Oxidative Stress Induces Differential Gene Expression in a Human Lens Epithelial Cell Line

Deborah A. Carper, Jennifer K. Sun, Takeshi Iwata, J. Samuel Zigler, Jr, Nobuhiro Ibaraki, Li-Ren Lin, and Venkat Reddy

Purpose. To identify differentially expressed genes in a human lens epithelial cell line exposed to oxidative stress.

Methods. Reverse transcriptase-polymerase chain reaction (RT-PCR) differential display was used to evaluate differential gene expression in a human lens epithelial cell line (SRA 01-04) when cells were exposed for 3 hours to a single bolus of 200 µM hydrogen peroxide. Differentially expressed genes were identified through DNA sequencing and a nucleotide database search. Differential expression was confirmed by northern blot and RT-PCR analyses.

Results. Using 18 primer sets, 28 RT-PCR products were differentially expressed between control and hydrogen peroxide-treated cells. In stressed cells, mitochondrial transcripts nicotinamide adenine dinucleotide (NADH) dehydrogenase subunit 4 and cytochrome b were downregulated 4-fold. Of the cytoplasmic mRNAs, glutamine cyclotransferase decreased 10-fold, whereas cytokine-inducible nuclear protein, alternative splicing factor 2, and β-hydroxyisobutyryl-coenzyme A hydrolase increased 2-, 4-, and 10-fold, respectively. Analysis of mitochondrial transcripts in a 24-hour time course showed that NADH dehydrogenase subunit 4 mRNA decreased by 2-fold as early as 1 hour after oxidative stress, whereas the rate of decrease was slower for cytochrome b, cytochrome oxidase III, and 16S rRNA.

Conclusions. Oxidative stress induced specific expressed gene changes in hydrogen peroxide-treated lens cells, including genes involved in cellular respiration and mRNA and peptide processing. These early changes may reflect pathways involved in the defense, pathology, or both of the lens epithelium, which is exposed to oxidative stress throughout life. (Invest Ophthalmol Vis Sci. 1999;40:400–406)

Oxidative stress is believed to be an important contributing factor in maturity-onset cataract. Reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂), superoxide anion, singlet oxygen, and the hydroxyl radical are postulated to contribute to this process. High concentrations of H₂O₂ have been reported in human cataracts and in the corresponding aqueous humor of the eye. In the organ-cultured lens, the presence of ROS causes a number of biochemical changes, which lead to an increase in water-insoluble lens proteins and the appearance of lens opacity.

The lens epithelium is especially vulnerable to oxidative stress. Damage to this single layer of cuboidal epithelial cells on the anterior surface of the lens can precede and contribute to lens opacification. For example, generation of ROS in a cultured rat lens system led to irreversible damage in the epithelial cell layer. Cellular and mitochondrial swelling, DNA fragmentation, and loss of cell viability were observed before cataract formation. These data emphasize the fact that the lens epithelium plays a crucial role in lens transparency. Generation of ROS can cause deleterious peroxidation of lipids, modification of proteins, and cleavage of DNA. However, at low concentrations, ROS, which are generated as by-products of normal mitochondrial electron transport, can serve a valuable function in cell signaling. For example, an increase in intracellular ROS via the increased expression of the GTP-binding protein Rac1 leads to activation of the transcription factor nuclear factor-kB. In prokaryotes, H₂O₂ acts as a second messenger activating OxyR protein, a transcriptional activator of many antioxidant genes, including hydroperoxidase I and glutathione reductase.

The mRNA differential display (DD) technique developed by Liang and Pardee permits simultaneous identification of upregulated and downregulated genes between two groups of cells, tissues, or conditions. This comparative method uses small amounts of RNA and is based on gel electrophoretic analysis of subpopulations of cDNAs produced by reverse transcriptase-polymerase chain reaction (RT-PCR) using 3' anchored oligo (dT) primers and short arbitrary 5' primers. For each primer set, cDNAs with differing signal intensities between experimental conditions can be isolated and further characterized. We have used this powerful method to compare the changes in gene expression between control and oxidatively stressed human lens epithelial cells. The goals of this study were to identify early changes in gene expression that are associated with oxidative damage in lens epithelial cells.

