Original Article

Altered retinol status and expression of retinol-related proteins in streptozotocin-induced type 1 diabetic model rats

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Diabetes is a metabolic disorder characterized by chronic hyperglycemia. Advanced diabetes is associated with severe complications and impaired nutritional status. Here, we assessed the expression of retinol-associated proteins, including β-carotene 15,15'-monooxygenase (BCMO), lecithin:retinol acyltransferase (LRAT), aldehyde dehydrogenase (ALDH), and cytochrome P450 26A1 (CYP26A1), and measured retinol levels in the plasma and liver of streptozotocin (STZ)-induced type 1 diabetic model rats. Compared to the levels in the control rats, retinol levels in the plasma and liver of STZ rats were decreased and increased, respectively. Hepatic expression of the LRAT gene in STZ rats was lower than that in the controls. In the liver of STZ rats, the expression of ALDH1A1, a retinal metabolizing enzyme was higher, whereas ALDH1A2 expression was lower than in the controls. Hepatic CYP26A1 expression in STZ rats was significantly higher than in the control rats. BCMO expression levels in the liver and intestine of STZ rats were much lower than those of the controls. Altered BCMO expression might affect retinol status. It is considered that the metabolic availability of retinol was lessened despite the accelerated catabolism of retinol; therefore, retinol mobilization may be unbalanced in the liver of rats in the type 1 diabetic state.

Key Words: **Type 1 diabetes, retinol, β-carotene 15,15'-monooxygenase, lecithin:retinol acyltransferase**

Retinol (vitamin A) deficiency is a public health problem in Africa and Southeast Asia.[1] In children, retinol deficiency is associated with xerophthalmia, night blindness, and an increased risk of mortality. Supplementation with retinol was conclusively shown to reduce mortality in children due to a reduced risk of severe infections. In addition to its roles in vision and immunity, retinol also has diverse functions in various processes, including development, reproduction, and cellular differentiation and proliferation. There are two main dietary sources of retinol: retinol or retinyl esters from animal origins, such as meat and eggs, and carotenoids from plant origins, such as vegetables and fruits.[1] Dietary retinyl esters are hydrolyzed to retinol, which is then absorbed in the intestine. In enterocytes, absorbed retinol is re-esterified, incorporated into chylomicrons, and subsequently secreted into the lymphatic circulation. Chylomicrons containing retinyl esters are taken up by the liver, where the retinyl esters are hydrolyzed in the hepatocytes. In hepatic stellate cells, retinol is re-esterified by lecithin:retinol acyltransferase (LRAT) for storage. Then, retinyl esters are hydrolyzed again and transferred back to the hepatocytes, where retinol binds to apo-retinol binding protein (apo-RBP), and the holo-RBP complex is secreted into the circulation.

In the liver and other peripheral tissues, retinol is oxidized to retinal by retinol dehydrogenase, and retinal is oxidized to retinoic acid by aldehyde dehydrogenase (ALDH).[1] Retinoic acid is the bioactive form of retinol and the ligand of retinoic acid receptors (RARs) and retinoid X receptors (RXRs). RARs and RXRs are members of the nuclear receptor superfamily and function as ligand-dependent transcriptional factors. Through RAR and RXR signaling, retinoic acid exerts diverse physiological functions. Retinoic acid is oxidized by the cytochrome P450 26 (CYP26) family of enzymes to 4-hydroxy retinoic acid and 4-oxo retinoic acid, which are conjugated by UDP-glucuronyl transferases for excretion into the bile.

