Hypoxia-induced changes in Ca\textsuperscript{2+} mobilization and protein phosphorylation implicated in impaired wound healing

Albert Lee, Kelsey Derricks, Martin Minns, Sophina Ji, Cheryl Chi, Matthew A. Nugent, and Vickery Trinkaus-Randall

1Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts; and 2Department of Ophthalmology, Boston University School of Medicine, Boston, Massachusetts

Submitted 29 April 2013; accepted in final form 24 March 2014

Damage to tissue activates intricate underlying mechanisms that mediate the healing process. Communication between cells is generated immediately after injury and continues during the migration phase and the later phases of proliferation and extracellular matrix reassembly (13, 14, 23). This reepithelialization requires the precise control of glutamatergic and purinergic signaling pathways. Injured cells release nucleotides that serve as ligands for cell-surface purinergic receptors, and injured neurons release components, including ATP and glutamate, that bind their respective receptors. The release of nucleotides can modulate Ca\textsuperscript{2+} homeostasis (4, 11, 43, 52, 54).

Neuronal transmitters such as glutamate bind to metabotropic G protein-coupled receptors or to ionotropic receptors, including 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid (AMPA), kainate, and N-methyl-D-aspartate (NMDA) receptors. It has been reported that epithelial cells do not respond to AMPA or kainate, but they do respond to glutamate in neuronal wound media and to NMDA. These observations led us to speculate that purinergic and NMDA receptors play modulatory roles in Ca\textsuperscript{2+} mobilization (43). While the activation of specific purinergic receptors provides an alarm signal to neighboring cells, the response appears to be modulated by the presence of NMDA receptors that facilitate cell-cell communication between the corneal epithelium and innervating neurons (43). Under different environmental conditions, tissues can respond in a number of ways. We hypothesize that hypoxia adversely affects repair pathways, such as nucleotide-induced Ca\textsuperscript{2+} mobilization and purinergic-glutamatergic signaling, leading to impaired wound closure.

The cornea is an ideal tissue for study of hypoxic events, as it is constantly exposed to chemical fluxes in the external environment (37). When healthy, the cornea is avascular, transparent, and innervated, and its epithelium undergoes constant renewal. A dense network of nerve endings derived from the stromal branches of trigeminal nerves penetrate the epithelium (41). The neurons and secreted products provide sensation and maintain homeostasis of the tissue (2, 29, 42). Because of its avascularity, the cornea requires atmospheric O\textsubscript{2} (45), and a reduction of O\textsubscript{2} availability impairs its physiology and function. Hypoxia may result from prolonged eyelid closure or extended use of impermeable contact lenses (35, 49), which can lead to a reduction of O\textsubscript{2} tension from 21% to 8%. The prolonged decrease in O\textsubscript{2} levels can induce an increase in corneal thickness, corneal edema, and acidification of the corneal environment resulting from an accumulation of CO\textsubscript{2} (8, 9, 26). However, the effect of hypoxia on wound-healing mechanisms is not well understood.

The goal of the present study is to determine changes in wound healing induced by hypoxia with use of in vitro and ex vivo corneal models. In epithelial cultures, there was a decrease in the distance that the Ca\textsuperscript{2+} wave propagated after injury due to diminished Ca\textsuperscript{2+} release from intracellular stores. Hypoxia also diminished the signaling between epithelial cells after they were stimulated with wound media from injured neurons. The decreased Ca\textsuperscript{2+} response and decreased phosphorylation of paxillin at Y118 may lead to a decrease in the rate of wound repair. These results demonstrate that hypoxia alters nucleotide/glutamate-induced Ca\textsuperscript{2+} mobilization, resulting in impaired cell-cell communication and delayed wound repair.
MATERIALS AND METHODS

Cell culture. A human corneal limbal epithelial (HCLE) cell line was used as described elsewhere (22). Previously, we demonstrated that HCLE cells and primary corneal epithelial cells respond similarly to injury (4). Briefly, cells were cultured in keratinocyte serum-free medium supplemented with 30 μg/ml bovine pituitary extract, 0.032 nM EGF (Invitrogen, Carlsbad, CA), 100 U/ml penicillin-streptomycin (Mediatech, Manassas, VA), and 0.3 mM CaCl₂. Incubation media were switched to quiescent media lacking EGF and bovine pituitary extract 24 h before the experiment.

Ex vivo culture. All experiments were conducted in voluntary compliance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Animal studies were performed according to an approved Institutional Animal Care and Use Committee protocol. An ex vivo cornea organ culture model system was used as described elsewhere (1). Briefly, Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were euthanized and decapitated. A 3-mm-diameter epithelial debridement was made on each cornea; then the eyes were enucleated and maintained at 4°C. Corneas were dissected from the eyes, leaving the scleral rim, and inverted, and endothelial concavity was filled with DMEM containing 0.75% low-melting-point agar. The constructs were placed, endothelial-side-down, in 35-mm₂ culture dishes, and DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% nonessential amino acids (Mediatech) was added. The corneal surface was moistened with media every 6 h.

Primary trigeminal ganglia cultures. Trigeminal neurons were isolated and cultured as described elsewhere (43). Briefly, neonatal Sprague-Dawley rats (Charles River Laboratories) were euthanized, and the trigeminal nerve was removed and incubated in a protease solution containing trypsin-EDTA, 1.5 mg/ml collagenase A, and 0.05 mg/ml papain for 1 h at 37°C on a nutator. Cells were triturated and dissociated, resuspended in neurobasal medium supplemented with B27, 0.1 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.5% amphotericin B (Fungizone), and 15 ng/ml neuronal growth factor (Invitrogen, Grand Island, NY), and plated on 100-mm₂ tissue culture dishes. Conditioned media were collected from the cultures before injury, and neuronal wound media were collected after cultures were scratch-wounded. The media were then centrifuged and the supernatant stored at 4°C.

Hypoxic incubation. Experimental in vitro and ex vivo samples were incubated in 1% O₂-5% CO₂-94% N₂ (hypoxia) using a hypoxic chamber (32). Control ex vivo samples were incubated under normoxic conditions (21% O₂-5% CO₂-74% N₂) for various times. Control ex vivo samples were incubated under normoxic conditions (21% O₂-5% CO₂-74% N₂) for various times.

