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Age-Related Cataracts: Role of unfolded protein response, Ca^{2+} mobilization, epigenetic DNA modifications, and loss of Nrf2/Keap1 dependent cytoprotection

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Abstract

Age-related cataracts are closely associated with lens chronological aging, oxidation, calcium imbalance, hydration and crystallin modifications. Accumulating evidence indicates that misfolded proteins are generated in the endoplasmic reticulum (ER) by most cataractogenic stresses. To eliminate misfolded proteins from cells before they can induce senescence, the cells activate a clean-up machinery called the ER stress/unfolded protein response (UPR). The UPR also activates the nuclear factor-erythroid-2-related factor 2 (Nrf2), a central transcriptional factor for cytoprotection against stress. Nrf2 activates nearly 600 cytoprotective target genes. However, if ER stress reaches critically high levels, the UPR activates destructive outputs to trigger programmed cell death. The UPR activates mobilization of ER- Ca^{2+} to the cytoplasm and results in activation of Ca^{2+} -dependent proteases to cleave various enzymes and proteins which cause the loss of normal lens function. The UPR also enhances the overproduction of reactive oxygen species (ROS), which damage lens constituents and induce failure of the Nrf2 dependent cytoprotection. Kelch-like ECH-associated protein 1 (Keap1) is an oxygen sensor protein and regulates the levels of Nrf2 by the proteasomal degradation. A significant loss of DNA methylation in diabetic cataracts was found in the *Keap1* promoter, which overexpresses the Keap1 protein. Overexpressed Keap1 significantly decreases the levels of Nrf2. Lower levels of Nrf2 induces loss of the redox balance toward to oxidative stress thereby leading to failure of lens cytoprotection. Here, this review summarizes the overall view of ER stress, increases in Ca^{2+} levels, protein cleavage, and loss of the well-established stress protection in somatic lens cells.

Keywords

ER stress; Unfolded protein response; Nrf2; Keap1; DNA methylation; Age-related cataracts

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1. Introduction

Protein misfolding and aggregation is considered as a key factor for various conformational diseases including cataract, a leading cause of blindness worldwide. Age-related cataracts (ARCs) have been characterized as conformational diseases as posttranslational modifications of lens proteins cause their conformational changes that lead to destabilization and eventual aggregation (Crabbe, 1998; Harding, 2002). It is well-documented that almost all cataract lenses have similar characters such as oxidation of lens constituents, loss of antioxidants, increased levels of cytosolic Ca^{2+} , activation of proteolysis, imbalances of ions, hydration and crystallin degradation and aggregation. The risk of ARCs significantly increases with age as many stressors play critical roles in the cataract formation.

Importantly, it has also been reported that the involvement of endoplasmic reticulum (ER) stress-mediated unfolded protein response (UPR) in human lens epithelial cells in the context of ARCs formation (Elanchezhian et al., 2012a; Elanchezhian et al., 2012b; Ikesugi et al., 2006a; Ikesugi et al., 2006b; Mulhern, 2012; Mulhern et al., 2006; Mulhern et al., 2007; Shinohara et al., 2006).

This review mainly discusses, 1) how environmental stressors induce misfolded protein conformation via ER stress; 2) the general UPR pathways; 3) how UPR drives Ca^{2+} release from ER to initiate a sequence of measures that culminates in the loss of various membrane-associated enzymes such as sarco/endoplasmic reticulum Ca^{2+} -ATPases (SERCA) and plasma membrane Ca^{2+} -ATPases (PMCA) and other proteins including transcriptional factors; 4) the link between reactive oxygen species (ROS) and ER stress 5) the importance of “Nrf2 dependent stress protection mechanism in the lens”, a major stress protection pathway during UPR; 6) the role of *Keap1* promoter DNA demethylation in the suppression of Nrf2 dependent stress protection; 7) the fact that ER stress is also induced in immature lens fiber cells. These discussions further define how different cataract types can be determined in human age-related cataracts.

2. Cataractogenic stress and the ER stress/UPR

Cells are well equipped to protect themselves against various stresses. Environmental stresses and mutant proteins are known to induce cataracts. Among the numerous environmental stresses, certain ones appear to induce misfolded protein conformation. Membrane, luminal or secretory proteins are synthesized in the rough ER and transported into the highly oxidized ER lumen. Stress appears to modify these proteins in the ER to generate misfolded proteins. Misfolded protein conformation in the ER initiates processes of cataract formation and full blown; chronic UPR further amplifies crystallin and protein degradation, modification, and aggregation in the downstream cascade.

2.1. Environmental stress

Cataracts can result from metabolic, nutritional or environmental insults, or they may be secondary to other ocular or systemic diseases such as retinal degenerative diseases, uveitis, glaucoma or diabetes (Harding, 1991). By far the most crucial risk factor is age; the prevalence of ARCs escalates from 4% at ages 52–64 to 50% at ages 75–85. The prevalence is expected to nearly double by the year 2020 due to an increased aging population. ARCs

constitute a vast majority of all cataracts and represent a major public health problem worldwide.

Most ARCs are thought to be caused by environmental stresses in combination with ecliptic genetic inheritance. Considering the fact that severe bilateral cataracts can be found in 40-year-old individuals, but a 90-year-old can have clear lenses, pathogenic processes, not age, are responsible for the formation of ARCs. Significant increases in the incidence of ARCs with age suggest cumulative processes or age-associated processes might play a vital role in ARCs. In the past several decades, large numbers of cataractogenic stresses have been characterized. Reviews of various aspects of ARCs have been published with lists of some of them in the last several years; posttranslational modification (Zhang and Lu, 2011), oxidation (Zoric et al., 2010), a model of normal and pathological aging (Michael and Bron, 2011), cataract-map (Beebe et al., 2010), redox regulation in the lens (Lou, 2003), oxidative damage (Bassnett and McNulty, 2003; Baynes and Thorpe, 1999; Brennan and Kantorow, 2009; Lou, 2003; Truscott and Augusteyn, 1977), alpha-crystallin on lens cell function (Andley, 2007, 2009), nutritional antioxidant (McGinty and Truscott, 2006), and presbyopia (Chiu and Taylor, 2007). A literature search indicates that most of the cataractogenic stresses in Table 1 induce ER stress in various tissues.

Among the many environmental stressors, very few are known to induce cataractogenic stress, but all environmental stressor-driven cataractogenic stress can cause ER stress that culminates in calcium imbalance, protein degradation, oxidative insults, perturbations of redox status, and loss of antioxidant defense mechanisms. Interestingly, the majority of cataractogenic stressors generate an accumulation of misfolded protein conformations in the ER, which is an essential requirement for activation of ER stress. Next, this review describes the literature related to the ER stressor that generates misfolded protein conformation in the ER lumen.

2.2. Cataractogenic stressor induces misfolded protein conformation

Here, the review describes how these stressors generate misfolded protein conformation in the lens epithelial cells thereby leading to cataract formation. Secretory, luminal and membrane proteins are primarily synthesized in the rough ER; ER lumen has developed a chemical environment which is more highly oxidized than the cytoplasm. The ER is equipped with a variety of molecular chaperones such as Ig binding protein (BiP), heat shock proteins and folding enzymes that assist the folding process, while at the same time exerting tight quality control measures. One of the common post-translational modifications of ER-synthesized proteins is disulfide bridge formation, catalyzed by the family of protein disulfide isomerases. This covalent modification provides unique structural advantages. However, the benefits that disulfide bonds impart to these proteins come at a high cost to the cell. Recent reports have demonstrated the mechanism by which the cell can deal with or even exploit the side reactions of disulfide bond formation to maintain homeostasis of the ER and its folding machinery (Feige and Hendershot, 2011).

The key question in cataractogenic ER stress is “does the cataractogenic stressor alter protein conformation in the ER?” Literature searches show that chronic alcoholics generate acetaldehyde, a highly reactive metabolite of ethanol, that forms covalent acetaldehyde-

