**Induction of p21\textsuperscript{Cip1}-mediated G\textsubscript{2}/M arrest in H\textsubscript{2}O\textsubscript{2}-treated lens epithelial cells**

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**Purpose:** Oxidative damage is one of the major factors associated with the formation of age-related cataract and with senescence of various cell types. Although the effects of oxidative stress are complex, we focused on whether oxidative damage affects control of the cell cycle in lens epithelial cells.

**Methods:** BrdU labeling and FACS analysis were used to investigate the effect of H\textsubscript{2}O\textsubscript{2} on the cell cycle of HLE B-3 cells. In addition, western and Northern blot analysis were performed to assess the expression of cell cycle regulatory proteins and transfection with siRNA was used to knock out expression of p21\textsuperscript{Cip1}. The activation of MAPK family members by oxidative stress was assessed using antibodies to detect the activated forms. To confirm the effect of H\textsubscript{2}O\textsubscript{2} on an ex vivo model, its effect on cultures of the lenses of 3-week-old SD rats were examined. The localization and expression of PCNA and p21\textsuperscript{Cip1} in the rat lenses were analyzed by immunohistochemistry.

**Results:** FACS analysis showed that H\textsubscript{2}O\textsubscript{2} treatment induced G\textsubscript{2}/M phase arrest of HLE B-3 cells. p21\textsuperscript{Cip1} was strongly induced by H\textsubscript{2}O\textsubscript{2}, whereas expression of other cell cycle genes was unchanged. Attenuation of p21\textsuperscript{Cip1} expression using siRNA reduced the H\textsubscript{2}O\textsubscript{2} induced G\textsubscript{2}/M arrest. Furthermore, JNK and ERK were activated by H\textsubscript{2}O\textsubscript{2} and their specific inhibitors SP600125 (for JNK) and U0126 (for ERK1/2) prevented p21\textsuperscript{Cip1} expression and blocked cell cycle arrest. H\textsubscript{2}O\textsubscript{2} treatment of a rat lens organ culture also caused an increase in p21\textsuperscript{Cip1}. However, H\textsubscript{2}O\textsubscript{2} treatment lowered the levels of p27\textsuperscript{Kip1}, cdc2, and PCNA in the rat lens culture, unlike in the HLE B-3 cells.

**Conclusions:** The accumulation of p21\textsuperscript{Cip1} in lenses exposed to oxidative stress may play a role as a defensive mediator of oxidative damage, an indicator for senescence or aging, or an inducer for the formation of cataract. This finding links oxidative stress with p21\textsuperscript{Cip1}-mediated control of the cell cycle in lens epithelial cells.

Reactive oxygen species (ROS), including the superoxide anion, hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), and hydroxyl radicals are natural byproducts of aerobic metabolism [1]. Oxidative stress caused by ROS leads to modification of proteins, lipid oxidation, DNA damage and other impairments of the physiological functions of cells and tissues [2]. It has also been suggested that oxidative stress is an important cause of aging, cancer, senescence (an irreversible cell arrest), and cell death [3].

The lens is derived from ectoderm and grows throughout life. This transparent organ consists of a single layer of epithelial cells on the anterior and equatorial surfaces, and elongated, terminally differentiated cells in the interior. The lens epithelial layer is essential for growth, differentiation, and homeostasis of the entire lens. The lens fiber cells obtain energy and nutrients (including water and ions) through metabolic communication with this monolayer [4]. It has been demonstrated that damage to lens epithelial cells (LECs) as a result of various stresses can be a major cause of the formation of cataract. Oxidative stress, in particular, combined with aging of the lens, is believed to contribute to the formation of age-related cataract [5]. This view is supported by the fact that the level of H\textsubscript{2}O\textsubscript{2} is high in the aqueous, vitreous, and lenses of patients with age-related cataract [6]. Moreover, this is correlated with extensive oxidation of the lens components. In addition, H\textsubscript{2}O\textsubscript{2} can induce opacification of the lens in rat organ cultures [7,8].

Age-related cataract is a multifactorial disease with a poorly understood etiology. Several lines of evidence suggest that changes in the regulation of the cell cycle in LECs are involved. The proliferative potential of the LECs decreases with age in rodents, suggesting that it is related to telomeric shortening, the induction of senescence, and the appearance of cataracts [9,10]. Furthermore, the abnormal regulation of the cell cycle in the lens, together with the accumulation of damaged proteins, is probably caused by an age-related decline in the ubiquitin-proteasome pathway that is associated with age-related cataract [11]. Despite numerous studies documenting biochemical and metabolic changes in the lens associated with age-related cataract, little is known about the involvement of the cell cycle. Recent reports have attempted to identify changes in gene expression associated with age-related cataract using microarrays [12,13]. Functional clustering of the identified genes revealed that levels of transcripts...
associated with the control of the cell cycle are reduced in age-related cataract.

