Source-Dependent Intracellular Distribution of Iron in Lens Epithelial Cells Cultured Under Normoxic and Hypoxic Conditions

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PURPOSE. Intracellular iron trafficking and the characteristics of iron distribution from different sources are poorly understood. We previously determined that the lens removes excess iron from fluids of inflamed eyes. In the current study, we examined uptake and intracellular distribution of ^{59}Fe from iron transport protein transferrin or ferric chloride (nontransferrin-bound iron [NTBI]) in cultured canine lens epithelial cells (LECs). Because lens tissue physiologically functions under low oxygen tension, we also tested effects of hypoxia on iron trafficking. Excess iron, not bound to proteins, can be damaging to cells due to its ability to catalyze formation of reactive oxygen species.

METHODS. LECs were labeled with ^{59}Fe -Tf or $^{59}\text{FeCl}_3$ under normoxic or hypoxic conditions. Cell lysates were fractionated into mitochondria-rich, nuclei-rich, and cytosolic fractions. Iron uptake and its subcellular distribution were measured by gamma counting.

RESULTS. ^{59}Fe accumulation into LECs labeled with ^{59}Fe -Tf was 55-fold lower as compared with that of $^{59}\text{FeCl}_3$. Hypoxia (24 hours) decreased uptake of iron from transferrin but not from FeCl_3 . More iron from $^{59}\text{FeCl}_3$ was directed to the mitochondria-rich fraction (32.6%–47.7%) compared with ^{59}Fe from transferrin (10.6%–12.6%). The opposite was found for the cytosolic fraction (8.7%–18.3% and 54.2%–46.6%, respectively). Hypoxia significantly decreased iron accumulation in the mitochondria-rich fraction of LECs labeled with ^{59}Fe -Tf.

CONCLUSIONS. There are source-dependent differences in iron uptake and trafficking. Uptake and distribution of NTBI are not as strictly regulated as that of iron from transferrin. Excessive exposure to NTBI, which could occur in pathological conditions, may oxidatively damage organelles, particularly mitochondria.

Keywords: iron trafficking, transferrin, NTBI, lens epithelial cells, hypoxia

Although significant advances have been made in studies of iron metabolism, the understanding of intracellular trafficking of iron delivered by transferrin and nontransferrin-mediated transport is still limited. Under physiological conditions, extracellular iron is chelated by transferrin (Tf) and internalized through transferrin receptor 1 (TfR1)-mediated endocytosis.¹ When iron content in plasma and extracellular fluids exceeds the binding capacity of transferrin, cells efficiently internalize nontransferrin-bound iron (NTBI), which consists of chelatable iron associated with low- M_r organic ligands, such as citrate and ATP, through mechanisms that are poorly understood.² Once inside the cell, iron is routed into different cell compartments or stored in cytosolic ferritin, the iron-storage protein.³ A small amount of “protein-free” cytosolic iron is defined as chelatable, redox-active iron bound by low molecular weight ligands and termed the labile iron pool (LIP). The composition and amount of iron in the LIP are not completely defined.⁴

A significant amount of iron taken up into the cell is trafficked to mitochondria, which play central role in iron metabolism, particularly in heme synthesizing erythroid cells. The mitochondrion is the sole site of heme biosynthesis⁵ and the location where the iron-sulfur (Fe-S) cluster is assembled. Assembled Fe-S clusters are incorporated not only into

mitochondrial but also cytosolic and nuclear apoproteins.⁶ Iron delivery to the mitochondria may depend on the form of iron internalized by a cell. It has been hypothesized that transferrin-delivered iron (Tf-iron) is sequestered through direct interaction of mitochondrial membranes with endosomes containing Fe-transferrin.⁷ NTBI is transported bound to unknown cytosolic chaperones in a form that is inaccessible to chelators.⁸

A separate pool of iron has been identified in cell nuclei. Nuclear iron is incorporated into proteins, which include nuclear ferritin,⁹ di-iron proteins, such as ribonucleotide reductase, iron-sulfur cluster proteins,¹⁰ and nuclear receptor hemoproteins.¹¹ It also may remain unbound as chelatable iron, which can exceed concentrations found in the cytosolic LIP.¹² The mechanisms by which iron enters nuclei are not well understood. It has been determined that transport of NTBI to nuclei is ATP-dependent and does not result from diffusion of iron through the nuclear pore.¹³