protein adducts with several proteins via lysine, cysteine, histidine and tyrosine residues (Stevens et al., 1981). Similarly, malondialdehyde-adducts or methylglyoxal adducts in diabetic patients react with arginine residues in proteins (Suji et al., 2008). Higher levels of urea induce a “direct interaction mechanism” with the protein backbone as well as side chains whereby urea has a stronger dispersion interaction with that protein than water thereby changing the protein conformation (Hua et al., 2008). Either extreme high or low-temperature changes protein conformation in mammalian cells (Caldwell, 1989). Dehydration or osmolality changes also affect protein conformation, and for several proteins, these conformational changes are at least partially irreversible (Prestrelski et al., 1993). Excess levels of molecular oxygen or other oxidative insults induce conformational changes by cysteine oxidation (Murray and Van Eyk, 2012). Similarly, ionizing radiation also changes protein conformation (Chapelier et al., 2001). The receptors of steroid hormones bind to ligands to induce significant conformational changes in the nucleus of the cell, which is central to steroid receptor activation (Allan et al., 1992). Reports have shown that steroid hormone induces ER stress in islet cells (Yusta et al., 2006; Yusta and Drucker, 2004) and prostate cancer cells (Yemelyanov et al., 2012). These results suggest that certain newly synthesized steroid receptors in the ER lumen bind excess levels of steroid hormone, activating ER stress and leading to cataract by small vacuole formation in the nucleus of the embryonic chick lens (Nishigori, 2006). Some drugs such as valproic acid (Cheema et al., 2007; Mandula et al., 2006), 5-Aza-2'-deoxycytidine (5-Aza) (Christman, 2002), thapsigargin (Davidson and Varhol, 1995), phenothiazine and tunicamycin (Lane et al., 1987), naphthoquinone, a toxic metabolite of naphthalene (Jali et al., 2014), bind to the proteins and disturb protein conformation. The well-known ER stressors, calcimycin (ionophore A23187) also induces a Ca^{2+} -dependent conformational change in SERCA (Champeil et al., 1986; Ohmiya and Kanazawa, 1991). Photosensitized paraquat has also reported to inducing protein structural alterations and free radical-mediated fragmentation of serum albumin (Jaiswal et al., 2002). Thapsigargin is a high-affinity inhibitor of SERCA and binds to SERCA and changes conformation as well as inhibits SERCA activity (Davidson and Varhol, 1995). Tunicamycin is an inhibitor of N-glycosylation thereby inhibiting the proper protein folding and induces the so-called UPR (Elbein, 1987). The deficiency of vitamins and amino acids are relatively slow to alter the protein conformation since they have some pooled materials in the cells. Tryptophan deficiency induces deficiency mutants and alters protein conformation (Kolvenbach et al., 1993), and vitamin deficiency results in the loss of cofactors of many enzymes and disturbs protein conformation. Homocysteine binds to cysteine by disulfide binding (Gonzalez et al., 2004), whereas sodium-selenite binds to cysteine by disulfide-selenite formation (Protein-S-Se-S-Protein). Soluble forms of mercury, copper, and lead also bind multiple cysteine-containing proteins such as papain (Guo et al., 2011), and change protein conformation. Environmental toxins such as paraquat (Dhakshinamoorthy and Jaiswal, 2002) and bisphenol A (Hiroi et al., 2006) also bind to proteins and interferes with functional roles as well as altering protein conformation. Thus, higher levels of cataractogenic stressors in the ER bind to newly synthesized membrane, luminal and secretory proteins. These bindings disturb protein conformation in the highly oxidized ER lumen, resulting in activation of ER stress. This is an important concept to elucidate cataractogenic stressors. It has been speculated that most of the stress damage in the lens may be recovered throughout life since most stresses are low enough to generate the

protective ER stress. Those lower levels of stress may be beneficial to enhance stress protection, which is called hormesis (Calabrese and Baldwin, 2003). However, continued activation of the ER stress indicates the inability to re-establish homeostasis. Chronic ER stress can be generated by either prolonged time with weak levels of stresses or severe stresses in short time, but the both ways provide similar cellular damage (Papa, 2012). Lens-specific cataract formation might be induced by a predominant interaction between lens specific protein(s) and the ER stressor(s). It has been demonstrated that human lens epithelial cells exposed to a certain oxygen environment activated a protective UPR while chronic hypoxic conditions induced ROS production and apoptotic UPR resulting in lens epithelial cell death and lens oxidation (Elanchezhian et al., 2012b; Zheng et al., 2015). Thus, cataract is one of the protein misfolding diseases, and misfolded protein conformation in the ER initiates pathogenic processes and results in misfolded crystallin aggregations. Such diversified cataractogenic stressors have a common principle to share a common pathway for cataract formation.

2.3. Mutant protein also induces ER stress/UPR

More than 30% of proteins are synthesized in the rough ER (Ghaemmaghami et al., 2003). Alterations in protein trafficking occur mainly in the ER, the central site for folding, post-translational modifications, and transport of these proteins. An imbalance between a load of misfolded proteins and the folding capacity of the ER leads to ER stress. Also, missense mutations may result in protein misfolding and disruption of protein trafficking. Many transgenic animals having mutant or chimeric genes also develop a cataract in the lens as well as dysfunctions in the other tissues (Firtina et al., 2009; Graw, 1999). Accumulating evidence also suggests that ER stress contributes to the development and progression of cystic fibrosis, α 1-antitrypsin deficiency, retinitis pigmentosa, and Alzheimer disease (Ogen-Shtern et al., 2016). This review focuses only on the relationship between environmental stresses-induced ER stress and cataracts. Gene mutant or inherited cataracts are beyond the scope of this review.

3. Activation of the ER stress/UPR

Cells induce a cascade of reactions, called the UPR, to cope with any perturbation by misfolded protein conformations (Kaufman et al., 2002; Schroder and Kaufman, 2005; Shen et al., 2004; Zhang et al., 2015; Zhang et al., 2005). Generally, the UPR responses are comprised of (1) translational attenuation to prevent further production of misfolded proteins (Banks et al., 1999) including degradation of mRNA (Narasimhan et al., 2012; Singh et al., 2013; Yang et al., 2011); (2) transcriptional induction of ER resident proteins to further assist protein folding (Gething and Sambrook, 1992); (3) induction of ER-associated degradation (ERAD) machinery to reduce the burden on ER folding capacity (Yoshida et al., 2000a); and (4) enlargement of the ER to deal with the massive load of unfolded proteins (Selye, 1985). The successful execution of compensatory responses to stress by these mechanisms restores normal cell function and ensures survival. Alternatively, irreversibly stressed cells would undergo senescence or apoptosis in response to chronic UPR (Haeri and Knox, 2012). There are three intertwined pathways that comprise the UPR (Walter and Ron, 2011). Each pathway is initiated by an integral ER membrane protein such as the protein

kinase R (PKR)-like endoplasmic reticulum kinase (PERK), the ER resident, inositol-requiring kinase 1 (IRE1), and activating transcription factor 6 (ATF6) that serves as a sensor for unfolded proteins in the ER lumen (Fig. 1).

PERK

Essentially all ER stressors that induce the UPR's transcriptional components can also transiently attenuate translation via phosphorylation of the eukaryotic translation initiation factor 2 α (eIF2 α), which is mediated by the PERK signaling pathway (Harding et al., 1999; Shi et al., 1998). PERK, an ER stress sensor, is a type I transmembrane protein kinase receptor which is activated by ER stress with the release of BiP from its luminal domain. The release of BiP from PERK results in its oligomerization with subsequent autophosphorylation of its cytosolic domain (Banks et al., 1999; Yoshida et al., 2000b). P-PERK receptors then reduce the activity of ribosomal initiating factor (eIF2 α) by phosphorylation of its α -subunit, which inhibits 80S ribosome assembly, and consequently attenuates protein synthesis/translation (Banks et al., 1999). In contrast, phosphorylation of eIF2 α promotes the translation of ATF4 upon ER stress (Zhang et al., 2005).

Similarly, P-PERK, (Cullinan and Diehl, 2004; Cullinan et al., 2003) protein kinase C (PKC) (Bloom and Jaiswal, 2003; Chiu et al., 2002), PI3 kinase (Kang et al., 2002) and/or MAP kinase (Zipper and Mulcahy, 2000) phosphorylates the nuclear factor-erythroid-2-related factor 2 (Nrf2) (Fig. 1). Phosphorylation of Nrf2 disrupts its association with the Kelch-like ECH-associated protein 1 (Keap1) and the P-Nrf2 is then translocated to the nucleus where it binds to the antioxidant response element (ARE) in the promoter (Desagher et al., 2005) to activate 600 target genes, including 20 for antioxidant associated enzymes such as glutathione (GSH)-reductase, GSH-S-transferase, thioredoxin, thioredoxin reductase, and many more antioxidant defense genes (Aldana et al., 2003; Datta et al., 2017; Kwak et al., 2003a; Thimmulappa et al., 2002). This system is called as Nrf2 dependent stress protection (Fig. 1). Human lens epithelial cells exposed to hypoxic/hypoglycemic conditions (Elanchezhian et al., 2012b), sodium selenite (Palsamy et al., 2014b), high or low glucose (Ikesugi et al., 2006a; Ikesugi et al., 2006b), homocysteine (Elanchezhian et al., 2012a), tunicamycin (Ikesugi et al., 2006b), thapsigargin, streptozotocin-induced diabetes and methylglyoxal (Palsamy et al., 2014a), and galactose (Mulhern et al., 2006) activate PERK and P-PERK and apparently activate ER stress. Of the three membrane signaling receptors, PERK is most strongly activated by the above ER stressors in human lens epithelial cells.

IRE1

Two isoforms (IRE1 α and IRE1 β) are found in mammalian ER membranes (Tirasophon et al., 1998) (Ding et al., 2011). In unstressed cells, the IRE1 protein kinase is maintained in an inactive monomeric form through association with BiP (Bertolotti et al., 2000). Once liberated from BiP, IRE1 is free to dimerize, *trans*-autophosphorylate and thereby activate its RNase activity (Tirasophon et al., 1998). IRE1 is required for splicing of the X-box transcription factor-1, XBP-1 (Yoshida et al., 2001) (Fig. 1). The spliced form of XBP1 is a potent transcriptional activator and plays a key role in the transcriptional induction of a broad range of UPR target genes, including several ER-resident chaperones (Lee et al.,

2003a; Yoshida et al., 2001). The UPR-dependent transcriptional program in mammalian cells does not rely solely on IRE1 signaling. Human lens epithelial cells exposed to hypoxic/hypoglycemic conditions (Elanchezhian et al., 2012b), high or low glucose (Ikesugi et al., 2006b), galactose (Mulhern et al., 2006), homocysteine (Elanchezhian et al., 2012a), tunicamycin (Ikesugi et al., 2006b), thapsigargin, streptozotocin-induced diabetes and methylglyoxal (Palsamy et al., 2014b), sodium selenite (Palsamy et al., 2014b), and valproic acid (Palsamy et al., 2014c), activated IRE1 suggesting that the ER stress is activated by cataractogenic stressors.

