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Hypoxia controls iron metabolism and glutamate secretion in retinal pigmented epithelial cells

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ABSTRACT

Background: Blood-barrier systems are essential in controlling iron levels in organs such as the brain and eye, both of which experience hypoxia in pathological conditions. While hypoxia's effects on numerous iron regulatory and storage proteins have been studied, little is known about how hypoxia affects iron metabolism. Iron also controls glutamate production and secretion; therefore the effects of hypoxia on iron metabolism and glutamate secretion were studied in polarized retinal pigmented epithelial (RPE) cells.
Methods: Primary canine RPE were cultured in Millicells to create polarized cell cultures. Iron uptake and efflux were measured in hypoxic and normoxic conditions. RPE were loaded with ^{59}Fe-transferrin. Glutamate concentrations in the cell conditioned media were also measured.
Results: Hypoxia induced a large increase in iron efflux from RPE in the basolateral direction. Glutamate secretion occurred mainly in the basolateral direction which is away from the retina and out of the eye in vivo. Glutamate secretion was doubled under hypoxic conditions.
Conclusions: Hypoxia is known to induce oxidative damage. The current results show that iron, a key catalyst of free radical generation, is removed from RPE under hypoxic conditions which may help protect RPE from oxidative stress. Results obtained here indicate the importance of using polarized tight junctional cells as more physiologically relevant models for blood-barrier-like systems.
General significance: While the effects of hypoxia on iron efflux and glutamate secretion may be protective for RPE cells and retina, increased glutamate secretion in the brain could cause some of the damaging neurological effects seen in stroke.

1. Introduction

Iron metabolism in cells is complex and involves numerous proteins with specific roles in iron uptake, storage, efflux and movement between intracellular compartments. Furthermore, there are as yet unidentified proteins which regulate this elegant and essential web of processes. Essential for the activity of dozens of enzymes, iron also can cause damage to cells by its ability to catalyze free radical reactions. Hence, the movement and levels of iron in cells and tissues are highly controlled. Considering the importance of this regulation to normal physiological functions, it is surprising that there is limited information available about how blood-barrier systems control iron concentrations of the tissues which they guard, such as those in the brain and the eye.

While it is clear that the iron concentration in intraocular tissues and fluids is tightly regulated [1,2] and its dysregulation has pathological consequences [3–6] very little is known about how intraocular iron concentrations are maintained. It has been clearly demonstrated that iron efflux in the retina is dependent upon the concerted activity of hephaestin and ceruloplasmin, since the knock-out of these ferroxidase proteins results in iron accumulation and a pathology resembling age-related macular degeneration (AMD) in mice [7]. Additionally, another study using intravitreally injected ^{59}Fe-transferrin showed iron uptake by the retina and axonal transport of transferrin by retinal ganglion cells [8]. But the overall functions of the blood–ocular barriers (BOB) in regulating iron movement in and out of the eye under normal circumstances and in pathological conditions are still unknown. It is the purpose of this investigation to begin to unravel the mechanisms controlling iron transport into and out of the eye using a model system of the BOB, cultured retinal pigmented epithelial (RPE) cells that are polarized and tight-junctional. In vivo, the basolateral surface of the RPE faces the blood supply in the choroid and the apical surface faces the retina.

Iron is removed from cells by the membrane protein, ferroportin, presently the only known specific pathway for iron efflux [9]. Ferroportin levels are regulated translationally by the binding of an iron regulatory protein to an iron responsive element in its mRNA, which decreases ferroportin translation when cellular iron levels are low, thus decreasing iron efflux [10,11]. There is also an important post-translational regulation of ferroportin. The small peptide hormone, hepcidin is made by RPE cells [12] and controls ferroportin levels by

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binding and targeting it to the proteasome for degradation [13,14]. Hence, when hepcidin levels are high, ferroportin levels and thus iron efflux are decreased. Ferroportin requires the presence of a ferroxidase such as ceruloplasmin and/or hephaestin to enable iron efflux and its subsequent binding to the iron transport protein transferrin for its removal from the site [15].

Hypoxia is integrally related to iron metabolism. Iron regulates the hypoxic response and hypoxia alters levels of iron regulatory and storage proteins [16]. The activity of the transcription factor, hypoxia-inducible factor-1 (HIF1) is regulated by both oxygen and iron. HIF1 is a dimer consisting of the constitutively produced nuclear HIF1β and the highly regulated cytoplasmic HIF1α. Under normoxic conditions and in the presence of iron, HIF1α is targeted for degradation by the proteasome [17,18]. Under hypoxic or low iron conditions, HIF1α moves to the nucleus where it forms a dimer with HIF1β producing active HIF1. HIF1 controls the transcription of dozens of genes, including most of the iron regulatory and storage proteins. Importantly, even under normoxic conditions iron chelators can prevent HIF1α degradation which underscores the dominant role played by iron in the regulation of HIF activity.

