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Momordica charantia and Its Novel Polypeptide Regulate Glucose Homeostasis in Mice via Binding to Insulin Receptor

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Supporting Information

ABSTRACT: *Momordica charantia* (MC) has been used as an alternative therapy for diabetes mellitus. This study analyzed and elucidated therapeutic targets contributing to the hypoglycemic effect of aqueous extract of MC seeds (MCSE) by transcriptomic analysis. Protein ingredients aimed at the hypoglycemic target were further identified by proteomic, docking, and receptor-binding assays. The data showed that MSCE (1 g/kg) significantly lowered the blood glucose level in normal and diabetic mice. Moreover, MCSE primarily regulated the insulin signaling pathway in muscles and adipose tissues, suggesting that MCSE might target insulin receptor (IR), stimulate the IR-downstream pathway, and subsequently display hypoglycemic activity in mice. It was further revealed that inhibitor against trypsin (TI) of MC directly docked into IR and activated the kinase activity of IR in a dose-dependent manner. In conclusion, the findings suggested that MCSE regulated glucose metabolism mainly via the insulin signaling pathway. Moreover, TI was newly identified as a novel IR-binding protein of MC that triggered the insulin signaling pathway via binding to IR.

KEYWORDS: *Momordica charantia, diabetes mellitus, inhibitor against trypsin, insulin receptor*

INTRODUCTION

Momordica charantia Linn. (MC), also named bitter gourd or bitter melon, is a common vegetable in Asia, Africa, and Latin American. MC displays hypoglycemic effects in cells, rodents, and humans. For instance, water-soluble fractions of MC enhance glucose uptake in 3T3-L1 adipocytes and L6 muscle cells. The aqueous extract of MC exhibits hypoglycemic effects in various animal models, such as normal mice, cyproheptadine-induced diabetic mice, streptozotocin (STZ)-induced diabetic rats, alloxan-induced diabetic rats, and *db*/*db* mice. Moreover, clinical studies with human subjects showed that extracts from fruits or seeds of MC decrease the fasting blood glucose levels in patients with diabetes mellitus.[1−3]

Approximately 228 different compounds have been isolated from MC, and some phytochemicals exhibit hypoglycemic effects.[4] For example, cucurbitane triterpenoids, such as 5β,19-epoxy-3β,25-dihydroxycucurbita-6,23(*E*)-diene and 3β,7β,25-trihydroxycucurbita-5,23(*E*)-dien-19-al, lower the blood sugar in diabetic mice.[5] Cucurbitane glycosides, such as momordicosides Q, R, S, and T and karaviloside XI, stimulate the translocation of glucose transporter 4 (GLUT4) to the cell membrane and increase the AMP-activated protein kinase (AMPK) activity, a key pathway mediating glucose uptake and fatty acid oxidation.[6] Saponins and cucurbitane triterpenoids, such as 3β,7β,25-trihydroxycucurbita-5,23(*E*)-dien-19-al, momordicine I, momordicine II, 3-hydroxycucurbita-5,24-dien-19-al-7,23-di-*O*-β-glucopyranoside, and kuguaglycoside G, stimulate insulin secretion in vitro.[7] Triterpenoid glycosides, such as momordin Ic, oleanolic acid 3-*O*-monodesmoside, and oleanolic acid 3-*O*-glucuronide, suppress the transfer of glucose from the stomach to the small intestine and inhibit glucose transport at the brush border of the small intestine.[8,9] In addition to these phytochemicals, few proteins exhibiting hypoglycemic effects have been identified from MC.[10] For example, polypeptide-p, M.Cy protein, and MC6 display hypoglycemic or antihyperglycemic effects in normal or diabetic mice.[11−13] Although several constituents of MC reveal hypoglycemic effects in vivo, the therapeutic targets of these constituents are still unclear so far.

