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Immunoreactive Hephaestin and ferroxidase activity are present in the cytosolic fraction of rat enterocytes

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Abstract

Discovered over a decade ago, hephaestin (Heph) has been implicated as a ferroxidase (FOX) vital for intestinal iron absorption. Stringent structural or kinetic data derived from purified, native protein is however lacking, leading to the hypothesis that an alternate, undiscovered form of Heph could exist in mammalian enterocytes. This possibility was tested using laboratory rodent and cell culture models. Cytosolic and membrane fractions were obtained from rat enterocytes and purity of the fractions was assessed. Western blot analyses revealed Heph in cytosol obtained by three different methods, ruling out the possibility of a method-induced artifact being the major contributor to this observation. Absence of two different membrane-proteins, ferroportin 1 and Menke's copper ATPase in cytosol, and the absence of lipids in representative cytosolic samples tested by thin layer chromatography, eliminated significant membrane contamination of cytosol. Further, immunohisto- and immunocyto-chemical analyses identified Heph in rat enterocytes and in two intestinal epithelial cell lines, IEC-6 and Caco-2, intracellularly. Additionally, cytosolic Heph increased upon iron-deprivation but more important, decreased significantly upon copper-deprivation, mimicking the response of membrane-bound Heph. Moreover, FOX activity was present in rat cytosol, and was partly inhibited by anti-Heph antibody. Finally, lack of immunodetectable ceruloplasmin (Cp) by western blot precluded Cp as an underlying cause of this activity. These data demonstrate that rat enterocytes contain a soluble/cytosolic form of Heph possibly contributing to the observed FOX activity.

Keywords

Intestine; Iron; Copper; Absorption

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Perungavur N. Ranganathan and Yan Lu both contributed equally to this work.

Introduction

Hephaestin (Heph) and ceruloplasmin (Cp) typify mammalian cupro-enzymes deemed necessary for the oxidation of iron to facilitate intestinal iron absorption (Heph), and iron release from various organs (e.g. liver and brain) (Cp). Cp has been extensively investigated since its discovery (Holmberg and Laurell 1948) at biochemical, kinetic and structural levels including by X-ray crystallography (Bielli and Calabrese 2002). Conversely, Heph, first identified as the mutant gene in the sex-linked anemia (*sla*) mouse (Grewal 1962; Vulpe et al. 1999), has not been thoroughly investigated at structural or functional levels. With no unequivocal structural/biophysical evidence, the protein was established to be membrane-inserted and considered to be the vital ferroxidase (FOX) essential for Fe oxidation in the intestine (Kuo et al. 2004; Hellman and Gitlin 2002). Several important caveats to these assumptions should be considered: (1) wild type Heph was never purified to homogeneity from natural sources: (2) neither partial amino acid (AA) sequence nor an experimentally derived AA composition is available: (3) the purported protein "structure" is based entirely on homology modeling compared to Cp (which is a soluble protein, derived from a different gene, synthesized at a different site, and found in different locations). This is further complicated because the AA sequence of Heph is a derived one (from cDNA), which is hypothetical: (4) western blot analyses were based on total cell/tissue lysates, the major constituent of which is the cytosol: (5) the same ambiguity applies to FOX activity measurements since they were also done in total lysates: and (6) elegant work by Griffiths et al. (2005) demonstrated FOX activity in a recombinant Heph devoid of the membrane-spanning segment (i.e., soluble). Such a lack of experimental structural/functional data on intestinal Heph raises questions about its site of function in enterocytes, particularly since the immunoreactive protein has been observed in a "supranuclear" compartment (Frazer et al. 2001; Kuo et al. 2004) and in endosomes (Kuo et al. 2004) of intestinal epithelial cells.

Previous studies determined that the cytosolic/soluble fraction of rat and mouse enterocytes contains a functional FOX (Ranganathan et al. 2012). Given the above delineated ambiguities regarding various aspects of Heph, the possibility that an alternative form of Heph could exist was considered. Experiments described in this manuscript indeed provide evidence for a cytosolic/soluble form of Heph expressed in rat enterocytes, possibly contributing to the observed FOX activity in this cellular fraction.

Materials and methods

Chemicals and reagents

Chemicals were of analytical grade or high purity, purchased from Sigma-Aldrich (USA) and Fisher Scientific (USA). Other sources are listed as appropriate.

