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Hepcidin expression in mouse retina and its regulation via lipopolysaccharide/Toll-like receptor-4 pathway independent of Hfe

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Summary

Hepcidin is a hormone central to the regulation of iron homeostasis in the body. It is believed to be produced exclusively by the liver. Ferroportin, an iron exporter, is the receptor for hepcidin. This transporter/receptor is expressed in Müller cells, photoreceptor cells, and retinal pigment epithelium (RPE) within the retina. Since the retina is protected by the retinal-blood barriers, we asked whether ferroportin in the retina is regulated by hepcidin in the circulation or whether the retina produces hepcidin for regulation of its own iron homeostasis. Here we show that hepcidin is expressed robustly in Müller cells, photoreceptor cells, and RPE, closely resembling the expression pattern of ferroportin. We also show that bacterial lipopolysaccharide (LPS) is a regulator of hepcidin expression in Müller cells and RPE, both in vitro and in vivo, and that the regulation occurs at the transcriptional level. The action of LPS on *hepcidin* expression is mediated by the Toll-like receptor-4. The upregulation of *hepcidin* by LPS occurs independent of Hfe (Human leukocyte antigen-like protein involved in Fe homeostasis). The increase in hepcidin levels in retinal cells in response to LPS treatment is associated with a decrease in ferroportin levels. The LPS-induced upregulation of hepcidin and consequent downregulation of ferroportin is associated with increased oxidative stress and apoptosis within the retina in vivo. We conclude that retinal iron homeostasis may be regulated in an autonomous manner by hepcidin generated within the retina and that chronic bacterial infection/inflammation of the retina may disrupt iron homeostasis and retinal function.

Keywords

hepcidin; retina; hemochromatosis; lipopolysaccharide; ferroportin; retinal pigment epithelium

INTRODUCTION

Iron, an essential nutrient obligatory for vital cellular functions, can induce oxidative stress and cellular dysfunction when accumulated excessively. Hereditary hemochromatosis is a genetic disorder associated with iron overload [1-5]. The tissues commonly affected in this disease are the liver, pancreas, kidney, pituitary, and heart, resulting in a myriad of diseases including liver cirrhosis, hepatocarcinoma, diabetes, cardiomyopathy, nephropathy, and endocrine dysfunction [1-5].

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Hepcidin is an important regulator of iron homeostasis. It is considered to be a circulatory hormone, secreted almost exclusively by the liver [6]. Recent studies have identified ferroportin, an iron exporter expressed in specific cell types, as the receptor for this iron-regulatory hormone [7]. The binding of hepcidin to ferroportin leads to internalization of the iron exporter with subsequent degradation in lysosomes. This results in the prevention of iron release from cells which express ferroportin. Duodenal enterocytes express ferroportin at the basolateral membrane, and the transporter is involved in the intestinal absorption of dietary iron [1-5]. Macrophages also express ferroportin and play a critical role in the recycling of heme-iron from aged erythrocytes. A decrease in circulating levels of hepcidin is a common feature in hemochromatosis, irrespective of whether the disease is caused by mutations in *HFE* (the gene coding for HFE, human leukocyte antigen-like protein involved in Fe homeostasis), *Tfr2* (the gene coding for transferrin receptor 2), *hemojuvelin* (*HJV*), or *hepcidin* [8]. The decrease in hepcidin prevents internalization of ferroportin in duodenal enterocytes, enhancing intestinal iron absorption and thus causing iron overload. In contrast, an increase in circulating levels of hepcidin leads to iron deficiency, resulting from decreased intestinal iron absorption and decreased recycling of heme-iron [9]. It has been documented that increase in hepcidin expression leads to iron deficiency and microcytic anemia [10, 11]. Inflammation upregulates *hepcidin* and increases circulating levels of hepcidin [11], providing a molecular basis for the anemia associated with chronic inflammation.

Iron overload has been postulated to play a role in the pathogenesis of various neurodegenerative diseases such as Parkinson's disease [12], Alzheimer's disease [13, 14], and amyotrophic lateral sclerosis [15]. This suggests that the central nervous system is susceptible to iron-induced oxidative damage. Recent studies have shown that hepcidin and its target ferroportin are expressed in the central nervous system, indicating that iron homeostasis within the brain may be regulated by hepcidin which is produced locally [16, 17]. Retina is constantly exposed to oxidative stress caused by photo-oxidation, and regulation of iron homeostasis within this tissue is important to prevent exacerbation of this oxidative stress by iron overload [18]. Retinal pigment epithelium (RPE), which constitutes the outer blood-retinal barrier, is responsible for phagocytosis of photoreceptor outer segments. These membrane segments are rich in iron, thus exposing this cell layer to increased risk for iron overload [19]. Müller cells play a critical role as a support cell to retinal neurons to maintain normal retinal function. Recent studies have shown that ferroportin is expressed in photoreceptor cells, Müller cells, and RPE [20], suggesting that this transporter may play an obligatory role in the maintenance of iron homeostasis within the retina. We recently reported that Hfe and Tfr2, two other proteins involved in the regulation of iron homeostasis, are also expressed in the retina [21]. Despite the fact that hepcidin may be the most crucial regulator of iron homeostasis and that an exquisite regulation of iron levels within the retinal cells is obligatory for optimal visual function, the expression and function of hepcidin have not been studied in the retina. Ferroportin, the receptor for hepcidin, is expressed in the basolateral membrane of RPE and thus has access to hepcidin in the circulation. This is not true in the case of ferroportin expressed in Müller cells and photoreceptor cells. Since the retina is surrounded by retinal-blood barriers, ferroportin present in these cells may not be subject to regulation by circulating hepcidin unless the 25-amino acid peptide can pass through the retinal-blood barriers. Therefore, we were interested to know whether ferroportin expressed within the retina is regulated by hepcidin produced within the retina. The present investigation was undertaken to study the expression, function, and regulation of hepcidin in mouse retina.