To address these questions, we prepared the a protein-rich extract of MC seeds (MCSE) with hypoglycemic effect and identified the protein composition of MCSE by proteomic analysis. Transcriptomic analysis was carried out to predict the hypoglycemic targets of MCSE. Proteins of MCSE aiming at the targets were further identified by docking analysis and receptor-binding assay. Our findings showed that MCSE exhibited hypoglycemic activity mainly via the insulin signaling transduction pathway. Moreover, inhibitor against trypsin (TI) targeting to the insulin receptor (IR) was newly identified as the hypoglycemic ingredient of MCSE.

MATERIALS AND METHODS

Chemicals. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless indicated. RNeasy Mini kit was obtained from Qiagen (Valencia, CA, USA). MessageAmp aRNA kit was purchased from Ambion (Austin, TX, USA), and Cy5 dye was obtained from Amersham Pharmacia (Piscataway, NJ, USA). Protein assay and IPG strips were obtained from Bio-Rad (Hercules, CA, USA). Trypsin Gold was purchased from Promega (Madison, WI, USA).

Preparation of MCSE and TI. Fresh fruits of MC were kindly provided by Hualien District Agricultural Research and Extension

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Station (Hualien, Taiwan). Seeds of MC were collected and ground to fine powders using a sample grinder. Powdered seeds (20 g) were extracted with phosphate-buffered saline (PBS) (137 mM NaCl, 1.4 mM KH_2PO_4, 4.3 mM Na_2HPO_4, 2.7 mM KCl, pH 7.2) (60 mL) by stirring at 4 °C. After an overnight stirring, the mixture was centrifuged at 15000*g* for 15 min, the lipid layer was removed, and the supernatant was then collected and lyophilized. The amount of dried MCSE recovered was approximately 400−500 mg/20 g MC seeds. The dried MCSE was kept in airtight containers and stored at −70 °C for further analysis. TI, a 68 amino acid polypeptide of 7 kDa, was synthesized by solid-phase peptide synthesis. The purity and identity of synthesized TI were analyzed by high-performance liquid chromatography and mass spectrometry (MS), respectively.

Animal Experiment and Glucose Tolerance Test. Six-week-old male BALB/c mice and 8-week-old male *ob*/*ob* mice (B6.V-*Lep*^{ob}/J) were obtained from National Laboratory Animal Center (Taipei, Taiwan). Mice were maintained under a 12 h day−12 h night cycle and had free access to water and food. Mouse experiments were conducted under ethics approval from the China Medical University Animal Care and Use Committee (Permit No. 99-205-N).

A glucose tolerance test was performed as described previously.[14] Briefly, mice were fasted for 4 h, and MCSE or PBS was then orally given 15 min before intraperitoneal administration of a glucose solution (1 g/kg body weight). Blood samples were collected from tails at 15 min before and 15, 30, 60, 90, 120, 150, 180, and 240 min after glucose challenge. Blood glucose was measured by glucose oxidase method using a glucometer (ACCU-CHEK Advantage, Roche Diagnostics, Basel, Switzerland). Mice were sacrificed 4 h after glucose challenge. Muscles, adipose tissues, and livers were collected for microarray analysis.

Microarray Analysis. Total RNAs were extracted from muscle, adipose tissue, and liver using an RNeasy Mini kit. Microarray analysis was performed as described previously.[15,16] Briefly, total RNA was amplified by MessageAmp aRNA kit, Cy5-labeled RNA targets were hybridized to Mouse Whole Genome OneArray (Phalanx Biotech Group, Hsinchu, Taiwan), and slides were scanned by the Axon 4000 scanner (Molecular Devices, Sunnyvale, CA, USA). The Cy5 fluorescent intensity of each spot was analyzed by Genepix 4.1 software (Molecular Devices). The signal intensity of each spot was corrected by subtracting background signals in the surrounding. We filtered out spots having a signal-to-noise ratio of <0 or control probes. Spots that passed aforementioned criteria were normalized by the R program in the Limma package using quantile normalization.[17] Fold changes of genes were calculated by dividing the normalized signal intensities of genes in MCSE-treated mice by those in untreated mice. Furthermore, we used the "geneSetTest" function to test which Kyoto Encyclopedia of Genes and Genomes (KEGG) biological pathways (http://www.genome.jp/kegg/pathway.html) were affected by MCSE. This function computes a *p* value to test the hypothesis that selected genes in a pathway tend to be differentially expressed. The signal transduction pathway associated with MCSE treatment in the adipose tissue was further manually built on the basis of gene expression profiles, KEGG pathway database, and the literature.[18,19] Microarray data are MIAME compliant, and raw data have been deposited in the Gene Expression Omnibus (accession no. GSE39689).

