Biochemical and Biophysical Research Communications 391 (2010) 346–351

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Methylglyoxal induces cellular damage by increasing argpyrimidine accumulation and oxidative DNA damage in human lens epithelial cells

Junghyun Kim, Nan Hee Kim, Eunjin Sohn, Chan-Sik Kim, Jin Sook Kim *

Diabetic Complications Research Center, Division of Traditional Korean Medicine (TKM) Integrated Research, Korea Institute of Oriental Medicine, Daejeon, South Korea

ARTICLE INFO

Article history:
Received 3 November 2009
Available online 12 November 2009

Keywords:
Apoptosis
Argpyrimidine
Lens epithelial cell
Methylglyoxal
Oxidative stress

ABSTRACT

Methylglyoxal (MGO) is a cytotoxic metabolite and modifies tissue proteins through the Maillard reaction, resulting in advanced glycation end products (AGEs), which can alter protein structure and functions. Several MGO-derived AGEs have been described, including argpyrimidine, a fluorescent product of the MGO reaction with arginine residues. Herein, we evaluated the cytotoxic role of MGO in human lens epithelial cell line (HLE-B3). HLE-B3 cells were exposed to 400 μM MGO in the present or absence of pyridoxamine for 24 h. We then examined the formation of argpyrimidine, apoptosis and oxidative stress in HLE-B3 cells. In MGO-treated HLE-B3 cells, the accumulation of argpyrimidine was markedly increased, and caspase-3 and 8-hydroxydeoxyguanosine (8-OHdG) were highly expressed, which paralleled apoptotic cell death. However, pyridoxamine (AGEs inhibitor) prevented the argpyrimidine formation and apoptosis of MGO-treated HLE-B3 cells. These results suggested that the accumulation of argpyrimidine and oxidative DNA damage caused by MGO are involved in apoptosis of HLE-B3 cells.

Advanced glycation end products (AGEs) are the late products of non-enzymatic glycation. Chronic hyperglycemia leads, through non-enzymatic glycation such as Schiff base and Amadori reactions, to the formation of AGEs [1]. AGEs bind to specific receptors on cells, where they can damage the affected tissues by causing intracellular oxidative stress as well as the synthesis of growth factors and cytokines [2]. Evidences suggest that AGEs contribute to the development of diabetic cataract [3]. In addition, AGEs stimulate a variety of cellular responses on several cell types including lens epithelial cells (LECs) [4].

Methylglyoxal (MGO, 2-oxopropanal) is a reactive dicarbonyl precursor of AGEs [5]. MGO originates from various biochemical pathways, including the dephosphorylation of glycolytic intermediates, metabolites of the polyol pathway, and from aminoacetone metabolism [6]. The concentration of MGO is much higher in the plasma of diabetic patients [7] and diabetic animal tissues [8]. MGO can modify amino acids, nucleic acids and proteins, and arginine appears to be a primary target in protein modification [9,10]. Several arginine modifications have been described and one major modification is argpyrimidine which is a blue fluorescent AGE [11]. It has been detected in the human lens and in cultured bovine retinal endothelial cells [12,13]. MGO modification increases the susceptibility of cataract development [14]. In addition, MGO is a cytotoxic compound and can induce stress in cells. It shows significant anti-proliferative properties as it can bind to nucleic acid and proteins, forming stable adduct [15]. MGO also induces apoptosis of rat Schwann cells [16], human vascular endothelial cells [17], rat mesangial cell [18] and bovine retinal pericytes [19]. It was reported that MGO-induced reactive oxygen species (ROS) production may trigger apoptosis [15,20].

The vertebrate lens is a nonvascularized and noninnervated organ and contains only a single layer of epithelial cells in its anterior surface. This epithelium is thought to protect underlying fibers from injury [21] and maintain the transparency of lens [22]. The damage of LECs is considered to be important in perturbing lens homeostasis. The exposure to high glucose has deleterious effects on LEC biochemistry and function [23]. Li et al. revealed that the blockade of apoptosis reduced the formation of cataract [22,24]. These previous results support the importance of LECs in cataract formation, but the exact mechanisms that underlie the alteration of these cells are not completely understood.

