Published in final edited form as:

Biochem Biophys Res Commun. 2011 February 11; 405(2): 244–249. doi:10.1016/j.bbrc.2011.01.018.

Transport of hepcidin, an iron-regulatory peptide hormone, into retinal pigment epithelial cells via oligopeptide transporters and its relevance to iron homeostasis

Paresh P. Chothe^a, Jaya P. Gnana-Prakasam^a, Sudha Ananth^a, Pamela M. Martin^a, Ram Kannan^b, David R. Hinton^b, Sylvia B. Smith^c, and Vadivel Ganapathy^{a,*}

^a Department of Biochemistry and Molecular Biology, Medical College of Georgia, Augusta, GA, USA

^c Department of Cellular Biology and Anatomy, Medical College of Georgia, Augusta, GA, USA

^b Department of Ophthalmology, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA

Abstract

Retinal pigment epithelial cells (RPE) expresses two transport systems (SOPT1 and SOPT2) for oligopeptides. Hepcidin is an iron-regulatory peptide hormone consisting of 25 amino acids. This hormone binds to ferroportin, an iron exporter expressed on the cell surface, and facilitates its degradation. Here we investigated if hepcidin is a substrate for SOPT1 and SOPT2 and if the hormone has any intracellular function in RPE. Hepcidin inhibited competitively the uptake of deltorphin II (a synthetic oligopeptide substrate for SOPT1) and DADLE (a synthetic oligopeptide substrate for SOPT2) with IC_{50} values in the range of 0.4 – 1.7 μ M. FITC-hepcidin was taken up into RPE, and this uptake was inhibited by deltorphin II and DADLE. The entry of FITC-hepcidin into cells was confirmed by flow cytometry. Incubation of RPE with hepcidin decreased the levels of ferroportin mRNA. This effect was not a consequence of hepcidin-induced ferroportin degradation because excessive iron accumulation in RPE, which is expected to occur in these cells as a result of ferroportin degradation, did not decrease but instead increased the levels of ferroportin mRNA. This study reveals for the first time a novel intracellular function for hepcidin other than its established cell surface action on ferroportin.

Keywords

Oligopeptide transporters; Hepcidin; Retinal pigment epithelium; Iron homeostasis; Ferroportin

1. Introduction

Recently our laboratory has indentified two novel Na^+ -coupled transport systems for oligopeptides in mammalian cells [1–5]. We named these two transport systems as sodium-coupled oligopeptide transport system 1 and 2 (SOPT1 and SOPT2) [5]. SOPT1 is completely Na^+ -dependent while SOPT2 is partially Na^+ -dependent. Both transport systems

*Corresponding author. vganapat@mcg.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

have overlapping substrate specificity and can recognize a wide variety of oligopeptides irrespective of their amino acid sequence. These oligopeptides include endogenous and synthetic opioid peptides [1–4] and exogenous oligopeptides such as HIV-Tat_{47–57} [5]. To date, the only endogenous oligopeptides recognized by these transport systems are the opioid peptides.

Hepcidin is a peptide hormone whose primary physiological function is to regulate iron homeostasis [6,7]. The mature and biologically active hepcidin consists of 25 amino acids. This hormone regulates iron homeostasis by binding to ferroportin, an iron exporter, on the cell surface of target cells and facilitating ferroportin degradation [6,7]. This results in decreased release of iron from cells such as the duodenal epithelial cells and macrophages, thus reducing the levels of iron in circulation. This is the only mechanism of hepcidin known to date that is involved in iron regulation. Recently we have shown that hepcidin is also synthesized in a variety of cell types in the retina, including the retinal pigment epithelium (RPE) [8]. Thus, RPE is exposed to hepatic hepcidin present in the circulation as well as to hepcidin present in the subretinal space arising from retinal cells. RPE also expresses ferroportin, the target of hepcidin [9]. Since the oligopeptide transporters SOPT1 and SOPT2 are expressed in RPE, we asked the question whether hepcidin, a peptide hormone, is recognized as a substrate by these transporters, and if it is, whether the hormone possesses any, hitherto unrecognized, intracellular function in RPE with regard to its role as an iron-regulatory hormone.