Animals, diets, and tissue collection

Animal studies utilizing male Sprague–Dawley rats, were approved by the Institutional Animal Care and Use Committee, University of Florida. Diets used were control (Ctrl; ~200 ppm Fe, 6 ppm Cu), iron-deficient (FeD; <3 ppm Fe, 6 ppm Cu) or copper-deficient (CuD; ~200 ppm Fe, ~1 ppm Cu), manufactured according to AIN-93G standards (Dyets Inc., Bethlehem, PA). Rats were weaned onto respective diets and sacrificed ~35 days later. All aspects of animal housing, tissue collection, and storage were carried out following standard procedures (Ranganathan et al. 2011).

Enterocyte isolation and subcellular fractionation

Enterocytes were isolated from duodenum and proximal jejunum of rats as described (Jiang et al. 2011).

Subcellular fractionation

Method I (grinding): Cytosolic and solubilized, particulate membrane fraction preparations were as described (Collins et al. 2008). All steps were per formed at 4°C. Briefly, enterocytes were homogenized by a tissue grinder in buffer 1 (0.025 M Tris–HCl, pH 7.4, 0.025 M NaCl, plus protease inhibitor cocktail) and centrifuged at 16,000*g* for 15 min. Cytosolic fractions were obtained by re-centrifuging the supernatants at 110,000*g* for 1 h. The pellets were resuspended in buffer 2 (buffer 1 with 0.25% [v/v] Tween-20), sonicated 2 × 5 s at 5 watts in an ice-water slurry with 15 s chilling in between and re-centrifuged at 16,000*g* for 30 min. These supernatants were termed solubilized membrane fraction.

Method II (hypotonic lysis): Enterocytes were incubated in buffer 1 on ice for 30 min with frequent mixing with 1 ml pipette tips and centrifuged at 16,000*g* for 15 min. Subsequent steps were identical to method 1.

Method III (freeze/thaw): Enterocytes were suspended in buffer 1 and snap-frozen in liquid nitrogen, then quickly thawed in a 37°C water bath. This temperature-change cycle was repeated five times and lysis of cells was confirmed visually under a microscope. Subsequent steps were identical to method I. Protein concentrations were estimated using the Pierce 660 nm protein assay reagent (Thermo Fisher Scientific, Rockford, IL).

Thin layer chromatography

Following the general method of Bligh and Dyer (1959), membrane and cytosolic fractions were extracted with 3:2 C_6H_{14}:$(CH_3)_2CHOH$, and developed on a silica gel plate, using 2:1 $CHCl_3$:CH_3OH as the mobile phase, along with purified phospholipids that were run as a positive control.

Western blot analysis

Experiments were performed using 30 μg of cytosolic and membrane-proteins as described earlier (Collins et al. 2008), using standard methods. The anti-Atp7a (Menke's copper ATPase) antibody (Ravia et al. 2005) (called 54–10), and one anti-Heph antibody (Chen et al. 2009) (called D4) are well-validated. The other anti-Heph antibody was from GeneTex (Cat # GTX115300, Irvine, CA). The D4 antibody recognizes an epitope in the central portion of the Heph protein (domain 4) while the GeneTex antibody recognizes the N-terminus. Anti-ferroportin Ab was from Santa Cruz Biotechnology (Cat# sc-49668, Santa Cruz, CA). Anti-Cp Ab was from Sigma (Cat # GW20074F, St. Louis, MO). KNRK (normal rat kidney [NRK] cell line transformed by Kirsten murine sarcoma virus) lysate, a kind gift (Cat# sc-2214, from Santa Cruz Biotech, CA) was used as a positive control. Goat anti-rabbit HRP-conjugated secondary antibody was from Bethyl Laboratories, Montgomery, TX (Cat # A120-101P). Donkey anti-goat HRP-conjugated secondary Ab was also a kind gift from Santa Cruz Biotechnology (Cat # sc-2020, Santa Cruz, CA). Rabbit anti-chicken IgY-HRP Ab was a kind gift from Sigma-Aldrich (Cat # A9046, St. Louis, MO). Blots were processed following a standard protocol (Ranganathan et al. 2011). To exemplify equal loading and transfer of proteins, in some figures, Ponceau S stained blots are shown below the chemiluminescent images on film. This approach avoids potential confounding variation in any single "housekeeping" protein.

