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Regulation of the cholesterol efflux transporters ABCA1 and ABCG1 in retina in hemochromatosis and by the endogenous siderophore 2,5-dihydroxybenzoic acid ☆

Sudha Ananth [a], Jaya P. Gnana-Prakasam [a], Yangzom D. Bhutia [a], Rajalakshmi Veeranan-Karmegam [a], Pamela M. Martin [a], Sylvia B. Smith [b], Vadivel Ganapathy [a,*]

[a] Department of Biochemistry and Molecular Biology, Georgia Regents University, Augusta, GA 30912, USA
[b] Department of Cellular Biology and Anatomy, Georgia Regents University, Augusta, GA 30912, USA

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ABSTRACT

Hypercholesterolemia and polymorphisms in the cholesterol exporter ABCA1 are linked to age-related macular degeneration (AMD). Excessive iron in retina also has a link to AMD pathogenesis. Whether these findings mean a biological/molecular connection between iron and cholesterol is not known. Here we examined the relationship between retinal iron and cholesterol using a mouse model ($Hfe^{-/-}$) of hemochromatosis, a genetic disorder of iron overload. We compared the expression of the cholesterol efflux transporters ABCA1 and ABCG1 and cholesterol content in wild type and $Hfe^{-/-}$ mouse retinas. We also investigated the expression of Bdh2, the rate-limiting enzyme in the synthesis of the endogenous siderophore 2,5-dihydroxybenzoic acid (2,5-DHBA) in wild type and $Hfe^{-/-}$ mouse retinas, and the influence of this siderophore on ABCA1/ABCG1 expression in retinal pigment epithelium. We found that ABCA1 and ABCG1 were expressed in all retinal cell types, and that their expression was decreased in $Hfe^{-/-}$ retina. This was accompanied with an increase in retinal cholesterol content. Bdh2 was also expressed in all retinal cell types, and its expression was decreased in hemochromatosis. In ARPE-19 cells, 2,5-DHBA increased ABCA1/ABCG1 expression and decreased cholesterol content. This was not due to depletion of free iron because 2,5-DHBA (a siderophore) and deferiprone (an iron chelator) had opposite effects on transferrin receptor expression and ferritin levels. We conclude that iron is a regulator of cholesterol homeostasis in retina and that removal of cholesterol from retinal cells is impaired in hemochromatosis. Since excessive cholesterol is pro-inflammatory, hemochromatosis might promote retinal inflammation via cholesterol in AMD.

1. Introduction

There is an overwhelming evidence for the involvement of oxidative stress and inflammation in the pathogenesis of age-related macular degeneration (AMD), a leading cause of blindness in adults in developed countries [1–4]. There are two forms of AMD, dry and wet, the former with accumulation of drusen deposits underneath the basal side of the retinal pigment epithelium and the latter with abnormal development of new blood vessels, which invade into the retina through the Bruch's membrane. There is no consensus on whether or not a similar mechanism participates in the pathogenesis of both forms, but the involvement of oxidative stress and inflammation has been implicated in dry as well as wet AMD. Accordingly, most of the animal models of AMD are based either on disruption of inflammatory pathways such as the complement system or chemokine signaling or on oxidative damage [5].

Iron is essential for the survival of all cells, but excessive iron is detrimental because free iron generates potent reactive oxygen species such as hydroxyl radicals. Numerous studies have suggested a role for excessive accumulation of iron in the retina as an important contributor to the pathogenesis of AMD [6–8]. Retina expresses most of the proteins that are known to participate in the regulation of iron homeostasis, a feature obligatory for maintenance of iron levels optimal for cellular function without the risk of oxidative stress [9]. Disruptions in the expression and function of these iron-regulatory proteins alter iron levels in the retina, involving all retinal cell types. Hemochromatosis is a hereditary disorder caused by loss-of-function mutations in five different genes coding for iron-regulatory proteins, namely *HFE*, *hemojuvelin* (also called *HFE2*), *hepcidin* (also called *HAMP*), *ferroportin*, and *transferrin receptor 2* (*TfR2*) [10–13]. This disorder represents one of the most prevalent genetic diseases in humans, with homozygosity or compound heterozygosity accounting for 1 in 300 in the general population. Mutations in *HFE* are

Abbreviations: RPE, retinal pigment epithelium; ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; Bdh2, β-hydroxybutyrate dehydrogenase-2; 2,5-DHBA, 2,5-dihydroxybenzoic acid; AMD, age-related macular degeneration; pRPE, primary cultures of mouse retinal pigment epithelial cells; pMC, primary cultures of mouse Muller cells; pGC, primary cultures of mouse retinal ganglion cells; 5-Aza-C, 5-azacytidine; TfR, transferrin receptor; HAMP, hepatic anti-microbial peptide

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* Corresponding author. Tel.: +1 706 721 7652; fax: +1 706 721 9947.
E-mail address: vganapat@gru.edu (V. Ganapathy).