The metabolism of iron is closely interconnected with the metabolism of oxygen. Cells adapt to low oxygen environments by activating hypoxia-inducible transcription factors (HIFs),¹⁴ which subsequently increase expression of more than 60 genes, including those involved in iron homeostasis, such as Tf, TfR1, ferroportin, divalent metal ion transporter (DMT1), and

hepcidin. HIF is a heterodimeric transcription factor composed of HIF α and HIF β subunits. There are three isoforms of each subunit: 1 α , 2 α , and 3 α and 1 β , 2 β , and 3 β . Beta subunits are expressed constitutively. Under normoxic conditions, α subunits are hydroxylated by prolyl hydroxylases in a reaction that requires iron, and are degraded by the ubiquitin-proteasome system.¹⁵ Regulation of cellular iron and oxygen homeostasis includes the same factors, HIF and iron responsive proteins (IRPs), which regulate iron transport and storage. The activity of these factors is controlled by both iron and oxygen signals.¹⁶

Many ocular tissues function in a very low oxygen environment. Oxygen pressure at the surface of the lens is 2.8 mm Hg, and is lower than in most other tissues.¹⁷ To our knowledge, there are no published studies on iron trafficking in ocular tissues. Iron is essential for many metabolic processes; however, if not stored or utilized properly, iron generates reactive oxygen species (ROS), which are implicated in the pathogenesis of oxidative-stress-related diseases, such as glaucoma,¹⁸ macular degeneration,¹⁹ and age-related cataracts.²⁰ A better understanding of the regulation of iron uptake and intracellular trafficking and the involvement of oxygen in these processes could lead to a more comprehensive understanding of the molecular background of pathologies in which there is a known dysregulation of iron metabolism.

METHODS

Cell Cultures

Lenses were obtained from eyes of mixed breed dogs euthanized in the Johnston County, North Carolina, Animal Shelter. The anterior capsules were removed and placed in 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). Adherent lens epithelial cells (LECs) that grew out of the capsules were dispersed with trypsin and after reaching confluence were plated in six-well plates at the density 200,000 cells per well.

Cell Count and Trypan Blue Viability Assay

The media over confluent LECs plated in six-well plates were replaced with serum-free MEM (Mediatech). Plates were incubated for 24 hours either in a hypoxia chamber (0.5% O₂, 5% CO₂ in INVIVO₂ 300 hypoxia chamber [Ruskinn, Pencoed, UK]) or in the cell culture incubator. LECs from each well were trypsinized, mixed with serum-free MEM, and centrifuged at 1.2g for 5 minutes. The cell pellets were resuspended in 1 mL PBS, and 20 μ L cell suspension was incubated for 3 minutes with 0.4% trypan blue (1:1 mixture). Both viable and nonviable LECs were counted in a hemocytometer.

Cell Proliferation Assay

LECs were grown to 70% confluency on 12-well plates, washed with serum-free MEM (Mediatech), and incubated for 24 hours with 1 μ Ci ³H-thymidine (ICN Radiochemicals, Irvine, CA) in 1 mL MEM in either normoxic or hypoxic conditions. After 24 hours, the cells were washed with ice-cold PBS, incubated twice for 10 minutes each time with 5% trichloroacetic acid, washed three times with ice-cold PBS and incubated with 500 μ L 0.2N NaOH for 30 minutes. Lysed cells were scraped and radioactivity in 400 μ L of the lysate was measured in a liquid scintillation counter (1409 Wallac; Perkin Elmer, Waltham, MA).

Labeling Tf With ⁵⁹FeCl₃

Human apotransferrin (Sigma-Aldrich, St. Louis, MO) was labeled according to the method of Bates and Wernicke,²¹ as described before.²² To label 6 mg Tf, 100 μ Ci ⁵⁹FeCl₃ (Perkin Elmer, Waltham, MA), of specific activity 35.08 to 63.35 mCi/mg, was used. The saturation of Tf with Fe was 75% to 100%, as determined by measuring the A₄₆₅/A₂₈₀ nm ratio, which is 0.046 to 0.048 for fully saturated transferrin.

