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Metformin prevents methylglyoxal-induced apoptosis of mouse Schwann cells

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Abstract

Methylglyoxal (MG) is involved in the pathogenesis of diabetic complications via the formation of advanced glycation end products (AGEs) and reactive oxygen species (ROS). To clarify whether the antidiabetic drug metformin prevents Schwann cell damage induced by MG, we cultured mouse Schwann cells in the presence of MG and metformin. Cell apoptosis was evaluated using Hoechst 33342 nuclear staining, caspase-3 activity, and c-Jun-N-terminal kinase (JNK) phosphorylation. Intracellular ROS formation was determined by flow cytometry, and AMP-activated kinase (AMPK) phosphorylation was also examined. MG treatment resulted in blunted cell proliferation, an increase in the number of apoptotic cells, and the activation of caspase-3 and JNK along with enhanced intracellular ROS formation. All of these changes were significantly inhibited by metformin. No significant activation of AMPK by MG or metformin was observed. Taken together, metformin likely prevents MG-induced apoptotic signals in mouse Schwann cells by inhibiting the formation of AGEs and ROS.

Keywords: Methylglyoxal; Metformin; Apoptosis; Advanced glycation end products; Reactive oxygen species; Oxidative stress

The pathogenesis of chronic diabetic complications is complex and has not been fully elucidated. Among various mechanisms suggested up to this point in time, glycation and its related reactions have drawn attention as playing a pivotal role [1].

Methylglyoxal (MG) is a reactive dicarbonyl precursor of advanced glycation end products (AGEs) [1]. MG is formed from a glycolytic intermediate glyceraldehyde 3-phosphate [2], from the early glycation process by degradation of glucose or Schiff's base, or from Amadori products in the intermediate stages of glycation [3]. MG is considered an important focal point at which glucose can go on to form AGEs [4]. It is also known that MG is a potent source of reactive oxygen species (ROS) [5,6] and inactivates antioxidative enzymes [7], causing enhanced intracellular oxidative stress.

Of interest, recent papers have reported that MG induces apoptosis of rat Schwann cells [8] and human vascular

Abbreviations: AGEs, advanced glycation end products; AMPK, AMP-activated kinase; BSA, bovine serum albumin; DCFH, 2′,7′-dichlorofluorescin diacetate; IMS, immortalized mouse Schwann cells; JNK, c-Jun-N-terminal kinase; MG, methylglyoxal; MTS, 3-(4,5- dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; ROS, reactive oxygen species.

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endothelial cells [9]. It is also notable that the concentration of methylglyoxal is increased not only in diabetic animal tissues [10], but also in the plasma of diabetic patients, particularly in those showing evidence of early diabetic retinopathy [11] and nephropathy [12,13]. These observations indicate that MG plays a significant role in the etiology of diabetic complications, and thus compounds that decrease MG may be beneficial in preventing the disease.

The guanidino compound aminoguanidine, the most extensively investigated inhibitor of AGE formation [14], is known to capture carbonyl compounds with its amino groups. Oral hypoglycemic biguanides, such as metformin and buformin, are guanidine compounds with chemical structures similar to aminoguanidine, and a previous report suggested that metformin directly reacted *in vitro* with glyoxal and MG leading to the formation of stable triazepinone derivatives [15]. Therefore, it may be postulated that metformin is able to trap reactive carbonyl species and lower their concentrations under physiological conditions. As a matter of fact, metformin has been reported to reduce systematic MG levels in patients with type 2 diabetes [16]. Because the results of large-scale clinical investigations have provided evidence that metformin has preventive effects on diabetic complications independent of its hypoglycemic action [17], the reaction between metformin and carbonyl species may account at least in part for the favorable effects of the agent. At present, the detailed mechanisms of the MG-metformin interaction in each complication remain to be clarified.

The present study was devised in order to elucidate the mechanisms of MG-induced dysfunction of Schwann cells and to clarify whether metformin is able to rescue it. The signaling pathway of apoptosis and the involvement of oxidative stress were investigated.