Recently, MGO has been proposed for the potential causative factor of diabetic cataract [25]. In diabetic rat lenses its levels have been shown to be raised [26]. Some studies have suggested that LECs undergo apoptosis in sugar cataract [27]. A recent study has suggested a direct role for AGEs in such LECs dysfunction [4]. However, the underlying mechanisms of MGO cytotoxicity in LECs have remained uncertain. Thus, in this study, we investigate the cytotoxic role of MGO in human lens epithelial cells (HLE-B3). We further examine the formation of argpyrimidine in HLE-B3 cells that

* Corresponding author. Address: Diabetic Complications Research Center, Division of Traditional Korean Medicine (TKM) Integrated Research, Korea Institute of Oriental Medicine (KIOM), 483 Exporo, Yuseong-gu, Daejeon 305-811, South Korea. Fax: +82 42 868 9471.
E-mail address: jskim@kiom.re.kr (J.S. Kim).

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doi:10.1016/j.bbrc.2009.11.061

were cultured with MGO. Moreover, we verify the hypothesis that argpyrimidine deposits in LECs and promotes the formation of oxidative DNA damage, thus fostering their apoptosis in LECs.

Materials and methods

Cell culture and treatment. HLE-B3 cells, a human lens epithelial cell line immortalized by SV-40 viral transformation, were obtained from the ATCC. Cells were cultured in minimal essential media (MEM) with 10% FBS at 37 °C in an incubator with 5% CO_2. Cells were plated onto appropriate culture dishes and used for experiments upon reaching 80% confluence. Standard culture medium was replaced with fresh serum-free medium 16 h before experiments. To induce the accumulation of MGO, cells were then incubated with the indicated concentration of MGO for 24 h. Various concentrations of pyridoxamine (Sigma, MO, USA) were added into the culture medium 1 h before MGO.

Cytotoxicity assays. Cytotoxicity assays were performed using the Cell Counting Kit-8 (CCK-8) as described by the manufacturer (Dojindo Laboratory, Kumamoto, Japan). Briefly, cells were seeded onto 96-well plates and grown in a final volume of 100 μl media per well. After treating as indicated in the text, 10 μl of kit reagent was added and incubated for an additional 3 h. Absorbance was measured at a wavelength of 450 nm using a microplate reader (Synergy HT, BioTek, USA).

Argpyrimidine formation. Intracellular formation of argpyrimidine was assessed by immunohistochemistry [12,28]. Cells were cultured as described above in eight-well chamber slides (Nunc International, IL, USA). The cells were fixed with 4% paraformaldehyde and permeabilized with 80% methanol. The wells were incubated with mouse anti-argpyrimidine antibody (Cosmo bio, Tokyo, Japan) followed by rhodamine-conjugated anti-mouse IgG (Santa Cruz, CA, USA). The slides were observed under a fluorescence microscope (Olympus).

Assessment of apoptosis. Cells were seeded on eight-well chamber slides and treated as indicated in the text and fixed with 4% paraformaldehyde. Apoptosis was assessed by a TUNEL kit (Dead-End apoptosis detection system, Promega). The numbers of apoptotic cells were was determined by counting five randomly selected microscopic fields.

In-cell Western blotting. Cells were plated on black-walled 96-well plates and treated as indicated in the text and fixed with 4% paraformaldehyde. Cells were then permeabilized with 0.1% Triton X-100 and were blocked with Li-Cor Odyssey blocking buffer. Cells were then incubated with cleaved caspase-3 antibody (Abcam, MA, USA) overnight at 4 °C. Cells were then washed and incubated with the appropriate IRDye800-labeled secondary antibody (Li-Cor, NE, USA) and Alexa-fluor 680-coupled phalloidin (Invitrogen, CA, USA). Plates were washed three times with PBS–0.1% Tween20, followed by analysis with the Li-Cor Odyssey system according to manufacturer's instructions.

