Interplay between lipoproteins and bee venom phospholipase A$_2$ in relation to their anti-plasmodium toxicity

Carole Guillaume,* Catherine Calzada,† Michel Lagarde,† Joseph Schrélé,* and Christiane Deregnaucourt1,*

USM 0504, Biologie Fonctionnelle des Protozoaires,* Muséum National d’Histoire Naturelle, Paris, France; and INSERM UMR 585, INSA-Lyon, Physiopathologie des Lipides et Membranes,† Villeurbanne, France

Abstract We previously showed that the in vitro intra-erythrocytic development of the malarial agent Plasmodium falciparum is strongly inhibited by secreted phospholipases A$_2$ (sPLA$_2$s) from animal venoms. Inhibition is dependent on enzymatic activity and requires the presence of serum lipoproteins in the parasite culture medium. To evaluate the potential involvement of host lipoproteins and sPLA$_2$s in malaria, we investigated the interactions between bee venom phospholipase A$_2$ (bvPLA$_2$), human triglyceride-rich lipoproteins, and infected erythrocytes. Even at high enzyme concentration (100 x IC$_{50}$), bvPLA$_2$ binding to Plasmodium-infected or normal erythrocytes was not detected. On the contrary, tight association with lipoproteins was observed through the formation of buoyant bvPLA$_2$/lipoprotein complexes. Direct involvement of the hydrolysis lipid products in toxicity was demonstrated. Arachidonic acid (C20:4), linoleic acid (C18:2), and, to a lesser extent, docosahexaenoic acid (C22:6) appeared as the main actors in toxicity. Minimal oxidation of lipoproteins enhanced toxicity of the lipolyzed particles and induced their interaction with infected or normal erythrocytes. Fresh or oxidized lipolyzed lipoproteins induced the parasite degeneration without host cell membrane disruption, ruling out a possible membranolytic action of fatty acids or peroxidation products in the death process. In conclusion, our data enlighten on the capability of secreted PLA$_2$s to exert cytotoxicity via the extra-cellular generation of toxic lipids, and raise the question of whether such mechanisms could be at play in pathophysiological situations such as malaria.

1 To whom correspondence should be addressed.

e-mail: deregnau@mnhn.fr

Malaria is a widespread parasitic disease occurring in over 100 countries. Its annual incidence has been estimated at 350 to 500 million clinical cases with 1.5 to 2.7 million deaths (1). Plasmodium falciparum and, to a lesser extent, Plasmodium vivax are the main causes of disease and death from malaria. The erythrocytic stage of the parasite life cycle is responsible for the malaria symptoms. The burden of malaria is increasing, especially in sub-Saharan Africa, because of drug and insecticide resistance, as well as social and environmental changes (2). Thus, there is an urgent need for new drugs, vaccines, and insecticides, as well as for a better understanding of the pathophysiological processes at play in malaria.

Phospholipases A$_2$ enzymes (EC 3.1.1.4) exhibit a variety of physiological activities in addition to intrinsic lipolytic action. Those enzymes catalyze the hydrolysis of the sn-2 ester bond of glycerophospholipids, leading to the production of NEFAs and lysophospholipids (lysoPLPs). Secreted phospholipases A$_2$ (sPLA$_2$s) form a large family of low-molecular-mass (13–19 kDa), water soluble, and structurally conserved enzymes that have primarily been identified in animal venoms, but are also distributed in mammalian tissues, fluids, and secretions (3), plants (4), bacteria (5), and viruses (6, 7). Interestingly, despite common catalytic properties, venom sPLA$_2$s differ greatly in their pharmacological effects, such as neurotoxic, myotoxic, cardiotoxic, or anticoagulant properties, and some sPLA$_2$s have been shown to display antibacterial (8, 9), antiviral (HIV) (10), or anti-Plasmodium properties (11, 12).

We previously showed that sPLA$_2$s from snake, scorpion, or bee venoms are potent inhibitors of the in vitro intra-erythrocytic development of P. falciparum (11, 12). Inhibi-
tion at low enzyme concentration occurs only in the presence of serum phospholipids, suggesting that hydrolysis of exogenous phospholipids, rather than hydrolysis of the infected red blood cell (RBC) membrane phospholipids, is required for sPLA2 toxicity.

Here, we were interested in deciphering the molecular and cellular interplays between sPLA2 enzyme, human lipoproteins, and infected erythrocytes, as an approach to a better understanding of what may occur in pathophysiological situations such as malaria, in which alterations of the host lipoproteinogram (13) and sPLA2 production (14, 15) are encountered.

To understand what governs the indirect toxicity of sPLA2, the distribution of the bee venom phospholipase A2 (bvPLA2) between the infected erythrocytes and the triglyceride-rich fraction [chylomicrons (chyls) and VLDLs] of human lipoproteins was analyzed. Incidence of lipoprotein oxidation on bvPLA2 particle binding and anti-Plasmodium activity was also analyzed, because peroxidation has been shown to increase the susceptibility of lipoproteins to hydrolysis by sPLA2 (16) and oxidized lipoproteins have been found in malaria patients (17). Finally, we attempted to establish which of the lipolyzed particles or lipid products are responsible for parasite killing. LysoPLs and NEFAs generated by the bvPLA2 activity were identified, and their individual involvement in parasite killing was established, exemplifying the major role of PUFAs.

Our results illustrate the capacity of sPLA2 to be active on biological targets via the generation of exogenous NEFAs and raise the question of a potential role for oxidized lipoproteins and/or endogenous sPLA2 in the host defense against malaria.

EXPERIMENTAL PROCEDURES

Materials

Phospholipase A2 from Apis mellifera venom (bvPLA2), BSA (fraction V), fatty acid (FA)-free BSA, and butylated hydroxytoluene (BHT) (2,6-di-tert-butyl-p-cresol), as well as individual FAs and lysoPLs were purchased from Sigma (St Quentin-Fallavier, France). 3H-hypoxanthine (37 MBq/ml) was from Amersham Biosciences (Orsay, France). The Bio-Rad DC protein assay and Affi-Gel10 gel were from Bio-Rad. Anti-bvPLA2 polyclonal antibody was a gift from Dr. G. Lambeau (Centre National de la Recherche Scientifique; Sophia-Antipolis, France). Anti-human apolipoprotein B (apoB) polyclonal antibodies were from Dade Behring S. A. (Paris, France). The 1,2-bis-(1-pyrenedecanoyl)-sn-glycero-3-phosphocholine (β-β-C18:0HPC) fluorescent substrate was obtained from Molecular Probes (Invitrogen SARL; Cergy Pontoise, France).

