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Endogenous hydrogen peroxide production in the epithelium of the developing embryonic lens

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Purpose: Hydrogen peroxide (H_2O_2) is an endogenously produced reactive oxygen species (ROS) present in a variety of mammalian systems. This particular ROS can play dichotomous roles, being beneficial in some cases and deleterious in others, which reflects the level and location of H_2O_2 production. While much is known about the redox regulation of ROS by antioxidant and repair systems in the lens, little is known about the endogenous production of H_2O_2 in embryonic lens tissue or the physiologic relevance of endogenous H_2O_2 to lens development. This gap in knowledge exists primarily from a lack of reagents that can specifically detect endogenous H_2O_2 in the intact lens. Here, using a recently developed chemoselective fluorescent boronate probe, peroxyfluor-6 acetoxymethyl ester (PF6-AM), which selectively detects H_2O_2 over related ROS, we examined the endogenous H_2O_2 signals in the embryonic lens.

Methods: Embryonic day 10 chick whole lenses in ex vivo organ culture and lens epithelial cells in primary culture were loaded with the H_2O_2 probe PF6-AM. To determine the relationship between localization of mitochondria with active membrane potential and the region of H_2O_2 production in the lens, cells were exposed to the mitochondrial probe MitoTracker Red CMXRos together with PF6-AM. Diphenyleneiodonium (DPI), a flavin inhibitor that blocks generation of intracellular ROS production, was used to confirm that the signal from PF6-AM was due to endogenous ROS production. All imaging was performed by live confocal microscopy.

Results: PF6-AM detected endogenous H_2O_2 in lens epithelial cells in whole lenses in ex vivo culture and in lens epithelial cells grown in primary culture. No endogenous H_2O_2 signal could be detected in differentiating lens fiber cells with this probe. Treatment with DPI markedly attenuated the fluorescence signal from the peroxide-specific probe PF6-AM in the lens epithelium, suggesting that basal generation of ROS occurs in this region. The lens epithelial cells producing an endogenous H_2O_2 signal were also rich in actively respiring mitochondria.

Conclusions: PF6-AM can be used as an effective reagent to detect the presence and localization of endogenous H_2O_2 in live lens cells.

Reactive oxygen species (ROS), once considered deleterious to cells, are now known to be involved in redox signaling pathways that contribute to normal cell functions, such as cell proliferation and differentiation [1]. In living systems the ROS family encompasses several molecules, such as hydrogen peroxide (H_2O_2), superoxide ($[O_2]^-$), hypochlorous acid (HOCl), singlet oxygen (1O_2), lipid peroxides (ROOH), ozone (O_3), and hydroxyl radical ($[OH]^\cdot$) [2]. Certain ROS, including H_2O_2 , are now known to function as key regulators of normal cell physiology when present at subtoxic concentrations [3-6].

H_2O_2 is an abundantly produced ROS in the cell and can act as a secondary messenger molecule by redox regulation of cellular targets [7]. One important source of endogenously

produced H_2O_2 in the cell is the mitochondria [8]. H_2O_2 is generated in mitochondria during aerobic respiration when superoxide $[O_2]^-$ is produced from complexes I and III in the electron transport chain, which is then rapidly converted to H_2O_2 by the enzyme superoxide dismutase [9]. The generated H_2O_2 can play quite distinct roles in the cell depending on its level of production. While high concentrations of H_2O_2 have toxic effects on the cell [10], at low concentrations H_2O_2 can drive cell proliferation [11], differentiation [4,12], and migration [13]. Without tight regulation, H_2O_2 can damage biomolecules and cause misregulation of cellular signaling pathways, contributing to aging, disease, and even cell death [14]. As H_2O_2 is a byproduct of aerobic respiration, to modulate levels of H_2O_2 (and other ROS species) and prevent oxidative damage, the cell has evolved several layers of antioxidant protective mechanisms. The H_2O_2 enzymatic detoxification systems include catalase, glutathione peroxidases, and peroxiredoxins, and the nonenzymatic systems include glutathione, vitamins A, C, E, and bilirubin [15,16]. In

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addition to the mitochondria, both the endoplasmic reticulum and peroxisomes can be sources of H_2O_2 in the cell [2,17]. H_2O_2 can also be generated at specific microdomains at the plasma membrane in signaling events involving NADPH oxidase (NOX) enzymes [18-20]. Indeed, there is increasing evidence that various growth factors and cytokines, such as platelet derived growth factor (PDGF), epidermal growth factor (EGF), insulin, angiotensin II, and tumor necrosis factor (TNF) alpha, generate H_2O_2 as a proliferation signal in target cells by stimulating the activation of specific Nox proteins [21,22].

