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Tracing heme in a living cell: hemoglobin degradation and heme traffic in digest cells of the cattle tick *Boophilus microplus*

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Summary

Heme is present in all cells, acting as a cofactor in essential metabolic pathways such as respiration and photosynthesis. Moreover, both heme and its degradation products, CO, iron and biliverdin, have been ascribed important signaling roles. However, limited knowledge is available on the intracellular pathways involved in the flux of heme between different cell compartments. The cattle tick *Boophilus microplus* ingests 100 times its own mass in blood. The digest cells of the midgut endocytose blood components and huge amounts of heme are released during hemoglobin digestion. Most of this heme is detoxified by accumulation into a specialized organelle, the hemosome.

We followed the fate of hemoglobin and albumin in primary cultures of digest cells by incubation with hemoglobin and albumin labeled with rhodamine. Uptake of hemoglobin by digest cells was inhibited by unlabeled globin, suggesting the presence of receptor-mediated endocytosis. After endocytosis, hemoglobin was observed inside large digestive vesicles. Albumin was exclusively associated with a population of small acidic vesicles, and an excess of unlabeled albumin did not inhibit its uptake. The intracellular pathway of the heme moiety of hemoglobin was specifically monitored using Palladium–mesoporphyrin IX (Pd-mP) as a fluorescent heme analog. When pulse and chase experiments were performed using digest cells incubated with Pd-mP bound to globin (Pd-mP-globin), strong yellow fluorescence was found in large digestive vesicles 4 h after the pulse. By 8 h, the emission of Pd-mP was red-shifted and more evident in the cytoplasm, and at 12 h most of the fluorescence was concentrated inside the hemosomes and had turned green. After 48 h, the Pd-mP signal was exclusively found in hemosomes. In methanol, Pd-mP showed maximal emission at 550 nm, exhibiting a red-shift to 665 nm when bound to proteins *in vitro*.

The red emission in the cytosol and at the boundary of hemosomes suggests the presence of heme-binding proteins, probably involved in transport of heme to the hemosome. The existence of an intracellular heme shuttle from the digestive vesicle to the hemosome acting as a detoxification mechanism should be regarded as a major adaptation of ticks to a blood-feeding way of life. To our knowledge, this is the first direct observation of intracellular transport of heme in a living eukaryotic cell. A similar approach, using Pd-mP fluorescence, could be applied to study heme intracellular metabolism in other cell types.

Key words: palladium mesoporphyrin, endocytosis, hematophagy, hemosome, cattle tick, *Boophilus microplus.*

Introduction

Iron protoporphyrin IX (heme) performs an extensive array of beneficial roles as the prosthetic group of enzymes involved in the catalysis of redox reactions in all types of cells. On the other hand, the unique reactivity of this molecule makes heme a powerful catalyst of the generation of reactive oxygen species (Van der Zee et al., 1996; Deterding et al., 2004), which are capable of promoting oxidative damage to lipids, proteins and DNA (Schmitt et al., 1993; Vincent, 1989; Aft and Muller, 1983). As a consequence, the toxic effects of heme have been related to diverse pathologies such as inflammation, atherosclerosis, Alzheimer's and Parkinson's diseases (Graça-Souza et al., 2002; Balla et al., 2003; Castellani et al., 2000, 1996).

The final step of heme biosynthesis, the insertion of the metal in the porphyrin ring, catalyzed by ferrochelatase, occurs inside the mitochondria; assembly of hemeproteins occurs not only in the mitochondria, but also in the cytosol and inside the endoplasmic reticulum (Asagami et al., 1994; Ponka, 1999). In addition, heme degradation by the microsomal heme oxygenase plays a critical role in cellular metabolism (Maines, 2003), being induced in response to stress in several organisms (Shibahara et al., 1987). Heme oxygenase acts not only as a

protection against the potential toxicity of heme, but also as a source of CO and biliverdin, which are important in cell signaling and as free radical scavengers (Dore et al., 1999; Stocker and Peterhans, 1989). However, very little information is available on the precise pathways involved in heme traffic inside eukaryotic cells.

Hematophagous arthropods face a very special situation in relation to heme metabolism since they ingest several times their own mass in vertebrate blood, which contains approximately 10 mmol l^{-1} heme bound to hemoglobin. Insects such as Triatominae bugs and mosquitoes digest their blood meals extracellularly, and most of the heme is retained in the gut lumen by means of extracellular protective matrixes secreted by gut epithelial cells. In the gut lumen of the kissing bug *Rhodnius prolixus*, a vector of Chagas disease, perimicrovillar phospholipid membranes convert heme into a special aggregate, hemozoin, also found in the malaria parasite (Oliveira et al., 1999, 2000). In the mosquito *Aedes aegypti*, heme is retained in the peritrophic matrix, a layer of protein and polysaccharides that covers the midgut and separates gut cells from the blood bolus (Páscoa et al., 2002). In contrast, the luminal region of tick midgut is crowded with digest cells, which degrade blood components intracellularly. Hemoglobin is thought to be hydrolyzed in the digestive vacuoles of these cells (Agbede et al., 1985; Walker and Fletcher, 1987) by the action of acid proteases (Mendiola et al., 1997), therefore releasing huge amounts of heme inside these cells. We have recently shown that most of the heme produced by this process is ultimately accumulated inside a specialized organelle called a hemosome, forming a unique type of heme aggregate (Lara et al., 2003). Using globin reconstituted with Pd-mP, a fluorescent metalloporphyrin employed here as a heme analog, we studied hemoglobin degradation in living digest cells of the cattle tick *Boophilus microplus*. We present evidence characterizing an intracellular pathway that starts with hemoglobin endocytosis, followed by hydrolysis of the globin polypeptide chain in a specific population of vesicles, then transport mediated by cytosolic proteins, and finally concentration of heme inside hemosomes.