Fluorescence recovery after photobleaching. HCLE cells were plated onto glass-bottom dishes coated with FBS. When HCLE cells reached ~80% confluency, the complete medium was replaced with quiescent medium, and the cells were incubated under normoxic or hypoxic conditions. HCLE cells were loaded with the fluorescent dye 5-carboxyfluorescein diacetate acetoxymethyl ester in the presence of 0.02% pluronic acid for 20 min at room temperature (Invitrogen). The cells were washed with HEPES-buffered saline (pH 7.3; Fisher Scientific, Pittsburgh, PA). Fluorescence recovery after photobleaching was performed as described previously (43). One cell in the field was photobleached using repeated laser iterations, and another cell was monitored for the same time and used to normalize the rate of recovery of the photobleached cells. Fluorescence recovery over time was monitored for 400 frames (1 frame per 0.789 s).

Western blot analysis. Lysates from HCLE cells were harvested, homogenized, and centrifuged at 11,000 g, and supernatant was collected as described previously (10). Protein concentration was determined using a bichinchoninic acid assay, and an equivalent amount of protein was loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and transferred to a polyvinylidene difluoride membrane (Millipore, Tullagreen, Ireland). Immunoblots were blocked with 5% BSA in Tris buffer (10 mM Tris, 100 mM NaCl, and 0.1% Tween 20, pH 7.5; Fisher Scientific) for 1 h at room temperature andCells were incubated with the specific primary antibody on overnight at 4°C. The membrane was incubated with HCLE cells and primary trigeminal neuronal cultures were cultured to 80–90% confluency, and medium was replaced with quiescent medium lacking any supplement. Cells were then incubated under normoxic or hypoxic conditions for 24 and 48 h. For determination of ATP concentration in the whole cell populations, cells were harvested in boiling Milli-Q water and vortexed for 2 min (57). For measurement of released ATP upon injury, scratch wounds were made, and medium was immediately collected in ice-cold PBS. Total ATP amount was measured using an ATP determination kit according to the manufacturer’s protocol (Invitrogen). ATP was normalized to protein using bichinchoninic acid (Pierce, Rockford, IL) (7).

Immunohistochemistry and confocal imaging. Rat corneas and HCLE cells were fixed in freshly prepared 4% paraformaldehyde for 20 min and stained for indirect immunofluorescence, as previously described (33). Cross sections of rat corneas and HCLE cells were blocked with 4% BSA in PBS and incubated with the primary antibody of choice in 1% BSA overnight at 4°C. The mouse monoclonal antibodies to anti-NMDA receptor 1 (NR1) and paxillin were used at dilutions of 1:100 and 1:250, respectively (BD Pharmingen, San Jose, CA). The mouse monoclonal antibody to fibronectin was used at 1:50 dilution (Millipore, Billerica, MA). After the samples were washed with PBS, they were incubated overnight with the appropriate Alexa Fluor secondary antibody containing 1% BSA (Invitrogen). Samples were counterstained with rhodamine phalloidin (Invitrogen) and a mounting medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). In vitro and organ cultures were imaged using an inverted laser scanning confocal microscope (700M Axiowert, Zeiss). For visualization of innervating neurons in the cornea, green fluorescent protein (GFP)-tagged Thy1 (Thy-1-GFP) transgenic rat corneas were imaged using an upright multiphoton-confocal microscope system (LSM 710 NLO, Carl Zeiss). Z sections were collected, and a three-dimensional image was constructed. Negative controls were imaged first, and experimental samples were imaged at the same settings to minimize nonspecific background fluorescence.

For extraction of soluble proteins and stabilization of the cytoskeletal framework, cells were washed in ice-cold PBS and incubated with cytoskeleton buffer (50 mM NaCl, 30 mM sucrose, 10 mM PIPES, 3 mM MgCl₂, 1 mM PMSF, and 0.05% Triton X-100, pH 6.8) on ice and fixed in 4% paraformaldehyde in PBS (21, 44).

Imaging and analysis. Ca²⁺ mobilization was performed on epithelial cells, as described previously (10). For the live cell-imaging experiments, cultures were preincubated for 24 h under hypoxic or normoxic conditions. Cultures were transferred to HEPES-buffered saline solution containing 137 mM NaCl, 5 mM KCl, 4 mM MgCl₂, 3 mM CaCl₂·2H₂O, 25 mM glucose, and 10 mM HEPES (pH 7.6). Cells were preloaded with 10 μM fluo 3-AM (Invitrogen) in a HEPES-buffered saline solution. Fifty frames were taken to establish background fluorescence.

For extraction of soluble proteins and stabilization of the cytoskeletal framework, cells were washed in ice-cold PBS and incubated with cytoskeleton buffer (50 mM NaCl, 30 mM sucrose, 10 mM PIPES, 3 mM MgCl₂, 1 mM PMSF, and 0.05% Triton X-100, pH 6.8) on ice and fixed in 4% paraformaldehyde in PBS (21, 44).

Imaging and analysis. Ca²⁺ mobilization was performed on epithelial cells, as described previously (10). For the live cell-imaging experiments, cultures were preincubated for 24 h under hypoxic or normoxic conditions. Cultures were transferred to HEPES-buffered saline solution containing 137 mM NaCl, 5 mM KCl, 4 mM MgCl₂, 3 mM CaCl₂·2H₂O, 25 mM glucose, and 10 mM HEPES (pH 7.6). Cells were preloaded with 10 μM fluo 3-AM (Invitrogen) in a HEPES-buffered saline solution. Fifty frames were taken to establish background fluorescence.

For extraction of soluble proteins and stabilization of the cytoskeletal framework, cells were washed in ice-cold PBS and incubated with cytoskeleton buffer (50 mM NaCl, 30 mM sucrose, 10 mM PIPES, 3 mM MgCl₂, 1 mM PMSF, and 0.05% Triton X-100, pH 6.8) on ice and fixed in 4% paraformaldehyde in PBS (21, 44).

