Placental ceruloplasmin homolog is regulated by iron and copper and is implicated in iron metabolism

RUTH DANZEISEN,1 CEDRIC FOSSET,1 ZEHANE CHARIANA,1 KENNETH PAGE,2 SAMUEL DAVID,3 AND HARRY J. McARDLE1
1The Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB;
2Department of Biomedical Sciences, Institute of Medical Sciences, University of Aberdeen, Aberdeen AB25 2ZD, Scotland, United Kingdom; and 3Center for Research in Neuroscience, The Montreal General Hospital Research Institute and McGill University, Montreal, Quebec, Canada H3G 1A4

Received 16 January 2001; accepted in final form 18 October 2001

DURING PREGNANCY, iron (Fe) is transported from mother to fetus across the placenta. The amount of Fe transported is considerable (300–400 mg) and equivalent to the total maternal body stores (4, 5). The mechanism of transport and putative gene products involved in the process are beginning to be elucidated. Transfer can be considered as occurring in three main stages: uptake across the microvillar membrane, transfer across the cytoplasm of the syncytiotrophoblast, and release into the fetal circulation.

In the placenta, Fe is taken up from Fe-transferrin (Fe-Tf) (3, 23, 36). The protein binds to the transferrin (Tf) receptor and is accumulated in endosomes. Fe is released from Tf after acidification of the endosome and is transferred to the cytoplasm (22). Fe efflux into the cytoplasm is probably through divalent metal transporter-1 (14, 33).

The mechanism of Fe transfer from the apical to the basolateral side of the placenta is not understood. Chaperone proteins, analogous to those described for copper (Cu) (19), may be involved, or perhaps the metal binds to moieties such as citrate. Explanation of these processes awaits further research.

Until recently, we also knew little about the mechanism of efflux. With the recent identification of ferroportin/iron-regulated transporter-1 as the putative Fe exporter on the basolateral side of placental syncytiotrophoblast and duodenal enterocytes, one step of the release process has been clarified (1, 11, 25). Furthermore, recent studies investigating Fe transfer across the gut have provided some valuable information. In mice, the sla mutation, which results in increased accumulation of Fe in the intestine, was shown to be the result of mutation in a protein called hephestin. Hephaestin is a Cu oxidase with a high degree of homology to serum ceruloplasmin (Cp) (37). Many studies have shown that Cp is essential for Fe release from a variety of tissues. For example, patients with aceruloplasminemia, a mutation that results in complete absence of serum Cp, accumulate Fe in brain and liver (18). Similarly, in animals with severe Cu deficiency, Fe is trapped in various tissues, causing hypoferrremia (21, 28, 30). It was hypothesized that Cp, a ferroxidase, was required to oxidize Fe(II) released from cells to Fe(III) for incorporation into Tf. Absence of the protein resulted in a failure of Fe release. Vulpe and colleagues (37) suggested that hephestin fulfilled the same function in the gut, and mutations in the protein resulted in loss of the ability to transfer Fe from the intestine to the portal circulation.

To study placental Fe transport, we use a placental cell line (BeWo). These cells are a choriocarcinoma...
with properties similar to those of human placental trophoblast, such as differentiation into a syncytiun and formation of a mature brush border on treatment with forskolin (38). When grown on porous filters, BeWo cells have the ability to form a polarized monolayer and display directional transport features, both of which cannot be observed in cells grown on a plastic surface (7, 8, 10, 27). Furthermore, BeWo cells have been used extensively as a model to study placental Fe uptake and flux (7, 8, 34–36). Using the BeWo cell line, we have presented evidence for an endogenous membrane-bound homolog to Cp, similar to hephaestin in the gut (10). The placental protein cross-reacts with antibodies against serum Cp, giving a main band at 100 kDa and a weaker band at 140 kDa. We have not determined whether this protein is derived from the gene for Cp or hephaestin or whether it might be a new third member in a family of homologous Cu oxidases. We therefore refer to this protein as “placental Cu oxidase.”

We previously showed that exogenous Cp does not stimulate Fe efflux from the placental cell and that there is an endogenous Cu oxidase (10). Here we present data showing that the placental Cu oxidase is regulated by Fe and Cu status. We show that p-phenylene diamine (PPD) activity in BeWo cells is enriched in the nuclei and the non-brush-border membranes, consistent with the localization of the Cu oxidase. Finally, we show that, depending on the environment of the cell, the endogenous Cu oxidase may be critical for normal Fe release.

