Retinol and its active derivative retinoic acid have an important role in development, reproduction, immunity, and cell proliferation/differentiation (1). Retinoic acid exerts physiological actions through retinoic acid receptors, including the retinoic acid receptor (RAR) and the retinoid X receptor (RXR) (2). These receptors are members of the steroid/thyroid hormone nuclear receptor superfamily, which act as ligand-dependent transcriptional factors. There are three subtypes (α, β, and γ) of the RAR and RXR; the RAR subtypes bind with all-trans retinoic acid (ATRA) and 9-cis retinoic acid (9-cis RA), while the RXR subtypes only bind with 9-cis RA.

Dietary retinol is absorbed through the intestinal epithelium and is enfolded as retinyl esters in nascent chylomicrons, which enter the systemic circulation through mesenteric lymphatics and reach the liver (3). Retinol in the liver is esterified by lecithin:retinol acyltransferase (LRAT), which is the enzyme responsible for catalyzing retinyl ester formation from retinol (4). Retinyl ester is stored in hepaticstellate cells and the transfer of retinol to extrahepatic tissues is mediated through cellular retinol binding protein-I (CRBP-I) in hepatocytes (5). CRBP-I delivers retinol to retinol-binding protein (RBP) for secretion from the liver into the circulation. Retinol bound to RBP circulates until it reaches the target tissues, where retinol is metabolized to retinoic acid as a ligand of the RARs and RXRs or is esterified to form retinyl ester for storage. Retinoic acid is catabolized by cytochrome P450 hydroxylases (CYP26A1, 26B1, and 26C1). LRAT catalyzes the esterification of retinol in several organs and tissues (intestine, liver, retina, skin, testis, and lung) (4). An important dietary source of retinol for humans is β-carotene, which is known as provitamin A. β-Carotene is converted to retinol through cleavage by β-carotene 15,15′ monooxygenase (BCM), which is expressed in the testis, lung, and kidney besides the liver and intestine (6). Thus, retinol is converted from β-carotene in several tissues where retinol is required.

Obesity is characterized by the excessive accumulation of body fat and is recognized as a risk factor for cardiovascular disease (7). Obesity is often accompanied by dyslipidemia, hypertension, and insulin resistance, all of which are components of metabolic syndrome. This syndrome is increasingly recognized as a major risk factor for cardiovascular disease, kidney disease, and diabetes (8), and its prevalence has been increasing worldwide.

Obese Zucker rats (fa/fa rats) are a well recognized and widely used experimental model of genetic obesity (9). These rats have a mutation of the leptin receptor (10). Leptin is one of the adipokines, which are secreted...
by adipose tissue and have an important role in regulating energy consumption and modulating lipid and glucose metabolism (11). Fa/fa rats develop severe obesity associated with overeating and then present with dyslipidemia, hepatic steatosis, and hyperinsulinemia, which are similar features to those of human metabolic syndrome (9).

In human studies, obesity shows an inverse correlation with the nutritional retinol status (12, 13). Retinol intake is inversely correlated with the body fat composition of healthy adults (14), and low retinol intake is associated with the development of metabolic syndrome in adults (15). In animal studies, retinoic acid activates peroxisome proliferator activated factor (PPAR) δ, which is the nuclear receptor coordinating lipid metabolism, or modulates adipogenesis (16, 17). However, there are few animal studies demonstrating the retinol metabolism under conditions of obesity or dyslipidemia. Although retinol-related molecules like BCM and LRAT are known as the key enzymes in retinol metabolism, it is unclear whether the expression of these enzymes is affected and associated with the retinol status in various pathological conditions. Therefore, the aim of this study was to investigate the retinol status and expression of retinol-related molecules in fa/fa rats with obesity and dyslipidemia.

