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Endogenous semicarbazide-sensitive amine oxidase (SSAO) inhibitor increases 1-methyl-4-phenylpyridinium ion (MPP^+)-induced dopamine efflux by immobilization stress in rat striatum

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Abstract

The present study examined whether or not immobilization stress (IMMO)-inducible semicarbazide-sensitive amine oxidase (SSAO) inhibitor by separated gel filtration from 105,000 g supernate in rat brain cytosol contribute to the dopamine (DA) efflux by 1-methyl-4-phenylpyridinium ion (MPP^+) in the rat striatum. The isoelectric point (p*I*) value of this inhibitor was determined by isoelectric focusing (IEF)-gel electrophoresis to about 3.8. The application of IMMO-induced SSAO inhibitor (3 μg) by IEF-gel electrophoresis increased DA efflux by MPP^+ in rat striatum. These results suggest that IMMO-inducible endogenous SSAO inhibitor enhances DA efflux by MPP^+.

Keywords: Semicarbazide-sensitive amine oxidase (SSAO); Immobilization stress (IMMO); Endogenous semicarbazide-sensitive amine oxidase inhibitor; Isoelectric point (p*I*); 1-Methyl-4-phenylpyridinium ion (MPP^+)

1. Introduction

Stress is one of the major risk factors responsible for the increased incidence of a number of common life-threatening disorders (Micutkova et al., 2004). Axelrod and Reisine (1984) reported that immobilization stress (IMMO) of rats causes an increase in plasma epinephrine and norepinephrine concentration. Catecholamine synthesising enzymes are modulated by IMMO (Kubovcakova et al., 2004). Although IMMO can cause a reduction in monoamine oxidase (MAO) (EC; 1.4.3.4) activity, the physiological role of semicarbazide-sensitive amine oxidase (SSAO) (cooper-containing) (EC; 1.4.3.6) activity remain far from clear (Lyles, 1994). Tissue-bound and soluble form of SSAO (Deng and Yu, 1999; Kinemuchi et al., 2001) differs from MAO in the inhibitor sensitivity and cofactor requirement and oxidizes only aliphatic and aromatic primary monoamines (Clarke et al., 1982; Eriksson and Fowler, 1984). SSAO is highly sensitive to inhibition by carbonyl reagents such as semicarbazide, but is insensitive to inhibition by similar concentrations of the selective MAO inhibitors clorgyline, deprenyl and pargyline (Lyles, 1996). The molecular weight of SSAO was estimated to be about 90,000 (Obata and Yamanaka, 1990). Although a dopaminergic neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), produces a parkinsonian syndrome after its conversion to dopamine (DA)-selective neurotoxin, 1-methyl-4-phenylpyridinium ion (MPP^+) by the type B MAO in the brain (Markey et al., 1984), the etiology of idiopathic Parkinson's disease remains obscure. Intracerebral administration of MPP^+ elicits an accumulation of Ca^{2+} (Lyles, 1984), a sustained increase in striatal dopamine (DA) efflux (Obata, 2006a). IMMO can increase DA and cause oxidative damages to the DA neurons in vivo, suggesting relevance to Parkinson's disease (Kim et al., 2005). In the present study, in order to investigate the DA efflux by the action of IMMO, I examined whether or not IMMO-inducible SSAO inhibitor by separated gel filtration from 105,000 g supernate in rat brain cytosol contribute to the DA efflux by MPP^+ in the extracellular fluid of rat striatum.

Abbreviations: IEF, isoelectric focusing; IMMO, immobilization stress; MAO, monoamine oxidase; MPP^+, 1-methyl-4-phenylpyridinium ion; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; SSAO, semicarbazide-sensitive amine oxidase

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2. Experimental procedure

2.1. Materials

The radioactive substrate [7-^{14}C]-benzylamine hydrochloride (1.85–2.29 Gbq/mmol) was obtained from Amersham International (Amersham, UK). Concentrations of compounds, used here were expressed as salt forms. MPP^+ was purchased from Research Biochemicals Inc. (Natic, MA, USA). Sodium salicylate and its hydroxylated metabolites were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Animals

Male Wistar rats, weighing 200–250 g at the start of the experiments, were kept at room temperature (control group). Rats from the IMMO group were immobilized for 2 h after being kept under the same conditions as the control group. This study was approved by the Ethical Committee for Animal Experiments, Oita Medical University, Japan. IMMO was carried out by taping all four limbs of the rat to metal mount attached to a board (Kvetnansky and Mikulaj, 1970).

