カキ果皮抽出物摂取による肝臓でのインスリンシグナル伝達経路に関する遺伝子発現の変化：2型糖尿病 Goto-Kakizaki ラットを用いた DNA マイクロアレイ解析

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Hepatic Gene Expression of the Insulin Signaling Pathway Is Altered by Administration of Persimmon Peel Extract: A DNA Microarray Study using Type 2 Diabetic Goto-Kakizaki Rats

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Persimmon (*Diospyros kaki*) is a very popular fruit in East Asian countries, but its peels are not consumed despite the fact that they contain many antioxidants such as carotenoids and polyphenols. We prepared a fat-soluble extract from persimmon peel (PP) and fed type 2 diabetic Goto-Kakizaki (GK) rats an AIN-93G rodent diet supplemented with persimmon peel extract (PP diet) for 12 weeks. Compared with the control AIN-93G diet, the PP diet significantly reduced plasma glutamic-pyruvate transaminase activity, with accumulation of β-cryptoxanthin in the liver. DNA microarray analysis revealed that the PP diet altered hepatic gene expression profiles. In particular, expression of insulin signaling pathway-related genes was significantly enriched in differentially expressed gene sets. Moreover, Western blotting analysis showed an increase in insulin receptor beta tyrosine phosphorylation in rats fed the PP diet. These data suggest that the PP diet improves insulin resistance in GK rats.

Key words: persimmon peel; type 2 diabetes mellitus; Goto-Kakizaki rat; carotenoid

INTRODUCTION

The persimmon (*Diospyros kaki*) is a fruit, which is cultivated primarily in East Asian countries such as China, South Korea and Japan. Persimmon leaves are consumed as a tea and have antihypertensive effects [1], and the calyx has been used as a traditional Chinese medicine treatment for hiccups [2]. The fruit is rich in vitamins, minerals, carotenoids and polyphenols [3], and it has been reported that the concentration of nutrients and other functional components is higher in the peel than in the pulp [4]. However, the persimmon peel (PP) is usually discarded during dried persimmon processing. We have prepared a fat-soluble PP extract enriched in polyphenols and carotenoids comprising β-cryptoxanthin at a concentration of 13.4 mg/g [5].

β-Cryptoxanthin is one of the main carotenoids found in human blood [6]. Previous studies have shown a relationship between the intake of carotenoids and a reduced risk of diabetes [7] and cancer [8]. Intake of β-cryptoxanthin is associated with a reduced risk of type 2 diabetes mellitus (T2DM) [9] and 10-week administration of freeze-dried Satsuma mandarin (*Citrus unshiu* Marc.) juice containing this carotenoid decreased plasma glucose levels in the Goto-Kakizaki (GK) rat, a T2DM animal model [10]. Administration of β-cryptoxanthin via a stomach tube for 7 days also decreased serum glucose in a streptozotocin-induced type 1 diabetic rat model [11]. Since PP extract contains carotenoids, primarily β-cryptoxanthin, we hypothesized that it would also improve diabetes.

T2DM is a complicated disorder that is influenced by both genetic and environmental factors [12]. Glycemic control through dietary management is very important to prevent progression of this disorder. However, it is difficult for many patients to adhere to a strict diet. Hence, glycemic control through a normal diet supplemented with a functional food component could improve quality of life for patients.

GK rats were created by selective breeding of animals selected from a population of normal Wistar rats using lowered glucose tolerance as an index [13], and have been used for research as a human T2DM model. Although GK rats show insulin resistance in the liver [14], this symptom is relatively mild.

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Therefore, this rat is a useful model to evaluate the effects of a functional food component on the prevention and treatment of diabetes.

To understand the mechanism by which PP extract exerts its antidiabetic effects, we used GK rats and examined the effects of a PP diet in these rats using DNA microarray technology [15, 16]. In addition, we measured phosphorylation level of some proteins using Western blotting to confirm the results of the microarray data.

MATERIALS AND METHODS

Animals and feeding

Male GK rats (90–107 g), aged 5 weeks, were purchased from Japan SLC Co. (Hamamatsu, Japan). The rats were maintained at 22±1°C under a12-h light-dark cycle (lights on at 08:00), and housed individually using wire cages. They were administered a commercial diet (AIN-93G, Research Diets Inc) for one week. The rats were allowed free access to food and water.

After one week, the rats were divided into two groups with similar average body weights. Control group (n = 7) continued to receive the commercial diet (control diet) and PP group (n = 7) was fed the same diet containing PP extract (PP diet; Table 1). In both groups, the feeding period was 12 weeks, and body weights and food intake were measured every other day during the test period.

After 12 weeks of feeding, each rat was anesthetized with pentobarbital after fasting for 3 hours. The blood was collected from the carotid artery. The liver was excised, a portion was treated with RNAlater (Ambion, Austin, TX) for subsequent RNA analysis, and the remainder was frozen in liquid nitrogen and stored at -80°C until use. The protocol for the animal experiments was approved by the Animal Use Committee of the Faculty of Agriculture at The University of Tokyo.

Table 1 Composition of experimental diets

Ingredients	Control diet (g)	PP diet (g)
Casein	200	200
L-Cystine	3	3
Corn starch	397.486	397.486
Sucrose	100	100
Maltodextrin	132	132
Cellulose	50	50
Soybean oil	70	70
t-Butylhydroquinone	0.014	0.014
Mineral mix S10022G	35	35
Vitamin mix V10037	10	10
Choline bitartrate	2.5	2.5
PP extract	—	0.0373[a]
Total	1000	1000.0373

[a] PP extract was added to the AIN-93G diet. 0.0373 g of the PP extracts contained 0.5 mg of β-cryptoxanthin.

Measurement of biochemical parameters in plasma.

Plasma glucose, triglyceride, glutamate oxaloacetate transaminase (GOT), glutamic-pyruvate transaminase (GPT), creatinine, HDL cholesterol (HDL-C), total cholesterol (TC), and non-esterified fatty acid (NEFA) were measured using a colorimetric enzymatic assay kit from WAKO Pure Chemical Industries (Osaka, Japan), and plasma alkaline phosphatase (ALP) activity was measured using an assay kit from Kainos Laboratories (Tokyo, Japan). Plasma insulin was measured using a commercially available ELISA kit (Morinaga Institute of Biological Science Inc., Yokohama, Japan).

