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Hypoxia Increases Corneal Cell Expression of CFTR Leading to Increased *Pseudomonas aeruginosa* Binding, Internalization, and Initiation of Inflammation

Tanweer Zaidi¹, Mary Mowrey-Mckee², and Gerald B. Pier¹

¹ From the Channing Laboratory, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts; and

² CIBA Vision Corporation, Division of Novartis, Duluth, Georgia.

Abstract

Purpose.—To investigate the effect of hypoxia-induced molecular responses of corneal epithelial cells on the surface of rabbit and human corneas and corneal cells in culture on interactions with *Pseudomonas aeruginosa* that may underlie increased susceptibility to keratitis.

Methods.—Organ cultures of rabbit and human corneal tissue, primary rabbit and human corneal cells, and transformed human corneal cells from a patient with cystic fibrosis and the same cell line corrected for expression of wild-type cystic fibrosis transmembrane conductance regulator (CFTR), the cellular receptor for *P. aeruginosa*, were exposed to hypoxic conditions for 24 to 72 hours. Changes in binding and internalization of *P. aeruginosa* were measured using cellular association and gentamicin-exclusion assays, and expression of CFTR and activation of NF- κ B in response to hypoxia were determined by confocal laser microscopy and quantitative measurements of NF- κ B activation.

Results.—Hypoxia induced in a time- and oxygen-concentration-dependent manner increased association and internalization of clinical isolates of *P. aeruginosa* in all cells tested. Hypoxia increased CFTR expression and NF- κ B nuclear translocation in rabbit and human cells with wild-type CFTR. Corneal cells lacking CFTR had reduced NF- κ B activation in response to hypoxia. Hypoxia did not affect the increase in corneal cell CFTR levels or NF- κ B activation after *P. aeruginosa* infection.

Conclusions.—Hypoxic conditions on the cornea exacerbate the binding and internalization of *P. aeruginosa* due to increased levels of CFTR expression and also induce basal NF- κ B activation. Both of these responses probably exacerbate the effects of *P. aeruginosa* infection by allowing lower infectious doses of bacteria to induce disease and promote destructive inflammatory responses.

To develop effective interventions for infectious diseases of the human eye, we must understand the molecular and cellular basis for the initiation and progression of microbial infection and the host response leading to resolution or disease. In regard to infectious bacterial keratitis, the reasons that extended-wear contact lenses are a major risk factor for the development of microbial keratitis are not fully understood.^{1–6} The incidence of microbial keratitis associated with contact lenses during the 1950s and 1960s was 0% with increases to 32% in the 1970s and to 52% in the 1980s.⁷ Among those who use contact lenses, ulcerative keratitis due to *P. aeruginosa* is one of the most common complications.^{4–6,8}

Corresponding author: Gerald B. Pier, Channing Laboratory, Brigham and Women's Hospital, Harvard Medical School, 181 Longwood Avenue, Boston, MA; gpier@channing.harvard.edu..

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The intact cornea is highly resistant to bacterial infection. Some of this resistance is thought to involve both antimicrobial components and physicochemical properties of the tear film and mucus layer. Microbial pathogens that penetrate mucus layers and reach epithelial cells can be further controlled by ingestion of the microbe, followed by shedding and/or an appropriate inflammatory response that effectively eliminates pathogens before significant tissue disease caused by inflammation ensues.^{9,10} When pathogens gain access to non-surface epithelial cells after scratching or similar damage to the cornea, they can become entrapped in the epithelial cells, and the normal host protective response can become a pathologic one, as the organisms now have a safe haven within which to replicate, whereas host effectors such as phagocytes are stymied in their attempts to ingest and kill the microbes. Similarly, if epithelial cells with ingested microbes become trapped underneath a contact lens for an extended period the organisms within the cells are shielded from host immune effectors, a process that could also promote progression of infection and development of significant tissue disease.

One of the consequences of extended wear of contact lenses that has been proposed to contribute to susceptibility to infectious keratitis is the development of a hypoxic state on the corneal surface, a process thought to have a direct link with keratitis. In a rabbit model, a lens contaminated with *P. aeruginosa* placed on a closed eye was more effective at inducing corneal infection than was corneal wounding or direct inoculation of bacteria into the cornea.¹¹ Studies by Ren et al.¹² also showed that the physical oxygen transmissibility properties of different contact lenses and not the lens type inversely correlates with binding of *P. aeruginosa* to human exfoliated corneal epithelial cells after overnight and extended wear of lenses. There was a significant decrease in surface epithelial cell desquamation and a significant increase in surface cell size after wearing of all types of lenses. Although hydrogel extended-wear contact lenses with high gas permeability have been approved and appear to be generally safe,¹³ it is still not clear whether these lenses reduce the incidence of keratitis,¹⁴ and case reports of *P. aeruginosa* keratitis among users of such lenses have been published.^{15,16}

Another component of corneal cells implicated in the development of *P. aeruginosa* keratitis is the ability of this organism to enter into corneal cells through the cystic fibrosis transmembrane conductance regulator (CFTR).¹⁰ In transgenic mice that lack functional CFTR protein, there was decreased epithelial cell uptake of *P. aeruginosa* and associated decreases in levels of infection and corneal disease. *P. aeruginosa* lipopolysaccharide (LPS) is specifically recognized and bound by CFTR,^{9,10,17} using a portion of the first predicted CFTR extracellular loop composed of amino acids 108–117.¹⁸ The binding of *P. aeruginosa* to CFTR leads to a rapid activation and nuclear translocation of the transcription factor NF- κ B,¹⁹ which could form the basis for development of a strong inflammatory response that in the eye could significantly contribute to corneal destruction.

