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Transcriptome Analysis of the Duodenum in Wistar Rats Fed a Trypsin Inhibitor Derived from Squid Viscera

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Supporting Information

ABSTRACT: To investigate the effects of oral administration of a trypsin inhibitor (TI), normal Wistar rats were fed a TI derived from squid (*Todarodes pacificus*) for 10 weeks and gene expression profiles in the duodenum, pancreas, liver, and muscle were then analyzed using DNA microarrays. Although no significant changes could be observed in growth, food intake, tissue weight, or blood tests among the tissues tested, the duodenum showed the most remarkable changes in the global gene expression profile. Significant up-regulation of mRNAs encoding gastrin, gastrokine, cholecystokinin and somatostatin in the duodenum was validated by qPCR analysis. In gene ontology (GO) analysis of the up-regulated differentially expressed genes (DEGs), GO terms related to keratinization and innate mucosal defense were enriched ($p < 0.001$) in the category of biological processes in addition to assumable terms such as regulation of secretion and response to nutrients, vesicle-mediated transport, and so forth. In the same analysis, calcium ion binding was listed at the deepest hierarchy in the category of molecular function. These results indicate that the duodenum responds to TI treatment by a wider range of physiological processes than previously assumed such as keratinocyte differentiation and innate mucosal defense, in which calcium plays a crucial role.

KEYWORDS: trypsin inhibitor, transcriptome, Wistar rat, duodenum, gene ontology

INTRODUCTION

It is generally accepted that trypsin inhibitors (TIs) have many pharmaceutical and nutritional effects. The Bowman−Birk inhibitor (BBI) and the Kunitz inhibitor (KI) are the most well characterized TIs, which are purified from soybeans.[1−3] Pharmaceutical and nutritional functions reported for the BBI and KI include lowering cholesterol levels, anticarcinogenic and protective effects against obesity, and treatment for diabetes and irritants of the digestive tract.[1−3] There have also been many reports of the hypoglycemic effects of a synthetic TI called camostat. Reportedly, oral administration of camostat improves glucose tolerance and insulin release as well as suppresses pathological development in a diabetic WBN/Kob rat model.[4] Ingestion of camostat also reduces the deposition of visceral adipose tissue and triglycerides, free fatty acids, and cholesterol in the blood.[5]

With regard to TI stimulation, it is generally accepted that gastrointestinal hormones, such as cholecystokinin (CCK), are secreted from the duodenum.[1,6−8] Subsequently, the secretion of insulin from the pancreas is induced by the effects of CCK.[9,10] Insulin can act on cells in the liver, muscle, and fat tissue to facilitate the entry of glucose from the blood, which plays a central role in regulating carbohydrate and fat metabolism.[9,10] Thus, the effects of TIs may involve multiple tissues; however, little is known about the patterns of gene regulation in various tissues and their communication via hormones except for CCK and insulin.

In our previous study, we partially purified a TI from the viscera of Japanese common squid (*Todarodes pacificus*), which has a molecular weight of approximately 6300 and is acid- and heat-stable.[11] In a single-glucose tolerance test, the squid TI (sqTI) fraction significantly stimulated insulin secretion by normal Wistar rats,[12] although details of how this occurred remain unclear, especially with regard to the global gene expression profiles in related tissues. In this study, we performed transcriptome analysis of the duodenum, pancreas, liver, and muscle in normal Wistar rats fed sqTI for 10 weeks. After the identification of differentially expressed genes (DEGs) from the DNA microarray data, gene ontology (GO) analysis was performed to identify functional groups of genes of interest in each tissue to investigate the global gene expression patterns and the communication of the tissues in Wistar rats fed sqTI.

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MATERIALS AND METHODS

Animals. Male isogenic Wistar rats (7 weeks of age), purchased from Japan SLC (Hamamatsu, Japan), were housed at two rats per cage in an air-conditioned room (temperature, 23 ± 1 °C; humidity, 30 ± 10%) under a 12 h dark/12 h light cycle (lights on at 7:00 a.m.), with free access to tap water and formulated MF diet (Oriental Yeast, Tokyo, Japan). Experiments were started at the age of 11 weeks after a 4 week adaptation period. All animal experiments were performed according to the Guidelines Concerning Animal Experiments at Hokkaido University.

Preparation of sqTI. The sqTI was prepared following the method we reported previously.[11] Briefly, the minced viscera of Japanese common squid (*T. pacificus*) was suspended in an equal volume of distilled water and was stirred at 5 °C for 2 h, after which the slurry was centrifuged (20000*g* for 10 min) to remove precipitates. The pH of the obtained supernatant was adjusted to 5.0 using 1 M HCl and was centrifuged again to remove precipitates. The pH was then readjusted to 7.0 using 0.5 M NaOH, after which it was recentrifuged to obtain the supernatant as the sqTI fraction. One unit (U) of porcine trypsin activity is defined as the amount of enzyme causing an increase of 1.0 in absorbance at 247 nm/min using *p*-toluenesulfonyl-L-arginine methyl ester (TAME) as the substrate.[13] One IU of TI activity is defined as the amount of inhibitor that decreases the activity of 1 mg of trypsin from the pancreas by 50%.

Oral Administration of sqTI. The rats in the experimental group ($n = 8$; 11 weeks old) were given an oral injection of sqTI (2.4 IU) every morning (10:00 a.m.) using a probe for 10 weeks. In the control group ($n = 8$; 11 weeks old), distilled water was injected orally instead of the sqTI.