Analysis of carotenoids in livers

Frozen liver samples (0.5−0.8 g) were treated with 1 ml of methanol containing 0.1% dibutyl hydroxyl toluene (BHT) and homogenized with 0.2 g of zirconia beads. Aliquots of the homogenates were added to 9 ml of methanol containing 0.1% BHT and 10 ml of 12% KOH, and saponified under N_2 gas for 45 min at 60°C. Aliquots of the reaction solution were added to 10 ml of hexane containing 0.1% BHT and 10 ml of H_2O, maintained at 4°C, and then centrifuged at 1700 × *g* for 10 min. The hexane layer was then washed with 10% saline and evaporated to dryness under reduced pressure. Before HPLC analysis, each residue was dissolved in ethanol containing 0.1% BHT and filtered (Duo-Filter, YMC Co. Ltd., Kyoto, Japan).

The levels of β-cryptoxanthin in liver samples were quantified using a Shimadzu (Kyoto, Japan) LC-20A HPLC system equipped with a UV detector (LC-20AV). The mobile phases used in this study were: (A) methanol-MTBE-H_2O (81:15:4, v/v/v) containing 0.1% (w/v) ammonium acetate and (B) methanol-MTBE (10:90, v/v) containing 0.1% (w/v) ammonium acetate. HPLC separation was carried out on a C_{30} column (250 mm × 4.6 mm, 5 μm particle size, YMC Co. Ltd.) with mobile phases (A) and (B) in a linear gradient for 60 min. The flow rate was set at 1 ml/min, detection was performed at 450 nm, and the column temperature was maintained at 32°C. β-Cryptoxanthin was identified by comparing the retention time of the peak with that of the authentic sample (Extrasynthese, Genay, France).

DNA microarray experiments

Total RNA was isolated from the liver using ISOGEN (Nippon Gene Co., Toyama, Japan) and then purified with an RNeasy mini kit (QIAGEN K.K., Tokyo, Japan). The quality and quantity of total RNA were assayed by agarose gel electrophoresis and spectrophotometry, respectively. In each group, four of the seven rats exhibiting mutually similar plasma GPT levels were selected. Total RNA was collected from these individuals, and then a DNA microarray analysis was performed

as described previously [17]. In brief, cDNA was synthesized from 2 μg of purified total RNA, and biotinylated cRNA was then transcribed using T7 RNA polymerase for fragmentation and added to an Affymetrix Rat Genome 230 2.0 Array (Affymetrix, Santa Clara, Ca). Following hybridization at 45°C for 16 h, the array was washed and labeled with phycoerythrin. Fluorescence signals were scanned using the Affymetrix GeneChip System. All microarray data were submitted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/, GEO Series ID GSE21417).

DNA microarray data analysis

Affymetrix GCOS software was used to reduce the array images to the intensity of each probe (CEL files). CEL files were quantified with Factor for Robust Microarray Summarization (FARMS) [18], using the statistical language R [19] and Bioconductor [20]. The annotation file for the Rat Genome 230 2.0 Array was downloaded from Affymetrix (cited on 8th July, 2008, from http://www.affymetrix.com/). Hierarchical clustering was performed by the pvclust() function in R. The RankProducts (RP) method [21] was used to extract differentially expressed genes from the two groups according to guidelines from a previous study [22]. Taking the false discovery rate (FDR) into account, we determined the differentially expressed genes (FDR < 0.05). Up- and downregulated genes were submitted to gene functional enrichment analysis using the Functional Annotation Tool from the Database for Annotation, Visualization, and Integrated Discovery (DAVID) [23, 24] (cited on 11th March, 2009, from http://david.abcc.ncifcrf.gov/).

Real-time PCR

Total RNA from the liver was extracted and purified as described earlier. cDNA was synthesized from 200 ng of the total RNA using PrimeScript 1st strand cDNA synthesis kit (Takara Bio. Inc., Shiga, Japan). Real-Time PCR analysis was performed using SYBR Green method and Thermal Cycler Dice Real Time System (Takara Bio Inc.). The following primer sequences were used for target genes. Sterol regulatory element-binding protein 1c (*Srebp-1c*): forward 5'-AGT TCC AGC ATG GCT ACC AC-3', reverse 5'-CTT GGG GAA TGT GCT CTA CC-3'; liver X receptor alpha (*Lxrα*): forward 5'-ACC CTT GCA TGG CAC TAA AG-3', reverse 5'-AAG AAC CCT GCA CAA AGT GG-3'; carnitine palmitoyltransferase 1 (*Cpt1*): forward 5'-ATG ACG GCT ATG GTG TCT CC-3', reverse 5'-GGC TTG TCT CAA GTG CTT CC-3'; protein tyrosine phosphatase receptor type sigma (*Ptpσ*): forward 5'-TGG CTC TAG TGA GAA GCG TGA G-3', reverse 5'-CGC AGA AAC GCC AGG AA-3'; hypoxanthine phosphoribosyltransferase (*Hprt*): forward 5'- GGC CAG TAA AGA ACT AGC AGA CG-3', reverse 5'-AAA GGG ACG CAG CAA CAG A-3'. Thermal cycling was carried out with under the following condition (95°C, 10s; and 45 cycle at 95°C, 5s; 55°C, 30s; 72°C, 20s). Relative expression quantity of each sample was calibrated by the standard curve method and the expression quantity of *Hprt* was used for normalization.

