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Accumulation of argpyrimidine, a methylglyoxal-derived advanced glycation end product, increases apoptosis of lens epithelial cells both *in vitro* and *in vivo*

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Abbreviations: AGEs, advanced glycation end products; LEC, lens epithelial cell; MGO, methylglyoxal; PDTC, pyrrolidine dithiocarbamate; PM, pyridoxamine; TRITC, tetramethyl rhodamine isothiocyanate; ZDF, Zucker diabetic fatty; ZL, Zucker lean

Abstract

The formation of advanced glycation end products (AGEs) has been considered to be a potential causative factor of injury to lens epithelial cells (LECs). Damage of LECs is believed to contribute to cataract formation. The purpose of this study was to investigate the cytotoxic effect of AGEs on LECs both *in vitro* and *in vivo*. We examined the accumulation of argpyrimidine, a methylglyoxal-derived AGE, and the expression of apoptosis-related molecules including nuclear factor-kappaB (NF-κB), Bax, and Bcl-2 in the human LEC line HLE-B3 and in cataractous lenses of Zucker diabetic fatty (ZDF) rats, an animal model of type 2 diabetes. In cataractous lenses from twenty-one-week-old ZDF rats, LEC apoptosis was markedly increased, and the accumulation of argpyrimidine as well as subsequent activation of NF-κB in LECs were significantly enhanced. The ratio of Bax to Bcl-2 protein levels was also increased. In addition, the accumulation of argpyrimidine triggered apoptosis in methylglyoxal-treated HLE-B3 cells. However, the presence of pyridoxamine (an AGEs inhibitor) and pyrrolidine dithiocarbamate (a NF-κB inhibitor) prevented apoptosis in HLE-B3 cells through the inhibition of argpyrimidine formation and the blockage of NF-κB nuclear translocalization, respectively. These results suggest that the cellular accumulation of argpyrimidine in LECs is NF-κB-dependent and pro-apoptotic.

Keywords: apoptosis; argpyrimidine; cataract; diabetes mellitus, type 2; epithelial cells; glycosylation end products, advanced; lens, crystalline; NF-κB

Introduction

Cataract development is the leading cause of blindness worldwide. Several factors, such as the polyol pathway, advanced glycation end products (AGEs) and oxidative stress have been implicated in diabetic cataract development (Kyselova *et al*., 2004). Lens epithelial cell (LEC) damage is thought to contribute to perturbation of lens homeostasis. LECs are believed to protect the underlying fiber cells from injury (Hightower *et al*., 1994) and maintain the transparency of the lens (Li *et al*., 1995). The death of LECs has been induced by ultraviolet radiation (Li and Spector, 1996), oxidative stress (Goswami *et al*., 2003) and hyperglycemia (Takamura *et al*., 2003) and has led to cataract formation. These results suggest that the lens epithelium has a protective role against cataract formation. However, the exact mechanisms that underlie the alteration of these cells are not completely understood.

AGEs can be derived not only from sugars but also other glycation agents (e.g., methylglyoxal, glyoxal, and glycolaldehyde). Argpyrimidine is the end product of a protein modification at the arginine residue by methylglyoxal (MGO) (Shipanova *et al*., 1997). Argpyrimidine has been detected in the human lens (Padayatti *et al*., 2001a). MGO modification has increased the susceptibility of cataract development (Oya-Ito *et al*., 2006). AGEs ex-

Table 1. Blood glucose levels

	ZL	ZDF
Blood glucose (mg/dl)	92.9 ± 10.8	489.8 ± 38.5*

ZL indicates normal Zucker lean rats; ZDF indicates vehicle-treated Zucker diabetic fatty rats. All data were expressed as mean ± SE. The asterisk (*) indicates a value of $P < 0.01$ vs. normal ZL rats.