### Materials and Methods

1) Animal experiments. Homozygous obese (fa/fa) male Zucker rats (n = 5) and their corresponding lean littermates (n = 5) were obtained at 9 wk of age from Japan SLC, Inc. (Shizuoka, Japan) and housed for 1 wk with chow and water provided ad libitum. The composition of the diets is shown in Table 1. Care and handling of the experimental animals were done according to Osaka Medical College guidelines for the ethical treatment of laboratory animals. At 10 wk of age, obese and lean Zucker rats were sacrificed by exsanguination under isoflurane anesthesia after being fasted overnight. Blood was collected into heparinized tubes and plasma was separated for storage at −80°C. The liver, jejunum, testis, and kidney were removed, immediately frozen in liquid nitrogen, and stored at −80°C.

2) Biochemical analysis. The plasma concentrations of glucose, total lipids, cholesterol, triglycerides, and alanine aminotransferase (ALT) were measured by enzymatic colorimetric methods. The retinol concentrations of plasma and liver homogenates were assayed by high-performance liquid chromatography, as described previously (18). For analysis, liver tissue was homogenized and saponified with one-twentieth volume of 60% potassium hydroxide in distilled water, after which the saponified liver samples were extracted with hexane. Plasma was not saponified and extracted with hexane. Then the protein content was measured according to the method of Bradford (19).

3) Immunoblotting. Anti-rat LRAT antibody (Immunobiological Laboratories, Gunma, Japan), and anti-CRBP-I antibody (Santa Cruz Biotechnology Inc., CA, USA) were purchased for use as the primary antibodies. LRAT is a microsomal protein, so liver microsomes were prepared as described previously (20). For detection of CRBP-I, the cytosolic fraction was prepared from the homogenate by ultracentrifugation at 100,000 × g for 60 min and its protein content was measured as mentioned above. Each primary antibody was appropriately diluted with Tris-buffered saline containing Tween-20 (TBS-T), and the appropriate horseradish peroxidase-conjugated secondary antibodies were used (Bio-Rad Laboratories, CA, USA). Target bands were detected with the ECL Western blotting Detection System (GE Healthcare UK Ltd., Buckinghamshire, UK).

4) Real-time reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated by the acid guanidium thiocyanate/phenol/chloroform (AGPC) method (21). Then quantitative real-time RT-PCR was performed to determine the levels of BCM and β-actin in RNA samples. The RT reaction was carried out using Omniscript (Qiagen, CA, USA) following the manufacturer’s protocol. Subsequently, one tenth (2 µL) of each RT reaction mixture was amplified in a LightCycler PCR (Roche’s Diagnostics, Switzerland), as previously described (22). Real-time PCR for BCM and β-actin was done with the Lightcycler HybProbe quantification kit, and that for CYP26A1 with the SYBR Green I quantification kit. The RT reaction was carried out using Omniscript (Qiagen, CA, USA) following the manufacturer’s protocol. Subsequently, one tenth (2 µL) of each RT reaction mixture was amplified in a LightCycler PCR (Roche’s Diagnostics, Switzerland), as previously described (22).

#### Table 1. Composition of the diets.

<table>
<thead>
<tr>
<th>Component</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>8.0%</td>
</tr>
<tr>
<td>Crude protein</td>
<td>20.1%</td>
</tr>
<tr>
<td>Crude fat</td>
<td>4.4%</td>
</tr>
<tr>
<td>Crude fiber</td>
<td>5.2%</td>
</tr>
<tr>
<td>Crude ash</td>
<td>8.8%</td>
</tr>
<tr>
<td>Nitrogen free extracts</td>
<td>53.5%</td>
</tr>
<tr>
<td>Vitamin mixture (containing vitamin A 1,000 IU/100 g and β-carotene 0.02 mg/100 g)</td>
<td></td>
</tr>
<tr>
<td>Total energy</td>
<td>4.09 kcal/g</td>
</tr>
</tbody>
</table>

The diet (MM-3) was purchased from Funabashi Farm (Chiba, Japan).
Table 2. Retinol and biochemical data.

