METHODS. Lenses from rats fed a standard diet containing 50% galactose with or without an aldose reductase inhibitor (ARI) were investigated. Transformed human LECs were cultured in standard 10% FCS-DMEM containing various concentrations of sugar. UPR-specific proteins from both the rat lenses and lens cultures were quantified by protein blot analysis. Cell death was evaluated with TUNEL staining and ethidium homodimer-1 (EthD) dyes. Reactive oxygen species (ROS) were quantified with H$_2$DCF, and free glutathione (GSH) levels were measured with a commercial GSH quantification kit.

RESULTS. Increased apoptosis of the LECs was observed in the lenses of rats fed the galactose diet for 5 to 9 days, and nuclear cataracts subsequently developed in these lenses after 13 to 15 days. Protein blot analysis of the LECs from these galactose-fed rats showed higher levels of the UPR-specific proteins Bip/GRP78, ATF4, and CHOP. These LECs also demonstrated activation of the UPR-specific procaspase-12 and the increased presence of ROS, whereas GSH was reduced. Because these results indicate that the UPR is activated in LECs along with the production of ROS and apoptosis during cataract formation in the galactose-fed rats, subsequent studies were conducted to determine the role of nonenzymatic glycation, osmotic stress, and oxidative stress on these biochemical processes. In vitro cultures of human LECs showed that the UPR was induced by osmotic and oxidative stress, but not by glycation. In addition, the UPR and apoptosis in LECs was induced by glucose deprivation. The ARI blocked the induction of the UPR, cell death, and cataract formation.

CONCLUSIONS. The UPR that is induced by abnormally high or low concentrations of sugar is linked to the production of ROS, increased apoptosis in LECs, and cataract formation. The inhibition of the UPR induction by ARI suggests that osmotic stress may be the primary inducer of the UPR. Modulation of the UPR pathways may offer novel methods for the development of therapeutic tools to delay cataracts. (Invest Ophthalmol Vis Sci. 2006;47:3951–3959) DOI:10.1167/iovs.06-0193

The incidence of diabetes mellitus (DM) has significantly increased in recent years. Poor control of DM is linked to the formation of several systemic and ocular complications, which include vision loss. Both chronic hyperglycemia and hypoglycemia are associated with cataract development. The incidence of hypoglycemia increases with insulin therapy, tight control of blood glucose levels, with exercise, and with anorexia. Hypoglycemia induces an unfolded protein response (UPR) that leads to lens epithelial cell (LEC) death through activation of specific death pathways. Uncontrolled DM results in hyperglycemia, which is associated in ocular tissues with nonenzymatic protein glycation, osmotic stress, and oxidative stress.

Studies in both aged and diabetic lenses suggest that increased glycation of crystalline proteins, the Maillard reactions, play an important role in cataract formation. The lenticular accumulation of advanced glycation end products (AGEs) in these lenses has been proposed to lead to increased light scatter. Methyglyoxal (MGO) and glyoxyl (GXL) are two highly reactive alpha-carbonyl metabolites of the glucose degradation pathway that can also strongly interact with cellular proteins, lipids, and nucleic acids. Glycation may induce loss of cellular function, structural alterations, and the induction of cell death. MGO has been proposed to be an important precursor of AGE formation that is catalyzed by aldose reductase (AR) and detoxified by the glyoxalase system. Glycation is accompanied by auto-oxidation, the overall process of which is referred to as glyco-oxidation.

Extensive investigations on the role of osmotic stress on the lens by sorbitol or galactitol accumulation have been conducted over the past three decades. AR is a widely expressed aldehyde-metabolizing enzyme that reduces aldonic sugars to their corresponding sugar alcohols. Its reduction of glucose to sorbitol is linked to the development of secondary diabetic complications, the induction of osmotic stress in the lenses, and the subsequent induction of apoptosis in LECs. Aldose reductase inhibitors (ARIs) block the reduction of galactose and glucose to galactitol and sorbitol, respectively, and inhibit the subsequent destruction of lens fibers and cataract formation.

Apoptosis in LECs is linked to cataract formation. LEC death induced by cataractogenic stress disturbs homeostasis and eventually induces the oxidation and hydration that result in cataract formation. It has been hypothesized that stressors associated with cataract formation are also endoplasmic reticulum (ER) stressors that activate the UPR, produce reactive oxygen species (ROS), and induce LEC death. This hypothesis is supported by in vitro studies with transformed human LECs which, when treated with ER stressors such as tunicamycin-
cint, homocysteine, calcium ionophore (A23178), and glucose deprivation induce UPR, ROS production, decreased glutathione (GSH) levels, and apoptosis. If the accumulation of misfolded proteins is not resolved, then apoptosis is activated through cell death pathways that include multiple caspases, m-calpain, and the release of produced ROS from LECs. The ER is a highly oxidized cellular compartment where multiple stressors can affect the conformation of proteins similar to those in oxidized lenses. Many cataractogenic stressors have also been observed to be ER stressors and to induce UPR-specific proteins, among which Bip/GRP78, CHOP, ATF4, and caspase-12, are mediated by an activation of the UPR pathway. In addition, the UPR activates the expression of lens epithelium-derived growth factor (LEDF, our unpublished observation).

