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Iron Increases APP Translation and Amyloid-Beta Production in the Retina

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Abstract

Age-related macular degeneration (AMD) is the most common cause of blindness among older adults in developed countries, and retinal iron accumulation may exacerbate the disease. Iron can upregulate the production of amyloid precursor protein (APP). Since amyloid- β (A β), a byproduct of APP proteolysis, is found in drusen, the histopathological hallmark of AMD, we tested the role of iron in regulating APP and A β levels in the retinal pigment epithelial cell line ARPE-19. We found that treatment with ferric ammonium citrate (FAC) increases APP at the translational level. FAC treatment also results in increased generation of APP C-terminal fragments C83 and C99, the products of APP proteolysis by α - and β -secretase, respectively, as well as levels of A β 42, a highly aggregative amyloid species. Additionally, retinal tissue sections from a patient with aceruloplasminemia, a disease causing iron overload in the retinal pigment epithelium (RPE), showed increased A β deposition in the RPE and drusen. Overall, our results suggest that RPE iron overload could contribute to A β accumulation in the retina.

Keywords

age-related macular degeneration; iron; amyloid precursor protein; amyloid-beta; aceruloplasminemia

1. Introduction

Age-related macular degeneration (AMD) causes progressive central vision distortion and loss and is the leading cause of irreversible blindness in the elderly worldwide. Although the causes of AMD are incompletely understood, there is evidence that iron toxicity contributes to AMD pathogenesis. Even though iron is essential for life, iron accumulation in tissues leads to free radical production and oxidative damage. The retina is highly vulnerable to photo-oxidative stress, which is exacerbated by perturbations in iron homeostasis. The body

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has no physiologic mechanism of iron excretion, thus tissue accumulation of iron rises with age. It is known that AMD retinas contain higher levels of iron compared to normal retinas (Wong et al., 2007), and patients with aceruloplasminemia (lacking the ferroxidase ceruloplasmin) exhibit retinal iron accumulation and early-onset macular degeneration (Dunaief et al., 2005; Wolkow et al., 2011). Consistent with this hypothesis, double knockout mice lacking the ferroxidases ceruloplasmin and hephaestin exhibit retinal iron accumulation and retinal degeneration that resemble AMD pathology (Hahn et al., 2004), and are protected from such retinal degeneration by treatment with the iron chelator deferiprone (Hadziahmetovic et al., 2011b).

Iron overload is also implicated in other age-related neurodegenerative diseases such as Alzheimer's disease (Sipe et al., 2002), and there exist other parallel pathologic findings between Alzheimer's disease and AMD (Ohno-Matsui, 2011). Amyloid precursor protein (APP), a main player in the pathogenesis of Alzheimer's disease, is expressed in inner retinal neurons and required for inner retinal function (Ho et al., 2012). Amyloid β ($A\beta$), the cleavage product of amyloid precursor protein (APP), has long been known to be the main constituent of senile plaques in brains of AD patients. It has been shown by multiple groups that $A\beta$ is also present in drusen, the extracellular deposits that accumulate beneath the retinal pigment epithelium (RPE) and are a pathologic hallmark of AMD (Johnson et al., 2002; Dentchev et al., 2003; Isas et al., 2010). In addition to $A\beta$, drusen also contain iron, apolipoproteins, and inflammatory mediators such as acute phase reactants and complement components (Mullins et al., 2000). $A\beta$ likely induces direct toxicity to the retinal pigment epithelium and contributes to AMD progression. In RPE cells, $A\beta$ reduces mitochondrial redox potential and increases production of reactive oxygen species (Bruban et al., 2009). It also induces an increase in vascular endothelial growth factor (VEGF) (Yoshida et al., 2005), a pro-angiogenic factor that drives the choroidal neovascularization of late-stage AMD. Anti- $A\beta$ antibodies protect a mouse model of AMD caused by a high fat diet and ApoE allele from degeneration (Ding et al., 2011, 2008).

Although there have been no published reports of iron directly affecting $A\beta$ production, the 5' untranslated region (UTR) of APP mRNA contains an iron-responsive element (IRE) stem loop with sequence homology to the ferritin IRE, suggesting that iron may regulate APP levels in the same way as it controls translation of the mRNA of the iron-storage proteins ferritin L and H (Rogers et al., 2002). While iron may regulate APP, conversely, APP may regulate iron. Iron levels in primary neurons are increased by APP ablation (Duce et al., 2010). In this study, we show that increased iron in cultured RPE cells leads to an elevation in APP protein and is accompanied by an increase in APP cleavage products – C-terminal fragments as well as $A\beta_{42}$, a highly aggregative amyloid species. These findings suggest that elevation of $A\beta$ is another mechanism by which iron overload contributes to retinal pathology. Taking these findings together with the literature, we propose a model, in which APP is upregulated by iron, but chronic iron overload causes $A\beta$ generation, a maladaptive stress response that may contribute to AMD susceptibility.