Materials and methods

Animals

Boophilus microplus were obtained from a colony maintained at the Faculdade de Veterinária of Universidade Federal do Rio Grande do Sul (UFRGS), Brazil. *Boophilus microplus* of the Porto Alegre strain, free of *Babesia* spp., were reared on calves obtained from a tick-free area. Engorged adult females were kept in Petri dishes at 28°C and 80% relative humidity until use. Animals were treated in compliance with UFRGS review committee for animal care.

The partially engorged females were mechanically detached from *Babesia* spp.-free cattle infested by *Boophilus microplus*, in the Centro de Pesquisas em Sanidade Animal (CPPAR), UNESP-Jaboticabal, São Paulo, Brazil.

Protein labeling

Globin and albumin were labeled for 1 h with rhodamine isothiocyanate (Molecular Probes, Eugene, OR, USA) in 0.1 mol l^{-1} sodium bicarbonate buffer, pH 9.0. The reaction was stopped by adding 1.5 mol l^{-1} hydroxylamine, pH 8.5 (www.probes.com/media/pis/mp00143.pdf). Unbound probe was removed by sequential dialysis against 0.15 mol l^{-1} NaCl, 10 mmol l^{-1} sodium phosphate, pH 7.2 (PBS).

Pd-mP-globin

The globin solution was prepared as follows: bovine hemoglobin was denatured and heme removed in acetone–HCl 0.02 mol l^{-1} kept in an ethanol–dry ice bath, as described (Ascoli et al., 1981). Precipitated protein was washed once and renaturated by sequential dialysis against water. An equimolar amount of Pd-mP (Frontier Scientific, Logan, UT, USA) was added to the refolded protein at a final concentration of 100 μmol l^{-1} and a new dialysis against water was performed, in order to remove unbound Pd-mP. The Pd-mP-globin solution was centrifuged at 12 000 ***g*** and the supernatant was filtered using a sterile 0.22 μm pore size membrane and stored at 4°C.

Artificial feeding of Boophilus microplus

Partially engorged females were fed by means of a 100 μl glass capillary filled with 100 μmol l^{-1} Pd-mP-globin prepared as described above. Ticks were fed for 12 h at 38°C and 20% relative humidity, as described by de la Vega et al. (2000). Subsequently, the females were dissected in PBS after different intervals, and anterior midguts were fixed and processed as described below.

Digest cells culture

Fully engorged females from the second day after a blood meal (ABM) were rinsed in 70% ethanol for 1 min, and dissected in sterile PBS containing 200 U ml^{-1} streptomycin and penicillin. To obtain primary cultures, midguts were isolated in sterile Petri dishes and digest cells were detached from the gut wall with sterile tweezers. Cells were carefully collected using a 1 ml pipette tip, washed three times in the same buffer and then placed in a 12-well culture plate with L-15 Leibowitz's medium supplemented with 150 mmol l^{-1} NaCl plus 100 U ml^{-1} each streptomycin and penicillin. Bovine fetal serum proved to be cytotoxic and was omitted from all incubations. Cells were kept at 28°C until use and were viable for several weeks under these conditions. In endocytosis experiments, cells were incubated with culture medium containing rhodamine–albumin, rhodamine–hemoglobin (20 μmol l^{-1}) or Pd-mP-globin (100 μmol l^{-1}) for 2 or 4 h, washed twice and observed immediately after the incubations. Alternatively, in pulse-chase experiments, cells were washed and incubated in medium without Pd-mP-globin at different time intervals as described in the figure legends.

Histological preparation and fluorescence microscopy

Engorged females were dissected in modified Karnovsky's

fixative (2.5% glutaraldehyde, 4% paraformaldehyde, 0.1 mol l^{-1} $CaCl_2$ and 0.1 mol l^{-1} sodium cacodylate buffer, pH 7.3). Tissues were transferred to fresh fixative and kept at 4°C for 12 h. Segments of the anterior portion of the midgut were then washed in 0.1 mol l^{-1} sodium cacodylate buffer pH 7.3. The tissues were dehydrated in ethanol and embedded in Historesin (Leica). Semi-thin (5 μm) sections were observed by differential interference contrast (DIC) using an Axioplan 2 microscope (Zeiss). The fluorescence images were obtained using a filtered 100 W mercury lamp as the excitation light source with a filter set Zeiss-09 (BP450–490 nm/FT 510 nm/LP 515 nm).

Digest cells were observed in culture using a coverslip with a 10 μm spacer. Fluorescence images were obtained using the same filter set described above or, alternatively, using a filter set Zeiss-02 (G 365 nm/FT 395 nm/LP 420 nm). The confocal images were acquired in a Zeiss Meta 510 laser scanning microscope, with an excitation laser of 514 nm. Emission spectra from selected areas of the image (hemosome, cytosol and digestive vesicle) were obtained using the Zeiss Meta 510 software.

