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Iron Loaded Ferritin Secretion and Inhibition by CI-976 in *Aedes aegypti* larval cells

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Abstract

Ferritin is a multimer of 24 subunits of heavy and light chains. In mammals, iron taken into cells is stored in ferritin or incorporated into iron-containing proteins. Very little ferritin is found circulating in mammalian serum; most is retained in the cytoplasm. Female mosquitoes, such as *Aedes aegypti* (yellow fever mosquito, Diptera), require a blood meal for oogenesis. Mosquitoes receive a potentially toxic level of iron in the blood meal which must be processed and stored. We demonstrate by ⁵⁹Fe pulse-chase experiments that cultured *A. aegypti* larval CCL-125 cells take up iron from culture media and store it in ferritin found mainly in the membrane fraction and secrete iron-loaded ferritin. We observe that in these larval cells ferritin co-localizes with ceramide-containing membranes in the absence of iron. With iron treatment, ferritin is found associated with ceramide-containing membranes as well as in cytoplasmic non-ceramide vesicles. Treatment of CCL-125 cells with iron and CI-976, an inhibitor of lysophospholipid acyl transferases, disrupts ferritin secretion with a concomitant decrease in cell viability. Interfering with ferritin secretion may limit the ability of mosquitoes to adjust to the high iron load of the blood meal and decrease iron delivery to the ovaries reducing egg numbers.

Keywords

Aedes aegypti; CI-976; ferritin; iron; mosquito; secretion

1. Introduction

Most species require iron for survival. The capacity of iron to coordinate with a variety of ligands and to donate and to receive electrons enables it to participate in numerous vital reactions within organisms (Koorts and Viljoen, 2007a). These properties also allow iron to promote the formation of free radicals in the presence of oxygen (Koorts and Viljoen, 2007b). Therefore, iron availability and processing are tightly controlled such that iron deficiency is avoided, while oxidative stress is limited (Hentze et al., 2004; Dunn et al., 2007).

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Ferritin is a stable, multimeric protein that is highly conserved among plants, animals and microorganisms (Nichol et al., 2002; Strickler-Dinglasan et al., 2006; Colbourne et al., 2007; De Zoysa and Lee, 2007; Li et al., 2008; Qiu et al., 2008). It serves to store and to transport iron and as a cytoprotective agent. In vertebrates, ferritin consists of 24 subunits configured as a hollow cage where iron is held in complex with oxygen and phosphate as a ferrihydrite mineral (Chasteen and Harrison, 1999; Hamburger et al., 2005; Theil et al., 2006; Koorts and Viljoen, 2007a). Iron loading occurs by oxidation of ferrous to ferric at ferroxidase sites on the molecule with subsequent transfer of ferric to the core via pores formed by subunit symmetry (Bou-Abdallah et al., 2005). Nucleation follows with growth of the mineral core (Koorts and Viljoen, 2007a). Ferritin allows concentration of iron in a protected environment inside cells. A single ferritin molecule can retain more than 2500 atoms of iron (Chasteen and Harrison, 1999; Theil et al., 2006). In *Pyrococcus furiosus*, an anaerobe and hyperthermophilic archaeon (living optimally at 100°C), it was recently found that the hyperthermal stability of ferritin reflects a high number of intramolecular hydrogen bonds that are preserved in ferritin structures (Tatur et al., 2007).

In mammals, ferritin is found in the cytoplasm, mitochondria and nucleus, as well as in blood. Cytoplasmic ferritin is the primary iron storage protein (Recalcati et al., 2008) and consists of heavy (H) and light (L) chain subunits. The H chains have ferroxidase sites and isoforms high in H chains are found in tissues with a demand for iron turnover and access (Bresgen et al., 2007). L subunits contribute to core nucleation and structure, and are found in isoforms in tissues involved in long term iron storage. Knockout of the H subunit is lethal in mammals (Ferreira et al., 2000; Thompson et al., 2003); while mutations of the L subunit result in adverse neurological effects (Levi et al., 2005; Vidal et al., 2008). Nuclear ferritin consists of only the H subunit, while mitochondrial ferritin is composed of an H subunit that is encoded by a separate intronless gene (Koorts and Viljoen, 2007a; Santambrogio et al., 2007). Blood ferritin is found at very low concentrations, and secretion is increased from hepatic cells under conditions of inflammation, iron stimulation and cell damage (Tran et al., 1997; Beard et al., 2006; Yamanishi et al., 2007; Recalcati et al., 2008). Secreted ferritin is relatively iron poor, (Worwood et al., 1976) and consists primarily of glycosylated L chains (Osterloh and Aisen, 1989; Adams and Chau, 1990; Aisen, 1991; Koorts and Viljoen, 2007a). Secretion is a controlled process and the destination of a given subunit is determined co-translationally (reviewed in Recalcati et al., 2008).

Ferritin also has been sequenced and studied in a variety of invertebrates including *Daphnia pulex* (freshwater zooplankton; Colbourne et al., 2007), *Haliotis discus* (disk abalone; De Zoysa and Lee, 2007), *Macrobrachium rosenbergii* (freshwater giant prawn; Qiu et al., 2008), *Branchiostoma belcheri* (lancelet; Li et al., 2008) and several insects (Dunkov and Georgieva, 2006; Missirlis et al., 2006; Strickler-Dinglasan et al., 2006). An early paper by Huebers et al. (1988) reported iron-loaded ferritin in larval hemolymph and the reproductive tract of newly emerged female *Manduca sexta* (tobacco hornworm, Lepidoptera; Huebers et al., 1988). Since that time it has been shown that most orders of insects have an abundance of ferritin in hemolymph and much less in the cell cytoplasm (Dunkov et al., 2002; Vierstraete et al., 2004; Paskewitz and Shi, 2005). The structure of *Trichoplusia ni* (cabbage looper, Lepidoptera) secreted ferritin has been resolved and the 24-subunit cage was found to contain pores that are more open than those of the mammalian ferritins encouraging the movement of iron into and out of the core (Hamburger et al., 2005). Recent work in *Glossina morsitans* (tsetse flies, Diptera) suggests that, in contrast to vertebrates, ferritin in these animals is involved in both iron transport and storage (Strickler-Dinglasan et al., 2006).

Aedes aegypti (yellow fever mosquito, Diptera) express ferritin throughout the life cycle and in most tissues. Mosquito ferritin increases in the ovaries, gut and hemolymph following a blood meal or iron intake (Dunkov et al., 2002; Nichol et al., 2002; Pham et al., 2003). Iron

that is required to meet the needs of reproduction must be rapidly delivered to the ovaries because oviposition occurs three to four days after a blood meal. In our previous work, we found that female mosquitoes are exposed to very high iron loads in the blood meal as heme and ferric-transferrin (Zhou et al., 2007). The majority of heme iron is excreted, but iron supplied as ferric-transferrin is released and ~90% is absorbed by gut tissues, and loaded into ferritin that is secreted into hemolymph. More than 50% of absorbed iron is delivered to the ovaries via ferritin within 24 hours post-blood meal (PBM) indicating that meal iron is required for egg development. Taken together, available evidence supports that mosquito secreted ferritin is an iron transport and a vitellogenic protein. Our overall hypothesis is that interference with secreted ferritin will limit the ability of the mosquito to adjust to the high iron load of the blood meal and decrease iron delivery to the ovaries reducing egg numbers.

