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Lower Mevalonate Pyrophosphate Decarboxylase Activity Is Caused by the Reduced Amount of Enzyme in Stroke-Prone Spontaneously Hypertensive Rat

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Spontaneously hypertensive rat (stroke-prone) (SHRSP) has a low serum cholesterol level as compared with the normotensive Wistar Kyoto rat (WKY). We previously indicated that the lower activity of mevalonate pyrophosphate decarboxylase (MPD) was responsible for the reduced cholesterol biosynthesis in the liver of SHRSP [Sawamura *et al.* (1992) *J. Biol. Chem.* 267, 6051-6055]. To elucidate the mechanism of the reduced activity, we purified liver MPD from SHRSP treated with cholestyramine and pravastatin in this study. We compared its enzymatic properties with those of the enzyme from WKY, and also measured the amounts of MPD in the crude extract of various tissues in WKY and SHRSP by Western blot analysis. Results indicated that (i) MPD of SHRSP has essentially the same properties as MPD of WKY, except for a difference in the dependency on divalent cations. (ii) The amount, as well as the activity, of MPD in the crude extract of brain and liver was reduced in SHRSP. (iii) There was no difference between SHRSP and WKY, in the ratio of the enzyme activity to the amount of MPD in the crude extract. These data led us to conclude that the lower activity of MPD was caused by the reduced amount of this enzyme in SHRSP.

Key words: cholesterol, mevalonate pyrophosphate decarboxylase, pravastatin, SHRSP.

The spontaneously hypertensive rat (stroke-prone) (SHRSP) is a rat that suffers from severe hypertension and cerebral hemorrhage (*1, 2*). The serum cholesterol level of this rat is lower than that of normotensive WKY (*3*). Epidemiological studies have indicated a negative association between serum cholesterol level and the incidence of cerebral hemorrhage in man (*4*). Since cholesterol is a major constituent of cellular membranes, the reduced cholesterol content in the cell would make the plasma membrane fragile. In fact, red blood cells in SHRSP were shown to have reduced resistance to osmotic pressure (*5, 6*). Therefore, the low level of serum cholesterol in SHRSP may cause the cerebral stroke in this rat. The following investigation demonstrated that the activity for biosynthesis of cholesterol was decreased in SHRSP due to the reduced activity of MPD (*7*). However, the mechanism underlying the reduced activity of this enzyme remains unclear.

In the first steps of the biosynthesis of cholesterol from acetic acid, MPD catalyzes a bimolecular reaction between MVAPP and ATP to form isopentenyl pyrophosphate, inorganic phosphate, ADP, and CO_2. Although HMG-CoA reductase is the rate-limiting enzyme of this pathway (*8-10*), MPD is also considered as a regulatory enzyme based on the effects of dietary cholesterol or cholesterol-lowering drugs (*11-14*). The enzyme has been purified from various sources, including yeast (*15, 16*), latex of Hevea brasiliensis (*17*), pig liver (*18, 19*), rat liver (*20-22*), and chicken liver (*23*). Recently, Toth and Huwyler reported the cDNA sequence of MPD from human and yeast (*24*).

We also purified two MPDs (45 and 37 kDa) from the liver of WKY fed on a diet containing 5% cholestyramine and 0.1% pravastatin (CP diet) (*22*). The CP diet-induced new species of 37 kDa MPD was characteristically and immunologically very similar to the well-known 45 kDa MPD. This led us to consider several questions: (i) Is 37 kDa MPD induced by CP diet in SHRSP? (ii) Are the kinetic properties of MPDs of SHRSP identical to those of WKY? (iii) If there is some difference of the enzymatic properties, is it responsible for the reduced enzymatic activity in SHRSP? (iv) Does SHRSP have the same amount of 45 kDa MPD as WKY? In order to answer these questions, we purified hepatic MPD from SHRSP fed on a CP diet, and compared its characteristics with those of WKY. We also measured the amount of MPD in the crude extract of various tissues in SHRSP and WKY by using a Western blot transfer procedure.