Loading LECs With Iron From ⁵⁹FeTf or ⁵⁹FeCl₃

LECs were plated in six-well plates and preincubated for 1 hour in serum-free MEM (Mediatech) in normoxic conditions to remove transferrin bound to the membrane. The media were replaced by fresh, serum-free MEM (1 mL/well) containing 70 to 180 ng of Fe as ⁵⁹FeTf or ⁵⁹FeCl₃. LECs were exposed to hypoxia (0.5% O₂, 5% CO₂) or incubated in normoxic conditions for 6 or 24 hours.

Subcellular Fractionation of LEC Lysates

LECs labeled with ⁵⁹Fe were lysed on ice with 10 mM Tris/HCl buffer containing 15% sucrose and 6 μ L/mL protease inhibitor cocktail for use with mammalian cells (Sigma-Aldrich) in a volume of 500 μ L/well. Lysates from two wells were combined and centrifuged at 600g for 5 minutes. The pellets containing nuclei and cell debris were called nuclei-rich fraction. Supernatants (S1) were centrifuged at 15,000g for 1 hour, generating mitochondria-rich pellets, and supernatant (S2) containing cytosol and microsomes (cytosolic fraction). The aliquots of each fraction were stained with MitoTracker Green FM and Hoechst 33342 to confirm enrichment of nuclei or mitochondria compared with the total cell lysates. The total radioactivity of whole lysates and each cell fraction was measured in a gamma counter 1480 Wallac Wizard (Wallac, Turku, Finland).

Fluorescent Labeling of Mitochondria and Nuclei in Cultured LEC and LEC Subcellular Fractions

LECs were plated in four-well chamber slides at 50,000 cells per well and grown to confluence. Cells were then switched to serum-free MEM and cultured either under hypoxic or normoxic conditions for 24 hours as detailed above, and then incubated for 30 minutes at 37°C in MEM containing 200 nM MitoTracker Green FM and 500 nM Hoechst 33342 to label mitochondria and nuclei, respectively. After labeling, the cells were immediately washed with MEM and imaged with a $\times$ 20 objective lens under normoxic or hypoxic conditions on a Leica AF7000 wide-field fluorescence microscope system equipped with an on-stage incubator. Images were captured with an Andor Clara interline charge-coupled device using the same exposures for normoxic and hypoxic cells.

At each fractionation step, a sample of LEC subcellular fraction was stained for 30 minutes with 50 nM MitoTracker Green FM and 100 ng/mL Hoechst 33342 and observed microscopically. The relative purity of each fraction was assessed based on the absence or presence and the enrichment of the target organelles for each fraction.

Separation of ⁵⁹Fe-Labeled Tf From the Cytosolic Fraction of LEC Lysates

The whole isolated cytosolic fractions of ⁵⁹Fe-Tf-loaded LECs were precipitated with 50% acetone (10 minutes on ice) and centrifuged at 15,000g. Protein precipitates were dissolved in nondenaturing PAGE loading buffer and separated on 8% PAGE.

TABLE 1. LEC Count and Viability

	Normoxia	Hypoxia
Cell count/well	$14.9 \pm 1.6 \times 10^4$	$17.5 \pm 1.8 \times 10^4$ *
Viable cells, %	97.0 ± 1.2	97.7 ± 1.6
Proliferation, %	100	88.8 ± 2.0 *

* Significantly different from normoxia; $P \leq 0.05$. Values are means $\pm$ SE.

The dried gels were imaged and quantified in a radioactivity detector (Instant Imager; Packard-Canberra, Rockville, MD). The only radioactive bands were that of transferrin and ferritin, which were identified based on their mobility in comparison with Kaleidoscope Protein Standards (Bio-Rad, Richmond, CA).