2. Materials and Methods

2.1. Materials

The human RPE cell lines ARPE-19 and HRPE were obtained from the American Type Culture Collection (Manassas, VA). The development of the stable ARPE-19 cell line expressing the HIV-1 Tat gene has been described previously [1]. Deltorphin II was obtained from American Peptide Company, Inc. (Sunnyvale, CA). DADLE and the tripeptide Gly-Gly-Ile were from Bachem Americas Inc. (Torrance, CA). Hepcidin (Asp-Thr-His-Phe-Pro-Ile-Cys-Ile-Phe-Cys-Cys-Gly-Cys-Cys-His-Arg-Ser-Lys-Cys-Gly-Tyr-Cys-Cys-Lys-Thr) was obtained from AnaSpec, Inc. (San Jose, CA). FITC-hepcidin was from Neopeptide (Cambridge, MA). Naloxone and naltrexone were obtained from Sigma-Aldrich (St. Louis, MO). [tyrosyl-3,5-³H(N)]Deltorphin II (sp. radioactivity, 38.5 Ci/mmol) and [tyrosyl-3,5-³H(N)]DADLE (sp. radioactivity, 45.7 Ci/mmol) were purchased from PerkinElmer (Boston, MA).

2.2. Cell Culture

ARPE-19 and HRPE cells were cultured in DMEM-F12 medium containing 10 % fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine. Tat-ARPE-19 cells were also cultured in the same medium but in the presence of the antibiotic G418 (0.1 mg/ml).

2.3. Primary cultures of mouse RPE cells

Three-week-old mice (C57/Bl6) were used for preparation of primary RPE cell cultures as described previously [8,10]. Briefly, enucleated mouse eyes were rinsed in 5% Povidone-Iodine solution, followed by rinsing with sterile Hank's Balanced Salt Solution. Eyes were placed in ice-cold RPE cell culture medium, which consisted of DMEM:F12 medium, supplemented with 25% fetal bovine serum, gentamicin (0.1 mg/ml), penicillin (100 U/ml), and streptomycin (100 µg/ml). Eyes were then incubated in the same medium, but now in the presence of collagenase (19.5 U/ml) and testicular hyaluronidase (38 U/ml) for 40 min at 37 °C, followed by incubation with 0.1% trypsin again in the same medium for 50 min at 37

°C. Eyes were dissected to separate RPE from neural retina. Isolated RPE cells were collected by centrifugation and cultured in RPE cell culture medium. Purity of the cultures was verified by immunocytochemistry using antibodies specific for RPE65 (retinal pigment epithelial protein 65) and CRALBP (cellular retinaldehyde binding protein), proteins widely used as markers of RPE. The experimental protocol was approved by the institutional Committee for Animal Use in Research and Education.

2.4. Primary cultures of human fetal RPE cells

The protocol for preparation and use of cultured human RPE cells was approved by the University of Southern California Institutional Review Board and adhered to the tenets of the Declaration of Helsinki. RPE cells were isolated from human fetal eyes (gestational age, 18–20 weeks) obtained from Advanced Bioscience Resources, Inc (Alameda, CA). Informed consent was obtained by the Advanced Bioscience Resources, Inc from the mothers of the eye tissue donors. Eyes were collected by the personnel at the Advanced Biosciences Resources, Inc; the time span between death of the donor and tissue preservation was 2–4 h. The eyes were shipped in RPMI medium at 4 °C to the University of Southern California on the same day and processed immediately. Primary cultures of RPE cells were established as described previously [11]. Purity of the cultures was established by immunohistochemical staining of cytokeratin, a marker for RPE cells. Greater than 95% of cells were cytokeratin-positive, indicating epithelial origin, whereas no cells were found positive for macrophage marker CD11 or endothelial cell marker von Willebrand factor. Frozen vials of RPE cells were then shipped to the Medical College of Georgia where the cells were used for uptake measurements. Experiments were performed using RPE cells that had been passaged 2–4 times.

