Comparison of Receptors and Enzymes Regulating Cholesterol Levels in Liver between SHR/NDmcr-cp Rats and Normotensive Wistar Kyoto Rats at Ten Weeks of Age

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The spontaneously hypertensive rat (SHR)/NDmcr-cp (SHR-cp), which is a metabolic syndrome model rat, was reported to show hypercholesterolemia, as compared with lean littermates. The serum total cholesterol level in SHR-cp at 18 weeks of age is higher than that of normotensive Wistar Kyoto rat (WKY), but that in SHR-cp at 10 weeks of age is the same. The objective of this study is to clarify whether there are differences in the system regulating serum cholesterol levels between SHR-cp and WKY at 10 weeks of age. Total serum cholesterol levels, and cholesterol levels of high density lipoprotein (HDL), low density lipoprotein (LDL), and very low density lipoprotein (VLDL) were similar in the two strains. However, the cholesterol levels in the liver of SHR-cp were lower than those of WKY. Next, mRNA levels of receptors (scavenger receptor class B type 1 [SRB1], LDL receptor [LDLR]) involved in uptake from serum to liver or enzymes of cholesterol catabolism (CYP7A1 and CYP8B1) and biosynthesis (mevalonate pyrophosphate decarboxylases [MPD]) in liver were compared between SHR-cp and WKY. High levels of MPD and LDLR and low levels of SRB1 were shown in SHR-cp, as compared with WKY. CYP7A1 and CYP8B1 levels were similar between SHR-cp and WKY. These results suggest that the serum cholesterol level in SHR-cp by the balance or regulation between the rise in cholesterol uptake and reduction in cholesterol biosynthesis in the liver is the same as that in WKY.

Key words metabolic syndrome; cholesterol; mevalonate pyrophosphate decarboxylase; Wistar Kyoto rat; SHR/NDmcr-cp

Spontaneously hypertensive rat (SHR)/NDmcr-cp (cp/cp) rat (SHR-cp) is a congenic SHR rat with nonsense mutation introduced into the leptin receptor.¹⁾ Metabolic syndrome, characterized by the concomitant presence of obesity, hypertension, hyperlipidemia and diabetes, develops spontaneously in the SHR-cp,^{2–4)} along with age-related histologically evident glomerular injury and tubulointerstitial damage, including mesangial activation, podocyte injury, and inflammatory cell infiltration in the tubulointerstitium.⁵⁾ SHR-cp was reported to show hypercholesterolemia and hyperglyceridemia, as compared with lean littermates (SHR-lin) that have hypertension.²⁾ The serum total cholesterol level in SHR-cp at 18 weeks of age is higher than that of Wistar Kyoto rat (WKY),⁶⁾ but that in SHR-cp at 10 weeks of age is the same.⁷⁾ It remains unclear whether there are differences in the system regulating serum cholesterol levels between SHR-cp and WKY at 10 weeks of age. Thus, we compared cholesterol levels in serum and liver between SHR-cp and WKY. Furthermore, mRNA levels of receptors or enzymes involved in uptake from serum to liver, cholesterol catabolism, or cholesterol biosynthesis in liver were compared between SHR-cp and WKY.

MATERIALS AND METHODS

Materials The Cholesterol E-test Wako was obtained from Wako (Osaka, Japan). The high density lipoprotein (HDL) and very low density lipoprotein (VLDL)/low density lipoprotein (LDL) Quantification Kit was from BioVision, Inc. The Biomasher was obtained from Assist (Tokyo, Japan), QuickGene RNA tissue kit SII from Fujifilm (Tokyo, Japan), and SYBR Ex Script reverse transcription-polymerase chain reaction (RT-PCR) kit from TaKaRa (Shiga, Japan). All other chemicals were of reagent grade and pur-

chased commercially.

Animals Male (6-week-old) WKY and SHR-cp were obtained from the Disease Model Co-operative Research Association, Japan. The rats were fed standard chow for 4 weeks and used at 10 weeks of age. The experimental protocol was reviewed and approved by the Animal Care and Use Committee of Fukuyama University.