Hypoxia causes oxidative damage to cells due to disruption of normal mitochondrial function [19]. We recently demonstrated that hypoxia decreased iron uptake in lens epithelial cells and also caused significant changes in iron movement between organelles including a large decrease in mitochondrial iron content [20]. Hypoxia also affects hepcidin production and therefore ferroportin levels in cells [21,22]. We previously demonstrated that changes in cellular iron metabolism directly affected glutamate production and secretion [23,24]. Since low tissue oxygen levels are found in certain ocular pathologies, we examined the effects of hypoxia on iron uptake by and efflux from polarized and non-polarized RPE cell cultures. Additionally, the secretion of glutamate from these cells was determined.

2. Methods

2.1. Tissue culture

Canine cadavers were collected from Johnston County Animal Shelter in North Carolina immediately after euthanasia. Within 3 h of death, eyes were removed and RPE cells carefully isolated from the posterior section of the globe by trypsinization followed by centrifugation to collect the cells. The RPE cells were resuspended in medium containing equal amounts Ham's F12 and Dulbecco's modified Eagle medium (cat# 31765-035 and #10566-016, Invitrogen, Rockville, MD) and supplemented with 10 or 20% fetal bovine serum (Hyclone, Logan, Utah) and 1% antibiotic–antimycotic solution (Mediatech, Manassas, VA). Cultures were incubated at 37 °C, 5% CO_2, 95% humidity in 60 mm dishes until confluence was reached in 7–10 days. For experiments, RPE were trypsinized and plated in 6-well tissue culture plates or on Millicell hanging cell culture inserts (EMD Millipore, Ballerica, MA).

2.2. Millicell culture model

Primary RPE cells were plated on 6-well (200,000 cells/insert) or 12-well (50,000 cells/insert) Millicell hanging tissue culture inserts (polyethylene terephthalate membrane; 1.0 μm pore size) in DMEM:F12 (1:1) medium containing 10% FBS and 1.0% penicillin–streptomycin–amphotericin B. After cell attachment, the apical medium was switched to serum-free DMEM:F12 (1:1) medium containing 1 × B27 supplement (Invitrogen, cat# 0080085S) the following day. Apical and basal media were replaced every other day. Transepithelial electrical resistance (TER) readings were measured once a week. Cultures were used for experiments once they exhibited TER readings over 300 Ω cm^2 and directional transport of secreted glutamate to the basal compartment (at least 5 × more to basal than apical compartments). Cultures typically achieved these parameters in 4–8 weeks.

2.3. HCN cell cultures

Human cortical neuron cells (CRL-10742) were obtained from ATCC (Manassas, VA) and grown according to the provided instructions. Medium used was DMEM (ATCC #30-2002) supplemented with 15% fetal bovine serum and 1% antibiotic–antimycotic. When flask cultures reached 70–80% confluency, cells were plated in 6-well tissue culture plates and grown to similar confluency, and then used in experiments to determine glutamate secretion under normoxic and hypoxic conditions, to replicate what was done in RPE cultures.

2.4. Iron uptake and efflux

Apical and basal compartments of 6-well Millicell RPE cultures were gently rinsed three times with warm MEM (serum- and glutamine-free, Invitrogen), then pre-incubated for 1 h in the same medium to try to remove as much transferrin as possible from both compartments. This is important as the cells are grown with the apical compartment containing serum-free medium and the basal compartment containing medium with serum. Serum contains a high concentration of transferrin. In addition, transferrin is secreted by RPE, and therefore this rinse and pre-incubation in serum-free medium is meant to substantially reduce and normalized the amount of transferrin present at the beginning of each experiment. ^{59}Fe-labeled transferrin (^{59}FeTf, prepared as previously described; Goralska, Harned et al. 1998) was then added to either apical or basal chambers containing 1 ml and 3.5 ml MEM, respectively, for a final concentration of 320 μg/ml transferrin. After thorough mixing, samples of the loading media were taken to measure zero time radioactivity in each loading compartment. Millicell cultures were then incubated for 24 h in either normoxic (20% oxygen in a standard incubator) or hypoxic (0.5% oxygen in a Ruskinn INVIVO2 300 workstation) conditions. After this period of iron loading, the non-loading chamber CCM was collected for counting and the RPE cultures were again rinsed as above and moved to a new plate containing fresh serum-free MEM and again incubated for 24 h in either normoxic or hypoxic conditions. Following this 24 h efflux period, CCM from both apical and basal compartments was collected for counting, and the RPE were rinsed with cold PBS and lysed in non-denaturing lysis buffer (10 mM Tris/HCl, pH 7.4, containing protease inhibitors) on ice. The cells were scraped off of the membrane, and radioactivity in the cell lysates determined. After centrifugation, protein in the supernatants was measured by the bicinchoninic method (BCA, Pierce, Rockford, IL). Similar experiments were also conducted using traditional RPE cultures on 6-well plates. Some experiments were also terminated after the initial 24 h of iron uptake. The effect of hypoxic conditions (24 and 48 h) on tight junctions was determined by ZO-1 immunofluorescence and TEER measurements in a parallel set of Millicell cultures, which were unchanged compared to control normoxic RPE cultures.