ATF6

Two isoforms (90 kDa ATF6 α and 110 kDa ATF6 β , also known as CREB-RP) are maintained at the ER membrane by their binding to BiP (Haze et al., 1999; Thuerlauf et al., 2007). Upon BiP release, both isoforms transit to the Golgi compartment where they are cleaved by site-specific proteases, S1P and S2P to generate ATF6 (50–60 kDa) protein (Abraham et al., 1999; Haze et al., 1999). The cleaved product then transits to the nucleus and promotes the transcription of many UPR target genes, including BiP and XBP-1 (Li et al., 2000; Okada et al., 2002). The overlap in targets between IRE1 and ATF6-dependent signaling demonstrates the convergence of the two pathways and the ability of the UPR to amplify its signaling potential. Transcriptional activation of ER-resident proteins is mediated by ATF6 and IRE1 receptors (Yoshida et al., 1998). One of the clearest demonstrations of ATF6 activation by ER stressor was observed in human lens epithelial cells treated with 10 μ M sodium selenite (Palsamy et al., 2014b). Also, human lens epithelial cells treated with methylglyoxal upregulated both the ATF4 and the phosphorylation of IRE1 (Palsamy et al., 2014a). Furthermore, the fact that BiP and XBP-1 are also increased in these cells further confirmed the finding that ER stress is induced by methylglyoxal (Palsamy et al., 2014a). Consistent with these observations, human lens epithelial cells treated with homocysteine (Elanchezhian et al., 2012a), non-physiologically high and low glucose (Ikesugi et al., 2006b), galactose (Mulhern et al., 2006), hypoxic/hypoglycemic conditions (Elanchezhian et al., 2012b), valproic acid (Palsamy et al., 2014c), thapsigargin (Palsamy et al., 2014a), tunicamycin (Ikesugi et al., 2006b), sodium selenite (Palsamy et al., 2014b) and calcium ionophore-A23187 (Ikesugi et al., 2006b) are also upregulated the UPR pathway.

4. ER stress/UPR initiates a Ca²⁺ imbalance by releasing ER-Ca²⁺

Cortical cataracts are the main type of cataract that exhibits elevated levels of Ca²⁺ ranging from 0.1 to 64 mM. However, nuclear cataracts possess normal levels of calcium and sodium (Baruch et al., 2001; Duncan and Jacob, 1984; Harding, 1991; Marcantonio et al., 1986). Ca²⁺ levels in human aqueous humor are approximately 1 mM, and there is a large, 10,000-fold-inwardly directed gradient across the plasma membrane. An increased Ca²⁺ level in the ER compartment generates a large Ca²⁺ gradient across the ER membrane. ER stressors such as methylglyoxal are also found to induce an ER-Ca²⁺ release spike for 2–39 seconds (Palsamy et al., 2014a), and valproic acid does the same for 2–183 seconds (Palsamy et al., 2014c) whereas homocysteine-induced ER-Ca²⁺ release and uptake occur several times within a few minutes. Sodium selenite provokes ER-Ca²⁺ release and induces severe chronic UPR (Palsamy et al., 2014b). SERCA, a family of proteins responsible for transporting Ca²⁺

from the cytosol into the lumen of the ER, is a major regulator of ER stress (Park et al., 2010). Repeated treatment with selenite gradually reduces the spontaneous ER- Ca^{2+} release, suggesting either a loss of ATP from the ER or decrease the rate of SERCA pumps. Reports also showed that SERCA was gradually decreased within 12 h and it was further degraded by 24 h in human lens epithelial cells exposed with sodium selenite (Palsamy et al., 2014b). These results suggest that ER stress initiates a spontaneous ER- Ca^{2+} release, and inhibition of SERCA can prevent the refilling of Ca^{2+} to the ER stores causing a measured release of Ca^{2+} into the cytosol through leak channels (Hofer et al., 1996). The basal Ca^{2+} leak occurs via the translocon, a transmembrane aqueous pore located in the ER (Giunti et al., 2007; Ong et al., 2007) that is responsible for the transport of newly synthesized secretory proteins into the ER lumen (Swanton and Bulleid, 2003). Gating of this translocon is found to be facilitated by BiP and Ca^{2+} -Calmodulin (Erdmann et al., 2011; Schauble et al., 2012). A research report by Paredes et al., suggests that Ca^{2+} may be both a cause and a consequence of ER protein misfolding and it appears that ER Ca^{2+} leak is a significant co-factor for the initiation of the UPR (Paredes et al., 2013).

The ER- Ca^{2+} store is entirely depleted by chronic UPR in lens cells, for example by exposure to thapsigargin, a widely used SERCA inhibitor and a well-known ER stressor (Foldi et al., 2013; Treiman et al., 1998). Cells treated with thapsigargin exhibit a marked decrease in protein synthesis. Net leakage of proteins was associated more strongly with an increase in ER- Ca^{2+} than with an increase in sodium (Abraham et al., 1999; Duncan and Jacob, 1984; Duncan and Wormstone, 1999; Shearer et al., 1997). Because ER- Ca^{2+} release under ER stress takes a matter of seconds, it has been speculated that ER- Ca^{2+} release is sufficient to activate ERAD components or m-calpain and induce SERCA degradation for slowing down Ca^{2+} uptake from the cytoplasm to ER (Palsamy et al., 2014a). The loss of SERCA generates higher Ca^{2+} levels in the cytoplasm for prolonged times. ERAD components and m-calpain further damage the PMCA, which might induce Ca^{2+} influx from aqueous humor. Since human lens cells normally possess very low Ca^{2+} permeability, depletion of intracellular stores by thapsigargin initiates a Ca^{2+} entry pathway. As an outcome, this phenomenon potentially provides a signal transduction mechanism with minimal risk of Ca^{2+} overload to the lens, whereas over-activation of the store-operated channel is a possible way of increasing Ca^{2+} in the lens (Park et al., 2010). Similar to this, studies have also reported that methylglyoxal (Palsamy et al., 2014a), homocysteine (Elanchezhian et al., 2012b), valproic acid (Palsamy et al., 2014c), sodium selenite (Palsamy et al., 2014b), and tunicamycin, calcium ionophore-A23187 (Ikesugi et al., 2006b) induce ER- Ca^{2+} verload and increasing ER- Ca^{2+} levels in the lens cytoplasm. Higher levels of Ca^{2+} activate ERAD components and m-calpain and several proteases including caspases, resulting in degradation of many important enzymes including SERCA (Palsamy et al., 2014b), and more importantly PMCA in the lens. This observation further suggested that sodium selenite induces a significant elevation of Ca^{2+} in the rat lens (Hightower et al., 1987). Lenses from rats injected with sodium selenite showed a 50% decrease in PMCA activity (Wang et al., 1993). Ca^{2+} homeostasis is, therefore, recognized as having a fundamental importance in lens pathophysiology. Theoretically, increased lens Ca^{2+} could be due to inhibition of outwardly directed PMCA pumps. The only path for Ca^{2+} to leave from the intact lens is by transporting it against its electrochemical gradient into fiber cells,

and then passing it by electrodiffusion from cell to cell through gap junctions to surface cells, where PMCA pump activity and $\text{Na}^+/\text{Ca}^{2+}$ exchange can transport it back to the aqueous or vitreous humors (Gao et al., 2004). The $\text{Na}^+/\text{Ca}^{2+}$ exchanger and PMCA actively remove Ca^{2+} from the lens epithelial cells, whereas SERCA sequesters Ca^{2+} in the ER, which contains the largest intracellular store of Ca^{2+} (mM range) and then the Ca^{2+} store is replenished by a high affinity, low capacity SERCA uptake system (Berridge, 1993; Pozzan et al., 1994).

5. Increased Ca^{2+} activates m-calpain, caspases, cysteine proteases and ERAD components

The accumulation of unfolded proteins within the ER, while promoting the UPR, also elicits ERAD activation (Jarosch et al., 2003). Highlighted by an increase in ubiquitin-mediated proteolysis, this response encourages the removal of misfolded proteins from the ER lumen (Jarosch et al., 2003) and ERAD activation prevents promotion of the chronic UPR (Haynes et al., 2004). Further, recent reports demonstrated that Nrf2 is one of the important transcription factors in the UPR, and can directly promote the transcription of several cytoprotective target genes encoding proteasome subunits, thereby promoting proteasome assembly, during oxidative stress conditions (Kwak et al., 2003a; Kwak et al., 2003b). This suggests that PERK-dependent Nrf2 activation may contribute to protein degradation, during the UPR. Thus, mild UPR promotes cell survival, but chronic UPR leads toward self-destruction. One can expect that severity and duration of ER stress must determine this delicate balance.