2-Dimensional Electrophoresis (2-DE). The chemical composition of MCSE was analyzed by sequential wavelength scanning with the spectrophotometer (Beckman Coulter, Fullerton, CA, USA). For 2-DE analysis, MCSE was precipitated by mixing with 10% trichloroacetic acid in acetone/20 mM dithiothreitol (DTT), incubating at −30 °C for 1 h, and centrifuging at 15000*g* for 15 min. The pellet was washed three times with 20 mM DTT in acetone and lyophilized. The resulting powder was resuspended in rehydration buffer (9.8 M urea, 4% CHAPS, 40 mM DTT, 0.2% biolytes), and the protein concentration was quantified according to the Bradford method (Bio-Rad). Protein samples (200 μg) were applied to IPG strips (7 cm, pH 3−10) and incubated overnight at 20 °C for rehydration. Isoelectric focusing was performed using a Protean IEF Cell (Bio-Rad) by the following program: 0−250 V over 250 Vh, 250−4000 V over 4000 Vh, and 4000 Vh for 20000 V. Focused IPG strips were then equilibrated in equilibration buffer I (75 mM Tris-HCl, pH 8.8, 6 M urea, 2% sodium dodecyl sulfate (SDS), 20% glycerol, 1% DTT) for 15 min and in equilibration buffer II (75 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS, 20% glycerol, 135 mM iodoacetamide) for another 15 min and subsequently separated by SDS−polyacrylamide gel (SDS-PAGE) on the 15% acrylamide gels using the Protean II Cell (Bio-Rad). Protein spots on the gels were visualized by Coomassie Brillant Blue R-250.

Liquid Chromatography Coupled with Tandem MS (LC-MS/MS) Analysis. Protein spots were excised from stained gels, and in-gel digestion was performed with Trypsin Gold. Proteins were identified using an Ultimate capillary LC system (LC Packings, Amsterdam, The Netherlands) coupled to a QSTARXL quadrupole-time-of-flight mass spectrometer (Applied Biosystem/MDS Sciex, Foster City, CA, USA). MS/MS data were matched against NCBI Inr and Swiss-Prot using the MASCOT search program (http://www.matrixscience.com).[20] Green plants (*Viridiplantae*) taxonomy restriction was used. Probable protein candidates were determined by the average theoretical molecular weight, isoelectric point (p*I*), and sequence coverage.

Docking Calculations. The PatchDock web server (http://bioinfo3d.cs.tau.ac.il/PatchDock/patchdock.html) was used for the prediction of ligand-binding sites.[21] The PDB files of IR (PDB code 2DTG) and TI (PDB code 1VBW) were obtained from Protein Data Bank (http://www.rcsb.org/pdb/).

IR Kinase Activity Assay. IR was purchased from Sigma (St. Louis, MO, USA), and the IR kinase activity assay was performed as described previously with a slight modification.[22] Briefly, mixtures containing IR and various amounts of synthesized TI were incubated on ice for 30 min. Mixtures were then mixed in kinase buffer (25 mM HEPES, pH 7.6, 25 mM $MgCl_2$, 100 μM ATP, 100 μM sodium orthovanadate, 2.5 mg/mL poly (Glu,Tyr), 25 μCi/mL [γ-^{32}P]ATP), incubated at 30 °C for 10 min, and spotted on the chromatography papers. Poly(Glu,Tyr) was then precipitated on papers by soaking the papers in 10% TCA solution, and the radioactivity incorporated into the precipitated poly(Glu,Tyr) was counted by the scintillation counter (Beckman Coulter). One unit of kinase activity is determined as transferring 1 pmol of phosphate from [γ-^{32}P]ATP to poly-(Glu,Tyr) per minute at 30 °C.

