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Methylglyoxal Induces Apoptosis Mediated by Reactive Oxygen Species in Bovine Retinal Pericytes

One of the histopathologic hallmarks of early diabetic retinopathy is the loss of pericytes. Evidences suggest that the pericyte loss in vivo is mediated by apoptosis. However, the underlying cause of pericyte apoptosis is not fully understood. This study investigated the influence of methylglyoxal (MGO), a reactive α-dicarbonyl compound of glucose metabolism, on apoptotic cell death in bovine retinal pericytes. Analysis of internucleosomal DNA fragmentation by ELISA showed that MGO (200 to 800 μM) induced apoptosis in a concentration-dependent manner. Intracellular reactive oxygen species were generated earlier and the antioxidant, N-acetyl cysteine, inhibited the MGO-induced apoptosis. NF-κB activation and increased caspase-3 activity were detected. Apoptosis was also inhibited by the caspase-3 inhibitor, Z-DEVD-fmk, or the NF-κB inhibitor, pyrrolidine dithiocarbamate. These data suggest that elevated MGO levels observed in diabetes may cause apoptosis in bovine retinal pericytes through an oxidative stress mechanism and suggests that the nuclear activation of NF-κB are involved in the apoptotic process.

Key Words : *Retina; Pericytes; Methylglyoxal; Pyruvaldehyde; Apoptosis; Reactive Oxygen Species; NF-kappaB*

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INTRODUCTION

Diabetic retinopathy is a debilitating complication of diabetes mellitus. It is a major cause of all blindness, despite the effective therapeutic modalities for diabetes. Retinal microvascular cells, both endothelial cells and pericytes, die prematurely and undergo apoptosis in both human and experimental diabetes (1). Loss of retinal pericytes is a hallmark of early diabetic retinal changes because pericytes do not replicate in the adult retina and their loss leads to the development of retinal histopathology including thickening of the basement membrane, formation of microaneurysms, and retinal hemorrhages (2, 3). However, the underlying cause of pericyte apoptosis is not fully investigated. Several lines of evidence indicate that hyperglycemia itself (4) and a number of glycation products formed secondary to chronic hyperglycemia might contribute to pericyte death. Previous studies demonstrated that advanced glycation end products (AGEs) caused apoptosis of retinal pericytes in vitro (5, 6). We have previously reported that glycated albumin, which is an early glycation product, has a role in pericyte apoptosis (7).

Methylglyoxal (MGO), a highly reactive α-dicarbonyl metabolite of glucose degradation pathways, interacts strongly with cellular proteins and nucleic acids, thereby inducing cytotoxicity (8, 9) and is known to be one of the sources of intracellular and plasma AGEs in diabetes (10). MGO can increase oxidative stress by inactivating glutathione reductase (11) with or without accompanying cell death (12, 13). It is elevated in the blood and tissues of diabetic patients in proportion to the degree of hyperglycemia and associated with the severity of diabetic complications (14-16). However, the role of the MGO in the development of diabetic retinopathy has not been investigated. In this study, we investigated whether MGO can increase oxidative stress and contribute to the induction of apoptosis in bovine retinal pericytes, and further explored whether activation of the transcription factor nuclear factor-κB (NF-κB) was associated with apoptosis.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO-BRL (Gaithersburg, MD, U.S.A.). MGO, N-acetyl cysteine (NAC) and pyrrolidine dithiocarbamate (PDTC) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The caspase-3 inhibitor, Z-DEVD-fmk, was purchased from Calbiochem (San Diego, CA, U.S.A.) and 2′, 7′-dichlorofluorescin diac-

etate (DCF-DA) from Molecular Probes (Eugene, OR, U.S.A.). Unless otherwise indicated, all other reagents were purchased from Sigma Chemical Co.

Cell culture

Primary cultures of retinal capillary pericytes were made from isolated bovine retina by homogenization and a series of filtration steps as described previously (7). Cells were maintained in DMEM supplemented with 10% FBS. The cells were used at the 3rd to 4th passages.

