Development and Characterization of an H$_2$O$_2$-Resistant Immortal Lens Epithelial Cell Line

Abrabam Spector, Ren-Rong Wang, Wanchao Ma, and Norman J. Kleiman

PURPOSE. To determine how nature would protect lens epithelial cells from H$_2$O$_2$ stress, an immortal murine lens epithelial cell line, aTN4+1, was subjected to gradually increasing H$_2$O$_2$ levels over a period of approximately 8 months. The resultant conditioned cells grew normally when exposed daily to 125 µM H$_2$O$_2$, whereas normal cells died within 9 hours. This communication describes changes in the cell biology of the conditioned cells that allowed them to remain viable. The manner in which critical biochemical parameters were affected in both conditioned and normal cells is also reported.

METHODS. Conditioned cells were obtained by gradually increasing the concentration of H$_2$O$_2$ over a period of approximately 8 months, introducing an aliquot of H$_2$O$_2$ every 24 hours. A wide spectra of biological parameters were evaluated, including catalase, GSH peroxidase and other antioxidative enzyme activities, cell number and cell viability, non-protein thiol, ATP, transport systems, thymidine incorporation, and DNA cleavage.

RESULTS. Surprisingly, the conditioned cells did not degrade the medium H$_2$O$_2$ more rapidly than normal cells. However, analyses of the antioxidative defenses indicated that catalase activity was increased 60-fold and glutathione peroxidase (GSH Pxn) approximately 2.7-fold. Glucose-6-phosphate dehydrogenase, GSH S-transferase, and GSSG reductase also had increased activity. Using one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis, in situ trypsin digestion and matrix-assisted laser desorption/ionization mass spectrometry, a highly amplified doublet in the conditioned cell preparation was shown to be GSH S-transferase α-1 and α-2 isomers. Examination of key biochemical parameters indicated that while most such parameters in the conditioned cells showed marked decay in the first hour or so after stress, recovery was then observed and within a few hours, these parameters were back in the normal range. In contrast, damage to the normal cells was not repaired. The damage to DNA was shown to involve Fenton chemistry. In the presence of a metal ion chelator, normal cells survive H$_2$O$_2$ stress.

CONCLUSIONS. The overall conclusion from this investigation is that nature has chosen to respond to the H$_2$O$_2$ stress by not only increasing the activity of enzymes degrading H$_2$O$_2$ but also the systems involved in repair, generation of reducing potential, and detoxification. All but one system of those evaluated appears to be permanently modified.

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The usual approach for developing an effective methodology to prevent or retard a disease process is to first investigate normal and diseased tissue. From an examination of the biological changes caused by the disease, it may be possible to understand the cause of the disorder and how to prevent its development. The lens of the eye has received extensive examination for a number of decades, resulting in a substantial body of information. In a similar manner, cataractogenesis, the process by which the lens loses its transparency, has also been studied. From such investigation, it can be concluded that cataract probably has more than one initiating event. Cataract associated with development and in the juvenile is frequently linked to gene defects. On the other hand, maturity onset cataract, the development of cataract in the older population, may have a number of causes, including oxidative stress, diabetes, the intake of certain drugs, inflammation, mutation, and degenerative disease in other parts of the eye. There is now considerable information that suggests that whatever the initiating cause of maturity onset cataract, oxidation is a major factor in its development. Extensive oxidation of lens components is seen in cataract. Furthermore, these oxidative changes can be shown to begin before significant loss of transparency is observed. Correlated with the oxidative changes is an elevation of H$_2$O$_2$ in the aqueous, vitreous, and the lens. These observed elevated H$_2$O$_2$ levels can cause cataract development in organ culture and produce the same pattern of oxidative damage as is found in the in vivo cataractous lens.

The two major enzyme systems used by the lens to degrade peroxides are catalase (CAT) and glutathione peroxidase.
(GSH Px). GSH Px-1 is the major GSH Px present primarily in the cytoplasm. It has a high affinity for H$_2$O$_2$ ($K_m \approx$40 $\mu$M), degrades lipid peroxides as well as H$_2$O$_2$, and requires a high concentration of a cofactor, GSH, for maximum activity. $^{20,21}$ Catalase, in contrast, is specific for H$_2$O$_2$, requires no cofactor, and becomes progressively more effective with increasing H$_2$O$_2$ concentration. $^{22}$ It should also be noted that the high concentration of GSH found in the lens makes it an attractive nonenzymatic alternative for degrading peroxides. In examining cultured lenses from normal and GSH Px-1 knockout subjected to 300 or 80 $\mu$M H$_2$O$_2$, it was found that GSH is a primary factor in degrading H$_2$O$_2$. $^{19}$ And, furthermore, surprisingly GSH Px-1 only accounted for approximately 15% of the H$_2$O$_2$ degraded and CAT approximately 25% at 300 $\mu$M H$_2$O$_2$ and approximately 10% at 80 $\mu$M H$_2$O$_2$. $^{19}$ Examination of GSH Px-1 knockout suggests that when cultured lenses were subjected to H$_2$O$_2$ stress, the knockout lenses responded in a similar manner to normal lenses. However, the knockout lenses were more sensitive to lipid peroxide-induced oxidation. $^{23,24}$ Other reports suggest that H$_2$O$_2$-induced damage in lenses were more sensitive to lipid peroxide–induced oxidation. $^{40}$ This confused picture of the effectiveness of the different defense systems in protecting the lens against peroxide stress encouraged us to ask how nature would protect a cell population from peroxide stress? The observations of Darwin in finding a relatively rapid biological response to environmental pressure $^{28}$ suggest that by subjecting a normal cell population to gradually increasing H$_2$O$_2$ concentration, a resistance to normally lethal concentrations of H$_2$O$_2$ could be achieved. This communication demonstrates the successful results of such an experiment and defines how nature manipulated the antioxidative and other biological defenses of the cell to achieve cell populations that grow normally at the remarkably high H$_2$O$_2$ concentration of 125 $\mu$M.