2. Materials and methods

2.1. Cell culture and treatments

The spontaneously immortalized human RPE cell line, ARPE-19 (ATCC, Manassas, VA) (Dunn et al., 1996) was cultured until confluent in 24-well plates (Falcon; BD Biosciences, Bedford, MA) in 1:1 DMEM/F12, 20 μ M L-glutamine (Invitrogen, Carlsbad, CA) supplemented with heat-inactivated 10% FBS (Hyclone, Logan, UT) and 1% penicillin-streptomycin. The cells were grown in 5% CO₂ at 37°C. After confluency, the cells were maintained in low-serum medium (1% FBS) and allowed to differentiate for one month. To assess effects of iron, ARPE-19 cells were treated with 250 μ M of ferric ammonium citrate (Sigma, St. Louis, MO) in DMEM/F12 with 1% FBS and harvested after 4 days for analysis. To assess effects of an iron chelator, ARPE-19 cells were treated with 100 μ M deferiprone (ApoPharma, Toronto, Canada) each day, and harvested after 4 days for analysis.

2.2. Western blot analysis

ARPE-19 cell lysates were collected in Laemmli buffer, and protein concentrations were quantified using a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). For Western blots of secretase proteins (in Fig. 4A), the general metalloprotease inhibitor 1,10-phenanthroline (at 10mM) was added to the lysis buffer to prevent TACE autoproteolysis. Protein lysates were separated on 4-12% SDS-PAGE and transferred to a nitrocellulose membrane. Blocking was achieved by incubation in phosphate buffered saline containing 5% milk and 0.1% Tween. Membranes were incubated overnight at 4°C with antibody to transferrin receptor (1:1000, clone H68.4; Invitrogen, Carlsbad, CA), APP N-terminus (1:1000, clone 22c11; Millipore, Billerica, MA), APP C-terminus (1:1000, cat. 171610, Millipore, Billerica, MA), TACE/ADAM17 (1:1000, cat. ab2051; Abcam, Cambridge, MA), ADAM10 (1:1000, cat. ab1997; Abcam, Cambridge, MA), or BACE1 (1:1000, cat. ab2077; Abcam, Cambridge, MA). After washes in PBS with 0.1% Tween, membranes were incubated with anti-rabbit or anti-mouse horseradish peroxidase (HRP) at a 1:10,000 dilution (GE Healthcare, Little Chalfont, UK) and were developed using a detection reagent (ECL Plus; GE Healthcare, Little Chalfont, UK). Images were acquired (Typhoon 9400 Variable Mode Imager; GE Healthcare, Little Chalfont, UK), and densitometry analysis was performed (ImageJ) such that the intensity of each band of interest was background-subtracted, divided by the intensity of the loading control band in the identical lane (i.e., α -tubulin), and then normalized to the value of the lowest-intensity band within the dataset.

2.3. Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) analysis was done using the TaqMan Custom Array (Applied Biosystems, Carlsbad, CA). RNA isolation/quantification and synthesis of cDNA were done as previously described (Hadziahmetovic et al., 2011a). Gene expression assays were obtained (TaqMan; Applied Biosystems, Foster City, CA) and used for PCR analysis. Probes specific to transferrin receptor (Tfrc, Hs00174609_m1) and APP (Hs01552283_m1) were used. Eukaryotic 18S rRNA (Hs99999901_s1) served as an internal control. qRT-PCR raw data were analyzed using the C_T method (Pfaffl, 2001), which provides normalized gene expression values. The amount of target mRNA was compared among the groups of

interest. All reactions were performed in biological (3 wells of cells) and technical (3 qPCR replicates per well of cells) triplicates.

2.4. Quantification of A β by enzyme-linked immunosorbent assay (ELISA)

ARPE-19 cells were grown in 75cm² flasks and treated with 250 μ M FAC. After 4 days, conditioned medium was collected and concentrated 70-fold using Amicon Ultra-15 Centrifugal Filter Units (Millipore, Billerica, MA). Then, 50 μ l of conditioned medium was used for A β 40 and A β 42 quantification by colorimetric BetaMark β -Amyloid x-40 and x-42 ELISA kits (Covance, Denver, PA) according to manufacturer's instructions.

