Methylglyoxal (MG), a highly reactive dicarbonyl, is a natural metabolite in glucose metabolism. MG is often found at high levels in the blood of diabetic patients, and such elevated MG levels in diabetes patients are believed to contribute to diabetic complications by inducing oxidative stress. MG can cause the nonenzymatic glycation of proteins to yield irreversible advanced glycation endproducts (AGEs), leading to the cross-linking or degradation of proteins. Accumulations of crosslinked AGEs in body tissues are believed to be responsible for the long-term complications of diabetes and ageing. In addition, these glycation reactions of MG with amino acids can induce oxidative stress and free radical generation, implicating them in the development of chronic complications such as nephropathy, retinopathy, and neuropathy in diabetic patients. MG can also increase oxidative stress by inactivating antioxidant enzymes such as glutathione reductase and glutathione peroxidase via their glycation. Therefore, attenuation of oxidative stress induced by MG may prevent or reverse abnormalities associated with diabetes mellitus and its complications.

Lipoic acid, a naturally occurring nutraceutical, is well known as an essential cofactor in metabolic reactions involved in energy utilization. Lipoic acid can be supplied by both de novo synthesis and dietary intake. Lipoic acid is reported to be an ideal antioxidant that directly quenches free radicals, inhibits reactive oxygen-generating, and regenerates other antioxidants. Lipoic acid has been shown to be beneficial in the prevention of different diseases that may be related to oxidative stress, such as neurodegeneration, ischemia-reperfusion, polyneuropathy, diabetes, AIDS, and hepatic disorder status. Other studies have shown the potential benefits of lipoic acid in conditions involving autoimmune disease, exercise-induced stress, and tissue ageing. Lipoic acid has also been shown to improve glucose uptake in patients with type 2 diabetes mellitus and exhibit antiobesity effects. In addition, lipoic acid has been used extensively in the treatment of diabetic complications, and linked to the possible prevention of long-term diabetic toxicity. In particular, lipoic acid has long been used clinically for treating diabetic neuropathy as a powerful antioxidant. Recent studies have reported that lipoic acid is a potent antioxidant in various drug-induced toxicities in experimental rat models. However, the effect of lipoic acid on MG-induced oxidative stress related to diabetic complications, especially diabetic nephropathy, has not been investigated to date.

Thus, in this study, we investigated the protective effects of lipoic acid against oxidative stress induced by MG using renal tubular epithelial cells, which are proximal tubule cells known to be susceptible to oxidative stress, and the potential of lipoic acid as a useful agent for diabetic nephropathy.

Materials and Methods
Materials. Racemic alpha-lipoic acid, approved as a
food-grade material in Japan, was supplied by Pharma Foods International Co., Kyoto, Japan. According to the product specification, the purity is more than 98% by the absorptiometric method, and the optical rotation is −1.0° to +1.0°. Dulbecco’s modified Eagle medium/nutrient mixture F-12 (DMEM/F-12) and fetal calf serum (FCS) were purchased from Life Technologies Inc. (Grand Island, NY) and Cell Culture Laboratories (Cleveland, OH), respectively. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) and 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). 2’,7’-Dichlorofluorescin-diacetate (DCFH-DA), dihydrorhodamine 123, and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG were obtained from the Sigma Chemical Co. (St Louis, MO). All other chemicals and reagents used were of analytical grade and obtained from commercial sources.

Cell culture. LLC-PK1, a porcine proximal tubule cell line, was maintained on culture plates containing 5% FCS-supplemented DMEM/F-12 medium (pH 7.2) at 37°C in a humidified atmosphere of 5% CO2 in air. All subsequent procedures were carried out under these conditions.

Cell viability. For cell viability analysis, LLC-PK1 cells were seeded at 2 × 10^4/mL in 96-well plates and preincubated for 24 h. The cells were treated with 500 μM MG and lipoic acid for 24 h. Cell viability was evaluated by a colorimetric MTT assay, which is based on the conversion of MTT to MTT-formazan by mitochondrial enzymes, as described previously (16). A 50 μL aliquot of MTT solution (1 mg/mL) was added to each well of a 96-well culture plate (n=6/group), incubated for 4 h at 37°C, and the medium containing MTT was removed. The incorporated formazan crystals in the viable cells were solubilized with 100 μL dimethyl sulfoxide, and the absorbance at 540 nm of each well was read using a microplate reader (Model 3550-UV, Bio-Rad, Tokyo, Japan).

