Comparison of Subcellular Distribution of Mevalonate Pyrophosphate Decarboxylase between Stroke-Prone Spontaneously Hypertensive Rat and Wistar Kyoto Rat

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We previously reported that the lower activity of mevalonate pyrophosphate decarboxylase (MPD) was caused by the reduced amount of this enzyme in stroke-prone spontaneously hypertensive rat (SHRSP) by immunoblot analysis using 20000×g supernatant containing cytosol and microsomes. A recent study showed that at least three different subcellular compartments, including peroxisomes, are involved in cholesterol synthesis. In this study, we examined the subcellular distribution of 45- and 37-kDa MPD in the liver of SHRSP and compared normotensive Wistar Kyoto rat (WKY) and SHRSP. 45-kDa MPD was detected in the cytosol and peroxisomes of SHRSP, while 37-kDa MPD was detected in the cytosol of SHRSP, but not in the peroxisomes. The relative enrichment of 45-kDa MPD in peroxisomes was lower than that of LDH, suggesting the possibility that 45-kDa MPD of SHRSP did not exist in the peroxisomes. Also, 45-kDa MPD was decreased in the crude extract containing 1% Triton X-100, cytosol and peroxisomes of SHRSP, and 37-kDa MPD was decreased in the crude extract containing 1% Triton X-100 and cytosol of SHRSP, as compared with WKY. These data indicate that the cholesterol synthesis in the liver of SHRSP by the reduced amount of MPD is significantly reduced.

Key words mevalonate pyrophosphate decarboxylase; cell fractionation; liver; peroxisome

One of the first steps in the biosynthesis of cholesterol from acetic acid is catalysis by mevalonate pyrophosphate decarboxylase (MPD). This decarboxylase catalyzes a bimolecular reaction between mevalonate pyrophosphate (MVAPP) and ATP to form isopentenyl pyrophosphate, inorganic phosphate, adenosine 5'-diphosphate (ADP) and CO₂.

Epidemiological studies have indicated a negative association between serum cholesterol level and the incidence of cerebral hemorrhage in human.¹⁾ The stroke-prone spontaneously hypertensive rat (SHRSP) is a widely used animal model for hypertension and stroke.²⁾ Iritani *et al.* reported that the serum cholesterol level in SHRSP was lower compared with normotensive age-matched Wistar Kyoto rat (WKY).³⁾ We previously reported that the lower serum cholesterol level in SHRSP than in WKY may therefore be attributable to the reduced activity of MPD and not to 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase.⁴⁾ Moreover, we found that the lower activity of MPD was caused by the reduced amount of this enzyme in SHRSP.⁵⁾ However, in the above study, we carried out a comparative using 20000×g sup (this sup containing cytosol and microsome fraction) in liver of WKY and SHRSP. A recent study showed that at least three different compartments (cytosol, microsomes and peroxisomes) are involved in cholesterol synthesis.⁶⁾ We previously purified two MPD (45- and 37-kDa MPD) from the liver of rats fed 12 d on a CP diet (which contained 0.1% pravastatin and 5% cholestyramine).⁷⁾ The CP diet-induced new species of 37-kDa MPD was characteristically and immunologically very similar to the well-known 45-kDa MPD.⁷⁾ We also reported that 45- and 37-kDa MPD is predominantly located in the cytosol and a small amount of 45-kDa MPD exists in peroxisomes, although there is no 37-kDa MPD in peroxisomes.⁸⁾ This led us to consider several questions: 1) Is the amount of 45-kDa MPD in the peroxisomes of SHRSP the same as in WKY? 2) Is 37-

kDa MPD induced by CP diet in the peroxisomes of SHRSP? 3) Is the total amount of MPD in the liver of SHRSP less than in WKY? To answer these questions, we carried out cell fractionation to separate the two compartments (cytosol and peroxisomes) involved in the distribution of MPD, and compared the amount of MPD in the crude extract, cytosol and peroxisomes of the liver between WKY and SHRSP fed a CP diet by immunoblot analysis.

MATERIALS AND METHODS

Materials and Animals Pravastatin was kindly provided by Sankyo, cholestyramine by Bristol Laboratories. All other chemicals were of reagent grade and purchased from commercial sources. Male Wistar Kyoto rats and stroke-prone spontaneously hypertensive rats (8 weeks old) were used for cell fractionation.