Materials and methods

Materials. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was purchased from Promega (Madison, WI, USA). Hoechst 33342 was obtained from Dojindo (Kumamoto, JAPAN). Antibodies against caspase-3 (Asp175), c-Jun-N-terminal kinase (JNK) and AMP-activated kinase (AMPK) were purchased from Cell Signaling (Beverly, MA, USA) and 2′,7′-dichlorofluorescin-diacetate (DCFH-DA) was from Calbiochem (Darmstadt, Germany). Anti-argpyrimidine antibody was obtained from NOF (Tokyo, Japan) and fetal bovine serum (FBS) was from Moregate (BioTech, Australia). DMEM and other chemicals were purchased from Sigma (St. Louis, MO, USA).

Cell culture condition. Immortalized mouse Schwann cells (IMS), established from adult mouse dorsal root ganglia [18], were used because of their suitable properties as mature Schwann cells [19]. The cells in passages between 34 and 39 were cultured in DMEM containing 5.5 mmol/L glucose, penicillin (100 U/ml)/streptomycin (100 mg/ml) with 5% FBS, pH 7.40 at 37 °C in a humidified 10% CO_2/90% air atmosphere, until IMS reached 80% confluency. Then the cells were starved in DMEM with 2% FBS for 24 h, followed by incubation with 0–1000 μmol/L methylglyoxal in the presence or absence of 250 –1000 μmol/L metformin for the indicated periods in each experiment.

Cell proliferation assay. We examined cell proliferation by means of MTS [20]. In brief, IMS were seeded in 96-well plates at a density of 7000 cells/well and grown for 24 h in DMEM with 5% FBS. The cells were incubated with MG and metformin in 5% FBS for 48 h at each concentration. Ten microliter of MTS solution was then added to 100 μl of medium in each well. After 3 h incubation, the absorbance at 405 nm was determined using a Powerscan HT spectrophotometer (Dainippon pharmaceutical, Osaka Japan).

Detection of apoptotic cells by Hoechst 33342. Morphological evidence of apotopsis in IMS was assessed by means of the fluorescent DNA-binding dye, Hoechst 33342. IMS were starved by reducing FBS in the medium to 2% for 24 h, and cultured under experimental conditions for 48 h. The cells were harvested using a cell scraper, washed with PBS and fixed with 4% paraformaldehyde for 30 min. After centrifugation, the supernatant was discarded and the cells were stained with 3 μl of 1 mg/ml Hoechst 33342 solution in 20 μl of PBS. The sample was put on a slide-glass and apoptotic cells were detected by means of a fluorescent microscopy (Olympus BX51, Tokyo, Japan). The numbers of apoptotic and total cells were counted in each visual field, and the percentages of apoptotic cells were calculated in 15 visual fields for each condition.

Immunoblot analysis for caspase-3, JNK and AMPK. IMS grown in 10 cm dishes were treated with 1000 μmol/L MG in combination with various concentrations of metformin for 6 h (JNK), 14 h (caspase-3) or 18 h (AMPK) after 24 h starvation in 2% FBS medium. Cells were lysed on ice in a buffer containing 50 mmol Tris–HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mmol/L Na_3VO_4 and 1 mmol/L NaF. After determination of the protein concentration using a BCA assay (Sigma, St. Louis, USA), 30 μg proteins were electrophoresed on an SDS–PAGE gel (15% acrylamide gel for caspase-3, 10% for JNK and AMPK) and transferred to a nitrocellulose membrane. The membrane was blocked overnight with ovalbumin and incubated with a polyclonal cleaved caspase-3 (Asp175) antibody, phospho-SAPK/JNK (Thr183/Tyr185) antibody or with a polyclonal anti-phospho AMPK-α antibody overnight at 4 °C, followed by incubation with an anti-rabbit polyclonal IgG antibody. The binding antibodies were visualized by using an ECL chemiluminesence detection kit (Amersham, Buckinghamshire, UK).