2.3. Assay of SSAO activity

The rats were killed by decapitation with a guillotine, and their brain were quickly removed and homogenized in 10 volumes of 10 mM phosphate buffer, pH 7.4 containing 0.32 M sucrose. An endogenous inhibitor of SSAO was separated by gel-filtration from 105,000 g supernate in rat brain cytosol following IMMO. The SSAO activity was measured radiochemically using the radioactive labelled substrates [7-^{14}C]-benzylamine hydrochloride (1.85–2.29 Gbq/mmol; Amersham International, UK), as described previously (Obata and Yamanaka, 2000a,b). The protein concentrations of the enzyme preparations were measured according to the methods of Lowry et al. (1951) using bovine serum albumin as the standard. Gel isoelectric focusing (IEF) was performed by the method of Fawcett (1968). The final composition of the gel was 5% acrylamide, 0.2% methylene bisacrylamide, 0.75% Triton X-100, 2% servalyte (pH 2–11), 0.0002% riboflavin, 0.01% ammonium persulfate, 0.05% TEMED (*N*,*N*,*N*′,*N*′-tetramethylenediamine). The gel was mounted on a vertical apparatus containing 0.01 M H_3PO_4 in the upper tank (anode) and 0.02 M NaOH in the lower tank (cathode). The current was at 100 V for the first 1 h, 200 V for the next 2 h and then 300 V for 2 h. After electrophoresis, the gel was cut into 4 mm thick slices and each sliced gel was placed in a test tube, and incubated for 1 h at room temperature by adding 1 ml of distilled water and bubbled with N_2 gas. After the measurement of slice pH, a minimum amount of 0.5 M H_3PO_4 was added to adjust the pH to 7.4. An aliquot of each slice suspension was then assayed for SSAO inhibition activity with benzylamine as a substrate.

2.4. Microdialysis experiments

In the preliminary experiments, the recovery rate of 0.1 μM DA were 20.8 ± 0.9%, respectively, at flow rate of 1 μl/min, used here for other experiments. The drugs were dissolved in Ringer's solution containing 147 mM NaCl, 2.3 mM $CaCl_2$ and 4 mM KCl, pH 7.4 for perfusion (1 μl/min) through a microdialysis probe into the striatum. The microdialysis probe was pre-washed with Ringer's solution for at least 30 min prior to stereotaxical implantation in the striatum (stereotaxic coordinates: AP, 1.0; R/L, 2.5; H, −7 mm from dura matter) (Paxinos and Watson, 1982). Brain dialysate (1 μl/min) was collected every 15 min into small collecting tubes containing 15 μl of 0.1 N $HClO_4$ to prevent amine oxidation and assayed immediately for DHBA, as described by an HPLC-EC procedure (Chiueh et al., 1992; Obata, 2006a).

2.5. Statistical analysis

All values are presented as means ± S.E.M. The significance of difference was determined by using ANOVA with Fisher's post hoc test. A *P*-value of less than 0.05 was regarded as being statistically significant.

3. Results

The present study was examined the presence of IMMO-inducible SSAO inhibitor in rat brain by IEF-gel electrophoresis. After adjusting pH to 7.4, the SSAO activity for 1 μM benzylamine was determined in the presence of each gel slice. Enzyme activity were assayed radiochemically by addition of 1 μM benzylamine as substrate at 37 °C for 20 min. The remaining activity is expressed as percentage of the control activity. The isoelectric point (p*I*) value of this inhibitor was determined by IEF-gel electrophoresis. However, SSAO inhibition of control group was not observed (Fig. 1A). In contrast, SSAO inhibition activity of IMMO-treated animals was found as a single peak at a position 2.0 cm the top of the gel. The pH measurement after IEF revealed that endogenous SSAO inhibitor has a p*I* value of about 3.8 (Fig. 1B). Time dependent changes in the level of DA were monitored in the dialysates from rat brain after MPP^+ treatment. As we and others reported, MPP^+ induces a massive release of DA in the striatum (Chiueh et al., 1992; Obata, 2006a).

Following the scheduled 45-min washout with Ringer's solution, MPP^+ (5 mM) was infused into striatum for 15 min (total dose, 75 nmol) to evoked a sustained, voltage-regulated