Immunofluorescence staining. The cells were blocked with PBS containing 0.2% Triton X-100 and 10% normal goat serum for 1 h. Slides were then incubated with mouse anti-8-hydroxygluanine

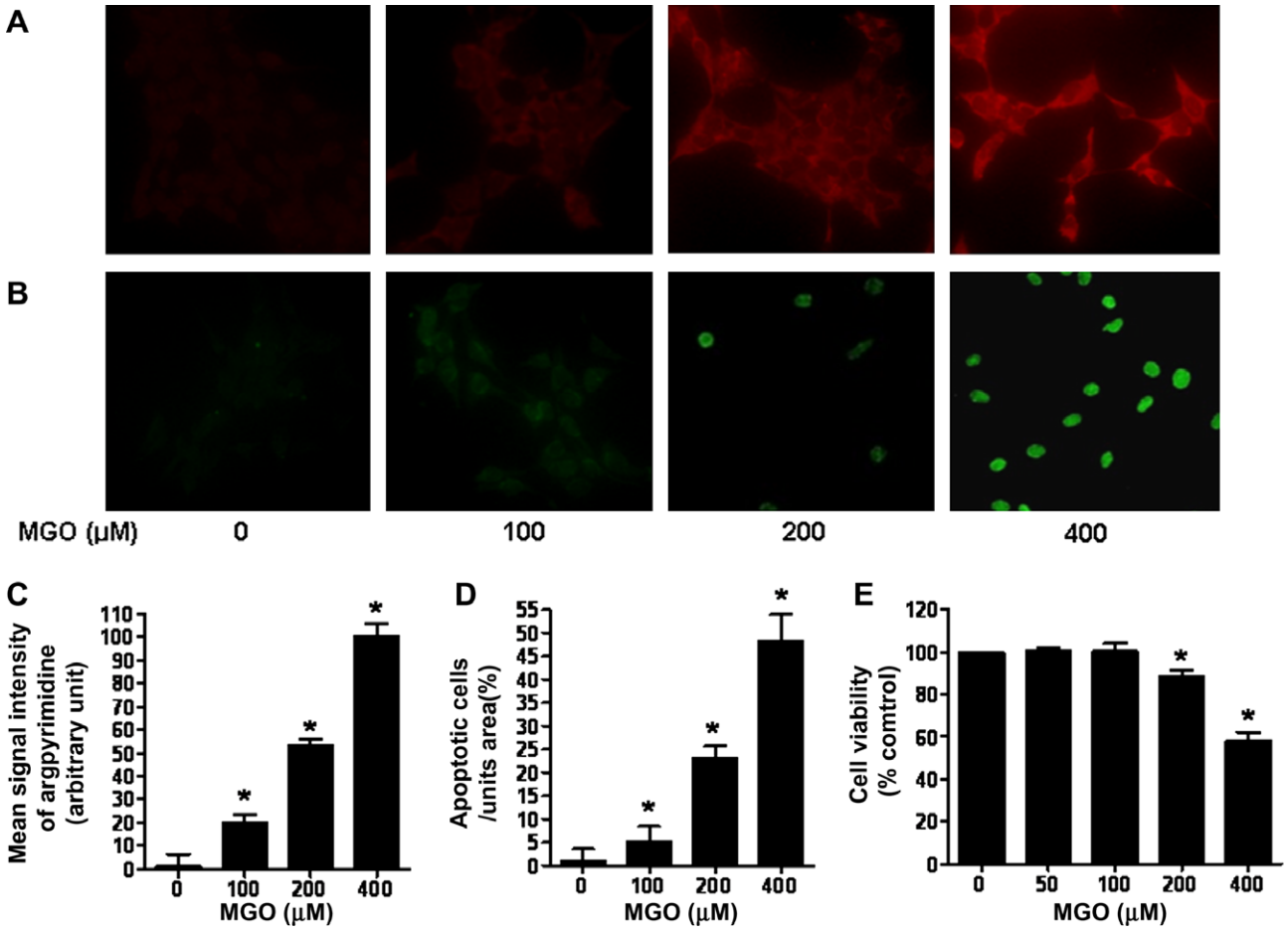

Fig. 1. MGO-induced argpyrimidine formation and cytotoxic effects of MGO in HLE-B3 cells. (A) Immunofluorescence staining of argpyrimidine. (B) TUNEL staining to detect apoptotic cells. (C) Quantitative analysis of positive signal intensity of argpyrimidine. (D) Quantitative analysis of TUNEL-positive cells. (E) Dose-dependent cytotoxic effects of MGO in HLE-B3 cells. Cytotoxicity was measured by CCK-8 assay. Values in the bar graphs represent means ± SE, $n = 4$. $^{*}p < 0.01$ vs. control group.