Determination of intracellular ROS. Intracellular ROS generation was measured using a flow cytometric assay by a modification of previous methods [21]. IMS were cultured in 6 cm dishes for 48 h with 5% FBS. The cells were incubated with metformin for 1.5 h followed by the addition of 1000 μmol/L MG. After additional incubation for 1 and 2 h, 10 μmol/L DCFH-DA was then added to the wells and the wells were incubated for 45 min. After washing with ice-cold PBS, the cells were collected with a scraper and were applied to flow cytometry. The generation of ROS was detected as changes in fluorescence due to the oxidation of DCFH.

In vitro AGE production from MG. Ten mg/ml BSA and 1000 or 100 μmol/L MG were incubated with metformin at the concentrations of 10 μmol/L to 10 mmol/L at 37 °C for 48 h or 14 days after sterilization using a MILLEX GV filter (Millipore, Cork, Ireland). AGE production was detected by fluorescence at excitation/emission wavelengths of 320/383 and 335/385 nm using the RF-1500 Spectro fluorophotometer (Shimadzu, Kyoto, Japan). Ten microgram proteins, after 14 days of incubation, were also applied to SDS–PAGE gels (10% acrylamide) and analyzed by Western blotting using anti-argpyrimidine antibody.

Statistical analysis. The differences among each experimental condition were assessed by analysis of variance (ANOVA).

Results

Cell proliferation and apoptosis

Fig. 1A shows the absorbance of culture media after incubation with MTS for 3 h, which is believed to be proportional to the number of living cells. MG suppressed the MTS-derived increase in absorbance by up to 80% in a dose-dependent manner. Metformin significantly attenuated the cytotoxicity of MG in a clear dose-dependent manner (Fig. 1B).