MATERIALS AND METHODS

Cell culture. BeWo cells were obtained from American Type Culture Collection and routinely cultured in 80-cm² flasks (Nunc) at 37°C in 5% CO₂-95% air and 100% humidity with complete medium [Ham’s F-12 Glutamax nutrient mixture with 10% fetal bovine serum (FBS) and 2% penicillin-streptomycin; all from Life Technologies]. For some experiments, the cells were incubated in 5% CO₂-5% O₂-90% N₂. The control cells were incubated for 18 h with 59Fe-Tf (0.5 Ci/plate). The Cu-deficient cells were incubated for 18 h simultaneously with 20 μM diamsar and 59Fe-Tf (0.5 μCi/plate). The control cells were incubated for 18 h with 59Fe-Tf (0.5 μCi/plate) alone. The 18-h incubation was carried out in 5% CO₂-95% air (−20% O₂) or 5% CO₂-5% O₂-90% N₂. We refer to these two conditions as “20% O₂” and “5% O₂,” respectively. After completion of the 18-h incubation, the cells were washed three times with ice-cold BSS and harvested using 1 M NaOH. Samples were counted in a gamma counter (Cobra 3100, Packard). Where necessary, DNA values were estimated from these and previous data (21 ± 0.4 and 21.4 ± 0.5 μg/plate for controls and diamsar, respectively, n = 131).

Because the cells have accumulated 59Fe from complete medium overnight, we cannot know the true specific activity of the labeled Tf. Consequently, we cannot give accumulation in terms of Fe. Instead, we normalize to the volume equivalent. Briefly, we counted known volumes of incubation medium and derived a specific activity for the medium (cpm/ml medium). We used this value to normalize the data and to allow comparison between experiments.

59Fe efflux experiments (low O₂). BeWo cells were seeded into dishes and incubated with diamsar and 59Fe-Tf in 5% O₂ as described above. Subsequently, the cells were washed three times in O₂-free BSS, and release was measured into BSS containing 10 μg/ml apo-Tf. O₂ concentrations in the BSS were reduced by preequilibration with N₂ for ≥18 h before the experiment. Low environmental O₂ levels were

AJP-Cell Physiol • VOL 282 • MARCH 2002 • www.ajpcell.org
maintained by incubation in a container with a continuous flow of N₂. Released \(^{59}\text{Fe}\) is expressed as percentage of total \(^{59}\text{Fe}\) (cells and medium).

**Statistical analysis.** The statistical analyses (Student’s \(t\)-test and 95% confidence level) were performed using Excel (Microsoft). For statistical analysis of the changes in Cu oxidase expression in relation to Fe or Cu status, a trend analysis was performed using Genstat (Statistics Department, IACR-Rothamsted, Harpenden, Herts, UK). A variable (“LevelY”) was introduced to represent metal status in the cells, as indicated in Table 1. A trend, linear in LevelY, was fitted to the expression measurements.

**RESULTS**

We previously showed that azide-sensitive PPD oxidase activity is detectable in BeWo cell homogenate (10). Here, we show that this enzyme activity is primarily associated with the nuclei and the non-brush-border membranes. Very little enzyme activity is detected in the brush-border membranes (Fig. 1).

Fe status in BeWo cells was altered by incubation with the Fe chelator DFO or addition of FeCl₃ to the complete culture medium. Cu status was altered by addition of the Cu chelator diamsar or CuHis₂ᵀ. To determine whether expression of the placental Cp homolog is regulated by Fe status, we performed immunoblotting on cell homogenates from Fe-deficient and Fe-loaded cells (see MATERIALS AND METHODS). Levels of immunoreactive polypeptide are proportional to Fe status (Fig. 2, A and B). As reported previously, we detected two bands at 140 and 100 kDa (10). The 100-kDa polypeptide yields the strongest signal and was used

