Original Article

Reduced Membrane-Bound Catechol-*O*-Methyltransferase in the Liver of Spontaneously Hypertensive Rats

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We have previously reported that methylation of catecholamines by catechol-*O*-methyltransferase (COMT) was attenuated in spontaneously hypertensive rats (SHR) with acute hypotension as compared with that of Wistar-Kyoto (WKY) rats. Here we examined the soluble (S-) and membrane-bound (MB-) COMT activities and COMT protein in the liver, kidney, and erythrocytes in both strains. Both the activities and the amounts of MB-COMT in the liver were lower in SHR than in WKY rats, but no such trend was found in the kidney or erythrocytes. Nor was such a trend observed in any of these three tissues for S-COMT. These results indicate that liver MB-COMT may be a relevant factor in blood pressure regulation in rats. (*Hypertens Res* 2003; 26: 923–927)

Key Words: catecholamine, catechol-*O*-methyltransferase, liver, membrane-bound

Introduction

The baroreflex-mediated sympathetic nervous system plays a critical role in short term blood pressure regulation. When the blood pressure decreases, the baroreceptor is activated and norepinephrine (NE) is released from sympathetic nerve endings. The released NE acts on α -receptors and blood pressure increases. Since plasma NE is mainly derived from sympathetic nerve endings, the concentration of NE could be used as an index of the baroreflex-mediated sympathetic nerve activity (1, 2). For example, hypotensive responses caused by calcium antagonists in rats have been shown to correlate with the increases of plasma NE concentrations. Furthermore, we previously reported that the response was reduced in spontaneously hypertensive rats (SHR) as compared with that in normotensive Wistar-Kyoto (WKY) rats (3–6).

We have also previously shown that the conversion of released NE to normetanephrine (NMN) by catechol-*O*-methyltransferase (COMT) was linked to blood pressure regulation, and this conversion could be estimated by utilizing a

highly sensitive method to measure NE and NMN (7–9). Using this method, we demonstrated that methylation of catecholamines by COMT in SHR was attenuated during acute hypotension, when compared to that in WKY rats. Furthermore, intravenous administration of *S*-adenosyl-L-methionine (SAME), a coenzyme for COMT, lowered blood pressure in SHR with a concomitant increase of NMN (10).

Since COMTs exist in most tissues in a soluble form (S-COMT) as well as a membrane-bound form (MB-COMT), it is important to examine whether one of these subtypes plays a more crucial role than the other in the inactivation of released NE. Therefore, we determined S- and MB-COMT enzyme activities and the amount of protein in two representative rat tissues—the liver and kidney—as well as in erythrocytes. To examine the correlation between these enzymes and hypertensive states, pre-hypertensive SHR (4 weeks of age), hypertensive adult SHR (23 weeks of age), and age-matched normotensive WKY rats were used.

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Methods

Reagents and Animals

NE, NMN, and SAME chloride salt were from Sigma Chemical Co. (St. Louis, USA). All other reagents were of analytical grade.

Male WKY rats and SHR were purchased from Charles River Japan Inc. (Kanagawa, Japan). The rats were kept at 22–24°C under a 12-h light-dark cycle, and they had free access to tap water and commercial chow. All animals received human care in compliance with the National Institutes of Health guidelines. Blood pressure was measured with the indirect tail-cuff method (BP-98A; Softron, Tokyo, Japan).

Determination of Plasma NE

Plasma NE concentrations were determined with high-performance liquid chromatography (HPLC)-peroxyoxalate chemiluminescence reaction detection using 50 µl of rat plasma (7–9).

COMT Activity and Kinetics Measurement

COMTs are found in most tissues. We chose rat liver and kidney tissues for this examination because they have been shown to have the highest COMT activities (11). Erythrocytes were also examined because erythrocyte COMT activity is used as an index of enzyme activity in humans (12).