Cell viability assay

To assess cell viability, a 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) assay was used as described previously (7). Briefly, pericytes were seeded at a density of 8×10^3 cells per well into 96-well culture plates. After attachment, the culture medium was changed to DMEM containing 0.5% FBS (DMEM/0.5% FBS) and grown for 24 hr. The medium was then changed to DMEM/0.5% FBS in the absence (control) or presence of various MGO concentrations (200, 400, 600 and 800 μM). After 6 hr, cell viability was assessed using MTT. Absorbance was measured at 570 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Pharmacia Biotech, Uppsala, Sweden). Untreated cells representing 100% viability were used as a control to estimate cytotoxicity (%) of the each sample treated with various MGO concentrations based on the following calculation.

Cytotoxicity (%)=(1-absorbance of treated cells/absorbance of control cells) $\times$ 100.

Analysis of internucleosomal DNA fragmentation by ELISA

Cells were plated on 12-well plates at 10^5 cells per well. MGO was applied at various concentrations and cell death was determined after 6 hr with the Cell Death Detection ELISA^{PLUS} (Roche Diagnostics Corp., Indianapolis, IN, U.S.A.) according to the manufacturer's recommendations. Briefly, after removing the supernatant containing necrotic DNA fragments, cell lysis was done. The lysates contain cytoplasmic nucleosomes from apoptotic cells. Both the supernatant and lysates were incubated with monoclonal anti-histone-biotin and monoclonal anti-DNA-POD in a streptavidin-coated microtiter plate for 2 hr at room temperature. After incubation the POD substrate ABTS was added for 10 min, then peroxidase activity was determined photometrically at 405 nm. Culture supernatant and lysates of untreated cells served as controls. Data are presented in percent of control.

Caspase-3 activity assay

The activity of caspase-3 was measured with the use of the commercially available ApoAlert Caspase-3 Colorimetric Assay Kits (BD Biosciences Clontech, Palo Alto, CA, U.S.A.) according to the manufacturer's instructions. Briefly, after 6 hr treatment with 800 μM MGO, 2×10^6 cells were lysed, and a colorimetrically labeled substrate (DEVD-pNA) was incubated with cell lysate at 37°C for 2 hr. The samples were then read on the ELISA plate reader at a wavelength of 405 nm. Comparison of the absorbance of pNA from a treated sample with an untreated control allowed determination of the percent increase in caspase activity.

Measurement of intracellular reactive oxygen species (ROS) generation by flow cytometry

The probe DCF-DA, a lipid-permeable nonfluorescent compound that could be oxidized by intracellular ROS to form a lipid-impermeable fluorescent compound, was employed (17). Cells in PBS were incubated for 15 min at 37°C with 1 μM DCF-DA. Cells were washed, re-suspended in DMEM, and incubated in the presence of 400 or 800 μM MGO with or without 2 mM NAC for 2 hr. Dye oxidation (increase in fluorescence) was measured using a FACStar flow cytometer (BD, San Jose, CA, U.S.A.) with excitation and emission settings of 488 and 530 nm, respectively. For analysis, 10,000 events were recorded.

Detection of NF-κB Activation

For immunocytochemical detection of NF-κB, cells were cultured on a chamber slide in the presence or absence of 800 μM MGO for 6 hr. The cells were then washed once with PBS and fixed for 1 hr in 70% ethanol. The chamber slides were then rinsed with three changes of PBS and incubated with 1:100 rabbit anti-NF-κB p65 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) for 12 hr at 4°C and subsequently incubated with biotinylated antibody at 4°C for 1 hr. Finally, the specimens were visualized using diaminobenzidine as the chromogen and counterstained with hematoxylin.

To undertake electrophoretic mobility shift assay (EMSA) for NF-κB, nuclear extracts from pericytes were prepared from 1×10^6 cells as described previously with minor modifications (18). Protein content was measured and 5 μg portions of the extracts were used for the binding reaction. A consensus double-stranded NF-κB probe was obtained from Promega (Madison, WI, U.S.A.), and end-labeled by using [γ-^{32}P]adenosine-5-triphosphate. Nuclear extracts were incubated in 10 μL of a binding buffer. Afterwards, the end-labeled probe was added (100,000 cpm/sample). Samples were then incubated for 20 min and loaded onto 4% non-denaturing polyacrylamide gel. Electrophoresis was run for 3 hr in a cold room. Protein complexes were identified by autoradiography. A 100-fold molar excess of unlabeled NF-κB probe was added 30 min prior to incubation to verify that the bands from the MGO-treated samples were indeed activated NF-κB.

