

Preventive Effect of Siphonaxanthin, a Carotenoid from Green Algae, against Diabetic Nephropathy and Lipid Metabolism Insufficiency in Skeletal Muscle

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Abstract

Diabetic nephropathy is a complication of diabetes mellitus characterized by the gradual progression of renal insufficiency, resulting in renal failure. Approximately 15% or more of patients with type 2 diabetes mellitus have diabetic nephropathy. Siphonaxanthin is a green algal carotenoid noted for its strong biological activities, including anti-obesity effects. In this study, we aimed to evaluate the preventive effects of siphonaxanthin on diabetic nephropathy using db/db mice as a type 2 diabetes mellitus and diabetic nephropathy model. Ingestion of AIN-93G containing 0.004% w/w siphonaxanthin did not improve plasma creatinine and urine albumin levels but significantly mitigated renal morphological changes in diabetic mice. Moreover, siphonaxanthin restored the decreased mRNA expression of fatty acid β -oxidation-related proteins in the skeletal muscle. These results indicate that siphonaxanthin can potentially ameliorate type 2 diabetes mellitus-induced kidney damage and lipid metabolism insufficiency in skeletal muscle. This study provides a possible daily nutraceutical solution for treating diabetic nephropathy and lipid metabolic abnormalities.

Keywords

Carotenoid, diabetic nephropathy, metabolic abnormality, siphonaxanthin, Type 2 diabetes mellitus

1. Introduction

Diabetes mellitus has gained global attention owing to its high prevalence and increased incidence. Among the three subtypes of diabetes, type 2 diabetes mellitus (T2DM) has been reported in more than 90% of clinical cases. The suffering from T2DM is barely limited to itself but can simultaneously come from its complications. Hypertension, stroke, and cardiovascular and microvascular diseases are among the most common T2DM complications. Diabetic nephropathy is a major microvascular disease characterized by the gradual

progression of renal insufficiency, resulting in renal failure. Approximately 15% or more of the patients with T2DM develop diabetic nephropathy, which is the leading cause of end-stage renal disease [1].

Transforming growth factor- β 1 (TGF- β 1) is one of the most important cytokines in the pathogenesis of diabetic nephropathy [2]. It binds to the type II membrane receptor for TGF- β (T β RII) and thus activates suppressor of mothers against decapentaplegic (Smad) transcription factors, which regulate the transcription of extracellular matrix proteins, including collagen and fibronectin (FN) [3]. Extracellular matrix accumulation in mesangium decreases glomerular filtration rate; hence, TGF- β 1/T β RII/Smad signaling is considered important. Treatment with neutralizing anti-TGF- β 1 antibody and Smad3 deficiency attenuated extracellular matrix deposition and ameliorated kidney injury [4, 5]. TGF- β 1 expression level is reported to be negatively related to renal function and positively related to diabetic nephropathy [6].

A pathogenic metabolic shift related to initial insulin resistance and later diabetic morbidity is a trigger for TGF- β 1 production. For example, hyperglycemia causes NAD(P)H oxidase 4 (NOX4) expression, which in turn induces TGF- β 1 expression by generating reactive oxygen species (ROS) [7, 8]. ROS also activates TGF- β 1 by releasing it from the inactivating protein complex [9]. Abnormalities in lipid metabolism also contribute to diabetic nephropathy [10, 11]. Kidney TGF- β 1 expression is induced under hyperlipidemia[12].

Advanced glycation end-products (AGEs) are also involved in diabetic nephropathy. AGEs are degenerated proteins found in the plasma of healthy individuals but are more prevalent in the plasma of patients with diabetes. The binding of AGEs to the receptor for AGEs (RAGE) induces TGF- β 1 expression and Smad activation independent of TGF- β 1/T β RII [11]. AGEs deposition and high RAGE expression have been observed in human diabetic kidneys [13], and an inhibitor of AGEs formation has been reported to mitigate diabetic nephropathy in db/db mice [14].

Siphonaxanthin is a carotenoid found in green algae, including *Codium* spp. and *Caulerpa lentillifera*, an edible seaweed popular in Japan [15]. The chemical structure of siphonaxanthin is characterized by the presence of a carbonyl group at C8 position and a hydroxy group at C19 position (Fig. 1A). Especially, carotenoids with a hydroxy group at C19 or C19' position are rare, with only 24 out of 1,204 carotenoids listed in the carotenoid database [16]. Similarly, only 50 carotenoids with a carbonyl group at either C8 or C8' position are listed in the database, highlighting the highly characteristic chemical structure of siphonaxanthin. Regarding its biological activities, a hydroxy group at C19 position appears to be nonessential for anti-inflammatory activity of siphonaxanthin because siphonein, a 2-lauroleic acid ester at C19 position of siphonaxanthin, also exhibited comparable activity [17]. Whereas carotenoids with a carbonyl group at C8 position exhibits strong suppressive effects on cellular inflammatory responses, and especially, siphonaxanthin shows strong activity [18, 19]. In our previous studies, only siphonaxanthin was able to suppress the inflammatory response induced by AGEs and RAGE, which is involved in the pathogenesis of diabetes and its complications [19, 20]. In animal experiments, oral administration of 1.3 mg siphonaxanthin per day (equivalent to 0.03% siphonaxanthin in the diet) for six weeks significantly reduced adipose tissue weight in KK-Ay obese diabetic mice [21]. Although siphonaxanthin did not downregulate mRNA expression of lipid metabolism-related genes in liver, it significantly upregulated mRNA expression of uncoupling protein 3 (UCP3) in skeletal muscle, suggesting an enhancement of energy metabolism in skeletal muscle. In Ob/Ob obese diabetic mouse study, feeding a 0.016% siphonaxanthin-supplemented diet for 43 days significantly decreased hepatic oxidative stress markers without affecting mRNA expression of lipid metabolism-related genes [22]. A renal oxidative stress marker was also significantly improved by siphonaxanthin ingestion; however, renal function assessment and histomorphological analysis of kidney tissue were not performed in that animal study. Given these findings, in this study, we aimed to examine the effects of siphonaxanthin on renal function and skeletal muscle energy

metabolism in db/db mice, which are well-investigated diabetic nephropathy and another T2DM model.