ert their main cellular effects through the interaction with the RAGE receptor (Kislinger *et al*., 1999). The interaction of AGEs and RAGE activates a downstream inflammatory response that includes the production of cytokines and chemokines (Hofmann *et al*., 1999). AGEs have enhanced apoptosis in retinal pericytes, corneal endothelial cells, neuronal cells and renal mesangial cells (Kasper *et al*., 2000; Denis *et al*., 2002; Kaji *et al*., 2003). AGEs may enhance apoptosis indirectly through increasing oxidative stress or *via* induced expression of pro- apoptotic cytokines (Yamagishi *et al*., 2002a; Kaji *et al*., 2003). Recently, we showed that the accumulation of argpyrimidine and oxidative DNA damage caused by MGO are involved in apoptosis of human LECs (Kim *et al*., 2010). In addition, AGEs stimulate the activation of nuclear factor-kappaB (NF-κB) in LECs *in vitro* (Hong *et al*., 2000). In unstimulated cells, NF-κB resides in the cytoplasm in an inactive complex with the inhibitor kappaB (IκB). Pathogenic stimuli cause the release of IκB and allow NF-κB to enter the nucleus, bind to DNA recognition sites in regulatory regions of target genes and influence the transcription of specific genes that determine cellular function (Schreck *et al*., 1992a; Baeuerle and Henkel, 1994; Boileau *et al*., 2003). Although *in vitro* studies have demonstrated that the activation of NF-κB in various LECs might play an important role in modulating the function of LECs (Dudek *et al*., 2001; Lee *et al*., 2005), the relationship between AGEs-mediated activation of NF-κB and apoptotic cell death of LECs remains unclear. Thus, the aim of this study was to determine the exact role of argpyrimidine in apoptosis of LECs using both *in vitro* and *in vivo* experiments.

We confirmed the accumulation of AGEs in LECs and investigated the activation of NF-κB using a human LEC line and Zucker diabetic fatty rats. In addition, the expression patterns of the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2 were investigated to confirm the role of activated NF-κB.

Results

Blood glucose and cataract formation

At 21 weeks of age, all ZDF rats developed hyperglycemia compared to the normal ZL rats. As shown in Table 1, the untreated ZDF rats had more than a four-fold increase of fasting blood glucose levels. We monitored the progression of opaque areas by slit-lamp microscopy and observed that lens opacity appeared at 15 weeks of age and progressed linearly up to 21 weeks of age in ZDF rats. In contrast, ZL rats had normal, clear lenses at 21 weeks of age. The mean grade of cataract formation is illustrated in Figure 1A. The grade of the normal ZL rats remained 0 for the duration of the study. However, the value of the ZDF rats was more than 3, which indicated a moderate to severe lens opacity.

Argpyrimidine accumulation and apoptosis of LECs

By western blotting, we detected multiple and robust immunoreactive bands for argpyrimidine in cataractous lenses from ZDF rats (Figure 1B). Moreover, we observed that numerous TUNEL-positive cells localized within the vicinity of argpyrimidine accumulation. ZL rats had weaker immunoreactivity for argpyrimidine and fewer TUNEL-positive cells in the lens epithelium (Figure 1C).

Activation of NF-κB in cataractous lenses

The NF-κB signaling pathway is affected by AGEs (Yamagishi *et al*., 2005) and plays an important role in apoptosis (Romeo *et al*., 2002; Kowluru *et al*., 2003). Thus, we investigated NF-κB activity in cataractous lenses. By immunohistochemical staining, we found the activated NF-κB mainly in the nuclei of LECs in cataractous lenses. In ZL rats, the activated NF-κB was rarely detected (Figure 2A). To evaluate NF-κB activation in a quantitative way, we also performed an ELISA-based NF-κB assay. ZDF rats presented a significantly higher activity of NF-κB than normal ZL rats (Figure 2B, $P < 0.01$).

Expression of Bcl-2 and Bax in cataractous lenses

High glucose has enhanced Bax expression and apoptosis in human LECs (Wu *et al*., 2008). In retinal pericytes, NF-κB activation by high glucose has increased Bax expression (Podesta *et al*., 2000; Romeo *et al*., 2002). Moreover, the Bax promoter contains an imperfect NF-κB consensus sequence (Dixon *et al*., 1997). Therefore, to further investigate the potent pro-apoptotic role of NF-κB activation in LECs, we focused on the expression of the pro-