Methods

Culture and synchronization of P. falciparum. The Colombian strain FcB1 of P. falciparum was used throughout the work. Cultures were grown in complete medium consisting of RPMI 1640 (Life Technologies, Inc.) supplemented with 11 mM glucose, 27.5 mM NaHCO3, 100 UI/ml penicillin, 100 μg/ml streptomycin, adjusted to pH 7.4 before addition of heat-inactivated human serum (8%) final), according to the procedure of Trager and Jensen (18). Parasites were grown at 37°C in human O+ or A+ RBCs at a 2% hematocrit and a 3–6% parasitemia, in a 3% CO2, 6% O2, and 91% N2 atmosphere. Cell cultures were synchronized by successive Plasmagel® (19) and sorbitol (20) treatments.

Purification and lipolysis of the chyl/VLDL fraction. The d<1.006 g/ml lipoprotein fraction comprising chyls and VLDLs was purified by centrifugation (24 h, 160,000 g, 4°C) of nonfasted human serum diluted 1:5 in sterile NaCl 0.9 %, according to the technique by Havel, Eder, and Bragdon (21). The protein content of the fraction was determined using the Bio-Rad DC protein assay according to the manufacturer’s recommendations.

Coupling of bvPLA2 to Affi-Gel 10 beads (Bio-Rad) was achieved following the instructions of the manufacturer. Efficiency of the coupling and enzymatic activity of the immobilized bvPLA2 were measured as in (12). For enzymatic lipolysis, 1 ml chyl/VLDLs (approximately 0.15 mg/ml in PBS), either freshly prepared (F-chyl/VLDL) or oxidized (Ox-chyl/VLDL), was mixed with 130 pM active immobilized bvPLA2 in the presence of 1 mM CaCl2 and incubated at 37°C for 17 h.

Chyl/VLDL oxidation. To induce air/light minimal oxidation of lipoproteins, chyl/VLDLs in PBS or in NaCl (9 g/l) were stored in a transparent flask at ambient temperature and under sterile air exchange for 18 days. Lipid peroxides were measured in terms of thiobarbituric acid-reactive substances (TBARS) according to the method of Morlière et al. (22). Results are expressed in malondialdehyde equivalents.

In experiments comparing properties of fresh and oxidized lipoproteins, chyl/VLDLs were isolated from plasma that had been aliquotted and frozen at −20°C just after blood drawing. One aliquot was thawed for chyl/VLDL purification and air/light oxidation. At the end of the 2 1/2 week oxidation period and just prior to the experiment, a second aliquot was thawed for purification of F-chyl/VLDLs.

Analysis of bvPLA2 binding to erythrocytes. Binding of bvPLA2 to RBCs was quantified by sedimenting the cells and measuring the fraction of enzyme remaining in the supernatant. RBCs were sedimented by centrifugation at 90 g for 3 min. The amount of bvPLA2 in the supernatant was measured using the fluorimetric assay with β-β-C18:0HPC as substrate (23). Binding experiments were performed either in PBS (10 mM NaPO4, 150 mM NaCl, pH 7.4) or in RPMI. Binding reactions (200 μl) contained 8.0 ng (2.5 nM) bvPLA2 and 2.5 × 107 RBCs, either normal or parasitized by mature forms of P. falciparum. Parasitized erythrocytes were highly enriched (70–80%) in schizont forms (36–48 h of age) recovered by Plasmagel® treatment. The procedure was as follows: RBCs in PBS were distributed in the BSA-coated wells of a 96-well microplate at the rate of 100 μl per well, then 100 μl bvPLA2 (5 nM) in PBS or in PBS + 0.15 mg/ml chyl/VLDLs was added. In the experiment with oxidized lipoproteins, bvPLA2 + chyl/VLDLs were incubated for 45 min at 37°C prior to addition. After 45 min incubation at 37°C, the plate was gently centrifuged at 90 g and supernatants were collected for the spectrofluorimetric assay.

The reaction medium was 50 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 6 mM CaCl2, 0.1% BSA, and 2 μM β-β-C18:0HPC. Two hundred microliters of substrate was added to 100 μl of supernatant. The pyrene monomer fluorescence, corresponding to the phospholipid hydrolysis upon bvPLA2 activity, was measured continuously for 10 min with a luminescence spectrometer (AMINCO; Bowman, Series 2) using 345 nm (excitation) and 398 nm (emission) wavelengths. PLA2 activity is expressed in fluorescence variation per second. BvPLA2 100% activity in PBS and in PBS + lipoproteins, and in the absence of cells, was mea-
sured at time zero of the incubation. Residual activity after 45 min incubation in the absence of cells was measured to estimate bvPLA2 binding to coated BSA. Negative controls were without bvPLA2. Detection limits were 25 pM enzyme in the absence and 250 pM enzyme in the presence of chyl/VLDLs.

**Lipid sequestration by BSA.** Two milliliters of chyl/VLDLs (0.15 mg/ml in PBS, 250 μM CaCl2) was incubated with 130 pM active Affi-Gel-immobilized bvPLA2 for 17 h at 37°C. After a brief centrifugation to pellet Affi-Gel beads, the supernatant was mixed with FA-free BSA (20 mg/ml) for a 2 h incubation at 37°C under gentle agitation. PBS was added up to 8 ml and chyl/VLDLs were separated from the lipid-charged BSA by ultracentrifugation (160,000 g, 20 h, 4°C). The buoyant lipoproteins were recovered from the top fraction, and the pelleted BSA was recovered from the bottom fraction. Both fractions were tested in dose-response assays toward the in vitro intraerythrocytic development of *P. falciparum*.

**NEFA and lysopl identification.** Lipid-enriched BSA was supplemented with 50 μM BHT and extracted twice with chloroform-methanol-water (2.5:2.5:1.25; v/v/v) using the Bligh and Dyer protocol (24). Extracted lipids were dried in a rotary evaporator and stored at −20°C under argon for further analysis. Extracted lipids in chloroform-methanol (1:1; v/v) were separated by TLC on silica gel 60G plates. Samples were duplicated, and migration was realized in chloroform-methanol-water (65:25:4; v/v/v) with standard markers. Lipids were fractionated into lysophosphatidylcholine (lysoPC) (RP 0.20), lysophosphatidylethanolamine (lysoPE) (RP 0.40), and NEFAs (RP 0.91). They were either revealed by 2′,7′-dichlorofluorescin, 0.02% in ethanol-water (95:5; v/v) vaporization, or left unstained for recovery before gas chromatography (GC) analysis.

Lipids were collected by plate scraping. LysoPLs were resuspended in 1 ml H2SO4 (5% in MeOH), and incubated for 1 h at 100°C in a dry bath. The reaction was stopped by transfer of 0°C, lipids were neutralized by the addition of 1.5 ml K2CO3 (5% in H2O) and extracted by the addition of 2 ml isooctane. The organic phase was collected upon centrifugation at 900 g for 10 min and dried, and lipids were resuspended in isoctane (2 ml) for GC analysis.

NEFAs were extracted twice with 1 ml diethyl ether-methanol (9:1; v/v). The organic phase was collected after centrifugation (10 min, 450 g), dried under argon, and derivatized by a 15 min incubation in the dark with 250 μl diazomethane. Upon derivatization, the fraction was dried under argon and resuspended in 250 μl isoctane for GC analysis.

