Reoxygenation Injury in a Cultured Corneal Epithelial Cell Line Protected by the Uptake of Lactoferrin

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Purpose. To investigate whether reoxygenation after extended hypoxia causes cellular damage in cultured corneal epithelial cells and to demonstrate the protective effects of lactoferrin.

Methods. Immortalized human corneal epithelial cells (T-HCECs) were cultured to confluence in 96-well culture plates, subjected to stringent hypoxia (1% O2, 5% CO2, 94% N2 at 37°C) for 24 hours, and returned to normoxic conditions (5% CO2, 95% air at 37°C). Cell viability was observed by 1 μM propidium iodide staining 0, 2, 4, and 6 hours after reoxygenation. Inhibition studies were performed after 2 hours' reoxygenation, using 2 mM iron chelator desferrioxamine and 0.2 mg/ml lactoferrin. Confocal immunocytochemistry for human lactoferrin and western blot analysis for lactoferrin-induced ferritin were performed in cultured T-HCECs to demonstrate the internalization of lactoferrin after application.

Results. After 2 hours, reoxygenation of T-HCECs after hypoxia produced an increase in cell death that was significantly greater than that observed in normoxic control cells or in cells subjected to hypoxia for the same time span without reoxygenation. The addition of desferrioxamine and lactoferrin at the time of reoxygenation significantly attenuated cellular damage. Confocal immunocytochemistry revealed that lactoferrin is taken into the cytoplasm of T-HCECs as early as 30 minutes after application. This was also demonstrated in western blot analysis by the upregulation of intracellular ferritin at 18 hours by the addition of iron-bound lactoferrin but not by iron-free lactoferrin.

Conclusion. Reoxygenation is responsible for increased cellular damage after extended hypoxia, which is attenuated by chelators of free iron in the cytosol, including the major tear protein lactoferrin. (Invest Ophthalmol Vis Sci. 1998;39:1346-1351)

Reoxygenation injury, more commonly referred to as reperfusion injury in vascularized organs, is a common clinical paradox observed in ischemic heart and cerebral disease1,2 and after the transplantation of such vascularized organs as the liver.3 Extensive research has revealed the role of reactive oxygen species (ROS) in the pathogenesis of reperfusion injury.4-6 In which the hydroxyl radical (OH·) has been suggested as the final effector because of its high reactivity. Hydroxyl radicals are physiologically produced by the Fenton-type reaction, which involves the spontaneous reduction of hydrogen peroxide by ferrous iron (Fe2+) or by ferric iron (Fe3+) in the presence of reducing agents such as the superoxide anion. Iron and copper are released after myocardial ischemia,7 and iron-chelating agents protect against reperfusion injury in vivo.8 Other mechanisms have also been suggested in the pathogenesis of reperfusion injury such as the xanthine dehydrogenase-oxidase system and infiltrating polymorphonucleocytes that recirculate into reperfused tissue.9,10 Both mechanisms may contribute to ROS formation.

Reoxygenation injury is distinguished from hypoxic injury that is characterized by cell death subsequent to adenosine triphosphate depletion, cell swelling, and bleb formation.11 Cell death caused by hypoxia can be prevented by hyperosmotic agents such as mannitol and polyethylene glycol,12 which indicates that the swelling of cells plays a significant role in bleb formation and cell death. However, cells sustaining reoxygenation injury do not exceed the threshold for hypoxic damage, and cell death occurs only after reoxygenation. The same cells would not have sustained the same damage had they remained in continuous hypoxic conditions until a threshold was reached. Because the most potent agents that protect cells from reoxygenation injury are iron-chelating agents that sequester cytoplasmic iron,8,13 the underlying mechanisms are distinct from hypoxic injury.