An important consequence of ER- Ca^{2+} mobilization in lenses is activation of cysteine proteases such as m-calpain which is a major calcium-activated non-lysosomal, cysteine protease in human (Wang et al., 1992), cows (David et al., 1989), rodent (David and Shearer, 1986; Morishima et al., 2002; Nakagawa et al., 2000; Shearer et al., 1997), rabbits (Shearer et al., 1997), and guinea pigs (Shearer et al., 1997). Ca^{2+} influx mediated calpain activation results in caspase-3-mediated apoptosis in the retina degeneration model (Sharma and Rohrer, 2004). The ER- Ca^{2+} elevation also activates multiple caspases including caspase-12, caspase-9, and caspase-3 in the mouse (Nakagawa et al., 2000). The human caspase-12 gene has frame-shifts and a premature stop codon and therefore identifies with human caspase-4, which has sequence homology with mouse sequences (Hitomi et al., 2004a; Hitomi et al., 2004b). In general, caspase-mediated apoptosis begins with signaling from an initiator caspase-12 (mouse) or caspase-4 (human), which, subsequently promotes the release of cytochrome *c* and SMAC/Diablo from the mitochondria as well as the activation of a series of effector caspases, such as caspase-3, that cleave many other proteins. While many caspases are localized in the cytoplasm, in human cells caspase-4 is found in the ER membrane (Hitomi et al., 2004a; Yukioka et al., 2008). UPR induction results in the cleavage of procaspase-4, followed by the proto-typical activation of downstream effector caspases, although the exact mechanism remains uncertain (Hitomi et al., 2004a; Kahns et al., 2003; Kalai et al., 2003; Yukioka et al., 2008). Further increasing Ca^{2+} activates the Ca^{2+} -dependent m-calpain and proteases and results in crystallin degradation, denaturation, and aggregation and are considered to cause cataract in rodents (Marcantonio, 1996; Shearer

et al., 1997). Also, the loss of cytoskeletal proteins was also reported from lenses of selenite-treated rats (David and Shearer, 1984). Sodium selenite accelerated the loss of the cytoskeletal proteins and unidentified nuclear proteins of 49, 60 and 90 kDa (Matsushima et al., 1997). Interestingly, calcium electrode studies, carried out on human lenses, have shown that in those with localized cataracts, the free calcium only rises in the opaque areas, whereas surrounding clear regions have near-normal free calcium levels (Duncan and Jacob, 1984). In a nutshell as a key regulator of cell survival Ca^{2+} can induce ER stress-mediated apoptosis regulated at both the early and late apoptotic stages in response to various conditions (Bahar et al., 2016).

6. Protein folding and assembly generate ROS

In aerobic respiratory tissues, 1) ROS are usually created by leakage of superoxide from the mitochondrial respiratory chain due to perturbation of mitochondrial structure and function. 2) ROS are also generated from subsequent reduction of molecular oxygen. Molecular oxygen usually accepts a total of four electrons, one at a time, and the equivalent quantity of protons to produce two molecules of water. During this process, different oxygen radicals are continuously formed as intermediate products, including superoxide and peroxide. 3) Also, ROS are generated inside ER by hyperactivity of the so-called oxidative protein folding.

Since the lens is localized in an hypoxic environment (Elanchezhian et al., 2012b; Hockwin, 1971; McNulty et al., 2004; Neelam et al., 2013; Siegfried et al., 2010), the low levels of the enzymes associated with the aerobic oxidation of glucose restrict metabolism mainly to anaerobic glycolysis (Kinoshita, 1965). Even in the epithelium, anaerobic glycolysis appears to be the principal source of bioenergy, nearly 50% of the ATP in the lens epithelial cells is generated by oxidative metabolism (Elanchezhian et al., 2012b; Kinoshita, 1965; Winkler and Riley, 1991). The anaerobic respiration does not generate ROS, therefore certain ROS must be produced from other sources such as the chronic UPR, and the oxidative protein folding might play a significant role in ROS production in the lens.

The ER lumen is a highly oxidized environment and is favorable for disulfide formation (Hwang et al., 1992). Disulfide formation generates ROS as a byproduct of thiol/disulfide exchange reactions (Zhang and Kaufman, 2004). This response involves ER oxidoreductin 1 (Ero1), protein disulfide isomerase (PDI) and ER client proteins (Higa and Chevet; Pagani et al., 2000). There are two Ero1 isoforms in humans, Ero1 α , and Ero1 β (Cabibbo et al., 2000; Pagani et al., 2000) and both proteins facilitate oxidative folding (Mezghrani et al., 2001). Ero1 is a flavoenzyme, which may interact directly with molecular oxygen, producing ROS in the form of hydrogen peroxide and thereby contributes to the burden of oxidative stress. Protein disulfide bond formation by PDIs is dependent on the redox status in the ER, which is in part maintained by glutathione. Glutathione is synthesized in the cytosol and presented as the main redox buffer in the ER lumen. It has been shown that oxidized glutathione is essential to provide oxidizing equivalents for disulfide bond formation and glutathione-mediated oxidation of PDI is the major pathway of thiol-disulfide exchange (Cai et al., 2000; Hwang et al., 1992). However, Ero1 (Cabibbo et al., 2000) is now thought as the primary electron acceptor for disulfide bond formation. In oxidized conditions, Ero1 oxidizes the

active cysteinyl thiol groups in PDI. PDI subsequently oxidizes the client proteins to create disulfide bonds. Ero1 transfers electrons from PDI to molecular oxygen and produces ROS. The reduced form of PDI breaks and rearranges disulfides in the nascent proteins. Also, reduced glutathione may be required for the disulfide isomerization by reducing the disulfides in PDI to the thiol (Csala et al., 2010). Recent reports have also revealed that PDI is the major substrate of Ero1 α (Hwang et al., 1992) and the interaction between PDI and Ero1 α is mediated through binding between the hydrophobic pocket in the b' domain of PDI and the protruding β -hairpin of Ero1 α (Masui et al., 2011).

To deal with ROS formed during protein folding in ER stress, the PERK pathway induces the Nrf2 dependent stress/antioxidant cytoprotection pathway (Fig. 1) which in turn activates the transcription of glutathione-S-transferase, NAD(P)H:quinone oxidoreductase-1, γ -glutamate cysteine ligase, and heme oxygenase-1 to protect the cells from oxidative stress (Itoh et al., 1997). Intriguingly, the ROS production associated enzymes, PDI, and Ero1-L β are constant in human lens epithelial cells treated with methylglyoxal and other ER stressors suggesting that ER stress-mediated ROS production must be constant. However, the continued ROS production diminishes the Nrf2 dependent antioxidant protection. On the other hand, human lens epithelial cells treated with sodium selenite produce significantly higher levels of ROS (Palsamy et al., 2014b). Similarly, human lens epithelial cells treated with homocysteine (Elanchezhian et al., 2012a), non-physiologically high and low levels of glucose (Ikesugi et al., 2006b), hypoxia with hypoglycemia (Elanchezhian et al., 2012b), valproic acid (Palsamy et al., 2014c), thapsigargin, tunicamycin, and calcium ionophore-A23187 (Ikesugi et al., 2006b) produce significant levels of ROS in human lens epithelial cells. Also, the levels of PDI and Ero1-L β are consistently elevated in human lens epithelial cells treated with each of these ER stressors.

7. Nrf2 is a central transcriptional activator for cytoprotective target genes

When cells are challenged by ER stressors, they must quickly augment their antioxidant capacity to counteract increased ROS production and maintain homeostasis. Nrf2 is a central transcription factor that functions as the key controller of the redox homeostatic gene regulatory network (Fig. 1). Under ER stresses, the Nrf2 signaling pathway is activated to enhance the expression of a multitude of cytoprotective enzymes that restore redox homeostasis. Keap1, a negative regulatory protein for Nrf2, is fastened to cytosolic actin and interacts with Nrf2 by acting as an adaptor protein. This interaction is a comparatively quick incident, with Nrf2 exhibiting a short half-life of 13–21 minutes (Hong et al., 2005; Katoh et al., 2005; Kobayashi and Yamamoto, 2006). Such a rapid turnover maintains a low, basal level of Nrf2, under unstressed conditions. An accumulation of phosphorylated-Nrf2 (P-Nrf2) in the nucleus that is associated with its transcriptional partner, Maf protein, forms a heterodimer that binds to ARE core sequence (GTGACNNNGC) on the DNA of target cytoprotective genes (Alam et al., 1999; Itoh et al., 1997; Itoh et al., 1999; Kobayashi et al., 1999; Motohashi and Yamamoto, 2004; Taguchi et al., 2011). Nrf2 protein is known to regulate nearly 200–600 cytoprotective genes (Enomoto et al., 2001; Kwak et al., 2003a; Lee et al., 2003b; Nguyen et al., 2000; Thimmulappa et al., 2002; Venugopal and Jaiswal, 1996; Wild et al., 1999), and are mainly associated with the expression of antioxidant enzymes, cellular transporters (Hayashi et al., 2003; Sasaki et al., 2002), enzymes that exclude the

entry of xenobiotic metabolites and toxic compounds (Hayashi et al., 2003), detoxification enzymes, several components of the proteasome as well as those involved in protein folding and degradation (Kwak et al., 2003c). Also, Nrf2 promotes the expression of genes implicated in cell growth, cell adhesion, protein folding, cell signaling, cell-cycle control, survival, and glucose metabolism (Kwak et al., 2003c; Lee et al., 2003b). Nrf2 also promotes the expression of molecular chaperones/heat shock proteins and wound healing response proteins (Braun et al., 2002). (Fig. 2)