2. Methods

2.1. Preparation of the siphonaxanthin-rich fraction

The siphonaxanthin-rich fraction was prepared as described previously [22]. The purity of the siphonaxanthin used in the *in vivo* study was 63%. Of the total, 4.6% consisted of neoxanthin and its isomer based on HPLC-PDA analysis as described previously (Fig. 1B) [23]. The remaining 32.4% was presumed to be mainly monogalactosyldiacylglycerol (MGDG) based on our previous studies [22].

2.2. Animals and diets

All animal experimental protocols were approved by the Animal Experimentation Committee of Kyoto University for the care and use of experimental animals (approval no. 30-39). Male C57BLKS/J-db/db mice and C57BLKS/J-db/m mice (5 weeks old) were obtained from Japan SLC (Shizuoka, Japan). All mice were housed individually and maintained on an alternating 12-h light/dark cycle at $23 \pm 1^\circ\text{C}$. After a one-week acclimatization period, db/db mice were randomly divided into control and siphonaxanthin groups, and db/m mice were designated as the normal group ($n = 4$ in each group). All mice had ad libitum access to drinking water and a normal diet (AIN93G) supplemented with or without 0.004% w/w siphonaxanthin (calculated by siphonaxanthin weight equivalent) dissolved in soybean oil. Blood samples were collected from the tail vein at 8 and 12 weeks of age to measure glucose levels. Body weight and food intake were monitored throughout the study period. At 14 weeks old, the mice were starved for 8 h and sacrificed under isoflurane anesthesia. Blood was collected, and the organs were rapidly removed, weighed, and immediately frozen in liquid nitrogen. Kidney and muscle were

partially stored in RNAlaterTM solution (Thermo Fisher Scientific, Waltham, MA, USA) at -80°C until analysis.

2.3. Biochemical analysis

The plasma was prepared by centrifuging at $1,000 \times g$ for 15 min at 4°C and stored at -80°C until use. Plasma concentrations of glucose, triacylglycerol, and total cholesterol were measured using commercially available kits (Glucose CII test Wako, Triglyceride E-test Wako, and Total cholesterol E-test Wako, respectively; FUJIFILM Wako Pure Chemical, Osaka, Japan). Plasma aspartate transaminase and alanine transaminase activities were evaluated by using Transaminase CII Test Wako (FUJIFILM Wako Pure Chemical). Plasma and urine creatinine concentrations were measured by using Creatinine Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI, USA). Liver triacylglycerol and total cholesterol levels were assessed in the total lipid fraction prepared from the liver by using commercial kits as described above, respectively. Urine albumin concentration was determined using a mouse albumin enzyme-linked immunosorbent assay kit (Assaypro, St. Charles, MO, USA).

2.4. Histomorphology analysis

The left kidney tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned into 3- μm slices. Periodic acid-Schiff (PAS) was performed to observe mesangial expansion and fibrosis in the kidney tissues. Coded cross-sections were read by an observer who was blinded to the experimental group from which they were derived. Twenty-five to thirty-five glomeruli were randomly selected from each slice, and the extent of the extracellular mesangial matrix was identified by PAS-positive and nuclei-free areas in the mesangium using a light microscope BZ-9000 (Keyence, Osaka, Japan). The relative mesangial area was calculated as the ratio of the mesangial area to the glomerular area.

2.5. Real-time quantitative reverse-transcription PCR (qRT-PCR)

RNA extraction, cDNA synthesis, and qRT-PCR were performed as described previously [22]. The primers used for quantifying mRNA expression of each protein are listed in Supplementary Table S1. All data were normalized to β -Actin mRNA levels as an internal control.

2.6. Western blot analysis

Western blot analysis was performed as described previously [22]. Anti-phosphorylated Akt (Thr-308) rabbit monoclonal antibody (catalog number#2965), anti-phosphorylated Akt (Ser-473) rabbit monoclonal antibody (#4060), anti-Akt rabbit monoclonal antibody (#4691), and horseradish peroxidase (HRP)-linked anti-rabbit IgG goat polyclonal antibody (#7074) were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA) and used to detect each protein.