Imaging and analysis. Ca²⁺ mobilization was performed on epithelial cells, as described previously (10). For the live cell-imaging experiments, cultures were preincubated for 24 h under hypoxic or normoxic conditions. Cultures were transferred to HEPES-buffered saline solution containing 137 mM NaCl, 5 mM KCl, 4 mM MgCl₂, 3 mM CaCl₂·2H₂O, 25 mM glucose, and 10 mM HEPES (pH 7.6). Cells were preloaded with 10 μM fluo 3-AM (Invitrogen) in a HEPES-buffered saline solution. Fifty frames were taken to establish background fluorescence.
(Thornwood, NY). The field was continuously monitored for 600 frames (1 frame per 0.789 s).

An algorithm was written using MATLAB (MathWorks, Natick, MA) to analyze Ca\(^{2+}\) mobilization. After images were collected from each experiment, they were exported in a Tag Image File format. Each cell was segmented and identified by the algorithm, which enabled calculation of the fluorescence intensity of each cell. Cells were segmented by using nuclear pixel minima in combination with cell perimeters to independently identify cells. The average diameter of each cell was calculated with the assumption that each cell was circular. For each frame, fluorescence intensity values were determined for each cell and corrected for the average of their individual background during the control frame. The average fluorescence intensity was calculated from an average derived from all the cells in one frame. Calculated intensity values from each cell were combined to yield the following outputs: percent change in average fluorescence intensity over time, number of activated cells, number of clusters, and percentage of cells participating in cluster formation. Cell clusters were defined as more than three activated cells within one cell distance of each other.

Changes in mitochondrial membrane potential under normoxic and hypoxic conditions were evaluated. HCLE cells were incubated with tetramethylrhodamine ethyl ester (TMRE; Abcam, Cambridge, MA) for 30 min, basal images were obtained for 200 frames, and freshly prepared carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; 20 \(\mu\)M) was added. FCCP is an ionophore uncoupler, and its addition results in decreased TMRE fluorescence. Images were taken over time, and fluorescence intensity was quantitated and graphed.

Statistical analysis. Values are means ± SE of a minimum of three independent experiments. Statistical significance was determined by Student’s t-test with a stringency of \(P < 0.05\) or a repeated ANOVA with a stringency of \(P < 0.05\).

RESULTS

Cell-cell signaling between neurons and epithelial cells is attenuated under hypoxic conditions. Previous studies in our laboratory and others have shown that Ca\(^{2+}\) mobilization occurs upon injury to epithelia. As the cornea is highly innervated, experiments were performed to elucidate the response to factors secreted by nerves upon injury and demonstrated that there was feedback between the two cell types (43). While pain and nociceptive receptors have been identified (3, 42), studies have not determined how the sensory nerves signal the epithelial cells under different environmental conditions. En face and cross-sectional views of Thy-1-GFP rat corneal nerve branches were imaged (Fig. 1). These corneas intrinsically express fluorescent labeling in nerves and demonstrate the integrity of innervation in the basal epithelium.

To determine if cell-cell communication between neuronal and epithelial cells is altered under hypoxic conditions, primary trigeminal ganglia cell cultures and epithelia were preincubated under normoxic or hypoxic conditions. Normoxic neuronal wound medium was added to epithelia cultured under normoxic conditions (Fig. 2B); similarly, hypoxic neuronal wound medium was added to epithelia cultured under hypoxic conditions (Fig. 2C). Conditioned medium collected before scratch wounding of normoxic neuronal cell cultures was used as control and did not elicit Ca\(^{2+}\) mobilization above the basal level in epithelial cells (Fig. 2A). When epithelial cells were cultured under normoxic conditions, addition of normoxic neuronal wound medium to epithelial cells mobilized a bimodal Ca\(^{2+}\) response, which was illustrated by a sharp initial response followed by a complex oscillatory Ca\(^{2+}\) mobilization (Fig. 2B). In contrast, when epithelial cells were incubated under hypoxic conditions and stimulated with hypoxic neuronal wound medium, Ca\(^{2+}\) mobilization was attenuated (Fig. 2C). Two additional controls were performed: epithelia cultured under normoxic conditions were stimulated with hypoxic neuronal wound medium (Fig. 2D), and epithelia cultured under hypoxic conditions were stimulated with normoxic neuronal wound medium (Fig. 2E). As shown in Fig. 2D, the epithelial cells retained the ability to induce Ca\(^{2+}\) mobilization. In contrast, in Fig. 2E, Ca\(^{2+}\) mobilization was attenuated. The results suggest that hypoxic stress of the "host" epithelial cells was a major contributor to changes in Ca\(^{2+}\) mobilization after injury.

Because of the complexity of Ca\(^{2+}\) dynamics between neurons and epithelia under hypoxic and normoxic conditions, we developed algorithms to analyze the communication between cells. A signal-sorting algorithm was used to determine location and cross talk between activated cells. Cells were segmented and identified as described previously. The average fluorescence intensity of all the individual cells was calculated from each cell’s maximums and minimums. Analysis was also performed to determine if an activated cell induced the activation of neighboring cells and the subsequent formation of cell clusters and additional cell communication (Fig. 3).

Analysis of data under normoxic and hypoxic conditions revealed marked differences. When cells were cultured under...
normoxic conditions, an initial sharp increase in average normalized fluorescence intensity (151%) was followed by a secondary mobilization for the duration of each run (Fig. 3A). Overall, there were significant differences between all three conditions ($P < 0.05$ by repeated ANOVA). Previously, we showed that the first peak was initiated by ATP and inhibited with apyrase, while the second peak was attenuated by NMDA inhibitors (43). Normalized percent change in fluorescence was analyzed at 68.6 s for the first peak and at 370.8 s for the second peak. At each of these two peaks, the cellular response under the normoxic condition was significantly different from that in cells cultured in hypoxic or control conditioned medium ($P < 0.05$ by Student’s $t$-test). The normoxic neuronal wound medium elicited Ca$^{2+}$ mobilization in $>20\%$ of the cell population in the first peak compared with $10\%$ under hypoxic conditions (Fig. 3B). To further assess intercellular communication, we analyzed the percentage of cells in a cluster and the number of clusters per 7.89 s or 10 frames under the three conditions (Fig. 3, C and D). When normoxic neuronal wound medium was added, $>10\%$ of the total cells became activated to form clusters in the first and second set of peaks (Fig. 3C). We then demonstrated that neuronal wound medium induced an increase in the number of clusters per 7.89 s compared with hypoxic wound medium and control medium (Fig. 3D).

