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HYPOXIA INDUCED CHANGES IN EXPRESSION OF PROTEINS INVOLVED IN IRON UPTAKE AND STORAGE IN CULTURED LENS EPITHELIAL CELLS

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Abstract

Hypoxia inducible factor (HIF) regulates expression of over 60 genes by binding to hypoxia response elements (HRE) located upstream of the transcriptional start sites. Many genes encoding proteins involved in iron transport and homeostasis are regulated by HIF. Expression of iron handling proteins can also be translationally regulated by binding of iron regulatory protein (IRP) to iron responsive elements (IREs) on the mRNA of ferritin chains and transferrin receptor (TfR). Lens epithelial cells (LEC) function in a low oxygen environment. This increases the risk of iron catalyzed formation of reactive oxygen species (ROS) and oxidative cell damage. We examined changes in expression of ferritin (iron storage protein) and Tf/TfR1 (iron uptake proteins) in LEC cultured under hypoxic conditions. Ferritin consists of 24 subunits of two types, heavy (H-chain) and light (L-chain) assembled in a cell specific ratio. Real-time PCR showed that 24 h exposure to hypoxia lowered transcription of both ferritin chains by over 50% when compared with normoxic LEC. However it increased the level of ferritin chain proteins (20% average). We previously found that 6 h exposure of LEC to hypoxia increased the concentration of cytosolic iron which would stimulate translation of ferritin chains. This elevated ferritin concentration increased the iron storage capacity of LEC. Hypoxic LEC labeled with ⁵⁹FeTf incorporated 70% more iron into ferritin after 6 h as compared to normoxic LEC. Exposure of LEC to hypoxia for 24 h reduced the concentration of TfR1 in cell lysates. As a result, hypoxic LEC internalized less Tf at this later time point. Incorporation of ⁵⁹Fe into ferritin of hypoxic LEC after 24 h did not differ from that of normoxic LEC due to lower ⁵⁹FeTf uptake. This study showed that hypoxia acutely increased iron storage capacity and lowered iron uptake due to changes in expression of iron handling proteins. These changes may better protect LEC against oxidative stress by limiting iron-catalyzed ROS formation in the low oxygen environment in which the lens resides.

Keywords

lens; iron; iron proteins; hypoxia

1. INTRODUCTION

Metabolism of iron and oxygen are interconnected by complex and incompletely understood mechanisms. We have previously shown that hypoxia significantly altered iron uptake and trafficking in cultured LEC. In order to gain further insight into these findings, we examined how iron-handling proteins function under normoxic and hypoxic conditions.

Mammalian cells adapt to a low oxygen environment by activating hypoxia inducible factor (HIF), a transcriptional factor which subsequently regulates expression of over 60 genes (Wang and Semenza, 1993). HIF is a heterodimeric protein which consists of constitutively expressed HIF- β and HIF- α subunits, the latter is regulated by availability of cellular oxygen. Each subunit has three isoforms: 1,2 and 3 α and 1,2 and 3 β (see (Chepelev and Willmore, 2011) for review). Under normoxic conditions HIF- α subunits are ubiquitinated by a mechanism involving prolyl hydroxylases (Ivan et al., 2001) (Jaakkola et al., 2001) while β subunits are expressed constitutively. Prolyl hydroxylases require iron in their active sites and are inactivated by low levels of cytosolic oxygen or iron.

HIF-1 can directly regulate gene expression by binding to hypoxia response elements (HRE), located upstream of transcriptional start sites of target genes (Semenza and Wang, 1992). HREs were found on many genes involved in iron transport and homeostasis including transferrin (Tf), transferrin receptor (TfR), ferroportin, hepcidin, ceruloplasmin, divalent metal transporter (DMT1) and iron regulatory protein-1 (IRP1) (see (Chepelev and Willmore, 2011) for reviews). Expression of these genes can be transcriptionally modulated by oxygen through binding of HIF to HRE. There is no consensus on how hypoxia affects expression of ferritin, transferrin and TfR. Most studies, often contradictory, were conducted on cells with high iron storage capacity.