Immunoprecipitation and western blot analysis

Frozen liver tissue, weighing approximately 100 mg per sample, was homogenized with a polytron homogenizer (Kinematica Inc., Bohemia, NY) in 1 ml of lysis buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol) containing 1% proteinase inhibitor cocktail, phosphatase inhibitor cocktail, and phosphatase inhibitor cocktail 2 (SIGMA, St. Louis, MO). The lysates were maintained at 4°C and centrifuged at 18000 × *g* for 10 min to remove tissue debris, and the resulting protein concentrations were measured using the Bradford method [25]. For immunoprecipitation, the protein concentration of the lysate was adjusted to 1 mg/ml with the lysis buffer containing the inhibitors, and then incubated with 4 μg of specific antibody against insulin receptor beta (IRβ), insulin receptor substrate (IRS) 1, or IRS2 (Upstate Biotechnology Inc., Lake Placid, NY), for 1 h at 4°C. The antibodies were added to 20 μl of a 50% protein A sepharose bead slurry in lysis buffer and purified after incubation at 4°C overnight. Immunoprecipitates were then washed 5 times with the lysis buffer. The beads were suspended in SDS-loading buffer (17 mM Tris-HCl, pH 6.8, 1.7% SDS, 7.6% glycerol, 0.0025% bromophenol blue, 5% β-mercaptoethanol) and heated for 5 min at 100°C. The samples were then subjected to 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to a PVDF membrane, and the membrane was blocked with 5% BSA in Tris-buffered saline (150 mM NaCl, 10 mM Tris, pH 7.4) for 1 h. It was then incubated with anti-IRβ, anti-IRS1, anti-IRS2, or anti-phosphotyrosine (4G10, Upstate Biotechnology Inc.) antibody and washed prior to reaction with peroxidase-conjugated secondary antibody (anti-rabbit IgG or anti-mouse IgG, GE Healthcare). Signals were visualized by chemiluminescence reactions with the Western Lightning System (PerkinElmer, Waltham, MA), and the images were processed using an LAS-4000 lumino-image analyzer (Fujifilm, Tokyo, Japan). The band strengths were measured with ImageQuant TL software (Fujifilm, Tokyo, Japan) for image analysis.

Statistical analysis

Body weight, food intake, biochemical parameters of the plasma, and carotenoid concentration in the liver were expressed as means ± SEM. Between-group differences were considered significant at $p < 0.05$, using a non-paired Student's *t*-test.

RESULTS

Changes in body weight, food intake, and plasma biochemical parameters of GK rats during feeding

We analyzed the effects of PP extract on body weight, food intake, and plasma biochemical parameters and did not find any significant between-group differences in body weight (Fig. 1A) or food intake (Fig. 1B). There were no significant between-group differences in plasma glucose or triglyceride levels (Table 2). Plasma insulin, GOT activity, HDL-C, TC, ALP activity, creatinine, and NEFA also showed no significant differences (Table 2). Plasma GPT activity was significantly lower in animals fed the PP diet, although it remained within the normal range. After PP administration for 11 weeks, intraperitoneal glucose tolerance was also similar between the 2 groups.

HPLC was used to confirm whether carotenoids in the PP extract were absorbed from the intestine and accumulated in the liver. β-Cryptoxanthin was detected only in livers of the PP group (Table 2).

Changes in gene expression profiles and pathway analysis

Plasma GPT activity, a marker of hepatic function, was significantly lower in the PP group than in the control group, and accumulation of β-cryptoxanthin was found to occur only in the livers of the PP group, suggesting that PP extract induces physiological changes in the liver. Therefore, the effects of PP extract on hepatic gene expression profile were evaluated by DNA microarray. We applied FARMS (qFARMS) to quantify the raw data (Affymetrix CEL files). Hierarchical clustering analysis revealed that each group formed a distinct cluster except for one sample in the PP group. To detect genes differentially expressed in the control and PP groups, the RP method was applied to qFARMS-quantified data. 4Taking into account an FDR lower than 0.05, we selected 937 genes upregulated and 1263 genes downregulated by PP extract administration.

To identify overrepresented pathways in the selected genes, we used the online software DAVID. Significantly enriched pathways were classified according to the KEGG pathway database (cited on 11th March, 2009, from http://www.genome.jp/kegg/pathway.html). The upregulated gene set showed enrichment in genes involved in 7 pathways, and the downregulated gene set showed enrichment in genes involved in 28 pathways (Tables 3 and 4, respectively). As shown in Fig. 2, the relationships between these pathways can be summarized by focusing on common genes in the different pathways. In the downregulated gene set, the key pathway was “signal transduction”. Other pathways such as “cancer”, “endocrine system”, “cell growth and death”, “cell communication”, “development”, and “immune system” were involved in signal transduction. In the upregulated gene set, the key pathway

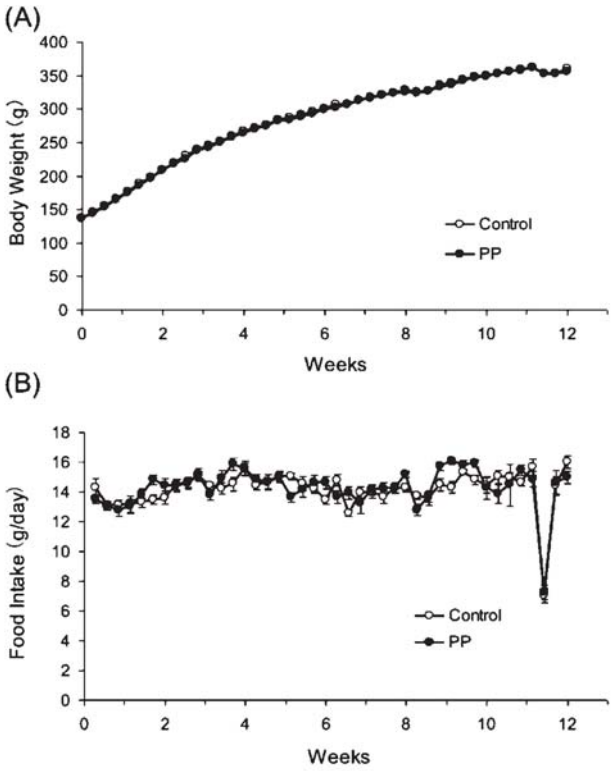

Fig. 1 Changes in body weight (A) and food intake (B) of GK rats fed a diet containing PP extract for 12 weeks. On week 11 after starting the test diet, body weight and food intake decreased because rats were fasted for an IPGTT. Body weight and food intake were measured every other day. Values are represented as means ± S.E.M.

was the “endocrine system”, including the “insulin signaling pathway”. Endocrine system-related pathways, including “Metabolic disorders”, “lipid metabolism”, and “cell motility” were also upregulated. Among significantly overrepresented pathways, only the insulin signaling pathway appeared in both the up- and downregulated gene sets. Moreover, “maturity onset diabetes of the young (MODY)”, included in metabolic disorders and one of the significantly enriched pathways in the upregulated gene set, is a type of genetic non-insulin-dependent diabetes mellitus developed young, usually by 25 years of age in humans [26]. These results indicate that administration of PP extract has antidiabetic effects. Therefore, we next focused on insulin signaling pathway-related genes, and evaluated the effects of PP extract on T2DM.