ROS (H$_2$O$_2$, O$_2^-$, and OH$^-$) in the UPR may be derived from two sources. One is the UPR-regulated oxidative protein-folding machinery in the ER. The second source of ROS (O$_2^-$) is the mitochondrial electron transport chain, where ROS production increases as a result of increased intracellular glucose metabolism. Clearly, the UPR may serve as a substantial source of ROS in LECs and significantly oxidize GSH in lenses.

Because cataracts are more common in DM where blood glucose levels fluctuate with glycemic control, it is hypothesized that either higher or lower concentrations of glucose levels increase ER stress, which in turn generates the UPR. In the present study, the importance of sugar fluctuation as a potential ER stressor that may induce the UPR and, as a result, induce cataract formation in animals was investigated. These studies indicate that UPR-dependent cell death is induced in both the lenses of galactose-fed rats and in LECs cultured in high glucose or glucose-deficient medium. This provides a link between ER stress, apoptosis, and sugar cataract formation in rat lenses.

**METHODS**

**Galactosemic Rats**

Young female Sprague-Dawley rats (65–100 g) were purchased from Charles River Laboratories Inc. (Wilmington, MA). Eighteen rats were fed a standard diet containing 50% galactose (wt/wt; BioServe, French-town, NJ), with or without 0.0125% of the ARI AL1576. Nine similar rats serving as normal control subjects were fed a standard diet. All studies were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**LEC Culture**

Transformed human LECs were obtained from Venkat Reddy (University of Michigan, Ann Arbor) and used as previously described. The human LECs (5 × 10$^4$ cells/mL in 6- or 10-cm dishes) were cultured in DMEM or glucose-free DMEM with 10% FBS and various added sugars for 3 days (Fig. 1D, 1E). After 15 days, vacuoles appeared to be differentiating lens fiber cells appeared relatively intact after 7 days (Fig. 1C), they became liquefied by 9 days of a galactose feeding. Highly vacuolated lens fiber cells were observed after 7, 9, and 15 days of galactose feeding. Young (65–100 g) Sprague–Dawley rats fed standard diet containing 50% galactose showed rapid development of cortical opacities, which appeared by days 7 to 10 and mature cataracts with nuclear involvement between 13 and 15 days. Lenses were examined by histology after 7, 9, and 15 days of galactose feeding. Highly vacuolated lens fiber cells were observed after 7 to 9 days of galactose feeding, and some of the LECs appeared differentiating from the capsule (Figs. 1B–E). Although some of the differentiating lens fiber cells appeared relatively intact after 7 days (Fig. 1C), they became liquefied by 9 days of a galactose diet (Figs. 1D, 1E). After 15 days, vacuoles appeared to be

**Protein Blot Analysis**

LEC obtained from lenses or harvested from cell culture plates were homogenized in RIPA buffer (20 mM Tris-HCl [pH 7.4] 1.0% Triton X-100, 150 mM NaCl, 100 μM leupeptin, 100 μM aprotinin, 1 mM EDTA, and 1 mM EGTA). The insoluble materials were removed by centrifugation. After electrophoresis, the separated proteins were blotted onto nitrocellulose filters. Filters were blocked for 1 hour in a buffer containing 5% nonfat milk. The primary antibody was applied overnight at 4°C and secondary antibody at room temperature for 1 hour. All antibodies were obtained from commercial sources; antibody to Bip/GRP78, LEDGF, (BD Biosciences, Franklin Lakes, NJ), CHOP (Santa Cruz Biotechnology, Santa Cruz, CA), ATF4 (Santa Cruz Biotechnology), Pro-caspases-12 (BD Biosciences), and GAPDH (Novus Biologicals, Littleton, CO). A luminescent reagent (Luminol; Santa Cruz Biotechnology) was used to make specific protein bands visible by incubating the filter and exposing the filter to x-ray film.

**TUNEL Staining**

TUNEL staining was conducted with an in situ fluorescent cell death detection kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer’s protocol.