Expression of proteins involved in iron homeostasis can also be regulated transcriptionally by changes in binding of IRP1 and IRP2 to iron responsive elements (IREs) located on either the 5' or 3' terminal of target mRNA. Binding of these IRPs to IREs is regulated by cytosolic levels of intracellular iron. Increases in cytosolic iron decreases binding of IRPs to 5' IRE and activates expression of ferritin H- and L-chains and ferroportin. Depletion of cytosolic iron increases IRP binding to the 3'IRE and elevates expression of TfR1 and DMT1. IRE-binding activity of IRP1 and IRP2 is also affected by the concentration of oxygen. IRP1/IRE binding activity decreases with hypoxia in many cell types (Hanson and Leibold, 1998) (Kuriyama-Matsumura et al., 1998) (Meyron-Holtz et al., 2004); (Luo et al., 2011) but increases in Hep3B cells (Toth et al., 1999). Hypoxia has the opposite effect on IRP2 binding activity; it increases its binding to IRE (Meyron-Holtz et al., 2004) (Hanson et al., 1999; Schneider and Leibold, 2003).

To our knowledge, there are no published studies on oxygen regulated expression of iron storage and transport proteins in an ocular tissue. Lens epithelial cells (LEC) function in a low oxygen environment, lower than most other tissues (Siegfried et al., 2010). Hypoxia increases levels of reactive oxygen species (ROS) the formation of which is catalyzed by iron. Therefore, in order to avoid extensive oxidative damage, intracellular iron trafficking and storage in this low oxygen environment must be strictly controlled. Furthermore,

regulation of iron uptake by lenticular tissue may differ from that of hematopoietic cells and other cells responsible for regulating systemic iron levels.

We examined ferritin, Tf and TfR1 in cultured LEC to determine how hypoxia affected expression of these proteins and subsequently the delivery and storage of iron. Ferritin is the main cytosolic storage protein for iron. It consists of 24 subunits of two types, heavy (H-chain) and light (L-chain) that are assembled in a cell specific ratio. Ferritin plays an important role by storing excess “free” iron in a metabolically inert form and preventing excessive ROS formation. Tf and TfR1 are involved in iron uptake and internalization to cytosol. Hypoxia could modify expression of iron-handling proteins in two ways; by altering translation by changing IRP2/IRE binding and/or altering the transcriptional effect of HIF-1 on the HRE.

2. METHODS

2.1 Cell cultures

The eyes were obtained from mixed breed dogs, estimated ages 1–7, euthanized at the Johnston County Animal Shelter in North Carolina. The anterior lens capsules were dissected from the lenses and placed on a tissue culture plates containing Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY) with 10% fetal bovine serum (FBS;Hyclone, Logan, UT) and 1% antibiotic-antimycotic solution (Mediatech, Manassas, VA). After adherent LEC grew out of the capsules, they were dispersed with trypsin, grown to confluence and plated in six-well plates at a density of 200,000 cells per well. Confluent LEC were cultured under normoxic (21% O₂; 5% CO₂) or hypoxic conditions (0.5% O₂; 5% CO₂) for 6 h or 24 h in an INVIVO₂ 300 hypoxia chamber (Ruskin, Pencoed, UK). Each experiment was repeated at least three times and was conducted on cell populations in the first passage, from one or two donors. The n refers to the number of samples processed in the combined experiments.

2.2 Reverse transcription and quantitative real-time PCR

Total RNA was extracted from cultured LEC using an RNeasy kit and Qiashredders (Qiagen, Valencia, CA) and following the kit protocol. RNA concentration was determined with NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA). One microgram of RNA was reverse transcribed using oligo dT primers and the ImProm-II Reverse Transcription System (Promega, Madison, WI). Real-time PCR was performed in an iCycler iQ (Bio-Rad, Richmond, CA) using a Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA). The reaction mixture (25 µl total volume) included primers at 0.3 µM concentration and 5 µl of template corresponding to 0.05% RT product. The custom-made primers (Invitrogen, Carlsbad, CA), were design using Oligo Analysis Tool software (Eurofins MWG Operon, Huntsville, AL).