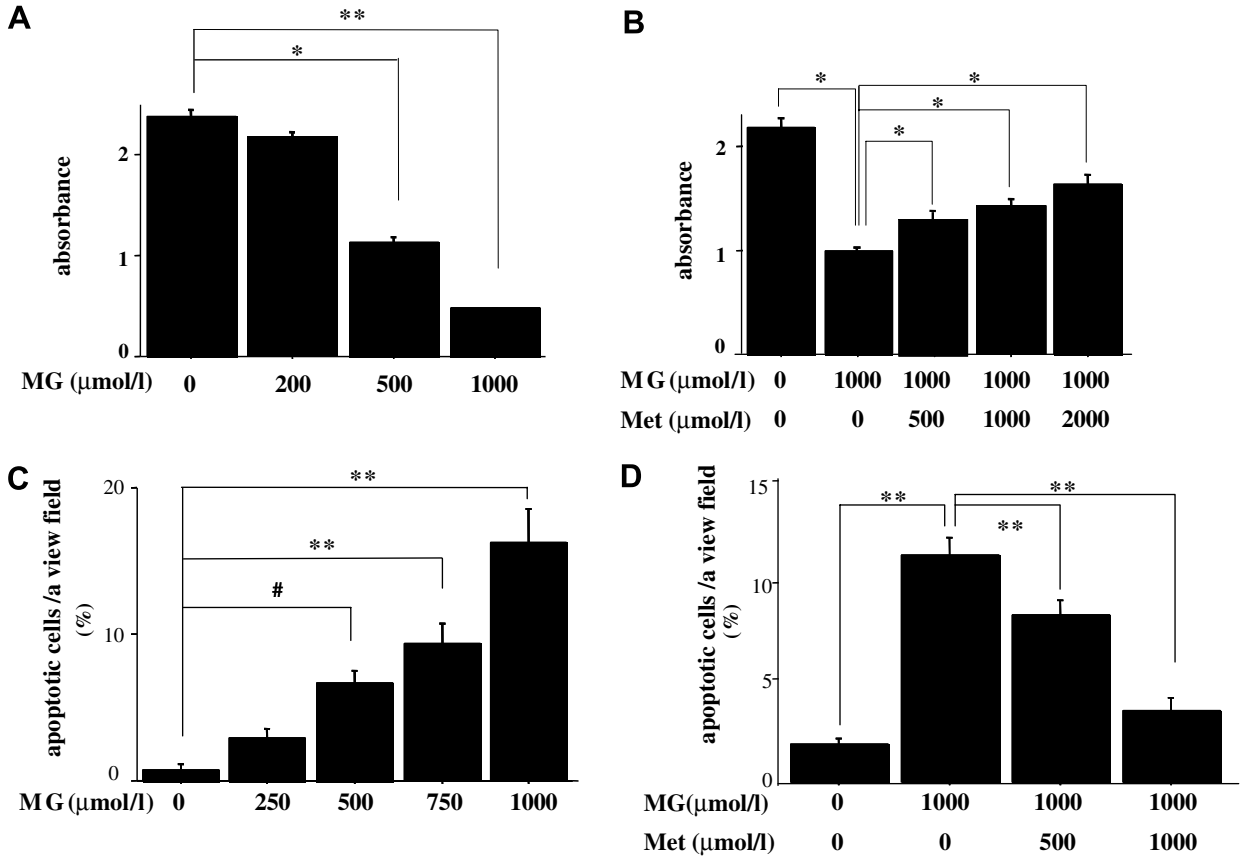

Fig. 1. Cell proliferation and apoptosis of IMS. (A) MTS assay after 24 h culture in the presence of MG; (B) the effect of metformin on the MG suppression of cell proliferation; (C) the percentage of apoptotic cells measured after 48 h incubation with MG; (D) the attenuation of the MG-induced apoptosis by metformin. Error bars express SD. $^{*}p < 0.001$, $^{**}p < 0.0001$, $^{\#}p < 0.01$.

Apoptotic cells were identified as those with nuclear condensation. In the control group, $0.7 \pm 1.39\%$ of the cells per one view were scored as apoptotic. The ratio of the apoptotic cell number to the total cell number was increased to $16.2 \pm 8.89\%$ when treated with 1000 μmol/L MG, with an apparent dose-dependency (Fig. 1C). This enhanced apoptotic cell death by MG was significantly suppressed by 26% with 500 μmol/L metformin and by 69% with 1000 μmol/L metformin, as shown in Fig. 2D.

Activation of caspase-3 and JNK

Caspase-3, a representative signaling molecule in the apoptosis pathway, is known to be activated by cleavage of itself. Western blot analysis of caspase-3 in IMS demonstrated that MG induced the cleavage of caspase-3 in a dose-dependent manner, and the induction by MG was clearly inhibited by treatment with metformin (Fig. 2A).

JNK is located upstream of caspase-3 and its activation has been linked to intracellular ROS production [22]. As shown in Fig. 2B, JNK was phosphorylated by incubation with 1000 μmol/L MG, and the addition of metformin dose-dependently suppressed the activation of JNK.

We also examined AMPK phosphorylation in order to clarify the possible involvement of AMPK in IMS apoptosis, because AMPK activation has been suggested to induce apoptosis in beta cells [23] and both oxidative stress and metformin may influence AMPK activity [24,25]. In contrast to caspase-3 and JNK, no significant increase of phosphorylated AMPK was observed by MG or metformin treatment in IMS under this experimental condition (Fig. 2C).

Intracellular ROS generation

Fig. 3 depicts the intracellular DCF fluorescence on flow cytometry. The cells incubated with MG for 1 and 2 h exhibited evidently enhanced fluorescence as compared with non-treated cells. The treatment with metformin normalized the fluorescence in cells incubated with MG.