**Cell Viability–Death Staining Method**

The rat lenses or cultured cells were stained with a mixture of two probes, calcein and ethidium homodimer-1 (EthD; Viability/Cytotoxicity Assay Kits; Biotium Inc., Hayward, CA), for 30 to 45 minutes according to the company’s protocol. Staining was observed by fluorescence microscopy with either a red (EthD; for cell death) or green (Calcein; for live cells) filter.

**ROS Assay**

Immediately after rats were euthanatized, the lenses were dissected. The lenses or cultured LECs were washed with PBS twice. Cytosolic ROS levels were assessed by adding 1 μg/mL of 2′,7′-dichlorodihydrofluorescein diacetate (H$_2$DCFH-DA; Invitrogen, Carlsbad, CA) in PBS for 30 minutes at room temperature, washed twice with PBS, and observed by fluorescence microscopic imaging (Eclipse TE2000-U; Nikon, Tokyo, Japan).

**Free GSH Assay**

Free GSH was determined with a quantification kit (Dojindo Molecular Technologies Inc., Kumamoto, Japan) according to the manufacturer’s instructions. Briefly, dissected lenses were lysed by the addition of 80 μL of 10 mM HCl and by freezing and thawing two times. To the homogenate was added 20 μL of 5% salicylsalicylic acid (SSA), and the mixture was centrifuged at 8000g for 10 minutes. GSH levels in the supernatant were determined according to the manufacturer’s protocol by measuring absorbance at 405 nm with a microtiter plate ELISA reader.

**Histology**

Eyes from 7-, 9-, and 15-day galactose-fed rats were fixed in 2% paraformaldehyde and 1% glutaraldehyde overnight. The eyes were embedded in paraffin, sectioned (10 μM), and stained with hematoxylin and eosin (Histology Core Facility at the University of Nebraska Medical Center [UNMC]).

**RESULTS**

**Galactosemic Rat Lenses**

Young (65–100 g) Sprague-Dawley rats fed standard diet containing 50% galactose showed rapid development of cortical opacities, which appeared by days 7 to 10 and mature cataracts with nuclear involvement between 13 and 15 days. Lenses were examined by histology after 7, 9, and 15 days of galactose feeding. Highly vacuolated lens fiber cells were observed after 7 to 9 days of galactose feeding, and some of the LECs appeared differentiating from the capsule (Figs. 1B–E). Although some of the differentiating lens fiber cells appeared relatively intact after 7 days (Fig. 1C), they became liquefied by 9 days of a galactose diet (Figs. 1D, 1E). After 15 days, vacuoles appeared to be
observed among the animals. The results represent the average development of cataract, cell death, and vacuole formation was capsule (Fig. 1F). In this study a 2- to 3-day variation in the

longer stained with EthD (Fig. 2I). The remaining live cells no longer stained with EthD (Fig. 2H). Most dead cells no longer stained with EthD (Fig. 2I). The remaining live cells appeared to proliferate and covered most of the anterior portion of the lens, as previously reported.56

Figure 1. Histology of galactose-fed rat lenses. The lenses from rats fed either control or galactose diet for 7, 9, or 15 days were sectioned and stained with hematoxylin and eosin. (A) The central region; (B, C) the cortical and equatorial regions of the lens, respectively, from a 7-day galactose-fed rat; (D, E) the cortical and equatorial regions of the lens, respectively. (F) The central region of lens from a 15-day galactose-fed rat. Note the increased degeneration of the epithelium with galactose feeding. Intensity and color tone were autoadjusted by computer.

reduced, with significant numbers of LECs present under the capsule (Fig. 1F). In this study a 2- to 3-day variation in the development of cataract, cell death, and vacuole formation was observed among the animals. The results represent the average of four to six lenses.

LEC death in these lenses was evaluated with a commercially available cell-viability assay kit containing a mixture of EthD and calcine. Although most of the cells at day 5 were alive, death-indicative red EthD staining was observed among the central LECs (Fig. 2B). By days 7 to 9, the lenses appeared hydrated, with some LECs separated from the lens capsule and floating between the capsule and lens fiber cells. By day 7, a significant numbers of LECs in the equatorial region had become calcine positive (Figs. 2C, 2D), and increased clusters of dead cells were present in the central anterior epithelial regions of the lenses (Fig. 2G). However, clusters of live, green-stained cells covering the center of the lens epithelial layers axis were still present between days 7 and 9 (Figs. 2E, 2F). By day 15, the majority of LECs in the entire lens were dead, with only scattered live cells observed (Fig. 2H). Most dead cells no longer stained with EthD (Fig. 2I). The remaining live cells appeared to proliferate and covered most of the anterior portion of the lens, as previously reported.56