2.5. Uptake measurements

Cells were seeded in 24-well culture plates at an initial density of 0.1×10^6 cells/well and uptake measurements were carried out on 3rd day. The medium was removed by aspiration and the cells washed with uptake buffer once. Uptake was initiated by adding 0.25 ml of uptake buffer containing 0.1 – 0.25 μ Ci of [³H]-deltorphin II or [³H]-DADLE. Concentration of these peptides during uptake was 10–25 nM depending on the experiment. Initial experiments were carried out to determine the time course of uptake. Subsequent uptake measurements were made with 30 min incubation representing initial uptake rates. Uptake was terminated by aspiration of the uptake buffer from the wells. The cell monolayers were quickly washed with ice-cold uptake buffer without the radiolabeled substrates. The cells were then dissolved in 1% SDS/0.2 M NaOH and radioactivity associated with the cells was measured. The uptake buffer in most experiments was 25 mM HEPES/Tris (pH 7.5), containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgSO₄, and 5 mM glucose. For uptake under Na⁺-free conditions, the uptake buffer was modified by iso-osmotically replacing NaCl with *N*-methyl-D-glucamine (NMDG) chloride. Non-mediated diffusional component was determined by measuring the uptake of [³H]-deltorphin II or [³H]-DADLE in the presence of excess (1 mM) of unlabeled deltorphin II and DADLE respectively. For both peptides, the diffusional component was <5% of measured total uptake. The IC₅₀ values of hepcidin (i. e. concentration of hepcidin necessary to cause 50% inhibition of deltorphin II uptake or DADLE uptake) were calculated from dose-response experiments.

2.6. Fluorescence microscopy for the assessment of FITC-hepcidin uptake

ARPE-19 cells were seeded in chamber slides (Nalge Nunc International, Chicago, IL) at a density of 5000 cells/chamber and cultured for 24 h. Cells were then washed with phosphate-buffered saline twice and subsequently incubated with fluorescein isothiocyanate-conjugated hepcidin (FITC-hepcidin; 10 nM) for 15 min in the absence or presence of 250

μM deltorphin II and DADLE. Cells were washed with phosphate-buffered saline and then fixed with 4% paraformaldehyde for 5 min at room temperature. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) for 10 min. Cells were then washed with water and the slides were mounted with Gel Mount (Sigma-Aldrich). The entry of FITC-hepcidin into cells was detected by epifluorescence using a fluorescence microscope.

2.7. Flow cytometry for the assessment of FITC-hepcidin uptake

ARPE-19 cells were seeded in 6-well plates at a density of 0.15×10^6 cells/well. After 48 h, cells were washed with NaCl-containing uptake buffer, followed by incubation with FITC-hepcidin for 15 min. For dose-response studies, cells were incubated with FITC-hepcidin in a concentration range of 0.5–2.5 μM . For time-dependence and Na^+ -dependence studies, cells were incubated with FITC-hepcidin for 5, 15, 30, or 45 min in the presence or absence of Na^+ . For inhibition studies, cells were preincubated with and without deltorphin II (2 mM), DADLE (2 mM), and naloxone (1 mM) for 15 min, after which FITC-hepcidin was added and incubated for an additional 15 min. Cells incubated in uptake buffer without FITC-hepcidin served as negative controls. Cells were then trypsinized to remove the membrane-bound peptide and washed with phosphate-buffered saline containing fetal calf serum. Finally, cells were treated with trypan blue (40 $\mu\text{g}/\text{ml}$) to quench residual membrane-associated fluorescence signals, followed by quantification of intracellular fluorescence by flow cytometry.

2.8. Treatment of mouse RPE cells with hepcidin and ferric ammonium citrate

RPE cells were seeded in 6-well culture plates and cultured for 24 h. Cells were then treated with and without hepcidin (2 $\mu\text{g}/\text{ml}$) or ferric ammonium citrate (100 $\mu\text{g}/\text{ml}$). The treatment time was 8 h for hepcidin and 72 h for ferric ammonium citrate. Cells were then used for RNA extraction.

2.9. RT-PCR

RT-PCR was carried out under optimal conditions for the primer pairs used; mouse ferroportin: 5'-TTG CAG GAG TCA TTG CTG CTA-3' (forward), 5'-TGG TCC AGT GAT TCT GCA CAC CAT TGA T-3' (reverse); mouse HFE: 5'-GGC TTC TGG AGA TAT GGT TAT-3' (forward), 5'-GAC TCC ACT GAT GAT TCC GAT A-3' (reverse). A 224-bp product for mouse ferroportin and a 540-bp product for mouse HFE were obtained with these primer pairs. HPRT1 (hypoxanthine/guanine phosphoribosyltransferase 1) was used as an internal control. Each PCR experiment was repeated at least three times with similar results. Real-time RT-PCR was carried out using the same primers for mouse ferroportin.

