Original Articles

Effect of Lipopolysaccharide and Bleeding on the Expression of Intestinal Proteins Involved in Iron and Haem Transport

(iron / haem / intestine / Hcp1 / Cybrd1 / lipopolysaccharide)

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Abstract. Haem carrier protein 1 (Hcp1) is a component of the haem-iron uptake pathway in the small intestine. Using quantitative real-time PCR, we examined the expression of Hcp1 and other intestinal iron-transporting proteins in male C57BL/6 mice with experimentally altered iron homeostasis. Intestinal *Hcp1* mRNA content was not significantly changed by iron overload (600 mg/kg); however, it was increased to 170 % of controls 72 h after withdrawal of 0.7 ml of blood; the same treatment increased intestinal *Cybrd1* mRNA to 900 % of controls. LPS treatment (1 mg/kg, 6 h) decreased intestinal *Hcp1* mRNA content to 66 % of controls and *Flvcr* mRNA content to 65 % of controls, while *Cybrd1* mRNA, *Dmt1* mRNA and *Fpn1* mRNA decreased to 6 %, 43 % and 32 %, respectively. In 129SvJ mice with targeted disruption of the hemojuvelin (*Hfe2*) gene, which display very low expression of liver hepcidin, *Cybrd1* mRNA content increased to 1040 %, *Dmt1* mRNA content to 200 % and *Fpn1* mRNA to 150 % when compared to wild-type mice; changes in *Hcp1*, *Abcg2* and *Flvcr* mRNA content were only minor. Overall, these results suggest that, during inflammation, the intestinal haem-iron uptake pathway is not as strongly transcriptionally downregulated as the non-haem iron uptake pathway. A decrease in circulating hepcidin increases the expression of proteins participating in non-haem iron uptake, but has no significant effect on *Hcp1* mRNA content.

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Abbreviations: Abcg2 – ATP-binding casette, subfamily G, member 2; Cybrd1 – cytochrome b reductase 1; Dmt1 – divalent metal transporter 1; Fpn1 – ferroportin 1; Flvcr – feline leukaemia virus subgroup C cellular receptor; Hcp1 – haem carrier protein 1; Hfe2 – hemojuvelin; LPS – lipopolysaccharide.

Introduction

Iron is a crucial constituent of many enzymes and oxygen carrier proteins. It is estimated that an adult male human loses about 1 mg of iron daily, and this loss must be compensated by absorption of dietary iron in the small intestine (Hentze et al., 2004).

Dietary iron exists in two forms – as haem iron, or as non-haem iron. The absorption of non-haem iron is relatively well understood (Frazer and Anderson, 2005). In the intestinal lumen, dietary ferric iron is reduced by the brush-border ferrireductase Cybrd1 to ferrous iron, which is transported across the enterocyte apical membrane by the divalent metal transporter Dmt1 (official symbol Slc11a2). At the basolateral membrane of the enterocyte, ferrous iron is exported by the iron exporter ferroportin1 (Fpn1, official symbol Slc40a1), and oxidized to ferric iron by hephaestin.

In contrast to the proteins involved in the uptake of non-haem iron, the transporter mediating absorption of haem iron by the enterocyte has been characterized only very recently (Shayeghi et al., 2005). Haem carrier protein 1 (Hcp1) has been postulated to transfer haem across the enterocyte apical membrane. Iron from haem is then probably released by haem oxygenase, and exported across the basolateral membrane by Fpn1 (Andrews, 2005).

In addition to Hcp1, two other proteins possibly participating in haem transport in the enterocyte have been described. Haem binds to the ABC transporter Abcg2, which has been proposed to play a role in the export of excess porphyrins from cells (Jonker et al., 2002). In addition, the presence of another putative haem exporter, Flvcr, has been reported in the intestinal epithelial Caco-2 cell line (Quigley et al., 2004).

Body iron homeostasis is a finely regulated process, and the expression of intestinal iron transporters must therefore sensitively respond to a number of stimuli. Expression of the *Cybrd1* gene, encoding the first enzyme participating in non-haem iron absorption, is transcriptionally upregulated in response to hypoxia and iron deficiency (McKie et al., 2001). On the other

hand, transcription of the *Hcp1* gene is not regulated by the iron status, and its activity is regulated by a post-translational mechanism: in iron–overloaded mice, Hcp1 protein has been observed in the cytoplasm, while iron deficiency resulted in its localization to the entero-cyte apical membrane (Shayeghi et al., 2005). Iron defi-ciency has been reported to increase *Abcg2* mRNA content in rat duodenum (Collins et al., 2005); the effects of iron overload on the expression of *Abcg2* or *Flvcr* are not known.