Oral Glucose Tolerance Test. An oral glucose tolerance test was performed before and after each experiment. Wistar rats were fasted for 16 h and then given an oral injection of glucose (2 mg/g body weight) and sqTI (2.4 IU) using a probe. Distilled water was injected orally instead of the sqTI in the control group. Blood was sampled periodically (0, 15, 30, 60, and 120 min after injection) to measure glucose and insulin levels using Glucocard Diameter (Arkray, Tokyo, Japan) and insulin enzyme-linked immunosorbent assay kits (Morinaga Institute of Biological Science, Inc., Yokohama, Japan), respectively.

RNA Extraction. Total RNAs were extracted from the duodenum, pancreas, liver, and muscle using an SV Total RNA Isolation System (Promega, Carlsbad, CA) following the manufacturer's instructions. RNA quantity, purity, and concentration were determined using a spectrophotometer and agarose gel electrophoresis.

DNA Microarray Analysis. DNA microarray analysis was performed following the Two-Color Microarray Gene Expression Analysis protocol using the dye swap design (Agilent Technologies, Santa Clara, CA). 3′- or 5′-cyanine-labeled cRNA was synthesized from total RNA ($n = 8$; 500 ng) using the Low RNA Input Linear Amplification Kit Plus, Two Color (Agilent Technologies), which was hybridized to whole rat genome (4 × 44 K) Oligo Microarray chips (Agilent Technologies). Scanning was performed using a GenePix 4000B scanner (Agilent Technologies), and imaging analyses of primary spot intensities were performed using Agilent's Feature Extraction software. The list of DEGs for each tissue that were up- or down-regulated by >2-fold after the TI treatment is given in the Supporting Information (file 1.

Gene Ontology Analysis. Gene ontology (GO) analysis was performed[14] on DEGs that were up- or down-regulated >2-fold. DAVID (Database for Annotation, Visualization and Integrated Discovery; david.abcc.ncifcrf.gov) was adopted for the functional annotation by statistical significance ($p < 0.01$). To visualize the results for GO terms, Cytoscape 2.6.3 (www.cytoscape.org/) was used.

Quantative Real-Time PCR (qPCR). qPCR analysis was performed using Power SYBR Green PCR Master Mix in an ABI PRISM 7000 Sequence Detection system (Applied Biosystems). The analysis was performed using the following optimized assay conditions: 10 min at 94 °C followed by 15 s at 94 °C and 1 min at 60 °C for 40 cycles.

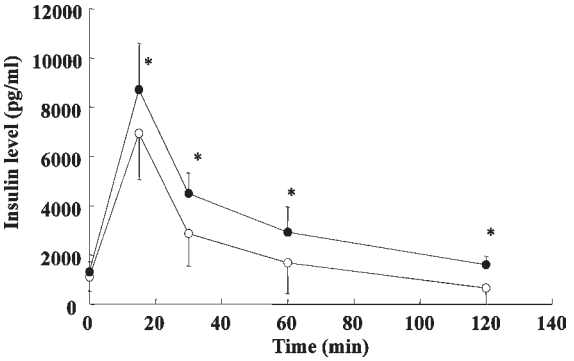

Figure 1. Blood insulin levels in the glucose tolerance test. Solid and open circles represent data for TI-treated ($n = 8$) and control ($n = 8$) groups, respectively. Each point is expressed as the mean ± SE. *, significantly different ($p < 0.05$) from the control.

Amplifications were performed in duplicate wells. Melting curve analysis was performed after each run to confirm the specificities of the primers used. The primers used for each gene were as follows: insulin sense, 5′-TGTGGTTCTCACTTGGTGGA-3′; insulin antisense, 5′-ATGCTGGTGCAGCACTGAT-3′; glucagon sense, 5′-TCGTGGCTGGATTGTTTGTA-3′; glucagon antisense, 5′-CAATGTTGTTCCGGTTCCTC-3′; gastrin sense, 5′-TACGGATGGATGGACTTTGG-3′; gastrin antisense, 5′-AGATGGCTGGGCTCTGGAA-3′; gastrokine sense, 5′-GCTCCTGGCTTTGCTTACAC-3′; gastrokine antisense, 5′-GTTGATGCTCACCGACTGCT-3′; somatostatin sense, 5′-GACCCCAGACTCCGTCAGTT-3′; somatostatin antisense, 5′-GGCATCGTTCTCTGTCTGGTT-3′; cholecystokinin sense, 5′-CACGACCCCTCGCCTCTAA-3′; cholecystokinin antisense, 5′-GGCTGCATTGCACACTCTGA-3′; *β*-actin sense, 5′-AAGTCCCTCACCCTCCCAAAAG-3′; *β*-actin antisense, 5′-AAGCAATGCTGTCACCTTCCC-3′.

RESULTS

Physiological Effects of sqTI Treatment. No significant differences between the sqTI-treated group and the control group were found in growth, food intake, weight of tissues, or blood tests (Supporting Information, files 1 and 2). In the single-glucose tolerance test, insulin levels were significantly higher in sqTI-fed rats at 30, 60, and 120 min after injection (Figure 1), although no significant difference was observed in the glucose level at the same time points (not shown). The peak value of blood insulin level (8724 pg/mL) was highly enhanced compared to pretreatment (2952 pg/mL), as reported previously.[12]

Transcriptome Analysis of Each Tissue.

Duodenum. After the filtration by Feature Extraction, 14807 spots were judged as positive from the 44000 spots on the array. Among them, 547 and 460 were DEGs that were up-regulated (>2.0) or down-regulated (<0.5) by sqTI treatment, respectively (Supporting Information, file 3). Table 1 shows the list of highly up-regulated DEGs (with -fold changes >5.0) following sqTI treatment and the assigned GO terms in the biological process category. Response to insulin stimulus genes included resistin 1 (-fold change = 17.79) and uncoupling protein 3 (-fold change = 8.07). Transglutaminase 1 (-fold change = 9.04) has been associated with keratinization and keratinocyte differentiation. Some GO terms about calcium regulation were allocated into calcium sensing receptor (-fold change = 7.43). As shown in Figure 2, CCK, gastrin, gastrokine, and somatostatin were significantly up-regulated in sqTI-treated Wistar rats as assessed by qPCR analysis, although those genes were not listed as DEGs after the filtration.