3. Results

3.1. Interaction of hepcidin with SOPT1 and SOPT2 in RPE cells

To determine whether hepcidin interacts with SOPT1 and SOPT2 in RPE cells (ARPE19, Tat-ARPE, and HRPE cell lines, mouse and human primary RPE cells), we examined the effects of hepcidin (25 μM) on [^3H]-deltorphin II and [^3H]-DADLE uptake. At a concentration of 25 μM , hepcidin almost completely inhibited deltorphin II and DADLE uptake in all cells (Fig. 1A). The inhibition was dose-dependent (Fig. 1B and C). The IC_{50} values for the inhibition of deltorphin II uptake in various RPE cells were as follows: 0.42 ± 0.12 μM in ARPE-19, 0.89 ± 0.25 μM in Tat-ARPE, 0.35 ± 0.09 μM in HRPE, 1.71 ± 0.5 μM in mouse primary RPE and 1.33 ± 0.31 μM in human primary RPE. The corresponding IC_{50} values for the inhibition of DADLE uptake were 0.39 ± 0.14 μM in ARPE-19, 1.02 ± 0.24 μM in Tat-ARPE, 0.34 ± 0.01 μM in HRPE, 1.87 ± 0.67 μM in mouse primary RPE and 0.49 ± 0.15 μM in human primary RPE.

We have already reported that the transport activity of SOPT1 is markedly stimulated by small peptides [2]. To further confirm that hepcidin really interacts with SOPT1, we investigated the effect of this peptide on [^3H]deltorphin II uptake in the absence and presence of the tripeptide Gly-Gly-Ile (a potent stimulator of SOPT1) in mouse RPE cells. Gly-Gly-Ile stimulated deltorphin II uptake, and hepcidin inhibited the uptake effectively in the absence and presence of the stimulating tripeptide (Fig. 1D), showing that hepcidin does interact with SOPT1. The IC_{50} values for the inhibition of deltorphin II uptake in the absence and presence of Gly-Gly-Ile were $1.5 \pm 0.1 \mu\text{M}$ and $4.2 \pm 1.3 \mu\text{M}$, respectively. The difference between the two IC_{50} values was statistically significant ($p < 0.01$).

3.2. Transport FITC-hepcidin into ARPE-19 cells

Although hepcidin inhibited deltorphin II uptake via SOPT1 and DADLE uptake via SOPT2 effectively, it is still not clear whether this peptide is a transportable substrate for these transporters. The observed inhibition does not rule out the possibility that hepcidin simply functions as a blocker of the transporters without itself getting transported into cells. Therefore, we examined the cellular uptake of hepcidin directly by using FITC-hepcidin in which case we could follow the peptide entry by monitoring the fluorescent signals. These studies showed that FITC-hepcidin was transported into cells as indicated by green fluorescence (Fig. 2). The transport was almost completely blocked in the presence of excess amounts of deltorphin II and DADLE, showing that deltorphin II and DADLE competed with FITC-hepcidin for the transport process. Since this was the first time we showed hepcidin transport into RPE cells via SOPT1 and SOPT2, we characterized this transport process further. We studied FITC-hepcidin transport in ARPE-19 cells by flow cytometry. We used flow cytometry because it can quantitatively measure the actual amount of FITC-labeled hepcidin transported into the cells. First, we examined the uptake of FITC-hepcidin in the concentration range of $0.5 - 2.5 \mu\text{M}$ to determine whether the peptide is transported in a dose-dependent manner. Representative flow cytometry results are given for $0.5 \mu\text{M}$ (Fig. 3A), $1 \mu\text{M}$ (Fig. 3B), and $2.5 \mu\text{M}$ (Fig. 3C), and the combined quantitative data from three independent experiments are given in Fig. 3D. The results from these studies showed dose-dependent FITC-hepcidin uptake in ARPE-19 cells. Since SOPT1 and SOPT2 are either totally (SOPT1) or partially (SOPT2) Na^+ -coupled transport systems, we wanted to see whether FITC-hepcidin is also transported into the cells in a Na^+ -dependent manner. We examined the uptake of FITC-hepcidin in the presence or absence of Na^+ for 5, 15, 30 and 45 min. The uptake of FITC-hepcidin was stimulated in the presence of Na^+ and the uptake increased in a time-dependent manner (Fig. 3E). There was however considerable uptake even in the absence of Na^+ . We interpret these data as evidence for the ability of both SOPT1, a transporter that is almost completely dependent on Na^+ for its activity, and SOPT2, a transporter that is dependent only partially on Na^+ for its activity, to transport hepcidin. We have already shown by fluorescence microscopy that uptake of FITC-hepcidin into ARPE-19 cells is completely blocked by excess amounts of deltorphin II and DADLE. To confirm this observation, we measured FITC-hepcidin uptake by flow cytometry in the absence and presence of deltorphin II (2 mM) and DADLE (2 mM). In agreement with our previous observations, both deltorphin II and DADLE significantly inhibited FITC-hepcidin uptake ($35-40\%$; $p < 0.001$) in ARPE-19 cells (Fig. 3F). The non-peptide opiate antagonist naloxone did not alter the uptake. Thus, the results from these studies led us to conclude that hepcidin does indeed get transported into the cells via SOPT1 and SOPT2.