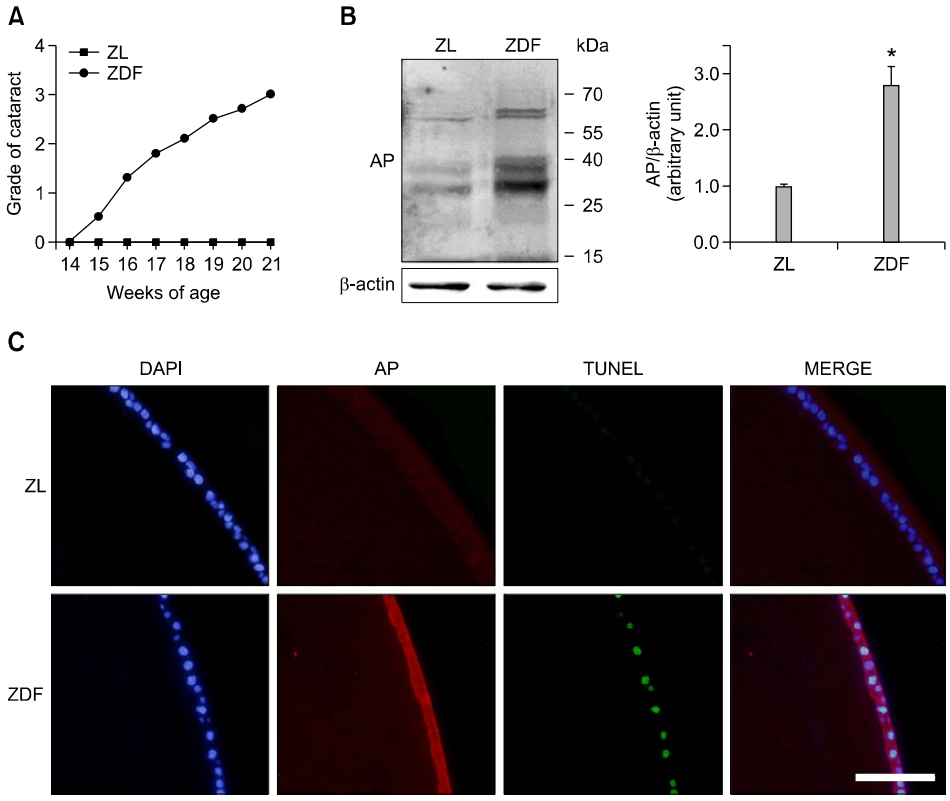


Figure 1. Argpyrimidine formation and apoptosis in LECs. (A) Grade of cataract formation in the normal ZL rat (■) and ZDF rat (●). (B) Western blot analysis of argpyrimidine. (C) Double staining for argpyrimidine and TUNEL-positive apoptotic cells. The lens sections from the normal ZL rats and ZDF rats are stained with argpyrimidine (AP, red), TUNEL (green) and DAPI (blue). Almost all TUNEL-positive cells coincide with argpyrimidine-positive cells. The scale bar = 50 μm. All data are expressed as means ± SE, $n = 8$. The asterisk (*) indicates a value of $P < 0.01$ vs. normal ZL rats.

apoptotic Bax protein and the anti-apoptotic Bcl-2 protein. We detected strong immunoreactivity of the Bax protein in the cytoplasm of LECs in the ZDF rats by immunofluorescence staining. However, Bcl-2 immunoreactivity did not differ between normal and diabetic rats (Figure 2A). By western blot analysis, the expression of the Bax protein was greatly increased in the ZDF rats compared to the normal ZL rats. However, the expression level of Bcl-2 did not differ between ZDF and ZL rats. The ratio of Bax to Bcl-2 protein levels in the ZDF rats was significantly higher than the ratio in the normal ZL rats (Figure 2C).

NF-κB activation and apoptosis in LECs exposed to MGO *in vitro*

We next investigated whether argpyrimidine accumulation induced a nuclear translocation of NF-κB p65 and apoptotic cell death in HLE-B3 cells by performing immunofluorescence staining. As shown in Figures 3 and 4, MGO treatment increased argpyrimidine formation in a dose-dependent manner (Figure 3A) and elicited robust apoptotic cell death in HLE-B3 cells (Figure 4A). In untreated cells, positive staining for NF-κB p65 was hardly detectable in the nucleus. Not surprisingly, there was more positive staining for NF-κB p65 in the nucleus of the cells after treatment with 400 μM MGO. The MGO-induced increase in nuclear staining of NF-κB p65 was significantly attenuated by the treatment with 25 μM PDTC, as shown Figure pannels 3C and 3D. Interestingly, pretreatment with 0.5 μM PM not only blocked argpyrimidine formation but also inhibited the nuclear translocalization of the NF-κB p65 subunit. Moreover, apoptotic cell death by MGO was significantly suppressed with the addition of 25 μM PDTC, as shown in Figure 4A. Unlike PM treatment, however, PDTC did not inhibit argpyrimidine formation. In addition, the ratio of Bax to Bcl-2 protein levels in the MGO-treated HLE-B3 cells was significantly higher than the ratio in the non-treated cells. However, in the PDTC-treated group, this ratio was similar to normal controls (Figure 4C). These results suggested that NF-κB might play an important role in regulation of AGEs-mediated apoptosis in LECs.