Table 1. GO Analysis of Biologicalprocesses for Highly Up-regulated DEGs in the Duodenum

gene name	GO term	fold change
carboxypeptidase (CA2) gene, exon 11 [M23721]		46.39
	GO:0006508: proteolysis	
albumin (Alb), mRNA [NM_134326]		32.84
	GO:0009267: cellular response to starvation	
	GO:0019836: hemolysis by symbiont of host erythrocytes	
	GO:0051659: maintenance of mitochondrion location	
	GO:0043066: negative regulation of apoptosis	
	GO:0046010: positive regulation of circadian sleep/wake cycle, non-REM sleep	
	GO:0046689: response to mercury ion	
	GO:0007584: response to nutrient	
	GO:0010033: response to organic substance	
	GO:0070541: response to platinum ion	
	GO:0006950: response to stress	
	GO:0043252: sodium-independent organic anion transport	
	GO:0006810: transport	
	GO:0042311: vasodilation	
resistin (Retn), mRNA [NM_144741]		17.79
	GO:0045444: fat cell differentiation	
	GO:0032868: response to insulin stimulus	
myelin and lymphocyte protein, T-cell differentiation protein (Mal), mRNA [NM_012798]		16.64
	GO:0006917: induction of apoptosis	
	GO:0006886: intracellular protein transport	
	GO:0042552: myelination	
prostaglandin D2 synthase (Ptgds), mRNA [NM_013015]		13.44
	GO:0006633: fatty acid biosynthetic process	
	GO:0001516: prostaglandin biosynthetic process	
	GO:0045187: regulation of circadian sleep/wake cycle, sleep	
	GO:0051384: response to glucocorticoid stimulus	
	GO:0006810: transport	
cellular retinoic acid binding protein 2 (Crabp2), mRNA [NM_017244]		11.12
	GO:0035115: embryonic forelimb morphogenesis	
	GO:0006869: lipid transport	
	GO:0042573: retinoic acid metabolic process	
transglutaminase 1 (Tgm1), mRNA [NM_031659]		9.04
	GO:0031424: keratinization	
	GO:0030216: keratinocyte differentiation	
	GO:0009887: organ morphogenesis	
	GO:0018149: peptide cross-linking	
calpain 8 (Capn8), mRNA [NM_133309]		8.18
	GO:0006508: proteolysis	
uncoupling protein 3 (mitochondrial, proton carrier) (Ucp3), mRNA [NM_013167]		8.07
	GO:0007568: aging	
	GO:0032870: cellular response to hormone stimulus	
	GO:0006631: fatty acid metabolic process	
	GO:0006839: mitochondrial transport	
	GO:0015992: proton transport	
	GO:0014823: response to activity	
	GO:0009409: response to cold	
	GO:0051384: response to glucocorticoid stimulus	
	GO:0001666: response to hypoxia	
	GO:0032868: response to insulin stimulus	
	GO:0007584: response to nutrient	
	GO:0048545: response to steroid hormone stimulus	
	GO:0000303: response to superoxide	
	GO:0055085: transmembrane transport	

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Table 1. Continued

gene name	GO term	fold change
growth differentiation factor 10 (Gdf10), mRNA [NM_024375]		7.72
	GO:0021549: cerebellum development	
	GO:0040007: growth	
	GO:0001649: osteoblast differentiation	
	GO:0042698: ovulation cycle	
	GO:0045669: positive regulation of osteoblast differentiation	
	GO:0030278: regulation of ossification	
	GO:0071559: response to transforming growth factor beta stimulus	
calcium-sensing receptor (Casr), mRNA [NM_016996]		7.43
	GO:0006816: calcium ion transport	
	GO:0019722: calcium:mediated signaling	
	GO:0006874: cellular calcium ion homeostasis	
	GO:0005513: detection of calcium ion	
	GO:0007186: G-protein coupled receptor protein signaling pathway	
	GO:0007254: JNK cascade	
	GO:0001503: ossification	
	GO:0032781: positive regulation of ATPase activity	
	GO:0008284: positive regulation of cell proliferation	
	GO:0051924: regulation of calcium ion transport	
	GO:0042311: vasodilation	
interleukin 1 receptor-like 1 (Il1rl1), mRNA [NM_013037]		6.83
	GO:0008150: biological process	
	GO:0019221: cytokine-mediated signaling pathway	
	GO:0045087: innate immune response	
	GO:0043124: negative regulation of I-κB kinase/NF-κB cascade	
	GO:0032689: negative regulation of interferon-γ production	
	GO:0002826: negative regulation of T-helper 1 type immune response	
	GO:0090197: positive regulation of chemokine secretion	
	GO:0050729: positive regulation of inflammatory response	
	GO:0032754: positive regulation of interleukin-5 production	
	GO:0043032: positive regulation of macrophage activation	
	GO:0007165: signal transduction	
thiosulfate sulfurtransferase (Tst), mRNA [NM_012808]		6.07
	GO:0016226: iron−sulfur cluster assembly	
	GO:0008272: sulfate transport	
complement component 3 (C3), mRNA [NM_016994]		5.51
	GO:0007596: blood coagulation	
	GO:0006935: chemotaxis	
	GO:0006956: complement activation	
	GO:0006957: complement activation, alternative pathway	
	GO:0006954: inflammatory response	
	GO:0001970: positive regulation of activation of membrane attack complex	
	GO:0045766: positive regulation of angiogenesis	
	GO:0050766: positive regulation of phagocytosis	
	GO:0001798: positive regulation of type IIa hypersensitivity	
	GO:0010575: positive regulation vascular endothelial growth factor production regulated endocrine-specific protein 18 (Resp18), mRNA	
	GO:0008150: biological process	
FXYD domain-containing ion transport regulator 3 (Fxyd3), mRNA [NM_172317]		5.03
	GO:0006811: ion transport	
	GO:0050790: regulation of catalytic activity	