The general procedure for the COMT activity measurement in tissues and erythrocytes was similar to that previously described (13, 14). Tissues were removed from anesthetized rats, weighed, and homogenized in homogenizing buffer (50 mmol/l sodium phosphate buffer (pH 7.5) with 0.5 mmol/l dithiothreitol). The tissue homogenates were centrifuged at 100,000 × *g* for 30 min at 4°C. The supernatant was used as the S-COMT sample. The pellet for MB-COMT was washed with homogenizing buffer. In the S-COMT assay, a 100 µl aliquot of the supernatant was incubated in 50 mmol/l sodium phosphate buffer (pH 7.8) containing 2 mmol/l MgCl₂, 200 µmol/l SAME, and 2 mmol/l NE in a total volume of 500 µl. In the MB-COMT assay, a 100 µl aliquot of the suspension was incubated in 50 mmol/l sodium phosphate buffer (pH 7.4) containing 2 mmol/l MgCl₂, 200 µmol/l SAME, and 100 µmol/l NE in a total volume of 500 µl. After 10 min incubation at 37°C, the reaction was stopped with 50 µl of 4 mol/l perchloric acid. After the removal of the protein precipitates, the 3-*O*-methyl metabolite (NMN) was analyzed by HPLC-fluorescence or chemiluminescence detection. Protein concentrations of the lysates were determined according to Bradford (15) using bovine serum albumin as a standard.

Kinetic parameters of S- and MB-COMT in the liver were determined using varying concentrations of the substrate, NE

Table 1. Mean BP and Plasma NE Concentrations of 4- and 23-Week-Old SHR and WKY Rats

Age	Rat strain	Mean BP (mmHg)	Plasma NE (pmol/ml)
4-week	SHR	114 ± 3 **	1.20 ± 0.15
	WKY rats	99 ± 5	1.14 ± 0.17
23-week	SHR	180 ± 5 **	1.39 ± 0.20 *
	WKY rats	122 ± 6	0.94 ± 0.17

The values represent the mean ± SD, *n* = 8. **p* < 0.05, ***p* < 0.01 vs. WKY rats. BP, blood pressure.

(10–2,000 µmol/l).

Sample Preparation for Immunoblotting

Rat tissues were homogenized in 10 mmol/l Tris-HCl buffer (pH 7.4) containing 0.25 mol/l sucrose and 1 mmol/l EDTA, and centrifuged at 700 × *g* for 10 min at 4°C. The supernatant was centrifuged at 100,000 × *g* for 60 min at 4°C for separation of the S- and MB-forms. The pellet was re-suspended in buffer and centrifuged again. The supernatant and the pellet were then separated on 15% SDS-PAGE, followed by electrophoretic transfer onto polyvinylidene difluoride membranes. The filters were treated with a guinea pig anti-COMT antiserum (16) (1:500 dilution) in phosphate-buffered saline (PBS)/0.1% Tween 20 and 5% non-fat dry milk for 12 h at 4°C, followed by peroxidase-conjugated goat anti-guinea pig IgG for 1 h at room temperature.

Data Analysis

Data are expressed as the means ± SD. The statistical significance was determined by analysis of variance (ANOVA) followed by a multiple comparison test with a Bonferroni adjustment, or Student's *t*-test. *P* values of less than 0.05 were considered statistically significant.

Results

Basal Blood Pressure and Plasma NE Levels in SHR and WKY Rats

The mean blood pressure and plasma NE levels in 4- and 23-week-old SHR and WKY rats are shown in Table 1. In 4-week-old SHR, the mean blood pressures were at pre-hypertensive levels but higher than those in age-matched WKY rats, whereas the plasma NE levels were similar in both rat strains.

At 23 weeks, both mean BP and plasma NE levels were significantly higher in SHR than WKY rats.

Table 2. S- and MB-COMT Activities in Liver, Kidney and Erythrocyte of 23-Week-Old SHR and WKY Rats

	Tissue	SHR	WKY rats
S-COMT	Liver	3,600 ± 110 **	2,180 ± 290
	Kidney	3,740 ± 680 **	1,790 ± 170
	Erythrocyte	36.9 ± 1.6	24.2 ± 3.2
MB-COMT	Liver	114 ± 10 *	164 ± 14
	Kidney	84.3 ± 3.2	78.3 ± 8.3
	Erythrocyte	8.95 ± 2.80	4.62 ± 0.55

The values represent the mean ± SD (pmol/min/mg protein), $n = 8$. * $p < 0.05$, ** $p < 0.01$ vs. WKY rats.

