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Age-related cataracts: Homocysteine coupled endoplasmic reticulum stress and suppression of Nrf2-dependent antioxidant protection

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Abstract

To determine whether high levels of homocysteine (Hcy) induce endoplasmic reticulum (ER) stress with suppression of the nuclear factor-erythroid-2-related factor 2 (Nrf2)-dependent antioxidant protection in lens epithelial cells (LECs). ER stress was acutely induced by exposure of LECs to 100 μM Hcy without FCS and also by exposure to 5 mM Hcy with 10% FCS. After exposure to Hcy, significant changes were found in P-PERK, P-eIF2α, XBP1, Nrf2, and Keap1 within 24 h. The production of reactive oxygen species (ROS) was increased after Hcy exposure. The downstream enzymes of Nrf2 like, catalase, and glutathione reductase, were significantly decreased. These results suggested that the Hcy-induced ER stress suppressed the Nrf2-dependent antioxidant protection and simultaneously generated ROS which resulted in further oxidation and death of LECs. The loss of Nrf2 is mainly due to proteosomal degradation and m-calpain activation by the increased levels of cytoplasmic Ca^{++}. The caspases also play a role in the degradation of Nrf2. Our findings demonstrated that high levels of Hcy induce ER stress, chronic UPR, alter the levels of UPR specific proteins, increase the production of ROS, degrade Nrf2 and block the Nrf2-dependent antioxidant defense protection in LECs. Thus, the upregulation of ROS might exceed the Nrf2 dependent antioxidant defense protection in the LECs and result in the highly oxidized lenses and resulted in ARCs.

Keywords

Homocysteine; ER stress; Nrf2; Antioxidants; Age-related cataract

1. Introduction

Age-related cataracts (ARCs) are a major type of cataracts and are considered to be due to the normal aging processes. Aging, combined with environmental and genetic stresses, is considered to be the main contributor to the pathogenesis of lens oxidation, crystallin modification, and aggregation. However, many of the pathogenic mechanisms involved in ARC remain undetermined.

One of the cellular conditions which have been related to the development of ARC is oxidative stress [12,22]. Oxidative stress is induced by a wide range of factors including

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diabetes, malnutrition, systemic and ocular disease processes, pollutants, drugs, heavy metals, ionizing radiation, glucose, and changes in the oxygenation of cells [13,22]. All of these stress factors lead to the generation of ROS [17,18,26,38]. The ROS include free radicals, such as the superoxide anion and the hydroxyl radical, and oxidants such as hydrogen peroxide [12,22].

High level of serum homocysteine (Hcy) is closely associated with juvenile cataracts and ARCs. Recent studies showed statistically significant increases of Hcy and decreases in vitamin B_2, B_6, B_{12}, and folic acid in the plasma of 40 adult patients with ARCs [50]. In addition, 24 of 29 children with homocystinuria type 1 had cataracts [40]. Adult homocystinuria can be acquired by chronic kidney disease, cigarette smoking, and alcoholism in addition to dietary deficiencies of vitamin B_2, B_6, B_{12}, and folic acid [9,44]. The Framingham Heart Study showed that the levels of these vitamins are the main determinant (67%) of high Hcy in the elderly [40]. In addition, 20–50% of older adults have atrophic gastritis type B which results in decreased gastric acid and pepsinogen secretion and hence decreased intestinal digestion and absorption of these vitamins [46].

Hcy is biosynthesized exclusively from methionine by a multistep process [34,44,45]. The co-enzymes involved in this process are folate and vitamin B_2, B_6, and B_{12}. Under normal metabolism, there is a strict balance between Hcy formation and elimination. With higher levels of Hcy, misfolded proteins are accumulated in the endoplasmic reticulum (ER) which can then induce the unfolded protein response (UPR) [1,32,33,42]. The initial responses to the UPR are the modification of three ER-associated transmembrane proteins; phosphorylation of PKR-like eukaryotic initiation factor 2α kinase (PERK), and inositol-requiring enzyme-1 (IRE1), and activating transcription factor-6 (ATF6) which is cleaved and separated from the ER membrane. These responses lead to translational and transcriptional arrest, and decrease protein production to try to recover from the overload of unfolded proteins in the ER. However in the presence of continued ER stress, the apoptotic pathway is activated by the central transcriptional activators, ATF6 and ATF4, which then activate the death factor, CCAAT/-enhancer-binding protein homologous protein (CHOP) [31]. The UPR also activates caspase-4 (caspase-12 in rodents) resulting in apoptosis (See Fig. 1) [1,33].

The UPR also generates excess levels of ROS which is driven by proteins involving new protein disulfide isomerase (PDI) and ER oxidoreductin 1 (Ero1-Lα and Lβ) [14,25,43]. The produced ROS decrease the levels of cytosolic glutathione and contribute to an additional source of ROS from the mitochondria [15] and result in cell death.

One of the most important cellular defense mechanism against excess ROS is regulated by NF-E2-related factor 2 (Nrf2), a PERK-dependent master transcriptional activator [7,8], which regulates many of the antioxidant defense genes, including glutathione reductase (GR), glutathione-S-transferase, thioredoxin, thioredoxin reductase, and other antioxidant enzymes [48]. In addition, there are several cytosolic kinases, including PERK [7,8], protein kinase [3,15], phosphatidylinositol 3-kinase [19], mitogen-activated protein kinase [47] that have been shown to modify Nrf2. Under unstressed conditions, the Kelch-like ECH associated protein 1 (Keap1), a negative regulator of Nrf2, interacts with Nrf2 and forms the Nrf2/Keap1 complexes, thereby Nrf2 is ubiquitinated and degraded by proteosomal degradation (Fig. 1). However, under stressed conditions, P-Nrf2/Keap1 complexes are translocated into the nucleus and P-Nrf2 binds to the antioxidant response element (ARE) to activate the multiple antioxidant genes [48].

The purpose of this study was to determine whether high levels of Hcy induce the UPR, ROS production, and activates the antioxidant defense system, but high Hcy eventually failed to activate the Nrf2-dependent antioxidant defense protection mechanism.

2. Materials and methods

2.1. Human lens epithelial cells (HLECs) culture

For cell growth, HLECs were cultured overnight in 25 mM glucose- Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen Corp., Carlsbad, CA) with 10% fetal calf serum (FCS) at 37 °C under 20% atmospheric oxygen. The HLECs were pre-cultured overnight in 5 mM glucose DMEM under 4% atmospheric oxygen and these cells were used for all experiments. Some cells were cultured in glucose-free (GF)-DMEM with 10% or no FCS at 37 °C at 1% and 4% atmospheric oxygen. The 1% and 4% atmospheric oxygen conditions were generated in an O_2/CO_2 incubator (Sanyo, Osaka, Japan), and 20% atmospheric oxygen was generated in a tissue culture incubator (ThermoForma, San Diego, CA).

