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ORIGINAL CONTRIBUTION

Transepithelial heme-iron transport: effect of heme oxygenase overexpression

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Abstract
Background Heme iron is found in the diet mainly in the form of hemoglobin and myoglobin. It is known that heme iron (heme-Fe) and inorganic iron are absorbed differently. Intracellularly, heme oxygenase-1 (HO1) participates in the cleavage of the heme ring producing biliverdin, CO and ferrous iron. Iron released from heme becomes part of labile iron pool, and it can be stored in ferritin or released through the basolateral membrane. The mechanism by which heme-Fe is metabolized within cells is not completely understood.
Objective This study focused on the uptake and transport of heme iron and on the role of heme oxygenase-1 on heme iron metabolism.
Design Caco-2 cells were incubated with different concentrations of heme-Fe. A full-length heme oxygenase-1 cDNA was expressed in Caco-2 cells and intracellular iron and heme-Fe content, heme uptake, heme and iron transport and heme oxygenase-1 immunolocalization were assessed in these cells.
Results Heme-Fe was bioavailable and induced an intracellular increase in iron, ferritin and HO1 levels and a decrease in DMT1 expression. In cells overexpressing HO1, heme-Fe uptake and transepithelial Fe transport was higher than in controls. Most heme-Fe was metabolized to free iron, most of which was found mainly in the basolateral chamber. However, there is a fraction of heme that is delivered intact to the basolateral side. In a high heme-Fe condition, HO1 is found near the plasma membrane.
Conclusions These results suggest that heme oxygenase-1 catabolizes most of the heme-Fe and favors iron influx and efflux in intestinal cells.

Keywords Heme-Fe · Transepithelial transport · Caco-2 cells · Heme oxygenase

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Introduction

One of the defense mechanisms most widely used in nature is enzyme heme oxygenase-1 (HO-1). This microsomal enzyme performs the seemingly lackluster function of catabolizing heme to generate bilirubin (an antioxidant), carbon monoxide, and free iron (a potent pro-oxidant) [37]. Three heme oxygenase (HO) isoforms have been identified, HO-1, HO-2, and HO-3. HO-1 is a 32-kDa heat shock protein, which is inducible by numerous noxious stimuli. The common characteristic of many of these inducers is their ability to cause oxidative stress. These include, but are not limited to: heme and heavy metals [14], hyperoxia [24], hypoxia [7, 25], UV light, hydrogen peroxide [19, 23], lipopolysaccharide [5], hyperthermia [13] and endotoxin [6]. HO-1 expression is primarily regulated at the transcriptional level [1, 9, 11, 15]. HO-2 is a constitutively synthesized 36-kDa protein, which is abundant in brain and testis [36, 39]. The third isoform, HO-3, has been reported as a pseudogene derived from HO-2 transcripts [17].

Remarkably, the activity of this enzyme results in profound changes in the ability of cells to protect themselves against oxidative injury. HO-1 has been shown to have anti-inflammatory, anti-apoptotic, and anti-proliferative effects, and it is now known to have salutary effects in diseases as diverse as atherosclerosis and sepsis. Stocker [35] has proposed that this enzyme might provide cellular

protection. The mechanism by which this enzyme confers cellular protection is only beginning to be unraveled. The appeal is readily apparent: if we can understand how cells are able to protect themselves from oxidative stress, then our understanding and ability to intervene in disease processes will be immeasurably advanced [28].

Both HO and its substrate, heme, are highly conserved molecules across almost all forms of life, from algae to mammals. Molecules so evolutionarily conserved and ubiquitous generally serve a necessary and fundamental purpose [28]. There are relatively few studies describing the mechanism of intestinal heme iron absorption despite the importance of heme iron as a highly bioavailable source of dietary iron. Populations that consume meat as a significant component of their diet are normally iron replete. In fact, it has been determined that two-thirds of absorbed dietary iron in North America and Europe is derived from heme iron, although it only comprises one-third of dietary iron. Intestinal absorption of heme iron is higher than that of non-heme iron, suggesting that heme may be a preferred iron source in iron deficiency; it may also be a source of dietary iron to avoid when iron status is high, such as in hemochromatosis [31]. In this study, we determined the effect of HO1 over-expression on heme-Fe bioavailability and intracellular iron transport.

Materials and methods

Cell culture

Caco-2 cells (American Type Culture Collection HTB37 Rockville, MD) (1×10^5 cells) were cultured at 37 °C and 5% CO_2 in 25-cm^2 flasks with Iscove′s media (Gibco Life technologies, Grand Island, NY) supplemented with 10% FBS, 10 kU/L penicillin/streptomycin, and 25 mg/L fungizone (Gibco). The cells were trypsinized and re-seeded in either 24- or in 6-well plates ($0.2–0.5 \times 10^5$ cells) or on polycarbonate membranes of 0.33 μM pore size and 6.5 mm diameter (0.2×10^5 cells) (Transwells, Corning, Costar, Cambridge, MA). The medium was changed every 2–3 days.