(8-OHdG) antibody (1:200, Santa Cruz, CA, USA) for 1 h. To detect 8-OHdG, the slides were incubated with rodamine-conjugated goat anti-mouse antibody (Santa Cruz). The cells were then counterstained with 4,6-diamidino-2-phenylindole. The intensity of the fluorescence was analyzed in five randomly selected mm^2 areas using ImageJ software (NIH).

Statistical analysis. The data were analyzed with the paired-*t* test and a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Statistical analysis was performed by GraphPad Prism 4.0 (GraphPad, CA, USA).

Results

MGO-induced argpyrimidine formation

To establish the formation of argpyrimidine by MGO in HLE-B3 cells, the immunostaining was performed in HLE-B3 cells. As shown in Fig. 1A and C, MGO caused an increase of argpyrimidine formation in a dose-dependent manner. A striking increase in immunoreactive straining for argpyrimidine was observed in the cytoplasm of HLE-B3 cells with 400 μM MGO (Fig. 1).

MGO-induced apoptosis of HLE-B3 cells

Fig. 1E shows the absorbance of culture media in CCK-8 assay, which is believed to be proportional to the number of living cells. MGO treatment elicited dose-dependent cytotoxicity on HLE-B3 cells. MGO reduced mildly cell survival as measured at a concentration of 200 μM. In contrast, cell death was readily apparent following incubation with 400 μM MGO. We investigated whether this MGO-induced cell death was apoptosis. Apoptotic cell death was identified by TUNEL assay. In TUNEL assay, the ratio of the apoptotic cell number to the total cell number was increased 48.5 ± 7.1% when treated with 400 μM MGO with an apparent dose-dependency (Fig. 1B and D).

Pyridoxamine prevented MGO-induced argpyrimidine formation and apoptosis in HLE-B3 cells

We examined the cytoprotective effects of pyridoxamine (AGEs formation inhibitor) on MGO-treated HLE-B3 cells. HLE-B3 cells were incubated with pyridoxamine and 400 μM MGO, and examined argpyrimidine formation, cell viability and apoptosis. As shown in Fig. 2, the pretreatment of pyridoxamine inhibited the formation of argpyrimidine in MGO-treated HLE-B3 cells. The viability of cells incubated with MGO alone was approximately 60% compared to that of controls, whereas pyridoxamine significantly suppressed MGO-induced cell death in a dose-dependent manner. In. TUNEL assay, pyridoxamine also significantly inhibited the enhanced apoptosis of MGO-treated HLE-B3 cells.

Oxidative DNA damage in HLE-B3 cells exposed to MGO

To confirm the oxidative stress status in MGO-treated HLE-B3 cells, we assessed the formation of oxidative stress specific marker, 8-OHdG. In untreated cells, positive staining for 8-OHdG was hardly detectable in the cytoplasm or nucleus. Obviously, there was more positive staining for 8-OHdG in the cytoplasm and nu-