Assay of intracellular reactive oxygen species (ROS) levels. Intracellular ROS levels were measured by the dichlorofluorescein-diacetate assay, as described by Wang and Joseph (17). Cells were plated on 96-well plates and treated with 500 μM MG and/or lipoic acid for 24 h. Afterward, the cells were washed with calcium- and magnesium-free phosphate-buffered saline (PBS) and incubated in 100 μM DCFH-DA-containing medium. After 15 min, the medium was removed, and the cells were incubated with fresh medium for 1 h. Fluorescence was measured using a fluorescence plate reader (Tecan, Switzerland; excitation was read at 485 nm, and emission was read at 535 nm).

Assay of nitric oxide (NO) levels. The amount of NO produced was assayed by measuring the accumulation of nitrite, using a microplate assay method based on the Griess reaction. Briefly, 100 μL culture supernatant was allowed to react with 100 μL Griess reagent [0.1% N-(1-naphthyl) ethylenediamine, 1% sulfanilamide, 2.5% H3PO4] (18) and then incubated at room temperature for 5 min. The optical density of the assay samples was measured at 540 nm using a microplate reader.

Immunohistochemical analysis of nuclear factor-kappa B (NF-κB) translocation. After incubation and treatment, cells were fixed with 3.7% paraformaldehyde for 30 min at 4°C, washed with PBS, and permeabilized with 0.2% Triton X-100 for 30 min at 4°C. The cells were washed with PBS, blocked with 2% bovine serum albumin for 1 h, and treated with anti-NF-κB antibody for 2 h at 4°C. The anti-NF-κB-bound cells were washed with PBS, incubated with FITC-conjugated anti-rabbit IgG for 1 h at 4°C, washed again with PBS, and then stained with DAPI for 5 min at room temperature. The cells were washed twice more with PBS and analyzed using a fluorescence microscope.

Data analysis. The results for each group are expressed as mean±SE of 6 values. The effect of each parameter was examined using one-way analysis of variance. Individual differences between groups were evaluated using Dunnett’s test, and those at p<0.05 were considered significant.

Results

Cell viability

Figure 1 shows the effects of MG on the viability of renal tubular LLC-PK1 cells. The exposure of LLC-PK1 cells to MG led to a significant loss of cell viability in a

![Fig. 1. Effects of methylglyoxal on cell viability. The results for each group are expressed as mean±SE of 6 values. *p<0.001 compared with non-treated methylglyoxal values.](image-url)

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![Fig. 2. Effects of lipoic acid on cell viability. The results for each group are expressed as mean±SE of 6 values. *p<0.001 compared with non-treated lipoic acid values.](image-url)

Fig. 2. Effects of lipoic acid on cell viability. The results for each group are expressed as mean±SE of 6 values. *p<0.001 compared with non-treated lipoic acid values.
concentration-dependent manner. Particularly, 2,000 μM MG treatment induced cell death to nearly 50% compared with non-treated control cells, while 500 μM MG treatment maintained cell viability at 64% of control cells.

Figure 2 shows the effects of lipoic acid on the viability of LLC-PK1 cells. Lipoic acid did not show any cytotoxic activity under the concentration of 50 μM in LLC-PK1.

The viability of 500 μM MG-treated cells significantly decreased compared with non-treated cells. However, lipoic acid treatment together with MG significantly and concentration-dependently protected the cells against damage induced by MG, as shown in Fig. 3.

**Intracellular ROS and NO**

As shown in Table 1, the generation of intracellular ROS in LLC-PK1 cells increased significantly after treatment with 500 μM MG compared with non-treated cells. However, lipoic acid treatment together with MG significantly and concentration-dependently reduced the intracellular ROS from 464 to 233%.

Table 1 also shows the effects of lipoic acid on MG-induced NO generation in LLC-PK1 cells. The NO level of 500 μM MG-treated cells was elevated significantly compared with that of non-treated cells. However, lipoic acid-treated cells showed significant and concentration-dependent decreases in the level of NO, in particular, 50 μM lipoic acid treatment together with MG significantly decreased the NO level from 349 to 160%.

**NF-κB nuclear translocation inhibitory effect**

Figure 4 shows the effects of lipoic acid on the MG-mediated nuclear translocation of NF-κB. Immunofluorescence staining was observed in the cytosol of non-treated cells, whereas an all-heavy nuclear staining in cells treated with 500 μM MG was observed. However, treatment with 50 μM lipoic acid inhibited NF-κB nuclear translocation from the cytosol. We used DAPI staining for the confirmation of nuclear translocation.