GC separation of FA methyl esters was carried out on a Supelco SP2380 capillary column (30 m × 0.25 mm) and analyzed on an HP 6890 G1530 chromatograph. Heptadecanoic acid (C17:0) and heptadecanoyl-lysoPC were used as internal standards. FAs were quantified according to the known amount of added internal standard.

**Toxicity assay (dose-response assay).** The diverse lipid preparations described above, as well as commercial lipids, were tested for their capacity to inhibit the in vitro intraerythrocytic development of *P. falciparum*. Dry preparations of commercial lysoPLs and NEFAs, to be tested either individually or upon mixing, were solubilized in 20 μl ethanol; then 1 ml RPMI 16% serum was added, and the lipid solution was shaken for 2 h at ambient temperature before being tested for parasite growth inhibition. Dose-response assays based upon [3H]-hypoxygen incorporation by growing parasites were performed as in (12). Radioactivity was measured with a 1450 Microbeta counter (Wallac, Perkin Elmer). Percentage of growth inhibition was calculated from the parasite-associated radioactivity compared with the control (25). Values for the IC50 and IC100 minimum (IC100min) were determined from dose-response curves.

**PLA2 association with lipoproteins.** Five milliliters of PBS containing bvPLA2 (3 μg/ml), BSA fraction V (1 mg/ml), and CaCl2 (1 mM) were supplemented with chyl/VLDLs, either fresh or oxidized (70 μg/ml final concentration), or supplemented by the same volume of PBS. Each preparation was centrifuged at 160,000 g for 24 h. Fractions (0.6 ml) were collected from the top to the bottom of each tube. They were analyzed by SDS-PAGE on an 8% polyacrylamide gel under reducing conditions. The gel was then stained by Coomassie blue or blotted for immunodetection of bvPLA2 with anti-bvPLA2 antibodies (1:5,000).

**Interaction between chylomicrons and erythrocytes.** F-chyl/VLDLs or Ox-chyl/VLDLs were hydrolyzed for 45 min at 37°C by 6 nM bvPLA2 in a 300 μl final volume of PBS containing 1 mM CaCl2, or treated equally in the absence of enzyme. Three hundred microliters of a 6% hematocrit suspension of normal or *Plasmodium* infected RBCs (trophozoites/schizonts at a 70–80% parasitemia) were added, then 50 μl aliquots were taken at time zero and after 0.5, 1, 2, 3, 5, 10, 15, and 30 min incubation at room temperature, and immediately centrifuged (briefly) to pellet the cells. The supernatants were boiled in SDS/β-mercaptoethanol buffer and subjected to SDS-PAGE on an 8% polyacrylamide gel. Proteins from gels were blooted onto nitrocellulose and analyzed for the presence of apoB-48 using anti-apoB antibodies diluted 1:10,000. ApoB-48 (molecular mass = 250 kDa) is the main and specific apolipoprotein of chyls. Because of its high molecular mass (about 500 kDa), apoB-100, the main apolipoprotein of the VLDLs, was confined to the stacking gel, and could not be visualized on the Western blot under our experimental conditions.

**Morphological analysis of *P. falciparum* in the presence of fresh or oxidized lipoproteins hydrolyzed by bvPLA2.** A *P. falciparum* culture was synchronized on a 4 h time window. After 16 h of growth under classical culture conditions, 100 μl of culture (parasite age: 16–20 h; parasitemia: 1%; hematocrit: 4%) in RPMI + 16% serum was distributed in a 96-well plate. PLA2-hydrolyzed F- and Ox-chyl/VLDLs diluted in RPMI alone were added at their respective IC100min (100 μl/well). As a control, the effect of nonhydrolyzed lipoproteins (fresh and oxidized) at the same concentration was analyzed. Culture was carried out in a candle jar at 37°C. Aliquots of the culture were taken every 2 h for 20 h (until parasites were 36–40 h old), then after 24 h of culture (40–44 h-old parasites) and 48 h (16–20 h-old reinvaded parasites). The parasitemia, stage distribution, and morphological development of the parasites were followed by optical examination of Giemsa-stained smears and by the counting of 4,000 cells. Images were captured by the Canon Power Shot S40 camera coupled to the Canon Utilities Remote Capture 2.1.0.10 software (magnification ×1140).

**RESULTS**

**BvPLA2 associates preferentially with lipoproteins as compared with erythrocytes.**

To determine why the anti-*Plasmodium* activity of most bvPLA2s results from hydrolysis of exogenous phospholipids and not from a direct action on the infected erythrocyte membrane, we analyzed the distribution of bvPLA2 between the lipoproteins and the erythrocytes.
Experiments were carried out at an enzyme-to-cells ratio 100-fold higher than the IC$_{50}$ toxic ratio (enzyme-to-cells ratio at the bvPLA$_2$ IC$_{50}$: 1.2 ng bvPLA$_2$:4.5 $\times 10^8$ RBCs). Noninfected or infected erythrocytes with mature forms of the parasite were incubated with bvPLA$_2$ with or without chyl/VLDLs in BSA-coated plastic wells; then enzymatic activity in the supernatant was measured. Initial velocity at time zero of the incubation (100% enzymatic activity), as well as residual activity after incubation in the BSA-coated wells in the absence of cells, was determined. The initial velocity in the presence of chyl/VLDLs could not be determined without preincubation, because phospholipids from lipoproteins competed strongly with $\beta$-py-C$_{10}$-HPC for hydrolysis by bvPLA$_2$.

In PBS alone, a net decrease (by approximately 56%) in enzyme activity was observed after the bvPLA$_2$ had been incubated in the BSA-coated wells, indicating that the enzyme had adsorbed to the BSA (see Fig. 1A). By contrast, when incubation was performed in the presence of healthy or parasitized erythrocytes, 100% activity was recovered in the supernatant, showing that under these conditions, the enzyme does not bind (or not at a detectable rate) to BSA or to erythrocytes. The same results were obtained when experiments were carried out in RPMI (not shown), indicating that in culture medium also, the enzyme does not bind to erythrocytes. When the experiment was performed in the presence of chyl/VLDLs and in the absence of cells, bvPLA$_2$ activity in the supernatant was found to be high (Fig. 1B), suggesting that the lipoproteins had largely prevented the enzyme adsorption to BSA. The presence of erythrocytes, either infected or not, did not lower the activity, indicating that in the presence of lipoproteins, as well as in PBS or RPMI alone, the bulk of enzyme does not bind to erythrocytes.

In a second step, we looked for bvPLA$_2$ association with chyl/VLDLs in a mixture of enzyme, lipoproteins, and BSA. The mixture was ultracentrifuged so that buoyant lipoproteins were separated from proteins, and top to bottom fractions were analyzed for the presence of bvPLA$_2$ by SDS-PAGE and immunoblotting. A large amount of enzyme was found in the top fractions containing lipoproteins (Fig. 2A, B for control), demonstrating that bvPLA$_2$ associates tightly with the lipoprotein particles (affinity is high enough to prevent total enzyme dissociation from the particles under a 160,000 g centrifuge force applied for 24 h), even in the presence of BSA. Also under the same constraints, BSA was not found associated with the lipoproteins (it is not present in the upper fractions), indicating that in the presence of lipoproteins, as well as in PBS or RPMI alone, the bulk of enzyme does not bind to erythrocytes.