Ferritin H-chain:

upstream: 5'– CGATGATGTGGCTTTGAAGA – 3'

downstream: 5' – AAGATTTCGACCACCTCGTTG – 3'

Ferritin L-chain:

upstream: 5' – AAACCGTCCCAAGATGAGTG – 3'

downstream: 5' – TGGTTCTCCAGGAAGTCACA – 3'

Expression of ferritin H- and L-chain genes (copy numbers) was quantified based on a standard curve generated using a mammalian expression pTargetT vector (Promega) containing cloned coding regions of canine H- and L-ferritin chain DNAs (Goralska et al., 2001). The cloned DNA fragments were excised with ECoRI (Promega), separated by low-melting-point agarose electrophoresis and purified with QIAquick Gel Extraction Kit (Qiagen). Isolated DNA fragments were quantified with NanoDrop 1000 Spectrophotometer (Thermo Scientific). Samples of known DNA concentration were diluted serially to generate standard curves. The data were analyzed using the thermal cycler's system software (ver. 3.0, iCycler™ iQ optional System Software; Bio-Rad).

2.3 Western Blot Analysis of H- and L-ferritin chains

LEC were lysed with 10 mM Tris/HCl buffer pH 7.3 containing 2% sodium dodecyl sulfate (SDS) and 6 µl/ml of Protease Inhibitor Cocktail for use with mammalian cells (Sigma-Aldrich, St Louis, MO). The protein concentration of the lysates was determined using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Samples containing 30–50 µg of protein were separated by 12 % Tris-tricine SDS-PAGE under denaturing conditions and transferred to nitrocellulose membranes (Hydrobond-ECL; GE Healthcare, Munich, Germany) using semi-dry blotting transfer system Trans-Blot® SD Cell (Bio-Rad). Blots were blocked in Tris buffer (10 mM Tris pH 7.4 containing 100mM NaCl, 0.1% Tween-20 and 5% dry milk) for 1 h and incubated with custom-made antibodies (Open Biosystems, Huntsville, AL), produced in rabbits immunized with peptides corresponding to H- and L-chain ferritin specific amino acid sequences. Blots were washed with Tris buffer without milk and were incubated with TrueBlotHRP-conjugated anti-rabbit IgG 1:1000 diluted antibodies (eBioscience, San Diego, CA). Immunoreactivity was determined with an ECL Western Blot Analysis System (GE, Healthcare). Blots were blocked with Tris buffer overnight and reprobed with 1:5,000 diluted, HRP-conjugated goat anti-human β-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). The blots were exposed to film and images were digitized and quantified with UN-SCAN-IT gel software (Silk Scientific, Orem, UT).

2.4 Metabolic labeling of de novo synthesized ferritin

LEC were incubated under normoxic or hypoxic conditions in methionine-free DMEM containing 20 % dialyzed serum (HyClone Laboratories, Logan, Utah) and 80 µCi of translabel-³⁵S-methionine (MP Biomedicals, Solon, OH). After 24 h incubation LEC were lysed with 0.05 M Tris/HCl buffer pH 8.0, containing 0.15 M NaCl, 1% Triton X-100, 0.02% sodium azide and Protease Inhibitor Cocktail for use with mammalian cells (Sigma-Aldrich). The lysates were centrifuged at 15 000 g and ferritin was immunoprecipitated from supernatants with goat anti-horse ferritin antibodies (Bethyl Laboratories, Montgomery, TX) and 10% Pansorbin (Calbiochem, La Jolla, CA). Newly synthesized ferritin chains were

separated by 12 % Tris-tricine SDS-PAGE under reducing conditions. The gels were quantified in an Instant Imager (Packard-Canberra, Rockville, MD) and autoradiographed.