Cell culture, immunocytochemistry (ICC) and immunohistochemistry (IHC) analyzes

IEC-6 and Caco-2 cells, obtained from ATCC, were cultured according to recommendations (www.atcc.org). Cells were seeded onto poly-D-lysine (Cat # P6407, Sigma-Aldrich, St. Louis, MO) coated cover slips in six well plates. Upon reaching ~80% confluence, medium was removed, cells were washed twice with ice-cold PBS and fixed with freshly made 4% formaldehyde in PBS at room temperature for 20 min. After three washes with PBS, the cells were incubated with IHC blocking buffer (Bethyl Laboratories) for 30 min, followed by anti-Heph antibody (GeneTex [1:500] or D4 [1:1,000]) or PBS, for 2 h at room temperature with gentle rocking. After three brief washes with PBS, the cells were incubated with a fluorescent-tagged secondary antibody (Alexa Fluor 647 goat anti-rabbit IgG [1:2,000]; Invitrogen Molecular Probes) in PBS for 30 min at room temperature. After three washes with PBS, the cover slips were drained, mounted on glass slides with fluorescent mounting medium and dried in the dark. The fluorescent signals were visualized with a Leica SP5 confocal microscope at the Interdisciplinary Center for Bio-technology (ICBR) of the University of Florida. Confocal settings were kept identical across different samples in the same experiment, enabling direct comparison of fluorescence intensity. Transverse segments from the proximal rat duodenum were fixed in formalin and embedded in paraffin. Ten micrometer sections were cut and affixed onto positively charged microscope slides. After de-paraffinization, tissue sections were reacted with IHC blocking buffer for 30 min followed by reaction with anti-Heph antibody (D4) overnight at 4°C in a humidified chamber. The remaining steps were as stated above for ICC analysis.

Enzyme activity assays

Transferrin-coupled FOX initial velocity assay—Equal amounts (60 μg, unless stated otherwise) of cytosolic or membrane-protein from isolated enterocytes of each animal, in 0.125 M CH_3COONa buffer, pH 5.0, were mixed with 50 μM bovine apo-transferrin (Sigma-Aldrich, St. Louis, MO). Reaction was initiated by adding $(NH_4)_2Fe(SO_4)_2$ to a 50 μM final concentration, in 200 μl total volume. Initial velocities from 30 to 120 s were obtained by following dA/dt at 460 nm in a Beckman DU 640 spectrophotometer, at room temperature. Blanks were run for each experiment. Biological inhibition was by pre-incubation of the protein with the anti-Heph antibody (D4) or an unrelated antibody (anti-Alox15) for 16 h at 4°C prior to running assays.

Results and discussion

Heph expression in rat intestinal cytosol and fraction purity

To eliminate chances of a method-induced artifact being a major contributor to the observed outcome, at the outset, cytosolic and membrane portions were obtained from enterocytes lysed by three different methods, i.e., grinding, hypotonic lysis, and freeze/thaw, and results from two representative samples from each method are shown. The membrane fractions contained the expected immunoreactive Heph in all six samples (Fig. 1a, bottom row). Interestingly, the cytosolic fraction also clearly revealed Heph in all six samples (Fig. 1a, top row). As there are no published reports of a soluble/cytosolic Heph in rodent enterocytes, additional experiments were done to rule out membrane contamination of cytosol. Cytosolic nature was first ascertained by the presence of lactate dehydrogenase (LDH, marker enzyme) activity (Ranganathan et al. 2012). Experiments performed to assess fraction purity by employing immunoblotting against a dilution series of a well-documented membrane-protein (Atp7a)-spiked cytosol, revealed >95% purity (Fig. 1b), while detecting strong bands in membrane, as expected. To reconfirm this data, western blot analysis was repeated using antibody against a second membrane-protein, ferroportin 1 (Fpn1); no Fpn1 was detected in either cytosol, while it was present as expected in both membrane fractions, with KNRK cell lysate serving as a positive control (Fig. 1c). Finally, as a third precautionary step, two

representative cytosolic and membrane fractions were also analyzed by TLC. As shown in Fig. 1d, both membrane fractions contained phospholipids as expected ("P-lipid" used as a [+] control), while both cytosols were negative, further supporting the results obtained by immunoblotting. Thus, the results from these four different experimental approaches not only validate the authenticity of the cytosolic fraction, but also remove any doubt that the observed location of Heph could have resulted from either a method-induced artifact or from contamination by membrane. The presence of two versions of the same protein is not unique. For example, a GPI-anchored form of Cp exists in mammalian astrocytes (Patel and David 1997) and a truncated transferrin receptor has been described in sheep reticulocytes (Ahn and Johnstone 1993).

