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Mechanism of Visceral Fat Reduction in Tsumura Suzuki Obese, Diabetes (TSOD) Mice Orally Administered β-Cryptoxanthin from Satsuma Mandarin Oranges (*Citrus unshiu* Marc)

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ABSTRACT: The carotenoid β-cryptoxanthin (β-CRX) is abundant in Satsuma mandarins (*Citrus unshiu* Marc). Several studies have shown a relationship between Satsuma mandarin consumption and a low risk of several diseases, for example, diabetes, gout, and hypertension, suggesting β-CRX involvement in disease prevention. We investigated the effect of β-CRX on mildly obese males. β-CRX administration reduced visceral adipose tissue, body weight, and abdominal circumference. However, the detailed mechanism by which β-CRX mediates these changes remains unknown. To identify this mechanism, we used an obese model mouse (TSOD). Oral β-CRX administration repressed body weight, abdominal adipose tissue weight, and serum lipid concentrations in TSOD; these results are identical to previous human trial results. β-CRX administration significantly repressed adipocyte hypertrophy. Gene expression analysis strongly indicated that β-CRX can alter cytokine secretion and cell proliferation. These results suggest that β-CRX derived from Satsuma mandarins can help prevent obesity by repressing hypertrophy of abdominal adipocytes.

KEYWORDS: β-cryptoxanthin, Satsuma mandarin (*Citrus unshiu* Marc), TSOD, adipocyte

INTRODUCTION

Carotenoids are widely accepted for their nutritional importance and are known as natural antioxidants and anticancer effectors.[1,2] In most cases, carotenoids refer to the abundant carotenoids, β-carotene, lutein, and lycopene. As such, only limited functional analyses of the minor carotenoids have been performed.

β-Cryptoxanthin (β-CRX) is a carotenoid classified as a xanthophyll; its chemical structure is similar to that of β-carotene with the addition of a hydroxyl group at the number 3 carbon atom (Figure 1).

Unlike the abundant carotenoids, β-CRX is not found in most fruits or vegetables but only in specific ones such as hot peppers, persimmons, and Satsuma mandarins (*Citrus unshiu* Marc).[3] Despite the low consumption of β-CRX, it is found in human blood together with the abundant carotenoids, thus suggesting the high bioavailability of β-CRX.

Satsuma mandarin, which is also known as the table orange in Western countries, is one of the most popular and highly consumed citrus fruits in Japan. As mentioned above, it is also one of the most β-CRX-rich fruits in the world. The edible part of the Satsuma mandarin contains about 1.8 mg of β-CRX per 100 g of fruit. In contrast, the Valencia orange has 0.2 mg per 100 g, and the grapefruit has been found to be devoid of it. Collectively, these factors may explain why the Japanese population has higher serum β-CRX concentrations than Western populations. It has been suggested that Satsuma mandarin consumption may be a good index of serum β-CRX concentrations in the Japanese people.[4,5]

The absorption, metabolism, and nutritional functions of β-carotene and lycopene are well studied.[6−10] Although these functions of β-CRX have not been adequately examined, several important studies have reported its potential health-promoting effects. These studies showed a significant negative correlation between serum β-CRX concentrations and disease morbidity, including liver disorders,[11,12] cancer,[13−15] postmenopausal osteoporosis,[16−18] and diabetes.[19,20] These results suggest that a high serum β-CRX concentration is beneficial to human health.

Obesity is one of the notorious symptoms of metabolic syndrome, because the accumulation of visceral fat results in a number of adverse health effects. A previous study indicated that β-CRX can normalize serum lipid levels.[21] Moreover, we have reported that the continuous oral intake of β-CRX may improve the symptoms of metabolic syndrome by reducing visceral adipose tissue, body weight, and abdominal circumference, in mildly obese males.[22] However, the mechanism by which β-CRX can help to prevent obesity and metabolic syndrome remains undetermined.

In this paper, we investigate the mechanism of the antiobesity effect of β-CRX using Tsumura Suzuki obese, diabetes (TSOD),[23,24] an obese mouse model. TSOD mice rapidly accumulate visceral fat after 4 weeks of age and show symptoms similar to human metabolic syndrome, making it a suitable model for studying how β-CRX reduces obesity.

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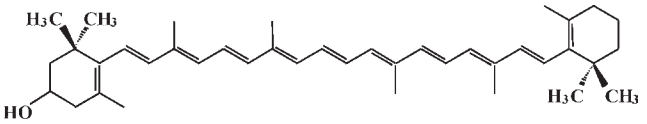

Figure 1. Chemical structure of β-cryptoxanthin.

Table 1. Nutrient Composition of Enzyme-Processed Satsuma Mandarin

	water (%)	protein (%)	lipid (%)	carbohydrate (%)	ash (%)	energy (kcal/100 g)
EPSM	3.3	21.3	29.3	43.0	3.1	521

MATERIALS AND METHODS

β-Cryptoxanthin. Enzyme-processed Satsuma mandarin (EPSM), manufactured by UNITIKA Ltd. (Osaka, Japan), was used as the source of β-CRX in this study. EPSM is an orange powder derived from Satsuma mandarin pulp after juicing and contains a minimum of 0.2% (w/w) β-CRX (Table 1).