2.2. Rat lens organ culture

The experimental procedures used on the rats were approved by the University of Nebraska Animal Care and Use Committee and were in compliance with the Animal Welfare Act (Public Law 91–579) as mandated by the NIH Guide for Care and Use of Laboratory Animals. Clinical veterinary services were provided through the University Nebraska Medical Center, and the procedures recommended by the Association for Research in Vision and Ophthalmology resolution on the use and treatment of animals in ophthalmic and vision research were followed. Sprague Dawley (SD) rats (Charles River Laboratories Inc., Wilmington, MA) were euthanized by CO_2 inhalation, the eyes were enucleated and the lenses were isolated while viewing the eye under a microscope. The isolated lenses were cultured in 2 mL of modified medium-199 (Lonza eShop, Melnik, Czech Republic) [17] with penicillin (100 units/mL), and streptomycin (100 units/mL) (Invitrogen Corp., Gland island, NY) for 24 or 48 h at 37 °C under 4% atmospheric oxygen.

2.3. Intraocular injection of Hcy

One drop of local anesthetic solution, 0.5% proparacaine hydrochloride, was applied. The pupils of the SD rats were dilated with 1% Mydriacyl, 1% Cyclopentolate hydrochloride and then a mixture of ketamine (40–87 mg/kg) and xylazine (5–13 mg/kg) was used to anesthetize the rats. Three microliters of 10 mM Hcy was injected intravitreally into the eyes 1 times/day for 3 days. After 7 days, the animals were killed by CO_2 inhalation, and the eyes were enucleated for further examination.

2.4. Protein blot analysis

LECs were lysed in RIPA buffer (Cell Signaling Tech., Danvers, MA). Then 10–20 μg/μL of soluble proteins separated by SDS–PAGE were blotted onto polyvinylidene fluoride membranes. Then, the membranes were blocked with 5% nonfat dry milk for 1 h at room temperature before an overnight incubation with primary antibodies at 4 °C. After rinsing the membranes, they were incubated with secondary antibodies for 1 h at room temperature. Luminol reagent (Thermo Fisher Scientific Inc. Rockford, IL) was applied to the membrane, and the bands were detected using Bio-rad XRS system. Antibodies to Bip, caspase 4 (BD Biosciences, Franklin Lakes, NJ), P-PERK, IRE1α, P-eIF2α, ATF6, ATF4, CHOP, Ero1-Lα, Ero1-Lβ, Nrf2, P-Nrf2, glutathione reductase (GR), catalase, X-box binding protein 1 (XBP1), PDI (Santa Cruz Biotechnology, Santa Cruz, CA), and GAPDH (Novus Biological, Littleton, CO) were purchased. The intensity of each band was normalized to that of GAPDH, and the relative intensities were analyzed with ImageJ software [39].

2.5. Cell viability/death and ROS staining

Rat lenses or cultured HLECs were stained with a mixture of two probes; calcein AM and ethidium homodimer-1 (EthD) (Viability/Cytotoxicity Assay Kits; Biotium Inc., Hayward, CA) for 30 min as recommended by the manufacturer. The cytosolic ROS level was determined by adding 1 μg/mL of 2′,7′-dichlorodihydrofluorescein diacetate (H_2-DCFH-DA) (Invitrogen, Carlsbad, CA) in phosphate buffered saline (PBS) for 40 min at 20 °C, then washed twice with PBS. The specimens were examined with a fluorescent microscopic (Nikon, Eclipse TE2000-U, Tokyo, Japan). The oxidation of H_2-DC FH-DA requires either cytochrome *c* or both redox-active transition metals [20] which are presenting in the LECs.

2.6. Real-time PCR

Total RNA was extracted from the HLECs treated with or without varying concentrations of Hcy by Quick-RNA™ MicroPrep solution (Zymo Research Corporation, Orange, CA) following the manufacturer's instructions. Then the purified total RNA was reverse transcribed by iScript™ Reverse Transcription Supermix for real-time PCR (Bio-Rad, Hercules, CA) following the manufacture's protocol. The reverse transcribed RNA was analyzed by real-time PCR using the SsoFast™ EvaGreen® supermix (Bio-Rad, Hercules, CA). The primer sequences were designed using the OligoPerfect™ Designer software with instructions of Invitrogen for optimal primer design and were synthesized commercially. The primer sequences for *Nrf2, Keap1*, and β-actin are; Nrf2-F (5-ACACGGTCCA CAGCTCATC-3), Nrf2-R (5-TGCCTCCAAAGTATGTCAATCA-3), Keap 1-F (5-GGGTCCC CTACAGCCAAG-3), Keap1-R (5-TGGGGTTCCAGAAGATAAGC-3), β-actin-F (5-CCAACCGCGAGAAGATGA-3) and β-actin-R (5-CCAGAGG CGTACAGGGATAG-3). Each reaction was carried out in triplicate and three independent experiments were run. A standard curve was prepared using a serial dilution of a reference sample and was included in each real-time run to correct for possible variations in product amplification. The relative copy numbers were obtained from the standard curve and were normalized to the values obtained for β-actin, the internal control. The fold change in expression was then obtained by the $2^{-\Delta\Delta CT}$ method.

2.7. Proteasomal degradation and m-calpain cleavage of Nrf2

The HLECs were cultured with or without 15 μM proteasomal inhibitor, MG132, (Selleck Chemicals, Houston, TX) for 24 h. Then the cells were harvested and the lysate was prepared using RIPA buffer (Cell Signaling Tech., Danvers, MA). The protein samples were analyzed by western blots using the antibodies specific to Nrf2 and P-Nrf2 (Santa Cruz Biotechnology, Santa Cruz, CA). Similarly, the protein samples from mouse LECs cultured in the presence or absence of 100 μM m-calpain inhibitor, SJA6017, (Merck KGaA, Darmstadt, Germany) for 24 h were analyzed. The intensity of each band was normalized with that of GAPDH and presented as a relative intensity using the ImageJ analysis software [39].

2.8. Histology and DAPI staining

Rat lenses were fixed in 4% glutaraldehyde, embedded in paraffin, and sectioned at 7 μm (Histology Core Facility at UNMC). Photographs were taken after the thin sections were stained with DAPI. The images were examined with a fluorescent microscope filter.

2.9. Statistical analysis

The results were expressed as the mean ± SD, and statistical significance was evaluated by student's *t* test using the SPSS (version 15.0) program. Values were considered statistically significant when $p < 0.05$.

3. Results

3.1. Hcy induces lens opacity, cell death, and ROS production in rat and human LECs

Two sets of 4 isolated lenses from 5-month-old rats were cultured in 25 mM glucose-DMEM with/without 5 mM Hcy for 24 h and 48 h. The clarity of these lenses incubated with 5 mM Hcy decreased after 24 h in culture (Fig. 2A), and the opacity of these lenses became more dense with increased hydration at 48 (data not shown). However, lenses cultured without Hcy remained clear and not hydrated during this period.