Since iron is needed by rapidly dividing cells, sequestration of iron, as well as a decrease of iron absorption from the diet, are important host defence mechanisms against infection. Hence, expression of many proteins involved in iron metabolism is regulated by cytokines. In the liver, inflammatory cytokines increase the expression of hepcidin, the crucial iron reg-ulatory hormone (Pigeon et al., 2001), and decrease the expression of Fpn1 (Yang et al., 2002). In the intestine, administration of lipopolysaccharide (LPS) has been reported to downregulate both *Cybrd1* and *Fpn1* (Anderson et al., 2002; Yeh et al., 2004) mRNA. How-ever, there is no information about a possible role of inflammation in the expression of genes involved in intestinal haem transport.

The purpose of the present study was to examine, by quantitative real-time PCR, the effect of iron overload, iron deficiency and LPS treatment on the expression of genes encoding putative intestinal haem transporters, as compared to the expression of genes involved in non-haem iron transport. To determine whether the expres-sion of the haem transport proteins was influenced by hepcidin or hemojuvelin, we examined their mRNA levels in mice with targeted disruption of the hemoju-velin gene, which display a very low level of hepatic hepcidin mRNA.

Material and Methods

All animal experiments were approved by the Animal Care Committee of the 1st Faculty of Medicine. Male C57BL/6N mice (Charles River, Sulzfeld, Germany), 19-23 g, were maintained on a standard laboratory diet (ST-1, Bergman, Jesenice u Prahy, Czech Republic), containing about 200 ppm of iron. In iron overload stud-ies, mice were administered a single subcutaneous injec-tion of iron-polyisomaltosate (Ferrum Lek, Ljubljana, Slovenia, 600 mg iron/kg) and euthanized after one week. In iron deficiency experiments, 0.7 ml of blood was withdrawn by retrobulbar puncture and mice were euthanized 72 h later. Lipopolysaccharide (LPS, serotype 055:B5, Sigma Aldrich s. r. o., Prague, Czech Republic, 1 mg/kg i.p.) was administered 6 h before euthanasia. Mice with targeted disruption of the *Hfe2* gene, strain 129SvJ, were a generous gift from the labo-ratory of Silvia Arber, Basel, Switzerland (Niederkofler et al., 2005); disruption of the *Hfe2* gene was verified on

mouse tail-isolated DNA samples by real-time PCR, using a primer pair targeting exon 2 of the *Hfe2* gene (forward ACTCCCAGTGCAAGATCCTC, reverse CCTGGACAAAGAGGAAGTCGT); $Hfe2^{+/-}$ mice were identified by the presence of enhanced green fluo-rescent protein DNA (forward primer GCTGACCCTGAAGTTCATCTG, reverse TCGTCCTTGAAGAAGATGGTG).

Mice were euthanized by cervical dislocation. Tis-sues were stored in RNAlater (Sigma Aldrich s. r. o), RNA was extracted using RNA Blue (Top-Bio, Prague, Czech Republic), treated with DNase I (Gibco BRL, Life Technologies, Gaithersburg, MD), and 1 µg of total RNA was reverse transcribed by RevertAid First Strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania).

The levels of *Cybrd1*, *Dmt1*, *Fpn1*, *Hcp1*, *Abcg2* and *Flvcr* mRNA were determined by real-time PCR in a Roche LightCycler instrument, using LightCycler FastStart DNA Master SYBR Green I kit (Roche Diag-nostics GmbH, Mannheim, Germany). Primer sequences (forward and reverse) were:
β-actin GACATGGAGAAGATCTGGCA and GGTCTTTACGGATGTCAACG;
Cybrd1 CGATACAGCCATGTGCATTC and TCACAGGCCTCAGTCAACAC;
Dmt1 CAATGGAATAGGCTGGAGGAT and ACAGACCCAATGCAATCAAAC;
Fpn1 TCGGTTCCTCTCACTCCTGT and GTGGAGAGAGAGTGGCCAAG;
Hcp1 ATACGCAGCATTTTGTTTTGG and ATATCTTGAGCCCCAAAATGC;
Abcg2 ATCAGCCTCGGTATTCCATCT and CCTTGGAAGGCTCTTCAGTCT;
Flvcr CTGCACATCAACTGGCTGTC and AGCATGGTGACCCAGAAGAG.

