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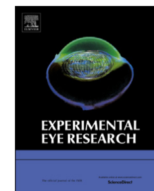

Hypoxia induced changes in expression of proteins involved in iron uptake and storage in cultured lens epithelial cells

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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 ^{59}FeTf 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 ^{59}Fe into ferritin of hypoxic LEC after 24 h did not differ from that of normoxic LEC due to lower ^{59}FeTf 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.

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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 3α and 1,2 and 3β (see (Chepelev and Willmore, 2011) for review). Under normoxic conditions HIF-α subunits are ubiquinated 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

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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 and Wang, 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% O_2; 5% CO_2) or hypoxic conditions (0.5% O_2; 5% CO_2) for 6 h or 24 h in an INVIVO$_2$ 300 hypoxia chamber (Ruskinn, 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′ – AAGATTCGACCACCTCGTTG – 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 pTargeT 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, iCyclerTM 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, Münich, 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 100 mM 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 1:5000 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

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