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Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; IPP, isopentenyl pyrophosphate; MVAPP, mevalonate pyrophosphate; MPD, mevalonate pyrophosphate decarboxylase; QAE, quaternary aminoethyl (diethyl mono-2-hydroxybutylaminoethyl); SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SHRSP, stroke-prone spontaneously hypertensive rat; WKY, Wistar Kyoto rat.

MATERIALS AND METHODS

Materials—Pravastatin was purchased from Sankyo (Tokyo), and cholestyramine from Bristol Laboratories. All other chemicals of reagent grade were purchased from commercial sources.

Animals—Male WKY (280 g) and SHRSP (240 g) (10 weeks old) were housed in a light-controlled room (light phase, 6:00-18:00). The rats were fed on a powdered chow with or without cholestyramine and/or pravastatin as indicated in the figure legends. The average food intake was 21 g/day. Rats were fasted for one day before being killed (10:00).

Purification of MPD—Purification of liver MPD of WKY and SHRSP was carried out as described by Michihara *et al.* (*22*), except for a minor modifications in the case of SHRSP. All active fractions of the first hydroxyapatite chromatography (10-40 mM) were combined and applied to a column of Phenyl Sepharose. Separation of 45 and 37 kDa MPD was achieved by the second hydroxyapatite chromatography.

Immunoblot Procedures—The crude extracts (20,000× *g*, sup) of various tissues were subjected to Western blot analysis using anti-MPD antiserum raised against the purified 45 kDa MPD of WKY as described previously (*22*). When the amount of MPD in the crude extract was measured, we carried out a parallel experiment with known amounts (5-110 ng) of the purified 45 kDa MPD from WKY to prepare a standard curve. The signals were quantitated with a Shimadzu Chromatoscanner (S-910) (Shimadzu, Tokyo) and an amount of 45 kDa MPD in the crude extract was quantitatively estimated from the standard curve.

The procedures, including the assays of MPD and molecular weight determination by Superose 12 column chromatography, and pI determination by isoelectric focusing were carried out as described in the previous report (*22*).

Statistics—Statistical analysis was carried out by the use of Student's *t* test. Data are presented as mean±SD.

RESULTS AND DISCUSSION

First, we purified hepatic MPD from SHRSP fed on a CP diet as described in the previous paper (*22*). Treatment of SHRSP with CP diet increased the MPD activity in the liver crude extract (20,000×*g*, sup) by ten fold (0.41 nmol/min/mg) as compared with non-treated rats. However, the maximum value was about a half of that in WKY fed on a CP diet (data not shown). Table I summarizes the results of the overall purification of hepatic MPD from SHRSP fed on a CP diet. As described for the MPDs of WKY (*22*), two

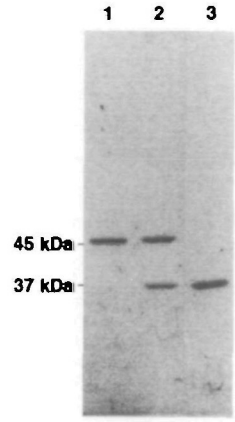

Fig. 1. **SDS-PAGE of the purified MPDs from SHRSP.** MPD obtained from the second hydroxyapatite fraction A (1 μg, lane 1), C (1 μg, lane 3), or the purified 45 and 37 kDa from WKY (1 μg, lane 2) were analyzed by SDS-PAGE.

TABLE I. **Purification of the hepatic MPDs from SHRSP.** MPD was purified from 20 livers (154 g) of rats fed on a CP diet. The second hydroxyapatite column resolved the 45 kDa (A) and the 37 kDa (C) MPD, while fraction B contained both (see Fig. 1).