(a) GO Analysis of Up-regulated DEGs. Up-regulated DEGs (>2.0) in GO biological process terms were enriched with genes involved in cell differentiation, tissue development, response to wounding, response to extracellular stimulus, regulation of cell death, regulation of transport, and coagulation (Figure 3A). In the cellular component category, GO terms related to vesicle,

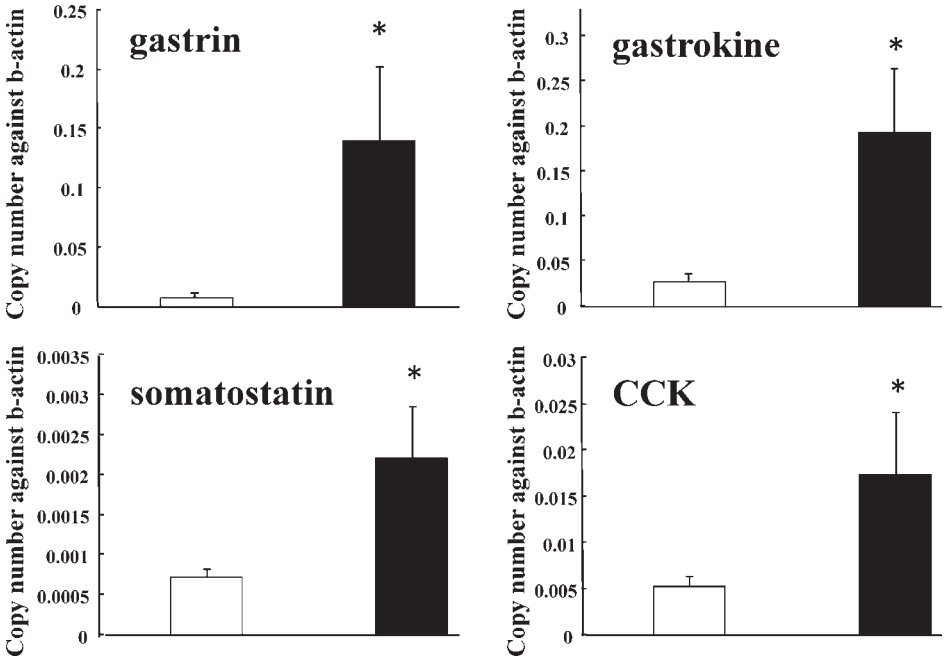

Figure 2. qPCR analysis of CCK, gastrin, gastrokine, and somatostatin mRNAs in the duodenum. The vertical axis indicates the relative expression ratio of each gene normalized against β-actin. Black and white bars represent the results for the TI-treated and control groups, respectively. Data are the mean ± SE for eight observations. *, $p < 0.05$ compared with control mice by Student's t test.

membrane, extracellular region, and Golgi apparatus were enriched (Figure 4A). Analysis of the molecular function category showed that polysaccharide binding, calcium ion binding, peptidase activity, and acetylgalactosaminyltransferase activity were enriched (Figure 5A).

(b) GO Analysis of Down-regulated DEGs. GO terms enriched in the biological process category included programmed cell death, ion homeostasis, antigen processing and presentation, and response to metal ion (Figure 3B). For cellular component analysis, GO terms identified were MHC protein complex, microsome, cytosol, and endoplasmic reticulum (Figure 4B). In the category of molecular function, specific GO molecular functions identified included inorganic cation transmembrane transporter activity, ATPase activity, coupling to transmembrane movement of substances, iron ion binding, and lipid binding (Figure 5B).

Summaries of the enriched GO categories ($p < 0.001$) for up- and for down-regulated DEGs are shown in the Supporting Information (file 4).

Pancreas. After the filtration, 1220 spots were judged as positive with 7 (>2.0) and 14 (<0.5) up- or down-regulated DEGs, respectively (Supporting Information, file 3). Glucagon was identified as the most highly up-regulated gene in the analysis; however, no significant differences were observed between the groups in qPCR analysis (not shown), suggesting that the microarray results were due to outliers. In the GO analysis for up-regulated DEGs, no significant ($p < .0.01$) enrichment could be found. For down-regulated DEGs, the enriched GO terms in the biological process category included immune response genes such as inflammatory response, innate immune response, immunoglobulin-mediated immune response, complement activation, and classical pathway. A summary of the GO analysis including other categories is shown in the Supporting Information (file 5).

Liver. After the filtration, 13567 spots were judged as positive with 78 (>2.0) and 52 (<0.5) up- or down-regulated DEGs, respectively (Supporting Information, file 3). For the analysis of up-regulated DEGs in the category of biological process, GO terms enriched included cellular amino acid catabolic process, cellular amino acid biosynthetic process, serine family amino acid metabolic process, fatty acid metabolic process, and response to glucocorticoid stimulus. Among the GO biological process terms for down-regulated DEGs, response to estrogen stimulus and positive regulation of macromolecule biosynthetic process were listed. A summary of GO analysis including other categories is shown in the Supporting Information (file 6).