Table 2. Plasma parameters and concentration of β-cryptoxanthin in the livers of GK rats after administration of PP extract for 12 weeks

Parameters	Control	PP
Plasma		
Glucose (mg/dl)	339.84 ± 7.16	332.84 ± 6.96
Triglyceride (mg/dl)	56.81 ± 10.95	69.02 ± 9.14
Insulin (ng/ml)	1.13 ± 0.03	1.18 ± 0.02
GOT (KU)	42.30 ± 1.07	40.94 ± 1.94
GPT (KU)	16.63 ± 0.87	13.54 ± 0.67[a]
HDL-C (mg/dl)	64.82 ± 2.06	64.16 ± 2.98
TC (mg/dl)	89.54 ± 4.03	93.85 ± 3.31
ALP activity (K-A)	0.84 ± 0.13	0.93 ± 0.07
Creatinine (mg/dl)	0.88 ± 0.03	0.93 ± 0.02
NEFA (mEq/l)	0.41 ± 0.03	0.42 ± 0.05
Liver		
β-Cryptoxanthin (ng/g)	<DL[b]	132.9 ± 11.8

[a] Student’s *t*-test, $P < 0.05$. [b] DL indicates detection limit. Values are represented as the means ± S.E.M. of 7 animals in each group.

Table 3. Significantly enriched KEGG pathways in the upregulated gene set

Pathway	Number of upregulated genes [a]	All genes in pathway [b]	*P*-value [c]
Human disease			
Metabolic Disorders			
Maturity onset diabetes of the young	5	26	0.093
Metabolism			
Metabolism of Other Amino Acids			
Glutathione metabolism	8	32	0.004
Lipid Metabolism			
Fatty acid biosynthesis	3	5	0.039
Cellular process			
Endocrine system			
Insulin signaling pathway	14	120	0.057
Cell Motility			
Regulation of actin cytoskeleton	19	190	0.083
Behavior			
Circadian rhythms	4	11	0.033
Genetic information processing			
Translation			
Ribosome	11	80	0.040

[a] The number of upregulated gene in the pathway by PP-treated. [b] The number of all genes in the pathway. [c] *P*-values are represented as EASE scores, a modified Fisher’s Exact *P*-value.

Table 4. Significantly enriched KEGG pathways in the downregulated gene set

Pathway	Number of downregulated genes[a]	All genes in pathway[b]	*P*-value [a]
Human disease			
Cancer			
Colorectal cancer	16	75	0.001
Pancreatic cancer	13	68	0.008
Renal cell carcinoma	13	68	0.008
Endometrial cancer	9	48	0.037
Non-small cell lung cancer	9	55	0.074
Chronic myeloid leukemia	11	77	0.092
Metabolism			
Amino acid metabolism			
Glutamate metabolism	8	24	0.002
Arginine and proline metabolism	7	26	0.016
Glycine, serine, and threonine metabolism	7	33	0.047
Lipid metabolism			
Fatty acid metabolism	9	42	0.018
Biosynthesis of steroids	5	22	0.098
Cellular processes			
Cell communication			
Adherens junction	15	66	0.001
Focal adhesion	25	175	0.007
Cell growth and death			
Apoptosis	16	76	0.001
Endocrine system			
Insulin signaling pathway	19	120	0.007
PPAR signaling pathway	12	72	0.029
Immune system			
Leukocyte transendothelial migration	18	101	0.003
Toll-like receptor signaling pathway	14	78	0.009
B cell receptor signaling pathway	11	60	0.021
Natural killer cell-mediated cytotoxicity	12	87	0.092
Development			
Axon guidance	17	100	0.006
Environmental Information Processing			
Signal transduction			
MAPK signaling pathway	30	238	0.016
VEGF signaling pathway	12	67	0.018
Wnt signaling pathway	18	124	0.021
mTOR signaling pathway	9	46	0.030
ErbB signaling pathway	13	86	0.044
Jak-STAT signaling pathway	14	108	0.097
Genetic Information Processing			
Folding, sorting and degradation			
Ubiquitin-mediated proteolysis	15	117	0.090

[a] The number of downregulated gene in the pathway by PP-treated. [b] The number of all genes in the pathway. [c] See Table 3 for *P*-value definition.