2.5 ^{59}Fe incorporation into ferritin

Human apotransferrin (Sigma-Aldrich, St Louis, MO) was labeled according to the method of Bates and Wernicke (Bates and Wernicke, 1971) as described before (Goralska et al., 1998). LEC were preincubated for 1 h in serum free MEM (Mediatech) under normoxic conditions to remove transferrin bound to the membrane and then labeled with $^{59}\text{FeTf}$ (70–180 ng of Fe) in 1ml fresh, serum-free MEM. LEC were exposed to hypoxia (0.5% O_2 ; 5% CO_2) or incubated under normoxic conditions for 6 or 24 h and lysed with 10 mM Tris/HCl buffer containing 15% sucrose and 6 $\mu\text{l}/\text{ml}$ of Protease Inhibitor Cocktail. Lysates were centrifuged at 15,000 g. The radioactivity of collected supernatants was measured in a gamma counter 1480 Wallac Wizard (Wallac, Turku, Finland). The supernatants were subsequently precipitated with 50% acetone (10 min on ice) and proteins were pelleted at 15 000 g. Pellets were dissolved in non-denaturing PAGE loading buffer and proteins were separated on native 8% PAGE. The dried gels were exposed to film and quantified in a radioactivity detector (Instant Imager, Packard-Canberra, Rockville, MD). The radioactive bands of ferritin were identified based on their mobility in comparison to Kaleidoscope Protein Standards (Bio-Rad, Richmond, CA).

2.6 Quantification of transferrin

Sandwich enzyme linked immunosorbent assay (ELISA) was used to measure transferrin concentrations in LEC lysates and cell-conditioned media (CCM). The goat anti-dog transferrin antibodies (Bethyl Labs, Montgomery, TX), at 1:500 dilution, and HRP-conjugate goat anti-dog antibodies (Bethyl Labs), at 1:10,000 dilution, were used in the assay as described previously (McGahan et al., 1995). Serially diluted dog apo-transferrin (Sigma-Aldrich) was used as a standard. Media from hypoxic and normoxic LEC were collected and cells were lysed with 0.01% digitonin. CCM and cell lysates were centrifuged at 15,000 g for 5 min and the supernatants were used in the assay. Protein content of the cell lysates was determined using the BCA Protein Assay Kit (Pierce Biotechnology).

2.7 Western Blot Analysis of transferrin receptor

LEC cultured under normoxic or hypoxic conditions for 6 h or 24 h were lysed with 0.01% digitonin. Lysate samples containing 25 μg proteins were separated by 8% Tris-tricine SDS-PAGE under denaturing conditions together with purified human placenta TfR1 (Alpha Diagnostics, San Antonio, TX) used as a control. The proteins were transferred to a nitrocellulose membrane (Hydrobond-ECL) for 30 min at 20 V using semi-dry blotting transfer system Trans-Blot® SD Cell. Blots were blocked in Tris buffer (10 mM Tris pH 7.4 containing 100mM NaCl, 0.1% Tween-20 and 5% dry milk) for 1 h and incubated with 1:2000 diluted, monoclonal mouse anti-human TfR1 antibodies (Invitrogen). After several washes with Tris buffer without milk, blots were incubated with 1: 750 diluted, HRP-labeled goat anti-mouse antibodies (BD Biosciences, Palo Alto, CA) and washed with Tris buffer. Immunoreactivity was determined with ECL and blots were reprobbed with 1:500 diluted, HRP-conjugated goat anti-human β -actin antibodies (Santa Cruz Biotechnology). The images were digitalized and evaluated as described above.

2.8 Statistical analysis

The significance of differences was determined by using paired T-test. The null hypothesis was rejected at $P < 0.05$. Data represent mean $\pm$ SEM.

3. RESULTS

3.1 Effect of hypoxia on ferritin H- and L- chain gene expression

The copy number of ferritin H-chain m-RNA was significantly higher (7.6 fold) than the L-ferritin chain in LEC cultured under normoxic conditions (Fig. 1A). Twenty four hour exposure to hypoxia significantly reduced the copy number of mRNA for both proteins but more so for the H-chain (5.0 fold) relative to the normoxic control (Fig. 1B).

3.2 Western blot analysis of ferritin H- and L- chain levels in LEC cultured under normoxic and hypoxic conditions

Six Hour Incubation: Ferritin L-chain levels did not change following 6 h exposure to hypoxia. In contrast, H-chain levels increased by 30% as compared to LEC cultured under normoxic conditions (Fig. 2).

Twenty Four Hour Incubation: Ferritin L-chain levels in hypoxic LEC increased by 28% whereas ferritin H-chain levels were unchanged when compared to normoxic LEC. However, 24 h exposure of LEC to hypoxia significantly decreased the level of ferritin H-chain as compared to the 6 h time point (Fig. 2).