To examine whether there is a connection between hypoxia and CFTR expression, binding, and internalization of *P. aeruginosa* and NF- κ B nuclear translocation that could establish a molecular and cellular basis for increased susceptibility in individuals who use extended-wear contact lenses, we exposed rabbit and human corneal tissues, as well as primary corneal cells from these two species, to hypoxic conditions and analyzed the changes in CFTR expression, bacterial interactions, and NF- κ B nuclear translocation. In addition, we established a transformed corneal cell line from corneal tissue obtained at autopsy from a patient with CF, along with a derivative of this cell transfected with a retrovirus-expressing wild-type CFTR, to analyze more closely the effect of CFTR on binding and internalization of *P. aeruginosa* by corneal cells. Our results indicated that hypoxia rapidly increased CFTR expression on corneal cells, leading to increased *P. aeruginosa* association with and internalization in these cells and enhanced NF- κ B nuclear translocation, establishing a link between one of the physical consequences of contact lens wear with the molecular response of corneal cells associated with increased susceptibility to *P. aeruginosa* keratitis.

MATERIALS AND METHODS

Bacterial Strains

For in vitro studies, we used the *P. aeruginosa* strains 6294 and 6206 isolated from human corneal infections, and laboratory strain PAO1-V (sensitive to chloramphenicol), provided by Michael Vasil (University of Colorado Health Science Center, Denver, CO). Strains were grown for inoculation into corneal cell cultures and rabbit and human eyes in organ culture, as described previously.²⁰

Organ Culture of Rabbit and Human Corneas

Rabbit eyes were obtained for bacterial association and internalization assays from Pel-Freeze Biologicals (Rogers, AR), and human corneal rims were obtained during corneal transplant surgery (courtesy of Kenneth Kenyon, Cornea Consultants, Boston, MA). Research involving human tissues adhered to the tenets of the Declaration of Helsinki. Rabbit eyes were placed in organ culture medium containing DMEM, 10% fetal calf serum, and a 1% solution of antibiotic-antimycotic and placed in a 37°C incubator for 24 to 72 hours in either atmospheric (20%) oxygen or an atmosphere of 0%, 5%, or 15% oxygen with 5% carbon dioxide, with the balance made up with nitrogen. Specialty gas blends were obtained from Medco Gases (Boston, MA).

Cell Culture

Corneas of rabbit eyes were excised and individual cells obtained for culture in supplemented hormonal epithelial medium (SHEM) to establish primary cultures in flasks, as described elsewhere.¹⁷ Primary cultures of human corneal cells were established from corneal rims prepared and cultured in flasks as described by Zaidi et al.¹⁰

Monolayer cultures of human and rabbit corneal cells were established in tissue culture wells (15 mm; Corning Costar, Inc., Corning, NY) by treating the initially cultured cells in the flasks with 1% trypsin to release them and then seeding the wells at 2×10^5 corneal cells per well. Cells were then placed into incubators with various concentrations of oxygen for bacterial association and ingestion assays.

Human Corneal Epithelial Cells Isogenic for Expression of Either the $\Delta F508$ or the Wild-Type *CFTR* Alleles

Primary cultures of epithelial cells from a human cornea obtained at autopsy from a patient with CF and homozygous for the $\Delta F508$ allele of *CFTR* were established and cultured as described by Zaidi et al.¹⁰ Transformed cell lines were then established from the primary cells by transduction with a retroviral vector expressing either an inserted *lacZ* gene or a wild-type copy of *CFTR*, as described,²¹ with confirmation of the proper production of wild-type CFTR by acquisition of a cyclic-adenosine monophosphate (AMP)-inducible chloride ion conductance.

P. aeruginosa Association Assays

Whole rabbit corneas or cell cultures were placed in incubators for 24 to 72 hours in atmospheres with different concentrations of oxygen—0%, 5%, 15%, or 20%—and 5% carbon dioxide, with the balance made up of nitrogen. For some experiments the corneas were scratch-injured, as described,^{17,22,23} just before infection. Corneas were infected with clinical keratitis isolates of *P. aeruginosa* for 30 minutes to measure the total amount of *P. aeruginosa* associated with the cornea, as described.^{22,24} “Association” denotes both adherent and internalized bacteria.