High levels of H_2O_2 generated from Nox proteins can have deleterious effects on tissues, [1,4,23], with particularly toxic effects on the ocular lens [24]. The lens is a transparent tissue primarily composed of lens epithelial cells and lens fiber cells. As differentiated lens fiber cells are not renewed and must last throughout an individual's lifetime, systems to maintain homeostasis and prevent oxidative damage are especially important to this tissue. Oxidative stress induces lens cataract [25,26], and excess H_2O_2 signaling is a leading cause of age-related cataracts [27]. The lens epithelium is particularly susceptible to oxidative damage [27,28]. However, recent evidence [11,14,29,30] point to the physiologic importance of low levels of H_2O_2 in the lens and indicate that this factor is an important regulator of key enzymes, including phosphatases and kinases. Although much is known about the repair systems that respond to oxidative stress in the lens and their role in maintaining lens homeostasis [14,24,31-33], there is little evidence of the physiologic role of H_2O_2 in the lens or its function as a signaling agent in lens epithelial cell differentiation. This knowledge gap has resulted from the lack of reagents to specifically detect H_2O_2 at endogenous basal levels within the intact lens. The development of chemosensitive fluorescent probes, such as peroxyfluor-6 acetoxymethyl ester (PF6-AM) [34,35], offers the potential to monitor H_2O_2 levels in intact tissue samples and even in vivo [36-38]. PF6-AM features a boronate chemical switch that allows for selective detection of H_2O_2 over other ROS, which is combined with acetoxymethyl (AM) ester-protected phenol and carboxylic acid groups for enhanced cellular retention and therefore sensitivity. PF6-AM is a membrane-permeable cell-trappable probe shown to be extremely sensitive and specific to H_2O_2 [39].

In this study we investigated whether the PF6-AM fluorescent probe can detect endogenous production of H_2O_2 in the lens and if there is differentiation-state specificity to H_2O_2 generation during embryonic lens development. Our data showed that healthy lens epithelial cells in the

embryonic lenses produce endogenous H_2O_2 , while no signal was detected in the lens fiber zone.

METHODS

Peroxyfluor-6 acetoxymethyl ester: The PF6-AM fluorescent probe is an effective tool for detecting cellular H_2O_2 [39]. PF6-AM is a carboxyfluorescein-based probe combining a boronate-masked phenol for H_2O_2 detection [35,40] and AM ester groups that mask phenol and carboxylic acid functionalities for enhanced cellular retention. The lipophilic AM esters allow the probe to pass readily through cell membranes, where nonspecific cytosolic esterases quickly deprotect the AM groups to reveal PF6. PF6, which is dianionic and therefore membrane impermeable, is "trapped" inside the cell where it can then respond to changes in intracellular H_2O_2 levels [39].

Primary lens cell culture preparation: Primary lens cell cultures were prepared as described previously [41,42]. The IACUC of Thomas Jefferson University approved the protocol for the studies with avian embryos. Briefly, following decapitation of Day 9 quail embryos and removal of lenses by dissection, lens epithelial cells were isolated by trypsinization followed by agitation, plated on laminin, and cultured in Medium 199 (Life Technologies, 11150-059, Grand Island, NY) containing 10% fetal bovine serum, 1% Penicillin and 1% Streptomycin. After 2 days in culture, cells were exposed to PF6-AM (5 μ M) for 30 mins in Medium 199 without serum at 37 °C, washed and imaged live by confocal microscopy in the presence of PBS.

Preparation of ex vivo embryonic lens organ cultures: E10 chick lenses were isolated from the eye, cleaned free of the ciliary body, and incubated with the PF6-AM probe (5 μ M) in Medium 199 (Life Technologies) without serum for 30 min at 37 °C in a 35-mm Petri dish. Next, the lenses were washed carefully and placed in Dulbecco's Phosphate Buffered Saline (DPBS, Corning, 21-0310CV, Tewksbury, MA) in a 35-mm culture dish sitting on a drop of 1% agarose that immobilizes the lens for live imaging. The lenses were imaged live using confocal microscopy.

Diphenyleneiodonium and MitoTracker Red CMXRos treatment: E10 lenses in organ culture or lens epithelial cells in primary culture were pre-incubated with PF6-AM in Medium 199 for 30 min. Following incubation, lenses or lens cells in culture were washed in DPBS buffer and were either exposed to diphenyleneiodonium (DPI; 5 μ M) for 20 min or the vehicle dimethyl sulfoxide (DMSO). Whole lenses or lens cultures were imaged live by confocal microscopy. For co-localization of active mitochondria with PF6-AM, lens cells in organ or primary culture were exposed to both

PF6-AM (5 μ M) and MitoTracker Red CMXRos (1 μ M) in Medium 199 for 30 min, washed with DPBS, and imaged live by confocal microscopy.