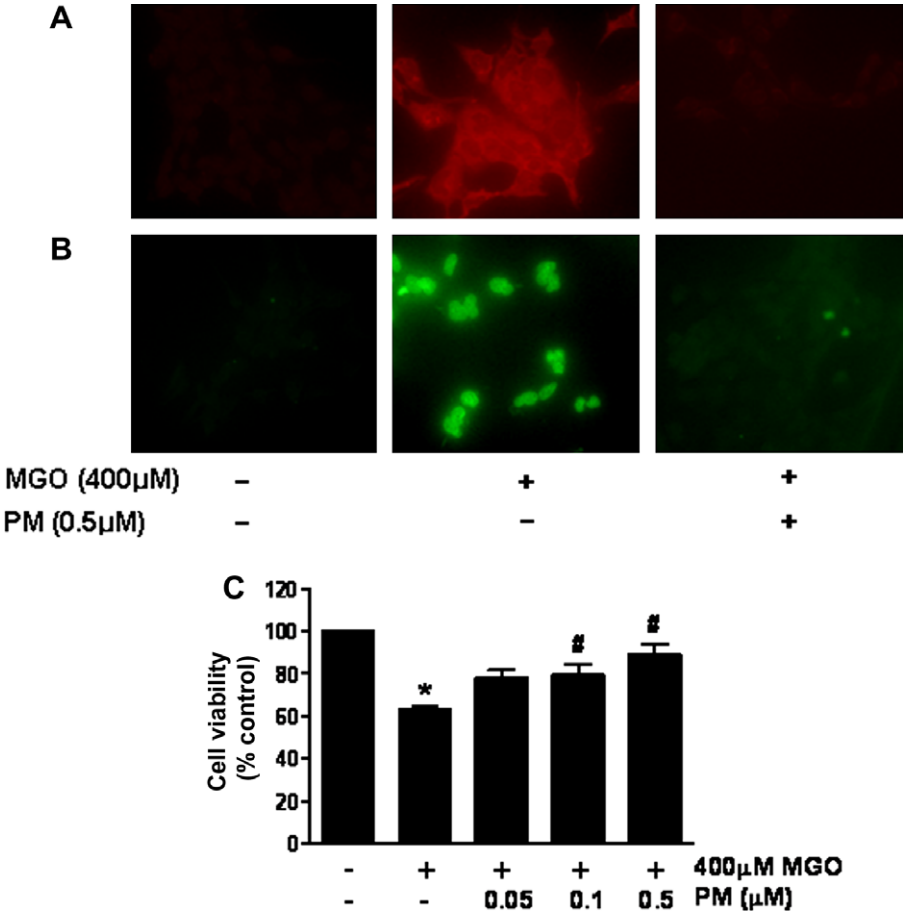

Fig. 2. Effects of pyridoxamine (PM) on apoptosis of MGO-treated HLE-B3 cells. (A) Immunofluorescence staining of argpyrimidine. (B) TUNEL staining. (C) CCK-8 assay. HLE-B3 cells were incubated with the indicated concentration of MGO in the present or absence of pyridoxamine. Pyridoxamine prevented not only the formation of argpyrimidine but also apoptosis of HLE-B3 cells. Values in the bar graphs represent means ± SE, $n = 4$. $^{*}p < 0.01$ vs. control group, $^{\#}p < 0.01$ vs. MGO-treated group.

cleus of the cells after treatment with 400 μM MGO (Fig. 3). The MGO-induced increases in positive staining of 8-OHdG was significantly also attenuated by the treatment with 0.5 μM pyridoxamine as shown in Fig. 3.

Caspase-3 activation in HLE-B3 cells exposed to MGO

To monitor caspase-3 activation in MGO-treated HLE-B3 cells, we performed the plate-based in cell western for cleaved-caspase-3. As shown in Fig. 4, we detected the cleaved-caspase-3 activation with an IRDye800-labeled antibody. We counterstained f-actin with Alexa-fluor 680-labeled phalloidin to control for cell numbers. We detected an increase in the cleaved-caspase-3 signal after MGO treatment, which decreased at a concentration of 400 μM. Similar to the cleaved-caspase-3 signal, the f-actin signal declined with 400 μM MGO. We believe the later loss of caspase-3 and f-actin staining is a result of apoptosis-induced detachment of cells. Of note, we detected a steady increase in the ratio of active-caspase-3 to f-actin over the entire concentration range, indicating an overall increase in caspase-3 activation on a per cell basis. However, this enhanced active-capase-3 signal by MGO was also suppressed by pyridoxamine in a dose-dependent manner.

Discussion

Apoptosis of LECs plays an important role in the development of several types of cataract. Oxidative stress, ultraviolet radiation and calcium influx are implicated in this apoptotic change [24,29,30]. In addition, apoptosis of LECs is also induced when LECs are cultured with high glucose or galactose [31,32]. The histological abnormalities of LECs, such as cell edema, rounding of nuclei and aberrant intracellular vacuoles, were detectable in the early stage of sugar cataract formation [33]. Apoptotic LECs were observed at 24 h after galactose exposure [27]. These findings suggest that the damage of LECs may occur first during the development of diabetic cataract.