Cell Fractionation Cell fractionation of WKY and SHRSP livers (8 g) fed on a CP diet was carried out as described by Michihara *et al.*⁸⁾

Marker Enzyme The catalase activity (peroxisomal marker) was measured according to Abei.⁹⁾ The activity of lactate dehydrogenase (LDH; cytosol marker) was measured according to Bergmyer *et al.*¹⁰⁾ and that of NADPH-cytochrome P-450 reductase (microsome marker) according to Kreibich *et al.*¹¹⁾ Cytochrome-c oxidase (mitochondria marker) activity was measured according to Polakis *et al.*¹²⁾ and the activity of acid phosphatase (lysosome marker) according to Shibko *et al.*¹³⁾

Protein Assay Proteins were determined by the method of Lowry *et al.*¹⁴⁾ using bovine serum albumin as the standard.

Preparation of Antibody MPD was purified from rat liver and polyclonal antiserum raised against the 45-kDa MPD as described by Michihara *et al.*⁷⁾

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Western Blot Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% slab gels according to Laemmli.¹⁵ Proteins in SDS-slab gels were transferred to a nylon membrane by electrophoresis using a modified version of the procedure of Towbin *et al.*¹⁶ The positive bands were visualized by ECL Western blotting kits (Amersham Pharmacia) which contain a sensitive chemiluminescence substrate for horseradish peroxidase.

We previously reported two MPD (45- and 37-kDa MPD) from liver of SHRSP fed on a CP diet.⁷ When the purified 45- or 37-kDa MPD from SHRSP was subjected to Western blot analysis using anti-45-kDa MPD antiserum, 5 ng of 45- or 37-kDa MPD was detected, but not 2 ng of 45- or 37-kDa MPD. Then, when the signal of 5 ng of 45- or 37-kDa MPD was measured by Gel-Pro analyzer, the arbitrary value of 45- or 37-kDa MPD was 5, respectively.

An experiment comparing MPD between WKY and SHRSP used, cytosol and peroxisomes in WKY isolated by cell fractionation as described by Michihara *et al.*⁸

RESULTS

Isolation of Peroxisomes by Density Gradient Centrifugation of SHRSP Liver To examine whether 45- or 37-kDa MPD exist in the peroxisomes of liver in SHRSP, the intact peroxisomes were prepared by cell fractionation using liver of SHRSP fed on a CP diet. Peroxisomes in SHRSP were isolated on a sucrose gradient as described by Michihara *et al.*⁸ Figure 1 illustrates the separation of peroxisomes from mitochondria, microsomes, and cytosol as determined by the distribution of marker enzymes on the gradient. The mitochondria, measured by cytochrome oxidase, and the microsomes, determined by NADPH-cytochrome P-450 reductase, are located close to the center of the gradient. The cytosolic marker LDH is located at the light end of the gradient and is well removed from the intact peroxisomes. The intact peroxisomes, as measured by catalase activity, are separated from the above organelle fraction and are located at higher densities. In agreement with earlier reports, a protein of the catalase activity is solubilized as a result of rupture of the peroxisomes during the isolation procedure, and migrates to the light end of the gradient. As shown in Table 1, the specific activities of catalase in fraction 19 (peroxisomal fraction) are approximately 150 times higher than that in the SHRSP liver homogenate. From these data, fraction 19 is indicated to contain intact peroxisomes.

Comparison of MPD in Peroxisomes between WKY and SHRSP When the peroxisome fraction was subjected to immunoblot analysis, a small amount of 45-kDa MPD was detected in peroxisomes of SHRSP, but not 37-kDa MPD (Fig. 2). When the amount of 45-kDa MPD in peroxisomes was compared between WKY and SHRSP, 45-kDa MPD in peroxisomes of SHRSP was one-fourth that of WKY.

Comparison of MPD in Cytosol between WKY and SHRSP Cytosol of SHRSP was isolated by cell fractionation as described by Michihara *et al.*⁸ As shown in Fig. 3, when the cytosol fraction was subjected to immunoblot analysis, the 45- and 37-kDa MPD were detected in the fraction of SHRSP, and the amount of both in the cytosol fraction of SHRSP was half that of WKY.