Image analysis and quantification: The whole embryonic lens and lens cells in primary culture were imaged live using the Zeiss LSM510 META confocal microscope. The water-immersion objective 40X Zeiss achroplan, water, 0.8 NA, IR, DIC was used for these live imaging studies. Line scans across image profiles were drawn using the LSM5 Image Examiner software to determine the intensity profile of the PF6-AM probe. Surface plot histograms were also used to compare intensity levels in the lens epithelium versus the lens fiber cell zone at E10.

RESULTS

Endogenous H₂O₂ was generated in lens epithelial cells during lens development, particularly by cells in the equatorial zone: To study H₂O₂ signaling in the normal lens, we used the boronate-based fluorescent probe PF6-AM (5 μ M) to detect endogenous H₂O₂ by live confocal microscopy imaging. For these experiments, we examined both whole lenses placed in organ culture at E10 and lens epithelial cells in primary culture [41]. Related small-molecule dyes have been shown to be readily taken up by the ocular lens and can circulate throughout the entire lens [43] along the endogenous lens microcirculation system [44-47]. Embryonic lenses exposed to PF6-AM were imaged from two angles—from the side to provide an anterior–posterior view and en-face providing a top to bottom view. Our results established that lens cells produced H₂O₂ and suggested that the fluorescent signal for H₂O₂ was cell-type specific. H₂O₂ generation was observed from lens epithelial cells, including those in the lens equatorial zone (Figure 1B,C), where lens cells withdraw from the cell cycle and initiate their differentiation [48,49] (modeled in Figure 1A). Endogenous H₂O₂ production was also observed from the undifferentiated cells of the central-anterior region of the lens (Figure 1B,D). Similarly, lens epithelial cells produced H₂O₂ in primary culture (Figure 1G). As the PF6-AM dye is not ratiometric and is dependent on the level of esterase activity within the cells, it was not possible to extrapolate these results to a quantifiable difference between the expression of endogenous H₂O₂ in different regions of the lens epithelium. However, despite the presence of esterase activity in the lens fiber zone [50], no detectable H₂O₂ signal was observed in the lens fiber cell zone with the PF6-AM probe. Line scans across the image profiles and a surface plot histogram were produced to illustrate these differences in observed PF6-AM fluorescence between the epithelial and fiber cell regions of the embryonic lens (Figure

1D). Previous studies from our laboratory with the mitochondrial potentiometric dye JC-1 showed the ease at which such dyes are able to penetrate through to the central fiber cells of embryonic lenses [42]. However, to ensure that the absence of a PF6-AM H₂O₂ signal in lens fiber cells did not result from an inability of PF6-AM to effectively reach all cells of the embryonic lens, we performed studies in which the lenses were punctured before incubation with PF6-AM. For these studies lenses were punctured along the lens equator in the direction of the central region of the lens through to the center of the fiber cell mass, with the tips of a number 5 forceps (Fine Science Tools, Foster City, CA). Under these conditions where the dye was able to come in direct contact with lens fiber cells, the results remained unchanged, with the observed fluorescent PF6-AM signal concentrated in lens epithelial cells and no signal detected in the fiber zone (Figure 1F). These findings strongly supported our findings with the intact lens that H₂O₂ was produced by lens epithelial cells. We further noted that exposure of whole lenses in ex vivo culture to the PF6-AM dye did not affect their transparency as no opacities or toxicity were observed with the probe applied at concentrations of 5 μ M.

Studies in which lens epithelial cells in primary culture were exposed to the PF6-AM probe also showed production of endogenous H₂O₂ in lens epithelial cells similar to that observed in the intact lens (Figure 1G). The PF6-AM signal was variable among the individual lens epithelial cells in the cultures, with some cells exhibiting more intense fluorescence than others.

Diphenyleneiodonium attenuated the endogenous lens H₂O₂ signal: To confirm that the fluorescent signal detected by PF6-AM in the intact lenses in ex vivo culture or lens epithelial cells in primary culture was due to endogenous ROS production, cells were exposed to the broad-spectrum flavin inhibitor DPI. DPI blocks common intracellular sources of H₂O₂, including mitochondrial-generated H₂O₂ and H₂O₂ generated by flavoenzymes, particularly NADPH oxidase [39]. For these experiments, E10 lenses or isolated lens epithelial cells were preloaded with PF6-AM before their exposure to DPI (5 μ M). Relative to basal states, DPI treatment caused a marked attenuation in the H₂O₂-induced PF6-AM fluorescence response in both the lens organ cultures (Figure 2A) and the primary lens epithelial cell cultures (Figure 2B). In control cultures exposed to PF6-AM alone, the lens epithelial cells exhibited the same pattern of fluorescence signal as described in the studies above. These results strongly suggested that PF6-AM detected endogenous H₂O₂ produced in lens epithelial cells (Figure 2A).