Discussion

In this study, in order to verify the cytotoxic role of AGEs in LEC injury both *in vitro* and *in vivo*, we investigated whether argpyrimidine-induced apoptosis occurs *via* the induction of NF-κB in HLE-B3 cells and ZDF rats. The ZDF rat is a well-estab-

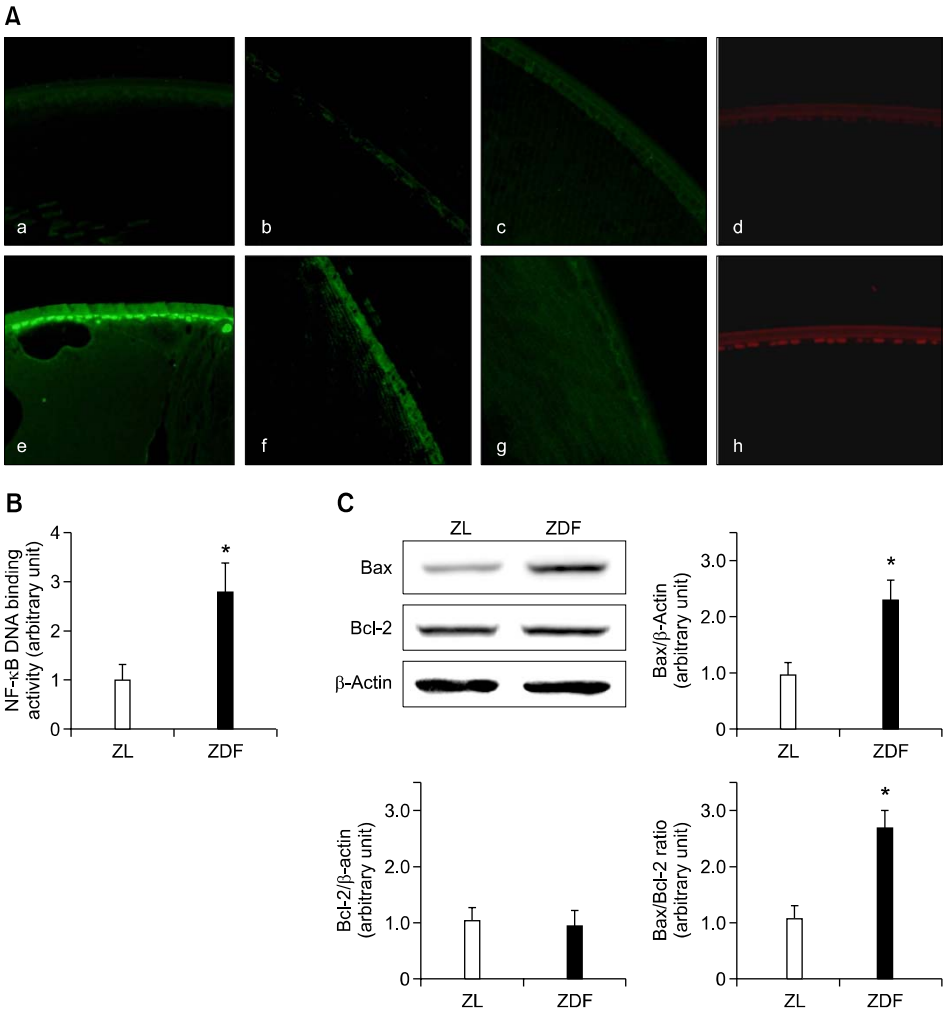


Figure 2. NF-κB activation and expression of Bax and Bcl-2 in LECs. (A) Immunofluorescence staining of NF-κB (a, d), Bax (b, e) and Bcl-2 (c, f). Representative photomicrographs of lenses from the normal ZL rat (a-c) and ZDF rat (d-f). Positive signals (green) for activated NF-κB are mainly detected in the nucleus of diabetic LECs. The lens epithelium of ZDF rats shows strong immunoreactivity for Bax. However, Bcl-2 immunoreactivity does not differ between normal and diabetic rats. The scale bar = 50 μm. (B) Analysis of NF-κB DNA binding activity by an ELISA-based assay. (C) Expression of Bax and Bcl-2 protein by western blotting and the ratio of Bax to Bcl-2 protein expression levels. Values in the bar graphs represent means ± SE, $n = 8$. The asterisk (*) indicates a value of $P < 0.01$ vs. normal ZL rats.

lished model for type 2 diabetes and is based on impaired glucose tolerance caused by an inherited insulin-resistance gene. ZDF rats exhibited hyperglycemia and diabetic cataract formation at 21 weeks of age, which is in agreement with previous reports (Shibata *et al*., 2000). We demonstrated that LECs of cataractous lenses accumulated argpyrimidine and underwent apoptosis. Moreover, NF-κB DNA binding activity was markedly increased in cataractous