Short refereed paper

HNE produced by the malaria parasite *Plasmodium falciparum* generates HNE–protein adducts and decreases erythrocyte deformability

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In *Plasmodium falciparum*-parasitized erythrocytes, hemozoin (HZ) formation was accompanied by enhanced formation of 4-hydroxynonenal (HNE)–protein adducts on the cell surface, reaching in the HZ-rich schizont forms the 16.8-fold amount of control non-parasitized cells. The addition of 1–100 μM exogenous HNE to control non-parasitized cells generated HNE-adducts on surface proteins in amounts similar to those found in schizonts. Parasitized as well as HNE-treated non-parasitized erythrocytes showed decreased cell deformability (measured as decreased filterability through cylindrical-pore filters) related to the amount of HNE adducts. *In vivo*, the HZ-containing trophozoites and schizonts are phagocytic targets for monocytes/macrophages. The reduced deformability of circulating erythrocytes carrying HNE-adducts may increase their phagocytic elimination. Uncontrolled HNE production by parasitized erythrocytes may additionally modify non-parasitized bystander erythrocytes, induce their phagocytosis, and contribute to malarial anemia, which is predominantly due to the removal of large numbers of indirectly damaged non-parasitized erythrocytes.

Keywords: Malaria, 4-hydroxynonenal, erythrocytes, hemozoin

INTRODUCTION

The malaria parasite digests up to 75% of erythrocyte hemoglobin during its maturation from the ring to the trophozoite form and polymerizes the undigested heme moiety to hemozoin (HZ) in close contact to the membrane of the digestive vacuole of the parasite, provoking lipid peroxidation. *In vitro*, isolated HZ catalyzes the peroxidation of polyunsaturated fatty acids (PUFAs) with subsequent degradation to 4-hydroxynonenal (HNE) and other products.[1,2] In the host erythrocytes, lipoperoxidation increased during the maturation process of the parasite and was accompanied by accumulation of HNE up to 51 μM in HZ isolated from mature trophozoites.[2] In non-parasitized erythrocytes, exogenously added HNE concentrated within the membranes and formed covalent adducts with cysteine, lysine and histidine residues in proteins,[3,4] and was a potent mediator of a variety of biological effects.[5]

Here, we show the formation of surface protein HNE adducts by endogenously formed/generated HNE during the process of intracellular parasite maturation. The formation of HNE and the appearance of HNE adducts on the erythrocyte surface co-incided with the onset of HZ formation in the parasite and was also apparently correlated with decrease of erythrocyte deformability.

MATERIALS AND METHODS

HZ was obtained from *in vitro* grown *Plasmodium falciparum* parasites (Palo Alto strain, *Mycoplasma*-free). Different maturation stages of the parasite were harvested from synchronized cultures and deprived of exogenous HZ by Percoll separation.[1] HNE–protein adducts were detected by flow cytometry (FACS) after labeling the intact cells with a specific anti-HNE–adduct antibody (Alexis Biochemicals, Lausen, Switzerland).[2]

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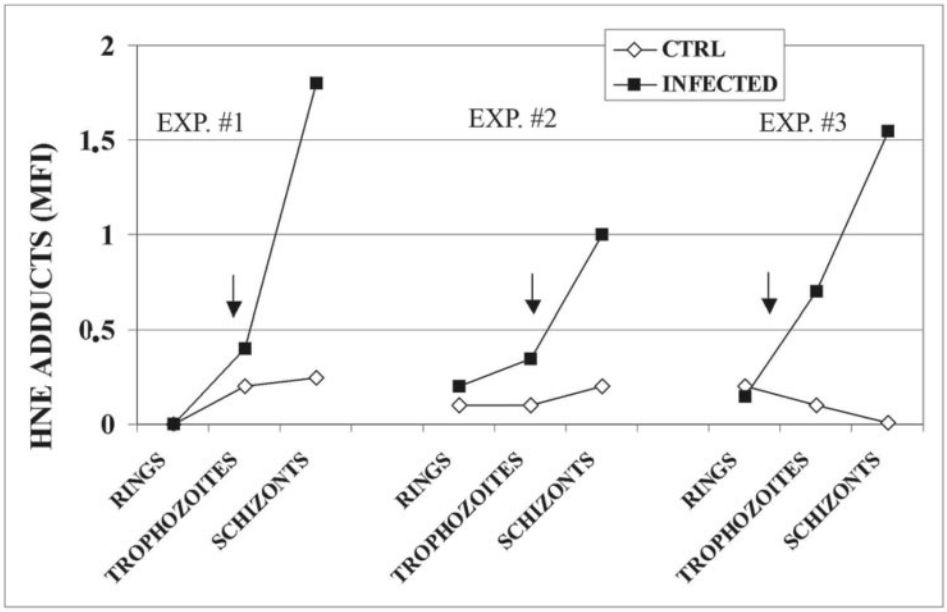