MATERIALS AND METHODS

Materials

Reagents were obtained from the following sources: RNA extraction reagent (TRIzol; Invitrogen-Gibco Corp., Grand Island, NY, U.S.A.), GeneAmp RT PCR kit (Applied Biosystems, Inc., Foster City, CA, U.S.A.), *Taq* polymerase kit (TaKaRa, Tokyo, Japan), and Power Block (Biogenex, San Ramon, CA, U.S.A.). DMEM/F12 medium (Gibco) with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin was used for growing retinal cell lines. Antibodies used were obtained from the following sources: rabbit polyclonal anti-ferroportin, rabbit polyclonal anti-4-hydroxynonenal (HNE), rabbit polyclonal anti-hepcidin, and hepcidin control/blocking peptide (Alpha Diagnostic International, San Antonio, TX, U.S.A.), goat anti-rabbit IgG coupled to Alexa Fluor 555 (Molecular Probes, Carlsbad, CA, U.S.A.), Vectashield Hardset Mounting Media with DAPI (Vector Laboratories, Burlingame, CA, U.S.A.), lipofectin transfection reagent (Invitrogen, Carlsbad, CA, U.S.A.), ApopTAG fluorescein in situ Apoptosis Detection Kit (Chemicon International, Temecula, CA, U.S.A.), and ketamine, xylazine, proparacaine, and bacterial lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO, U.S.A.).

Animals

C57BL/6 mice (6-week-old) were used for the preparation of total RNA from neural retina and RPE/eyecup, and 3-week-old mice were used for establishment of primary RPE cell cultures. *Hfe* knockout mice were obtained from The Jackson laboratory (Bar Harbor, ME, U.S.A.). Albino Balb/c mice (3-week-old) were used for immunofluorescence and in situ hybridization analyses. These mice (3-week-old) were also used for intravitreal injection of LPS to induce inflammation in the eyes. Mice were purchased from Harlan-Sprague Dawley and maintained in our facility.

Establishment of primary RPE cell cultures from mouse eyes

Three-week-old C57BL/6 mice were used for preparation of primary RPE cell cultures using a method adapted from that described for isolation of rat RPE [22]. Briefly, enucleated mouse eyes were rinsed in 5% Povidone-Iodine solution, followed by rinsing with sterile Hank's Balanced Salt Solution (HBSS). Eyes were then placed in cold RPE cell culture media 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). To aid in the degradation of extracellular matrix components and enhance the dissociation of RPE from neural retina and choroid, eyes were then incubated in HBSS, containing collagenase (19.5 U/ml) and testicular hyaluronidase (38 U/ml) for 40 min at 37 °C, followed by incubation in HBSS containing 0.1% trypsin (pH 8.0) for 50 min at 37 °C. Eyes were then dissected to separate RPE from neural retina. Isolated RPE cells were collected in a 15 ml centrifuge tube and centrifuged at 1200 rpm for 10 min, followed by resuspension in RPE cell media. RPE cells were then cultured at 37 °C. Purity of the culture was verified by immunodetection of RPE-65 (retinal pigment epithelial protein 65) and CRALBP (cellular retinaldehyde binding protein), proteins known to be expressed in RPE.

Primary cultures of Müller cells

Primary cultures of retinal Müller cells were established from retinas of C57BL/6 mice according to our previously published method [23]. Purity of the culture was verified by immunodetection of vimentin, glutamine synthetase, CRALBP, and the glutamate transporter EAAT1, proteins known to be expressed in Müller cells.

Intravitreal injection

Female Balb/c mice (age, 3 weeks) were weighed and anesthetized by intraperitoneal injection using 15 μ l (1 μ l per gram body weight) of a solution of ketamine (80 mg/ml) and xylazine (12 mg/ml). Immediately before the intraperitoneal injection, 5 μ l of 5% proparacaine was administered topically to the eyes. One microliter of LPS (10 μ g/ μ l prepared in PBS) was then injected into the vitreous body of the right eyes at the limbus, and the left eye received an equal volume of PBS. Control mice received an equal volume of PBS in both the eyes. Twenty four hours after injections, mice were euthanized by CO₂ inhalation. One set of the mouse eyes were harvested for cryosectioning and immunohistochemistry. Neural retina and RPE were dissected from remaining eyes for isolation of RNA.

RT-PCR

Neural retina and RPE/eye cup were prepared according to our previously published method [24] and used for preparation of total RNA. RT-PCR was carried out under optimal conditions depending on the nature of the specific PCR primer pairs listed in Table I. HPRT1 (hypoxanthine phosphoribosyl transferase 1) was used as an internal control for PCR reaction. The hepcidin PCR product was subcloned into the pGEM-T Easy vector and sequenced to confirm its molecular identity. The subcloned plasmid was also used for generation of sense and antisense riboprobes for in situ hybridization.

In situ hybridization

Mouse eyes were frozen in Tissue-Tek OCT, and sections were made at 10- μ m thickness and fixed in 4% paraformaldehyde. Treatment of tissue sections and hybridization with digoxigenin-labeled sense and antisense riboprobes were carried out as described previously [25-27]. The hybridization signals were detected with anti-digoxigenin antibody, conjugated to alkaline phosphatase. The color reaction was developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate. Cryosections were hybridized with the sense (negative control) riboprobe to determine nonspecific binding. For the preparation of antisense and sense riboprobes, a 269-bp product specific for mouse hepcidin was amplified by RT-PCR and subcloned into the pGEM-T Easy vector, and the orientation of the insert was identified by sequencing. The specificity of the probe for hepcidin was confirmed by searching the GenBank database with the nucleotide sequence of the segment encompassed by the primers as the query. The probes were prepared by in vitro transcription with appropriate RNA polymerases after linearizing the plasmid with suitable restriction enzymes.