Isotopic labeling and digestion of hemoglobin (Hb)

An iron isotope (^{55}Fe) of high specific activity was used as a tracer (NEN, Life Science Products, Boston, MA). Labeled hemoglobin (Hb) was prepared from red blood cells obtained from New Zealand rabbits that had received an intravenous injection of 74 MBq of ^{55}Fe as ferric citrate diluted in saline solution. The rabbits were bled through a cardiac puncture 15 days later. The radioactive red blood cells were centrifuged ($1{,}000 \times g$ for 15 min at 22 °C) and washed with saline solution, then hemolyzed by freezing and dehydrated by lyophilization. The cell extract was labeled with a specific activity of 2,460 kBq of ^{55}Fe per mg of heme-Fe obtained. Partial digestion of Hb solution was performed. Briefly, Hb solution containing 2 mM ^{55}Fe or ^{56}Fe were digested with 0.1% pepsin at pH 2.0 for 1 h at 37 °C. This solution was diluted by adding HEPES buffer (pH 7.2) to increase the pH to 6.8. A digestion of $52 \pm 3\%$ was estimated in the Hb-digest by measuring Hb content in the supernatant, which was used as a source of heme-Fe. Elemental Fe was determined by atomic absorption spectrometry with graphite furnace (SIMAA 6100, Perkin-Elmer, Norwalk, CT).

Caco-2 cells with different heme or non-heme iron concentrations

Caco-2 cells were grown for 7 days in the presence of heme-Fe, as described earlier. The incubations were made using a stock heme-Fe solution with the following concentrations: 0.1, 5, 10, 20, and 50 μM of heme-Fe. The medium was changed every 2–3 days. After 7 days, the cells were trypsinized and re-seeded (5–10% initial cells $= 1 \times 10^5$), as previously described, for another 7 days. After the treatment, a cell lysate was prepared with lysis buffer (in mM: 10 HEPES, pH 7.5, 3 $MgCl_2$, 40 KCl, 1 PMSF, 1 DTT, and 5% glycerol, 0.5% Triton X-100 and $1 \times$ protease inhibitor cocktail (Sigma, St Louis, MO). The mix was incubated for 15 min on ice and centrifuged for 10 min at 4 °C and 15,000 rpm. The supernatant was aliquoted and stored at −70 °C. Protein concentration was determined by Lowry method [26], intracellular ferritin by ELISA (rabbit anti-human ferritin code A0133 and peroxidase-conjugated rabbit anti-human ferritin code P0145, Dako Corp, Denmark) and total iron by spectrometric atomic absorption with graphite furnace (SIMAA 6100, Perkin-Elmer, Norwalk, CT).

Antibodies and immunodetections

Western blotting assays were performed on cell lysate to study the expression of HO-1, DMT1 (Divalent Metal Transporter 1) and Ireg1 (also, ferroportin). Fifty micrograms of cell lysate were loaded and separated on 14% (HO-1) and 8% (DMT1 and Ireg1) SDS–PAGE, and transferred to a nitrocellulose membrane. The primary antibody for HO1 was a rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc). DMT1 and Ireg1 antibodies were provided by Dr. MT Núñez, Faculty of Science, University of Chile. DMT1 and Ireg1 antibodies were rabbit polyclonal antibodies prepared against COOH-terminal peptide. The secondary antibody was peroxidase-labeled goat anti-rabbit immunoglobulin G (Sigma Chemical). For membrane

examination, the enhanced chemiluminescence Western blotting detection system (Amersham, Arlington Heights, IL) was used. Membranes were stripped with 100 mM citric acid (pH 3.0) and then re-blotted with anti-actin (Sigma Chemical).

Subcellular localization of HO1 in Caco-2 cells

Cells were grown in polyester membrane Transwells (Costar) for 14 days, incubated with or without 50 μM heme-Fe for the last 5 days, then fixed with 4% paraformaldehyde, permeated with 0.2% Triton X-100 in saline, and reacted with anti-HO1 antibody. A second antibody was FITC-labeled anti-rabbit IgG antibody (Sigma Chem. Co.). Fluorescence was determined in a Zeiss MP40 confocal microscope. For co-immunolocalization analysis, cells were incubated over night with mouse anti-HO1 (1:250, US Biological) and rabbit anti-Glut1 (1:200, US Biological). Then, cells were washed 6× with PBS-BSA for 5 min and then incubated with Alexa 546 anti-mouse IgG (1:500, Molecular Probes) and Alexa 488 anti-rabbit IgG (1:500, Molecular Probes). Fluorescence was determined as mentioned earlier.

