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Methylglyoxal Induces Mitochondrial Dysfunction and Cell Death in Liver

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Degradation of glucose is aberrantly increased in hyperglycemia, which causes various harmful effects on the liver. Methylglyoxal is produced during glucose degradation and the levels of methylglyoxal are increased in diabetes patients. In this study we investigated whether methylglyoxal induces mitochondrial impairment and apoptosis in HepG2 cells and induces liver toxicity *in vivo*. Methylglyoxal caused apoptotic cell death in HepG2 cells. Moreover, methylglyoxal significantly promoted the production of reactive oxygen species (ROS) and depleted glutathione (GSH) content. Pretreatment with antioxidants caused a marked decrease in methylglyoxal-induced apoptosis, indicating that oxidant species are involved in the apoptotic process. Methylglyoxal treatment induced mitochondrial permeability transition, which represents mitochondrial impairment. However, pretreatment with cyclosporin A, an inhibitor of the formation of the permeability transition pore, partially inhibited methylglyoxal-induced cell death. Furthermore, acute treatment of mice with methylglyoxal increased the plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), indicating liver toxicity. Collectively, our results showed that methylglyoxal increases cell death and induces liver toxicity, which results from ROS-mediated mitochondrial dysfunction and oxidative stress.

***Key words*:** Methylglyoxal, Reactive oxygen species, Mitochondrial dysfunction, Oxidative stress, Liver

INTRODUCTION

Hyperglycemia associated with metabolic diseases leads to the generation of excessive levels of glucose metabolites (1). Methylglyoxal, as a dicarbonyl metabolite of glucose, is produced in hyperglycemic conditions. (1,2). Increase in the plasma level of methylglyoxal has been reported in various metabolic diseases, including obesity and fatty liver (3,4). In addition, treatment of mice with methylglyoxal showed an increase in adipose tissues and obesity-related symptoms (5). However, metformin, an oral hypoglycemic biguanide, which is used as a therapeutic agent for metabolic diseases, decreased the level of methylglyoxal (6). The dicarbonyl group within methylglyoxal can react with DNA, RNA, and proteins. In particular, methylglyoxal modifies proteins with arginine, cysteine, and lysine residues, which produce advanced glycation end products (AGEs) (1,7). Accumulation of methylglyoxal and AGEs cause repetitive inflammation and oxidative stress, and finally induce toxicity in various organs (1,3,7,8).

The human body has developed mechanisms to prevent the formation of AGEs. The glyoxalase system is responsible for detoxification of methylglyoxal (3,9). Therefore, an increase in glyoxalase activity can decrease the levels of methylglyoxal. Overexpression of glyoxalase in rats with diabetes showed a decrease in the levels of methylglyoxal and AGEs and oxidative damage in kidney were diminished (9). Furthermore, pharmacological agents against metabolic diseases affect the glyoxalase system, which contributes to their beneficial effects (6,10,11). Taken together, increased levels of methylglyoxal and AGEs are associated with the development of metabolic diseases.

The liver is responsible for regulation of the blood glucose level by glucose production and metabolism (12). Therefore, it is critical to maintain normal liver function under metabolic stress. However, metabolic disorders, including diabetes and obesity, are associated with hepatic dysfunc-

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Abbreviations: AGEs, advanced glycation end products; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; GCL, glutamate cysteine ligase; GSH, glutathione; PARP, poly (ADP) ribose polymerase; RAGE, receptor for advanced glycation end products; Rh123, rhodamine123; ROS, reactive oxygen species; SOD, superoxide dismutase.

tion because of the high levels of nutrients and metabolites (3,13). The mitochondrion is a major intracellular site for metabolism of nutrients; therefore, it is crucial to preserve the function of mitochondria in metabolic diseases (14,15). However, an increase in the metabolism of nutrients leads to an increase in the generation of reactive oxygen species (ROS) from the mitochondrial respiratory chain, and thus, increases the levels of metabolites within the mitochondria (14). Excessive ROS cause loss of the mitochondrial membrane potential, which represents mitochondrial dysfunction. These detrimental effects on the mitochondria consequently decrease the cellular ATP production and finally cause apoptosis (16,17). Therefore, the mitochondria are believed to play an important role in the apoptotic process in the liver during metabolic disease.

In view of the importance of the liver function in metabolic disease, we investigated the toxicological aspects of methylglyoxal in the liver. In particular, we determined whether methylglyoxal causes cell death from mitochondrial impairment and ROS production and *in vivo* liver injury.