To study the effect of Hcy *in vivo*, we injected 3–4 μL of 10mM Hcy (estimated from concentration of cell culture study presented in Figs. 4 and 5) in PBS into the vitreal cavity of 5 rats once a day for 3 days. Seven days after the last injection, the animals were killed and the extracted lenses were fixed in 4% formaldehyde. Thin sections were cut and stained with DAPI (Fig. 2B), and the number of DAPI-positive nuclei in the control and Hcy treated lens sections were compared (Fig. 2C).

Our results showed that the Hcy injected eyes had approximately 20% fewer LECs suggesting that Hcy had induced LEC death. We concluded that 10 mM Hcy is toxic for LECs and induced cell death, i.e., experimental Hcy-induced cataract in rat lenses.

Rat lenses exposed with 5 mM Hcy (determined by cell culture study presented in Figs. 4 and 5) or without Hcy (control) for 24 h in culture medium had significantly higher levels of ROS in the cortical region of the LECs but not in the lens fiber cells. This further suggested that higher levels of Hcy increase the levels of ROS in rat LECs (Fig. 3A).

HLECs were cultured in 5 mM Hcy in DMEM with 10% FCS for 10 h. The level of ROS and death of HLECs were significantly increased, and the increased level of ROS was found prior to the cell death (Fig. 3B and C), but no apparent cell death and the ROS production was found in LECs not treated with Hcy.

3.2. HLECs treated with higher levels of Hcy activate UPR-specific proteins

To determine when the UPR specific proteins were expressed in HLECs, the cells were cultured with 5 mMHcy for 3, 6, 12, and 24 h. Protein blot analyses showed that 12–24 h exposure was sufficient to induce the levels of UPR specific proteins in the HLECs (data not shown). Next, we determined the concentration of Hcy which would activate the expression of UPR specific proteins. HLECs were cultured in 5 mM glucose-DMEM with 10% FCS containing 0, 0.06, 0.18, 0.55, 1.66, 5, 15, and 45 mM Hcy for 24 h under 1% atmospheric oxygen. Protein blots were analyzed with antibodies specific to the UPR signaling proteins, viz., P-PERK, P-eIF2α, and IRE1α, showed no significant changes with 0.06 and 1.66 mM Hcy but significant decrease was seen (for P-PERK and IRE1α) with 5, 15 and 45 mM of Hcy, contrary, P-eIF2α was significantly increased (Fig. 4A). The antioxidant defense proteins, viz., Nrf2, P-Nrf2, Keap1, GR, and catalase and XBP1 did not change significantly with <5 mM Hcy. However they were significantly suppressed by 15 and 45 mM Hcy except Keap1 which was significantly increased (Fig. 4B). On the other hand, the ROS-associated enzymes, viz., PDI and Ero1-Lβ, were not change significantly at 15 and 45 mM Hcy. However, Ero1-Lα was decreased significantly which was expected because mild ER stress is known to suppress the Ero1-Lα but to activate Ero1-Lβ (Fig. 4C). The UPR mediator proteins, ATF4, CHOP, and caspase-4, were not changed significantly at all Hcy concentrations suggesting that the UPR pathway must be active even at 15 and 45 mM Hcy (Fig. 4D). These results indicated that UPR was activated in the cultured HLECs with higher levels of Hcy within 24 h.

3.3. Prolonged exposure of Hcy also suppresses Nrf2-dependent antioxidant protection

ER stress is induced by combination between incubation time and the concentration of ER stressors. Here, we would like to show that low level of Hcy with prolonged exposure also induce the ER stress. We cultured HLECs with 0.06, 0.18, 0.55, 1.66, and 50 mM Hcy for 48 and 72 h and found that the levels of Nrf2, catalase, and GR were decreased after 48 h of incubation and significantly reduced after 72 h in 5 mM Hcy (Fig. 5).

3.4. HLECs treated with lower levels of Hcy without FCS activate UPR-specific proteins

We used very high concentrations of Hcy in our above experiments (Fig. 4), because 80% of Hcy binds to serum proteins, albumin with disulfide bond and free Hcy are taken up by HLECs. HLECs were cultured in 100 μM Hcy in serum-free DMEM for 0.5, 1, 2, and 4 h, and the levels of UPR-specific proteins were determined. The levels of Nrf2, Keap1, and ATF6 were significantly up-regulated at 1 h (Fig. 6) and then decreased. P-PERK was also slightly increased at 2 h but did not decrease later (Fig. 6C). Thus, removal of FCS from the culture medium generated the UPR in HLECs by Hcy at near physiological concentrations. Thus, near physiological concentrations of Hcy without FCS in the culture medium generated the UPR in HLECs. Control experiments (FCS free medium without Hcy) did not induce the UPR (data not shown) suggesting that serum starvation might not affected within 4 h culture.

3.5. Quantification of the mRNA of Nrf2/Keap1 in HLECs treated with Hcy

One of our interests is why the levels of Nrf2 decreased but the Keap1 protein was increased in HLECs treated with higher levels of Hcy. We examined the effects of Hcy on the transcription of the *Nrf2* or *Keap1* genes in HLECs. HLECs were cultured in different concentrations of Hcy and total mRNAs were extracted. The mRNAs of Nrf2 and Keap1 were determined quantitatively by RT-PCR analysis (Fig. 7). The levels of Nrf2 and Keap1 mRNAs were found to be relatively constant between 0.06 and 1.66 mM Hcy. The mRNA of Nrf2 increased by 1.5 folds in 15 and 45 mM Hcy and Keap1 was slightly increased in 45 mM. These results suggested that the suppression of Nrf2 and significant increase of Keap1 might not be at the transcriptional level.

3.6. Effect of decreased Nrf2 on phosphorylation and nuclear translocation of Nrf2

Under unstressed condition, Nrf2 is known to bind with Keap1, become ubiquitinated, and is constantly eliminated by proteasomal degradation [41]. However, under stressed conditions, the Nrf2 is phosphorylated and dissociated from the Keap1 protein, then translocated into the nucleus where P-Nrf2 binds to the AREs present in the promoter region of several antioxidant genes to activate them. To study the nuclear translocation of P-Nrf2, HLECs were cultured for 1, 4, and 8 h in DMEM containing 5 mM Hcy, and then the nuclear and cytoplasmic proteins were separated. These protein preparations were immunoblotted with antibodies specific to P-Nrf2. P-Nrf2 (150 kDa) was predominantly found in the nucleus and not in the cytoplasm (Fig. 8A). This suggested that P-Nrf2 was translocated into the nucleus from the cytoplasm.