COMT Activity in the Liver, Kidney, and Erythrocytes in 23-Week-Old Rats

COMT activities in the liver, kidney, and erythrocytes in 23-week-old SHR and their age-matched WKY rats are summarized in Table 2. In 23-week-old SHR and WKY rats, S-COMT activities in all tissues examined were 30–50 times higher than MB-COMT activities. The liver MB-COMT activities were lower in SHR than in WKY rats ($p < 0.05$; $n = 8$ each). However, no such trend was found in the kidney or erythrocytes or for S-COMT in any of these three tissues.

COMT Activity in the Liver in 4-Week-Old Rats

Liver S-COMT activities were 3.46 ± 0.43 and 3.52 ± 0.29 nmol/min/mg protein ($n = 8$) for 4-week-old SHR and WKY rats, respectively. Liver MB-COMT activities were 83.6 ± 6.5 and 78.9 ± 6.9 pmol/min/mg protein ($n = 8$) for 4-week-old SHR and WKY rats, respectively. It is of interest to note that, in 4-week-old pre-hypertensive SHR and age-matched normotensive WKY rats, the liver S- and MB-COMT activities were comparable. In addition, MB-COMT activities in 4-week-old SHR were lower than those in 23-week-old rats ($p < 0.01$).

K_m Values for NE in the Liver

The affinity for the substrate (NE) was thought to influence catecholamine metabolism *in vivo*. Therefore, we also examined the affinity for NE in the liver in 23-week-old SHR and WKY rats. In S-COMT, the K_m values for NE were 478 ± 44 and 464 ± 51 $\mu\text{mol/l}$ for SHR and WKY rats, respectively. In MB-COMT, the K_m values for NE were 8.58 ± 0.60 and 6.64 ± 1.45 $\mu\text{mol/l}$ for SHR and WKY rats, respectively. There were no differences in the affinity for NE in both isozymes. In both SHR and WKY rats, the K_m values for NE in S-COMT were about 80 times higher than those in MB-COMT, which is in agreement with the previous reports (13, 14, 17).

The $V_{\max}/K_m$ values, *i.e.*, the specificity constant values were calculated based on the above data. In the liver of SHR,

Fig. 1. Western-blotting analysis of rat liver samples. Sample preparation was performed as described in the Methods section. In brief, rat liver homogenates were centrifuged at $100,000 \times g$ for the separation of S-COMT and MB-COMT. The supernatant and the pellet were separated on 15% SDS-PAGE, followed by electrophoretic transfer onto polyvinylidene difluoride membranes. The filters were treated with a guinea pig anti-COMT, followed by peroxidase-conjugated goat anti-guinea pig IgG. Lanes 1 and 3, WKY rats; lanes 2 and 4, SHR. The positions of a molecular-mass standard and 24.8 kDa S-COMT and 29.6 kDa MB-COMT proteins are indicated.

the $V_{\max}/K_m$ values were 0.0075 and 0.013 for S- and MB-COMT, respectively. S-COMT showed two-fold lower $V_{\max}/K_m$ values for catecholamines than MB-COMT.

Western Blotting of Liver COMT

The amounts of 24.8 kDa S-COMT and 29.6 kDa MB-COMT were determined by Western blotting in the liver of 23-week-old SHR and WKY rats using anti-COMT antiserum (16). As shown in Fig. 1, the amount of hepatic S-COMT in SHR was higher than that in WKY rats. On the other hand, the amount of hepatic MB-COMT in SHR was less than that in WKY rats. The ratios of COMT protein in SHR vs. WKY rats were 1.61 and 0.69 for S-COMT and MB-COMT, respectively, and the ratios of COMT activities in SHR vs. WKY rats were 1.66 and 0.76 for S-COMT and MB-COMT, respectively. The data indicate that COMT proteins in SHR and WKY rats have similar specific activity.