From the 1Laboratory of Mechanisms of Ocular Diseases, National Eye Institute, National Institutes of Health, Bethesda, Maryland; the 2Department of Ophthalmology, Nippon Medical School 1-1-5 Sendagi, Bunkyo-ku, Tokyo, Japan; and the 3Eye Research Institute, Oakland University, Rochester, Michigan.

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Reprint requests: Deborah A. Carper, Building 6, Room 232, National Eye Institute, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892.

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and also to evaluate functional systems that may be involved in cell signaling and defense. We have identified several genes with altered expression in stressed cells that are involved in signaling and posttranslational processing. In addition, we observed decreased expression of genes for specific mitochondrial enzymes involved in electron transport.

METHODS

Cell Culture and Isolation of RNA

Cells from the SRA 01-04 human lens epithelial cell line were grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco-BRL Life Technologies, Gaithersburg, MD) supplemented with 20% fetal bovine serum (Gibco-BRL) and 0.05 mg/ml gentamicin (Gibco-BRL) in a 5% CO2 37°C incubator. These cells attach to the surface of the plastic flask and appear cuboidal in shape when confluent. The viable cells were counted using trypan blue stain (Gibco-BRL), and equal aliquots were placed into 75-cm² flasks. Once the cells reached approximately 95% confluence (2 x 10⁶ cells/75-cm² flask), the cells were washed with DMEM that did not contain supplements. A 10-ml volume of DMEM, containing either water or 200 μM H2O2 (Mallinckrodt, Paris, KY), and not containing any other supplements, was then added to each 75-cm² flask. The cells were incubated for an additional 3 hours for the DD or for 0 to 24 hours for the time course experiments. Flasks were then washed twice with 1× PBS (Gibco-BRL), and the cells were harvested by lysis with 1 mL RNA denaturing solution (guanidinium thiocyanate; Stratagene, La Jolla, CA). The number of attached viable control or H2O2-treated cells did not change (2 x 10⁶ cells/75-cm² flask by trypan blue exclusion) after 3 hours of treatment but was reduced to 85% of the original cell number by 24 hours. The RNA was processed using the Stratagene RNA Isolation kit (Stratagene) followed by RNase-free DNase treatment (Boehringer-Mannheim, Indianapolis, IN). The quality of the RNA was evaluated by agarose gel electrophoresis.

Differential Display Analysis

RNA populations from control and H2O2-treated cells were compared using the Differential Display Hypoliglyph mRNA Profile Kit (Genomyx, Foster City, CA). Eighteen primer sets were used, which represents approximately 10% to 20% of the more than 10,000 genes expressed in a cell. Anchor primers 1 and 2 were combined with arbitrary primers 1, 2, and 4. Anchor primers 8, 9, and 10 were used with arbitrary primers 1 through 4. First-strand synthesis of cDNA was performed with 200 ng RNA, 0.2 μM anchor primer (Genomyx), 20 U RNasin (Promega, Madison, WI), dNTP mix (25 μM each, Genomyx), 10 mM dithiothreitol, 40 U SuperScript II RT Enzyme, and SuperScript II RT Buffer (the last three were from Gibco-BRL). After first-strand synthesis, PCR was performed in duplicate for each condition using the Hypoliglyph kit and protocol (Genomyx), the AmpliTaq enzyme (Perkin-Elmer, Foster City, CA), and [α-32P]dCTP (DuPont-NEN, Wilmington, DE) in a Perkin-Elmer GeneAmp 9600 thermocycler (Perkin-Elmer) as described previously. Reamplified bands were directly sequenced using the Perkin-Elmer 310 fluorescent sequencer and the DNA FS sequencing kit and protocol (Perkin-Elmer). The M13 universal reverse primer provided by the Genomyx system was used to initiate DNA amplification, the PCR products were purified by Wizard PCR preparation (Promega, Madison, WI) and analyzed by agarose gel electrophoresis.