2.5. Glutamate determination

Confluent RPE in traditional 6-well plates or in 6 or 12-well Millicell format were incubated for 1–24 h in serum-free glutamine-free MEM in normoxic or hypoxic conditions with or without additional treatments. Glutamate was measured in the CCM using an Amplex Red Assay kit (Invitrogen). Standards and diluted CCM were added to a 96-well black-walled plate, and after addition of the reaction mixture and incubation at 37 °C, fluorescence was measured (Fluoroskan Ascent FL, Thermo Electron, Milford, MA) at 530 nm excitation and 590 nm emission. Protein concentration was measured in the cell lysates by the BCA assay.

2.6. Ferroportin knockdown/Fe uptake and efflux

RPE were plated at a density of about 100,000 cells per well on 6-well plates in the normal RPE tissue culture medium described above,

without the antibiotic–antimycotic and incubated at 37 °C overnight, typically reaching a confluency of 30–50%. Ferroportin-1 siRNA (#sc-60633(h) Santa Cruz Biotechnology, Dallas, TX), a pool of 3 target-specific 20–25 nucleotide siRNAs, was used to transfect the cells, at a final concentration of 50 nm. Included on each plate was an equal number of wells transfected with a control non-targeting pool of siRNA (#D-001810-1005, Thermo Scientific Dharmacon, Lafayette, CO) at the same concentration, to account for any non-sequence-specific cell responses. Lipofectamine RNAiMAX (Invitrogen) was used as transfection reagent at 1 μl per well. RNAiMAX–siRNA complexes were formed in serum-free DMEM according to the manufacturer's protocol, then added to the cells bathed in 1 ml fresh RPE medium without antibiotic supplementation. After 24 h, fresh complete medium was added and cultures were incubated for another 48 h to achieve maximum knockdown. At this point, as described in detail above, cells were rinsed and loaded with ^{59}FeTf in either normoxic or hypoxic conditions for 24 h, rinsed again, and incubated another 24 h in normoxia or hypoxia to measure the efflux of Fe from the loaded cells under different oxygen concentrations.

2.7. Determination of ^{59}Fe in ferritin

After iron loading and efflux, cells were lysed in non-denaturing hypotonic Tris–HCl buffer and lysates centrifuged at 30,000 ×*g*, cytosolic proteins collected by acetone precipitation, and the protein pellet dissolved in PBS and analyzed using 8% native PAGE. Radioactivity in the ferritin bands was quantified using an Instant Imager (Perkin Elmer).

3. Results

The RPE are polarized in vivo and play important roles in providing nutrients and removing wastes from the retina, as well as forming an important part of the blood–retinal barrier. Since little is known about how iron levels in the eye are maintained, polarized cultures of RPE cells were used to study iron movement into and out of these cells. Additionally, these cells were exposed to a pathological situation, hypoxia, to determine how iron movements are altered by the changes in the concentration of oxygen. RPE cultured using the Millicell culture model have polarized localization of Na^+–K^+–ATPase, have tight junctions, and maintain their high transepithelial electrical resistance under both normoxic and hypoxic conditions for the duration of the experiments.

3.1. Iron uptake

RPE cells were exposed to the physiological form of iron by adding ^{59}Fe-transferrin (Fe-TF) to the medium bathing the apical or basolateral surface. Under normoxic conditions, more iron was taken up from the basolateral side than the apical side. When the cells were exposed to hypoxia (0.5% oxygen) for 24 h, significantly less iron was taken up into the RPE from either side. However, more iron was still taken up across the basolateral side than the apical side (Fig. 1).

3.2. Iron efflux

RPE cells were loaded with Fe-Tf from either the apical or basolateral side for 24 h. RPE were then washed to remove the Fe-Tf and were exposed to either normoxic or hypoxic conditions for another 24 h. The total counts from the media and cells were determined. Under normoxic conditions, cells loaded from the apical side had iron efflux across the basolateral side equivalent to that crossing into the apical medium. However, cells loaded from the basolateral side had significantly greater efflux from that side (20% of total iron loaded) versus the apical side (5% of the total iron loaded). Hypoxia caused a large and significant increase in iron efflux in the BL direction whether ^{59}Fe-Tf was loaded from the apical or basolateral side (22% and 33% of the total iron loaded, respectively; Fig. 2).