TSOD and TSNO Mice. Three-week-old male TSOD and Tsumura Suzuki non-obese, diabetes (TSNO) control mice were obtained from the Institute for Animal Reproduction (Ibaraki, Japan). After an acclimatization period of a week, the TSOD and TSNO mice were divided into experimental and control groups (n = 6 for each group). For 8 weeks, the experimental groups were orally administered EPSM suspended in olive oil at a dose of 400 mg/kg/day (equivalent to 0.8 mg of β-CRX/kg/day), whereas the control groups were orally administered only olive oil.

Each group of mice was housed in one cage under a 12 h light/dark cycle and fed Labo MR Stock (Nosan, Kanagawa, Japan). Body weight and food and water consumption were monitored during the experimental period. After the experimental period, three adipose tissues (epididymal, perirenal, and mesenteric), liver, femoral muscle, and serum were isolated.

This experiment was approved and carried out in accordance with UNITIKA's Guidelines for Animal Experimentation, which are based on the notification of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Serum Lipid Assays. The serum concentrations of triglyceride (TG), total cholesterol (TC), and nonesterified fatty acid (NEFA) were analyzed using a diagnosis kit (Wako, Osaka, Japan) following the manufacturer's instructions.

Adipose Tissue Analysis. Portions of epididymal adipose tissues were fixed in formalin, paraffin embedded and sectioned, and hematoxylin−eosin (HE) stained. Adipocytes were analyzed under a microscope using an Image Pro Discovery computer-assisted analysis system (Media Cybernetics, Bethesda, MD).

DNA Microarray Analysis. Epididymal adipose tissue, liver, and femoral muscle were isolated, quickly placed in RNAlater (Ambion, Austin, TX), and stored at −80 °C prior to use. Total RNA was isolated from these tissues using the RNeasy kit (Qiagen, Duesseldorf, Germany) according to the manufacturer's manual. Equal quantities of total RNA for each experimental group were pooled prior to the labeling reaction. Biotin-labeled cRNA was synthesized and fragmented using the GeneChip 3′-IVT labeling kit (Affymetrix, Santa Clara, CA). Fragmented cRNA was hybridized at 45 °C for 16 h to a GeneChip Mouse Genome 430A 2.0 Array (Affymetrix), which contains about 14000 well-characterized genes. The gene chips were washed and stained using a fluid station and scanned using a GeneChip scanner (Affymetrix). The data were analyzed by Expression Console (Affymetrix) and imported into the ArrayStar software program (DNAstar, Madison, MI) files. These data were normalized, and the expression levels were compared using ArrayStar. For the biological interpretation of the differentially expressed genes, the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.2 (http://david.abcc.ncifcrf.gov/) was used.[25] GO biological processes with a $p < 0.01$ were considered to be changed in a statistically significant manner. Moreover, differentially expressed genes were categorized and mapped onto pathways using KEGG tools (http://www.genome.jp/kegg/).[26]

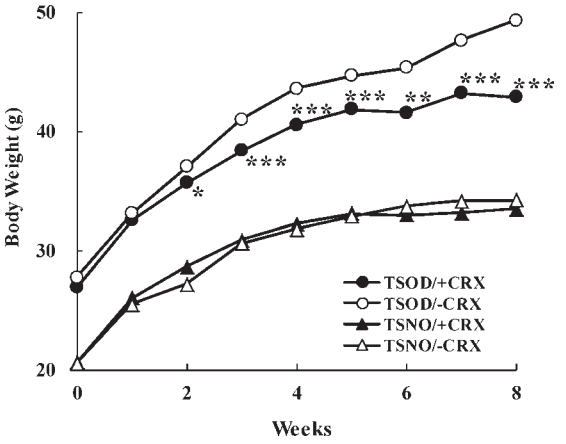

Figure 2. Effect of β-cryptoxanthin on body weight: (●) TSOD experimental group (obese mice); (○) TSOD control group; (▲) TNSO experimental group (normal mice); (△) TSNO control group. Asterisks indicate statistical significance between the TSOD experimental and control groups; ∗, ∗∗, and ∗∗∗ indicate the p values were less than 0.05, 0.01, and 0.005, respectively.

Real-Time Quantitative Reverse-Transcriptase Polymerase Chain Reaction (Real-Time RT-PCR) Analysis. The mRNA expression was quantitated by real-time RT-PCR analysis. Total RNA from each tissue was isolated as described under DNA Microarray Analysis. cDNA was synthesized from the total RNA using a PrimeScript RT kit (Takara Bio Inc., Shiga, Japan). Real-time RT-PCR analysis was performed using Sybr Premix Ex taq (Takara Bio Inc.) and automated sequence detection systems, StepOne (Applied Biosystems Japan Ltd., Tokyo, Japan). The amplification conditions were incubation at 95 °C for 15 s followed by 60 °C for 1 min for 40 cycles. The primers for PCR (adiponectin, MCP-1, and TNF-α) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). The sequences of the primers were as follows: adiponectin/sense (5′-AGAGAAGGGAGAGAAAGGAGATGC-3′) and antisense/(5′-TGAGCGATACACATAAGCGGC-3′),[27] MCP-1 sense (5′-TTAAAAACCTGGATCGGAACCAA-3′) and antisense (5′-GCATTAGCTTCAGATTTACGGGT-3′),[28] and TNF-a sense (5′-TCCCCAAAGGGATGAGAAGTTC-3′) and antisense (5′-TCATACCAGGGTTTGAGCTCAG-3′),[29] respectively.