**METHODS**

**Cell Conditioning**

Cells were conditioned to resist H$_2$O$_2$ stress in the following manner. $\alpha$TN-4-1 cells (generously provided by Paul Russell, National Eye Institute, Bethesda, MD) were initially subcultured to give approximately 200,000 cells in a 35-mm dish with 2 ml of minimum essential medium, MEM (GIBCO BRL 41500-034) supplemented with NaHCO$_3$ 2.2 g/l medium (pH 7.2), 100 U penicillin/ml medium and 100 $\mu$g streptomycin/ml medium (GIBCO 15140-122), fungizone 2.5 mg/l medium (GIBCO 15295-017), and fetal bovine serum (Hyclone) to give a final concentration of 10% (the standard medium). After overnight incubation at 37°C in a 5% CO$_2$ incubator, the medium was replaced with 4 ml of the standard medium, and various amounts of 10 mM H$_2$O$_2$ were added to give the desired concentration. Every 24 hours, an additional aliquot of H$_2$O$_2$ was placed in the dish. The culture medium was changed every 3 days. When cells were approximately 80% confluent, they were subcultured, and after 16 hours, exposure to H$_2$O$_2$ was reinitiated. Over an approximately 8-month period, the H$_2$O$_2$ concentration was gradually increased from 25 $\mu$M H$_2$O$_2$ to 125 $\mu$M. (Attempts to accelerate this process led to considerable cell death.) The conditioned cells were then maintained in 125 $\mu$M H$_2$O$_2$ as described above. Normal $\alpha$TN-4-1 cells were maintained in an identical fashion but without H$_2$O$_2$.

**Cell Staining**

Cells were stained with trypan blue in the following manner to determine viability. A 25-$\mu$l aliquot of trypan blue solution 0.4% (Sigma T-8154) was added to a suspension of 25 $\mu$l of cells (ideally 80-100 $\times$ 10$^5$ cells/ml MEM containing 10% serum). After 2 minutes, the cells were examined. Unstained and stained cells were then counted with a hemocytometer.

**Enzyme Determinations**

GSH peroxidase, GSSG reductase (GSSG Red), and CAT were assayed as previously described. $^{29}$ Two hundred microliters of 0.15% Triton X-100 in the appropriate buffer was added at 0°C to a 35-mm dish containing 6 to 8 $\times$ 10$^5$ cells and after scraping transferred to an Eppendorf tube, manually homogenized briefly, and then centrifuged at 14,000 rpm at 4°C for 5 minutes. Aliquots were then taken for assay. Glucose-6-phosphate dehydrogenase was determined as described by a modification of the method described by Reddan et al. $^{30}$ The assay was conducted in 50 mM triethanolamine (pH 7.5), 1 mM EDTA, 7.5 mM MgCl$_2$, 1 mM glucose-6-phosphate, and 0.4 mM NADP, at 37°C in a total volume of 0.5 ml.

Glyceraldehyde-3-phosphate dehydrogenase (GPD) was assayed after removing cells from the plate with 0.15% Triton X-100, 0.05 M bicine (pH 8.5), 1 M Na acetate, 1 mM EDTA, 0.5 ml. After 20 minutes, the preparation was centrifuged at 14,000 rpm at 4°C for 8 minutes. The determination of GPD was conducted using a modification of the method of Byers. $^{31}$ Eighty microliters of the cell extract was added to 720 $\mu$l of a 0.05 M bicine buffer (pH 8.5), 50 $\mu$l sodium arsenate (pH 8.5), 0.5 M, 10 $\mu$l phosphoglycolic acid, 10 $\mu$m, and 100 $\mu$l NAD, 10 $\mu$m (pH 4.0). After a 2-minute equilibration, 50 $\mu$l of glyceraldehyde-3-phosphate, 20 $\mu$m, was added, and the change in 340 nm absorption between 30 and 60 seconds after addition was used to determine the rate of the reaction. An E$_{340}$ at 340 nm = 6.20 was used.