RESULTS

Effect of Hypoxia on LECs Growth and Viability

Exposure of LEC to hypoxia increased the number of cells by 17.4 % and did not change the number of viable cells during a 24-hour exposure as determined by trypan blue exclusion (Table 1).

However, hypoxia decreased the proliferation rate of LECs by 11.2% (Table 1) during the same period of time as determined by thymidine incorporation.

^{59}Fe Uptake and Efflux in Cultured LECs

The total uptake of iron by LECs, measured after 24-hour labeling, was 55-fold higher from $^{59}\text{FeCl}_3$ than from transferrin-dependent transport (Figs. 1, 2). Hypoxia did not affect iron uptake from $^{59}\text{FeCl}_3$ (NTBI, non-transferrin bound iron), but decreased the amount of ^{59}Fe delivered by transferrin ($^{59}\text{Fe-Tf}$) detected at the 24-hour time point. To determine if this effect was caused by increased efflux of ^{59}Fe , LECs were treated with $^{59}\text{Fe-Tf}$ for 24 hours. The media were then removed and cells were incubated with fresh media for 24 hours. Iron content in cell-conditioned media (CCM), collected 24 hours after

FIGURE 1. Total uptake of ^{59}Fe from $^{59}\text{FeCl}_3$ by LECs cultured under normoxic (N) or hypoxic (H) conditions. LECs were loaded with iron for 6 hours or 24 hours and lysed with 10 mM Tris/HCl buffer containing 15% sucrose and protease inhibitor cocktail as described in the Methods section. The whole lysates were counted in a gamma counter. Iron uptake was normalized to the protein content. Results are expressed as the mean $\pm$ SEM of at least nine samples.

FIGURE 2. Total uptake of ^{59}Fe from $^{59}\text{Fe-Tf}$ by LECs cultured in N or H conditions. LECs were loaded with iron for 6 hours or 24 hours and lysed with 10 mM Tris/HCl buffer containing 15% sucrose and protease inhibitor cocktail as described in the Methods section. The whole lysates were counted in a gamma counter. Iron uptake was normalized to the protein content. Results are expressed as the mean $\pm$ SEM of at least nine samples. *Significantly different from N at the same time point, $P < 0.05$.

removing the label, was the same for hypoxic and normoxic LECs (Table 2).

These results indicated that hypoxia reduced the influx of $^{59}\text{Fe-Tf}$ but did not increase the efflux of iron from cultured LECs. PAGE analysis of proteins from the cytosolic fraction of $^{59}\text{Fe-Tf}$ -labeled LEC lysates confirmed that after 24-hour exposure to hypoxia, LECs contained significantly less $^{59}\text{Fe-Tf}$ as compared with cells cultured under normoxic conditions (Fig. 3).

The Distribution of $^{59}\text{Fe-Tf}$ and $^{59}\text{FeCl}_3$ Delivered Iron in the Subcellular Compartments of LECs Cultured in Normoxic Conditions

The ^{59}Fe content of nuclei-rich, mitochondria-rich, and cytosolic fractions of LEC, were determined after 6-hour and 24-hour exposure to $^{59}\text{Fe-Tf}$ or $^{59}\text{FeCl}_3$. When expressed as percentage of total uptake, there were significant Fe-source dependent differences in the subcellular distribution of Fe in cultured LECs (Figs. 4, 5). LECs labeled for 6 hours with $^{59}\text{FeCl}_3$ showed only 9% incorporation of iron into the cytosolic fraction (Fig. 4). Twenty-four-hour exposure increased cytosolic content of Fe to 18% (Fig. 4). However, the percentage incorporation was still below that measured for $^{59}\text{Fe-Tf}$ -labeled LECs at either time point (Fig. 5). Most iron in $^{59}\text{FeCl}_3$ -labeled LECs was detected in nuclei-rich and mitochondria-rich fractions after 6-hour exposure. After 24 hours of labeling with $^{59}\text{FeCl}_3$, iron content in the mitochondria-rich fraction declined and more iron was detected in the cytosolic fraction (Fig. 4). After 6 hours of labeling, more than 50% of internalized ^{59}Fe from $^{59}\text{Fe-Tf}$ was found in the cytosolic fraction (Fig. 5). Prolonged labeling (24 hours) reduced the iron content in the cytosolic fraction and increased iron incorporation into the nuclear-rich fraction of the LECs (Fig. 5).

