Thyroxine Ameliorates Oxidative Stress by Inducing Lipid Compositional Changes in Human Lens Epithelial Cells

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Purpose. Lipid saturation and sphingolipids make model membranes less susceptible to oxidation. A human lens epithelial cell line, HLE B-3, was treated with thyroxine to determine whether this treatment increases lipid saturation and membrane sphingolipids, as it does in other tissues, and if so, to see whether the treatment ameliorates the affects of lipid oxidation.

Methods. One group of HLE B-3 cells was treated with thyroxine, and another group was not. Cells were then grown in a normoxic (20% O_2), or hyperoxic (80% O_2), atmosphere. Phospholipid composition was determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry and ^{31}P nuclear magnetic resonance spectroscopy. Cell viability was determined with a trypan blue dye assay. A chromogenic reagent was used to measure the secondary products of lipid oxidation.

Results. After 6 days of growth in a hyperoxic atmosphere, the thyroxine-treated cells were 20 times more viable than were the untreated cells. As a result of thyroxine treatment, the phosphatidylcholine (PC)-to-sphingolipid molar ratio decreased significantly (by 52%), and the PCs were eight times more unsaturated than were the sphingomyelins. The decrease in the amount of PCs coupled with a 33% decrease in the average unsaturation of the sphingolipids resulted in a phospholipid membrane with fewer double bonds. Products of lipid oxidation were three times higher in untreated cells after growth in a hyperoxic atmosphere than in untreated cells grown in a normoxic atmosphere. Thyroxine treatment reduced the amount of lipid oxidation products by approximately 60% compared with that in untreated cells. A 100% increase in cardiolipin with thyroxine treatment may contribute to a decrease in reactive oxygen species generated by the mitochondria. The total antioxidant power was not affected by thyroxine. Therefore, thyroxine-induced fluctuations in antioxidant levels are unlikely to influence increased cell viability and a concomitant decrease in the amount of lipid oxidation products in thyroxine-treated cells.

Conclusions. The results support the idea that membranes containing more cardiolipin and more sphingolipids and having higher levels of saturation are more resistant to oxidation and protect cells from oxidative stress. Development of a therapy to increase sphingomyelins and lipid saturation in the lens may delay the onset of cataract. (*Invest Ophthalmol Vis Sci.* 2007;48:3698–3704) DOI:10.1167/iovs.06-0908

Hyperoxic damage to HLE B-3 cells[1] is produced by enhanced generation of reactive oxygen species (ROS) from the mitochondrial electron transport chain,[2] and is a consequence of the oxidation of the phospholipid cardiolipin and the subsequent collapse of mitochondrial membrane potential.[2] ROS cause the oxidation of unsaturated lipids, which produce potentially harmful secondary products of lipid oxidation.[1] Among the many products of lipid oxidation produced, malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) are elevated in human lenses with age.[3] These oxidized species may contribute to cataract.[4–8]

Lipid saturation, composition, and susceptibility to oxidation relationships are important, not only in pathologic events in the aging lens but in our understanding of life span and aging in other tissues. There are currently more than 300 theories regarding the mechanisms of aging.[9] One of the prevailing theories is that free radicals produced by the mitochondria damage cellular components and accelerate the aging process.[9] Membrane lipid composition, structure, lipid peroxidization index, and life span have been correlated in the lens[10] and other tissues.[11] There is a correlation between life spans and higher levels of phospholipid saturation and sphingolipid content and lower PC content in the lens.[10]

In organs other than the lens, thyroid hormone regulates cell viability and may make tissues less susceptible to oxidative damage[12] by decreasing the susceptibility of the lipids[13] and proteins[14] to oxidation. When thyroid hormone levels are elevated or animals ingest fats with altered saturation, the ratio of sphingolipid and PC change and inversely correlate with the susceptibility of mitochondrial protein and lipids, to become oxidized.[15,16] In this study, we treated HLE B-3 cells with thyroxine and quantified the expected changes in lipid saturation and membrane sphingolipids and the amelioration of lipid oxidation due to these alterations.

Materials and Methods

A cell viability reagent (Alamar blue) was purchased from Biosource (Camarillo, CA). Triton X-100, Hanks' balanced salt solution, magnesium sulfate, calcium chloride, cesium chloride, trifluoroacetic acid (TFA), 2,5-dihydroxybenzoic acid (DHB), 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), L-thyroxine sodium salt (T4), sodium hydroxide solution, and all the solvents (methanol, chloroform, D-chloroform) were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. Minimum essential medium (MEM), trypsin-EDTA (EDTA), fetal bovine serum (FBS), L-glutamine, gentamicin, and phosphate-buffered saline (7.4; PBS) were obtained from Invitrogen-Gibco (Grand Island, NY). 4-Nitroaniline, or *para*-nitroaniline (PNA), was obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI). A cell-viability analyzer (Vi-CELL reagent Pak) was purchased from Beckman Coulter (Hialeah, FL). A lipid peroxidation assay kit was obtained from Calbiochem (San Diego, CA).