<table>
<thead>
<tr>
<th>LevelY</th>
<th>Fe Status</th>
<th>Cu Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>-5</td>
<td>20 (\mu\text{M} \text{ DFO})</td>
<td>20 (\mu\text{M} \text{ diamsar})</td>
</tr>
<tr>
<td>-3</td>
<td>10 (\mu\text{M} \text{ DFO})</td>
<td>10 (\mu\text{M} \text{ diamsar})</td>
</tr>
<tr>
<td>-1</td>
<td>5 (\mu\text{M} \text{ DFO})</td>
<td>5 (\mu\text{M} \text{ diamsar})</td>
</tr>
<tr>
<td>1</td>
<td>20 (\mu\text{M} \text{ FeCl}_3)</td>
<td>5 (\mu\text{M} \text{ CuHis}_2)</td>
</tr>
<tr>
<td>3</td>
<td>50 (\mu\text{M} \text{ FeCl}_3)</td>
<td>10 (\mu\text{M} \text{ CuHis}_2)</td>
</tr>
<tr>
<td>5</td>
<td>100 (\mu\text{M} \text{ FeCl}_3)</td>
<td>20 (\mu\text{M} \text{ CuHis}_2)</td>
</tr>
</tbody>
</table>

---

**Table 1. Mathematical representation of metal status**

---

Fig. 1. Activity of \(p\)-phenylenediamine (PPD) oxidase in BeWo cells is associated with membranes. BeWo cell fractions were isolated, and PPD oxidase activity and protein content were measured in each fraction. H, cell homogenate; N, nuclei; nBBM, non-brush-border membranes; BBM, brush-border membranes; Cp, ceruloplasmin.

Fig. 2. The Cp homolog is inversely regulated by Fe status. BeWo cells were grown to 80% confluence and incubated with the Fe chelator desferrioxamine (DFO; A) or FeCl₃ (B). Cell homogenate was solubilized, equal amounts of protein were loaded on 7.5% SDS gels, and the protein was transferred onto nitrocellulose and incubated. A and B represent typical results for each condition. Intensity of each band was measured and compared with the respective control (untreated cell homogenate) on each blot. C: summarized data (means ± SE). The relationship between Fe status and Cu oxidase expression is linear and significant (\(P < 0.001\)). Values in parentheses represent the number of replicates.
for the quantification (see MATERIALS AND METHODS). A summary of the effect of Fe deficiency or Fe loading on levels of Cp-immunoreactive polypeptide is shown in Fig. 2C. The relationship between Fe status and Cu oxidase expression is linear and significant \( (P < 0.001) \).

Similarly, regulation of the Cp homolog by Cu was investigated. Protein levels are increased by Cu loading (Fig. 3A) and decreased by Cu deficiency (Fig. 3B). As described for Fe levels, Cu oxidase immunoreactivity is proportional to Cu status (Fig. 3C). The relationship is linear and significant \( (P < 0.001) \). Importantly, PPD oxidase activity is also downregulated in Cu-deficient BeWo cells. Cu-deficient cells show a significant reduction in enzyme activity to 64\% of the respective controls \( (P < 0.05; \) Fig. 3D), consistent with a downregulation in immunoreactive protein.

Having established that Cu status alters expression of the Cu oxidase, we were able to examine the physiological function of this protein. We tested the hypothesis that it is involved in cellular Fe metabolism by measuring \(^{59}\text{Fe}\) accumulation in Cu-deficient and control BeWo cells. The cells were exposed simultaneously to \(^{59}\text{Fe}\)-Tf and diamsar (Cu-deficient group) or to \(^{59}\text{Fe}\)-Tf alone (control group). This incubation was initially carried out in 5\% CO\(_2\)-95\% air \( \sim 20\% \text{O}_2 \). Under such conditions, \(^{59}\text{Fe}\) accumulation is equal in control and Cu-deficient cells (Fig. 4A).

In a low-O\(_2\) environment \( (5\% \text{O}_2) \), different results are obtained. BeWo cells were exposed simultaneously to \(^{59}\text{Fe}\)-Tf and diamsar (Cu-deficient group) or to \(^{59}\text{Fe}\)-Tf alone (control group) as described above but, this time, in 5\% O\(_2\) (Fig. 4B). Because we cannot be certain of the specific activity of exogenously added \(^{59}\text{Fe}\), we have expressed the data as nanoliters of medium to compare different experiments (see MATERIALS AND METHODS). Under these conditions, the Cu-deficient BeWo cells retain significantly more \(^{59}\text{Fe}\) than their control counterparts \( (P < 0.001, \) unpaired Student’s \( t\)-test, Excel). Interestingly, the control cells accumulate more Fe in low O\(_2\) than in atmospheric O\(_2\) \( (P < 0.001; \) Fig. 4, A and B).