To determine whether the Nrf2/Keap1 complex was present in HLECs, Nrf2 and Keap1 were immunoprecipitated from the HLEC lysate with polyclonal antibody specific to Nrf2. This precipitate was separated by protein blot analysis and stained with monoclonal antibody specific to Nrf2. The predominant band was native Nrf2 (66 kDa; Fig. 8B). The precipitate obtained with polyclonal Keap1 antibody and blotted with monoclonal Nrf2 antibody had only the Nrf2 band suggesting that the Nrf2/Keap1 complex had definitely formed. Additionally, the lysate was immunoprecipitated with Keap1 polyclonal antibody, and then blotted with monoclonal antibody specific to Keap1. Protein blot analysis showed a

77 kDa Keap1 band (Fig. 8B). These results clearly indicated that Keap1/Nrf2 complexes were present and were transported into the nucleus in the HLECs.

3.7. Decline of Nrf2 was predominantly proteosomal degradation with minor activation of m-calpain and caspase under chronic ER stress in HLECs

We next investigated whether Nrf2 might be rapidly degraded by proteasomal degradation. To do this, HLECs were cultured with or without a proteosomal inhibitor (MG132) for 24 h, and then the levels of Nrf2 and P-Nrf2 were determined by protein blot analysis (Fig. 9A and B). Exposure to MG 132 clearly blocked the degradation of Nrf2 and P-Nrf2 indicating that Nrf2 must undergo proteosomal degradation.

Because the UPR releases Ca^{++} into the cytoplasm from the ER [41], we hypothesized that m-calpain [2,36] might cleave the Nrf2 protein in the HLECs. To test this, HLECs were treated with 15 mM Hcy with an addition of 1 mM $CaCl_2$ in DMEM (DMEM contains 1.25 mM of Ca^{++}), and the m-calpain inhibitor, SJA6017, for 24 h. The cleavage of native Nrf2 was not increased to detectable levels after the exposure (data not shown). Because the human lens contains only 3% of the m-calpain activity of rodents [36], we speculated that m-calpain activity in the human lens is not high enough to detect Nrf2 cleavage under our experimental conditions.

We used mouse LECs culture to further evaluate the m-calpain activity. Our results showed that the degradation of the Nrf2 and P-Nrf2 was significantly suppressed by the m-calpain inhibitor, SJA6017 (Fig. 9C and D). There are 48 cleavage sites for Nrf2 by m-calpain but Keap1 has only 3 sites (evaluated by the GPS-CCD program) [21]. This indicated that m-calpain played a role in Nrf2 degradation in mouse LECs as well as in HLECs.

Activated m-calpain also cleaves procaspase-4 to generate an enzymatically active form of caspase-4, which further activates multiple caspases including caspase-3. Nrf2 is a substrate of caspase- 3 and it has two caspase-3 cleavage sites at D208 and D366 in the total 589 aminoacid residues. The cleavage generates a 30 kDa and a 50 kDa peptide (Fig. 10C), which can be detected by antibody specific to the C-terminus peptide of Nrf2 [28]. HLECs lysates were prepared and the lysates were incubated with or without a caspase inhibitor, e.g., Ac-YVAD-CHO or Ac-DEVD-CHO. There was no detectable degradation in native Nrf2 protein (66 kDa), and the cleaved 30 kDa protein was not present after exposure to either Ac-YVAD-CHO or Ac-DEVD-CHO inhibitors. These findings suggested that caspase-3 does not play a significant role in cleaving native Nrf2, but it is clear that the levels of degraded product (30 kDa) were suppressed by caspase-3 and -1 inhibitors (Fig. 10A). To validate this observation, the lens lysate was incubated at 37 °C and bands of 50 and 30 kDa were increased after 80 min (Fig. 10B) of incubation suggesting that caspase-3 and -1 activity are present in the HLECs at very low levels.

4. Discussion

Higher levels of Hcy are associated with adverse results in patients although a direct causal relationship was not found between high levels of Hcy and ARCs. Our results showed that 100 μM of Hcy is sufficient to induce the UPR within 1–2 h in HLECs cultured without FCS but >5 mM Hcy is required for HLECs cultured in 10% FCS. There was also an increased expression of ROS, and significant degradation of Nrf2 which resulted in an accumulation of ROS in the HLECs. These results combined with the findings in ARC patients with higher levels of Hcy [50] and in young homocystinuria patients with juvenile cataracts [40] suggest that elevated levels of Hcy are closely associated with cataracts.

Systemic disorders such as diabetes, obesity, malnutrition including vitamin deficiencies, and other systemic disorders [44] increase with increasing age. These changes with increasing age are consistent with the findings that supplements of the vitamin B groups and antioxidants protect or postpone ARCs [4,5,9,31]. The results of the Framingham Heart Study showed that the vitamin levels and vitamin intake are primary determinants (67%) of high Hcy in the elderly [40].

The level of Hcy in patients with hereditary homocystinuria due to methylene-tetrahydrofolate-reductase polymorphism is significantly elevated [4,5], and these patients have a higher incidence of thrombosis and cardiovascular disease [4,5], lens dislocation, glaucoma, and cataracts [9,31,49]. In addition, there is strong evidence to support the idea that Hcy accelerates the aging process at the cellular and at the organismic levels [30]. The Hcy level in the plasma ARC patients has been found to be between 16 and 34 mM which are lower than the concentrations used in the present experiments. In addition, about 80% of Hcy in the plasma is bound to albumin [35] or fibronectin [23] by a disulphide bond. However, the remaining 20% exists as free from disulphide [19]. The Hcy in the plasma remains in the patient for long time, and the disulfide bond between albumin and Hcy can be dissociated intraocularly because it is a highly reduced environment. Then, the free-Hcy can be internalized into LECs by the membrane transporter of cysteine [16]. Thus, elevated levels of Hcy in the lens can induce significant damage to the lens cells which results in the development and progression of cataracts. The lens generates 80% ATP from anaerobic and 20% aerobic respiration. We would like to emphasize that anaerobic respiration does not generate ROS from the mitochondria, however we are not sure how much aerobic respiration is present in the old HLECs.

The activation of UPR induces the Ca^{++} release from the ER, thereby increase the concentration of cytosolic Ca^{++}, and up-regulates the m-calpain activity. These changes then lead to cleavage of procaspase-4 into active caspase-4 which in turn activates multiple caspases including caspase-3 and -1. These caspases also cleave crystalline proteins [27,37]. Most ARCs contain elevated levels of Ca^{++} in the lens, and the Ca^{++} influx is considered to be due to the activation of Ca^{++} dependent proteinases including m-calpain [2,10,36]. On the other hand, our results suggest that the cataractogenic stress induced the UPR which increase the release of Ca^{++} from the ER. The amount of Ca^{++} was sufficient to activate m-calpain to cleave the lenticular proteins. Increased Ca^{++} in the culture medium further activates m-calpain. Thus, Ca^{++} released from the ER must be the initial event in the cascade and repeated ER stress/UPR further induces damage in the lenses of the elderly. These changes further upset the Ca^{++} homeostasis and result in higher levels of Ca^{++} and protein degradation in the lens.