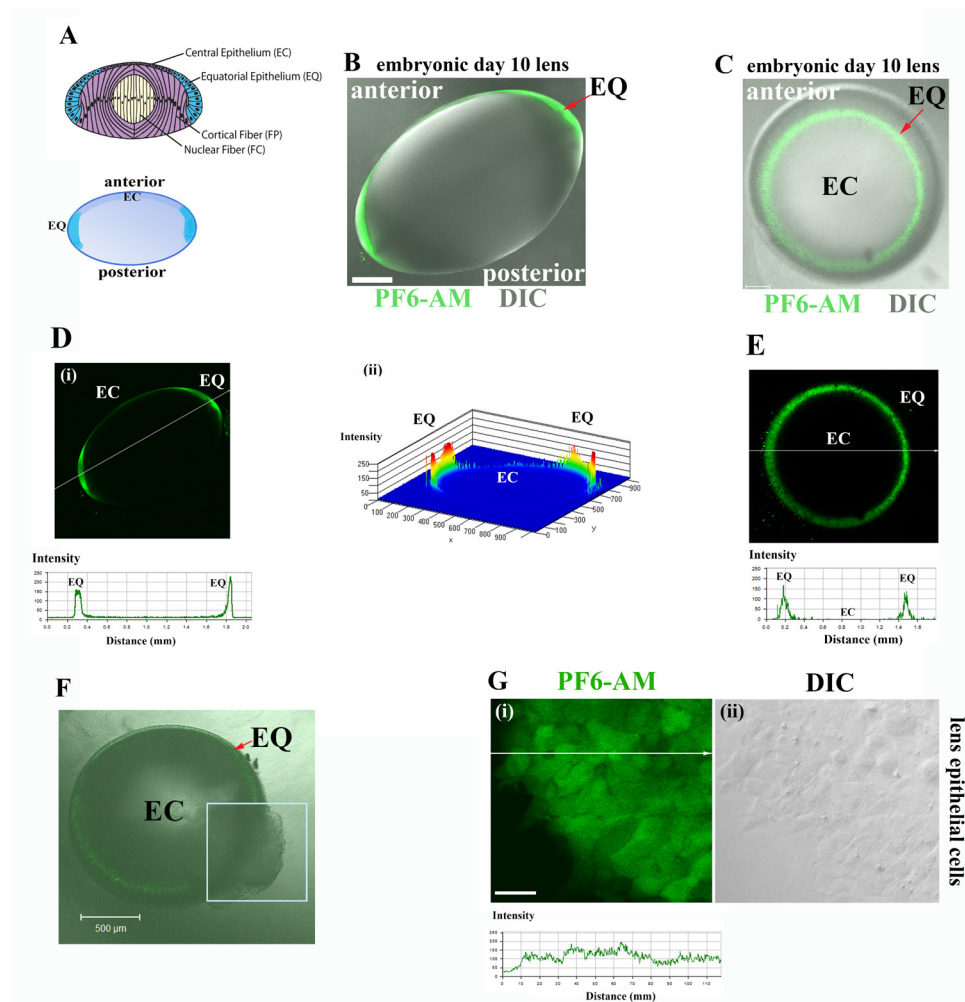


Figure 1. Detection of endogenous hydrogen peroxide (H_2O_2) in lens cells with peroxyfluor-6 acetoxymethyl ester (PF6-AM). **A:** Diagram of the embryonic day 10 (E10) chick lens depicting four distinct regions of differentiation including: EC, undifferentiated lens epithelial cells; EQ, equatorial epithelium which is the zone of differentiation initiation; FP, the zone of lens fiber cell morphogenesis; and FC, the zone of fiber cell maturation. Lens model highlighting the EC and EQ zones (blue, bottom panel). **B:** E10 chick lens placed in organ culture and loaded with PF6-AM (green) to detect endogenous H_2O_2 production, shown as an overlay of PF6-AM (green) to detect endogenous H_2O_2 production, and the differential interference contrast (DIC) image. Whole lens was viewed live from the equatorial region providing an anterior–posterior view. The detection of H_2O_2 production was limited to the lens epithelium and was highest in the EQ zone. **C:** E10 chick lens exposed to PF6-AM (green) and viewed en-face as an overlay with DIC, demonstrating