Target gene cycle threshold (CT) numbers were nor-malized to β-actin ($\Delta CT = CT_{Target} - CT_{Actin}$), and target gene mRNA content was calculated as the relative amount of target mRNA compared to β-actin mRNA, assuming exact doubling of both target and β-actin cDNA in each PCR cycle (relative mRNA content = $2^{-\Delta CT}$). Sta-tistical analyses were performed on the respective paired relative amounts of target mRNAs (control vs. treated), using paired two-tailed t-test.

Results

Administration of iron polyisomaltosate increased liver non-haem iron content from 48 ± 10 µg/g wet weight to 2328 ± 98 µg/g wet weight (N = 3), liver hep-cidin 1 mRNA content increased to 477 ± 242 % of con-trols (results not shown). In agreement with previously reported data (McKie et al., 2001; Shayeghi et al., 2005), iron overload decreased the amount of *Cybrd1* mRNA, and did not significantly influence *Hcp1* mRNA (Fig. 1). *Abcg2* and *Flvcr* mRNA levels were only slightly affected by iron overload. Compared to
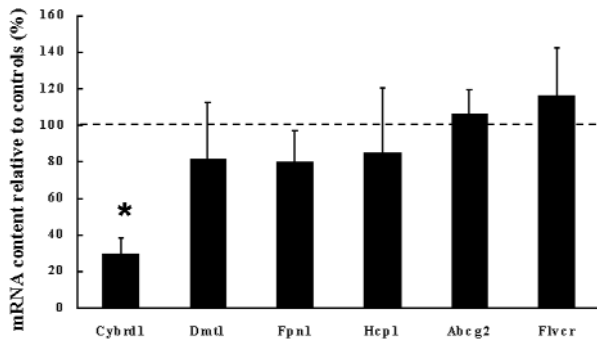
Fig. 1. Effect of iron overload on the expression of intestinal proteins participating in iron and haem transport. Male C57BL/6 mice were treated with iron polyisomaltosate (600 mg/kg) one week before sacrifice. Target mRNA content in treated animals is expressed relative to the target mRNA content of control animals. * significantly different from controls (P < 0.05, N = 5)

Fig. 2. Effect of bleeding on the expression of intestinal proteins participating in iron and haem transport. The amount of 0.7 ml of blood was withdrawn from male C57BL/6 mice by retrobulbar puncture 72 h before sacrifice. Target mRNA content in treated animals is expressed relative to the target mRNA content of control animals. * significantly different from controls (P < 0.05, N = 4)

the relatively high basal levels of *Hcp1* and *Abcg2* mRNA (about 8 % and 4 % of β-actin mRNA, respectively), the amount of intestial *Flvcr* mRNA was very low (about 0.1 % relative to β-actin mRNA).

Seventy-two h after withdrawal of 0.7 ml of blood, haematocrit was 0.34 ± 0.05 (controls 0.48 ± 0.02); liver non-haem iron content was not significantly affected. Hepatic hepcidin 1 mRNA content decreased to 26 ± 17 % of controls. Intestinal *Cybrd1* mRNA content displayed marked upregulation (Fig. 2), intestinal *Hcp1* mRNA levels increased to 170 ± 27 % (N = 4). *Abcg2* mRNA levels were not significantly changed.

Six h after LPS administration, plasma iron levels dropped from 21.9 ± 3.0 to 8.9 ± 4.9 μmol/l (N = 6, P < 0.001); hepatic hepcidin 1 mRNA content increased to 272 ± 167 % of control values (results not shown). LPS treatment dramatically decreased *Cybrd1* mRNA content and, to a lesser extent, *Dmt1* and *Fpn1* mRNA content (Fig. 3). Interestingly, LPS also decreased the

amount of *Hcp1* and *Flvcr* mRNA, while *Abcg2* mRNA levels were not significantly changed (Fig. 3).