LECs have strong antioxidant defense mechanisms that can neutralize ROS and maintain the redox balance. One of the important cellular defense mechanisms against ROS is mediated through the activation of the nuclear transcriptional factor, Nrf2, which binds to the ARE present in the promoter DNA of many antioxidant genes. Nrf2 has been shown to function as a molecular switch to turn on and off the Nrf2-mediated antioxidant response. Under normal conditions, the Nrf2 inhibitor, Keap1, is in the off position and is constantly targeting the ubiquitination and degradation of Nrf2, and the level of Nrf2 in the lens is very low (Fig. 1). When cells are under ER stress, the switch is turned on which then inhibits the activity of Keap1/Nrf2 ubiquitin ligase, resulting in increased levels of Nrf2 and activation of its downstream antioxidant target genes (Fig. 1). However, the switch is turned off again upon elimination of the UPR, and Keap1 is transported back into the nucleus to remove Nrf2 from the ARE. The Nrf2/Keap1 complex is then transported out of the nucleus by Keap1. In the cytosol, the Nrf2/Keap1 complex is associated with the core ubiquitin machinery

resulting in the degradation of Nrf2. Thus, these switches have to be blocked under strong ER stress.

We recently reported that the Nrf2-dependent antioxidant protection was decreased in diabetic eyes with ARCs [29] due to the promoter DNA demethylation of the Keap1 gene. Keap1 is a negative regulatory protein of Nrf2 and increased Keap1 stimulates the Nrf2 degradation due to proteosomal degradation, and the reduced Nrf2 suppresses the Nrf2-dependent antioxidant protection. The results of this study demonstrated an analogous Nrf2 degradation pathway in which higher levels of Hcy stimulated the proteosomal degradation of Nrf2. Thus, the antioxidant protection might be regulated at the transcriptional level and proteosomal degradation levels, and a failure of the Nrf2-dependent oxidative stress protection resulted in oxidation of the HLECs and cataract formation.

A decrease of Hcy can be achieved by increased uptake of the vitamin B groups in the diet, and it is known that vitamin B_6, B_{12}, and folate delay the development of ARCs in humans. The use of multivitamin-mineral supplements increases the plasma vitamin levels and increases the GSH reductase activity in the elderly population [6,24].

5. Conclusion

Aging, combined with environmental and genetic stresses, are considered to be the main contributors to the oxidation, modification, and aggregation of lenticular proteins. The cause of the generation of ROS in cataractogenic stresses remains unclear. Our findings revealed that higher levels of Hcy led to chronic UPR, production of ROS, proteosomal degradation of Nrf2 and release of Ca^{++} from the ER, activation of m-calpain, further proteolysis of Nrf2 and resulted in the failure of the Nrf2-dependent antioxidant defense protection. Thus, Hcy can be considered to be a cataractogenic stressor, and its elevation with increasing age generates the lens oxidation and proteolysis of lenticular proteins ultimately leading to the formation of ARC.

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Abbreviations

ARC	age-related cataract
ARE	antioxidant response element
ATF4	activating transcription factor 4
ATF6	activating transcription factor 6
Bip	ER-resident chaperone protein
CHOP	C/EBP homologous protein
DAPI	4′, 6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
eIF2α	eukaryotic translation initiation factor 2α
EthD	ethidium homodimer-1
Ero1-Lα and -Lβ	ER oxidoreductin 1-Lα and -Lβ

ER	endoplasmic reticulum
FCS	fetal calf serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GR	glutathione re ductase
GSH	free glutathione
H_2-DCFH-DA	2′,7′-dichlorodihydrofluorescein diacetate
Hcy	homocysteine
HLECs	human lens epithelial cells
IRE	ER resident, transmembrane endoribonuclease
Keap1	Kelch-like ECH-associated protein 1
LEC	lens epithelial cells
Nrf2	nuclear factor-erythroid-2-related factor 2
PDI	protein disulfide isomerase
PERK	PKR-like endoplasmic reticulum kinase
PKC	protein kinase C
ROS	reactive oxygenspecies
RT-PCR	real time-polymerase chain reaction
UPR	unfolded protein response
XBP1	X-box-binding protein 1

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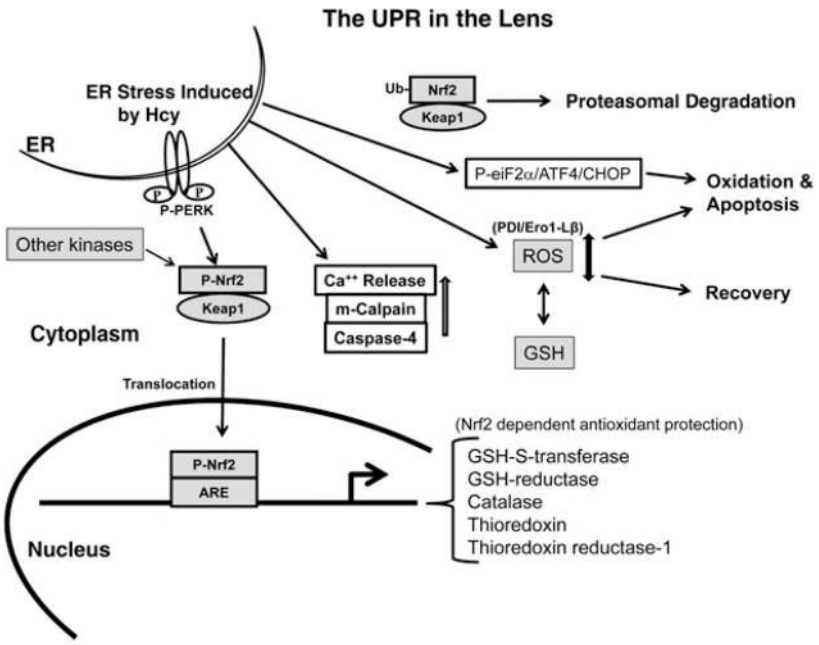

Fig 1.

Schematic diagram of our working hypothesis for the development of Hcy induced cataracts. Homocysteine induces the UPR. The UPR is a protective mechanism against ER stress, and its activation leads to the expression of P-PERK. Prolonged or severe ER stress induces the apoptotic UPR which generates excess levels of ROS and releases Ca^{++} from the ER to activate m-calpain. Under basal conditions, Nrf2 binds to Keap1 and is ubiquitinated for proteasomal degradation. P-PERK phosphorylates Nrf2, the P-Nrf2 dissociates from Keap1 and translocates into the nucleus to bind to the antioxidant response element (ARE), which activates the transcription of more than 200 stress associated genes including 20 antioxidant enzymes such as glutathione, glutathione reductase (GR), thioredoxin, thioredoxin-S-transferase, and catalase. These antioxidant enzymes regenerate GSH, and the resultant GSH eliminates ROS to survive or recover from the stress. We hypothesized that high Hcy suppresses the levels of Nrf2, which down-regulates the antioxidant enzymes and GSH, and these changes result in oxidation of the lens. Thus, high Hcy activates the UPR, production of ROS, and dysfunction of the Nrf2 dependent antioxidant defense protection leading to lens oxidation.