Cell cycle arrest plays an important role in development and differentiation [14], and it is a well-known indicator of aging, senescence, and apoptosis [3,15]. Positive regulation of the cell cycle is mediated by the cyclin-dependent kinases (CDKs). Acting in opposition to the CDKs are two families of CDK inhibitors (CKIs); the Cip/Kip family (p21Cip1, p27Kip1, and p57Kip2) and the INK4 family (p15, p16, p18, and p19) [16]. The biochemical activities and patterns of expression of CKIs during development and differentiation implicate these proteins as primary effectors controlling cell cycle exit [17].

Several lines of evidence indicate that temporal and spatial control of the cell cycle is important in differentiation and development of the lens. In the embryonic lens, cell cycle withdrawal is correlated with the expression of p57Kip2 [18], and with coordinate functioning of p27Kip1 and p57Kip2 [19]. Consistent with this view, expression of both p27Kip1 and p57Kip2 is upregulated in cultured LECs by inhibition of Src family tyrosine kinases, which causes withdrawal from the cell cycle [20]. In addition, p21Cip1 and p27Kip1 are upregulated during bFGF-induced lens cell differentiation [21]. The LECs in the vicinity of the visual axis do not divide, but cell division occurs in the equatorial region and terminal differentiation is initiated in the bow region, which generates the fiber cells. Proper execution of the differentiation program and the formation of mature fibers seem to be essential for lens transparency. This means that abnormalities that result in the incomplete degradation of intracellular organelles are associated with various forms of cataract [12,22].

Because the control of the cell cycle in LECs plays an essential role in lens differentiation, development, and maintenance, an alteration in this control could be directly or indirectly involved in the formation of cataract. Furthermore, the cell cycle can be regulated by oxidative stress, which induces multiphase arrest and subsequent cell death in various types of cells [3,23,24]. These observations, together with the potential effect of ROS as inducers of age-related cataract, prompted us to investigate whether oxidative stress induced by ROS can affect the cell cycle of LECs and, if so, what mechanism is involved. We found that a sublethal dose of oxidative stress to an established line of human LECs (HLE B-3) induced p21Cip1 expression, followed by G1/M phase arrest as a consequence of activation of c-Jun amino-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK). Furthermore, in a rat lens organ culture, lens opacification by H2O2 was accompanied by abnormal expression of cell cycle regulators such as p21Cip1, p27Kip1, cdc2, and proliferating cell nuclear antigen (PCNA).

**METHODS**

**Cell culture conditions and H2O2 treatment:** Human lens epithelial B-3 (HLE B-3) cells were kindly provided from Usha P. Andley of Washington University [25]. The cells were cultured in minimum essential medium (MEM, Gibco, Rockville, MD) with 20% fetal bovine serum (FBS, Gibco) and 50 µg/ml gentamycin (Gibco) at 37 °C with 5% CO2.

For experiments, the cells were grown to 70% confluence and then were treated with various concentrations of H2O2 (Sigma-Aldrich, St. Louis, MO) in serum free media for the indicated times. For the cell counting experiment, H2O2 treated or untreated cells in 35 mm culture dishes were washed with MEM without serum, and then 0.25%Trypsin-1 mM EDTA (Welgene, Daegu, Korea) was added for 5 min at room temperature. MEM+20% FBS (1 ml total) was added then the cells were collected. After centrifugation at 2,000 rpm for 5 min, the cells were resuspended in the 1 ml of medium and total cell number was counted. For the inhibitor study, JNK/SAPK specific inhibitor SP600125, MEK inhibitor U0126, p38 inhibitor SB203580, PI-3 kinase inhibitor Wortmannin (Calbiochem, San Diego, CA) and PKC inhibitor GF109203X (Sigma-Aldrich) were added 30 min before H2O2 treatment.