To determine superoxide dismutase (SOD) activity, one 35-mm dish containing approximately 6 to $\times$ 10$^5$ cells was used. Three hundred fifty microliters of a solution containing 0.15% Triton X-100, 50 mM phosphate (pH 7.8), 0.1 mM EDTA, and 0.01 mM NaN$_3$ was added at 0°C. The cells were removed from the plate, homogenized, and then centrifuged at 14,000 rpm (4°C) for 5 minutes. To remove low-molecular-weight components and concentrate the preparation, 80 to 150 $\mu$l of the supernatant was added to a Microcon 30 concentrator (Amicon) and centrifuged at 10,000g at 4°C for 5 minutes. The resultant 30 to 50 $\mu$l above the filter was diluted with 300 $\mu$l of the above-described buffer minus Triton X-100, and filtration was again conducted for approximately 25 minutes. After repetition of this procedure, the solution above the filter was removed and saved. The filter was washed with a small volume of the same buffer and combined with the same solution to give a final volume of 80 to 150 $\mu$l. Thirty microliters was then assayed for SOD as described by Flohé and Otting $^{32}$ with 0.01 mM NaN$_3$ using ferricytochrome C reduction. Assays were carried out in 0.5 ml at 25°C.
Glutathione S-transferase was assayed as described by Habig and Jakoby54 using 1.0 mM 1-chloro-2,4-dinitrobenzene as a substrate in a total volume of 0.5 ml at 37°C. Six to 8 × 10^5 cells were removed from a Petri dish with 200 µl of a 0.1 M phosphate (pH 6.5) and 0.15% Triton X-100 at 0°C and centrifuged for 5 minutes at 14,000 rpm at 4°C. Five to 10 µl was used for assay.

**H_2O_2** and Protein Determinations

H_2O_2 was determined by modification of the method described by Spector et al.29 Aliquots of the cell medium were removed at various times and 100% trichloroacetic acid (TCA) was added to give a final concentration of 10% TCA. After 2 minutes at 25°C, the preparation was centrifuged at 14,000 rpm for 1 minute at 25°C, and then 50 µl was taken for assay.

Protein was determined as described by Spector et al.29

**Choline and Calcium Uptake**

[^14C]choline uptake was determined with 2 × 10^5 cells in 1 ml of standard medium minus serum containing 0.14 µCi[^14C]choline and nonradioactive choline to give a final choline concentration of 5 µM as described by Yang et al.29 After a 90-minute incubation at 37°C in 5% CO_2, the solution was removed, the cells were rapidly washed with isotonic saline two times, and then 250 µl of 0.1 N NaOH was added. The solution was transferred to a counting vial and the dish was washed with an additional 250 µl of 0.1 N NaOH, which was also added to the vial. Five milliliters of Ecolite (+) (ICN Pharmaceuticals) was added and the preparation was counted.

For ^45Ca uptake, 2 µCi[^45Ca]Cl_2 was used. The methodology is the same as that described for choline uptake except that a 30-minute incubation time was used.

**Thymidine Incorporation**

For[^3H]thymidine incorporation, 2 × 10^5 cells were incubated in 1 ml of standard medium minus serum containing 15 µCi[^3H]thymidine at 37°C, 5% CO_2 for 90 minutes.29 The medium was then removed, and the preparation was washed with the same medium and then incubated with 2 ml of standard medium minus serum for 10 minutes at 37°C, 5% CO_2. Another 10-minute incubation was then carried out with fresh medium minus serum. After removal of the medium, 250 µl of cold 5% TCA was added, and the cells were scraped into an Eppendorf tube. An additional wash with 250 µl 5% TCA was pooled with the initial solution and then prepared for counting as previously described by Spector et al.29

**ATP and Nonprotein Thiol Determinations**

Two times 10^5 cells were used for ATP determinations. After H_2O_2 stress or with controls, the medium was removed and the reaction was quickly stopped with liquid N_2. The dish was placed on an ice bath and 300 µl of Somatic Cell ATP Releasing Reagent (Sigma, FL-ASC) at 0°C was added. Twenty microliters was used for the ATP bioluminescent assay as described in the Sigma technical bulletin #BSCA-1. An LKB luminometer 1250 was used to measure luminescence.

Nonprotein thiol (NP-SH) was assayed as described by Spector et al. (1996)29 with the following modification: 200 mM Tris (pH 8.8) containing 2 mM EDTA was used and the sample volume was increased to 160 µl.

**Alkaline Elution of DNA**

Alkaline elution was usually performed in the following manner. Approximately 2 × 10^5 cells were labeled in 35-mm dishes containing 2 ml of the standard MEM and 0.14 µCi/ml of[^3H]thymidine, specific activity 60 Ci/mmol (ICN Pharmaceuticals) and 0.5 µM of cold thymidine. After 48 hours in a 5% CO_2 incubator at 37°C, the cells were subcultured and approximately 2 × 10^5 cells were incubated in 2 ml standard MEM, 10 µM cold thymidine, and cultured overnight. Then the medium was removed and replaced with 4 ml of standard MEM, and some of the cells were stressed with 125 µM H_2O_2 for various periods. The medium was removed, the cells were washed with 2 ml of the same medium minus H_2O_2 and 4 ml of standard medium added, and the cells were incubated for specified post-insult periods. After removing the medium, the DNA single-strand breaks were analyzed as previously described.33,35

**Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis**

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with approximately 6 to 7 × 10^5 cells. The cells were homogenized in 70 µl of 0.15% Triton X-100, 50 mM phosphate (pH 7.0) at 0°C, then centrifuged at 14,000 rpm at 4°C for 5 minutes. Only a trace of insoluble material was usually observed. The supernatant was mixed with an equal volume of 2X sample buffer for SDS-PAGE, as described by Smith,36 boiled 3 minutes, and then approximately 20 µl equivalent to 30 µg protein was used for electrophoresis as described by Laemmli37 with slight modification as indicated by Wang and Spector.38