Statistical Analysis. Individual value was determined as the mean of triplicates and used for the statistical analysis. The data are shown as the mean ± SD and were analyzed using Student's t test for two groups; p values <0.05 were considered to be statistically significant.

RESULTS

Body Weight. The body weights of each group during the experimental period are shown in Figure 2. There were no differences in the body weights of the TSNO mice administered β-CRX or the untreated, control TSNO mice. The average weight of the control TSOD mice was significantly higher than that of the control TSNO mice, presumably because of the differences in genetic backgrounds.

In contrast, the body weight of TSOD mice administered β-CRX was notably repressed compared to that of control TSOD

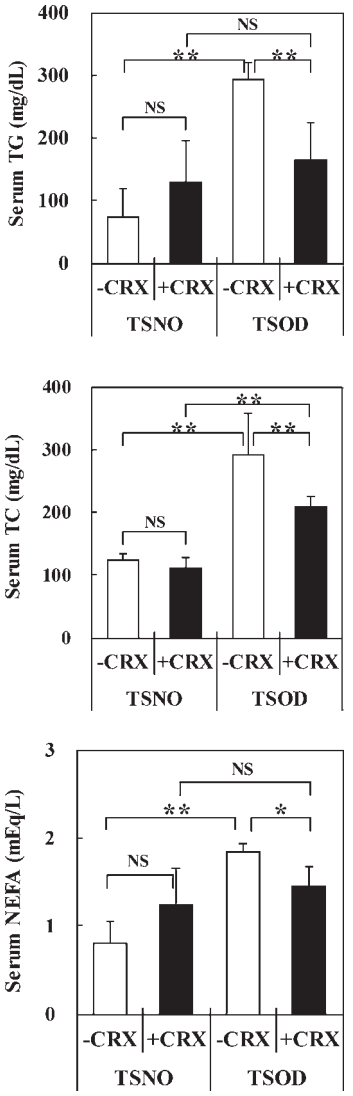

Figure 3. Effect of β-cryptoxanthin on serum lipid levels in mice. Serum triglyceride (TG), total cholesterol (TC), and nonesterified fatty acid (NEFA) concentrations were assayed. * and ** asterisks indicate the p value was less than 0.05 and 0.01, respectively, and NS indicates no significance between the groups.

mice, with the differences statistically significant after the second week. As the food and water consumptions of both TSOD groups were almost the same during the experimental period (data not shown), these differences were not due to the food consumption but to the administration of β-CRX. Moreover, β-CRX consumption did not affect the body weight of nonobese mice, suggesting that β-CRX may contribute to body weight reduction only in obese individuals.

Serum Lipids. The serum concentrations of TG, TC, and NEFA were assayed (Figure 3). There were no differences in the serum lipid concentrations between the TSNO control and experimental groups. By contrast, the levels of all three lipids were significantly elevated in the TSOD control group relative to the other three groups. Thus, the administration of β-CRX in the TSOD mice resulted in the repression of the serum lipid elevation. The TG and NEFA concentrations in the experimental TSOD group were significantly reduced relative to those of the TSOD control group and were repressed to the same levels observed in

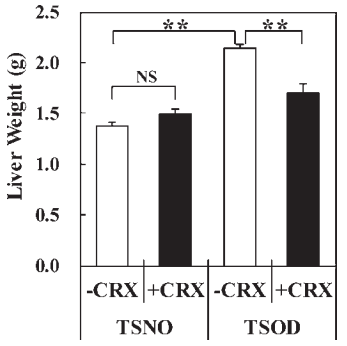

Figure 4. Effect of β-cryptoxanthin on liver weight. ** indicate the p value was less than 0.01, and NS indicates no significance between the groups.

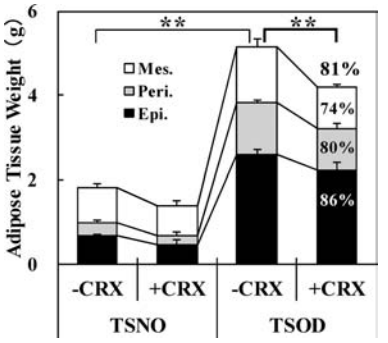

Figure 5. Effect of β-cryptoxanthin on the weight of visceral adipose tissues. Three visceral adipose tissues were removed, and their weights between groups were compared. Percentages in the TSOD/+CRX column represent the rate of weight reduction compared to TSOD/−CRX. Statistical difference was analyzed against the total weight of three tissues; ** indicate the p value was less than 0.01, and NS indicates no significance between the groups.

the TSNO control group. Although the TC concentration in the experimental TSOD group was also significantly repressed compared to that of the control TSOD group, it was still significantly higher than that of the TSNO experimental group. The administration of β-CRX significantly repressed the elevation of serum lipid concentrations in TSOD mice but had no discernible effect on the nonobese TSNO mice. These results indicate that β-CRX administration does not simply reduce the serum lipid levels but normalizes them by repressing their elevation.