H_2O_2 production was greatest in the EQ zone. **D:** Line scan intensity analysis across the image profile from (B) (created using LSM5 Image Examiner along [i] an EQ to EQ line) to quantify the level of H_2O_2 production showed that highest intensity labeling for endogenous H_2O_2 occurred in the EQ zone of the lens. (ii) Surface plot histogram (plotted against EC and EQ zones of the lens in B) showing the relative intensities of the H_2O_2 signal across the lens confirmed that the greatest endogenous H_2O_2 production occurred in the EQ zone. **E:** Line scan intensity analysis across the image profile from (C) demonstrated that the greatest production of endogenous H_2O_2 occurred in the EQ zone. **F:** E10 lens with a puncture in the equatorial region (boxed area) incubated with PF6-AM and imaged en-face as an overlay with the DIC image. Results confirmed the specificity of H_2O_2 production to the lens epithelium, as detected by PF6-AM. **G:** (i) Primary lens epithelial cells in culture loaded with PF6-AM showed production of endogenous H_2O_2 (PF6-AM, green); (ii) DIC of image shown in (i). Panel just below (i) shows the line scan intensity analysis for H_2O_2 in lens epithelial cells in (i). Images were acquired live by confocal microscopy. Each optical slice is 3 μ m in thickness in (B) and (C); scale bar = 200 μ m. Optical slice thickness for images (F) and (G) is 1 μ m; scale bar in (F) is 500 μ m and in (G) is 20 μ m. Results are representative of three independent experiments. Studies provided direct evidence for the presence of endogenous H_2O_2 in lens epithelial cells.

Lens cells that expressed endogenous H_2O_2 were rich in actively respiring mitochondria: As mitochondria are the major source of H_2O_2 in the cell, we investigated if H_2O_2 -generating cells have a higher abundance of actively respiring mitochondria. Our previous studies using the potentiometric dye JC-1 showed that at E10, lens epithelial cells but not fiber cells are rich in actively respiring mitochondria

[42]. To examine directly the relationship between lens cells whose mitochondria exhibit active membrane potential at E10 and those producing an H_2O_2 -dependent PF6-AM fluorescent signal, whole lenses were placed in organ culture and exposed concomitantly to both the mitochondrial potentiometric dye MitoTracker Red CMXRos and PF6-AM. Live confocal imaging of whole E10 lenses showed a closely overlapping

