The Role of Hypoxia-Inducible Factor 1α in the Regulation of Human Meibomian Gland Epithelial Cells

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Optimal meibomian gland (MG) function is critically important for the health and wellbeing of the ocular surface.1,2 These glands synthesize and secrete a proteinaceous lipid mixture (i.e., meibum) that enhances the stability and prevents the evaporation of the tear film.1,3 MG dysfunction (MGD), in turn, leads to a loss of meibum, destabilization and hypervaporation of the tear film, and dry eye disease.1,4 Indeed, MGD is the most common cause of dry eye disease.1,2

There is no known cure for MGD. This fact is likely due to the relative lack of information concerning the physiological regulation of the human MG in health and disease. Recently, we discovered that a hypoxic environment is beneficial for MG function.5 More specifically, we found that human and mouse MGs exist in a hypoxic environment in vivo and that low oxygen (O2) stimulates differentiation of immortalized human meibomian gland epithelial cells (IHMGECs) in vitro.8 This hypoxic response involves an increased expression of sterol regulatory element binding protein 1 (SREBP1), an enlargement of lysosomes, and a rise in deoxyribonuclease (DNase) II activity.8 SREBP1 is a key controller of lipid synthesis9 and DNase II is a biomarker for terminal differentiation and holocrine secretion.10,11 In contrast, the local hypoxic environment may be lost in MGD.8 Our results suggest that re-induction of this hypoxic status may be a breath of fresh air for the treatment of MGD in future.

The mechanism(s) underlying this effect of hypoxia on the MG is unknown, but we hypothesize that it is due to an increase in the levels of hypoxia-inducible factor 1α (HIF1α). In other tissues, HIF1α is the primary regulator of cellular responses to hypoxia, and HIF1α expression can be induced by multiple stimuli, including hypoxia and hypoxia-mimetic agents. The objective of this study was to test our hypothesis.

Methods. Human eyelid tissues were stained for HIF1α. Immortalized human MG epithelial cells (IHMGECs) were cultured for varying time periods under normoxic (21% O2) or hypoxic (1% O2) conditions, in the presence or absence of the hypoxia-mimetic agent roxadustat (Roxa). IHMGECs were then processed for the analysis of cell number, HIF1α expression, lipid-containing vesicles, neutral and polar lipid content, DNase II activity, and intracellular pH.

Results. Our results show that HIF1α protein is present in human MG acinar epithelial cells in vivo. Our findings also demonstrate that exposure to 1% O2 or to Roxa increases the expression of HIF1α, the number of lipid-containing vesicles, the content of neutral lipids, and the activity of DNase II and decreases the pH in IHMGECs in vitro.

Conclusions. Our data support our hypothesis that the beneficial effect of hypoxia on the MG is mediated through an increased expression of HIF1α.

Keywords: hypoxia, meibomian gland epithelial cells, hypoxia-inducible factor 1α, roxadustat, differentiation

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HIF1α-mediated hypoxic response, albeit under normoxic conditions, both in vivo and in vitro. These drugs have been used to facilitate wound healing, treat retinopathy of prematurity, protect corneal endothelial cells, and regenerate tissues. Given this background, we chose to test our hypothesis. First, we evaluated whether HIF1α is present in the human MG. Second, we determined whether exposure to 1% O2 (hypoxia) or to Roxa at 1% or 21% O2 (normoxia) increases HIF1α expression in IHMGECs. Third, we examined whether Roxa can mimic a hypoxic condition by stimulating differentiation in IHMGECs.

METHODS

Terminology

There are several definitions for hypoxia, such as physiological, modest, moderate, severe, and anoxia, which are used to designate 10%–14%, 2.5%, 0.5%, 0.1%, and 0% O2, respectively. For this paper we use the terms “hypoxia” or “hypoxic environment,” defined as an O2 concentration of less than 5%. This usage is consistent with that of other studies.

We utilized 1% O2 for the experiments reported in this paper. This contrasts with the 3% O2 used in our previous study of relative hypoxia and the MG. Our rationale for this change was that we discovered that the mouse MG stains positively for pimonidazole. Pimonidazole is a biomarker for hypoxia in vivo and stains tissues that contain O2 levels below 1.3%. Consequently, we chose to better imitate the O2 conditions in the MG in vivo.

Human Tissues

Human eyelid tissues were collected after ectropion surgery from patients without MGD (1 male, 2 females; age range, 70–82 years) and processed for frozen section preparation as previously described. All samples were deidentified according to Health Insurance Portability and Accountability Act requirements. Our use of human tissues was approved by the Institutional Review Board of the Massachusetts Eye and Ear Infirmary and Schepens Eye Research Institute and adhered to the tenets of the Declaration of Helsinki.

