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β-Hematin (Hemozoin) Mediated Decompostion of Polyunsaturated Fatty Acids to 4-Hydroxy-2-nonenal

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β-Hematin is an important heme metabolite of malarial infection. Its role as an agent mediating the formation of the reactive electrophile 4-hydroxynonenal (HNE) from polyunsaturated fatty acids was investigated. In vitro formation of HNE was found to be facilitated by the presence of hemozoin in a concentration-dependent fashion. The reactivity of HNE derived from reaction with *β*-hematin was confirmed through its ability to form protein adducts on myoglobin.

β-Hematin (hemozoin, malaria pigment) is a heme detoxification biomineral produced in a variety of blood-feeding organisms including *Plasmodium falciparum*, the most common and deadly form of the malaria parasite, as a result of hemoglobin catabolism.[1−3] The structure of hemozoin is that of a dimer of five-coordinate ferric protoporphyrin IX [Fe^{III}PPIX] linked by the binding of the central iron of the first porphyrin to one of the propionic acid side chains of the reciprocating porphyrin in a monodentate carboxylate linkage (Figure 1). Hydrogen bonding between the free propionic acid groups allows the dimer to form an extended network. Powder diffraction X-ray (XRD) studies have unambiguously demonstrated that native hemozoin is identical to synthetic *β*-hematin, providing an important synthetic route for studies of this material.[4]

The presence of hemozoin in circulating leukocytes and monocytes has been correlated to the severity of malaria.[5] Under physiological conditions, hemozoin has been shown to form immunomodulatory hydroxylated fatty acids (hydroxyeicosatetraenoic acids, HETEs) from arachidonic acid.[6] Additionally, in cultures of peripheral blood monocytes that contain phagocytosed hemozoin, monocyte function appears to be impaired,[7] and increased levels of the highly reactive electrophile 4-hydroxy-2-nonenal (HNE) have been detected.[8] In these monocytes, HNE protein adducts have been identified on protein kinase C, a key enzyme involved in the regulation of oxidative burst.[9] Consequently, it has been speculated that hemozoin plays a critical role in the modulation of the host innate immune system during malaria infection by disrupting macrophage function.[10,11] Herein, we demonstrate that hemozoin can directly mediate the formation of HNE.

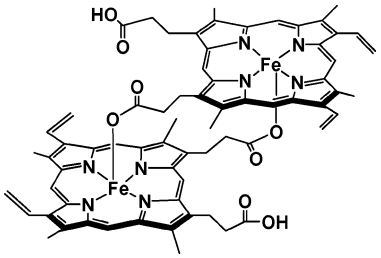

Figure 1. Dimeric heme unit of the *β*-hematin aggregate.

β-Hematin was synthesized via the dehydrohalogenation strategy of Bohle et al.[12] In contrast to alternate literature routes, the advantages of this synthetic method include both high yields and low reaction temperatures, minimizing any contaminants from free or degraded hemin starting material. Reaction yields for *β*-hematin were 95%. Relying on the differential solubility of hemin chloride and *β*-hematin, exhaustive washings in MeOH, 0.1 M $NaHCO_3$ (pH 9.0), and DMSO removed excess free heme and small aggregates (Supporting Information).[13] The resulting *β*-hematin was characterized by IR spectroscopy, SEM, and XRD (Sup-

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porting Information), and the results matched previously reported spectra.[4,14,15]

The heterogeneous reaction between β-hematin and polyunsaturated fatty acid (PUFA) substrates involves a number of radical intermediates, resulting in a complex distribution of lipid peroxidation products. For previously identified hydroxylated lipid products in reactions between β-hematin and a PUFA, a key intermediate is the corresponding hydroperoxypolyunsaturated fatty acid.[16] β-Hematin has been shown to have a pro-oxidant effect on the formation of such intermediates.[17] This same hydroperoxide intermediate has also been identified as a precursor of HNE.[18,19]

In examining the β-hematin mediated production of HNE, arachidonic acid (AA), linoleic acid (LA), and their respective methyl esters (MeAA and MeLA, respectively) were used as substrates. In a typical reaction, the appropriate fatty acid substrate (24 mM) was incubated with β-hematin (1.2 mM) in chelexed phosphate buffer (2 mL, 25 mM, pH 7.4) for 4 h at 25 °C. The reaction mixture was extracted with diethyl ether and centrifuged, and the supernatant was decanted for analysis. Recovery of β-hematin following a typical reaction indicated that, within experimental error, β-hematin did not significantly decompose during the course of the experiment. Further control experiments with hemin chloride demonstrated that the minimal amounts of free heme possibly present would account for less than 1% of the observed reactivity.

Formation of HNE was confirmed with LC/APCI/MS (Supporting Information). The isolated product demonstrated an LC retention time and product ion spectra of MH^+ [mass-to-charge ratio (*m*/*z*) 157] and MH^+-H_2O [*m*/*z* 139] identical to those of an authentic standard of HNE (Supporting Information). Subsequent quantitation of HNE was performed by UV analysis of normal phase liquid chromatograms.

Experiments demonstrated that the production of HNE increased linearly (Figure 2A) with the concentration of β-hematin. Relative to the auto-oxidation of the substrates, β-hematin resulted in an overall 14-fold increase in the production of HNE (Supporting Information). Under anaerobic conditions, no HNE was produced, suggesting that the hydroxide oxygen is derived from atmospheric O_2. Additionally, the formation of HNE increased linearly over 12 h (Figure 2B). Addition of antioxidants such as methyl trolox significantly reduced the amount of HNE formed (Supporting Information). Similar reactivity patterns were observed for all substrates.