Discussion

In this study, we attempted to search for the main COMT(s) that inactivate(s) released NE during acute hypotension. We have previously demonstrated that the methylation of catecholamines in 23-week-old SHR was attenuated when compared to that in age-matched WKY rats (10). In general, COMT activities in SHR are attenuated. In this study, we demonstrated that liver MB-COMT shows exactly the same pattern. To our surprise, no such pattern existed for liver S-COMT, kidney and erythrocyte. The higher activities in SHR may be attributable to the higher sympathetic nervous system activity in SHR.

In the present study, we only examined COMTs in the liver, kidney, and erythrocytes. COMTs in other tissues could potentially play major roles in blood pressure regulation. Several lines of evidence suggest that the methylation by MB-COMT could regulate tissue levels of catecholamines in the rat: the K_m value of MB-COMT for catecholamines has been shown to be approximately 100 times smaller than that of S-COMT (17, 18); and the liver has been shown to play a major role in the removal and the 3-*O*-methyl metabolism of circulating catecholamines in mice (19), rabbits (20), and humans (21). Furthermore, the specificity constant for S-COMT was two-fold lower than that for MB-COMT, which suggests that catecholamines are methylated more rapidly by MB-COMT. We therefore believe that the hepatic MB-COMT has an important function in blood pressure regulation.

It is interesting to note the effect of aging on liver MB-COMT activities. In young pre-hypertensive SHR and age-matched WKY rats, hepatic MB-COMT activities were similar. As they grew older, the MB-COMT activity in WKY rats increased two-fold, from 78.9 ± 6.9 to 164 ± 14 pmol/min/mg protein by 23 weeks of age ($p < 0.01$; $n = 8$). In SHR, the increases of MB-COMT activity were significant but not as large as those in WKY rats (from 83.6 ± 6.5 to 114 ± 10 pmol/min/mg protein; $p < 0.01$; $n = 8$). It is therefore possible that the attenuated increases of hepatic MB-COMT activity in SHR with age may account for the reduced ability of MB-COMT to methylate catecholamines. This is also consistent with the finding that the plasma NE concentrations in 23-week-old SHR were significantly higher than those in age-matched WKY rats. We considered that it would be of greater interest to determine the COMT activity at the onset of hypertension, *i.e.*, at 12–14 weeks of age. We therefore investigated this activity in our next assay.

When we measured COMT protein by immunoblotting, we found that the amount of hepatic MB-COMT protein in SHR at the age of 23 weeks was less than that in age-matched WKY rats, whereas the amount of hepatic S-COMT protein in SHR was higher than that in WKY rats. These observations are consistent with the findings of COMT activities in the liver. Thus, it is possible that the lower COMT activity in SHR may be due to a lower amount of hepatic MB-COMT protein rather than lower specific activity.

S- and MB-COMT protein are coded by a single gene, and the expression of the COMT gene is said to be controlled by two distinct promoters (P1 and P2) (11). The P1 promoter expresses S-mRNA, which solely encodes S-COMT protein. The P2 promoter expresses MB-mRNA, which has the capacity for encoding both S-COMT and MB-COMT proteins by the leaky scanning mechanism of translation initiation (22). The production of S- and MB-COMT proteins can thus be regulated on both the transcriptional and translational levels. Therefore, the lower amount of hepatic MB-COMT protein in SHR may be ascribable to the difference of COMT transcription factors and/or the translation initiation between

the two strains.

In summary, we have found that in the liver of SHR, both the activities and the amounts of MB-COMT were lower than those of WKY rats. No such trend was found in the kidney or erythrocytes or for S-COMT in any of the three tissues. It is plausible that hepatic MB-COMT may be a dominant factor in blood pressure regulation, and it would be of interest to examine whether idiopathic human hypertension is due to a reduced production of MB-COMT.

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