Analysis of Liver and Adipose Tissues. The liver weights were compared between the groups (Figure 4). There were no differences between the experimental and control TSNO groups, whereas the liver weight of the TSOD control group was significantly higher than those of the TSNO groups. Although the liver weight of the experimental TSOD group was higher than those of the TSNO groups, it was significantly reduced compared to the TSOD control group. This indicates that obesity-induced liver hypertrophy was repressed by the administration of β-CRX.

A similar tendency was observed in the three adipose tissues (epididymal, perirenal, and mesenteric) (Figure 5). The weights of the epididymal, perirenal, and mesenteric tissues of the experimental TSOD group were reduced 86, 80, and 74%, respectively, compared to those of the TSOD control group. The weight reduction in the mesenteric adipose tissue, commonly called the intraperitoneal adipose tissue, was the greatest among the three tissues.

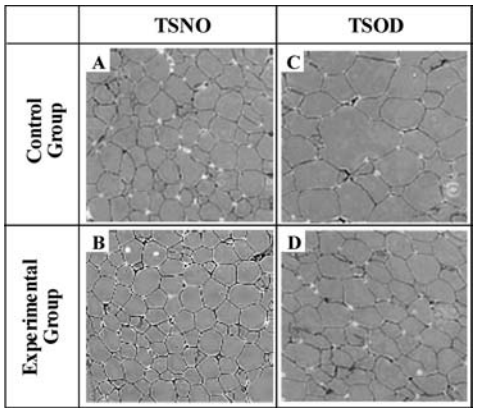

Figure 6. Effect of β-cryptoxanthin on the size of adipocytes: HE-stained epididymal adipose tissue sections derived from (A) the TSNO control group, (B) the TSNO experimental group, (C) the TSOD control group, and (D) the TSOD experimental group.

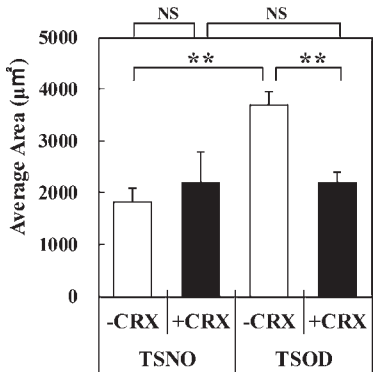

Figure 7. Effect of β-cryptoxanthin on the area of individual adipose cells. The average areas of individual epididymal cells were calculated and compared. ** indicate the p value was less than 0.01, and NS indicates no significance between the groups.

Moreover, the total weight of the three adipose tissues was reduced by 81% in the TSOD experimental group compared to that of the respective control group.

Histological specimens were prepared from epididymal adipose tissues and examined microscopically (Figure 6). The sizes of the adipocytes in the TSNO experimental and control groups were nearly the same. Meanwhile, the adipocyte cells in the TSOD control group were enlarged relative to those of the TSNO control group but were significantly reduced in the TSOD experimental group. The average areas of the adipocyte cells were calculated to evaluate them objectively (Figure 7), and the results were consistent with the visual inspections. Together, these observations showed that the mature adipocytes of TSOD mice were hypertrophic compared to those of TSNO mice and that the administration of β-CRX may be able to prevent the hypertrophy of adipocytes and thus normalize them.

Gene Expression Analysis by DNA Microarray and Real-Time RT-PCR. The effect of β-CRX on the gene expression patterns in TSOD mice was evaluated by Affymetrix GeneChip analysis (Table 2). The numbers of genes with expression fluctuating more than 2-fold between the TSOD and TSNO control groups and between the TSOD control and experimental groups in the liver, muscle, and adipocytes were 270, 56, and 217, respectively.

Table 2. Summary of the Microarray Analysis of Gene Expression Changes in Mice Fed β-CRX[a]

	TSNO/−CRX vs TSOD/−CRX			TSOD/−CRX vs TSOD/+CRX			
organ	up	down	total (A)	up	down	total (B)	A and B
liver	920	424	1344	146	260	406	270
adipocyte	728	1229	1957	71	181	252	217
muscle	190	677	867	30	59	89	56

[a] Number of genes differentially expressed (>2-fold change) in the boldfaced groups.