Detection of Heph by immunofluorescence

To determine Heph protein location in cells and tissues, immunohistochemistry (IHC) and immunocytochemistry (ICC) analyses were performed using the same D4 anti-Heph antibody. In rat intestinal sections, a specific signal was detected in the basolateral membrane of enterocytes in addition to a non-descript staining pattern inside epithelial cells (Fig. 2a). Secondary antibody alone did not lead to any noticeable signal (panel b), attesting to the specificity of the staining in panel A. Moreover, the D4 antibody produced a similar, intracellular punctuate staining pattern in IEC-6 (panel c) as well as Caco-2 cells (panel d). Similar results were observed with Genetex anti-Heph antibody (data not shown). Since (i) Heph was also detected in a native state and (ii) in all three different biological sources tested (i.e., rat intestine and rat- and human intestine-derived cell lines), this set of data provides further support for the existence of a putative, intracellular form of Heph. It is worth reiterating that Heph has been documented in a supra-nuclear location (Frazer et al. 2001; Kuo et al. 2004) and in mouse endosomes (Kuo et al. 2004).

Heph expression in response to iron and copper deficiency

Since membrane Heph is known to respond differently when rodents are subjected to deprivation of dietary iron or copper (Chen et al. 2004), the next logical step was to test if this putative, soluble Heph responded likewise. Rats weaned onto the deficient diets had drastic reductions in serum hemoglobin and hematocrit levels (>80% in the FeD group and >50% in the CuD group for both parameters) indicating the effectiveness of the dietary regimen, (data not shown). Denaturing western blot data from two representative samples using anti-Heph (D4) antibody showed an increase in Heph protein upon Fe withdrawal compared to control rats, similar to the membrane-bound form (Fig. 3a). Notably, repeating the immunoblotting under native conditions yielded very similar results (Fig. 3b), indirectly supporting results shown in Fig. 3a. Further, being a multi-Cu FOX, membrane Heph is known to decrease in low Cu conditions, presumably due to the requirement of Cu as a prosthetic group. As shown in Fig. 3c, a clear decrease in cytosolic Heph upon Cu withdrawal was noted as compared to control rats, again mimicking the membrane-bound form. To obtain further validity, the experiment was repeated with three representative samples using a different anti-Heph antibody (GeneTex), which also yielded identical results in all three samples (Fig. 3d). Thus, results shown in Fig. 3, representing samples obtained from multiple experimental animals all tested individually using two anti-Heph antibodies, showing parallel response to withdrawal of two physiologically relevant metal ions, Fe and Cu, strengthens the link with membrane Heph.

Ferroxidase activity in rat enterocyte cytosol

Since catalytic activity is the ultimate proof for an enzyme, FOX activity was tested in cytosol using *apo*-transferrin in a coupled-assay, with diferric-transferrin being the end-product. Rat cytosol clearly exhibited FOX activity, as evidenced by the progress curve shown in Fig. 4a. This enzymatic activity was reduced by >30% by anti-Heph antibody (Fig.

4b), suggesting that at least part of this activity could be attributed to a soluble/cytosolic Heph or an immunologically related protein.

These data raise two important points: (1) a cytosolic FOX would only be of physiologic relevance if its substrate (Fe^{2+}) was also present, and (2) a soluble FOX could function within intracellular, iron-containing membrane-bound structures. With regard to the initial point, several lines of evidence support the presence of free iron in the cytosol, existing predominantly as Fe^{2+}, the substrate for FOX. First and most important, the cytosolic environment is reducing, favoring any iron present as Fe^{2+} (Hider and Kong 2011; Williams 1982). Millero (1985) has shown that Cl^{-} and CO_3^{2-}, which are physiologic anions, retard oxidation. Moreover, a cytosolic pool of free iron has been proposed, constituting ~5% of the total (Andrews 2004), being buffered as glutathione (GSH)-complexed iron (Fe^{2+}–GS) due to cytoplasmic GSH. Moreover, cytosolic electrode potential (E^0, the potential difference between the metal and the solution, in *V)* is considered to favor Fe^{2+}over Fe^{3+}(Morgan and Lahav 2007). In addition, ~80% of the iron pool in K562 (human, myelogenous leukemia) cells is known to exist as Fe^{2+} (Breuer et al. 1995). These facts delineate how an intracellular environment can support the existence of Fe^{2+}, the substrate for FOX. Although, the cytosolic environment favors Fe^{2+}, oxidized iron could interact with other proteins/biological molecules that could stabilize it in the ferric form.