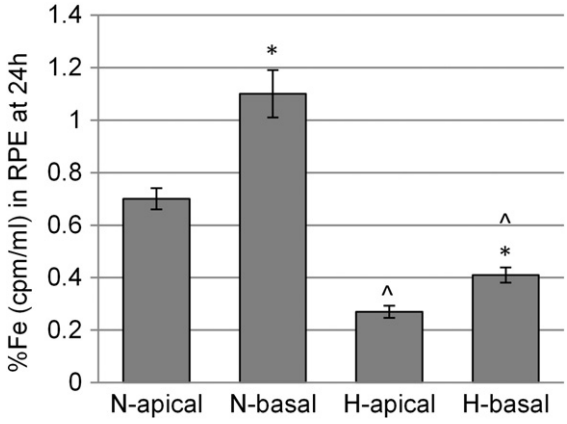

Fig. 1. The effect of hypoxia on iron uptake in polarized RPE cultures. Apical or basal compartments of polarized RPE Millicell cultures were loaded with ^{59}Fe-Tf and incubated for 24 h in normoxic or hypoxic (0.5% O_2) conditions. Cells were lysed and radioactivity in the lysates was measured using a gamma counter. Data is expressed as a percentage of the ^{59}Fe loaded (cpm/ml) that was taken up and present in the cells after 24 h. Each histogram bar represents the mean +/− SEM of at least 4 experiments. Statistical analysis used was a paired *t*-test, 95% confidence level, $p < 0.01$. *Significantly different from apical under same oxygen conditions. ^Significantly different from corresponding normoxia treatment.

We also determined whether hypoxia had this effect on iron efflux in non-polarized monolayers of RPE in standard tissue culture dishes. In fact, whether the cells were loaded with ^{59}Fe-Tf under normoxic or hypoxic conditions, there was greater efflux of loaded iron from the cells when efflux was measured under hypoxia (Fig. 3). However, the total efflux did not exceed 14% under these conditions; total efflux (apical + basolateral) from polarized RPE loaded from the BL side was 43% of the total iron loaded.

3.3. Hypoxia affected the iron content of ferritin

Cytosolic extracts were prepared from cells incubated with ^{59}Fe-Tf for 24 h then exposed to hypoxic or normoxic conditions for 24 h. The proteins in these extracts were separated by gel electrophoresis and the radioactivity in the band corresponding to ferritin was quantified. Ferritin is the ubiquitous storage protein for iron and is an indicator of iron levels in cells and tissues. There was a large decrease in the amount of iron present in ferritin in RPE cultured under hypoxic conditions (Fig. 4).

3.4. Ferroportin

In order to determine if an increase in ferroportin was responsible for the increase in iron efflux under hypoxic conditions RPE cells were transfected with siRNA for ferroportin. As noted earlier (Fig. 1; [20]), hypoxia decreased the uptake of iron and the amount of iron available for efflux. Therefore the results are reported as percent of total uptake. The total amount of iron efflux was increased under hypoxic conditions by about 170%. Treatment with ferroportin siRNA decreased efflux by 20% under either hypoxic or normoxic conditions (Fig. 5).

3.5. Effect of hypoxia on glutamate secretion

We have previously demonstrated that iron controls glutamate production and secretion. Therefore the dramatic effect of hypoxia on iron transport and efflux led us to examine if hypoxia can affect glutamate metabolism and if iron is involved. Even after 1 h of hypoxia, a significant increase in glutamate secretion was found. This increase continued

