Low Catechol-*O*-methyltransferase Activity in the Brain and Blood Pressure Regulation

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Catecholamines (CAs) are important hormones in regulating blood pressure both in centrally and peripheral sympathetic nerve endings. Production of CAs, release and inactivation are three components to regulate CAs level. We have reported that the inactivation of CAs by catechol-*O*-methyltransferase (COMT) in the liver is important in high blood pressure in spontaneously hypertensive rats (SHR). In the present study, we investigated central role of COMT in hypertension. We investigated COMT activities in cerebral cortex, cerebellum, hippocampus, brain stem, hypophysis, and hypothalamus of SHR and Wistar-Kyoto (WKY) rats. COMT activities were assessed by measuring normetanephrine with the use of norepinephrine as an endogenous substrate. Membrane-bound COMT activities in cerebral cortex were significantly reduced in SHR (19.1±1.8 pmol/min/mg protein) compared with WKY rats (25.0±3.3 pmol/min/mg protein). The ratio of concentrations of normetanephrine/ norepinephrine in cerebral cortex was also lower in SHR than in WKY rats. Our results suggest that there is an association between MB-COMT in cerebral cortex and blood pressure regulation.

Key words catecholamine; hypertension; spontaneously hypertensive rat

Catechol-*O*-methyltransferase (COMT; EC2.1.1.6) is an enzyme which inactivates the released catecholamines from nerve endings by methylating their catechol moieties using *S*-adenosyl-L-methionine (SAMe) as a methyl donor.[1—4) COMT is found in most mammalian tissues, with highest activity in the liver and the kidney. There are two COMT isoforms: in the cytoplasm as soluble COMT (S-COMT) and in association with membranes as membrane-bound COMT (MB-COMT). S-COMT protein is more prevalent than MB-COMT in all tissues in rats.[5]

Catecholamines, norepinephrine (NE), dopamine and epinephrine, play important roles in the central nervous system as in the periphery,[6] and in central regions, catecholamines-related gene expression was correlated with blood pressure.[7]

We have previously reported an assay method for rat brain COMT activities, using NE as an endogenous substrate.[8] The use of the endogenous substrate provides significant information of COMT *in vivo* as compared with formerly reported method which uses an artificial substrate, 3,4-dihydroxybenzoic acid (DBA). Endogenous NE has higher affinity for COMT than DBA, and our method is more sensitive to measure COMT activity. In this study, COMT activities were evaluated in cerebral cortex, cerebellum, hippocampus, brain stem, hypophysis, and hypothalamus in order to evaluate the role of COMT in blood pressure regulation using spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats. In addition, in order to investigate the contribution of COMT activities to NE metabolism, NE and its 3-*O*-methyl metabolite, normetanephrine (NMN), concentrations were examined in discrete areas of SHR and WKY rats.

MATERIALS AND METHODS

Reagent NE, NMN, SAMe chloride salt and 4-methoxytyramine (4-MT) were obtained from Sigma (St. Louis, MO, U.S.A.). Ethylenediamine was obtained from Sigma-Aldrich (Milwaukee, WI, U.S.A.). Imidazole and 1,4-dithiothreitol were from Merck (Darmstadt, Germany). Acetonitrile and ethanol, both of HPLC grade, were purchased from Wako Pure Chemicals (Osaka, Japan). All other reagents were of analytical grade.

Animals Male WKY rats (22 weeks old, 405—415 g) and SHR (22 weeks old, 330—350 g) were purchased from Charles River Japan Inc. (Kanagawa, Japan), and housed under controlled environment (22—24 °C and a 12-h light–dark cycle) with free access to tap water and diet for at least 1 week before study. All animals received animal care in compliance with the National Institute of Health guideline.

Preparation of Brain COMT Samples In anesthetized rats with pentobarbital, blood was removed from inferior vena cava, and cerebral cortex, cerebellum, hippocampus, brain stem, hypophysis and hypothalamus were immediately removed and chilled on ice. All further procedures were conducted at 4 °C. Each tissue was weighed and then homogenized with four volumes of 50 mmol/l sodium phosphate buffer containing 0.5 mmol/l 1,4-dithiothreitol (pH 7.5). The homogenates were centrifuged at 100000×g for 30 min and the supernatants were stored for the determination of S-COMT. The pellet was washed with 2 ml of homogenizing buffer (pH 7.5) and re-centrifuged at 100000×g for 30 min. Then the pellet was suspended in four volumes of homogenizing buffer for the MB-COMT. Each fraction was frozen at −80 °C until the COMT assay.