2.7. Statistical analysis

Data analyses were performed using the statistical program SPSS 23 for Mac. Statistical significance was verified between groups of normally distributed data using a 1-factor analysis of variance followed by Tukey's post hoc analysis. Variance homogeneity was examined using Levene's test. When the variances between groups were unequal, the data were transformed to logarithms before analysis using a 1-factor analysis of variance. Data are represented as mean $\pm$ standard error of the mean (SEM). Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Physiological and biochemical analysis

Body weight, food intake, liver weight, and perirenal and epididymal white adipose tissue (WAT) weights of mice in the control group were significantly higher than those of mice in the normal group and were as high as those of the siphonaxanthin-fed group (Table 1). No

significant differences were observed in the mesenteric WAT weights between the groups. The skeletal muscle weight in the control group was significantly lower than that in the normal group and was as low as that in the siphonaxanthin-fed group. In contrast, the left and right kidney weights in the control group were significantly higher than those in the normal group, and siphonaxanthin ingestion tended to decrease. Total kidney weight was significantly higher in the control group than in the normal and siphonaxanthin groups, indicating significant improvement following siphonaxanthin ingestion. At 8- and 12-week-old, the plasma glucose levels of mice in the control and siphonaxanthin-fed groups were higher than 13.3 mmol/L, indicating that they were in a diabetic condition and that siphonaxanthin could not ameliorate plasma glucose levels (Table 2). Endpoint plasma glucose levels showed the same trend (Table 3). Plasma creatinine levels significantly increased in the control group and remained comparable in the siphonaxanthin-fed group. A significant elevation in plasma aspartate transaminase and alanine transaminase levels in the control group suggested liver damage, whereas aspartate transaminase levels tended to recover after siphonaxanthin ingestion. Both triacylglycerol and total cholesterol levels in the plasma and liver were significantly elevated in the control group compared with those in the normal group, and only plasma triacylglycerol levels tended to recover after siphonaxanthin ingestion (Tables 3 and 4). The elevated urine concentration of albumin, urine albumin-to-creatinine ratio, and plasma creatinine levels in the control group indicated impaired kidney function, which was not improved by siphonaxanthin (Table 5).

3.2. Renal histological analysis

The onset of diabetic nephropathy was confirmed using histological analysis of the kidneys, with typical phenotypes of renal hypertrophy, mesangial expansion, and glomerular dilation (Fig. 2A). The mesangial and glomerular areas were significantly increased in the control group compared with those in the normal group, which were significantly mitigated in the

siphonaxanthin-fed group (Figs. 2B and C). Whereas the increased mesangial area ratio was not reduced by siphonaxanthin ingestion (Fig. 2D).

3.3. Renal mRNA expression of fibrogenesis-related proteins

Renal mRNA expression results represented a marginal significant or significant recovery of upregulated profibrotic proteins, including TGF- β 1, collagen, type I, alpha 1 (COL1 α 1), collagen, type IV, alpha 1 (COL4 α 1), and collagen, type I, alpha 1 (COL4 α 3), except for FN in the siphonaxanthin-fed group compared with the control group (Fig. 3). COL1 α 1 is a subunit of type I collagen, and COL4 α 1 and COL4 α 3 are subunits of type IV collagen. There were no significant differences in the FN mRNA expression. The mRNA expression of NOX4, a ROS-generating enzyme, was significantly higher in the control group than in the normal group and was compatible with that of the siphonaxanthin-fed group. Increased mRNA expression of glutathione S-transferase alpha 4 (GSTA4) and NAD(P)H quinone oxidoreductase (NQO1), which are oxidative stress markers, tended to further increase in the siphonaxanthin-fed group. RAGE mRNA levels were significantly increased in the control group and were significantly downregulated by siphonaxanthin ingestion.

3.4. Skeletal muscle mRNA expression associated with fatty acid β -oxidation and the antioxidant system

Since siphonaxanthin ingestion did not decrease plasma glucose levels but tended to decrease plasma triacylglycerol levels, we focused on the effects of siphonaxanthin on lipid metabolism. Consistent with our previous studies using KK-Ay [21] and Ob/Ob [22] mice, siphonaxanthin did not decrease lipid accumulation in the liver. Additionally, weight of white adipose tissues in siphonaxanthin-fed mice were compatible to that of control mice. These data suggested that effects of siphonaxanthin on lipid metabolism of liver and white adipose tissue are minimal. Then, we next focused on skeletal muscle, another metabolically important tissue. Western blot

analysis showed no significant effect of siphonaxanthin on the phosphorylation levels of Akt at Thr-308 and Ser-473, which are critical steps in insulin signaling leading to glucose uptake from blood stream (Suppl. Fig. S1). Although the phosphorylation level of Thr-308 was significantly higher in the control group than in the normal group and tended to decrease with siphonaxanthin ingestion ($p = 0.075$), it is known that the phosphorylation level of Akt at Thr-308 is generally reduced under diabetic conditions. While siphonaxanthin restored the phosphorylation level to that of the normal group, suggesting a potential mitigating effect on diabetes-induced changes, it is reasonable to consider that siphonaxanthin did not exhibit a significant effect on insulin signaling under diabetic conditions. Based on this, we further examined the mRNA expression levels of genes related to lipid catabolism. mRNA expression of fatty acid β -oxidation-related proteins, including peroxisome proliferator-activated receptor (PPAR) gamma coactivator 1-alpha (PGC-1 α), PPAR α , PPAR δ , and acyl-CoA oxidase 1 (ACOX1) showed lower levels in the control group compared with the normal group, whereas their expression was significantly restored in the siphonaxanthin-fed group (Fig. 4A). UCP3 is a protein predominantly expressed in skeletal muscle and is involved in energy balance and lipid metabolism. Its mRNA expression declined in the control group and significantly recovered to a normal level by siphonaxanthin ingestion. mRNA expression of carnitine palmitoyltransferase-1 beta (CPT1 β), the muscle isoform of CPT1 required for fatty acid β -oxidation in mitochondria, specifically showed higher expression in the siphonaxanthin-fed group. mRNA expression of NRF1, a mitochondria biogenesis-related gene, and DRP1, a mitochondria fission-related gene, did not differ significantly among the three groups, whereas that of MFN2, a mitochondria fusion-related gene, was reduced in the control group but was significantly restored to normal levels by siphonaxanthin ingestion (Fig. 4A).

