Increased levels of 4-hydroxynonenal in human monocytes fed with malarial pigment hemozoin

A possible clue for hemozoin toxicity

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Abstract In human monocytes, lipoperoxides were increased 3-fold at 2 h, 6-fold at 5 h and 7.5-fold at 12 h after hemozoin phagocytosis. 4-Hydroxynonenal (HNE) was also increased, reaching 40 nmol/10^{10} cells at 2 h (approximate intracellular concentration [AIE] 8 μM), 230 nmol/10^{10} cells at 5 h (AIE 46 μM) and 79 nmol/10^{10} cells (AIE 16 μM) at 12 h. A moderate increase in HNE, approx. 20 nmol/10^{10} cells (AIE 4 μM) was also observed after phagocytosis of anti-D IgG-opsonized erythrocytes. HNE in unfed controls was approx. 5 nmol/10^{10} cells (AIE 1 μM) during the whole incubation period. An increased amount of protein kinase C (PKC)/HNE adduct was demonstrated in hemozoin-fed monocytes. Purified PKC was profoundly inhibited at HNE > 10 μM. The impairment of PKC previously observed in hemozoin-fed monocytes can thus be explained by direct interaction with increased HNE levels.

Key words: 4-Hydroxynonenal; Malaria (*Plasmodium falciparum*); Hemozoin; Monocyte (human); Phagocytosis; Protein kinase C inhibition

1. Introduction

During the 48 h intra-erythrocytic life cycle of *Plasmodium falciparum*, between 80 and 100% host hemoglobin is degraded, most intensely during the short period of trophozoite development [1]. The parasite is unable to degrade heme, which aggregates to an insoluble polymer, called hemozoin [1–4]. Upon RBC rupture hemozoin is dispersed in the vasculature. In vivo and in vitro studies have shown that human monocytes and macrophages avidly ingest free hemozoin or hemozoin-containing trophozoites and schizonts [5,6].

In vitro studies have shown that hemozoin-fed human monocytes are viable but functionally impaired. They are unable to digest hemozoin, to repeat phagocytosis and to generate oxidative burst upon appropriate stimuli [5]. In addition, membrane-localized PKC was precociously and severely impaired [7], as was the activity of NADPH oxidase, the enzyme complex responsible for burst generation [8]. Moreover, hemozoin-fed human and murine monocytes/macrophages released high levels of tumor necrosis factor-α [9–11], interleukin-1β [9,10], MIP-1α and MIP-1β [11].

The presence in hemozoin of large quantities of ferric heme together with small amounts of free iron [2,3] makes hemozoin a likely generator of oxidative radicals that may form lipoperoxides from polyunsaturated fatty acids present in membranes. A constant byproduct of lipoperoxidation is HNE, a strong electrophilic alkylating agent which spontaneously reacts with thiols and amino groups in proteins and purine/pyrimidines [12–15]. We report here a marked increase in membrane lipoperoxides and HNE levels in isolated hemozoin-fed human monocytes. We also report distinct inhibition of purified PKC by HNE at concentrations comparable to those found in hemozoin-fed monocytes, and demonstrate for the first time increased amounts of PKC-HNE adduct in hemozoin-fed monocytes. Thus, the effects observed in hemozoin-fed monocytes may result from direct interaction of HNE with PKC and, possibly, with other enzymes.

2. Materials and methods

Adherent and suspended human monocytes were prepared as indicated [5,7,8] from freshly collected platelet-poor buffy coats discarded from blood donations of healthy adult donors of both sexes. Hemozoin was prepared from *P. falciparum* cultures (strain FCR-3), opsonized with fresh human serum and quantified by heme luminescence as described [16]. Freshly drawn human RBC were isolated and opsonized with anti-D IgG (Rhesogam S, Behring) as indicated [8] (briefly, opsonized RBC). Phagocytosis of opsonized hemozoin and RBC was performed and quantified as described [16]. Lipoperoxides were quantified by measuring generation of luminol-dependent luminescence catalyzed by cytochrome P-450 as described [5]. Details are given in the legend to Fig. 1.