Human Lens Epithelial-B3 Cell Line

An extensively studied human lens epithelial-B3 cell line (HLE B-3) was provided by Usha Andley (Washington University, St. Louis, MO). This

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immortalized cell line was derived from infant human lens tissue and transformed with adenovirus 12-simian virus (SV40).[17] The HLE B-3 cells used in our experiments were at passages 18 to 26. All human tissue samples were obtained in accordance with the provisions of the Declaration of Helsinki for the use of human tissue in research.

Cell Culture Conditions

For routine passage, cells were washed with PBS and detached with trypsin-EDTA at 1 mL/25 cm^2 and incubated for 5 minutes. Fresh culture medium was then added, and the cell suspensions were transferred to sterile centrifuge tubes and gently centrifuged at 3000 rpm for 5 minutes. Homogeneous suspensions of HLE B-3 cells were prepared by adding fresh medium to the cell pellet and passing it repeatedly through the tip of a glass pipette (Fisherbrand, St. Louis, MO).

Cells were seeded at density of 1×10^5/75 cm^2 in polystyrene T-75 culture flasks (BD Bioscience, Bedford, MA) and cultured in a normoxic atmosphere for 5 days, until they reached 50% confluence. These cells were separated into two groups: Group C cells, the control group, were grown in MEM containing 5% FBS, 1% gentamicin, and 1% glutamine in a normoxic atmosphere (Table 1). Group T cells, the thyroxine group, were treated under the same conditions as those in group C, except that the culture medium always contained 0.335 nM free thyroxine (36 nM total thyroxine). After approximately 3 days of growth, when the cells reached 80% confluence, they were subcultured in identical conditions for another 7 days, until they reached 80% confluence. Cells from groups C and T were each divided into two more groups, groups CN, CH, TN, and TH (Table 1). Groups CN and TN were placed in a normoxic (N) atmosphere. Groups CH and TH were placed in a hyperoxic (H) atmosphere (Table 1). Cell growth, cell viability, and phospholipid composition were quantified in theses four groups of cells, as described later.

The cells grown in a normoxic atmosphere were in surface-modified, polystyrene, tissue-culture flasks (BD Bioscience). The cells grown in a hyperoxic atmosphere were in 10-cm polypropylene tissue-culture plates (Corning Inc., Acton, MA), to allow them to fit in a gas-tight modular incubator chamber (Billups-Rothenberg, Inc., Del Mar, CA). All cells were grown in an incubator (VWR Scientific; Sheldon MFC, Inc., Cornelius, OR). Because cell susceptibility to hyperoxia may vary with cell density,[18] the initial number of cells was routinely adjusted to $\sim 1 \times 10^5$ in MEM. The sealed chamber was placed in a standard tissue-culture incubator, and the atmosphere was replenished every 48 hours.

Testing the Cytotoxicity of Thyroxine by the Alamar Blue Assay

Cells were seeded at a density of 1×10^5/75 cm^2 in polystyrene T-75 culture flasks (BD Bioscience) and cultured in normoxic atmosphere for 7 days, until they reached 80% confluence. To test whether thyroxine was cytotoxic, the cells were harvested by washing them with PBS and detached after a 5-minute incubation with trypsin-EDTA. Fresh culture medium containing trypsin inhibitor (5% in PBS) 30 mL was then added, and cell suspensions were transferred to sterile centrifuge tubes and centrifuged at 3000 rpm for 5 minutes. The cells were resuspended in the growth medium and counted. The cell suspensions were adjusted to a concentration of 1×10^5 cells/mL, and 250 μL of each cell suspension was added to each well of a 96-well plate. The cells were incubated at 37°C in a 5% CO_2, 20% O_2, and 75% N_2 atmosphere for 5 days until they reached 80% confluence. The culture medium was then replaced by a serum-free culture medium containing a range (nanomolar to micromolar) of thyroxine concentrations for 10 hours at 37°C. Alamar blue (25 μL; AlamarBlue cell viability assay kit; Biosource) was added to each sample and incubated for 4 hours. The fluorescence of the reduced dye was measured with a spectrofluorometric plate reader (Molecular Devices, Sunnyvale, CA). The fluorescence intensity was measured at an excitation wavelength of 530 nm and an emission wavelength of 590 nm.[18–21] Cell viability was calculated as the ratio of surviving cells to the original cell population. Cell viability was also confirmed with the trypan blue assay.

Table 1. Grouping of Cells by Experimental Conditions

Group	Growth Atmosphere: Normoxic (20% O_2)	Growth Atmosphere: Hyperoxic (80% O_2)	Thyroxine Treatment
CN	+	−	−
CH	−	+	−
TN	+	−	+
TH	−	+	+

Measurement of Cellular Growth Curves for Control and Thyroxine-Treated Cells Grown under Normoxia and Hyperoxic Atmosphere

The four groups of cells described in the Cell Culture Conditions section (Table 1) were harvested after various periods of growth of 0 to 7 days, by washing them with PBS. They were detached after a 5-minute incubation period with trypsin-EDTA. Fresh culture medium containing 10 mL of trypsin inhibitor (5% in PBS) was then added, and the cells were suspended. One milliliter of the cell suspension was placed in a vial provided in a cell-viability assay kit (Beckman Coulter). The kit included trypan blue, cleaning reagent, disinfectant, and buffer solution. Viability was determined using a cell analyzer (Beckman Coulter).