**Molecular Mass Analyses**

For identification of unknown bands found after SDS-PAGE, molecular mass technology was used. The gels were stained with 0.05% Coomassie brilliant blue G, 0.5% acetic acid, 20% methanol for 15 minutes, and then destained for 10 minutes with shaking in 30% methanol. The bands of interest were cut out, tryptic digested, and extracted as previously described.39 After digestion, the preparation was treated as described previously40,41 and the subpicomolar level peptides with masses in the 1000- to 3000-Da range were determined by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry using PerSeptive Voyager DE-RIA mass spectrometer in the linear mode available in the Protein Core Facility in the College of Physicians & Surgeons of Columbia University.40,41

**RESULTS**

**Effects of H_2O_2 on Cell Viability and Growth**

Conditioned αTN-1 cells prepared as described in the previous section survived and grew in the presence of 125 µM H_2O_2 (added as a concentrated aliquot every 24 hours) (Fig. 1A). In contrast, normal αTN-1 cells not previously exposed to H_2O_2 were all dead by 24 hours. When the conditioned cells were allowed to double four times in the absence of H_2O_2 and then subjected to H_2O_2 stress under conditions identical to those described above, essentially the same growth of the cell pop-
ulation was observed as that with the cells regularly exposed to H$_2$O$_2$ (Fig. 1A, conditioned [without H$_2$O$_2$]). It would, therefore, appear that at least in the short-term, the cells retain their ability to resist H$_2$O$_2$ stress. Recent experiments indicate that based on long-term growth in the absence of H$_2$O$_2$, the conditioned cells retain their ability to survive and grow normally in the presence of H$_2$O$_2$ (authors’ unpublished observations).

When the growth rate of normal cells was compared with that of conditioned cells in the absence of H$_2$O$_2$, similar results were also obtained. This suggests no significant change in the ability of the cells to divide as a result of H$_2$O$_2$ conditioning.

As shown in Figure 2, the appearance of the conditioned cells was similar to that of the unexposed cells. Also, little difference in appearance was found between conditioned cells grown with or without H$_2$O$_2$.

Examination of the decay of H$_2$O$_2$ in the presence of approximately 220 to 250 $\times$ 10$^3$ cells indicated surprisingly little difference between conditioned and normal cells in their ability to degrade medium H$_2$O$_2$ (Fig. 1B). With both cell types, by 2 hours after exposure to H$_2$O$_2$, most of the peroxide was gone and by 4 hours it was all gone. It is unlikely that the small difference of approximately 15 $\mu$M H$_2$O$_2$ consistently observed at the 2-hour point is meaningful.

In Figure 1A (insert), a closer inspection of the change in the normal cell number observed after the addition of H$_2$O$_2$ is shown. It is interesting that at 4 hours, most of the cells were still present and then in the following 2.5 hours, there was a marked drop in their number. The decrease in the cell population continued so that at 9 hours, only a few cells were detectable. Thus, it was in the period immediately after the disappearance of H$_2$O$_2$ that the major change in cell number occurred.

To characterize the state of the cells, they were treated with trypan blue, a stain that measures cell viability. As shown in Table 1, in the conditioned cell preparations, approximately 4% of the cells stained after the first 4 hours of stress and then staining decreased to approximately 2%. In contrast, although the percent cell staining of the normal cells was comparable to the conditioned cells in the first hour, it then increased markedly so that at 4 hours 18.0% and at 6.5 hours 23.3% of the cells were stained. It was assumed that the cells that did not adhere to the plates had died. To confirm this assumption, the media from the dishes containing the 4-, 6.5- and 9-hour normal cell preparations were gently centrifuged, stained, and then examined. It was found that in all cases, almost all the cells were stained (data not shown). Unstressed cells released from the plates with trypsin and treated in a similar manner had only a few percent stained cells. Thus, the disappearance of the cells from the plates indicated cell death. Therefore, the data on total cell number (see Fig. 1A, insert) overstate the number of viable cells. The major discrepancy was at 4 hours, when approximately 40,000 of the counted cells were stained.

**Cell Viability after H$_2$O$_2$ Stress**

To assess the effect of different periods of exposure to the H$_2$O$_2$ stress, the normal cells were removed from the H$_2$O$_2$ at various times after initiation of the insult and grown in an H$_2$O$_2$-free medium for an additional 16 hours. As shown in...
number of cells present on a given plate declined at the rate of approximately 8000 cells/min, whereas in the second phase with cells withdrawn from H2O2 after 30 minutes, the rate of cell loss was 1450 cells/min. In the first phase, the total number of cells was greater than or equal to the starting population; and since little cell death was detected, the decay in total cell number probably represents an inhibition of cell division. The second phase was indicative of cell death because the total number of cells at the end of the incubation was less than the initial number and because significant cell death was detected by trypan blue staining.

To examine the fate of cells subjected to H2O2 more closely, cells exposed to H2O2 for 30 minutes or 2 hours were examined over a longer postinsult period (Fig. 3B). With a 30-minute stress, little increase in cell number was found in the first 16 hours; however, in the following days, the cell number began to increase but at a rate considerably slower than observed with normal cells not exposed to H2O2. After 2 hours of H2O2 stress, about half the cells died in the first 16 hours postinsult (Fig. 3B). The remaining cells slowly died so that by 96 hours postinsult, almost all the cells were gone. Thus, during the first 30 minutes of stress, the cells were able to recover and with longer H2O2 exposure, the cell number gradually declined.