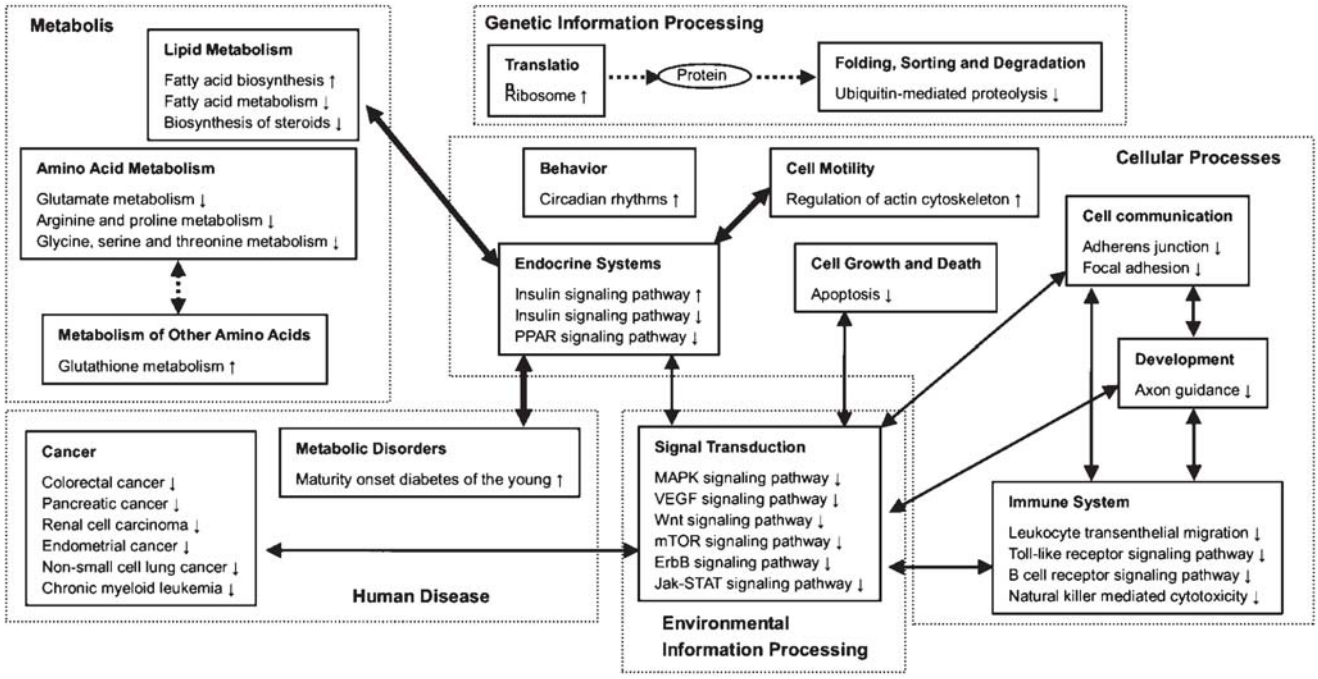

Fig. 2　Relationship between up- and downregulated pathways.　The dotted- and solid-lined squares indicate broad categories and subcategories of KEGG pathways, respectively.　The heavy- and thin-lined arrows indicate the relationships between pathways containing common up- and downregulated genes, respectively.　The relationships between pathways containing one or no common genes are not shown.　The dotted-lined arrows indicate mutually related pathways that have no common genes.

Insulin signaling pathway

As shown in Tables 5 and 6, insulin signaling pathway-related genes were extracted from the differentially expressed gene set. In the upregulated gene set, the transcription factors *Srebp-1c, Lxrα,* and *Foxa2*; glycolysis-related genes *Pyk*, *Pfk* and *Gk*; and genes for key enzymes in fatty acid synthesis, *Acc* and *Fas*, were identified, while the β-oxidation-related gene *Cpt1* and gluconeogenesis-related gene *G6pc* were found among the downregulated genes. Moreover, expression of *Ptpσ* was significantly decreased in the PP group. PTPσ is a transmembrane protein that dephosphorylates IRβ *in vitro* [27], and may negatively regulate insulin signaling. Expression of *Ptpδ/σ* is higher in the livers of GK rats than in normal Wistar rats [28]. This implies that downregulation of *Ptpσ* can improve insulin receptor signaling. We confirmed the changes in the expression of *Ptpσ, Srebp-1c, Lxrα and Cpt1* by real-time PCR. The results were consistent with the microarray data (Fig. 3). The insulin signaling pathway in hepatocytes is schematically represented in Fig. 4.

Tyrosine phosphorylation of IRβ and IRS

If the increased expression of PTPσ in the liver of GK rats promotes dephosphorylation of IRβ and/or IRS, we hypothesized that suppression of PTPσ expression by PP extract should activate tyrosine phosphorylation of these proteins. In order to examine this hypothesis, IRβ and IRS tyrosine phosphorylation in the liver were measured by Western blotting. Total IRβ protein was not different in the PP group than in the control group, but tyrosine-phosphorylated IRβ protein was elevated in the PP group (Fig. 5). However, the levels of total and tyrosine-phosphorylated IRS-1 and IRS-2 protein did not differ between the two groups (Fig. 5).

Table 5. Insulin signaling pathway-related genes upregulated by PP extract administration

Probe set ID	Gene title	Gene symbol	Rank[a]	Fold Change	FDR[b]	Entry name
1386945_a_at	protein kinase, AMP-activated, beta 1 non-catalytic subunit	Prkab1	114	1.41	0.002	AMPK
1375349_at	similar to sorbin and SH3 domain containing 1 isoform 3 / sorbin and SH3 domain containing 1	Sorbs1	115	1.46	0.002	CAP
1368035_a_at	protein tyrosine phosphatase, receptor type, F	Ptprf	119	1.32	0.002	LAR
1398834_at	mitogen activated protein kinase kinase 2	Map2k2	287	1.23	0.008	MEK1/2
1371578_at	protein kinase, cAMP-dependent, catalytic, alpha	Prkaca	435	1.17	0.012	PKA
1367746_a_at	flotillin 2	Flot2	541	1.16	0.016	Flotillin
1369936_at	calmodulin 1	Calm1	650	1.15	0.023	PHK
1386863_at	protein phosphatase 1, catalytic subunit, alpha isoform	Ppp1ca	653	1.12	0.023	PP1
	Transcription factors					
1388426_at	sterol regulatory element binding factor 1	Srebf1	41	1.44	0.001	SREBP-1 c
1371104_at			183	1.26	0.004	
1387365_at	nuclear receptor subfamily 1, group H, member 3	Nr1h3	281	1.22	0.008	LXRα
1368711_at	forkhead box A2	Foxa2	627	1.23	0.021	FOXA2
	Glycolysis					
1387263_at,	pyruvate kinase, liver and red blood cell	Pklr	116	1.47	0.002	PYK
1368651_at			438	1.26	0.012	
1367743_at	phosphofructokinase, liver, B-type	Pfkl	564	1.22	0.017	PFK
1387312_a_at	Glucokinase	Gck	623	1.08	0.021	GK
	Fatty acid synthesis					
1373778_at	acetyl-coenzyme A carboxylase beta	Acacb	78	1.39	0.001	ACC
1370893_at	acetyl-coenzyme A carboxylase alpha	Acaca	495	1.27	0.013	
1367707_at	fatty acid synthase	Fasn	199	1.46	0.004	FAS
1367708_a_at			345	1.35	0.009	