Muscle. After the filtration, 16251 spots were judged as positive with 79 (>2.0) and 63 (<0.5) up- or down-regulated DEGs, respectively (Supporting Information, file 3). GO terms enriched for up-regulated DEGs included regulation of intracellular protein kinase cascade, response to insulin stimulus, and apoptosis. For down-regulated DEGs, a wide variety of GO terms were found to be enriched including regulation of transcription, negative regulation of phosphorylation, regulation of cyclin-dependent protein kinase activity, negative regulation of protein kinase activity, chromatin modification, interphase of mitotic cell cycle, and muscle cell differentiation. A summary of GO analysis including other categories is shown in the Supporting Information (file 7).

DISCUSSION

It is generally accepted that in the duodenum, TIs stimulate the secretion of CCK,[1,6−8] which has been extensively studied as a gastrointestinal hormone and neuropeptide. CCK can enhance insulin secretion in vivo or in pancreatic perfusions in rodents.[9,10] Insulin works on cells in the liver, muscle, and fat tissue and plays a crucial role in regulating carbohydrate and fat metabolism.[9,10] Thus, the effects of TI on metabolism involve a wide variety of

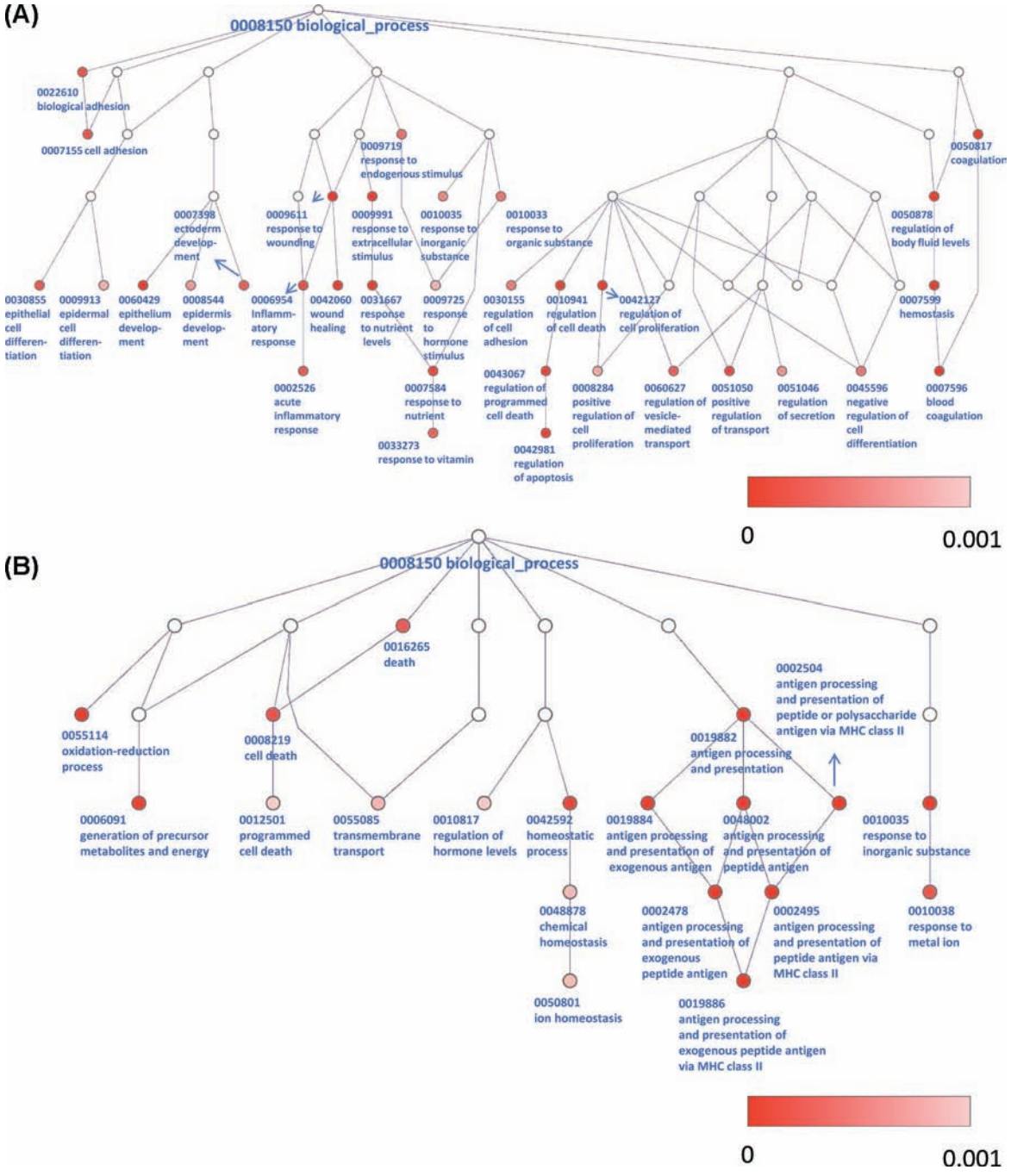

Figure 3. (A) GO analysis of up-regulated DEGs in the duodenum for the category of biological process. Significantly enriched GO terms ($p < 0.001$) were found in the top 547 up-regulated DEGs. (B) GO analysis of down-regulated DEGs in the duodenum for the category of biological process. Significantly enriched GO terms ($p < 0.001$) were found in the top 460 down-regulated DEGs. The color of the node and the p value in functional annotation clustering in DAVID are indicated in the bar. The arrow was used in cases when there was no space for the GO term.

signals in multiple tissues; however, little information has been available. Especially in the duodenum, the first stage of the hormonal system, the detailed mechanisms of recognition for TI, intracellular signaling, and secretion of hormones are poorly understood, and much remains to be investigated. The investigation of the secretion mechanism for CCK and gastrin from their specific cells (I cells and G cells) upon sqTI stimulation is now in progress.