AGE production in vitro

The fluorescence at excitation/emission wavelengths of 320/383 and 335/385 nm is thought to reflect the amount of argpyrimidine and pentosidine, respectively. The incubation of BSA with 1000 μmol/L MG for 48 h resulted in an apparent increase in the fluorescence at both wavelengths, and metformin inhibited the fluorescent augmentation in a dose-dependent manner (Fig. 4A). Similar results were observed after 14 days incubation of BSA with 100 μmol/

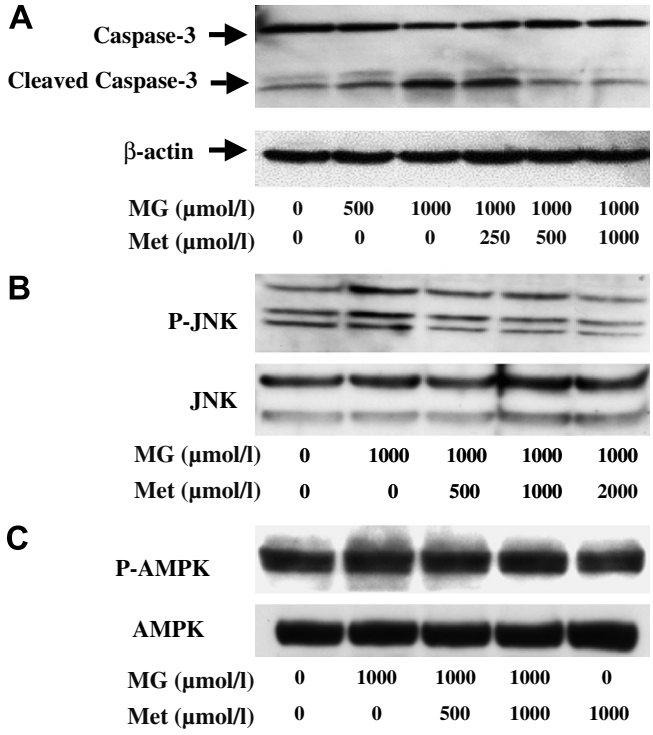

Fig. 2. Effects of MG and metformin (Met) on caspase-3 cleavage (A), JNK phosphorylation (B) and AMPK phosphorylation (C) in IMS incubated with MG and Met for 14 h (A), 6 h (B) or 18 h (C).

L MG (Fig. 4B). In the presence of MG and metformin at equal concentrations, the inhibition of fluorescent increases was approximately 20%. Western blot analysis using anti-argpyrimidine antibody showed comparable results to the fluorescence examination, with complete disappearance of argpyrimidine by 10 mmol/L metformin against 100 μmol/L MG (Fig. 4C).

Discussion

The present results of the MTS assay, nuclear staining and caspase-3 assay clearly indicated that MG is a potent inducer of apoptosis in IMS. The results seem comparable with previous observations that MG directed Schwann cells or vascular endothelial cells to apoptosis [8,9]. Given the fact that MG is increased in diabetic tissues [12] and the loss of Schwann cells plays a pivotal role in diabetic nerve dysfunction [26–28], these data appear to suggest the probable involvement of MG in the pathogenesis of diabetic neuropathy.

To elucidate the mechanisms of MG-induced apoptosis in IMS, we focused on JNK. This kinase is located upstream of caspase-3 [29] and is activated by oxidative stress [22]. MG and its products, AGEs, are known to be potent sources of ROS that, in theory, stimulate JNK. As expected, MG caused obvious phosphorylation of JNK