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Recovery (%)
QAE	466	96.9	0.208	1.0	100
Blue Sepharose	37.4	30.5	0.816	3.9	31.5
Hydroxyapatite	3.5	17.9	5.1	24.5	18.5
Phenyl Sepharose	0.42	7.38	17.6	84.6	7.60
2nd Hydroxyapatite A	0.016	0.18	11.3	54.3	0.18
B	0.202	3.11	15.4	74.0	3.20
C	0.053	0.70	13.2	63.5	0.72

TABLE II. **Comparison of characteristics of MPDs in WKY and SHRSP.**

	45 kDa MPD		37 kDa MPD	
	WKY*	SHRSP	WKY*	SHRSP
MVAPP (K_m; μM)	22.7±2.2	22.7±2.5	20.0±1.4	19.6±1.2
(V_{max}; μmol/min/mg)	7.4±0.2	6.3±0.5	6.1±0.1	4.4±1.5
ATP (K_m; mM)	0.71±0.05	0.62±0.03	0.81±0.02	0.54±0.16
(V_{max}; μmol/min/mg)	5.5±0.2	4.0±0.8	5.2±0.05	3.5±1.2
Affinity for Mg^{2+} (K_m; mM)**	1.5±0.1[a]	0.52±0.1[a]	1.14±0.06[b]	0.68±0.03[b]
Optimum pH	7.0	7.0	7.0	7.0
pI	5.6	5.0	5.4	5.0
Native M.W. (kDa)	90	90	90	90

*The values of WKY were from our previous data (*22*). **The concentration at which half maximum activity was observed. [a,b]Significantly different, $p<0.0005$ ($n=3$).

species of MPDs were purified from SHRSP, and they co-migrated with the respective MPDs of WKY on SDS-PAGE (45 and 37 kDa, Fig. 1). At present we do not know whether 37 kDa MPD is a proteolytic fragment of 45 kDa MPD or the two forms are produced by alternative splicing of the same mRNA. Peptide mapping and Northern blot analysis will be necessary to address these questions.

Next, a set of experiments was carried out to examine the enzymatic properties of the MPDs of SHRSP as compared to those of WKY (Table II). Both MPDs of SHRSP showed apparent molecular weights of 90 kDa on Superose 12. The pI values for both MPDs were 5.0, and the K_m values for MVAPP and ATP were about 20 μM and 0.6 mM, respectively. The optimal pH was 7.0 for both enzymes. These results demonstrated that MPDs of SHRSP have essentially the same properties as those of WKY.

However, the affinity for Mg^{2+} was significantly higher in the case of the SHRSP enzyme when compared with WKY. The concentration at which half maximum activity was observed was about a third for 45 kDa MPD, and a half for 37 kDa MPD in SHRSP, as compared with those of WKY. Therefore, we further tested the dependency of the MPDs on other cations (Table III). For 45 kDa MPD of WKY, Mn^{2+} and Co^{2+} gave slightly higher activity than Mg^{2+} at 5 mM, whereas in SHRSP, 45 kDa MPD showed markedly less activity in the presence of Mn^{2+}. On the other hand, 37 kDa MPD of WKY showed 149 and 91% activity in the presence of Mn^{2+} and Co^{2+}, respectively, as compared to Mg^{2+}. The SHRSP enzyme again showed decreased activity in the presence of Mn^{2+}, whereas markedly enhanced activity was observed in the presence of Co^{2+}. These results suggested a structural difference of the cation-binding domain of MPD between WKY and SHRSP. Indeed, there is a difference in the Mg^{2+} dependency, but it cannot account for the decreased MPD activity in SHRSP. The affinity for Mg^{2+} was higher, not lower, in SHRSP, and the enzymatic activity at 5 mM, a physiological intracellular concentration, was only slightly less in SHRSP than in WKY (Table III).

Our previous study using liver crude extract demonstrat-

TABLE III. **Effect of cations on the activity of the liver 45 and 37 kDa MPD purified from SHRSP.** Activities in the presence of Mg are referred to as 100%. Data are the means of four identical experiments and each value varies within 10%.

Cation (5 mM)	Relative activity (%)			
	45 kDa MPD		37 kDa MPD	
	WKY*	SHRSP	WKY*	SHRSP
$MgCl_2$	100[a]	100[b]	100[c]	100[d]
$MnCl_2$	126	39	149	87
$CoCl_2$	125	98	91	166
$CaCl_2$	55	48	38	41
$CdCl_2$	33	7	25	15
$BaCl_2$	21	10	16	12
KCl	16	11	15	12
NaCl	21	10	13	12
None	9	11	9	10

[a-d]The actual values in the presence of 5 mM each of ATP and $MgCl_2$ are: a, 5.6; b, 4.1; c, 4.8; d, 3.9 (μmol/min/mg). *For the values of WKY we referred to our previous report (*22*).