Fluorescence and absorption spectroscopy

Palladium meso-porphyrin IX stock solution (10 mmol l^{-1}) was prepared in 0.1 mol l^{-1} NaOH and diluted to 1 mmol l^{-1} in methanol. 10 μmol l^{-1} Pd-mP solutions were made in water, methanol or PBS with globin (10 μmol l^{-1}) or albumin (10 μmol l^{-1}). Absorption spectra were acquired in a GBC 920 (Camberra, Melbourne, Australia) spectrophotometer. Fluorescence spectra and fluorescence decay time course were recorded on a Varian Eclipse spectrofluorimeter (Palo Alto, CA, USA). Liposomes of soybean phosphatidylcholine (Sigma, St Louis, MO, USA) were prepared as described (Persechini et al., 1992) and used at 0.015% (w/v) in 10 mmol l^{-1} Hepes buffer, pH 7.2.

Results

Endocytosis of hemoglobin and albumin by midgut digest cells

Taken together, hemoglobin and albumin account for more than 80% of the total protein content of vertebrate blood and therefore constitute the main sources of amino acids for blood-feeding animals. In order to investigate how the cattle tick *Boophilus microplus* handles these two major components of its blood meal, we developed a protocol for keeping midgut digest cells in culture for extended periods. Digest cells were incubated with rhodamine-labeled bovine hemoglobin and albumin and examined by DIC and fluorescence microscopy. Both proteins were taken up by digest cells (Fig. 1), but by routes that were clearly distinct. Hemoglobin was preferentially associated with a group of large (3–12 μm) vesicles (Fig. 1A–D), in contrast to albumin, which was directed exclusively to a population of smaller (1–2 μm) vesicles (Fig. 1E–H). Addition of a 100-fold molar excess of unlabeled albumin to the incubation medium did not prevent accumulation of rhodamine–albumin (Fig. 1G,H), in contrast to the result obtained with rhodamine–hemoglobin, where uptake was effectively blocked by unlabeled hemoglobin (Fig. 1D,H), suggesting the existence of a specific receptor for hemoglobin on the surface of digest cells. These two types of vesicles could also be distinguished by their internal pH, as most of the small vesicles showed some degree of acidification when digest cells were incubated with Acridine Orange, but only a few of the larger, hemoglobin-containing vesicles showed low internal pH (Fig. 2).

Palladium mesoporphyrin IX (Pd-mP) fluorescence

Most metalloporphyrins do not display fluorescence (Falk, 1964). Pd-porphyrin phosphorescence, but not fluorescence, has been used to study oxygen transport *in vivo* (Sinaasappel et al., 1999). In preliminary experiments we found that Pd-mP was fluorescent and could be used to study the fate of the heme moiety of hemoglobin inside the digest cells. The Pd-mP absorption spectrum in water had a Soret band at 375 nm and showed α and β bands at 513 and 545 nm, respectively (Fig. 3A). Binding to globin or changing the solvent to methanol increased the intensity of absorption of all three peaks and red-shifted the Soret band to 390 nm. Fluorescence of Pd-mP can be observed using all three absorption peaks as excitation wavelengths (not shown). The fluorescence spectra, however, proved to be much more sensitive to the molecular environment of Pd-mP than light absorption. Excitation of Pd-mP in water at 400 nm resulted in a weak green emission peak at 545 nm, with barely detectable peaks at 595 nm and 665 nm. Fluorescence spectra of Pd-mP in water was not sensitive to pH (not shown). Adding methanol, phosphatidylcholine liposomes or globin to the solution greatly increased Pd-mP fluorescence intensity at 545 nm, compared to water (Fig. 3B). Pd-mP in acetonitrile showed a fluorescence spectrum similar to that obtained in methanol (not shown), suggesting that this increase in the 545 nm peak might be attributable to a reduction in water activity of the environment of the metaloporphyrin. However, when Pd-mP was bound to the globin polypeptide, its red emission at 665 nm greatly increased in comparison to Pd-mP in methanol or water (Fig. 3B). Several other proteins were tested, including bovine albumin, HeLP, a heme-binding lipoprotein from the *Boophilus microplus* hemolymph (Maya-Monteiro et al., 2000) and RHBP, a heme-binding protein from *Rhodnius prolixus* (Oliveira et al., 1995), and all of them induced a fluorescence peak at 665 nm, while proteins not capable of binding heme, such as bovine trypsin, did not alter the emission profile (not shown). Phospholipid liposomes, on the other hand, showed an intermediate profile, with a 665 nm fluorescence peak higher than that obtained in water or methanol, but much less intense than that of protein-bound Pd-mP (Fig. 3B).

The marked differences between the fluorescence spectra of free and globin-bound Pd-mP suggested that we might use them to follow the degradation of hemoglobin inside the cells. In order to test this possibility *in vitro*, the time course of hemoglobin degradation by trypsin was followed using 400 nm

Hb-R (1 μmol l^{-1})	+	+	–	–
BSARh (1 μmol l^{-1})	–	–	+	+
Hb (100 μmol l^{-1})	–	+	–	–
BSA (100 μmol l^{-1})	–	–	–	+