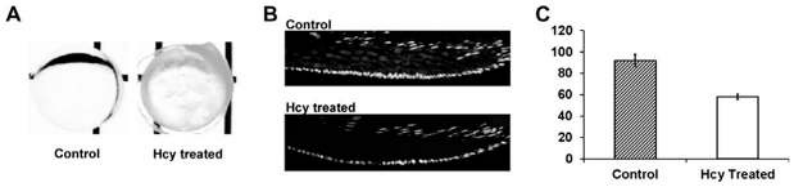

Fig 2.

Cataract formation and loss of LECs in rat lenses treated with Hcy. Lens opacity in SD rat lenses treated with 5 mM of Hcy for 24 h (A). Lens epithelial cells death in rats treated with intravitreal injection of 3 μL of 10 mM Hcy. Fixation, histology, DAPI staining, and photography were done 7 days after the last Hcy injection (B). Relative lens epithelial cell numbers in the 5 rat lens treated with Hcy and 5 untreated control lens (C). These results revealed that Hcy is highly toxic to HLECs.

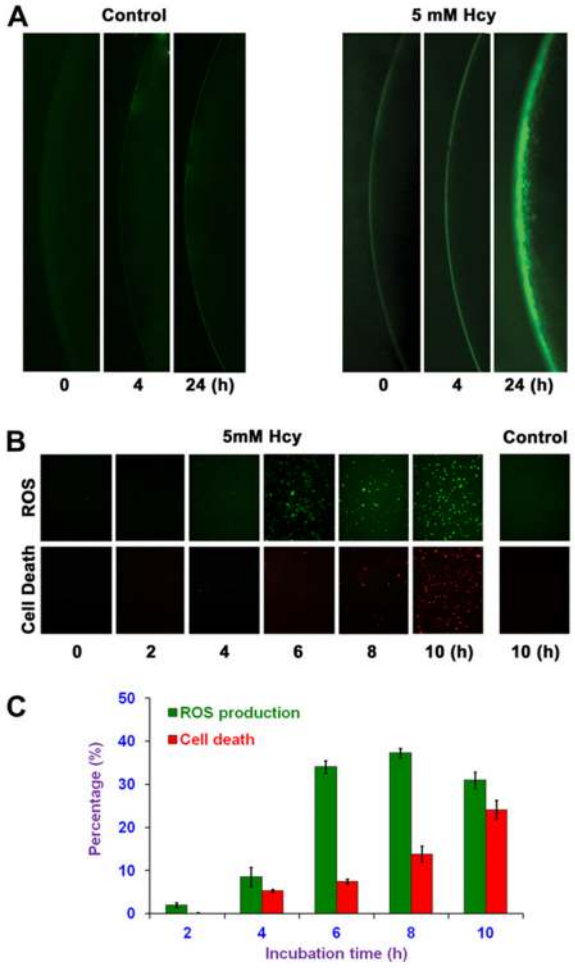

Fig 3.

Production of ROS in rat lenses and HLECs exposed to Hcy. (A) Results of representative experiments of ROS production in rat lens cultured with 5 mM Hcy under 1% atmospheric O_2 for 0, 4, and 24 h and then stained with H_2-DCFH-DA. The photographs were taken by a Nikon Fluorescent microscope with a green filter as described [11]. (B) Results of representative experiments of ROS production and cell death in HLECs cultured under 1% atmospheric O_2 for 0, 2, 4, 6, 8, and 10 h and then stained with H_2-DCFH-DA for ROS production or EthD for cell death. (C) Percentage of ROS and EthD positive cells. The results are the mean ± SD of three experiments for each staining.

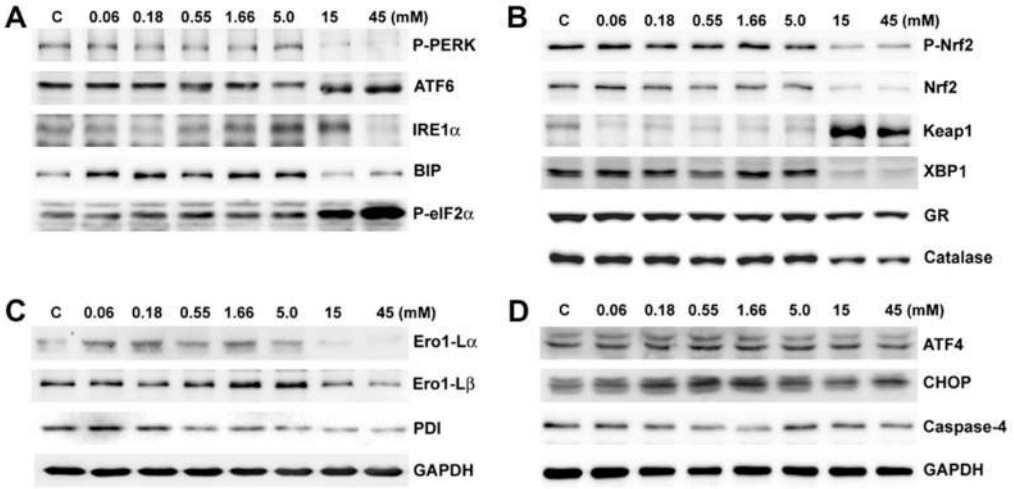

Fig 4.

Protein blot analysis of UPR specific proteins in HLECs treated with Hcy for 24 h. (A) Representative Western blots for UPR signaling proteins such as P-PERK, ATF6, IRE1α, Bip and P-eIF2α of HLECs cultured with different concentrations of Hcy (0, 0.06, 0.18, 0.55, 1.66, 5, 15 and 45 mM) for 24 h. (B) Representative Western blots of antioxidant proteins such as, P-Nrf2, Nrf2, Keap1, XBP1, GR, and catalase. (C) Representative Western blots of ROS associated enzymes, Ero1-Lα, Ero1-Lβ and PDI of HLECs. (D) Representative Western blots of apoptosis associated enzymes such as ATF4, CHOP, and caspase-4. Intensity of each band is normalized to intensity of GAPDH. GAPDH shown in (C) is common to (A). Similarly, GAPDH of (D) is common to (B).