lenses. The accumulation of argpyrimidine and subsequent NF-κB activation occurred in the same LEC layer. In addition, our previous *in vitro* work demonstrated that the formation of argpyrimidine contributed to the injury of HLE-B3 cells, and apoptosis of HLE-B3 cells was prevented by the inhibition of argpyrimidine formation (Kim *et al*., 2010). In the present study, we showed that PDTC treatment blocked the nuclear translocalization of NF-κB and dramatically decreased apoptosis. Based on these results, it is likely that the damage to LECs induced by argpyrimidine accumulation could also be linked to increases in NF-κB activation. PDTC has been widely used as a NF-κB inhibitor. The mechanism of action of PDTC has been ascribed to its antioxidant or metal chelator activity (Schreck *et al*., 1992b). Although the dithiocarboxyl group on PDTC is expected to exert an antioxidant effect (Bessho *et al*., 1994), PDTC is a far more potent NF-κB inhibitor than most other antioxidants (Kim *et al*., 1999). This observation suggests that the anti-apoptotic effect of PDTC treatment in LECs is attributable, at least in part, to its antioxidative and NF-κB-inhibitory properties.

Lens opacification is a complex phenomenon. Glycation is one of the contributory factors in lens opacification. This nonenzymatic glycation process, also known as the Maillard browning reaction, results in the formation of AGEs. We observed that the level of lens protein glycation in diabetic rats was significantly higher than the level in normal rats. AGEs have been reported to induce apoptosis