Regarding the second point mentioned above, cytosolic FOX is active at pH 5.0, which is consistent with the pH of acidified, intracellular vesicles. Interestingly, Heph has been localized in endosomes, presumably within the membrane (Kuo et al. 2004). Ferroportin 1, the only intestinal iron exporter, has also been observed within intracellular compartments (Yeh et al. 2011). Cytosolic FOX could thus be located within Fe^{2+} containing vesicles, perhaps participating in the proposed *trans*-cytosis of dietary iron (Ma et al. 2002). Thus, data presented so far provide preliminary rational, immunological and enzymatic support for a cytosol-based FOX activity similar to membrane FOX activity, possibly contributed at least in part by a soluble form of Heph.

Could intestinal Cp explain cytosolic FOX activity?

Intestinal Cp has been noted in mice (Cherukuri et al. 2005), and if it is true in rats, at least part of the anti-Heph Ab-resistant activity (>60%) could be attributed to Cp. To test this possibility, enterocyte cytosols were immunoprobed with a well-established anti-Cp antibody. Western blot data from a representative sample shown in Fig. 5 clearly demonstrates that no Cp could be detected in rat cytosol. Presence of the expected band in serum (positive control) and its absence in membrane (negative control) in addition to authenticating the data, suggests that Cp may not contribute to this activity.

Conclusions

The objective of this study was to test the hypothesis that an alternative form of Heph exists in rat enterocytes. This supposition was precipitated by a lack of rigorous and cohesive structural and kinetic data pertaining to the presumed membrane-bound form of Heph. To this end, after establishing the purity of rat enterocyte-derived cytosolic preparations by six different approaches, data presented in this manuscript show the presence of cytosolic Heph by: (1) immunolocalization in a native form from three different (one animal and two cell lines) sources/samples; (2) clear presence of the expected (~130 kD) band not only by standard denaturing (SDS) PAGE but also under native conditions, thereby also supporting the IHC and ICC data; (3) responding to two different dietary conditions, i.e. increasing during iron deficiency and decreasing during copper deficiency, similar to membrane-bound Heph; (4) most important, displaying FOX enzymatic activity in all rat samples studied consistent with the presence of immunoreactive Heph; and finally, (5) indicating the lack of

involvement of Cp in this phenomenon. The data presented here when considered in its entirety suggest that a cytosolic/soluble form of Heph may exist in rat enterocytes. From a functional standpoint, given the low catalytic efficiency of FOXs in general and rodent FOX in particular, the cytosolic FOX envisaged here could augment the limited iron oxidizing capacity of enterocytes.

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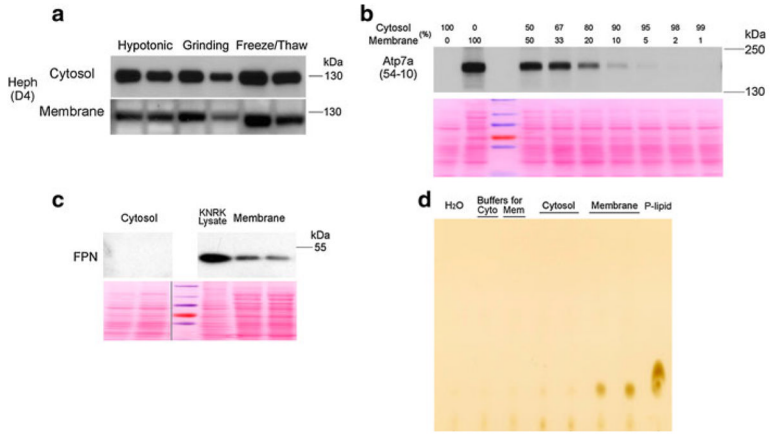

Fig 1.