Immunofluorescence analysis

Cryosections of mouse eyes were fixed in 4% paraformaldehyde for 10 min, washed with 0.01M PBS (pH 7.4), and blocked with 1X Power Block for 60 min. Sections were then incubated overnight at 4°C with one of the following primary antibodies: polyclonal anti-hepcidin (1:50 dilution), polyclonal anti-ferroportin (1:100 dilution) or polyclonal anti-HNE (1:500 dilution). The specificity of hepcidin antibody was confirmed by using the peptide-neutralized antibody. The hepcidin antibody was mixed with the antigenic peptide, and incubated for 2 h at 37°C and 24 h at 4°C. The mixture was centrifuged at 15,000 rpm for 15 min at 4°C to pellet the immune complex. The supernatant was used to incubate with the sections overnight at 4°C to serve as negative controls. Additional negative controls involved the omission of the primary antibodies. Sections were rinsed and incubated for 1 h with goat anti-rabbit IgG coupled to Alexa Fluor 555 at a dilution of 1:1000. Coverslips were mounted with Vectashield Hardset mounting media with DAPI (a nuclear stain) and sections were examined by epi-fluorescence using an Axioplan-2 microscope, equipped with an HRM camera and the Axiovision imaging program (Carl Zeiss, Germany).

Retinal cell lines and primary retinal cell cultures were grown on 12-mm cover slips for 24 h in 24-well cell culture plates at 37°C in a 5% CO₂ incubator. Medium was removed and the cells were fixed in ice-cold methanol for 10 min after air drying. Cells were then washed with 0.01M PBS (pH 7.4) and blocked with 1X Power Block for 120 min. Cells were incubated overnight at 4°C with polyclonal anti-hepcidin (1:50 dilution), polyclonal anti-ferroportin (1:100 dilution), or polyclonal anti-HNE (1:500 dilution). After incubation of the cells with primary antibody, the same protocol was followed as used for staining the tissues. Negative control cells were treated likewise but in the absence of the primary antibodies.

Generation of *hepcidin*-specific promoter-reporter constructs

The human *hepcidin* promoter-EGFP construct was generated by first subcloning the 2-kb *hepcidin* promoter (obtained by PCR using human genomic DNA as the template) into the pGEM-T Easy vector and then using the HindIII/XhoI-digested promoter insert into pUIIR3-EGFP vector. The primers used for PCR were: 5'-ATACTCGAGACTCTCACCCAGGCTGGG-3' (sense) and 5'-AAGCTTCATCGTGCCGTCTGTCTGGCT-3' (antisense). After transfection with the *hepcidin* promoter-EGFP construct, cells were treated with LPS for 4 h and enhanced green fluorescent protein (EGFP) expression was monitored by epifluorescence under the fluorescence microscope.

In situ detection of DNA fragmentation by TUNEL assay

The TUNEL assay was performed using the ApopTAG fluorescein in situ apoptosis detection kit. After staining, the tissue sections were viewed by epifluorescence using standard fluorescence excitation and emission filters. Each section was scanned systematically from the temporal to the nasal ora serrata for fluorescent cells indicative of apoptosis. To distinguish between structures that autofluoresced versus those that were TUNEL-positive, all slides were examined first with the rhodamine filter and then with the FITC filter. Autofluorescent structures were visible under both filters, whereas TUNEL-positive cells were detectable only with the FITC filter.

Primary cultures of RPE from wild type and *Hfe*^{-/-} mouse retinas

Wild type and *Hfe*^{-/-} mice were obtained from the same litter originating from the mating of heterozygous mice. Genotyping was performed according to the protocol supplied by The Jackson Laboratory. 3-week-old mice were then used to establish primary cultures of RPE.

RESULTS

Expression of *hepcidin* in retina

To investigate whether *hepcidin* is expressed in the retina, RT-PCR was done with RNA isolated from neural retina and RPE/eye cup of normal mouse. We found evidence for the expression of *hepcidin* mRNA in RPE/eye cup as well as in the neural retina (Figure 1A). The PCR product was subcloned in pGEM-T Easy vector and sequenced to confirm the molecular identity of the product using BLAST analysis. We further analyzed the expression pattern of *hepcidin* mRNA and protein in the retina by in situ hybridization and immunofluorescence. In situ hybridization revealed the expression of *hepcidin* mRNA in the inner nuclear layer, inner segment of photoreceptor cells, and RPE cell layer (Figure 1B). The signals observed were specific because no positive signals were detected with a sense riboprobe. Immunofluorescence analysis of *hepcidin* protein showed positive signals throughout the retina (Figure 1C). The cells in the inner nuclear layer, which include Müller cells, were immunopositive. The expression was also evident in photoreceptor cells and RPE (Figure 1C). Negative controls with omission of the primary antibody or with peptide-

neutralized antibody did not yield positive signals, indicating the specificity of the signals obtained with the antibody. The expression pattern of hepcidin protein corroborates the expression pattern of hepcidin mRNA. The inner nuclear layer contains, among other cell types, the nuclei for Müller cells, the inner segment of photoreceptor cells is enriched in RNA, and the RPE cell layer contains the nuclei for RPE. Thus, there is a good correlation between the expression pattern of hepcidin mRNA and that of hepcidin protein.

Expression of hepcidin in specific retinal cell types

RT-PCR was performed using hepcidin-specific primers with RNA isolated from primary cultures of RPE and Müller cells. RPE and Müller cells showed significant expression of hepcidin mRNA (Figure 1D). To confirm further the expression of hepcidin in RPE and Müller cells, immunolocalization studies were done. Immunofluorescence analysis confirmed hepcidin expression not only in primary cultures of RPE and Müller cells but also in ARPE-19 (a human RPE cell line) and rMC1 (a rat Müller cell line) cells (Figure 1E).

Regulation of hepcidin expression by LPS

To assess the role of hepcidin in retina during inflammation, primary cultures of RPE and Müller cells as well as RPE and Müller cell lines were treated with and without LPS (10 and 100 ng/ml in culture medium) for 4 h. The steady-state levels of hepcidin mRNA increased with LPS treatment in a dose-dependent manner in primary cultures of RPE and Müller cells (Figure 2A). The same was true for rMC1, a rat Müller cell line. Interestingly, hepcidin expression did not respond to LPS treatment in ARPE-19 cells, a human RPE cell line, but the LPS induction of hepcidin expression was evident in HRPE cells, also a human RPE cell line. LPS produces its effects by acting as a ligand to the Toll-like receptor-4 (TLR4). To examine the potential reasons for the differential effects of LPS on hepcidin expression in ARPE-19 cells versus HRPE cells, we monitored the expression of TLR4 in these cells. The expression of TLR4 was several-fold higher in HRPE cells than in ARPE-19 cells (Figure 2B). RNA from human colon was used as a positive control. These data suggest that ARPE-19 cells failed to respond to LPS treatment because of the markedly reduced levels of TLR4 expression. The increase in hepcidin mRNA in primary cultures of RPE and Müller cells following LPS treatment was associated with an increase in hepcidin protein levels as detected by immunofluorescence (Figure 2C and 2D).