**5-Bromo-2′-deoxy-uridine (BrdU) Staining:** HLE B-3 cells were treated or untreated with 200 µM H2O2, incubated for 24 h, and then BrdU was added and incubated for a further 40 min. The cells were fixed with 70% ethanol in 50 mM Glycine pH 2, then immunofluorescence detection of BrdU was performed using the 5-Bromo-2′-deoxy-uridine (BrdU) Labeling and Detection Kit I (Roche Applied Science, Germany) according to the manufacturer’s protocol. Nuclear stain was done by addition of Hoechst 33342 (Molecular probes, Eugene, OR).

**Flow cytometric cell cycle analysis of HLE B-3 cells:** HLE B-3 cells were seeded on 100 mm culture dish at a density of 1x10^6 cells per dish. The H2O2 treated (24 h) or untreated cells were washed twice with ice cold PBS. After treatment of 0.25% Trypsin-1 mM EDTA for 5 min, the cells were collected. The cells were washed twice with PBS then resuspended in 0.5 ml of PBS. Fixation was done by the addition of 5 ml of ice-cold 70% ethanol and incubated overnight at -20 °C. The next day, the cells were washed twice with PBS and resuspended in 500 µl of PBS. After pretreatment with 50 µg/ml of RNase A (Sigma-Aldrich) for 15 min at 37 °C, the cells were stained with addition of 0.5 ml propidium iodide (100 µg/ml in PBS; Sigma-Aldrich) for 30 min at 37 °C. Stained cells were dispersed with a pipette and cell cycle analysis was carried out using a FACSVantage SE (BD Biosciences Immunocytometry Systems, San Jose, CA) using excitation at 536 nm and detection at 617 nm for red fluorescence. The percentage of the cells in each cell cycle phase was determined using the ModFit LT software (Becton-Dickinson) based on the DNA histogram. Ten thousand cells per sample were analyzed.

**Antibodies and western blot analysis:** Antibodies used in this study were obtained as follows: phospho-JNK, ERK1/2 and JNK from Cell signaling (Beverly, MA). Caspase-3 and p21Cip1 was from Upstate cell signaling solutions (Charlottesville, VA). p27Kip1, cdc2, PCNA, caspase-9, PARP, Cyclin A, Cyclin D1, Cyclin E, and phospho-ERK1/2 were from Santa Cruz (Santa Cruz, CA). Actin and α-tubulin were purchased from Sigma-Aldrich. Goat anti-rabbit and anti-mouse antibody conjugated to horseradish peroxidase was purchased from Zymed (South San Francisco, CA).

For western blot analysis, lens epithelial cells were harvested in protein lysis buffer (25 mM Tris-HCl, pH 7.4, 1%
Tween-20, 0.1% SDS, 0.5% sodium deoxycholate, 10% Glyc- erol, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 50 mM NaF, 1 mM Na₂VO₄, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin). The cell lysates were incubated on ice for 15 min with occasional mixing and cleared of cell debris and large molecules by centrifugation at 14,000 rpm for 20 min at 4 °C. Protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL). The lysates containing 10 µg or 20 µg of proteins were boiled for 5 min in 1X SDS sample buffer, loaded and separated on a 10% or 12% SDS-polyacrylamide gel electrophoresis (PAGE) gel, and transferred to a nitrocellulose membrane (Amersham Life Science, Cleve-
land, OH) using an electrotransfer apparatus (Amersham). Skim milk (5%) in Tris-buffered Saline (50 mM Tris-Cl, 150 mM NaCl, pH 7.4)-Tween-20 (0.1%) was used as a blocking and antibody dilution buffer. The membrane was developed by enhanced chemiluminescence (Santa Cruz). Prestained molecular weight standards were purchased from Elpis-Biotech (DaeJeon, Korea).

**Northern blot analysis:** HLE B-3 cells were incubated with MEM+1% FBS. The next day, the medium was changed to MEM without serum and then 200 µM of H₂O₂ was added at the indicated times. Total cellular RNA was isolated using TRIZOL reagent (GIBCO). Total RNA was quantified by absorbance at 260 nm. The integrity of RNA was checked on 1% agarose/formaldehyde gel. Total RNA (20 µg) was applied to each lane. The RNA was transferred to a positively charged Nylon membrane (Schleicher&Scuell, Germany) and fixed to the membrane using a UV cross-linker (Stratagene, La Jolla, CA) with exposure of 120 000 µJ/cm².