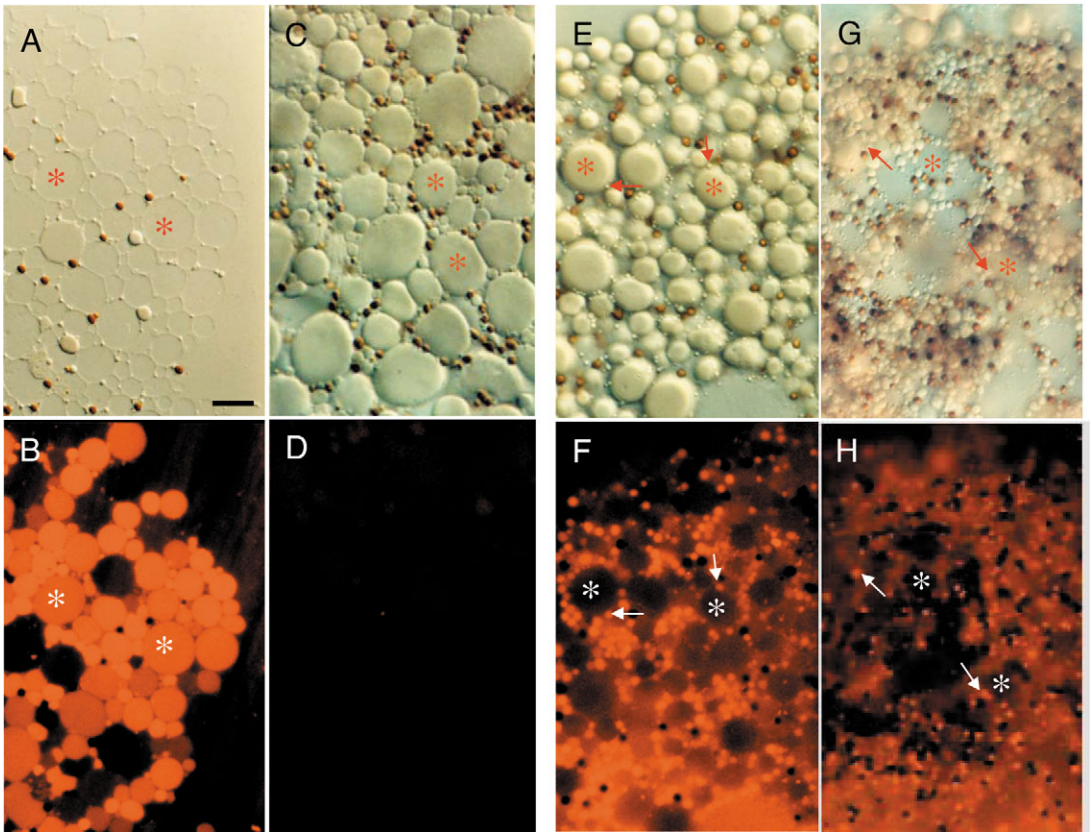

Fig. 1. Uptake of albumin and hemoglobin by tick midgut digest cells. Digest cells were incubated for 4 h in culture medium containing albumin or hemoglobin labeled with rhodamine. Incubation conditions were (A,B) 1 μmol l^{-1} rhodamine–hemoglobin; (C,D) 1 μmol l^{-1} rhodamine–hemoglobin plus 100 μmol l^{-1} unlabeled hemoglobin; (E,F) 1 μmol l^{-1} rhodamine–albumin; (G,H) 1 μmol l^{-1} rhodamine–albumin plus 100 μmol l^{-1} unlabeled albumin. (A,C,E,G) Differential interference contrast (DIC) images; (B,D,F,H) rhodamine fluorescence. Scale bar, 10 μm. Large hemoglobin-accumulating vesicles (asterisks) and small albumin-accumulating vesicles (arrows) are indicated in the figure. Hemosomes are seen as brown-colored structures on DIC panels and are not labeled by any of the proteins conjugated to rhodamine.

as excitation wavelength and monitoring fluorescence emission at 545 nm (green) and 665 nm (red). Addition of globin to the cuvette immediately produced the spectrum of Pd-mP-globin, characterized by an increase in fluorescence at both wavelengths (Fig. 4A). Addition of trypsin resulted in progressive loss of the bound Pd-mP spectrum, and the time course of proteolysis could be followed by the decrease in the 665 nm emission, which was more sensitive than the 545 nm emission (Fig. 4B,C). A new addition of globin partially restored the spectrum to the initial emission (Fig. 4D).

Pd-mP-globin uptake by digest cells in vivo

Partially engorged adult females were fed using bovine plasma enriched with Pd-mP-globin for 12 h, using an artificial feeding system. Ticks were dissected 2 and 6 h after feeding and midguts were fixed and embedded in methacrylate. At 2 h (Fig. 5A), Pd-mP was visible as a yellow-green fluorescence, associated mainly with the large vesicles of digest cells (the vesicles implicated in hemoglobin uptake in Fig. 1A), but some fluorescence was also found in the cytosol, in contrast to hemosomes, which were almost devoid of label at this time (Fig. 5A). At 6 h (Fig. 5B), the Pd-mP fluorescence had reached the hemosomes, showing that the Pd-mP generated from digestion of Pd-mP-globin *in vivo* followed the same path suggested previously for heme accumulation in the digest cell (Lara et al., 2003).