HNE levels were measured in adherent or suspended human monocytes fed with opsonized hemozoin or RBC. HNE analysis was performed as described [17–20] after derivatization of HNE with dinitrophenylhydrazine and separation of dinitrophenylhydrazones by thin-layer chromatography and subsequently by HPLC. Peak identification was performed by comparison of retention times of peaks of biological extracts and of standard extracts (pure HNE was a kind gift from Professor H. Esterbauer, Graz, Austria), by co-elution of biological extracts with the reference compounds and by comparison of spectra [19,20]. Cell viability was checked by the trypan blue exclusion test. Details are given in the legend to Fig. 2.

PKC activity was determined by measuring the transfer of the [^{32}P]phosphate from [γ-^{32}P]ATP (Amersham) to the PKC substrate

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Abbreviations: BSA, bovine serum albumin; ECL, enhanced chemiluminescence; HNE, 4-hydroxy-2,3-*trans*-nonenal (4-hydroxynonenal); HPLC, high-performance liquid chromatography; IgG, immunoglobulin G; luminol, 5-amino-2,3-dihydro-1,4-phthalazinedione; MIP-1α/-1β, macrophage inflammatory protein-1α/-1β; PKC, protein kinase C; RBC, erythrocytes; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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histone H1 (Boehringer Mannheim). Details are given in the legend to Fig. 3. PKC-HNE adduct was demonstrated in immunoprecipitated PKC from hemozoin-fed monocytes and unfed controls making use of monoclonal antibody 1H4H12. This antibody (a kind gift from Professor H. Esterbauer, Graz, Austria) preferentially recognizes covalent adduct between HNE and histidine (80%) and also adducts between HNE and lysine (18%) and cysteine (2%). Details are given in the legend to Fig. 4.

3. Results

As shown in Fig. 1, lipoperoxides measured in lipid extracts of hemozoin-fed monocytes were increased 3-fold at 2 h, 6-fold at 5 h and 7.5-fold at 12 h compared to time-matched unfed controls, and persisted high until 48 h (not shown) after hemozoin phagocytosis. Lipoperoxides measured in monocytes fed with anti-D IgG-opsonized RBC used as positive controls (briefly, RBC-fed monocytes) were moderately increased at 12 h (Fig. 1). In contrast to phagocytosed hemozoin which is not digested by the monocyte, phagocytosed hemoglobin of positive controls is rapidly and totally catabolized by monocytes [5].

HNE was measured in isolated monocytes at different time points after hemozoin or RBC phagocytosis as the sum of intracellular HNE and HNE liberated in the supernatant. As shown in Fig. 2, HNE was increased, reaching 40 nmol/10^{10} cells at 2 h, 230 nmol/10^{10} cells at 5 h and 79 nmol/10^{10}