Comparison of Total Amount of MPD between WKY

Fig. 1. Isolation of Peroxisomes in SHRSP Liver by Sucrose Density Gradient Centrifugation

A peroxisomal-enriched fraction prepared by differential centrifugation of the liver homogenate from CP diet-treated SHRSP was further purified by sucrose density gradient centrifugation to separate peroxisomes from other cell organelles. Twenty-three fractions were collected from the top to the bottom of the tubes. A: Protein; B, C: the distribution of catalase (marker enzyme for peroxisome), oxidase (cytochrome-c oxidase; marker enzyme for mitochondria), LDH (lactate dehydrogenase; marker enzyme for cytosol), and reductase (NADPH-cytochrome P-450 reductase; marker enzyme for endoplasmic reticulum) were plotted as % of total.

Table 1. Enzymic Composition of SHRSP Liver Peroxisomes

Enzyme	Specific activity (Unit/mg)	Yield (% of homogenate)	Relative enrichment
Reductase	0.002	0.052	0.07
Oxidase	0.027	0.28	0.68
LDH	0.026	0.6	1.8
Catalase	1.356	25.1	152.1
Acid phosphatase	0.042	0.31	0.67

Relative enrichment was defined as the ratio of specific activity of the peroxisomes to that of the homogenate. The values are the means of three identical experiments and each value varies within 10%.

Fig. 2. Analysis of MPD in Peroxisomal Fraction of WKY and SHRSP by Immunoblot

Peroxisomal fraction prepared from WKY (W, 80 μ g) or SHRSP (SP, 80 μ g) was subjected to immunoblot analysis.

Fig. 3. Analysis of MPD in Cytosol Fraction of WKY and SHRSP by Immunoblot

Cytosol fraction prepared from WKY (W, 10 μ g) or SHRSP (SP, 10 μ g) was subjected to immunoblot analysis.

Fig. 4. Analysis of MPD in Crude Extract of WKY and SHRSP by Immunoblot

WKY and SHRSP livers fed a CP diet or normal chow were washed with 100 mM sodium phosphate (pH 7.0) containing 10 mM mercaptoethanol and 1 mM EDTA (buffer A), and homogenized with 3 volumes of ice-cold buffer A containing 1% Triton X-100 and 0.5 mM phenylmethylsulfonyl fluoride and protease inhibitors (1 mM leupeptin, 1 mM pepstatin A, 1 mM chymostatin, and 1 mM antipain) using a Polytron motor driven homogenizer. The homogenate was centrifuged at 100000 $\times g$ for 1 h, and the supernatant was used as crude extract. W: WKY, SP: SHRSP. A: Crude extracts in the liver of WKY (30 μ g) and SHRSP (30 μ g) fed a normal chow were subjected to immunoblot analysis. B: Crude extracts in the liver of WKY (10 μ g) and SHRSP (10 μ g) fed a CP diet were subjected to immunoblot analysis. Mean $\pm$ S.D., $n=3$.

and SHRSP As shown in Fig. 4A, the amount of 45-kDa MPD was decreased in 100000 $\times g$ sup containing 1% Triton X-100 of SHRSP fed a normal chow compared with WKY fed a normal chow, while no change was observed in 20000 $\times g$ sup (data not shown). As shown in Fig. 4B, the amount of both 45- and 37-kDa MPD were decreased more in 100000 $\times g$ sup containing 1% Triton X-100 of SHRSP fed a CP diet than in WKY fed the same diet, while no change was observed in 20000 $\times g$ sup (data not shown). Thus the difference in amount of 45-kDa MPD between WKY and SHRSP fed a CP diet reflects the same difference of 45-kDa

Table 2. Relative Enrichment of 45-kDa MPD in WKY and SHRSP Liver Peroxisomes

Rats	Arbitrary unit (Arbitrary value/mg)	Yield (% of homogenate)	Relative enrichment
WKY	0.9	0.3	3.25
SHRSP	0.22	0.1	1.21

After the homogenates or peroxisomes were subjected to immunoblot analysis using anti-45-kDa MPD antiserum, signals of 45-kDa MPD were measured by a Gel-Pro Analyzer. Relative enrichment was defined as the ratio of the arbitrary unit (arbitrary value of signal of 45-kDa MPD/protein (mg)) of the peroxisomes to that of the homogenate. The values are the means of three identical experiments and each value varies within 10%. The values of WKY were from our previous data.⁸⁾

MPD between the two groups fed a normal chow. The amount of MPD in liver of SHRSP was significantly lower than WKY, and the amount of 37-kDa MPD in crude extract of SHRSP was half that of WKY (Fig. 4B).