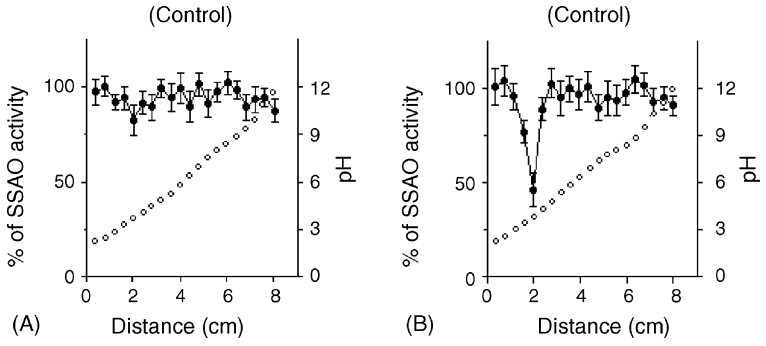

Fig. 1. Estimation of isoelectric point (p*I*) value of endogenous SSAO inhibitor in rat brain cytosol by gel isoelectric focusing (IEF)-gel electrophoresis. SSAO inhibitor fraction was collected and was solubilized 0.75% Triton X-100. After IEF-gel electrophoresis, the gel cut into 4-mm slices and the pH (open circle) of each gel slice was determined. After adjusting pH to 7.4, the SSAO activity for 1 μM benzylamine was determined in the presence of each gel slice (closed circle). Enzyme activity were assayed radiochemically by addition of 1 μM benzylamine as substrate at 37 °C for 20 min. The remaining activity is expressed as percentage of the control activity. Each point represents the mean percentages (±S.E.) on the control triplicate experiments.

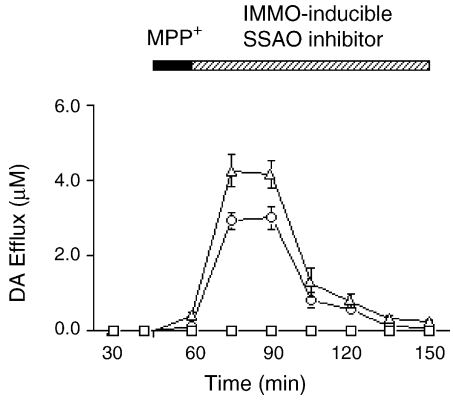

Fig. 2. Effects of MPP^+ on the release of dopamine (DA) after immobilization stress (IMMO)-inducible semicarbazide-sensitive amine oxidase (SSAO) inhibitor treatment. After a 45-min washout with Ringer's solution (pH 7.4), striatum was infused with MPP^+ (5 mM) for 15 min (solid bar; total dose, 75 nmol) to evoke the release of DA (A). Brain dialysate was collected every 15 min in 0.1N $HClO_4$ and immediately assayed by an HPLC-EC procedure. MPP^+-only group (open circle), IMMO-inducible SSAO inhibitor (total amount, 3 μg)-treated MPP^+ group (triangle) and non-treatment (square) were compared. Values are mean ± S.E.M. for six animals.

and calcium-dependent release of DA. The dependent changes in the level of DA efflux were monitored in the dialysate from the striatum after MPP^+ treatment. MPP^+ evoked a significant DA efflux in the striatum. The application of IMMO-inducible SSAO inhibitor (total amount, 3 μg) increased MPP^+-evoked DA efflux at 75 and 90 min, 145.2 ± 14.5 and 137.6 ± 12.2%, respectively ($n = 6$, $P < 0.05$), compared with the MPP^+-only-treated animals (100%) (Fig. 2). The steady-state levels of DA determined by applying various concentration (0, 0.5, 1, 2, 3 and 4 μg) of SSAO inhibitor by IEF-gel electrophoresis through the probe are summarized in Fig. 2, which shows that the concentrations of DA measured at 60–90 min after probe implantation increased in an MPP^+ concentration-dependent manner. The maximum attainable concentration of dialysate DA (E_{max}) by MPP^+ was 3 μg (Fig. 3).

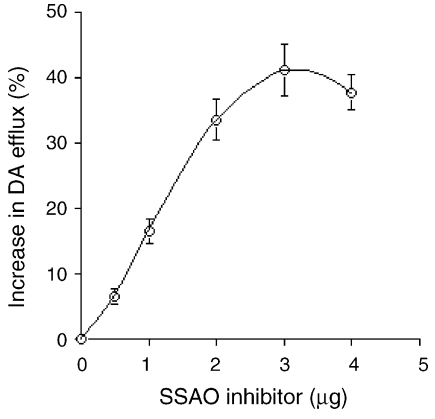

Fig. 3. Concentration-dependent effect of DA efflux by MPP^+-treated rat. The total amount of IMMO-inducible SSAO inhibitor was increased gradually from 0 to 40 μg. Ordinate scale: DA concentration after introduction of MPP^+ (5 mM) containing Ringer's solution through the probe. Abscissa scale: total amount of IMMO-inducible SSAO inhibitor added. Dialysate samples were immediately assayed for DA using an HPLC-EC procedure. Values are mean ± S.E.M. for five animals.