3.3. Downregulation of ferroportin mRNA by hepcidin in mouse RPE cells

As hepcidin is transported into RPE cells via SOPT1 and SOPT2, we asked whether this iron-regulatory hormone has any intracellular function. To address this question, we treated mouse primary RPE cells with hepcidin for 8 h at a concentration of $2 \mu\text{g/ml}$ and then measured the steady-state levels of ferroportin mRNA and HFE mRNA. Ferroportin as well

as HFE are expressed in RPE, and HFE is an important regulator of iron homeostasis in the retina [12,13]. We found that hepcidin treatment caused a significant reduction in ferroportin mRNA levels in mouse RPE cells (Fig. 4A). There was no effect on HFE mRNA levels. The decrease in ferroportin mRNA levels was confirmed by real-time RT-PCR (Fig. 4B). We also monitored the steady-state levels of other genes (transferrin receptor 1, transferrin, and hemojuvelin) that are involved in iron regulation, and found that the expression of none of these genes was altered by hepcidin treatment (data not shown). These data demonstrate that hepcidin downregulates the expression of ferroportin specifically. We envisaged two possible explanations for these findings. The first explanation was based on what is already known as the sole function of hepcidin, namely the binding to and subsequent internalization and degradation of ferroportin. Treatment of RPE cells with hepcidin is therefore expected to decrease the density of ferroportin in the plasma membrane of these cells, thus reducing the efflux of iron and consequently causing iron accumulation in cells. It is possible that the observed effect of hepcidin treatment on ferroportin mRNA levels in RPE cells is an indirect effect due to changes in intracellular levels of iron. Alternatively, hepcidin may function intracellularly by a mechanism, hitherto unrecognized, by directly influencing the expression of ferroportin. To differentiate between these two mechanisms, we examined the influence of excess iron in RPE cells on ferroportin mRNA. The rationale for this was that if the first mechanism was responsible for the effects of hepcidin on ferroportin mRNA, excess iron should decrease the levels of ferroportin mRNA. We treated mouse RPE cells with ferric ammonium citrate to increase the cellular levels of iron and then monitored the steady-state levels of ferroportin mRNA. We found that the levels of ferroportin mRNA did not decrease but instead increased as a result of this treatment (Fig. 4C). These data were confirmed by real-time RT-PCR (Fig. 4D). These results show that the observed decrease in ferroportin mRNA in RPE cells with hepcidin treatment was not due to the extracellular action of the hormone on cell-surface ferroportin but instead due to an intracellular action.

4. Discussion

We have already shown that SOPT1 and SOPT2 handle a variety of endogenous and synthetic opioid peptides and other non-opioid peptides like HIV-1 Tat peptide [1–5]. Here we report for the first time that hepcidin, a 25-amino-acid peptide hormone, secreted by hepatocytes and various retinal cell types, serves as a high-affinity substrate for these two oligopeptide transport systems in RPE cells. These findings are important and novel for three reasons: (i) hepcidin is the longest peptide examined thus far which is recognized as a transportable high-affinity substrate by these transport systems, (ii) this is the first report describing the transport of hepcidin peptide into cells via specific transport mechanisms, and (iii) this is the first time hepcidin has been shown to have an intracellular function in iron regulation by regulating ferroportin mRNA levels. Our studies show that this 25-amino-acid hormonal peptide interacts with SOPT1 and SOPT2 in RPE cells. The interaction occurs with high affinity. Direct evidence for the transport of hepcidin into RPE cells via SOPT1 and SOPT2 comes from the uptake characteristics of FITC-hepcidin. The entry of this fluorescently labeled peptide into RPE cells is almost completely blocked by deltorphin II and DADLE. In addition, FITC-hepcidin uptake into RPE cells, analyzed by flow cytometry, shows that this peptide is transported into the cells in a Na^+ -dependent and dose-dependent manner.