DISCUSSION

We previously reported that the lower activity of MPD was caused by the reduced amount of this enzyme in SHRSP. However, comparison in this study used 20000 $\times g$ sup (containing cytosol and microsome fraction) in liver of WKY and SHRSP, and found that at least three compartments (cytosol, microsomes and peroxisomes) are involved in cholesterol synthesis. We examined the subcellular distribution of 45- and 37-kDa MPD in SHRSP for comparison. 45-kDa MPD was detected in the cytosol and peroxisomes of SHRSP. As shown in Fig. 1C and Table 1, since the peroxisome fraction contains a small amount of LDH, a small amount of 45-kDa MPD in cytosol is believed to be contained in peroxisomes. Therefore, following estimation of the relative enrichment of 45-kDa MPD in peroxisomes of SHRSP from an arbitrary unit of the homogenate and peroxisomes (Table 2), it was compared and found lower than that of LDH of SHRSP (Table 1). This confirmed that the 45-kDa MPD in cytosol was contained in the peroxisomes, suggesting that 45-kDa MPD of SHRSP may not exist in the peroxisomes. Three pathways of cholesterol synthesis were considered; I) from cytosol to endoplasmic reticulum (ER), II) from peroxisomes through cytosol to ER, and III) in peroxisomes only. Our data also suggested that cholesterol of SHRSP was synthesized by the pathway from cytosol to endoplasmic reticulum. Further study is necessary to understand whether cholesterol synthesis in peroxisomes is important or whether other enzymes involved in cholesterol synthesis exist in peroxisomes.

37-kDa MPD was detected in the cytosol of SHRSP, but not in the peroxisomes. When the crude extract (100000 $\times g$ sup) of SHRSP liver containing 1% Triton X-100 was subjected to Western blot analysis, the ratio of 45- to 37-kDa MPD was 6:1. If the ratio in the peroxisome fraction were the same, 37-kDa MPD in peroxisomes should have been detected when 80 μ g of peroxisome fraction (arbitrary value in 80 μ g of the peroxisomes was 35) was subjected to Western blot. However, when 250 μ g of an excess intact peroxisomal fraction (arbitrary value in 250 μ g of peroxisomes was 110) was subjected to Western blot, 37-kDa MPD was not detected in the peroxisome fraction (data not shown). This suggested that 37-kDa MPD did not exist in peroxisomes.

Western blot analysis showed the total amount of 45-kDa

Fig. 5. Relative Amount of MPDs

Signals in Fig. 2, 3 or 4 were measured using a Gel-Pro analyzer. A: Relative amount of 45-kDa MPD illustrated as total amount in the crude extract, cytosol and peroxisomes of WKY (■) and SHRSP (□) fed a CP diet. B: Relative amount of 37-kDa MPD illustrated as total amount in the crude extract, cytosol and peroxisomes of WKY (■) and SHRSP (□) fed a CP diet. Total amount of 45-kDa MPD in crude extract of WKY liver fed a CP diet is taken as 100% for both A and B. T: crude extract, C: cytosol, P: peroxisome. Mean $\pm$ S.D., $n=3$.

MPD in the crude extract, cytosol and peroxisomes was less in SHRSP than in WKY (Fig. 5A); this was also true of 37-kDa MPD in the extract and cytosol (Fig. 5B). The amount of 45-kDa MPD was significantly decreased in liver of SHRSP, and the production effect of 37-kDa MPD also decreased in the liver of SHRSP, as compared with WKY.

This study suggested a difference in subcellular distribu-

tion of 45-kDa MPD between WKY and SHRSP. Further study is necessary to understand the physiological role of peroxisomes involved in cholesterol synthesis and difference in subcellular distribution of 45-kDa MPD between the two rat groups.

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