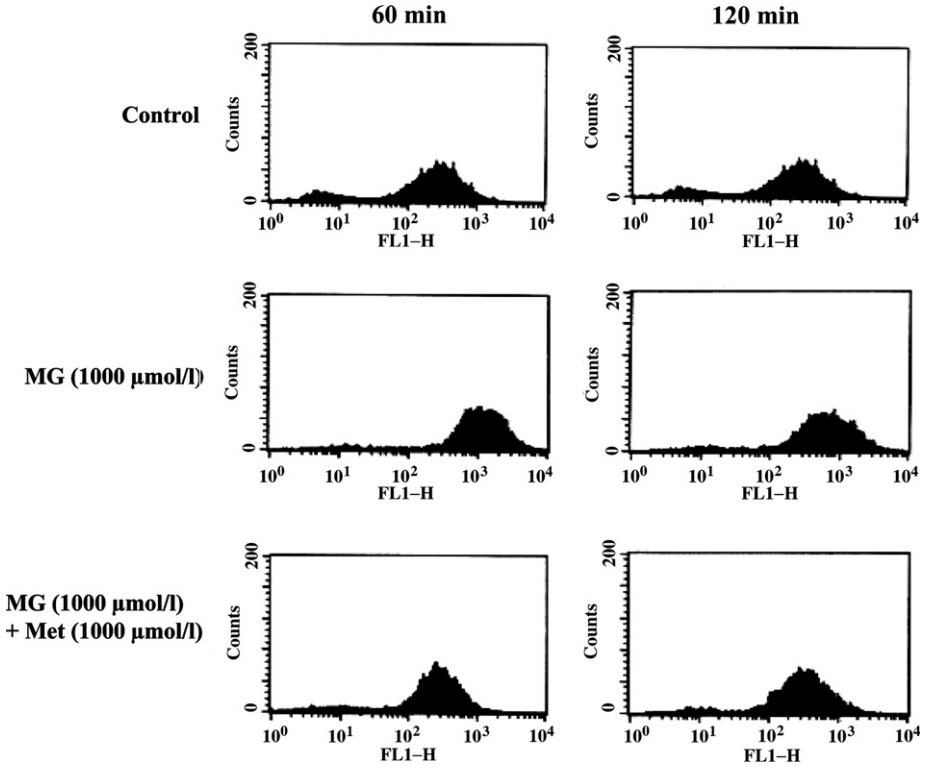

Fig. 3. Intracellular oxidative stress assessed by means of DCFH on flow-cytometry. IMS were incubated with metformin for 1.5 h followed by the addition of 1000 μmol/L MG. After additional incubation for 1 and 2 h, 10 μmol/L DCFH-DA were added to the wells. The cells were incubated for another 45 min, washed and applied to flow cytometry.