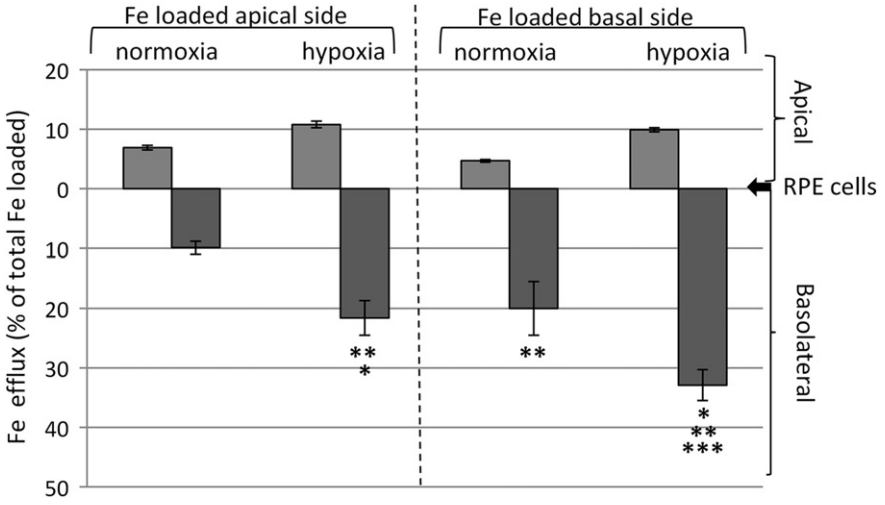

Fig. 2. Effect of hypoxia on directional iron efflux in polarized RPE cultures. RPE were loaded with ^{59}Fe-Tf from either the apical or basal compartments for 24 h under normoxic conditions. Apical and basal chambers were rinsed thoroughly 3 times and fresh serum-free MEM added back to each chamber and then plates were incubated for an additional 24 h efflux period in either normoxia or hypoxia. CCM from both chambers was collected and cells were rinsed and lysed, and radioactivity in the media and lysates was measured using a gamma counter. Data is expressed as the percentage of total ^{59}Fe taken up by the cells that moved to either the apical compartment (bars above the zero line) or the basal compartment (bars below the zero line) during the 24 h efflux period. Each histogram bar represents the mean +/− SEM of at least 3 samples. Statistical analysis used was ANOVA with Tukey's, $p < 0.05$. *Significantly different than corresponding normoxia efflux. **Significantly different than efflux to corresponding apical compartment under same O_2 conditions. *** Significantly different than all other groups.

to grow at 6 h and 24 h from cells cultured as non-polarized monolayers (Fig. 6). Importantly, these results were repeated in polarized Millicell cultures (Fig. 7). Glutamate secretion is very polarized with about 25-times more glutamate being secreted in the BL direction than in the apical direction under either normoxic or hypoxic conditions. Hypoxia increased glutamate secretion in both directions, and interestingly doubled the amount secreted to either side.

In order to determine whether or not hypoxia-induced glutamate secretion was a more global phenomenon, a completely different cell type, human cortical neurons (HCN2) were exposed to hypoxia and glutamate secretion was determined. Hypoxia increased glutamate secretion by a statistically significant 37% ($p = 0$, ANOVA).

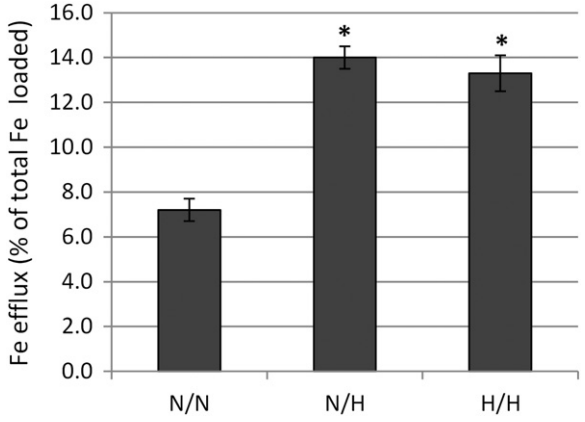

Fig. 3. Effect of hypoxia on iron efflux in non-polarized RPE. Confluent RPE cultures in traditional 6-well plates were treated with ^{59}Fe-Tf, a sample of the loading media saved for counting, and cultures incubated for 20 h under normoxic or hypoxic conditions for Fe loading. Cells were rinsed 3 times with warm serum-free MEM, fresh serum-free media added, then incubated another 24 h in either normoxia (N/N) or hypoxia (N/H, H/H) for determination of iron efflux. CCM was removed and ^{59}Fe counted on a gamma counter. Cells were rinsed with cold PBS and lysed in 10 mM Tris–HCl, pH 7.3, with 2% SDS and protease inhibitors, and radioactivity in the lysates was determined. Data is expressed as the percentage of total ^{59}Fe taken up that left the cells during the 24 h efflux period. Each histogram bar represents the mean +/− SEM of at least 9 samples. *Significantly different than N/N by ANOVA and Tukey's test, $p = 0$.

Because glutamate production and secretion is regulated by iron, it was important to determine if hypoxia's effect on glutamate secretion was dependent upon iron. RPE were treated with either an iron chelator (Dp44mT) or an inhibitor of the iron-dependent aconitase pathway responsible for glutamate production (oxalomalate; OMA). Both compounds significantly decreased the effect of hypoxia-induced glutamate secretion, thereby underscoring iron's involvement in this process (Fig. 8).

4. Discussion and conclusions

Comprehensive elucidation of the mechanisms regulating movement of iron across the blood ocular barriers as well as its metabolism in ocular tissues is an essential yet understudied topic. The purpose of this study was to obtain key and fundamental information about iron uptake and efflux from RPE cells. Additionally, the effects of hypoxia on these processes and on iron-dependent glutamate secretion were examined. Since much of the literature is based upon studies using RPE grown as traditional monolayers on plastic tissue culture plates, we compared results using this type of culture to those from RPE grown on permeable tissue culture inserts (Millicells) that produce polarized epithelial cell layers that more closely resemble the in vivo