[a] Ranking by Rank products statistic. The smaller rank means the higher upregulation. [b] Genes with a false discovery rate (FDR) of < 0.05 were considered to be significantly changed. Abbreviations: AMPK, AMP-activated protein kinase; CAP, c-Cbl-associated protein; LAR, leukocyte common antigen-related; MEK1/2, extracellular signal-regulated kinase 1/2; PKA, protein kinase A; PHK, phosphorylase kinase; PP1, protein phosphatase 1; SREBP-1c, sterol regulatory element-binding protein 1c; LXRα, liver X receptor alpha; FOXA2, forkhead box A2; PYK, pyruvate kinase; PFK, phosphofructokinase; GK, glucokinase; ACC, acetyl coenzyme-A carboxylase; FAS, fatty acid synthase.

Table 6. Insulin signaling pathway-related genes downregulated by PP extract administration

Probe_ID	Gene Title	Gene Symbol	Rank[a]	Fold Change	FDR	Entry name
1371161_at	protein phosphatase 1, regulatory (inhibitor) subunit 3B	Ppp1r3b	31	0.346	0.000	PP1
1395236_at	protein phosphatase 1, regulatory (inhibitor) subunit 3C	Ppp1r3c	55	0.379	0.000	
1373108_at			367	0.582	0.002	
1386950_at	protein phosphatase 1, catalytic subunit, beta isoform	Ppp1cb	1028	0.732	0.031	
1368646_at	mitogen-activated protein kinase 9	Mapk9	185	0.527	0.000	JNK
1371776_at	phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1	Pik3r1	237	0.558	0.001	PI3K
1387847_at	phosphatidylinositol 3-kinase, catalytic, beta polypeptide	Pik3cb	527	0.679	0.006	
1374232_at	phosphatidylinositol 3-kinase, catalytic, alpha polypeptide	Pik3ca	817	0.746	0.019	
1372727_at	suppressor of cytokine signaling 2	Socs2	411	0.829	0.011	SOCS
1384772_at	protein kinase, cAMP dependent, catalytic, beta	Prkacb	274	0.578	0.001	PKA
1370746_at			531	0.677	0.006	
1378415_at	protein kinase, cAMP dependent, regulatory, type 2, alpha	Prkar2a	1110	0.773	0.036	
1369078_at	mitogen activated protein kinase 1	Mapk1	317	0.602	0.002	ERK1/2
1369912_at	v-crk sarcoma virus CT10 oncogene homolog (avian)	Crk	354	0.600	0.002	CRK2
1368116_a_at	ribosomal protein S6 kinase, polypeptide 1	Rps6kb1	364	0.612	0.002	P70S6K
1369654_at	protein kinase, AMP-activated, alpha 2 catalytic subunit	Prkaa2	384	0.650	0.003	AMPK
1369157_at	phosphodiesterase 3B	Pde3b	463	0.661	0.004	PDE3
1387353_at	thymoma viral proto-oncogene 2	Akt2	577	0.687	0.008	AKT
1371091_at	insulin receptor substrate 2	Irs2	925	0.707	0.024	IRS2
1368385_a_at	growth factor receptor bound protein 2	Grb2	940	0.777	0.025	GRB2
1370488_a_at	protein tyrosine phosphatase, receptor type, D	Ptprd	1171	0.837	0.042	PTPσ
	β-Oxidation					
1367836_at	carnitine palmitoyltransferase 1a, liver	Cpt1a	19	0.310	0.000	CPT1
	Gluconeogenesis					
1370725_a_at	glucose-6-phosphatase, catalytic	G6pc	413	0.589	0.003	G6PC
	Fatty acid synthesis					
1387538_at	acetyl-coenzyme A carboxylase alpha	Acaca	432	0.604	0.003	ACC

[a] Ranking by Rank products statistic. The smaller rank means the higher downregulation. Abbreviations: JNK, c-Jun N-terminal kinase; PI3K, phosphatidylinositol-3-kinase; SOCS, suppressor of cytokine signaling; CRK2, cdc2-related kinase 2; P70S6K, p70 ribosomal S6 kinase; PDE3, phosphodiesterase 3; AKT=PKB, protein kinase B; IRS2, insulin receptor substrate 2; GRB2, growth factor receptor-binding protein 2; PTPσ, protein tyrosine phosphatase receptor type delta/sigma; CPT1, carnitine palmitoyltransferase 1; G6PC, glucose-6-phosphatase.

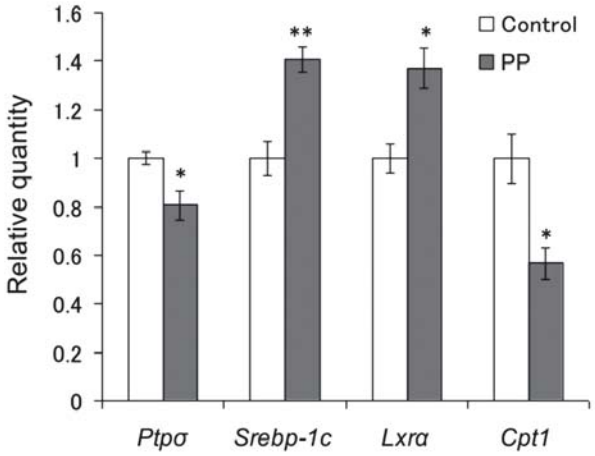

Fig. 3　Real-time quantitative PCR analysis of *Ptpσ*, *Srebp-1c*, *Lxrα* and *Cpt1* from liver of GK rats of control and PP group.　Each value are represented as mean ± S.E.M. (n = 4).　Significant difference were observed at *p<0.05 and **p<0.01 using a non-paired Student's *t*-test.