Fig. 1. Analysis of bee venom phospholipase A$_2$ (bvPLA$_2$) binding to normal and *P. falciparum*-infected erythrocytes. Erythrocytes in PBS (2.5 $\times 10^7$), either normal (triangles) or parasitized by mature forms (24–48 h of age) of *P. falciparum* (closed circles), were distributed in BSA-coated wells of a 96-well microplate (100 µl per well). BvPLA$_2$ in PBS (A) or in PBS containing 0.15 mg/ml chylomicron (chyl)/VLDLs (B) was added 100 µl/well; final concentration 2.5 nM enzyme. After a 45 min incubation at 37°C, the microplate was centrifuged (2 min, 90 g), and supernatant from each well was transferred to another BSA-coated plate and analyzed for enzymatic activity. Kinetic analysis of the enzymatic activity was carried out using the $\beta$-py-C$_{10}$-HPC substrate. Squares: enzymatic activity at time zero of the incubation (100% activity) in PBS alone. Open circles: activity upon incubation in the BSA-coated wells in the absence of cells. $\times$: Incubation in the absence of bvPLA$_2$. Three independent experiments using red blood cells (RBCs) and lipoproteins from different donors were performed in triplicate. Values are the mean ± SD. A representative experiment is presented.

Fig. 2. Analysis of the physical association of the bvPLA$_2$ with lipoproteins. Two ultra-centrifuge tubes were filled with 5 ml of a PBS solution containing 3 µg/ml bvPLA$_2$, 1 mg/ml BSA, and 1 mM CaCl$_2$, supplemented (A) or not (B) with 70.0 µg/ml chyl/VLDLs. Solutions were centrifuged at 160,000 g for 24 h at 4°C. Fractions (0.6 ml) were collected from the top to the bottom of the tubes and analyzed by SDS-PAGE on an 8% polyacrylamide gel, either stained with Coomassie blue for detection of apolipoprotein B (apoB)-48 (250 kDa) and BSA (65 kDa), or blotted for immunodetection of bvPLA$_2$ (16 kDa) with specific antibodies. Fractions are numbered from 1 (top fraction) to 8 (bottom fraction) along the tube. *, high-molecular-mass polymers of BSA.
Fig. 3. Anti-Plasmodium activity of the hydrolysis products from bvPLA2-digested lipoproteins. Chyl/VLDLs from human serum were hydrolyzed by bvPLA2 for 20 h at 37°C, then incubated for 2 h at 37°C under gentle agitation with 20 mg/ml fatty acid (FA)-free BSA. Lipoproteins were separated from BSA by centrifugation at 100,000 g for 24 h at 4°C. Buoyant lipid-discharged lipoproteins and pelleted lipid-charged BSA were recovered separately and resuspended in PBS. A second run of delipidation was carried out on discharged chyl/VLDLs, following the same procedure. Native (non-hydrolyzed) chyl/VLDLs were processed in parallel. The different fractions were tested for their ability to inhibit the intraerythrocytic growth of P. falciparum in a dose-response assay. A: Analysis of the lipoprotein particle toxicity: native chyl/VLDLs (squares); bvPLA2-hydrolyzed chyl/VLDLs (open triangles); lipid-discharged chyl/VLDLs after one run of extraction (closed triangles), and after two runs of extraction (circles). B: Analysis of the lipid-charged BSA toxicity: lipid-free BSA (squares); lipid-charged BSA (triangles). Error bars represent SD of experiment performed in triplicate.

suggesting that the protein does not exhibit high affinity for chyl/VLDLs. The absence of BSA in the bvPLA2-lipoprotein complexes also demonstrates that the enzyme associates freely with the lipoprotein particles, despite its capability of binding to BSA. From this, we can infer that bvPLA2 displacement from the coated BSA observed previously in the presence of lipoproteins resulted from a higher enzyme affinity for the lipoproteins than for the BSA, rather than from a competition between the enzyme and the chyl/VLDLs for binding to BSA.

Taken together, these results indicate that the bvPLA2 associates more readily with lipoproteins than with erythrocytes, and strongly suggest that the indirect toxicity of the enzyme toward infected erythrocytes is mainly dictated by its distribution in favor of lipoproteins.

Lipid products are essential to the anti-Plasmodium toxicity of bvPLA2-hydrolyzed lipoproteins

To assess whether lysoPLs and NEFAs are involved in the anti-Plasmodium toxicity of the lipolyzed lipoproteins, lipids were sequestered from particles by using lipid-free BSA, then both the resulting lipid-charged BSA and the delipidated lipoproteins were tested for their capacity to inhibit the parasite intraerythrocytic growth.

As can be seen in Fig. 3A, toxicity of the bvPLA2-hydrolyzed chyl/VLDLs was decreased after one round of lipid extraction and almost totally lost after two rounds, indicating that lipid products are obligatory elements in the hydrolyzed lipoproteins’ toxicity. In good correlation with this, the lipid-charged BSA became toxic to Plasmodium (Fig. 3B).

Molecular analysis of NEFAs and LysoPLs from sPLA2-hydrolyzed chyl/VLDLs

To improve our understanding of the molecular events at play in the lipoprotein-derived bvPLA2 toxicity, we identified and quantified the molecular species of lipid products. Chyl/VLDLs were hydrolyzed by bvPLA2, then lysoPLs and NEFAs from one BSA extraction cycle were analyzed by TLC/GC. Three independent experiments were performed with chyl/VLDLs from different sera. The results are reported in Table 1.

Phospholipid hydrolysis was confirmed by a net increase in lysoPLs and NEFAs. In accordance with the well-known NEFA binding properties of BSA (26), the vast majority of the lipids identified on BSA were NEFAs. LysoPC was the prevalent lysoPL, as expected, because phosphatidylcholine (PC) is the main glycerophospholipid in human LDLs, HDLs, and VLDLs (27). Contrasting with LysoPC, the lysoPE amount was not increased upon hydrolysis of chyl/VLDLs, suggesting that hydrolysis of phosphatidylethanolamine did not occur to a large extent. Also, lysophosphatidylinositol and lysophosphatidylserine were not

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<th>TABLE 1. Quantitative analysis of the lipids produced by bvPLA2 digestion of chyl/VLDLs</th>
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Values are in μg/mg chyl/VLDLs. The chyl/VLDL fractions from sera of different donors were purified and individually digested overnight at 37°C by bee venom phospholipase A2 (bvPLA2) (bvPLA2-hydrolyzed chyl/VLDLs) or incubated without bvPLA2 (native chyl/VLDLs), then NEFAs and lysophospholipids were back-extracted by lipid-free BSA. Lipids from the resulting lipid-charged BSA were analyzed by TLC/gas chromatography. Values are the means of three independent results (±SD). Chyl, chylomicron; lysoPC, lysophosphatidylcholine; lysoPE, lysophosphatidylethanolamine.
detected. Molecular species analysis revealed that lysoPC contained almost exclusively C16:0 (palmitic acyl) and C18:0 (stearic acyl) FAs, and lysoPE contained C16:0, C18:0, and C18:2 n-6 (linoleic acyl) (not shown).