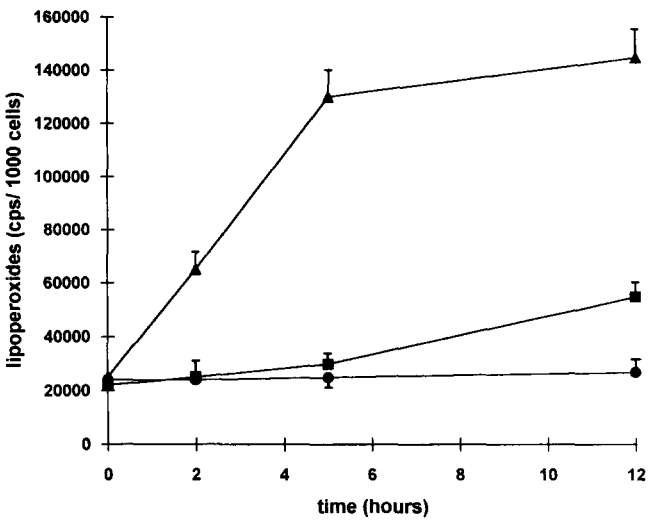

Fig. 1. Generation of lipoperoxides in human adherent monocytes after phagocytosis of hemozoin or opsonized RBC. Approx. 5×10^6 adherent monocytes covered with 10 ml RPMI 1640 medium supplemented with 24 mM $NaHCO_3$ and 5% (v/v) autologous decomplemented serum/6.5 cm diameter Petri dish fed with opsonized hemozoin (▲) or anti-D IgG-opsonized RBC (■), and unfed controls (●) were incubated in a humidified 5% CO_2/95% air incubator at 37°C. At selected time points and after removal of noningested material, monocytes were detached and a small aliquot removed for cell counting. Detached cells (approx. 25000 cells) were solubilized in 0.5 ml of 0.1 M NaOH containing 0.025% (v/v) Triton X-100 and 3 mM EDTA. Lipids were extracted with 1 ml chloroform/methanol. 10 μl of the chloroform/methanol phase were added to 300 μl of solution A (0.3 μg cytochrome P-450/ml dissolved in 3 mM (final) EDTA in 0.1 M NaOH) and 300 μl of solution B (10 μg luminol (Sigma)/ml dissolved in 3 mM (final) EDTA in 0.1 M NaOH). Luminol-dependent luminescence generation was measured with a Magic Lite Analyzer (Ciba Corning) 2 s after injection of solutions A and B, and expressed as cps/1000 monocytes. Mean values ± S.D. from 5 experiments.

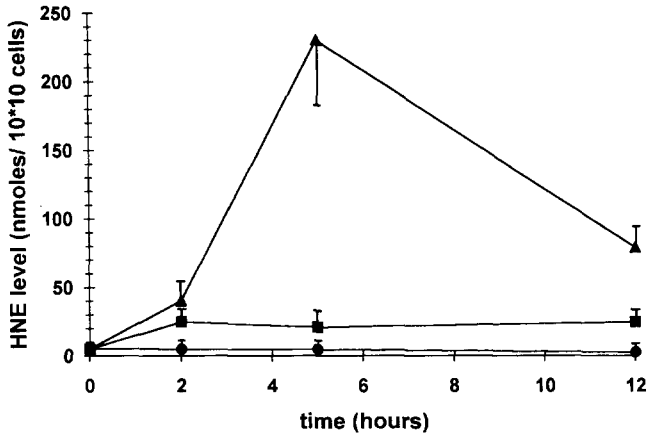

Fig. 2. Generation of HNE in human adherent monocytes after phagocytosis of hemozoin or opsonized RBC. Monocytes fed with hemozoin (▲) or with opsonized RBC (■), and unfed controls (●) were incubated in a humidified 5% CO_2/95% air incubator at 37°C (see legend to Fig. 1 for details). At selected time points, supernatants and detached monocytes were collected for HNE analysis. HNE levels are expressed as nmol HNE generated per 10^{10} cells. Mean values ± S.D. from 5 experiments.

cells at 12 h after hemozoin phagocytosis. A moderate increase in HNE (approx. 20 nmol/10^{10} cells) was also observed in RBC-fed monocytes during the whole incubation period. HNE level in unfed controls was approx. 5 nmol/10^{10} cells during the whole incubation period. Assuming an average cellular volume of 500 fl for the human monocyte and even distribution of HNE within the cell, the estimated HNE level would be approx. 1 μM in unfed controls during the whole experimental period, approx. 8 μM at 2 h, approx. 46 μM at 5 h and approx. 16 μM at 12 h after hemozoin phagocytosis. Interestingly, the increase in HNE was time-related with impairment of oxidative burst [5] and inhibition of NADPH oxidase observed previously [8] in hemozoin-fed monocytes, whereby increase in lipoperoxides seems to precede HNE increase. Phagocytosis of opsonized RBC was also accompanied by rapid though moderate increase in HNE level, which remained rather constant until 12 h after phagocytosis at an estimated cellular level of approx. 4 μM.

The dose-dependent effect of HNE on activity of purified PKC is shown in Fig. 3. 10 nM HNE reduced PKC activity by approx. 30%. Abrupt drop in activity of PKC to almost zero was evident at HNE concentrations higher than 10 μM.

An indication that PKC inhibition is due to direct binding of HNE to the enzyme molecule is documented by the increased presence of PKC-HNE adduct in immunoprecipitated PKC in hemozoin-fed relative to control monocytes. As shown in Fig. 4, immunoprecipitation of the same amounts of PKC with anti-pan PKC antibodies and subsequent immunoblot analysis with monoclonal antibody 1H4H12 indicated an increased amount of adduct in hemozoin-fed monocytes.