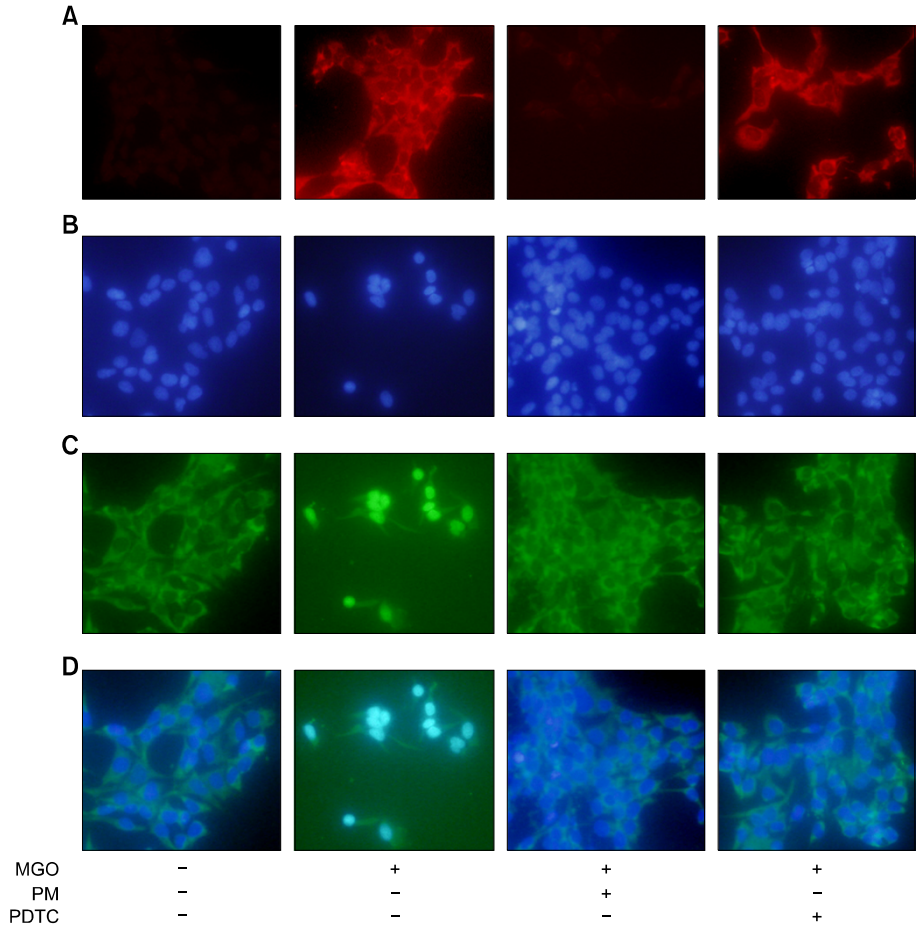
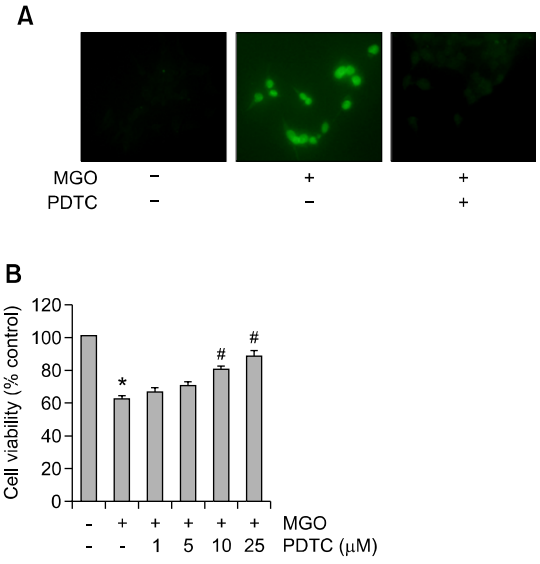


Figure 3. Argpyrimidine formation and subcellular localization of the NF-κB p65 subunit in HLE-B3 cells. (A) Immunofluorescence staining of argpyrimidine. (B) Fluorescent counterstaining of nuclei with DAPI. (C) Subcellular localization of the NF-κB p65 subunit. (D) Merge of the signals for NF-κB p65 subunits and DAPI. HLE-B3 cells are incubated with the indicated concentration of MGO in the presence or absence of PDTC for 24 h. In control cells, NF-κB is located in the cytoplasm. In cells treated with MGO for 24 h, argpyrimidine (AP) formation is induced by MGO and NF-κB is translocated into the nuclei. However, both PM and PDTC inhibit NF-κB nuclear translocation.



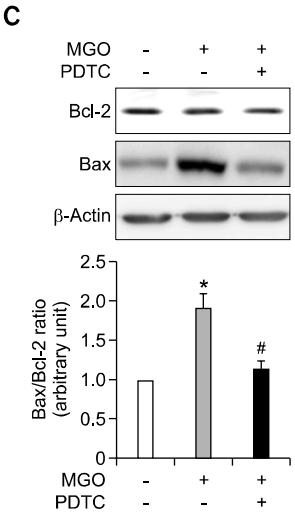


Figure 4. Effects of PDTC on apoptosis and expression of Bax and Bcl-2 in HLE-B3 cells. (A) TUNEL staining. (B) CCK-8 assay. (C) Expression of Bax and Bcl-2 protein by western blotting and the ratio of Bax to Bcl-2 protein expression level. HLE-B3 cells are incubated with the indicated concentration of MGO in the presence or absence of PDTC for 24 h. PDTC inhibits apoptosis of HLE-B3 cells in a dose-dependent manner. Values in the bar graphs represent means ± SE, n = 4. The asterisk (*) indicates a value of $P < 0.01$ vs. the control group; the pound sign (#) indicates a value of $P < 0.01$ vs. the MGO-treated group.