4. Discussion

It is known that IMMO cause an increase in DA in the brain (Kim et al., 2005; Swanson et al., 2004). I previously reported (Obata and Yamanaka, 2000a,b) that the differences in the brain SSAO might exist due to evidence of multiple catalytic site. Inhibition of brain SSAO activity either inhibitor is the most likely, since SSAO inhibitor cause increase in DA level. This MPP^+ evoked DA overflow was enhanced by IMMO-inducible SSAO inhibitor. Accordingly, IMMO-inducible SSAO inhibitor may play a key role in MPP^+-induced enhancement of evoked release of DA from nigrostriatal neurons in the brain. It seems likely that this large contribution of SSAO to the total benzylamine deamination at the assay concentration may be mainly due to the difference in the K_m values of SSAO (around 5 μM) (Lyles, 1984) and MAO (around 160 μM) (Andree and Clark, 1981) for this substrate. Selective discrimination between MAO and SSAO on the basis of substrate concentration was only possible using 1 μM benzylamine at the assay concentration which may be mainly due to the difference in K_m value of SSAO (Clarke et al., 1982). Consequently, low benzylamine concentration can be used for studying benzylamine deamination by SSAO alone in tissues, even though MAO may be present. The data without the MAO inhibitor pretreatment indicated benzylamine deamination as being predominantly by SSAO at the concentration used (1 μM).

Increased SSAO-mediated deamination on monoamine levels in several rat brain regions has been implicated in some pathophysiological conditions (Yu et al., 2003). SSAO activity is suspected to cause damage, such as diabetes in human (Boomsma et al., 1995; Yu, 1998). Despite this enzyme widespread tissue distribution (Castillo et al., 1998; Grönvall et al., 1998), the physiological roles remain far from clear (Lyles, 1994). MPP^+ is known to be actively taken up into dopaminergic neurons via the DA transporter (Kitamura et al., 2000). Therefore, low doses of MPP^+ may increase Ca^{2+} uptake leading to enhancement of DA release in dopaminergic terminals rather than other monoaminergic system. The calcium channel antagonist affect the enhanced DA release by MPP^+-induced calcium uptake into striatal nerve terminals. Parkinson's disease is a progressive neurodegenerative disease of nigrostriatal DA neurons that project from the substantia nigra pars compacta to the striatum. An increase in extracellular DA levels indicate that bioenergetic defects may contribute to the pathogenesis of chronic neurodegenerative diseases through a mechanism involving DA on monoaminergic systems (Moy et al., 2000). DA- and α-receptors may play a role in behavioral response (Rogoz et al., 2004). Stress stimulates a rapid influence on central catecholamines (Waters et al., 2005) and physical activity separately influence monoaminergic function. IMMO causes increases in DA and neuromelanin and oxidative damage in the nigrostriatal system (Swanson et al., 2004; Kim et al., 2005). The primary mechanism for termination of monoaminergic neurotransmission is through reuptake of

released neurotransmitter. Modulation of DA transporter activity may be a physiological mechanism for regulating the concentration of DA that reaches receptors, following periods of stress (Copeland et al., 2005).

My findings suggest that modulators induced by IMMO may have an important role in SSAO activity. The p*I* value of this inhibitor was determined by IEF-gel electrophoresis to about 3.8 (Fig. 1B). The present study showed that this result indicates that this inhibitor is an acidic compound. Accumulation of endogenous catecholamine(s), which are also substrate for SSAO present in the microvessels in the brain (Obata, 2006a). Obata (2006b) reported that a definite mechanism is not clear at the moment, after inhibition of SSAO activity, with consequent increases in bioactive amine levels due to MPP^+. Although the physiological role of this inhibitor still remains unclear, I consider that this modulator may play some role in regulating the SSAO activity in rat brain. Cerebrovascular disorders are well known to be associated with stress related behaviors (Yu et al., 1997). Stress enhances excretion of adrenaline, which is deaminated by MAO, and methylamine is formed. This product can be further deaminated by SSAO and converted to toxic formaldehyde, hydrogen peroxide and ammonia. Such chronic ''formaldehyde'' stress may be involved in the initiation of endothelial injury and subsequently angiopathy. SSAO catalyzes the deamination of not only longer chain aliphatic amines but also short chain aliphatic amines including methylamine. Inhibition of the SSAO activity completely protects brain cells from the methylamine-SSAO induced damage. While the role of this inhibitor is no doubt important, their mechanism remain to be elucidated. These results suggest that IMMO-inducible endogenous SSAO inhibitor enhances DA efflux by MPP^+ using microdialysis technique.

In conclusion, IMMO enhanced DA efflux in extracellular spaces of the striatum during the catecholamine release by MPP^+. These findings may be useful in elucidating the actual mechanism of free radical formation in the pathogenesis of neurodegenerative brain disorders, including Parkinson's disease, Alzheimer's disease and traumatic brain injuries.

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