Among the carotenoids that exist in nature, β-carotene possesses exquisite activity as a retinol.[1] Dietary β-carotene is incorporated into micelles along with other lipids, which are absorbed in the intestine. In the enterocytes, β-carotene is converted to two molecules of retinal by β-carotene 15,15'-monooxygenase (BCMO). Retinal is either reduced to retinol by retinal reductases or oxidized to retinoic acid by ALDH. Alternatively, intact carotenoids in the enterocytes can be packed into chylomicrons along with dietary lipids and secreted into the lymphatic circulation. A study of BCMO knockout mice showed that BCMO acts as a regulator of lipid metabolism via peroxisome proliferator-activated receptor (PPAR)γ signaling and functions as a controller of body adiposity.[3,4] In addition, it also facilitates β-carotene and retinol metabolism from embryonic development through the adult stage.[5]

Diabetes is a metabolic disorder characterized by chronic hyperglycemia that leads to severe complications, including ketoacidosis, renal failure, cardiovascular disease, and retinopathy.[6] Type 1 diabetes is characterized by the lack of islet β-cell function due to cellular and hormone-mediated autoimmune responses. Type 1 diabetes presents insulin secretion dysfunction, and patients with type 1 diabetes are dependent on insulin administration for life. Diabetic microvascular complications, including nephropathy, retinopathy, and polyneuropathy, develop as a result of hyperglycemia-induced oxidative stress. There are a number of human and animal studies on the relationship between retinol status and diabetes.[7,8] However, the association between retinol and retinol-related proteins in the type 1 diabetic state has not been well studied. Here, we assessed retinol status and the levels of retinol-related proteins, including BCMO, metabolic enzymes, cytochrome P450, and binding protein, in the type 1 diabetic state using streptozotocin (STZ)-treated rats.

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Material and Methods

Animal experiments. The animals used in this study were Wistar rats (male, age: 4 weeks) purchased from Japan SLC Inc. (Shizuoka, Japan). The control and STZ groups of rats ($n = 5$, each group) were fed with regular chow, given free access to tap water, and housed in a temperature-controlled, humidity-controlled, and light-controlled facility. Diet containing retinol (10,000 IU/kg) and β-carotene (0.2 mg/kg) was purchased from Funahashi Farm (Chiba, Japan). Rats in the STZ group were intraperitoneally injected with a single dose of STZ (70 mg/kg in 0.1 M acetate buffer at pH 4.4), and control rats received an injection of the acetate buffer as previously described.[9] After 4 weeks, the rats were sacrificed by exsanguination under isoflurane anesthesia after overnight fasting. Blood was collected in heparinized tubes, and plasma was separated before storage at −80°C. Tissues were removed, immediately frozen in liquid nitrogen, and stored at −80°C. All care and handling of experimental animals were done in accordance with the Osaka Medical College guidelines for the ethical treatment of laboratory animals.

Biochemical examination. Plasma levels of glucose and cholesterol were analyzed using the oxygen electrode method and the enzymatic colorimetric method, respectively. Retinol levels in plasma and liver homogenates were assayed using high-performance liquid chromatography, as described previously.[10] For analysis, liver tissue was homogenized and saponified with one-twentieth volume of 60% potassium hydroxide in distilled water, and then the saponified liver samples were extracted with hexane. Protein content was then measured according to the method of Bradford.[11]

Immunoblotting. Anti-cellular retinol binding protein (CRBP)-I (Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-ALDH1A2 (Santa Cruz Biotechnology Inc.), anti-ALDH1A1 (ProSci Incorporated, Poway, CA), and anti-β-actin (Medical & Biological Laboratories Co. Ltd., Nagoya, Japan) antibodies were used as the primary antibodies. The cytosolic fraction of the liver homogenate was prepared by ultracentrifugation at $100,000 \times g$ for 60 min, and the protein content was measured using the method of Bradford.[11] The extracted protein was subjected to electrophoresis, transferred to a PVDF membrane, and immunoblotted with the appropriate primary antibody. Each primary antibody was appropriately diluted with Tris-buffered saline containing Tween-20 (TBS-T), and then the membrane was blotted with the appropriate horseradish peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories, Hercules, CA). Target bands were detected using the ECL Western Blotting Detection System (GE Healthcare UK Ltd., Buckinghamshire, UK). Relative protein intensities were determined using ImageJ 1.46r software (National Institutes of Health, Bethesda, MD). The ratio of the intensity of each cytosolic protein band to that of a β-actin standard was determined, and the mean and standard deviation of these ratios were calculated. An anti-human RBP antibody (Santa Cruz Biotechnology Inc.) was used to assess plasma RBP levels as described previously.[12]