UPR-Generated ROS and UPR-Specific Proteins in Galactosemic Rat LECs

Evidence suggests that the UPR generates ROS.32-34 To confirm this observation, ROS generation in the lenses was evaluated (Fig. 3). Cytosolic ROS levels were assessed by loading the lens cells with DCF, followed by fluorescence imaging. Overall, DCF intensity increased with the progression of galactose feeding. In the lenses from the galactose-fed rats, the intensity significantly increased between days 5 and 9 (Fig. 3A). This increase in intensity paralleled the increase in LEC death. These results support previous observations.12,13,19

Free GSH levels were measured in the lenses of galactose-fed rats because ROS oxidizes free GSH and levels of GSH are therefore a marker of ROS production. Compared with GSH levels in the normal control rats, GSH levels were reduced nearly 60% in the lenses of galactose-fed rats after 7 days of galactose diet and the GSH levels remained reduced after 15 days of galactose feeding (Fig. 3B). These results support the premise that the UPR generates ROS in LECs (Fig. 4).5

To confirm that the UPR was initiated in the LECs, after 9 and 15 days LECs were evaluated for the presence of UPR-specific proteins by protein blot analysis. Total soluble LEC proteins from eight lenses were prepared. Protein analysis by SDS-PAGE indicated that significant levels of crystallins were present on day 9 (data not shown) but not on day 15 in the LECs from galactose-fed rats. (Fig. 4A). This suggests possible lens fiber cell contamination in the 9-day LEC preparation. Because isolating capsule-attached LECs without fiber cell contamination is difficult, protein blot analyses were normalized with GAPDH as the internal protein marker. In the protein blot analyses from the 15-day preparation shown in Figure 4B, Bip/GRP78 was significantly upregulated in LECs from the galactose-fed animals. Similarly, CHOP; a central death pathway protein, ATF4; and LEDGF were significantly elevated. Upregulation of LEDGF has also been observed when LECs are treated with other ER stressors5 but not in normal LECs. In contrast, procaspase-12 levels decreases, because activation of the UPR cleaves procaspase-12 to generate a mature caspase-12. Therefore, protein blot analysis strongly supported the contention that the UPR is activated in LECs from galactose-fed rats after 9 and 15 days of galactose feeding.

Effect of Glucose Deprivation and High Concentrations of Hexoses on the UPR in LEC Culture

To investigate further the effects of glucose deprivation and increased hexose levels on induction of the UPR, in vitro cell culture studies were conducted. Human LECs (5 × 10^4 cells) after 1 day of attachment with standard 10% FBS/DMEM were cultured for 24 and 72 hours in sugar-free 10% FBS/DMEM containing increasing concentrations of glucose (0, 5, 25, 125, or 250 mM). At the conclusion of culture, the number of living cells was estimated with EthD staining (Fig. 5). EthD staining decreased in cells grown for 72 hours in both glucose-deprived medium (0 mM) and medium containing high glucose concentrations (125 and 250 mM), whereas the highest viability was observed with cells grown in medium containing 25 mM of glucose. Staining for cell death (Fig. 5; calcine- and TUNEL-positive staining) was significantly higher in the cells grown in glucose-deprived (0 mM) and high-glucose (250 mM) medium. The TUNEL-positive staining suggests that these cells are apoptotic. These cells also demonstrated higher DCF fluorescence (Fig. 5), suggesting that ROS production was linked to the UPR. Levels of UPR-specific proteins in LECs treated with glucose for 24 hours were evaluated by protein blot analysis. As shown in Figure 6, Bip/GPR78, CHOP, and LEDGF were significantly
elevated, whereas procaspase-3 was decreased in the cells cultured with either glucose-deprived medium (0 mM) or high-glucose (125 or 250 mM) media, but not with similar cells cultured in media containing 10 or 25 mM of glucose. Equal amounts of proteins were applied to each gel, as indicated by the internal protein marker, GAPDH. These results indicate that UPR was induced in cells cultured for 24 hours in either glucose-deprived or high-glucose (125 mM) media.

To determine whether the UPR can be preferentially induced by different hexoses, similarly precultured LECs were cultured for 24 hours in medium containing 100 mM of mannose or 25 mM of glucose; 125 mM of galactose; 125 mM of glucose alone; or insulin (2.0 pM) 125 mM of glucose. Cell death increased in all media containing a total of 125 mM of hexose compared with control media (Fig. 7A). Protein blot analysis indicated that Bip/GPR78 levels were significantly increased in all hexose-cultured LECs compared with those cultured in control medium (Fig. 7B). These results indicate that the UPR can be induced by higher levels of glucose, galactose, or mannose.