Induction of hepcidin promoter activity by LPS

The effect of LPS on *hepcidin* expression in the liver occurs via transcriptional regulation [26]. To examine whether a similar mechanism operates in the regulation of *hepcidin* expression in retinal cells, we monitored the effects of LPS on *hepcidin* promoter activity using EGFP as the reporter. For this, a 2-kb *hepcidin* promoter region was cloned into an EGFP vector, and the promoter-reporter construct was transfected into rMC1, ARPE-19, and HRPE cell lines. Twenty four hours following transfection, the cells were treated with LPS (100 ng/ml culture medium) for 4 h. The promoter activity was then monitored using a fluorescence microscope to detect the expression of the EGFP reporter. Vector-transfected cells served as the negative controls. The induction of *hepcidin* promoter activity by LPS was detectable in rMC1 cells and HRPE cells but not in ARPE-19 cells (Figure 3). This corroborates the findings on the effects of LPS treatment on hepcidin mRNA levels in these cell lines.

Regulation of hepcidin expression by LPS in retina in vivo

To determine whether the effects of LPS on hepcidin mRNA and protein levels seen in vitro in retinal cell lines and in primary cell cultures are also demonstrable in vivo, we injected LPS into mouse eyes intravitreally. Twenty four hours following LPS injection, neural retina

and RPE/eyecup were isolated for preparation of RNA, and retinal sections were prepared for analysis of oxidative stress and apoptotic cell death. RT-PCR showed an increase in hepcidin mRNA in both neural retina and RPE/eyecup in response to LPS treatment. Interestingly, the induction of hepcidin mRNA was seen not only in the eyes that were injected with LPS but also in the contralateral eyes from the same mice that were injected with PBS instead of LPS even though the effects were considerably lower (Figure 4A). The induction of *hepcidin* expression by LPS was independent of *Hfe* or *Hjv* because the steady-state levels of mRNAs specific for these genes were not altered by LPS treatment in the retina. The lack of involvement of *Hfe* in LPS-induced expression of hepcidin was confirmed with primary RPE cell cultures established from retinas of wild type mice and *Hfe*-null mice. The genotype identity was confirmed by analysis of *Hfe* mRNA in wild type RPE and *Hfe*^{-/-} RPE (Figure 4B). Treatment of *Hfe*^{-/-} RPE cells with LPS induced hepcidin expression as evidenced from an increase in mRNA levels (Figure 4C) and protein levels (Figure 4D).

Consequences of LPS-induced increase in hepcidin expression on ferroportin levels in retina

Hepcidin is known to bind ferroportin and induce lysosomal degradation of the transporter [7]. To determine whether similar changes occur in steady-state levels of ferroportin in intact retina following LPS treatment, we monitored the expression of hepcidin and ferroportin by immunofluorescence in retinal sections from control mouse eyes, PBS-treated contralateral eyes from mice treated with LPS on one eye, and from LPS-treated eyes. To compare the expression levels among different tissue sections, the exposure time on camera was held constant. These studies showed that LPS treatment increased the levels of hepcidin protein and that this increase was associated with a substantial decrease in ferroportin protein levels (Figure 5). Similar changes, but to a smaller extent, were seen in PBS-injected contralateral eyes.

Induction of hepcidin expression by LPS in retinal cells may result in the down-regulation of ferroportin, causing iron overload in these cells in vivo. To determine whether there were any consequences to the retina from such effects, we monitored the levels of oxidative stress in control and LPS-treated retinas by using an antibody capable of detecting 4-hydroxynonenal (HNE), a metabolic product of lipid peroxidation with hydroxyl radicals. Increased accumulation of free iron is expected to generate hydroxyl radicals via the Fenton reaction, which would then lead to enhanced lipid peroxidation and increased generation of HNE. Thus, HNE serves as a surrogate for detecting increased iron levels. These studies showed that the levels of HNE were significantly higher in LPS-treated retinas compared to normal retinas (Figure 6A). We then investigated whether these changes in oxidative stress lead to apoptosis in the retina. We used TUNEL assay to detect apoptosis. There was considerably more apoptosis in retinal sections from eyes that were injected with LPS compared with retinal sections from control mice (Figure 6B). The increased TUNEL-positive signals were detectable in the outer nuclear layer and in the inner nuclear layer. PBS-injected contralateral eyes had more apoptosis in outer nuclear layer compared to the mice that received PBS in both eyes. Interestingly, there was no evidence of increased apoptotic cell death in RPE cell layer and in the ganglion cell layer. Ganglion cells do not express hepcidin and hence the absence of apoptosis in these cells following intravitreal injection of LPS is not surprising. To check if RPE was resistant to LPS-induced oxidative stress in spite of the expression of hepcidin, we treated primary RPE cells with LPS for 24 h and checked for ferroportin levels and found that it was downregulated (Figure 6C). The downregulation of ferroportin was associated with increased oxidative stress as monitored by immunofluorescence with anti-HNE antibody (data not shown).