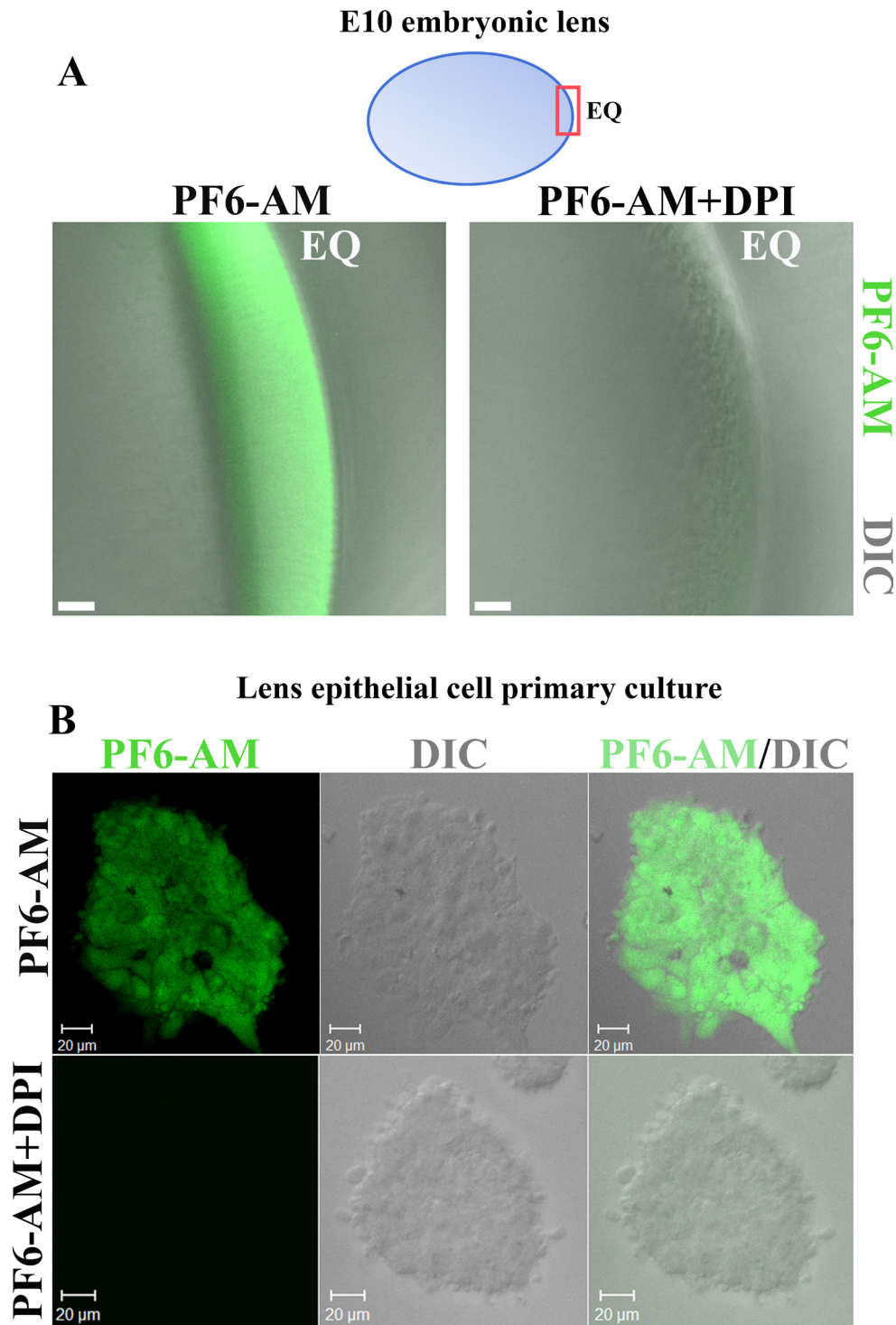


Figure 2. Diphenyleneiodonium (DPI) inhibitor markedly attenuated the hydrogen peroxide (H_2O_2) generated signal in lens cells detected by peroxyfluor-6 acetoxy-methyl ester (PF6-AM). **A**, top panel: Model indicating (red box) the region of E10 chick embryonic lens containing the equatorial zone (EQ) that is imaged below. **A**, bottom left panel: Lens placed in organ culture incubated with PF6-AM (green) showing H_2O_2 production in the EQ zone. **A**, bottom right panel: Lens placed in organ culture incubated with PF6-AM (green) before exposure to the inhibitor diphenyleneiodonium (DPI) attenuates PF6-AM fluorescence, suggesting the presence of an endogenous H_2O_2 signal. Both images are shown as an overlay with the differential interference contrast (DIC) image. **B**: Primary cultures of lens epithelial cells. (Top panels) Cultured lens epithelial cells incubated with PF6-AM (green, left panel) showed similar H_2O_2 production by cultured lens cells as occurs in the EQ region of the intact lens. Cells also shown imaged by DIC (middle panel) and presented as a PF6-AM/DIC overlay (right panel). (Bottom panels) Cultured lens epithelial cells incubated with the PF6-AM H_2O_2 probe (green, left panel) before exposure to DPI show a decrease in signal from PF6-AM. Cells also shown imaged by DIC (middle panel) and presented as a PF6-AM/DIC overlay (right panel). DPI results suggested that the PF6-AM signal is due to H_2O_2 production in cultured lens epithelial cells. Images were acquired live

using confocal microscopy. Each image is a single optical slice of 1 μm; scale bar = 20 μm. Results are representative of three independent experiments.

profile of cells with actively respiring mitochondria (labeled red with the MitoTracker probe) and those producing endogenous H_2O_2 (labeled green with the PF6-AM probe). MitoTracker-labeled cells were detected throughout the epithelium at E10 in both the anterior and equatorial epithelium, with no signal detected in lens fiber cells (Figure 3A). The finding that the PF6-AM fluorescent signal for H_2O_2 overlapped with

that of the Mitotracker dye showed that regions of the lens containing active mitochondria corresponded directly with the regions that generated H_2O_2 (Figure 3A). Similarly, lens epithelial cells in primary culture that produced endogenous H_2O_2 , as detected by PF6-AM, were rich in active mitochondria (Figure 3B).

