Methylglyoxal increases dopamine level and leads to oxidative stress in SH-SY5Y cells

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More and more studies have suggested that methylglyoxal (MGO) induced by type-2 diabetes is related to Parkinson’s disease (PD). However, little is known about the molecular mechanism. In this study, we explored the MGO toxicity in neuroblastoma SH-SY5Y cells. Neurotoxicity of MGO was measured by mitochondrial membrane potential, malondialdehyde, and methylthiazoletetrazolium assays. The levels of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), and 1-methyl-4-phenyl-1,2,3,4-tetrahydroisoquinoline (salsolinol) were detected by liquid chromatography–mass spectrometry/mass spectrometry. The expressions of tyrosine hydroxylase (TH) and dopamine transporter (DAT) were detected by reverse transcriptase polymerase chain reaction and western blot analysis. The results showed that MGO induced an increase in TH and DAT expressions in SH-SY5Y neuroblastoma cells, while the levels of dopamine, DOPAC, and endogenous neurotoxin salsolinol also increased. Aminoguanidine (AG) is an inhibitor of MGO. It was found that AG could decrease the reactive oxygen species (ROS) level induced by MGO, but could not inhibit an increase of TH, DAT and dopamine. The increase of dopamine, DOPAC and salsolinol levels could lead to high ROS and mitochondrial damage. This study suggests that ROS caused by dopamine could contribute to the damage of dopaminergic neurons when MGO is increased during the course of diabetes.

Keywords methylglyoxal; tyrosine hydroxylase; dopamine transporter; dopamine; Parkinson’s disease

Introduction

Parkinson’s disease (PD) is an age-related neurodegenerative disease [1]. The pathology of PD is associated with the death of dopaminergic neurons in the nigrostriatal pathway and the presence of Lewy bodies in the surviving substantia nigral neurons [2,3]. However, its etiology is still unknown [4]. Environmental factors, such as pesticide exposure [5,6], adiposity [7,8], and diabetes [9,10], have been reported to be related to PD. Recent studies supported a strong link between type-2 diabetes and PD [11,12]. Furthermore, motor symptoms of PD are worse in individuals with co-morbid type-2 diabetes than that in the normal [13]. However, the underlying molecular mechanisms responsible for the relation between diabetes and PD are still unknown. Methylglyoxal (MGO) is a well-known toxic compound that is found to be increasing under certain pathophysiological conditions, particularly in diabetes. Therefore, it has implication in diabetic neuropathy [14,15]. MGO cross-links amino groups in proteins forming advanced glycation end-products, which are found in various intra-neuronal protein deposits such as neurofibrillary tangles in Alzheimer’s disease and Lewy bodies in PD [16–18]. Furthermore, MGO may directly damage neurons through increasing reactive oxygen species (ROS) production and by inhibiting mitochondrial respiration [18]. Previous studies have confirmed that oxidative stress involved in lipid peroxidation may contribute to the pathogenesis of PD [19]. The brain depends on mitochondrial energy supply which is related to the production of highly ROS, and the substantia nigra under specific circumstances produces high oxidative stress. Previous studies have proved that mitochondrial dysfunction and oxidative stress could contribute to neuronal cell death in PD [20]. An increased dopamine (DA) turnover, resulting in peroxide formation in excess [21], could induce oxidative stress. In addition, DA as a catechol, generates ROS non-enzymatically that cause toxicity in neuronal cells, and finally leads towards PD [22]. The dopamine is synthesized by tyrosine hydroxylase (TH) and metabolizes into 3,4-dihydroxyphenylacetic acid (DOPAC) by monoamine oxidase (MAO). TH synthesizes sufficient amount of DA which ultimately ensures the homeostasis of DA. Most DA is re-uptaken to the cytoplasm by dopamine transporter (DAT), and transported to vesicles by the vesicular monoamine transporter 2, thus avoiding DA auto-oxidation. Both TH and MAO generate ROS, which further endangers...
nigrostriatal neurons [23,24]. The steady state and recycling of DA is very important. The initial loss of nigrostriatal neurons would result in a homeostatic response of augmented DA turnover. 1-Methyl-4-phenyl-1,2,3,4-tetrahydrossoquinoline (sal-solinol) is one of the toxic metabolites of dopamine, which is produced by DA and acetaldehyde [22], and involved in the pathogenesis of PD [25,26]. Toxicity of sal-solinol, which is similar to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), results in the inhibition of complex I of the electron transport chain and subsequent oxidative stress [27], and causes neuronal cell death in substantia nigra.