Vector construction and transfection of Caco-2 cells with ho1 cDNA

Total RNA was isolated from Caco-2 cells with Trizol reagent (Invitrogen) according to manufacturer instructions. Briefly, Caco-2 cells seeded in 25-cm^2 bottles were cultured for 7 days and lysed with 2.5 mL of Trizol. RNA was resuspended in DEPC water, aliquoted and stored at −80 °C. cDNA was obtained by reverse transcription. The reaction contained 5 μg of RNA and 0.5 μg oligo-dT. The mix was incubated at 70 °C for 10 min and then at 4 °C for 1 min. Two microliters of 10× PCR buffer, 1 μL of 50 mM $MgCl_2$, 1 μL of 10 mM dNTPs, and 2 μL of 0,1 M DTT were added to a final volume of 20 μL and incubated at 42 °C for 5 min. Then 200 U of MMLV Reverse Transcriptase (Invitrogen Corporation, Carlsbad, CA, USA) was added, and the mix was incubated at 42 °C for 50 min. The reaction was stopped by incubation at 70 °C for 15 min. One microliter of RNase H (Invitrogen) was added, and the mix was incubated at 37 °C for 20 min. *ho1* full-length cDNA (GenBank accession number: NM_002133) was amplified by PCR using the following primers: HO1s 5′-GAACGAGCCAAGCTTCGGCCGGATG-3′ (position 59-83) and HO1a 5′-GGAGCCAGCGCGGCCGCATACACAT-3′ (position 942-966). Underlined letters indicate nucleotide changes with respect to the *ho1* mRNA sequence in order to introduce restriction sites for HindIII and NotI. The cDNA was used as a template for PCR amplification using the following cycles: 94 °C for 35 min, 94 °C for 15 seg, 60 °C for 1 min, 72 °C for 1 min, and 72 °C for 10 min. A band of 908 pb was obtained. The PCR product was digested with HindIII and NotI and purified with Gene Clean II Bio 101 using silica (Sigma–Aldrich, St Louis, MO). *ho1* cDNA was cloned in the pcDNA3.1myc-his (Invitrogen) expression vector. *E. coli* DH5α were transformed, and positive clones were verified with restriction analysis and sequencing. Caco-2 cells were grown at subconfluence (50–70%) and transfected with *pcDNA3.1myc-his-ho1* vector (HO1 cells) or *pcDNA3.1myc-his* (control cells) using Lipofectamin 2000 (Invitrogen). Transfected cells were selected using 800 μg/mL G418 (Gibco) for 48 h, and then the cells were maintained in Iscove′s media as earlier with 400 μg/mL G418.

Intracellular total levels of iron and ferritin

HO-1 and control cells were grown in 12-well plates in selection medium for 7 days. A cell lysate was prepared, then digested with 65% nitric acid (1:1) and incubated at 60 °C overnight. Total iron was determined by spectrometric atomic absorption with graphite furnace Simaa 6100 (Perkin Elmer). Intracellular ferritin was determined in cell lysate using ELISA (rabbit anti-human ferritin Code A0133 and peroxidase-conjugated rabbit anti-human ferritin code P0145, Dako Corp, Denmark).

Heme-Fe and iron uptake and transport

HO1 and control cells were plated onto 0.33 cm^2 polycarbonate inserts for 12 days and grown as previously described. The medium was changed every 3 days. Inserts were used when they attained stable resistance values between 250 and 280 Ω cm^2. On the day of the experiment, the cells were washed with 1× PBS, and 50 μM heme-^{55}Fe or 25 μM $^{55}FeCl_3$ (Fe:ascorbic acid 1:5) was added to the apical side in transport buffer (in mM: 50 MOPS-Na; 94 NaCl; 7.4 KCl; 0.74 $MgCl_2$; 1.5 $CaCl_2$; 5 glucose, pH 6.5) at different times (0–60 min) at 37 °C. The reaction was stopped washing the inserts 3 times with cold PBS/1 mM EDTA. Afterward the membranes were cut out and 1 mL of scintillation liquid was added to each tube. Also, 100 μL of the basolateral medium was diluted with scintillation liquid. The radioactivity from ^{55}Fe in both the membrane and the basolateral medium was measured in a gamma counter (Beckman LS 5000 TD).

Determination of non-heme iron and protoporphyrin transport at the basolateral side

For protoporphyrin transport determination, HO1 and control cells were grown in bicameral chambers (0.66 cm^2) in

Iscove′s media with 10% low-Fe FBS and G418 (400 μg/mL). On the day of the experiment, 50 μM heme-Fe was added to the apical chamber, and the cells were incubated at 37 °C for different periods of time (0–60 min). The transepithelial electric resistance (TEER) was monitored during the experiment. Inserts with TEER lower than 240 Ω cm^2 were eliminated. Basolateral media was collected and protoporphyrin concentrations were measured in a Shimadzu UV-1601 spectrophotometer at 398 nm. We used a molar extinction coefficient of $\varepsilon = 1.56 \times 10^5$ M^{-1} cm^{-1} for this calculation. For non-heme iron transport determination, control and HO1 cells were grown in bicameral inserts (1 cm^2). On the day of the experiment, inserts were washed with MOPS-saline buffer. Afterward, 0.1 μM calcein in MOPS-glucose buffer and MOPS-glucose were added to the basolateral and apical chamber, respectively. Calcein fluorescence was measured, then heme-Fe (10 μM) was added to the apical compartment and the decrease in fluorescence was measured again in 30 cycles of 1 min each. Finally, 10 μL of 10 μM SIH were added to chelate Fe.

