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Original Contribution

Bilirubin inhibits *Plasmodium falciparum* growth through the generation of reactive oxygen species

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Abstract

Free heme is very toxic because it generates highly reactive hydroxyl radicals (•OH) to cause oxidative damage. Detoxification of free heme by the heme oxygenase (HO) system is a very common phenomenon by which free heme is catabolized to form bilirubin as an end product. Interestingly, the malaria parasite, *Plasmodium falciparum,* lacks an HO system, but it forms hemozoin, mainly to detoxify free heme. Here, we report that bilirubin significantly induces oxidative stress in the parasite as evident from the increased formation of lipid peroxide, decrease in glutathione content, and increased formation of H_2O_2 and •OH. Bilirubin can effectively inhibit hemozoin formation also. Furthermore, results indicate that bilirubin inhibits parasite growth and induces caspase-like protease activity, up-regulates the expression of apoptosis-related protein (Gene ID PFI0450c), and reduces the mitochondrial membrane potential. •OH scavengers such as mannitol, as well as the spin trap α-phenyl-*n*-*tert*-butylnitrone, effectively protect the parasite from bilirubin-induced oxidative stress and growth inhibition. These findings suggest that bilirubin, through the development of oxidative stress, induces *P. falciparum* cell death and that the malaria parasite lacks an HO system probably to protect itself from bilirubin-induced cell death as a second line of defense.

Keywords: Bilirubin; Hydroxyl radical; Apoptosis; Malaria parasite; Hemozoin; Free radicals

The malaria parasite (*Plasmodium* spp.), during intraerythrocytic stages, digests huge quantities of hemoglobin and releases a large amount of highly toxic redox-active free heme [1]. To overcome the free heme toxicity, the malaria parasite is equipped with unique heme detoxification systems. Among these, hemozoin formation is considered to be the main heme detoxification system and the inhibition of hemozoin formation leads to parasite death [1,2]. In contrast, detoxification of free heme by the heme oxygenase-1 (HO-1) system is a very common process in higher eukaryotes, including mammals [3]. To degrade heme, HO-1 requires the microsomal NADPH-cytochrome P-450 reductase [4] and shunts reducing equivalents from NADPH-cytochrome P-450 reductase to the α-methene bridge and cleaves the tetrapyrrolic ring of heme, causing the liberation of CO plus an equimolar amount of biliverdin. Biliverdin is converted into bilirubin by biliverdin reductase [5]. Interestingly, the malaria parasite lacks this common heme oxygenase (www.plasmodb.org) to catabolize free heme.

Bilirubin has both antioxidant and pro-oxidant properties [6]. Bilirubin, at low physiological concentrations (0.01–10 μM), scavenges reactive oxygen species (ROS), reduces oxidant-induced cellular injury, and attenuates oxidative stress [7,8]. Unconjugated bilirubin (UCB) is a scavenger of ROS such as hydrogen peroxide, peroxynitrite, and peroxyl radicals, both in vivo and in vitro, and plays a key physiological role in cytoprotection against oxidant-mediated cell damage [9,10]. In contrast, elevated concentrations (>20 μM) of UCB have

Abbreviations: HO, Heme oxygenase; •OH, Hydroxyl radical; PBN, α-phenyl-*tert*-butylnitrone.

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deleterious effects in both neuronal and nonneuronal tissues [11]. It develops oxidative stress by generating intracellular ROS in Hepa1c1c7 cells and causes lipid peroxidation [12]. It causes brain damage in newborn piglets by increasing lipid peroxidation [13]. Oxidative stress is frequently associated with the induction of apoptosis [14,15]. UCB induces apoptosis in astrocytes at moderately elevated concentrations [16]. UCB-mediated programmed cell death was also reported in cultured rat aortic smooth muscle cells and bovine brain endothelial cells [17,18]. Thus the concentration as well as the type of cell is vital for bilirubin to exert when or where it will be pro-oxidant or antioxidant. Here, we report that bilirubin develops oxidative stress in *Plasmodium falciparum* and inhibits parasite growth. Further, we give evidence that bilirubin-induced oxidative stress-mediated parasite death is associated with the induction of caspase-like protease activity, up-regulation of the expression of apoptosis-related protein (Gene ID PFI0450c), and reduction of the mitochondrial membrane potential ($\Delta\Psi_m$).