We (unpublished observations) and others (36) have shown that Fe uptake is linear for 1 h after exposure to \(^{59}\text{Fe}\)-Tf. To investigate whether the increase in \(^{59}\text{Fe}\) accumulation was due to an increase in uptake, we measured uptake of \(^{59}\text{Fe}\)-Tf in control and Cu-deficient BeWo cells in 20\% O\(_2\) and 5\% O\(_2\). In both conditions,
uptake was equal in both groups (data not shown). To investigate whether the increase in $^{59}$Fe accumulation was due to a decrease in $^{59}$Fe release, we measured $^{59}$Fe efflux from control and Cu-deficient cells in 5% O$_2$ and 20% O$_2$. In 20% O$_2$, efflux is unchanged in the Cu-deficient cells (data not shown). In 5% O$_2$, however, there is a decrease in $^{59}$Fe release from the Cu-deficient cells (Fig. 5). This decrease is small in absolute terms (2% intracellular $^{59}$Fe) but represents a 20% difference between control and treated cells and is significant at 10 and 30 min ($P < 0.05$). It is possible that this impairment in Fe release could account for the significant accumulation of Fe observed over 18 h.

**DISCUSSION**

We have investigated the subcellular localization of Cu oxidase activity, the regulation of the placental Cu oxidase by Fe and Cu, and, in parallel, the effect of the same treatments on Fe uptake and release. The data suggest, through parallel changes in protein expression, enzyme activity, Fe levels within the cell, and Fe efflux in low O$_2$, that there may be a role for Cu oxidase in Fe release similar to that hypothesized for hephaestin in the intestine.

We previously showed that Cu oxidase activity (measured as azide-sensitive PPD oxidation) (32) is detectable in BeWo cell homogenate (10). The present data give lower values for oxidase activity than we previously reported. To perform subcellular fractionation, homogenization must be more vigorous than normally used for enzyme assays. This procedure results in a decrease in activity and a decrease in the proportion of the 140-kDa band on Western blots (data not shown). Despite this, the relative distribution remains consistent with our previous results, where we showed that the immunoreactivity was located primarily in intracellular compartments. We localize this enzyme activity to the nuclear fraction and the non-brush-border membranes, which include all intracellular membranes. Interestingly, we could not colocalize any of the conventional membrane markers with the enzyme (10). Intriguingly, Kuo and colleagues (20) suggest that hephaestin may also be located in an intracellular compartment, rather than on the basolateral membrane.

We previously proposed that the placental Cu oxidase may have a role in Fe metabolism. Consequently, we have tested whether this protein is regulated by Fe status as an indication of its possible function. We demonstrate that expression of the Cp homolog is increased in Fe deficiency and decreased in Fe loading. Such a regulation might be expected for a protein that plays a role in placental Fe transport. If the placenta receives the signal of low Fe availability, all steps of transplacental Fe flux should be increased (uptake,
transcellular movement, and release) to protect the fetus from Fe deficiency. This regulatory response can indeed be observed in vivo (13).

Maternal Fe deficiency, which results in a decrease in Fe levels in the maternal liver, results in a smaller decrease in the placenta and an even smaller decrease in fetal liver Fe levels (13). This adaptation is caused by compensatory changes in the Fe transport mechanisms of the placenta, minimizing the level of Fe deficiency in the fetus. For example, there is a rise in Tf receptor protein and mRNA, an increase in divalent metal transporter-1 protein and mRNA, and, importantly, an increase in placental Cu oxidase enzyme activity (13). Clearly, these data strongly support the results presented here.

Another regulator of Cp expression and activity is Cu, but very little is known about the underlying mechanisms. Cp activity is reduced in the serum of Cu-deficient animals (12, 30). In Cu-deficient rats, serum Cp activity is almost abolished, although hepatic Cp mRNA levels remain unchanged (15, 24). Similarly, the enzyme activity of the placental Cp homolog is decreased in Cu-deficient cells. The downregulation in response to Cu deficiency can also be observed on the immunologic level. This could mean that the protein is less stable and more prone to breakdown if the Cu binding sites are unoccupied. However, we did not detect any further smaller bands on the immunoblots, even after longer exposure (up to 18 h).