DISCUSSION

Previous studies have shown that Hfe is expressed in the RPE basolateral membrane [21]. Since this membrane is in contact with systemic blood via choroidal circulation, the expression of Hfe in this membrane suggests that the protein might play a critical role in the sensing of systemic iron status. Hahn et al. [20] have shown that ferroportin is expressed abundantly in RPE, Müller cells, and photoreceptor cells. Ferroportin is the target for hepcidin [7], the critical iron-regulatory hormone currently believed to be produced primarily by the liver [29-32]. In RPE, ferroportin is expressed in the basolateral membrane; thus ferroportin in RPE may be subject to regulation by hepatic hepcidin present in the circulation. In contrast, ferroportin expressed in Müller cells and photoreceptor cells does not have access to circulating hepcidin. Regulation of ferroportin in these cells by hepatic hepcidin would require the transfer of hepcidin across the blood-retinal barriers. Alternatively, hepcidin may be produced within the retina to exert local control on ferroportin in retinal cells independent of hepatic hepcidin. There are no reports in the literature on the expression of hepcidin in the retina. In the present study, we found that hepcidin is expressed abundantly in Müller cells, photoreceptor cells, and RPE. These are the same cell types which also express ferroportin [20]. This represents the first report on the expression of this important iron-regulatory hormone within the retina, suggesting that ferroportin expressed in cells within the retina may be subject to regulation by hepcidin produced locally within the tissue.

The expression of hepcidin in retina raises an important question: Do changes in the expression levels of hepcidin regulate ferroportin levels in retinal cells? Hepatic expression of hepcidin is induced by inflammation [28]. The expression of iron-regulatory proteins in different retinal cell types has not been studied in retinal infections. In the present study, we mimicked bacterial infection of the retina by intravitreal injection of LPS, and examined the changes in the expression of hepcidin within the retina. Our studies show that hepcidin expressed in retinal cells is subject to regulation by LPS with obligatory involvement of the Toll-like receptor-4. Hfe and HJV regulate hepcidin synthesis in the liver [33, 34], but are not involved in the inflammation-mediated regulation of hepcidin expression [28]. Our findings in the retina are similar to those in the liver. The increase in hepcidin expression in response to LPS in the retina is associated with a decrease in ferroportin levels in Müller cells, photoreceptor cells, and RPE.

It is interesting that injection of LPS in one eye leads to changes in the expression of hepcidin and ferroportin in the contralateral eye. It is possible that LPS injected in one eye may have access to the contralateral eye via circulation. Alternatively, proinflammatory mediators, released in response to LPS in one eye, may travel to the contralateral eye via circulation and elicit their effects. It has been shown that LPS may produce its effects on hepcidin expression in the liver through IL-6 and/or TNF- α in an autocrine manner [35]. Such intermediates may also be released into circulation from the retina of the LPS-injected eye and subsequently elicit their effects on hepcidin expression in the contralateral eye. Exposure of the retina to LPS leads to increased oxidative stress as monitored by the levels of HNE. Several studies have shown that iron overload causes oxidative stress and that HNE levels increase under these conditions [36-39]. The LPS-induced oxidative stress in vivo in the retina is accompanied with apoptosis in specific cell types. A significant increase in apoptotic nuclei is seen in the outer nuclear layer, which contains the nuclei of photoreceptor cells, and in the inner nuclear layer, which contains, among other cell types, the nuclei of Müller cells. Interestingly, there is no evidence of increased apoptosis in RPE in response to LPS in vivo even though this cell expresses hepcidin and ferroportin. RPE is expected to respond to intravitreally injected LPS because the Toll-like receptor-4 is present at the apical membrane of these cells [40]. Therefore, the findings that there was no increase in apoptosis

in this cell layer in response to LPS treatment in vivo are intriguing. It is possible that RPE may possess robust anti-oxidant mechanisms to fight against oxidative stress. Though we observed a significant increase in oxidative stress and apoptosis in specific cell types in the retina in response to LPS injection, obvious morphological changes in the retina were not readily apparent. This could be due to the fact that the retina was exposed to LPS only for 24 h. Exposure for longer periods may increase the severity of the phenotype. Iron deficiency and anemia are observed in chronic, but not in acute, inflammation [41].

Since loss-of-function mutations in hepcidin cause a severe form of hemochromatosis, known as juvenile hemochromatosis, our findings on the expression of this protein within the retina are of clinical relevance. To our knowledge, there have been no reports on the iron status within the retina in patients with juvenile hemochromatosis. Since ferroportin, the molecular target of hepcidin, is expressed in several retinal cell types, it is expected that the levels of this iron transporter will be increased in patients with loss of hepcidin function. What this means in terms of iron homeostasis within the retina remains to be investigated. Studies by Hahn *et al* [42] showed that a combined disruption of the function of ceruloplasmin and hephaestin in mice leads to iron overload in retina, and that the retinal accumulation of iron causes a retinal phenotype similar to that of age-related macular degeneration. Since iron is a pro-oxidant, iron-induced oxidative stress may be an etiological factor in age-related macular degeneration in humans. Chronic retinal inflammation in humans is expected to cause iron overload within the retina, but whether such effects have any relevance to the pathogenesis of age-related macular degeneration remains to be seen. It is interesting to note that genetic variations in genes involved in inflammation and oxidative stress, including the Toll-like receptor-4 [43], have been implicated in the pathogenesis of age-related macular degeneration [44-46].

The currently prevailing notion that hepcidin is produced exclusively in the liver as a hormone which has its biologic effects distantly on other cells expressing ferroportin needs to be revisited in the light of more recent findings. There is evidence that hepcidin is made in non-hepatic tissues such as the brain [16], heart [47] and lung [48]. Here we have shown that various cell types within the retina express this peptide. The emerging evidence that hepcidin and its molecular target ferroportin may be expressed in the same cell (e.g., RPE, Müller cell, and photoreceptor cell) raises several interesting issues. It is currently believed that the plasma membrane ferroportin functions as the receptor for circulating hepcidin and that the binding of hepcidin to ferroportin leads to the internalization and degradation of the transporter [7]. If the same cell type expresses hepcidin and ferroportin, it is possible that hepcidin may regulate the levels of ferroportin in the cell even prior to the recruitment of the transporter to the plasma membrane by binding to the transporter at intracellular sites. The consequences of such binding inside the cell remain to be investigated. The binding of ferroportin to hepcidin leads to phosphorylation of specific tyrosine residues on the transporter, a process obligatory for internalization [49]. After internalization, the transporter is dephosphorylated and subsequently ubiquitinated to initiate degradation. It is not known at present whether the binding of hepcidin to the transporter at intracellular sites in retinal cells would lead to these covalent modifications of the transporter without involving other signaling components associated with the plasma membrane.