Superoxide dismutase 2 (SOD2) is an antioxidative enzyme that scavenges intramitochondrial free radicals. Decreased SOD2 expression has been shown to result in lipid accumulation in the mouse liver [24], and SOD2 mRNA expression has been

reported to be decreased in the skeletal muscle of patients with T2DM [25]. We evaluated the mRNA expression of SOD2 in db/db mice and found that it was significantly downregulated in control mice and that siphonaxanthin ingestion almost completely recovered it (Fig. 4B). We also assessed the mRNA expression of other antioxidant-related proteins, including GSTA4, NQO1, glutamate-cysteine ligase catalytic subunit (GCLC), and glutathione peroxidase 4 (GPX4). Their mRNA expression decreased in the control group but was restored in the siphonaxanthin-fed group.

4. Discussion

In the present study, we investigated the preventive effects of siphonaxanthin on diabetic nephropathy in db/db mice. We used siphonaxanthin-rich fraction prepared from *Codium cylindricum* as the test sample, and the purity of siphonaxanthin was 63%. A major impurity was presumed as MGDG, with a maximum of 32.4% of the total. We previously showed that dietary MGDG is degraded into two fatty acid molecules and one monogalactosylglycerol, which is not absorbed to the body [26]. The maximum concentration of MGDG in the diet (AIN-93G) was 0.002% calculated from the amount of siphonaxanthin, and AIN-93G contains 7% soybean oil. Therefore, the amount of fatty acids produced by the degradation of MGDG in the intestine is negligibly small compared to those by the degradation of soybean oil. MGDG in the siphonaxanthin-rich fraction would not affect our results. The other impurities were neoxanthin and its isomer. Although the bioavailability of neoxanthin is extremely low in humans [27, 28], neoxanthin and its acid-converted form, neochrome, were detected in the plasma of mice after the oral administration of neoxanthin [29]. A detailed analysis of the biological activities of neoxanthin and neochrome in db/db mice will be necessary in future studies. However, given the low neoxanthin content in the siphonaxanthin-rich fraction, its contribution to the results of this study is expected to be minimal.

The urine concentration of albumin, urine albumin-to-creatinine ratio, and plasma creatinine levels were significantly increased in diabetic mice, indicating decreased renal function. The worsened renal function was not improved by siphonaxanthin ingestion. However, siphonaxanthin significantly improved other markers of diabetic nephropathy, including kidney weight increase, mesangial area expansion, and glomerular area dilation. Moreover, the increased renal mRNA expressions of COL1 α 1 and COL4 α 1 tended to decrease, and renal mRNA expression of COL4 α 3, which tended to be elevated in diabetic conditions, was significantly decreased by siphonaxanthin ingestion. We conclude that 0.004% w/w siphonaxanthin ingestion has a weak preventive effect on diabetic nephropathy. The dose of siphonaxanthin used in this study (0.004% w/w) was much lower than that used in our previous study. Daily ingestion of 1.3 mg of siphonaxanthin ameliorated weight gain in the mesenteric WAT of KK-Ay mice [21]. Feeding a diet containing 0.016% w/w siphonaxanthin significantly suppressed hepatic oxidative damage in Ob/Ob mice [22]. The administered dose of siphonaxanthin was insufficient to improve renal function. Contrary to our expectations, there was no significant difference between db/db (diabetic) and db/m (non-diabetic) mice regarding mesenteric WAT weight and renal mRNA expression of TGF- β 1, COL4 α 3, and FN. Optimization of the experimental conditions, including dose, could clarify the preventive effects of siphonaxanthin against diabetic nephropathy. Moreover, since db/db mice lack leptin receptors—a hormone that plays a crucial role in interactions between adipose tissue and other organs—future studies should employ alternative models to further investigate the effects of siphonaxanthin on diabetic nephropathy in detail, with focus on organ-organ interactions.

During the development and progression of diabetic nephropathy, the extracellular matrix deposition in the kidney is one of the major features and is regulated mainly by TGF- β 1 [11]. Hence, a decrease in TGF- β 1 mRNA expression may be one of the mechanisms underlying the preventive effects of siphonaxanthin on diabetic nephropathy. TGF- β 1 mRNA expression is reported to be upregulated by NOX4-derived ROS and AGEs [7, 8, 11].

Siphonaxanthin did not decrease NOX4 mRNA expression or oxidative stress markers (GSTA4 and NQO1 mRNA) and significantly suppressed the mRNA expression of RAGE, a receptor for AGEs. Therefore, suppression of AGEs/RAGE rather than NOX4/ROS signaling might be responsible for the decrease in TGF- β 1 mRNA and, thus, the preventive effects of siphonaxanthin. This idea is supported by our previous findings that siphonaxanthin potently suppresses AGEs/RAGE signaling [19, 20].