Effect of Hypoxia on ^{59}Fe Content in LECs Subcellular Fractions

Interestingly, the subcellular distribution of ^{59}Fe in LECs exposed to hypoxia was similar to that of normoxic LECs in

TABLE 2. ^{59}Fe Efflux From LECs Labeled With $^{59}\text{Fe-Tf}^*$

	Normoxia	Hypoxia
cpm/ μg proteins	0.90 ± 0.09	0.93 ± 0.06

* Expressed as cpm/ μg protein LEC lysates. Values are means $\pm$ SE.

both $^{59}\text{Fe-Tf}$ and $^{59}\text{FeCl}_3$ -labeled cells at both the 6-hour and 24-hour time points (data not shown). However, when iron incorporation was normalized to the protein content of each subcellular fraction, the results reveal significant iron source- and oxygen-dependent differences in iron sequestration into cell fractions (Figs. 6, 7). The most significant were the differences in incorporation of iron into the mitochondria-rich fraction. Under normoxic conditions, incorporation to that fraction was 400-fold (at 6 hours) and 140-fold (at 24 hours) higher for LECs labeled with $^{59}\text{FeCl}_3$ in comparison with cells labeled with $^{59}\text{Fe-Tf}$. Taking into consideration that total uptake of iron from $^{59}\text{FeCl}_3$ was only 55-fold higher than from $^{59}\text{Fe-Tf}$ (Figs. 1, 2), there is a much quicker and more efficient trafficking of NTBI into the mitochondrial fraction as compared with iron delivered by transferrin. Hypoxia significantly reduced ^{59}Fe content in the mitochondria-rich fraction after 6 hours and 24 hours of labeling but only when $^{59}\text{Fe-Tf}$ was the source of ^{59}Fe (Fig. 7).

The nuclei-rich fraction showed a relatively high content of iron in comparison with cytosolic fractions in both $^{59}\text{Fe-Tf}$ and $^{59}\text{FeCl}_3$ -labeled LECs (Figs. 6, 7). When LECs were labeled with $^{59}\text{Fe-Tf}$, hypoxia reduced the content of iron in the nuclei-rich fraction but had no effect on the level of nuclear iron when $^{59}\text{FeCl}_3$ was the source.

LECs cultured under hypoxic conditions retained from 37% ($^{59}\text{FeCl}_3$) to 62% ($^{59}\text{Fe-Tf}$) more iron in the cytosolic fraction at 6-hour exposure in comparison with normoxic LECs. There were no differences in cytosolic iron levels at 24 hours when LECs were cultured under normoxic or hypoxic conditions regardless of the source of iron (Figs. 6, 7).

FIGURE 3. The effect of hypoxia on internalization of holotransferrin ($^{59}\text{Fe-Tf}$) into cytosolic fraction of LECs after 6 hours or 24 hours loading under N and H conditions. The cytosolic fractions, obtained after discarding the 15,000g organelle-containing pellets, were precipitated with 50% acetone and protein precipitates were pelleted at 15,000g. Proteins were dissolved in PAGE loading buffer and separated on 8% PAGE. The dried gels were imaged and quantified with an Instant Imager radioactivity detector. Results are expressed as the mean $\pm$ SEM of five samples. *Significantly different from N at the same time point, $P < 0.05$.

FIGURE 4. Comparison of the subcellular distribution of ^{59}Fe in LECs loaded with $^{59}\text{FeCl}_3$ for 6 hours or for 24 hours under N conditions. LECs were loaded with iron and lysed with 10 mM Tris/HCl buffer containing 15% sucrose and protease inhibitor cocktail as described in the Methods section. The lysates were centrifuged at 600g for 5 minutes, generating nuclei-rich fraction (pellets; n). The supernatants were centrifuged at 15,000g for 1 hour, generating mitochondria-rich fraction (pellets; m) and the cytosolic fraction (supernatant; c). The radioactivity of whole lysates (total uptake) and each cell fraction was counted in a gamma counter. Results are expressed as the mean $\pm$ SEM of nine samples. *Significantly different from 6-hour time point, $P < 0.05$.