8. Loss of DNA methylation in the *Keap1* promoter

Extensive studies of protein, lipid and DNA modifications by oxidative insults have been investigated, and multiple review papers have been published in the past (Bassnett and McNulty, 2003; Cvekl and Duncan, 2007; Kowluru et al., 2015; Lou, 2003; Sobrin and Seddon, 2014; Truscott and Augusteyn, 1977; Zoric et al., 2010). Oxidative insults are also well known to induce DNA modifications, and DNA methylation is a most common epigenetic modification in somatic cells during aging that limits the activity of gene regulatory elements, including cell type-specific gene promoters and enhancers. Further, the patterns of DNA methylation are non-random, well-regulated, and tissue-specific (Eckhardt et al., 2006). Cell type-specific DNA methylation patterns are established during mammalian development and maintained in adult somatic cells (Cridder et al., 2012). In essence, DNA methylation is a biochemical process that occurs predominantly at CpG dinucleotides where DNA methyltransferases (Dnmts) transfer the methyl group to cytosine nucleotides, generating 5-methylcytosine (5mC) which is found almost entirely within CpG dinucleotides (about 70–80%) present in DNA of mammalian somatic cells. Interestingly, CpG DNA methylation is highly prevalent in repetitive sequences but rare in CpG islands within housekeeping promoters. A detailed literature search supports the concept that DNA methylation of a gene functions to stabilize or lock in the silent state of certain genes (Razin and Riggs, 1980). However, DNA methylation patterns are perturbed in human diseases, such as imprinting disorders, degenerative diseases, and cancer (Jaenisch and Bird, 2003; Kagey et al., 2010). Also, studies with *in vitro* and animal models have shown that loss of genomic DNA methylation is associated with cellular senescence and aging of organisms (Oakes et al., 2003; Wilson and Jones, 1983). In humans, loss of genomic DNA methylation, measured in blood or cancer DNA samples (Guz et al., 2008; Hsiung et al., 2007; Moore et al., 2008) has been found in a variety of age-related diseases (Fraga et al., 2005; Fraga and Esteller, 2007), but little information is available on changes in promoter DNA methylation patterns during normal lens aging and ARCs (Palsamy et al., 2012). The importance of an epigenetic event is that it represents a mechanism by which the gene function is selectively altered in response to environmental and aging stresses. The *Nrf2* and *Keap1* genes have 60, and 90 CpG dinucleotides in their promoters, respectively, and methylated DNA sequence analyses showed that the CpG dinucleotides in the *Keap1* gene are indeed epigenetically modified but not in the *Nrf2* gene (Palsamy et al., 2012). Thus, age-dependent environmental stresses appear to lead to epigenetic DNA modifications, which cause aberrant gene expression. Specifically, loss of DNA methylation in the promoter of *Keap1* gene decreases Nrf2-dependent antioxidant protection and results in a redox imbalance altered towards lens oxidation (Elanchezhian et al., 2012a; Elanchezhian et al., 2012b;

Palsamy et al., 2012) (Palsamy et al., 2014a; Palsamy et al., 2014b, c). To be precise, there is a significant loss of methylcytosine in diabetic cataractous lenses (possibly more than 90%), and it appears to be very rapid. This notion is substantiated by human lens epithelial cell culture experiments with ER stressors such as methylglyoxal or sodium selenite (Palsamy et al., 2014a; Palsamy et al., 2014b). These findings indicate that diabetic cataracts are not induced by aging *per se*, but rather by age-associated diabetic stresses. In contrast, it has been shown that loss of DNA methylation in the *Keap1* promoter in clear lenses is at a rate of 1.25% per year. Interestingly, diabetic lenses with cortical, nuclear, posterior subcapsular, and mixed type cataracts demonstrated significant loss of DNA methylation in the *Keap1* promoter (Palsamy et al., 2014a). These data are of huge importance as they suggest that most of the population, with appropriate preventive care, would not develop a cataract in their natural lifetime. Characterization of dysregulation in DNA methylation during aging is at a very early stage and even understudied research area. This signifies the importance of studying the epigenetic DNA remodeling during age-related variations, including ARCs. In contrast, DNA is methylated with age in repetitive DNA segments such as Alu repeats, where Alu elements exhibit a striking tissue-specific pattern of methylation (Xie et al., 2009). Further studies are required to explore how some genes are methylated, while other genes are demethylated.

9. Regulation of DNA methylation

Although Ca^{2+} dependent proteolysis through ERAD might be responsible for the decreased levels of Nrf2, reports have also shown that loss of DNA methylation in the *Keap1* gene also plays a major role in suppression of Nrf2 and loss of Nrf2-dependent cytoprotection. There are two known DNA demethylation pathways; one is the passive demethylation pathway in the germinative zone of replicating lens epithelial cells, and the other is the active demethylation pathway in the remaining non-replicating lens epithelial cells. Epigenetic modifications of DNA can last for a lifetime, and some of them might be imprinted (Kagey et al., 2010), a fact which can be used as a new tool to look at the history of and the correlations between the various stresses that are encountered throughout life and their pathogenic consequences. Since age-dependent cataractogenic stresses reprogram a broad range of genes by the epigenetic loss of promoter DNA methylation, this research study may predict the no-return pathway for ARCs formation and senescence.

It is thought that DNA methylation patterns are established during embryonic development and are then faithfully inherited in the cells by a 'maintenance' mechanism in somatic cells. Failure of such 'maintenance' mechanism leads to DNA demethylation, which can result from passive demethylation via Dnmt1, Dnmt3a and Dnmt3b following DNA replication (Fig. 3), or from an active, replication-independent process of enzymatic removal of the 5mC by ten-eleven translocation 1 (TET1), activation-induced cytidine deaminase (AID), and thymine-DNA glycosylase (TDG). This suggests that promoter DNA methylation dynamics play an important part in human disease development and aging (Fig. 4).

It has been hypothesized that ER stress-mediated chronic UPR further degrades/inactivates Dnmt1, Dnmt3a and Dnmt3b and lower levels of Dnmts no longer methylate the hemimethylated DNA through 'maintenance methylation' and result in loss of DNA

methylation (Palsamy et al., 2012; Palsamy et al., 2014a). In contrast, chronic UPR could stimulate active DNA demethylation, which can be a major demethylation pathway in the rest of lens epithelial cells of diabetic cataractous lenses since there is no apparent DNA replication in those cells throughout life (Palsamy et al., 2012; Palsamy et al., 2014a).

In addition to promoter DNA demethylation of the *Keap1* promoter in ARCs formation, there are reports demonstrated the involvement of promoter DNA hypermethylation in the context of ARCs formation. For instance, promoter DNA hypermethylation of *CRYAA* has been characterized in ARCs, thereby correlating the increased DNA methylation with decreased gene as well as protein expression (Zhou et al., 2012). Interestingly, treatment with zebularine, an inhibitor of DNMTs, was reported to increase the *CRYAA* expression levels in the lens epithelial cells (Zhou et al., 2012). Also, the lens epithelial-derived growth factor, *LEDGF* is expressed by lens epithelial cells, upregulates heat shock proteins and β -crystallins, and responds to environmental stress. *LEDGF* is an oncogene, and its expression is regulated by a promoter CpG island methylation (Bhargavan et al., 2013; Shinohara et al., 2002). Histone modification is also known to regulate the *LEDGF* response to UV damage, one of the environmental exposures that lead to ARCs formation (Bhargavan et al., 2013). DNA damage is an additional consequence of oxidative stress, and there are several reports have demonstrated the association between DNA repair genes with ARCs formation. More specifically, the promoter of the DNA repair genes, such as *O6-methylguanine DNA methyltransferase (MGMT)*, and *8-oxoguanine DNA glycosylase 1 (OGG1)* have been shown to hypermethylated in the cataractous tissues (Li et al., 2014; Wang et al., 2015). However, to understand the major mechanisms and function of DNA methylation in ARCs formation needs further research in this field.

10. ER stressor alters levels of DNA methylation in the *Keap1* promoter

Diabetes is a well-documented cataract risk factor that accelerates early cataract formation. DNA methylation in the *Keap1* gene is significantly decreased in diabetic cataractous lenses (Palsamy et al., 2012), and it is unknown what induces this loss in the lenses of these patients. It is speculated that loss of DNA modification enzymes (Dnmts and TET1) in the active and passive DNA demethylation pathways might be responsible for the loss of DNA methylation. mRNA and protein expressions studies confirmed that methylglyoxal, valproic acid, 5-Aza and sodium selenite treatment significantly activates the transcription of *Nrf2* and *Keap1* genes, while significantly suppressing *Nrf2* and increasing *Keap1* protein (Palsamy et al., 2014a; Palsamy et al., 2014b, c). Furthermore, DNA methyltransferases, Dnmt1, Dnmt3a and Dnmt3b and the active DNA demethylation enzymes TET1, AID, and TDG are over-expressed in human lens epithelial cells treated with methylglyoxal, valproic acid, and sodium selenite (Palsamy et al., 2014a; Palsamy et al., 2014b, c). Bisulfite-converted DNA sequencing results revealed a significant loss of DNA demethylation in the *Keap1* promoter with notable increases in *Keap1* mRNA and protein in human lens epithelial cells treated with methylglyoxal, sodium selenite, and valproic acid, but not in the *Nrf2* promoter (Palsamy et al., 2012; Palsamy et al., 2014a; Palsamy et al., 2014b, c). Similarly, all other ER stressors listed in Table 1 might induce the loss of DNA methylation as shown (Fig. 5).