To determine whether the effects of hypoxia on *P. aeruginosa* infection were reversible, we incubated rabbit eyes in 15% oxygen for 24 hours and then moved a portion of the eyes to 20% oxygen for an additional 24, 48, or 72 hours. Another portion was continuously incubated in reduced oxygen, and control eyes were continuously maintained in 20% oxygen. After these incubation periods, *P. aeruginosa* was applied to the whole eyes and an association assay performed to measure the colony-forming units (cfu) of *P. aeruginosa* that bound to the corneas.²²

Cellular Ingestion Assays

Rabbit corneas in organ culture or primary or transformed corneal cells were incubated in a 37°C incubator with atmospheres containing 0% to 20% oxygen as described above for 12 to 72 hours. Ingestion of *P. aeruginosa* by cultured cells, was measured by gentamicin exclusion assay, as described previously.^{9,17,22} In brief, approximately 10⁶ cfu of *P. aeruginosa* was added to monolayers of 10⁵ cultured corneal cells. Infected cells were placed for 3 hours at 37°C in 5% CO₂. Nonadherent bacteria were washed away, and 300 µg of gentamicin/mL was added to kill extracellular bacteria. After 1 hour of exposure to antibiotic, cells were washed, and intracellular bacteria were released from the cells by lysis with 0.5% Triton X-100, diluted, and plated for bacterial enumeration. In each assay a control was run wherein cells were first lysed with 0.5% Triton X-100, and then antibiotic was added followed 1 hour later by plating of the centrifuged bacterial pellet to ensure sufficient antibiotic had been added to kill all potential extracellular bacteria.

For analysis of the role of CFTR in internalization of *P. aeruginosa* into corneal epithelial cells, we used as an inhibitor of ingestion a 10-amino-acid peptide composed of amino acids 108–117 of the CFTR molecule. This was synthesized along with a 10-amino-acid peptide composed of the same residues but in a scrambled order, as described.¹⁸ Twenty-five nM of peptide was added to cell cultures along with *P. aeruginosa* strain 6294, and cellular ingestion assays were completed, as described in the preceding paragraph.

Immunohistochemical Staining of Corneas and Cells for NF-κB and CFTR

For immunohistochemical staining of whole corneas, corneal tissue was excised from the surface of uninfected (control) or infected eyes and fixed in 4% formaldehyde for NF-κB and CFTR immunohistochemical analysis. For microscopic analysis of the immunohistochemical staining reactions, tissues were embedded in paraffin and sections cut on a microtome.

Corneal cells in culture were infected with *P. aeruginosa* for 15 minutes to 3 hours in 35-mm glass-bottomed microwell dishes (MatTek Corp., Ashland, MA), the unbound and uningested bacteria removed by washing three times, and the cells fixed and permeabilized with 1% paraformaldehyde and 0.2% Tween-20.

Fixed corneal tissues and cells were processed for immunohistochemical analysis of NF-κB nuclear translocation and CFTR expression, as described.¹⁹ Tissue sections were first deparaffinized then rehydrated for staining. Fixed cells and tissue sections were then incubated with rabbit anti-human (p65) NF-κB (Santa Cruz Biotechnology, Santa Cruz, CA) for 90 minutes at 37°C, washed, and incubated with Alexa Fluor 594 (Molecular Probes, Eugene, OR) dye-labeled goat anti-rabbit IgG at 37°C for 90 minutes. After extensive washing, the nucleic-acid stain 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) was added, to visualize the nucleus and also to visualize the translocation of NF-κB into the nucleus. Deparaffinized, rehydrated tissue sections and fixed, permeabilized cells in dishes were stained for CFTR expression by incubation with the mouse anti-CFTR monoclonal antibody CF3²⁵ for 90 minutes and counterstained with FITC-conjugated anti-mouse IgM. Sections were visualized by confocal laser microscopy.

Scanning Electron Microscopy

After incubation in 20% or 15% oxygen for 24 to 72 hours, rabbit corneas were fixed in 0.2 M cacodylate buffer (pH 7.4) containing 2.5% glutaraldehyde for analysis by scanning electron microscopy. Microscopy was performed as has been described.²⁶

Quantitation of Cellular NFκB

Quantification of the amount of activated cellular NF-κB present in different cells incubated in 20% or 15% oxygen was performed with an NF-κB p65 ELISA kit (Trans-Am; Active Motif, Carlsbad, CA) according to the manufacturer's instructions. Cellular lysates from different treatments that contained identical protein amounts were analyzed with the kit.

Statistical Analysis

Two-group comparisons were performed with unpaired *t*-tests and multigroup comparisons were made by analysis of variance (ANOVA), followed by the Fisher protected least-significant difference (PLSD) test for pair-wise comparisons. Statistical analyses were performed on a computer (Stat-View software package; SAS, Cary, NC; Macintosh computer; Apple Computer, Cupertino, CA).