Measurement of Free Thyroxine Concentration in the Culture Medium

An 18-μL aliquot of 10^{-4} M thyroxine was added to 500 mL MEM containing 5% FBS, and 5 mL was taken from the medium to measure the free thyroxine concentration. Free thyroxine was measured with an automated electrochemiluminescence immunoassay (cat. no. 1731297; Modular Analytics E 170; Roche Diagnostics Corp., Indianapolis, IN).

Measurement of Phospholipid Compositional Changes with Thyroxine Treatment

Two groups of cells, CN and TN, were prepared as described in the Cell Culture Conditions section. There were seven flasks of cells in each group. The cells were harvested by washing them with PBS. They were detached after a 5-minute incubation period with trypsin-EDTA. Fresh culture medium containing 30 mL of trypsin inhibitor (5% in PBS) was then added, and the cell suspensions were transferred to sterile centrifuge tubes and centrifuged at 3000 rpm for 5 minutes. A 1-mL aliquot was taken from the culture flask to assay cell viability by the trypan protocol. The remainder was centrifuged at 5000 rpm for 5 minutes. The supernatant was decanted. Lipid was extracted from the pellet containing HLE B-3 cells, by using a monophasic extraction protocol, and phospholipid composition was measured with ^{31}P nuclear magnetic resonance (NMR) spectroscopy and matrix-assisted desorption ionization, time of flight mass spectrometry (MALDI-TOF/MS).

Phospholipid Compositional Analysis with Cell Growth by MALDI-TOF/MS

The protocol for measuring phospholipid composition with MALDI-TOF/MS is identical with the detailed published protocol.[1] Briefly, cells pooled from seven flasks were washed with PBS and lifted from the bottom of the culture flasks. An aliquot was taken from the culture flask for cell-viability analysis. The remainder was centrifuged at 5000 rpm for 1 hour. The pellet of cells was put in a glass centrifuge tube containing 30 mL of methanol and sonicated with a microprobe sonicator (Branson Sonic Power Co., Danbury, CT) three to four times for 15 seconds, with a 5-minute pause between sonication bursts. The

solution was centrifuged at 5000 rpm for 1 hour and the supernatant decanted into another centrifuge tube. The methanol in the supernatant was evaporated with a rotary evaporator (Buchi Rotavapor 011; Brinkman Instruments, Inc., Westbury, NY). Hexane and isopropanol (2:1 vol/vol, 10 mL) were added to the dry lipid film and sonicated with a microprobe for 15 seconds. The solution was transferred to a centrifuge tube and centrifuged at 5000 rpm for 1 hour. The lipid-containing supernatant was decanted into another tube, and the solvent was evaporated by a flow of nitrogen. Methanol (150 μL) was added to solubilize the lipid. A 50-μL aliquot was taken for NMR analysis, and the remainder was used for MALDI-TOF/MS analysis.

The mass spectral analysis was performed (Voyager-DE Pro MALDI-TOF mass spectrometer; Applied Biosystems, Inc. [ABI], Foster City, CA)with the system in the positive mode.[22,23] CsCl crystals (3 mg) were added to reduced spectral complexity. DHB (0.5 M) prepared in methanol containing 0.1% of TFA was used to analyze the samples in the positive mode. To obtain negative-ion spectra, no CsCl was added, and a 0.085-M solution of PNA in chloroform-methanol (2:1) was used as the matrix.

Ten aliquots (1-μL each) of each of the methanolic lipid extracts were spotted onto 10 wells of a stainless-steel MALDI plate. The matrix (1 μL per spot) was added on top of each spot.

Phospholipid Compositional Changes with Hyperoxia Quantified by ^{31}P NMR Spectroscopy

Chloroform-D (500 μL) was added to dissolve the film of extracted lipid. The samples were heated at 40°C for 20 minutes and then allowed to return to room temperature before NMR spectral acquisition. A 250-μL aliquot of cesium EDTA (Cs^{+}-EDTA) reagent (pH 6.0; 4:1, vol/vol) was added to each sample. The NMR instrument's setting and spectral process were the same as described in Yappert et al.[24]

Quantification of Lipid Peroxidation, with and without T4 Treatment

Four groups of cells were cultured as described in the Cell Culture Conditions section (Table 1). Cells were harvested after 0, 24, and 48 hours of normoxic or hyperoxic oxygen exposure. Secondary products of lipid oxidation, MDA and HAE, were measured exactly as described in detail by Huang et al.[1]

Briefly, the cells were diluted with 1 mL of ice-cold 20 mM PBS with BHT and homogenized with three 1-second bursts from an ultrasonication probe (Branson Sonic, Power Co.). The diluted homogenates were centrifuged at 3000*g* for 10 minutes at 4°C. The supernatant was collected, and lipid oxidation was measured in 200 μL of supernatant with a lipid peroxidation assay kit (cat. no. 437634; Calbiochem, Inc.). The assay was performed according to the protocol provided with the kit. This assay takes advantage of a chromogenic reagent, *N*-methyl-2-phenylindole that reacts with MDA and HAE. Condensation of one molecule of either MDA or HAE with two molecules of the reagent phenylindole yields a stable chromophore with maximum absorbance at 586 nm.[25]