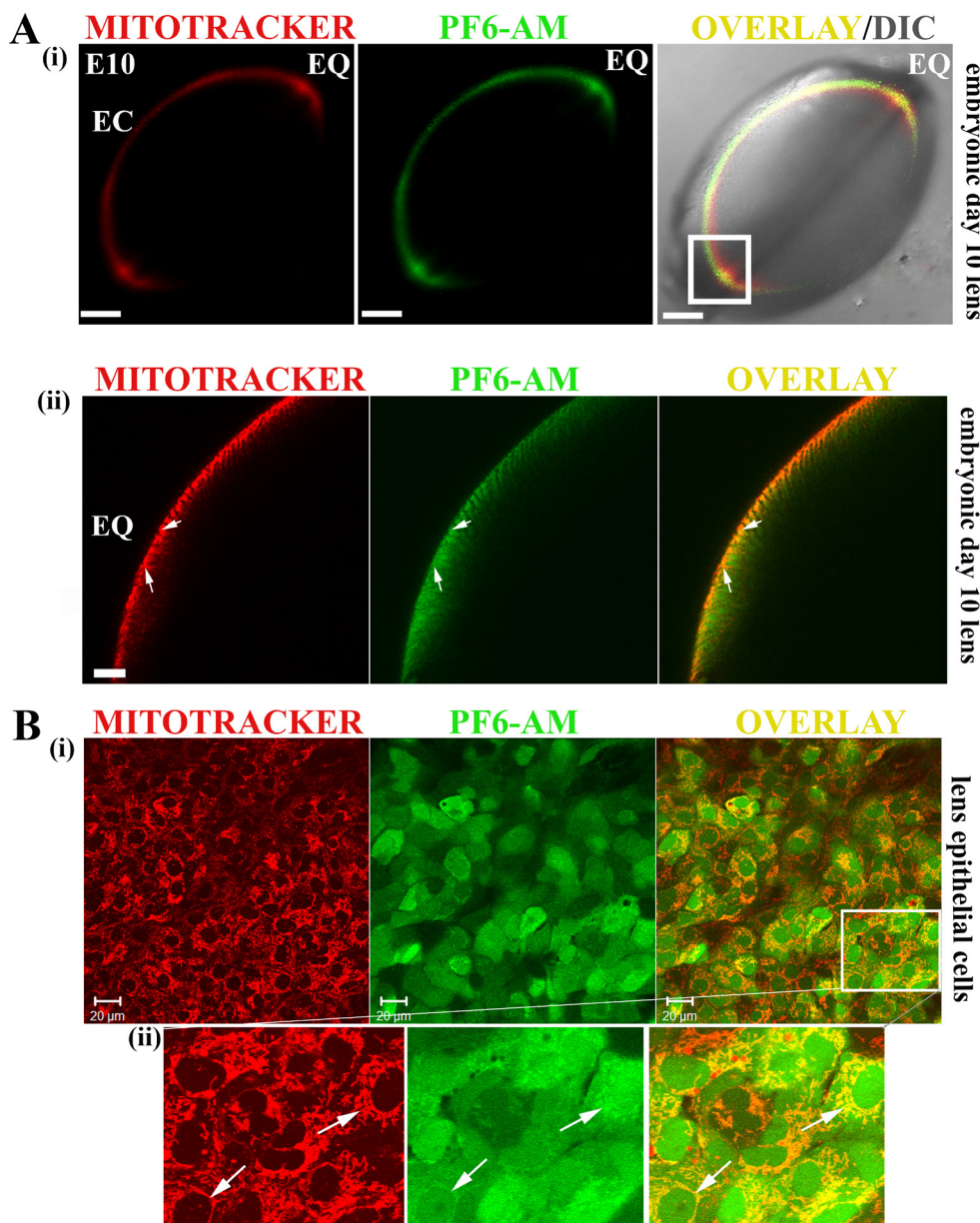


Figure 3. Lens cells rich in actively respiring mitochondria have high endogenous hydrogen peroxide (H_2O_2). **A:** E10 chick embryonic lens in organ culture exposed simultaneously to both MitoTracker Red CMXRos, a mitochondrial probe that detects mitochondria with active membrane potential (red), and PF6-AM (green). Image shows close overlap of actively respiring mitochondria and production of endogenous H_2O_2 in the EQ zone of the lens. (i) Low magnification image in which the whole lens was viewed in an anterior–posterior direction. Co-localization also occurred in the EC zone, but the label was most intense in cells of the EQ zone. The right panel in (i) is an overlay of MitoTracker Red (red), peroxyfluor-6 acetoxymethyl ester (PF6-AM, green) and differential interference contrast (DIC) imaging (greyscale). The boxed region in (i) is shown in (ii) at higher magnification. (ii) EQ zone of the E10 lens showing overlap of H_2O_2 production (PF6-AM, green) with cells rich in active mitochondria (MitoTracker Red CMXRos, red), (arrows). Overlay of PF6-AM and Mitotracker is shown in the right panel. Note fiber cell regions are negative for both probes. **B:** Primary lens epithelial cells in culture exposed simultaneously to MitoTracker Red CMXRos (red)

and PF6-AM (green) often showed a pattern where cells rich in active mitochondria have the highest levels of endogenous H_2O_2 (i). Right panel shows overlay of MitoTracker Red CMXRos (red) and PF6-AM (green). Boxed area in (i) is shown below at a higher magnification in (ii), to highlight the mitochondria (red) in lens epithelial cells with production of endogenous H_2O_2 (green). Right panels show the mitotracker/PF6-AM overlay. Arrows indicate individual active mitochondria. Results suggested that the majority of the lens cells rich in actively respiring mitochondria are generating endogenous H_2O_2 . Images were acquired live using confocal microscopy. Each image is a single optical slice of 1 μm thickness; scale bar = 20 μm . Results are representative of three independent experiments.