A. aegypti CCL-125 larval epithelial cells are a model system for studying the intracellular mechanisms involved in mosquito iron metabolism (Geiser et al., 2003; Geiser et al., 2006; Geiser et al., 2007). Such systems are widely used in mammalian work to discern intracellular iron processing (Koorts and Viljoen, 2007a). The current work was done with three goals in mind: 1) to determine whether ferritin secreted from CCL-125 cells is loaded with iron, 2) to evaluate the effects of extreme iron exposure and iron restriction on iron and ferritin secretion and distribution, and 3) to explore the effects of various inhibitors on ferritin secretion. We found that ferritin secreted from CCL-125 cells is loaded with iron acquired from the culture medium. In contrast to mammalian cells, mosquito cells limit iron exposure by removing excess iron in association with ferritin. Unexpectedly, exposure of mosquito cells to iron restriction did not alter viability or deplete cytoplasmic iron, but reduced iron in the membrane fraction without altering ferritin levels. Finally, we report the effect of various chemical reagents on ferritin secretion.

2. Materials and Methods

2.1. Cell Culture and Experimental Protocol

A. aegypti larval, epithelial cells (CCL-125) were obtained from the American Type Culture Collection (Manassas, VA). Stock cell cultures were maintained as described elsewhere (Geiser et al., 2007) in medium supplemented with 15% heat-inactivated fetal bovine serum (Gemini Bio-Products, Calabasas, CA) and 0.15% antibiotics/antimycotics (Invitrogen), in a water-jacketed incubator (10% humidity, 95% air-5% CO₂ atmosphere, 28°C). All experiments were performed on cells at >80% confluence under sterile conditions. At the start of each experiment the complete medium was removed, the cells were washed twice with Hanks' Balanced Salt Solution (HBSS; Invitrogen) and serum-free, antibiotics/antimycotics-free medium (Geiser et al., 2007) placed on the cells. The cells were incubated for 1 h (28°C) and the medium replaced with fresh serum-free, antibiotics/antimycotics-free medium. For the iron dose experiment, HBSS (control), 100–500 µM ferric ammonium citrate in HBSS (FAC, Sigma, St Louis, MO, 18.3% iron, 1 µg Fe/µg FAC), 200 µM FAC with 200 µM deferoxamine mesylate salt (DES, Sigma) or 500 µM FAC plus 500 µM DES in HBSS (Fe/DES) or 200 µM or 500 µM DES in HBSS was added to the medium and cells were incubated for 18 h (28°C) in vented 75 cm² tissue culture flasks (Corning Incorporated, Corning, NY). For the inhibition of ferritin secretion experiment, 100 µM FAC with either 0.1% ethanol (FAC/EtOH, F/E; control), 10 mg/mL Brefeldin A/EtOH (FAC/BFA, F/B; Sigma), 50 µM CI-976/EtOH (FAC/CI-976, F/C; Tocris Cookson Inc., Ellisville, MO), 10 µM Cytochalasin D/EtOH (FAC/CytoD, F/CD; Sigma), 0.1% HBSS (FAC/HBSS, F/H; control) or 1 nM PLTX-II/HBSS (FAC/PLTX-II, F/P; Alomone Labs Ltd., Jerusalem, Israel) was added to the media and cells were incubated for 18 h (28°C) in vented 75 cm² tissue culture flasks (Corning). Since all cells at the time of harvest do not adhere, the medium was removed from the flask of cells, transferred to a 15 mL conical tube and centrifuged at 900 g (10 min, 4°C). The supernatant was removed, flash frozen

in liquid nitrogen and stored at -80°C for subsequent use. The remaining cells in the flasks were scraped into 5 mL HBSS, added to the cell pellet from the medium and suspended. The cell suspension was centrifuged at 900 g (10 min, 4°C). The supernatant was removed and the cells were suspended in 5 mL fresh HBSS. Cells were harvested and prepared as described previously (Geiser et al., 2007). Aliquots were taken for RNA isolation, measurements of cell viability and total cell number, calcein quench and iron assays as well as protein for Western blot analyses as formerly described (Geiser et al., 2007).

2.2. Cell Viability and Cell Number

Cell viability was determined in triplicate for all flasks by CellTiter 96[®] Aqueous One Solution Cell Proliferation Assay per manufacturer's instructions (Promega Corporation, Madison, WI, USA). Total cell number was measured in triplicate for all flasks by the LIVE/DEAD[®] Viability/Cytotoxicity Assay per manufacturer's instructions (Molecular Probes, Eugene, OR, USA).

2.3. Media, Cytoplasmic Extracts and Membrane Fractions

Media were concentrated five-fold by centrifuging samples in Centricon[®] Ultracel YM-30 centrifugal filter devices (Millipore, Billerica, MA, USA) at 5,000 g (~ 20 min, 4°C), and frozen in small aliquots held at -80°C . Cell extracts were prepared as previously described with some modification (Ausubel, 1998). Briefly, cell pellets suspended in hypotonic buffer and stored at -80°C were thawed on ice and homogenized. The cell suspensions were centrifuged at 100,000 g (30 min, 4°C) to remove debris and membranes. Cytoplasmic extract supernatants were frozen in small aliquots held at -80°C . The remaining pellet was suspended in hypotonic buffer (Geiser et al., 2007) and frozen in small aliquots held at -80°C until use. These pellets contain plasma membranes, Golgi and other cell membrane components; they are referred to hereafter as the membrane fraction. All protein concentrations were determined by the method of Bradford (1976), except the membrane fraction protein that was determined by SDS-Lowry (Markwell et al., 1978).

2.4. Iron Determination

Iron Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) was conducted as described elsewhere (Geiser et al., 2007). MilliQ H₂O and 1% HNO₃ were used as negative controls. The Elan Dynamic Reaction Cell (DRC) II (Perkin ElmerSCIEX, Shelton, CT, USA) was used to quantitate iron in our samples. The DRC compartment was purged with NH₃ to avoid polyatomic complexes formed by the presence of argon. To reduce oxide formation, a hotter than normal plasma was used. The temperature of the argon plasma is a function of the flow rate of the nebulizer gas. If the flow is increased (i.e. 1 L/min or higher) the situation is referred to as a "cool plasma," though it can still be close to 6000°C . The lower flow rates (i.e. 0.9 L/min or less) is referred to as "hotter plasma." Our samples were run at 0.82 L/min, with minor adjustments optimized before each set of samples were analyzed. Oxide formation was monitored by the CeO/Ce ratio. The ICP-MS readings were corrected to $\mu\text{g Fe}/\mu\text{g total protein}$.