Heme oxygenase enzymatic activity

A cell lysate from control and HO1 cells was prepared using a non-denaturing lysis buffer (20 mM Tris–HCl; pH 7.4; 0.5% Triton X-100; protein inhibitor cocktail). Two hundred fifty micrograms of cell lysate were incubated with 600 μL of B buffer (100 mM KH_2PO_4, pH 7.4), 100 μL of 150 μM hemin, 100 μL of 100 μg/mL rat liver extract containing biliverdin reductase and 100 μL of 10 mM NADPH for 1 h at 37 °C in the dark. Bilirubin formed in the reaction was extracted with 1 mL of chloroform for 1 h at room temperature in a shaker (100 rpm). Then, absorbance was measured at 530 nm (molar extinction coefficient of bilirubin: 43.5 mM^{-1} cm^{-1}). HO enzymatic activity was expressed as nmole of bilirubin/mg protein/hr.

Bilirubin reductase isolation

Rat livers (*Rattus norvegicus*) were perfunded in situ with saline (0.9% NaCl, pH 7.2) until complete discoloration, dissected, homogenized in lysis buffer A (0.1 M sodium citrate, pH 5.0; 10% glycerol) and centrifuged for 20 min at 10,000×*g* and 1 h at 105,000×*g*. The supernatant was diluted in 20 mM KH_2PO_5; 135 mM KCl; 0.1 mM EDTA; pH 7.4. Protein concentration was determined. The extract was aliquoted and stored at −20 °C.

Statistical analysis

Variables were tested in triplicate, and the experiments were repeated at least twice. Variability among experiments was <20%. One-way ANOVA and *T* test were used to test differences in mean values, and Bonferroni′s post hoc test was used for comparisons (SAS 8.0E, SAS Institute Inc., Cory, NC). Differences were considered significant if $p < 0.05$.

Results

Intracellular iron and ferritin content in Caco-2 cells incubated with heme-Fe

To determine the bioavailability of heme-Fe, we measured total intracellular iron (Fig. 1a) and ferritin (Fig. 1b) in Caco-2 cells incubated with different extracellular heme-Fe concentrations for two passages. Intracellular Fe increased (2.8 ± 0.4–10.8 ± 0.8 nmole Fe/mg protein) when extracellular heme-Fe increased from 0.5 to 100 μM (one-way ANOVA: $p < 0.001$). Intracellular ferritin also increased (1.3 ± 0.2–43.6 ± 0.6 nmole Fn/mg protein) in the same range of extracellular heme-Fe (one-way ANOVA: $p < 0.001$). HO1 expression was induced at high extracellular heme-Fe concentrations ($p < 0.01$). As expected, DMT1 expression decreased when intracellular Fe increased ($p < 0.05$). No change was observed in Ireg1 expression (Fig. 1c, d).

Immunolocalization of heme oxygenase

To determine the intracellular localization of HO1 enzyme, Caco-2 cells were incubated with or without 50 μM heme-Fe for 5 days and subjected to confocal microscopy. In Caco-2 cells incubated with heme-Fe, HO1 changed its localization from perinuclear to a domain close to the plasma membrane (Fig. 2A:a) compared with control cells (Fig. 2A:c). To confirm this result, a co-immunolocalization was performed in cells incubated with GLUT1 (2B:a) and HO1 (2B:b) antibodies. We observed that HO1 co-localizated with GLUT1 transporter (a basolateral marker in Caco-2 cells), which suggests that HO1 could be associated to an inner plasma membrane domain.

Characterization of Caco-2 cells over-expressing HO1

To enhance the expression of HO1, Caco-2 cells were transfected with *pcDNA3.1myc-his-ho1* vector (HO cells), and HO1 overexpression was confirmed by Western blotting (Fig. 3a). Under this condition, HO1 enzymatic activity increased from 6.4 ± 2.1 to 10.2 ± 0.4 nmole bilirubin/hr/mg protein, in control and HO1 cells, respectively ($p < 0.05$; Fig. 3b). Intracellular ferritin concentration also increased from 0.9 ± 0.1 to 6.4 ± 1.9 ng ferritin/mg protein, in control and HO1 cells, respectively ($p < 0.01$, Fig. 3c).