When compared to the autocatalytic conversion of the purported intermediate 15-HPETE to HNE, the β-hematin

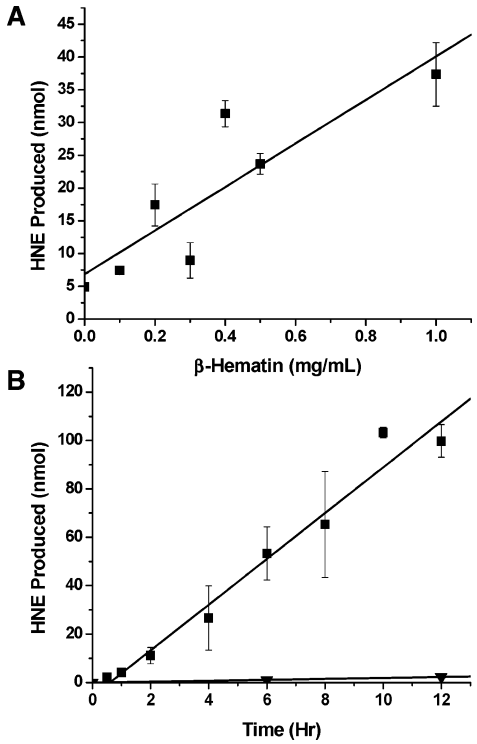

Figure 2. (A) Increased HNE production from arachidonic acid as a function of β-hematin concentration. (B) Time dependence of HNE production from hemozoin mediated arachidonic acid oxidation (■, aerobic; ▼, anaerobic). Reaction conditions as above.

mediated reaction yielded a 3-fold increase in levels of HNE (Supporting Information). Transition metal mediated degradation of lipid hydroperoxides (Cu^{2+},[20] Fe^{2+},[20−22] Fe^{3+},[22] hematin[23]) derived from both arachidonic and linoleic acids to HNE is well documented and thought to occur by one-electron reduction to an alkoxyl radical. EPR experiments using spin trap labels revealed that the reaction of β-hematin with *tert*-BuOOH formed both methoxyl and *tert*-butoxyl radicals.[24] Although additional mechanistic details are difficult to access given the heterogeneous nature of the reaction, the formation of HNE from β-hematin likely proceeds through a similar pathway.

To confirm the reactivity of HNE generated by the reaction of β-hematin and arachadonic acid, the isolated, pooled product was reacted with horseheart myoglobin (Mb) at a molar ratio of 7:1 (HNE/Mb) overnight at 37 °C. Analysis of the sample by ESI-MS (Finnigan TSQ 7000 with positive ionization) resulted in the multiply charged envelope of Mb with adducts of HNE (Figure 3). The $M + 18^+$ (*m*/*z* 942) region provides an example of the development of two prominent HNE adducts on the protein with *m*/*z* of 951.5 and 962.4. Analysis of the multiply charged envelope revealed up to five HNE additions onto the protein (Sup-

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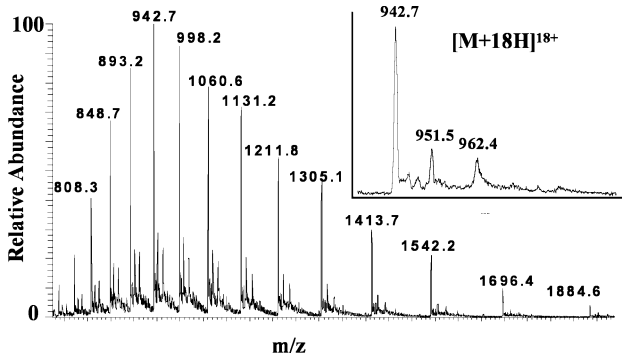

Figure 3. ESI−MS of horseheart myoglobin modified with 4-hydroxy-2-nonenal. Inset: Expansion of the $[M+18H]^{18+}$ charge state showing the addition of two HNE adducts.

porting Information). The adduction pattern is consistent with a Michael addition of the aldehyde to histidine or lysine, similar to recent literature reports of the in vitro reaction of excess HNE with Mb.[25,26] Such reactivity of β-hematin derived HNE is important, as HNE adducts have been found on key regulatory proteins, such as protein kinase C, within dysfunctional macrophages that contain phagocytosed hemozoin.[9] These results suggest that HNE adduction might be a useful tag for the identification of potentially disrupted proteins within host monocytes using proteomic analysis.

In vitro, β-hematin promotes the decomposition of arachidonic and linoleic fatty acids to 4-hydroxy-2-nonenal in two ways: (1) it serves as the pro-oxidant agent of PUFA substrates to their reactive hydroperoxide intermediates, and (2) it promotes the formation of the alkoxyl radical leading to HNE formation. Although the relative total conversion of PUFA to HNE is low under these in vitro conditions (2%), physiologically, levels of hemozoin accumulate to significant concentrations within the liver and spleen.[27] Consequently, significant concentrations of secondary lipid peroxidation products would be generated during the course of disease. Additionally, the reactivity of β-hematin might be linked to the observed pathogenic concentrations of HNE that disrupt protein function through adduct formation.

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Supporting Information Available: IR, SEM, and XRD data for β-hematin, LC/APCI/MS spectra, antioxidant data, ESI-MS spectra, and HPETE decomposition data. This material is available free of charge via the Internet at http://pubs.acs.org.

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