Fig. 2. Hypoxia diminishes cross talk between epithelia and nerves in the cornea. Human corneal limbal epithelial (HICLE) cells were cultured under normoxia or hypoxia for 24 h, washed, and incubated with fluo 3-AM. Conditioned or neuronal wound medium was collected from cultures incubated under normoxia or hypoxia. After basal levels were obtained prior to stimulation, the epithelial response was monitored for $>600$ frames (0.789 s/frame). Pseudocolored 2.5-dimensional images show the cell response over time using a 6-color intensity scale. A: Ca$^{2+}$ mobilization of epithelial cells in response to control conditioned media. B: Ca$^{2+}$ mobilization of epithelial cells incubated under normoxic conditions in response to normoxic neuronal wound media. C: Ca$^{2+}$ mobilization of epithelial cells incubated under hypoxic conditions in response to hypoxic neuronal wound media. D: Ca$^{2+}$ mobilization of epithelial cells incubated under normoxic conditions in response to hypoxic neuronal wound media. E: Ca$^{2+}$ mobilization of epithelial cells incubated under hypoxic conditions in response to normoxic neuronal wound media. Images are representative of $>22$ independent experiments for each condition.
Previously, we showed a decrease in the secondary response when the normoxic neuronal media were incubated with NMDA inhibitors and then added to epithelial cells (43). To correlate changes in Ca\(^{2+}\)/H\(_{11001}\) mobilization caused by hypoxia with changes in glutamatergic signaling, we examined effects of hypoxia on localization of NR1 in epithelial cells. NR1 is known to be localized in lipid rafts under control, unwounded conditions (5). After culture under hypoxic conditions, its localization was altered (Fig. 3E). NR1 was more diffuse and cytosolic in cells exposed to hypoxia than in cells maintained in normoxia, where it was more focused at the cell junctions (Fig. 3E). In addition, there was a decrease in the fluorescence of NR1 under hypoxic conditions compared with normoxic conditions (Fig. 3E). Together, the data suggest that a decrease in ATP-induced Ca\(^{2+}\) mobilization and a change in NR1 localization under hypoxic conditions resulted in diminished communication between cells.

**Hypoxia impairs Ca\(^{2+}\) mobilization upon injury in epithelial cells.** After examining the response to neuronal medium, we examined the effect of hypoxia on Ca\(^{2+}\) mobilization or propagation in response to injury of epithelial cultures. For each experiment, 50 frames were collected prior to injury to establish the baseline; then the cells were injured under various conditions, and changes in fluorescence were collected for 500 frames (0.789 s/frame). We found a 38.6% decrease in the distance traveled by the Ca\(^{2+}\) wave from the origin of the

---

**Fig. 3.** Ca\(^{2+}\) mobilization between epithelial cells in response to neuronal wound media. HCLE cells were cultured under normoxia or hypoxia for 24 h, washed, and incubated with fluo 3-AM. Conditioned (CM) or neuronal wound media were collected from cultures incubated under normoxia (Nx) or hypoxia (Hx). Epithelial response to the media was monitored for >600 frames at 0.789 s/frame. Data were exported into MATLAB software written for analysis. A: percent change in average fluorescence of HCLE cells in response to neuronal wound media. B: percentage of cells activated over time in response to neuronal wound media. C: percentage of cells participating in cluster formation in response to neuronal wound media. D: number of clusters per 7.89 s (10 frames) in response to neuronal wound media. E: N-methyl-D-aspartate (NMDA) receptor 1 localization is altered under hypoxic conditions in HCLE cells. Unwounded HCLE cells were incubated under normoxia or hypoxia for 24 or 48 h and stained for NMDA receptor 1 (green) and counterstained with DAPI (blue) and rhodamine phalloidin (red). Tiled confocal images were collected, and representative images are shown. NC, negative control. Scale bar, 50 \(\mu\)m. Traces in A–D are representative of \(\geq\)22 independent experiments; images in E are representative of 3 independent experiments.
wound under hypoxia compared with normoxia (Fig. 4A; P < 0.05 by Student’s t-test).

Since hypoxia diminished the injury-induced propagation, we asked if there were changes in Ca^{2+} mobilization in the absence of extracellular Ca^{2+}. Cells were incubated under normoxic or hypoxic conditions and incubated in medium lacking Ca^{2+} (Fig. 4B). Under normoxic conditions, propagation of the Ca^{2+} wave was attenuated compared with Fig. 4A (P < 0.05 by Student’s t-test), and this supported previous studies (54). In contrast, when cells were injured under hypoxic conditions, Ca^{2+} mobilization was completely abrogated (Fig. 4, A and B; P < 0.05 by Student’s t-test).

In the next set of experiments, cells were cultured under normoxic or hypoxic conditions and then incubated with 25 μM BAPTA-AM, a cell-permeant Ca^{2+} chelator, for 20 min. There was no significant change between the two conditions in Fig. 4C. However, when the cultures were compared with control conditions (Fig. 4A), differences were revealed. Under normoxic conditions, there was a significant decrease in the distance traveled by the Ca^{2+} wave (Fig. 4C) compared with normoxic control (Fig. 4A; P < 0.05 by Student’s t-test) (32, 54). However, when the cells were cultured under hypoxic conditions and then incubated with BAPTA-AM, there was no additional decrease in the distance traveled by the Ca^{2+} wave from the origin of the wound compared with hypoxic control (Fig. 4, A and C).