On the basis of a functional clustering analysis (Table 3), the genes involved in steroid metabolism (nine genes) were affected by β-CRX in the liver of TSOD mice. Among them, the expression levels of 3-hydroxy-3-methylglutaryl-CoA synthase 1 (Hmgcs1), lanosterol 14-α-demethylase (Cyp51), and isopentenyl-diphosphate δ isomerase 1 (Idi1), which are involved in the synthesis of steroid, were up-regulated by β-CRX in TSOD mice. By contrast, their mRNA levels were decreased in the TSOD control group relative to those of the TSNO control one (Table 4). Moreover, the expression levels of high-density lipoprotein binding protein (Hdlbp) and ATP-binding cassette subfamily A member 1 (Abca1), which are involved in the transport of steroid, were down-regulated by β-CRX in TSOD mice, whereas their expression was increased in the TSOD control group compared to that of the TSNO control. DNA replication initiation (four genes), which is involved in the first step of cell proliferation, was also affected by β-CRX. In addition, the expression levels of the members of the mini-chromosome maintenance (MCM) replication initiating complex, Mcm2, Mcm4, Mcm5, and Mcm6, were repressed in the liver by β-CRX in TSOD mice, although their mRNA levels were increased in the control TSOD group relative to those of the TSNO control group.

In adipocytes, the expression of genes involved in the cell cycle, chemotaxis, and immune system development was affected by β-CRX (Table 3). Cyclin dependent kinase (Cdk1) and cyclins (Ccna2, Ccnb1, and Ccnb2), which are well-known key regulators of the cell cycle, were down-regulated by β-CRX, although their mRNA levels were increased in the TSOD control group compared to those of the TSNO control one (Table 5). Moreover, the chemokines CXCL (Cxcl2 and Cxcl10) and CCL (Ccl2, Ccl3, Ccl4, Ccl7, and Ccl12) were also repressed by β-CRX. However, their mRNA levels were increased in the TSOD control group relative to those of the TSNO control one.

The expression of important adipokines, namely, adiponectin, MCP-1, and TNF-α, was precisely investigated by quantitative RT-PCR (Table 7). The mRNA expression of adiponectin was significantly up-regulated by β-CRX in the TSOD group, whereas that of MCP-1 was significantly down-regulated. TNF-α expression was also down-regulated but was not found to be statistically significant.

In muscle, the expression of genes involved in (cardiac) muscle contraction, fatty acid biosynthesis, lipid transport, and wound response was affected by β-CRX (Table 3). The genes involved in muscle contraction (Myh7, My2, Tpm3, and Tnnc1) were up-regulated by β-CRX. Meanwhile, their mRNA levels were increased in the TSOD control group relative to those of the TSNO control one (Table 6). The expression levels of the elongation of very long chain fatty acids protein 6 (Elovl6), stearoyl-CoA desaturase (Scd1), and fatty acid synthase (Fasn), which are involved in fatty acid

Table 3. GO Biological Processes Affected by Oral Ingestion of β-CRX

	GO ID	GO biological process	changed genes	total genes	%	p value (DAVID)
liver						
1	GO:0006270	DNA replication initiation	4	20	20.0	3.10×10^{-4}
2	GO:0045455	steroid metabolic process	9	224	4.0	2.00×10^{-3}
3	GO:0045859	regulation of protein kinase activity	10	372	2.7	2.10×10^{-3}
4	GO:0019220	regulation of phosphate metabolic process	16	611	2.6	2.50×10^{-5}
5	GO:0006468	protein amino acid phosphorylation	19	1020	1.9	4.20×10^{-3}
adipocyte						
1	GO:0050000	chromosome localization	4	17	23.5	8.60×10^{-5}
2	GO:0007049	cell cycle	36	1000	3.6	3.40×10^{-15}
3	GO:0006935	chemotaxis	10	291	3.4	5.40×10^{-6}
4	GO:0002520	immune system development	12	466	2.6	7.40×10^{-4}
5	GO:0042981	regulation of apoptosis	16	988	1.6	2.20×10^{-3}
muscle						
1	GO:0060048	cardiac muscle contraction	4	33	12.1	6.20×10^{-3}
2	GO:0006633	fatty acid biosynthetic process	4	122	3.3	1.70×10^{-3}
3	GO:0006869	lipid transport	5	159	3.1	4.00×10^{-4}
4	GO:0042060	wound healing	3	187	1.6	4.30×10^{-2}
5	GO:0010817	regulation of hormone levels	4	295	1.4	6.30×10^{-3}

synthesis, were down-regulated by β-CRX. In contrast, their mRNA levels were decreased in the TSOD control group compared to those of the TSNO control one. Furthermore, ApoA (Apoa1 and Apoa2) and ApoC (Apoc1 and Apoc3) were down-regulated by β-CRX in TSOD mice. The wound healing-related genes (Fga, Kng, and Serpinc1) were likewise down-regulated by β-CRX in TSOD mice. However, their mRNA levels were increased in the TSOD control group relative to those of the TSNO control one.

DISCUSSION

Obesity is closely associated with chronic diseases such as diabetes, cardiovascular diseases, hyperlipidemia, and hypertension, which are well-known symptoms of metabolic syndrome. Because metabolic syndrome is becoming a serious social problem, many scientists are contributing to the effort to decrease its prevalence.

We previously reported that the administration of β-CRX resulted in visceral fat reduction in mildly obese Japanese males.[22] Despite this clear result, the mechanism of the visceral fat reduction was not examined. Consistent with our previous human study, the present study showed that β-CRX administration repressed the elevation of serum lipid levels and body and adipose tissue weight in TSOD mice. These results indicate that β-CRX administration may be able to prevent metabolic syndrome in both mice and humans.