Confirmation of Expressed Gene Changes

Northern blot analysis and RT-PCR were used to verify the expressed gene changes observed with the DD gels. For northern blot analysis, 3 μg RNA from the original DD experiment or 5 μg or 10 μg from two subsequent experiments was run in a standard formaldehyde agarose gel. After the gel was blotted with Biotrans nitrocellulose according to protocol (ICN, Costa Mesa, CA), the blot was probed with 32P-labeled DNA subcloned DD bands or subcloned DNA. To generate a probe from the subcloned DNA, primers specific for each target gene were synthesized (Applied Biosystems) and used to produce specific PCR products (260 bp to 430 bp) using AmpliTaq enzyme (Perkin-Elmer). These products were subsequently separated by agarose gel electrophoresis, extracted from the gels (Qiagen Gel Extraction Kit; Qiagen, Chatsworth, CA), and sequenced to verify the correct target gene. An 18S rRNA probe (Ambion, Austin, TX) was used to normalize for variations in RNA loading. Radioactive labeling was carried out using a random primer kit (Gibco-BRL) and [α-32P]dATP (DuPont). After standard hybridization and wash protocols at 42°C (ICN), the blot was exposed to X-Omat film (Kodak) and also to fluorescent detection and quantitation using Storm and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). To evaluate expressed gene changes by RT-PCR, target genes were compared between the two cell conditions using 300 ng RNA pretreated with DNase (Boehringer-Mannheim), 200 nM specific gene primers generated on a DNA synthesizer (Applied Biosystems), and the One Step RT-PCR system and protocol (Gibco-BRL). GAPDH was used as a housekeeping enzyme normalization control. Controls lacking reverse transcriptase (heat-inactivated) were performed under identical conditions. PCR was carried out with a single set of specific gene primers or in combination with GAPDH. PCR products were verified by size...
FIGURE 1. Micrographs of human lens epithelial cell line SRA 01-04. Cells were incubated for 3 hours in DMEM without (Control) or with 200 μM H₂O₂ (Hydrogen Peroxide).