These studies have established that treatment of human lens epithelial cells with ER stressor diminishes DNA methylation in the *Keap1* promoter, similar to what occurs in human diabetic cataractous lenses. This finding indicates that loss of DNA methylation in the CpG islands of the *Keap1* promoter in lenses of diabetic patients activates expression of the Keap1 protein, which then increases Nrf2 proteasomal degradation. As an outcome, decreased Nrf2 activity further represses the transcription of many cytoprotective target genes and alters the redox balance towards lens protein oxidation and blocks cytoprotection against cataractogenic stresses. Thus, the failure of antioxidant protection due to demethylation of CpG islands in the *Keap1* promoter is linked to diabetic cataracts and possibly ARCs.

11. ER stressor activates proteasomal degradation of Nrf2 in lens epithelial cells

The mRNA levels of Nrf2/Keap1 in human lens epithelial cells are increased significantly, but the levels of Nrf2 protein is decreased significantly in human lens epithelial cells treated with ER stressor, especially cataractogenic stressors (Elanchezhian et al., 2012a; Palsamy et al., 2014a; Palsamy et al., 2014b, c). This discrepancy might be due to proteasomal degradation or ERAD, and this was confirmed in human lens epithelial cells that were pretreated with MG-132, an inhibitor of proteasome protease, followed by methylglyoxal treatment for another 24 h. The protein profiles of Nrf2 and Keap1 revealed that these ER stressors such as methylglyoxal treatment augmented significant proteasomal degradation of Nrf2, but not of Keap1 protein. However, when MG-132 pretreated human lens epithelial cells were then cultured with methylglyoxal revealed a substantial increase in the levels of Nrf2 and Keap1 protein compared to that of human lens epithelial cells treated with methylglyoxal alone (Palsamy et al., 2014a). These results further confirm that ER stressors augment the degradation of Nrf2 and Keap1 proteins through activation of proteasomal degradation. Also, the passive DNA demethylation enzymes, Dnmts, also decreased in human lens epithelial cells treated with ER stressor, but human lens epithelial cells pretreated with MG-132 augment degradation of Dnmts through activation of proteasomal degradation.

12. Lower levels of Nrf2 suppress expression of *Nrf2-target cytoprotective genes*

Since levels of Nrf2 decrease in human lens epithelial cells treated with ER stressor, levels of mRNA expression profiles of Nrf2-target cytoprotective genes such as glutamate-cysteine ligase catalytic subunit, glutamate-cysteine ligase modifier subunit, quinone reductase, thioredoxin reductase 1 and sulfiredoxin-1 must be significantly decreased. These Nrf2-target genes in human lens epithelial cells treated with methylglyoxal also decrease because Nrf2 protein is suppressed by higher levels of Keap1 protein leading to repression of the availability of Nrf2 for this transcription of Nrf2-target genes (Enomoto et al., 2001; Kwak et al., 2003a; Lee and Ye, 2004; Nair et al., 2007; Palsamy et al., 2014a; Palsamy et al., 2014b, c). Glutathione reductase and catalase levels also decrease in human lens epithelial cells treated with ER stressors such as methylglyoxal, sodium selenite and valproic acid

(Palsamy et al., 2014a; Palsamy et al., 2014b, c). These findings strongly suggest that *Nrf2*-target cytoprotective genes may be suppressed upon exposure to either ER stressors or cataractogenic stressors in lens epithelial cells. Further, cultured lens epithelial cells of *Nrf2* knockout (*Nrf2*^{-/-}) mice treated with varying concentrations of methylglyoxal showed enhanced cell death, compared to cultured lens epithelial cells of wild-type mice. These results demonstrate the importance of retaining normal levels of *Nrf2* and *Nrf2* target cytoprotective genes for the survival of the lens under stressed conditions (Palsamy et al., 2014a).

13. UPR activation in immature lens fiber cells

Induction of ER stress in lens epithelial cells is well documented, but it is still obscure in the case of immature lens fiber cells. As lens opacity develops in these fiber cell layers, it is crucial to identify whether ER stress is induced in immature lens fiber cells and generates *Nrf2*-deficiency in these cells. Immature lens fiber cells contain ER, mitochondria and intact DNA and mRNAs (Bassnett and Sikic, 2017). Crystallin mRNAs are present for at least 1 month from the time of initiation of lens fiber differentiation in the chicken lens (Shinohara and Piatigorsky, 1976). Proliferating lens epithelial cells in the lens germinative zone remain as the most sensitive to ER stress (Elanchezhian et al., 2012b), and immature lens fiber cells appear to overexpress ROS in suckling rat lenses (Palsamy et al., 2014b). Firtina et al. reported that the ER-resident chaperones, BiP and PDI, were expressed at high levels in the newly forming fiber cells of mouse embryonic lens (Firtina and Duncan, 2011). These fiber cells also expressed the UPR-associated molecules; XBP-1, ATF6, P-PERK, and ATF4 during embryogenesis. Moreover, spliced XBP-1, cleaved ATF6, and P-eIF2 α were detected in embryonic mouse lenses suggesting that the UPR pathway is active in this tissue (Firtina and Duncan, 2011). Also, the expression of Ero-1 β , an ER stress-mediated ROS-producing enzyme, was found in the cortical lens fiber cells of an ARC lens from a 57-year-old individual, but the rest of UPR proteins, such as *Nrf2*, BiP, and Keap1, were not detected (Elanchezhian et al., 2012b). Lens epithelial cells and immature lens fiber cells are partially liquefied in selenite injected 14-day suckling rat lenses (Palsamy et al., 2014b; Shearer et al., 1997) suggesting significant induction of UPR induced damage in immature lens fiber cells. Also, detection of string-like DNA staining with 2',7'-dichlorodihydrofluorescein diacetate (H₂-DCFH) in the lens fiber cells suggests that ER stressors-induced increased the permeability of the plasma membrane and binding of H₂-DCFH to DNA, resulting in detection of DCF fluorescence on DNA (Palsamy et al., 2014b).

Based on these observations, this review summarizes the possibility that lens fiber cells that form under ER stress might develop cataract due to a deficiency in *Nrf2* dependent cytoprotection in the multi-layer of the cells. Interestingly, the localization of these cells determines the type of cataract to develop. An unsettled report that nuclear cataract patients have significantly higher mortality than those with other types of cataracts (Wang et al., 2014) suggests that nuclear cataract patients may have mild levels of ER stress in the lens throughout life.

14. Prevention of ARCs

It can be expected that almost all the ER stressors listed in Table 1 generate a misfolded protein conformation which triggers an activation of ER stress. Loss of DNA methylation in somatic cells is considered irreversible, as is aging, and ARCs, but these are preventable. Avoiding ER stressors by changing one's lifestyle, improving systemic diseases, preventing nutritional deficiencies, and avoiding toxic drugs, environmental toxins, and irradiations may prevent ARCs formation. Those are the best strategies to protect the lens from initiation of chronic UPR. Many reports indicate that intake of excess levels of antioxidants may help to prevent cataract formation. It is also important to block the loss of DNA methylation in somatic lens cells in order to maintain the preprogrammed methylated DNA status for a prolonged period of time. Huang et al. recently reported that the stable knockdown of *Tet1* results in a decrease of *Keap1* mRNA when compared to control cells (Huang et al., 2014). From their report, one can speculate that if *Tet1* is suppressed, the *Keap1* gene retains its methylation status and Keap1 expression is also suppressed. The bisulfite genomic DNA sequencing of the human *Keap1* gene suggests that if promoter DNA demethylation is avoided, ARCs can be preventable up to ages over 100. Also, although the *Keap1*^{-/-} mouse dies postnatally, the lung-specific knockdown of *Keap1* significantly enhances antioxidant protection (Blake et al., 2010; Wakabayashi et al., 2003) suggesting that *Keap1* suppression can be a potential therapeutic approach for the prevention of ARCs. These strategies protect the lens from cataract formation, though they are also beneficial for longevity.

15. Future directions

Oxidative stress is associated with DNA hypomethylation in the *Keap1* gene in the cataractous lens, but DNA hypermethylation is associated with colorectal (Hanada et al., 2012) and lung cancers (Muscarella et al., 2011). Interestingly, the lens is the only organ in the human body which does not develop cancer (Bhat, 2001). A better understanding of the DNA methylation status in the *Keap1* promoter might reveal links between cellular malignancy and senescence.

The DNA methylation status of the *Keap1* promoter suggests that aging directly affects ARC formation in term of the loss of Nrf2 dependent cytoprotection. Loss of DNA methylation in the *Keap1* promoter is nearly 0% at age 17, but at the ages 60 and 75, it is 40% and 50%, respectively (Palsamy et al., 2012; Palsamy et al., 2014a). A 40–50% loss of DNA methylation stress in elderly populations can increase to an average of 90% loss with cataractogenic stress in those who develop ARCs (Palsamy et al., 2012; Palsamy et al., 2014a). Thus, the incidence of ARCs shows significant increases with loss of DNA methylation. Also, loss of DNA methylation is known to occur in multiple genes, such as hypoxia-inducible factor-1 (HIF-1) (Ke and Costa, 2006). HIF-1 is the first gene involved in homeostatic processes in the lens and plays an integral role in the response of the lens to low oxygen concentrations or hypoxia. HIF-1 activates transcription of numerous genes including vascular endothelial growth factor (VEGF), glucose transporter-1 (GLUT-1), and genes involved in glucose metabolism (Willam et al., 2002). HIF-1 interacts with the von Hippel-Lindau (pVHL) ubiquitin E3 ligase complex (Masson et al., 2001; Srinivas et al., 1999). In normoxic conditions, hydroxylation at two proline residues and acetylation at a

lysine residue in the oxygen-dependent degradation domain of HIF-1 triggers its connotation with pVHL E3 ligase complex, leading to HIF-1 degradation via the ubiquitin-proteasome pathway. Methylation of pVHL regulates the levels of HIF-1 in patients with metastatic clear-cell renal cell carcinoma (Choueiri et al., 2013). One can speculate that it is not HIF-1, but instead the pVHL promoter (HIF-1/pVHL), which can significantly lose DNA methylation and reprogram the oxygen response pathway in the lens. Extensive analysis of the DNA methylation status of these genes might yield a significant contribution. Also, nuclear factor- κ B (NF- κ B) activation is induced by a wide variety of agents including stress, cigarette smoke, other carcinogens, bacteria, inflammatory stimuli, cytokines, free radicals, tumor promoters, and endotoxins. Upon activation, NF- κ B regulates the expression of almost 400 different genes, which include angiogenic factors, cell cycle regulatory molecules, cytokines, enzymes, and viral proteins. NF- κ B is sequestered in the cytoplasm in an inactive form through its association with one of the several inhibitory molecules, including I κ B- α , which is the most abundant form; NF- κ B in NF- κ B/I κ B- α complexes are ubiquitinated and undergo proteasomal degradation (Oeckinghaus and Ghosh, 2009).