Immunofluorescence Staining

Frozen sections of human MGs were incubated overnight at 4°C in a moist chamber with an antibody specific for HIF1α (28b, 1:50, SC-13515; Santa Cruz Biotchnology, Dallas, TX). After three phosphate buffered saline rinses, the sections were incubated with donkey anti-mouse secondary antibody (1:200, 2492098; EMD Millipore, Temecula, CA) for 1 hour at room temperature. The primary antibody was replaced with mouse IgG (Cell Signaling Technology, Danvers, MA) for negative controls. All slides were mounted with ProLong Gold antifade containing 4',6-diamidino-2-phenylindole blue nuclear stain (Invitrogen; Thermo Fisher Scientific, Waltham, MA).

Cell Culture

Authenticated IHMGECs were grown and passaged in keratinocyte serum-free medium with 5 ng/mL epidermal growth factor and 50 μg/mL bovine pituitary extract (Thermo Fisher Scientific) under 21% O2 (normoxia). Upon reaching 60% to 70% confluence, culture media were replaced by a 1:1 mixture of Dulbecco’s Modified Eagle’s Medium and Ham’s F12 (DMEM/F12; Corning Life Sciences, Tewksbury, MA), supplemented with 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA). Cells were cultured for 4 to 10 days under 21% O2 (normoxic) or 1% O2 (hypoxic) conditions. Culture plates of cells in the hypoxic condition were incubated inside hypoxia chambers filled with a premade 1% O2, 5% CO2, and 94% N2 gas mixture (Linde Gas North America, Hammond, IN), according to published protocols. Following cell medium changes, the chambers were re-flushed with the gas mixture before returning to the incubator. Cells were cultured in the presence or absence of 10-μM Roxa (Selleck Chemical, Houston, TX) under normoxic or hypoxic conditions for 4 to 10 days. Cell culture experiments (n = 3 wells/treatment) were repeated at least three times.

Western Blot

After culture in DMEM/F12 +10% fetal bovine serum under normoxic conditions for 7 days, cells were exposed to 21% O2, 1% O2, and 10-μM Roxa, alone or in combination, for an additional 6 hours. The cells were lysed in 2x Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) and sonicated to extract nuclear protein. Human hepatocellular carcinoma cell lines (HepG2 cells; America Type Culture Collection, Manassas, VA), which express high concentrations of HIF1α under CoCl2 stimulation for 4 hours, were used as a positive control for HIF1α antibody. The CoCl2 was purchased from Sigma-Aldrich Corp. (St. Louis, MO). Cell lysates were then evaluated for HIF1α (1:1000; BD Biosciences, San Jose, CA) or β-actin (1:10,000; Cell Signaling Technology) expression. Secondary antibodies were horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG (both 1:5000; Sigma-Aldrich). Densitometry was performed using ImageJ software (http://rsb.info.nih.gov/ij).

Lipid Analyses

After 4 days of incubation, cells seeded on chamber slides (Corning, Rochester, NY) were stained with 0.3% Oil Red O (ORO; Fisher Biotec, Wembley, Australia) according to our published protocol. Slides were viewed using a Nikon (Tokyo, Japan) Eclipse E800 microscope. The size of the stained area was quantified using ImageJ.

Cells seeded in 10-cm polystyrene-coated dishes (Corning Falcon) were extracted with chloroform and methanol, and the lipid extractions were separated by high-performance thin-layer chromatography (HPTLC), as previously described. In brief, for nonpolar lipid analysis, the plate (Silica gel 60, Merck, Darmstadt, Germany) was developed in hexane:diethyl ether:acetic acid (80:20:1.5, vol/vol/al alone. For phospholipids analysis, the plate was developed sequentially in chloroform:methanol:water (60:30:5 vol/vol) and then hexane:diethyl ether:acetic acid (80:20:1.5, vol/vol/vol). Bands were visualized according to published protocols, and the intensities of bands were quantified using ImageJ.
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Single Radial Enzyme Diffusion Assay

After being treated with 21% O2, 1% O2, 10-μM Roxa, or a combination for 10 days, cell numbers were counted with a hemocytometer and the cell lysates and cell culture supernatants were collected, as previously described.8 DNase II activity in fresh samples was analyzed by single radial enzyme diffusion assay in agarose gels according to published protocols.8,56 Cell lysates and culture media were used to measure the DNase II activity inside and outside of the cells, respectively. After incubation for 3 to 24 hours at 37°C, the area of the dark circle in the gel was measured under an UV transilluminator at 312 nm. For comparison among conditions, values were normalized to cell number.

Statistical Analysis

The significance of the differences between groups was determined by using ANOVA and Fisher’s least significant difference multiple comparisons test (Prism 5; GraphPad Software, Inc., La Jolla, CA). Values with $P < 0.05$ were considered statistically significant.