Statistical analysis

The data are expressed as the mean±SD. Statistical analyses were performed using a Student's t-test. A value of $p<0.05$ was considered to be significant (SPSS for Windows, version 11.5, SPSS, Chicago, IL, U.S.A.).

RESULTS

MGO showed cytotoxicity in bovine retinal pericytes

MGO produced a progressive cytotoxic effect on retinal pericytes with increasing concentration, reaching maximum cytotoxicity with 40% damaged cells at 800 μM after 6 hr incubation (Fig. 1). A statistically significant cytotoxicity occurred at a concentration of 200 μM ($p<0.05$) or greater ($p<0.01$).

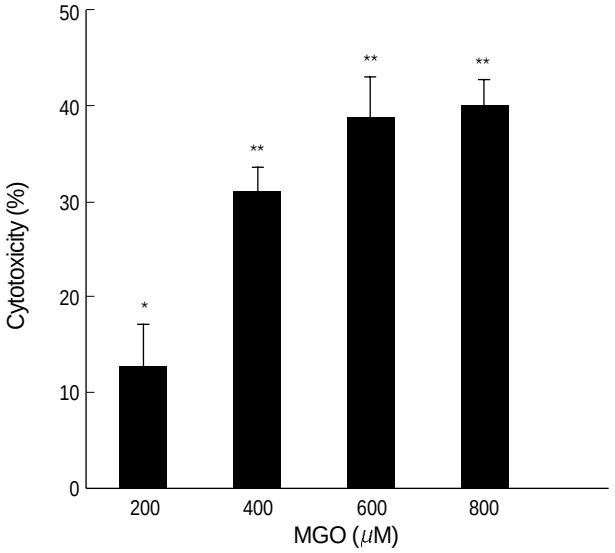


Fig. 1. Dose-dependent cytotoxic effects of MGO in retinal pericytes. Cytotoxicity was measured by MTT assay after 6 hr. Data are means±SD of triplicate experiments. *$p<0.05$, **$p<0.01$.

MGO induced cell death through apoptosis, not through necrosis and caspase-3 activity was increased

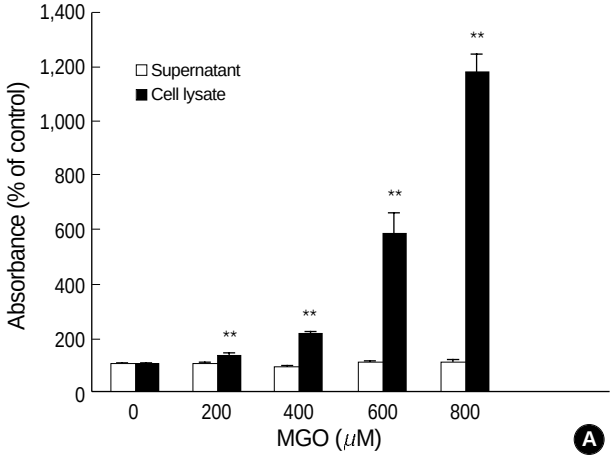
After 6 hr of incubation with MGO, the concentration of intracellular nucleosomes increased, and peaked at 800 μM ($p<0.01$), whereas no significant amount of nucleosomes was detected in the cell culture supernatant (Fig. 2A). This reflected the intracellular degradation of DNA during apoptosis. To further elucidate the mechanisms involved in the observed apoptosis, we measured the intracellular caspase-3 activity. MGO (800 μM) induced 1.5-fold increase of caspase-3 activity after 6 hr incubation ($p<0.05$) (Fig. 2B).