Hypoxic injury to the corneal epithelium is also a well-established entity, which is often observed during use of contact lenses with poor oxygen transmissivity. The classical signs of hypoxic corneal epithelial damage include thinning, micro cyst formation, and reduced cell adhesion.14 It is still unknown whether corneal epithelial cells sustain reoxygenation injury as a possible mechanism for certain types of contact lens-induced damage. The corneal epithelium is subjected to a harsh environment of increased oxygen tension (Po2), which can range from atmospheric values of 155 mm Hg when eyes are open to 37.4 ± 20.9 mm Hg when they are closed.15 Pressure is even lower when contact lenses are in place. We therefore postu-
Vacuum pump
US sonicator

FIGURE 1. Deoxygenation apparatus. Minimum essential medium was placed in a 50-ml conical tube sealed with an airtight silicon rubber cap. The air within the tube was withdrawn using a vacuum pump while ultrasound was applied to facilitate degassing of the medium. The clamp on the N₂-CO₂ balloon was slowly released to return pressure to atmospheric levels.

Related that corneal epithelial cells can sustain reoxygenation injury after sublethal hypoxia and that tear lactoferrin may play a protective role, because the major tear protein has been demonstrated to protect epithelial cells against oxidative stress caused by UV-B exposure.¹⁶

MATERIALS AND METHODS

Cell Culture
Immortalized human corneal epithelial cells (T-HCECs) were cultured as described previously,¹⁷ with a few modifications.¹⁸ in supplemented hormone epithelial medium consisting of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 with 10% fetal calf serum, 5 μg/ml insulin, 0.1 μg/ml cholera toxin, 10 ng/ml human epithelial growth factor, and 40 μg/ml gentamicin. Reagents were purchased from Life Technologies (Gaithersburg, MD), except for insulin (Sigma, St Louis, MO). Cells were cultured in 250-ml flasks and passaged in 1:4 split ratios (approximately 10⁴ cells/cm²) after confluence, using 0.05% trypsin, 0.02% EDTA in Ca²⁺, Mg²⁺-free phosphate-buffered saline (PBS[-]), pH 7.4. Forty-eight hours before experiments, T-HCECs were passaged into 96-well plates at a density of 10⁴ cells/well. After 24 hours' incubation, cells were subjected to serum deprivation by replacement of supplemented hormone epithelial medium with minimum essential medium (MEM; 50% Dulbecco's modified Eagle's medium/50% Ham's F-12 with 0.1% fetal calf serum) for another 24 hours to arrest growth.

Hypoxic Stress

Minimum essential medium was deoxygenated by placing 20 ml medium in a 50-ml conical tube (Sumilon, Tokyo, Japan) with an airtight silicone rubber cap fitted with two glass tubes to provide a vacuum and repressurization (Fig. 1). A rubber balloon containing 95% N₂-5% CO₂ gas was clamped and attached to the end of the input tubing, and the output tube was attached to a motorized vacuum pump (MDA 015; Sinku Kiko, Tokyo, Japan). The entire tube was bathed in an ultrasound sonicator (Sono Cleaner; Kajo Denki, Tokyo, Japan) filled with tap water that assists in degassing the medium while in the vacuum. The medium was placed in a vacuum of less than 10 mm Hg for 30 minutes, by which time bubbles were no longer observed lining the inner tube wall. The interior of the tube was returned to normal atmospheric pressures by slowly releasing the clamp on the balloon containing N₂ and CO₂. This procedure reduces Po₂ of the medium to less than 10 mm Hg.

FIGURE 2. Propidium iodide (PI) staining of a confluent sheet of immortalized human corneal epithelial cells. (A) Normoxic control cells. Two hours of reoxygenation after 24 hours of hypoxia (B) produces more propidium iodide-positive cells than in normoxic control cells or hypoxic control cells (C). Scale bar, 100 μm.

A: normoxic control
B: hypoxia/reoxygenation
C: hypoxic control
FIGURE 3. Time course of propidium iodide-positive cells after reoxygenation. Cells in the hypoxia-reoxygenation group sustained significantly greater damage (P < 0.05) than cells in the normoxic control and the strictly hypoxic groups at 2, 4, and 6 hours.