Materials and methods

Hemin, RPMI 1640, saponin, glutathione (GSH), caspase-3 assay kit, Triton X-100, proteinase K, mannitol, α-phenyl-*n*-*tert*-butylnitrone (PBN), Nonidet P-40, dichlorofluorescein diacetate, thiobarbituric acid, trichloroacetic acid (TCA), tetraethoxypropane, antimycin A, Hoechst 33342, and bilirubin were purchased from Sigma (St. Louis, MO, USA). Albumax II was procured from Gibco BRL (Grand Island, NY, USA). Giemsa stain was purchased from Qualigens Fine Chemicals (India). [^{3}H]Hypoxanthine and Ready to Go RT-PCR beads were purchased from Amersham Biosciences (Arlington Heights, IL, USA). RNeasy kit was purchased from Qiagen (Valencia, CA, USA). 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC1) was purchased from Molecular Probes (Eugene, OR, USA). All other chemicals were of analytical grade purity. Bilirubin (Sigma Chemical Co.) was further purified to remove biliary lipids as described earlier [19]. The purified bilirubin was dissolved in 0.01 N NaOH followed by dilution with triple-distilled water to make 1 ml of a 1 mM stock and prepared fresh each time before use. Immediately after the preparation, the bilirubin stock solution was used. The stock solution was kept on ice protected from light and all incubations containing bilirubin were carried out in the dark to avoid degradation.

Parasite culture

The ring-synchronized *P. falciparum* (clone NF-54) was grown as described [20] at a hematocrit level of 5% in complete RPMI medium (CRPMI; RPMI 1640 medium supplemented with 25 mM Hepes, 50 μg ml^{-1} gentamycin, 370 μM hypoxanthine, and 0.5% (w/v) Albumax II) in tissue-culture flasks (25 and 75 cm^2) with loose screw caps. Used medium was replaced with fresh medium once in 24 h and the culture was routinely monitored through Giemsa staining of thin smears. The ring-synchronized *P. falciparum* was usually cultured for 48 h to complete one cycle from ring to schizont stage [21]. As the effect of drug or agent may be stage specific, to see the effect of bilirubin on the growth of the parasite at any stage, it was cultured for 48 h in the presence or absence of different concentrations of bilirubin.

Free (unbound) bilirubin in culture medium containing Albumax II (0.5% (w/v); a lipid-rich albumin from bovine serum, generally used to culture *P. falciparum*) [22] was measured at different total bilirubin levels using the peroxidase method as described [23] in a Shimadzu UV/Vis 1700 spectrophotometer.

Isolation of parasites from infected erythrocytes and preparation of parasite lysate

Parasites were isolated as described previously [24]. Briefly, erythrocytes with ~10% parasitemia were centrifuged at 800 ×*g* for 5 min, washed, and resuspended in cold phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 5.3 mM Na_2HPO_4, and 1.8 mM KH_2PO_4). An equal volume of 0.5% saponin in PBS (final concentration 0.25%) was added to the erythrocyte suspension and kept on ice for 15 min. It was centrifuged at 1300 ×*g* for 5 min to get the parasite pellet, and finally the pellet was washed with PBS and the isolated parasites were lysed in PBS by mild sonication (30-s pulse, bath-type sonicator) at 4°C. The whole lysate was stored at −20°C for future use. The protein concentration in the parasite lysate was estimated as described [25].

Measurement of lipid peroxidation as an index of oxidative damage

P. falciparum culture (4% parasitemia, ring synchronized) was incubated in the absence and presence of different concentrations of bilirubin for 48 h. After incubation, parasites were isolated and resuspended in PBS (500 μl) to prepare parasite lysate as described above and the lipid peroxidation product from the lysate was measured as described earlier [26,27]. In brief, an aliquot (50 μl) of the parasite lysate was allowed to react with 100 μl of trichloroacetic acid–thiobarbituric acid–HCl reagent containing 0.01% butylated hydroxytoluene, heated in a boiling water bath for 15 min, cooled, and centrifuged, and the supernatant was used for thiobarbituric acid-reactive substances determination at 535 nm using tetraethoxypropane as standard and expressed as nanomoles of lipid peroxide/milligram of lysate protein.

Measurement of reduced glutathione

P. falciparum (4% parasitemia) was cultured in the presence or absence of different concentrations of bilirubin. After 48 h of treatment, the culture was washed twice with PBS and the parasite was isolated from the infected erythrocytes as described above. GSH content from control and bilirubin-treated parasites was determined as described earlier [15,27,28]. In brief, parasite lysate (50 μl) was mixed with an equal volume of 10% TCA and the protein precipitate was removed by centrifugation. The supernatant was added to an equal volume of 0.8 M Tris–Cl, pH 9, containing 20 mM 5,5′-dithionitrobenzoic acid to yield the