3.3 Effect of 24 h exposure to hypoxia on de novo synthesis of ferritin L- and H-chains

Hypoxia increased levels of ferritin chains synthesized during 24 h of labeling. L-chain content was increased by 13% and H-chain by 20% (Fig. 3). The H/L-ferritin chain ratio (1.35–1.48) was not significantly changed. There seemed to be a discrepancy between the decrease ferritin H-chain levels detected by Western blotting at the 24 h time point (Fig. 2) and the increase in de novo synthesis of H-chain following 24 h of labeling of LEC under hypoxic conditions (Fig. 3).

3.4 Effect of iron supplementation or depletion on ferritin H- and L-chains levels in LEC cultured under normoxic or hypoxic conditions for 24 h

Cytosolic iron concentration regulates expression of both ferritin chains through the IRP/IRE interaction. The iron chelator, desferrioxamine (DFO) decreased the level of ferritin H- and L-chain at 24 h, whereas supplementation with ferric ammonium citrate (FAC) had the opposite effect. These treatments had the same effect under normoxic or hypoxic conditions (Fig. 4).

3.5 Effect of hypoxia on incorporation of ^{59}Fe into ferritin of LEC labeled with $^{59}\text{FeTf}$

We previously determined that hypoxia changes iron trafficking within the LEC increasing its level in cytosol at the 6 h time point and lowering ^{59}Fe uptake from transferrin after 24 h of labeling (Goralska et al., 2013). In the current study we determined how these changes affect iron storage in ferritin. There was more ^{59}Fe incorporated into ferritin at 24 h as compared to 6 h time regardless of the oxygen level (Fig. 5). Hypoxic LEC stored more iron

in ferritin at 6 h as compared to LEC cultured under normoxia. At the 24 h time point there were no difference in iron incorporation into ferritin between normoxic and hypoxic LEC despite our previous finding that hypoxia significantly lowered iron uptake from transferrin at this time point (Goralska et al., 2013).

3.6 Transferrin in the LEC lysates and CCM (ELISA)

LEC synthesize and secrete Tf in order to capture iron in the intraocular fluids (McGahan et al., 1995). We determined that 24 h exposure to hypoxia significantly increased the Tf concentration in the CCM by 13% (Fig. 6A). The opposite was true for Tf levels in LEC lysates (Fig. 6B). Hypoxia decreased Tf levels in LEC lysates by 3.6% at 6 h and 7.3% at 24 h compared to normoxic LEC.

3.7 Western blot analysis of TfR1 in LEC cultured under normoxic and hypoxic conditions

The difference in Tf distribution between normoxic and hypoxic LEC suggested that hypoxia altered the internalization of Tf by way of the TfR. To test this hypothesis we examined TfR1 levels in LEC lysates and found 11% reduction in TfR1 after 24 h exposure (Fig. 7).

3.8 Effect of iron supplementation or depletion on TfR1 levels in LEC cultured under normoxic or hypoxic conditions for 24 h

Cytosolic iron levels regulate expression of TfR1 through modification of IRE/IRP binding. A decrease in intracellular iron stabilizes the mRNA of TfR1 by increasing IRP binding to IRE located at the 3' terminal. An increase in iron content had an opposite effect. DFO treatment of normoxic LEC significantly increased the level of TfR1 and FAC had the opposite effect (Fig. 8). Expression of TfR1 in LEC cultured under hypoxia did not respond to DFO or FAC (Fig. 8).