RESULTS AND DISCUSSION

Human lens epithelial cells from the immortalized SRA 01-04 cell line were incubated in DMEM without bovine serum albumin or antibiotics for 3 hours in the presence or absence of a single bolus of 200 μM H₂O₂. These conditions were chosen on the basis of previous findings that these parameters elicit morphologic and biochemical changes in cultured human lens epithelial cells. In addition, it has been shown that H₂O₂ levels can be quite high, varying from 10 μM to 660 μM in the aqueous humor of cataract patients. However, with other methods of measurement, H₂O₂ levels have been reported to be less than 10 μM. For this reason, a recent study has attempted to address this issue by investigating the ability of aqueous humor (bovine) to generate high concentrations of H₂O₂, which lack long polyA tails, nevertheless can be reverse-transcribed, as has been reported in previous DD studies. Other bands identified by direct sequencing were ribosomal protein S10 and mitochondrial nicotinamide adenine dinucleotide (NADH) dehydrogenase subunit 4. Four of the DD bands gave multiple signals on nucleotide sequencing, suggesting that two or more expressed genes were contained in the excised DD bands. Thus, the remaining 4 unidentified bands were subcloned to obtain pure populations of expressed genes. Twenty clones were picked from each subcloned DD band and sequenced. From 2 to 4 expressed genes were identified for each subclone, some of which were identical with the directly sequenced DD bands. Northern blot analyses were run to confirm the expressed gene changes detected by DD (Fig. 3). Glutamine cyclotransferase (QC) changed the most, with a 10-fold decrease in length, were observed to be either upregulated (n = 10) or downregulated (n = 18) in the H₂O₂ treatment group (Fig. 2). Fourteen of the 28 differentially expressed bands, those showing the strongest intensities and best resolutions, were amplified and sequenced. Analysis using nucleotide database search identified 10 of the 14 genes. For example, the band designated by the top arrow in Figure 2 was glutamine cyclotransferase by BLAST search. Of the genes identified by direct sequencing (>96% match), 2 were mitochondrial cytochrome b, 3 were mitochondrial 16S rRNA, and 2 were cytoplasmic glycoprotein 130, a receptor component for interleukin-6. This emphasizes the fact that arbitrary primers, being only 10 bp in length, can prime at several sites within a given RNA species, resulting in redundancy of cDNA products. In addition, rRNAs, which lack long polyA tails, nevertheless can be reverse-transcribed, as has been reported in previous DD studies. Other bands identified by direct sequencing were ribosomal protein S10 and mitochondrial nicotinamide adenine dinucleotide (NADH) dehydrogenase subunit 4. Four of the DD bands gave multiple signals on nucleotide sequencing, suggesting that two or more expressed genes were contained in the excised DD bands. Thus, the remaining 4 unidentified bands were subcloned to obtain pure populations of expressed genes. Twenty clones were picked from each subcloned DD band and sequenced. From 2 to 4 expressed genes were identified for each subclone, some of which were identical with the directly sequenced DD bands. The lower band (bottom arrow) in Figure 2 turned out to be a mixed population consisting of mitochondrial NADH dehydrogenase subunit 5 and cytochrome b. Table 1 shows a list of the differentially expressed genes identified by direct or subcloned DNA sequencing.

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FIGURE 2. Autoradiograph of a DD gel. RT-PCR products from control (—) and H2O2-treated (+) cells in duplicate were compared. Arrows indicate downregulation of expressed genes. Top arrow, glutamine cyclotransferase; bottom arrow, a mixture of mitochondrial NADH dehydrogenase subunit 5 and cytochrome b. This autoradiograph represents approximately 25% of the length of the gel for anchor primer 8/arbitrary primer 1. Expressed gene changes were not observed with this primer set in other areas of the gel.

expression in the H2O2-treated cells, similar to that observed in the original DD gel (Fig. 2, top arrow). This protein is responsible for posttranslational modification of N-terminal pyroglutamyl residues of neuropeptide precursors.25,28 The cyclization of the N-terminal glutaminyl residue converts these precursors into biologically active hormones. QC has been identified in a variety of tissues, including pituitary, brain, B-lymphocytes, and retina and also in plants. The peptide precursors for QC in the human lens epithelial cells are unknown; however, the presence of QC mRNA indicates a distinct mechanism of peptide processing in lens cells. The dramatically lowered level of QC mRNA in oxidatively stressed cells may indicate a reduced demand for processing enzymes either due to lower peptide concentrations or turnover.

Cytokine-inducible nuclear protein, which is involved in signal transduction and is considered to be a member of the primary response gene family,27 increased twofold in stressed cells. Cytokine-inducible nuclear protein was first identified from a cDNA library prepared from interleukin-1 and tumor necrosis factor-α-stimulated human dermal microvascular endothelial cells. Its location in the nucleus, the presence of an ankyrin-like repeat structure, and its similarity to 1Xβ-like proteins suggest that this protein plays a role in the regulation of gene expression.

By northern blot analysis, glycoprotein 130 decreased 1.5-fold, alternative splicing factor (ASF) increased 4-fold (data not shown because of faintness of the band), and ribosomal protein S10 mRNA did not change between the two conditions. The latter finding emphasizes one of the inherent problems of DD, in which low annealing temperatures that are necessary to obtain PCR products can produce false positives (~10% in our study).