2.5. Human tissue

Iron-overloaded eyes were obtained from a 60-year-old male donor with aceruloplasminemia, and normal control eyes were obtained from a 60-year-old male donor with no history of retinal disease. Eyes from both donors were collected following a 7-hour postmortem period. All specimens were obtained in accordance with institutional review board regulations and the provisions of the Declaration of Helsinki for research involving human tissues. Formalin-fixed eyes were embedded in paraffin and sectioned at 7- μ m thickness.

2.6. Immunohistochemistry

Rehydrated paraffin sections were stained with a rabbit polyclonal antibody raised against A β (1-14) (1:200, cat. Ab2539, Abcam, Cambridge, MA) using the Vectastain ABC Alkaline Phosphatase Kit and the Vector BCIP/NBT Kit (Vector Laboratories, Burlingame, CA).

2.7. Perls' Prussian Blue stain for iron

Rehydrated paraffin sections were stained with 5% K₄Fe(CN)₆ and 5% HCl in the dark for 45 min at 65°C. Sections were bleached with 0.25% KMnO₄ and 0.5% oxalic acid for 5 min each and counterstained with Nuclear Fast Red (Vector Laboratories, Burlingame, CA).

2.8. Secretase activity assays

ARPE-19 cells were maintained and treated as described above. Samples were collected and processed for fluorometric secretase activity assays. SensoLyte 520 TACE Activity Assay Kit (Anaspec, Fremont, CA) and β -Secretase Activity Fluorometric Assay Kit (BioVision, Milpitas, CA) were used to measure α - and β -secretase activities, respectively, according to manufacturers' instructions.

2.9. Statistical analysis

The mean $\pm$ SD were calculated for each comparison pair. The means between each pair were compared by t-test. P<0.05 was considered statistically significant. All statistical analysis was performed on GraphPad statistical software (GraphPad Software, San Diego CA).

3. Results

3.1. Iron increases APP protein at the translational level in ARPE-19 cells

We used the well-characterized human retinal pigment epithelium cell line ARPE-19 to investigate the effects of iron on APP levels. The cells were treated with 250 μ M ferric ammonium citrate (FAC) or 100 μ M iron chelator, deferiprone, and analyzed after 4 days. APP protein levels increased almost 10-fold in response to FAC treatment and decreased in response to treatment with the iron chelator deferiprone (Fig. 1A, B). As validation of the effect of both treatments on iron physiology, we showed that protein levels of transferrin receptor exhibited the opposite trend, by decreasing with FAC and increasing with DFP (Fig. 1C, D). The mRNA levels of APP did not change in response to FAC (Fig. 2A), whereas mRNA levels of transferrin receptor decreased (Fig. 2B), consistent with the well-known phenomenon that iron promotes the degradation of transferrin receptor mRNA (Hentze and Kuhnt, 1996; Rao et al., 1985). Altogether, these results show that in ARPE-19 cells, iron upregulates APP protein at the translational level, consistent with prior reports of APP having an iron response element in its 5' UTR (Rogers et al., 2002).

3.2. Iron increases the abundance of APP processing products in ARPE-19 cells

APP is a type I transmembrane protein that can undergo sequential proteolysis, first by α - or β -secretase (diagrammed in Fig. 3A). Cleavage by α -secretase occurs within the A β domain of APP (thus precluding A β generation) and releases a soluble ectodomain (sAPP α) and an 83-residue C-terminal fragment (C83), known to be 10kD in size. Cleavage by β -secretase releases a soluble fragment (sAPP β) into the extracellular space, and leaves the 99-amino acid C-terminal stub (C99) within the membrane (known to be 12kD). Subsequent cleavage of C99 by the intramembrane protease γ -secretase generates A β . Most A β peptides are 40 residues in length (A β 40), but a variant consisting of 42 residues (A β 42) is more hydrophobic and prone to aggregation, and is found in cerebral plaques (LaFerla et al., 2007).