Calcein fluorescent quench was used to measure iron uptake into cells and measured in triplicate for all flasks by the LIVE/DEAD[®] Viability/Cytotoxicity Assay per manufacturer's instructions (Molecular Probes) by adding calcein to a final concentration of 1 μM and incubating for 45 min (28°C).

2.5. Western blots

Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) 17% or 18.5% homogeneous slab gels, run at 60 volts overnight (~ 1000 Vh, 4°C) or 4–20% Native-PAGE gradient slab gels run at 100 volts for 3 h (300 Vh, RT). Cytoplasmic

extracts and membrane fractions were loaded at 15 µg total protein and concentrated media samples at 3 µg total protein or as indicated. Since no endogenous secretory protein is available as a loading control, FLAG protein (Sigma) at 100 ng was added to the media samples as an exogenous loading control. Purified *A. aegypti* ferritin (1 µg, Geiser et al., 2003) served as a positive control for ferritin. Cytoplasmic extract (15 µg total protein) of human type II alveolar cells (A549, ATCC), served as a positive control for actin. Purified FLAG-containing bacterial alkaline phosphatase (100 ng, Sigma) served as a positive control for FLAG. Secreted proteins were visualized by Coomassie blue staining of the gel. Proteins were transferred to nitrocellulose membranes as formerly described (Geiser et al., 2007) and transfer was confirmed by SYPRO[®] Ruby protein blot stain (BIORAD, Hercules, CA, USA) or Ponceau staining and Kaleidoscope molecular mass markers (BIORAD). The nitrocellulose membranes were cut for analysis of the individual proteins. Specific proteins were detected as previously described (Geiser et al., 2007): ferritin using anti-*A. aegypti* ferritin-specific rabbit serum (1:4000 v/v, a kind gift from Dr. John Law) and FLAG using anti-FLAG-specific rabbit serum (1:4000 v/v; Sigma) with anti-rabbit alkaline phosphatase conjugate antibody (1:1000 v/v; Jackson Immuno, West Grove, PA, USA); actin using anti-β-actin-specific mouse serum (1:2000 v/v; Abcam, Cambridge, MA, USA) with anti-mouse HRP conjugate antibody (1:2000 v/v; Pierce, Rockford, IL, USA). Digital images were accessed using the VersaDoc[™] 3000 Imaging System (BIORAD) and quantified with Quantity One 4.5.1 software (BIORAD). Data were analyzed by densitometry of a defined volume of all the ferritin subunits, FLAG and actin proteins and corrected for background. Ferritin concentration was evaluated by taking its volume in ratio to an exogenous (FLAG) or endogenous (actin) loading control. Volume units are arbitrarily assigned.

2.6. Real-Time RT-PCR

Total RNA was isolated from $\sim 1.5 \times 10^6$ CCL-125 cells using the RNeasy[®] Mini Kit (Qiagen Inc., Valencia, CA, USA). Purified total RNA was treated with DNase (Invitrogen) according to the manufacturer's instructions. Reverse transcription was done according to the manufacturer's instructions using SuperScript[™] II RNase H⁻ Reverse Transcriptase (Invitrogen). The primers for the PCR reactions were designed to obtain specific PCR products of similar size for the ORF of each message: Ferritin Heavy Chain (198 bp): 5'-ccagggccaggagacaacag-3' and 3'-tcaaaaagaaggtgcggcgg-5'; Ferritin Light Chain (166 bp): 5'-tttcaccgccagtttttctca-3' and 3'-gacatggcgttctagaggctg-5'; S7 (202 bp): 5'-ccaggctatcctggagttg-3' and 3'-caagaggccgttcgtgcag-5'. Real-time RT-PCR reactions were conducted using iQ[™] SYBR[®] Green Supermix (BIORAD) with the buffers provided at: 94°C, 3 min, 1 cycle; 94°C, 10 s; 60°C, 30 s and 72°C, 30 s, 40 cycles; with a melt curve over a temperature range starting at 55°C and ending at 95°C in a MyiQ[™] Cyclor (BIORAD). PCR product quality was monitored using post-PCR melt curve analysis. While a standard curve for each product showed the experimental samples fell within the linear range. Data analysis for fold change was quantified using the Pfaffl method to calculate for relative quantification (Pfaffl, 2001). All PCR products were cloned and sequenced by the Genomic Analysis and Technology Core Facility at the University of Arizona (<http://gatk.arl.arizona.edu/about/equipment/sequencing.php>) to determine that the product sequence represents that of the desired message (Geiser et al., 2003).

2.7. ⁵⁹Fe Pulse-Chase Experiment

Conversion of FeCl₃ or ⁵⁹FeCl₃ (PerkinElmer Life and Analytical Sciences, Boston, MA, USA) to ferric citrate or ⁵⁹ferric citrate was accomplished by mixing ferric chloride with sodium citrate in 1:50 molar ratio and incubating for 1 h at RT.

CCL-125 larval cells were maintained and prepared as described in section 2.1. Following the 1 h incubation as described in section 2.1, the medium was replaced with fresh serum-free

medium with 0.15% antibiotics/antimycotics (Invitrogen) and either 33.6 nM ferric citrate and 100 μ M FAC in HBSS (Fe/FAC) or 33.6 nM 59 ferric citrate (0.9 μ Ci) and 100 μ M FAC in HBSS (59 Fe/FAC) and incubated for 4 h (28°C) in vented 25 cm² tissue culture flasks (Becton Dickinson Labware, Franklin Lakes, NJ, USA). After incubation the medium was removed and the cells were washed three times with HBSS. Fresh serum-free medium with 0.15% antibiotics/antimycotics (Invitrogen) was placed on the cells with 100 μ M FAC and incubated an additional 14 h (28°C). Cells and media were harvested as described in section 2.1. Aliquots of cells were taken for cell viability, total cell number and calcein quench assays described in sections 2.2. and 2.4. The remaining cells were centrifuged at 17,000 g (1 min, 4°C). The supernatant was removed and the cell pellet was prepared for cytoplasmic extracts and membrane fractions as described in section 2.3.

Proteins were resolved by a Native-PAGE 4–20% gradient slab gel, run at 90 volts overnight (~1300 Vh, RT). Membrane fractions were loaded at 10 μ g total protein and concentrated media samples at 3 μ g total protein. One gel was transferred to a nitrocellulose membrane and the other gel was stained by SYPRO[®] Ruby gel stain (BIORAD) according to the manufacturer's instructions (see section 2.5). Ferritin was detected as described in section 2.5. The nitrocellulose membrane was exposed to BioMax MR film (Eastman Kodak Company, New Haven, CT, USA) to detect proteins associated with 59 Fe. The SYPRO[®] Ruby stained gel was dried and proteins associated with 59 Fe were assessed by autoradiography exposure to BioMax MR film (Eastman Kodak Company). Digital images were obtained using the VersaDoc[™] 3000 Imaging System (BIORAD).