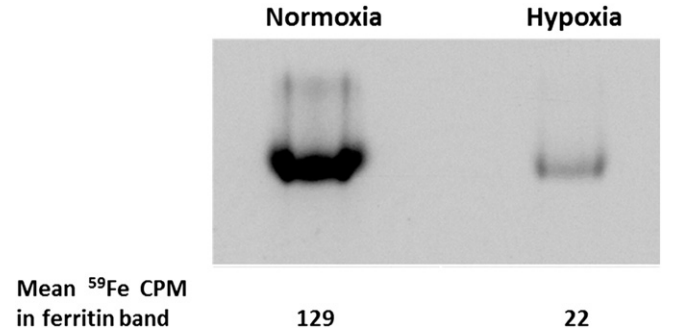

Fig. 4. Effect of hypoxia on the iron content of ferritin in RPE cells. RPE were labeled with ^{59}Fe-Tf for 24 h, rinsed, and incubated an additional 24 h in serum-free MEM in normoxic or hypoxic conditions. Cells were lysed and proteins in lysates separated on a native 8% gel. Radioactivity in the bands was quantified using an Instant Imager (Perkin Elmer).

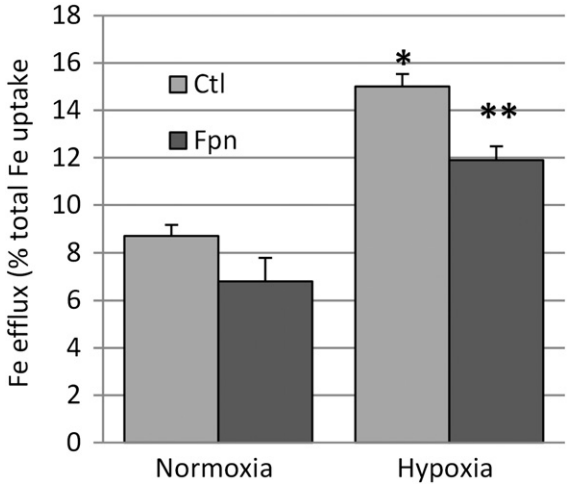

Fig. 5. Effect of ferroportin siRNA on iron efflux. RPE (at 30–50% confluency) were transfected with ferroportin-1 siRNA (#sc-60633 h, Santa Cruz Biotechnology) at a final concentration of 50 nM. Some cells were transfected with a non-targeting control pool of siRNA (#D-001810-1005, Thermo Scientific Dharmacon, Lafayette, CO). 48 h after transfection, cells were rinsed and loaded with ^{59}Fe-Tf in normoxia for 24 h, rinsed again, and incubated another 24 h in serum-free, glutamine-free MEM under normoxic or hypoxic conditions. ^{59}Fe efflux into the CCM and ^{59}Fe in the cell lysates was measured in a gamma counter (cpm). Data is expressed as the percentage of total ^{59}Fe uptake (cpm in cell lysates and in CCM) that was in the CCM. Histogram bars represent the mean +/− SEM of at least 9 samples. Statistical analysis used was ANOVA with Tukey's, $p = 0$. *Significantly different from normoxic counterpart. **Significantly different from hypoxic control.

condition. The use of these polarized cultures in the current study clearly shows that there are highly significant quantitative as well as qualitative differences in iron uptake and efflux as well as glutamate secretion compared to non-polarized RPE cultures. These differences underscore the importance of determining directionality of iron movement in order to obtain a better understanding of the normal physiology of the RPE as part of the blood–ocular barrier and how pathological situations alter barrier function.

Iron uptake from the basolateral surface was greater than from the apical surface of RPE cells. This is not surprising since the basolateral surface of polarized RPE in vivo are exposed to the choroidal circulation and therefore to transferrin as an iron source. RPE express the transferrin receptor on both the apical and basolateral membranes [25,26].

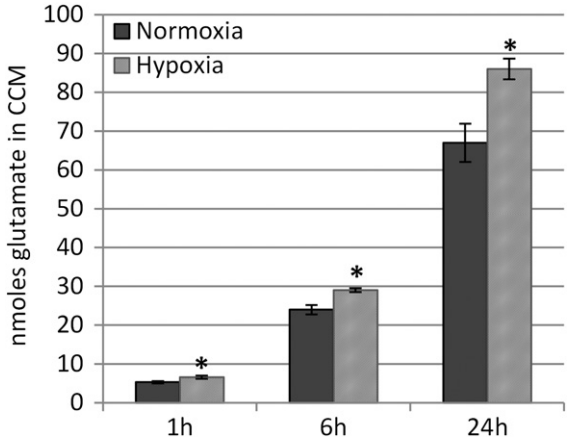

Fig. 6. The effect of hypoxia on non-polarized glutamate secretion. RPE were plated in 6-well culture plates in complete RPE medium (1:1 Ham's F12 and DMEM supplemented with 20% FBS and 1% antibiotic–antimycotic solution) and grown to confluence. Cultures were rinsed twice with serum-free glutamine-free MEM and incubated in fresh MEM under normoxic or hypoxic conditions for 1, 6, or 24 h. Glutamate was measured in the CCM with an Amplex Red Assay kit (Invitrogen). Data is expressed as nanomoles glutamate in the CCM and was analyzed using a paired *t*-test, $p < 0.01$. Each histogram bar represents the mean +/− SEM of at least 12 samples. *Significantly different than normoxia.