**FIGURE 2.** LEC viability in galactosemic rat lenses. Lenses from galactose-fed rats were isolated, stained with a cytotoxicity assay kit and visualized under a fluorescent microscope. Calcein (green) and ethidium homodimer-1 (EthD, red) indicate live and dead cells, respectively. (A) The staining of a normal control lens. By the fifth day of galactose feeding no cell death was apparent in the anterior LECs. Since these animals are young, mitotic LECs is still scattered in the central LECs, which appear the first to die (B). By day 7, massive cell death was observed in LECs covering the equatorial region of the lenses (perhaps the mitotic zone of LECs) (C, D). By days 7 and 9, live LECs covering the central lens remained (E, F). By the 15th day of galactose feeding, most LECs were killed, the scattered live LECs appeared to be rapidly dividing and increasing in number (H). The average changes between four to six lenses are presented. Onset of cell death varied between 2 and 3 days.

**FIGURE 3.** Lenses from the galactose-fed rats were increasingly oxidized (A), whereas free GSH levels were reduced (B). (A) Fluorescent staining of lenses from 5-, 7-, and 9-day galactose-fed rats stained with DCF for 30 to 45 minutes to visualize the presence of ROS. (B) The lenticular levels of GSH in the lenses after 0, 7, and 15 days of galactose feeding (mean ± SD).

**FIGURE 4.** Activation of UPR-specific proteins. Lenses from galactose-fed rats were isolated after 9 and 15 days and the LECs attached to the lens capsule were removed. (A) The Coomassie staining patterns from the rats fed a diet with or without 50% galactose for 15 days. (B) The corresponding protein blot analysis with antibodies against Bip/GPR78, CHOP, LEDGF, ATF4, procaspase-12, and GAPDH with a luminescent reagent. Staining for the UPR-specific proteins was apparently much higher from the lenses of rats fed galactose diet compared with control diet. Similar results were obtained with LECs from 9-day fed rats.
Figure 5. Staining of human LECs cultured for 72 hours with medium containing 0, 5, 25, 125, and 250 mM of glucose. Calcein is a live cell marker and EthD is a cell death marker. The cells treated with 0, 5, 125, and 250 mM of glucose exhibited increased cell death with few remaining live cells. The number of live cells dramatically decreased in glucose deficient medium and medium containing glucose levels more than 25 mM. Similarly, staining for cell death increased when cells were cultured in the glucose deficient and high glucose medium. Similar cultured cells stained with TUNEL and DCF after 24 hours showed increased apoptosis and ROS in cells cultured with 250 mM of glucose. A slightly higher number of apoptotic cells were observed in cells cultured in the glucose-deprived medium.

Figure 6. Protein blot analysis showing changes in Bip/GRP78, CHOP, LEDGF, and pro-caspase-3 in LECs cultured for 24 hours in 10% FCS/DMEM containing no glucose or 5, 25, 125, and 250 mM of glucose. Cells were harvested and 10 µg of protein were separated by SDS-PAGE, blotted on a nitrocellulose filter, and quantified by protein blot analysis with antibodies to Bip/GRP78, CHOP, LEDGF and pro-caspase-3, a luminescent reagent. In each experiment, GAPDH was used as an internal marker and the intensity of each band was normalized by the intensity of GAPDH.

Figure 7. Cell viability and expression of Bip/GRP78 in LECs. Cells were cultured for 24 hours in glucose-free 10% FCS/DMEM containing with 100 mM of mannose and 25 mM of glucose, 125 mM of glucose, and insulin (2.0 pM), or 125 mM of glucose. (A) Cell viability while (B) is a protein blot illustrating that the levels of GRP78/Bip in LECs cultured with different hexoses are all similarly increased compared to cells grown in glucose-deficient medium. The protein blots represent results obtained from three independent experiments.
Effect of Osmotic and Oxidative Stress on the UPR

Because the UPR is induced in the lenses of galactose-fed rats, attempts were made to identify the type of stress (nonenzymatic glycation, oxidative, and osmotic) associated with UPR induction in the cultured cells. LECs, precultured for 1 day, were cultured for 24 hours in 10% FBS/DMEM containing 125 or 250 mM mannitol or sodium chloride. Cell death resulted in 20% to 40% of the cells cultured with the 125 or 250 mM of mannitol or 125 mM of NaCl, and complete cell loss was observed with 250 mM NaCl. Protein blot analysis was used to measure the levels of Bip/GRP78, a central UPR marker and AR, a marker for osmotic stress. Both the levels of Bip/GRP78, and AR increased (Fig. 8A), suggesting that UPR and AR induction was the result of response to extracellular osmotic stress in LECs in culture. In addition, AR levels in the LECs of galactose-fed rats and LECs cultured with 1, 25, 50, 150, or 250 mM of glucose were investigated. As summarized in Figures 8B and 8C, AR levels were apparently elevated in the galactose-fed LECs and LECs treated with 125 or 250 mM of glucose but not in LECs cultured with 5 or 25 mM of glucose or in glucose-deprived medium (0 mM). The lack of AR elevation in the LECs cultured with glucose-deprived medium suggests that AR levels are upregulated by osmotic stress but not by the UPR.