Statistical Analysis. Data were presented as the mean ± standard error. Student's *t* test was used for comparisons between two experiments. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

Hypoglycemic Effect of MCSE in Mice. To elucidate the hypoglycemic target of MCSE, we first determined the hypoglycemic activity of MCSE by glucose tolerance test and then assessed the gene expression profiling of MCSE by transcriptomic analysis. Various amounts of MCSE were orally administered to normal mice. As shown in Figure 1A, fasting blood glucose levels of normal mice were approximately 40 mg/dL, and the blood glucose concentration reached a maximal level (600 mg/dL) at 15 min after glucose challenge. The mock group displayed a poor glucose clearance because the blood glucose level decreased slowly. In contrast, oral administration of MCSE resulted in a more rapid clearance of glucose than that observed in the mock group. Moreover, the hypoglycemic activity of MCSE displayed a dose-dependent manner. We further administered 1 g/kg MCSE to *ob*/*ob* mice, obese diabetic mice having hyperglycemia and hypercholesterolemia. As shown in Figure 1B, MCSE also displayed a more rapid clearance of glucose in *ob*/*ob* mice, which was consistent with data in normal mice. These findings indicated that MCSE exhibited the hypoglycemic effect in normal and diabetic mice.

Signal Transduction Pathways of MCSE on Glucose Metabolism in Diabetic Mice. To elucidate pathways and therapeutic targets contributing to the hypoglycemic effect of

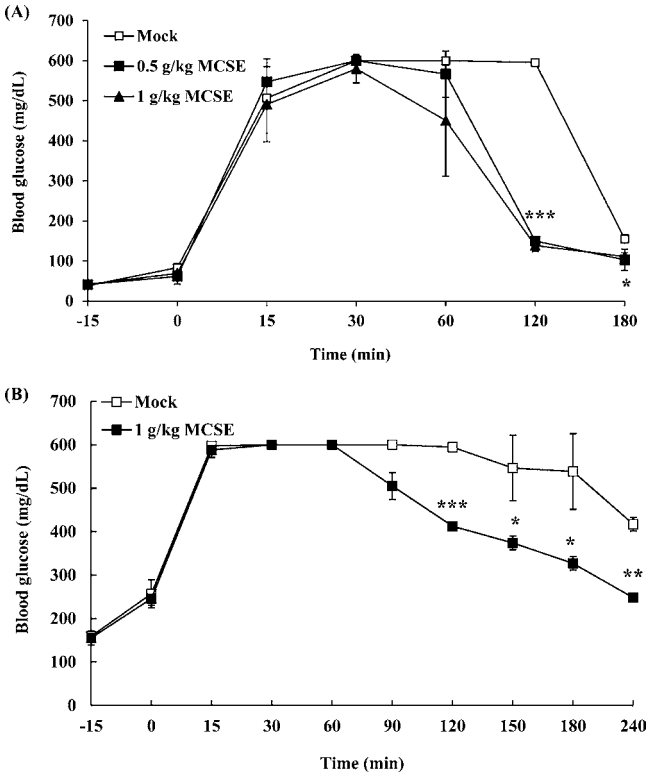

Figure 1. Hypoglycemic effect of MCSE in mice by glucose tolerance test. BALB/c mice (A) and *ob*/*ob* mice (B) were orally administered PBS (mock) or MCSE. Fifteen minutes later, glucose solution (1 g/kg) was administered intraperitoneally, blood samples were collected at the indicated time points, and blood glucose levels were measured by a glucometer. Data are expressed as the mean ± standard error of six mice. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, compared with mock.

