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Report

Hepatic Expression of the Na$^+$-Coupled Citrate Transporter (NaCT/Slc13a5) and Cellular Uptake of Citrate in a Mouse Model of Type 1 Diabetes Induced by Streptozotocin

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Non-alcoholic fatty liver disease (NAFLD) is the most common hepatic disorder, characterized by the fat accumulation in hepatocytes without significant alcohol assumption. In lipogenesis in hepatocytes, the tricarboxylic acid cycle citrate plays a crucial role as a carbon source. Citrate is transported into hepatocytes via Na$^+$-coupled citrate transporter, NaCT. It has been demonstrated that knockdown of NaCT expression ameliorates diet-induced NAFLD in mice. In addition, NaCT expression in the liver has been reported to be induced in type 2 diabetic mice. Based on these findings, NaCT is considered to be involved in the high prevalence of NAFLD in patients with type 2 diabetes. On the other hand, it is still unclear for the expression level of NaCT under type 1 diabetic condition and its relationship to hepatic lipid accumulation. In this study, we investigated the gene and functional expression level of NaCT in streptozotocin (STZ)-induced type 1 diabetic mice. The mRNA and protein expression levels of NaCT in STZ-treated mice were gradually decreased after STZ treatment. On the other hand, the Na$^+$-dependent citrate uptake activity in hepatocytes isolated from STZ-treated mice was not different from that isolated from non-treated mice. Nevertheless, the plasma triglyceride, cholesterol, and nonesterified fatty acid levels were much higher in STZ-treated mice. These results suggest that NaCT expression level is not closely related to the citrate uptake in hepatocytes under type 1 diabetic condition. In conclusion, unlike type 2 diabetes, NaCT may not be responsible for the pathogenesis of NAFLD in type 1 diabetes.

Key words citrate, Na$^+$-coupled citrate transporter, Slc13a5, type 1 diabetes, lipid accumulation

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common hepatic disorder, including the wide range of hepatic diseases from simple steatosis to hepatic fibrosis and cirrhosis.[1,2] Its prevalence reaches an average of 25% in adults, and continues to increase. Among NAFLD patients, approximately 25% of patients develop non-alcoholic steatohepatitis (NASH), and moreover, about 20% of NASH patients progress to cirrhosis.[1,2]

NAFLD is considered to be a hepatic manifestation of ectopic fat accumulation without significant alcohol assumption. Hepatic fat accumulation is known to occur via increased energy supply and lipogenesis together with suppressed fatty acid β-oxidation. The tricarboxylic acid (TCA) cycle intermediate citrate plays a crucial role in the generation of biochemical energy and lipogenesis.[3,4] For example, citrate is a prime carbon source for energy production and lipogenesis. Moreover, Citrate is capable of stimulating gluconeogenesis and reducing glycolytic flux. Furthermore, citrate can promote the *de novo* lipogenesis. Thus, it is plausible that citrate is a key metabolite in hepatic lipid accumulation.

Citrate has been reported to be transported from plasma membrane into cells via Na$^+$-coupled di/tricarboxylate transporters in mammal.[5,6] So far, three different functional transporters have been reported, which belong to a member of Na$^+$-coupled di- and tri-carboxylate cotransporter gene family (SLC13), NaDC1 (SLC13A2), NaDC3 (SLC13A3), and NaCT (SLC13A5). In particular, NaCT is the predominant citrate carrier mainly expressed in the liver of humans and rodents.[3,7,8] NaCT preferentially transports citrate over other intermediates in TCA cycle, such as succinate, malate, and fumarate. It has been reported that the NaCT mRNA expression level in the human liver is elevated in patients with NAFLD.[9] Moreover, previous study has demonstrated that increased NaCT expression enhances the citrate uptake and *de novo* lipid synthesis in hepatocytes.[10] In addition, several reports have shown that knockdown of NaCT expression ameliorates diet-induced NAFLD in mice.[11,12] Therefore, NaCT is proposed to be closely related to the development of NAFLD, and be a promising target for treating NAFLD.

NaCT has been reported to be induced in liver of type 2 diabetic rats.[10] In addition, type 2 diabetes is well recognized as an independent risk factor for NAFLD.[13,14] Individuals with type 2 diabetes have higher prevalence rate of NAFLD, more than 70%. Taking these into consideration, NaCT is assumed to play a crucial role for developing NAFLD under type 2 diabetic condition. On the other hand, it has also been report-

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ed that approximately 10-50% of patients with type 1 diabetes detected NAFLD, even though this proportion is quite less than that of the patients with type 2 diabetes.[15,16] These observation suggest that type 1 diabetes also correlates with the prevalence of NAFLD. However, the mechanisms of hepatic fat accumulation under type 1 diabetic condition, particularly with respect to the participation of NaCT, have remain unclear.

