation [42]. Such effect can not be exerted on BH because the central iron coordinates to the carboxyl group of the adjacent molecule. In addition, since Fe(II)PPIX is less apt to form μ-oxo bridges than Fe(III)PPIX [35], the equilibrium further shifts toward the more prooxidant monomers or dimers—as demonstrated by the U.V/Vis spectra of aggregated AH in the presence of GSH (Fig. 5b). In contrast, ascorbic acid did not affect AH prooxidant activity nor did it modify the UV/Vis spectra (Fig. 5c). In agreement with the Michaelis-Menten-like kinetics reported by Atamna and Ginsburg [43] no significant degradation of heme was seen in our experimental conditions (100 μM GSH). Thus, low concentrations of GSH, not only would not be able to detoxify heme [4], but could be also dangerous for the parasites because they can reduce the central iron atom of the porphyrin aggregates releasing highly prooxidant Fe(II) PPIX. In addition, Fe(II) PPIX does not form hemozoin and is an effective inhibitor of BH formation [44].

4.5. Effect of chloroquine

CQ (Fig. 3) significantly reduces the degree of LUVs peroxidation induced by BH. This finding could be due to the binding of CQ to both natural hemozoin and synthetic BH (“capping”) [45 and Egan, personal communication], thus preventing in part its interaction with membranes. Our findings are in agreement with a previous report with a similar experimental model [46] that CQ enhances AH prooxidant activity. We found that such effect is particularly marked at concentrations >2 μM, where AH is present in the aggregated form (Fig. 3). Hematin is known to form non-covalent high-affinity complexes with CQ [36,48] which cause erythrocytes and malaria parasites to lysate [48]. The observed shift in the UV/Vis spectra in the presence of CQ (Fig. 4b) suggests that CQ-monomer or CQ-dimer adducts may form [5,6,36,49], that would intercalate more easily into membranes and induce peroxidation more effectively. The same changes in the UV/Vis spectra occur at the two pH tested (5.7 and 7.4) (also the Ka of CQ with AH and HE varies insignificantly with pH [36,49]).

Our findings indicate that, in addition to preventing the crystallization of heme (highly pro-oxidant) into BH [5,36] (which has a lower pro-oxidant potential), CQ act also by enhancing the prooxidant capability of AH aggregates. It is worthwhile noting that AH peroxidase activity was shown

to be strongly increased by hydrogen peroxide; the effect was ascribed to porphyrin ring degradation [50]. The addition of CQ to AH in the presence of hydrogen peroxide proved to inhibit, rather than increasing, the peroxidase activity of AH, by protecting the porphyrin ring from degradation. Overall, in the parasite the CQ-mediated reduction of BH-induced peroxidation is most probably insignificant compared to the CQ-induced enhancement of AH pro-oxidant capability and the considerably higher toxicity of the heme-CQ adducts with respect to the BH crystals.

Taken together our data indicate that: (i) BH induces oxidative stress; its pro-oxidant activity depends upon pre-existing hydroperoxides in the membrane (i.e. on prior oxidative stress). This feature could be of significant advantage for the parasite, whose FV membrane has a relatively low content in unsaturated fatty acids. In contrast, the formation of hemozoin may not be favorable for the cells of the human host, such as red blood cells (due to the high oxygen tension and presence of hemoglobin) and phagocytes ingesting the pigment [20] because hemozoin, differently from AH, cannot be degraded [51] and, as such, its pro-oxidant activity, although lower than AH, is maintained almost indefinitely (in relation with the life-span of neutrophils, monocytes and macrophages); (ii) several factors, such as pH, levels of GSH content and presence of drugs such as CQ, regulate the equilibrium between toxic and less toxic forms of heme in the parasite microenvironment.

In summary, we believe that these data contribute to elucidate heme effects and toxicity to both the parasite and phagocytes, as well as to characterize the targets of the quinoline family of antimalarials and potentially to design newer compounds.

Acknowledgments

This work was supported in part by UNDP/World Bank/WHO Special Program for Research and Training on Tropical Diseases, Geneva (CH) N. 98-0363 and by the Italian Ministero dell'Università e Ricerca Scientifica e Tecnologica, Cofinanziamento 1999, Project "Molecular, immunological and pharmacological analysis of the interactions between parasite, hosts and vectors" to T.D.

References

[1] Francis SE, Sullivan DJ, Goldberg DE. Hemoglobin metabolism in the malaria parasite Plasmodium falciparum. Annu Rev Microbiol 1997;51:97–123.

[2] Slater AFG, Swiggard WJ, Orton BR, Flitter WD, Goldberg DE, Cerami A, Henderson GB. An iron-carboxilate bond links the heme units of malaria pigment. Proc Natl Acad Sci USA 1991;88:325–9.

[3] Pagola S, Stephens PW, Bohle DS, Kosar AD, Madsen SK. The structure of malaria pigment beta-haematin. Nature 2000;404:307–10.

[4] Kirk K, Tilley L, Ginsburg H. Transport and trafficking in the malaria infected erythrocytes. Paras Today 1999;15:355–9.