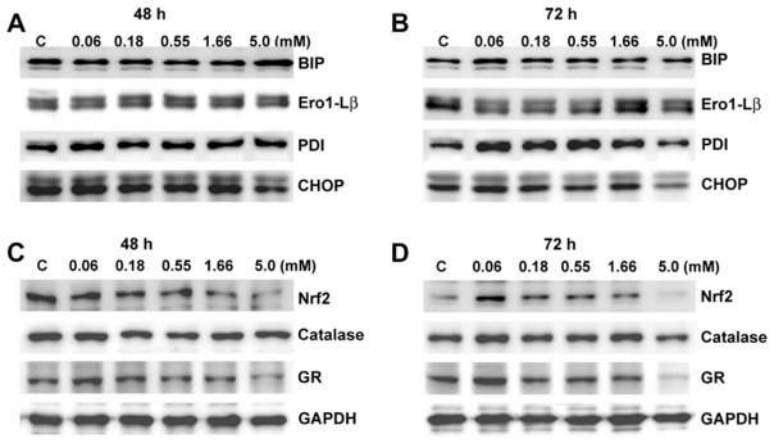

Fig 5.

Protein blot analysis of UPR specific proteins in HLECs cultured for 48 and 72 h. Representative Western blots for Bip, Ero1-Lβ, PDI, and CHOP of HLECs cultured in different concentrations of Hcy for 48 h (A) and 72 h (B). Representative Western blots of antioxidant proteins, Nrf2, GR, and catalase of HLECs cultured in different concentration of Hcy for 48 h (C) and 72 h (D). Intensity of each band was normalized to that of GAPDH. GAPDH shown in (C) is common to (A). Similarly, GAPDH of (D) is common to (B).

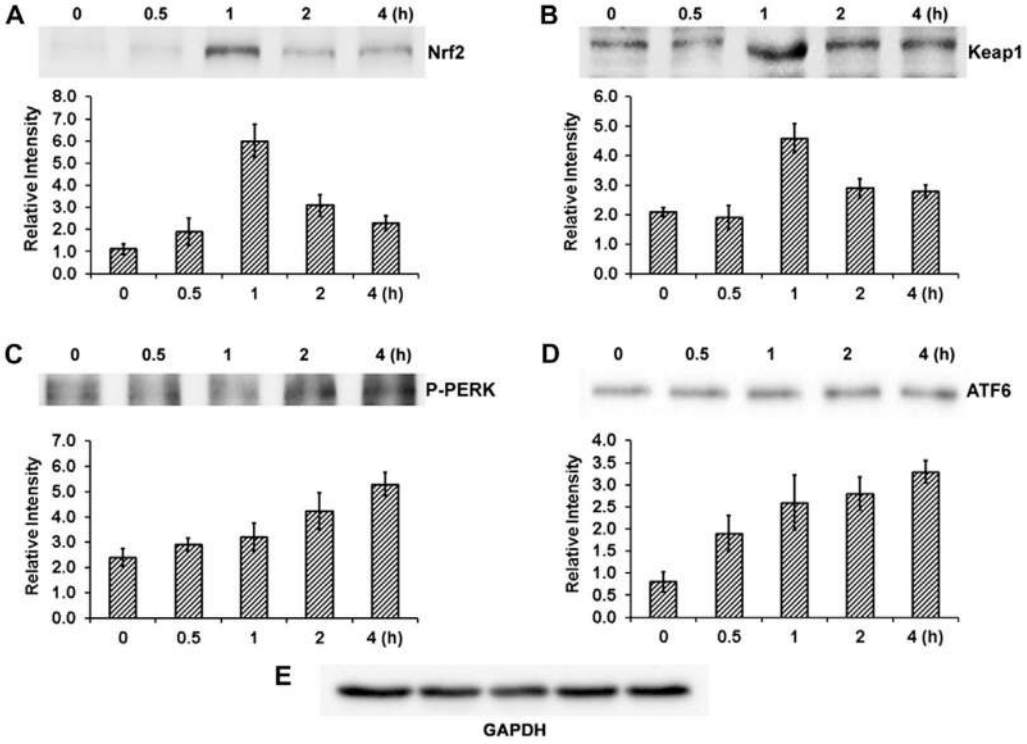

Fig 6.

Expression of UPR specific proteins in HLECs treated with 100 μM of Hcy without FCS. Representative Western blots of Nrf2 (A), Keap1 (B), P-PERK (C) and ATF6 (D) of HLECs cultured with 100 μM of Hcy for 0, 0.5, 1.0, 2 and 4 h. (E) Representative Western blot of GAPDH of HLECs cultured with 100 μM of Hcy for 0, 0.5, 1.0, 2 and 4 h. The intensity of each band was normalized to the intensity of GAPDH. The results are the mean ± SD of three experiments for each protein.

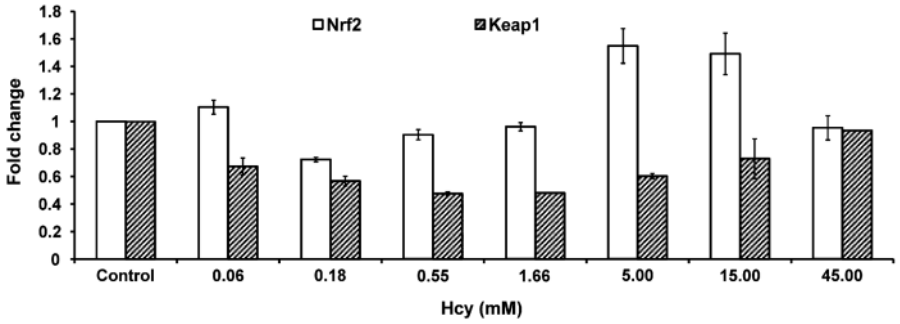

Fig 7.

Quantitative RT-PCR of Nrf2 and Keap1 mRNAs in HLECs treated with different concentrations of Hcy. Total RNA was extracted, treated with reverse-transcriptase, and amplified with two primers described in the Section 2.6 [11].

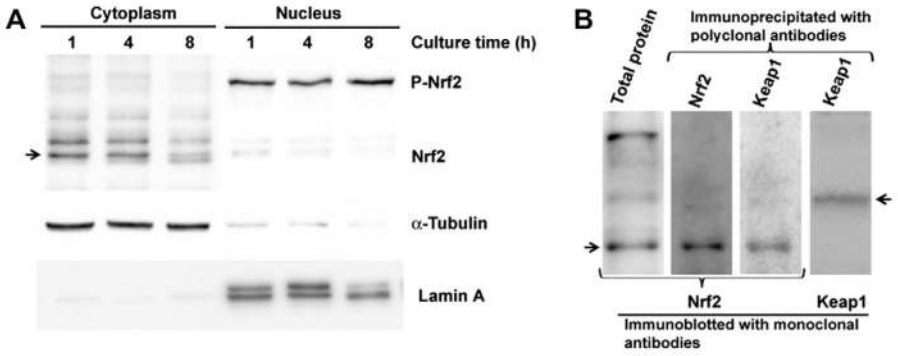

Fig 8.