2.8. Immunofluorescent Microscopy

CCL-125 cells grown on glass coverslips were treated as described in section 2.1 for 18 h. After treatment, the cells were rinsed in HBSS and staining was done on live cells using BODIPY TR C₅-ceramide (Invitrogen) for 30 min at 28°C. In mammals, this ceramide analog produces selective staining of the Golgi complex for visualization by fluorescence microscopy (Pagano et al., 1991; Celis, 1998; Cooper et al., 1999). However, ceramide can be found along with its catabolic metabolite, sphingosine, in association with other organelles (i.e. ER, mitochondria, nucleus). Ferritin staining was performed on the BODIPY TR C₅-ceramide stained cells after they were fixed in 2% paraformaldehyde/HBSS for 30 min at RT and then permeabilized with 0.2% Triton X-100 (Sigma). To reduce non-specific binding of antibodies, the cells were incubated in 2% BSA/1% donkey serum/PBS for 30 min at RT. Ferritin was detected using anti-A. *aegypti* ferritin-specific rabbit serum (1:150 v/v, a kind gift from Dr. John Law) for 1 h at RT and washed twice in HBSS for 15 min each. The primary antibody incubation was followed by incubating the coverslips in Cy 2 TM conjugated goat anti-rabbit IgG (Pierce) at a dilution of 1:200 for 45 min at RT. Cells were analyzed on a Leica TCS 4D laser scanning confocal microscope (Bio5, The University of Arizona) using a 100x (n.a. 1.4) oil immersion objective with simultaneous two channel recording. Images were derived from a single optical section. (We are unable to visualize DAPI stain for nuclei with the available microscope.) Cell fluorescence was imaged by LaserSharp2000 (BIORAD). Images were processed using ImageJ freeware version 1.42e (Collins, 2007).

2.9. Statistics

Data for protein and 59 Fe-associated protein were analyzed by autoradiography and the VersaDoc[™] 3000 Imaging System (BIORAD) and quantified with Quantity One 4.5.1 software (BIORAD). Data for subunit messages was collected by MyiQ[™] Cyclor (BIORAD) and analysis for fold change was according to the Pfaffl method (Pfaffl, 2001). Treatment differences were determined by one-way analysis of variance using the Tukey's multiple comparisons test or one-tailed unpaired t-test for comparison of selected data sets (Graph Pad

Software, Inc., San Diego, CA, USA). These experiments were conducted in triplicate and the data for a given variable were analyzed at the same time.

3. Results

3.1. CCL-125 cells secreted iron-loaded ferritin

We previously demonstrated that mosquitoes secrete ferritin into hemolymph that is loaded with iron taken in the blood meal as ferric-transferrin (Zhou et al., 2007). We also reported that CCL-125 cells secrete ferritin in response to iron dose (0–100 μ M FAC; Geiser et al., 2006). In order to continue to use these cells as a model, it is imperative that ferritin secreted in response to iron exposure be loaded with iron taken into cells from the culture medium. To evaluate this, we pulsed CCL-125 cells with ^{59}Fe -FAC for 4 h and chased with unlabeled FAC for 14 h. Numbers (Figure 1A) and viability of ^{59}Fe -FAC-treated cells (Fig. 1B) were >80% that of controls. Fig. 1C shows that Fe entered the cells. Calcein AM enters the cell cytoplasm where esterases cleave the molecule resulting in fluorescence. Although the mechanism is not known, labile iron prevents this reaction, and quench reflects iron levels of the labile pool (Ali et al., 2003; Tenopoulou et al., 2007). Samples from three separate cultures documented that ^{59}Fe that entered the cells from the culture medium during the pulse phase was associated with ferritin in the membrane fraction and secreted ferritin following the chase (Fig. 1D–E). Western blot and autoradiography confirmed the presence of ^{59}Fe -ferritin in the culture medium (Fig. 1F). These data indicate that CCL-125 cells take up iron from the culture medium, and it is subsequently found associated with membrane ferritin and secreted ferritin. This supports that CCL-125 cells can be used as model of mosquito intracellular iron metabolism with relevance to animals; it advances the hypothesis that the secretion of iron-loaded ferritin from mosquito cells in response to iron exposure is likely a mechanism used to prevent intracellular iron-overload.

3.2. Membranes are the primary site of iron uptake, turnover and removal in CCL-125 cells

Mosquito adult males receive very little dietary iron, while females receive a high iron load. Given these extremes, we evaluated the effects of excess iron as well as iron restriction on viability and ferritin levels of CCL-125 cells. Pig blood contains 0.6 μg Fe/ μl . Although female gut iron concentrations are unknown, we would expect concentrations to significantly increase as the animal undergoes diaphoresis during feeding. In our previous work, we evaluated the response of CCL-125 cells to 0–100 μM FAC (0–6 μg s iron/ μl) which is at maximum 10 times the concentration of iron in blood. For these studies, we exposed cells to 20 (200 μM FAC) and 50 (500 μM FAC) times these iron levels. Untreated cells, cells exposed to 100 μM FAC and cells exposed to FAC and DES served as negative, positive and rescue controls. The administration of DES, an iron-specific chelating agent, has been shown to deplete mammalian cells of iron (Richardson and Baker, 1992). We administered DES at two concentrations to CCL-125 cells in order to limit iron availability.

Cell numbers did not vary by more than 8% from those of untreated cells (Fig. 1SA); cell viability was >80% that of control cells for all treatment groups (Fig. 1SB). Although the addition of iron modestly compromised cell viability in a non-dose responsive manner, iron restriction did not decrease viability at either DES concentration.

Similar to findings in mammalian cells (Ali et al., 2003; Tenopoulou et al., 2007), calcein fluorescence of CCL-125 cells decreases significantly with exposure to iron at all concentrations relative to control cells ($p < 0.0001$, Fig. 2A). However, quench is maximal at 100 μM FAC and exposure to higher iron concentrations does not further expand the labile iron pool. The addition of DES to FAC rescues fluorescence indicating iron is responsible for quench. DES alone fails to enhance fluorescence suggesting very low levels of labile iron are

normally present in cytoplasm in the absence of an iron stimulus. Since it is not possible to quantify iron from calcein fluorescence analyses and these data indicate only labile iron, we measured total cytoplasmic iron by ICP-MS. In agreement with calcein fluorescence, total cytoplasmic iron increases significantly with medium iron concentration, but is dose responsive to 200 μ M FAC (Fig. 2B), and then plateaus. Also in agreement with calcein quench data, DES at either concentration does not significantly reduce total cytoplasmic iron. Taken together, these data support the presence of two cytoplasmic iron pools. In the absence of iron exposure, cytoplasmic iron of mosquito cells is very low and contained such that it is inaccessible to chelating by DES. Although exposure to high concentrations of iron increases a labile cytoplasmic iron pool, cytoplasmic iron remains low and is maximal at iron concentrations of less than 500 μ M FAC.