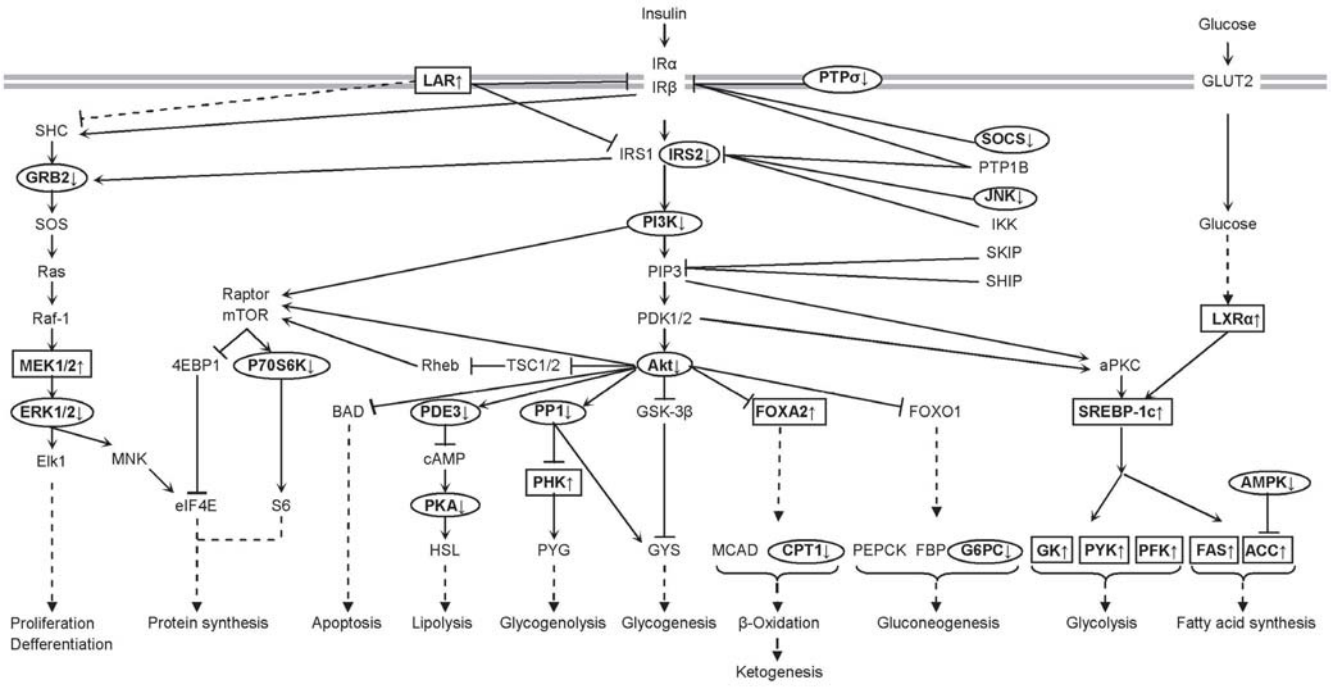

Fig. 4　Relationships between insulin signaling pathways in hepatocytes and insulin-regulated genes [31, 35, 45, 46].　PP1 decreased since Ppp1r3b and Ppp1r3c are more important in glycogen synthesis than Ppp1ca and Ppp1cb.　ACC increased since both Acaca and Acacb are associated with malonyl-CoA synthesis.　AMPK decreased since Prkaa2 is more important than Prkab1.　PKA is composed of regulatory and catalytic subunits, and the latter is separated by binding cAMP, leading to activation.　Thus, because catalytic subunit activity is more prominent, PKA is expected to decrease. Boxes: upregulated gene, round shapes: downregulated gene, solid arrows: activation of the downstream, T-shaped lines: inactivation of the downstream, dotted arrows: indirect activation.

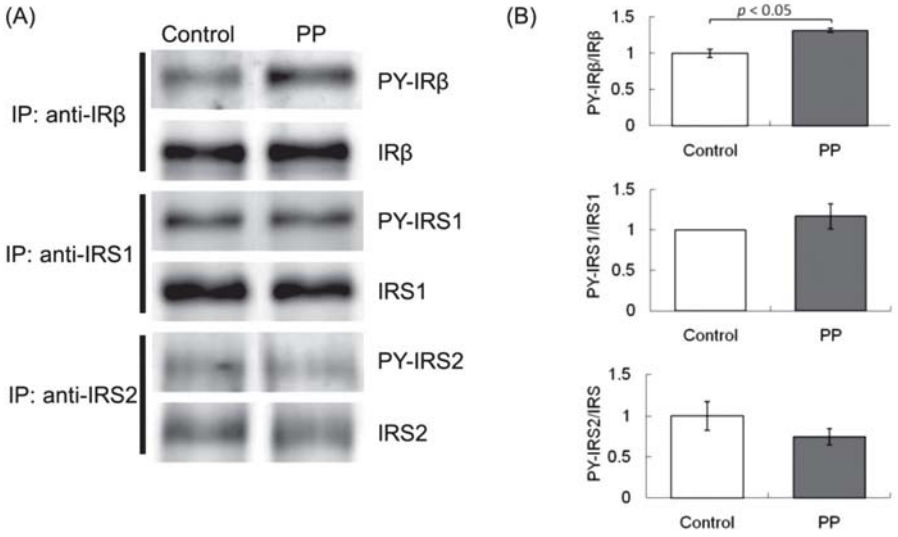

Fig. 5 (A) Protein expression and tyrosine phosphorylation of IRβ, IRS1, and IRS2 in the livers of GK rats fed the PP diet (right) and those fed the control diet (left). (B) Ratio of protein expression and tyrosine phosphorylation of IRβ, IRS1, and IRS2. PY: phosphorylated tyrosine. For details see Materials and Methods.