[5] Egan TJ, Ross DC, Adams PA. Quinoline anti-malarial drugs inhibits spontaneous formation of beta-haematin (malaria pigment). FEBS Letters 1994;352:54–7.

[6] Dorn A, Vippagunta SR, Matile H, Jaquet C, Vennerstrom JL, Ridley RG. An assessment of drug-haematin binding as a mechanism for inhibition of haematin polymerization by quinoline antimalarials. Biochem Pharmacol 1998;55:727–36.

[7] Phu NH, Day NPJ. Intraleucocytic malaria pigment and prognosis in severe malaria. Trans R Soc Trop Med Hyg 1995;89(2):200–4.

[8] Chou AC, Fitch CD. Hemolysis of mouse erythrocytes by ferriprotoporphyrin IX and chloroquine. Chemotherapeutic implications. J Clin Invest 1980;66:856–8.

[9] Tipping E, Ketterer B, Christodoulides L. Interactions of small molecules with phospholipid bilayers. Binding to egg phosphatidylcholine of some organic anions (bromosulphophtalein, oestrone sulphate, heme) that bind to ligandin and aminoazo-dye-binding protein A. Biochem J 1979;180:327–37.

[10] Ginsburg H, Demel RA. Interactions of hemin, antimalarial drugs hemin antimalarial complexes with phospholipid monolayers. Chem Phys Lipids 1984;35:331–47.

[11] Chou AC, Fitch CD. Mechanism of hemolysis induced by ferriprotoporphyrin IX. J Clin Invest 1981;68:672–7.

[12] Davies J. Detection of peroxyl and alcoxyl radicals produced by reaction of hydroperoxides with heme proteins by electron spin resonance spectroscopy. Biochim Biophys Acta 1988;964:28–35.

[13] Wheeler EL. Cytochrome P-450 mediated interaction between hydroperoxide and molecular oxygen. A proposed method for P-450 kineticstudies. Biochem Biophys Res Commun 1983;110:646–53.

[14] Golenser J, Marva E, Chevion M. The survival of Plasmodium under oxidant stress. Paras Today 1991;7:142–6.

[15] Elder GH. Update on enzyme and molecular defects in porphyria. Photodermatol, Photoimmunol & Photomed 1998;14:66–9.

[16] Hebbel RP. The sickle erythrocyte in double jeopardy: autoxidation and iron decompartimentalization. Seminars in Hematol 1990;27(1):51–69.

[17] Arese P, Schwarzer E. Malarial pigment (haemozoin): a very active "inert" substance. Annals of Trop Med and Parasitol 1997;91:501–16.

[18] Prada J, Malinowski J, Muller S, Bienzle U, Kremser P. Effect of Plasmodium Vinckei Haemozoin on the production of oxygen radicals and nitrogen oxides in murine macrophages. Am J Trop Med Hyg 1996;54:620–4.

[19] Taramelli D, Basilico N, Pagani E, Grande R, Monti D, Ghione M, Olliaro P. The heme moiety of malaria pigment (b-hematin) mediates the inhibition of NitricOxide and Tumor Necrosis Factor production by lipopolysaccharide-stimulated macrophages. Exp Parasitol 1995;81:501–11.

[20] Bohle SD, Dinnebier RE, Madsen SK, Stephens PW. Characterization of the products of the heme detoxification pathway in malaria late trophozoites by X-ray diffraction. J Biol Chem 1997;272:713–6.

[21] Omodeo-Sale' F, Basilico N, Folini M, Olliaro P, Taramelli D. Macrophage populations of different origins have distinct suceptibilities to lipid peroxidation induced by β-haematin (malaria pigment). FEBS letters 1998;433:215–8.

[22] Basilico N, Monti D, Olliaro P, Taramelli D. Non-iron porphyrins inhibit beta-haematin (malaria pigment) polymerization. FEBS Letters 1997;409(2):297–9.

[23] Kates M. In: Work TS, Work E, editors. Techniques of lipidology: Isolation, analysis and identification of lipids. Laboratory Techniques in Biochemistry and Molecular Biology, vol. 3, part II, New York: Elsevier Science, 1972. p. 353.

[24] Nourooz-Zadeh J, Tajaddini-Sarrmadi J, Wolff SP. Measurement of plasma hydroperoxide concentrations by the ferrous oxidation orange assay in conjunction with triphenylphosphine. Anal Biochem 1994;220:403–9.

[25] Folch J, Lees M, Sloane Stanley GM. A simple method for the isolation and purification of total lipids from animal tissue. J Biol Chem 1957;226:497–509.