PGC-1 α is a transcription factor regulating the expression of fatty acid β -oxidation-related genes [30], and its mRNA expression was reported to be decreased in the skeletal muscle of patients with T2DM [31-33]. Consistent with these human studies, PGC-1 α mRNA expression was clearly decreased in the skeletal muscle of db/db mice, and siphonaxanthin ingestion significantly recovered it. In addition to PGC-1 α mRNA, the downregulation of mRNA expression of the other fatty acid β -oxidation-related proteins (PPAR α , PPAR δ , ACOX1, and UCP3) was significantly restored by siphonaxanthin. mRNA expression of MFN2, which promotes mitochondrial fusion and improves mitochondrial quality—a key site of lipid metabolism—showed a similar trend, indicating that siphonaxanthin prevented diabetes-induced loss of mitochondrial quality. These transcriptional changes illustrate the possible function of siphonaxanthin in restoring metabolic capacity in diabetes mellitus. In addition to the transcriptional analysis, future analyses of biochemical markers related to skeletal muscle metabolism, including AMP-to-ATP ratio, activation levels of AMP-activated protein kinase, and mitochondrial DNA content, will provide further insight into the effects of siphonaxanthin on muscle function.

PPAR δ mRNA expression in the siphonaxanthin-fed group was slightly higher than that in the normal group. Moreover, mRNA expression of CPT1 β in the siphonaxanthin-fed group was greatly higher than that in the other groups. Since a PPAR δ agonist was reported to induce CPT1 β mRNA expression in the skeletal muscle [34, 35], siphonaxanthin could potentially activate PPAR δ . The mRNA expression of PGC-1 α , UCP3, and SOD2 is directly regulated by

PPAR δ [36-38]. Hence, the activation of PPAR δ could be one of the mechanisms by which siphonaxanthin restored the downregulation of β -oxidation-related genes.

Siphonaxanthin significantly ameliorated the reduction in mRNA expression of antioxidant-related proteins, including SOD2, GSTA4, NQO1, GCLC, and GPX4, in the skeletal muscles of db/db diabetic mice. These expression levels were regulated by nuclear factor erythroid 2-related factor 2 (Nrf2). We previously reported that siphonaxanthin increased Nrf2-regulating genes in a mouse macrophage-like cell line, RAW264, and human umbilical vein endothelial cells [20]. Siphonaxanthin also activated Nrf2 in human hepatocarcinoma HepG2 cells [22]. These results indicate that siphonaxanthin would activate Nrf2 regardless of cell type. Nrf2 expresses ubiquitously in somatic cells, including skeletal muscle cells, and dietary siphonaxanthin certainly reached to the skeletal muscle [39]. Furthermore, metabolites of siphonaxanthin, which accumulate to a similar extent as siphonaxanthin in skeletal muscle, were recently shown to activate Nrf2 more strongly than siphonaxanthin itself [40]. Altogether, one of the molecular mechanisms by which dietary siphonaxanthin ameliorates the reduction in antioxidant-related gene expression may be the activation of Nrf2 in the skeletal muscle.

5. Conclusion

In this study, we demonstrated that dietary supplementation with siphonaxanthin ameliorated mesangial expansion and glomerular dilation induced by diabetes mellitus in mice. Renal mRNA expression analysis indicated that the suppression of AGEs/RAGE signaling could be one of the underlying molecular mechanisms. Additionally, siphonaxanthin ingestion restored the reduction of mRNA expression of fatty acid β -oxidation-related proteins in the skeletal muscle. However, a limitation of this study is that the data supporting the mechanistic hypothesis are restricted to the transcriptional level, with no analysis of transcriptional regulators at the protein level. Detailed investigations are required to unveil the full picture of

335 the preventive effect of siphonaxanthin on diabetic nephropathy and lipid metabolism
336 insufficiency in the skeletal muscle.

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338 **Author contributions**

Jiawen Zheng: Data curation, Formal analysis, Investigation, Writing – original draft.

Yuki Manabe: Writing – original draft, Writing – review and editing, Visualization,
Methodology, Supervision.

Tatsuya Sugawara: Conceptualization, Methodology, Project administration, Resources,
Supervision, Writing – review and editing.

All authors reviewed and approved the final version of the manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

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Figure captions

Fig. 1. Analysis of siphonaxanthin-rich fraction

Chemical structure of siphonaxanthin (A). HPLC chromatogram at 450 nm of siphonaxanthin standard and siphonaxanthin-rich fraction (B). SPX, siphonaxanthin.

Fig. 2. Histological analysis of db/db mouse kidney.

Representative photographs of kidney tissue sections stained with PAS (A). Quantification of mesangial area (B), glomerular area (C), and mesangial area ratio (D). Values represent the mean $\pm$ SEM ($n = 4$). Different letters indicate significant differences among groups ($p < 0.05$). Normal, db/m mice fed AIN-93G; Control, db/db mice fed AIN-93G; SPX, db/db mice fed AIN-93G supplemented with 0.004% w/w siphonaxanthin.