Real-time PCR. Total RNA from the liver was prepared using ISOGEN (Wako Pure Chem. Ind., Ltd., Osaka, Japan) according to the manufacturer's protocol. Quantitative real-time PCR was performed to determine mRNA levels in the samples. Reverse transcription (RT) reactions were performed with Omniscript (Qiagen, Hilden, Germany). Subsequently, one-tenth (2 μl) of each RT reaction mixture was amplified by PCR using a LightCycler (F. Hoffmann-La Roche Ltd. Diagnostics Division, Basel, Switzerland) with the LightCycler FastStart DNA Master Hybridization Probe Kit or the FastStart DNA Master SYBR Green I Kit (F. Hoffmann-La Roche Ltd. Diagnostics Division) according to the manufacturer's instructions. Real-time PCR was performed using oligonucleotide primers for BCMO, CYP26A1, LRAT, and β-actin, as previously described.[13] The oligonucleotide primers and accession number of genes are listed in Table 1.

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

Biochemical data and RBP levels of plasma. Plasma glucose and cholesterol levels in STZ rats were higher than those in the control rats (Table 2), which was in agreement with the results of previous reports.[14,15] The plasma retinol levels of STZ rats lower were compared with those of the control rats, whereas the hepatic retinol levels of STZ rats were higher than those of the control rats. Similar to the plasma retinol levels, plasma RBP levels in STZ rats were lower than those in the control rats (Fig. 1).

Expression of the BCMO gene. BCMO mRNA expression levels in several tissues of the STZ and control rats as determined by real-time PCR are shown in Fig. 2. BCMO mRNA expression levels in both the liver and intestine of STZ rats were lower than those of the control rats. BCMO expression in both the testes and lungs of STZ rats were higher than those in the control rats, whereas there was no difference in BCMO mRNA expression levels in the kidneys of the two groups.

Expression of hepatic retinol-related proteins genes. The expression of retinol-related proteins, including processing enzymes and retinol binding protein, is shown in Fig. 3 and 4. The hepatic expression levels of CRBP-I in STZ and control rats were similar (Fig. 3). ALDH1A1 expression levels in the livers of STZ

Table 2. Retinol and biochemical data between control and STZ rats

	Control ($n = 5$)	STZ rats ($n = 5$)
Glucose (mg/dl)	132.0 ± 6.8	412.8 ± 111.8**
Cholesterol (mg/dl)	44.7 ± 6.6	268.8 ± 68.4***
Plasma retinol (mg/dl)	36.9 ± 6.0	10.7 ± 3.7***
Hepatic retinol (μg/mg protein)	0.47 ± 0.11	1.34 ± 0.45*

Values are mean ± SD; *p<0.01, **p<0.05, ***p<0.001.

Table 1. Sequences of primers for real-time PCR

Gene (Accession Number)	Forward	Reverse	Product (bp)
BCMO (NM_053648)	5'-CAAGTCCTCCTTAAAGTGGT-3'	5'-AATAAACCATGCAGGTCCA-3'	225
	5'-CATCATCTCTACAGATCCCCAAAAGC-3'-FITC		
	LC-5'-GCCCTTTTTACTTATTCTGGATGCG-3'		
β-actin (V01217 J00691)	5'-CCTGTATGCCTCTGGTCGTA-3'	5'-CCATCTCTTGCTCGAAGTCT-3'	260
	5'-CGGGACCTGACAGACTACCTCATG-3'-FITC		
	LC-5'-AGATCCTGACCGAGCGTGGCTAC-3'		
CYP26A1 (DQ266888)	5'-GTGCCAGTGATTGCTGAAGA-3'	5'-AGAGAAGAGATTGCGGGTCA-3'	213
LRAT (AF255060)	5'-GGAACAACTGCGAACACT-3'	5'-ACACTAATCCCAAGACAGC-3'	141