RESULTS

Effect of Hypoxia on *P. aeruginosa*'s Association with Corneal Cells on Intact Rabbit Eyes Cultured In Vitro

Rabbit eyes were incubated in organ cultures for 24, 48, or 72 hours in either atmospheric oxygen or various concentrations of oxygen (15%, 5%, or 0% oxygen balanced with 5% carbon dioxide, with nitrogen making up the remainder) followed by the addition of *P. aeruginosa* strain 6294 for 30 minutes to measure associated (adherent and invaded) as well as internalized bacteria. Hypoxic conditions led to consistent increases in the total association of *P. aeruginosa* 6294 to the corneal cells, starting as early as 24 hours after incubation in 15% oxygen (Fig. 1A). At 72 hours of incubation, bacterial association was almost 100 times higher than in eyes incubated in 20% oxygen. After 24 and 48 hours of incubation in 5% oxygen, there was up to a fivefold increase in association of *P. aeruginosa* 6294 with corneas compared with corneas kept in 20% oxygen, but after 72 hours of incubation, levels of bacterial association did not increase beyond those at 48 hours (Fig. 1B). When rabbit corneas were incubated for 24, 48, or 72 hours in the absence of oxygen, levels of bacterial association increased approximately 40-fold up to 48 hours (Fig. 1C), compared with corneas incubated in 20% oxygen; but, at 72 hours in 0% oxygen, considerable cellular toxicity was observed that was associated with decreased overall bacterial association.

We tested another clinical isolate of *P. aeruginosa*, strain 6206, an ExoU⁺ strain that is toxic to cultured cells, comparing not only total bacterial association but also corneal cell internalization in rabbit corneas incubated in either 20% or 15% oxygen for 24 to 72 hours. Both bacterial cell association (Fig. 1D) and internalization (Fig. 1E) were increased from 5- to 50-fold after 48 and 72 hours in corneas incubated in 15% oxygen compared with corneas incubated in 20% oxygen. Association, but not internalization, was also increased in corneas incubated in 15% oxygen for 24 hours with this strain of *P. aeruginosa*. We also noted a consistent increase in internalization, but not association, in corneas incubated in 20% oxygen from 24 to 72 hours (Fig. 1E), suggesting that some changes in the tissue that promotes internalization had occurred due to the ex vivo conditions.

Confirmation that incubation of rabbit corneas for 48 hours in 15% oxygen generally increased *P. aeruginosa* association and invasion was obtained by using a third strain, PAO1-V (Fig. 1F), whose genome has been sequenced.²⁷ Corneas in the lower oxygen concentration had

approximately 30-fold higher levels of associated bacteria and 4-fold higher levels of internalized bacteria. Strains PAO1 and 6294 had comparable levels of association and internalization by rabbit corneal cells incubated in the different conditions, whereas the cytotoxic strain 6206 (Figs. 1D, 1E) was less adherent and invasive, perhaps due to lysis of cells and loss of bacteria caused by the cytotoxic strain.

Scanning electron microscopy of rabbit corneas incubated in either 20% or 15% oxygen for 24 to 72 hours without addition of any bacteria showed progressive disruption of the corneal surface over time with incubation at the lower oxygen concentration (Fig. 2). Fields representative of the general effect of hypoxia on the corneas are shown in Figure 2. There was notable loosening of cellular junctions; exposure of basolateral epithelial cell surfaces, which are known to be sites for *P. aeruginosa* binding and internalization^{28,29}; and appearance of cells actively desquamating from the corneal surface. Thus, hypoxic conditions were associated with increasing disruption of the integrity of the corneal surface during longer incubation times in 15% oxygen.

We next examined whether the effect of hypoxia on *P. aeruginosa* corneal associations could be reversed by incubating rabbit corneas in 15% oxygen for 24 hours and then returning some of the corneas to 20% oxygen and comparing bacterial association with that in corneas incubated continuously in either 20% or 15% oxygen. In this experiment, eyes exposed to 15% oxygen for 24 hours had increased levels of association of *P. aeruginosa* compared with eyes incubated for 24 hours in 20% oxygen (Fig. 3). However, eyes incubated for 24 hours in 15% oxygen and returned to 20% oxygen for 24 or 48 hours had association levels of *P. aeruginosa* 6294 comparable to that in eyes incubated continuously in 20% oxygen and significantly lower ($P < 0.05$, ANOVA and Fisher PLSD) levels of bacterial association than eyes kept continuously in 15% oxygen. Effects of short-term hypoxia (24 hours) on *P. aeruginosa* interactions with corneal cells appear reversible.

Effect of Hypoxia on Association and Invasion of *P. aeruginosa* by Cultured Corneal Cells

To determine whether rabbit and human corneal epithelial cells in hypoxic cultures would respond to *P. aeruginosa* challenge in the same manner as did intact rabbit corneas, we compared the total and internalized *P. aeruginosa* 6294 by primary rabbit and human corneal epithelial cells incubated for 48 hours in either 20% or 15% oxygen. Figure 4 shows that there were increased levels of total and internalized *P. aeruginosa* in both rabbit and human corneal epithelial cells incubated for 48 hours in 15% oxygen compared with cells incubated for 48 hours in 20% oxygen.