Hepcidin is a physiologically important peptide hormone in the regulation of iron levels in the body. It has been well documented that hepcidin binds to ferroportin on target cells and that this complex gets internalized with subsequent degradation [14]. Here we described for the first time an active transport mechanism for the entry of this peptide into RPE cells, suggesting that this peptide might have some, hitherto unrecognized, intracellular function. In the present study, we observed that hepcidin downregulates ferroportin mRNA, thus

uncovering a novel function of hepcidin in iron homeostasis. Based on these novel findings, we conclude that hepcidin decreases the levels of ferroportin in target cells by two distinct mechanisms. The first mechanism involves an extracellular action of hepcidin in which the hormone binds to ferroportin at the cell surface and then facilitates the degradation of the transporter. The second mechanism involves an hitherto unrecognized intracellular function in which the hormone decreases the expression of ferroportin at the mRNA level. The oligopeptide transporters SOPT1 and SOPT2 are necessary for the second mechanism. The exact molecular events associated with this intracellular mechanism remain to be identified.

Acknowledgments

This work was supported by the National Institutes of Health grant EY019672.

Abbreviations

RPE	retinal pigment epithelium
SOPT	sodium-coupled oligopeptide transporter
deltorphin II	Tyr-D-Ala-Phe-Glu-Val-Val-Gly
DADLE	Tyr-D-Ala-Gly-Phe-D-Leu

References

1. Hu H, Miyauchi S, Bridges CC, Smith SB, Ganapathy V. Identification of a novel Na⁺ and Cl⁻-coupled system for endogenous opioid peptides in the retinal pigment epithelium and induction of the transport system by HIV-1 Tat. *Biochem J.* 2003; 375:17–22. [PubMed: 12924983]
2. Miyauchi S, Gopal E, Thakkar SV, Ichikawa S, Prasad PD, Ganapathy V. Differential modulation of sodium- and chloride-dependent opioid peptide transport system by small non-opioid peptides and free amino acids. *J Pharmacol Exp Ther.* 2007; 321:257–264. [PubMed: 17259448]
3. Ananth S, Karunakaran S, Martin PM, Nagineni CN, Hooks JJ, Smith SB, Prasad PD, Ganapathy V. Functional identification of a novel transport system for endogenous and synthetic opioid peptides in the rabbit conjunctival epithelial cell line CJVE. *Pharm Res.* 2008; 26:1226–1235. [PubMed: 18781380]
4. Thakkar SV, Miyauchi S, Prasad PD, Ganapathy V. Stimulation of Na⁺/Cl⁻-coupled opioid peptide transport system in SK-N-SH cells by L-kyotorphin, an endogenous substrate for H⁺-coupled peptide transporter PEPT2. *Drug Metab Pharmacokinet.* 2008; 23:254–262. [PubMed: 18762712]
5. Chothe P, Thakkar SV, Gnana-Prakasam JP, Ananth S, Hinton DR, Kannan R, Smith SB, Martin PM, Ganapathy V. Identification of a novel sodium-coupled oligopeptide transporter (SOPT2) in mouse and human retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci.* 2010; 51:413–420. [PubMed: 19643969]
6. Nemeth E, Ganz T. Regulation of iron metabolism by hepcidin. *Annu Rev Nutr.* 2006; 26:323–342. [PubMed: 16848710]
7. Lee PL, Beutler E. Regulation of hepcidin and iron-overload disease. *Annu Rev Pathol.* 2009; 4:489–515. [PubMed: 19400694]
8. Gnana-Prakasam JP, Martin PM, Mysona BA, Roon P, Smith SB, Ganapathy V. Hepcidin expression in mouse retina and its regulation via lipopolysaccharide/Toll-like receptor-4 pathway independent of Hfe. *Biochem J.* 2008; 411:79–88. [PubMed: 18042040]
9. Hahn P, Dentchev T, Qian Y, Rouault T, Harris ZL, Dunaief JL. Immunolocalization and regulation of iron handling proteins ferritin and ferroportin in the retina. *Mol Vis.* 2004; 10:598–607. [PubMed: 15354085]
10. Gnana-Prakasam JP, Zhang M, Martin PM, Atherton SS, Smith SB, Ganapathy V. Expression of the iron-regulatory protein hemojuvelin in retina and its regulation during cytomegalovirus infection. *Biochem J.* 2009; 419:533–543. [PubMed: 19191760]