Pd-mP-globin uptake by digest cells in vitro

Primary culture of digest cells allowed dynamic characterization of the fate of heme after endocytosis using pulse and chase experiments. Previous evidence (Lara et al., 2003) suggested that hemoglobin digestion is polarized in digest cells when attached to the epithelium, with the digestive vesicles at the luminal side and hemosomes concentrated at the

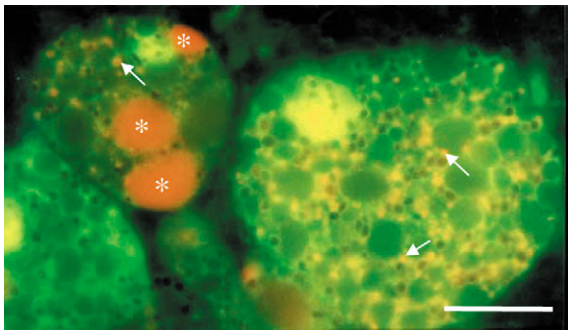

Fig. 2. Acidification of digestive vesicles. Digest cells were incubated with 100 µmol l^{-1} of globin for 2 h, and then exposed to 50 µmol l^{-1} of Acridine Orange for 1 min, washed and observed by fluorescence microscopy. Small (arrows) and large (asterisks) acid digestive vesicles are indicated. Scale bar, 10 µm.

perinuclear or basal side. Cultured cells retained this organization, and after a 2 h incubation with Pd-mP-globin, the fluorescent label was found in vesicles concentrated at the luminal side, opposite to the hemosomes, suggesting the concentration of endocytotic activity in that part of the cell (Fig. 6A). A 4 h incubation resulted in more intense labeling of the cells, which was extended to vesicles situated closer to the hemosomes, and displayed a more diffuse pattern, which might be explained by the presence of Pd-mP also in the cytosol (Fig. 6B). When a 4 h pulse was followed by a chase of 4 h, in addition to the yellow fluorescence in both digestive vesicles and in the cytosol, Pd-mP also reached the hemosomes, where the emission shifted to green (Fig. 6C). Extending the chase time produced a progressive accumulation of the Pd-mP in the hemosomes, which were almost the only structures labeled by 48 h (Fig. 6D–F).

In order to compare the fluorescence profile of the images obtained in the microscope with the emission spectra observed in Fig. 3, the fate of Pd-mP-globin after endocytosis by digest cells was evaluated by spectral analysis using confocal microscopy with excitation using a 514 nm laser (Fig. 7A). Images were acquired at a succession of defined wavelengths over the 530–700 nm range. Representative areas of the image

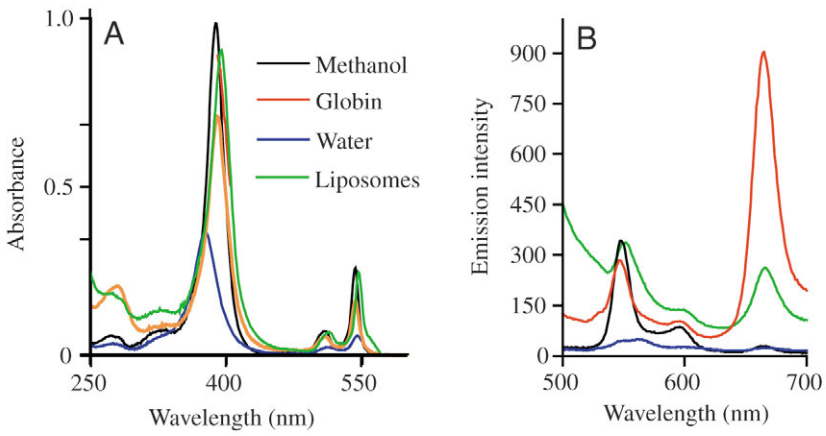

Fig. 3. Light absorption and fluorescence spectra of Pd-mesoporphyrin IX. Spectra of Pd-mP (5 µmol l^{-1}) were recorded in water, methanol, phosphatidylcholine liposomes or bound to 5 µmol l^{-1} globin. (A) Light absorption spectra; (B) fluorescence emission spectra, λ_{ex}=400 nm.

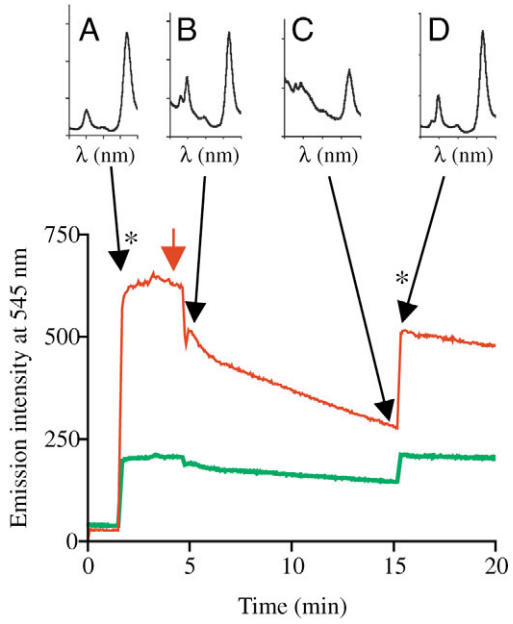

Fig. 4. Time course of proteolytic digestion of Pd-mP-globin. Fluorescence emission of Pd-mP (5 µmol l^{-1}) in water was recorded at 545 nm and 665 nm with excitation at 400 nm. Emission spectra A–D (insets) using the same excitation wavelength were acquired at the times indicated by black arrows, after addition of 5 µmol l^{-1} globin (asterisks) and 1% bovine trypsin (red arrow).

were selected and analysis of their emission spectra (Fig. 7B) showed the predominance of a red emission in the cytosol, contrasting with the higher intensity of the green emission in the hemosome. Digestive vacuoles exhibited an intermediate profile, probably as a consequence of Pd-mP-globin degradation, as described in Fig. 4.