Effect of Hypoxia on Association and Invasion of *P. aeruginosa* by Cultured Human Corneal Cells Isogenic for Wild-Type or $\Delta F508$ Alleles of *CFTR*

Our laboratory has provided evidence that CFTR can serve as a corneal epithelial cell receptor for internalization of *P. aeruginosa*,¹⁸ and loss of this receptor in transgenic CF knockout mice or transgenic mice with the $\Delta F508$ *Cfr* allele increases host resistance to *P. aeruginosa* corneal infection.¹⁰ In the current study, we sought to examine further the role of hypoxia on *P. aeruginosa* interactions with human corneal cells and the effect of CFTR expression on the interaction by using transformed human corneal epithelial cells initially obtained at autopsy from a patient with CF and the same cell line containing a wild-type copy of functional CFTR inserted through retroviral transposition. As shown in Figure 4C, growth in 15% oxygen for 48 hours increased both the associated and internalized *P. aeruginosa* 6294, with cells expressing either wild-type or $\Delta F508$ *CFTR* alleles compared with the same cells incubated in 20% oxygen. Regardless of the concentration of oxygen, corneal cells with the wild-type *CFTR* allele had greater overall association and internalization of *P. aeruginosa* than did the

cells with only $\Delta F508$ *CFTR* alleles ($P = 0.0001$ for both overall ANOVA and the Fisher PLSD test).

To confirm that CFTR interacts with *P. aeruginosa* in the corneal cell line expressing the wild-type allele for this protein, we determined the ability of a 10-mer peptide composed of amino acids 108–117 located in the first predicted extracellular loop of CFTR to inhibit internalization of *P. aeruginosa* 6294 by cells growing in 20% oxygen (Fig. 4D). Prior work had identified this region of CFTR as the one that binds to *P. aeruginosa*.^{10,18} When 25 nM of the cognate CFTR peptide was added to the wild-type *CFTR*-expressing human corneal cell cultures, there was significant inhibition of ingestion of *P. aeruginosa* by the wild-type *CFTR* cells, but no effect of this peptide on ingestion of bacteria by the $\Delta F508$ *CFTR* cells, which have little or no plasma membrane protein (Fig. 4D). This decrease in internalization was significant when results were analyzed either in cells with no added peptide or in cells to which 25 nM of a scrambled version of the CFTR peptide had been added.

A previously described property of lung epithelial cells indicative of a CFTR-dependent epithelial response to *P. aeruginosa* is rapid translocation of NF- κ B into the nucleus within 15 minutes of infection of cells.¹⁹ As shown in Figure 4E, translocation of NF- κ B into the nucleus of the corneal epithelial cells with the wild-type *CFTR* gene was readily apparent 15 minutes after *P. aeruginosa* infection, whereas no such response was observed in the cell line with only $\Delta F508$ *CFTR*. This result shows an effect of CFTR expression in corneal cells comparable to that in lung epithelial cells, with regard to the rapid nuclear translocation of NF- κ B in response to *P. aeruginosa* infection.¹⁹

Effect of Hypoxia on CFTR Expression in the Cornea

We next examined the effect of hypoxia on CFTR expression in rabbit and human corneas by incubating rabbit eyes in 15% or 20% oxygen for 24 or 48 hours or human corneas in 15% or 20% oxygen for 48 hours. Hypoxia dramatically increased levels of CFTR in the corneal epithelium of both rabbits and humans (Fig. 5). Similarly, hypoxia increased the levels of CFTR expression in primary cultures of rabbit or human corneal epithelial cells (Fig. 5). In the transformed human corneal cells expressing wild-type CFTR, there were also increased amounts of this protein after incubation in 15% O₂, whereas the cells with only $\Delta F508$ CFTR, as expected, had no detectable protein when incubated in 20% oxygen, but did have some detectable CFTR after 48 hours of incubation in 15% oxygen (Fig. 5), perhaps because the $\Delta F508$ CFTR protein can be expressed under abnormal cell culture conditions.³⁰

Effect of Hypoxia on Nuclear Translocation of NF- κ B

The translocation of NF- κ B into the nuclei of corneal epithelial cells in intact rabbit corneas after 24 to 72 hours of incubation in 20% or 15% oxygen is shown in Figure 6. No nuclear translocation of significance was observed after 24 hours of incubation in 15% oxygen, but after 48 or 72 hours of incubation in 15%, but not 20%, oxygen there was clear evidence of NF- κ B nuclear translocation in the rabbit cornea. Similarly, after 48 hours of incubation in 15% but not 20% oxygen, there was strong NF- κ B nuclear translocation in the human cornea (Fig. 6). In primary cultures of rabbit and human corneal cells, 48 hours of incubation in 15% oxygen also induced NF- κ B nuclear translocation, which was not observed in cells incubated in 20% oxygen. In the transformed human corneal cell lines isogenic for wild-type or mutant CFTR, hypoxia induced NF- κ B nuclear translocation in the cells with wild-type CFTR comparable to the effect observed with the primary cell lines (not shown), but somewhat less so in cells with only $\Delta F508$ CFTR (not shown). In this latter line, we observed some cells with obvious NF- κ B nuclear translocation, but the percentage of the cells responding to hypoxia with NF- κ B in the nucleus was lower in the absence of wild-type CFTR. Overall, it was difficult

to quantify by microscopy whether there was an effect of CFTR expression on NF- κ B nuclear translocation in corneal cells in response to hypoxia.