Prehybridization was performed in 5X SSPE, 50% formamide, 10X Denhardt’s solution, and 0.5% SDS for 2 h at 42°C. The membrane was hybridized with p21Cip1 or β-actin cDNA that was labeled with [α-³²P] dCTP by using a Random Primer DNA Labeling Kit (Roche) for 16 h at 42°C. The membrane was washed with 2X SSC, 0.1% SDS for 30 min at room tem-

Figure 2. H₂O₂ induces the expression of p21Cip1. A: HLE B-3 cells were treated with 200 µM H₂O₂ at the indicated times. Cell lysates were prepared for western blot analysis. The expression of p21Cip1, p27Kip1, cdc2, cyclin A, cyclin D1, cyclin E, and actin were analyzed. B: Northern blot analysis for the induction of p21Cip1 by 200 µM H₂O₂ at indicated times. C: HLE B-3 cells were treated for 16 h in either the absence or presence various concentrations of H₂O₂ as indicated. The cell lysates were prepared and analyzed in the level of p21Cip1 by western blot. The data shown are representative of three independent experiments.
perature, with 0.5X SSC, 0.1% SDS for 30 min at 55 °C, and then twice with 0.2X SSC, 0.1% SDS for 30 min at 55 °C. The membrane was exposed for autoradiography at -70 °C for 4 h on X-ray films.

**siRNA experiments:** Validated p21cip1 specific siRNA was obtained from Ambion (catalog number 51320; Austin, TX). HLE B-3 cells were plated with MEM+1% FBS. At 24 h, the medium was changed to Opti-MEM (GIBCO) and the cells (about 70% confluence) were transfected with the p21cip1 siRNAs using SiPORT™ Lipid siRNA transfection agent (catalog number 4505; Ambion) according to the manufacturer’s protocol. H2O2 (200 µM) was added after 1 day of transfection. After 24 h of H2O2 treatment, the cells were collected and subjected to western or FACS analysis.

**Rat lens organ culture and western blot analysis:** Whole lenses were carefully removed from 3-week-old male Sprague-Dawley rats and incubated in Medium 199 (Sigma-Aldrich) containing 0.1% BSA (GIBCO) and 50 µg/ml gentamycin at 37 °C with 5% CO2. The next day, the medium was changed and 200 µM of H2O2 was added for 1 day. The entire lens epithelium and fiber cells were carefully removed under the dissection microscope. The isolated lens epithelial cells and fiber cells in 100 µl of lysis buffer were extracted by sonication. Protein concentration was determined by BCA assay (Pierce). Total protein (10 or 40 µg) was subjected to western blot analysis.

**Rat lens section and immunohistochemistry:** For immunohistochemistry, whole rat lenses were fixed in Carnoy’s fixative at 4 °C overnight, and then embedded in paraffin. The paraffin-embedded lenses were sectioned on a microtome at a thickness of 7 µm. The lens sections were incubated in 5% normal horse serum and 0.5% BSA in PBS for 1 h at room temperature, then treated with a 1:100 dilution of mouse anti-PCNA or mouse anti-p21cip1 (Santa Cruz) for 2 h at room tem-

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**Figure 3.** H2O2 mediated p21cip1 and G2/M phase arrest is attenuated by p21cip1 siRNA. HLE B-3 cells were transfected with 100 nM of p21cip1 siRNA. After 24 h, the cells were incubated in the absence or presence of 200 µM H2O2 for an additional 24 h, collected, and subjected to both western blot analysis (A) and FACS analysis (B) to confirm the attenuation of p21cip1 and cell cycle arrest, respectively. The graph shown in A represented the quantification of p21cip1 expression (performed by scanning densitometry). The bars shown (A, lower) represent the means±SE of three independent expressions and are expressed as the fold induction above control (unstimulated) values.
perature. After 3 washs with PBS, the sections were incubated in 1:200 dilution of anti-mouse Alexa Fluor 488 (Molecular probes) in PBS for 40 min, then counter stained with Hoechst 33258 (Molecular probes). Immunolabeled lens sections were visualized and photographed digitally with an inverted fluorescence microscope (Axiovert S100, Carl Zeiss Meditec) and digital camera (Axiocam, Carl Zeiss Meditec).