MCSE, we analyzed the gene expression profiles of muscle, adipose tissue, and liver by DNA microarray. In a total of 29922 genes, the expression levels of 723, 7096, and 1478 genes in muscle, adipose tissue, and liver, respectively, were up-regulated by 2-fold, whereas the expression levels of 327, 8024, and 1081 genes in muscle, adipose tissue, and liver, respectively, were down-regulated by 2-fold by MCSE. These genes were further selected and tested for the biological pathways by "geneSetTest" function. Among 352 pathways, 6 pathways associated with glucose and lipid metabolism were significantly affected by MCSE (Table 1). Insulin, adipocytokine, peroxisome proliferator-activated receptors (PPAR), and free fatty acid signaling pathways were commonly and significantly regulated by MCSE

Table 1. Statistical Significances of Pathways Associated with Glucose and Lipid Metabolism in MCSE-Treated *ob*/*ob* Mice[a]

pathway	muscle	adipose tissue	liver
insulin signaling pathway	6.8×10^{-6}	0.0035	0.0094
adipocytokine signaling pathway	0.0022	0.0071	0.0451
PPAR signaling pathway	0.0008	1.9×10^{-8}	0.0226
oxidative phosphorylation	0.1120	1.1×10^{-16}	0.0050
glycolysis/gluconeogenesis	0.0081	0.0001	0.3020
free fatty acid signaling pathway	0.0047	9.6×10^{-5}	0.0209

[a]Values are p values calculated on the WebGestalt web site (http://bioinfo.vanderbilt.edu/webgestalt/login.php) by hypergeometric test.

in these tissues. Oxidative phosphorylation and glycolysis/gluconeogenesis were significantly affected by MCSE primarily in adipose tissue.

We further built signal transduction pathways contributing to the modulatory effect of MCSE on the glucose metabolism. As shown in Figure 2, MCSE primarily regulated the expression levels of genes in the insulin signaling pathway. MCSE treatment increased the expression levels of phosphoinositide 3-kinase (PI3K), Akt, AMPK, Cbl adaptor protein (CAP), CrkII, and TC10 genes, resulting in the translocation of GLUT4 and glucose transport. MCSE also stimulated the expression of genes encoding glycolytic and fatty acid synthetic enzymes, such as phosphofructokinase (PFK), pyruvate kinase (PK), and fatty acid synthase (FAS), leading to the utilization and storage of glucose as lipid. Moreover, MCSE activated the expression of mammalian target of rapamycin (mTOR), regulatory associated protein of mTOR (Raptor), p70 S6 kinase, and S6 ribosomal protein genes, resulting in the stimulation of protein synthesis. Furthermore, MCSE increased the expression levels of fatty acid binding protein 4 (FABP4) and PPARγ, contributing to the adipocyte differentiation and lipid metabolism (Supporting Information, Supplementary Table 1). Our findings showed that MCSE induced the insulin signaling transduction pathway and, in turn, resulted in increases of glucose transport, protein synthesis, and adipocyte differentiation. These data suggested that MCSE might target IR, stimulate the IR-downstream pathways, and subsequently display hypoglycemic activity in mice.

Analysis of Protein Constituents in MCSE by 2-DE and LC-MS/MS. To identify the constituents of MCSE targeting the IR, we first evaluated the chemical composition of MCSE by sequential wavelength scan ranging from 240 to 800 nm. As shown in Figure 3A, maximal absorbance was achieved at 280 nm, suggesting that the major ingredients in MCSE might be proteins. 2-DE and LC-MS/MS were further carried out to identify protein constituents of MCSE. We separated MCSE in the first dimension on IPG strips over a pH range of 3−10, followed by separation in the second dimension on 15% SDS-PAGE. After Coomassie Blue staining, we found that approximately 80% of protein content in MCSE was small proteins with molecular masses of <7 kDa (Figure 3B). Fifteen spots were excised from gels, incubated with trypsin for in-gel digestion, and then identified by LC-MS/MS. The identified protein spots of MCSE are summarized in Table 2. Two major low molecular weight proteins identified here were TI and napin-like protein large chain. Napin-like protein large chain, 11S globulin β subunit precursor, and glutelin type-B1 precursor are storage proteins. Some proteins are protective proteins, and their main physiological function is believed to protection of plants against insect predation and viral infection. For example, TI is resistant to trypsin digestion.[31,38] Superoxide dismutase exhibits the antioxidant effect, whereas β-momorcharin, momordin I precursor, and momordin II precursor are ribosome-inactivating proteins.[23,24] The percentage of TI in MCSE and TCA-precipitated MCSE proteins ranged from 21 to 34% by quantifying the protein spots on SDS-PAGE and 2-DE.

