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Original Contribution

Methylglyoxal induces endoplasmic reticulum stress and DNA demethylation in the *Keap1* promoter of human lens epithelial cells and age-related cataracts

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ABSTRACT

Age-related cataracts are a leading cause of blindness. Previously, we have demonstrated the association of the unfolded protein response with various cataractogenic stressors. However, DNA methylation alterations leading to suppression of lenticular antioxidant protection remains unclear. Here, we report the methylglyoxal-mediated sequential events responsible for *Keap1* promoter DNA demethylation in human lens epithelial cells, because Keap1 is a negative regulatory protein that regulates the Nrf2 antioxidant protein. Methylglyoxal induces endoplasmic reticulum stress and activates the unfolded protein response leading to overproduction of reactive oxygen species before human lens epithelial cell death. Methylglyoxal also suppresses Nrf2 and DNA methyltransferases but activates the DNA demethylation pathway enzyme TET1. Bisulfite genomic DNA sequencing confirms the methylglyoxal-mediated *Keap1* promoter DNA demethylation leading to overexpression of *Keap1* mRNA and protein. Similarly, bisulfite genomic DNA sequencing shows that human clear lenses ($n = 15$) slowly lose 5-methylcytosine in the *Keap1* promoter throughout life, at a rate of 1% per year. By contrast, diabetic cataractous lenses ($n = 21$) lose an average of 90% of the 5-methylcytosine regardless of age. Overexpressed Keap1 protein is responsible for decreasing Nrf2 by proteasomal degradation, thereby suppressing Nrf2-dependent stress protection. This study demonstrates for the first time the associations of unfolded protein response activation, Nrf2-dependent antioxidant system failure, and loss of *Keap1* promoter methylation because of altered active and passive DNA demethylation pathway enzymes in human lens epithelial cells by methylglyoxal. As an outcome, the cellular redox balance is altered toward lens oxidation and cataract formation.

Age-related cataracts (ARCs)[1] are a leading cause of visual impairment worldwide. The prevalence of ARCs escalates from 4% at age 52–64 to 50% at age 75–85. Diabetes is one of the potential risk factors, with fivefold increases in the incidence and progression of early cataract formation [1,2], influenced essentially by duration of the diabetes and the quality of glycemic control [1,3]. Being a non-insulin-dependent tissue, the lens is incapable of downregulating glucose transport. The increase in extracellular glucose concentrations leads to hyperglycemic deleterious effects [4], which include hyperosmolarity combined with oxidation [5], increased cytosolic Ca^{2+} [6], proteolysis [7], lens epithelial cell (LEC) death [8], LEC migration [9], and aging [10].

Hyperglycemia results in abnormal cellular accumulation of reactive aldehydes, including methylglyoxal (MGO), which is generated as

Abbreviations: H_2-DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; 5-Aza, 5-aza-2′-deoxycytidine; 5-mC, 5-methylcytosine; ATF, activating transcription factor; AID, activation-induced deaminase; AGE, advanced glycation end-product; ARC, age-related cataract; ARE, antioxidant-response element; CHOP, C/EBP-homologous protein; Dnmt1, DNA methyltransferase 1; eIF2α, eukaryotic translation initiation factor 2α; ER, endoplasmic reticulum; Ero1, ER oxidoreductin 1; GR, glutathione reductase; Glo-1, glyoxalase 1; HLEC, human lens epithelial cell; IRE1α, inositol-requiring enzyme 1α; Keap1, Kelch-like ECH-associated protein 1; LEC, lens epithelial cell; MGO, methylglyoxal; Nrf2, nuclear factor erythroid-2-related factor 2; PERK, PKR-like endoplasmic reticulum kinase; PSC, posterior subcapsular cataract; PDI, protein disulfide isomerase; ROS, reactive oxygen species; RT-qPCR, real-time quantitative PCR; TET1, 10-11-translocation 1; TDG, thymine DNA glycosylase; UPR, unfolded protein response

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a consequence of both nonenzymatic reactions of glucose with proteins [11] and the enhanced formation and resultant breakdown of triose phosphates within cells [12]. MGO reacts with arginine and lysine residues of lens proteins and generates protein adducts, resulting in MGO-derived advanced glycation end-products (AGEs), thereby altering the functions and conformation of lens proteins by crystallin aggregations, a key contributor to increased light-scattering in lens opacity [13–15]. In addition, MGO reacts with DNA, forming major DNA adducts linked to increased DNA strand breaks and frameshift mutation [16], mitochondrial dysfunction, and reactive oxygen species (ROS) formation [5,17,18].

Paradoxically, improper control of glycemia has been connected with activation of both oxidative and endoplasmic reticulum (ER) stress signaling pathways. A growing body of evidence suggests that ER stress triggers an evolutionarily conserved adaptive program known as the unfolded protein response (UPR), which combines the early inhibition of protein synthesis with a later upregulation of genes that stimulate protein folding or clearance [19,20]. UPR is mediated by three ER transmembrane proteins: inositol-requiring enzyme 1α (IRE1α), PKR-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6), thereby inducing overall gene expression changes to restore ER homeostasis [21,22]. Further, if ER stress is not alleviated, the prolonged UPR activates apoptosis by upregulating activating transcription factor 4 (ATF4), which promotes both the transcription of prosurvival genes and the expression of the proapoptotic transcription factor C/EBP-homologous protein (CHOP) and caspases [23,24]. Also, UPR upregulates intracellular ROS production and activates the transcriptional factor nuclear factor erythroid-2-related factor 2 (Nrf2), to protect the cellular redox homeostasis from oxidative damage by controlling the inducible expression of many cytoprotective genes [25,26].