4. Discussion

Monocytes and macrophages avidly ingest free hemozoin and hemozoin-rich trophozoites and schizonts [5,6]. Ingestion of hemozoin impairs important monocyte functions such as oxidative burst and the ability to repeat the phagocytic process [5]. Also impaired are membrane-localized PKC and NADPH oxidase, the enzyme complex responsible for oxida-

tive burst [7,8]. Ferri- and ferro-heme are abundantly present in hemozoin together with small amounts of free iron [1–5]. Thus, in the acidic milieu of monocyte phagolysosome and in the presence of oxygen and hydrogen peroxide, hemozoin is a likely generator of radical oxygen species that may produce lipoperoxides from membrane lipids carrying unsaturated fatty acids.

We show here that ingestion of hemozoin by monocytes is indeed accompanied by increased levels of lipoperoxides. This effect occurs early and persists up to 48 h after hemozoin phagocytosis. Since lipoperoxides may easily generate short-chain aldehydes [12], it was not unexpected to find increased levels of HNE in hemozoin-fed monocytes. HNE peaked at 5 h after hemozoin phagocytosis attaining 50-fold levels of unfed controls, and was still almost 15-fold the control levels at 12 h. Because of the unknown pattern of distribution of HNE in different cellular compartments, only a very rough estimate of cellular HNE concentrations could be made. Assuming a homogeneous intracellular distribution and 10^{10} cells/ml, approx. 1 μM HNE at rest and almost 50 μM HNE at peak after hemozoin feeding are plausible HNE concentrations. However, due to the marked lipophilic character of HNE, accumulation of HNE in membranes can be expected [12]. In view of the high HNE catabolic potency of control monocytes (approx. 8 nmol per 10^6 cells in 20 s), an increased residual HNE level observed in hemozoin-fed monocytes indicates additional impairment of HNE catabolism.

HNE exerts a wide array of effects depending on the concentration, cell type and time of contact. HNE is cytotoxic at widely different concentrations between 5 μM in human growth-arrested fibroblasts, 25 μM in human endothelial cells and 1000 μM in isolated mouse hepatocytes (see [12] for review). HNE affects growth and blocks cell proliferation at markedly lower concentrations than needed for a cytotoxic effect (see [12] for review). It modulates the expression of specific genes at very low, para-physiological concentrations [21,22]. HNE is a strong electrophilic alkylating agent which spontaneously reacts with thiols, and less so with amino groups in amino acids, proteins and purine/pyrimidines [12–15]. HNE-mediated inhibition of numerous enzymes resides in blockage of functionally important thiol or amino groups [12–15].

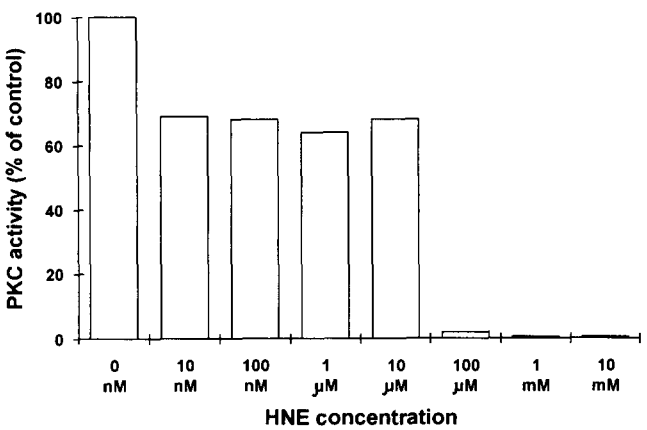

Fig. 3. Inhibition of PKC activity by HNE. PKC activity was determined by measuring [^{32}P]phosphate transfer from [γ-^{32}P]ATP (Amersham) to histone H1 (Boehringer Mannheim). Purified PKC (EC 2.7.1.37) from rat brain (Boehringer Mannheim) (0.05 mU) was preincubated with increasing concentrations of HNE in 20 mM Tris buffer containing 0.5 mM EDTA and 0.5 mM EGTA, pH 7.5, on ice for 5 min. The reaction mixture (10 mM $MgCl_2$, 10 μM ATP (Sigma), 500 μM $CaCl_2$, 0.25% BSA (type V, Sigma), 100 μg/ml phosphatidylserine, 20 μg/ml 1,2-dioleyl-*sn*-glycerol, 200 μg/ml (both from Sigma) histone H1 in 20 mM Tris-HCl, pH 7.5) was added to the preincubated tubes. Reaction was started by adding 5.5 μCi of [γ-^{32}P]ATP (specific activity 6000 dpm/pmol). After 3 min incubation at 37°C reaction was stopped by adding ice-cold 10% (w/v) trichloroacetic acid. The precipitated material was quantitatively transferred to glass fiber filters. After washing with excess trichloroacetic acid, filters were counted. PKC activity is given as percent of control PKC activity (0.56 μmol/mg protein per min) measured without HNE. Results are mean values of two independent experiments.