Interestingly, Nrf2, HIF-1, and NF- κ B are central transcriptional activators and Keap1, pVHL and I κ B- α are regulatory proteins which induce proteasomal degradation of these transcriptional factors under stress conditions. One can expect a loss of DNA methylation in the *Keap1*, *pVHL*, and *I κ B- α* promoters, which can ultimately result in an overexpression of these proteins, resulting in decreased levels of transcriptional activators and loss of cytoprotection. Elucidating the epigenetic mechanisms in the whole genome is an exciting challenge that is expected to lead to a clear understanding of the development of human senescence and malignancy through ARCs research.

16. Summary and conclusions

Most cataractogenic stress generates misfolded protein conformation of the membrane luminal and secretory proteins in the highly oxidized ER lumen. The conformational change of the protein is one of the most important and essential triggers to generate a cascade of cytoprotective and self-destructive chain reactions, the so-called ER stress/UPR. The protective ER stress eliminates misfolded proteins from the cell by ERAD pathway. Chronic UPR releases ER-Ca²⁺ to activate m-calpain and proteolysis enzymes. It also produces aggravated ROS, that are sufficient to oxidize cellular constituents, leading thereby to cellular apoptosis. Induction of apoptosis in lens epithelial cells have been reported in certain types of cataracts (Li et al., 1995), but not in nuclear cataracts (Konofsky et al., 1987). Cortical cataracts typically begin in a small region of the lens periphery and can spread around the lens circumference. Chronic and strong UPR might produce severe ROS thereby inducing apoptosis in lens epithelial cells. On the contrary, the weak UPR under mild ER stress conditions could be simply overcome by the lens epithelial cellular defense system. The ROS generated by these cells might cause little pathology (Papa, 2012). However, though ER is not present in the lens nucleus, it is speculated that nuclear cataracts, which do not require apoptosis in lens epithelial cells, can be induced by non-lethal exposures of ER stressors for extended times or recurrent times (Ikesugi et al., 2006b; Palsamy et al., 2014b). There are several experimental nuclear cataract models describing the involvement of UPR in the disease pathology, and none of them provided the detailed

mechanism. Thus, one can suggest that various ER stressors with different potential and exposure durations may affect the development of the different types of cataracts (Berthoud et al., 2016; Lyu et al., 2016; Mulhern et al., 2006; Palsamy et al., 2014b). Recently, it has been demonstrated the activation of the UPR through discrete pathways in the lens of age-related, high myopia-related and congenital cataracts (Yang et al., 2015). Specifically, all three UPR pathways are activated in age-related and high myopia-related cataractous lenses; however, only the IRE1/XBP-1 and PERK/eIF2 α /ATF4 pathways are activated in the congenital cataract lenses (Yang et al., 2015). Overall, the functional consequences of differential UPR activation in the context of the mechanism(s) of development of various cataracts are largely unknown but can be a topic of future research.

In addition, the expression of the *Keap1* gene is upregulated by the loss of DNA methylation. The demethylation is induced by the decreased level of Dnmts and increasing levels of TET1, AID, and TDG, which further induce the loss of DNA methylation in the *Keap1* promoter. The demethylated promoter of *Keap1* results in overexpression of the *Keap1* gene and produces higher levels of Keap1 protein. Higher levels of Keap1 induce the degradation of Nrf2 by ubiquitin-mediated proteasomal degradation and ERAD. As a result, Nrf2 dependent stress protection declines and the redox balance is altered towards lens oxidation leading to cataract formation. Thus, misfolded protein conformation initiates the production of misfolded crystallin aggregation (Fig. 6).

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List of abbreviations

5-Aza	5-aza-2'-deoxycytidine
5mC	5-methylcytosine
AID	activation-induced cytidine deaminase
ARCs	age-related cataracts
ARE	antioxidant response element
ATF6	activating transcription factor 6
BiP	Ig binding protein
Dnmts	DNA methyltransferases
eIF2α	eukaryotic translation initiation factor 2 α
ER	endoplasmic reticulum
ERAD	ER-associated degradation
Ero1	ER oxidoreductin 1

GSH	glutathione
H2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
HIF-1	hypoxia-inducible factor-1
IRE1	inositol-requiring kinase 1
Keap1	Kelch-like ECH-associated protein 1
NF-κB	nuclear factor- κ B
Nrf2	nuclear factor-erythroid-2-related factor 2
PDI	protein disulfide isomerase
PERK	protein kinase R (PKR)-like endoplasmic reticulum kinase
PKC	protein kinase C
PMCA	plasma membrane Ca^{2+} -ATPases
pVHL	von Hippel-Lindau
ROS	reactive oxygen species
SERCA	sarco/endoplasmic reticulum Ca^{2+} -ATPases
TDG	thymine-DNA glycosylase
TET1	ten-eleven translocation 1
UPR	unfolded protein response
XBP-1	X-box transcription factor-1.

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Fig. 1.

Schematic diagram for molecular mechanisms of activation of ER stress. Accumulation of misfolded protein triggers the phosphorylation of PERK and IRE1 and cleavage of ATF6 to activate the UPR. This rapidly reduces the misfolded protein load through reversible translational attenuation and transcriptional induction of ER resident proteins to further assist protein folding and through induction of chaperones, ER biosynthetic machinery, and ER-associated degradation (ERAD) components. Chronic ER stress induces the apoptotic UPR which generates excessive levels of ROS, through the involvement of Ero1-L α and -L β and PDI. Furthermore, the UPR releases Ca²⁺ from the ER to activate m-calpain and caspases. P-PERK phosphorylates eIF2 α which further activates ATF4 and the CHOP-death pathway. P-PERK, PKC, PI3 kinase and MAP kinase phosphorylate Nrf2; the P-Nrf2 dissociates from Keap1 and translocates into the nucleus to bind to the antioxidant response element (ARE), and this activates the transcription of more than 200 stress/antioxidant enzymatic genes such as glutathione, glutathione reductase, thioredoxin, thioredoxin-S-transferase, and catalase (see Fig. 2). These stress/antioxidant enzymes regenerate reduced glutathione, and the resultant reduced glutathione eliminates ROS so the cell can survive and

recover from the stress. Chronic UPR activates the death pathway and results in senescence or death.

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Fig. 2.

The Nrf2 target genes. The transcriptional factor, Nrf2 binds to the ARE site in the promoter of cytoprotective genes, and activates (>2.0 fold) or suppresses ($<1/2$ fold) more than 200–600 target genes in non-lens tissues.

Fig. 3.

Passive DNA demethylation pathway. Passive DNA demethylation is caused by a reduction in activity or absence of Dnmts. Dnmt3a and 3b are responsible for de novo DNA methylation of parent DNA. When methylated DNA is replicated, the daughter strands of DNA are unmethylated. This hemimethylated DNA is recognized by the maintenance DNA methyltransferase1 (Dnmt1) and restores parental DNA methylation patterns through successive rounds of cell division. If Dnmt1 is inhibited or absent when the cell divides, the newly synthesized strand of DNA will not be methylated, and successive rounds of cell division will result in passive demethylation. In this figure, unmethylated CpGs are shown by empty circles, and methylated CpGs are indicated by red circles.

Fig. 4.

Active DNA demethylation pathway. Active DNA demethylation takes place through the enzymatic replacement of 5mC with cytosine via the base-excision DNA repair pathway (BER) pathway. Cytosine is methylated by Dnmt1, 3a, and 3b and the resulting 5mC is oxidized to 5-hydroxymethylcytosine (5hmC) by TET1 proteins. 5hmC is then deaminated by AID into 5-hydroxy uracil (5hmU). Finally, 5hmU can be excised by TDG and repaired by the BER pathway with unmethylated cytosines. Active DNA demethylation pathway is induced in the non-replicating cells.

Fig. 5.