In this study, we determined the hepatic expression level of NaCT and cellular uptake of citrate in a mouse model of type 1 diabetes induced by streptozotocin (STZ). Moreover, we also investigated the plasma lipid levels in type 1 diabetic mice.

MATERIALS AND METHODS

Animals All experiments were carried out in accordance with the principles and procedures in the National Institute of Health Guide for the Care and Use of Laboratory. All animal experimental protocols were reviewed and approved by the Animal Care and Use Committee of Ritsumeikan University (BKC2017-049).

Six-week-old male C57BL/6J mice (Japan SLC, Shizuoka, Japan) were divided into nondiabetic (non-treated: NT) and type 1 diabetic (STZ-treated) groups (n=20 in each group). Type 1 diabetic mice were induced by intraperitoneal injection of a single dose of STZ (200 mg/kg) (FUJIFILM Wako Chemicals, Osaka, Japan) in saline (pH 7.0).[17] The nondiabetic mice were injected with saline. The development of diabetes was confirmed by blood glucose level of >400 mg/dL in fasting mice at 4 d after STZ injection.

Real-Time Reverse Transcription (RT)-PCR Analysis Total RNA was isolated from liver using Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions. First-strand cDNA was synthesized using ReverTra Ace (TOYOBO Co., Ltd, Osaka, Japan) with and oligo$(dT)_{20}$ primer. Total RNA (2 μg) was used for a reverse transcription reaction (20 μL). Real-time RT-PCR was performed on Applied Biosystems StepOne Real-time PCR System with PowerUp SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) according to the following conditions: 95°C for 10 min and 95°C for 15 s, 60°C for 60 s, repeated for 45 cycles. This was followed by the additional extension steps at 95°C for 15 s and 60°C for 60 s. The specific primer sequences were as follows: GAPDH (forward 5'-CCATCACCATCTTCCAGGAG-3'; reverse 5'-CCTGGTTCACCACCTTCTTG-3'), NaCT (forward 5'-GTCAGTCTCCCTTTCACGCG-3'; reverse 5'-CTCCACAGCTGTATTGGCGG-3'), NaDC3 (forward 5'-CTTCCTCGACACCAACTTCC-3'; reverse 5'-CTTGTTCTGCACGTTTGCCA-3'). The target mRNA expression levels were normalized to the mRNA expression level of GAPDH. The relative mRNA expression level was presented as a ratio of STZ-treated to NT mice (STZ/NT).

Western Blotting Western blotting of mouse liver was performed using monoclonal antibody against SLC13A5/NaCT (clone2G4, catalog #PA5-60679, SIGMA-Aldrich, St. Louis, MO, USA). Total protein were extracted with RIPA buffer (0.1% TritonX-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 150 mM NaCl, 50 mM Tris-HCl) containing protease inhibitor cocktail (Nacalai Tesque) and were quantified with a BCA protein assay kit (Nacalai Tesque). The samples were run on 10% SDS-PAGE and then transferred to a polyvinylidene difluoride membrane. After a blocking procedure, the membrane was reacted with anti-NaCT antibody (1:250) or anti-β-actin (1:1,000) (catalog #4967, Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4°C, washed and then incubated at room temperature with horseradish peroxidase (HRP)-conjugated secondary anti-mouse IgG antibody (1:1,000) (catalog #7076, Cell Signaling Technology) or anti-rabbit IgG (1:1,000) (catalog #7074, Cell Signaling Technology). The bands were detected using ImmunoStar LD (FUJIFILM Wako Chemicals, Osaka).

Uptake Experiment of Citrate Mouse primary hepatocytes were isolated by collagenase perfusion according to the previous report.[18] The cells were suspended at 1 x 10^5 cells/mL in transport buffer (25 mM HEPES/Tris (pH 7.4), 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5 mM glucose). Uptake of [^{14}C]citrate (specific activity: 116.4 mCi/mmol, PerkinElmer, Boston, MA, USA) was initiated by mixing 1 mL of cell suspension (1 x 10^5 cells) and 1 mL of uptake buffer containing [^{14}C]citrate. After 15 min incubation at 37°C, the mixture was then filtered using Whatman® glass microfiber filter (GF/F) and washed twice with an excess volume of ice-cold transport buffer. The washed filters were transferred to a counting vial, and the radioactivity associated with the cells was counted by liquid scintillation spectrometry (Model LSC6000, Beckmann, Palo Alto, CA, USA). Na^+-dependent uptake of [^{14}C]citrate was obtained by subtracting the uptake in the *N*-methyl-d-glucamine chloride-containing buffer from the uptake in the NaCl-containing buffer. The relative Na^+-dependent [^{14}C]citrate uptake ratio was presented as a ratio of STZ-treated to NT mice (STZ/NT).