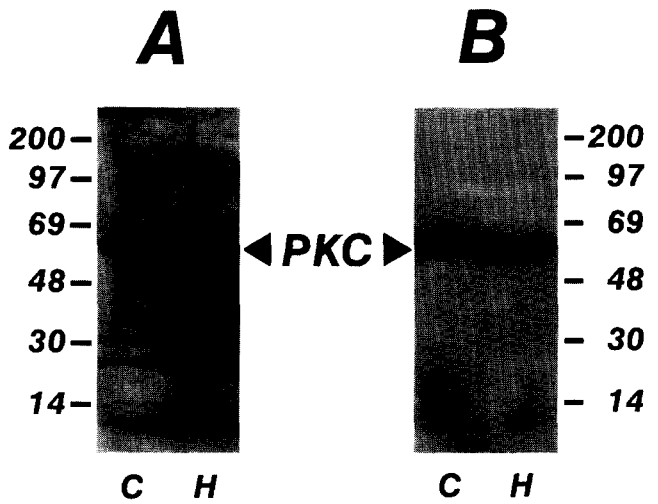

Fig. 4. Formation of PKC-HNE adduct in hemozoin-fed and unfed control monocytes. Hemozoin-fed and unfed control monocytes were incubated at 2×10^6 cells/ml in AIM V cell culture medium (Gibco) in 5 cm diameter Teflon dishes (Heraeus) in a humidified incubator (5% CO_2/95% air) at 37°C. After 12 h incubation, approx. 20×10^6 each of hemozoin-fed and unfed cells were harvested and sedimented at $400\times g$ for 8 min at 4°C. Packed cells were lysed by addition of 1.5 ml of 50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1 mM EDTA, standard protease inhibitors (Sigma) and 1% (w/v) Triton-X 100 (Sigma) (lysis buffer) for 20 min on ice. After pre-cleaning with Protein A-Sepharose 6MB beads (Sigma), 1 mg of supernatant protein was used for immunoprecipitation of PKC with anti-pan PKC (rabbit polyclonal IgG) (Boehringer Mannheim) as specified by the manufacturer. The immunoprecipitate was resuspended in 5-fold concentrated Laemmli sample buffer, proteins separated by 10% SDS-PAGE electrophoresis and transferred to nitrocellulose. The blocked nitrocellulose was probed with monoclonal antibody 1H4H12 (a kind gift of Professor H. Esterbauer, Graz, Austria) (A), and with anti-pan PKC (B) after stripping. C, unfed control monocytes; H, hemozoin-fed monocytes. HNE-PKC adduct was detected by ECL (Amersham) after incubation of membranes with peroxidase-linked second antibody. Numbers indicate molecular weight (kDa) of markers (Amersham).

For the first time we show here an increased amount of adduct established between the histidine/lysine free amino groups of PKC and HNE. In view of the distinctly higher reactivity of HNE towards thiol compared to amino groups [12], the formation of increased adduct between PKC thiol groups and HNE can also be assumed to occur in hemozoin-fed monocytes. PKC possesses important thiol groups that must be in the reduced state for correct functioning of the enzyme [23–25]. Thus, blockage of histidine/lysine amino groups as well as alkylation of thiol groups due to HNE/PKC adduct formation may lead to PKC inhibition, and explain the inhibition of purified PKC by micromolar concentrations of HNE observed here. The range between 10 and 100 μM

HNE seemed to be critical for down-modulation of PKC activity to almost zero. As stated above, it is realistic to assume that HNE concentrations above 10 μM were present for longer periods of time in hemozoin-fed monocytes in specific membrane compartments.

In conclusion, the present results have shown markedly and long-lasting increased levels of lipoperoxides and HNE for several hours after hemozoin phagocytosis, accompanied by increased HNE/PKC adduct. HNE concentrations in the same range as observed here were found to down-regulate PKC activity [26]. In view of the role played by PKC in a vast number of cellular functions [27], its inhibition may offer a clue to explaining monocyte incapacitation after hemozoin phagocytosis.

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