Presence of immunoreactive Heph in cytosolic and membrane fractions and fraction purity. *Panel a* western blot of Heph protein in cytosolic and membrane fractions of rat duodenal enterocytes prepared by three different methods. *D4* indicates the particular anti-Heph Ab used. *Panel b* western blot of Atp7a protein in 60 μg protein samples containing various ratios of membrane to cytosolic proteins. *54–10* indicates the particular anti-Atp7a Ab used. *Panel c* western blot of FPN protein in rat duodenal enterocytes and KNRK (a transformed rat kidney [NRK] cell line) lysate (positive control). Results from two individual rats are shown. Stained blots in *panels b* and *c* exemplify proper loading and transfer of proteins. The *blank space* and *black line* in *panel c* indicate removal of unrelated lanes. *Panel d* thin layer chromatography (TLC) of water, buffers, enterocyte cytosol and membrane fractions, and *P-lipid* (phospholipid, used as a positive control)

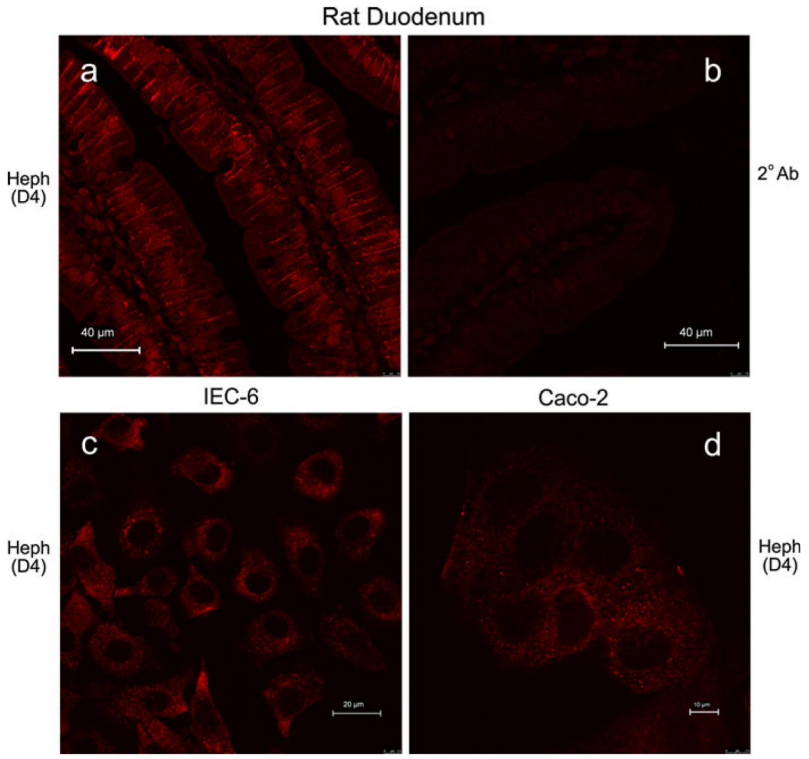

Fig 2.

Immunofluorescence analysis of rat duodenum, IEC-6 cells, and Caco-2 cells. *Panels a and b* immunohistochemical analysis of duodenal sections from an iron-deficient rat (**a**) with and (**b**) without the D4 anti-Heph antibody. *Panels c and d* immunocytochemical analysis of pre-confluent IEC-6 and Caco-2 cells. Data in all panels are representative of three independent experiments performed

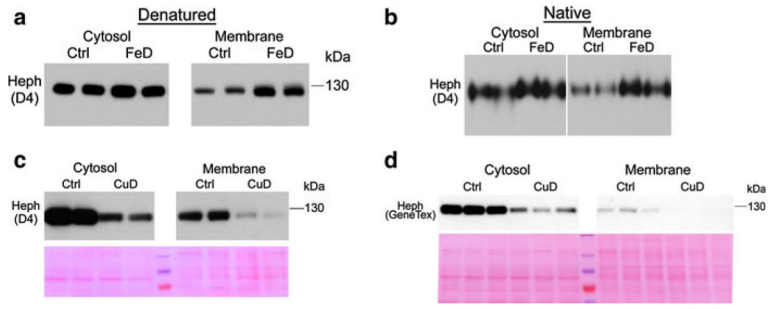

Fig 3.