DISCUSSION

The results of this study provide evidence of endogenously produced H_2O_2 in the developing lens, particularly in the lens epithelial cells. In addition, these studies established the utility of the PF6-AM fluorescent H_2O_2 probe as a live-imaging reagent to detect endogenous H_2O_2 in lens models. Live labeling with both PF6-AM and the mitochondrial potentiometric dye CMXRos indicated that mitochondria are a likely source of the H_2O_2 produced by lens epithelial cells. However, another potential generator of the endogenous H_2O_2 signal in the lens is the NADPH oxidases (Nox proteins), which are spatially restricted enzyme complexes [51,52]. Nox proteins are capable of generating H_2O_2 at the plasma membrane [20]. DPI inhibits Nox production of H_2O_2 [39,52] as well as the mitochondrial source of H_2O_2 . The H_2O_2 signal detected by PF6-AM in both whole lenses and lens epithelial cells grown in primary culture was markedly attenuated by DPI, verifying the specificity of the PF6-AM signal.

Whole lenses lend themselves well to dye studies. Gap junction connections between different compartments of the lens have been analyzed using dye transfer studies. The results suggest significant gap junction communication within the epithelial and fiber cell compartments but a lack of gap junction communication between the anterior lens epithelium and the underlying lens fiber cells [53]. The current understanding of solute transfer from the aqueous and vitreous compartments to the lens fiber cells *in vivo* suggest that several different mechanisms are at play. These include paracellular transport, and the transport of small molecules through ion channels and water channels [54]. Additional mechanisms of transport that could facilitate dye transfer include gap junctions, and vesicle-mediated transport [55]. Our previous studies using potentiometric dyes, such as JC1, have shown that isolated chick embryo lenses in organ culture readily take up dye from the media into all compartments of the lens [42]. These studies showed that the use of live dyes in the whole embryonic lens is a useful technique to examine cellular dynamics throughout the lens, in real time. In the current study, using the dye PF6-AM under similar conditions, we showed for the first time that the epithelial cells of the intact lens generate endogenous H_2O_2 under basal conditions and that this signal was coincident with the region of the lens containing actively respiring mitochondria. In contrast, the fiber cell zone, in which mitochondria do not exhibit active membrane potential at E10, did not produce an H_2O_2 signal even when the lens was punctured through to the fiber zone so that the fiber cells were exposed directly to the PF6-AM dye. While this result is strongly suggestive of an absence of an endogenous H_2O_2 signal in lens fiber cells and

consistent with the absence of mitochondria with an active membrane potential [42], we cannot rule out the possibility that there may be other factors interfering with the PF6-AM signal, such as the level of uncaging of the probe by esterases [39].

It is likely that the spatial and temporal fluxes of H_2O_2 generated in the lens determine its biologic outcome and that this redox signal is tightly regulated. While lower controlled fluxes of H_2O_2 can function as a cellular signal, higher concentrations of H_2O_2 can be toxic and lead to DNA damage, lipid peroxidation, and protein oxidation, causing oxidative stress [28,56]. Indeed, oxidative stress resulting from increasingly high levels of ROS is a leading cause of protein damage in the lens and age-related cataracts [24-26,28,33]. Therefore, conserved defense mechanisms that the lens cells have evolved, both nonenzymatic (e.g., glutathione, vitamin C, vitamin E, and carotenoids) and enzymatic (e.g., superoxide dismutase, glutathione peroxidase, and catalase), are involved in regulating H_2O_2 produced in these cells and maintaining it at low concentrations [24,33].

As studies in lens epithelial cell cultures have previously shown the production of H_2O_2 by these cells [14,57], the relevance of detecting an endogenous H_2O_2 signal in the lens epithelium takes on even more importance. For example, Lou and colleagues showed a connection between H_2O_2 production and lens epithelial cell proliferation, involving activation of the extracellular signal-regulated kinase (ERK), mitogen-activated protein (MAP) kinase-signaling pathway [14,58]. In other cell types, low-dose H_2O_2 signaling has been shown to induce cell adhesion, migration [13], differentiation [2,4], and proliferation [52,58]. Many features of lens differentiation, including the role of the mitochondrial death pathway as a nonapoptotic signal required for differentiation initiation [48,59], could involve an endogenous H_2O_2 signal, such as the current studies showed in the equatorial epithelium. The use of PF6-AM as a new reagent in the intact lens to visualize the live endogenous H_2O_2 signal opens up new avenues of study to determine the specific role of this signal in lens development, including the potential to use this dye in live studies *in vivo*.

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