COMT Assay Forty microliters of the S-COMT sample was incubated with 50 mmol/l sodium phosphate buffer (pH 7.8) containing 2 mmol/l NE, 2 mmol/l $MgCl_2$ and 200 μmol/l SAMe. On the other hand, the reaction mixture of the MB-COMT sample consisted of 50 mmol/l sodium phosphate buffer (pH 7.0) containing 100 μmol/l NE, 2 mmol/l $MgCl_2$ and 200 μmol/l SAMe. After incubation at 37 °C for 15 (cerebral cortex and cerebellum), 30 (hippocampus and brain stem) and 120 (hypophysis and hypothalamus) min, the reaction was terminated by the addition of 20 μl of 4.0 mol/l perchloric acid. The reaction mixtures were kept on an ice-

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bath for 10 min followed by centrifugation at 4000×*g* for 10 min. A 30 μl aliquot of the supernatant was added to 170 μl of the sample dilution buffer [10 mmol/l glutathione, 10 mmol/l citric acid, 0.1% Triton X-100, 100 mg/l EDTA-2Na and 50 nmol/l 4-MT (internal standard) (pH 4.5)] and 150 μl of aliquots were injected onto the HPLC system. The activities were not changed when monoamine oxidase inhibitors were added to the reaction mixture, suggesting that monoamine oxidase has no effect on the assay.

Protein concentrations of the samples were determined according to Bradford[9] using bovine serum albumin as the standards and calibration control samples.

HPLC Conditions COMT activity was calculated by measuring the amount of the methylation product, NMN, and values were converted to pmol/min/mg protein. NMN was determined with HPLC-fluorescence detection system.[10] The method involved in-line extraction of amines including NE and NMN with a cation-exchange column followed by reversed-phase mode separation on a separation column, coulometric oxidation to *o*-quinones and fluorogenic derivatization with ethylenediamine. The automatic system consisted of three HPLC pumps (PU-2080 and PU-980, JASCO, Tokyo, Japan), an autosampler (950-AS, JASCO), a rotatory six-way switching valve (HV-992-01, JASCO), a electrochemical coulometric reactor (Coulochem 5100A, ESA) and fluorescence detector (2025-FP, JASCO). A pre-column, SERUMOUT-CEX, 10×4.0 mm I.D. (Sekisui Co., Osaka, Japan) and a separation column, TSK-gel ODS-80Ts, 150×4.6 mm I.D. (TOSOH, Tokyo, Japan) kept at 40 °C in a column oven were used. The fluorescence detection was carried out at an emission wavelength of 505 nm with excitation at 430 nm.

The pretreatment buffer consisted of 10 mmol/l potassium phosphate buffer (pH 7.5)/ethanol (92/8, v/v). The buffer was pumped at a flow rate of 1.0 ml/min. The mobile phase was 75 mmol/l potassium acetate buffer (pH 3.2)/50 mmol/l potassium phosphate buffer (pH 3.2)/acetonitrile (90.25/4.75/5, v/v/v) containing 4 mmol/l sodium 1-hexanesulfonate and the flow rate was 0.5 ml/min. The fluorogenic reagent, 105 mmol/l ethylenediamine and 175 mmol/l imidazole in acetonitrile/ethanol/water (80/10/10, v/v/v), was pumped at a flow rate of 0.32 ml/min. The coulometric reactor potential was +0.6 V *versus* a H_2/H^+ reference electrode.

Determination of NE and NMN Concentrations in the Brains of SHR and WKY Rats The tissues were homogenized with four volumes of 0.4 mol/l perchloric acid containing 0.05% (w/v) EDTA-2Na and centrifuged at 4000×*g* for 20 min. After centrifugation, 10 μl of the supernatant was added to 50 μl of the sample dilution buffer [10 mmol/l glutathione, 10 mmol/l citric acid, 0.1% Triton X-100, 100 mg/l EDTA-2Na and 3.1 nmol/l 4-methoxytyramine (internal standard) (pH 4.5)] and 45 μl of aliquots were injected onto the automated HPLC-chemiluminescence detection system, and NE and NMN concentrations were determined.[10,11]

Data Analysis Values of all data in this paper are described as the mean±standard deviation (S.D.). For statistical comparison, analysis of variance (ANOVA) followed by a multiple comparison test with a Bonferroni adjustment or Student's *t*-test was used. When $p<0.05$ differences between two strains were considered statistically significant.