The present study determined the following. In qPCR analysis of the duodenum, CCK, gastrin, and gastrokine were significantly up-regulated (Figure 2). Subsequently, insulin secretion in sqTI-treated rats was up-regulated in the single-glucose tolerance test compared to the control group at the end of the test (Figure 1). The insulin level obtained (8724 pg/mL) was significantly higher than at pretreatment (2952 pg/mL), as we reported previously.[12] These results indicate that the events described above occurred in rats treated with sqTI, although no detectable physiological effects were observed (Supporting Information, files 1 and 2). Although the sqTI used was a crude fraction that contains several other proteins, sqTI (MW = 6300) is insensitive to pepsin/

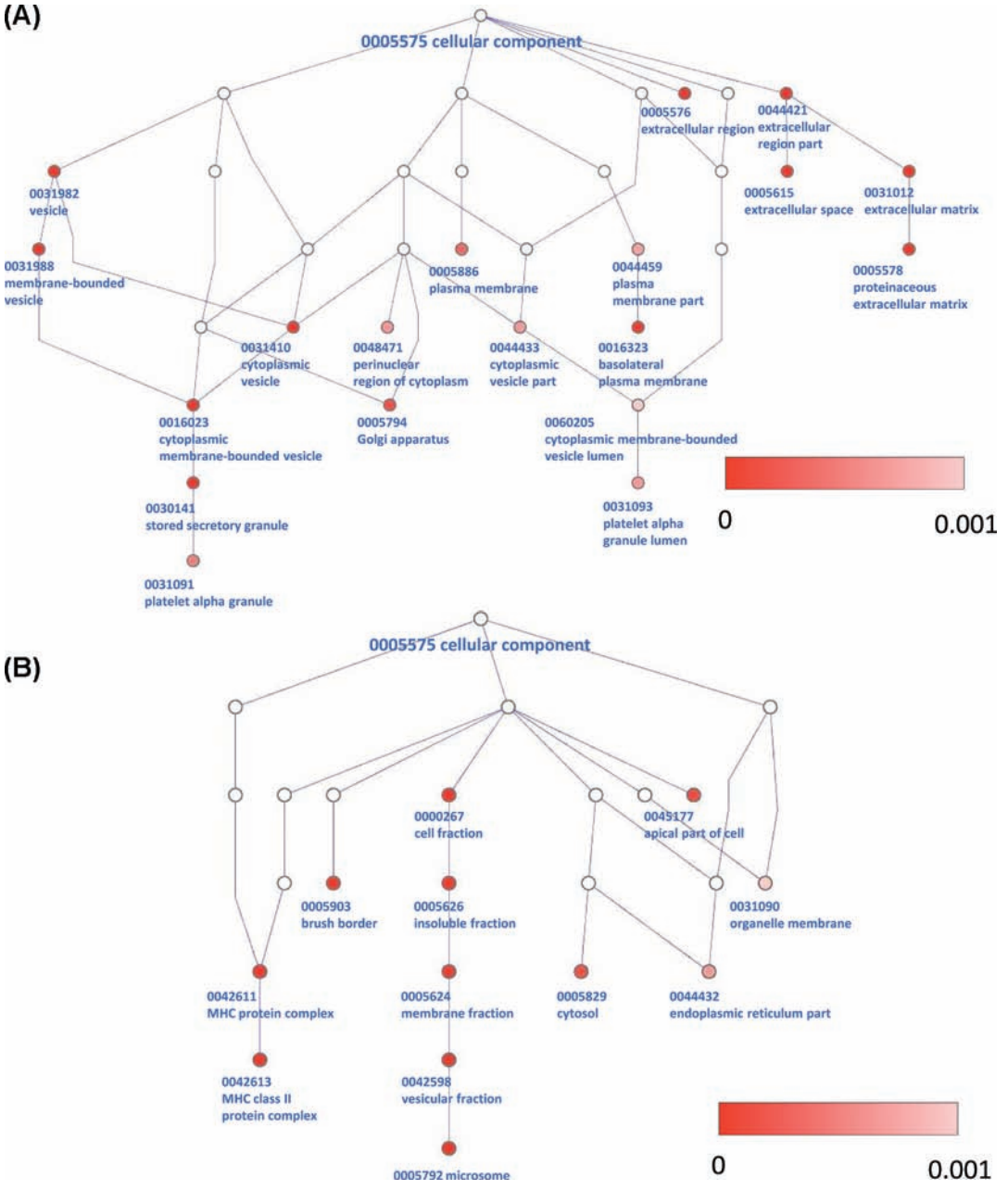

Figure 4. GO analysis of (A) up-regulated and (B) DEGs in the duodenum for the category of cellular component. The conditions for assignment are as described for Figure 3.

chymotrypsin treatment in vitro, whereas other proteins are completely digested (not shown). These facts indicate that sqTI is the sole component of the crude TI solution which authentically works on the duodenum. The stimulation of insulin secretion by CCK in normal rats has already been reported.[15] The reason remains controversial, although it has been ascribed to β cell proliferation in diabetic rats.[16]

In the category of biological process, response to extracellular stimulus was found to be enriched in up-regulated DEGs, which includes response to nutrient levels and response to nutrients at deeper levels of the category. Actually, the detailed mechanism of how the duodenum recognizes and responds to TI to evoke the release of CCK remains unclear, but the present results, including the DEGs assigned to these categories (Supporting Information, files 3 and 4), may provide clues to clarify that mechanism. The GO term regulation of transport was enriched in DEGs including regulation of vesicle-mediated transport, positive regulation of transport, and regulation of secretion (Figure 3A). In the analysis for cellular component GO terms, vesicle and Golgi apparatus (Figure 4A) terms were enriched, indicating that protein secretion is enhanced in this tissue. Calpain 8 is listed among the highly up-regulated DEGs (Table 1), and its function is reported to be vesicle trafficking between the ER and Golgi.[17,18] Epsin, another highly up-regulated DEG (Table 1), is in the family of membrane proteins that are important in creating membrane curvature.[18] These results indicate that several genes related to the secretion of proteins, including gastrointestinal hormones such as CCK and gastrin, were significantly up-regulated.