To determine whether the UPR can be induced by oxidative stress, human LECs were cultured for 24 hours in medium containing 0, 10, 30, or 50 μM H2O2. Cell death increased with increasing levels of H2O2, with 20% to 30% cell death observed with 50 μM of H2O2. As illustrated in Figure 9, time- and concentration-dependent increases in Bip/GRP78 levels were observed as a result of peroxide exposure to 30 or 50 μM of H2O2. These results suggest that oxidative stress from H2O2 (30–50 μM) also induces the UPR in human LECs in culture.

Exposing LECs to higher sugar concentrations should increase protein glycation that can initiate ER stress and subsequently the induction of the UPR. To determine whether glycation occurs, the human LECs were precultured for 24 hours in 10% FBS/DMEM without phenol red and then cultured an additional 24 hours in medium with or without 50, 100, 200, or 300 μM MGO. Although cell death increased in medium containing 300 μM of MGO, no significant change in Bip/GRP78 levels was detected (Fig. 10A). These results indicate that UPR was not induced by incubation with up to 300 μM of the potent glycating agent MGO for 24 hours.

UPR in LECs from ARI-Treated Galactose-Fed Rats

Because ARIs are known to suppress cataract formation in the galactose-fed rats,11–20 the ability of ARI to suppress the induc-

![Figure 8](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933235/)  
**Figure 8.** Protein blot analysis of AR in LECs. (A) Both AR and Bip/GRP78 protein levels were increased in LECs cultured for 24 hours in the 10% FCS/DMEM with increasing osmolarity from the addition of glucose that ranges from 0 mM (control) to 125 and 250 mM. LECs from galactosemic rat lenses and control LECs. (B) AR levels increased in the lenses from 15-day-old galactose-fed rats. (C) AR levels increased with 24-hour culture with increasing concentrations of glucose in the medium. GAPDH was used as an internal marker and the intensity of each band was normalized by the intensity of GAPDH. The protein blots represent results obtained from three independent experiments.

![Figure 9](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933235/)  
**Figure 9.** Protein blot analysis of LECs cultured with different concentrations of H2O2. (A) The protein blot of GRP78/Bip in LECs cultured for 24 hours in 10% FBS/DMEM containing 0, 10, 30, and 50 μM of H2O2. (B) The protein blots illustrating activation of GRP78/Bip in LECs within 24 hours. In all protein blots, equal amounts of protein were loaded in each well. The expression of GAPDH was used as an internal control. The protein blots represent results obtained from three independent experiments.

![Figure 10](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933235/)  
**Figure 10.** Protein blot analysis of cultured LECs treated with MGO and LECs isolated from the lenses of rats fed normal control diet or diet containing 50% galactose with/without 0.0125% of the ARI, AL1576. (A) Protein blot of Bip/GRP78 from LECs cultured with increasing concentrations of H2O2. (B) Protein blot of Bip/GRP78 illustrating that AR levels and CHOP levels are increased only in the lenses from untreated galactose-fed rats. In all protein blots, equal amounts of protein were loaded on each well with the expression of GAPDH used as an internal control. These protein blots are representatives of results obtained from three independent experiments.
tion of UPR in galactose-fed rats was investigated. Groups of four rats fed a normal control diet or diet containing 50% galactose with/without 0.0125% of the ARI AL1576 for 15 days were compared by evaluating the capsule-attached LECs for UPR-specific proteins, by protein blot analysis. In contrast to the untreated galactose-fed rats, addition of ARI to the galactose diet prevented the upregulation of either Bip/GRP78 or CHOP, the two major UPR marker proteins in the LECs (Fig. 10B). The lenses remained clear with no apparent signs of hydration or increased staining for cell death (photographs are not shown). The results suggest that the UPR activation is inhibited in galactose-fed rats by ARI treatment.