Intracellular ROS generation was an early event in MGO-induced retinal pericytes apoptosis

Considerable production of intracellular ROS was observed after 2 hr incubation with 400 or 800 μM MGO (Fig. 3A). The increase in intracellular ROS was suppressed by the addition of 2 mM NAC (Fig. 3B).

MGO-induced apoptosis was associated with activation of NF-κB

Subcellular immunolocalization of the p65 subunit of NF-κB demonstrated a cytoplasmic distribution in controls (Fig. 4A, B). However, after incubation with MGO, nuclear translocation of NF-κB was evident (Fig. 4C, D). The EMSA demonstrated more NF-κB binding was evident after 6 hr of MGO treatment (Fig. 5).



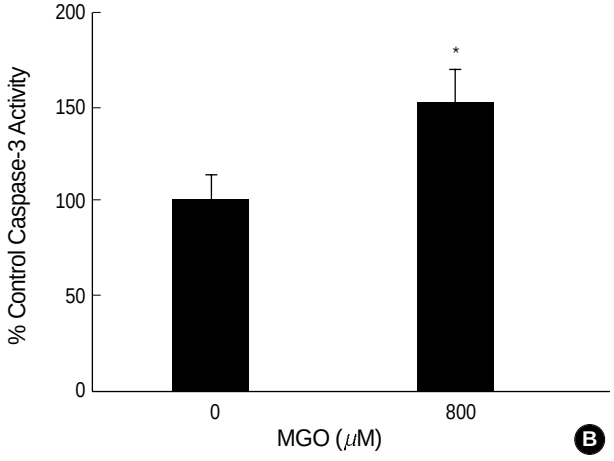


Fig. 2. (A) Effect of MGO on intracellular nucleosome enrichment. Cells were treated with 200, 400, 600, 800 μM MGO for 6 hr and the nucleosome concentration within the cell and in the cell culture supernatant was measured by ELISA. (B) Caspase-3 activity in retinal pericytes. Data are means±SD of triplicate experiments. *$p<0.05$, **$p<0.01$ compared to the value of the control.

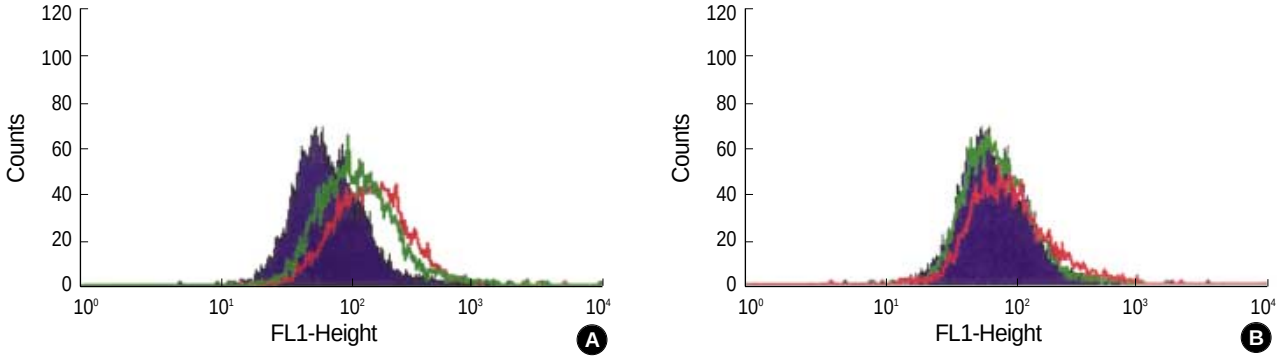


Fig. 3. Measurement of intracellular ROS production by flow cytometry using DCF-DA. (A) Cells incubated with or without MGO (control, purplish; 400 μM, green; 800 μM, red) for 2 hr. (B) Cells co-treated with 2 mM NAC were incubated with or without MGO for 2 hr. (control, purplish; 400 μM+NAC, green; 800 μM+NAC, red). Data are representative results from three separate experiments.

