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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).