Since the ferroportin-expressing cell types within the retina also express hepcidin, ferroportin in these cells may be regulated by hepcidin generated within these cells independent of hepcidin in the circulation. This is true even for RPE in which ferroportin is expressed on the basolateral membrane with access to circulating hepcidin. Thus, hepatic hepcidin is not the sole determinant of ferroportin density in the plasma membrane of retinal cells. Pathologic conditions such as retinal infections and local inflammation may increase the production of hepcidin within the retina and reduce the ferroportin density in the plasma

membrane in retinal cells without any observable changes in the circulating levels of hepcidin.

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References

1. Donovan A, Andrews NC. The molecular regulation of iron metabolism. *Hematol J*. 2004; 5:373–380. [PubMed: 15448662]
2. Fleming RE, Britton RS, Waheed A, Sly WS, Bacon BR. Pathophysiology of hereditary hemochromatosis. *Semin Liver Dis*. 2005; 25:411–419. [PubMed: 16315135]
3. Beutler E. Hemochromatosis: genetics and pathophysiology. *Annu Rev Med*. 2006; 57:331–347. [PubMed: 16409153]
4. Ganz T, Nemeth E. Iron imports. IV. Hepcidin and regulation of body iron metabolism. *Am J Physiol Gastrointest Liver Physiol*. 2006; 290:G199–G203. [PubMed: 16407589]
5. Fleming RE, Britton RS. Iron Imports. VI. HFE and regulation of intestinal iron absorption. *Am J Physiol Gastrointest Liver Physiol*. 2006; 290:G590–G594. [PubMed: 16537971]
6. Park C, Valore E, Waring A, Ganz T. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. *J Biol Chem*. 2001; 276:7806–7810. [PubMed: 11113131]
7. 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; 306:2051–2053. [PubMed: 15604397]
8. Vaulont S, Lou D, Viatte L, Kahn A. Of mice and men: the iron age. *J Clin Invest*. 2005; 115:2079–2082. [PubMed: 16075054]
9. Nemeth E, Ganz T. Regulation of iron metabolism by hepcidin. *Annu Rev Nutr*. 2006; 26:323–342. [PubMed: 16848710]
10. Nicolas G, Bennoun M, Porteu A, Mativet S, Beaumont C, Grandchamp B, Sirtio M, Swadogo M, Kahn A, Vaulont S. Severe iron deficiency anemia in transgenic mice expressing liver hepcidin. *Proc Natl Acad Sci USA*. 2002; 99:4596–4601. [PubMed: 11930010]
11. Andrews NC. Anemia of inflammation: the cytokine-hepcidin link. *J Clin Invest*. 2004; 113:1251–1253. [PubMed: 15124013]
12. Dekker MC, Giesbergen PC, Njajou OT, van Swieten JC, Hofman A, Breteler MM, Van Duijn CM. Mutations in the hemochromatosis gene (HFE), Parkinson's disease and parkinsonism. *Neurosci Lett*. 2003; 348:117–119. [PubMed: 12902032]
13. Pulliam JF, Jennings CD, Kryscio RJ, Davis DG, Wilson D, Montine TJ, Schmitt FA, Markesbery WR. Association of HFE mutations with neurodegeneration and oxidative stress in Alzheimer's disease and correlation with APOE. *Am J Med Genet*. 2003; 119:48–53. [PubMed: 12707938]
14. Connor JR, Milward EA, Moalem S, Sampietro M, Boyer P, Percy ME, Vergani C, Scott RJ, Chorney M. Is hemochromatosis a risk factor for Alzheimer's disease? *J Alzheimers Dis*. 2001; 3:471–477. [PubMed: 12214033]
15. Wang XS, Lee S, Simmons Z, Boyer P, Scott K, Liu W, Connor JR. Increased incidence of HFE mutations in amyotrophic lateral sclerosis. *J Neurol Sci*. 2004; 227:27–33. [PubMed: 15546588]
16. Zechel S, Huber-Wittmer K, Oliver von Bohlen und Halbach. Distribution of the iron-regulating protein hepcidin in the murine central nervous system. *J Neurosci Res*. 2006; 84:790–800. [PubMed: 16933319]
17. Wu LJ, Leenders AG, Cooperman S, Meyron-Holtz E, Smith S, Land W, Tsai RY, Berger UV, Sheng ZH, Rouault TA. Expression of the iron transporter ferroportin in synaptic vesicles and blood-brain barrier. *Brain Res*. 2004; 1001:108–117. [PubMed: 14972659]
18. Klomp LW, Farhangrazi ZS, Dugan LL, Gitlin JD. Ceruloplasmin gene expression in the murine central nervous system. *J Clin Invest*. 1996; 98:207–15. [PubMed: 8690795]