GC analysis of NEFAs identified seven main species of lipids (Fig. 4). Among them, PUFAs were the most prevalent, with linoleic acid (LA) (C18:2 n-6), arachidonic acid (AA) (C20:4 n-6), docosahexaenoic acid (DHA) (C22:6 n-3), and dihomo-γ-linolenic acid (C20:3 n-6), accounting, respectively, for 39.2, 15.1, 7.1, and 5.5% of the 35.4 μg NEFAs produced per mg chyl/VLDLs. The monounsaturated oleic acid (C18:1 n-9) accounted for 13.3%, and the saturated palmitic acid (C16:0) and stearic acid (C18:0) for 6.5% and 4.6%, respectively. It thus appeared that PUFAs, especially LA, are predominant products of the chyl/VLDLs hydrolysis. This was quite expected, inasmuch as phospholipids are preferentially esterified by a saturated FA at the sn-1 position and by an unsaturated FA at the sn-2 position. Also, it must be noted that the relative distribution of NEFAs in the BSA fraction reflected that of grafted FA in PC from VLDLs (27), suggesting that the FA molecular species at the sn-2 position in phospholipids has no effect on the hydrolyzing activity of bvPLA2 toward chyl/VLDLs.

AA, LA, and DHA are the main actors in the bvPLA2 anti-Plasmodium activity

To analyze whether lipolyzed lipoprotein toxicity might result from the specific action of one NEFA or lysoPL, toxicity of the individual lipids against Plasmodium was tested in dose-response assays. IC50 values reported in Table 2 indicate that all NEFAs are toxic to Plasmodium; however, PUFAs are clearly the most toxic. This is in agreement with previous data reporting the involvement of the FA structure in their anti-Plasmodium activity (28). AA and DHA are the most lethal, with a 0.8–2.4 μg/ml (5.3 ± 2.6 μM) and a 1.4–3.6 μg/ml (7.8 ± 3.2 μM) IC50, respectively. By comparison, LA, the most represented FA in the lipid fraction, is moderately toxic, with an IC50 of 6.8–18.4 μg/ml (45.2 ± 20.7 μM). LysoPC and lysoPE displayed relatively low toxicity, as compared with the most lethal NEFAs, with respective IC50s of 27.8 μg/ml and 14.6 μg/ml.

The seven NEFAs found to be prevalent in the lipid extract from bvPLA2-digested chyl/VLDLs were mixed together in the same proportions as in the original extract, and the synthetic lipid mixture was tested against Plasmodium. The mixture was found to be toxic, with a 36.5 μg lipid/ml IC50 (mean of two independent experiments; see Table 2). The respective internal concentrations of each FA at the IC50 concentration of the mixture were calculated and compared with their individual IC50s. Only AA was present in excess concentration in the mixture (√×3.6), and LA in an almost equivalent concentration (√×1.2), with respect to their individual IC50s. Only AA was present in excess concentration in the mixture (√×3.6), and LA in an almost equivalent concentration (√×1.2), with respect to their individual IC50s. Only AA was present in excess concentration in the mixture (√×3.6), and LA in an almost equivalent concentration (√×1.2), with respect to their individual IC50s. Only AA was present in excess concentration in the mixture (√×3.6), and LA in an almost equivalent concentration (√×1.2), with respect to their individual IC50s.

Fig. 4. Quantification and molecular identification of the NEFAs produced upon bvPLA2 hydrolysis of lipoproteins’ phospholipids. Lipids adsorbed onto BSA after back-extraction of bvPLA2-hydrolyzed chyl/VLDLs were extracted according to Bligh and Dyer (25) in the presence of butylated hydroxytoluene, dried under vacuum, resuspended in chloroform-methanol and subjected to TLC. NEFAs scraped from the silica plate were resuspended in diethyl ether-methanol (9:1; v/v) and centrifuged, and the organic phase was collected and dried under argon. After derivatization by diazomethane, NEFAs were solubilized in isooctane for HPLC-gas chromatography analysis. Margaric acid (C17:0) was added for internal standardization. White bars: NEFAs from native chyl/VLDLs; black bars: NEFAs from bvPLA2-digested chyl/VLDLs. Values are the mean of three independent experiments with chyl/VLDLs from three different sera. Despite similar qualitative profiles in the NEFA molecular species, variations in the total amount of NEFAs recovered from the different sera were observed, producing important standard deviation for each FA species amount. For a more comprehensive presentation, error bars were omitted.
suggesting that this PUFA might participate to some extent in the anti-Plasmodium activity of lipids produced by hydrolysis of lipoproteins.

**Albumin decreases the bvPLA2-hydrolyzed lipoprotein toxicity**

Because serum albumin is a well-known transporter of FAs in plasma and is able to capture lysoPLs and NEFAs from bvPLA2-hydrolyzed lipoproteins, we asked whether it might be involved in toxicity by facilitating the transport of the lipid products from the lipolyzed particles to the erythrocytes. We compared the anti-Plasmodium activity of the bvPLA2-hydrolyzed chyl/VLDLs in normal culture conditions and in a culture containing additional BSA (20 mg/ml final, i.e., approximately five times the normal culture concentration). The presence of BSA led to a net decrease in lipolyzed lipoprotein toxicity (IC50: 158.5 μg/ml vs. 45.7 μg/ml in the absence of BSA) (Fig. 5), indicating that BSA does not facilitate but rather prevents the anti-Plasmodium activity of the lipid products.

This antitoxic effect of BSA, added to the very low solubility of long-chain FAs in aqueous phase, making it unlikely that lipid products reach erythrocytes as free monomers, supported the idea that translocation of the lipids to erythrocytes is accomplished by the lipoprotein particle itself; this prompted us to analyze the toxicity in relation to the physicochemical status of the lipoprotein particle.

**Oxidative modification of chyl/VLDLs enhances the toxicity of the bvPLA2-digested lipoproteins**

Because it has been shown that minimal oxidation of lipoproteins increases the susceptibility to phospholipid hydrolysis by the human group IIA sPLA2 (16), we asked whether it might improve the toxicity of the bvPLA2-hydrolyzed lipoproteins. The chyl/VLDL fraction was purified from an aliquot of freshly drawn plasma and settled for 2 1/2 weeks under light and sterile air exchange so that minimal oxidation of lipids occurred. TBARS content was measured upon oxidation and was found to be approximately twice that in F-chyl/VLDLs; digestion by bvPLA2 did not affect the TBARS content in either fresh or oxidized lipoproteins (not shown).