One of the major consequences of hyperglycemia is the formation of AGEs. MGO is a major precursor of AGEs and increased in diabetic tissues [8]. Present study successfully showed that MGO

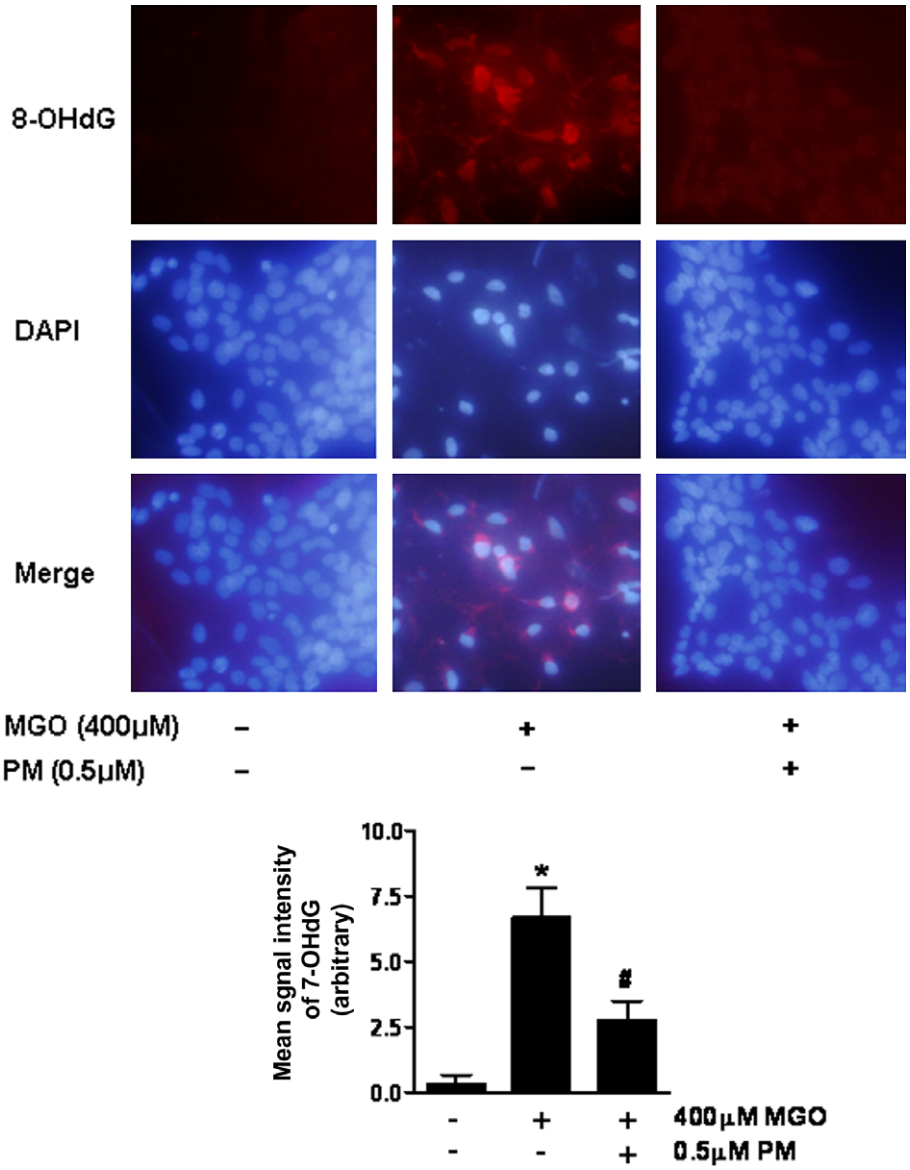

Fig. 3. Oxidative DNA damage of MGO-treated HLE-B3 cells. Immunofluorescence stained of 8-OHdG. HLE-B3 cells were incubated with the indicated concentration of MGO in the present or absence of pyridoxamine. Intracellular oxidative DNA damage was detected using an immunofluorescence stained with anti-8-OHdG monoclonal antibody. The images are representatives of four independent experiments. Middle panel indicates DAPI nuclear staining as a counterstain. Values in the bar graphs represent means ± SE, $n = 4$. $^{*}p < 0.01$ vs. control group, $^{\#}p < 0.01$ vs. MGO-treated group.