Enzyme Activities

It was of interest to examine differences in the antioxidative defenses of the normal and conditioned cells. As shown in Table 2, when a group of enzymes were assayed, marked enrichments in CAT and GSH Px of 60- and 2.7-fold, respectively, were observed. These enzymes provide the major defense against H2O2. Significant increases in the activity of GSSG Red, GSH S-transferase (using 1-chloro-2,4-dinitrobenzene), and glucose 6-phosphate dehydrogenase were also observed. GSSG Red with NADPH as a cofactor maintains reduced GSH, whereas GSH S-transferases detoxify a broad spectrum of compounds. Glucose-6-phosphate dehydrogenase is a hexose monophosphate shunt enzyme that provides a reducing capacity to the cell by generating NADPH.

Analyses of Protein from Normal and Conditioned Cells

An analysis of the protein composition of the normal and conditioned cells was also undertaken. SDS gel electrophoresis of Triton X-100 solubilized protein (almost all the protein) gave the profiles shown in Figure 4 when equal amounts of protein from normal and conditioned cells were examined. A band,
which appears as a doublet just below a 26-kDa reference band, was found to be markedly enriched in the conditioned cells. Scanning the gels with a Molecular Dynamics laser computing densitometer (model 300A) indicated a 2.8-fold enrichment in these cells. Although other changes were observed, none were as striking as this one. The upper and lower segments of the doublet were cut out and digested with trypsin, and then the masses of the mixtures were determined with a matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometer. The masses in the 1000- to

Table 2. Enzyme Activities of Normal and Conditioned Cells

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Normal (N)</th>
<th>Conditioned (C)</th>
<th>C/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH Px</td>
<td>24 ± 3</td>
<td>65 ± 14</td>
<td>2.7</td>
</tr>
<tr>
<td>CAT</td>
<td>156 ± 6</td>
<td>9,440 ± 1,200</td>
<td>60</td>
</tr>
<tr>
<td>SOD</td>
<td>4,500 ± 450</td>
<td>4,550 ± 390</td>
<td>1.0</td>
</tr>
<tr>
<td>GSSG Red</td>
<td>85 ± 22</td>
<td>125 ± 20</td>
<td>1.5</td>
</tr>
<tr>
<td>GSH-S-transferase</td>
<td>773 ± 72</td>
<td>1,342 ± 90</td>
<td>1.7</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>355 ± 24</td>
<td>718 ± 51</td>
<td>2</td>
</tr>
<tr>
<td>Glycerinaldehyde-3-phosphate dehydrogenase</td>
<td>844 ± 39</td>
<td>682 ± 80</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Cells were grown to 80% confluence in the usual manner and then taken for assay as described in the Methods section. All assays represent the average of 3 or more determinations ± SD. Enzyme units are defined as follows: GSH Px, one unit catalyzes degradation of 1 μMole of H$_2$O$_2$/min with 4 mM GSH at pH 7.0 and 37°C; CAT, one unit will degrade 1 μMole/min of H$_2$O$_2$ at pH 7.0, 37°C, utilizing 100 μM H$_2$O$_2$; GSSG Red, one unit will reduce 1.0 μMole of GSSG/min at pH 7.0 and 37°C with 0.25 mM NADPH; SOD, one unit will inhibit the rate of reduction of cytochrome C by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 at 25°C in a 3.0-ml reaction volume; GSH S-transferase, one unit will conjugate 1.0 μMole of 1-chloro-2,4-dinitrobenzene/min with 1 mM GSH at pH 7.5 and 37°C; glucose-6-phosphate dehydrogenase, one unit will reduce 1 μMole/min of 0.4 mM NADP in the presence of D-glucose-6-phosphate, 1 mM, at pH 6.5 and 37°C, glycerinaldehyde-3-phosphate dehydrogenase, one unit will reduce 1 μMole/min of 1 mM NAD in the presence of 1 mM D-glycerinaldehyde-3-phosphate and 0.1 mM phosphoglycolate at pH 8.5 at 25°C.

Figure 3. Effects of various periods of H$_2$O$_2$ exposure on normal αTN4-1 total cell number. (A) After a 16-hour postinsult period. Cells were prepared as in legend to Figure 1, but the overnight culture was 24 hours. H$_2$O$_2$ (125 μM) was then added to fresh medium at 0 time. At the designated times, the medium was removed. The cells were washed with 2 ml of standard medium, and then 4 ml of the standard medium was added and the incubations continued for 16 hours. The cells were then counted. (B) After a 30-minute or 2-hour insult. Cells were prepared as in (A) but after a 30-minute or 2-hour exposure to 125 μM H$_2$O$_2$ the medium was replaced and the cells were counted at the designated times. All values represent the average ± SD of 3 experiments.