Interestingly, in contrast to F-chyl/VLDLs, Ox-chyl/VLDLs were able to induce Plasmodium growth arrest in vitro, with a 117.3 ± 9.5 μg/ml IC50 (see Fig. 6). Upon di-

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**TABLE 2. Individual involvement of NEFAs in the anti-Plasmodium toxicity of lipolyzed lipoproteins**

<table>
<thead>
<tr>
<th>Lipid Name</th>
<th>IC50</th>
<th>µg/ml (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LysoPC</td>
<td>27.8* (= 54.0)</td>
<td></td>
</tr>
<tr>
<td>LysoPE</td>
<td>14.6* (= 33.0)</td>
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</tbody>
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A mixture of the seven major NEFAs from lipolyzed lipoproteins was made, with respective proportions (% lipid mixture) as in the original extract. IC50 of the mixture, as well as respective IC50s of commercial NEFAs were determined. To evaluate the involvement of each NEFA in the mixture toxicity, respective concentration at the mixture IC50 was calculated. IC50 values are the mean of n independent experiments.

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* n = 2.

† n = 3.

‡ n = 4.

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Fig. 5. BSA lowers the toxic activity of bvPLA2-digested lipoproteins. The inhibitory activity of lipolyzed chyl/VLDLs toward the intraerythrocytic development of P. falciparum was tested in the presence of BSA. Increasing concentrations of bvPLA2-hydrolyzed chyl/VLDLs in complete culture medium were distributed in a 96-well plate (100 μl/well) in the presence (open circles) or in the absence (open squares) of additional BSA (40 mg/ml final concentration). Then, an asynchronous culture of P. falciparum (1.5% parasitemia, 4% hematocrit) in complete medium was added at the rate of 100 μl per well, and the dose-response assay proceeded as described in Experimental Procedures. Toxicity of the BSA in excess was controlled (closed circles).
bvPLA2 were added to a suspension of healthy RBCs. An information about the toxicity of oxidized lipoproteins comes largely from studies of oxidized LDLs (Ox-LDLs). Ox-LDL toxicity is associated mainly with the lipid fraction, which contains a wide variety of oxidized lipids that are responsible for diverse biological effects, including cell death. However, the main part of the toxic lipids remains bound to Ox-LDL, and the toxicity of the particle involves its interaction with cell components at the cell surface or inside the cell (as reviewed in Ref. 29). Low-affinity binding sites for LDL, and to a lesser extent for HDL, have been described on erythrocytes; these correspond to sites distinct from the LDL receptors at the surface of other cells (30). To our knowledge, interaction of Ox-LDLs or triglyceride-rich lipoproteins with erythrocytes, which would be enhanced when the lipoproteins are further modified by enzymatic hydrolysis.

**BvPLA2 associates with oxidized lipoproteins**

The same approach used to visualize bvPLA2 association with F-chyl/VLDLs was carried out with Ox-chyl/VLDLs. Upon ultracentrifugation of a mixture of bvPLA2, Ox-chyl/VLDLs, and BSA, the enzyme was found to be associated with the buoyant oxidized lipoproteins to virtually the same extent as with F-chyl/VLDLs (not shown). Also, enzyme association with the erythrocytes was reconsidered in the presence of oxidized lipoproteins. The enzyme was incubated for 45 min at room temperature and centrifuged, and the supernatant was analyzed for the presence of apoB-48, the specific 250 kDa apolipoprotein of chyl. As can be seen in Fig. 7, lipoprotein oxidation induced the removal of apoB-48 from the incubation medium in a time-dependent manner. Removal was completed within approximately 1 h. ApoB-48 disappearance was not observed in the absence of cells (not shown), indicating that protein removal from the supernatant was not due to Ox-chyl adsorption on the tube wall, nor to any chyl/VLDL-dependent proteolytic degradation of the apoB-48 induced by oxidation. Upon Ox-chyl/VLDL incubation with bvPLA2, the apoB-48 removal occurred faster (noticeable within 10–15 min, completed within 30 min). Kinetic analysis of apoB-48 disappearance was similar regardless of whether incubation had been performed with noninfected or with P. falciparum-infected erythrocytes (not shown). Remarkably, no depletion inapoB-48 was observed with native lipoproteins, either enzymatically digested or not.

These results suggest that oxidation induces some sort of interaction between the oxidized lipoproteins and the erythrocytes, which would be enhanced when the lipoproteins are further modified by enzymatic hydrolysis.

**Ox-chyl/VLDLs interact with erythrocytes**

The information about the toxicity of oxidized lipoproteins comes largely from studies of oxidized LDLs (Ox-LDLs). Ox-LDL toxicity is associated mainly with the lipid fraction, which contains a wide variety of oxidized lipids that are responsible for diverse biological effects, including cell death. However, the main part of the toxic lipids remains bound to Ox-LDL, and the toxicity of the particle involves its interaction with cell components at the cell surface or inside the cell (as reviewed in Ref. 29). Low-affinity binding sites for LDL, and to a lesser extent for HDL, have been described on erythrocytes; these correspond to sites distinct from the LDL receptors at the surface of other cells (30). To our knowledge, interaction of Ox-LDLs or triglyceride-rich lipoproteins with erythrocytes infected by *Plasmodium* has never been reported. In this context, we investigated the ability of Ox-chyl/VLDLs to interact with erythrocytes, arguing that interaction might be involved in the lipoprotein toxicity mechanism.

Ox-chyl/VLDLs and F-chyl/VLDLs preincubated with bvPLA2 were added to a suspension of healthy RBCs. An aliquot of the suspension was taken at different times, then centrifuged, and the supernatant was analyzed for the presence of apoB-48, the specific 250 kDa apolipoprotein of chyl. As can be seen in Fig. 7, lipoprotein oxidation induced the removal of apoB-48 from the incubation medium in a time-dependent manner. Removal was completed within approximately 1 h. ApoB-48 disappearance was not observed in the absence of cells (not shown), indicating that protein removal from the supernatant was not due to Ox-chyl adsorption on the tube wall, nor to any chyl/VLDL-dependent proteolytic degradation of the apoB-48 induced by oxidation. Upon Ox-chyl/VLDL incubation with bvPLA2, the apoB-48 removal occurred faster (noticeable within 10–15 min, completed within 30 min). Kinetic analysis of apoB-48 disappearance was similar regardless of whether incubation had been performed with noninfected or with *P. falciparum*-infected erythrocytes (not shown). Remarkably, no depletion in apoB-48 was observed with native lipoproteins, either enzymatically digested or not.