MATERIALS AND METHODS

Materials. Anti-poly (ADP) ribose polymerase (PARP) antibody and rhodamine123 (Rh123) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-caspase-3 antibody was obtained from Cell Signaling (Danvers, MA). Mn-TBAP was supplied by Cayman Chemical (Ann Arbor, MI). Cyclosporin A was purchased from CalbioChem (Darmstadt, Germany). Methylglyoxal, PEG-catalase, trolox, 2′,7′-dichlorofluorescein diacetate (DCFH-DA), dimethylsulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), anti-β-actin antibody, and other reagents were obtained from Sigma Chemicals (St. Louis, MO).

Cell culture. HepG2 cells, a human hepatocyte-derived cell line, were purchased from American Type Culture Collection (ATCC, Rockville, MD) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO_2. For all experiments, the cells (1×10^5) were plated in six-well plates for 2~3 days (i.e. 80% confluency) and were depleted of serum overnight before treatments.

Animals and treatment. The animal experiments study were conducted according to the protocols approved by the Animal Care and Use Committee of Chosun University. Male ICR mice (6 week old) were supplied from Oriental Bio (Sungnam, Korea). Mice (N = 5/group) were maintained at 20 ± 2°C with 12 hr light/dark cycles and a relative humidity of 50 ± 5% under filtered, pathogen-free air, with food (Purina, Korea) and water available ad libitum. Methylglyoxal (400 mg/kg body weight, a single dose) was intraperitoneally injected. Control animals received saline only. Blood samples were collected 6.5 hr after methylglyoxal treatment.

MTT assay. Cells were plated at a density of 5×10^4 cells per well in a 48-well plate. After treatment, the MTT assay was performed according to the method described previously to measure cell death (18).

Immunoblot analysis. Preparation of cell lysates and immunoblot analysis were performed as previously reported (18). Equal loading of proteins was confirmed by immunoblotting for β-actin.

Determination of reduced glutathione (GSH). The level of GSH in the cells was measured using a commercially available GSH-400 determination kit (Oxis International, Portland, OR, USA) according to the method described in a previous study (18).

Measurement of ROS production. Cells were stained with 10 μM DCFH-DA for the last 1 hr of each treatment and then harvested by trypsinization. ROS generation was determined by increases in the fluorescence intensity of dichlorofluorescein. The intensity of fluorescence was measured with a fluorescence microplate reader (Gemini XPS, Molecular Device, Sunnyvale, CA).

Analysis of mitochondrial membrane permeability change. The changes in mitochondrial membrane permeability were determined using Rh123, a membrane-permeable cationic fluorescent dye. The cells were stained with 0.05 μg/ml Rh123 for 1 hr after each treatment, and were collected by trypsinization. The changes in fluorescence intensity indicative of mitochondrial membrane permeability were measured using the fluorescence microplate reader (Gemini XPS, Molecular Device, Sunnyvale, CA).

Blood biochemistry. The levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in plasma were analyzed using serum Transaminase assay kit (ASAN, Korea) based on colorimetric reaction (Reitman-Frankel method).

Statistical analysis. For each statistically significant effect of treatment, the two-tailed Student's *t*-test was used for comparisons between multiple group means. The data were expressed as means ± standard error (S.E.) from at least three independent experiments. The criterion for statistical significance was set at $p < 0.05$ or $p < 0.01$.

RESULTS

Induction of cell death by methylglyoxal. To verify whether methylglyoxal alters cell viability, HepG2 cells