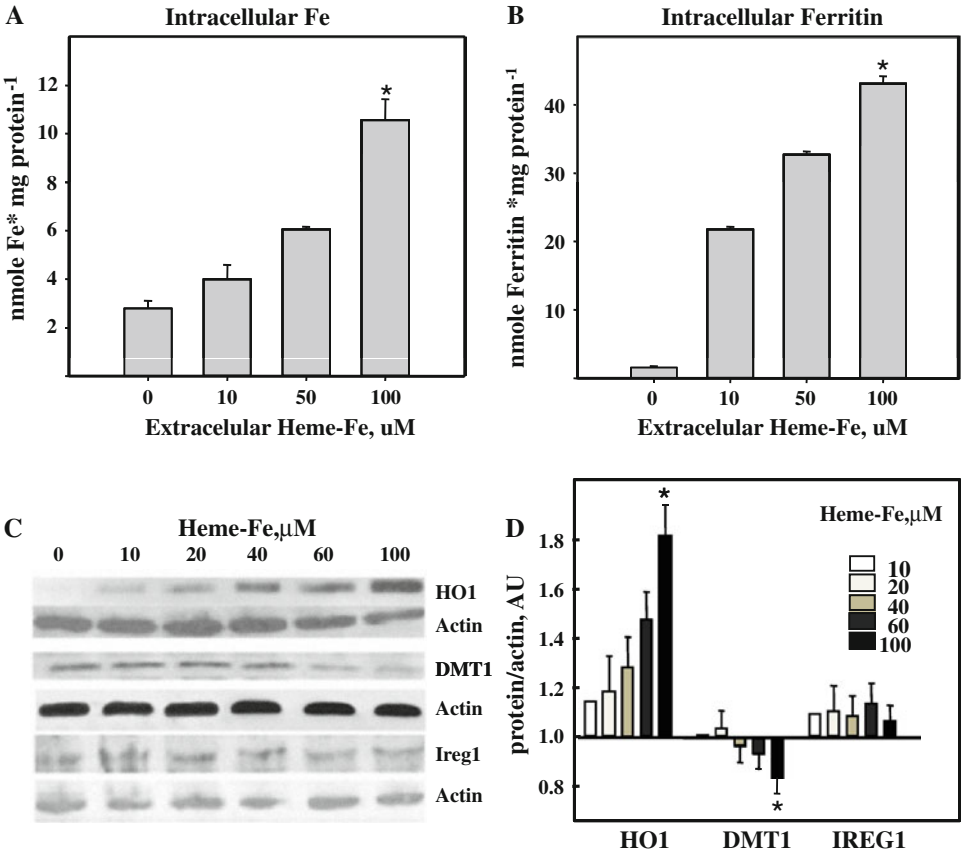

Fig. 1 Caco-2 cells were incubated with different heme-Fe concentration (range 0–100 μM). Intracellular total Fe was measured by spectrometric atomic absorption with graphite furnace Simaa 6100 (**a**), ferritin by ELISA (**b**), Western blot of HO1, DMT1, and Ireg1 (**c**) and densitometric analysis of C (**d**). (*One-way ANOVA, $p < 0.001$)

Heme-Fe and non-heme iron uptake and transport in HO cells

To determine the effect of HO1 over-expression on heme-Fe and iron uptake, HO1 cells seeded in bicameral inserts were incubated with 50 μM heme-^{55}Fe or 25 μM $^{55}FeCl_3$ added to the apical side, and radioactivity from the cell lysate and basolateral medium was measured to determine heme-Fe or iron uptake and iron transport, respectively. HO1 cells showed an increase in heme-Fe uptake ($p < 0.02$) (Fig. 4a) and transport of ^{55}Fe to the basolateral side, compared with control cells ($p < 0.01$) (Fig. 4b). There were no significant changes in apical non-heme iron uptake or apical to basolateral iron transport.

Apical to basolateral protoporphyrin and iron transport in HO cells

To elucidate which form of iron (i.e. heme-Fe or ferrous Fe) is the main contributor of apical to basolateral ^{55}Fe transport, heme (as a protoporphyrin) and non-heme iron were measured from the basolateral side after HO1 cells were incubated with 50 μM heme-Fe apically. Transport of protoporphyrin was significantly lower in HO1 cells compared to control cells ($p < 0.001$) (Fig. 5a). However, iron transport to the basolateral side, measured by calcein quenching, was higher in HO1 cells than in control cells (one-way ANOVA: $p < 0.02$) (Fig. 5b).

Discussion

The process of non-heme-Fe absorption by enterocytes is very well known [4, 10, 30, 34]. However, there are few studies regarding heme-Fe uptake and transport by intestinal cells. The movement of heme into and within cells was thought to occur by diffusion. However, the chemical properties of heme make diffusion too slow to keep pace with biological processes [21]. It has been suggested that heme enters cells as an intact molecule of metalloporphyrin [33], and three different mechanisms have been proposed for heme uptake: (1) pinocytosis [29, 42], (2) heme receptor on duodenal brush border [16] and (3) via the heme transporter HCP1 (heme carrier protein 1) [32], whose activity should be closely related to heme oxygenase enzyme. Iron released from heme is later found in the blood [10, 40, 41]. However, the mechanism of intracellular heme movement from apical to basolateral side has yet to be explained.

Heme-Fe was bioavailable for Caco-2 cells when they were incubated with different heme-Fe concentrations. Similar to what is observed in Caco-2 cells incubated with