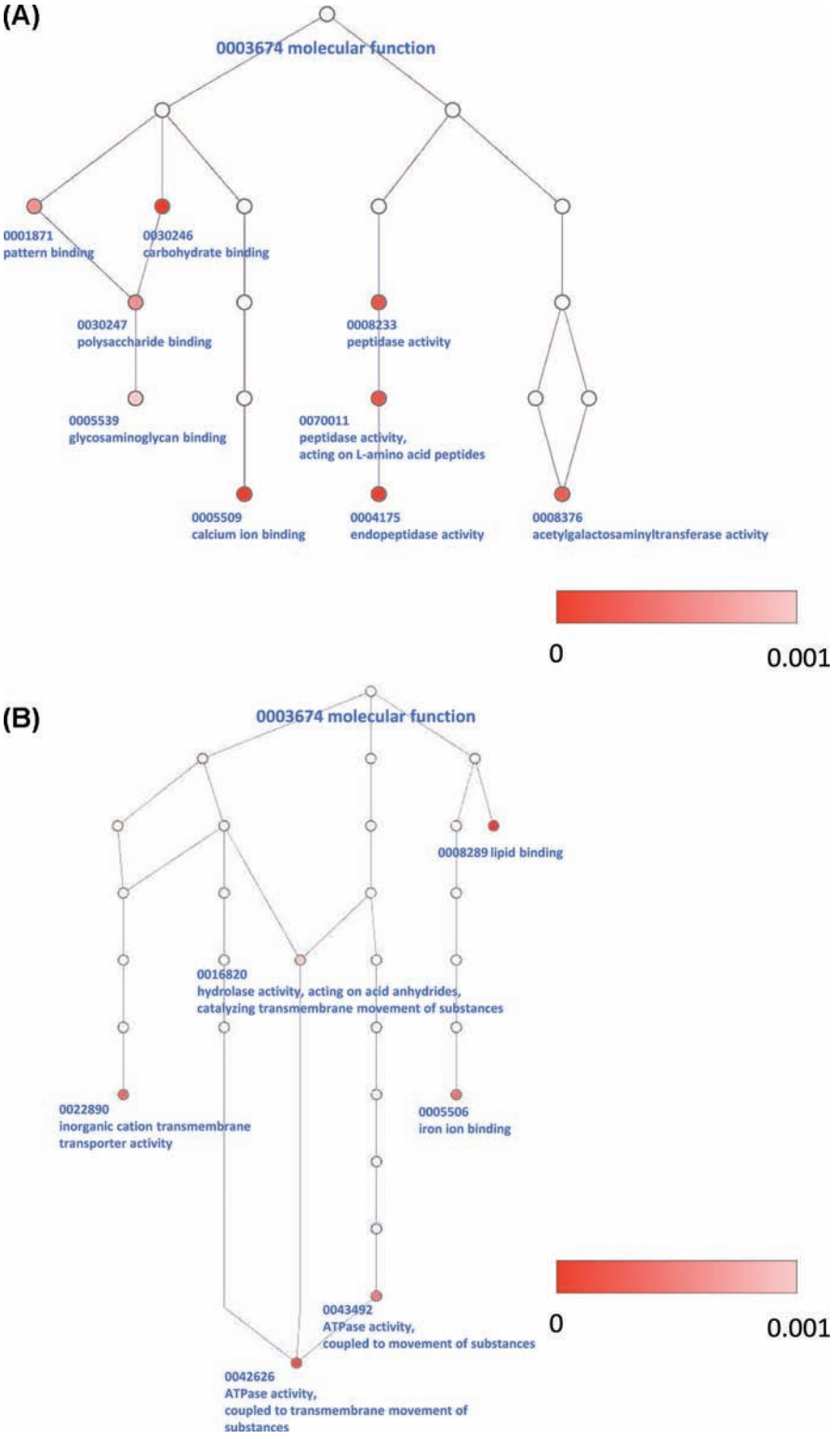

Figure 5. GO analysis of (A) up-regulated and (B) down-regulated DEGs in the duodenum for the category of molecular function. The conditions for assignment are as described for Figure 3.

GO analysis also suggested a previously unknown mechanism related to TI stimulation in the duodenum. For example, the up-regulation of transglutaminase 1 (Table 1) and the enrichment of the GO term regulation of apoptosis in the analysis for biological process category for up-regulated DEGs should be noted (Figure 3A). GO terms assigned for transglutaminase 1 include keratinization and keratinocyte differentiation (Table 1). In the up-regulated DEGs list for the duodenum, five genes related to keratin were also identified (ex Krtap3-3_predicted, mRNA in Supporting Information, file 3). Keratinocytes turn over in the epidermis with several differentiation

stages in various layers.[19] As keratinocytes migrate from the basal to the outermost layers of the epidermis, they form a biological barrier called the cornified layer, which is composed of structural proteins cross-linked by transglutaminase. Keratinocytes undergo apoptosis to form this layer, which functions in defense against wounding and against microbial invasion.[19] In the category of biological process, GO terms related to cell differentiation were enriched including epithelial cell differentiation and epidermal cell differentiation. These results clearly suggest that sqTI treatment induces differentiation, development, and apoptosis in the duodenum including the turnover of keratinocytes.