RESULTS

Expression of HIF1α in the Human MG

To determine whether HIF1α is present in the human MG, we evaluated eyelid tissues for HIF1α staining. Our results show that human MG acinar epithelial cells express HIF1α. This protein appeared to locate primarily within nuclei (Fig. 1, arrows), and its expression appears to be elevated near the central area of acinar complexes.

Influence of Low O2 Levels and Roxa on HIF1α Expression in IHMGECs

To determine whether HIF1α is also expressed in vitro and whether different treatments impact the expression of this protein, we exposed IHMGECs to Roxa (10-μM) in both normoxic (21% O2) and 1% O2 environments for a 6-hour period. As shown in Figure 2, we found that the administration of both low O2 and Roxa significantly increased the HIF1α expression in IHMGECs. Roxa by itself induced significantly more HIF1α than low O2 alone. The combination of both Roxa and low O2 stimulated the greatest accumula-
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Impact of Low O2 and Roxa on Lipid Accumulation and Composition in IHMGECs

To examine whether 1% O2 exposure and/or Roxa administration stimulate the accumulation of neutral lipids within IHMGECs, we treated IHMGECs with Roxa (10-μM) in either normoxic or 1% O2 conditions for 4 days. Analysis of nonpolar lipids by HPTLC demonstrated that Roxa, but not low O2 alone, increased the levels of triglycerides and free fatty acids under both normoxic and hypoxic conditions, as compared to controls (Fig. 3). The 1% O2 and Roxa treatments also appeared to enhance the expression of unidentified nonpolar (Fig. 4c) and polar (Fig. 4d) lipids, respectively. Neither Roxa nor 1% O2 had any effect on the amounts of cholesterol esters, wax esters, phosphatidylethanolamine, phosphatidylinositol, phosphatidylcholine, phosphatidic acid, or lysophosphatidylcholines in IHMGECs (data not shown).

Impact of Low O2 and Roxa on the Terminal Differentiation of IHMGECs

We have also previously found that low O2 increases DNase II activity in, and the terminal differentiation of, IHMGECs. To determine whether Roxa treatment can reproduce this hypoxic effect, we treated IHMGECs with Roxa in both normoxic and 1% O2 conditions for 10 days and then processed cells for counting and the analysis of DNase II activity in cell lysates and supernatants. As shown in Figure 5, we discovered that the administration of both Roxa and low O2 significantly increased the DNase II activity in IHMGEC lysates and their culture supernatants. The magnitude of this effect was greatest in the Roxa and low O2 combination, followed by 1% O2 alone and then by Roxa alone. These effects were paralleled by corresponding significant decreases in cell numbers. Such decreases would be expected, given that terminal differentiation of IHMGECs involves cellular disintegration.
Figure 4. The effect of low O₂ and Roxa on the expression of neutral lipids and phospholipids in IHMGECs. IHMGECs were exposed to 21% O₂, 1% O₂, or Roxa, alone or in combination, for 4 days and then the lipid extracts were analyzed by HPTLC. Band intensity was measured with ImageJ; control band intensity was set to 1, and data (mean ± SE) are reported as fold-change compared to controls. The data were pooled from three independent experiments (*P < 0.05, **P < 0.01). (a, b) Roxa significantly increased triglyceride and free fatty acid contents in IHMGECs in both 21% O₂ and 1% O₂ conditions. Arbitrary units stand for the normalized intensity of different bands to that of control. (c, d) One percent O₂ and Roxa appeared to increase the expression of unidentified bands in nonpolar (arrow) and polar (arrow head) lipids, respectively.

Discussion

We conducted this study to begin to test our hypothesis that HIF1α mediates the beneficial effects of hypoxia on HMGE function. Our findings demonstrate that HIF1α is expressed by human MG acinar epithelial cells in vivo. Further, our results show that exposure to 1% O₂ or to Roxa increases the levels of HIF1α, the number of lipid-containing vesicles, the content of neutral lipids, and the activity of DNase II in IHMGECs in vitro. These data support our hypothesis and suggest an important role for HIF1α in the regulation of MG function.

We found that HIF1α protein is located in vivo primarily within nuclei of HMGEcs and especially in those cells situated near the central areas of MG acini. This nuclear location is consistent with findings in other sebaceous glands but uncommon in non-sebaceous tissues. The accumulation of HIF1α in the center of acinar complexes may be because this area is likely the most hypoxic in the MG. The reason is that the O₂ source for the MG is the vasculature located beyond the MG basement membrane. By Krogh’s law, this would establish an O₂ gradient, with the highest O₂ concentration within the blood vessels and the lowest in the central acinar region. Such hypoxic conditions would be expected to prevent HIF1α degradation and promote its translocation to the nucleus. The nuclear location of HIF1α in HMGEcs indicates that this protein is active and able to stimulate gene transcription.