In this study, we extended our investigations into the pathological effects of MGO. In addition, we also measured the change of TH and DAT in MGO condition and further explored whether MGO could impair the dopamine metabolic balance and lead to oxidative stress in SH-SY5Y cells, which as a model of DA neuron has been used in experimental neurological studies.

**Materials and Methods**

**Chemicals**
The following chemicals were used: Dulbecco’s modified Eagle’s medium (DMEM) and trypsin-EDTA (GIBCO, Karlsruhe, Germany); fetal calf serum (Seromed, Berlin, Germany); sal-solinol, DA, DOPAC, and isoproterenol (ISOP) (Sigma, St Louis, USA); HPLC-grade fórmic acid and acetonitrile (Edmonton, Alberta, Canada). All other reagents were of analytical grade.

**Cell culture and cell viability assay**
SH-SY5Y cells (Xuanwu Hospital, Beijing, China) were cultured in DMEM supplemented with 10% fetal calf serum, penicillin/streptomycin (100 U/ml; 100 μg/ml), and 2 mM L-glutamine at 37°C in a humidified atmosphere containing 5% CO₂/95% air. Cell viability was determined by the MTT assay. Cells were seeded with 10³ cells. The cells were inoculated with different concentrations of drug. After treating for 24 h, 20 μl of MTT (Sigma) solution (5 mg/ml) was added in each well. Then cells were incubated at 37°C for 4 h, and 200 μl dimethyl sulfoxide was added to each well. The absorbance was measured at 570 nm.

**Intracellular ROS production assay and mitochondrial membrane potential detection**
The cellular level of malondialdehyde (MDA) was measured to indicate the oxidative stress level in cells. The MDA level was measured based on the thiobarbituric acid-based colorimetric method. Briefly, samples were prepared by collecting cells after centrifugation, and resuspended in phosphate buffered saline (PBS) containing protease inhibitor solution. Cells were lysed by sonication for 40 s (2 s ON, 1 s OFF). Protein concentration was determined by the Bradford method. The MDA level was measured using malondialdehyde assay kit (Northwest Life Science Specialties, Vancouver, USA). Mitochondrial membrane potential (MMP) was detected using a cell-permeable, mitochondrial-specific fluorescent probe JC-1 (BD Pharmingen, San Diego, USA). Cells were seeded on the glass bottom dish and grown to ~80% confluence. After 15 min loading in JC-1 working solution (1 μg/ml), cells were washed twice with 1× assay buffer and observed under the microscope. Fluorescence images were collected by a Leica TCS SP5 laser scanning confocal microscope (Leica Microsystems, Heidelberg, Germany). Fluorescence intensity was detected by a spectrofluorometer (Horiba, Kyoto, Japan) with excitation/emission wavelength of 488 nm/534 nm (green fluorescence) and 514 nm/594 nm (red fluorescence), respectively. The ratio of green/red fluorescence intensity was recorded.

**Reverse transcriptase polymerase chain reaction analysis**
Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, USA). The cDNA was synthesized using M-MLV reverse transcriptase (RT, Promega, Madison, USA) and oligo dT primer. Polymerase chain reaction (PCR) was performed using cDNA as a template. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. The primers used in PCR were as follows [28]: DAT (106 bp) sense 5’-AGCCTGCGTGCGGTCTTTTCG-3’ and antisense 5’-AGTGGCCGACGTGAAGTGG-3’; TH (142 bp) sense 5’-CGA CCCTGACCTGAGTTGGA-3’ and antisense 5’-GGGATCT TCCTCGGGCG GTG-3’; GAPDH (307 bp) sense 5’-AGTTGC CACCAGCTGAC ACGTT-3’ and antisense 5’-GCCTCAAGA TCATCAGAAT-3’ [29]. Amplified products were separated on a 1.5% agarose gel via electrophoresis, stained with ethidium bromide and photographed under ultraviolet light. Finally, the expression levels of the examined mRNAs were quantified by calculating the intensities of RT-PCR products via quantity one program (Bio-Rad, Hercules, USA).

**High-performance liquid chromatography-electrospray ionization–tandem mass spectrometry analysis of DA, DOPAC, and sal-solinol**
The cells were collected by centrifugation, and resuspended in PBS containing protease inhibitor. The cells were broken by sonication for 40 s (2 s ON, 1 s OFF). The protein concentration was determined, followed by addition of 0.1 M perchloric acid to remove the protein. The prepared cells were centrifuged at 4°C 17,000 g for 20 min. The sample was filtered through 0.22 μm membrane prior to high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–MS/MS). The conditions of
LC–MS/MS were; chromatographic separation was achieved on a Discovery HS-F5 column (3 µm, 150 mm × 2.1 mm; Sigma) maintained at 30 °C. The mobile phase consisted of methanol–water (25/75; v/v) with 10 mM ammonium formate (pH 3.5) and was delivered at a flow rate of 0.15 ml/min. The injection volume was 10 µl. The column eluent was coupled to an electrospray-ionization triple quadrupole mass spectrometer (Agilent, Santa Clara, USA) that was run in the multiple-reaction-monitoring mode (MRM), where a precursor ion is fragmented in the second quadrupole (Q2), and the resulting fragments are detected in the third quadrupole (Q3). MS was operated in the positive mode with a capillary voltage of 3500 V. The nebulizer gas was set at 35 psi, drying gas was set at 6 l/min, and drying gas temperature was kept at 300 °C. The [M+H]^+ precursor ions were used for salsolinol (m/z 180.1), DA (m/z 137), DOPAC (m/z 166.9), and ISOP (m/z 212.1). The product ions selected for the MRM scans were m/z 117.1 for salsolinol, m/z 91 for dopamine, m/z 122.8 for DOPAC, and m/z 107.1 for ISOP. The fragmentor and collision energy (CE) were optimized for each analysis.

Western blot analysis
The cells were lysed in radioimmunoprecipitation buffer and mixed with loading buffer (200 mM Tris-Cl, pH 6.8, 50% glycerol, 2% sodium dodecyl sulfate, 20% β-mercaptoethanol, 0.04% bromophenol blue), and boiled for 5 min. Aliquots of cell lysates (100 µg total protein/sample) were separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, electro transferred to polyvinylidene difluoride membrane (Bio-Rad), blocked with 5% non-fat milk in Tris-buffered saline-Tween buffer for 1.5 h at room temperature, and incubated overnight at 4 °C with the primary antibody against DAT (1 : 250; Biosynthesis, Beijing, China), TH (1 : 250; Biosynthesis, Beijing, China), and β-actin (1 : 7000; Abcam, Cambridge, USA), then with corresponding secondary antibody (Biosynthesis, Beijing, China) for 1 h at room temperature. After extensive washing, the bands were visualized with enhanced chemiluminescence reagents (Thermo Scientific, Madison, USA) and exposed to X-ray film (Kodak, New York, USA). The densitometry of the bands was analyzed by Bio-Rad imaging system Quantity One®.

Statistical analysis
Data were expressed as mean ± standard error of the mean (SEM) from at least three independent experiments. Statistical differences between mean values in two groups were evaluated using Student’s t-test analysis. The differences among more than two groups were analyzed by one-way analysis of variance and Tukey’s post-hoc test. The differences were considered statistically significant at P < 0.05.

Results

Dose-dependent effect of MGO on cell viability in SH-SY5Y cells
The effect of MGO on cell viability was measured by MTT assay. Cells were exposed to MGO for 24 h at 37 °C in concentrations ranging from 0 to 1000 µM. The results showed a significant dose-dependent decrease in cell viability (Fig. 1). When cells were exposed to 600 µM MGO, cell viability was decreased to 68%.

Effect of MGO on the expressions of TH and DAT
The levels of TH and DAT are related to intracellular metabolism of DA, and indirectly reflect the content of dopamine. In order to examine the effect of MGO on the mRNA expressions of TH and DAT in SH-SY5Y neuroblastoma cells, RT-PCR was performed. As shown in Fig. 2A,B, compared with control, MGO enhanced the expressions of DAT and TH at mRNA levels. The effect of MGO on protein levels of TH and DAT in SH-SY5Y neuroblastoma cells were determined by western blot analysis. As shown in Fig. 2C,D, MGO increased the expressions of TH and DAT compared with control. The expressions of DAT and TH reached the maximum at 400–600 µM MGO and decreased continuously with increasing concentration of MGO. The results in Fig. 2 clearly showed that MGO (0–600 µM) increased the mRNA levels and protein expressions of DAT and TH. The content of dopamine was the highest at 600 µM MGO compared with that at the other concentrations, so we selected 600 µM MGO for the subsequent experiments.

Figure 1. Effect of MGO on cell viability of SH-SY5Y neuroblastoma cells. SH-SY5Y neuroblastoma cells were exposed to MGO concentration up to 1000 µM for 24 h. Cell viability was decreased in a dose-dependent manner. **P < 0.01 vs. control.
Effect of MGO on mitochondrial dysfunction and oxidative stress

The cellular level of MDA was measured to indirectly indicate the level of oxidative stress in cells. The damage of mitochondria was examined by measuring the MMP using the fluorescent JC-1. Cells were exposed to 600 μM MGO for 24 h at 37°C for the subsequent experiments. The results showed that 600 μM MGO increased intracellular oxidative stress, while causing a decrease of MMP and seriously damaged the mitochondrial function (Fig. 3).

MGO increased DA and DA metabolites

The effects of MGO on dopamine, DOPAC, and salsolinol were measured using the LC–MS/MS in SH-SY5Y neuroblastoma cells. Cells were exposed to 600 μM MGO for 24 h at 37°C. The results showed that MGO increased the levels of dopamine, DOPAC, and salsolinol (Fig. 4A–C). Aminoguanidine (AG) is a nucleophilic hydrazine compound that quickly reacts with dicarbonyl compounds such as glyoxal and MGO [30]. The inhibitory activity of AG against MGO-induced cell death has been reported previously [31]. Cells were pre-incubated with the carbonyl scavengers (AG) for 1 h at the concentration 250 μM, and then exposed to 600 μM MGO. AG, as an inhibitor of the MGO, reduced the intracellular oxidative stress induced by MGO (Fig. 4D), and it also significantly decreased MGO-induced DOPAC and salsolinol (Fig. 4B,C). But it cannot affect the increase of DA (Fig. 4A).

Effect of AG in MGO-induced DAT and TH

The levels of TH and DAT were related to the content of dopamine. Next, we detected the effect of carbonyl scavengers (AG) on MGO-induced TH and DAT by western blot analysis. The results showed that 600 μM MGO induced an increase of TH and DAT levels, and 250 μM AG could not significantly inhibit the increase of TH and DAT (Fig. 5A,B).

Discussion

Previous epidemiological studies have shown that more than 50% of PD patients exhibit abnormal glucose tolerance or diabetes [32]. Diabetes is estimated to impact 300 million individuals by 2025 [33]. Nevertheless, great number of population is also at high risk for neurodegenerative diseases like PD. Therefore, it is critical to understand the potential of
diabetes to contribute to PD. Recent studies have suggested that excessive carbohydrate (glucose or fructose) catabolism in diabetic patient’s brain causes mitochondrial dysfunction that leads towards PD. The scavenger of MGO can also influence some of the biochemical events, i.e. signal transduction, stress protein synthesis, glycation, and toxin generation that are associated with PD pathology [34]. However, the role of MGO in dopamine neurons apoptosis and its function for the increased risk of developing PD is still unclear.

In this study, we found that MGO increased the expression of TH and DAT. TH can synthesize sufficient amount of DA and DAT can reuptake dopamine to the cytoplasm. The DA metabolic system specifically exists in the dopaminergic neurons. Moreover, DA as a catechol has been suggested as a toxin, as it is involved in the generation of ROS non-enzymatically [22]. The steady state and recycling of dopamine is very important for PD. Here, MGO was found to cause continuous increase of DA, which resulted in high oxidative stress in neurons. In addition, salsolinol as an endogenous neurotoxin is a product of dopamine and acetaldehyde. As well-known endogenous neurotoxin involved in PD, it could induce apoptosis in dopaminergic cells. MGO increased endogenous neurotoxin salsolinol that is more stable and difficult to clear out. Furthermore, its accumulation in cells leads to the storage of toxins. Hence, this study suggested that the production of endogenous neurotoxins could be a reason of neuronal cell death.

ROS produced by MGO have also been suggested as a strong neurotoxin inducer of free radicals and glycation, which consequently leads to Parkinson’s and Alzheimer’s diseases [18]. ROS are removed rapidly by antioxidant system. In this study, AG represented as an inhibitor of MGO, reduced the intracellular oxidative stress induced by
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Figure 6. The neurotoxin of MGO for dopaminergic neurons Here we found that MGO increased the levels of TH and DAT in SH-SY5Y neuroblastoma cells, which were two important enzymes for the endogenous increase of dopamine. Furthermore, MGO induced the increase of dopamine and toxic metabolites of dopamine. It suggested that MGO could increase dopamine-mediated oxidative stress, and contributed to damage of dopaminergic neurons during the course of diabetes.

MGO, and it also decreased MGO induced DOPAC and salvinol. However, it cannot change the increase of DA, TH and DAT. MGO increased dopamine-induced oxidative stress which contributed to targeted damage to dopaminergic neurons.

In summary, this is the first time to explore the relationship between diabetes and PD, and find that cytotoxicity of DA was induced by MGO. The conclusive representation is summarized in Fig. 6. The neurotoxin of MGO in dopaminergic neurons may specifically increase the DA level and other toxic metabolites such as salvinol. DA-mediated oxidative stress could contribute to damage of dopaminergic neurons during the course of diabetes. This study provides a logical explanation about the role of diabetes as risk factors for PD.

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References