Measurement of Total Antioxidant Power

Two groups of cells, CN and TN, were grown as described in the Cell Culture Conditions section (Table 1). When the cells reach 80% confluence, they were harvested. Antioxidant power was measured by using an assay that measures the oxidation of cuprous ions exactly as detailed by Huang et al.[1]

Briefly, the protocol provided in the TA01 kit was used (Oxford Biomedical Research, Oxford, UK). Cells were lysed with Triton X-100 by ultrasonification for 5 minutes in a bath sonicator (Branson Sonic Power Co.) and a 3-second pulse from a probe sonicator. The assay was run at room temperature. Samples and standards were both diluted 1:40 in the dilution buffer provided, and the absorbance of 200-μL aliquots of diluted samples or standards were measured at 490 nm in a plate reader (Power Wave X; Bio-tek Instruments, Inc. Winooski, VT). A Cu^{+2} solution (50 μL) was added to each well and after 3 minutes, 50 μL of stop solution was added to each well. The plates were read a second time at 490 nm. The optical density readings for each sample were compared with the standard curve.[26,27]

Results

Thyroxine Toxicity

After 10 hours of thyroxine treatment of HLE B-3 cells with thyroxine levels between 10^{-12} and 10^{-6} M, cell viability decreased by only 10% (Fig. 1). Subsequent treatment with 0.335 nM free thyroxine for 1 to 9 days had no affect on cell growth (Fig. 2). This result indicates that thyroxine is not toxic to these cells. Because proteins present in the FBS culture medium can bind thyroxine, the free thyroxine was carefully measured and found to be 0.335 nM, approximately 100 times lower than the total thyroxine content of 36 nM.

Growth Curves of Control and Thyroxine-Treated Cells Grown in Normoxic and Hyperoxic Atmospheres

Uptake of trypan blue dye by HLE B-3 cells was measured to assess cell viability and growth. Only nonviable cells take up trypan blue. Cells treated with thyroxine (Fig. 2, circles and dotted line) and cultured in a normoxic atmosphere grew linearly up to approximately 6 days, after which growth diminished and confluence was reached. The growth curve for these cells was not significantly different from the curve of cells grown without thyroxine (Fig. 2, top solid line). This confirms the results presented in the section above showing that thyroxine at 0.335 nM is not toxic to the HLE B-3 cells. Cell-doubling time was approximately 1.8 days for cells grown with or without thyroxine in a normoxic atmosphere. Cells treated with thyroxine and grown in a hyperoxic atmosphere (Fig. 2, square and bottom dotted line) increased linearly for approximately 7 days, in sharp contrast to the cells grown under identical conditions but without thyroxine (Fig. 2, bottom solid line). The number of cells increased linearly for approximately 3 days and then stopped growing and progressively died.

Phospholipid Compositional Changes with Thyroxine Treatment

Eleven phospholipids were detected and identified in the ^{31}P NMR spectra of lipids from HLE B-3 cells. The relative amount

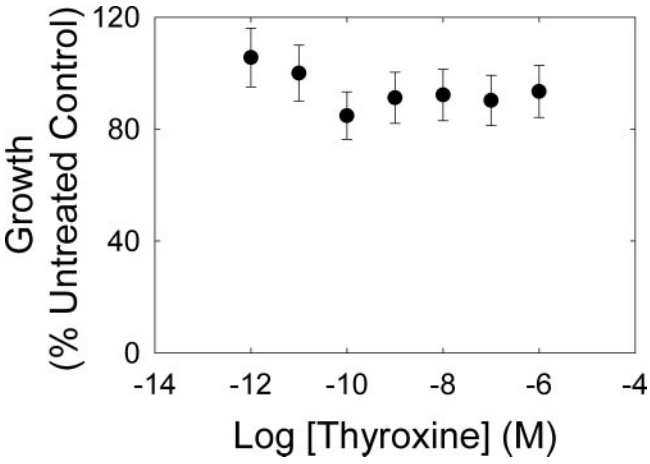

Figure 1. Thyroxine toxicity was tested with a cell-viability assay that uses Alamar blue dye. HLE B-3 cells were treated with thyroxine for a period of 10 hours. Thyroxine was not significantly toxic to the cells at 3.35×10^{-10} M, and that concentration was used in subsequent studies. In each case, results are the mean ± SD of results from three independent cultures.