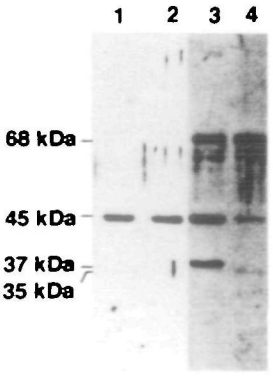

Fig. 2. **Immunoblot analysis of hepatic MPDs in SHRSP.** The purified 45 kDa from WKY (lane 1, 60 ng) and SHRSP (lane 2, 60 ng) and liver crude extract of either CP diet-treated (lane 3, 10 μg) or non-treated (lane 4, 10 μg) SHRSP was subjected to immunoblot analysis according to the method described in "MATERIALS AND METHODS."

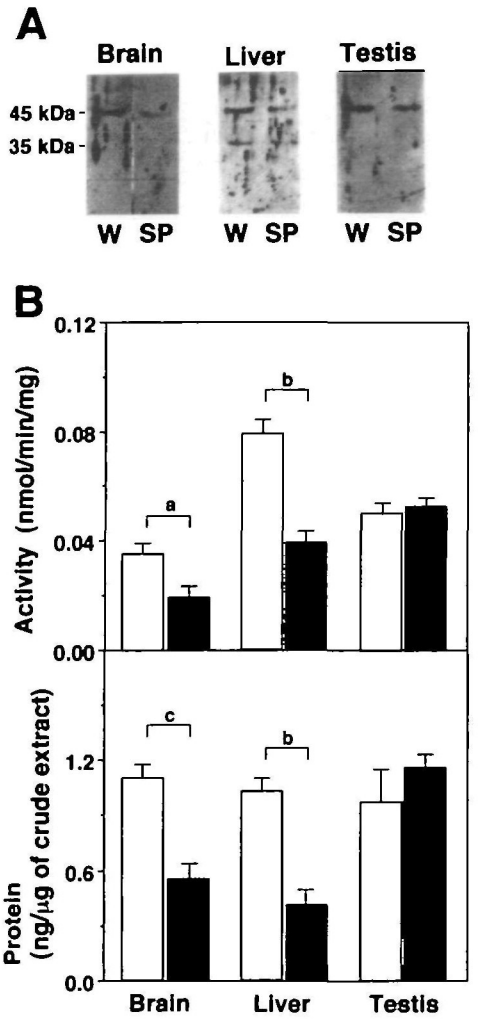

Fig. 3. **Quantification of the activity and the amount of MPD in the crude extract.** A: Twenty-four micrograms of the liver crude extract and 8 μg of the brain and testis were subjected to immunoblot analysis. B: Upper panel, 4 mg of crude extract was incubated with [^{3}H]MVAPP (250 nmol) for 15 min. IPP formed was measured as described under "MATERIALS AND METHODS." Lower panel, signals of A were quantified by the use of chromatoscanner. WKY (□) and SHRSP (■). Significantly different: $p<0.05$ (a); $p<0.001$ (b); $p<0.01$ (c). $n=3$.

TABLE IV. **Comparison of the ratio of the activity to the amount of 45 kDa MPD in various tissues between WKY and SHRSP fed on a normal chow.**

	WKY			SHRSP		
	Activity/tissue (nmol/min/g)	Amount/tissue (μg/g)	Activity/amount (nmol/min/mg)	Activity/tissue (nmol/min/g)	Amount/tissue (μg/g)	Activity/amount (nmol/min/mg)
Brain	0.43±0.03	15.6±3.60	28.0±4.30[a]	0.27±0.05	8.1±1.57	33.4±0.90[c]
Liver	3.74±0.36	54.2±5.70	69.1±4.90[a,b]	1.78±0.27	24.2±1.95	73.9±5.30[c,d]
Testis	1.62±0.02	38.5±1.90	43.0±1.50[b]	1.53±0.08	37.5±1.50	43.1±1.35[d]

Significantly different: $p<0.001$ (a, c); $p<0.05$ (b, d). $n=3$.