Figure 4. SDS-PAGE of normal and conditioned αTN4-1 cells. Thirty micrograms of protein was separated by SDS-PAGE and then stained with Coomassie brilliant blue G. Standard protein markers (Std) ranged from 66 to 14.2 kDa. N, normal αTN4-1 protein; C, conditioned αTN4-1 protein; arrow, a doublet that was present in much higher amounts in the conditioned preparation.
3000-kDa range were matched with a database containing Swiss-Prot, the protein identification resource, as well as translation of the GenBank. Both samples matched to GSH S-transferase, the top band to the α-1 isomer with a mass of 25,477 and the lower band to the α-2 isomer with a mass of 25,402. The α-1 polypeptide contains 222 residues with the sequence shown in Table 3. Tryptic digestion would be expected to give 10 peptides in the 1000- to 3000-Da range, corresponding to 44% of the sequence assuming one possible missed cleavage. Ten peptides were found with masses that match the theoretical masses in 9 cases within 0.5 Da and with one peptide within 0.9 Da. The peptides match sequences throughout the polypeptide. There also were a number of significant peaks that did not match the theoretical masses. The experimental and theoretical masses are shown in Table 3. GSH S-transferase α-2 is similar to the α-1 form containing 221 residues with a mass of 25,402 Da. The α-2 polypeptide gave results similar to those of α-1 with matches to 45% of the polypeptide sequence. Thus, this experiment strongly suggests that GSH S-transferase α-1 and α-2 are significantly enhanced in the conditioned cells, confirming the enzyme activity observations.

**Evaluation of Metabolic Parameters**

To obtain a better understanding of the impact of the H₂O₂ stress on the conditioned and normal αTN4-1 cells, a number of critical parameters were examined. Nonprotein thiols (NP-SH) is essentially a measure of GSH, a critical antioxidative defense component. Conditioned and normal cells were exposed to 125 μM H₂O₂ under conditions previously described and then assayed at designated times. As shown in Figure 5A, with both normal and conditioned cells, there is an early rapid drop in NP-SH; however, in contrast to the normal cells, the conditioned cells recover gradually, whereas the normal cells maintain their NP-SH level for a short while and then decline further. It would appear that normal αTN4-1 cells are not able to maintain GSH under H₂O₂ stress. A similar but more dramatic change was found in ATP levels (Fig. 5B). Although ATP declined slightly in the conditioned cells before recovering, in the normal cells ATP levels were down more than 50% within 1 hour. By 3 hours, no ATP could be found in these cells. Thus, even though a substantial concentration of H₂O₂ was present at the 1-hour point, the conditioned cells reversed the decline in ATP. And even though little cell death could be detected at 3 hours in normal cells, hardly any ATP remained.

The H₂O₂ is added outside the cell and requires diffusion into the cell to initiate cellular oxidative stress. It was, therefore, of interest to determine the impact of the H₂O₂ on the cell membrane. Two important indicators of membrane function that are sensitive to oxidative stress are the choline transport system and the Ca²⁺ pump. These membrane systems were, therefore, evaluated. [³⁵Ca] influx did not appear to be affected in normal cells during the first 2 hours of stress, but by 3 hours, influx was increased twofold (Table 5). Unexpectedly, the conditioned cells were affected earlier, but by 2 hours the system had returned to normal. It appeared that the effect of H₂O₂ varies considerably depending on the parameter under investigation.

It was also of interest to examine the effect of the H₂O₂ stress on thymidine incorporation because this reflects DNA synthesis and repair and is indicative of the effect of a stress introduced extracellularly on the nucleus, a region in the interior of the cell. In the first half hour after stress, a 50% or more decrease in thymidine incorporation was observed in both cell preparations (Fig. 6). In the conditioned cells, there was a slightly further decrease and then a rapid return to unstressed levels, whereas thymidine incorporation continued to decrease and essentially ceased by 2 hours in the normal cells.

### Table 3. Comparison of Experimental and Theoretical Masses in the 1000 to 3000-Da Range Obtained after Tryptic Digestion

<table>
<thead>
<tr>
<th>Major Experimental Masses</th>
<th>Expected Theoretical Masses</th>
<th>Peptide Sequence</th>
<th>Position</th>
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</thead>
<tbody>
<tr>
<td>1060.75</td>
<td>1061.228</td>
<td>KFLQPGSQR</td>
<td>195-203</td>
</tr>
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<td>1205.86</td>
<td>1206.357</td>
<td>FIRQSPDEDL</td>
<td>33-42</td>
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<tr>
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<td>1246.324</td>
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Sequence: AGKPVLYHIP YRN ARGRMECIRW LLLAAAGVEFE EKFIQSPEDL EKIKKDGNL MFDQPVMPVEIDGMKLAQTRAI LNYIATKLYD ILGGKM ERAL IDMSIEGILD LTEMIGQVL CPPDQREAKT ALAKDRTRKNR YLPPEFKVLK SHGQDLYVGN RLLTRVDHILL EVILLYVEED ASLTPPPL KAFKSRSSLI PNVKKFLQPG SQRPMDA AKIQEARKAFK IQ. GSH S-transferase-α1, mass 25,477 Da. Bold letters in the above sequence indicate tryptic cleavage points.

*C is assumed to be a propionamide cysteine derivative.
Alkaline Elution

To gain insight into the integrity of the DNA after insult, alkaline elution assays were carried out. The cells were labeled with [3H]thymidine and subjected to 125 μM H2O2 for various periods. DNA single-strand breaks were then analyzed by alkaline elution through 2-μm filters. Fractions were collected every hour as shown in Figure 7A. When normal cells were exposed to H2O2 for 0.5 hours, most of the DNA rapidly passed through the filter, indicating significant single-strand DNA cleavage. With further exposure to H2O2, net repair was not observed. In contrast, in the conditioned cells, although some damage was observed after 0.5-hour H2O2 exposure, repair began to predominate over damage and the DNA size gradually increased so that by 6.5 hours, it was not possible to discriminate between native and H2O2-stressed preparations. Thus, even though H2O2 levels of 80 μM remained after 0.5 hours, the conditioned cells were able to significantly repair DNA breaks during the following 90 minutes as the H2O2 levels dropped further to approximately 20 μM.