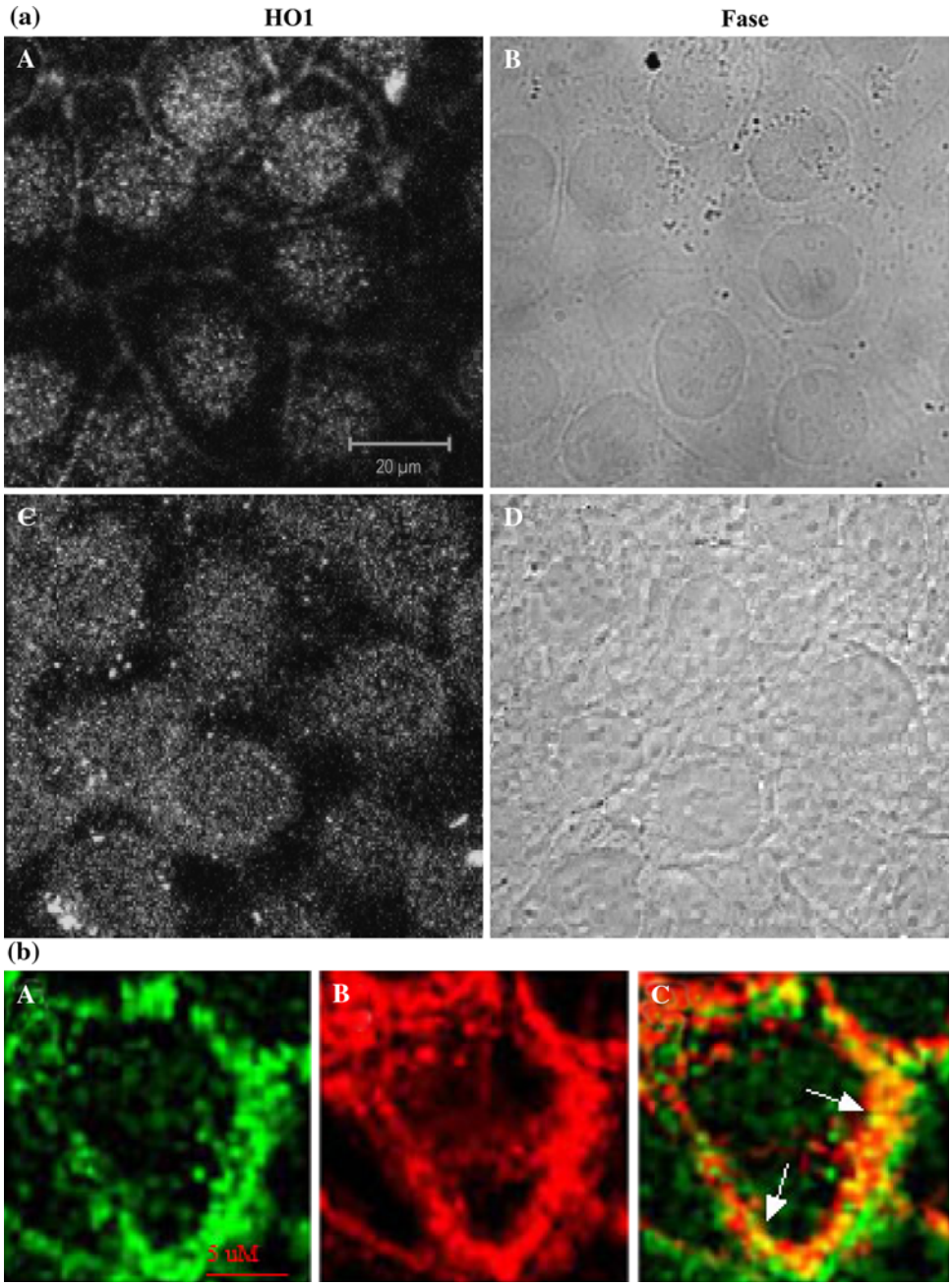

Fig. 2 Cellular HO1 distribution in Caco-2 cells. **a** Caco-2 cells were reacted with anti-HO1 antibody followed by FITC-labeled anti-rabbit IgG antibody as described in Methods (*A* and *C*). Caco-2 cells were incubated with (*A*) or without (*B*) 50 µM heme-Fe for 5 days. HO1 localization was assessed following FITC fluorescence in a confocal microscope. Phase contrast of Caco-2 cells (*B* and *D*) (**b**) Co-immunolocalization of HO1 and GLUT1 transporter. Caco-2 cells were incubated with rabbit anti-GLUT1 (*A*) and mouse anti-HO1 (*B*). Then cells were incubated with Alexa 546 anti-mouse IgG and Alexa 488 anti-rabbit IgG. Fluorescence was determined as mentioned earlier

non-heme iron [2], in cells incubated with heme-Fe, total intracellular iron and intracellular ferritin concentration increased. Furthermore, HO1 protein expression was dependent of heme-Fe bioavailability, as we had previously shown [3]. As a result of the increased intracellular Fe, DMT1 transporter expression decreased. However, Ireg1 protein expression did not change in the present experimental conditions. These results indicate that heme-Fe was available for the cell and that once iron is released it becomes part of the intracellular Fe pool. Similar results have been shown by Eisenstein et al. [12], who observed that release of iron from heme is necessary for maximal induction of ferritin synthesis and that direct donation of iron to the intracellular iron pool induced ferritin synthesis significantly, but it was not a good inducer of HO. In humans, Pizarro et al. [31], demonstrated that heme-Fe absorption is a saturable process post-ingestion of physiological doses of either hemoglobin or myoglobin.