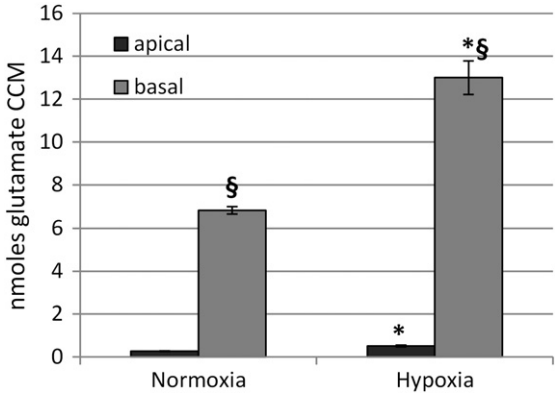

Fig. 7. The effect of hypoxia on glutamate secretion in polarized RPE cultures. RPE were grown on 12-well Millicell inserts as described in Methods. At the time of the experiment, the transepithelial electrical resistance readings averaged 400 Ω × cm^2. Apical and basal chambers were rinsed 3 times with serum-free glutamine-free MEM, fresh MEM was added to each chamber and cultures incubated for 24 h in normoxia or hypoxia. Glutamate concentration was determined in the cell-conditioned medium from each compartment using an Amplex Red Assay kit (Invitrogen). Data is expressed as the nanomoles glutamate secreted into the CCM of each compartment. Each histogram bar represents the mean +/− SEM of at least 6 samples. Statistical analysis used was ANOVA and Tukey's, $p = 0$. § Significantly different than apical glutamate. *Significantly different than normoxia.

Since the basolateral surface area of these cells is much larger than the apical surface area, it is not surprising that there is greater iron uptake across this surface. Hypoxia-induced decreases in iron uptake have been reported for lens cells [20]. In contrast, hypoxia increased iron levels in microglial and neuronal cell cultures [27,28]. However, these studies were of hypoxia conditioning and reperfusion and did not include an analysis of iron uptake or efflux. Also, the increase in iron levels mainly occurred after a period of oxygen reperfusion. Therefore it is difficult to compare these experiments to the current study.

It is important to note that iron efflux from polarized cells was always greater in the basolateral direction and that under hypoxic conditions efflux in this direction was significantly increased. This finding is significant as it would be physiologically beneficial to divert potentially

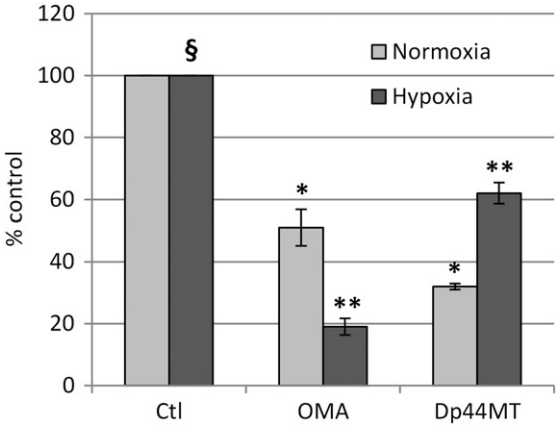

Fig. 8. The iron/aconitase pathway is responsible for the hypoxia induced increase in glutamate secretion. Confluent RPE cultures on 6-well plates were rinsed and treated with either 5 mM oxalomalic acid (Caymen Chemical, Ann Arbor, Michigan) or 0.3 μM Dp44mT (EMD Millipore, Billerica, MA) in serum-free, glutamine-free MEM for 24 h in either normoxic or hypoxic conditions. CCM was collected and cells were lysed in 10 mM Tris–HCl, pH 7.4. Glutamate was measured in the CCM and the protein content of the lysates was determined using the BCA assay. Data is expressed as a percentage of the non-treated control at the corresponding oxygen concentration. Each histogram bar represents the mean +/− the SEM of 7 samples. Statistical analysis used was ANOVA and Tukey's test, $p < 0.01$. *Significantly different from normoxia control. ** Significantly different from corresponding normoxia treatment and from hypoxia control, § although shown here as 100% for comparison to cells treated in hypoxia with OMA or Dp44mT, the glutamate secretion from untreated hypoxia control cells was actually 206% (+/− 11.1%) higher than that of untreated normoxia control cells, and significantly different as well.

damaging excess iron away from the retina and back to the circulation under conditions of hypoxia. This would provide protection against iron-induced free radical damage. A comparison of results from non-polarized monolayer cultures to polarized RPE shows that only about 14% of loaded iron was transported out of non-polarized cells under hypoxia, whereas in polarized cells under the same hypoxic conditions 43% of the loaded iron left the polarized cells. The current results are further evidence for the physiological relevance of the polarized cell culture system employed in our studies. While our results show polarized secretion in the presence of an intact barrier system, it should be noted that the blood ocular barrier is often disrupted in ocular disease, thereby adding another layer of dysfunction which may contribute to disease pathogenesis.