We tested the effects of iron on APP processing in ARPE-19 cells by examining levels of APP C-terminal fragments using an antibody against amino acids 751-770. FAC treatment results in an increase in both C83 and C99 (Fig. 3B, C). The level of C83 is much higher than that of C99, which is expected since non-amyloidogenic cleavage by α -secretase is the predominant pathway for APP processing (Postina, 2008). We tested the effect of FAC on A β levels using a validated colorimetric ELISA (Dasari et al., 2010; Jayaraman et al., 2012; Thakker et al., 2009), which showed an increase in A β 42, with no detectable change in A β 40 (Fig. 3D). From the A β ELISA data, the A β 42/40 ratio is calculated to be 0.42 ± 0.07 without FAC treatment, and 0.64 ± 0.18 with FAC treatment; conversely, the A β 40/42 ratio is 2.37 ± 0.42 without FAC treatment, and 1.55 ± 0.42 with FAC treatment.

3.3. Iron does not affect α - and β -secretase levels or activities

Prior studies suggest that iron may affect secretase enzymes involved in APP processing (Bodovitz et al., 1995; Kim and Yoo, 2013; Li et al., 2013; Xiong et al., 2007). To determine whether this effect contributes to the observed iron-induced generation of APP cleavage products, we measured protein levels and cleavage activities of α - and β -secretases in

ARPE-19 cells treated with iron (FAC) or the iron chelator, deferiprone (DFP). Western analysis revealed no appreciable difference in the protein levels of these secretases (Fig. 4A). In line with this finding, fluorometric assays showed no change in α - and β -secretase activities in response to the treatments (Fig. 4B). Furthermore, a microarray analysis of FAC-treated versus untreated ARPE-19 cells found no appreciable changes in gene expression of any α -, β -, or γ -secretase components: specifically, ADAM10, TACE, BACE1, presenilin 1 and 2, Aph1a, Aph1b, nicastrin, and Pen2 (data not shown). Altogether, these data suggest that iron-induced accumulation of APP cleavage products is a direct consequence of APP overproduction, with no observed change in secretase levels or cleavage activities.

3.3. A β deposition is increased in the retinal pigment epithelium in aceruloplasminemia

To test our *in vitro* findings in disease-relevant human tissue, we used retinal sections from a patient with aceruloplasminemia, a systemic iron-overload condition known to cause iron deposition in the RPE. As expected, Perls' Prussian Blue stain for iron detected notable iron deposits in the macular RPE of the aceruloplasminemia eye, as compared to an age/gender-matched normal control (Fig. 5A), consistent with our previous report (Wolkow et al., 2011). We then performed A β immunohistochemistry using an antibody raised against the N-terminal, β -cleavage-specific epitope A β (1-14), which had been validated by other studies for A β immunohistochemistry (Bernstein et al., 2014; Tajiri et al., 2013). We observed that A β immunostaining is markedly elevated in the aceruloplasminemia RPE compared to that of an age/gender-matched control (Fig. 5A) and is seen in drusen (Fig. 5B), providing *in vivo* evidence that increased RPE iron is associated with A β accumulation.

4. Discussion

The pathogenesis of age-related macular degeneration (AMD) shares multiple mechanisms with Alzheimer's disease (AD), including the presence of extracellular deposits, which are drusen in AMD and senile plaques in AD. While it is believed that small amounts of A β accumulate in the normal brain and retina with age, pronounced A β accumulation has long been associated with AD pathogenesis, and has been found in drusen of AMD patients by multiple groups (Anderson et al., 2004; Dentchev et al., 2003; Isas et al., 2010). Additionally, oxidative stress and iron overload are other factors implicated in the pathogenesis of both diseases (Wong et al., 2007; Zecca et al., 2004). Furthermore, *in vitro* studies have shown that iron directly binds to A β peptides (Bousejra-ElGarah et al., 2011; Nair et al., 2010), raising the possibility that A β could potentiate iron accumulation with drusen.

Our present findings demonstrate that treatment of ARPE-19 cells with ferric ammonium citrate increases translation of amyloid precursor protein (APP) in a manner independent of mRNA levels, consistent with previous reports in other cell types (Duce et al., 2010; Rogers et al., 2002). We also show that ferric ammonium citrate increases the abundance of APP cleavage products, specifically APP C-terminal fragments (both C83 and C99), as well as A β 42. The overproduction of these cleavage products appears to be a consequence of increased APP protein levels, and not a result of increased secretase levels or activity. It is

possible that normal secretase levels are sufficient to process the increased APP, or that a change in secretase localization may contribute to increased APP processing. We also demonstrate elevated A β deposition in the RPE from a patient with aceruloplasminemia, an iron-overload condition with ocular manifestations similar to those of AMD. Overall, our data suggest a mechanism whereby iron overload could exacerbate processes of amyloid-related pathology in AMD.