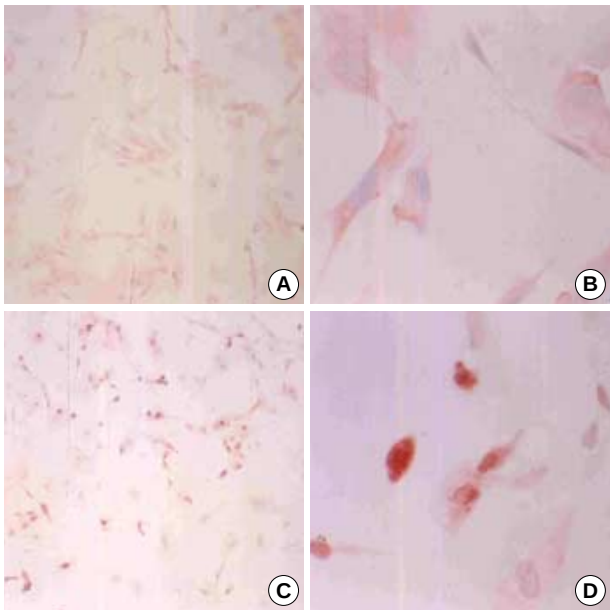


Fig. 4. Subcellular localization of NF-κB p65 subunits. (A, B) In control cells, NF-κB is located in the cytoplasm. (C, D) In cells treated 800 μM MGO for 6 hr, NF-κB is translocated into the nuclei (red). Magnification, (A, C) $\times$100; (B, D) $\times$400.

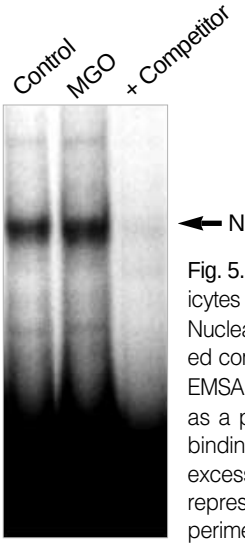


Fig. 5. Effects of MGO on NF-κB binding. Pericytes were treated with MGO (800 μM) for 6 hr. Nuclear extracts from the treated and untreated control cells were isolated and used in an EMSA with ^{32}P-labeled NF-κB oligonucleotide as a probe. The arrow indicates the NF-κB binding complex. Competitor, 100-fold molar excess of unlabeled NF-κB probe. Data are representative results from three separate experiments.

Involvement of oxidative stress, NF-κB and caspase-3 in induction of apoptosis by MGO

Co-treatment with 2 mM NAC significantly reduced MGO-induced apoptosis by 36% and 35% (400 μM, $p<0.01$; 800 μM, $p<0.01$, respectively). Apoptosis was also suppressed by the addition of 10 μM PDTC, a reagent which inhibits NF-κB, by 22% and 33% (400 μM, $p<0.05$; 800 μM, $p<0.01$, respectively). After co-incubation with the caspase-3 inhibitor, Z-DEVD-fmk, DNA fragments were also suppressed by 14% and 29% (400 μM , $p<0.05$; 800 μM, $p<0.01$, respectively) (Fig. 6).

DISCUSSION

MGO is generated by nonoxidative mechanisms from triose phosphate intermediates in the glycolytic pathway (19). In hyperglycemic conditions, MGO formation is increased due to elevated levels of triose phosphate precursors like glucose, fructose, and D-glyceraldehyde. The AGEs derived from the reaction of MGO with protein cause retinal pericyte death in vitro. Denis et al. reported that MGO-modified bovine serum albumin leads to pericyte apoptosis and oxidative stress has a critical role (5). Although MGO is produced by a nonoxidative mechanism, it can induce oxidative stress and apoptosis in some cells (8, 12, 20). In the present study we demonstrated that MGO decreased viability of cultured bovine retinal pericytes as assessed by the MTT assay. This cell death involved apoptosis in a dose-dependent manner measured by intracellular enrichment of nucleosomes as an apoptotic parameter.