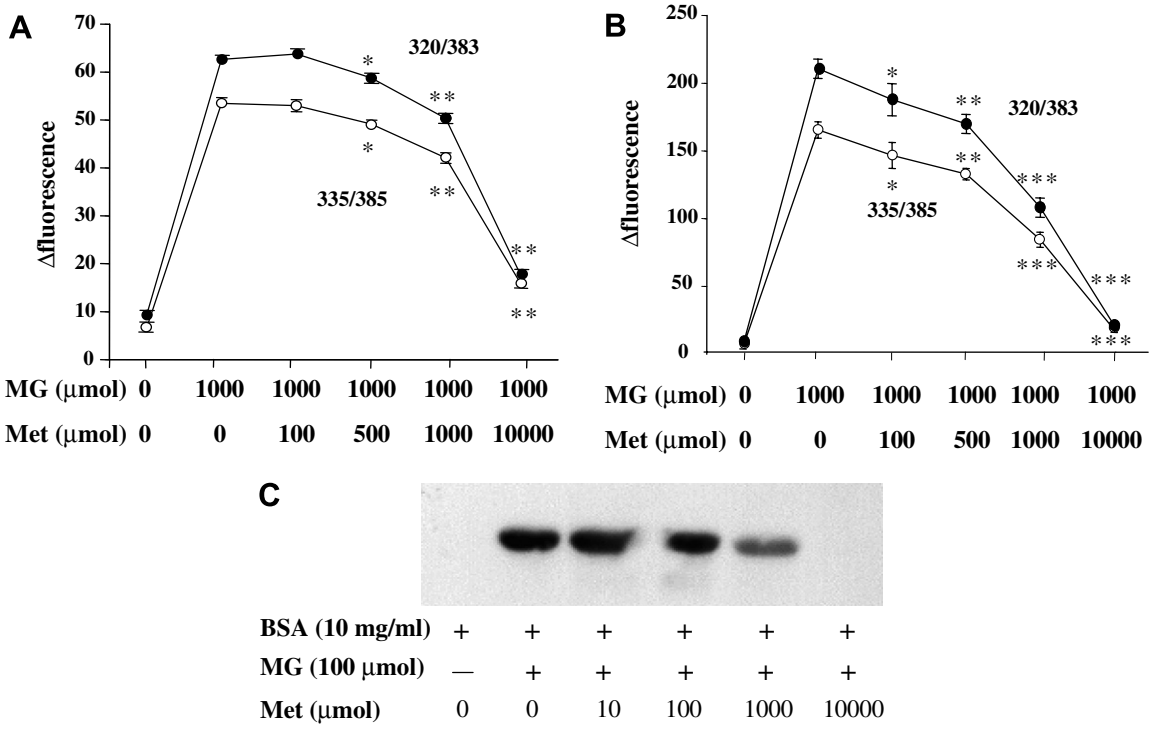

Fig. 4. Suppressive effects of metformin (Met) on AGE formation from BSA and MG after 48 h (A) or 14 days (B). AGE formation was evaluated by fluorescence (A,B) and Western blotting (C). $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0001$ vs MG group.

in a dose-dependent manner. Although there is a report that MG induces apoptosis in rat Schwann cells through the activation of p38 MAPK [8], our findings indicate that JNK is also involved in the signaling pathway of MG-related apoptosis in IMS.

Of interest, metformin apparently prevented IMS from apoptotic cell death caused by MG treatment. The inhibition of apoptosis was accompanied by the suppression of JNK phosphorylation, suggesting that metformin may block apoptotic signals toward JNK. The results of flow cytometry after incubating cells with DCFH-DA gave a possible explanation. The intercellular ROS formation enhanced by MG was explicitly attenuated by the treatment with metformin.

These anti-oxidant effects of metformin would be attributable to its structure as a biguanide [15]. Metformin, like aminoguanidine, reacts, in theory, with MG to form triazepinone derivatives [15], and our *in vitro* experiment clearly demonstrated that the AGE formation from MG was inhibited by the presence of metformin. The results may be relevant to previous observations of metformin suppression of AGE formation from MG or glucose [30,31] and seem to account for its antioxidant action, because MG exerts itself as a potent pro-oxidant substance [5–7]. Nevertheless, the metformin scavenging of MG may not be an exclusive mechanism for cell protection, considering that the suppression of AGE formation from MG was partial (Fig. 4). Another possible explanation may be a direct suppression of oxidative stress by metformin independent of trapping MG. Previous studies have shown that metformin directly scavenges ROS or modulates the intracellular production of superoxide anion [32,33].

These favorable characteristics of metformin against carbonyl and oxidative stress in Schwann cells seem to give theoretical support to previous data showing that metformin ameliorated the peripheral nerve dysfunction in diabetic rats [34].

One may raise a question about the contribution of AMPK activity in the present experiments, because metformin is recognized as an AMPK activator [24] and an interaction between AMPK and oxidative stress has been reported [25]. However, AMPK was not activated in our study after 24 h incubation with either metformin or MG, when apparent apoptosis was observed in IMS. We thus reached the conclusion that AMPK was not involved in the MG-related apoptosis process and its prevention by metformin observed in IMS.

In conclusion, the results of the present study indicate that metformin may have beneficial effects on chronic diabetic neuropathy, not only through blood glucose reduction, but also through the prevention of AGE formation or ROS production from dicarbonyl compounds in Schwann cells. Because metformin is widely used as a hypoglycemic agent, its suppressive effects on diabetic complications warrant further investigation.

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