DISCUSSION

Effects of PP extract in liver

GK rats have been widely used as a reliable animal model of T2DM. A lack of obesity and modest hyperglycemia are characteristic features of GK rats [13, 29]. We used GK rats to examine the effects of PP extract administration on diabetes and found that it did not affect body weight, food intake, or plasma glucose or triglyceride levels. However, gene expression profiles differed in the PP and control groups. β-Cryptoxanthin accumulation was observed in the liver, with a significant decrease in plasma GPT activity (Table 2). Blood GPT activity is used as a liver injury marker because GPT is specifically expressed in the liver, and blood GPT levels are elevated by the destruction of hepatocytes. Since PP extract is rich in antioxidants such as carotenoids, oxidation-derived injury and destruction of hepatocytes can be suppressed by PP administration. It has reported that the β-cryptoxanthin and quercetin in PP extract have a carcinogenesis-preventing effect [8, 30, 31]. Our data showed a decrease in the expression of cancer-related genes, suggesting that PP extract suppresses oxidative stress that can result in carcinogenesis, although further investigation is needed to clarify the mechanism underlying this anti-cancer effect.

Effects of carotenoids in PP extract

Ingested β-carotene and β-cryptoxanthin are converted to vitamin A and then to retinoic acid, which regulates ribosome- and ubiquitin-mediated proteolysis-related genes by binding to the retinoic acid receptor (RAR) [32]. The immune system is also regulated by retinoic acid [33]. These biological events may be induced by retinoic acid produced from carotenoids found in PP extract. It has been reported that when β-carotene is administered to rats long-term, reduced-form glutathione content slightly increases and glutathione S-transferase levels in the liver significantly increase [34]. Glutathione metabolism gene expression was upregulated by administration of PP extract, suggesting that β-carotene may have the ability to activate glutathione.

Relationship between the insulin signaling pathway and PP extract

The insulin signaling pathway is related to diabetes and regulates glucose and lipid homeostasis [35]. The insulin receptor (IR) is comprised of two extracellular α subunits (IRα) and two intracellular β subunits (IRβ). Insulin binds to the IRα and induces tyrosine phosphorylation of IRβ. Tyrosine-phosphorylated IRβ transduces the insulin signal through IRS and other downstream factors, thereby maintaining glucose homeostasis [35]. We found that tyrosine-phosphorylated IRβ was increased in the livers of the PP group (Fig. 5). This increase in phosphorylated IRβ was likely caused by decreased expression of *Ptpσ* caused by the PP diet.

It is known that GK rats have hepatic insulin resistance [14]. Expression of *Ptpσ* in the livers and islets of GK rats is higher than in its genetically background Wistar rats [28]. PTPσ belongs to the LAR family of tyrosine phosphatases [36]. In rats, *Ptpσ* is expressed predominantly in the liver, heart, and testis, and *Ptpσ* dephosphorylates IRβ *in vitro* [27]. Whole-body insulin sensitivity is increased in *Ptpσ*$^{-/-}$ mice [37] and decreased in GK rats [14]. Thus,

it is likely that overexpression of *Ptpσ* contributes to insulin resistance in the livers of GK rats. Elevation of phosphorylated IRβ might improve insulin sensitivity in these animals.

Insulin promotes fatty acid synthesis, glycolysis, glycogenesis, and protein synthesis, and suppresses β-oxidation, gluconeogenesis, glycogenolysis, and apoptosis (*35, 38*). In our microarray data, expression of genes related to fatty acid synthesis and glycolysis was upregulated, while those related to β-oxidation and gluconeogenesis were downregulated in the livers of GK rats fed the PP diet. Moreover, apoptosis-related genes showed decreased expression, while expression of ribosome-related genes was increased in the PP group (Tables 3 and 4). These results suggest that the insulin signaling pathway was activated in the PP group, with a corresponding improvement in insulin sensitivity.

Since *Srebp-1c* and *Lxrα* mRNA are increased in the livers of rats by insulin stimulation [39], enhanced insulin receptor signaling could lead to activation of downstream SREBP-1c and LXR. Activation of SREBP-1c suppresses the expression of *Irs-2* [40, 41] and enhances the expression of *Gk, Pyk, Pfk, Fas,* and *Acc* mRNA, promoting glycolysis and fatty acid synthesis [42, 43]. Furthermore, LXR activates transcription of the *Srebp-1c* gene [44, 45]. Activation of glycolysis may promote glucose uptake in the liver through GLUT2. However, downregulation of *G6pc* expression can decrease gluconeogenesis in the liver. Taken together, it seems likely that the alterations in the gene expression profiles of rats fed the PP diet would result in reduced plasma glucose. However, the plasma glucose of the PP group remained unchanged. There are two possible explanations for this phenomenon. First, given that improvement of hyperglycemia by administration of PP extract to diabetic rats occurs slowly, it is possible that our study duration of 12 weeks was not sufficient to see an improvement in plasma glucose levels. Alternatively, the dose of PP extract used for the experimental diet may have been too low to cause a change in plasma glucose.

Previous studies have reported that dietary intake of Satsuma mandarin juice containing β-cryptoxanthin decreased blood glucose in GK rats during a non-fasting period [10], and that forced administration of β-cryptoxanthin decreased blood glucose in streptozotocin-induced type 1 diabetic rats [11]. Since the PP extract used in this study was rich in β-cryptoxanthin, it may have also had an effect on hyperglycemia. However, there may be synergistic effects with other components, such as quercetin, which has strong antioxidant activity [46] and may have effects on diabetes [47]. Further investigation is required to elucidate the most medically beneficial components of PP extract for hyperglycemia.

In this study, we attempted to evaluate the effects of PP extract on diabetes. Although a resulting phenotypic change was not observed, gene expression profiles obtained from DNA microarray analysis clearly showed between-group differences. Furthermore, our data suggest that the PP extract affects the expression of genes related to the insulin signaling pathway. Thus, we conclude that downregulation of *Ptpσ* through administration of PP extract promotes tyrosine phosphorylation of IRβ, leads to activation of the insulin signaling pathway, and upregulates genes related to both glucose homeostasis and lipid homeostasis. Our results thus suggest that dietary intake of PP extract can help maintain euglycemia.

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