The continuing presence of H2O2 is a major factor in the normal cells inability to repair the DNA. When the H2O2 was removed after a 0.5-hour stress, the DNA appeared to be repaired, as shown by the 3-hour postinsult data (Fig. 7B). Even after a 2-hour H2O2 stress, significant repair was observed after a 3-hour recovery period (Fig. 7B), although the cells were gradually dying (Fig. 3B). No further repair was noted at 24 hours postinsult (data not shown). And by 48 hours postinsult, the remaining cells had a considerable breakdown of their DNA. This is not surprising considering the loss of ATP (Fig. 5B) and the inability to maintain cell viability.

Effects of Ortho-1,10-Phenanthroline on DNA Cleavage and Cell Viability

The most likely mechanism by which H2O2 causes single-strand breaks involves Fenton chemistry, in which metal ion bound to the DNA reacts with H2O2 to produce hydroxyl radical which then cleaves the DNA.35,44 It was, therefore, of interest to examine the effect on H2O2 stress of ortho-1,10-phenanthroline (OP), an effective chelator of metal ion. Cells were treated with 100 μM OP and then subjected to 125 μM H2O2. As shown in Figure 8A, in the presence of OP, DNA cleavage was not observed after 0.5-, 2-, or 6.5-hour stress. The DNA also continues to appear intact at 48 hours postinsult.

Does this mean that OP prevents cell death? As shown in Figure 8B, cells subjected to 2 hours of H2O2 stress in the presence of OP recover and after a 1-day stationary phase grow but at a slower rate than normal cells. However, OP is toxic and if cells are subjected to OP for 6.5 hours, cell death occurs even though no significant single-strand breaks are observed (Fig. 8A). Even a 2-hour exposure to OP alone causes a pause in significant cell growth. Thus, it can be concluded that OP

Table 4. Effect of Various Periods of H2O2 Stress on [14C]Choline Uptake

<table>
<thead>
<tr>
<th>H2O2 Exposure, h</th>
<th>αTN4-1, cpm/mg protein × 10⁻³</th>
<th>Normal</th>
<th>Conditioned</th>
</tr>
</thead>
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<tr>
<td>0</td>
<td></td>
<td>471 ± 6</td>
<td>477 ± 6</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>320 ± 7</td>
<td>309 ± 15</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>156 ± 33</td>
<td>476 ± 9</td>
</tr>
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</table>

Results are the average of 3 experiments ± SD.

Table 5. Effect of Various Periods of H2O2 Exposure on [45Ca] Influx

<table>
<thead>
<tr>
<th>H2O2 Exposure, min</th>
<th>αTN4-1, cpm/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Conditioned</td>
</tr>
<tr>
<td>0</td>
<td>17,400 ± 1,600</td>
</tr>
<tr>
<td>30</td>
<td>15,100 ± 1,100</td>
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<tr>
<td>60</td>
<td>15,900 ± 800</td>
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</tr>
<tr>
<td>180</td>
<td>35,000 ± 3,200</td>
</tr>
</tbody>
</table>

Results are the average of 2 experiments ± SD.
causes cell death in a manner that does not initially include DNA breaks, whereas it appears that with H₂O₂ stress if the DNA is protected, the cells can recover. Because the OP experiments indicate that the cells will survive a 2-hour stress with OP, this strongly suggests that OP also prevents Fenton-type reactions directed at other critical aspects of the cells' metabolic system. However, it should be noted that normal cells die after 2 hours of H₂O₂ stress even though the DNA appears to have returned to the normal size range in the first few hours of the postinsult period (Fig. 7B). This observation suggests that in this case, DNA repair is either not sufficient or contains lethal errors. Another possibility is that there are two stages to the cell death process. In the first phase represented by the first 24 hours following a 2-hour stress, the more susceptible cells are overwhelmed and die. The remaining cells then slowly die, perhaps by a programmed cell death triggered by the prior stress. Such observations suggest a considerable heterogeneity in the normal αTN4-1 population.

DISCUSSION

From the observations reported in this article, it is apparent that nature has chosen to increase more than one antioxidative defense system in response to H₂O₂ stress. Although, as might be expected, there was a striking increase in the activity of CAT and a large increase in GSH Px activity, other systems such as GSH S-transferase were also affected. The complex changes in the biology of these conditioned cells reported in this article raised the question of whether all these modifications are necessary to resist H₂O₂ stress. It is conceivable that nature has responded conservatively, increasing more systems than are

![Graph showing thymidine incorporation](image)

**Figure 6.** The effect of H₂O₂ stress on thymidine incorporation. Cells were prepared and stressed as described in legend to Figure 1. At indicated times, the medium was removed, the cells were washed with 2 ml of standard medium without serum, and cells were then incubated with 1 ml of standard medium minus serum containing 15 μCi [³H]thymidine for 90 minutes at 37°C and in 5% CO₂. Preparations were then treated as described in the Methods section. Results represent a typical experiment.