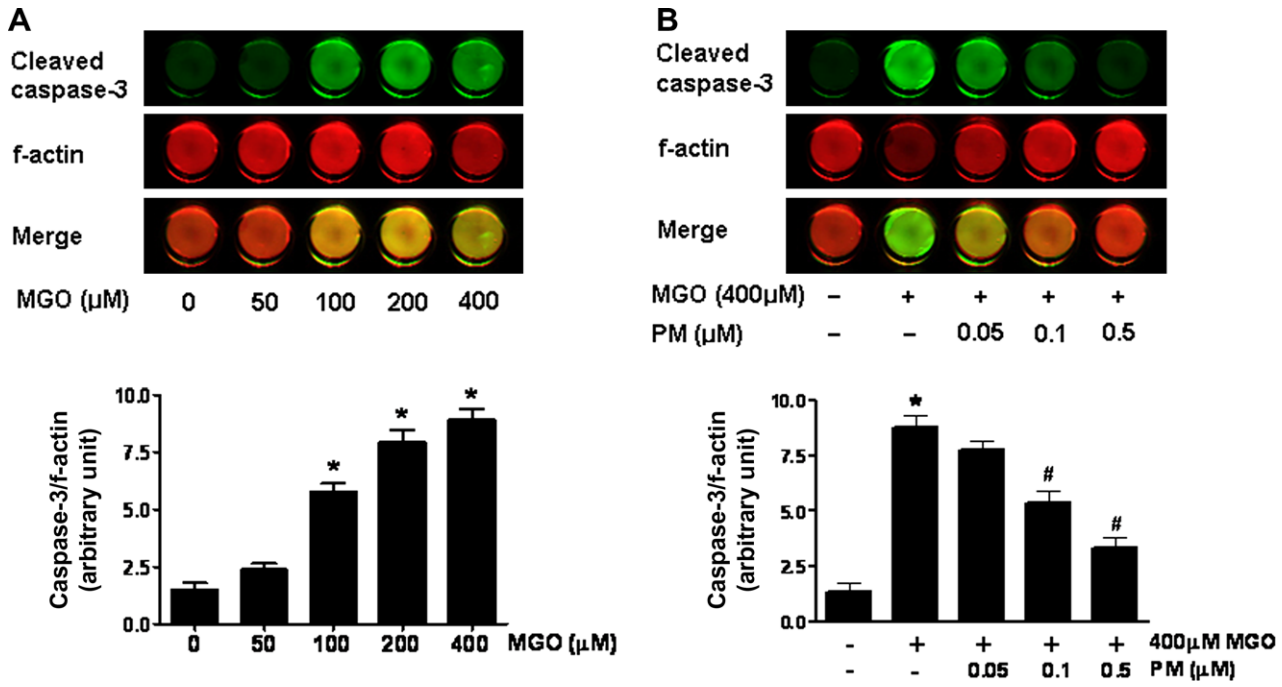

Fig. 4. Caspase-3 activation in MGO-treated HLE-B3 cells. (A,B) In-Cell quantitative analysis of HLE-B3 cells treated with the indicated concentration of MGO in the present or absence of pyridoxamine (PM). HLE-B3 cells stained for anti-cleaved-caspase-3 (green) and f-actin (red) and merged. Cleaved-caspase-3 protein levels were quantified and normalized to f-actin levels. Exposure of HLE-B3 cells to MGO increased the relative levels of cleaved-caspase-3, whereas its enhanced expression was inhibited in a dose-dependent manner by treatment with pyridoxamine. Values in the bar graphs represent means ± SE, $n = 4$. $^{*}p < 0.01$ vs. control group, $^{\#}p < 0.01$ vs. MGO-treated group.