Fluorescent Labeling of Mitochondria and Nuclei in Cultured LECs

We were interested to see if there were any changes in mitochondrial morphology induced by hypoxia, such as swelling or enlargement due to irregular fusion, as have been observed in other cell types.²³ We imaged mitochondria in live LECs by using the mitochondrion-specific dye MitoTracker Green, the accumulation of which is independent of mitochondrial membrane potential. A comparison of images of

FIGURE 5. Comparison of the subcellular distribution of ^{59}Fe in LECs loaded with $^{59}\text{Fe-Tf}$ for 6 hours or for 24 hours under N conditions. LECs were loaded with iron and lysed with 10 mM Tris/HCl buffer containing 15% sucrose and protease inhibitor cocktail as described in the Methods section. The lysates were centrifuged at 600g for 5 minutes, generating nuclei-rich fraction (pellets; n). The supernatants were centrifuged at 15,000g for 1 hour, generating mitochondria-rich fraction (pellets; m) and the cytosolic fraction (supernatant; c). The radioactivity of whole lysates (total uptake) and each cell fraction was counted in a gamma counter. Results are expressed as the mean $\pm$ SEM of 15 samples. *Significantly different from 6-hour time point, $P < 0.05$.

FIGURE 6. The effect of hypoxia on accumulation of ^{59}Fe in subcellular fractions of LECs loaded with $^{59}\text{FeCl}_3$ for 6 hours or 24 hours under N or H conditions. LECs were loaded with $^{59}\text{FeCl}_3$ and lysed with 10 mM Tris/HCl buffer containing 15% sucrose and protease inhibitor cocktail as described in the Methods section. The lysates were centrifuged at 600g for 5 minutes generating nuclei-rich fraction (pellets; n). The supernatants were centrifuged at 15,000g for 1 hour generating mitochondria-rich fraction (pellets; m) and the cytosolic fraction (supernatant; c). The radioactivity of whole lysates and each cell fraction was counted in a gamma counter and normalized to the protein content of each fraction. Results are expressed as the mean $\pm$ SEM of nine samples. *Significantly different from N at the same time point, $P < 0.05$.

LECs grown under normoxia and hypoxia confirmed the expected targeted accumulation of MitoTracker Green in mitochondria, but no significant detectable morphological differences in mitochondria were observed between normoxic and hypoxic cells (Fig. 8).

DISCUSSION

Based on the cell counts (Table 1), 24 hours of hypoxia increased the number of LECs and did not decrease cell viability. However, hypoxia lowered the proliferation rate of LECs by

11.2% during the same exposure time. Hypoxia can affect cell proliferation and apoptosis in a diverse, cell-specific manner; these effects are mediated by HIF-1 α (see Refs. 24 and 25 for reviews). We previously determined that HIF-1 α is expressed in cultured canine LECs and that hypoxia increased it by 75% after 24 hours (data not shown). In murine lenses, HIF-1 α decreased proliferation of LECs, but only in older animals²⁶ and did not increase apoptosis in the cultured epithelial cells of human and rabbit lenses.²⁷ To function in a low-oxygen environment, most cell types have to reduce oxygen consumption by adjusting metabolic pathways and limiting cell population. However, LECs

FIGURE 7. The effect of hypoxia on an accumulation of ^{59}Fe in subcellular fractions of LEC loaded with $^{59}\text{Fe-Tf}$ for 6 hours or 24 hours under N or H conditions. LECs were loaded with $^{59}\text{Fe-Tf}$ and lysed with 10 mM Tris/HCl buffer containing 15% sucrose and protease inhibitor cocktail as described in the Methods section. The lysates were centrifuged at 600g for 5 minutes generating nuclei-rich fraction (pellets; n). The supernatants were centrifuged at 15,000g for 1 hour generating mitochondria-rich fraction (pellets; m) and the cytosolic fraction (supernatant; c). The radioactivity of whole lysates and each cell fraction was counted in a gamma counter and normalized to the protein content of each fraction. Results are expressed as the mean $\pm$ SEM of at least eight samples. *Significantly different from N at the same time point, $P < 0.05$.