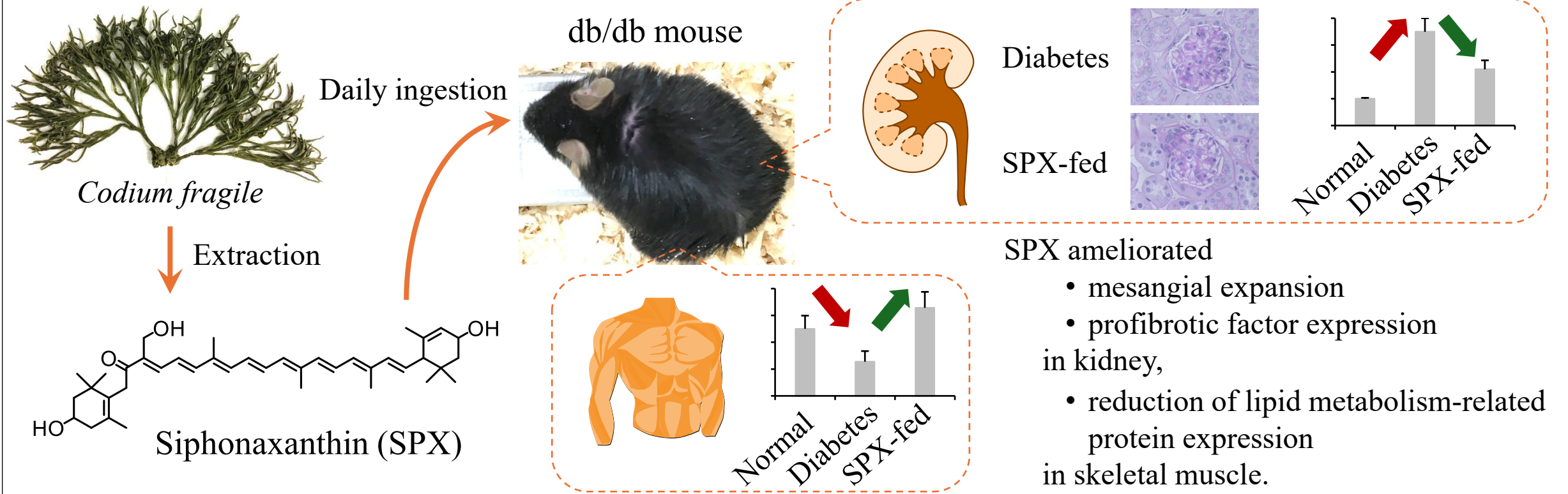
Fig. 3. Effects of siphonaxanthin on renal mRNA expression of fibrogenesis-related proteins.

The mRNA expression in kidney samples was evaluated using real-time qRT-PCR analysis. Values represent the mean $\pm$ SEM ($n = 4$). Different letters for each gene indicate significant differences among groups ($p < 0.05$). Normal, db/m mice fed AIN-93G; Control, db/db mice fed AIN-93G; SPX, db/db mice fed AIN-93G supplemented with 0.004% w/w siphonaxanthin.

Fig. 4. Effects of siphonaxanthin on skeletal muscle mRNA expression of factors related to lipid metabolism and antioxidant systems.

mRNA expressions of proteins related to β -oxidation (A) and the antioxidant system (B) in skeletal muscle samples were evaluated using real-time qRT-PCR analysis. Values represent the mean $\pm$ SEM ($n = 4$). Different letters for each gene indicate significant differences among groups ($p < 0.05$). Normal, db/m mice fed AIN-93G; Control, db/db mice fed AIN-93G; SPX, db/db mice fed AIN-93G supplemented with 0.004% w/w siphonaxanthin.

Preventive Effect of Siphonaxanthin, a Carotenoid from Green Algae, against Diabetic Nephropathy and Lipid Metabolism Insufficiency in Skeletal Muscle



- Siphonaxanthin ameliorates diabetes mellitus-induced kidney damage in db/db mice.
- Renal mRNA of TGF- β 1 and RAGE was significantly decreased by siphonaxanthin.
- Suppression of AGEs/RAGE signaling could be one of the underlying mechanisms.
- Siphonaxanthin might restore lipid metabolism insufficiency in skeletal muscle.