Table 1. Expressed Genes Identified by Differential Display

<table>
<thead>
<tr>
<th>Mitochondrial</th>
<th>Cytoplasmic</th>
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<tr>
<td>NADH dehydrogenase subunit 4</td>
<td>Glutamine cyclotransferase</td>
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<tr>
<td>NADH dehydrogenase subunit 5</td>
<td>Cytokine-inducible nuclear protein</td>
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<tr>
<td>Cytochrome b</td>
<td>Glycoprotein 130</td>
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<tr>
<td>16S rRNA</td>
<td>Ribosomal protein S10</td>
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<tr>
<td></td>
<td>Alternative splicing factor</td>
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<tr>
<td></td>
<td>β-hydroxyisobutyryl-Coenzyme</td>
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<tr>
<td></td>
<td>A hydrolase</td>
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<td></td>
<td>Cathepsin</td>
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comparison with control cells after normalization to 18S rRNA using imageQuaNT Software. No degraded RNA bands were observed. All RNA hybridizations were at the expected size for the full-length cDNAs reported in the nucleotide database and original publications.

**Figure 3.** Composite of northern blot analysis using DD bands as probes. All RNAs were analyzed on blot A (2 µg total RNA/lane) except cytokine-inducible protein, which was analyzed on blot B (10 µg total RNA/lane) from a separate experiment. X represents the relative increase or decrease in specific RNAs in the H2O2-treated cells when compared with control cells after normalization to 18S rRNA using ImageQuaNT Software. No degraded RNA bands were observed. All RNA hybridizations were at the expected size for the full-length cDNAs reported in the nucleotide database and original publications.

Mitochondrial NADH dehydrogenase 5

Mitochondrial NADH dehydrogenase 4

Mitochondrial cytochrome b

Mitochondrial 16S rRNA

Cytoplasmic 18S rRNA

Cytoplasmic 18S rRNA

Glutamine cyclotransferase

Cytokine inducible protein

Glycoprotein130

Ribosomal protein S10

Mitochondrial NADH dehydrogenase 4

Mitochondrial NADH dehydrogenase 5

Mitochondrial cytochrome b

Mitochondrial 16S rRNA

Cytoplasmic 18S rRNA

Cytoplasmic 18S rRNA

Cont. H2O2

0.1 X

2 X

0.67 X

1 X

0.25 X

0.15 X

0.25 X

0.8 X

Blot A

Blot B

able to assume that splice-site selection via the upregulation of ASF is one of the mechanisms important in the rapid recruitment of functional proteins during stress conditions, such as seen in the human lens epithelial cells in our study.

β-Hydroxyisobutyryl-coenzyme A (CoA) hydrolase (E.C. 3.1.2.4; β-H) increased 10-fold in H2O2-treated cells, whereas cathepsin increased slightly (1.4-fold after normalization to GAPDH; Fig. 4). In light of our observations on the differential expression of mitochondrial gene products, it is interesting to note that β-H, although derived from the nuclear not the mitochondrial genome has a mitochondrial leader sequence and is located in the mitochondrial matrix space.33 This enzyme catalyzes the hydrolysis of S-hydroxyisobutyryl-CoA, an intermediate in the valine catabolic pathway.35 It has been postulated that β-H plays a role in cellular defense, because it protects cells from the effects of the highly toxic metabolite methacrylyl-CoA, which is another intermediate in the catabolism of valine upstream of S-hydroxyisobutyryl-CoA. Methacrylyl-CoA is a thiol-reactive molecule that could inactivate numerous enzymes in the absence of a mechanism designed to minimize its intramitochondrial concentration.34 It has been reported that an infant born with a deficiency of β-H exhibited large amounts of cysteine/cysteamine conjugates of methacrylic acid, indicating that conjugation between methacrylyl-CoA and glutathione had occurred.34 Such a loss of glutathione could be expected to create an imbalance in the redox state of the mitochondria if not the entire cell. The increase in β-H transcripts could reflect an increase in valine catabolism and could also include an enhanced metabolism of methacrylyl-CoA. The association between the increase in β-H transcripts and the decrease in mitochondrial gene products is not clear; however, it is plausible that, although mitochondrial electron transport function is downregulated, defense mechanisms are being induced to protect mitochondrial integrity.