elevated the formation of argpyrimidine and induced apoptosis in HLE-B3 cells. Lens argpyrimidine were highly accumulated in diabetic rats than nondiabetic controls [34]. Argpyrimidine is directly and rapidly formed from MGO. One month of diabetes may be enough to produce argpyrimidine in diabetic animal tissue [34]. Argpyrimidine is only one of the three known arginine modifications of MGO. The other two modifications, *N*-δ-(4-carboxy-4,6-dimethyl-5,6-dihydroxy-1,4,5,6-tetrahydropyri-midine-2-yl)-L-ornithine and *N*-δ-(5-hydro-5-methyl-4-imidazolon-2-yl)-L-ornithine, have not yet been detected in tissues [13]. The formation of AGEs in ocular tissue closely correlated with the development of diabetic cataract [3]. A variety of AGEs inhibitors, such as aminoguanidine, LR-90 and pyridoxamine, have been developed to reduce the accumulation of AGEs. The treatment with aminoguanidine reduced glycated protein level in the diabetic rat lenses [35]. AGEs have been reported to induce apoptosis in mesangial cells [36], endothelial cells [37] and retinal pericytes [38]. It was recently reported that oxidative stress or pro-apoptotic cytokine through interaction AGEs/RAGE were involved in this process [37,39]. In our in vitro work demonstrated that the treatment of MGO elicited the formation of argpyrimidine and the apoptosis of HLE-B3 cells. The inhibition of argpyrimidine formation by 0.5 μM pyridoxamine leaded to a dramatic decrease in apoptosis. Pyridoxamine, a member of the B6 vitamer family, is a potent scavenger of reactive carbonyls, inhibiting the late stages of glycation reactions that lead to AGE formation [40]. When lens proteins were incubated with MGO, pyridoxamine inhibits formation of MGO-derived AGEs concentration dependently [41]. These results suggest that MGO plays a crucial role in the apoptotic changes of HLE-B3 cells and that the formation of argpyrimidine by MGO is a cause of the injury of HLE-B3 cells.

Cytotoxicity induced by MGO has been already reported in various cells, including rat Schwann cells, human vascular endothelial cells, rat mesangial cell and bovine retinal pericytes [16–19]. MGO is knows as the source of ROS [42]. MGO and its potent precursor, glyceraldehyde 3-phosphate, have been reported to augment the formation of 8-OHdG or to induce DNA fragmentation [43]. In present study, the results of 8-OHdG immunofluorescence clearly demonstrated the presence of oxidative DNA damage in MGO-treated HLE-B3 cells. Oxidation of guanine to form 8-hydroxy-2′-deoxyguanosine acts as a marker of oxidative DNA damage [44]. When DNA is damaged, cells initiate a response, such as DNA repair, cell cycle delay, or induction of apoptosis [45]. This indicates that the cytotoxic effect of MGO on HLE-B3 cells could be linked to increases in oxidative stress.

Caspase-3 is activated by proteolytic processing and is responsible for the cleavage, and therefore the breakdown, of cellular components that are involved in DNA repair, and thereby plays a key role in the execution of apoptosis [46,47]. ROS are potent inducers of apoptosis in various cell types including mouse and human LECs [48,49]. The cleavage of caspase-3 was increased in concert with increased LECs death following exposure to hydrogen peroxide [50]. Our current observations are also showed that the cleavage of caspase-3 was increased in LECs following exposure to MGO, and that pyridoxamine reduced oxidative DNA damage and caspase-3 activation in HLE-B3 cells. It was reported that pyridoxamine does not appear to function as an antioxidant [51]. Thus, we suspect that an indirect effect of pyridoxamine partly accounts for a decrease of oxidative DNA damage by MGO.

In conclusion, our study shows that MGO may enhance the formation of argpyrimidine in HLE-B3 cells, which leads to apoptosis of these cells. Pyridoxamine has the ability to attenuate the increase in argpyrimidine formation, oxidative DNA damage and caspase-3 activation, resulting in the prevention of apoptosis. Taking together, it seems likely that MGO plays a critical role in regulation of HLE-B3 cells apoptosis.

Acknowledgment

This research was supported by a grant [L08010, K09030] from the Korea Institute of Oriental Medicine (KIOM).

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