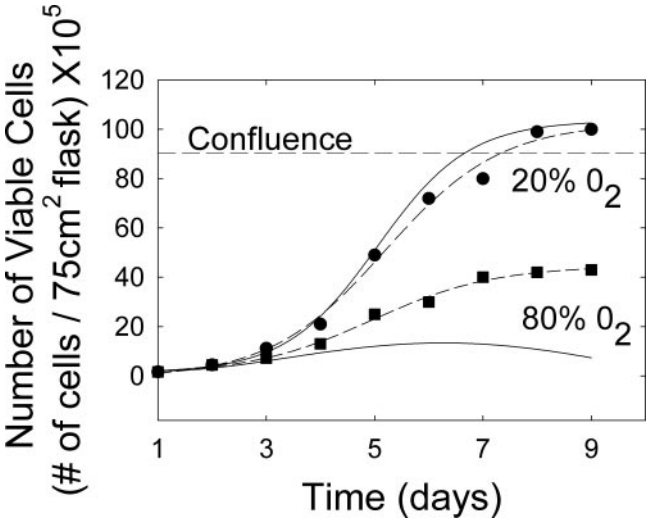

FIGURE 2. HLE B-3 cells were treated with 3.3×10^{-10} M thyroxine for 10 days and then allowed to grow in an atmosphere of (●) 20% O_2, group TN, Table 1 or (■) 80% O_2, group TH, Table 1. Cell viability was determined with a trypan blue assay. *Top solid line*: data from group CN, Table 1, is the curve fit to the data presented in Huang et al.[2] *Bottom solid line*: data from group CH, Table 1, is the curve fit to the data presented in Huang et al.[2] *Dashed line*: fit of data to a three-parameter sigmoidal curve. Results are expressed as the mean ± SD of results from three independent cultures. Error bars are not shown, because they are smaller than the symbol size.

of each phospholipid is presented in Figure 3. Numerous changes in the relative phospholipid composition induced by thyroxine treatment were measured, including a decrease in the relative amount of PCs and an increase in the relative amounts of sphingomyelins and cardiolipins.

The most abundant sphingomyelin species contained a palmitoyl acyl chain and is represented as SM(16:0), where the 16 refers to the number of carbons and the 0 the number of sites of unsaturation. In the case of PCs, there are two acyl chains, and the total number of carbons and sites of unsaturation in both chains is used to denote the different species. In general, the *sn*-1 chain is saturated and the *sn*-2 chain is unsaturated.[28] MALDI-TOF/MS data showed numerous sites of unsaturation in the hydrocarbon chains of lipids extracted from HLE-3 cells (Fig. 4). These sites could serve as targets of oxidative damage and a source for MDA formation. The thyroxine treatment of HLE B-3 cells increased significantly the relative amount of several sphingomyelins and decreased the unsaturated PC(36:1) (Fig. 4). Decreases in other PC species were seen that contributed to the decrease in the PC-to-sphingolipid molar ratio (Table 2).

Lipid Peroxidation in the Thyroxine-Treated Cells Compared with the Nonthyroxine-Treated Cells

MDA and HAE levels were significantly higher (~3 times; $P < 0.01$, ANOVA test) after 24 or 48 hours of growth in a hyperoxic atmosphere (Fig. 5, open squares) compared with cells grown in a normoxic atmosphere (Fig. 5, open and filled circles). Although the increase in secondary products of lipid oxidation in thyroxine-treated cells was significant ($P < 0.05$) between 0 and 48 hours of growth in a hyperoxic atmosphere (Fig. 5, filled square), thyroxine treatment reduced the amount of lipid oxidation products by approximately 60% compared with untreated cells (Fig. 5, open squares).

Antioxidant Power in Thyroxine-Treated Cells Compared with Nonthyroxine-Treated Cells

The total antioxidant power was detected by evaluation of Cu^+ derived from Cu^{2+} by the combined action of all antioxidants present in the sample. The lens contains antioxidants such as ascorbate, carotenoids, glutathione, and pyruvate. These antioxidants and any others present in the lens contribute to the total antioxidant power measured. The total antioxidant power assay that we used is advantageous because identification and quantification of each and every antioxidant would be difficult, and measuring just a few could be misleading.[29]

In the total antioxidant assay, Cu^+ is detected after complex formation with bathocuprine. This complex is stable and has an absorption maximum between 480 and 490 nm. For both treated and untreated cells, the total antioxidant power of the cells increased identically after 36 hours of growth in a hyperoxic atmosphere followed by a gradual decrease (Fig. 6). There were no differences between the total antioxidant power of cells treated with thyroxine (Fig. 6, filled squares) and that of untreated cells (Fig. 6, open squares) at any time studied. To determine whether thyroxine contributed to or interfered with our antioxidant power measurements, standard curves were measured, with and without 36 nM thyroxine in the uric acid reaction mixture used as a standard. Standard curves were plotted as optical density at 490 nm versus uric acid concentration (in millimolar) on the *x*-axis. Thyroxine, even at a concentration 100 times greater than that of the free thyroxine in our cell culture medium, did not contribute to or interfere with the assay as evident by the lack of changes in the standard