[26] Vance DE, Sweeley CC. Quantitative determination of the neutral glycosylcermides in human blood. J Lipid Res 1967;8:621–30.
[27] Bartlett GR. Phosphorous assay assay in column chromatography. J Biol Chem 1959;234:466–8.
[28] Buege JA, Aust SD. Micrososomal lipid peroxidation. In: Fleischer S, Packer S, editors. Methods in Enzymol, vol 52 New York: Academic Press, 1978. p. 302–10.
[29] Brown SB, Dean TC, Jones P. Aggregation of ferriheme. Dimerization and protolytic equilibria of protoferriheme and deuteroferriheme in aqueous solution. Biochem J 1970;117:733–9.
[30] Dix T, Marnett LJ. Conversion of linoleic acid hydroperoxide to hydroxy, keto, epoxyhydroxy and trihydroxy fatty acids by hematin. J Biol Chem 1985;260:5351–7.
[31] Dix TA, Fontana R, Panthani A, Marnett LJ. Hematin-catalyzed epoxidation of 7,8-dihydroxy-7,8-dihydrobenzo (a) pyrene by polyunsaturated fatty acid hydroperoxides. J Biol Chem 1985;260:5358–65.
[32] Nakumura T, Maeda HA. Simple assay for lipid hydroperoxides based on triphenylphosphine oxidation and high performance liquid chromatography. Lipids 1991;26:765–8.
[33] Kumaratilake LM, Robinson BS, Ferrante A, Poulos A. Antimalarial properties of n-3 and n-6 polyunsaturated fatty acids: in vitro effects on Plasmodium falciparum and in vivo effects on P. berghei. J Clin Invest 1992;89:961–7.
[34] Kuzelova K, Mrhalova M, Hrkal Z. Kinetics of heme interaction with heme-binding proteins: the effect of heme aggregation state. Biochim Biophys Acta 1997;1336:497–501.
[35] White WI. Aggregation of porphyrins and metallo-porphyrins. In: Dolphin D, editor. The porphyrins, Vol. V, Physical Chemistry. New York: Academic Press, 1978. p. 303–39.
[36] Egan TJ, Mavuso WW, Ross DC, Marques MJ. Thermodynamic factors controlling the interaction of antimalarial drugs with ferriprotoporphyrin IX. J Inorg Biochem 1997;68:137–45.
[37] Kaschnitz RM, Yatefi Y. Lipid oxidation in biological membranes. Electron transfer proteins as initiators of lipid autoxidation. Arch Biochem Biophys 1975;171:292–304.
[38] Schmitt TH, Frezzatti WA, Schreier S. Hemin-induced lipid membrane disorder and increased permeability: a molecular model for the mechanism of cell lysis. Arch Biochem Biophys 1993;307:96–103.
[39] Vincent SH, Grady RW, Shaklai N, Snider JM, Muller-Eberhard U. The influence of heme-binding proteins in heme-catalyzed oxidations. Arch Biochem Biophys 1988;265:539–50.
[40] Cannon JB, Kuo FS, Pasternack RF, Wong N, Muller-Eberhard U. Kinetics of the interaction of hemin liposomes with heme binding proteins. Biochemistry 1984;23:3721–5.
[41] Barclay LR, Artz JD, Mowat J. Partitioning and antioxidant action of the water-soluble antioxidant Trolox, between the aqueous and lipid phases of phosphatidylcholine membranes: ^{14}C tracer and product studies. Biochim Biophys Acta 1995;1237:77–85.
[42] Tang LX, Yang JL, Shen XJ. Effects of additional iron chelators on Fe^{2+} initiated lipid peroxidation: evidence to support the $Fe^{2+\cdots}Fe^{3+}$ complex as initiator. J Inorg Biochem 1997;68:265–72.
[43] Atamna H, Ginsburg H. Heme degradation in the presence of glutathione. J Biol Chem 1995;270:24876–83.
[44] Monti D, Vodopivec B, Basilico N, Olliaro P, Taramelli D. A novel endogenous antimalarial: Fe (II)-protoporphyrin IX α (heme) inhibits hematin polymerization to β-hematin (malaria pigment) and kills malaria parasites. Biochemistry 1999;38:8858–63.
[45] Sullivan DJ, Gluzman IY, Russell DG, Goldberg DE. On the molecular mechanism of chloroquine's antimalarial action. Proc Natl Acad Sci USA 1996;93:11865–70.
[46] Sugioka Y, Suzuki M. The chemical basis for the ferriprotoporphyrin IX-chloroquine complex induced peroxidation. Biochim Biophys Acta 1991;1974:19–24.
[47] Bray PG, Janneh O, Raynes KJ, Mungthin M, Ginsburg H, Ward SA. J Cell Biol 1999;145:363–76.
[48] Fitch CD, Chevli R, Banyal HS, Phillips G, Pfaller MA, Krogstad DJ. Lysis of Plasmodium falciparum by ferriprotoporphyrin IX and a chloroquine-ferriprotoporphyrin IX complex. Antimicrobial Agents & Chemotherapy 1982;21:819–22.
[49] Vippagunta SR, Dorn A, Ridley RG, Vennerstrom JL. Characterization of chloroquine-hematin μ-oxo dimer binding by isothermal titration calorimetry. Biochim Biophys Acta 2000;1475:133–40.
[50] de Almeida Ribeiro MC, Augusto O, da Costa Ferreira AM. Inhibitory effect of chloroquine on the peroxidase activity of ferriprotoporphyrin IX. J Chem Soc Dalton Trans 1995:3759–66.
[51] Fitch CD, Kanjananggulpan PJ. The state of ferriprotoporphyrin malaria pigment. J Biol Chem 1987;262:15552–5.