Membrane iron also is very low in untreated cells, but increases directly with iron exposure (Figure 2C). In contrast to cytoplasmic iron, membrane iron levels increase dramatically, by more than two orders of magnitude, when cells are exposed to 200 μ M FAC. Surprisingly, exposure to 500 μ M FAC results in levels that are comparable to those of cells exposed to 100 μ M FAC. Also unexpectedly, we found that membrane iron is accessible to DES and depleted by DES treatment in a dose responsive manner to levels below those of control cells. Taken together from our data we conclude that mosquito cells take up iron from the culture medium and distribute it primarily to a membrane iron pool that expands with iron exposure, is finite and is accessible to chelating agents. When iron retained in the membrane pool reaches a maximum, as occurs with exposure to concentrations >200 μ M FAC, it is removed from the cells. Although exposure to very high levels of iron also could result in limiting iron uptake, our data show a greater increase in iron levels for cells exposed to 200 μ M FAC than for cells exposed to 500 μ M FAC indicating iron loss from the membrane pool, presumably in association with secreted ferritin.

3.3. Iron alters CCL-125 cell ferritin distribution and levels

In our previous work, we reported that CCL-125 cells increase ferritin in response to iron dose administered in the range of 0–100 μ M FAC. Here we observe that cytoplasmic ferritin is low in the absence of an iron stimulus and is unresponsive to DES at either concentration. Cytoplasmic ferritin increases significantly and directly with iron dose relative to untreated cells ($p<0.02$, Fig. 3A).

In contrast, membrane ferritin levels remain relatively stable in the presence of high levels of iron or its relative absence (Fig. 3B). Despite the dramatic increase in membrane iron we observed (Fig. 2C), membrane ferritin was only modestly increased (Fig. 3B). Further, although membrane iron was reduced by DES, membrane ferritin was not. These data indicate that the membrane ferritin pool is maintained irrespective of membrane iron levels and that some factor other than iron is the primary stimulus for maintaining this ferritin pool. The modest increase in membrane ferritin in response to high levels of iron suggests that these cells maintain sufficient membrane ferritin in the absence of iron to meet the iron storage or transport requirements imposed by a high iron load.

Membrane ferritin also remains stable in the presence of a significant increase in ferritin secretion from the cells following iron exposure ($p<0.0004$ relative to control cells, Fig. 3C). In our previous work, we found a low dose (50 μ M FAC; Geiser et al., 2006) results in maximum ferritin secretion. Here we show that ferritin secretion was not iron-dose responsive, even following exposure to very high iron levels, but is abrogated by the addition of DES to FAC indicating iron is a stimulus for ferritin secretion. On the other hand, in the absence of iron, ferritin secretion is low but constitutive, and unhindered by treatment with DES. We interpret these data to indicate that in the absence of iron low levels of ferritin are secreted and

iron is not the stimulus for constitutive secretion. However, iron exposure of CCL-125 cells increases iron in the membranes and stimulates ferritin secretion.

A. aegypti ferritin consists of subunits of 24, 26, 28 and 30- kDa (Dunkov et al., 1995). The 24 and 26 kDa subunits are homologues of the mammalian H chain (heavy chain homologue (HCH); Dunkov et al., 1995), and subunit synthesis is subject to transcriptional and translational control (Geiser et al., 2006). The 28 kDa subunit is considered a light chain homologue (LCH; Geiser et al., 2003) and synthesis reflects mRNA levels (Geiser et al., 2003; Pham and Chavez, 2005; Geiser et al., 2006; Geiser et al., 2007).

Cytoplasmic ferritin consists of both subunits, while in membrane and secreted ferritin the HCH predominates. Since cytoplasmic ferritin mirrors cytoplasmic iron levels, but membrane ferritin does not, we analyzed transcript levels for the LCH and HCH (Fig. 4A–B). LCH transcript increases in an iron-dose responsive manner to about 9-fold that of control cells and becomes stable at concentrations above 200 μ M FAC; levels reflect total cytoplasmic iron (Fig. 2B); whereas, HCH transcript increases approximately 3-fold with iron exposure and is greatest at 500 μ M FAC (Fig. 4B). The HCH, but not the LCH, message has an iron responsive element (IRE) in 5'-UTR (Dunkov et al., 1995). The mosquito iron regulatory protein (IRP) interaction with this element represses HCH translation and HCH levels reflect, in part, this level of translational control (Geiser et al., 2006; Zhou et al., 2007). It would seem that in the absence of iron, maintenance of the HCH for constitutive secretion represents balanced translational control, while HCH synthesis in response to iron requires an up-regulation of transcription (Pham et al., 2005), as well as de-repression of synthesis by the mosquito IRP (Geiser et al., 2006). In agreement with total cytoplasmic iron levels, in the absence of iron or in the presence of DES, both transcripts are maintained at low levels.

3.4. Mosquito ferritin is found in vesicles in several cell locations following iron stimulation

Ferritin of mammals and some insects is found in cytoplasm. Although we found a cytoplasmic pool, database analysis indicated that the ferritin subunits of *A. aegypti* are targeted to different cellular organelles. We were curious as to the location of ferritin in mosquito cells. Previously, we reported the coalescence of ferritin with a ceramide marker in CCL-125 cells as determined by fluorescence microscopy. We evaluated untreated cells or cells treated with iron by confocal imaging. Images show that in the absence of iron, as expected, ferritin (green, Fig. 5A) in CCL-125 cells is coalesced (yellow, Fig. 5C) primarily with organelles containing ceramide (Fig. 5B, red), but also is present at the edges of the cell in the absence of ceramide (Fig. 5C, green). Further, there is coalescence (Fig. 5C, yellow) of ferritin and the fluorescent ceramide marker in the nucleus where ferritin subunits have been observed by others (Surguladze et al., 2005). However, following iron treatment, ferritin (green, Fig. 5D) increases and is found coalesced with ceramide (Fig. 5F, yellow, upper white arrow), in cytoplasmic, non-ceramide vesicles (Fig. 5F, green, middle white arrow) and on the margins of the cell also without ceramide (Fig. 5F, green, lower white arrow).

3.5. CI-976 inhibits ferritin secretion from CCL-125 cells and reduces cell viability

The absence of the typically diffuse staining and the presence of discrete vesicles indicate that CCL-125 cytoplasmic ferritin is contained in vesicles in cytoplasm. Further, the cytoplasmic isoform is distinct as it contains a significant portion of LCH subunits (Geiser et al., 2006; Geiser et al., 2007; Fig. 3). In order to shed some light on the pathways involved in ferritin secretion and to obtain a secretion blocking reagent for further studies, we evaluated the capacity of different chemicals to block secretion of ferritin.