4. DISCUSSION

Our results suggested that LEC expressed higher levels of H-chain gene transcripts as compared to L-chain transcripts (7.6 fold see Fig. 1A). Ferritin H- and L-chains belong to a multigene family and are coded by separate genes located on different chromosomes, which include several pseudogenes for each chains (Quaresima et al., 1994). These pseudogenes have high sequence identity which could complicate interpretation of the PCR results. Recent release of an updated version of the whole-genome shotgun assembly of the canine genome (Dog Genome Sequencing Consortium) allowed us to identify genes, pseudogenes and ferritin chain-like sequences which could be recognized by the primers used in the current study. Using the nucleotide sequence database of the GenBank, we determined that the canine genome includes two copies of a protein-coding ferritin H-chain gene (NC_006600.3; chromosome 18 and NC_006593.3; chromosome 11) one copy of a protein coding ferritin H-chain-like gene (NC_006613.3; chromosome 31), and two different ferritin H-chain pseudogenes (NC_006590.3; chromosome 8 and NC_006605.3; chromosome 23) which could be recognized by our ferritin H-chain PCR primers. With respect to ferritin L-chain, only one ferritin L-chain gene was identified (NC_006583.3; chromosome 1) and was the only sequence our ferritin L-chain PCR primers recognized since none of the ferritin L-

chain-like or pseudogene sequences matched both primers. Thus we concluded that in contrast to a single gene transcript of ferritin L-chain gene, mRNA for ferritin H-chain contained transcripts of more than one gene. Therefore it cannot be concluded that there was 7.6 times more H-chain mRNA available for translation. In fact we could not ascertain how much of the amplified sequence codes for full length H-chain. The real-time quantitative RT-PCR is commonly used to quantify transcripts of a single-gene, however considering the abundance of pseudogenes (Pink and Carter, 2013), gene-like sequences and multicopy genes (Sudmant et al., 2010) in all eukaryotic organisms strict scrutiny of all possible sequences present in the cell should be applied before making final conclusions regarding the obtained data.

Hypoxia significantly reduced transcription of the ferritin L-chain gene by over 60% at the 24 h time point. The effect on ferritin H-chain genes was even greater (by 80%) at the same time point (Fig. 1B). Transcription of both ferritin chain type genes is mediated by several transcription factors. The transcriptional enhancer, antioxidant responsive element (ARE), is found in the promoter region of several genes involved in antioxidant defense (Wasserman and Fahl, 1997) (Tsuiji, 2005), including ferritin genes, and the transcriptional nuclear factor-E2-related factor 2 (Nrf2) is a potent activator of ARE. Nrf2 enhances expression of human ferritin genes (Nguyen et al., 2000) (Huang et al., 2013) and transcriptional repressor Bach1 has the opposite effect (Hintze et al., 2007). HIF-1 α down-regulates Nrf2 and simultaneously increases Bach1 activity (Kitamuro et al., 2003) (Loboda et al., 2009). Ferritin genes, proteins and ferritin translation mechanisms are highly conserved in all species (Harrison and Arosio, 1996). Since 24 h exposure of cultured LEC to hypoxia elevated HIF-1 α by 75% (data not shown) this suggests that reduction in the transcription rate of canine ferritin genes could be a consequence of an HIF-1 mediated increase in Bach1 and/or decrease Nrf2 activities.

The hypoxia-induced reduction in ferritin gene transcripts (Fig. 1B) did not correspond with expression of ferritin proteins (Fig. 2 and 3). That is consistent with a global gene expression study where only 40% of the variance in protein levels was due to changes in corresponding transcripts and protein expression was predominantly regulated at the translational level (Schwanhauser et al., 2011). H-chain protein increased at 6 h and L-chain protein at 24 h based upon Western blot analysis (Fig. 2) and de novo synthesis of both ferritin chains increased at 24 h (Fig. 3). These results can be explained in the following manner. The goat anti-horse ferritin antibodies used to immunoprecipitate metabolically labeled ferritin (i.e. de novo synthesis) had high affinity for assembled ferritin but not for “free”, unassembled chains. However, all chains (assembled or free) would be detected by the chain-specific antibodies used in the Western blot assay. Furthermore, we have shown that LEC strictly control H-chain levels through increased secretion (Goralska et al., 2003) or degradation (Goralska et al., 2005) whereas the L-chain can freely accumulate in the cytosol. This suggests that hypoxia stimulated synthesis of both chains and increased the content of assembled ferritin and L-chain after 24 h exposure. However, newly synthesized H-chain which was not unassembled with L-chain at the ratio specific for LEC would be secreted or proteolyzed thus reducing its level at 24 h time point (Fig. 2).