Effects of iron- and copper-deprivation on Heph protein expression. *Panel a* shows denaturing and *panel b* shows native western blot analysis of Heph in cytosolic and membrane fractions of Ctrl and FeD rat enterocytes. Data in panel a are representative of experiments done with 12 individual rats per group. *Panels c and d* show denatured western blot analysis of Heph in cytosolic and membrane fractions of Ctrl and CuD rat enterocytes by (**c**) D4 and (**d**) GeneTex anti-Heph Abs. Stained blots below show equal loading and transfer of protein samples. In *panels a–c* data from two rats per group are shown, and in *panel d* data from three rats per group are shown. *Ctrl* control, *FeD* iron-deficient, *CuD* copper-deficient

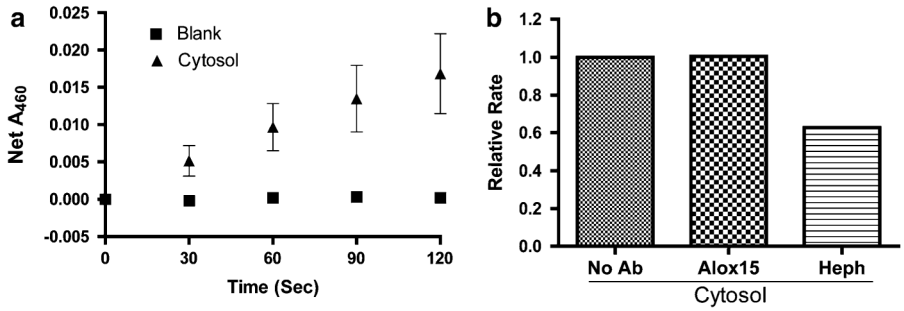

Fig 4.

FOX activity of rat duodenal enterocyte cytosol and antibody inhibition. *Panel a* shows the enzyme progress curve of rat cytosol FOX activity by Tf assay. Data represent three individual rats, mean ± SD is shown. *Panel b* effect of anti-Heph (D4) and anti-Alox15 (against an unrelated protein) Abs on cytosolic FOX activity by Tf assay. $n = 2$ individual rats, mean is shown

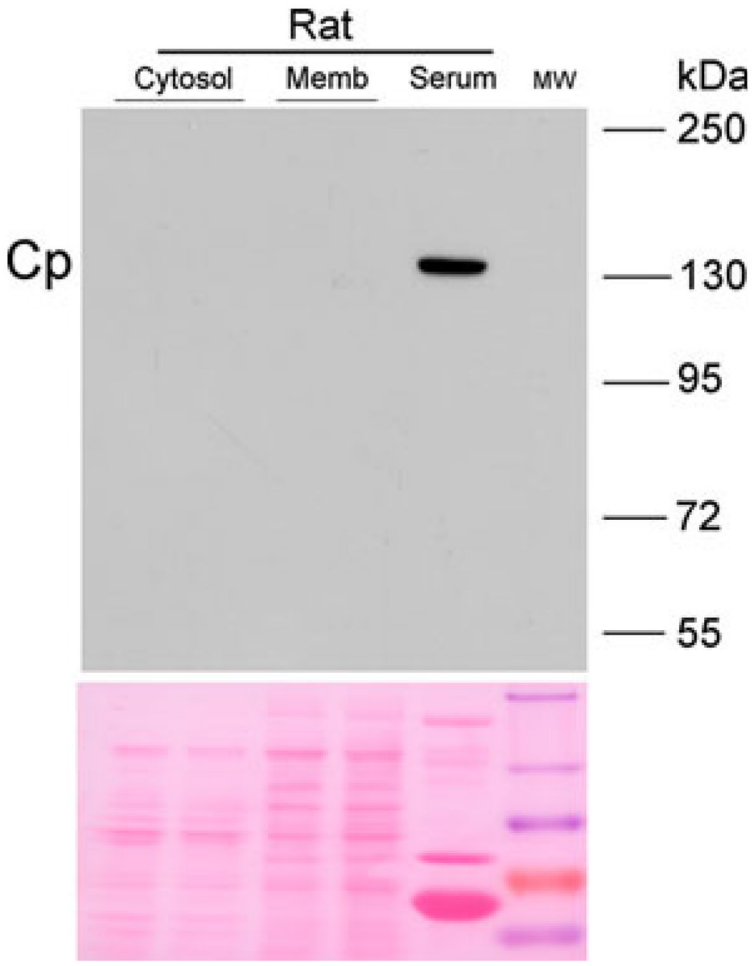

Fig 5.

Ceruloplasmin expression in rat duodenal enterocyte fractions and serum. A western blot for Cp is shown above. The *stained blot* below exemplifies equal loading and transfer of protein samples. Data are representative of two independent experiments that were performed with identical results. MW, molecular weight markers. Memb, membrane