**RESULTS**

**H₂O₂ induces cell cycle arrest in HLE B-3 cells:** In order to determine the effect of H₂O₂ on cell growth, HLE B-3 cells were exposed to various concentrations of H₂O₂ for 24 h and counted. H₂O₂ (5-200 µM) had only a small effect on cell numbers and no effect on their morphology (data not shown) whereas higher doses (400-800 µM) caused a sharp drop in cell number (Figure 1A). To investigate the effects of sublethal doses of H₂O₂ on the cell cycle, BrdU labeling and FACS analysis were employed, as described in Methods. Cells were treated with 200 µM H₂O₂ for 24 h and fixed following 4 h incubation with BrdU. The cells were then immunostained with anti-BrdU antibody and the ratio of BrdU/Hoechst double-positive cells to the total number of Hoechst-positive cells was determined (Figure 1B). BrdU incorporation was dramatically decreased in the H₂O₂ treated cells, without morphological changes. To determine whether phases of the cell cycle in addition to S phase are affected by H₂O₂, under these conditions, we examined nuclear DNA content by PI staining and FACS analysis (Figure 1C). There was a significant shift toward G₂/M (from 21% to 68.4%), in the H₂O₂ treated cells while the proportion of cells in G₀/G₁ and S decreased (from 48.2% to 13.3% and from 30.8% to 18.3%, respectively Figure 1C, lower). These data demonstrate that treatment of HLE B-3 cells with sublethal doses of H₂O₂ for 24 h induced G₂/M phase arrest.

To test whether H₂O₂ induced cell cycle arrest at a sublethal dose is independent of apoptosis, we examined the activation of caspases and the cleavage of PARP, which is cleaved by activated caspases during apoptosis. As shown as Figure 1D, the expression of caspase-3, caspase-8, and caspase-9 did not change; in addition we did not detect the activated forms of these enzymes, or cleavage of PARP (data not shown). We conclude that 50-200 µM of H₂O₂ induced G₂/M arrest of the HLE B-3 cells without cell death.

**p21^Cip1 is induced in H₂O₂ treated HLE B-3 cells:** The expression of proteins related to the cell cycle was investigated in H₂O₂ treated HLE B-3 cells by western blot analysis. There were no obvious changes in the expression of cdc2, cyclin A, cyclin D1, cyclin E, p27Kip1, and p57Kip2 (data not shown) in response to H₂O₂. However, p21^Cip1 increased significantly 8 h after H₂O₂ treatment and remained

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Figure 4. H₂O₂ induced p21^Cip1 accumulation is mediated by JNK and ERK activations. A: HLE B-3 cells were treated for 30 min with specific kinases inhibitors before treatment with 200 µM H₂O₂. The kinase inhibitors used were 20 µM SB203580 (SB), 20 µM SP600125 (SP), 1 µM Wortmannin (W), 20 µM U0126 (U), and 10 µM GF109203X (GF) which inhibit p38, JNK, PI-3 kinase, MEK, and PKC, respectively. p21^Cip1 and actin levels were measured after 16 h of H₂O₂ treatment. B: HLE B-3 cells were stimulated with 200 µM of H₂O₂ for the indicated time period. After lysis, 10 µg (for ERK) or 30 µg (for JNK) of total proteins were loaded on 10% PAGE gel. Phosphorylated (p-ERK and p-JNK) or nonphosphorylated levels of JNK and ERK were measured by western blot analysis using specific antibodies. C: FACS analysis showed that SP600125 and U0126 blocked G₂/M phase arrest caused by H₂O₂. The data shown are representative of two to three independent experiments.
high for 24 h. Northern blot analysis revealed an increase in p21\textsuperscript{Cip1} transcripts 4 h after treatment with 200 µM H\textsubscript{2}O\textsubscript{2}, reaching a maximum after 16 h (Figure 2B). The data in Figure 2C demonstrate a dose-dependent increase in p21\textsuperscript{Cip1} with a maximum at 200 µM, paralleling the G\textsubscript{2}/M arrest induced by H\textsubscript{2}O\textsubscript{2} (Figure 1C). When cells were exposed to 400 µM H\textsubscript{2}O\textsubscript{2}, total protein decreased and most of the cells died within 36 h (data not shown).

Regulation of H\textsubscript{2}O\textsubscript{2} induced G\textsubscript{2}/M phase arrest by p21\textsuperscript{Cip1}: Accumulating data demonstrate that p21\textsuperscript{Cip1} induces G\textsubscript{2}/M phase arrest in fibroblast, colorectal cancer cells, and lung cancer cells [23,24]. We therefore asked whether p21\textsuperscript{Cip1} is

![Image](http://www.molvis.org/molvis/v11/a92/)