**Table 1. Effects of lipoic acid on ROS and NO levels.**

<table>
<thead>
<tr>
<th>Methylglyoxal (μM)</th>
<th>Lipoic acid (μM)</th>
<th>ROS (%)</th>
<th>NO (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>100±7.9 *</td>
<td>100±12.0 *</td>
</tr>
<tr>
<td>500</td>
<td>0</td>
<td>464±4.7 *</td>
<td>349±5.5 *</td>
</tr>
<tr>
<td>500</td>
<td>6.25</td>
<td>330±4.8 *</td>
<td>217±9.4 * #</td>
</tr>
<tr>
<td>500</td>
<td>12.5</td>
<td>293±3.5 *</td>
<td>196±5.0 * #</td>
</tr>
<tr>
<td>500</td>
<td>25</td>
<td>266±3.4 #</td>
<td>180±10.4 * #</td>
</tr>
<tr>
<td>500</td>
<td>50</td>
<td>233±5.9 #</td>
<td>160±9.9 * #</td>
</tr>
</tbody>
</table>

The results for each group are expressed as mean±SE of 6 values. *p<0.001 compared with non-treated values; # p<0.001 compared with 500 μM methylglyoxal-treated values.

![Fig. 3. Effects of methylglyoxal and lipoic acid on cell viability. The results for each group are expressed as mean±SE of 6 values. *p<0.001 compared with non-treated values: # p<0.001 compared with 500 μM methylglyoxal-treated values.](image1)

![Fig. 4. Effects of lipoic acid on the nuclear translocation of NF-κB. The anti-NF-κB-bound cells were incubated with FITC-conjugated anti-rabbit IgG and then stained with DAPI. Fixed cells were double-immunostained for NF-κB (green) and DAPI (blue). Magnification: ×400.](image2)
Discussion

Increasing evidence has indicated that elevated MG levels may play a role in the development of a number of diabetic complications, including diabetic nephropathy (19). MG is well known to be a reactive dicarbonyl compound produced mainly from glycolytic intermediates in the cell, and is often found at high levels in blood from diabetic patients. MG readily reacts with arginine, lysine, and sulfhydryl groups of proteins and nucleic acids (20), inducing the formation of a variety of structurally identified AGEs. AGE formation promoted by MG appears to be associated with diabetic complications (21). As the major precursor of AGE formation, both in the cell and plasma, MG can influence multiple aspects of cellular biology in diabetes (22). Recently, a great deal of attention has been focused on compounds capable of inhibiting AGE formation and exhibiting antioxidant properties for protection against diabetic complications.

Lipoic acid, due to its antioxidant activity, has been shown to be beneficial in various forms of oxidative stress, and is of interest as a therapeutic agent in ischemia/reperfusion injury and diabetic complications (23). Here, we examined the effects of lipoic acid on MG-induced oxidative stress using renal tubular epithelial cells (LLC-PK1). As most commercial alpha-lipoic acid in Japan is racemate, we used racemic alpha-lipoic acid in this study. However, it is reported that enantiomers of lipoic acid have different physiological activities. Breithaupt-Grogler et al. (24) reported that plasma enantiomer concentrations increased after the oral administration of racemic lipoic acid in healthy volunteers. Both enantiomers were rapidly absorbed and showed peak concentrations within 0.5 to 1 h. Maximum plasma concentrations of R(+)-enantiomer were about 40 to 50% higher than those of S(-)-enantiomer. The effects of enantiomers of lipoic acid on the enzymatic response are different. R(+)-Enantiomer has a greater effect on lipoamide dehydrogenase than S(-)-enantiomer, while the activity of glutathione reductase was higher with S(-)-enantiomer (25). Therefore, S(-)-alpha-lipoic acid might also contribute to antioxidant protection by activating glutathione reductase. To clarify the contributions of enantiomers for MG-induced oxidative stress, further study is needed.

ROS are oxygen-containing molecules having either unpaired electrons or the ability to cleave electrons from other molecules. The development of chronic human disease is associated with increased ROS generation, and excessive ROS O2·-, H2O2, hydroxyl radical (-OH) production is an important cause of cell damage, such as that occurring in renal ischemia (26). In addition, ROS generation has an adverse effect on cell viability (27). It was reported that glycation reactions of amino acids caused by MG generated ROS (28). A recent study also reported that MG induces ROS such as both O2·- and H2O2 in neutrophils (29). Our results also showed that MG-treated LLC-PK1 cells significantly increased the total intracellular ROS level compared with non-treated cells. However, lipoic acid significantly decreased the elevated ROS level induced by MG (Table 1). These findings imply that lipoic acid alleviates oxidative stress by inhibiting ROS production induced by MG.