Translation of both ferritin chains is controlled by binding of iron-regulatory proteins (IRP1 and IRP2) to IRE; either IRP blocks translation of these proteins. Hypoxia can modify this binding (Kuriyama-Matsumura et al., 1998) (Toth et al., 1999) (Schneider and Leibold, 2003) (Meyron-Holtz et al., 2004) (Luo et al., 2011) and it's generally agreed that IRP-1 is not active at low oxygen levels and that hypoxia increases binding of IRP-2 to IRE. However, IRP-2 binding to IRE also responds to iron levels even at low oxygen concentration (Meyron-Holtz et al., 2004) and decreases as iron content increases most likely through iron-mediated proteolysis of IRP2 (Guo et al., 1995). This could explain why ferritin chain levels in cultured LEC responded to iron depletion (DFO) or supplementation (FAC) similarly, regardless of the oxygen level (Fig. 4). This suggests that increased trafficking of iron into the cytosol under hypoxic conditions (Goralska et al., 2013) caused a translational stimulation of ferritin chain synthesis in LEC exposed to hypoxia for 24 h by decreasing IRP2 binding to IRE (Fig. 2).

LEC secrete Tf into the medium under normoxic conditions (McGahan et al., 1995). Twenty-four hours of hypoxia increased Tf levels in the CCM (Fig. 6A) and decreased the cytosolic content of Tf (Fig. 6B). We have shown that internalization of ⁵⁹FeTf was also significantly lower after 24 h of hypoxia (Goralska et al., 2013) indicating reduced uptake of ⁵⁹Fe-Tf through the Tf/TfR. The current finding (10 mM Tris pH 7.4 containing 100mM NaCl, 0.1% Tween-20 and 5% dry milk) of a reduction in TfR1 in hypoxic LEC after 24 h, corroborated this earlier finding (Fig. 7). Depletion or supplementation of hypoxic LEC with iron did not modify expression of TfR1 through IRP/IRE binding, contrary to what was determined for normoxic LEC (Fig. 8). This suggests transcriptional rather than translational regulation of TfR1 expression in a low oxygen environment.

Hypoxia has profound effects on systemic iron metabolism through regulation of expression of hepcidin, the main factor controlling central iron homeostasis. There is an extensive literature on the relationship between oxygen concentration and Tf iron uptake and delivery to erythroid cells (Hintze and McClung, 2011) (Liu et al., 2012) (Piperno et al., 2011). In the current investigation we have extended our understanding of the relationship between oxygen concentration and iron metabolism at the cellular level of a non-erythroid cell, the lens epithelium. We have shown that hypoxia elevated cytosolic iron content by altering intracellular iron trafficking in LEC and decreasing iron uptake through the Tf/TfR1 (Goralska et al., 2013). In this study we documented that hypoxia increased iron storage capacity and lowered iron uptake by LEC through changes in expression of iron handling proteins. These changes could represent protective mechanisms against iron-catalyzed damaging free radical formation which is increased in the low oxygen environment in which the lens resides.

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Fig. 1.

A Real time quantitative RT-PCR analysis of ferritin H- and L-chains mRNA levels in normoxic LEC. Result represent the mean $\pm$ SEM; n=7

B. Real-time quantitative RT-PCR analysis of the effects of hypoxia on L- and H-chain expression by LEC. Data are expressed as fold-change of LEC exposed to hypoxia relative to normoxic LEC at the same time point. N-normoxia; H-hypoxia Results represent the mean $\pm$ SEM; n=6 p<0.05*

Fig. 2.

Western blot analysis of ferritin L- and H-chains in LEC cultured under normoxic (N) or hypoxic (H) conditions for 6 h or 24 h.

LEC lysates containing 30–50 μ g of protein were separated by 12 % Tris-tricine SDS-PAGE under reducing conditions and transferred into nitrocellulose membranes. Canine liver and heart ferritins were used as standards (st) for L- and H-chains respectively. Ferritin H- and L-chains were immunodetected with anti-chain specific antibodies. The blots were reprobbed with HRP-goat-anti-human β -actin as a loading control and exposed to film. Images were quantified with UN-SCAN-IT gel software

Data represent the mean $\pm$ SEM; n=at least 4 samples; statistically different from corresponding N $p < 0.05$ *; statistically different from H 6 h ^

Fig. 3.