To identify the antiobesity mechanism, we initially focused on the visceral adipose tissue. Adipose tissue has two roles: energy storage and endocrine function. Both of these roles are closely related to one other. When mature adipocytes accumulate adequate amounts of TG, they secrete several beneficial adipokines, including adiponectin and leptin. Obesity leads to the overaccumulation of TG in the adipocytes, with the subsequent hypertrophic adipocytes secreting inflammatory messengers such as TNF-α, IL-1β, and IL-10 instead of the beneficial ones.[30] This evokes a strong immune response, and the resultant inflammation of the adipocytes causes even worse symptoms. Finally, these events can result in insulin resistance and deterioration into diabetes or worsened obesity.

In this study, we found that β-CRX reduced both the weight and the cell size of adipocytes in TSOD mice (Figures 5−7). These results indicate that β-CRX can inhibit the proliferation of adipocytes and the accumulation of TG in the cells. According to the microarray results, the mRNA levels of genes involved in the cell cycle, chemotaxis, and immune system development were affected by β-CRX in the adipose tissues of TSOD mice (Table 3). Microarray analysis showed that the expression levels of 36 genes involved in the cell cycle were higher in TSOD mice than in TSNO mice, but were decreased by β-CRX intake (Table 5). Tang et al. reported that CDK inhibitor can repress both proliferation and differentiation of adipocytes.[31] These results suggest that β-CRX may suppress the cell cycle and lead to the decreased cell proliferation and hypertrophy of the adipocytes.

The mRNA levels of 10 genes involved in chemotaxis, including 7 chemokines, were higher in TSOD mice than in TSNO mice but were decreased by β-CRX intake (Table 5). Chemokines are proteins that are induced during an immune response to recruit cells from the immune system to the site of infection, thereby resulting in inflammation. The up-regulation of the chemokines indicates that obesity in TSOD mice may result in chronic inflammation in adipose tissues and β-CRX may inhibit it. Among these chemokines, CXCL10 was reported to increase in adipocytes treated with lipopolysaccharide, via the IFN-β pathway.[32]

The quantitative RT-PCR experiment confirmed the DNA microarray results (Table 7). MCP-1 is a chemotactic protein and is known as a strong inducer of inflammation. Adipocytes in TSOD control mice exhibited an increase in MCP-1 expression, indicating severe inflammation of adipocytes. TNF-α up-regulation and adiponectin down-regulation are also consistent with the inflammatory state. β-CRX administration resulted in

MCP-1 repression and adiponectin up-regulation, indicating the severe inflammation of adipocytes was reduced by the administration of β-CRX.

Our results suggest that the oral administration of β-CRX can reduce proliferation, hypertrophy, secretion of inflammatory chemokines, and excess immune responses in adipocytes. It is well-known

Table 4. Genes Regulated in the Liver by the Oral Ingestion of β-CRX in TSOD Mice

GO ID symbol	GO biological process gene name	fold change TSOD/−CRX vs TSNO/−CRX	fold change TSOD/+CRX vs TSOD/−CRX
GO:0006270	**DNA replication initiation**		
Mcm2	minichromosome maintenance deficient 2	3.25	0.45
Mcm4	minichromosome maintenance deficient 4	3.32	0.45
Mcm5	minichromosome maintenance deficient 5	4.82	0.28
Mcm6	minichromosome maintenance deficient 6	4.82	0.29
GO:0045455	**steroid metabolic process**		
Sult2a2	sulfotransferase family 2A2	3.70	0.28
Pctp	phosphatidylcholine transfer protein	5.85	0.31
Hdlbp	high-density lipoprotein binding protein	4.26	0.33
Mbtps1	membrane-bound transcription factor peptidase, site 1	4.22	0.37
Nr3c1	nuclear receptor subfamily 3, group C, member 1	4.05	0.44
Abca1	ATP-binding cassette, subfamily A, member 1	3.58	0.45
Cyp51	cytochrome P450, family 51	0.48	2.07
Hmgcs1	3-hydroxy-3-methylglutaryl-coenzyme A synthase 1	0.39	2.16
Idi1	isopentenyl-diphosphate δ isomerase	0.25	3.95
GO:0045859	**regulation of protein kinase activity**		
Aplp2	amyloid β (A4) precursor-like protein 2	4.69	0.36
Hgf	hepatocyte growth factor	3.14	0.37
Met	met proto-oncogene	2.54	0.40
Ceacam1	carcinoembryonic antigen-related cell adhesion molecule 1	4.12	0.42
Ptpn11	protein tyrosine phosphatase, nonreceptor type 11	2.17	0.47
Shc1	src homology 2 domain-containing transforming protein C1	2.50	0.48
Nf2	neurofibromatosis 2	2.41	0.49
Tgfbr2	transforming growth factor, beta receptor II	2.12	0.49
Lats2	large tumor suppressor 2	0.43	2.17
Gtpbp4	GTP binding protein 4	0.35	2.91

Table 5. Genes Regulated in the Adipocytes by the Oral Ingestion of β-CRX in TSOD Mice