rats were higher than those of controls rats, whereas hepatic ALDH1A2 expression in STZ rats was lower than in the control rats. LRAT expression in the liver and hepatic CYP26A1 expression in STZ rats were lower and higher than those of the controls, respectively (Fig. 4).
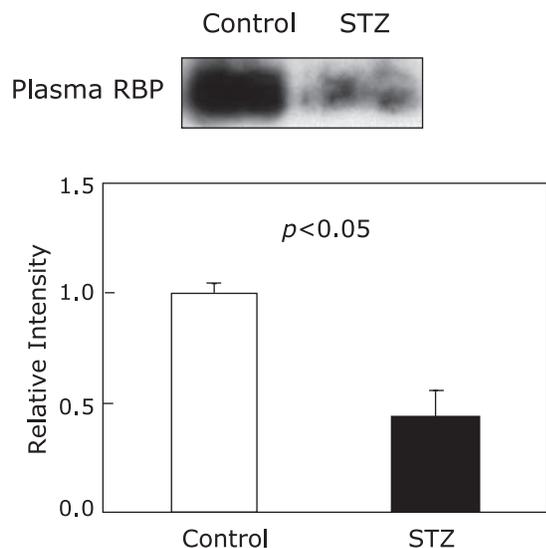
Fig. 1. RBP expression in plasma samples from in STZ-induced type 1 diabetic model rats and control rats ($n = 3$/group) as detected by immunoblotting and analyzed as described in Materials and Methods. Values are reported as mean ± SD. Significant differences are indicated in the graph.

Discussion

In several human studies, a relationship between retinol status and insulin sensitivity has been reported. The levels of plasma retinol and retinol-binding proteins, including RBP and transthyretin, are decreased in patients with type 1 diabetes,[7,8] however, the mechanism of retinol deficiency is unclear. It is speculated that the retinol metabolism may be altered be altered in type 1 diabetic state. Granado *et al.*[16] reported that type 1 diabetic patients exhibited higher carotenoid and retinyl ester levels, which suggested decreased conversion of carotenoids to retinal. In contrast, type 2 diabetic patients present elevated levels of plasma retinol and RBP. Therefore, it seems that circulating retinol levels are higher in the hyperinsulinemic state, and lower in the insulin-depleted state.[8] In the current study, both the plasma retinol and RBP levels in type 1 diabetic model STZ rats were significantly lower than those in the control rats, which was in agreement with the findings of previous animal and human studies. In addition, hepatic retinol levels were higher in STZ rats, which was also in agreement with published reports.[7] Hepatic retinyl ester hydrolase activity in type 1 diabetic model rats was repressed, which agreed with the elevated retinol levels detected in the liver.[17] The previous reports speculate impaired metabolic availability of retinol and disturbed secretion of retinol from the liver in the type 1 diabetic state.[18,19] In contrast, Goto-Kakizaki (GK) rats, a type 2 diabetes model, presented higher plasma retinol and RBP levels than the control rats.[12] The mechanism underlying the difference in the plasma retinol status between type 1 and 2 diabetes remains to be determined.