None of the inhibitors reduced cell numbers (Fig. 6A). CI-976, a small, hydrophobic, membrane-permeant compound which inhibits COP II vesicle budding from the ER (Chambers

and Brown, 2004; Chambers et al., 2005), reduced cell viability by more than 50% relative to control cells ($p < 0.0001$), while CytoD, a fungal metabolite that inhibits actin polymerization in the cell cytoskeleton (Ramm et al., 1994), reduced viability by 25% ($p < 0.0006$; Fig. 6B). None of the reagents we tested altered intracellular ferritin levels significantly, except PLTX-II, a spider venom toxin which inhibits exocytosis by blocking a primary presynaptic voltage-gated Ca^{2+} channel in *Drosophila melanogaster* (fruit fly, Diptera) motor-nerve terminals (Kuromi and Kidokoro, 2005; $p < 0.05$; Fig. 7A, upper panel). Analysis of ferritin from three separate samples of culture medium showed that only CI-976 effectively blocked secretion, while secretion was partially inhibited by CytoD (Fig. 7B, upper panel). The presence of FAC in control cell medium established 100% quench (Fig. 6C), and our data show that both BFA, a fungal metabolite that disrupts the Golgi apparatus by inhibiting COP I vesicle formation (Dinter and Berger, 1998; Ghosh et al., 2004; Donaldson et al., 2005), and CytoD sequestered a portion of the available iron reducing quench. Despite this, sufficient iron entered the cells exposed to these reagents to provoke ferritin secretion and to establish that the reagents did not block this process (Fig. 7B, upper panel). Enhanced calcein quench is observed with CI-976 treatment (Fig. 6C). This could suggest that inhibition of ferritin secretion increases the levels of iron in a pool that is available to Calcein AM, or alternatively, it could reflect reduced cell viability (Fig. 6B). However, media proteins resolved on Native-PAGE gels that were subsequently stained with Commassie blue (Fig. 7B, lower panel) indicates that the cells continued to secrete protein in the presence of CI-976 and suggest that inhibition of ferritin secretion by CI-976 was selective.

4. Discussion

Mosquitoes transmit devastating diseases that kill millions of people each year (Jacob, 2001; Edmunds, 2005). Since disease transmission efficiency is low, rates reflect the great numbers of mosquito vectors in endemic regions. Female mosquitoes blood feed in order to complete their life cycle. These species are unusual in that in the blood meal the adult female encounters a high iron load, while adult males receive only low levels of iron supplied in waters and nectars (Nichol et al., 2002; Winzerling and Pham, 2006). In *D. melanogaster*, ferritin is crucial for iron balance and the absence of either HCH or LCH subunits results in embryonic lethality (Missirlis et al., 2007). We are interested in mapping how the female mosquito processes the iron from a blood meal as a means to develop control strategies and to hinder egg development.

Our data indicate that in the absence of an iron stimulus, mosquito cells maintain low levels of ferritin in cytoplasmic vesicles and in the membrane fractions and constitutively secrete ferritin. When presented with a high iron load, CCL-125 cells take up iron and distribute a minor amount to the cytoplasm, but retain the majority in the membrane fraction, at least a portion of which is in association with ferritin. Our data also indicate that although ferritin secretion is increased at low levels of iron exposure, iron taken into mosquito cells is allowed to accumulate in membranes to a finite level, above which it is removed in association with secreted ferritin. Ferritin secreted from the membrane compartment is replaced, and synthesis is sufficient to maintain ferritin levels in the membrane fraction in the presence of low constitutive secretion and to increase secretion in response to iron stimulation. These findings support our previous work in mosquitoes where iron taken in the blood meal is loaded into ferritin that is secreted into hemolymph and delivered to the eggs and ovaries. These data agree with observations in *D. melanogaster*, where constitutive expression of ferritin in the midgut occurs in the absence of iron stimulation (Missirlis et al., 2007). Taken together available evidence supports, that in contrast to mammalian cells which limit iron entry, mosquito cells and those of other insects, likely maintain iron homeostasis by secreting iron-loaded ferritin. Thus, secreted ferritin, in addition to serving as the primary iron transport protein, could play a role in preventing cellular iron overload, and thereby in limiting iron-mediated oxidative stress (Geiser et al., 2006). We suggest that this depot serves as the primary site of iron storage.

We speculate that cytoplasmic ferritin serves a role in making iron available for use by the cells, and in this respect, mosquito cells are similar to those of mammals.

It is intriguing to think of ferritin as an antioxidant because of its capacity to sequester vast quantities of iron in a non-toxic form. In mammals, induction of ferritin expression by oxidative-stress was observed in the liver and erythroid cells of mice (MacKenzie et al., 2008). In some disease states characterized by an increase in reactive oxygen species (ROS), ferritin H subunit is preferentially up-regulated to allow for H-rich ferritin isoforms to accumulate and ferritin rich in H subunits increases endothelial cell resistance to oxidative challenges by rapidly sequestering iron (Balla et al., 2007; Koorts and Viljoen, 2007b). These observations could help explain why we detect little ROS formation in CCL-125 cells treated with iron (Geiser et al., 2006) as large amounts of ferritin, rich in HCH, are present in the membranes.

Results for iron deprivation as imposed by DES were surprising. DES is a siderophore produced by *Streptomyces pilosus* that specifically chelates ferric (De Domenico et al., 2006; Kidane et al., 2006). When administered to mammalian cells, it lowers cytoplasmic iron and ferritin levels (De Domenico et al., 2006; Kidane et al., 2006; Sammarco et al., 2008). DES at the concentrations used here did not reduce cytoplasmic iron or ferritin of mosquito CCL-125 cells. Although this could indicate that DES access to cytoplasmic ferritin is restricted in mosquito cells, it seems more likely that in the absence of an iron challenge, very little iron is present in a labile cytoplasmic pool in these cells. This notion is supported by the iron ICP-MS data as well as the very low levels of cytoplasmic ferritin. If DES crosses membranes directly or enters cells by way of endocytosis remains a subject of some debate. However, our data indicate that it enters the membrane fraction of CCL-125 cells and decreases membrane iron. This implies that iron in the membrane fraction is susceptible to DES chelation. How this occurs we do not know. Possibly, DES enhances membrane ferritin degradation with iron release. Alternatively, labile iron pools accessible to DES could exist in the membrane fractions. If this is the case, then the form of retention must be such that oxidative stress is avoided. It is interesting that membrane, but not cytoplasmic, iron was reduced by DES. If our hypothesis is correct and cytoplasmic ferritin serves to supply the cells with iron when needed, then these cells could protect against the potential for intracellular iron deprivation from invading pathogens by iron storage in cytoplasmic ferritin in a location impervious to bacterial siderophores (Ong et al., 2006).