GO ID symbol	GO biological process gene name	fold change TSOD/−CRX vs TSNO/−CRX	fold change TSOD/+CRX vs TSOD/−CRX
GO:0007049	**cell cycle**		
Ereg	epiregulin	8.44	0.14
Birc5	baculoviral IAP repeat-containing 5	6.78	0.25
Mki67	antigen identified by monoclonal antibody Ki 67	4.85	0.27
Cenpf	centromere protein F	3.21	0.28
Cdk1	cell division cycle 2 homologue A	4.62	0.29
Ccnb2	cyclin B2	4.15	0.29
Mcm6	minichromosome maintenance deficient 6	4.26	0.30
Bub1	budding uninhibited by benzimidazoles 1	4.69	0.32
Cdca5	cell division cycle associated 5	4.56	0.32
Cep55	centrosomal protein 55	6.53	0.33
Ccna2	cyclin A2	3.83	0.34

Table 5. Continued

GO ID symbol	GO biological process gene name	fold change TSOD/−CRX vs TSNO/−CRX	fold change TSOD/+CRX vs TSOD/−CRX
Kif11	kinesin family member 11	4.01	0.35
Prc1	protein regulator of cytokinesis 1	3.78	0.36
Aspm	asp (abnormal spindle)-like, microcephaly associated	3.75	0.37
Nusap1	nucleolar and spindle associated protein 1	3.25	0.40
Hells	helicase, lymphoid specific	2.64	0.40
Ccdc99	coiled-coil domain containing 99	2.25	0.40
Brca1	breast cancer 1	2.53	0.42
Spag5	sperm associated antigen 5	2.41	0.42
Ccnb1	cyclin B1	2.73	0.43
Tpx2	TPX2, microtubule-associated protein	2.82	0.43
C79407	expressed sequence C79407	2.82	0.44
Ska1	RIKEN cDNA 2810433K01 gene	2.44	0.44
Nuf2	NDC80 kinetochore complex component	3.11	0.45
Mad2l1	mitotic arrest deficient-like 1	2.48	0.45
Spc25	SPC25, NDC80 kinetochore complex component	4.45	0.45
Trip13	thyroid hormone receptor interactor 13	3.09	0.45
Plk2	polo-like kinase 2	3.23	0.46
Uhrf1	ubiquitin-like, containing PHD and RING finger domains, 1	2.27	0.46
Aurkb	aurora kinase B	4.03	0.46
Cdca3	cell division cycle associated 3	2.99	0.47
Cdt1	chromatin licensing and DNA replication factor 1	2.42	0.47
Dbf4	DBF4	3.14	0.47
Ndc80	NDC80 homologue, kinetochore complex component	2.35	0.48
Ckap2	cytoskeleton associated protein 2	2.01	0.49
Pttg1	pituitary tumor-transforming gene 1	3.94	0.49
GO:0006935	**chemotaxis**		
Cxcl10	chemokine (C−X−C motif) ligand 10	12.58	0.31
Ccl12	chemokine (C−C motif) ligand 12	2.46	0.33
Ccl7	chemokine (C−C motif) ligand 7	30.74	0.33
Ccl2	chemokine (C−C motif) ligand 2	22.65	0.33
S100a8	S100 calcium binding protein A8	2.15	0.36
Ccl3	chemokine (C−C motif) ligand 3	4.56	0.46
C5ar1	complement component 5a receptor 1	4.77	0.46
Cxcl2	chemokine (C−X−C motif) ligand 2	2.76	0.46
Ccl4	chemokine (C−C motif) ligand 4	3.02	0.47
C3ar1	complement component 3a receptor 1	2.85	0.49
GO:0002520	**immune system development**		
Timp1	tissue inhibitor of metalloproteinase 1	5.98	0.24
Ccnb2	cyclin B2	4.15	0.29
Stap1	signal transducing adaptor family member 1	8.90	0.32
Il7r	interleukin 7 receptor	4.96	0.40
Hells	helicase, lymphoid specific	2.64	0.40
Tnfrsf11a	tumor necrosis factor receptor superfamily, member 11a	2.63	0.45
Runx1	runt related transcription factor 1	3.29	0.46
Bcl3	B-cell leukemia/lymphoma 3	3.40	0.47
Vav1	vav 1 oncogene	3.03	0.48
Tiparp	TCDD-inducible poly(ADP-ribose) polymerase	0.29	2.08
Zbtb16	zinc finger and BTB domain containing 16	0.36	2.41
Pik3r1	phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1	0.31	2.90

Table 6. Genes Regulated in the Muscle by the Oral Ingestion of β-CRX in TSOD Mice