Normally, Nrf2 is found in the cytosol by its binding with its negative regulator, Kelch-like ECH-associated protein 1 (Keap1). Under basal conditions, Nrf2 is constantly ubiquitinated by the E3-ubiquitin ligase-like domain of Keap1, an oxygen free radical sensor protein [27], followed by 26S proteasomal degradation [28]. Upon oxidative/ER stress, Nrf2 dissociates from Keap1 and then translocates into the nucleus and activates the transcription of antioxidant-response element (ARE)-containing genes [29]. Under terminal UPR, the level of Nrf2 decreases owing to proteasomal degradation and proteolysis by m-calpain and caspase-3 and caspase-1 [30,31]. Nrf2 also regulates the expression of the glyoxalase 1 (Glo-1) gene [32], which is a major MGO detoxification enzyme.

In addition, ER-induced oxidative stress targets Ca^{2+} release from ER calcium stores, which activates m-calpain in the lens [33]. Recent findings have suggested a direct link between the production of ROS and protein folding and oxidation, because oxidative stress and ROS production are integral components of ER stress and are not just consequences of ER stress induction [34]. The key enzymatic machinery of ROS production during UPR induction is driven by a protein relay involving a protein disulfide isomerase (PDI), ER oxidoreductin 1 (Ero1)-Lα and Ero1-Lβ, and molecular oxygen, a terminal electron acceptor [35,36].

Moreover, ROS produced by oxidative/ER stress stimulate the alterations in DNA methylation patterns, without changing the DNA base sequence. Such alterations in DNA methylation patterns are known to strongly control the expression of several genes [37]. DNA methylation patterns are established during embryonic development and are then accurately inherited in the cells with a "maintenance" mechanism, which involves the methylation of DNA daughter strands after replication by DNA methyltransferase 1 (Dnmt1). Failure of this maintenance mechanism leads to DNA demethylation, which can result from passive demethylation in the absence of Dnmt1 or from the prevention of Dnmt1 action after DNA replication [38]. In contrast, active DNA demethylation occurs through the enzymatic substitution of 5-methylcytosine (5-mC) with cytosine [39], in which the 10-11-translocation 1 protein (TET1) and the activation-induced deaminase (AID) convert 5-mC to thymine followed by base-excision repair by thymine DNA glycosylase (TDG) in nonreplicating cells [32,40–42].

Here, we hypothesize that MGO might induce overproduction of ROS, ER-stress-mediated activation of the UPR, suppression of Nrf2-dependent antioxidant protection, and alteration of passive and active DNA demethylation pathway enzymes, leading to reprogramming of gene expression. We further hypothesize that this sequence of events is responsible for *Keap1* promoter DNA demethylation in diabetic ARC lenses. To test this hypothesis, we have studied the cell death, ROS overproduction, Ca^{2+} release, and UPR induction and evaluated the levels of Nrf2/Keap1, active and passive DNA demethylation pathway enzymes, and promoter DNA methylation status of the *Keap1* gene in human lens epithelial cells (HLECs) treated with MGO. Our findings highlight the associations between induction of ER stress and UPR activation, ROS overproduction, Nrf2-dependent antioxidant system failure, and loss of *Keap1* promoter methylation because of altered active and passive DNA demethylation pathway enzymes in HLECs by MGO.

Materials and methods

Ethics statement

All animal experiments 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 the Care and Use of Laboratory Animals,* 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. Further, the approval and oversight of the Institutional Review Board are not required to conduct research on human lens samples for bisulfite genomic sequencing of human *Keap1* promoter DNA because this study did not obtain data through intervention or interaction with the human subjects and did not receive any identifiable private information from them. Therefore, Institutional Review Board approval for research involving leftover (excess) human biological material is not required. Human clear and diabetic cataractous lenses were obtained from National Disease Research Interchange (Philadelphia, PA, USA).

Cell culture

HLECs (SRA 01/04) [43] were cultured overnight in Dulbecco's modified Eagle's medium (DMEM), high glucose (Life Technologies, Gaithersburg, MD, USA), with 10% fetal bovine serum (Gemini Bio-Products, Calabasas, CA, USA) under 20% atmospheric oxygen at 37 °C. Cells were plated 24 h before experiments in DMEM, low glucose (Life Technologies), under 4% atmospheric oxygen and cultured with 10 μmol/L 5-aza-2′-deoxycytidine (5-Aza; Sigma–Aldrich, St. Louis, MO, USA) for 7 days or with varying concentrations of MGO, a generous gift from Dr. Keshore R. Bidasee (University of Nebraska Medical Center, Omaha, NE, USA), for 24 h. A 4% atmospheric oxygen environment was generated in an O_2/CO_2 incubator (Sanyo, Osaka, Japan) with an attached 50-gallon liquid nitrogen gas tank, because human lens is located in a hypoxic environment [44]. At the end of the experiment, the cells were harvested and used for cell death and intracellular ROS production assays, bisulfite genomic DNA sequencing, real-time quantitative PCR (RT-qPCR), and Western blotting.

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