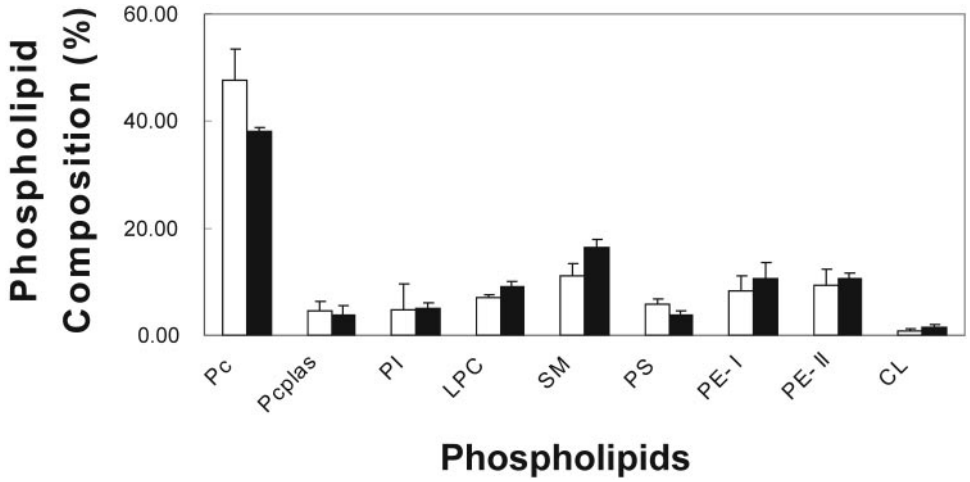

FIGURE 3. Phospholipid compositional changes in HLE B-3 cells that were 80% confluent, using ^{31}P NMR. (□) Untreated cells, group CN, Table 1; (■) cells treated for 10 days with 3.35×10^{-10} M thyroxine, group TN, Table 1. Data represent the mean ± SD of three samples. *Statistically significant change ($P < 0.05$, Student's *t*-test). Pc, phosphatidylcholines; Pcplas, phosphatidylcholine plasmalogens; PI, phosphatidylinositols; LPC, lysophosphatidylcholines; SM, sphingomyelins; PS. phosphatidylserines; PE-I, a PE-related phospholipid formally assigned to phosphatidylethanolamine plasmalogen; PE-II, is an unidentified band with possibly a PE headgroup; CL, is cardiolipins.

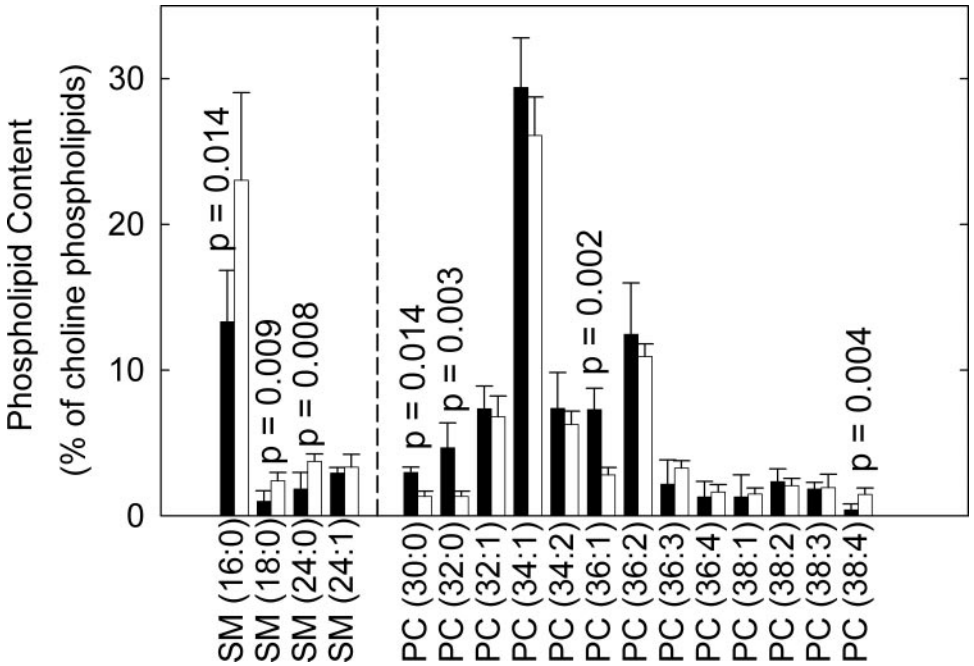

FIGURE 4. Hydrocarbon chain composition of choline containing phospholipids extracted from HLE B-3 cells. Composition was determined by MALDI-TOF MS. (■) Untreated cells, group CN, Table 1; (□) cells treated for 10 days with 3.35×10^{-10} M thyroxine, group TN, Table 1. Data were normalized to SM (24:1). Results are expressed as means ± SD of five samples. PC, phosphatidylcholine; SM, sphingomyelin.

curves obtained with and without thyroxine: (intercept of 0.041 ± 0.007, slope of 0.557 ± 0.008, $n = 5$, $r^2 = 0.999$, compared with the standard curve with thyroxine, intercept of 0.03 ± 0.01, slope of 0.57 ± 0.02 $n = 5$, $r^2 = 0.997$. Data are average ± SEM).