To test whether the increase of intestinal *Cybrd1* and *Hcp1* mRNA content observed in bled animals could be related to decreased hepcidin levels, we examined the amount of intestinal mRNA in 129SvJ mice with targeted disruption of hemojuvelin (*Hfe2*), which display very low hepcidin expression (Niederkofler et al., 2005). Hepatic hepcidin 1 mRNA content in these mice was only about 3 % when compared to wild-type animals (results not shown). As indicated in Fig. 4, intestinal *Cybrd1*, *Dmt1* and *Fpn1* mRNA levels were increased, but *Hcp1*, *Abcg2* and *Flvcr* mRNA levels did not differ between wild-type and *Hfe2*-mutant animals.

Discussion

Iron absorption in the intestine is a finely regulated process. Whereas the regulation of the enzymes and transporters participating in intestinal non-haem iron
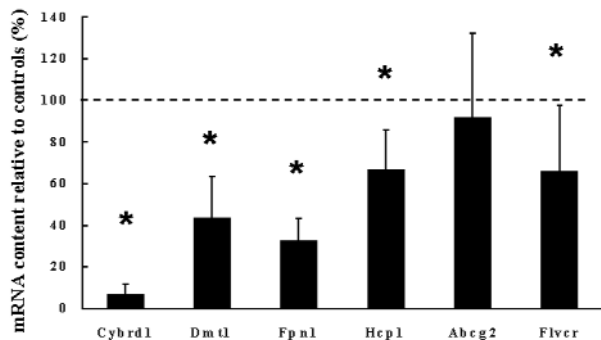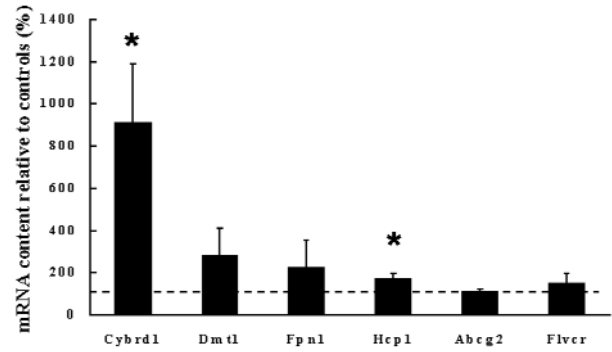

Fig. 3. Effect of lipopolysaccharide on the expression of intestinal proteins participating in iron and haem transport. Male C57BL/6 mice were treated with lipopolysaccharide (1 mg/kg) 6 h before sacrifice. Target mRNA content in treated animals is expressed relative to the target mRNA content of control animals. * significantly different from controls (P < 0.05, N = 8)
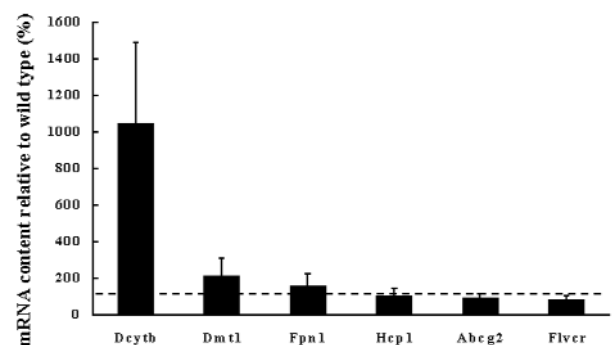

Fig. 4. Expression of intestinal proteins participating in iron and haem transport in 129SvJ mice with targeted disruption of the hemojuvelin (*Hfe2*) gene. Target mRNA content is expressed relative to the target mRNA content of wild-type animals (N = 3).

uptake has been relatively well characterized, information regarding the uptake of dietary haem iron is still incomplete.

It has already been reported that the Hcp1 protein responds to iron deficiency by localization to the apical membrane. Iron overload does not significantly change the amount of *Hcp1* mRNA (Shayeghi et al., 2005), an observation confirmed by our data in Fig. 1. While administration of iron-deficient diet has been reported to increase the amount of *Hcp1* mRNA only marginally (Shayeghi et al., 2005), bleeding resulted in a statistically significant increase (Fig. 2). *Hcp1* mRNA content has been reported to respond to hypoxia, and it is therefore possible that hypoxia, in addition to iron loss, contributes to the increase in *Hcp1* mRNA content observed in this study.