We discovered that exposure to hypoxic conditions, as well as to the hypoxia-mimetic Roxa, increases the levels of HIF1α in IHMGECs. The mechanisms underlying these low O₂ and drug responses may be the same. To explain, under normoxic conditions HIF-prolyl hydroxylase (PH) domain enzymes destabilize HIF proteins by hydroxylating two prolyl residues in the α subunit. However, both low O₂ levels and Roxa inhibit HIF-PH, thereby enhancing the steady-state levels of HIF1α. We found that Roxa treatment was more effective than hypoxia alone in stimulating the accumulation of HIF1α. This result may be due to a stronger HIF-PH inhibiting effect or to a delay in achieving the desired O₂ concentration in the culture media during the 6-hour time course of our experiments. O₂ has a relatively low solubility and diffusion rate in aqueous solutions, and its concentration in culture media may not reach equilibrium with the hypoxic chamber air for...
up to 3 hours. Thus, the IHMGECs may have been exposed to a final 1% O₂ environment, as compared to Roxa, for less time. Of interest, the combination of both low O₂ and Roxa induced the highest levels of HIF1α.

The 1% O₂ environment and Roxa administration led to a significant accumulation of neutral lipid-containing droplets in IHMGECs. This effect may have been mediated through the increased content of HIF1α, given that this protein is involved in modulating lipid signaling and synthesis, lipid uptake and transport, free fatty acid and sterol metabolism, and lipid droplet biogenesis. These droplets are the major lipid storage organelle in most eukaryotic cells and contain variable amounts of neutral lipids, including triglycerides and free and esterified sterols, enclosed by a phospholipid monolayer. The enhanced lipid droplet content in IHMGECs in response to low O₂ and/or Roxa exposure is consistent with the effect of a hypoxic environment in other cell types.

In contrast to our present results, we did not observe a change in the amount of neutral lipids in response to low O₂ in IHMGECs in a previous study. These inconsistent findings may be due to several factors, including differences in experimental design and staining procedures. In our earlier study, we exposed IHMGECs to 3% O₂, unlike the 1% O₂ in the current experiments. Our rationale for this decrease was that we had discovered that mouse MGs stain positively for pimonidazole, indicating that the O level in MGs is less than 1.3%. In addition, we previously used LipidTOX (Thermo Fisher Scientific) reagent to identify neutral lipids in IHMGECs, and found that most staining occurred within lysosomes. These lysosomes may well be lamellar bodies, which contain cholesterol, neutral lipids, phospholipids, and various enzymes. In the current study, we utilized ORO staining and bright-field microscopy, which permit the visualization of neutral lipids in lipid droplets. Both lamellar bodies and lipid droplets are organelles specialized for lipid storage, and there is evidence that lamellar bodies may be derived from, or transform into, lipid droplets. Considering that both lipid droplets and lamellar bodies exist in the human MG, their specific roles in lipid dynamics await clarification. Finally, it is important to note that we used different methods to quantitate
the extent of neutral lipid expression in our previous versus current studies. In our earlier study, we measured the intensity of LipidTOX staining,76 whereas in our present experiments we calculated the area of ORO staining. Intensity and area measurements do not necessarily lead to equivalent results (Yang Liu, unpublished data).

We discovered that the administration of 1% O2 and/or Roxa promoted the terminal differentiation of IHMGECs, as indicated by the significant increase in DNa II activity in cell lysates and culture supernatants. DNa II is a lysosomal enzyme74 that is typically activated by acidic conditions (pH 4.5–5.5) and then translocates from lysosomes to the nucleus.75 Within that location, DNa II triggers programmed cell death and ultimately holocrine secretion.11,75 Once again, the heightened levels of HIF1α may have mediated this process. Activation of HIF1α stimulates glycolysis and lactic acid production, which decreases the intracellular pH.76 HIF1α also reduces the intracellular pH through modulation of carbonic anhydrase (CA) IX,78 CA IX expression, which we have recently identified in HMGECs in vivo (Yang Liu, unpublished data), is increased by HIF1α.79 The CA enzymes, in turn, could increase intracellular H+ mobility and promote cellular acidification.80 These HIF1α effects on pH would destabilize lysosomal membranes, cause the release of lysosomal enzymes, and lead to the activation of DNa II.81–84 For comparison, hypoxic environments and mimetics also induce such lysosomal disruption in other cell types.84–87

Overall, our data support our hypothesis and suggest an important role for HIF1α in the regulation of MG function. Our findings also suggest that the local administration of hypoxia mimetics may be beneficial for the treatment of MGD.

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A provisional patent has been filed around this technology. The intellectual property for this application is owned by the Schepens Eye Research Institute of Massachusetts Eye and Ear.

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