Fig. 1. Onset of HZ formation (late ring stage, arrow) co-incides with enhanced formation of HNE–protein adducts on the surface of parasitized erythrocytes, represented as MFI (see Materials and Methods). Progressive HZ generation during parasite growth was accompanied by the continuous increase of HNE adducts as shown in three distinct representative experiments. Parasitemia was kept constant between 15–25% during parasite maturation in each experiment.

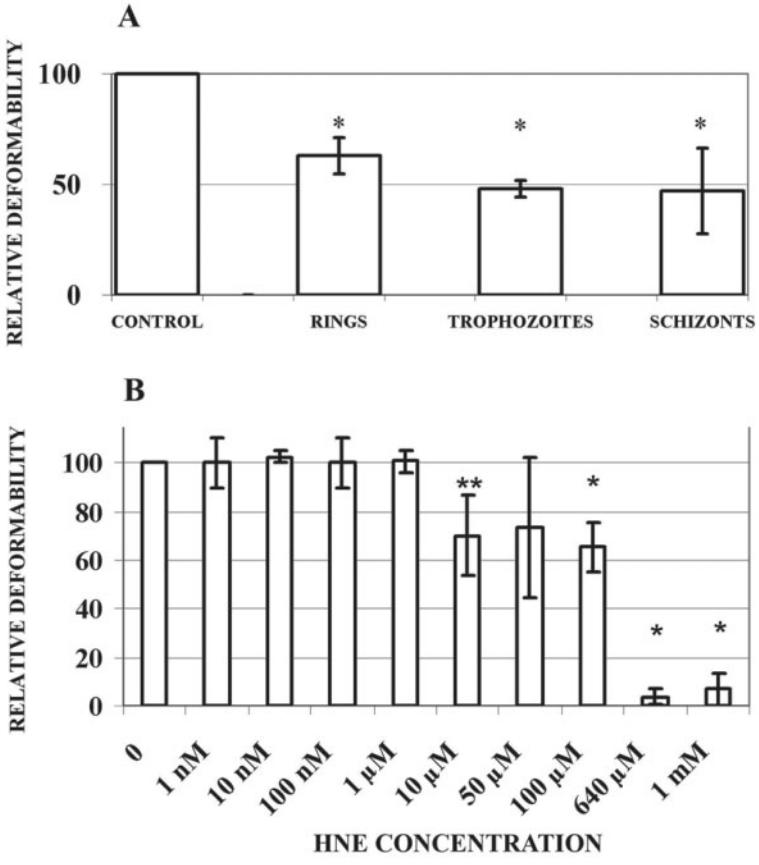

Fig. 2. Decrease in erythrocyte deformability during parasite maturation (**A**) and after exogenous HNE treatment (**B**). Mean ± SEM of 4 independent experiments. Significance: **P* < 0.05; ***P* < 0.1.

The amount of HNE adducts is presented as mean fluorescence intensity (MFI) defining background as zero. To examine the effect of HNE on erythrocyte deformability, erythrocytes were treated with 0–1 mM HNE for 30 min at 37°C. Prior to the deformability measurement, parasitized and HNE-treated erythrocytes were washed 3 times with PBS-G, then the erythrocytes were diluted with PBS-G to a hematocrit of 1%. Non-parasitized, fresh erythrocytes from the same donor served as controls. Erythrocyte deformability was assessed at room temperature by the filtration technique using the equipment and methods described elsewhere.[6] Briefly, aliquots (250 μl) of PBS-G and the erythrocyte suspension were allowed to pass through a filter with cylindrical pores 3 μm in diameter, and their passage times (T-PBS and T-suspension, respectively) were recorded. The T-PBS/T-suspension ratio was used to define erythrocyte deformability.