Ferritin secreted in response to iron exposure at lower iron levels was sufficient to store and to remove iron that was taken into the cells at higher iron levels. This no doubt reflects the capacity of ferritin to accommodate numerous atoms of iron (Chasteen and Harrison, 1999). We found that ferritin, not transferrin, is the primary transport protein of mosquitoes (Zhou et al., 2007). More than half the iron absorbed from the blood meal goes to the ovaries and eggs indicating iron demand for the development process. Transport of iron via ferritin rather than transferrin would assure delivery of high levels of iron in a form that precludes iron-mediated oxidative stress and can be easily accessed.

The various ferritin isoforms of mosquito cells reflect the presence of constitutive HCH and LCH transcript, as well as up regulation of transcription with iron stimulation (Pham and Chavez, 2005; Pham et al., 2005). Our data indicate that ferritin in CCL-125 cells is located in membranes associated with ceramide. The mosquito HCH and LCH have targeting signals for secretion (Dunkov et al., 1995; Geiser et al., 2003) and both are post-translationally processed. Missirlis et al. (2007) reported that 1 h post iron feeding of *D. melanogaster*, GFP-tagged HCH was induced and found in compartments resembling the ER, while GFP-tagged LCH was observed in Golgi. At 4 h post-iron induction, there was robust expression of both GFP-HCH and GFP-LCH and the two subunits were in complex with each other in Golgi. Ferritin

associated with the Golgi could represent primarily the LCH or both subunits, while that found in vesicles and at the cell margins could reflect primarily the HCH. This latter notion would be in agreement with the findings in *D. melanogaster*. The location of the various subunits in mosquito tissues remains to be studied using antiserum specific for either subunit.

Ferritin secretion was not inhibited by BFA or by other COPI vesicle inhibitors that we tested (monensin and nocodazole, data not shown). However, secretion was blocked by CI-976. CI-976 promotes tubulation of the Golgi with return of proteins to the ER and disrupts membrane trafficking in eukaryotic cells by inhibiting COPII vesicle fission from the ER at ER exit sites (Drecktrah et al., 2003; Brown and Schmidt, 2005; Chambers et al., 2005; Brown et al., 2008). CI-976 blocks bud fission by inhibiting a lysophospholipid acyl transferase (LPAT) that transfers an acyl group from a donor to a lysophospholipid (Chambers and Brown, 2004). In contrast to other reagents, CytoD inhibits transport by interference with microfilament associated transport and partially reduced ferritin secretion (Ramm et al., 1994). The movement of cargo from the ER or SER destined for different locations in the cell is governed by proteins that modulate the function of COPII-based cargo selection and vesicle formation (Tang et al., 2005). Sorting and targeting can occur distal or proximal to the Golgi complex and also directly from the ER or SER to the plasma membranes. Our confocal images support that both the Golgi and COPII vesicles are involved in ferritin processing, but our secretory studies indicate that inhibition of COPI vesicle transport will not deter secretion. Thus, we speculate that ferritin that consists of both subunits exists in the cytoplasm in discrete, non-ceramide containing particles in the absence of iron represents ferritin that stores iron for use by the cells. We suggest that ferritin that consists predominately of HCH is that associated with COPII vesicles, and such is subsequently loaded with iron and secreted following iron stimulation. If our speculations are correct, then ferritin assembly and iron loading likely occurs prior to vesicle distribution from the ER. Further, definitive pulse-chase experiments as well as imaging studies with other markers and subunit specific antiserum are needed to elucidate the intracellular pathway of ferritin secretion. Although our data indicate the presence of iron-loaded ferritin in the membrane fraction, studies examining where iron loading occurs also are beyond the scope of the present work. However, our findings do support the early work of Locke (2003) whose electron microscopy studies indicated that iron is loaded directly into ferritin in ER or SER, moved to the plasma membrane and secreted (Locke, 2003).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

H	heavy
L	light
PBM	post-blood meal
FAC	

	ferric ammonium citrate
DES	deferoxamine mesylate salt
HBSS	Hanks' balance salt solution
BFA	Brefeldin A
EtOH	ethanol
CytoD	Cytochalasin D
HCH	heavy chain homologue
LCH	light chain homologue
LPAT	lysophospholipid acyl transferase

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Figure 1. *A. aegypti* larval cell ferritin is present in the membrane fraction and secreted into the medium loaded with iron

CCL-125 cells were treated with 33.6 nM ferric citrate and 100 μ M FAC (Fe/FAC, control) or 33.6 nM ⁵⁹ferric citrate (0.9 μ Ci) and 100 μ M FAC (⁵⁹Fe/FAC) and incubated for 4 h. Cells were washed and subsequently treated with 100 μ M FAC for an additional 14 h. Media and cells were recovered as described in the methods. Protein samples were resolved by a Native-PAGE 4–20% as previously described. (A–C) Cell number, viability and calcein fluorescent quench are similar between Fe/FAC and ⁵⁹Fe/FAC-treated cells. Cell number by ethidium bromide fluorescence, cell viability by Formazan absorbance and calcein fluorescent quench were assayed as described in the methods. *Significantly different relative to control ($p < 0.001$). (D) SYPRO® Ruby gel stain of proteins from the membrane fraction (Mb, 10 μ g protein) and concentrated media samples (3 μ g protein). (E) The gel in D was dried and ⁵⁹Fe assessed by autoradiography with exposure to BioMax MR film. (F) Iron-loaded ferritin in culture medium. (Lane 1) Western blot of culture medium with ⁵⁹Fe detected by autoradiography with exposure to BioMax MR film as described in the methods. (Lane 2) Ferritin subsequently detected by Western blot using anti-*A. aegypti* ferritin-specific rabbit serum as described in the methods. M = molecular mass markers (BIORAD). Graphed data represent means $\pm$ SEM of triplicates.

Figure 2. Iron treatment alters iron levels of CCL-125 cells

CCL-125 cells were treated with 0, 100–500 µM FAC, 200 or 500 µM FAC/DES (F/D), and 200 or 500 µM DES (D) for 18 h. **(A)** Iron is taken up by cells exposed to iron as measured by calcein fluorescent quench. *Significantly different relative to 0 ($p < 0.0001$). ^aSignificantly different relative to 200 µM FAC ($p < 0.0001$). ^bSignificantly different relative to 500 µM FAC ($p < 0.0001$). **(B)** Cytoplasmic iron levels increase in response to iron exposure as determined by iron ICP-MS. *Significantly different relative to 0 ($p < 0.04$). [∞]Significantly different relative to 100 µM FAC ($p = 0.054$). ^aSignificantly different relative to 200 µM FAC ($p < 0.005$). **(C)** Iron treatment alters membrane iron levels as determined by iron ICP-MS. *Significantly different relative to 0 ($p < 0.02$). [∞]Significantly different relative to 100 µM FAC

($p < 0.0002$). ^ΦSignificantly different relative to 200 μ M FAC ($p < 0.0009$). ^aSignificantly different relative to 200 μ M FAC ($p < 0.0001$). ^bSignificantly different relative to 500 μ M FAC ($p < 0.0002$). Data represent means $\pm$ SEM of triplicates.