For a more quantitative estimate of the effect of CFTR expression and hypoxia on NF- κ B activation in human corneal cells, we quantified the total level of the p65 subunit of this transcription factor in the nuclei of the transformed cells expressing either wild-type or Δ F508 CFTR in primary human corneal cells in culture. Incubation in 15% oxygen significantly increased the measured levels of the nuclear NF- κ B p65 subunit in both transformed cells with wild-type CFTR and primary human corneal epithelial cells (Fig. 7A). Although there was an increase in the p65 subunit of NF- κ B in the nuclei of transformed corneal cells expressing the Δ F508 allele of *CFTR*, it was not significant ($P > 0.05$), but was consistent with the microscopic observations that some of these cells had activated NF- κ B. Of note, cultured cells with the Δ F508 *CFTR* allele grown in either 20% or 15% oxygen had decreased levels of activated NF- κ B p65 in the nucleus compared with cells with wild-type CFTR (Fig. 7A).

Effect of Hypoxia on CFTR Expression and NF- κ B Nuclear Translocation after *P. aeruginosa* Infection

We next determined whether there were differences in CFTR expression or NF- κ B nuclear translocation after 3 hours of *P. aeruginosa* infection of rabbit or human corneas and primary corneal cells grown either in 15% or 20% oxygen for 48 hours (Fig. 8). When examined by immunohistochemistry and microscopy, bacterial infection was shown to induce comparable levels of CFTR expression and NF- κ B nuclear translocation in cells exposed to either atmospheric or reduced oxygen conditions. When we quantified NF- κ B p65 nuclear translocation in transformed cells expressing either wild-type or Δ F508 alleles of *CFTR*, as well as in primary human corneal epithelial cell cultures, we found no significant differences in the NF- κ B activation response of cells to *P. aeruginosa* based on prior culture for 48 hours in 20% or 15% oxygen (Fig. 7B). However, the transformed cells with wild-type CFTR had an overall greater degree of NF- κ B activation in response to *P. aeruginosa* than did the transformed cells with Δ F508 CFTR, identical with effects previously reported in lung epithelial cells isogenic for wild-type or Δ F508 *CFTR* alleles.¹⁹

DISCUSSION

It is generally accepted among practitioners that extended wear of certain types of contact lenses increases the risk of development of microbial keratitis and significant vision loss,^{5, 31,32} although some have questioned the adequacy of the clinical and epidemiologic studies that support this conclusion.³³ Molecular mechanisms studied to account for the increased susceptibility to keratitis include enhanced binding of bacterial pathogens to corneal cells^{34–38} in individuals who use extended-wear lenses or certain lens care solutions, decreased cellular exfoliation and turnover associated with decreased ability to enter into an apoptotic state,^{39, 40} and changes in the homeostatic regulation of corneal epithelial cell turnover.^{40–43} One factor thought to promote many of these responses is the production of a hypoxic state on the corneal epithelium during extended wear of low-oxygen-permeable lenses.^{40,43,44} The recently introduced high and hyper-oxygen-permeable lenses seem, in the early phases of their use, to reverse corneal epithelial changes associated with hypoxia and to decrease the incidence of microbial keratitis.^{13,34,35,44,45} However, case reports of *P. aeruginosa* microbial keratitis associated with extended wear of high-oxygen-permeable lenses continue to accrue,^{15,16} and definitive epidemiologic studies assessing the true risk of keratitis among individuals who wear high- or hyper-oxygen-permeable lenses for extended periods have not yet been published.^{14,31}

Whereas prior studies correlating hypoxic conditions to increased binding of *P. aeruginosa* to corneal cells have clearly shown an effect,^{34,36–38,44} specific cellular and molecular

mechanisms to account for this have not been addressed with any degree of intensity. We have shown that a key receptor for binding and internalization of *P. aeruginosa* by corneal epithelial cells is CFTR,¹⁰ which recognizes the conserved outer core of the bacterial LPS.^{9,18,23} In contrast to patients with CF without functional CFTR, who are hypersusceptible to chronic lung infections with *P. aeruginosa*,⁴⁶ the loss of this protein in the eye increases resistance to *P. aeruginosa* corneal infection in a murine model of scratch injury.¹⁰ Uniting these apparent disparate observations is the likelihood that on both the lung and corneal epithelial surface CFTR-mediated recognition, binding and ingestion of *P. aeruginosa* is normally a host protective mechanism, leading to cellular desquamation of the internalized bacteria, activation of subclinical, protective innate immunity and inflammation, and resolution of the infection. In the CF-affected lung, failure of this mechanism allows bacterial colonization and ultimately infection of the airways with bacteria trapped in mucus plugs. On the eye of an individual with an extended-wear lens, entrapment of the epithelial cells with ingested bacteria inhibits clearance, promotes bacterial growth, and promotes elaboration of toxins and other virulence factors, and, along with lens-induced changes on the corneal surface, becomes a mechanism whereby the bacteria escape host immune effectors and promote destructive disease. In the absence of CFTR expression on corneal cells, there is less internalization of *P. aeruginosa*, leaving the extracellular bacteria susceptible to host defense effectors, even when an extended-wear contact lens is present.