in mesangial cells (Yamagishi *et al*., 2002b), endothelial cells (Kaji *et al*., 2003) and retinal pericytes (Yamagishi *et al*., 2005). It was recently reported that the interaction of AGEs and RAGE leads to activation of NF-κB (Kowluru, 2005). The activated NF-κB binds to its target DNA in cellular nuclei to

activate the genes for either induction or inhibition of cellular death. AGEs induced a dose-dependent activation of NF-κB in LEC culture (Hong *et al*., 2000). NF-κB activation due to ultraviolet irradiation has induced accelerated LECs apoptosis (Lee *et al*., 2005), and NF-κB activation in ocular tissue has induced accelerated pericyte loss (Romeo *et al*., 2002) and retinal capillary cell death (Kowluru *et al*., 2003). In addition, we found that the accumulation of argpyrimidine in LECs significantly increased the expression of the pro-apoptotic Bax protein but not the anti-apoptotic Bcl-2 protein. Bax and Bcl-2 are considered the principal factors in determining whether or not the execution of apoptosis proceeds *via* the activation of caspases (Carambula *et al*., 2002). The ratio of the pro-apoptotic Bax protein to the anti-apoptotic Bcl-2-like protein is believed to be important in determining cell survival versus cell death (Oltvai *et al*., 1993). High glucose has been shown to increase the ratio of Bax to Bcl-2 protein levels and promote apoptosis (Allen *et al*., 2005). It has also been demonstrated that an increased ratio of Bax to Bcl-2 protein levels damages the integrity of mitochondria, causing the release of cytochrome c from the mitochondria and leading to the activation of caspase-3 and caspase-9 (Desagher and Martinou, 2000). In LECs from patients with anterior polar cataracts, the mRNA and protein levels of Bcl-2 were decreased. Similarly, the ratio of Bax to Bcl-2 protein levels was increased in human LECs treated with high glucose (Wu *et al*., 2008). In this study, we also observed an increased ratio of Bax to Bcl-2 protein levels that favored apoptosis. Furthermore, we previously found that the cleavage of caspase-3 was increased in HLE-B3 cells that accumulated argpyrimidine and were exposed to MGO (Kim *et al*., 2010). Caspase-3 is a key regulatory enzyme of apoptosis in human LECs (Wu *et al*., 2008). Therefore, the increase of Bax protein likely promotes apoptosis of LECs in diabetic cataractous lenses. Thus, the activation of NF-κB might result in the expression of the Bax protein and an increased ratio of Bax to Bcl-2 protein levels in cataractous lenses.

The main conclusion from this study is that LECs undergo apoptotic changes in diabetic cataractogenesis. The increased accumulation of argpyrimidine, NF-κB activation, Bax expression and caspase-3 activation were found in the LECs of diabetic cataractous lenses. These results support the hypothesis that NF-κB activation is induced by AGEs accumulation and plays an important role in LEC apoptosis.

Methods

Animals and experimental design

Eight male 6-week-old ZDF rats (ZDF/Gmi-fa/fa) and eight Zucker lean (ZL) counterparts (ZDF/Gmi-lean) were purchased from Charles River Laboratory (Waltham, MA). Rats were individually housed in plastic cages and maintained at 24 ± 2°C with a 12-h light:dark cycle and received a diet of Purina 5008 (Ralston Purina, St. Louis, MO) and tap water ad libitum. Fasting plasma glucose levels were analyzed using an enzymatic assay based on glucose oxidase and peroxidase (Glucose B-Test Wako, Wako Pure Chemical, Osaka, Japan). At 21 weeks of age, the right eye from each rat was enucleated under deep anesthesia, following an intraperitoneal injection of pentobarbital sodium (30 mg/kg body weight), and fixed in 10% neutralized formalin for 24 h and embedded in paraffin. The left eyes were enucleated and stored until assayed. All procedures involving rats were approved by the Institutional Animal Care and Use Committee at the Korea Institute of Oriental Medicine.

Evaluation of cataract development

Lenses were examined weekly using a slit lamp (Kowa Genesis, Tokyo, Japan) after dilating the pupils using a tropicamide solution. Lens opacity was scored according to the classification by Ao *et al*. (1991): grade 0, clear normal lens; 1, peripheral vesicles; 2, peripheral vesicles and cortical opacities; 3, diffuse central opacities; and 4, matured nuclear cataract. The mean grade of cataract formation was calculated as the average of grades in all eyes.