Biomedical Analyses in Blood Samples Blood samples were collected from NT and STZ-treated mice at 0, 1, 2, 4, 6 and 8 week after STZ injection. Plasma glucose, triglyceride, cholesterol and nonesterified fatty acid (NEFA) levels were measured by using LabAssay Glucose, LabAssay Triglyceride, LabAssay Cholesterol and LabAssay NEFA (FUJIFILM Wako Chemicals), respectively.

Data Analysis ANOVA was used to test the statistical significance of differences between groups. Two-group comparisons were performed with Student's *t* test. Multiple comparisons among control groups and other groups were performed with Dunnett's test.

RESULTS

NaCT Expression in Liver of Type 1 Diabetic Model Mice Initially, we determined the expression level of NaCT in STZ-induced type 1 diabetic model mice. As shown in Fig. 1A, the mRNA expression level of NaCT in STZ-treated mice was gradually decreased after STZ treatment, and its expression level at 8 week was about 5-fold lower than that at 0 week. On the other hand, other Slc13a transporter family NaDC3 mRNA expression was increased at 2 week after STZ treatment, and its expression level was kept up to 8 week (Fig. 1B). As well as mRNA expression, we also observed that NaCT protein expression in STZ-treated mice was decreased in a time-dependent manner (Fig. 2).

[^{14}C]Citrate Uptake in Hepatocytes of Type 1 Diabetic Model Mice We also assessed the citrate uptake in hepatocytes isolated from STZ-treated mice. In preliminary experiment, we confirmed that the cellular uptake of [^{14}C]citrate in hepatocytes was linear for up to 15 min (data not shown). Therefore, all uptake studies were performed with a 15-min

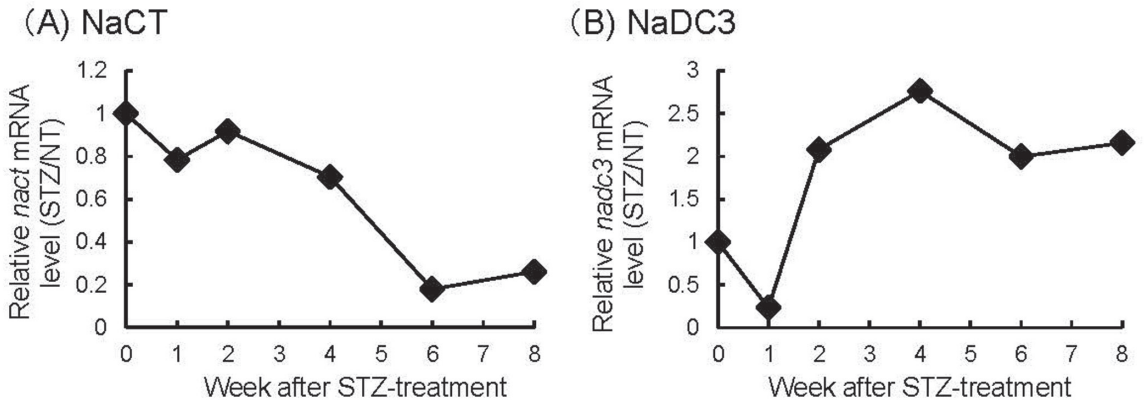

Fig. 1. mRNA Expression of NaCT (A) and NaDC3 (B) in Liver of Type 1 Diabetic Model Mice

Total RNA was isolated from liver of NT mice and STZ-treated mice at 0, 1, 2, 4, 6, and 8 weeks after STZ treatment. Real-time RT-PCR was performed using specific primers for NaCT, NaDC3 and GAPDH. Data were normalized to GAPDH. The relative mRNA expression level was presented as a ratio of STZ-treated to NT mice (STZ/NT).

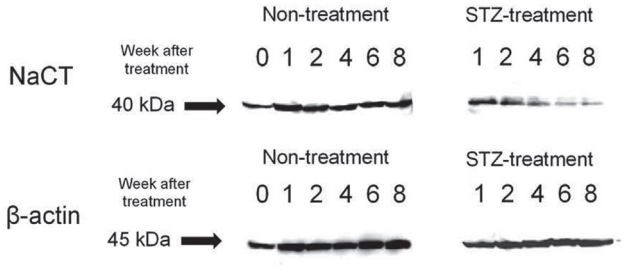

Fig. 2. Protein Expression of NaCT in Liver of Type 1 Diabetic Model Mice.

Protein was isolated from liver of NT mice and STZ-treated mice at 0, 1, 2, 4, 6, and 8 weeks after STZ treatment. NaCT and β-actin protein expression was detected by Western blot analysis.

incubation period. Since the citrate transport via NaCT is driven by an inward electrogenic Na^+ gradient, we determined the Na^+-dependent uptake of [^{14}C]citrate in hepatocytes. The Na^+-dependent citrate uptake of hepatocytes from STZ-treated mice was similar to that of hepatocytes from NT mice during the experimental period (Fig. 3).

Plasma Lipid Levels in Type 1 Diabetic Model Mice The plasma triglyceride, cholesterol, and NEFA levels in STZ-treated mice were evaluated. As shown in Table 1, the plasma triglyceride, cholesterol and NEFA concentrations in type 1 diabetic mice were much higher than those in NT mice during the experimental period. In particular, the plasma triglyceride level was approximately 2-3 fold higher in STZ-treated mice than in NT mice. We also confirmed that body weight of STZ-treated mice was significantly lower than that of NT mice.

DISCUSSION

In this study, we investigated the relationship between hepatic NaCT expression level and lipid accumulation in type 1 diabetic model mice. We observed that the mRNA and protein expression of NaCT in mouse liver were gradually decreased after STZ treatment (Figs. 1 and 2). These results differ from the previous report demonstrating the increased expression of hepatic NaCT in type 2 diabetic rats.[10] This may be due to the difference of the regulation of glucagon secretion between type 1 diabetes and type 2 diabetes. It has been reported that the increased hepatic NaCT expression in type 2 diabetic mice is induced by glucagon via the activation of cAMP-responsive element-binding protein.[10] Patients with type 2 diabetes showed higher day-long plasma glucagon levels,[19,20] and this would contribute to the increased expression of NaCT under type 2 diabetic condition. On the other hand, type 1 diabetes is associated with dysfunction of pancreatic alpha cells,[21] and the glucagon secretion profile in type 1 diabetes seems to be different from that in type 2 diabetes. For example, it has been reported that glucagon level in type 1 diabetes is suppressed by elevation of plasma free fatty acids levels, and its suppression level is comparable to that in nondiabetic individuals.[22] In addition, the increment of glucagon secretion in response to hypoglycaemia is absent in type 1 diabetes patients.[19,23] These specific condition in type 1 diabetes may contribute to the decreased expression of NaCT in liver. However, the difference of plasma glucagon concentration profile between type 1 and type 2 diabetic animal model is unknown. In addition, the downregulation mechanism of NaCT in liver remains poorly understood, although the upregulation mechanisms of NaCT have been elucidated.[5,24] Therefore, further studies are needed to clarify the glucagon secretion profile and glucagon-related mechanism for downregulation of hepatic NaCT in type 1 diabetic mice.

While the hepatic NaCT expression was decreased in STZ-treated mice, the citrate uptake of hepatocytes isolated from STZ-treated mice was not different from that isolated from NT mice (Fig. 3). We considered that this result would be attributed to the involvement of other transporters belonging to SLC13 family. Among the SLC13 family, NaDC3 is expressed in various tissues, including liver.[5,6] NaDC3 has high affinity for four carbon dicarboxylates, such as succinate, malate and fumarate, and it also transports citrate with lower affinity compared with succinate. NaCT is known as the dominant citrate transporter in human hepatocytes, whereas it has been reported that NaDC3 contributes to citrate uptake in mouse hepatocytes as well as NaCT.[4] When we evaluated the expression of hepatic NaDC3 in STZ-treated mice, the elevated mRNA expression was observed compared with NT mice (Fig. 1B). We assume that this is a compensatory response to restore the uptake level of citrate in hepatocytes, responding to the reduction of NaCT