RESULTS

COMT Activities in the Brains of SHR and WKY Rats Mean blood pressure in SHR was 175±10 mmHg, which was significantly higher than those in WKY rats (125±9 mmHg) ($p<0.01$).

V_{max} (COMT activity), K_m and V_{max}/K_m values of SHR and WKY rats in cerebral cortex, cerebellum, hippocampus, brain stem, hypophysis and hypothalamus are shown in Table 1. The V_{max} values had been used to calculate the turnover rate for the substrate while the V_{max}/K_m values were used to calculate the specificity constant. The highest S-COMT activities in both SHR and WKY rats were found in hippocampus (114±15 and 134±11 pmol/min/mg protein, respectively) followed by cerebellum (101±10 and 117±13 pmol/min/mg protein, respectively). Interestingly, MB-COMT activities in each rat brain area were similar (from 9.83±1.7 to 28.3±5.6 pmol/min/mg protein). These are in agreement with our previous report with Sprague-Dawley (SD) rats.[8] Also, K_m and V_{max}/K_m values in SHR and WKY rats were similar with SD rats.

Significantly lower MB-COMT activity in cerebral cortex was found in SHR (19.1±1.8 pmol/min/mg protein) compared to WKY rats (25.0±3.3 pmol/min/mg protein). In other areas, no significant differences in S- and MB-COMT activities were detected when compared SHR with WKY rats. K_m and V_{max}/K_m values were also similar between SHR and WKY rats in all areas of the brain.

NE and NMN Concentrations in the Brains of SHR and WKY Rats Since extraneuronal NE is metabolized by COMT, we determined the levels of NE and NMN in order to understand if COMT contributes to catecholamine metabolism. Table 2 shows the amounts of NE and NMN in six brain areas of both SHR and WKY rats. NE concentrations of WKY rats were in agreement with those reported previously.[12] In cerebral cortex and cerebellum, NE concentrations of SHR were significantly higher than those of WKY rats, whereas there was no difference in other areas. These results were consistent with the previous report, which measured at 14 week-old.[13] In cerebral cortex, although NE concentration in SHR (5.50±0.43 nmol/g tissue) was higher than WKY rats (3.87±0.51 nmol/g tissue), the ratio of NMN to NE of SHR (0.028) tended to be lower than that of WKY (0.034). However, the ratios in cerebellum and brain stem of SHR were similar to those of WKY rats.

DISCUSSION

We previously developed a method to measure both S- and MB-COMT activities (V_{max}) in six brain areas of rats (cerebral cortex, cerebellum, hippocampus, brain stem, hypophysis and hypothalamus) using NE as a substrate with HPLC-fluorescence detection.[8] In this study, to examine the differences in catecholamine metabolism which might be related to the pathogenesis of hypertension, we measured the brain COMT activities in SHR and WKY rats.

From the results obtained from the present study and a previous report,[8] MB-COMT activities were lower than S-COMT activities in all the brain areas as well as in liver and kidney.[14—16] However, there is a difference in their magnitudes: MB-COMT activities were only 2—5 times lower than

Table 1. V_{max}, K_m and V_{max}/K_m Values in Individual Brain Areas (Cerebral Cortex, Cerebellum, Hippocampus, Brain Stem, Hypophysis and Hypothalamus) of SHR and WKY Rats for (a) S-COMT or (b) MB-COMT

(a)

	WKY rats			SHR		
	V_{max} (pmol/min/mg protein)	K_m value (μM)	V_{max}/K_m	V_{max} (pmol/min/mg protein)	K_m value (μM)	V_{max}/K_m
Cerebral cortex	66.1±12.8	412	0.16	53.4±5.9	357	0.15
Cerebellum	117±13	358	0.33	101±10	369	0.27
Hippocampus	134±11	463	0.29	114±15	410	0.28
Brain stem	93.3±8.2	452	0.21	79.1±8.4	459	0.17
Hypophysis	40.9±5.4	331	0.12	40.2±4.6	310	0.13
Hypothalamus	49.3±9.5	413	0.12	40.1±4.1	392	0.10

Values shown are the means±S.D. (n=5).