Immunofluorescence staining

The following antibodies were used for immunofluorescence staining of the lens sections: rabbit anti-Bax (1:250, Santa Cruz, CA), rabbit anti-Bcl-2 (1:200, Santa Cruz) and mouse anti-NF-κB antibody MAB3026 (1:200, Chemicon International, CA). The MAB3026 antibody recognizes an epitope overlapping the nuclear localization signal of the p65 subunit of the NF-κB heterodimer (Kaltschmidt *et al*., 1995). Thus, this antibody selectively binds the IκB-free, activated form of NF-κB. NF-κB was visualized with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Santa Cruz). For detection of Bax and Bcl-2, the sections were incubated with FITC-conjugated goat anti-rabbit IgG (Santa Cruz).

Double staining

In order to confirm the apoptotic cell death in argpyrimidine-enriched LECs, a sequential immunostaining was performed. The sections were first stained using a TdT-mediated dUTP nick-end labeling (TUNEL) kit according to the manufacturer's instructions (DeadEnd apoptosis detection system, Promega, WI). Apoptotic cells were detected with FITC-conjugated streptavidin (Santa Cruz). The second sequence of immunostaining using anti-argpyrimidine (1:100, Cosmo bio, Tokyo, Japan) was performed on the same sections with tetramethyl rhodamine iso-

thiocyanate (TRITC)-conjugated goat anti-mouse IgG antibody (Santa Cruz). The slides were mounted in glycerol-based Vectashield media (Vector, CA) containing 4,6-diamidino-2-phenylindole (DAPI).

Evaluation of NF-κB DNA binding activity using the ELISA-based method

Nuclear proteins were isolated from lenses by using commercially available nuclear and cytoplasmic extraction kits (NE-PER™ Nuclear and Cytoplasmic Extraction Reagents, Pierce, IL). NF-κB DNA-binding activity was evaluated using the ELISA-based EZ-detected™ Transcription Factor Kit for NF-κB p65 (Pierce, IL) according to the manufacturer's instructions. The amount of active NF-κB p65 in each sample was obtained from a standard curve and normalized to the protein content.

Cell culture and treatment

HLE-B3 cells, a human LEC line immortalized by SV-40 viral transformation, were obtained from the ATCC (Manassas, VA). Cells were cultured in minimal essential media with 10% FBS at 37°C in an incubator with 5% CO_2. Cells were plated 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 formation of argpyrimidine, cells were then incubated with the indicated concentration of MGO for 24 h. Various concentrations of the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) (Sigma, MO) and the AGEs inhibitor pyridoxamine (PM) (Sigma) were added to the culture medium 1 h before MGO treatment.

Evaluation of argpyrimidine formation and NF-κB nuclear translocalization

Intracellular formation of argpyrimidine was assessed by immunohistochemistry as previously described (Padayatti *et al*., 2001b; Padival *et al*., 2003; Kim *et al*., 2010). To evaluate NF-κB nuclear translocalization, immunofluorescence staining for the NF-κB p65 subunit was performed on the cultured LECs with a mouse anti-NF-κB p65 antibody (Santa Cruz, CA). NF-κB was visualized with FITC-conjugated goat anti-mouse IgG (Santa Cruz). The nuclei of cultured LECs were then counterstained with DAPI.

Western blot analysis

Proteins were extracted from both the lenses and cultured LECs and then separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad, CA). Membranes were probed with the anti-argpyrimidine, anti-Bax or anti-Bcl-2 antibodies, and then the immune complexes were visualized with an enhanced chemiluminescence detection system (ECL; Amersham Bioscience, NJ). Protein expression levels were determined by analyzing the signals captured on the nitrocellulose membranes using an image analyzer (Las-3000, Fuji photo, Tokyo, Japan).

Cytotoxicity assay and assessment of apoptosis

Cytotoxicity assays were performed using the Cell Counting Kit-8 as described by the manufacturer (Dojindo Laboratory, Kumamoto, Japan). Apoptosis was assessed by using the TUNEL assay (DeadEnd apoptosis detection system, Promega).

Statistical analysis

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

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