**Figure 5.** H\textsubscript{2}O\textsubscript{2} induces lens opacification and the accumulation of p21\textsuperscript{Cip1} in rat lens. SD rat lenses were removed from eye and cultured on M199+0.1% BSA with or without 200 µM H\textsubscript{2}O\textsubscript{2} for 1 day. A: Lens opacification was observed in the equatorial region spreading throughout the superficial cortex. B: Rat LECs and fiber cells were separately removed and analyzed using antibodies against the cell cycle (left) and apoptosis (right) related proteins. C: Rat lenses were embedded in paraffin, sectioned, and then immunostained with anti-PCNA (green, upper) and anti-p21\textsuperscript{Cip1} (green, lower). Nuclear stain with Hoechst (blue) was carried out to show the localization of LECs. The original magnification was x200.
required for H$_2$O$_2$ induced G/M phase arrest in HLE B-3 cells, using transfection with p21$^{Cip1}$-specific siRNA to inhibit the expression of p21$^{Cip1}$. Expression of p21$^{Cip1}$ in response to H$_2$O$_2$ decreased (by 50%) in the siRNA-transfected HLE B-3 cells (Figure 3A). Under these conditions G/M phase arrest was partially inhibited (from 69.3% to 48.2%) with a parallel increase in G$_0$/G$_1$ phase (from 13.8% to 24.3%; Figure 3B). These results indicated that the H$_2$O$_2$ induced expression of p21$^{Cip1}$ was responsible for stimulating G$_2$/M phase arrest in HLE B-3 cells.

**JNK and ERK1/2 are involved in the induction of p21$^{Cip1}$ by H$_2$O$_2$:** Many protein kinases and transcription regulatory factors such as MAPK family, PI3K, NF-$k$B, and AP-1 are activated by oxidative stress [26,27]. To investigate which protein kinases are involved in H$_2$O$_2$ induced p21$^{Cip1}$ expression and G/M phase arrest, we tested specific inhibitors of several signaling kinases known to be activated by H$_2$O$_2$; p38 MAPK (SB203580), JNK (SP600125), PI-3 kinase (Wortmanin), MEK (U0126), and PKC (GF109203X). As illustrated in Figure 4A, pretreatment of HLE B-3 cells with 10 $\mu$M SP600125 or U0126 blocked the H$_2$O$_2$ induced increase of p21$^{Cip1}$ protein, suggesting that ERK and JNK, but not the p38 MAPK, PI-3 kinase, nor PKC pathways are involved in this process.

To confirm the involvement of the ERK and JNK pathways in H$_2$O$_2$ induced upregulation of p21$^{Cip1}$, we examined the kinetics of activation of ERK and JNK. As shown in Figure 4B, we observed bimodal ERK activation in response to H$_2$O$_2$. The first peak was rapid and transient, with a maximum at 15 min, followed by a decline toward baseline by 30 min. This was followed by a gradual rise to a secondary sustained rise. In contrast to ERK, JNK activation could be detected within 1 h, reaching a maximum at 2 h and subsequently declining gradually to almost baseline. In addition, p38 MAPK was not activated by H$_2$O$_2$ in the HLE B-3 cells (data not shown). Since the timing of the activation of ERK and JNK is closely related with that of p21$^{Cip1}$ expression (Figure 2B), these results support the idea that both the ERK and JNK pathways mediate H$_2$O$_2$ induced p21$^{Cip1}$ expression in HLE B-3 cells.

To see whether the ERK and JNK signaling cascades are actually required for H$_2$O$_2$ induced G/M arrest, we tested the effect of specific inhibitors of ERK and JNK. As shown in Figure 4C, these inhibitors completely prevented G/M phase arrest. We conclude that H$_2$O$_2$ induced G/M phase arrest in HLE B-3 cells proceeds via p21$^{Cip1}$ expression, which is dependent on the ERK and JNK pathways.

**The role of p21$^{Cip1}$ in H$_2$O$_2$ induced cataract formation:** To confirm these conclusions ex vivo we used the intact rat lens system, previously described by Spector [8]. When rat lenses were incubated in the presence of 200 $\mu$M H$_2$O$_2$, opacification was observed in the equatorial region, spreading throughout the superficial cortex (Figure 5A, right) as previously reported. No change in the morphology of the LECs was observed in response to 200 $\mu$M (data not shown) but exposure to 400 $\mu$M H$_2$O$_2$ or more caused severe lens opacity, together with death of LECs (data not shown). In addition, western blot analysis showed that levels of PCNA, cdc2, and p27$^{kip1}$ declined, while p21$^{Cip1}$ accumulated dramatically in the rat lens treated with 200 $\mu$M H$_2$O$_2$ (Figure 5B, left), and the reduction in PCNA and increase in p21$^{Cip1}$ were confirmed by immunohistochemistry (Figure 5C). Interestingly, PCNA was present in the differentiated LECs of control rat lenses and disappeared as a result of H$_2$O$_2$ treatment. Activation of caspases 3, 8, and 9 was not detected, again suggesting that treatment of rat lenses with 200 $\mu$M H$_2$O$_2$ does not induce apoptotic cell death (Figure 5B, right).