BCMO has an important role in β-carotene and retinol metabolism: preventing excess or deficits in retinol status.[20] The expression of the BCMO gene in peripheral tissues was altered in several pathological conditions, including obesity and dyslipidemia, nephrosis, type 2 diabetes, and non-alcoholic fatty liver disease (NAFLD).[12,13,21,22] The findings of these reports led to the hypo-
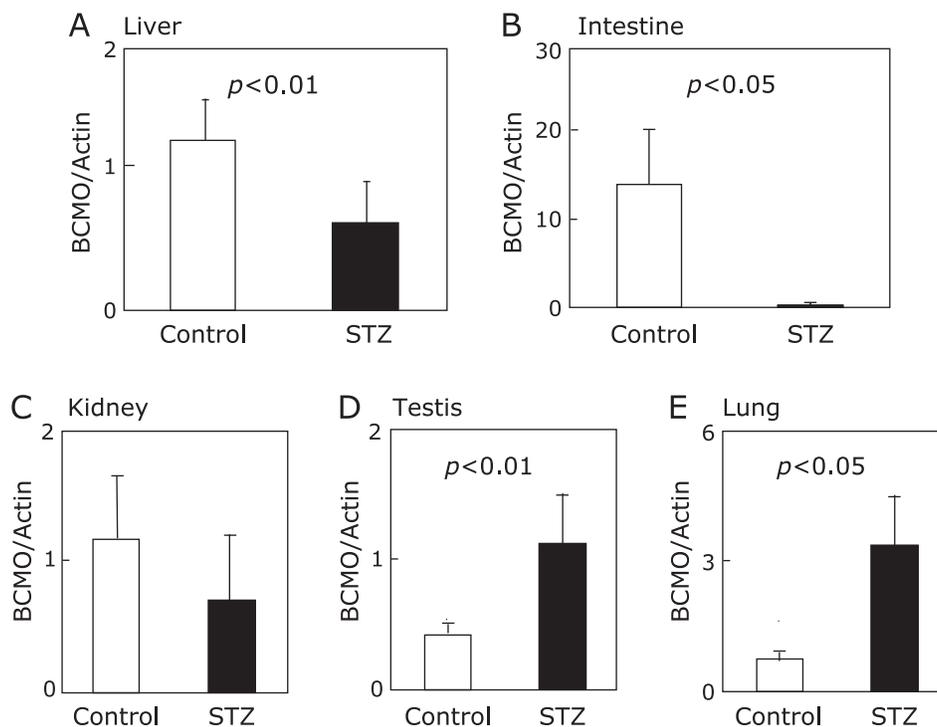
Fig. 2. Expression of the BCMO gene in samples from STZ and control rats ($n = 5$/group) as determined by real-time PCR and analyzed as described in Materials and Methods. Values are reported as mean ± SD. Significant differences are indicated in the graph.
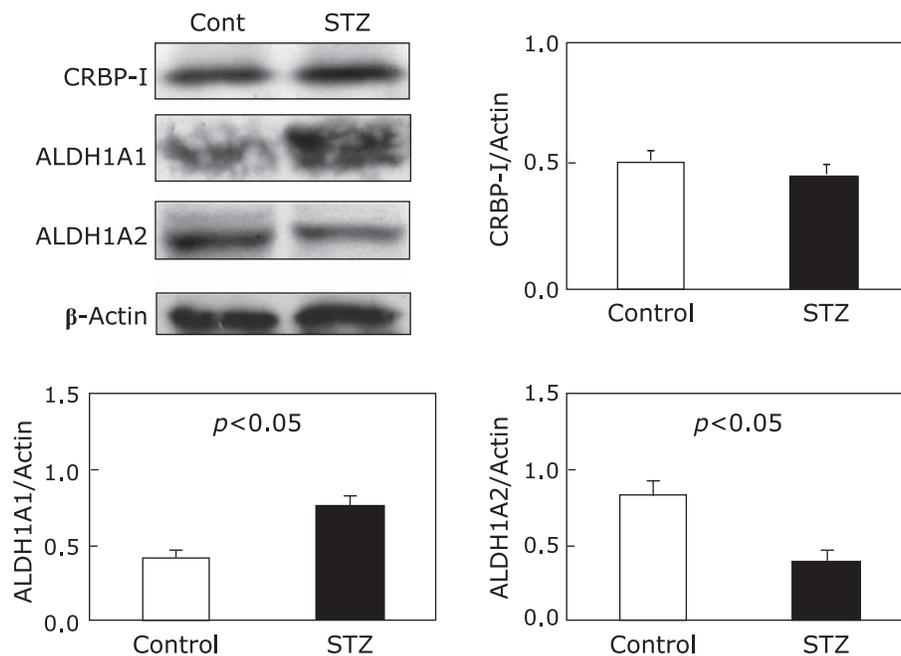
Fig. 3. Expression of CRBP, ALDH1A1, and ALDH1A2 in the liver of STZ and control rats ($n = 3$/group) as determined by immunoblotting and analyzed as described in Materials and Methods. Values are reported as mean $\pm$ SD. Significant differences are indicated in the graph.
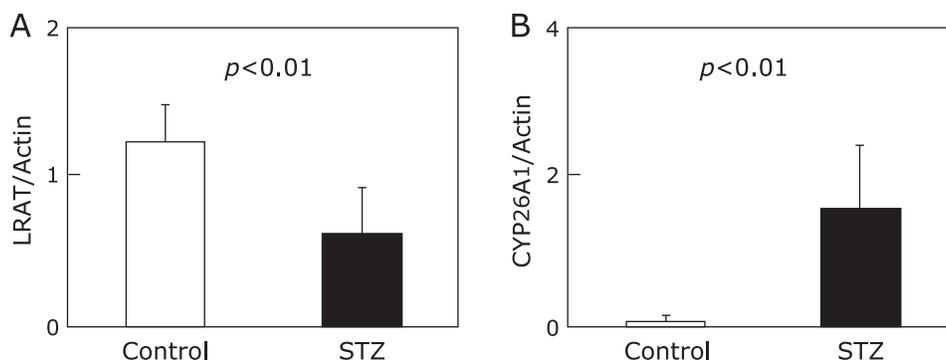
Fig. 4. Expression of LRAT and CYP26A1 mRNA in the liver of STZ and control rats ($n = 5$/group) as determined by real-time PCR and analyzed as described in Materials and Methods. Values are reported as mean $\pm$ SD. Significant differences are indicated in the graph.