DISCUSSION

HLE B-3 cells grown in a hyperoxic atmosphere have been shown to change their phospholipid composition.[1] These changes have been attributed to the uncoupling of the mitochondrial electron transport chain, which increases the generation of ROS.[2] Oxidative stress in the HLE B-3 cell line induces lipid changes that are similar to those observed with age for human lens epithelial and fiber cells.[1] The time course of these studies was 24 hours—too short to influence cell viability. The present study showed that with extended exposure (>3 days) to a hyperoxic atmosphere, HLE B-3 cell viability decreased. Our data support the idea that extended hyperoxic exposure generates ROS above the threshold of the antioxidant defense system. As a result, cell damage and, eventually, cell death occur.

In this study, we used thyroxine in hopes of changing the lipid composition of HLE B-3 cells to increase viability as reported in other tissues.[12–15] Indeed, cultures treated with thyroxine contained up to 20 times more viable cells after 3 days of growth in a hyperoxic atmosphere compared with control cultures (Fig. 2). This trend is similar to that reported for another cell culture system.[12] We were careful to measure and use an effective amount of free thyroxine in the medium, because thyroxine binds strongly to proteins and it is the free, not bound, thyroxine that is biologically active and crosses cell membranes and binds to receptors.[30,31] The concentration of free thyroxine was 100 times less than the total thyroxine in the medium in accordance with other studies.[32–34]

TABLE 2. Lipid Composition of Thyroxine-Treated Group versus the Untreated Group Determined by MADLI-TOF Spectroscopy

Thyroxine Treatment	No T4*	T4
Average SM unsaturation (C/mole)	0.15 ± 0.05	0.10 ± 0.02
Average PC unsaturation (C/mole)	1.13 ± 0.02	1.17 ± 0.06
Average PC and SM unsaturation (C/mole)	0.95 ± 0.09	0.83 ± 0.07
Average chain length for PCs (number of hydrocarbon chain carbons)	17.2 ± 1.02	17.3 ± 1.21
Length for SMs (number of hydrocarbon chain carbons)	18.1 ± 1.32	17.9 ± 2.17
SM 24:1 (% of SMs)	15.4 ± 1.27	10.2 ± 0.98†
PC/SM	4.2 ± 0.28	2.0 ± 0.16†

Results are expressed as the standard deviation of the mean of five samples. SM, sphingomyelin; PC, phosphatidylcholine.

* Also reported as a control for hyperoxic growth study by Huang et al.[1]

† Statistically significant ($P < 0.01$).

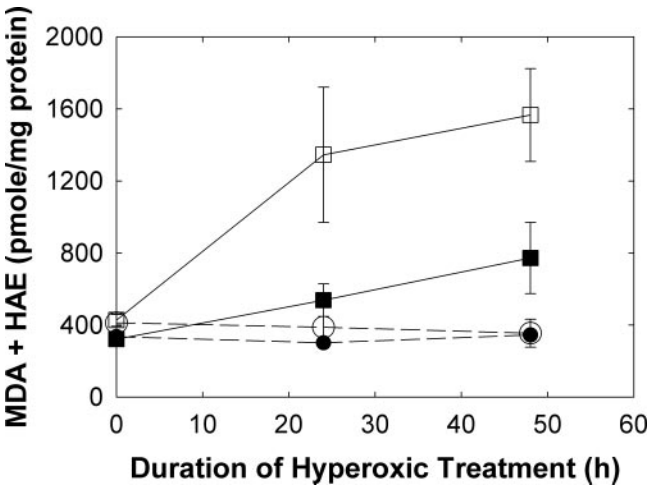

FIGURE 5. Lipid peroxidation was estimated by measuring MDA and HAE. (○) Cells grown in a normoxic atmosphere, group CN, Table 1. (□) Cells grown in a hyperoxic atmosphere, group CH, Table 1. (●) Cells treated with 3.35×10^{-10} M thyroxine and grown in a normoxic atmosphere, group TN, Table 1. (■) Cells treated with 3.35×10^{-10} M thyroxine and grown in a hyperoxic atmosphere, group TH, Table 1. Data represent the mean ± the SD, $n = 4$.

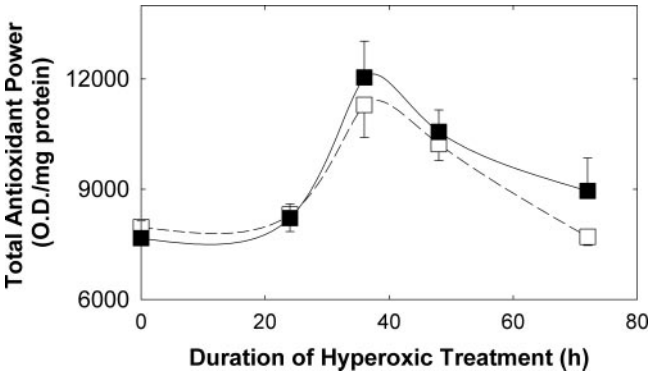

FIGURE 6. Total antioxidant power of HLE B-3 cells grown in a hyperoxic atmosphere and treated without thyroxine, CH group, Table 1 (□); grown in a hyperoxic atmosphere and treated with 3.35×10^{-10} M thyroxine TH group, Table 1 (■). Each data point represents a mean value ± SD in eight replicate experiments.