<table>
<thead>
<tr>
<th></th>
<th>Lean</th>
<th>fa/fa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>224.0±4.0</td>
<td>334.8±8.1***</td>
</tr>
<tr>
<td>Total lipid (mg/dL)</td>
<td>200.6±6.7</td>
<td>508.4±64.4***</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>77.2±4.2</td>
<td>89.6±5.4**</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>13.4±3.4</td>
<td>322.4±52.8***</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>63.8±7.9</td>
<td>272.8±41.7***</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>41.4±3.4</td>
<td>78.8±6.4***</td>
</tr>
<tr>
<td>Retinol (plasma) (µg/dL)</td>
<td>1.4±0.14</td>
<td>2.4±0.57*</td>
</tr>
<tr>
<td>Retinyl palmitate (plasma) (µg/dL)</td>
<td>1.5±0.1</td>
<td>1.33±0.2</td>
</tr>
</tbody>
</table>

Data are expressed mean±SD and asterisks indicate significant differences (*p<0.05, **p<0.01, ***p<0.001) between lean and fa/fa rats.

(CYP26A1) were used as the external PCR standards. Using LightCycler software, the amplification curves of the experimental samples were plotted against the standard curves to obtain an estimate of the number of specific mRNA copies. To compensate for differences in RT efficiency among the samples, BCM and CYP26A1 values were then normalized by the copy number for /H9252-actin.

5) Statistical analysis. Results are expressed as the mean±SD. To determine the significance of differences, Welch’s t-test was used. Differences between groups were considered significant at a p value of less than 0.05.

Results

1) Biochemical data and retinol levels

The lipid profiles and retinol levels of fa/fa rats and lean rats are shown in Table 2. It can be seen that hyperlipidemia, hyperglycemia, and hepatic dysfunction were present in fa/fa rats. The plasma retinol and retinyl ester levels were significantly higher in fa/fa rats than in lean rats, while there was no difference in the hepatic retinol level between the two groups.

2) BCM expression

Expression of the BCM gene in several tissues is shown in Fig. 1. BCM expression was increased in the jejunum of fa/fa rats compared with lean rats while BCM expression in the liver, testis, and kidney showed no differences between fa/fa rats and lean rats by real-time RT-PCR.

Fig. 2. Expression of the rat CYP26A1 and CRBP-I genes. Real-time RT-PCR for the hepatic CYP26A1 gene and immunoblotting for the hepatic CRBP-I gene were performed. The BCM/β-actin ratio was determined by real-time RT-PCR. Bars indicate the SD. Protein was subjected to electrophoresis, transferred to a PVDF membrane, and immunoblotted with the primary antibody as described in “Materials and Methods.”

Fig. 3. Expression of the rat LRAT gene. Real-time RT-PCR and immunoblotting for the hepatic LRAT gene were performed. The BCM/β-actin ratio was determined by real-time RT-PCR. Bars indicate the SD and asterisks indicate significant differences (**p<0.01).
3) Hepatic expression of CYP26A1, CRBP-I, and LRAT

The expression of genes for retinol-metabolizing enzyme, and binding protein is shown in Figs. 2 and 3. Hepatic expression of CYP26A1 and CRBP-I showed no difference between fa/fa rats and lean rats using real-time RT-PCR and immunoblotting, respectively (Fig. 2). Hepatic expression of LRAT was significantly reduced in fa/fa rats compared with lean rats using real-time RT-PCR and immunoblotting (Fig. 3).

Discussion

Previous human reports show that obesity has an inverse correlation with the nutritional retinol status (12–15). In contrast, the present study showed that plasma retinol levels were increased in obese fa/fa rats. The reason for increased plasma retinol levels in fa/fa rats is unclear; however, several explanations for this discrepancy can be considered, including the difference of species or the rodent genetic background. Obese fa/fa rats have dysfunction of the leptin receptor and are widely used as a model of obesity and dyslipidemia (9). Abnormal lipid metabolism and excessive dietary intake by fa/fa rats lead to hypertriglyceremia.