![Graph showing alkaline elution](image)

**Figure 7.** Alkaline elution of normal and conditioned cells after various periods of H₂O₂ stress after recovery periods. (A) Alkaline elution after various periods of stress with 125 μM H₂O₂ plus controls. (B) Alkaline elution of normal cells, after various periods of H₂O₂ insult and recovery.
necessary for survival or that responses are limited so that if there is a requirement for greater activity of a given system, other systems are automatically increased. It should be noted that when these cells conditioned to resist H_2O_2 are subjected to a lipid peroxide, cell death ensues even though GSH Px, an enzyme that degrades lipid peroxides, has been elevated approximately 2.7-fold (authors’ unpublished results). Thus, the present state of these conditioned cells does not give them the ability to handle all types of oxidative stress.

It is interesting that despite the marked increase in H_2O_2-degrading enzymes in conditioned cells, there was significant H_2O_2-induced damage in the first hour or so of exposure to H_2O_2. What is fundamentally different between these cell types is that even though a relatively high level of H_2O_2 remains in the medium in both cases, the conditioned cells begin to repair the damage, whereas there is a continued decline of metabolic activity in the normal cells. This is illustrated in dramatic fashion with respect to ATP concentration and thymidine incorporation. In conditioned cells, by the 4-hour point, ATP and thymidine incorporation rates have returned to their original levels, but the normal cells have lost their entire ATP supply and their DNA metabolism, as reflected in thymidine incorporation, is halted. It is not clear whether the disappearance of ATP is solely associated with damage to ATP-generating systems or whether the great demand for energy has contributed to the rapid decline in ATP. The examination of parameters involved with nuclear, cytoplasmic, and membrane components makes it apparent that from the initiation of the stress, all parts of the cell are affected.

Although the conditioned cells were able to sustain an H_2O_2 stress of 125 μM introduced every 24 hours, it should not be assumed that such cells can withstand any H_2O_2 stress. Higher concentrations of H_2O_2 will cause cell death. However, it is probable that by continuing the same procedures described in this communication, the cells could be conditioned to withstand higher levels of H_2O_2 and longer periods of high H_2O_2 exposure.

Most normal cells die after the toxic H_2O_2 levels are gone, and the conditioned cells require a recovery period before normal biological activity is resumed. This observation provides an important insight with respect to the investigation of human cataract in particular and with model systems in general. Namely, the absence of H_2O_2 does not mean that H_2O_2 was not responsible for the observed damage and loss of transparency in the cataractous eye.

A fundamental question concerning this work is how permanent are the observed changes in these conditioned cells? In unpublished experiments, this question has been addressed. Four single cells were obtained from the conditioned cell line and cultured independently in the absence of H_2O_2 stress to produce four cell populations. It was found that these four cell lines continue to express high levels of CAT and elevated levels of the other enzymes discussed in this article. Thus, the changes observed appear to be permanent. There is at least one exception to the permanence of the quantitative changes discussed above. In the early phases of this work when the cells first achieve resistance to 125 μM H_2O_2, GSH Px activity was more than fivefold greater than normal cell levels. Gradually, this value has decreased to the 2.7-fold level and in the clones grown without H_2O_2 to below twofold. These observations suggest that the control of GSH Px gene expression in response to stress may differ from that of other enzymes examined in this work. Although there is some variation between these lines, within about ±20%, all four lines are similar to the conditioned cells from which they arose. Such observations suggest that there probably is little heterogeneity between cells in the original conditioned cell population. At this point, it is not clear whether the observed changes in cell biology are due to natural selection of members of the cell population capable of resisting H_2O_2 stress or mutation. The actual mechanism producing the observed modifications remains to be determined.

It is surprising that despite the conditioned cells’ greatly enhanced ability to degrade H_2O_2, the H_2O_2 concentrations in the medium of conditioned and normal αTN4-1 cells decrease at approximately the same rate. This was found to be the case when either relatively large volumes of medium or volumes as small as 500 μl were used with the same number of cells (authors’ unpublished observations). Such observations suggest that the permeability of H_2O_2 is rate limiting despite the ability of H_2O_2 to move across both hydrophilic and hydrophobic barriers. This conclusion is supported by observations with
lenses from transgenic mice where CAT was elevated 50-fold in the lens fibers but was not increased in the epithelial cells. 27 H2O2 stress caused the development of cortical cataract that was attributed to epithelial cell death. Thus, even though there was vastly increased CAT activity in the fibers, one cell layer removed from the epithelial cells, H2O2 levels sufficient to cause damage are presumed to have accumulated in the epithelial cells. While it can be argued that increasing CAT is not sufficient to protect the system, it should be noted that αTN4-1 cells with elevated CAT are able to successfully withstand H2O2 stress. 26

The experiments with OP indicate that if the cellular DNA is protected from hydroxyl radicals arising from H2O2 by Fenton chemistry, the cells will survive. (It is interesting that nature has chosen to eliminate H2O2 and superoxide, the ton chemistry, the cells will survive. Such findings contribute to the cells resistance of O2 stress. However, when a fibroblast line has shown that conditioning the cells to high HA-1 cell line has shown that conditioning the cells to high levels of H2O2 using a methodology different from that reported in this article results in a large elevation in CAT activity as well as a number of other antioxidative and GSH dependent enzymes. 47-49 CAT activity was found to be very important with respect to resisting H2O2 stress. However, when a fibroblast line was exposed to high concentrations of oxygen, decreasing CAT activity more than 80%, cell viability did not appear to be affected. It was shown that GSH appeared to contribute to the cells resistance of O2 stress. Such findings may be pertinent with respect to the observation that hyperbaric O2 causes nuclear cataract. 50,51

Acknowledgments

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References