(b)

	WKY rats			SHR		
	V_{max} (pmol/min/mg protein)	K_m value (μM)	V_{max}/K_m	V_{max} (pmol/min/mg protein)	K_m value (μM)	V_{max}/K_m
Cerebral cortex	25.0±3.3	12.4	2.0	19.1±1.8*	12.2	1.6
Cerebellum	28.3±5.6	12.2	2.3	27.5±2.5	13.8	2.0
Hippocampus	25.2±4.3	14.7	1.7	25.4±2.7	12.9	2.0
Brain stem	13.7±2.2	10.5	1.3	14.2±2.0	9.6	1.5
Hypophysis	21.3±3.1	13.2	1.6	19.6±2.9	11.7	1.7
Hypothalamus	11.4±1.5	13.9	0.82	9.83±1.7	18.5	0.53

Values shown are the means±S.D. (n=5). * $p<0.05$ SHR *vs.* WKY rats.

Table 2. Norepinephrine (NE) and Normetanephrine (NMN) Concentrations and the Ratio of NMN to NE of Individual Brain Areas (Cerebral Cortex, Cerebellum, Hippocampus, Brain Stem, Hypophysis and Hypothalamus) of SHR and WKY Rats

	WKY rats			SHR		
	NE (nmol/g tissue)	NMN (nmol/g tissue)	NMN /NE	NE (nmol/g tissue)	NMN (nmol/g tissue)	NMN /NE
Cerebral cortex	3.87±0.51	0.131±0.023	0.034	5.50±0.43*	0.152±0.031	0.028
Cerebellum	0.654±0.108	0.0833±0.0244	0.13	1.41±0.34*	0.162±0.048	0.12
Hippocampus	1.17±0.27	0.150±0.042	0.13	1.15±0.13	0.144±0.039	0.13
Brain stem	6.08±1.08	0.123±0.021	0.20	8.11±0.68	0.187±0.027	0.23
Hypophysis	0.315±0.085	0.102±0.013	0.32	0.433±0.064	0.139±0.030	0.32
Hypothalamus	6.34±1.22	0.223±0.041	0.035	4.82±0.56	0.181±0.035	0.038

Values shown are the means±S.D. (n=5). * $p<0.05$ SHR *vs.* WKY rats.

S-COMT activities in the brain but were 15—25 times lower in the liver and the kidney. Considering the results obtained in the difference of affinities for COMT described below, MB-COMT in the brain may play a more important role than other peripheral tissues.

Contrary to the lower MB-COMT activities than S-COMT in the brain, the affinities of MB-COMT for catecholamines (K_m) were about 20—50 times higher than those of S-COMT, and specific constants of MB-COMT (V_{max}/K_m) were about 2—15 times higher than those of S-COMT. This indicates that MB-COMT is more important for the metabolism of catecholamines in the brain than S-COMT. Furthermore, we have shown that the specific constants of MB-COMT were similar to those of S-COMT in liver and kidney. It should be stressed again that the role of MB-COMT in the brain should be more significant than other tissues.

We found that MB-COMT activities in cerebral cortex in SHR were lower than those in WKY rats. Concordance with this, NE concentrations in cerebral cortex in SHR were significantly higher than those in WKY rats and the ratio of NMN to NE was lower in SHR than that in WKY rats. It is reported that NE release in the cerebral cortex affects the blood pressure by the activation of the sympathetic outflow to tissues.[17,18] These findings suggest that lower MB-COMT activity in the cerebral cortex may be one factor involved in the blood pressure regulation. However, it is not clear the mechanism of the difference of MB-COMT activity in cerebral cortex observed between SHR and WKY rats. Furthermore, catecholamines are also metabolized by monoamine oxidase. Therefore, further experiments should be necessary

to clarify the mechanism of metabolism and investigate more dynamic aspects of catecholamine metabolism in relation to the development of hypertension.

Acknowledgement We thank Dr. Chiho Lee for his valuable suggestions and discussion.

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