We then asked if impairment of ATP-dependent Ca^{2+} uptake into intracellular stores by hypoxia contributed to changes in Ca^{2+} mobilization. HCLE cells were preincubated with 1 μM thapsigargin for 20 min to inhibit Ca^{2+} release from intracellular stores. Under normoxic conditions, when cells were preincubated with thapsigargin, there was a decrease in Ca^{2+} propagation upon injury compared with control (absence of thapsigargin), as the mobilization was limited to 2–3 cells from the wound edge (Fig. 4D; P < 0.05 by Student’s t-test) (32, 39). When cells were cultured under hypoxic conditions in the presence or absence of thapsigargin, there was no additional decrease in Ca^{2+} propagation. These results indicate that disrupted ATPase-mediated cycles of Ca^{2+} release and uptake induce the attenuated injury-induced Ca^{2+} response (Fig. 4A). Taken together, the data suggest that hypoxia impairs Ca^{2+} mobilization of epithelial cells, which modulates early events of corneal wound healing upon injury.

**Hypoxia alters mitochondrial membrane potential.** To evaluate the role of the mitochondrial membrane potential, cells were cultured under normoxic or hypoxic conditions and imaged using TMRE. In Fig. 5A, cells were cultured under...
normoxic or hypoxic conditions for 24 h, incubated with 7.5 nM TMRE for 30 min, and imaged. While fluorescence was higher in the cells cultured under normoxic conditions than in those cultured under hypoxic conditions, the difference was not significant (Fig. 5A). When FCCP was added to cells cultured under normoxic conditions and the cells were loaded with TMRE, fluorescence decreased sharply, indicating depolarized or inactive mitochondria, resulting in a decline in mitochondrial potential (Fig. 5B). However, under hypoxic conditions, the rate of the decrease was slower and the drop was less intense. These results indicate that the depolarized or inactive mitochondria have decreased membrane potential and fail to sequester TMRE.

We then examined the effect of mitochondrial depolarization on Ca\(^{2+}\) mobilization. Cells were repeatedly stimulated with 100 \(\mu\)M ATP, as described previously (33). A decrease in peak fluorescence occurred over time in the controls, which was established previously (Fig. 5C) (33). Addition of 20 \(\mu\)M FCCP had no effect on the initial Ca\(^{2+}\) peak but caused a decrease in subsequent peaks. There was a 20% drop in fluorescence in the second peak and a 50% decrease in subsequent stimulations compared with control (Fig. 5C). A minor increase in fluorescence and return to baseline immediately after FCCP addition have been reported (16).

Previously, our laboratory demonstrated that ATP is released upon corneal injury, which induces Ca\(^{2+}\) mobilization to surrounding cells, leading to promotion of wound repair (32). On the basis of our previous results, we speculated that hypoxia decreased levels of ATP released, resulting in a diminished response. To evaluate this possibility, cells were cultured under normoxic or hypoxic conditions for 24 or 48 h and subjected to scratch wounding, and the level of ATP released into the media was measured immediately. There was a decrease in released ATP after cells were scratch-wounded following incubation under hypoxia compared with cells maintained in normoxic conditions (Fig. 6A; \(P<0.05\) by Student’s \(t\)-test). There was no detectable release of ATP in normoxic or hypoxic unwounded cultures (control) at equivalent time points. In parallel experiments performed on neuronal cultures after 4 h of incubation under normoxia or hypoxia (as they could not tolerate longer exposure to hypoxia), there was a similar significant decrease (Fig. 6B). In addition, we tested whether hypoxia altered the intracellular concentration of ATP. HCLE cells were again incubated in normoxia or hypoxia for 24 and 48 h. Hypoxia significantly reduced the amount of intracellular ATP (Fig. 6C; \(P<0.05\) by Student’s \(t\)-test). Additional control experiments were performed to assess lactate dehydrogenase (LDH) levels, and no significant difference in released LDH or in total LDH was found (data not shown). The results demonstrate that cells released less ATP under hypoxic conditions after injury, and this decrease may have led to changes in early events, such as Ca\(^{2+}\) mobilization, that occur during wound repair.

**Hypoxia decreases purinergic receptor-induced Ca\(^{2+}\) mobilization.** Previous studies demonstrated that ATP was released with injury and that knockdown of the P2Y\(_2\) receptor attenuated injury-induced Ca\(^{2+}\) mobilization. In contrast, knockdown of the P2Y\(_4\) receptor did not significantly affect the corneal epithelial wound response (10). Additional studies demonstrated that the P2X\(_7\) receptor was activated with 2',3'-\(O\)-(4-benzoylbenzoyl)-ATP (BzATP) and that distinct sources of Ca\(^{2+}\) adjacent to the wound margin were involved in the injury response (40). Since hypoxia altered the injury-induced mobilization of Ca\(^{2+}\), we examined if there were changes in the activation of purinergic receptors. HCLE cells were cul-
tured under normoxic or hypoxic conditions for 24 or 48 h, stimulated with 100 μM UTP or BzATP, and then monitored (Fig. 7). In these experiments, the concentration of agonist was used in excess compared with the concentration of nucleotide released with injury to determine if hypoxia affected specific purinergic receptor functions. Compared with cells treated under normoxic conditions, in cells stimulated with UTP under hypoxic conditions, Ca²⁺ mobilization was decreased by 22.9% after 24 h and 35.4% after 48 h, but the differences were not significant (Fig. 7). In contrast, BzATP-stimulated Ca²⁺ mobilization was significantly decreased by 72.4% and 56.2% after 24 and 48 h, respectively (Fig. 7; P < 0.05 by Student’s t-test).

**Hypoxia impairs cell-cell communication.** Because previous work demonstrated that communication between epithelia and neurons was inhibited using a cocktail of inhibitors (43), experiments were performed to determine if hypoxia altered the efficacy of gap junctions. Photobleached cells were monitored for 400 frames at 10 s/frame. Unbleached cells were monitored over the same period to normalize the rate of recovery over time. Under hypoxic conditions, the recovery rate was attenuated (Fig. 8). The rate of fluorescence return was significantly decreased in hypoxic cells compared with control cells incubated under normoxic conditions over the time course evaluated. The maximal recoveries under normoxic and hypoxic conditions were 61.1% and 40.3%, respectively (Fig. 8; P < 0.05 by Student’s t-test). The decrease in the rate of fluorescence return under hypoxia demonstrated that hypoxia impaired the function of gap junctions, which may subsequently contribute to the decrease in Ca²⁺ mobilization after injury to confluent HCLE cell cultures.