Table 1. Plasma Biochemical Parameters in STZ-Treated Mice

		week													
		0		1		2		4		6		8			
		AVE	SD	AVE	SD	AVE	SD	AVE	SD	AVE	SD	AVE	SD		
Body weight (g)	NT	19.04 ± 0.72		19.72 ± 0.59		20.83 ± 0.73		21.73 ± 0.98		23.48 ± 1.35		24.58 ± 1.11			
	STZ	19.90 ± 0.88	*	19.04 ± 0.94	**	18.84 ± 0.85	**	18.92 ± 0.92	**	20.43 ± 1.21	*	22.36 ± 1.19	*		
Glucose level (mg/dL)	NT	171.80 ± 16.87		155.41 ± 25.32		169.42 ± 44.72		158.62 ± 33.35		151.62 ± 0.63		151.13 ± 10.32			
	STZ	145.73 ± 34.49		329.63 ± 100.40	**	471.45 ± 103.10	**	476.70 ± 100.55		521.26 ± 288.45		439.85 ± 57.64	**		
Triglyceride (mg/dL)	NT	33.13 ± 10.12		29.85 ± 19.07		59.23 ± 30.80		44.15 ± 31.19		60.82 ± 40.97		94.38 ± 33.19			
	STZ	21.06 ± 8.12	*	71.34 ± 52.39	*	172.69 ± 157.65		47.81 ± 31.80		112.25 ± 126.26		181.61 ± 181.73			
Cholesterol (mg/dL)	NT	98.86 ± 10.56		93.55 ± 7.85		92.59 ± 11.97		68.16 ± 14.98		59.18 ± 15.72		65.62 ± 5.15			
	STZ	98.67 ± 27.77		77.56 ± 31.22	*	119.67 ± 27.04		97.40 ± 37.91	*	87.16 ± 25.13		116.65 ± 59.18			
NEFA (mEq/L)	NT	0.50 ± 0.16		0.53 ± 0.17		0.52 ± 0.17		0.43 ± 0.20		0.58 ± 0.16		0.46 ± 0.20			
	STZ	0.61 ± 0.29		0.90 ± 0.42	**	1.18 ± 0.60	**	0.76 ± 0.21	*	1.00 ± 0.35		0.76 ± 0.37			

Each value represents the mean ± SD (n = 10).
*P<0.05; **P<0.01, compared with the corresponding NT mice.

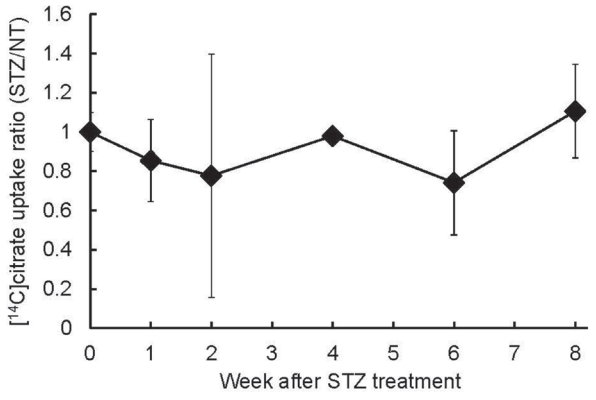

Fig. 3. Na^+-Dependent Uptake of Citrate in Hepatocytes of Type 1 Diabetic Model Mice

Mouse primary hepatocytes were isolated from NT mice and STZ-treated mice at 0, 1, 2, 4, 6, and 8 weeks after STZ treatment. The cells were incubated in transport buffer containing [^{14}C]citrate (0.43 μmol/L) with shaking for 15 min at 37°C. Na^+-dependent uptake was obtained by subtracting citrate uptake amount in the absence of Na^+ from that in the presence of Na^+. Each value represents the mean ± SD (n = 3). The relative [^{14}C]citrate uptake ratio was presented as a ratio of STZ-treated to NT mice (STZ/NT).

expression. This result supports our view that NaDC3 is participated in the citrate transport in liver of mice with type 1 diabetes.

We also observed the plasma triglyceride, cholesterol and NEFA levels were higher in STZ-treated mice than in NT mice (Table 1), although the citrate uptake in hepatocytes was not changed. Although it is not clear why the plasma lipid levels were increased without the enhancement of hepatocellular uptake of citrate, we assume that this would be due to the increased expression of NaDC3 in hepatocytes. NaDC3 transports not only citrate but also other intermediates in TCA cycle with high affinity into hepatocytes,[5,6] and these intermediates may contribute to the citrate and lipid synthesis in hepatocytes under type 1 diabetic condition.

Taken together, unlike type 2 diabetes, it seems that NaCT is not closely related to the pathogenesis of NAFLD under type 1 diabetic condition. However, it needs to pay attention that we determined the lipid accumulation by the plasma triglyceride, cholesterol and NEFA levels, not by hepatic lipid levels. In addition, mice used in this study were young (6-week-old) and the experimental period was relatively short (8 weeks). Therefore, we are proceeding the evaluation of the expression level of NaCT in liver and hepatic lipid accumulation in aged mice (20-week-old) with STZ-induced type 1 diabetes for longer period (up to 4 months). Nevertheless, our present findings make a contribution to the elucidation of the mechanisms of NAFLD development from type 1 diabetes.

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Conflict of interest The authors declare no conflict of interest.

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