In addition to Hcp1, two other proteins, Abcg2 and Flvcr, have recently been suggested to play a role in intestinal haem transport. Both proteins probably function as haem exporters, and their role in haem-iron transport is unclear, since the majority of haem entering the enterocyte is believed to be degraded by haem oxygenase (Andrews, 2005). On the other hand, export of a fraction of intact haem from the enterocyte still remains an interesting possibility (Andrews, 2005; Latunde-Dada et al., 2006). The amount of *Abcg2* mRNA in the intestine is relatively high, approximately equal to the amount of *Hcp1* mRNA. However, the Abcg2 protein has been described mainly at the apical membrane of enterocytes (Maliepaard et al., 2001; Xia et al., 2005; for reviews see Doyle and Ross, 2003; Štaud and Pávek, 2005). Consequently, Abcg2 could modulate the enterocyte free haem level by export of excess haem into the lumen, but its direct role in possible iron uptake from the diet appears unlikely. As can be seen in Figs. 1–3, *Abcg2* mRNA content is not significantly changed by iron overload, blood loss or inflammation. On the other hand, upregulation of *Abcg2* mRNA has recently been reported in iron-deficient rats, and the presence of a hypoxia-response element in the *Abcg2* gene (Krishnamurthy et al., 2004) suggests possible regulation of the intestinal Abcg2 protein by hypoxia. At present, the exact role of this protein in intestinal haem transport remains unclear.

The amount of *Fvlcr* mRNA in the intestine is low, and, like *Abcg2* mRNA, *Flvcr* mRNA did not display significant changes following iron overload or bleeding. However, *Flvcr* mRNA levels were significantly decreased by lipopolysaccharide treatment. The exact cellular localization of the Fvlcr protein in the enterocyte has not been reported, and it is therefore theoretically possible that Flvcr could be present at the basolateral membrane and participate in the export of intact haem from the enterocyte to portal circulation, as suggested by Andrews (2005). If so, the observed decrease in *Flvcr* mRNA following LPS treatment would be in agreement with the general strategy to decrease dietary iron uptake during inflammation.

Inflammation has been shown to influence the expression of a number of proteins participating in iron metabolism. A significant LPS-induced decrease of mRNA content, exceeding one order of magnitude, has been observed for hepatic and splenic *Fpn1* (Yang et al., 2002), intestinal *Cybrd1* (Anderson et al., 2002), and hepatic *Hfe2* (Krijt et al., 2004). In addition, inflammatory cytokines increase the expression of hepatic hepcidin (Pigeon et al., 2001), which in turn decreases ferroportin-mediated iron export from cells (Nemeth et al., 2004). Surprisingly, compared with the dramatic transcriptional downregulation of Cybrd1, which mediates non-haem iron entry into the enterocyte, the response of Hcp1, which mediates the entry of haem iron, is only modest (Fig. 3), suggesting that the intestinal haem-iron uptake pathway is not as strongly transcriptionally downregulated by inflammation as the non-haem iron uptake pathway.

Proteins participating in intestinal iron uptake are regulated by several mechanisms. One of them is hypoxia, which influences the expression of Cybrd1 (McKie et al., 2001) and Hcp1 (Shayeghi et al., 2005). Another mechanism is the regulation of mRNA translation or stability by the iron regulatory proteins. Yet another mechanism has recently been described for the regulation of the Fpn1 protein, which is degraded in the presence of the iron-regulatory hormone hepcidin (Nemeth et al., 2004). Since hepcidin is the crucial factor regulating intestinal iron absorption, it was of interest to determine whether the lack of hepcidin would influence the expression of iron-transporting proteins in the intestine. As can be seen in Fig. 4, mice with targeted disruption of the *Hfe2* gene, which have low hepcidin expression, displayed increased amounts of intestinal *Cybrd1*, *Dmt1* and *Fpn1* mRNA, but no changes in *Hcp1*, *Abcg2* or *Flvcr* mRNA. These results indicate that the expression of the putative intestinal haem transporters is probably not transcriptionally regulated by hepcidin. In addition, they also support the recently suggested possibility (Wang et al., 2005) that hepcidin not only mediates the degradation of the Fpn1 protein, but also regulates the expression of *Cybrd1*, *Dmt1* and *Fpn1* at the level of transcription.

In conclusion, the presented study shows that the expression of the recently described intestinal haem transporter Hcp1, as well as the putative intestinal haem exporter Flvcr, is downregulated by lipopolysaccharide treatment; however, the decrease in *Hcp1* and *Flvcr* mRNA is only slight in comparison with *Cybrd1* or *Fpn1* mRNA. Data from *Hfe2*-mutant mice suggest that *Hcp1* and *Flvcr* mRNA levels are not influenced by hepcidin.

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