**Hypoxia diminishes the activation of paxillin: in vitro and organ culture.** Because the cytoskeletal network supports the plasma membrane, we asked whether hypoxic conditions altered phosphorylation of paxillin, a critical component of the focal adhesion complex. It has been shown that exposing cells to nucleotides or subjecting them to injury altered phosphorylation of paxillin at Y118 (33, 50), indicating that paxillin is a...
downstream target of the ATP-induced P2Y2 receptor pathway. To determine if hypoxia is indicative of impaired motility, we examined the shape of the leading edge at the wound margin and the localization of paxillin in vitro. In the absence of injury (no wound), paxillin was predominantly localized in the cytoplasm (Fig. 9B). The wound margin at 1 h of hypoxia was similar to that in normoxia, but after 4 h of exposure to hypoxia the wound margin lacked lamellipodial extensions, and paxillin was not detected in focal adhesions at 1 h. To assess whether paxillin was recruited to the cytoskeletal framework, epithelial cells were incubated in the presence of cytoskeleton buffer at each time point (Fig. 9C) (21, 44). When scratch-wounded HCLE cells cultured under normoxic conditions were incubated in the presence of cytoskeleton buffer prior to fixation, paxillin was detected along the wound edge, with staining in lamellipodia of cells at 1 and 4 h (Fig. 9C). In contrast, paxillin was not detected at the wound edge in cells cultured under hypoxic conditions, and the overall levels of paxillin were decreased (Fig. 9C), indicating that more paxillin was soluble and less able to contribute to cell motility.

**Hypoxia delayed wound closure rate in organ cultures.** Wound repair using air-lifted organ cultures under hypoxic conditions was compared with the in vitro data. Rat corneas were subjected to epithelial debridement and then incubated under normoxic or hypoxic conditions for 2, 12, 18, or 24 h. To assess the effect of hypoxic stress on wound closure, the wound size was measured at each time point by staining with methylene blue (Fig. 10A). Under normoxic conditions, the wounds closed in 18 h. In contrast, under hypoxic conditions, the overall wound closure rate was reduced and epithelial abrasions did not heal until 24 h (Fig. 10A). At the 12-h time point, corneas were stained with phalloidin and 4’,6-diamidino-2-phenylindole, and the leading edge was imaged as a z-series and flattened. Under normoxic conditions, the leading edge displayed typical epithelial lamellipodial protrusions in an en face projection (Fig. 10B). In contrast, under hypoxic conditions, lamellipodial protrusions were minimal (Fig. 10B), and the wound margin resembled the flat edge detected in wounded HCLE cells under hypoxia (Fig. 9B).

When the corneas were imaged in cross section and stained for F-actin and paxillin, there were striking differences at the leading edge. Corneas incubated under normoxic conditions demonstrated a typical leading edge (Fig. 10C). Under hypoxic conditions, superficial cells were not detected at the leading edge for a distance of over five cells. In addition, paxillin decreased (Fig. 10C).

Numerous studies have shown that fibronectin is transiently expressed after injury along the basal lamina and acts as a provisional matrix to support epithelial migration by interacting with integrin-focal adhesion complexes (15, 24, 59). In our organ culture model, we examined the presence of fibronectin at 0, 2, 24, and 48 h after wounding (Fig. 11A). Under normoxic conditions, fibronectin was not detected at the wound margin (Fig. 11A). When scratch-wounded HCLE cells cultured under normoxic conditions were incubated in the presence of cytoskeleton buffer prior to fixation, paxillin was recruited to the cytoskeletal framework, epithelial cells were incubated in the presence of cytoskeleton buffer at each time point (Fig. 9C) (21, 44). When scratch-wounded HCLE cells cultured under normoxic conditions were incubated in the presence of cytoskeleton buffer prior to fixation, paxillin was detected along the wound edge, with staining in lamellipodia of cells at 1 and 4 h (Fig. 9C). In contrast, paxillin was not detected at the wound edge in cells cultured under hypoxic conditions, and the overall levels of paxillin were decreased (Fig. 9C), indicating that more paxillin was soluble and less able to contribute to cell motility.

**Hypoxia delayed wound closure rate in organ cultures.** Wound repair using air-lifted organ cultures under hypoxic conditions was compared with the in vitro data. Rat corneas were subjected to epithelial debridement and then incubated under normoxic or hypoxic conditions for 2, 12, 18, or 24 h. To assess the effect of hypoxic stress on wound closure, the wound size was measured at each time point by staining with methylene blue (Fig. 10A). Under normoxic conditions, the wounds closed in 18 h. In contrast, under hypoxic conditions, the overall wound closure rate was reduced and epithelial abrasions did not heal until 24 h (Fig. 10A). At the 12-h time point, corneas were stained with phalloidin and 4’,6-diamidino-2-phenylindole, and the leading edge was imaged as a z-series and flattened. Under normoxic conditions, the leading edge displayed typical epithelial lamellipodial protrusions in an en face projection (Fig. 10B). In contrast, under hypoxic conditions, lamellipodial protrusions were minimal (Fig. 10B), and the wound margin resembled the flat edge detected in wounded HCLE cells under hypoxia (Fig. 9B).

When the corneas were imaged in cross section and stained for F-actin and paxillin, there were striking differences at the leading edge. Corneas incubated under normoxic conditions demonstrated a typical leading edge (Fig. 10C). Under hypoxic conditions, superficial cells were not detected at the leading edge for a distance of over five cells. In addition, paxillin decreased (Fig. 10C).