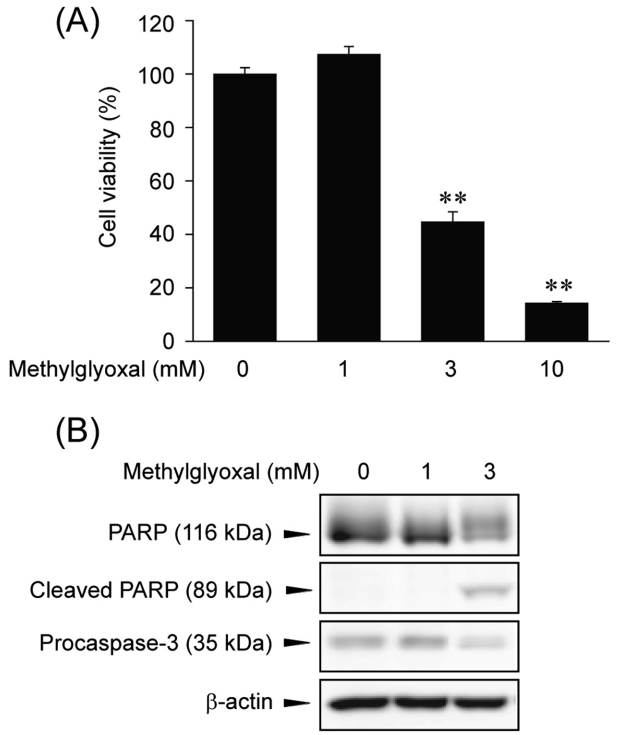

Fig. 1. Methylglyoxal-induced apoptotic cell death in HepG2 cells. (A) Cell viability assay. Cells were treated with methylglyoxal (1~10 mM) for 36 hr. The cytotoxic effect of methylglyoxal was assessed using the MTT assay. The data were expressed as means ± S.E. from at least three independent experiments. The statistical significance of differences between each treatment group and the vehicle-treated control (**$p < 0.01$) was determined. (B) Immunoblot analysis. Cells were treated with methylglyoxal (1 or 3 mM) for 36 hr. Cell lysates were immunoblotted for apoptotic proteins. These results are representative of at least three separate experiments.

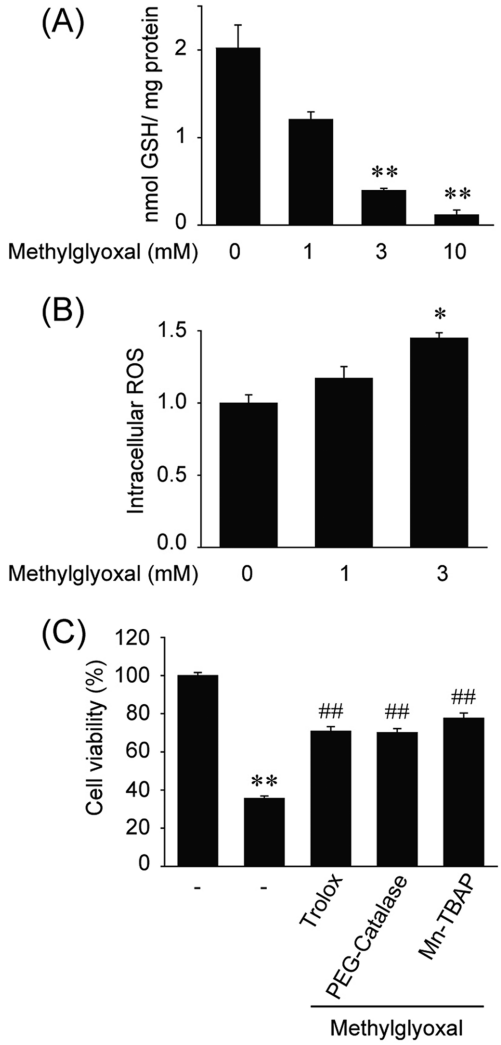

Fig. 2. Involvement of oxidative stress in methylglyoxal-induced cell death. (A) GSH content. The GSH content was assessed in HepG2 cells that had been treated with methylglyoxal (1~10 mM) for 12 hr. Data represent the mean ± S.E. of at least three replicates (significant compared to vehicle-treated control, **$p < 0.01$). (B) ROS production. HepG2 cells were treated with methylglyoxal (1 or 3 mM) for 12 hr and stained with DCFH-DA for the last 1 hr. The production of ROS was determined using the fluorescence reader. Data represent the mean ± S.E. of at least three replicates (significant compared to vehicle-treated control, *$p < 0.05$). (C) Cell viability assay. Cells were treated with trolox (100 μM), PEG-catalase (1000 U/ml), or Mn-TBAP (20 μM) and continuously incubated with methylglyoxal (3 mM) for 36 hr. The cytoprotective effect of the antioxidants was assessed using the MTT assay. Data were expressed as means ± S.E. from at least three independent experiments (significant compared to vehicle-treated control, ** $p < 0.01$ or methylglyoxal alone, ##$p < 0.01$).

were treated with different concentrations of methylglyoxal for 36 hr, and then, MTT assay was performed. Compared to vehicle-treated controls, cells treated with 3 or 10 mM methylglyoxal showed a significant decrease in the cell viability (Fig. 1A). To determine whether apoptotic cell death was involved in methylglyoxal-induced toxicity, we examined the changes in the levels of marker proteins for apoptotic death in methylglyoxal-treated cell lysates. Methylglyoxal treatment induced PARP cleavage and procaspase-3 activation (shown as a decrease in the level of procaspase-3, Fig. 1B). Caspase-3 is involved in PARP cleavage, and a cleaved form of PARP is responsible for DNA repair and apoptosis (19,20); therefore, a decrease in procaspase-3 and PARP levels indicate the induction of apoptosis. Collectively, these results indicate that methylglyoxal induces apoptotic cell death in HepG2 cells.