thesis that BCMO expression and retinol status may influence one another. In the present study, BCMO gene expression in both the liver and intestine of STZ rats was reduced. Morinobu *et al.*[19] also reported that hepatic BCMO activity in STZ rats was lower, whereas retinol levels in the plasma and liver were lower and higher, respectively. These results, obtained in a type 1 diabetes model, were in contrast with results obtained in type 2 diabetes model GK rats.[12] BCMO gene expression was higher in both the liver and intestine of GK rats, which may lead to increased plasma retinol levels.[12] The insulin resistance or insulin depletion might affect the expression of BCMO gene in both the liver and intestine. The mechanism of the regulation of BCMO gene under the condition of diabetes is unclear. Further studies will be required to clarify this point. Alternatively, in a study of NAFLD model rodents, plasma retinol levels and BMCO gene expression in both the liver and intestine were lower than those in the control animals.[22] This finding of NAFLD model rats was similar to the results of the present study. BCMO has a critical role in the conversion of β-carotene to retinal; thus, altered expression of

the BCMO gene might affect retinol status.

BCMO is widely distributed in the kidneys, testis, lung, liver, and intestine,[23,24] and it has an essential role in retinol production in peripheral tissues. Retinol plays a critical role in lung development and function.[25] The surfactant proteins generated in type II alveolar cells are absolutely essential for lung maturation, and retinoic acid, along with glucocorticosteroid, regulates the formation of these surfactant proteins. Retinoid is also essential for testicular function. Both retinol excess and deficiency induce impaired spermatogenesis. Male mice with mutant RARα and RXRβ present aspermia, which suggests that retinoid signaling via RAR and RXR modulates male reproduction.[26] In the present study, BCMO gene expression in the testes and lungs was higher in STZ rats, whereas that in the kidneys did not differ compared to that in the controls. Based on the finding of lower retinol levels in the blood, the retinol levels in the testes and lungs might be regulated at the level of β-carotene conversion to retinal. However, the reason that renal BCMO expression was not altered remains to be determined. In peripheral tissues, BCMO gene

expression may be regulated in a tissue-specific manner. Further investigation will be required to understand the mechanism of the variations in BCMO gene expression in different tissues.