Many chemical and physical treatments capable of inducing apoptosis are known to provoke oxidative stress via ROS generation in cells, suggesting a close relationship between oxidative stress and apoptosis. The association between MG and ROS generation is particularly noteworthy because ROS and oxidative stress can damage many biological molecules (e.g., DNA) and cause various cell injuries. In addition, glycation reactions of MG with amino acids can induce oxidative stress and free radical generation, leading to subsequent cytotoxicity and apoptosis. In particular, ROS generation and/or nitrogen species that have been implicated in the initiation and development of renal diseases involve the tubular cells, and such generation results in the eventual injury and death of renal cells (30). Since the renal functional changes of tubular cells mediated by free radicals are essentially related to renal injury, the protection of renal tubular cells against free radical damage may ameliorate renal injury and dysfunction. To investigate the effect of lipoic acid on renal tubular LLC-PK1 cellular damage induced by MG, we measured cell viability using the MTT assay in this study. Our findings demonstrated that the exposure of renal tubular LLC-PK1 cells to MG at a concentration of 500 µM resulted in the loss of cell viability (Fig. 1). Lipoic acid inhibited cell death, suggesting that it protected LLC-PK1 cells from MG-induced cytotoxicity.

However, this inhibition was not increased concentration-dependently, despite the concentration-dependent inhibition of ROS and NO. These results also imply that lipoic acid would prevent diabetic nephropathy, a diabetic complication induced by MG in diabetes, through inhibiting renal tubular injury and cell death. Thus, it is thought that cell death may be affected by not only oxidative stress but also many other factors, such as ATP depletion, increased intracellular Ca2+ and exposure to heavy metals (31).

NO, one of the reactive nitrogen species, is also considered a member of the ROS family. It rapidly reacts with O2·- to form peroxynitrite (ONOO−). ONOO− can cross cell membranes freely and is an extremely strong and reactive oxidant (32). NO is also responsible for deleterious effects on cell function, and it interacts with O2·- to form ·OH that leads to highly reactive oxidative damage associated with diabetes (33). Furthermore, NO targets intracellular antioxidative enzymes, resulting in the loss of their function (34). In the present study, we found that lipoic acid inhibited NO produced by MG treatment (Table 1). In support of this, lipoic acid was reported to inhibit LPS-induced activation of iNOS and NO in macrophages (35). These findings suggest that lipoic acid might protect against oxidative stress by inhibiting ROS and NO by MG.

NF-κB, an oxidative-stress-responsive transcription factor, is known to play an important role in the mechanism of cell injury and in the inductions of iNOS and COX-2, which are both expressed as a result of NF-κB
activation (36). Furthermore, NF-κB plays important roles in the expressions of a variety of genes involved in inflammatory responses, and in apoptosis in multiple tissues and cell types (37). NF-κB is normally sequestered in the cytoplasm as an inactive complex through binding to an inhibitory protein, inhibitory kappa B (IκB). The phosphorylation and subsequent ubiquitination of IκB induced by various extracellular stimuli causes the rapid degradation of this inhibitory subunit by proteosomes. Free NF-κB translocates into the nucleus, where it binds to its binding sites in the promoters of target genes, thereby controlling their expression (38). According to recent research, NF-κB translocation into the nucleus is inhibited by antiinflammatory agents (sodium salicylate and dexamethasone), antioxidants, and proteosome inhibitors (39, 40). Wu (41) reported that MG activated NF-κB in freshly isolated and cultured smooth muscle cells. Although lipoic acid was reported to be a potent inhibitor of NF-κB activation in human T cells and to inhibit NF-κB activation in osteoclast precursor cells (42, 43), we investigated the activity of lipoic acid in LLC-PK1 cells at concentrations from 6.25 to 50 μM in this study. Lipoic acid showed a protective effect at a lower concentration (6.25 μM), as shown in Fig. 3, and its inhibitory activity against ROS and NO was increased in a concentration-dependent manner (Table 1). Therefore, we conducted immunochemical analysis of NF-κB translocation at the concentration of 50 μM to obtain clear data, as shown in Fig. 4. Our data also clearly show that lipoic acid inhibited MG-induced NF-κB nuclear translocation, indicating that lipoic acid might protect against oxidative stress induced by MG and be useful in inhibiting NF-κB translocation into the nucleus.

To summarize, the present study demonstrated that lipoic acid may ameliorate oxidative stress induced by MG through regulation of the intracellular ROS and NO levels. Lipoic acid treatment also reduces MG-induced cytotoxicity by inhibiting NF-κB activation in LLC-PK1 cells. These results provide evidence of the potential benefits of lipoic acid in pathological conditions associated with diabetic complications, including diabetic nephropathy induced by MG in diabetes.

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