The time course of hemoglobin digestion was also followed using an excitation bandpass filter of 350–400 nm (Fig. 8). This excitation wavelength resulted in a higher level of autofluorescence of digest cells. However, the higher emission intensity allowed a brief Pd-mP labeling pulse of only 2 h, which resulted in a more precise discrimination of events during traffic of heme in the cell. By 6 h Pd-mP was clearly detected in the cytosol as a red fluorescence (Fig. 8), suggesting association of the probe with proteins. At the same time, Pd-mP labeling reached the hemosomes, several showing fluorescence concentrated at the border of the organelle as a bright red emission, suggesting interaction with proteins (Fig. 8,

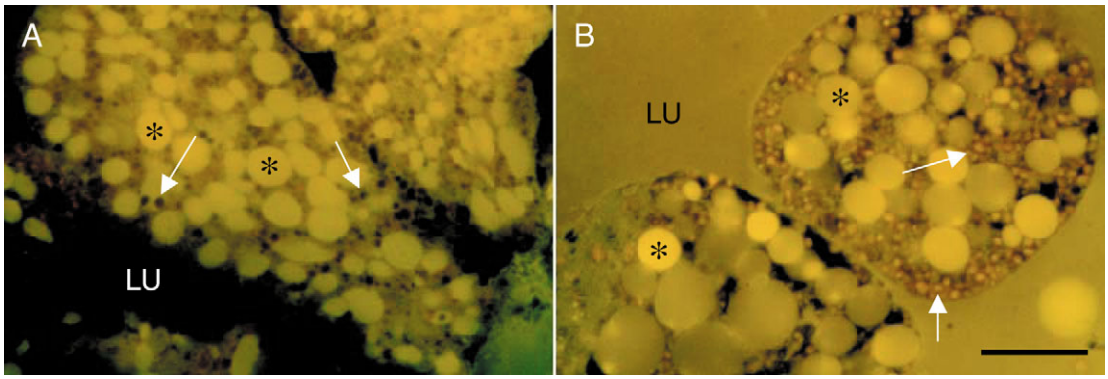

Fig. 5. Digestion of Pd-mP-globin *in vivo*. Partially engorged females were fed with serum enriched with 100 µmol l^{-1} Pd-mP-globin for 12 h, as described in Materials and methods. Anterior midguts were fixed and embedded in methacrylate, and semi-thin sections were observed under a fluorescence microscope, as described under Materials and methods. (A) Two digest cells 2 h after feeding; (B) two digest cells 6 h after feeding. *Digestive vesicles; arrows, hemosomes; LU, intestinal lumen. Scale bar, 30 µm.

inset). Interestingly, a few hemosomes already showed the characteristic green emission associated to the hemosome core, similar to what was observed in Fig. 6.

Discussion

Notwithstanding all the beneficial aspects of heme, its toxicity has been demonstrated in several *in vitro* and *in vivo* experimental conditions (Chiu and Lubin, 1989; Vincent, 1989; Hebbel and Eaton, 1989), and for that reason there is a general consensus about the need for heme-binding proteins to carry out the intracellular transport of heme and to target it to its proper intracellular locations, in harmony with heme utilization for synthesis of hemeproteins and heme degradation by heme oxygenase. In some pathogenic bacteria several proteins that were identified as virulence factors have been shown to bind heme and to participate in the uptake and intracellular trafficking of heme (Otto et al., 1992). Eukaryotic cells present an additional degree of complexity because heme must leave the mitochondria after biosynthesis. Several intracellular heme-binding proteins have been identified, such as fatty-acid-binding protein (Stewart et al., 1996), glutathione-S-transferase (Harvey and Beutler, 1982), heme-binding proteins HBP23 (Iwahara et al., 1995) and p22 HBP (Taketani