To determine the role of HO1 enzyme on intracellular iron transport, we transfected Caco-2 cells with the HO1 cDNA. We observed an increase in HO1 protein expression and enzymatic activity and in intracellular ferritin concentration in these cells. The increase in ferritin concentration is an indicator of increased iron availability. Under

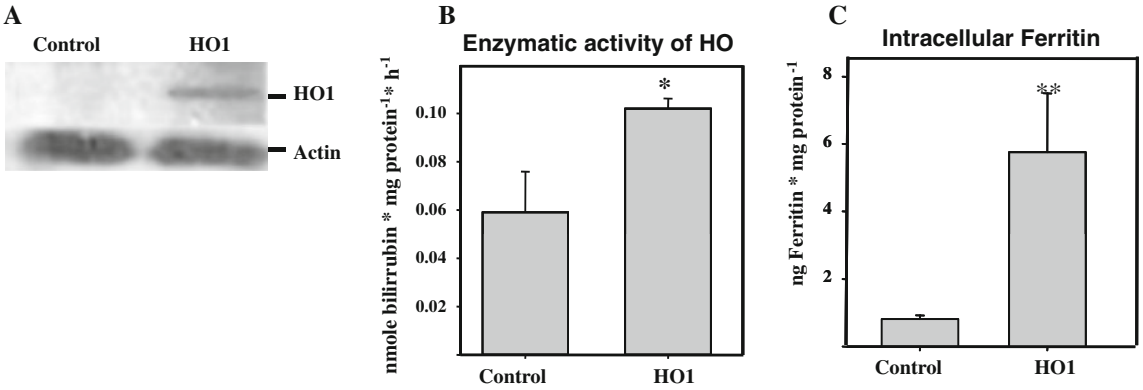

Fig. 3 Caco-2 cells were transfected with HO1 cDNA (HO1 cells). **a** HO1 Western blot in HO1 cells and control cells; **b** heme oxygenase activity; and **c** intracellular ferritin concentration. (*T* test: $*p < 0.01$; $**p < 0.001$)

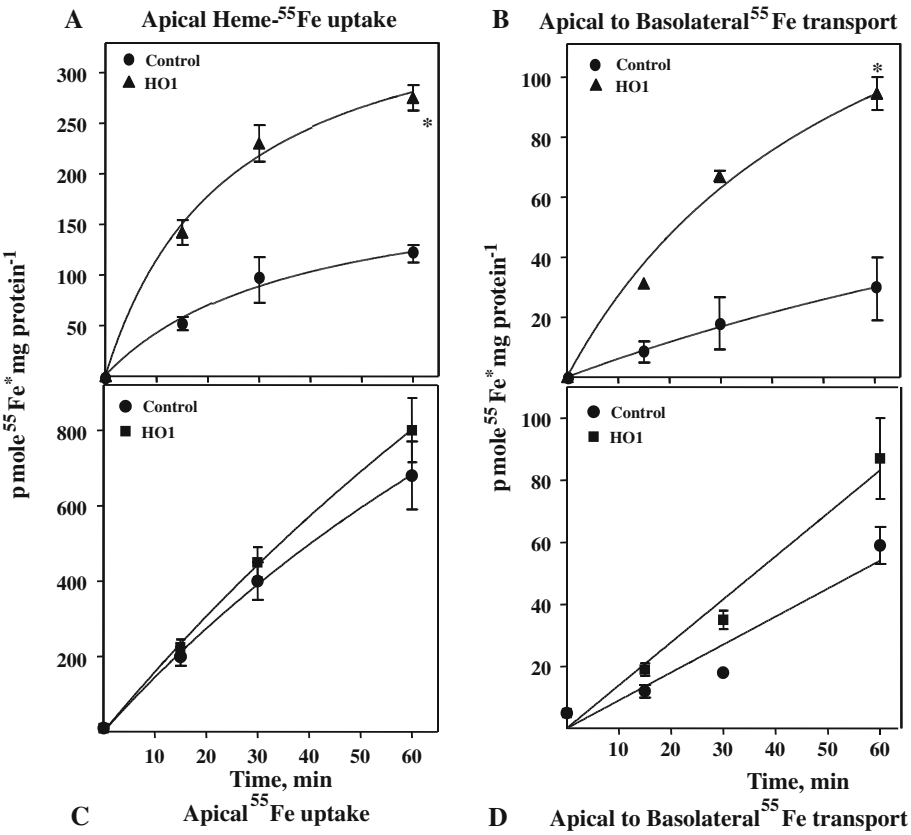

Fig. 4 Heme-Fe uptake (**a**) and apical to basolateral Fe transport (**b**) in HO1 cells and control cells. Caco-2 cells were incubated at 37 °C for 0–60 min with 10 μM heme-^{55}Fe. Radioactivity was measured in membranes and in basolateral media, (*two-way ANOVA, $p < 0.001$)

these conditions, heme-Fe uptake by HO1 cells was increased, which correlated positively with iron availability. This result suggests that in HO1 cells, which show a high HO1 enzymatic activity, the catabolism of intracellular heme is enhanced, resulting in a decrease in the intracellular heme/non-heme-Fe ratio. This is an indication that HO1 cells have a higher activity than controls cells that results in a decrease in heme transport and an increase in iron transport. Furthermore, the over-expression of HO1 triggers an increase in heme uptake, but does not modify non-heme-Fe uptake, which also suggests that the intracellular heme levels in these cells are lower, leading to a compensatory increment in heme uptake. The latter is possibly due to an up-regulation of the expression of a heme importer. Taken together, these results suggest that the increase in intracellular ferritin is due to an increase in heme uptake. In HO cells, heme-Fe uptake and apical to basolateral iron transport were higher than in control cells. Independent of iron concentration, the cells exposed to heme-Fe transported iron out of the cell at a higher rate.