Cholesterol Levels in Liver and Serum One hundred milligrams of liver was homogenized in 500 μ l of homogenization buffer (50 mM Tris-HCl, pH 7.5 containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM 2-mercaptoethanol, 1 mM ethylenediaminetetraacetic acid (EDTA), and protease inhibitors [1 μ M leupeptin, 1 μ M pepstatin A, 1 μ M chymostatin, and 1 μ M antipain]) and centrifuged at 1000 $\times g$ for 10 min. Forty microliters of postnuclear supernatant (PNS) was mixed with 5 ml of Folch extract (chloroform-methanol, 2 : 1), and the mixture was incubated for 10 min at 37 °C with shaking. After the mixture was centrifuged at 3000 $\times g$ for 10 min, 3 ml of the supernatant was evaporated dry by boiling at 100 °C, and then dissolved in 200 μ l of isopropyl alcohol containing 1% Triton-X-100. The cholesterol content of the solution or serum (20 μ l) was determined using the Cholesterol E-test Wako (optical density (OD) 600 nm).

Isolation of HDL and VLDL/LDL Fractions from Serum The HDL and VLDL/LDL fractions were isolated from serum with the HDL and VLDL/LDL Quantification Kit, and their cholesterol content measured (OD 570 nm).

Cholesterol Levels of LDL from Serum Low density lipoprotein (LDL) levels were determined using an enzymatic kit (Wako, Co., Ltd., Tokyo, Japan).

Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction (Real-Time PCR) Rat tissue (15 mg) was homogenized using the Biomasher. The ho-

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mogenate was dissolved using the QuickGene RNA tissue kit SII, and total RNA (60 μ l) was isolated using the same kit (total RNA extract kit) and QuickGene-810 (Nucleic Acid Isolation System; Fujifilm, Tokyo, Japan). One hundred nanograms sample of total RNA from each tissue was subjected to reverse transcription (RT) using reverse transcriptase in a 50 μ l reaction volume. After the RT reaction, the cDNA template was amplified by polymerase chain reaction with a SYBR Ex Script RT-PCR Kit (TaKaRa, Tokyo, Japan). SYBR Green was used for the real-time PCR analysis of mevalonate pyrophosphate decarboxylase. Real-time PCR was performed using a ABI7500 system (Applied

Biosystems Japan, Tokyo, Japan). Cycling conditions were 40 cycles of 96 °C for 35 s, 64 °C for 35 s and 72 °C for 35 s. Relative gene expression was quantified using GAPDH as an internal control. As shown in Table 1, the primer pairs of GAPDH, CYP7A1, CYP8B1, SRB1, LDLR, and mevalonate pyrophosphate decarboxylase (MPD) were used in real time-PCR. The cDNA products obtained by RT-PCR were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining under UV light.

Statistical Analysis The statistical analysis was carried out using Student's *t*-test. Data are presented as the mean $\pm$ S.D.

Table 1. Primers Used for PCR

Genes	Primer sequence
GAPDH	F5'-GGCACAGTCAAGGCTGAGAATG-3' R5'-ATGGTGGTGAAGACGCCAGTA-3'
CYP7A1	F5'-CTGTGTTCACTTTCTGAAGCCATG-3' R5'-CCCAGGCATTGCTCTTTGAT-3'
CYP8B1	F5'-GGCTGGCTTCCTGAGCTTGTT-3' R5'-ACTTCCTGAACAGCTCATCGG-3'
SRB1	F5'-GTTCCGTGAAGATGCAGCTGAG-3' R5'-AACCACAGCAATGGCAGGACTAC-3'
LDLR	F5'-CACAACGTCACGCAGCCTAGA-3' R5'-AGAACCCTTGCCTCACACCAG-3'
MPD	F5'-AGGACCGCATCTGGCTGAAC-3' R5'-TGACGCCACGTGCACCTTA-3'

RESULTS AND DISCUSSION

Comparison of Serum and Liver Cholesterol Levels between SHR-cp and WKY

Total serum cholesterol levels and cholesterol levels of LDL of SHR-cp were found to be similar to those of WKY (Figs. 1A, B). There were no significant differences in cholesterol levels of the HDL and VLDL/LDL fractions between SHR-cp and WKY (Figs. 1C, D). These findings suggest no differences in cholesterol levels of VLDL between SHR-cp and WKY (Figs. 1B, D). The cholesterol secreted from liver was released as VLDL, and changed to intermediate density lipoprotein (IDL), and LDL containing cholesterol was supplied to other tissues. Therefore, it was suggested that the hepatic secretion of cho-

Fig. 1. Total Cholesterol Levels and Cholesterol Levels of HDL, LDL, and VLDL in Serum of SHR-cp and WKY

Total cholesterol levels in serum (A), and the cholesterol levels of LDL (B), HDL (C) and VLDL/LDL (D) were measured as described in Materials and Methods. Data are the means for four identical experiments.

lesterol was the same in SHR-cp and WKY.