FIGURE 8. Fluorescent labeling of mitochondria and nuclei in LECs after exposure to N or H conditions. LECs were grown under N (A) or H (B) conditions for 24 hours and then labeled with MitoTracker Green (green) and Hoechst 33342 (blue). Scale bar: 100 μ m.

proliferate and differentiate in a normally hypoxic environment. Therefore, any negative effect of HIF-1 α on LEC proliferation could be balanced by expression of factors critically important for cell survival, such as VEGF, which was found to stimulate lens cell proliferation and differentiation.^{28,29}

Our previous studies found higher uptake and accumulation of iron from $^{59}\text{FeCl}_3$ than $^{59}\text{Fe-Tf}$.²² The present study confirmed those results (Figs. 1, 2). On average, ^{59}Fe accumulation into LECs labeled with $^{59}\text{Fe-Tf}$ was 55-fold lower as compared with that of $^{59}\text{FeCl}_3$, regardless of culture conditions (Figs. 1, 2). Uptake of iron from $^{59}\text{Fe-Tf}$ increased at 24 hours as compared with 6 hours of labeling (Fig. 2), and almost doubled at the 24-hour point when $^{59}\text{FeCl}_3$ was the source of ^{59}Fe (Fig. 1). Uptake of NTBI depends on the duration of labeling, concentration of extracellular iron, and composition of the medium, but is unaffected by iron requirements, growth rate, or division of cells.³⁰ This is in contrast to receptor-based uptake of transferrin iron, which is strictly controlled, through complex mechanisms, by intracellular iron levels. Lack of iron-controlled negative feedback may result in excessive intracellular accumulation of NTBI due to unregulated uptake, as supported by our previous and current results. NTBI accumulated in extracellular fluids as a result of iron-overload produced by excessive iron ingestion or by pathological conditions, such as hereditary hemochromatosis, hypotransferrinemia hemolytic anemias, and tissue injury.² In the eye, intraocular inflammation increased iron levels in the intraocular fluids.³¹ The elevated iron in the fluids of inflamed eyes, some of it likely NTBI, was efficiently absorbed by the lens,³² thereby reducing the iron content in the fluid surrounding it.

The intracellular distribution pattern of iron is also source dependent (Figs. 4, 5). Relative to total iron uptake, most ^{59}Fe from $^{59}\text{FeCl}_3$ was directed to the nuclei-rich (53.1%) and mitochondria-rich (47.7%) fractions during the first 6 hours of labeling. Subsequently, the iron levels in the mitochondria-rich fraction declined and the cytosolic content doubled after 24 hours of labeling (Fig. 4). Most $^{59}\text{Fe-Tf}$ was cytosolic during first 6 hours (Fig. 5). After 24 hours, the cytosolic iron content declined and sequestration of iron to the nuclei-rich fraction increased (Fig. 5). The most pronounced difference in source-dependent iron distribution was the high trafficking of ^{59}Fe from $^{59}\text{FeCl}_3$ to the mitochondria-rich fraction (32.6%–47.7%) as compared with much lower content of mitochondrial iron (10.6%–12.6%) in $^{59}\text{Fe-Tf}$ labeled LECs. The difference was

even more striking when results were presented as the ratio of counts per minute (cpm) of ^{59}Fe per/ μ g of protein (Figs. 6, 7). LECs labeled with $^{59}\text{FeCl}_3$ showed 140- to 400-fold higher levels of mitochondrial iron in comparison with the mitochondria-rich fraction of $^{59}\text{Fe-Tf}$ -labeled cells. It has been suggested that the mitochondrial demand for iron regulates cellular iron sequestration and metabolism through an unknown signaling mechanism.³³ Our results suggest this may be true only if Tf delivers the iron. Cells that synthesize large quantities of heme, such as erythroid cells, have high demand for mitochondrial iron. The low (12.6%) incorporation of ^{59}Fe from transferrin to the mitochondrial fraction of LEC may reflect the physiological iron requirements of mitochondria in this cell type. In contrast, higher accumulation of $^{59}\text{FeCl}_3$ iron could demonstrate a lack of control of NTBI uptake by mitochondria. The consequence of this may be oxidative damage to mitochondria due to increased ROS production. The mechanisms of source-dependent delivery of iron to mitochondria are not clear and there is no direct evidence to substantiate or eliminate the possibility of direct transfer of transferrin iron from endosomes to mitochondria⁷ and cytosolic chaperone transfer of NTBI to mitochondria.⁸ Mitochondrial iron content could be regulated by essential iron transporters mitoferrins 1 and 2, which facilitate iron influx to mitochondria.³⁴