Effect of hypoxia on de novo ferritin chain synthesis in cultured LEC. Cells were labeled with ^{35}S -methionine for 24 h under normoxic (N) or hypoxic (H) conditions. Ferritin chains were immunoprecipitated, separated by 12 % Tris-tricine SDS-PAGE under reducing conditions quantified in an Instant Imager, and autoradiographed. Data represent the mean $\pm$ SEM n=6; statistically different from normoxic LEC p<0.05*

Fig. 4.

The effect of DFO and FCA on expression of ferritin chains in LEC cultured under normoxic or hypoxic conditions. LEC were treated with 100 μM DFO (iron chelator) or 5 μg/ml FAC for 24 h under normoxic or hypoxic conditions. Cell lysates containing 30–50 μg of protein were separated by 12 % Tris-tricine SDS-PAGE under denaturing conditions. After Western transfer to a nitrocellulose membrane, ferritin H- and L-chains were immunodetected with chain specific antibodies. Blot was re probed with HRP-goat-anti-human β-actin as a loading control and imaged in Personal Molecular Imager System. Images are representative of three experiments.

Fig. 5.

Effect of hypoxia on ^{59}Fe ferritin levels in LEC labeled with $^{59}\text{FeTf}$. LEC were labeled for 6 h or 24 h under normoxic (N) or hypoxic conditions (H). Cells were lysed with 10 mM Tris/HCl buffer containing 15% sucrose and protease inhibitors. The cytosolic fraction was isolated by 15 000 g spin and its radioactivity was measured in a gamma counter. After concentrating by precipitation with acetone, all proteins of cytosolic fraction were loaded and separated on native 8% PAGE. The gels were dried and labeled ferritin was quantified. The dried gels were exposed to film and quantified in an Instant Imager radioactivity detector. Data represent the mean $\pm$ SEM; $n = 5$; $p < 0.05$ *statistically different from corresponding normoxic LEC

Fig. 6.

Effects of hypoxia on the levels of Tf in CCM (A) and LEC lysates (B). LEC were incubated under normoxic (N) or hypoxic (H) conditions for 6 h or 24 h. CCM were collected and cells were lysed with 0.01% digitonin. Transferrin levels were measured by sandwich enzyme linked immunosorbent assay (ELISA) with goat anti-dog transferrin antibodies and HRP-conjugated goat anti-dog antibodies. Results were expressed as ng Tf/ μ g protein in cell lysates. Data represent the mean $\pm$ SEM; n=at least 7 samples; $p<0.05$ * statistically different from corresponding normoxic LEC

Fig. 7.

Western blot analysis of TfR1 in LEC cultured under normoxic (N) or hypoxic (H) conditions for 6 h or 24 h. LEC lysates containing 25 μ g proteins were separated by 8% Tris-tricine SDS-PAGE under denaturing conditions together with purified human placenta Tf which was used as a control. The proteins were transferred to a nitrocellulose membrane and TfR1 was immunodetected with monoclonal mouse anti-human TfR1 antibodies. The blots were reprobed with HRP-goat-anti-human β -actin as a loading control, exposed to film, and images were quantified in Personal Molecular Imager System. Data represent the mean $\pm$ SEM $n=6$ for 6 h and $n=12$ for 24 h time points * statistically different from corresponding normoxic LEC * $p<0.05$

Fig. 8.

The effect of DFO and FCA on expression of TfR1 in LEC cultured under normoxic (N) or hypoxic (N) conditions.

LEC were treated with 100 μ M DFO (iron chelator) or 5 μ g/ml FAC for 24 h. Cell lysates containing 25–30 μ g of protein were separated by 8% Tris-tricine SDS-PAGE under denaturing conditions. After Western transfer to a nitrocellulose membrane TfR1 was immunodetected with specific antibodies. The blot was reprobed with HRP-goat-anti-human β -actin as a loading control and imaged and quantitated in a Personal Molecular Imager System. Data represent the mean $\pm$ SEM; n= 6; p<0.05*statistically different from corresponding normoxic LEC