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seen in >85% of patients with hemochromatosis, the remaining ~15% arising from mutations in the other four genes. Irrespective of the gene involved, hemochromatosis is associated with excessive iron accumulation in multiple systemic organs, leading to oxidative stress and consequent organ dysfunction. It was believed for a long time that the retina and the brain are spared in this disease because of the blood-retinal barrier or blood–brain barrier, but emerging evidence strongly indicates otherwise [9,14]. Mouse models of hemochromatosis (*Hfe*$^{-/-}$, *Hjv*$^{-/-}$, and *hepcidin*$^{-/-}$ mice) have provided unequivocal evidence for excessive iron accumulation in the retina in this disease [15–17]. These mice exhibit morphological and biological changes in the retina that are similar to those found in AMD [15–17], indicating that iron-mediated oxidative stress is likely to be a critical determinant in the pathogenesis and/or progression of AMD.

With regard to the role of inflammation in AMD, available evidence points to the involvement of diverse immune cell types, cytokines, and signaling pathways [3,4]. Cholesterol is receiving increasing attention in recent years as a critical determinant of inflammatory pathways [18–20], and two transporters, ABCA1 and ABCG1, both participating in the efflux of cholesterol from peripheral tissues to load it to on HDL, have been implicated in this process [21,22]. Furthermore, there is strong evidence from animal models of hypercholesterolemia for excessive cholesterol build-up in the retina as a causative factor in AMD [23–26].

From what has been described above, it is likely that excessive iron in retina may contribute to the pathogenesis/progression of AMD through oxidative damage and that excessive cholesterol in retina may also contribute to the pathogenesis/progression of AMD through inflammation. However, whether there is any link between iron and cholesterol in health and disease is not known. The purpose of the present study was to investigate the expression of the cholesterol efflux transporters ABCA1 and ABCG1 in the retina in normal mice and in a mouse model of hemochromatosis (*Hfe*$^{-/-}$ mouse) and also to determine how the iron status in retina and RPE influences the expression of these two transporters. Furthermore, there has been a recent development in the area of iron homeostasis in mammalian cells, which involves identification of an endogenous siderophore (i.e., iron carrier) and the enzyme critical for its synthesis [27]. The siderophore is 2,5-dihydroxybenzoic acid (2,5-DHBA) and the enzyme is the cytosolic β-hydroxybutyrate dehydrogenase, known as Bdh2. This siderophore binds iron and facilitates its entry through plasma membrane and mitochondrial inner membrane [27]. There is no information available at present on the expression of Bdh2 and on the role, if any, of the newly discovered endogenous siderophore in the retina; therefore, here we studied the expression of this important iron-regulatory enzyme in the retina in wild type and *Hfe*$^{-/-}$ mice and also examined the influence of the siderophore 2,5-DHBA on the expression of ABCA1 and ABCG1 in RPE cells.

2. Materials and methods

2.1. Materials

Antibodies were obtained from the following sources: rabbit polyclonal anti-ABCA1 and rabbit polyclonal anti-ABCG1 (Novus Biologicals, Littleton, CO, USA), goat polyclonal anti-Bdh2 (Abcam, Cambridge, MA, USA), mouse monoclonal anti-vimentin (Millipore, Billerica, MA, USA) and chicken anti-MCT1 (Alpha Diagnostic International, San Antonio, TX, USA), goat anti-rabbit IgG coupled to Alexa Fluor 568, donkey anti-goat IgG coupled to Alexa Fluor 568, goat anti-chicken IgG coupled to Alexa Fluor 568, and goat anti-rabbit and anti-mouse IgG coupled to Alexa Fluor 488 (Molecular Probes, Carlsbad, CA, USA). The dilutions of the antibodies used for immunofluorescence experiments were: 1:1000 for ABCA1, 1:25 for ABCG1, 1:50 for vimentin, 1:50 for Bdh2, and 1:1000 for MCT1. Rabbit polyclonal antibodies specific for L-ferritin and H-ferritin were provided by Professor P. Arosio (Dipartimento Materno Infantile e Tecnologie Biomediche, Universita di Brescia, Brescia, Italy).