Numerous studies have shown that fibronectin is transiently expressed after injury along the basal lamina and acts as a provisional matrix to support epithelial migration by interacting with integrin-focal adhesion complexes (15, 24, 59). In our organ culture model, we examined the presence of fibronectin at 0, 2, 24, and 48 h after wounding (Fig. 11A). Under normoxic conditions, fibronectin was not detected at the wound margin (Fig. 11A). When scratch-wounded HCLE cells cultured under normoxic conditions were incubated in the presence of cytoskeleton buffer prior to fixation, paxillin was recruited to the cytoskeletal framework, epithelial cells were incubated in the presence of cytoskeleton buffer at each time point (Fig. 9C) (21, 44). When scratch-wounded HCLE cells cultured under normoxic conditions were incubated in the presence of cytoskeleton buffer prior to fixation, paxillin was detected along the wound edge, with staining in lamellipodia of cells at 1 and 4 h (Fig. 9C). In contrast, paxillin was not detected at the wound edge in cells cultured under hypoxic conditions, and the overall levels of paxillin were decreased (Fig. 9C), indicating that more paxillin was soluble and less able to contribute to cell motility.

**Hypoxia delayed wound closure rate in organ cultures.** Wound repair using air-lifted organ cultures under hypoxic conditions was compared with the in vitro data. Rat corneas were subjected to epithelial debridement and then incubated under normoxic or hypoxic conditions for 2, 12, 18, or 24 h. To assess the effect of hypoxic stress on wound closure, the wound size was measured at each time point by staining with methylene blue (Fig. 10A). Under normoxic conditions, the wounds closed in 18 h. In contrast, under hypoxic conditions, the overall wound closure rate was reduced and epithelial abrasions did not heal until 24 h (Fig. 10A). At the 12-h time point, corneas were stained with phalloidin and 4’,6-diamidino-2-phenylindole, and the leading edge was imaged as a z-series and flattened. Under normoxic conditions, the leading edge displayed typical epithelial lamellipodial protrusions in an en face projection (Fig. 10B). In contrast, under hypoxic conditions, lamellipodial protrusions were minimal (Fig. 10B), and the wound margin resembled the flat edge detected in wounded HCLE cells under hypoxia (Fig. 9B).

When the corneas were imaged in cross section and stained for F-actin and paxillin, there were striking differences at the leading edge. Corneas incubated under normoxic conditions demonstrated a typical leading edge (Fig. 10C). Under hypoxic conditions, superficial cells were not detected at the leading edge for a distance of over five cells. In addition, paxillin decreased (Fig. 10C).

Numerous studies have shown that fibronectin is transiently expressed after injury along the basal lamina and acts as a provisional matrix to support epithelial migration by interacting with integrin-focal adhesion complexes (15, 24, 59). In our organ culture model, we examined the presence of fibronectin at 0, 2, 24, and 48 h after wounding (Fig. 11A). As expected, fibronectin was not detected when corneas were not wounded and had not been exposed to hypoxia (0 h in Fig. 11A). The lack of detectable fibronectin verified that our organ culture system responded in an equivalent way to the in vivo wound model (15, 24, 59). At 2 and 24 h after injury, fibronectin was detected at the wound margin and in the stroma under nor-
moxic and hypoxic conditions (Fig. 11A). However, fluorescence intensity was decreased under hypoxia compared with normoxia (Fig. 11B). After wound closure (48 h), fibronectin staining was not observed (Fig. 11), as expected (24). These results suggest that hypoxia caused a decrease in fibronectin deposition during corneal wound repair; thus the basement membrane zone was not able to act as a provisional matrix, contributing to delayed cell migration.

**DISCUSSION**

The cornea is an excellent model system to examine signaling and communication between epithelial and neuronal cells required for wound closure. Previously, we showed that when the epithelium is injured in vitro, ATP is released and a Ca\(^{2+}\)/H\(_{1001}\) wave is mobilized to neighboring cells (11, 32). However, proper wound closure in vivo does not occur in the absence of sensory nerves. Investigators have shown that innervation of tissue impacts the rate and integrity of wound repair. Denervated corneas are associated with a defective epithelial attachment to the basement membrane zone (2). In the epidermis, damage to sympathetic or sensory nerves is known to impair reepithelialization (46, 47). Innervation is also critical for scarless fetal wound healing of the skin (48). Moreover, peripheral neuropathies that occur in diabetic patients may result in retardation of the wound-healing process (30).

In our coculture system of epithelial cells and nerves, we found that factors, such as ATP and glutamate, that altered signaling at the level of Ca\(^{2+}\) mobilization and directed cell migration were released. The release of ATP from epithelial cells induced the release of glutamate from neuronal cells, which caused the release of ATP, indicative of a reciprocal response between two receptors (43). The aim of our current work was to determine how the signaling between epithelial cells is altered under hypoxia.

To facilitate our analysis of Ca\(^{2+}\) signaling in this study, we developed an algorithm to examine the complex communication between cells. This algorithm allowed us to evaluate how cells are activated and form multicell clusters that respond in a coordinated fashion postinjury. These clusters have the ability to excite other cells and form yet more clusters, propagating the signal over a large area of cells. We determined that the cells respond in very different patterns when activated by neuronal media that contained ATP and glutamatergic factors or by the release of ATP alone (from epithelial cells alone) (40). Furthermore, specific NMDA receptors are expressed in the epithelium, and inhibition with NMDA inhibitors attenuated only the intercellular communication detected in the second Ca\(^{2+}\) response peak. When the cells were cultured under hypoxic conditions, the products secreted by neurons elicited a modification of the first and second Ca\(^{2+}\) response peaks. Not only was fluorescence decreased with hypoxia, but there were fewer cells activated and fewer cells in each cluster. These data were supported by a change in localization of the NMDA receptor, the signaling function of which appears to
depend on its presence at the cell membrane. Previously, hypoxia was shown to induce changes in the activity of specific NMDA receptor isoforms (6). In addition, blocking the activation of NMDA receptors is thought to prevent hypoxic stress-induced excitotoxicity (55). Together, these factors indicate an association of impairment in NMDA receptor function and localization with Ca\textsuperscript{2+}/H\textsubscript{11001} signaling critical to wound repair under hypoxia.