19. Shichi H. Microsomal electron transfer system of bovine retinal pigment epithelium. *Exp Eye Res.* 1969; 8:60–68. [PubMed: 4388081]
20. 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]
21. Martin PM, Gnana-Prakasam JP, Roon P, Smith RG, Smith SB, Ganapathy V. Expression and polarized localization of the hemochromatosis gene product HFE in retinal pigment epithelium. *Invest Ophthalmol Vis Sci.* 2006; 47:4238–4244. [PubMed: 17003411]
22. Mayerson PL, Hall MO, Clark V, Tashka A. An improved method for isolation and culture of rat retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci.* 1985; 26:1599–1609. [PubMed: 4055292]
23. Umapathy NS, Li W, Mysona BA, Smith SB, Ganapathy V. Expression and function of glutamine transporters SN1 (SNAT3) and SN2 (SNAT5) in retinal Müller cells. *Invest Ophthalmol Vis Sci.* 2005; 46:3980–3987. [PubMed: 16249471]
24. Bridges CC, Hu H, Miyauchi S, Siddaramappa UN, Ganapathy ME, Ignatowicz L, Maddox DM, Smith SB, Ganapathy V. Induction of cystine-glutamate transporter x_c^- by human immunodeficiency virus type 1 transactivator protein Tat in retinal pigment epithelium. *Invest Ophthalmol Vis Sci.* 2004; 45:2906–2914. [PubMed: 15326101]
25. Chancy CD, Kekuda R, Huang W, Prasad PD, Kuhnel JM, Sirotnak FM, Ganapathy V, Smith SB. Expression and differential polarization of the reduced-folate transporter-1 and the folate receptor x in mammalian retinal pigment epithelium. *J Biol Chem.* 2000; 275:20676–20684. [PubMed: 10787414]
26. Rajan DP, Kekuda R, Chancy CD, Huang W, Ganapathy V, Smith SB. Expression of the extraneuronal monoamine transporter in RPE and neural retina. *Curr Eye Res.* 2000; 20:195–204. [PubMed: 10694895]
27. Ola SM, Moore P, Maddox D, El-Sherbeny A, Huang W, Roon P, Agarwal N, Ganapathy V, Smith SB. Analysis of sigma receptor (sigmaR1) expression in retinal ganglion cells cultured under hyperglycemic conditions and in diabetic mice. *Brain Res Mol Brain Res.* 2002; 107:97–107. [PubMed: 12425939]
28. Verga FMV, Vujic SM, Kessler R, Stolte J, Hentze MW, Muckenthaler MU. STAT3 mediates hepatic hepcidin expression and its inflammatory stimulation. *Blood.* 2007; 109:353–358. [PubMed: 16946298]
29. Rossi E. Hepcidin - the iron regulatory hormone. *Clin Biochem Rev.* 2005; 26:47–49. [PubMed: 16450011]
30. Verga FMV, Muckenthaler MU. Hepcidin: Iron-hormone and antimicrobial peptide. *Gene.* 2005; 364:37–44. [PubMed: 16203112]
31. Roy CN, Andrews NC. Anemia of inflammation: the hepcidin link. *Curr Opin Hematol.* 2005; 12:107–111. [PubMed: 15725899]
32. Ganz T, Nemeth E. Iron imports. IV. Hepcidin and regulation of body iron metabolism. *Am J Physiol Gastrointest Liver Physiol.* 2006; 290:199–203.
33. Ahmad KA, Ahmann JR, Migas MC, Waheed A, Britton RS, Bacon BR, Sly WS, Fleming RE. Decreased liver hepcidin expression in the Hfe knockout mouse. *Blood Cells Mol Dis.* 2002; 29:361–366. [PubMed: 12547226]
34. Babitt JL, Huang FW, Wrighting DM, Xia Y, Sidis Y, Samad TA, Campagna A, Chung RT, Schneyer AL, Woolf CJ, Andrews NC, Lin HY. Bone morphogenetic protein signaling by hemojuvelin regulates hepcidin expression. *Nat Genet.* 2006; 38:531–539. [PubMed: 16604073]
35. Constante M, Wang D, Raymond VA, Bilodeau M, Santos MM. Repression of repulsive guidance molecule C during inflammation is independent of Hfe and involves tumor necrosis factor- α . *Am J Pathol.* 2007; 170:497–504. [PubMed: 17255318]
36. Khan MF, Wu X, Tipnis UR, Ansari GAS, Boor PJ. Protein adducts of malondialdehyde and 4-hydroxynonenal in the livers of iron-loaded rats: Quantitation and localization. *Toxicology.* 2002; 173:193–201. [PubMed: 11960672]

37. Kaneko K, Yoshida K, Arima K, Ohara S, Miyajima H, Kato T, Ohta M, Ikeda SI. Astrocytic deformity and globular structures are characteristic of the brains of patients with aceruloplasminemia. *J Neuropathol Exp Neurol*. 2002; 61:1069–1077. [PubMed: 12484569]
38. Hammer A, Ferro M, Tillian HM, Tatzber F, Zollner H, Schauenstein E, Schaur RJ. Effect of oxidative stress by iron on 4-hydroxynonenal formation and proliferative activity in hepatomas of different degrees of differentiation. *Free Rad Biol Med*. 1997; 23:26–33. [PubMed: 9165294]
39. Hougum K, Filip M, Witztum JL, Chojkier M. Malondialdehyde and 4-hydroxynonenal protein adducts in plasma and liver of rats with iron overload. *J Clin Invest*. 1990; 86:1991–1998. [PubMed: 2123889]
40. Kindzelskii AL, Elner VM, Elner SG, Yang D, Hughes BA, Petty HR. Toll-like receptor 4 (TLR4) of retinal pigment epithelial cells participates in transmembrane signaling in response to photoreceptor outer segments. *J Gen Physiol*. 2004; 124:139–149. [PubMed: 15277575]
41. Ganz T. Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation. *Blood*. 2003; 102:783–788. [PubMed: 12663437]
42. Hahn P, Qian Y, Dentchev T, Chen L, Beard J, Harris ZL, Dunaief JL. Disruption of ceruloplasmin and hephaestin in mice causes retinal iron overload and retinal degeneration with features of age-related macular degeneration. *Proc Natl Acad Sci USA*. 2004; 101:13850–13855. [PubMed: 15365174]
43. Zarepari S, Buraczynska M, Branham KEH, Shah S, Eng D, Li M, Pawar H, Yashar BM, Moroi SE, Lichter PR, Petty HR, Richards JE, Abecasis GR, Elner VM, Swaroop A. Toll-like receptor 4 variant D299G is associated with susceptibility to age-related macular degeneration. *Hum Mol Genet*. 2005; 14:1449–1455. [PubMed: 15829498]
44. Ambati J, Ambati BK, Yoo SH, Ianchulev S, Adamis AP. Age-related macular degeneration: etiology, pathogenesis, and therapeutic strategies. *Surv Ophthalmol*. 2003; 48:257–293. [PubMed: 12745003]
45. Hageman GS, Luthert PJ, Victor Chong NH, Johnson LV, Anderson DH, Mullins RF. An integrated hypothesis that considers drusen as biomarkers of immune-mediated processes at the RPE-Bruch's membrane interface in aging and age-related macular degeneration. *Prog Retin Eye Res*. 2001; 20:705–732. [PubMed: 11587915]
46. Zarbin MA. Current concepts in the pathogenesis of age-related macular degeneration. *Arch Ophthalmol*. 2004; 122:598–614. [PubMed: 15078679]
47. Merle U, Fein E, Gehrke SG, Stremmel W, Kulaksiz H. The iron regulatory peptide hepcidin is expressed in the heart and regulated by hypoxia and inflammation. *Endocrinology*. 2007; 148:2663–2668. [PubMed: 17363462]
48. Nguyen NB, Callaghan KD, Ghio AJ, Haile DJ, Yang F. Hepcidin expression and iron transport in alveolar macrophages. *Am J Physiol Lung Cell Mol Physiol*. 2006; 291:L417–L425. [PubMed: 16648237]
49. De Domenico I, Ward DM, Langelier C, Vaughn MB, Nemeth E, Sundquist WI, Ganz T, Musci G, Kaplan J. The molecular mechanism of hepcidin-mediated ferroportin down-regulation. *Mol Biol Cell*. 2007; 18:2569–2578. [PubMed: 17475779]