**DISCUSSION**

The present studies provide critical evidence of the role of the UPR in LEC apoptosis and cataractogenesis in galactose-fed rats. The UPR pathway produces ROS and induced apoptosis in the LECs of the galactose-fed rat lenses. This suggests that the UPR can play an important role in LEC damage during the development of diabetic cataracts. The present studies show that in cultured LECs the UPR is induced by either glucose deprivation or high glucose concentrations. Intracellular osmotic stress and oxidative stress both can induce the UPR in LECs. Suppression of intracellular osmotic stress by treatment with ARI also suppresses the induction of the UPR in galactose-fed rat lenses, suggesting that the induction of UPR is linked to intracellular osmotic stress. Although glycation is well known to induce UPR-mediated cell death, no activation of the UPR was observed by culturing the LECs with the potent glycat ing agent MGO.

**Cell Death in the LECs of Galactose-Fed Rats**

The UPR induces the production of ROS and apoptosis in LECs of galactose-fed rats between 5 and 15 days. Because both high levels of sugars and glucose deprivation were observed to induce the UPR, one can speculate that wide fluctuations of hexose levels in diabetic patients, especially those with blood glucose under tight control where the incidence of hyperglycemia is often increased, may also induce the UPR, perhaps resulting in the increased production of ROS and cell death and contributing to cataract formation. LEC death has been observed during cataractogenesis and is suggested to be a cause of cataract formation.27,28 Our results showed that with galactose toxicity, mitotic LECs in the central and peripheral mitotic zone die first, followed by the central, nonmitotic LECs, and eventually the remaining LECs.27 The scattered, remaining LECs may rapidly proliferate to cover the entire anterior region of the lens.26 A small number of LECs appeared to migrate into the posterior lens regions, proliferating and remaining there.26 Although LECs have been reported to appear normal in galactose-fed rats after 20 to 30 days of galactose feeding26–28 most of these LECs appeared to have been killed and replaced by newly proliferating LECs.

The present results on cell death are consistent with previous reports that strong protein-synthesis inhibition occurs along with significant changes in mRNA populations, altered lens fiber differentiation, and the induction of cell death in lenses of 11-day galactose-fed rats and of fiber differentiation.28–30 In addition, free GSH and ATP levels along with amino acid transport are significantly reduced during sugar cataract development.40 In these lenses, the UPR activates m-calpain and caspases that cleave various crystallins and induce apoptosis.41 Because galactosemic cataracts in their initial stage are reversible by withdrawal of the galactose diet,42 the newly proliferated LECs must retain their functional capacity to maintain lens clarity. However, massive cell death, the loss of organelles, significant increases in ROS, and loss of GSH all may influence the cataractogenic process.

**Induction of the UPR and Production of ROS in Transformed LECs by Glucose Deprivation.** Glucose deprivation is an active death process because the transcription and translation of UPR-specific proteins are activated in LECs during glucose deprivation. Glucose deprivation is well known to trigger the UPR in several cell types by disrupting protein unfolding in the ER.42,43 In addition, ischemia is well known to induce the UPR.43–45 We have previously reported that glucose deprivation activates the expression of UPR-specific proteins in cultured LECs46 and retinal pericytes.47 Two mechanisms have been proposed for the observed induction of UPR by glucose deprivation. From studies in Xenopus oocytes, Nutt et al.,48 proposed that glucose deprivation reduces levels of NADPH, which depletes the key glucose metabolism intermediate glucose-6-phosphate (G-6-P). This causes the loss of an inhibitory phosphorylation of caspase-12 by Ca2+ dependent kinase II (CaMKII) that activates caspase-12 and induces apoptosis. Alternatively, glucose deprivation by depleting glucose from cells inhibits the glycosylation of membrane or secretory proteins in the ER. These nonglycosylated proteins, in turn, are recognized by Bip/GRP78 and induce ER stress and UPR-dependent apoptosis. This concept is supported by the observations that tunicamycin, an inhibitor of glycosylation strongly induces the UPR49 and that the hypoglycosylation in Walker-Warburg syndrome (WWS) induces cataract formation.44 The Diabetes Control and Complications Trial reported that tight control of hyperglycemia increases the risk of hypoglycemia.1–4 Our studies indicate that hypoglycemia is as serious as hyperglycemia in inducing the UPR and LEC death. Therefore, both hypoglycemia and hyperglycemia can contribute to diabetic cataract formation.

**Injection of UPR in LECs by Osmotic and Oxidative Stress.** In galactose-fed animals, osmotic and oxidative but not glycative stress induce the UPR. Plasma galactose concentrations in young rats fed 50% galactose diet reach levels of approximately 20 mM (350 mg/dL) sufficient to induce the UPR within 7 to 15 days and cataracts within 15 to 15 days.45 These cataracts are osmotic. AR catalyzes the reduction of galactose to galactitol and the intracellular accumulation of galactitol in LECs and increases intracellular osmolarity in rat lenses.11,26,46 By blocking the accumulation of galactitol, ARIs prevent increased intracellular osmolarity, the induction of the UPR, and cataract. This suggests that the induction of UPR is linked to polyol-associated osmotic changes. Because ARIs do not inhibit glycation, the higher galactose levels in the LECs should increase glyco-oxidation. Because the UPR is not induced, we can conclude that glyco-oxidation is not a major initiator of the UPR.