In the organ cultures, the rate of epithelial migration and wound repair was delayed under hypoxic conditions compared with normoxic controls. At 12 h of incubation in normoxia or hypoxia, rat cornea organ cultures were fixed and counterstained with rhodamine phalloidin (red) and DAPI (blue). Scale bar, 50 μm. Arrows denote wound edge. Images represent ≥3 independent experiments. C: hypoxia alters migrating epithelium. Cross sections of wounded corneas were stained for paxillin (green) and counterstained with DAPI (blue) and rhodamine phalloidin (red). Tiled images (1 × 4) of the wound margin are presented. Note decreased paxillin and altered stratification of superficial cells at the leading edge of corneas cultured under hypoxic conditions compared with normoxia. Scale bar, 25 μm. Images represent ≥3 independent experiments.

depend on its presence at the cell membrane. Previously, hypoxia was shown to induce changes in the activity of specific NMDA receptor isoforms (6). In addition, blocking the activation of NMDA receptors is thought to prevent hypoxic stress-induced excitotoxicity (55). Together, these factors indicate an association of impairment in NMDA receptor function and localization with Ca\textsuperscript{2+}/H\textsubscript{11001} signaling critical to wound repair under hypoxia.

In the organ cultures, the rate of epithelial migration and wound repair was delayed under hypoxic conditions compared with normoxic controls. At 12 h of hypoxia, there was a lack of lamellipodial extensions at the leading edge that correlated with a lack of superficial cells and a decrease in paxillin (34, 51, 58). In the in vitro and organ culture experiments, hypoxia appeared to delay recruitment of paxillin to the focal adhesion complex. Increased levels of the cytoskeletal complex-bound insoluble form of paxillin under normoxia in response to the injury, along with a decrease in detectable paxillin under hypoxic conditions, suggest that hypoxia disrupts the membrane-cytoskeletal linkage, which contributes to a decrease in the rate of repair. A similar decrease in focal adhesion proteins occurred rapidly when renal proximal tubule cells were exposed to a mitochondrial inhibitor or a 95% N\textsubscript{2}-5% CO\textsubscript{2} atmosphere (36). These findings indicate that the linkage of proteins with the membrane is dependent on the extracellular environment and cell type, as the corneal epithelium is exposed to fluxes in O\textsubscript{2} daily. Additionally, some investigators have performed reoxygenation experiments (53), while others have shown that, under hypoxia, O\textsubscript{2} consumption changes and cells maintain oxidative phosphorylation at 1% O\textsubscript{2} (19). In corneal epithelial cells that are naturally exposed to 8% O\textsubscript{2} for a sleep cycle every 24 h, it is unclear whether the low O\textsubscript{2} levels are completely responsible for changes in the mitochondrial membrane potential and subsequent reduction in ATP. Collins et al. (16) showed that FCCP inhibited responses to ATP, and their data indicate that depolarizing mitochondrial membrane potential prevents activation of inositol 1,4,5-trisphosphate receptor. In our studies with the uncoupling agent FCCP, there was only a partial reduction, indicating that mitochondrial depolarization played a contributory role but that it is more complex (19, 20).

Additional studies have shown that delayed reepithelialization after injury can precede stromal ulceration and may correlate with a change in substrate and increase in matrix metalloproteinases (18). In this study, we examined changes in
the substrate and found a decrease in fibronectin along the basement membrane zone in wounded cells under hypoxic conditions. However, the unwounded cells showed no change. Investigations to determine changes in integrin complexes, along with other focal adhesion proteins, are a goal of future work.

A number of laboratories, including ours, have shown that intracellular ATP becomes depleted under hypoxic conditions (19, 25). The hypoxia-induced decrease in ATP may attenuate activation of the purinergic signaling pathway upon injury. Because ATP has been shown to modulate intracellular Ca$^{2+}$ homeostasis via Ca$^{2+}$-ATPase expressed on the endoplasmic reticulum and plasma membrane, lower ATP levels may impede Ca$^{2+}$ mobilization (12). This possibility is supported by observations that cells preincubated with thapsigargin showed no additional decrease in Ca$^{2+}$ mobilization under hypoxic conditions. These results could be due to a depletion of Ca$^{2+}$ from intracellular stores under hypoxia or the inability to release Ca$^{2+}$ from the stores (31, 38, 39). Another contributing factor could be changes in the regulation of extracellular ATP levels, which may be mediated by membrane pores such as those formed by pannexins. Previous studies found a direct relationship between pannexin channels and the regulation of released ATP-mediated signal transmission (17, 27, 28, 56). Because we observed a decrease in released ATP levels after injury, it is possible that hypoxic stress may induce changes in the modulation of pannexin pore formation along with other downstream targets, ultimately contributing to impaired Ca$^{2+}$ mobilization. We speculate that depleted intracellular ATP levels and changes in ATP release to the extracellular space could affect injury-induced Ca$^{2+}$ mobilization.

In summary, our results provide evidence that complex Ca$^{2+}$-mediated signaling occurs between sensory neurons and epithelial cells after injury and is critical to wound healing. The signaling appears to require activation of purinergic and glutamatergic pathways and synergistic interactions between them to modulate Ca$^{2+}$ mobilization. The change that occurs after 24 h of hypoxia affects various events critical to the success of wound healing, including changes in localization of NMDA receptors, distance of Ca$^{2+}$ wave propagation, and phosphorylation of focal adhesion proteins. Information revealed by these studies will contribute to an enhanced understanding of wound repair under compromised conditions and may provide insight into ways to effectively stimulate proper epithelial repair in pathological conditions.

ACKNOWLEDGMENTS

We thank Gerald Coffman (Boston University School of Public Health) for statistical analysis, Dr. Orian Shirihai for discussions on mitochondrial uncoupling and use of TMRE, and Dr. Karen Symes and Celeste Rich for critical review of the manuscript.

GRANTS

This work was supported by National Institutes of Health Grants R01 EY-06000, R01 EY-06000S, and S10 RR-017967 and the New England Corneal Transplant Fund (to V. Trinkaus-Randall), National Heart, Lung, and Blood Institute Grant R01 HL-088672 and BrightFocus Foundation Grant M2012014 (to M. A. Nugent), and an Ophthalmology Departmental Grant from the Massachusetts Lions Eye Research Fund.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES


