Protective Effect of (4-Methoxybenzylidene)-(3-methoxynophenyl)amine against Neuronal Cell Death Induced by Oxygen and Glucose Deprivation in Rat Organotypic Hippocampal Slice Culture

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Oxygen and glucose are indispensable for the generation of energy. Because brain requires the highest amount of oxygen and glucose among all the organs in the body, it is very sensitive to fluctuations in the oxygen and glucose level. A deficient supply of oxygen and glucose causes fatal damage to neuronal cells, particularly the hippocampus region, leading to an impairment of learning and memory. Although the mechanism underlying neuronal cell death in ischemia is not fully understood, it is known that glutamate excitotoxicity, oxidative stress and postischemic inflammation are implicated in neuronal cell injury. However, in the early phase of ischemia, ATP depletion is believed to contribute to the neuronal cell death. Cellular ATP depletion following the onset of ischemia disrupts the ability of an ion-motive ATPase to eliminate intracellular Na+ and Ca2+, which impairs the ion balance between the intra- and extracellular region. Therefore, it is believed that the inhibition of ATP depletion has a beneficial effect on neuronal cell survival in ischemia.

Although the French population consumes a large amount of meat, the incidence of cardiovascular disease is low compared with other countries. This phenomenon is referred to as the “French paradox”. Experimental evidences have demonstrated that the “French paradox” is attributed to the antioxidant effect of resveratrol (trans-3,4’,5-trihydroxystilbene, Fig. 1A), which is an ingredient of grapes consumed in wine.2–4 Since oxidative stress has been associated with neurodegenerative disease as well as cardiovascular disease,5 a great deal of attention is given to the resveratrol as neuroprotective agent. Indeed, resveratrol has been reported to reduce the neuronal cell death induced by oxidative-generating agents in an in vitro experimental system.4,6,7 However, the physiological benefits of resveratrol on neurodegenerative diseases are not well known—The reason for this is unclear. The Blood-Brain Barrier (BBB) allows lipid-soluble molecules to enter the brain, and selectively impedes the penetration of water-soluble agents into the CNS.7,8 Therefore, it is assumed that only a small amount of resveratrol moves into the CNS as a result of its hydrophilic properties. Recent experimental evidence suggests that its extravascular level in the brain was the lowest among the other organs after the oral administration of resveratrol to animals.9 Therefore, in order to develop a resveratrol-related neuroprotective drug, it is necessary to synthesize a potent neuroprotective resveratrol derivative, in which the free phenolic groups are blocked, thus reducing the hydrophilic properties associated with resveratrol itself.

This study examined the neuroprotective ability of the resveratrol derivative, (4-methoxybenzylidene)-(3-methoxynophenyl)amine (MBMPA), which has blocked free phenolic groups and a transformed connection chain between the two benzene rings (Fig. 1 B). The examining methods involved the effect on the neuronal cell viability in an oxygen and glucose deprivation (OGD)-exposed rat-organotypic-hippocampal slice culture, and the ATP contents of the rat adult hippocampal slice injured by in vitro ischemia. Furthermore,

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this study compared the neuroprotective activity of MBMPA and resveratrol.

MATERIALS AND METHODS

Instrumentations and Materials The UV spectra were obtained using a Molecular Devices E09090 microplate reader. The 1H- (300 MHz) and 13C- (75 MHz) NMR spectra were run on a Gemini-2000 spectrometer. The MS spectra were measured on a Hewlett Packard mass spectrometer. TLC and column chromatography were carried out on precoated silica gel F254 plates (Merck) and Silica gel 60 (Merck, 70—230 mesh). The resveratrol was purchased from the Sigma Co. All the other chemicals and solvents were analytical grade and were used without further purification.

Synthesis of (4-Methoxybenzylidene)-(3-methoxyphenyl)amine The synthesis of MBMPA was performed using a method previously reported.10 p-Anisaldehyde (610 μl, 5.0 mmol) and m-anisidine (560 μl, 5.0 mmol) were dissolved in toluene (25 ml). The reaction mixture was equipped with a dean-stark apparatus and refluxed for 9 h. The toluene was evaporated and the products were recrystallized with 100 ml of methanol at −20°C. The yield of the compound was 145.7 mg (11%).

(4-Methoxybenzylidene)-(3-methoxyphenyl)amine: Yellowish solid; mp 152°C decomposed. 1H-NMR (CDCl 3, 300 MHz) δ: 8.39 (1H, s), 7.86 (1H, t), 7.83 (1H, t), 7.28 (1H, m), 7.00 (1H, t), 6.97 (1H, t), 6.79 (1H, t), 6.76 (2H, m), 3.88 (3H, s), 3.84 (3H, s). 13C-NMR (CDCl 3, 75 MHz) δ: 159.83, 131.99, 130.56, 129.83, 129.15, 114.19, 112.87, 111.49, 107.90, 106.60, 101.07, 55.43, 55.32. IR νmax 2994, 2936, 2836, 1593, 1512, 1464, 1309, 1252, 1165, 1142, 1034, 939, 833, 774 cm−1 MS (EI, m/z): 241 (M+, 100), 210 (3), 121 (2), 108 (7), 92 (12), 77 (22).

Organotypic Hippocampal Slice Cultures Organotypic hippocampal slices cultures were prepared using interface culture methods with minor modifications.11—13 Briefly, the hippocampal slices cultures were prepared using interface cultures methods with minor modifications.11—13) Briefly, the hippocampal slices were cut into 400 μm slices using a Mcllwain tissue chopper in a preincubated ice-cold artificial cerebrospinal fluid (ACSF) containing the following: 124 mM NaCl, 5 mM KCl, NaHCO3 and 10 mM glucose bubbled with 95% O2/5% CO2 gas. Subsequently, the slices were stored in an oxygenated ACSF at room temperature for 1 h. Within the time frame, the synaptic function of the slices had recovered. The ACSF solution was replaced with that without glucose, and the hippocampal slice was exposed to an anaerobic chamber (Forma Co. U.S.A.) equilibrated with 85% N2/10% O2/5% CO2 for 20 min. All the incubation conditions were performed at 37°C. The test samples were added to the reaction medium during the ischemia period.15)

Measurement of ATP Content Six hippocampal slices were homogenized in ice-cold 1 N perchloric acid and subsequently neutralized. The homogenate was then centrifuged at 4°C at 13000 rpm for 7 min. The supernatant was diluted 40 times in an ATP reagent (50 mM Tris acetate pH 7.8, 2 mM EDTA, 6 mM DTT, 0.075% BSA and 10 mM Mg-Acetate) without luciferin/luciferase. Fifty microliters of the supernatant was added to 100 μl of the ATP reagent with 0.035 mM luciferin and 0.4 mg luciferase. The luminescence was then measured using a luminometer (Lumat Lb 9051, Berthold) for one second. A known amount of ATP was used as a standard to calculate the ATP content in the hippocampal slice.

Measurement of Protein Content The protein concentration in the slice was measured using the modified Bradford method.16) Bovine serum albumin was used as the protein standard.

Statistical Analysis The data are presented as a mean±SD from three independent experiments. Statistical analysis was carried out using one-way ANOVA followed by Turkey’s test.

RESULTS

Protective Effect of MBMPA on Cultured Organotypic Hippocampal Slices in Oxygen and Glucose Deprivation

Cellular damage was assessed using the fluorescent image analysis PI uptake. The PI uptake was observed at pre-OGD and 48 h after reoxygenation by confocal laser-scanning microscopy (Carl Zeiss LSM 510, Germany) with a rhodamine filter (Texas red). The digital photos were analyzed directly using the public domain NIH image program (version 1.29). The cell death area of the 40 min OGD-exposed hippocampal slice culture was set to 100% cell death. The level of cell death in the MBMPA or resveratrol-treated hippocampal slice culture was calculated as a percentage of this value.