The cytosolic ALDH family consists of three enzymes, ALDH1A1, ALDH1A2, and ALDH1A3, all of which oxidize retinal to retinoic acid. In most tissues, all three enzymes are expressed, and there are no tissue-specific differences in the expression levels of these enzymes. However, ALDH1A1 is more predominantly expressed in the liver than the other ALDH enzymes. The expression of each ALDH family member is regulated in a different manner, such as by sex hormones and diet, including cholesterol and fatty acid levels.[27] In the current study, the expression of ALDH1A1 and ALDH1A2 in the liver of STZ rats was higher and lower, respectively, than in the liver of the controls, and the expression patterns of the two enzymes were different. This finding may be due to differences in the regulation of the expression of the two enzymes in the liver. ADH1A1 plays an essential role in hepatic gluconeogenesis and lipid metabolism, independent of adiposity.[28] Mice with ALDH1A1 gene disruption presented markedly decreased glucose levels. ALDH1A1 may be closely related to glucose metabolism; thus, hyperglycemia might affect the hepatic expression of ALDH1A1. Alternatively, the increased retinol levels in the liver of STZ rats may enhance the catabolism of retinol. Furthermore, hepatic ALDH1A2 expression in STZ rats might compensate for the altered expression of ALDH1A1. However, the mechanism underlying ALDH expression in several pathological conditions is unclear. Therefore, further studies are needed to elucidate this mechanism.

LRAT converts retinol to retinyl esters for storage in several tissues, including the liver, testis, lung, pancreas, intestine, and the retinal pigment epithelium (RPE). All-*trans* retinol (vitamin A) is isomerized to 11-*cis* retinol, which is then oxidized to 11-*cis* retinal in the RPE, and has a critical role in the vision cycle.[29] 11-*Cis* retinol combines with opsin to generate rhodopsin and iodopsin in photoreceptor cells. All-*trans* retinol is esterified by LRAT and stored in the RPE as it is in the liver. LRAT knockout mice showed severely impaired cone visual functions at 8 weeks.[30] Over time, STZ-treated diabetic rats developed reduced LRAT gene expression in the retina.[31] In the present study, STZ rats also exhibited lower LRAT expression in the liver despite the higher retinol levels observed in the liver. Thus, the hyperglycemic state may reduce hepatic LRAT expression.

Retinoic acid, the active form of retinol, has important properties for numerous physiological functions. Both deficiency and excess retinoic acid impair tissue homeostasis. Therefore, retinoic acid concentrations at the cellular and tissue levels, are tightly controlled by retinol-related metabolic enzymes.[32] Retinoic acid is metabolized by enzymes of the CYP26 family, which consists of CYP26A1, CYP26B1, and CYP26C1. All CYP26 family members have critical roles in embryonic development. CYP26A1 is expression mainly in the liver, but also less so in the testis and brain. Expression of CYP26A1 is transcriptionally regulated by retinoic acid via RAR and RXR.[33] In the current study, hepatic ALDH1A1 and CYP26A1 expression was enhanced in diabetic rats. Therefore, retinol catabolism may be accelerated in the type 1 diabetic state. Ashla *et al*.[34] reported that the expression of retinoid metabolism-related proteins, including binding proteins, enzymes, cytochrome P450, and nuclear receptors, in the liver of patients with NAFLD were enhanced, and hypothesized that the hypermetabolic state of retinoids exhibits in the liver of NAFLD. It is known that oxidative stress worsens both diabetes and NAFLD.[35,36] Hyperglycemia or oxidative stress might be implicated in the accelerated metabolism of retinol. Further studies are needed to fully address this issue.

In conclusion, retinol status and the expression patterns of retinol-related proteins, including enzymes and binding proteins, were altered in the livers of STZ rats. The altered expression of the BCMO gene might affect retinol status. The metabolic availability of retinol was lessened despite the accelerated catabolism of retinol; therefore, retinol mobilization may be unbalanced in the liver of rats in the type 1 diabetic state.

Acknowledgments

Abbreviations

ALDH	aldehyde dehydrogenase
BCMO	β-carotene 15,15'-monooxygenase
CRBP	cellular retinol binding protein
CYP	cytochrome P450
LRAT	lecithin:retinol acyltransferase
PPAR	peroxisome proliferator-activated receptor
RAR	retinoic acid receptor
RBP	retinol binding protein
RXR	retinoid X receptor
STZ	streptozotocin

Conflict of Interest

No potential conflicts of interest were disclosed.

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