Loss of *Keap1* promoter DNA methylation in human lens epithelial cells treated with methylglyoxal, sodium selenite, valproic acid (VPA) and 5-Aza-2'-deoxycytidine (5- Aza). Control human lens epithelial cells do not show any loss of DNA methylation of the *Keap1* promoter (A). However, significant loss of DNA methylation of the *Keap1* promoter is found in human lens epithelial cells treated with either 100 μ M methylglyoxal for 1 day (B), 1 μ M sodium selenite for 1 day (C), 20 mM of VPA for 5 days (D) and/or 10 μ M 5-Aza for 7 days (E). Ten individual clones of the bisulfite-converted DNA sequences of human lens epithelial cells were analyzed for DNA methylation in fragment-1 (contains 20 CpG dinucleotides) of the *Keap1* promoter using BISMA software with default filtering threshold settings (<http://biochem.jacobs-university.de/BDPC/BISMA/>). Each row of red squares represents methylated CpG dinucleotides. Blue squares represent unmethylated CpG dinucleotides, and white squares represent an undetermined CpG status. The color gradient bar shows that the red region contains more methylated CpG dinucleotides, and the blue region contains more unmethylated CpG dinucleotides. Fold changes in the mRNA expression levels of *Keap1* and *Nrf2* in control human lens epithelial cells and treated with methylglyoxal, sodium selenite, and 5-Aza (F). The corresponding fold variations in the protein levels of Keap1 and Nrf2 in human lens epithelial cells treated with methylglyoxal (G), sodium selenite (H), VPA (I) and 5-Aza (J).

Fig. 6.
A schematic diagram showing the involvement of various cataractogenic stressors mediating ER stress mediated Nrf2 dependent antioxidant system failure via *Keap1* promoter DNA demethylation.

Types of Stress	References for cataract induction	ER stress	References for ER stress activation
Other diseases			
Alcoholic	(Fang et al., 2013; Geng et al., 2013)	Yes	(Kaphalia and Calhoun, 2013; Petrasek et al., 2013)
Anorexia	(Archer, 1981; Pestka, 2010)	Yes	(Gillum et al., 2011)
Diabetes mellitus	(Palsamy et al., 2012)	Yes	(Palsamy et al., 2012)
Galactosemia	(Mulhern et al., 2006; Mulhern et al., 2007)	Yes	(Mulhern et al., 2006; Mulhern et al., 2007)
Hypercalcemia	(Hough et al., 2004)	Yes	(De Miguel and Pollock, 2013; Osada et al., 2010)
Hypertension	(Nemet et al., 2010)	Unknown	
Obesity	(Cheung and Wong, 2007)	Unknown	(Sato and Suzuki, 2007)
Uremia	(Harding and Rixon, 1980; Kuckel et al., 1993)	Yes	(Zhang et al., 1999)
Uveitis	(Clayton et al., 1980; Jancevski and Foster, 2010)	Unknown	
Nitric oxide (NO)	(Gerkowicz et al., 2005)	Yes	(Santi-Rocca et al.)
Environmental Stress			
Cold or high temperature	(Tanaka and Benedek, 1975a, b; Validandi et al., 2011)	Yes	(Jain et al., 2013)
Dehydration	(Alves et al., 2011)	Unknown in mammals	Well known in Plants
Hyperbaric Oxygen	(Schocket et al., 1972)	Unknown	
Oxidative insults	(Lou, 2003)	Yes	(Tan et al., 2009)
Ionizing Radiation (UVB, X-ray, Infrared, sunlight)	(Chiu et al., 2012; Hammer et al., 2013)	Yes	(Zhang, 2010)
Osmolality	(Zhang et al., 2012)	Yes	(Alves et al., 2011)
Traumatic	(Shah et al., 2013)	Yes	(Han et al., 2013)
Infection:			
Hepatitis B	(Cushnir et al., 1997; Kushnir et al., 1996)	Yes	(Vanz et al., 2012)
Rubella,	(Foster et al., 1997)	Unknown	
HSV-1,	(Mahalakshmi et al., 2010)	Unknown	
CMV	(Mahalakshmi et al., 2010)	Unknown	
measles, mumps, and rubella (MMR) vaccine	(Ferrini et al., 2013)	Unknown	
Trachoma	(Roba et al., 2010)	Unknown	
Drugs:			
Allopurinol	(Garbe et al., 1998; Jaanus, 1991)	Unknown	

Types of Stress	References for cataract induction	ER stress	References for ER stress activation
Amiodarone (Antiarrhythmic agent)	(Flach and Dolan, 1993; Kim et al., 2011b)	Yes	(Van Summeren et al., 2013)
Anticholinesterases	(Shaffer and Hetherington, 1966)	Yes	(Lopez et al., 2009)
5-Aza-2'-deoxycytidine	(Palsamy et al., 2012)	Yes	(Palsamy et al., 2014a)
Chloroquine	(Inada et al., 2005)	Yes	(Li et al., 2013)
Chlorpromazine	(Richa and Yazbek, 2010)	Yes	(Varadarajan et al., 2012)
Cholesterol Synthesis Inhibitor (Statins)	(Morek et al., 2009)	Yes	(Li et al., 2009)
Cholesterol biosynthesis inhibitor (U18666A)	(Lee and Ye, 2004; Pedruzzi et al., 2004)	Yes	(Friend, 1990)
Clozapine	(Alam and Praveen Kumar 2016).		
Corticosteroids	(Haas et al., 2012)	Yes	(Yusta et al., 2006; Yusta and Drucker, 2004)
Glucocorticoids	(Nishigori, 2006)	Yes	(Yemelyanov et al., 2012)
Diazepam	(van den Brule et al., 1998)	Yes	(Kim et al., 2009)
Miotics	(Zimmerman and Wheeler, 1982)	Unknown	
Phenothiazines	(De Filippi et al., 2007; Flach et al., 1985)	Yes	(Popov et al., 1979)
Valproate	Unknown	Yes	(Zhang et al., 2011) (Penas et al., 2011)
Well Known ER Stressors			
Calcimycin (Ionophore A23187)	(Ikesugi et al., 2006b)	Yes	(Horke et al., 2008)
Malondialdehyde (MDA)	(Bhuyan et al., 1986, 1991)	Yes	(Xue et al., 2013)
Paraquat (Herbicide)	(Bhuyan et al., 1991)	Yes	(Chen et al., 2012)
Thapsigargin (Ca ²⁺ -ATPase Inhibitor)	(Archer, 1981) (Duncan et al., 1997)	Yes	(Pyati et al., 2011)
Tunicamycin (Glycosylation inhibitor)	(Fatma et al., 2011)	Yes	(Fatma et al., 2011)
Deficiency:			
Cobalamin, Vitamin B12	(Sen et al., 2008)	Yes	(Ghemrawi et al., 2013)
Folate, Vitamin B9	(Chen et al., 2005)	Yes	(Gueant et al., 2013)
Niacin, Vitamin B3	(Athanasiadis et al., 2007)	Unknown	
Pyridoxine, Vitamin B6	(Yusuf et al., 2013)	Unknown	
Riboflavin	(Prechal et al., 1978; Skalka and Prechal, 1981a, b)	Yes	(Manthey et al., 2005)
Thymine, Vitamin B1	(Frederikse et al., 1999)	Unknown	
Vitamin C and E (antioxidant effects)	(Thiagarajan and Manikandan, 2013)	Unknown	
Vitamin D	(Blau, 1996)	Yes	(Weng et al., 2013)

Types of Stress	References for cataract induction	ER stress	References for ER stress activation
Tryptophan	(Raju et al., 2007; Salih et al., 1985; Vrensen et al., 2004)	Yes	(Barnes et al., 2009)
Excess levels			
Monosodium Glutamate (MSG)	(Kawamura and Azuma, 1992)	Unknown	
Toxic Intermediate Metabolites			
Naphthalene/Quinone	(Oliveira et al., 2010)	Yes	(Lien et al., 2008; Peyrou et al., 2007)
Methylglyoxal (Diabetes)	(Giacco and Brownlee, 2010)	Yes	(Palsamy et al., 2012)
Homocysteine (Homocystinemia)	(Elanchezhian et al., 2012a)	Yes	(Elanchezhian et al., 2012a)
Malondialdehyde (MDA)	(Bhuyan et al., 1986, 1991)		(Chang et al., 2013; Yuan et al., 2013)
Hypoxia/Hyperoxia	(Elanchezhian et al., 2012b)	Yes	(Elanchezhian et al., 2012b)
Hypoglycemia/Hyperglycemia	(Elanchezhian et al., 2012b)	Yes	(Elanchezhian et al., 2012b)
Metal ions:			
Mercury	(Minoia et al.) (Minoia et al., 2011)	Yes	(Nozaki et al., 2001)
Copper	(Meo and Al-Khlaifi, 2003)	Yes	(Tardito et al., 2011)
Lead	(Rosin, 2009)	Yes	(Shinkai and Kaji, 2012)
Sodium Selenite	(Azuma et al., 1997; Shearer et al., 1997)	Yes	(Guan et al., 2009; Palsamy et al., 2014b)
Environmental Toxins:			
Acetaldehyde	(Derham and Harding, 2002)	Yes	(Ji, 2012)
Bisphenol A (Epoxy glue)	(Yalon et al., 1988)	Yes	(Asahi et al., 2010; Kim et al., 2011a)
Dinitrophenol	(Bonashevskaja, 1970)	Yes	(Miyokawa-Gorin et al., 2012)
Diquat (Herbicide)	(Bhuyan et al., 1991; Bhuyan et al., 1992)	Unknown	
3-aminotriazole	(Bhuyan and Bhuyan, 1984)	Yes	(Racape et al., 2011)
Smoking	(Prokofyeva et al., 2013)	Yes	(Kenche et al., 2013)