These results suggest that oxidation induces some sort of interaction between the oxidized lipoproteins and the erythrocytes, which would be enhanced when the lipoproteins are further modified by enzymatic hydrolysis.
with Ox-chyl/VLDLs or with F-chyl/VLDLs at a similar enzyme/lipoprotein (protein) ratio, then incubation proceeded for another 45 min period in the presence of parasitized or healthy erythrocytes. Measurement of the enzymatic activity in the incubation supernatants is reported in Fig. 8. BvPLA₂ preincubated with fresh lipoproteins did not associate with erythrocytes, either infected or not (Fig. 8A), confirming the results reported above and ruling out the possibility that prehydrolysis by bvPLA₂ might have changed the binding properties of fresh lipoproteins. By contrast, a dramatic depletion in the supernatant activity (by approximately 88%) was observed when the enzyme had been preincubated with Ox-chyl/VLDLs (Fig. 8B). No loss in activity was observed upon incubation of bvPLA₂ with Ox-chyl/VLDLs alone, ruling out the possibility that bvPLA₂ depletion in the presence of cells might be due to an oxidized lipoprotein-dependent PLA₂ inactivation process. Altogether, our results thus strongly suggest that oxidation of lipoproteins leads to an interaction of yet unknown nature between the lipoprotein particle and the erythrocyte, which might be involved in the oxidation-mediated lipoprotein toxicity process toward *P. falciparum*.

**Morphological features of parasite death induced by the bvPLA₂-hydrolyzed chyl/VLDLs**

Alterations of parasite morphology in cultures incubated with toxic concentrations (IC100min) of lipolyzed F-chyl/VLDLs and lipolyzed Ox-chyl/VLDLs were analyzed by optical microscopic examination of Giemsa-stained smears. Incubation was initiated with parasites at the young trophozoite stage (16–20 h) and prolonged for 48 h in culture conditions, with collection of samples every 2 h. Parasitemia, as well as distribution of the developmental stages and abnormal parasites in the culture, was determined from each smear by counting 4,000 erythrocytes.

When incubated with lipolyzed chyl/VLDLs, either oxidized or not, trophozoites did not develop into schizonts, and presented increasing alterations ending mainly at the formation of a condensed parasite (pyknotic form) inside the host erythrocyte. Twenty-five to thirty percent abnormal forms were observed as soon as after a 4 h–6 h incubation period (Fig. 9). Parasitemia remained unaffected until approximately 16 h of incubation, then decreased to zero during the following period (not shown), in correlation with the observation of extra-erythrocytic parasites in the culture. Therefore, it appeared that bvPLA₂-hydrolyzed chyl/VLDLs, either native or oxidized, induced similar phenotypes of parasite death, independent of (i.e., preceding) erythrocyte membrane lysis.

**DISCUSSION**

**Distribution of bvPLA₂**

We have previously demonstrated that lipoprotein hydrolysis is an obligatory step in the anti-*Plasmodium* toxicity process of several secreted PLA₂s ([12](#)). By investigating the distribution of the bvPLA₂ between the host erythrocyte membrane and the serum lipoproteins, we now demonstrate that this indirect mechanism results from a likely exclusive association of the enzyme with lipoproteins. Under our experimental conditions, bvPLA₂ binding to normal erythrocytes or to parasitized erythrocytes was not detected. This is not unexpected, inasmuch as bvPLA₂, like other sPLA₂s, binds anionic phospholipids in preference to zwitterionic ones, and normal erythrocyte membrane exhibits a classical asymmetric distribution of phospholipids, with PC (zwitterionic) as the main phos-
pholipid class in the outer leaflet of the membrane (31). Also, M-type (also named PLA2R) and/or N-type receptors for secreted PLA2s, which were primarily identified as mediators of enzyme binding to muscular and neuronal cells, respectively (as reviewed in Ref. 32), have not been described in RBCs, although the M-type receptor is found in a variety of cell types, including neutrophils (33) and macrophages (34). More surprising is the absence of enzyme binding to Plasmodium-infected erythrocytes. Indeed, modifications in the asymmetric distribution of phospho-

Fig. 9. Morphological analysis of the intraerythrocytic development of *P. falciparum* in the presence of bvPLA2-lipolyzed chyl/VLDLs. Young rings (0 h–4 h) were grown for 16 h under normal culture conditions before bvPLA2-lipolyzed chyl/VLDLs were applied. Samples were taken every 2 h during a 48 h period for morphological analysis of the parasite development by examination of Giemsa-stained smears under a light microscope. Toxicity of F-chyl/VLDLs, bvPLA2-hydrolyzed F-chyl/VLDLs, Ox-chyl/VLDLs, and bvPLA2-hydrolyzed Ox-chyl/VLDLs was measured in a dose-response assay. IC<sub>100min</sub> was determined in each case. Enzymatically digested lipoproteins were the most toxic, with 20 µg/ml (bvPLA2-hydrolyzed Ox-chyl/VLDLs) and 38 µg/ml (bvPLA2-hydrolyzed F-chyl/VLDLs) IC<sub>100min</sub>. Nonhydrolyzed oxidized lipoproteins were toxic at higher concentrations, whereas fresh lipoproteins were not toxic. BvPLA2-hydrolyzed Ox-chyl/VLDLs and their concurrent nonhydrolyzed Ox-chyl/VLDLs were applied on the culture at 20 µg/ml; bvPLA2-hydrolyzed F-chyl/VLDLs and their concurrent nonhydrolyzed F-chyl/VLDLs were applied at 38 µg/ml. Representative images at different times of incubation (0 h, 6 h, 10 h, 16 h, 24 h, and 48 h) in the absence of lipoproteins (control), or in the presence of the different lipoprotein preparations are presented, as well as a schematic distribution of the developmental stages and abnormal forms among the parasitized erythrocytes out of a total of 4,000 erythrocytes. Stages are as following: rings (black), young trophozoites (dark gray), late trophozoites (medium gray), schizonts (light gray), segmenters (very light gray), abnormal forms (white). –, cultures containing only abnormal forms, mostly outside the RBCs.
Binding of free bvPLA₂ to chyl/VLDLs was observed through the formation of buoyant enzyme-lipoprotein complexes. These complexes were formed in PBS/BSA, as well as in RPMI, even in the presence of normal erythrocytes (unpublished observations), indicating that under the conditions of cell culture also, the bvPLA₂ associates primarily with lipoproteins. It is interesting to note that calcium is present in RPMI, inasmuch as calcium was demonstrated to increase the interfacial affinity of bvPLA₂ for mixed PS/PC/cholesterol/sphingomyelin (SM) vesicles with compositions reflecting those of mammalian cell membranes (37) and might have increased the enzyme binding to erythrocytes in RPMI. Yet, inconsistent with this, we could not detect any binding to healthy erythrocytes in the absence (PBS) or in the presence (RPMI) of calcium.

Why bvPLA₂ associates with lipoproteins rather than with erythrocytes remains an open question. However, some considerations can be put forward: First, the external leaflet of the erythrocyte membrane is enriched in SM (38), whereas the lipoprotein surface is not, and SM was shown to inhibit sPLA₂ activity by perturbing the enzyme anchorage to the membrane (39). Second, the erythrocyte membrane is largely composed of proteins and glycoproteins that might prevent optimal binding of the enzyme. Third, membrane curvature has been postulated to modulate the sPLA₂ activity (40, 41), and surface curvatures of disk-shaped erythrocytes and spherical lipoproteins are obviously different.