Even though the tissue level of MGO might be high and

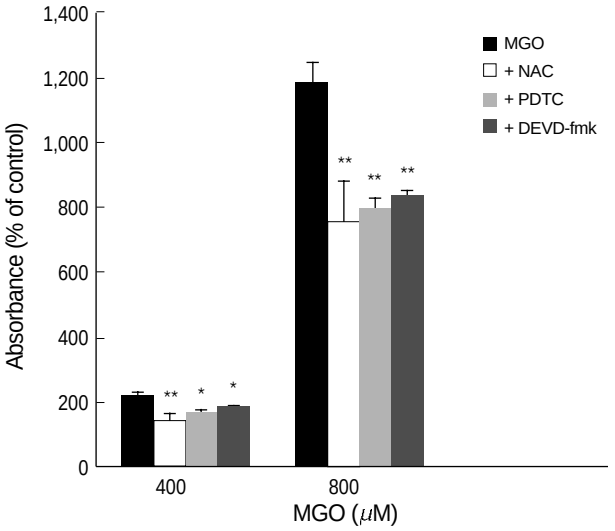


Fig. 6. Inhibitory effects of NAC, PDTC, and Z-DEVD-fmk on MGO-induced pericyte apoptosis. Apoptosis was measured by ELISA after 400 or 800 μM MGO treatment for 6 hr. Data are means±SD of triplicate experiments. *p<0.05, **p<0.01 vs without NAC or inhibitor.

as much as 310 μM can be found in Chinese hamster ovary cells (21), the concentrations of MGO that induced cell death in this study were higher than the plasma levels of diabetic patients (as high as 8 μM) (15). However, the high micromolar range of MGO used in this study might be possible to the pericytes in diabetic retinas because pericytes do not need insulin for glucose transport, thereby allowing entrance of glucose into the cells for production of MGO, even in mild hyperglycemic states (22).

ROS may induce cell death by themselves or act as intracellular messengers during the cell death induced by various other kinds of stimuli (23). To investigate whether ROS are involved in the apoptosis induced by MGO, intracellular ROS levels were measured prior to the time point that apoptotic cell death became evident. We demonstrated that ROS levels were increased in a dose-dependent manner by MGO and that the antioxidant NAC, which can raise intracellular GSH levels and thereby protect cells from the effects of ROS (24, 25), completely blocked the oxidative stress induced by MGO. Furthermore, NAC significantly inhibited the MGO-induced apoptosis. Therefore, it is obvious that MGO-induced apoptosis is mediated by ROS.

Caspase-3 has been demonstrated to be a major executor protease in apoptosis in response to oxidative stress (26, 27). We found that increased caspase-3 activity was associated with apoptosis and co-treatment with the selective caspase-3 inhibitor Z-DEVD-fmk provided significant reduction of apoptotic cells.

We next questioned whether MGO might be involved in the activation of NF-κB. The activation of NF-κB is known to be regulated by ROS (28), but the role of NF-κB as a promoter or inhibitor of apoptosis in the presence of oxidative stress remains controversial. A large body of experimental evidence now suggests that ROS co-modulates the activation of NF-κB in certain cell types under certain conditions (29, 30). Recently, Romeo et al. reported that increased numbers of pericyte nuclei positive for NF-κB were detected in the retinal capillaries of diabetic eye donors and that this was associated with pericyte apoptosis (31). Our data support the notion that NF-κB activation by oxidative stress is involved in apoptotic cell death. This assumption is supported by direct observation of NF-κB positive nuclei wherein we noted that this staining coincides with condensed apoptotic nuclei. By EMSA, NF-κB binding was greater to nuclear extracts from MGO-treated pericytes than control. NF-κB binding was also detected to some extent in nuclei from control cells but this finding can be explained that constitutive NF-κB activity in vitro results from serum exposure (32). Taken together with the finding that addition of PDTC, a putative inhibitor of NF-κB (33), inhibits MGO-induced apoptosis, these results suggest that NF-κB has a pro-apoptotic role in MGO-induced apoptosis mediated by ROS.

In conclusion, our study demonstrates for the first time that elevated MGO levels observed in diabetes may cause apoptosis in bovine retinal pericytes through oxidative stress, and suggests that the nuclear activation of NF-κB, is at least partially involved in the apoptotic process.

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