The presence of the IRE within the 5' UTR of APP is likely the mechanism for its iron-induced increase in protein levels (Duce et al., 2010; Rogers et al., 2002). It is worth noting that we observed an increase in A β 42, but did not observe changes in A β 40. It is unclear what mechanism drives this preferential elevation of A β 42, but interestingly, it was reported that treatment of ARPE-19 cells with the cholesterol oxidation metabolite 27-hydroxycholesterol also increased A β 42 levels but not A β 40 levels (Dasari et al., 2010), hinting that A β 42 generation could be linked to pathways of oxidative stress. While the magnitude of A β 42 increase is small, its gradual accumulation in the retina over years to decades could increase disease susceptibility, especially since AMD mostly afflicts the elderly.

Amyloid-containing vesicles likely originate from the retinal pigment epithelium (RPE), due to its proximity to drusen, and since A β immunoreactivity is observed within the RPE cell cytoplasm (Johnson et al., 2002). RPE cells constitutively express APP and the secretases responsible for its processing (Yoshida et al., 2005). Deposition of A β likely causes local inflammation that hastens drusen formation, eventually resulting in RPE atrophy and photoreceptor cell death (Johnson et al., 2002).

Overall, our results suggest a model of AMD pathogenesis, in which APP, as an iron regulatory protein (Duce et al., 2010), is upregulated by elevated iron, but chronic iron overload increases the byproducts of APP processing, leading to generation of toxic A β species. Therefore, this iron-induced increase in APP translation could be a maladaptive stress response that is insufficient to balance iron homeostasis, yet increases AMD susceptibility. Already, anti-amyloid therapy has been shown to protect against RPE damage and vision loss in a mouse model of AMD (Ding et al., 2011, 2008), and iron chelators have also shown promise (Hadziahmetovic et al., 2011b; Lukinova et al., 2009). Our present report lends support to these emerging modes of therapy.

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Figure 1. Ferric ammonium citrate (FAC) increases APP at the protein level in ARPE-19 cells
(A) Lysates of FAC-treated or DFP-treated ARPE-19 cells immunoblotted with antibody against the N-terminus of amyloid-precursor protein (APP), with **(B)** densitometry quantitation of both total APP (including both mature and immature APP) from n=2 experiments. **(C)** Lysate of FAC-treated or DFP-treated ARPE-19 cells immunoblotted with antibody against transferrin receptor (TFR), with **(D)** densitometry quantitation from n=3 experiments. α-tubulin serves as a loading control. Molecular weight markers are shown to the left of the blot (in kDa).

Figure 2. Ferric ammonium citrate does not affect APP mRNA levels

qRT-PCR of FAC-treated ARPE-19 cells with primers for APP (A) and TFR (B), with 18S rRNA used as a reference gene (n=3).

Figure 3. Ferric ammonium citrate (FAC) increases accumulation of APP cleavage byproducts, including $A\beta$

(A) Schematic of APP processing, showing sequential cleavage by α , β , and γ -secretases to generate C-terminal fragments (C83 and C99) and $A\beta$. (B) Lysates from FAC-treated or DFP-treated ARPE-19 cells immunoblotted with antibody against the C-terminus of APP. α -tubulin serves as a loading control. Molecular weight markers are shown to the left of the blot (in kDa). A contrast-enhanced image of the blot is provided to facilitate visualization of the C99 band. (C) Densitometry quantitation of B from n=2 experiments. (D) Levels of $A\beta$ 40 and $A\beta$ 42 in the culture media measured by colorimetric ELISA (n=3).

Figure 4. Ferric ammonium citrate does not affect protein levels or activity of α - and β -secretases
(A) Lysates from FAC-treated or DFP-treated ARPE-19 cells immunoblotted with antibodies against the α -secretases (TACE and ADAM10) or β -secretase (BACE1). α -tubulin serves as a loading control. **(B)** Activities of α - and β -secretases were analyzed by fluorometric assays (n=3) in FAC-treated or DFP-treated ARPE-19 cells

Figure 5. Increased amyloid β staining in the RPE of an aceruloplasminemia patient
(A) Immunohistochemistry of *post mortem* eyes from an aceruloplasminemia patient compared to that of an age/gender-matched control, with antibody against A β . Iron deposits in the RPE are detected with Perls' Prussian Blue Stain. Representative image is shown from $n=3$ experiments. **(B)** Immunohistochemistry of a section containing drusen in *post mortem* eyes from an aceruloplasminemia patient, with anti-A β antibody. Black arrows indicate A β deposits in drusen.