It should also be mentioned that mucin 1, transmembrane (Muc1), and (predicted) surfactant associated protein D (SP-D) are highly expressed following sqTI treatment (Supporting Information, file 3). In porcine tissues, Sfpd was shown to be present mainly in the lung and was less abundant in the duodenum; its function is to bind to carbohydrate structures on the surfaces of a variety of pathogens to initiate several immune responses.[20] Mucin is a transmembrane glycoprotein expressed at the apical surface of mucosal epithelial cells.[21] Among the 10 cell surface mucin genes identified to date, mucin 1 is generally expressed in mucosal tissues and plays a crucial role in mucosal defense.[21] In the GO analysis for up-regulated DEGs, acute inflammatory response was enriched with 10 DEGs including complement factor D (adipsin), complement component 3, and so forth. These results indicate that the duodenum in sqTI-treated rats shows increased expression of genes involved with innate mucosal defense including the inflammatory response, although no significant difference could be found in organ weight or in blood tests compared to the control group.

Liddle et al. reported that pancreatic secretory trypsin inhibitor interacts directly with cells to signal CCK release through an increase in intracellular calcium.[22] Calcium plays a crucial role in the response to TI in the duodenum in both intra- and extracellular regions. In the GO analysis for up-regulated DEGs in the duodenum, calcium ion binding was listed at the deepest hierarchy in the category of molecular function (Figure 5A), where 46 genes were assigned to this term (Supporting Information, file 4). Furthermore, the list of highly up-regulated DEGs (-fold change > 5.0) involved at least three genes associated with this ion (Table 1). Calcium-sensing receptors can detect levels of extracellular calcium to regulate homeostasis.[23] Calpain 8 is a protein belonging to the family of calcium-dependent, nonlysosomal cysteine proteases, which possibly participate in processes such as cell mobility and cell cycle progression within cells.[24] Transglutaminase is a well-known calcium-dependent enzyme that catalyzes the formation of bridges between proteins.[25] In the same analysis, surfactant protein D (SP-D), a calcium-dependent carbohydrate-binding protein,[26] was listed (-fold change = 19.34), although it is only a predicted gene (Supporting Information, file 3). Calcium also plays a crucial role in keratinocyte differentiation.[27] Rapid increases in extracellular calcium concentration lead to the accumulation of keratins,[28] precursor proteins of the cornified layer, and transglutaminase,[19] which cross-links proteins in a calcium-dependent manner to form the physical barrier of keratinocytes.[19] Intracellular calcium concentrations elevate in response to increases of extracellular calcium, which stimulates keratinocytes to differentiate.[29] Thus, upon TI stimulation in the duodenum, calcium seems to be involved in the innate mucosal defense system and in keratinocyte differentiation, although further investigation is required to characterize the detailed mechanism, including possible adverse effects of TI. There has been a report about the effects of nutritional factor on the expression profile on signal transduction;[30] however, little meaningful information was extracted using the DEGs after filtration except for those involved in calcium signaling.

In conclusion, in this study we investigated the global gene expression patterns in several tissues after sqTI treatment of Wistar rats. GO analysis of DEGs in the duodenum indicates that this event can be involved in keratinization and innate mucosal defense as well as response to nutrients, vesicle-mediated transport, and so forth. This analysis suggests that calcium is involved in the innate mucosal defense system and in keratinocyte differentiation.

■ ASSOCIATED CONTENT

Supporting Information. File 1, body and organ weights. Fle 2, blood test results. File 3, the DEGs and their-fold change values for the duodenum, pancreas, liver, muscle (Table 1, up-regulated DEGs in the duodenum; Table 2, down-regulated DEGs in the duodenum; Table 3, up-regulated DEGs in the pancreas; Table 4, down-regulated DEGs in the pancreas; Table 5, up-regulated DEGs in the liver; Table 6, down-regulated DEGs in the liver; Table 7, up-regulated DEGs in muscle; Table 8, down-regulated DEGs in muscle). File 4, GO terms assigned in DAVID using DEGs in the duodenum. The file contains six tab sheets which represent the category of biological process, cellular component and molecular function for up- and for down-regulated DEGs, respectively. For example, the tab sheets Biological Process (UP) means the GO list for up-regulated DEGs in the category of biological process. The tab sheet includes GO term, count (number of genes involved), percentage (involved genes/total genes), *p* value (modified Fisher exact *p* value; the smaller, the more enriched), gene list involved, and their Genbank accession number. File 5, the GO term assigned in DAVID using DEGs in the pancreas. Please note that no GO term was enriched in up-regulated DEGs in the 3 categories. File 6, the GO term assigned in DAVID using DEGs in the liver. Please note that no GO term was enriched in down-regulated DEGs in molecular function. File 7, the GO term assigned in DAVID using DEGs in muscle. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

BBI, Bowman−Birk inhibitor; CCK, cholecystokinin; DAVID, Database for Annotation, Visualization and Integrated Discovery; DEGs, differentially expressed genes; GO, gene ontology; KI, Kunitz inhibitor; TAME, *p*-toluenesulfonyl-L-arginine methyl ester; TI, trypsin inhibitor; SP-D, surfactant associated protein D; sqTI, squid trypsin inhibitor; SREBP, sterol regulatory element-binding protein.

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