Oxidation of lipoproteins

We show for the first time that air/light Ox-chyl/VLDLs are inhibitory to P. falciparum-infected erythrocytes (as reviewed in Refs. 35, 36). Because it has been shown that bvPLA₂ exhibits enhanced binding to mixed PS/PC vesicles when the mole percent of PS is increased (37), our results suggest that such membrane modifications, if they occur, are not marked enough to induce appreciable binding of bvPLA₂.

Interestingly, these results are in line with the previously published demonstration that tumor necrosis factor sera
containing peroxidized lipoproteins are inhibitory to the in vitro development of \textit{P. falciparum} (50).

**Role of NEFAs**

Phospholipids from \textit{chyl-VLDLs} are substrates of \textit{bvPLA}_{2}, as confirmed by TLC/GC analysis of the lipids produced. Here, we show that the generated NEFAs are obligatory actors in the anti-\textit{Plasmodium} toxicity of \textit{bvPLA}_{2}-digested lipoproteins, because BSA-delipidated lipoproteins are no longer toxic, whereas the lipid-charged BSA becomes toxic. However, the observation that FA-free BSA diminishes the toxicity of the lipolyzed particles suggests that the protein avidity for the lipid molecules prevents their toxic activity, and sustains the idea that lipid delivery to the erythrocytes is a prerequisite to parasite killing (because of BSA retention, fewer lipids would be available for toxicity). The lipid fraction sequestered by BSA comprises mostly NEFAs, especially PUFAs, with LA and AA representing 39.2\% and 15.1\% of total NEFAs, respectively. The highest anti-\textit{Plasmodium} activity was found with the long PUFAs: AA and DHA were individually the most lethal to the intraerythrocytic \textit{Plasmodium}, with \textit{IC}_{50}s of 5.3 \pm 2.6 \text{\mu M} and 7.8 \pm 3.2 \text{\mu M}, respectively. LA exhibited a higher \textit{IC}_{50}, at 45.2 \pm 20.7 \text{\mu M}. On the basis of the individual representation of each NEFA in the lipid fraction, it appeared that AA and LA may alone be responsible for parasite killing, and that AA is the best candidate, insofar as it is found at a 4-fold \textit{IC}_{50} concentration in the lipid fraction.

The in vitro and in vivo antimalarial effects of NEFAs have long been known (28, 51, 52). Our results are in accordance with the observation by Kumaratilake and co-workers (28) that the antimalarial activity of NEFAs is dependent on the number of double bonds of the carbon chain. These authors also showed that toxicity of the PUFAs might relate to their susceptibility to peroxidation. In our case, antioxidants (\alpha-tocopherol, BHT) did not significantly alter the toxicity of the \textit{bvPLA}_{2}-digested lipoproteins (unpublished observations), suggesting that peroxidation is not primarily responsible for their toxicity. It must be noted that intraerythrocytic degeneration (condensation) of \textit{P. falciparum} upon incubation with FAs was also observed in those studies (28, 51), reinforcing the idea that NEFAs are directly responsible for the parasitidal activity of the lipolyzed lipoproteins. Interestingly, the antimalarial activity of DHA, AA, and LA has been demonstrated in vivo: DHA and AA are capable of inhibiting the growth of \textit{Plasmodium berghei} in BALB/c mice and of preventing the malaria-induced anemia (28), whereas LA provokes a substantial reduction of parasitemia in Swiss mice infected with \textit{Plasmodium vinckeii petteri} (51). A massive supply in AA or LA is susceptible to being toxic via multiple pathways, either by perturbing the FA metabolism of the parasite, or by more specific effects, such as alteration of heme detoxification in the case of LA (53), or perturbation of the parasite prostaglandin production in the case of AA (54). Also, because many different NEFAs, with numerous bioactive properties, are generated by the enzymatic hydrolysis of lipoproteins, we cannot exclude a toxic mechanism implying all or several of these FAs. Interestingly, Rigoni et al. (55) recently reported similar effects of lysoPLs-FA mixtures and neurotoxic sPLA\textsubscript{2}s on the neuromuscular junction. LysoPL and NEFAs generated by enzymatic hydrolysis of the cell membrane are shown to act synergistically to paralyze the neuromuscular junction. In line with this, it would be worthwhile to measure the anti-\textit{Plasmodium} potencies of joined NEFAs and lysoPC, because lysoPC, despite moderate toxicity, represents a large part of the hydrolysis products.

**CONCLUSION**

We propose the following scheme for the indirect toxicity of the \textit{bvPLA}_{2} on the intraerythrocytic \textit{Plasmodium}. In an environment comprising serum and RBCs, parasitized or not, the \textit{bvPLA}_{2} will adsorb preferentially to lipoproteins and hydrolyze phospholipids at the particle surface, generating mostly polyunsaturated NEFAs, whose delivery to cells leads to parasite death. Polyunsaturated FA delivery would be favored by close contact between the free lipid-charged particle and the erythrocyte surface. Minimal oxidation would enhance lipoprotein toxicity by generating peroxidation products toxic to the parasite and by conferring adhesive properties on the particles.

It has been shown that the intrusion and development of the malarial parasite increase the systemic rate of inflammatory sPLA\textsubscript{2}-IIA in humans (14, 15), and implication of the enzyme in the host defense against the parasite has been suggested. Nine catalytically active sPLA\textsubscript{2}s have been identified in humans, with each sPLA\textsubscript{2} displaying a specific phospholipid affinity (56) and a specific pattern of expression (57, 58). Like venomous sPLA\textsubscript{2}s, human sPLA\textsubscript{2}s can hydrolyze lipoproteins. Groups V and X sPLA\textsubscript{2}s are capable of hydrolyzing anionic and neutral PLs and are 20-fold more effective in hydrolyzing lipoproteins, as compared with sPLA\textsubscript{2}-IIA, which hydrolyzes almost exclusively anionic PLs (59). However, as far as we know, no sPLA\textsubscript{2} different from the sPLA\textsubscript{2}-IIA in plasma, cells, or tissues from malarial patients has been reported. Remarkably, the hydrolysis efficiency of the sPLA\textsubscript{2}-IIA is enhanced 26-fold when the lipoproteins are oxidized (16), and it has been shown recently that lipoprotein fluidity and oxidative degree are enhanced in malaria patients, in relation to the severity of the case (17). If we place our results in the malaria context, the alteration of the physiological parameters in malaria patients might be considered as a modification of the host physiology toward an environment more favorable to the anti-\textit{Plasmodium} activity of sPLA\textsubscript{2}s and a direct participation of the oxidized lipoproteins in the host defense. Moreover, this innate defense mechanism deserves to be considered in a more general view, because elevation of the sPLA\textsubscript{2}-IIA concentration in plasma and oxidation of lipoproteins is a recurrent scheme in infections by pathogenic microorganisms and inflammatory diseases.\textsuperscript{44}

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