**DISCUSSION**

Oxidative stress is believed to be an important cause of aging, cancer, and cell death. It has been also suggested to be a key mediator of the formation of lens cataracts. However, investigating the role of oxidative stress is not simple, as it shuts down some metabolic pathways, increases protein aggregations and oxidation, inhibits ubiquitin-proteasome systems, induces cell senescence/arrest via telomere shortening and/or damage to telomere DNA, stimulates DNA repair systems, activates apoptosis, and mobilizes defenses. Among these complex responses to oxidative stress, the present study focused on control of the cell cycle and regulation of the CKIs in LECs, because it has been suggested that lens maintenance and ocular transparency depend on accurate control of the cell cycle, and that disregulation of the CKIs is associated with cataractogenesis induced by various stimuli. For example, low power microwaves induced G$_0$/G$_1$ arrest and p27$^{kip1}$ expression in rabbit LECs [28], and accumulation of p21$^{Cip1}$ was observed in a rat sugar cataract model [29]. Recently, Hawse et al. [12] examined human lenses with age-related cataracts and reported a decrease in the levels of cyclin D1 and cyclin G1, which function in the G$_0$ to S phase transition, and the DNA damage response, respectively. In the present study we found that oxidative stress induced G$_2$/M phase arrest in the LECs as a result of p21$^{Cip1}$ expression, thus providing a link between control of the cell cycle and cataractogenesis.

Oxidative stress may act on growth factor receptors, such as EGFR, to activate the ERK and JNK pathways [27,30,31]. Several lines of evidence indicate that oxidants activate the ERK and/or JNK pathways mainly by stimulating growth-factor receptors, mimicking the actions of natural ligands. This is probably the result of oxidant-mediated inactivation of phosphatases necessary for dephosphorylation of the growth factor receptors [31]. However, we found that pretreatment with AG1478, a specific inhibitor of EGFR, did not inhibit p21$^{Cip1}$ expression or ERK/JNK activation by H$_2$O$_2$ in HLE B-3 cells (data not shown), indicating that ERK/JNK activation is independent of EGFR transactivation, although we cannot rule out the involvement of other growth factor receptors. Another possibility is that oxidative stress causes the expression and release of cytokine/growth factors such as TGF-$\beta$, which activate the MAPK pathways. Because oxidative stress induces the expression of TGF-$\beta$ [32], we have examined the effect of TGF-$\beta$ using an antibody that blocks TGF-$\beta$ function. However, this did not affect p21$^{Cip1}$ upregulation and ERK/JNK activation by H$_2$O$_2$. Therefore, further studies are needed to investigate how H$_2$O$_2$ causes activation of ERK and/or JNK.

p21$^{Cip1}$ was originally identified as a gene regulated by
the tumor suppressor protein p53 [33], and the induction of p21Cip1 in response to X-rays and other DNA-damaging agents relies, to different extents, on its transcriptional upregulation by p53 [34]. However, induction of p21Cip1 in response to mitochondrial stress or to other stresses occurs via mechanisms that are independent of p53 [34,35]. In this study, we demonstrated that the induction of p21Cip1 by H2O2 in LECs requires both ERK and JNK, but we do not know if it is dependent on p53. The ERK and/or JNK pathways are involved in p21Cip1 expression in various cell types [36], but there is controversy over how the ERK pathway functions in p21Cip1 transcription. In p53-deficient human lung cancer cells, H2O2-dependent ERK pathways mediate p21Cip1 upregulation by activating AP-1 and G1/M phase arrest [24]. JNK, along with ERK, is considered a key mediator of p21Cip1 expression. JNK-1-responsive cis-acting regulatory elements are present between -127 and -64 of the p21Cip1 promoter, which contains six GC-rich Sp1-responsive elements known to play a major role in p53-independent transcription of p21Cip1 [37]. Consistent with these results, a previous study showed that c-Jun, a substrate for JNK, mediates the p53-independent activation of the p21Cip1 promoter by physical interaction with Sp1 [38]. These findings, together with the evidence for ERK/JNK activation by oxidative stress, support the view that the H2O2-mediated p21Cip1 induction in the LECs occurs via ERK/JNK activation.