An increase in efflux of iron from RPE cells during hypoxia could be due to an increase in ferroportin expression or activity as ferroportin is found in RPE cells [29]. While the effects of ferroportin knockdown did significantly reduce iron efflux in normoxia and hypoxia, however, the results were not dramatic (20% decrease in both cases). These results are not surprising since hypoxia is known to decrease hepcidin levels in other cells [30] and hepcidin is responsible for removal of ferroportin from the membrane and its subsequent degradation. Hepcidin is present in RPE cells [12]. Therefore a reduction in hepcidin production would decrease the removal of ferroportin from the membrane which would increase its stability. This could explain how ferroportin knockdown would be of limited effect, as was supported by the current results. However, our results do indicate that ferroportin is involved in iron efflux from RPE cells.

Hypoxia causes oxidative stress in cells when they are exposed to ischemic conditions that occur in a variety of disease states, including ocular diseases [31]. Furthermore, hypoxia induces significant changes in iron movement between cellular compartments, especially on the movement of iron out of the mitochondria and nuclei [20]. Once again these may represent protective responses of these cells that reduce oxidative damage to these intracellular organelles. Our current results which showed a large efflux of iron out of RPE cells under hypoxic conditions may explain why in the earlier study there was a decrease in iron in the mitochondria and nuclei that was not reflected by an increase in cytoplasmic iron. The decrease in uptake and the total amount of iron in the RPE could be a protective response because iron catalyzes free radical damage which is one of the devastating consequences of hypoxia [19]. In fact, it has recently been hypothesized that the shift from aerobic to anaerobic metabolism in hypoxia is important to inhibit the increased generation of reactive oxygen species by mitochondria which occurs in response to an environment with reduced oxygen levels [32–34]. The reduction in iron content of RPE in hypoxia as shown in the current study would add another protective layer to the cellular response to hypoxia. The reduction in RPE iron levels is clearly demonstrated by the very large reduction in iron stored in ferritin which is considered an indicator of cellular and tissue iron status. Iron chelators and antioxidants are promising treatment modalities for conditions that have hypoxia as a component [6].

Iron regulates glutamate secretion in RPE cells and conditions that alter intracellular iron metabolism affect glutamate secretion. Therefore it was not surprising to find that hypoxia dramatically increased glutamate secretion. It appears contradictory that while the cells are limiting/decreasing their iron content, glutamate secretion increased. However, glutamate accumulation in the cell-conditioned medium occurred over a 24 h period when the iron content in the cytoplasm was likely initially very high since the intracellular iron storage sites are clearing iron and the cytoplasm is the transit site for iron efflux. It is clear that the cytoplasmic iron-dependent aconitase pathway for glutamate production and secretion is participating in this process since both OMA (an inhibitor of aconitase) and an iron chelator significantly decrease the effects of hypoxia. It is important to again note that there are large differences in the effects of hypoxia on secretion from non-polarized and polarized RPE cells (22% vs. 100% increase, respectively). The very large polarization of glutamate secretion under both normoxia and hypoxia is notable and demonstrates that our RPE cultures are highly polarized and tight-junctional. We have demonstrated that RPE cells contain the X_c^- antiporter, which exchanges glutamate for cystine. Cystine is reduced in cells to cysteine, the rate limiting amino acid for glutathione synthesis. In RPE cells glutamate secretion was accompanied by an exchange for cystine resulting in an increase in production of the essential intracellular antioxidant, glutathione [24]. The basolateral surface is more likely to have access to cysteine from the choroidal circulation therefore the secretion of glutamate from this surface would provide an advantage, increasing the potential for the production of glutathione.

Importantly, we have now also shown that cultured neuronal cells (HCN2) had significantly increased glutamate secretion in response to hypoxia. This not only indicates that the effects of hypoxia on glutamate production and secretion are not limited to RPE cells, but also have physiological significance since glutamate is an excitatory neurotransmitter that can be damaging to brain cells; for example its levels are increased after stroke and accompanying ischemia [35]. The current results are consistent with our hypothesis that hypoxia-induced changes in iron metabolism in cells can cause a dramatic increase in glutamate production and secretion which could contribute to the devastating effects of neurological stroke.

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