The placental Cp homolog is upregulated in Cu-loaded cells. A possible explanation is that apoprotein is synthesized in excess, and any additional Cu will stabilize preexisting apoprotein. The regulation by Cu or Cu deficiency might also occur transcriptionally. Interestingly, Cp mRNA in Hep G2 cells is upregulated by Cu in a time-dependent manner (9), although after 20 h of incubation in primary hepatocytes (close to the conditions used in our experiments) no upregulation was observed (24).

The data in the literature clearly suggest a role in Fe efflux for Cp and its homologs (16, 17, 26, 37). We have suggested a role for the placental Cu oxidase in Fe metabolism but have not yet tested the possible physiological function of this protein. Here, we have used the regulation of the placental Cu oxidase by Cu as a tool to examine its role. We measured Fe accumulation in cells with downregulated protein. Under conditions of limited O2, Cu-deficient cells show a significant increase in 59Fe accumulation. Our data are consistent with observations in the sla mouse. These animals are deficient in the gut Cp homolog hephaestin and show excessive Fe accumulation in gut enterocytes. Although sla mice are severely anemic, they have sufficient body Fe to survive (2), indicating that a small proportion of the Fe absorbed by the gut is transported to the portal circulation. Similarly, we do not observe a total block of Fe release in the Cu-deficient BeWo cells. There are explanations for this observation. First, our experimental setup may not be sensitive enough to detect small differences in Fe movement. Our model does not involve a total knockout of the placental Cu oxidase. We can only induce a 60% decrease in enzyme activity to maintain good cell viability. This decrease might not be enough to cause a big change in release rates over short times. Second, it is conceivable that there may be alternative pathways of Fe release not involving a Cp homolog. The fact that sla mice have sufficient body Fe for survival would argue for this possibility.

Our hypothesis is also supported by the fact that low O2 increases, rather than decreases, Fe accumulation. This would not normally be expected, since reduced O2 would be expected to decrease metabolism, and it is well established that Fe uptake into placental cells requires metabolic energy (22, 34). In contrast, efflux does not require ATP (34). However, if an oxidative step is necessary and rate limiting for efflux, then the data make sense. In low O2, the oxidase cannot substitute completely for the increased O2 availability under control (20% O2) conditions. Downregulation of the oxidase by decreasing Cu levels, however, impairs the ability to oxidize Fe(II) markedly when other sources of oxidative capacity are lacking.

It is important to note that the increase in Fe accumulation in Cu-deficient cells can be observed only if the experiment is carried out in low O2. Under the conditions used initially (20% O2), the partial pressure of O2 is much higher than that normally seen in the placenta [18–20 mmHg (29)]. We reasoned that 20% O2 may be sufficient to allow nonenzymatic oxidation of the Fe(II). Therefore, we repeated our previous experiments in a low-O2 environment, mimicking the physiological environment of placental cells. Under such conditions, Cu-deficient cells accumulate excess Fe. This result indicates that some cells that are not normally exposed to high O2 tension may need an oxidative capacity to release Fe. Furthermore, this result underlines the importance of considering the normal physiological environment of the cell.

According to the present knowledge, Fe is thought to be released as Fe(II) through ferroportin (iron-regulated transporter-1) (1, 11, 25). We propose that in the placenta, analogously to hephaestin in the gut, Fe(II) is then oxidized by the Cp homolog before incorporation into fetal Tf. Where exactly in the cell this occurs is yet to be determined. Some data suggest that ferroportin is primarily located on the basolateral membrane (11); our own results, supported by Kuo and colleagues (20), suggest that the oxidase is an intracellular protein (10). We are carrying out colocalization experiments to try to resolve the controversy.

We thank L. Gambling, A. Finch, and J. Beattie for helpful discussion and S. Gair for technical support. BioStatistics Scotland provided excellent statistical advice. This work was supported by SERAD, Boehringer Ingelheim Fonds, and European Union Grant QLK1-1999-00337.

REFERENCES
REGULATION AND FUNCTION OF THE PLACENTAL Cu OXIDASE