Induction of oxidative stress by methylglyoxal. Previous studies have shown that methylglyoxal disturbs redox-homeostasis in cells (21,22). Therefore, we examined whether oxidative stress was involved in methylglyoxal-induced toxicity. Since GSH plays a role as a cellular antioxidant

(23), we first measured the level of GSH in cells. Methylglyoxal treatment markedly decreased the levels of GSH (Fig. 2A). In addition, we observed an increase in intracellular ROS accumulation caused by methylglyoxal (Fig. 2B). To confirm that overproduction of ROS accounts for methylglyoxal-induced apoptosis, we used various antioxidants. Pretreatment with trolox (a vitamin E analogue), PEG-catalase (a hydrogen peroxide scavenger), or Mn-TBAP (a superoxide dismutase [SOD] mimetic) enabled cells to survive against methylglyoxal (Fig. 2C). These results support our conclusion that methylglyoxal aberrantly increases ROS production under oxidative stress, which in turn induces apoptosis.

Induction of mitochondrial dysfunction by methylglyoxal. Recently, direct evidence of the presence of reactive dicarbonyls within the mitochondria in response to hyperglycemia has been reported (14). To establish a correlation between methylglyoxal-induced apoptosis and disruption of mitochondrial function, we measured the changes in mitochondrial membrane permeability using Rh123 staining. Methylglyoxal treatment significantly decreased the intensity of Rh123 fluorescence, which represents mitochondrial damage (Fig. 3A). To identify whether the loss of mitochondrial membrane permeability was involved in methylglyoxal-induced apoptosis, we pretreated the cells with cyclosporin A (an inhibitor of permeability transition pore formation) before methylglyoxal treatment. The viability of cells treated with methylglyoxal was restored by cyclosporin A treatment (Fig. 3B). These results suggest that methylglyoxal causes disruption of mitochondrial function, and mitochondrial permeability transition plays a role in methylglyoxal-induced apoptosis.

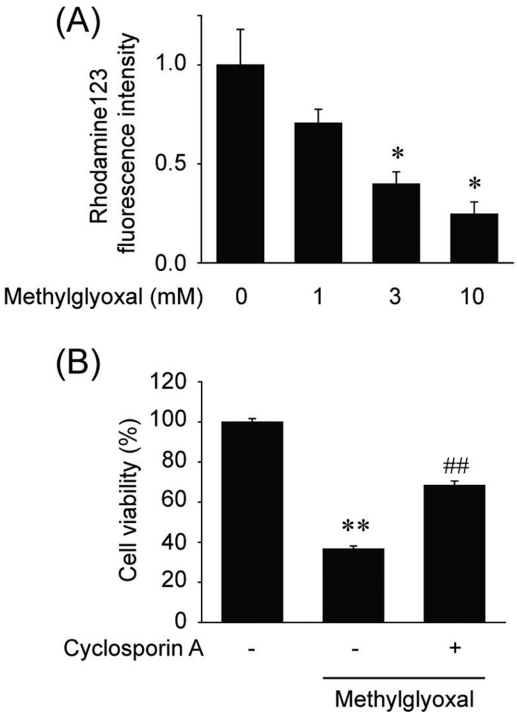

Fig. 3. Involvement of mitochondrial dysfunction in methylglyoxal-induced cell death. (A) Measurement of mitochondrial membrane permeability changes. Cells were treated with methylglyoxal (1~10 mM) for 18 hr. After staining with Rh123, the cells were harvested, and then, changes in the mitochondrial membrane permeability were determined using the fluorescence reader. Data represent the mean ± S.E. of at least three replicates (significant compared to vehicle-treated control, $^{*}p < 0.05$). (B) Cell viability assay. Cells were treated with cyclosporin A (10 μg/ml) and continuously incubated with methylglyoxal (3 mM) for 36 hr. The cytoprotective effect of cyclosporin A was assessed using the MTT assay. Data were expressed as means ± S.E. from at least three independent experiments (significant compared to vehicle-treated control, $^{**}p < 0.01$ or methylglyoxal alone, $^{\#\#}p < 0.01$).