Based on the data reported in this study, hypoxia clearly increases adherence of *P. aeruginosa* to rabbit corneas, primary rabbit and human corneal epithelial cells, and transformed human corneal epithelial cells. Mechanistically, modest hypoxia (15% oxygen) increases CFTR expression which increases bacterial association and internalization, and this leads to increased levels of corneal epithelial cell activation of NF- κ B. Hypoxia alone also increased NF- κ B activation, indicating that corneal cells exposed to low oxygen may already be in a state of activation for production of inflammatory cytokines, which can clearly participate in the onset of disease if infection then develops.^{47,48} Thus, hypoxic conditions on the corneal surface may exacerbate the development of *P. aeruginosa* keratitis by increasing the amount of CFTR, increasing the internalization of the bacteria into cells where they are protected from host phagocytes, and increasing the amount of NF- κ B-induced cytokine production, turning a normal protective host response into a pathologic one. However, it should be noted that the cultured corneas probably lacked an intact tear film, which, if present, could have modified some of the findings. Nonetheless, we believe this impact would mostly be manifest in absolute and not relative effects on *P. aeruginosa* binding and internalization in atmospheric or reduced oxygen conditions.

Few studies have examined the effects of hypoxia on CFTR expression in any cell. Transgenic *Cfr* knockout mice have been reported to have reduced ventilatory capacity in response to severe hypoxia.⁴⁹ Individuals susceptible to high-altitude pulmonary edema had decreased levels of mRNA for CFTR when they were at high altitude, whereas those who did not have pulmonary edema had no overall changes in transcription of mRNA for CFTR,⁵⁰ suggesting that an inability to maintain normal levels of this protein under hypoxic conditions could lead to pathologic responses. Pulmonary neuroendocrine cells in the lung contain oxygen sensors and also express CFTR, and decreased levels of CFTR expression in these cells abolishes hypoxia-induced release of the signaling molecule 5 hydroxy-tryptophan and reduces secretory responses to high potassium.⁵¹ Overall, it appears that in different tissues and cells, hypoxia affects CFTR expression and function and potentially affects or exacerbates pathologic responses associated with the hypoxia-induced changes in CFTR levels or function.

These results indicate additional molecular mechanisms that associate hypoxia with increased susceptibility of the cornea to *P. aeruginosa* infection. Hypoxia-induced increases in CFTR expression led to increased binding and internalization of the organism into corneal cells, both

in culture and on the surface of an intact cornea, providing a means for the organism to proliferate, elaborate toxins, exacerbate inflammation, and escape host defenses. Hypoxia also induced increased levels of NF- κ B activation, which, when combined with *P. aeruginosa* infection, could change a potential protective inflammatory response leading to bacterial clearance into a pathologic response leading to destruction of corneal tissue. However, factors such as reduced tear flow behind lenses and other effects of lenses on the integrity of the cornea likely also contribute to the increased incidence of ulcerative keratitis in individuals who use extended-wear lenses. Early studies conducted since the release of high- and hyper-oxygen-permeable contact lenses indicate that these lenses may lessen the impact of extended wear on the cornea and decrease the susceptibility to *P. aeruginosa* and other causes of microbial keratitis,^{13,34,45} consistent with the findings reported herein of effects of hypoxia on bacterial association and invasion. However, case reports continue to be published¹⁶ that indicate *P. aeruginosa* ulcerative keratitis can occur in individuals who use high-oxygen-permeable lenses, suggesting that some of the risk factors for *P. aeruginosa* keratitis are independent of the state of hypoxia on the eye during lens wear.

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Figure 1.

Effect of hypoxia on association and internalization of *P. aeruginosa* by the corneal epithelia on rabbit eyes in organ culture. (A–C) Levels of *P. aeruginosa* strain 6294 associated with corneal cells on intact rabbit corneas incubated in the indicated level of oxygen for the indicated time period. (D, E) Levels of cytotoxic *P. aeruginosa* strain 6206 associated (D) with or internalized (E) by corneal cells on an intact rabbit cornea incubated for the indicated times in 20% or 15% oxygen. (F) Associated and internalized levels of *P. aeruginosa* PAO1 by corneal cells on an intact rabbit cornea incubated for 48 hours in 20% or 15% oxygen. Bars, means of four to six samples; error bars, SEM. * $P < 0.05$, t -test when comparing cells kept in 20% oxygen with cells kept in lower concentrations of oxygen.