Figure 1. Expression of hepcidin in mouse retina

A, RT-PCR analysis of hepcidin mRNA in neural retina and RPE/eyecup from mouse eyes. **B**, Analysis of hepcidin mRNA in mouse retina by in situ hybridization. Right panel shows a representative section of retina hybridized with a digoxigenin-labeled antisense riboprobe specific for hepcidin. Left panel is a negative control with a sense probe. **C**, Immunofluorescence localization of hepcidin protein in mouse retina. Left panel is a negative control without the primary antibody. Middle panel shows the antibody specificity, using the hepcidin antibody which had been neutralized with the antigenic peptide. Right panel shows the pattern of expression of hepcidin in retina. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium. **D**, RT-PCR analysis of hepcidin mRNA in primary RPE cells and primary Müller cells from mouse eyes. HPRT1 was used as an internal control. **E**, Immunofluorescence showing hepcidin protein expression in primary Müller cells, primary RPE cells, rMC1 cells (a rat Müller cell line), and ARPE19 cells (a human RPE cell line). Right panel indicates negative control with the omission of the primary antibody.

Figure 2. Regulation of hepcidin expression by LPS

A, RT-PCR showing hepcidin mRNA levels in untreated and LPS-treated (10 ng/ml and 100 ng/ml) retinal cells. **B**, RT-PCR showing Toll-like receptor 4 (TLR4) expression levels in retinal cells. **C**, Hepcidin expression in primary RPE cells treated with or without LPS. **D**, Hepcidin expression in primary Müller cells treated with or without LPS. Left most panels in (C) and (D) indicate negative control with the omission of the primary antibody.

Figure 3. Effects of LPS on *hepcidin* promoter activity using EGFP reporter assay
Left panels show the effects of LPS on EGFP reporter in the absence of *hepcidin* promoter.
Right panels show the effects of LPS on *hepcidin* promoter-EGFP construct.

Figure 4. Effects of intravitreal injection of LPS on the expression of iron-regulatory proteins in the retina and non-involvement of Hfe in the process

A, PBS was injected in both the eyes of control mice (lane 1); LPS (10 μ g) was injected in the right eye (lane 3) and PBS in the left eye in experimental mice (lane 2). RT PCR was done for Hfe, HJV and hepcidin in neural retina and RPE/eyecup. HPRT1 was used as an internal control. **B**, RT-PCR showing Hfe expression in primary RPE cells from wild type and *Hfe*-null mice. **C**, RT-PCR analysis of hepcidin in control and LPS-treated (10 ng/ml and 100 ng/ml) primary RPE cells from *Hfe*-null mice. **D**, Hepcidin protein expression in control and LPS-treated (10 ng/ml and 100 ng/ml) primary RPE cells from *Hfe*-null mice.

Figure 5. Immunostaining for hepcidin and ferroportin in LPS-injected mouse retinal sections
 Top panel: negative control with no primary antibody. Middle panel: staining with hepcidin antibody in retinal sections from control, PBS- and LPS-injected mouse eyes. Bottom panel: staining with ferroportin antibody in retinal sections from control, PBS- and LPS-injected mouse eyes.

Figure 6. Oxidative stress and apoptosis in LPS-injected mouse retina

A, Immunostaining for 4-hydroxynonenal, showing positive staining in outer and inner nuclear layer of LPS-injected mouse retina compared to PBS-injected control retina. There was mild staining in PBS-injected contralateral eye of treatment mice. **B**, Number of apoptotic cells in each cell layer of retina in LPS-injected mouse retinal sections versus control retinal sections. **C**, Immunostaining for ferroportin levels in control and LPS-treated (100 ng/ml) primary RPE cells.

Table I

PCR primers

<u>Gene Name</u>	<u>NCBI Accession No.</u>	<u>Primers Sequence</u>
Mouse hepcidin	AF297664	Forward: GCACCACCTATCTCCATCAACAGA Reverse: GGTCAGGATGTGGCTCTAGGCTAT
Rat hepcidin	AF344185	Forward: GAAGGCAAGATGGCACTAAGCA Reverse: TCTCGTCTGTTGCCGAGATAG
Human hepcidin	NM_021175	Forward: ACTGTCACTCGGTCCCAGACA Reverse: TCCAAGACCTATGTTCTGGGG
Mouse Hfe	NM_010424	Forward: GGCTTCTGGAGATATGGTTAT Reverse: GACTCCACTGATGATTCCGATA
Mouse HJV	NM_027126	Forward: GGCTGAGGTGGACAATCTTC Reverse: GAACAAAGAGGGCCGAAAG
Mouse TLR4	BC029856	Forward: CTGCCAAGTCTCAGCTATCT Reverse: CTGCTAAGAAGGCGATACAA
Rat TLR4	NM_019178	Forward: ACAAGAGCCGAAAGTTATT Reverse: CTGCTAAGAAGGCGATACAA
Human TLR4	BC117422	Forward: ACAGACTTGCGGGTTCTACA Reverse: CTGCTGAGAAGGCGGTACAG