Because mitochondrial transcripts seemed disproportionately affected by oxidative stress, the rate of differential gene expression of several mitochondrial gene products was evaluated in cells at 1 to 24 hours after a single bolus of 200 µM H2O2 (Fig. 5). NADH dehydrogenase subunit 4 decreased two-fold as early as 1 hour after cells were treated with H2O2. The level of this transcript declined to 30% of control by 3 hours and 20% of control at 5 hours. The level rose to 53% of control at 24 hours. In contrast, cytochrome b transcripts showed a steady decrease, from 92% of control at 1 hour, to 42% at 3 hours and 37% at 5 hours, and ending at 20% of control at 24 hours. Cytochrome oxidase III, although not originally detected by DD, was also evaluated in the time course experiments. It followed a pattern similar to that of cytochrome b. Transcript levels of cytochrome oxidase III from H2O2-treated cells were 95% of control at 1 hour, 55% at 3 hours, 50% at 5 hours, and 20% of control at 24 hours. As observed in the original DD experiment, 16S rRNA transcripts were generally less affected by oxidative stress than the other mitochondrial gene products. The level of 16S rRNA was 92% at 1 hour, 57% at 3 hours, 65% at 5 hours, and 31% of control at 24 hours.

Mitochondria are sensitive to oxidative stress. In the presence of ROS, mitochondria undergo morphologic and biochemical changes, including swelling,35 loss of electron transport capacity,35 and decreased transcription of gene products.12,36 Our findings agree with a recent DD study on hamster fibroblasts, in which oxidative stress (4 µM to 10 µM

<table>
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<tr>
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<th>Cathespin</th>
<th>ASF</th>
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<tr>
<td>GAPDH</td>
<td>+</td>
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**Figure 4.** Ethidium bromide-stained agarose gel of RT-PCR differentially expressed genes. Expression levels of cathespin, ASF 2, and β-hydroxyisobutyryl-coenzyme A hydrolase (β-H) were evaluated in control (−) and H2O2-treated (+) cells. Each reaction contains additional primers for GAPDH as an internal standard. The same results were obtained for cathespin, ASF, and β-H using their respective primers alone. The PCR products were quantified and normalized to GAPDH using Kodak BioMax 1D Image Analysis Software.
H2O2/10^7 cells for 8 minutes to 10 hours) caused a decrease in a number of mitochondrial transcripts. However, this latter study reported multiple bands and degraded mitochondrial gene products, which were suggested to be due to a mediator of this oxidative stress. Several pathways, including cellular respiration and mRNA and peptidase processing, are involved in this stress response.

Using DD, we have shown that oxidative stress induces changes in gene expression in a human lens epithelial cell line. Several pathways, including cellular respiration and mRNA and peptidase processing, are involved in this stress response. Lenses cells use a number of strategies to maintain ROS at low levels, including activation of the ROS scavenger enzyme catalase and glutathione peroxidase. However, with age there is a diminution of these protective systems, placing the lens at some risk for oxidative damage and cataract. H2O2 is believed to play a role in oxidative damage of the lens and the development of maturity-onset cataract. Its involvement in cataract formation has been well documented in animal models. In addition, oxidative damage occurs in the lens epithelium, and the effects of this damage precede lens opacification.

The present DD study was carried out to assess the oxidative stress response of the lens, at the molecular level, using a transformed cell line originating from human lens epithelium. These cells maintain certain lens characteristics and are useful in that they are of human origin. The response of these cells to H2O2 may help reveal some of the same mechanisms and pathways that are involved in the development of maturity-onset cataract. Using the genes in these pathways as targets, we can assess their expression in normal and cataractous human lenses and monitor their response in the presence of therapeutic compounds.

Acknowledgments
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