There was a time-dependent movement of iron between the cytosolic and nuclei-rich fraction when ^{59}Fe was delivered by transferrin. Labeling for 24 hours increased the ^{59}Fe level in the nuclei-rich fraction with a concomitant decline in the cytosolic fraction (Fig. 5). It is difficult to explain this movement of iron because the mechanism regulating uptake of iron from transferrin to the nucleus is unknown.

Hypoxia did not affect total $^{59}\text{FeCl}_3$ uptake (Fig. 1). However, there is evidence that the lack of effect of hypoxia on NTBI uptake may be cell-specific. Human embryonic kidney cells (HEK293) incorporated more NTBI under hypoxic than normoxic conditions.³⁵ In contrast to what we found for $^{59}\text{FeCl}_3$, total uptake of iron from $^{59}\text{Fe-Tf}$ during 24 hours of labeling was decreased by hypoxia (Fig. 2). Because the partial pressure of oxygen did not affect the efflux of ^{59}Fe from LECs (Table 2), the reduced levels of Fe when transferrin was the source may be the consequence of reduced iron influx via the $^{59}\text{Fe-Tf}$ /TfR1 mechanism.

HIF-1 regulates expression of genes by binding to a hypoxia responsive element (HRE) located in the promoter region.³⁶ HRE sequences were found in both transferrin³⁷ and transfer-

rin receptor (TfR1) genes.³⁸ LECs express both proteins and secrete Tf, which contributes to the high concentration of Tf in the intraocular fluids.³⁹ Because the presence of an HRE sequence usually indicates increased expression of target genes,⁴⁰ the observation that hypoxia reduced rather than increased iron uptake via Tf/TfR1 was unexpected. However, analysis of iron-labeled, cytosolic proteins separated by PAGE confirmed that LECs incorporated less ⁵⁹Fe-Tf when cultured under hypoxic conditions (Fig. 3). We concluded that in canine LECs, hypoxia reduces iron uptake by limiting internalization of ⁵⁹Fe-Tf.

Hypoxia also affected the subcellular distribution of iron. Hypoxic LECs labeled with ⁵⁹Fe-Tf had lower iron content in mitochondria when compared with normoxic LECs (Fig. 7). Hypoxia also decreased trafficking of iron from ⁵⁹Fe-Tf to the nuclei-rich fraction (30%) (Fig. 7). DNA can bind redox-active iron, which can subsequently cause oxidative DNA damage by catalyzing formation of ROS.⁴¹ Limiting the iron trafficking to the nucleus may represent yet another adaptive mechanism of LECs cultured under hypoxic conditions. This adaptive response did not occur when ⁵⁹FeCl₃ was the iron source.

In conclusion, there are significant source-dependent differences in iron uptake and trafficking within the cell. The uptake and distribution of iron from nontransferrin sources (⁵⁹FeCl₃) is not as strictly regulated as from transferrin and may lead to excessive Fe accumulation in cells and subcellular fractions, particularly in mitochondria and nuclei. This may increase the probability of oxidative damage during conditions of tissue iron overload. For example, excessive Fe levels accompanying hemorrhage could lead to large amounts of iron being taken up from NTBI. This could result in iron accumulation in mitochondria and render cells more vulnerable to oxidative stress. Hypoxia limited cellular uptake of Tf iron, decreased iron content in mitochondria, and altered its trafficking into nuclei. This could result in less DNA damage due to reduced ROS production.

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