Results and Discussion

The onset of hemoglobin degradation and HZ formation at late-ring stage of the parasite co-incided with significantly enhanced formation of HNE-protein adducts on the surface of parasitized erythrocytes (2.6 ± 0.5-fold [mean ± SEM, n = 10]), compared to non-parasitized control erythrocytes (Fig. 1). The progressive HZ generation during intra-erythrocytic parasite growth was accompanied by the continuous increase of adducts reaching 16.8 ± 3.4-fold (mean ± SEM, n = 9) in the HZ-rich schizont forms compared to non-parasitized control erythrocytes. The addition of 1–100 μM exogenous HNE to non-parasitized erythrocytes generated HNE adducts of surface proteins in amounts similar to those found in parasitized erythrocytes (data not shown).

The amount of HNE–protein adducts on the surface of parasitized erythrocytes can be considered as: (i) a marker for elevated intracellular, most likely HZ-catalyzed, HNE production; and (ii) an indicator for persistent transmembrane diffusion of reactive HNE out of the cells in spite of up-regulated GSH levels in the parasites, as GSH should be able to scavenge all HNE produced, if accessible.[7] The occurrence of HNE adducts in parasitized erythrocytes and in HNE-treated non-parasitized erythrocytes resulted in decreased deformability, measured as decreased filterability through cylindrical-pore membranes (Fig. 2A,B).

Decreased erythrocyte deformability is present in malaria,[8,9] and was found to correlate with the degree of anemia.[10] The appearance of protein–HNE adducts in the membrane of parasitized erythrocytes could alter their deformability. Preliminary experiments from our laboratory (data not shown) have shown modifications in cytoskeleton and membrane proteins by HNE that may explain the decrease in erythrocyte deformability. *In vivo*, the HZ-containing trophozoite as well as isolated HZ are phagocytic targets for monocytes/macrophages.[1] The reduced deformability of circulating erythrocytes carrying HNE adducts should increase their phagocytic elimination in spleen, liver and bone marrow. Uncontrolled HNE production by parasitized erythrocytes may additionally modify bystander non-parasitized erythrocytes, induce their phagocytosis, and contribute to malarial anemia, which is predominantly due to the removal of non-parasitized erythrocytes.[11]

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References

1. Schwarzer E, Kühn H, Valente E, Arese P. Malaria-parasitized erythrocytes and hemozoin nonenzymatically generate large amounts of hydroxy fatty acids that inhibit monocyte functions. *Blood* 2003; **101**: 722–728.
2. Skorokhod A, Schwarzer E, Grune T, Arese P. Role of 4-hydroxynonenal in the hemozoin-mediated inhibition of differentiation of human monocytes to dendritic cells induced by GM-CSF/IL-4. *BioFactors* 2005; **24**: 283–289.
3. Uchida K, Hasui Y, Osawa T. Covalent attachment of 4-hydroxy-2-nonenal to erythrocyte proteins. *J Biochem (Tokyo)* 1997; **122**: 1246–1251.
4. Schaur RJ. Basic aspects of the biochemical reactivity of 4-hydroxynonenal. *Mol Aspects Med* 2003; **24**: 149–159.
5. Poli G, Schaur RJ. 4-Hydroxynonenal in the pathomechanism of oxidative stress. *IUBMB Life* 2000; **50**: 315–321.
6. Lisovskaia IL, Ataullakhanov FI, Tuzhilova EG, Vitvitsky VM. Analysis of geometric parameters and mechanical properties of erythrocytes by filtration through nuclear membrane filters. II. Experimental verification of a mathematical model. *Biofizika* 1994; **39**: 864–871.
7. Ayi K, Cappadoro M, Branca M, Turrini F, Arese P. *Plasmodium falciparum* glutathione metabolism and growth are independent of glutathione system of host erythrocyte. *FEBS Lett* 1998; **424**: 257–261.
8. Cooke BM, Mohandas N, Coppel RL. Malaria and the red blood cell membrane. *Semin Hematol* 2004; **41**: 173–188.
9. Dobbe JG, Hardeman MR, Streekstra GJ *et al.* Analyzing red blood cell deformability distributions. *Blood Cells Mol Dis* 2002; **28**: 373–384.
10. Dondorp AM, Angus BJ, Chotivanich K *et al.* Red blood cell deformability as a predictor of anemia in severe falciparum malaria. *Am J Trop Med Hyg* 1999; **60**: 733–737.
11. Price RN, Simpson JA, Nosten F *et al.* Factors contributing to anemia after uncomplicated falciparum malaria. *Am J Trop Med Hyg* 2001; **65**: 614–622.