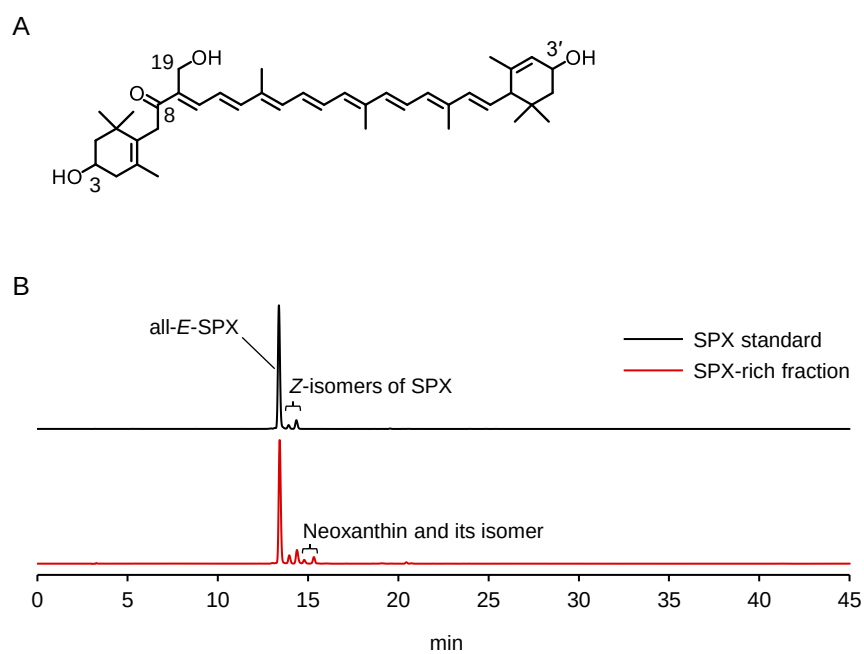


Figure 1

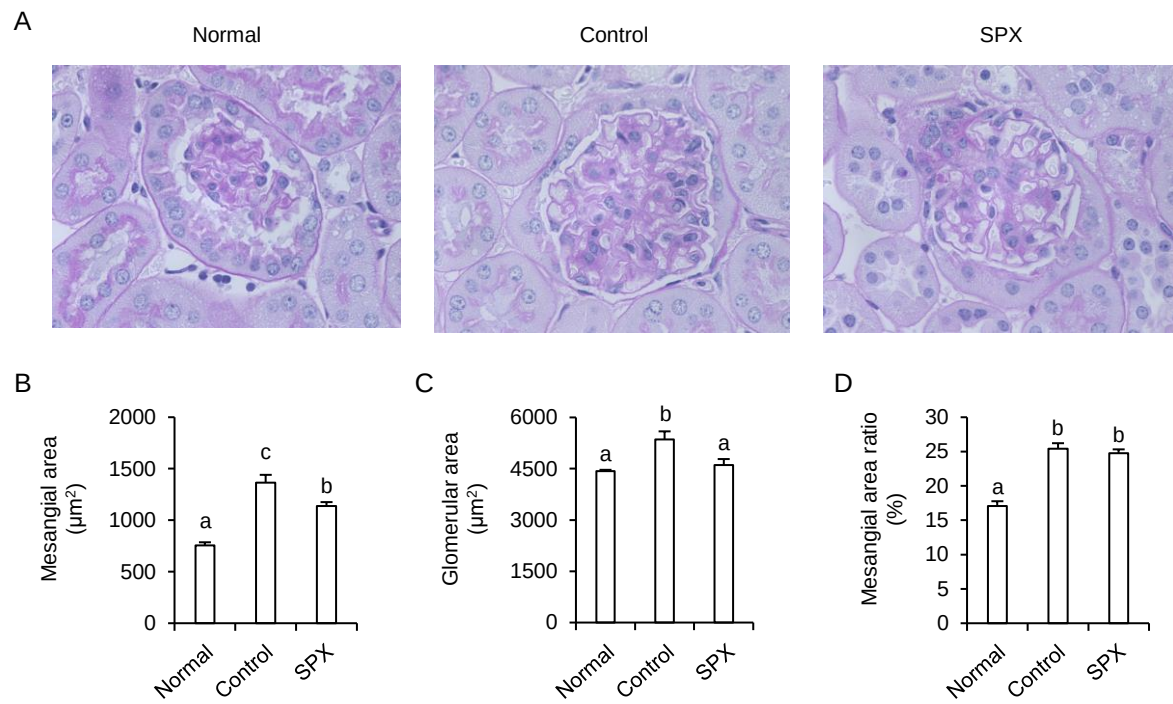


Figure 2

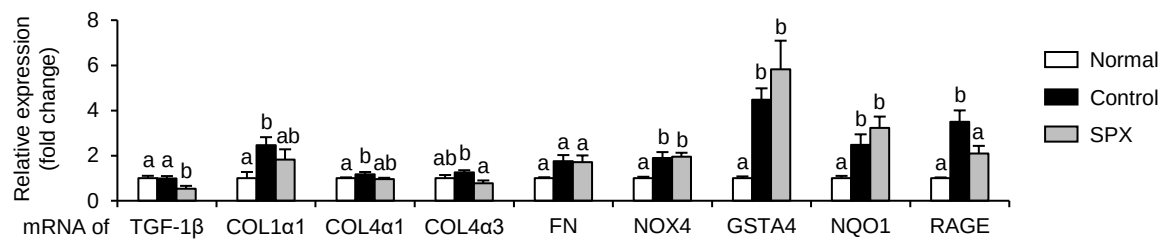


Figure 3

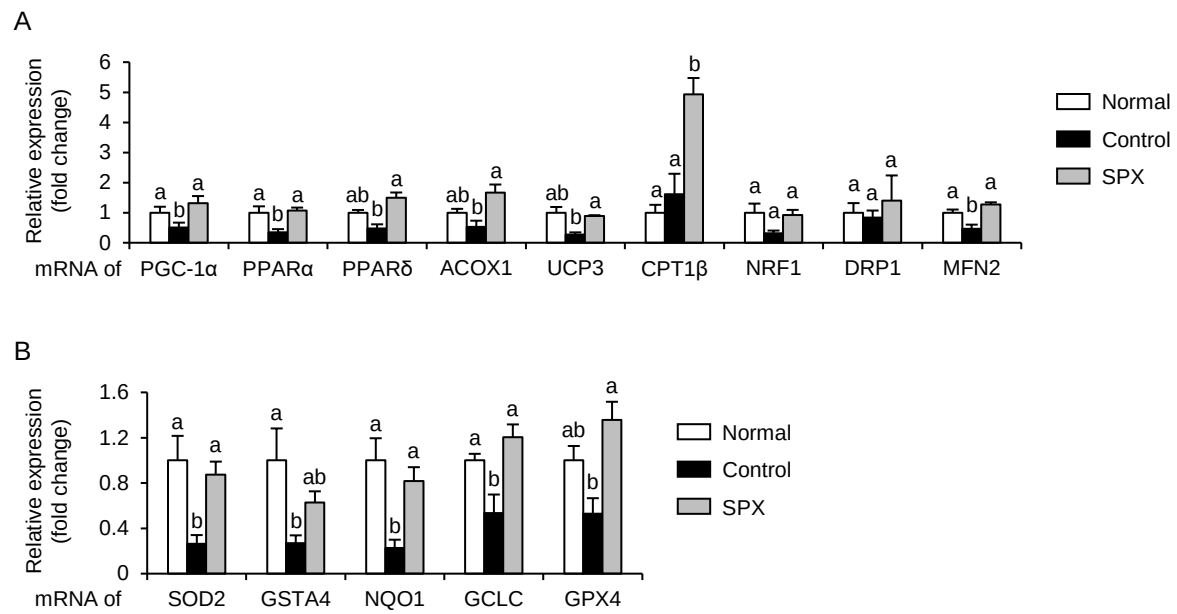


Figure 4

Supplementary Material

Preventive Effect of Siphonaxanthin, a Carotenoid from Green Algae, against Diabetic Nephropathy and Lipid Metabolism Insufficiency in Skeletal Muscle