Figure 2.

Effect of hypoxia on the corneal surface morphology rabbit eyes. Rabbit eyes were incubated for the indicated time in either 20% or 15% oxygen. Shown are areas representative of a significant portion of the corneal surface. Incubation in 15% oxygen showed cellular disruption, loosening of junctions and exposure of basolateral surfaces along with cellular desquamation. Magnification, $\times 400$.

Figure 3.

Reversibility of the effect of hypoxia on the association of *P. aeruginosa* strain 6294 with rabbit corneas. *P. aeruginosa* association and internalization into the corneal cells was determined after continuous incubation in 20% or 15% oxygen, or 15% oxygen for 24 hours then 20% oxygen for either an additional 24 or 48 hours, giving a total incubation time of 48 and 72 hours, respectively, for these corneas. Bars, means of four to six samples; error bars, SEM. * $P < 0.05$, t -test for two-group comparisons as well as ANOVA for three-group comparisons, along with $P < .05$ for the Fisher PLSD for pair-wise comparisons. Comparisons are for results from cells indicated by *asterisks* with results from cells in the same grouping incubated continuously in 20% oxygen.

Figure 4.

Effect of hypoxia on association and internalization by cultured cells of *P. aeruginosa* strain 6294. Associated (A on x-axes) and internalized (I on x-axes) *P. aeruginosa* 6294 in rabbit (A) or human (B) primary corneal epithelial cells in cultures incubated for 48 hours in either 20% or 15% oxygen. (C) Association and internalization of *P. aeruginosa* 6294 into transformed human corneal cells expressing either wild-type or ΔF508 CFTR incubated in either 20% or 15% oxygen. (D) Inhibition of internalization by wild-type or ΔF508 CFTR transformed human corneal cells in culture in 20% oxygen by peptide 108–117 of CFTR but not by a scrambled version of this same peptide. (E) NF-κB p65 subunit nuclear translocation 15 minutes after exposure to *P. aeruginosa* 6294 of transformed human corneal cells expressing either wild-type or ΔF508 CFTR. NF-κB p65 in the nucleus is shown in magenta, representing the colocalization of NF-κB p65, visualized by a red-dye-conjugated reagent, and the blue nucleus, visualized by staining with DAPI. Magnification, ×800.

Figure 5.

CFTR expression in rabbit and human corneas after 24 or 48 hours of incubation in atmospheric or 15% oxygen. Expression of CFTR in rabbit and human corneas, primary corneal cells, or transformed corneal cells expressing wild-type (WT) CFTR was increased after culture for 48 hours in 15% oxygen compared with culture in 20% oxygen. In transformed human corneal cells expressing $\Delta F508$ CFTR, a small but perceptible increase in the amount of the mutant protein in cells exposed to hypoxic conditions was noted.

Figure 6.

Effect of hypoxia on translocation of NF-κB in rabbit or human corneal epithelium or primary corneal cell cultures. After the indicated time of incubation in 20% or 15% oxygen, the presence of active NF-κB in the nucleus of both the corneal tissue and the cells was visualized by immunohistochemical staining. NF-κB p65 subunit in the nucleus is stained *magenta*, representing the colocalization of NF-κB p65, visualized by a *red*-dye-conjugated reagent, and the *blue* nucleus, visualized by staining with DAPI. Magnification: corneal epithelium, $\times 100$; primary epithelial cells, $\times 400$.

Figure 7.

Effect of oxygen concentration on the amount of activated NF- κ B p65 subunit present in the nuclei of corneal cells. Transformed human corneal cells, expressing either wild-type or Δ F508 *CFTR*, or primary human corneal cells were incubated for 48 hours in 20% or 15% oxygen without infection (A) or after infection (B) with *P. aeruginosa*. Bars, means of triplicate samples; error bars, SEM. Probabilities were derived by comparison of amount of activated NF- κ B in cells exposed to 15% oxygen with that in cells exposed to 20% oxygen, using the Fisher PLSD determination for pair-wise comparisons after ANOVA, which generated, overall, $P < 0.05$.

Figure 8.

Effect of hypoxia on expression of CFTR and NF-κB nuclear translocation after infection with *P. aeruginosa*. Comparable CFTR expression and NF-κB p65 subunit nuclear translocation were observed in rabbit or human corneal tissues, or primary rabbit or human corneal cells, incubated in either atmospheric (20%) or 15% oxygen for 48 hours followed by 3 hours of infection with *P. aeruginosa*. CFTR expression (*green*) and NF-κB p65 in the nucleus (*magenta*) were visualized by immunohistochemical staining. NF-κB p65 in the nucleus is stained *magenta*, representing the colocalization of NF-κB p65, visualized by a *red*-dye–conjugated reagent, and the *blue* nucleus, visualized by staining with DAPI. Magnification: corneas, ×100; corneal cells, ×400.