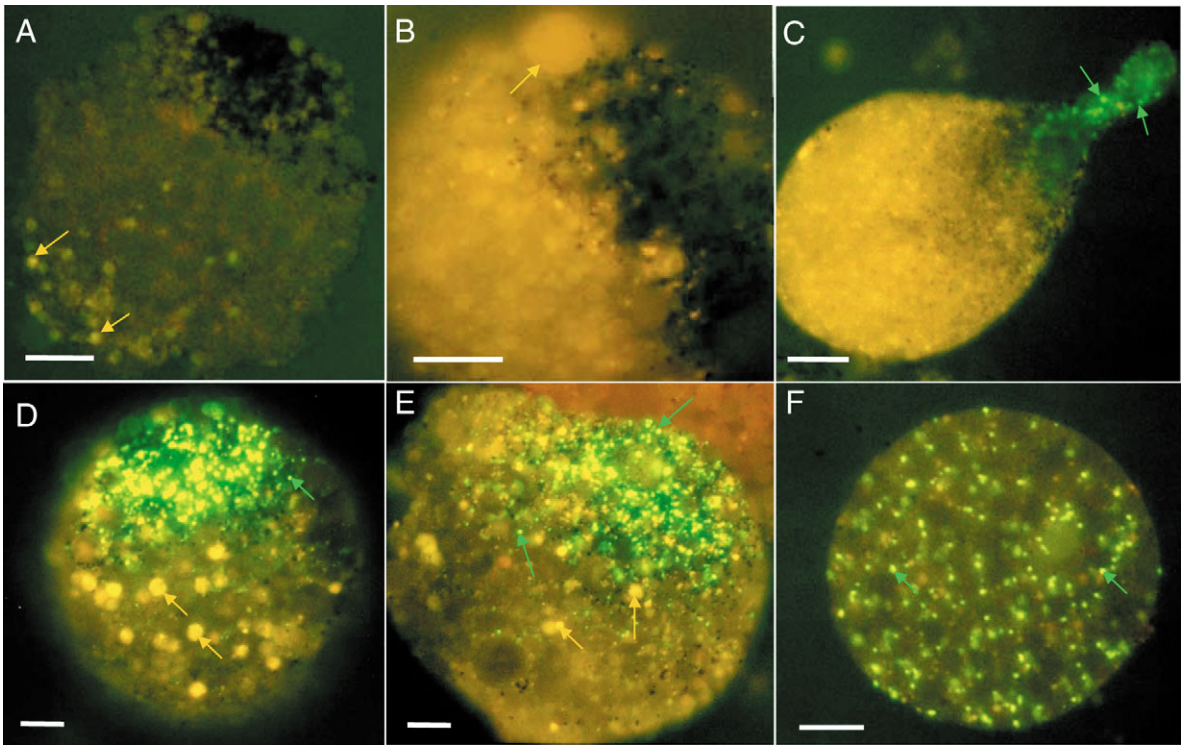

Fig. 6. Time course of degradation of Pd-mP-globin by digest cells *in vitro*. Digest cells were incubated in culture medium with 100 µmol l^{-1} Pd-mP-globin for (A) 2 h, (B) 4 h. (C–E) Pulse (4 h incubation with Pd-mP-globin) and chase experiments: (C) 4 h; (D) 8 h; (E) 20 h; (F) 44 h. Yellow arrows, digestive vesicles; green arrows, hemosomes. Fluorescence of Pd-mP was observed using a Zeiss-09 filter set. Scale bars, 40 µm.

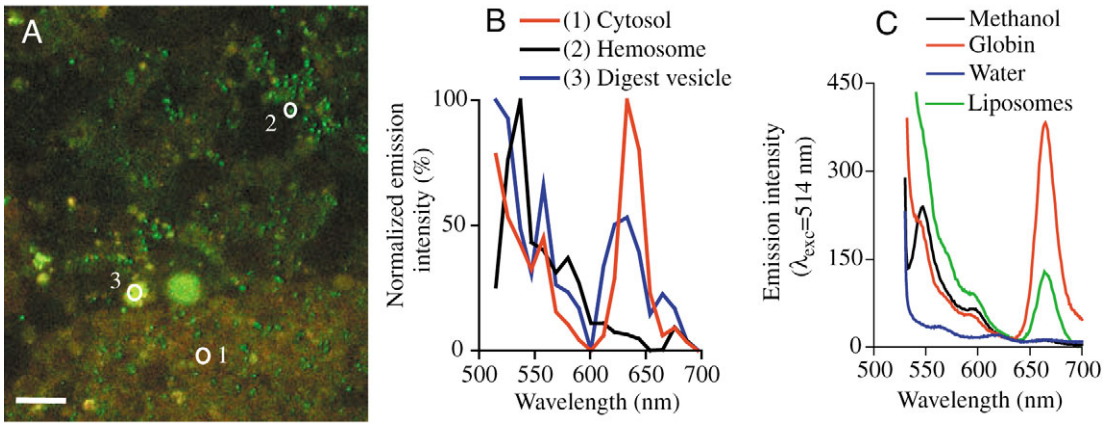

Fig. 7. Confocal fluoroscopy of Pd-mP emission spectra in digest cells *in vitro*. Digest cells were incubated for 4 h with Pd-mP-globin 100 µmol l^{-1}, and observed 8 h later using laser excitation at 514 nm. Images were collected from 530 to 700 nm, with ±10 nm intervals between each consecutive image. (A) Digest cell total emission image. Arrows indicate areas of the image selected for spectral analysis: (1) cytosol, (2) hemosome and (3) digest vesicle. Scale bar, 20 µm. (B) Emission spectra obtained from image analyses. (C) Pd-mP 5 µmol l^{-1} fluorescence spectra with λ_{ex}=514 nm in water, methanol, phosphatidylcholine liposomes, or bound to 5 µmol l^{-1} globin.

et al., 1998), but their precise role in heme trafficking is not yet clear. Recently, a plasma membrane protein, the FLVCR (feline leukemia virus cellular receptor), was shown to be responsible for exportation of cytoplasmic heme from human erythroblasts, a function attributed to a cellular defense against heme toxicity (Quigley et al., 2004). Here, for the first time, we provide direct observation of the intracellular route followed by heme in a living cell, using Pd-mP as a heme analog, and present evidence for the association of heme to heme-binding proteins in the cytosol of the digest cells of the midgut of *Boophilus microplus*.

Literature on the biology of ticks has described digest cells as phagocytic cells (Agbed and Kemp, 1985; Walker and Fletcher, 1987). However, the difficulty in obtaining evidence for phagocytosis of red blood cells led some authors to postulate that endocytosis of blood proteins may occur by pinocytosis (Koh et al., 1991). Here we show that albumin and hemoglobin, the two main proteins of vertebrate blood, are not handled in the same way (Fig. 1). Albumin seems to be taken up by non-specific, probably fluid-phase, endocytosis, as there is no competition between labeled and unlabeled protein. Once inside the cell, albumin is directed to a population of small acidic vesicles (Figs 1B and 2). Hemoglobin, on the other hand, seems to be recognized by specific cell-surface receptors, which target it towards a population of large vesicles (Fig. 1A) that are also capable of acidification (Fig. 2). This is consistent with the presence of proteases having acid pH optima in the midgut of ticks (Mendiola et al., 1996), but the presence of two independent endocytotic pathways points to the need for subcellular localization of each protease in order to establish its precise role in blood digestion. In mammals, macrophages are the main site of heme degradation, and an acute phase protein, CD163, has been recently identified as the macrophage hemoglobin scavenger receptor (Kristiansen et al., 2001). In this case, however, the receptor mediates endocytosis of the haptoglobin–hemoglobin complex, whereas tick cells are able to take up hemoglobin alone. Whatever the molecular nature of the digest cell receptor for hemoglobin, the existence of a distinct apparatus – receptor, cell compartments and possibly proteases and membrane transporters – dedicated to digestion of hemoglobin can be understood as a protection against the potential toxicity of the heme group, and it illustrates the complexity of adaptation to digestion of hemoglobin. In the case of mammalian macrophages, several other protein ligands are taken up by endocytosis (Cherukuri et al., 1998), but the possibility of a distinct path for hemoglobin hydrolysis has not been investigated.