Figure 3. Cytoplasmic, membrane and secreted ferritin increases in response to iron dose
 CCL-125 cells were treated with 0, 100–500 μ M FAC, 200 or 500 μ M FAC/DES (F/D), and 200 or 500 μ M DES (D) for 18 h. All protein samples were resolved by 17% SDS-PAGE and transferred to nitrocellulose membranes as described in the methods. **(A)** Ferritin and actin in cytoplasmic extracts (15 μ g protein) were detected by Western blot. *Significantly different for relative to 0 ($p < 0.02$). ^aSignificantly different relative to 200 μ M FAC ($p = 0.0557$). ^bSignificantly different relative to 500 μ M FAC ($p < 0.04$). **(B)** Ferritin and actin in membrane fractions (15 μ g protein) were detected by Western blot. *Significantly different relative to 0 ($p < 0.05$). ^bSignificantly different relative to 500 μ M FAC ($p = 0.0518$). **(C)** The medium was recovered and concentrated. Ferritin and FLAG in media samples (3 μ g protein)

were detected by Western blot. *Significantly different relative to 0 ($p<0.0004$). ^aSignificantly different relative to 200 μ M FAC ($p<0.003$). ^bSignificantly different relative to 500 μ M FAC ($p<0.0006$). Ferritin was detected using anti-*A. aegypti* ferritin-specific rabbit serum, endogenous actin was detected using anti- β -actin-specific mouse serum and exogenous FLAG was detected using anti-FLAG-specific rabbit serum. Purified *A. aegypti* ferritin (1 μ g) was used as a ferritin positive control (+); A549 cell cytoplasmic extract (15 μ g protein) served as a positive control for actin (A); purified FLAG-containing bacterial alkaline phosphatase (100 ng, Sigma) served as a positive control for FLAG (F). M = molecular mass markers (BIORAD). A representative Western blot is shown above the graphed data. Graphed data represent means $\pm$ SEM of triplicates.

Figure 4. Real-time RT-PCR analysis of mRNA for LCH and HCH

CCL-125 cells were treated with 0, 100-500 μ M FAC, 200 or 500 μ M FAC/DES (F/D), and 200 or 500 μ M DES (D) for 18 h. Ferritin subunit homologue mRNA levels were evaluated using real-time RT-PCR with S7 ribosomal RNA as an endogenous control for total RNA as described in the methods. (A) LCH mRNA increases in a dose dependent manner.

*Significantly different relative to 0 ($p < 0.02$). [∞]Significantly different relative to 100 μ M FAC ($p < 0.05$). ^aSignificantly different relative to 200 μ M FAC ($p < 0.02$). ^bSignificantly different relative to 500 μ M FAC ($p < 0.005$). (B) HCH mRNA increases and plateaus at the lowest iron concentrations. *Significantly different relative to 0 ($p < 0.006$). ^aSignificantly different relative to 200 μ M FAC ($p < 0.003$). ^bSignificantly different relative to 500 μ M FAC ($p < 0.002$).

Graphed data represent means $\pm$ SEM of triplicates.

Figure 5. Confocal images for localization of ferritin and ceramide-containing membranes in CCL-125 cells

CCL-125 cells were treated with 0 or 100 μM FAC for 18 h. Membranes were identified with BODIPY TR C_5 -ceramide (red) and ferritin with anti-*A. aegypti* ferritin-specific rabbit serum and visualized with Cy 2 TM conjugated goat anti-rabbit IgG (green). (A-C) Cells treated with 0 μM FAC for 18 h. (D-F) Cells treated with 100 μM FAC for 18 h. White arrows identify ferritin that is present in ceramide-containing membranes (yellow, upper arrow), cytoplasm (green, middle arrow) and on the margins of the cell (green, lower arrow).

Figure 6. CI-976 reduces viability of CCL-125 cells

CCL-125 cells were concurrently treated with a chemical blocking reagent and 100 μ M FAC for 18 h. (A) Cell numbers did not differ among treatment groups as determined by ethidium bromide fluorescence described in the methods. (B) Cell viability was compromised by CI-976 as measured by Formazan absorbance described in the methods. *Significantly different relative to F/E ($p < 0.0001$). **Significantly different relative to F/E ($p < 0.0006$). (C) Calcein fluorescent quench was decreased by CI-976 as described in the methods. *Significantly different relative to F/E ($p < 0.0002$). FAC/EtOH (F/E) was used as control for FAC/BFA (F/B), FAC/CI-976 (F/C) and FAC/CytoD (F/CD) and FAC/HBSS (F/H) served as a control for FAC/PLTX-II (F/P). Graphed data represent means $\pm$ SEM of triplicates.

Figure 7. CI-976 blocks ferritin secretion of CCL-125 cells

CCL-125 cells were concurrently treated with a chemical blocking reagent and 100 μ M FAC for 18 h. **(A)** Ferritin does not change in cytoplasmic extracts or the membrane fraction with CI-976 treatment. Cytoplasmic extracts (upper panel, 8 μ g protein) or membrane fractions (lower panel, 5 μ g protein) for each treatment were resolved by 17% SDS-PAGE and transferred to nitrocellulose membranes. Ferritin was detected by Western blot using anti-*A. aegypti* ferritin-specific rabbit serum and endogenous actin was detected using anti- β -actin-specific mouse serum. *Significantly different for cytoplasmic ferritin relative to F/H ($p < 0.05$). **(B)** CI-976 inhibits ferritin secretion. The medium was recovered and concentrated. Media samples for each treatment were resolved either by 18.5% SDS-PAGE and transferred to

nitrocellulose membranes (upper panel, 150 μ L) or by 4–20% Native-PAGE (lower panel, 250 μ L) and Coomassie blue stained for total protein. Ferritin was detected by Western blot using anti-*A. aegypti* ferritin-specific rabbit serum. *Significantly different relative to F/E ($p < 0.02$). FAC/EtOH (F/E) was used as control for FAC/BFA (F/B), FAC/CI-976 (F/C) and FAC/CytoD (F/CD) and FAC/HBSS (F/H) served as a control for FAC/PLTX-II (F/P). Purified *A. aegypti* ferritin (1 μ g) was used as a ferritin positive control (+); A549 cell cytoplasmic extract (15 μ g protein) served as a positive control for actin (A). M = molecular mass markers (BIORAD). Representative Western blots are shown above the graphed data. Graphed data represent means $\pm$ SEM of triplicates.