In contrast, LECs appeared to be less sensitive to extracellular osmolarity. In the transformed human LECs, 125 to 250 mM of hexose was necessary to induce the UPR within 24 hours of culture. These nonphysiological concentrations in the LECs induced hyperosmotic conditions that clearly show that the UPR is significantly induced.

Oxidative stress has been recognized as one of the major factors in cataract formation.27,47 In the present studies, galactose feeding resulted in the increased lenticular detection of oxidative stress. Increased oxidative stress such as that produced by 30 μM of H2O2, induced the UPR and cataract formation. In whole lens culture, 0.5 to 20 μM H2O2 does not appear to affect the lens adversely.27 However, lens cell culture appears to be more sensitive, with 15 μM of H2O2 inducing 40% apoptotic cell death.48 UPR proteins are regulated by cellular oxygen tension and it has been documented that hypoxia induces the UPR.49–51 It has recently been reported that the UPR induced by H2O2 generates different UPR pathways
than those induced by TNF-\(\alpha\) or tunicamycin in the murine fibrosarcoma cell line L929.\textsuperscript{50} They suggest that the UPR induced by \(\text{H}_2\text{O}_2\) is also a different pathway from that induced by arsenite.\textsuperscript{50} Although specific detailing of the UPR pathways induced by \(\text{H}_2\text{O}_2\) is beyond the scope of this study, the results the oxidative stress induced by galactosemia clearly plays an important role in activation of the UPR.

Although glycation may not induce the UPR in LECs, exposure of LECs to 300 \(\mu\text{M}\) of the potent glycating agent MGO caused cell death. Whereas plasma MGO levels in diabetic patients are approximately 8 \(\mu\text{M}\),\textsuperscript{51} intercellular MGO concentrations of 510 \(\mu\text{M}\) have been reported in Chinese hamster ovary cells.\textsuperscript{52} In the present studies, we used approximately 200 to 300 \(\mu\text{M}\) of MGO to induce LEC death. In the cultured lens cells, MGO may modify all types of proteins, which can subsequently result in the generation of dysfunctional multiple proteins, which may be sufficient to induce cell death.

**LEC Production of ROS under ER Stress**

Oxidative damage is the most common factor associated with cataract formation. Increased intracellular glucose metabolism can lead to increased ROS production through the mitochondrial electron transport chain.\textsuperscript{35} The UPR is known to generate ROS,\textsuperscript{32–34,53} which decreases free GSH and loss of this free GSH further damages the mitochondrial function to produce further ROS (\(O_2^-\)).\textsuperscript{53} Formation of disulfide bonds in the ER generates two electrons, which pass to molecular oxygen to generate ROS.\textsuperscript{32–34,53} Harding et al.\textsuperscript{51} have speculated that approximately 25% of cellular ROS is produced during protein synthesis. Depletion of GSH by the UPR subsequently induces mitochondrial dysfunction and consequential ROS accumulation.\textsuperscript{34,55} Thus, ROS from both the ER and mitochondria can contribute to the production of ROS under the UPR. Bioactive factors such as platelet-derived growth factor (PDGF),\textsuperscript{56} epithelial growth factor (EGF), b-FGF, and TGF-B\textsuperscript{57} have recently been reported to increase the production of ROS in cultured LECs. Studies with hyperbaric oxygen suggest that molecular oxygen contributes to cataract formation in patients\textsuperscript{58} and animals.\textsuperscript{59} In addition, peroxisomes, lipooxygenases, NADPH oxidase, and cytochrome \(P_{450}\) are all potential sources of ROS.\textsuperscript{60} Because the lens is avascular, the produced ROS remains localized within the lens rather than being removed through blood circulation. The generated ROS may further decrease the remaining GSH in the LECs or it may diffuse from LECs to the cortical lens fiber cells where it contributes to the formation of crystallin aggregates and large light-scattering particles.

Galactosemic and diabetic cataracts progress through similar stages with similar biochemical changes and changes in structural integrity. This suggests a common underlying mechanism for their formation.\textsuperscript{25} The present study demonstrates that the UPR activation is another common mechanism of cell death that is observed in both types of cataract. This cell death is first observed in the mitotic LECs in the central and peripheral mitotic zone, followed by the central, nonmitotic LECs, and eventually the remaining LECs.

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**References**