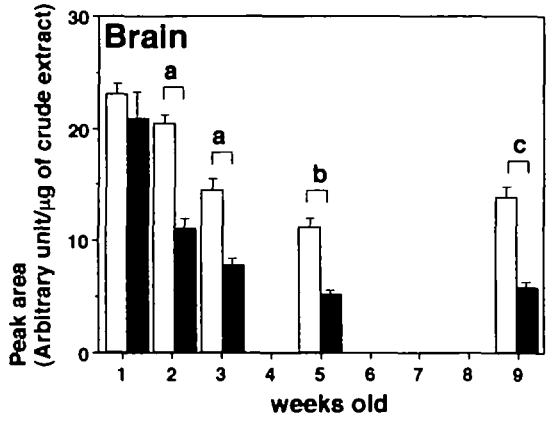

Fig. 4. **Age-dependent change in the amount of MPD in the brain.** Rats were killed at the indicated age and the amount of MPD in the brain crude extract was determined as described in "MATERIALS AND METHODS." WKY (□) and SHRSP (■). Significantly different: $p<0.05$ (a); $p<0.01$ (b); $p<0.001$ (c). $n=4$.

ed a lower V_{max} but an unchanged K_m for MVAPP in SHRSP, suggesting a decrease in the amount of MPD enzyme (*7*). In order to prove this hypothesis, we determined the amount of MPD in the crude extract by Western blot analysis. We had previously prepared an antiserum against 45 kDa MPD of WKY that also reacted to 45 kDa MPD of SHRSP with the same affinity (Fig. 2, lane 2). When the liver crude extract of SHRSP was subjected to Western blot analysis using this antiserum, 45 kDa but not 37 kDa MPD was detected (Fig. 2, lane 4). As described previously (*22*) the 68 and 35 kDa proteins were not MPD. When the liver of CP diet-treated SHRSP was examined, 37 kDa MPD was also found in the crude extract (Fig. 2, lane 3).

As shown in Fig. 3A, the amount of 45 kDa MPD was decreased in the crude extract of the brain and liver of SHRSP, while no change was observed in the testis. The signals were quantified by the use of chromatoscanner to compare the enzymatic activity in the crude extract (Fig. 3B). The amount of 45 kDa MPD was significantly decreased in the brain and liver in parallel with the enzymatic activity. However, in the testis, there was no difference in the amount or in the activity of MPD. To confirm this result we calculated the ratio of the enzyme activity to the amount of MPD in each tissue (Table IV). No significant difference was found between WKY and SHRSP in each tissue, demonstrating that the lower activity of MPD in SHRSP was caused by the decreased amount of the enzyme. The activity/amount ratio varied among tissues, being highest in the liver and lowest in the brain. This implies the presence of a tissue-specific regulatory mechanism of MPD in cells.

As cholesterol is a major constituent of myelin, there is much more in the brain than in other tissues. However, cholesterol is not supplied exogenously to this organ, so reduced cholesterol synthesis might affect important nerve functions. Duffard *et al.* reported that rapid myelination occurred in the rat brain at days of 15–25, and the treatment of these rats with dichlorophenoxy acetic acid to inhibit cholesterol synthesis resulted severe demyelination (*25*). As shown in Fig. 4, the decrease in the amount of MPD in the brain was observed at two weeks old and later in SHRSP. In addition, MPD activity is necessary to synthesize isoprenoids that covalently attached to many proteins to modulate their functions. Matthies Jr. *et al.* reported that the inhibition of isoprenoid synthesis decreased long-term potentiation in the CA1 region (*26*). Therefore, the reduced amount of MPD in SHRSP may result in the severe dysfunction of nerve systems and also cerebral stroke.

In conclusion, we found a reduced amount of MPD in SHRSP, and showed that this is responsible for the lowered activity of MPD in this rat. Further studies are necessary to understand the mechanism of this reduction and its role in the pathophysiology of SHRSP.

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