Figure 4. Inhibitory effects of desferrioxamine and lactoferrin on reoxygenation injury observed after 2 hours of reoxygenation (mean ± SE). DFO, desferrioxamine; LFO, lactoferrin; *P < 0.05 against hypoxia-reoxygenation.
Lactoferrin and desferrioxamine added at the time of reoxy-
genation inhibited cell death compared with untreated cells
(Fig. 4). These results are consistent with previous reports
demonstrating the effects of iron chelators in ischemia-reper-
fusion injury in the heart.8

**Lactoferrin Uptake**

Desferrioxamine is a low molecular weight iron chelator that is known to enter cells spontaneously at these concentrations.20 However, it is still unknown whether the lactoferrin found abundantly in tears enters corneal epithelial cells. The confocal immunocytochemistry study of T-HCECs revealed that lactoferrin-assisted fluorescence was greater in cells pretreated with human lactoferrin (Fig. 5). The confocal images in Figure 5 are 5-μm slices at the level of the nucleus, which appear black, suggesting that the fluorescence observed is within the cyto-
plasm. Little or no fluorescence was observed in nontreated cells or in control cells stained with nonspecific mouse IgG as the primary antibody. Western blot analysis for intracellular ferritin demonstrated that the protein is upregulated by pre-
treatment with iron-saturated lactoferrin but not by the un-
bound form (Fig. 6). This suggests that lactoferrin is taken into the cytoplasm, because ferritin is ubiquitously upregulated by increased intracellular iron content to sequester the potentially harmful cytoplasmic free iron.21

**DISCUSSION**

The corneal epithelium is unique compared with epithelia in other organs, in that the tissue is avascular and the oxygen required for metabolism is almost entirely supplied by the atmosphere. Partial pressures of O2 (PO2) are relatively high when the eye is open and decrease substantially when the eye is closed and when contact lenses are in place.15 The difference in PO2 during these two extremes in the cornea is therefore greater than in any tissue elsewhere in the body. Molecular oxygen is a highly reactive species that is capable of reacting with many organic molecules and is highly toxic for...
an anaerobic microbes. However, the benefits of using molecular oxygen as a source of adenosine triphosphate production are so great that most organisms have adopted respiration as a source of energy while evolving mechanisms to protect themselves from the toxicity of oxygen. However, molecular oxygen is toxic under certain circumstances, such as paraquat ported in the myocardium. Reoxygenation injury is another form of toxicity, and in concentrations exceeding atmospheric levels (hyperbaric oxygen). Reoxygenation injury is another form of oxygen toxicity observed during pathologic states, first reported in the myocardium.

Reoxygenation injury is also referred to as reperfusion injury, because it often involves vascular reperfusion of the heart or brain. However, an intact vascular system is not necessarily required for the phenomenon to occur, which makes the cornea a candidate for vulnerability to reoxygenation damage. Factors such as increased glucose, calcium channel blockers, and iron chelators inhibit reoxygenation injury, suggesting that a metabolic process is involved. The final common effector seems to be the hydroxyl radical (OH") produced by precursor ROS and free iron through a Fenton-type reaction. The protective effects of iron chelators were also demonstrated in our study, which, along with the presence of atmospheric oxygen, makes ROS production the likely cause of reoxygenation injury in the cornea. One question that remains is the time course of the observed phenomenon. Reoxygenation injury requires hours to become manifest, and a certain time span is required for desferrioxamine and lactoferrin to enter the cytoplasm before free iron can be sequestered. Because most ROS are short lived, including the hydroxyl radical, mechanisms leading to cell death do not seem to involve the direct action of ROS produced immediately after reoxygenation. Further studies are required to elucidate the precise mechanisms of reoxygenation injury in the cornea.

Lactoferrin is a major tear secretion protein occurring in concentrations as high as 2 mg/ml. It also enters a human myelogenous leukemia cell line (K562) and binds to a sequence-specific DNA site. Thus, lactoferrin taken into cells of lymphocyte lineage may also play a role in transcriptional activation. The results of the confocal immunocytochemistry and western blot analyses for ferritin suggest that human lactoferrin is taken into T-HCECs, and that the antioxidant activity in this and a previous study is caused by its iron-chelating properties within the cytoplasm.

The results of the this study do not show whether the uptake of lactoferrin is through a specific receptor or a non-specific mechanism such as pinocytosis. However, it is the first report to our knowledge that has suggested that lactoferrin in tears may be taken into corneal epithelial cells as iron transporters or antioxidants against excessive free iron. Further studies are required to characterize the existence of lactoferrin receptors, if any, in corneal epithelial cells, and we are currently investigating the possibilities.

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References

Protection against Reoxygenation Injury by Lactoferrin