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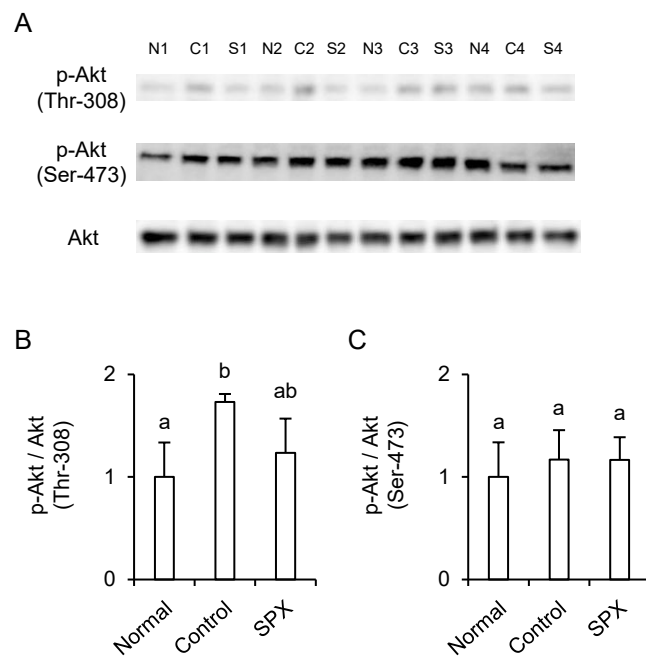
Supplementary Figure S1. Effects of siphonaxanthin on skeletal muscle Akt phosphorylation.

Supplementary Table S1. Real-time qRT-PCR primers.

Figure Caption

Supplementary Figure S1. Effects of siphonaxanthin on skeletal muscle Akt phosphorylation.

Protein expression levels were assessed using Western blot analysis (A–C). Values represent the mean $\pm$ SEM (n = 4). Different letters for each gene indicate significant differences among groups ($p < 0.05$). N, Normal, db/m mice fed AIN-93G; C, Control, db/db mice fed AIN-93G; S, SPX, db/db mice fed AIN-93G supplemented with 0.004% w/w siphonaxanthin, p-Akt, phosphorylated Akt.



Supplementary Figure S1

Supplementary Table S1. Real-time qRT-PCR primers

Gene	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')
β -Actin	CCTCTATGCCAACACAGTGC	GTACTTGCGCTCAGGAGGAG
TGF- β 1	GAGCCCGAAGCGGACTACTA	TGGTTTTCTCATAGATGGCGTTG
COL1 α 1	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGGG
COL4 α 1	CTGGCACAAAAGGGACGAG	ACGTGGCCGAGAATTTACCC
COL4 α 3	CAAAGGCATCAGGGGAATAACT	ATCCGTTGCATCCTGGTAAAC
FN	ATGTGGACCCCTCCTGATAGT	GCCCAGTGATTTTACGAAAAGG
NOX4	GAAGGGGTAAACACCTCTGC	ATGCTCTGCTTAAACACAATCCT
GSTA4	AGCTCAGTTGGGCAGACATC	TCCTGACCACCTCAACATAGG
NQO1	AGAGAGTGCTCGTAGCAGGAT	GTGGTGATAGAAAGCAAGGTCTT
RAGE	ACTACCGAGTCCGAGTCTACC	CCCACCTTATTAGGGACACTGG
PGC-1 α	AAGTGTFFAACTCTCTGGAAGTG	GGGTTATCTTGGTTFFCTTTATG
PPAR α	TACTGCCGTTTTTACAAGTGC	AGGTCGTGTTACAGGTAAGA
PPAR δ	AATGCGCTGGAGCTCGATGAC	ACTGGCTGTCAGGGTGGTTG
ACOX1	TAACTTCCTCACTCGAAGCCA	AGTTCCATGACCCATCTCTGTC
UCP3	CTGCACCGCCAGATGAGTTT	ATCATGGCTTGAAATCGGACC
CPT1 β	TTGCCCTACAGCTGGCTCATTTCC	GCACCCAGATGATTGGGATACTGT
SOD2	CAGACCTGCCTTACGACTATGG	CTCGGTGGCGTTGAGATTGTT
GCLC	GGCTACTTCTGTACTAGGAGAGC	TGCCGGATGTTTCTTGTTAGAG
GPX4	TGTGCATCCCGCGATGATT	CCCTGTACTTATCCAGGCAGA
NRF1	TATGGCGGAAGTAATGAAAGACG	CAACGTAAGCTCTGCCTTGTT
DRP1	CAGGAATTGTTACGGTTCCTAA	CCTGAATTAACCTGTCCCGTGA
MFN2	ATGGCAGAAACGGTATCTCCA	CTCGGATGCTATTTCGATCAAGTT