Circulating retinol is bound to RBP or contained in chylomicrons as retinyl esters (3). Most chylomicrons containing retinol are excluded from the circulation by the liver and the remaining (approximately 25%) chylomicrons containing retinol are removed from the circulation by extrahepatic tissues (5, 23). Thus, plasma retinyl ester levels may reflect dietary retinol. In the study, the elevated plasma retinol and retinyl ester levels in fa/fa rats may be caused by excessive dietary intake.

BCM gene expression was increased in the intestine, while that in other tissues was unaltered. We previously reported on the changes of BCM gene expression under several pathologic conditions (24–26). BCM expression is inversely regulated by retinoic acid in rats with vitamin A deficiency (25). Bachmann and colleagues reported that BCM expression in the chicken intestine is under feedback regulation by retinoic acid and that BCM activity is down-regulated in the rat intestine (27). These studies indicate that retinoic acid may repress BCM gene expression in the principal tissues for retinol metabolism. In type 2 diabetic Goto-Kakizaki rats, BCM gene expression in both the intestine and liver are increased while plasma retinol level is elevated (24). This report suggests that increased BCM gene expression causes elevated plasma retinol levels under conditions of insulin resistance. These findings taken together, BCM gene expression and retinol status may influence one another. The present study showed increased BCM gene expression in the intestine along with elevated plasma retinol levels in fa/fa rats. The reason for increased intestinal BCM gene expression remained unclear. However, increased BCM expression in the intestine, which is the principal tissue involved in the conversion of β-carotene to retinal, might contribute to increased plasma retinol levels in fa/fa rats even if only slightly, regardless of the small amount of β-carotene in the diets.

Fa/fa rats have leptin receptor mutation (9, 10), so abnormal adipokine signaling might impair BCM gene expression. BCM gene expression in human adipose tissue is transcriptionally upregulated by agonists of PPARγ, which controls energy, lipid, and glucose metabolism as a transcriptional factor (28, 29). PPARγ and leptin cooperate in the regulation of lipid metabolism and adipogenesis (30, 31). Therefore, impaired leptin signaling and dyslipidemia might affect PPARγ gene expression and lead to increased BCM gene expression of the intestine; however, further studies are needed to clarify this point.

CYP26A1 and CRBP-I gene expression in the liver was not different in the two groups. These genes are known as retinoic acid responsive genes, and the expression of these genes in rodents is transcriptionally regulated via RARα and RXRα (32–34). In the present study, the findings suggest that the RAR and RXR signaling pathway has not been altered in fa/fa rats regarding retinol metabolism.

The role of LRAT to convert retinol into retinyl ester for storage is well known (4). Mice with LRAT gene disruption show impaired absorption of retinol and susceptibility to retinol deficiency (35, 36). LRAT gene expression affects the retinol status in the liver. Hepatic LRAT expression is increased by retinol or retinoic acid administration in rats with vitamin A deficiency (37, 38). Although the rat LRAT promoter lacks the RAR-binding site, this region responds to RARs and RXRα in the luciferase assay (39). It is likely that RARs participate in the transcriptional regulation of the rat LRAT gene. In the present study, since hepatic expression of retinoic acid response genes including CYP26A1 and CRBP-I in fa/fa rats has not been changed as mentioned above, several factors related to obesity and dyslipidemia other than the RARα and RXRα signaling pathway might affect hepatic LRAT gene expression. At least reduced LRAT expression in the liver may affect plasma retinol levels. Further studies will be needed to assess LRAT gene expression under several pathologic conditions.

In conclusion, this study showed that obesity and dyslipidemia may influence plasma retinol levels and expression of the retinol metabolizing enzymes. Altered expression of BCM and LRAT might affect circulatory retinol status in fa/fa rats. Further investigations are required to assess the regulation of BCM and LRAT gene expression under obese and dyslipidemic conditions.

Acknowledgments

We are grateful to Ms. Kazumi Honda and Ms. Yumiko Yoshimura for their technical assistance. This work was supported by a Grant-in-Aid for Scientific Research (No. 21591336) from the Japan Society for the Promotion of Science, the Mitsui Life Social Welfare Foundation, and a grant from Kao Research Council for the Study of Healthcare Science.
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