In addition, p21Cip1 levels are also regulated post-transcriptionally, being subject to proteasome-dependent degradation [39] that can be modulated by interaction with CDKs or PCNA [40,41]. This suggests that the accumulation of p21Cip1 may be affected by a decrease in proteasome-dependent proteolysis. Normally, LECs and fiber cells possess a fully functional ubiquitin-proteasome pathway and ubiquitin conjugating activity [42,43]. However, it has been reported that a decrease in the ubiquitin conjugating activity in LECs is associated with the accumulation of oxidative damage, aging, and terminal differentiation [11,21,44,45].

The role of p21Cip1 in oxidative stress remains controversial. Although there is evidence that p21Cip1 is proapoptotic in certain situations, most studies have provided evidence that it functions as a protective factor during stress, due to its growth-inhibitory properties [46]. Therefore, the accumulation of p21Cip1 by H2O2 may activate a cell cycle checkpoint to rescue the cells from DNA damage. Another role is suggested to be an indicator of senescence in vitro or aging in vivo. At the molecular level, senescence is associated with changes in the expression of a large number of genes [47]. Senescent cells have increased levels of CKIs, p21Cip1, and p16, which are negative regulators of cell proliferation and cannot express c-fos, cdc2, and PCNA [48-50]. Furthermore, LECs in age-related cataract also display decreased levels of cyclins [12,13]. Therefore, these data support the idea that accumulation of p21Cip1 in the LECs is closely related to the formation of age-related cataracts. As in senescence, H2O2-treated rat lenses show a decrease in PCNA and cdc2, in contrast to the increase in the p21Cip1 (Figure 5B). Finally, p21Cip1 also functions as a transcriptional co-activator. It can regulate the activity of general transcriptional co-activators (CBP/p300) [51] and repress the activity of well-characterized transcription factors (E2F, c-Myc, and STAT3) [52-54]. Moreover, overexpression of p21Cip1 is sufficient to induce senescence-like growth arrest in many cell types and the expression of numerous genes associated with senescence and aging [55]. Therefore, it is possible that p21Cip1 induced by oxidative stress acts as an inducer of age-related cataract.

In the present study, the H2O2-treated rat lenses displayed a decrease in the levels of cdc2 and PCNA, and accumulation of p21Cip1, similar to features of senescence as mentioned above. However, it is not certain that this ex vivo situation is related to the G2/M phase arrest in rat LECs. Nevertheless, cdc2 plays an important role in entry to mitosis; inhibition of cdc2 activity results in G2/M phase arrest in various cell types [56] and reduction of its expression. Accumulating data shows that the function of cdc2 is regulated by p21Cip1, which inhibits cdc2 activity by inactivating CDK2 or PCNA [57,58], and by repressing its transcription together with p53 and Rb [59,60]. Therefore, the accumulation of p21Cip1 may inhibit cdc2 activity and cause its repression, thus possibly inducing G2/M phase arrest in the rat lens damaged by oxidative stress. Another point is that unlike the rat lens, H2O2-treated HLE B-3 cells did not show any changes in the level of cdc2. HLE B-3 cells were originally immortalized with SV-40 large T antigen [25], which can inactivate p53 and Rb [61]. Therefore, the repression of cdc2 could be attenuated by SV-40 large T antigen due to inactivation of either p53 or Rb in the HLE B-3 cells.

In conclusion, sublethal oxidative stress induces G2/M phase arrest in HLE B-3 cells via the induction of p21Cip1, and ERK and JNK pathways regulate the accumulation of p21Cip1. Furthermore, oxidative damage in intact rat lenses increases accumulation of p21Cip1, which is accompanied by a decrease in the levels of PCNA, p27Kip1, and cdc2. A role of p21Cip1 in the in vitro and ex vivo models is suggested as a defensive mediator against oxidative damage, an indicator of senescence or aging, or an inducer of the formation of cataract. This study provides a pathological mechanism for oxidative stress linked to the control of the cell cycle mediated by p21Cip1. Further studies will be necessary to elucidate the mechanism underlying ERK and JNK activation by H2O2, whether the induction of p21Cip1 is dependent on p53, and what the exact role of p21Cip1 accumulation is in the intact lens in response to oxidative damage.

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