11. Sonoda S, Spee C, Barron E, Ryan SJ, Kannan R, Hinton DR. A protocol for the culture and differentiation of highly polarized human retinal pigment epithelial cells. *Nat Protocols*. 2009; 4:662–673.
12. Martin PM, Gnana-Prakasam JP, Roon P, Smith RG, Smith SB, Ganapathy V. Expression and polarized localization of the hemochromatosis gene product HFE in the retinal pigment epithelium. *Invest Ophthalmol Vis Sci*. 2006; 47:4238–4244. [PubMed: 17003411]
13. Gnana-Prakasam JP, Martin PM, Smith SB, Ganapathy V. Expression and function of iron-regulatory proteins in retina. *IUBMB Life*. 2010; 62:363–370. [PubMed: 20408179]
14. Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM, Ganz T, Kaplan J. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science*. 2004; 306:2090–2093. [PubMed: 15514116]

Fig. 1. Inhibition of deltorphin II and DADLE uptake by hepcidin in RPE cells

(A) Uptake of [3 H]-deltorphin II (20 nM; 30 min incubation) and [3 H]-DADLE (10 nM; 30 min incubation) in the absence and presence of hepcidin (25 μ M). Data are presented as percentage of control uptake (100%). The difference between the uptake values in the absence and presence of hepcidin was statistically significant in all cases (a, $p < 0.001$). (B) Uptake of [3 H]-deltorphin II (20 nM; 30 min incubation) and (C) Uptake of [3 H]-DADLE (10 nM; 30 min incubation) was monitored in the presence of increasing concentrations of hepcidin. Data (means $\pm$ S. E.) are from three independent experiments, each done in duplicate. (D) Uptake of [3 H]-deltorphin II (20 nM; 30 min incubation) was monitored in the absence and presence Gly-Gly-Ile (1 mM) with increasing concentrations of hepcidin. Data (means $\pm$ S. E.) are from three independent experiments, each done in duplicate.

Fig. 2. Uptake of the FITC-hepcidin in ARPE-19 cells

Cells were incubated with FITC-hepcidin (10 nM) for 15 min in a Na⁺-containing medium in the absence or presence of deltorphin II and DADLE (250 μM). Cells incubated under similar conditions but in the absence of FITC-hepcidin served as the negative control. Following the incubation, cells were washed with ice-cold uptake buffer and stained with DAPI (nuclear stain) and observed with a fluorescence microscope (blue, nuclear stain; green, FITC-hepcidin).

Fig. 3. Characteristics of FITC-hepcidin uptake in ARPE-19 cells

Cells were incubated with FITC-hepcidin at a concentration of (A) 0.5 μ M, (B) 1 μ M and (C) 2.5 μ M for 15 min. Cells incubated in uptake buffer without FITC-hepcidin served as a negative control. Cells were trypsinized to remove the membrane-bound peptide and washed with phosphate-buffered saline containing fetal calf serum. Finally, cells were treated with trypan blue (40 μ g/ml) to quench any residual membrane-associated fluorescence signals, followed by quantification of intracellular fluorescence signals by flow cytometry. (D) Quantification of flow cytometry results from three independent experiments. (E) Cells were incubated with FITC-hepcidin at different time points in the presence or absence of Na⁺. (F) Cells were preincubated with and without deltorphin II (2 mM), DADLE (2 mM) and naloxone (1 mM). After 15 min, FITC-hepcidin (2.5 μ M) was added and incubated for 15 min. Cells were treated as described above and then used for quantification of intracellular fluorescence signals by flow cytometry. (a, $p < 0.01$ compared to control).

Fig. 4. Effects of hepcidin and ferric ammonium citrate (FAC) on ferroportin mRNA levels in mouse primary RPE cells

(A & B) Cells were treated with or without hepcidin (2 $\mu\text{g}/\text{ml}$) for 8 h. (C & D) Cells were treated with or without ferric ammonium citrate (100 $\mu\text{g}/\text{ml}$) for 72 h. RNA was isolated from these cells and used for semi-quantitative RT-PCR (A & C) and real-time RT-PCR (B & D). (a, $P < 0.001$).