GO ID symbol	GO biological process gene name	fold change TSOD/−CRX vs TSNO/−CRX	fold change TSOD/+CRX vs TSOD/−CRX
GO:0060048	**cardiac muscle contraction**		
Myh7	myosin, heavy polypeptide 7, cardiac muscle, β	4.67	5.03
Myl2	myosin, light polypeptide 2, regulatory, cardiac, slow	5.35	8.87
Tpm3	tropomyosin 3, γ	2.87	7.15
Tnnc1	troponin C, cardiac/slow skeletal	2.59	8.76
GO:0006633	**fatty acid biosynthetic process**		
Elovl6	ELOVL family member 6	0.08	0.29
Scd1	stearoyl-coenzyme A desaturase 1	0.09	0.33
Fasn	fatty acid synthase	0.05	0.48
Myo5a	myosin VA	0.50	2.32
GO:0006869	**lipid transport**		
Apoa1	apolipoprotein A-I	2.74	0.27
Apoa2	apolipoprotein A-II	2.34	0.36
Apoc3	apolipoprotein C-III	2.31	0.38
Apoc1	apolipoprotein C-I	0.28	0.44
Rbp4	retinol binding protein 4, plasma	0.20	0.48
GO:0042060	**wound healing**		
Fga	fibrinogen α chain	4.35	0.22
Kng1	kininogen 1	2.57	0.35
Serpinc1	serine peptidase inhibitor, clade C, member 1	2.42	0.48

Table 7. mRNA Expression in Adipocyte

	TSNO			TSOD		
	−CRX	+CRX	*p* value	−CRX	+CRX	*p* value
adiponectin	1.00 ± 0.01	1.52 ± 0.65	0.358	0.59 ± 0.07	1.13 ± 0.21	0.022 *
MCP-1	1.00 ± 0.31	1.00 ± 0.06	0.998	60.56 ± 6.10	16.67 ± 9.33	0.031 *
TNF-α	1.00 ± 0.24	1.47 ± 0.14	0.141	5.05 ± 0.07	2.92 ± 0.71	0.051

that adipocyte differentiation is regulated by PPARγ. PPAR is a nuclear receptor identified in *Xenopus*,[33] and the expression of the γ-subtype in preadipocytes accelerates their maturation.[34] Further investigation revealed that PPARγ activation is the key step in preadipocyte maturation.

Recently, PPARγ has become a major target for the treatment of metabolic syndrome or diabetes. For example, thiazolidinedione, a well-known synthetic PPARγ agonist, is widely used as a drug for diabetes.[35] PPARγ is also thought to be a regulator of inflammation because thiazolidinedione has also been reported to suppress the secretion of inflammatory cytokines and chemokines.[36] Our recent investigation revealed that β-CRX is able to negatively modulate PPARγ activity.[37] When these results are taken together, β-CRX may act to modulate PPARγ signaling, resulting in repression of adipocyte hypertrophy and its chronic inflammation.

In TSOD obese mice, the weight of liver was heavier than that in the TNSO control mice, whereas the TG, TC, and NEFA levels were lower than those in the TNSO mice. These differences may be the result of abnormalities in liver metabolism. Microarray analysis showed that steroid metabolism was altered in the liver of TSOD mice compared to that in TSNO ones. The genes involved in sterol synthesis (Hmgcs1, Idi1, and Cyp51) were down-regulated in TSOD obese mice, whereas those encoding cholesterol transporters (Abca1 and Hdlbp) were up-regulated. However, β-CRX uptake reversed these trends in the expression of these genes, suggesting that β-CRX can correct disordered lipid metabolism and transport in obese mice.

Together with dietary restriction, exercise is the first choice in the prevention of metabolic syndrome. Muscular development is important not only for increasing energy consumption but for improving insulin resistance.[38] The results of microarray analysis showed that genes involved in muscle contractions were up-regulated in TSOD mice compared to TSNO ones (Table 6). Moreover, these genes were up-regulated by the oral administration of β-CRX in TSOD mice. These results suggest that β-CRX may enhance muscular development and/or contraction. The genes involved in fatty acid metabolism and lipid transfer were also down-regulated in muscle by the intake of β-CRX.

Among them, Scd1, Apoa1, Apoa2, and Apoc3 are known to be regulated by PPARα transcription regulator.[39] The changes in the expression of these genes caused by β-CRX in muscle may result in the modulation of lipid transport and its metabolism via the PPARα signaling pathway. Together with the results in the adipocytes, the PPAR pathway may be a target of β-CRX in both muscle and adipose tissue.

In this study, we demonstrated that the oral administration of β-CRX repressed body weight and adipocyte hypertrophy in obese model mice. These changes may prevent the decline of beneficial adipokines and the escalation of inflammatory messengers; the findings from the microarray analysis were consistent with a healthier state of signaling. Serum lipid repression was also observed and corresponded well to the results of the microarray analysis. These results strongly suggest that β-CRX modulates the expression of a number of genes, including the PPAR signaling pathway.

These results were consistent with the previous human trial,[37] and the genes that were affected by β-CRX administration were mostly highly conserved between mice and humans, which suggests that there could be a common mechanism in humans as well.

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ABBREVIATIONS USED

β-CRX, β-cryptoxanthin; EPSM, enzyme-processed Satsuma mandarin; RT-PCR, reverse transcriptase-polymerase chain reaction; HE, hematoxylin−eosin; MCM, mini-chromosome maintenance; NEFA, nonesterified fatty acid; TC, total cholesterol; TG, triglyceride; TSNO, Tsumura Suzuki non-obese, diabetes; TSOD, Tsumura Suzuki obese, diabetes.

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