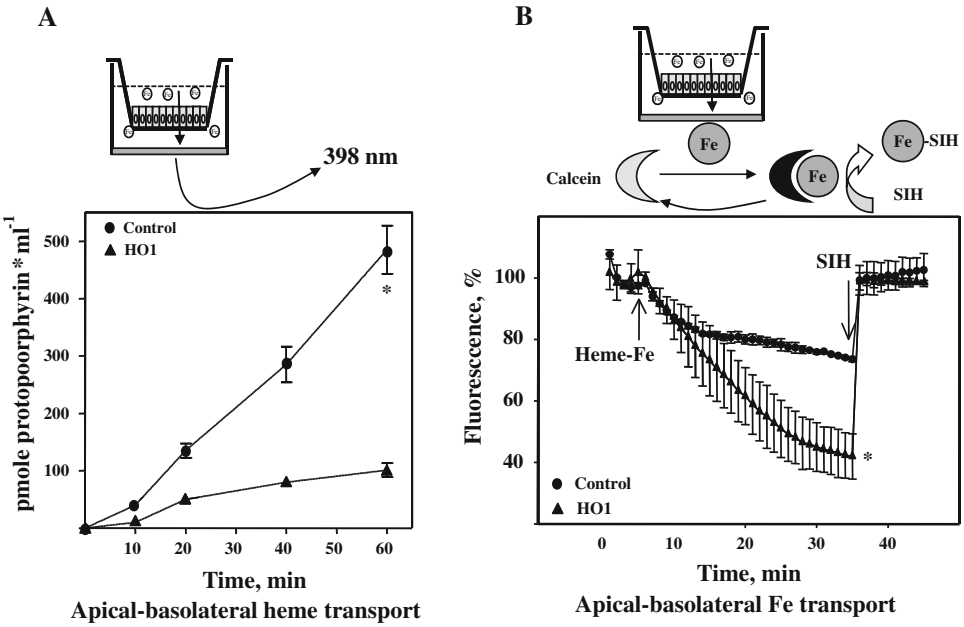

Fig. 5 Apical to basolateral heme transport (**a**) and apical to basolateral Fe transport. Caco-2 cells were cultured in bicameral chambers and incubated with heme-Fe in the apical side. Heme (**a**) and Fe (**b**) transport was measured in the basolateral chamber. (*Two-way ANOVA, $p < 0.001$)

It has been proposed that cells exposed to heme-Fe cannot sense iron uptake [8, 38]. However, as we were following ^{55}Fe, we cannot discriminate which form of iron (heme-Fe or Fe) was transported to the basolateral side.

We also studied whether heme is transported to the basolateral side. The results suggest that most of the heme-Fe was catabolized in the HO1 cells. However, a proportion of heme-Fe (as protoporphyrin) is transported intact to the basolateral side (control cells). Free heme and protoporphyrin are toxic to the cell; therefore, cells must balance their intracellular metabolism, and for this reason free heme must be transported out of the cells. This transport could be performed by FLVCR (Feline Leukemia Virus subgroup C Receptor) [18]. FLVCR protects erythroid cells from heme toxicity during differentiation. This heme-efflux protein is expressed in other cells and tissues, including the intestine, where they appear to function as apical/basolateral heme exporters to prevent toxicity within the enterocytes [22, 32]. In this study, iron in the basolateral media was threefold higher in HO1 cells than in control cells.

Heme oxygenase-1 distribution in control cells was mainly perinuclear, which corroborates previous results from this group [27]. However, in cells incubated with heme-Fe, the expression of HO1 was higher and detected at a peripheral compartment. In Caco-2 cells that were over-expressing HO1, the intracellular localization of HO1 changed from a perinuclear to a putative plasma membrane topology. Kim et al. [20], using inducers of HO1 or over-expression of HO1, demonstrated an increase in HO1 protein in a detergent-resistant fraction containing caveolin-1. Inducible HO activity appeared in plasma membrane, cytosol, and isolated caveolae. HO1-GLUT1 co-localization suggests a basolateral localization of HO1 in Caco-2 cells. However, it is necessary to take into account that most of the membrane surface in these cells corresponds to a basolateral membrane. It is probably that HO1 is associated to a structure that itself interacts with the plasma membrane. Further experiments are necessary to dilucidate this question.

In summary, our study shows that Caco-2 cells can be used as a model to study intestinal heme-iron metabolism when high specific activity heme ^{55}Fe is used. Heme-Fe is taken up by the cells, mostly degraded by HO1, and free iron forms part of the labile iron pool, which is delivered either to ferritin or to the basolateral side. A fraction of heme can be transported out of the cells intact.

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