Cholesterol levels in liver were significantly reduced by 20% in SHR-cp as compared with WKY (Fig. 2). These results suggested that the low level of cholesterol in the liver was due to a reduction in cholesterol biosynthesis, increased cholesterol uptake, or an acceleration of cholesterol catabolism.

Comparison of mRNA Levels of Receptors and Enzymes Involved in Cholesterol Uptake, Catabolism, and Biosynthesis in Liver between SHR-cp and WKY When mRNA levels of CYP7A1 and CYP8B1 involved in cholesterol catabolism were measured by real-time PCR, they were found to be similar in SHR-cp to those in WKY (Figs. 3A, B). This suggested that the low level of cholesterol in the

Fig. 2. Cholesterol Levels in Liver of SHR-cp and WKY

Cholesterol levels in the liver were measured as described in Materials and Methods. Data are the means for four identical experiments. Significantly different: * $p < 0.05$.

Fig. 3. mRNA Levels of Receptors and Enzymes Involved in Cholesterol Uptake, Catabolism, or Biosynthesis in the Liver of SHR-cp and WKY

RT-PCR was performed using the primers listed in Table 1 with total RNA from the liver of SHR-cp and WKY, as described in Materials and Methods. cDNA products (1 μ l) were electrophoresed on a 2% agarose gel and visualized with ethidium bromide staining under UV light. PCR resulted in a single band, and therefore real-time PCR was performed as described in Materials and Methods. Data are the mean for four identical experiments. Significantly different: * $p < 0.05$, ** $p < 0.001$.

liver was not responsible for the acceleration of cholesterol catabolism. Next, mRNA levels of SRB1 and LDLR involved in the uptake of cholesterol were measured. SRB1 and LDLR are considered to mediate the selective uptake of HDL and LDL into liver, respectively.⁸⁾ Thus, rise in SRB1 and LDLR may accelerate the uptake of HDL and LDL from serum to liver, respectively. In this study, significantly lower levels of LDLR and higher level of SRB1 were found in SHR-cp, as compared with WKY (Figs. 3C, D). More HDL than LDL is contained in serum, as the cholesterol ester transfer protein is not present in rats.⁹⁾ Namely, most of the cholesterol taken up into the liver from serum is HDL rather than LDL in rats. Therefore, the low level of cholesterol of the liver in SHR-cp was largely not responsible for the reduction in uptake of cholesterol, although it may be partly involved. Kudo *et al.* reported that mRNA levels of CYP51 involved in cholesterol biosynthesis were significantly reduced, but not those of HMG-CoA reductase, as compared with levels in WKY at 7 weeks of age.¹⁰⁾ When mRNA levels of MPD involved in cholesterol biosynthesis were measured, they were found to be significantly reduced (Fig. 3E). This finding suggested that the reductions of MPD and CYP51 in the liver were phenomena of SHR-cp, and the low level of cholesterol of the liver was caused by the decrease in cholesterol biosynthesis. These results suggest that serum cholesterol levels of young SHR-cp remain at the same level as in WKY by an acceleration of the uptake of HDL from serum to liver caused by the rise in SRB1, to supplementing the low level of cholesterol in the liver of SHR-cp caused by the reduction in cholesterol biosynthesis due to low levels of MPD. Namely, the serum cholesterol level in SHR-cp, balanced or regulated by increased cholesterol uptake and decreased cholesterol biosynthesis in the liver, is the same as that in WKY.

We previously reported that total cholesterol levels in serum of SHR-cp at 6 or 10 weeks of age were unchanged, as compared with levels in WKY.⁷⁾ SHR-cp was reported to show hypercholesteremia, as compared with WKY at 18 weeks of age.⁶⁾ Therefore, when the balance or regulation between the rise in cholesterol uptake and reduction in choles-

terol biosynthesis of liver disappears from 10 weeks of age onward, SHR-cp may develop hypercholesteremia.

Serum cholesterol level in SHR-cp was higher than that of SHR-ln,²⁾ but similar to that of WKY.⁷⁾ Therefore, the serum cholesterol level in SHR-ln is lower than that of WKY. It was reported that serum cholesterol level in SHR is also lower than that of WKY.¹¹⁾ As SHR-cp and SHR-ln were derived from SHR,¹⁾ the reduction of serum cholesterol level in SHR-ln may be derived from the genetic background of SHR. If the serum cholesterol level in SHR-cp with nonsense mutation of the leptin receptor is higher than that in SHR, the mutation may be responsible for the increase/recovery of serum cholesterol. The findings of this study obtained by comparative studies between SHR-cp and WKY may be useful in the elucidation of leptin receptor function.

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