2.2. Animals

Breeding pairs of *Hfe*$^{+/-}$ mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Age- and gender-matched wild type and *Hfe*$^{-/-}$ mice were obtained from the same litter originating from the mating of heterozygous mice. Albino Balb/c mice (6-week-old) were used for immunofluorescence analyses in some studies. Mice were purchased from Harlan–Sprague Dawley, Inc. (Indianapolis, IN, USA). All experimental procedures involving these animals adhered to the "Principles of Laboratory Animal Care" (National Institutes of Health publication #85-23, revised in 1985) and were approved by the Institutional Committee for Animal Use in Research and Education.

2.3. Immunofluorescence analysis

Eyes were embedded in OCT compound and frozen at –20 °C. Sections (8-μm thick) were used for immunostaining by fixing in 4% paraformaldehyde for 10 min, washed with 0.01 M phosphate-buffered saline (pH 7.4), and blocked with 1× Power Block for 60 min. Sections were then incubated overnight at 4 °C with appropriate primary antibodies. Negative controls involved omission of the primary antibodies. Sections were rinsed and incubated for 1 h with appropriate secondary antibodies. Coverslips were mounted after staining with Hoechst nuclear stain and sections were examined by epifluorescence microscopy (Axioplan-2 microscope, equipped with an HRM camera and the AxioVision imaging program; Carl Zeiss, Jena, Germany).

2.4. Primary RPE, Müller, and ganglion cell cultures from mouse eyes

Primary cultures of RPE were prepared as described previously [15,17]. Three-week-old wild type and *Hfe*$^{-/-}$ mice were used to establish primary cultures of RPE. The purity of the culture was verified by immunodetection of RPE-65 (retinal pigment epithelial protein 65) and CRALBP (cellular retinaldehyde binding protein). Müller cells were prepared from 7- to 10-day-old C57BL/6 mice by a method adapted from Hicks and Courtois [28] and described in one of our previous publications [29]. Staining for the Müller cell markers glutamine synthetase, glutamate transporter EAAT1, and CRALBP confirmed the purity of the primary cultures. Retinal ganglion cells were isolated by immunopanning from 2-day-old C57BL/6 mice by the method of Barres et al. [30] as described previously in our publications [31,32]. The purity of the cultures was confirmed by immunostaining for Thy-1, a ganglion cell marker.

2.5. Real time PCR

The following real time primers were used: 5′-AGTTTCGGTATGGC GGGTTT-3′ (forward) and 5′-AGCATGCCAGCCCTTGTTAT-3′ (reverse) for mouse ABCA1; 5′-ACCTACCACAACCCAGCAGACTTT-3′ (forward) and 5′-GGTGCCAAAGAAACGGGTTCACAT-3′ (reverse) for mouse ABCG1; 5′-GATGCAACTGTGTGTGTCCAGGAA-3′ (forward) and 5′-ACAGGGTTGCCAGTTACATAGGCT-3′ (reverse) for mouse Bdh2; 5′-GAAGTACATCAGAACATGGGC-3′ (forward) and 5′-GATCAAAGCCATGG CTGTAG-3′ (reverse) for human ABCA1; 5′-CAGGAAGATTAGACACTG TGG-3′ (forward) and 5′-GAAAGGGGAATGGAGAGAAG-3′ (reverse) for human ABCG1; 5″-GAGGACGCGCTAGTGTTCTT-3′ (forward) and 5′-TGTGACATTGGCCTTTGTGTT-3′ (reverse) for human transferrin receptor 1 (TfR1). 18S and hypoxanthine phosphoribosyl transferase 1 (HPRT1) were used as internal controls. Real-time amplifications using SYBR Green detection chemistry were run in triplicate on 96-well reaction plates. Reactions were prepared in a total volume of 25 μl containing: 5 μl cDNA, 0.5 μl of each 20 μM primer, 12.5 μl of SYBR® Green Supermix and 6.5 μl RNase/DNase-free sterile water.