Increase in liver toxicity by methylglyoxal. Choudhary *et al*. have reported that methylglyoxal decreases the activities of antioxidant enzymes, including SOD, catalase, and

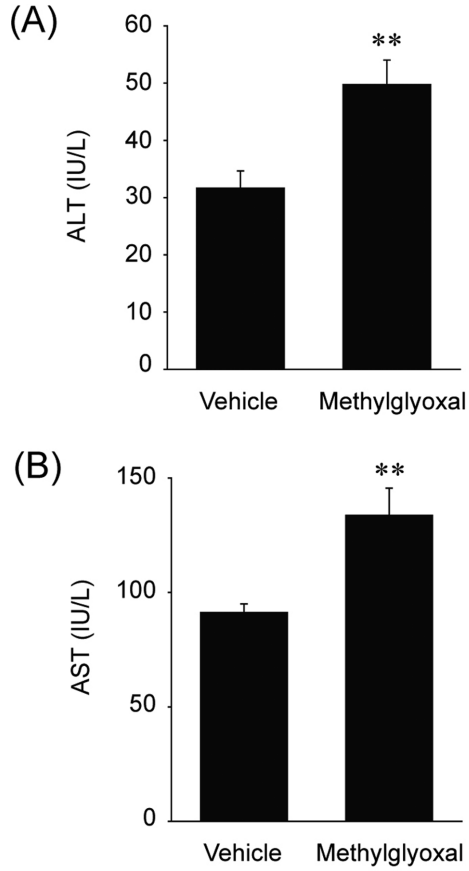

Fig. 4. Methylglyoxal-induced liver injury in mice. (A) Plasma ALT activity, (B) Plasma AST activity. After treatment with methylglyoxal for 6.5 hr, we measured the plasma ALT and AST activities. Data represent the mean ± S.E. of 5 mice in each group (significant compared to vehicle-treated mice, $^{**}p < 0.01$).

GSTs, in the liver (24). Previous studies showed that methylglyoxal affects antioxidant defense systems; thus, we evaluated the effect of methylglyoxal treatment on liver damage in mice. ALT and AST are cytosolic enzymes in the liver, and an increase in the plasma levels of these enzymes indicates liver injury (25). Blood biochemistry showed that plasma ALT and AST activities increased moderately 6.5 hr after treatment with 400 mg/kg of methylglyoxal. These results confirmed that methylglyoxal exerts liver damage *in vivo*.

DISCUSSION

In this study, we investigated the mechanism underlying the toxicity of methylglyoxal in the liver. The correlations between metabolic disease and mitochondrial dysfunction in the liver showed that methylglyoxal disturbed the mitochondrial function and induced apoptosis in the cells and liver injury in mice.

Several studies have indicated the pathophysiological roles of methylglyoxal in the liver (24,26). Exposure of mice to methylglyoxal induced significant changes in redox-homeostasis in the liver (24). Methylglyoxal decreased the GSH content and induced lipid peroxidation (24). However, GSH depletion in rats induced the accumulation of methylglyoxal in the liver (27). Moreover, the activities of various antioxidant enzymes decreased because of treatment with methylglyoxal (24). In HepG2 cells, methylglyoxal impaired insulin signaling by inhibition of phosphorylation of insulin receptor substrate 1 (IRS1) and activation of phosphoinositide 3-kinase (PI3K) which might be mediated ROS production (26).

Our results indicated that oxidative stress is involved in methylglyoxal-induced toxicity. In agreement with previous reports (28-30), methylglyoxal induced a significant but relatively small increase in ROS formation. However, experiments by using trolox, PEG-catalase, and Mn-TBAP supported the conclusion that ROS formation by methylglyoxal plays a key role in cell death. Moreover, cells treated with methylglyoxal showed significant decreases in the mitochondrial membrane potential. Furthermore, the data showing the prevention of methylglyoxal-induced apoptosis by cyclosporin A support the hypothesis that the toxic effects of methylglyoxal might be because of mitochondrial impairment. In addition, we detected a marked increase in the activities of blood transaminases after methylglyoxal injection in mice.

Methylglyoxal-induced deleterious effects on cells are also mediated by the formation of AGEs. AGEs activate the receptor for advanced glycation end products (RAGE) (31). Binding of AGEs with RAGE activates various transcription factors including nuclear factor-kappaB (NF-κB), activator protein-1 (AP-1), cAMP response element-binding protein (CREB), signal transducer and activator of transcription 3 (STAT3), or $p21^{ras}$, which produces proinflammatory cytokines, ROS, or impairs tissue remodeling (8,31,32). RAGE-engaged intracellular signalings cause autophagy, apoptosis, inflammation, or delayed tissue repair in various cells (31). However, the precise signaling pathway that mediates the harmful effect of methylglyoxal or AGEs on the mitochondria remains to be elucidated.

In conclusion, our results showed that methylglyoxal induces mitochondrial dysfunction and cell death by production of ROS. These findings would provide insights in understanding the mechanism underlying methylglyoxal-mediated toxicity in the liver.

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