Preparation of Adult Hippocampal Slices and Induction of in Vitro Ischemia Male Sprague-Dawley rats (220±20 g, Daehan Biolink Co. Korea) were sacrificed by decapitation and the forebrain slices were prepared as described elsewhere.14) The hippocampi were cut into 400 μm slices using a Mcllwain tissue chopper in a preincubated ice-cold artificial cerebrospinal fluid (ACSF) containing the following: 124 mM NaCl, 5 mM KCl, NaHCO3 and 10 mM glucose bubbled with 95% O2/5% CO2 gas. Subsequently, the slices were stored in an oxygenated ACSF at room temperature for 1 h. Within the time frame, the synaptic function of the slices had recovered. The ACSF solution was replaced with that without glucose, and the hippocampal slice was exposed to an anaerobic chamber (Forma Co. U.S.A.) equilibrated with 85% N2/10% O2/5% CO2 for 20 min. All the incubation conditions were performed at 37°C. The test samples were added to the reaction medium during the ischemia period.15)

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Measurement of Protein Content The protein concentration in the slice was measured using the modified Bradford method.16) Bovine serum albumin was used as the protein standard.

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RESULTS
MBMPA was added to the medium before and after 40 min OGD exposure. Two days later, the level of cell death was assessed using the PI uptake. The cell death area was analyzed using a public domain NIH image program (version 1.29). (A) Normoxia, (B) 40 min OGD and vehicle treatment, (C) 40 min OGD and MBMPA (1 μM) treatment, (D) 40 min OGD and MBMPA (10 μM) treatment, (E) 40 min OGD and MBMPA (100 μM) treatment, (F) 40 min OGD and resveratrol (100 μM). (G) Cell death in the OGD-exposed hippocampal slice culture in the presence or absence of MBMPA (n = 5). The three independent experiments were carried out. The data was expressed as a mean ± S.D. The asterisk indicates a significant difference from exposure to 40 min OGD (**p < 0.01, one-way ANOVA followed by Turkey’s test).

Fig. 2. Neuroprotective Effect of MBMPA on the OGD-Exposed Rat Hippocampal Slice Culture

MBMPA has the following advantage over resveratrol in some respects as neuroprotective agent. 1) MBMPA is synthesized via a single step process. However, resveratrol needs to pass many synthetic steps and only very small quantities of resveratrol are found in natural resources. Therefore, MBMPA can be more easily obtained than resveratrol. 2) MBMPA has a blocked free phenolic groups associated with resveratrol. Based on the relationship between the hydrophilic properties and the BBB permeability, MBMPA is expected to penetrate BBB more easily than resveratrol. However, this study did not investigate this difference using pharmacokinetic experiments, which remain to be demonstrated. 3) Based on the advantage of MBMPA, the MBMPA was applied to a 40 min OGD-exposed rat hippocampal slice culture to investigate its neuroprotective effect. MBMPA offers better protection against neuronal cell death than resveratrol (Figs. 2A—G).

DISCUSSION

MBMPA has the following advantage over resveratrol in some respects as neuroprotective agent. 1) MBMPA is synthesized via a single step process. However, resveratrol needs to pass many synthetic steps and only very small quantities of resveratrol are found in natural resources. Therefore, MBMPA was more easily obtained than resveratrol.
lease following the recovery of the ATP level. As the Fig. 2 and Fig. 3, the relationship between the protective effect on neuronal cell death and recovery of ATP content was explained that the neuronal cell death can be significantly protected with ATP recovery level of 100 μM MBMP A.

To the best of the authors’ knowledge, this is the first report of the ability of resveratrol and its derivative to inhibit the ATP depletion in the OGD condition. Further studies will be needed to demonstrate the mechanism by which MBMP A protects neuronal cells in the OGD condition. Overall, these results suggest that MBMP A can act as a neuroprotective agent via its ability to inhibit ATP depletion in ischemia. Furthermore, there is the possibility of resveratrol derivatives being used to treat stroke.

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