In heart and liver cells, thyroxine treatment results in changes in the phospholipid composition, decreasing the number of double bonds and thereby causing the lipids to be less susceptible to oxidation.[12–15] In the current study, thyroxine treatment similarly changed HLE B-3 cell lipid composition and decreased the number of double bonds. As a result of thyroxine treatment, the ratio of PCs to sphingolipids decreased by a factor of 2. The PCs in HLE B-3 cells were eight times more unsaturated than the sphingomyelins. A decrease in the amount of PCs coupled with a 33% decrease in the average unsaturation of the sphingolipids resulted in a membrane with fewer double bonds and thus more resistant to oxidative insult (Table 2, Fig. 3). More specifically, thyroxine treatment of HLE B-3 increased the relative amounts of SM(16:0), SM(18:0), and SM(24:0) and decreased the relative amounts of the most abundant PC species (Fig. 4). Oborina and Yappert,[35] have shown that sphingomyelin also decreases the susceptibility of polyunsaturated PC to oxidation in vitro. Therefore, an increase in sphingomyelin and decrease in membrane lipid unsaturation may both contribute to a membrane, which is less susceptible to oxidative damage. This notion is evident, in that HLE B-3 cells showed a 60% decrease in secondary products of lipid oxidation in response to thyroxine-treated cells compared with control cultures (Fig. 5).

Why were the thyroxine-treated cells more viable and with less secondary products of lipid oxidation? Were there more antioxidants in cells treated with thyroxine? It is possible that the thyroxine-treated cells had a survival advantage compared with the control group, because their membranes were less susceptible to oxidative damage, and/or because they had a hypertrophied antioxidant defense system. We found that there was no significant difference in the antioxidant power of cells treated with thyroxine or untreated cells. For both treated and untreated cells, the total antioxidant power of the cells increased identically after 36 hours of growth in a hyperoxic atmosphere followed by a gradual decrease (Fig. 6). Our data are in agreement with another study of lens epithelium, in which oxidative stress caused an increase in almost every antioxidant enzyme assayed and caused the upregulation of most antioxidants.[36] Our data are also in agreement with the study of Kosano et al.,[37] who showed that the level of glutathione, one of the major antioxidants in the lens, does not change with thyroxine treatment or with glucocorticoid-induced metabolic changes in thyroxine-treated chick lenses. A thyroxine-induced hypertrophied antioxidant defense system appears not to be a factor in the thyroxine-induced increased cell viability and decreased amount of lipid oxidation products. Rather, it appears that the thyroxine-treated cells had a survival advantage compared with the non–thyroxine-treated group, because their membrane lipids were less susceptible to oxidative damage.

Why were the thyroxine-treated cells more viable, with less secondary products of lipid oxidation? Was less ROS produced by the mitochondria in cells treated with thyroxine? Cardiolipin is a phospholipid exclusively distributed in the mitochondrial inner membrane.[38,39] The relative amount of cardiolipin increased by 100% in HLE B-3 cells treated with thyroxine. This may be important to the mitochondria, since cardiolipin stabilizes the electron transport chain.[39–42] In HLE B-3 cells, mitochondria produce 90% of the ROS, and cardiolipin and ROS produced are inversely related.[2] One would expect that the doubling in cardiolipin with thyroxine would cause less ROS to be produced in thyroxine-treated HLE B-3 cells. A decrease in cardiolipin-related ROS production may contribute to a thyroxine-related increase in viability and a decrease in secondary products of lipid oxidation when cells are stressed with oxygen.

Relationships between the Lens Epithelium and Fiber Cells

In the human lens, deleterious oxidation products, such as MDA, increase with age[3] and may contribute to cataract.[4–8] As a consequence of the production of these products over a lifetime, more than 30% of the lens phospholipids are degraded.[43] Age-related changes in the phospholipid composition of the HLE B-3 cells with oxidation are similar in nature, albeit more pronounced, to those observed in human lens fiber and epithelial cells,[24,43,44] suggesting that the lipid compositional changes share a common mechanism. We have shown that in the same system,[2] lipid changes may influence mitochondrial function, which leads to the generation of ROS and secondary products of lipid oxidation. If these deleterious products were to migrate to the fiber cells, it is possible that their prolonged accumulation could eventually lead to alterations in fiber cell structure and increased opacity.[3–8] Furthermore, fiber cells have relatively no intracellular organelles[45] or repair capacity and lower antioxidant concentrations,[46] and so it is likely that they are more susceptible to oxidative damage than are epithelial cells. The epithelium produces most of the antioxidants in the lens. If the antioxidant production by the epithelium were compromised, the fiber cells would become even more susceptible to oxidative insult.

The findings in the present study show that an increase in cardiolipin, unsaturation, and sphingolipid content cause lens epithelial cells to be more viable and less susceptible to oxidative stress and provide insight into the mechanisms responsible for the lifespan-lipid–saturation–sphingolipid correlations found in the lens[10] and other organs.[11]

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