Nuclear localization of P-Nrf2 and Keap1/Nrf2 complex formation. (A) Representative western blot analysis of P-Nrf2 and Nrf2. HLECs were treated with 5 mM Hcy for 1, 4, and 8 h, and the cells were harvested and the nuclear and cytoplasmic proteins separated. These fractions were submitted to protein blot analysis with antibodies specific to P-Nrf2 and Nrf2. Lamin A was used as a nuclear marker, and α-tubulin as a cytoplasmic marker for the protein blot analysis. (B) Representative protein blot analysis of Nrf2/Keap1 complex formation. HLECs were cultured for 24 h in the presence of 5 mM Hcy and then cell lysates were prepared. Initially, polyclonal antibody specific to Nrf2 or Keap1 was added to 100 mL of the HLECs lysate and immunoprecipitated. After 3 washings with PBS, the precipitates were applied onto SDS–PAGE electrophoresis and blotted and then immunostained with monoclonal antibodies specific to Nrf2 or Keap1. The intensity of each band was normalized to the intensity of GAPDH.

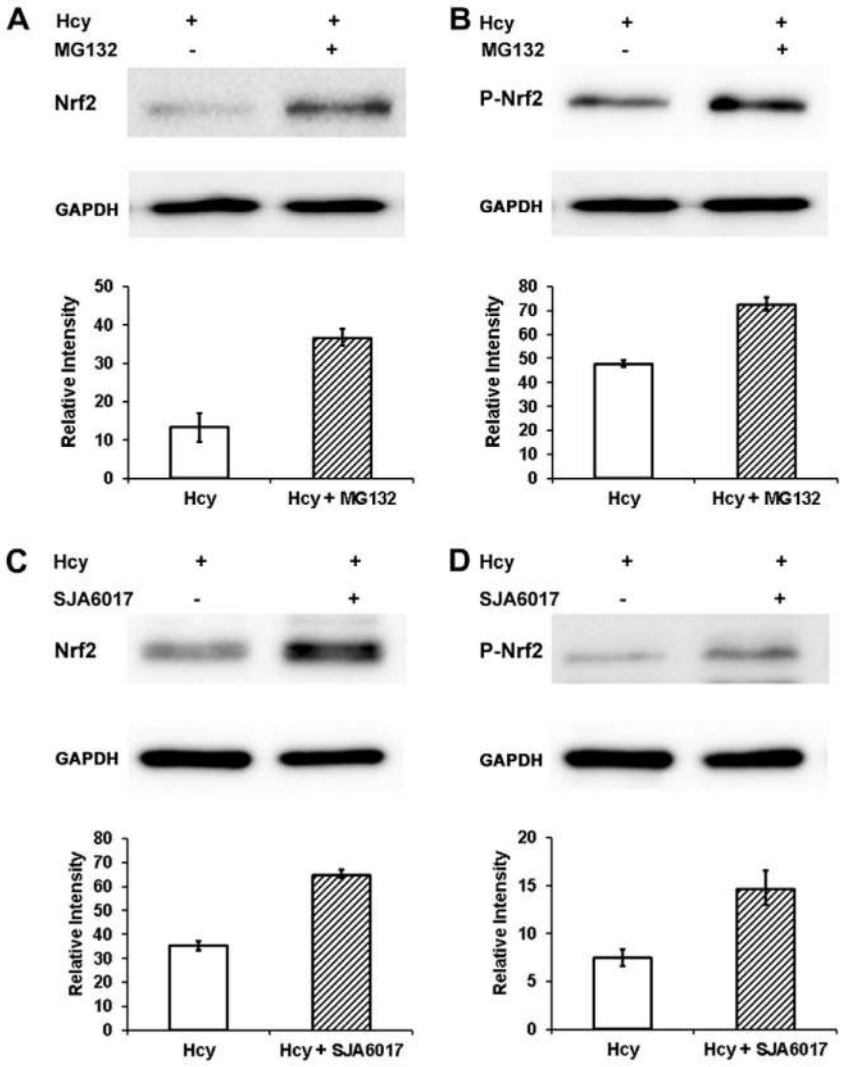

Fig 9.

Inhibitors of proteasome and m-calpain block Nrf2 or P-Nrf2 degradation. HLECs were treated with a proteosomal proteinase inhibitor, MG132 for 24 h and the resulting lysates were subjected to protein blot analysis with antibodies specific to Nrf2 (A) and P-Nrf2 (B). Mouse LECs were treated with m-calpain inhibitor, SJA6017 for 24 h and the resulting lysates were subjected to protein blot analysis with antibodies specific to Nrf2 (C) and P-Nrf2 (D).

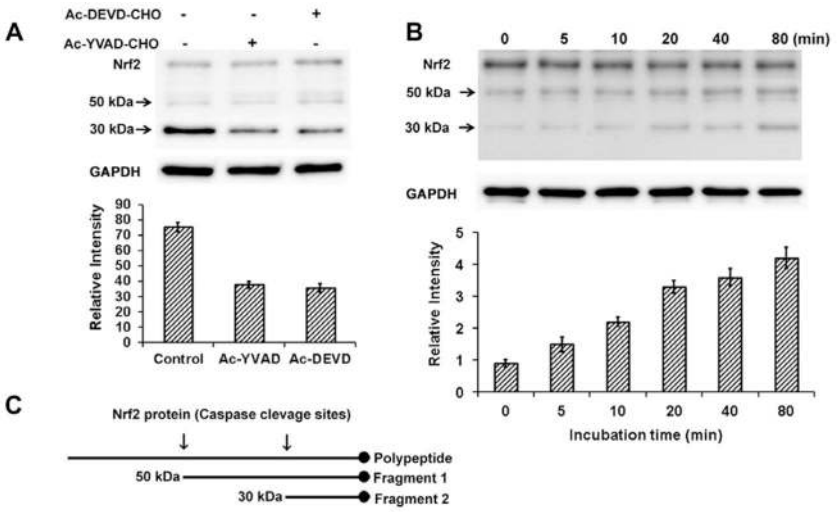

Fig 10.

Inhibitors of caspase-3 and -1 block Nrf2 or P-Nrf2 degradation. (A) Representative Western blot of Nrf2 showed the degraded products as 50 and 30 kDa polypeptides and normalized to the intensity of GAPDH. HLECs were treated with caspase-3 and -1 inhibitor (Ac-DEVD-CHO) (Ac-YVAD-CHO) for 24 h, and the resulting lysates were western blotted with antibody specific to Nrf2. (B) Representative Western blot of P-Nrf2 and Nrf2 in the LEC lysate incubated 0, 5, 10, 20, 40, and 80 min at 37 °C. (C) Schematic diagram of caspase-3 and -1 sites in the Nrf2. The Nrf2 antibody recognition site is indicated by black circle at the end of the carboxyl terminal.