The Pd-mP fluorescence emission is sensitive to its molecular environment and a marked red shift was produced

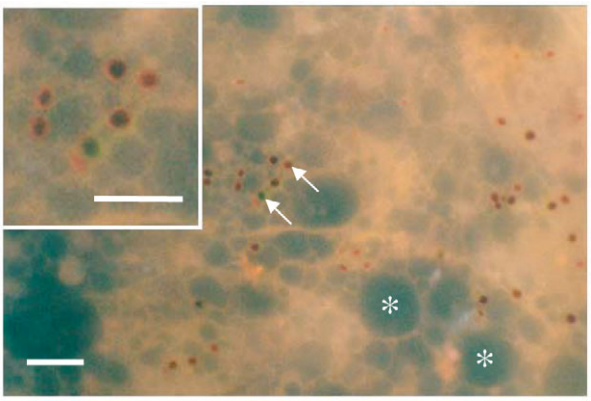

Fig. 8. Pd-mP uptake by the hemosomes. Digest cells were incubated for 2 h in culture medium with 100 µmol l^{-1} Pd-mP-globin, followed by 6 h of chase. Arrows indicate labeled hemosomes; asterisks, digestive vesicles. Inset shows labeled hemosomes at higher magnification. Fluorescence of Pd-mP was observed using a Zeiss-02 filter set. Scale bar, 20 µm (10 µm in inset).

when the probe is bound to globin/protein (Fig. 3). During *in vitro* proteolytic digestion of Pd-mP-globin, intermediate profiles were obtained (Fig. 4) due to a mixture of native, undigested globin, and the metalloporphyrin splitting out from the heme pocket upon loss of tertiary structure that follows cleavage of the globin polypeptide chain, similar to what happens during hemoglobin digestion in the digestive vacuole of the malaria parasite (Goldberg et al., 1990). In living tick digest cells, similar intermediate emission spectra were also observed during Pd-mP-globin digestion (Figs 6 and 7), resulting in the characteristic yellow fluorescence of the digestive vesicles. Being a mixture of green and red, a yellow emission might be due to association of Pd-mP with a phospholipid bilayer (Fig. 5). However, as the large digestive vesicle is the site of hemoglobin hydrolysis, a more plausible explanation may be that it contains both undigested Pd-mP bound to globin together with substantial amounts of free Pd-mP produced inside the large digestive vesicles. In contrast, when Pd-mP was released from the digestive vesicles into the cytosol, its emission spectrum shifted again to red, pointing to association with proteins (Figs 7 and 8), in accordance with the general assumption that heme in the cytosol is always bound to proteins. An even more intense red shift was observed when the fluorophore reached the edge of the hemosome (Fig. 8), suggesting that the initial events in the entrance of heme into the hemosome may include an integral membrane protein transporter. ABC cassette transporters have been implicated in translocation of heme across membranes both in bacteria and mitochondria (Köster, 2001). However, further Pd-mP accumulation into the hemosome resulted in replacement of the red emission by the green fluorescence that predominates at later chase times (Fig. 6C–F), which is in accordance with our previous result (Lara et al., 2003) showing that the hemosome core has a low protein content and is made up of 90% heme. Heme segregation in the hemosome is thought to be a protective mechanism, acting by reducing availability of heme to participate in redox reactions that generate free radical species (not shown), in a way analogous to the formation of hemozoin, the heme aggregate of the malaria parasite. Hemozoin formation inhibited free radical production by steric hindrance of substrate access to heme molecules in the interior of the aggregate particle (Oliveira et al., 2002).

Ticks are obligate ectoparasites that have vertebrate blood as their only source of nutrients. They are thought to have evolved a blood-feeding life style in the Paleozoic era, before the advent of mammals (Hoogstraal and Aeschlimann, 1982). The development of efficient heme detoxification mechanisms, in particular hemosomes, should be regarded as an essential aspect of their evolutionary track to hematophagy. Some of the protein components of the intracellular machinery used to transport heme to the hemosome may be new, tick-specific proteins; once identified, these may be the objects of future research directed to the development of new methods of population control of the parasite. On the other hand, it is plausible to speculate that conserved proteins related to heme trafficking in other organisms may also have been recruited in the tick to achieve safe, efficient heme accumulation in the hemosome. Accordingly, the tick digest cell may prove useful as a general model for studying the cell biology of heme.

List of abbreviations

ABM	after blood meal
BC	basophilic cell
BL	basement membrane
DC	digestive cell
DIC	differential interference contrast
DV	digestive vesicle
ESI-MS	electrospray ionisation mass-spectrometry
LP	long pass
NU	nucleus
TEM	transmission electron microscopy

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