Identification of IR-Binding Protein of MCSE by Docking and IR Kinase Activity Assays. Transcriptomic analysis indicated that IR might be the hypoglycemic target of MCSE. Docking analysis was therefore performed to identify which proteins in MCSE might interact with IR. The crystal structure of IR ectodomain monomer is shown in Figure 4A.

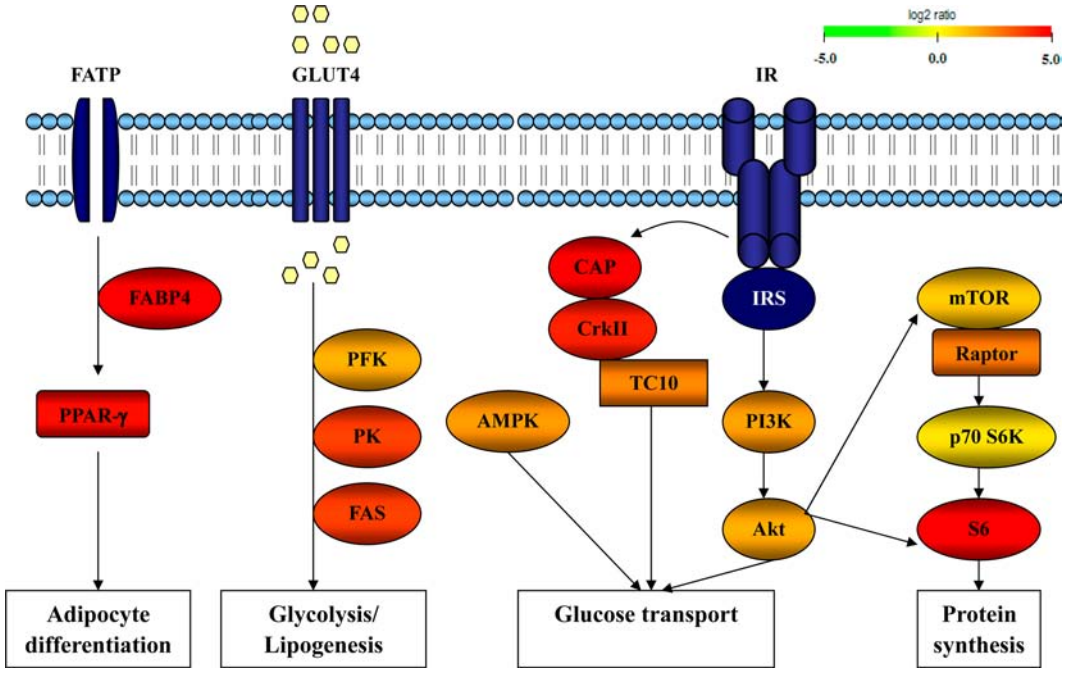

Figure 2. Signal transduction pathways contributing to the modulatory effect of MCSE on glucose metabolism in diabetic mice. Normalized $\log_2$ expression levels are color-coded according to the legend at the top. Increased levels are colored red and decreased levels, green. AMPK, AMP-activated protein kinase; CAP, Cb1 adaptor protein; FABP4, fatty acid binding protein 4; FAS, fatty acid synthase; FATP, fatty acid transport; GLUT4, glucose transporter 4; IR, insulin receptor; IRS, insulin receptor substrate; mTOR, mammalian target of rapamycin; p70 S6K, p70 S6 kinase; PFK, phosphofructokinase; PI3K, phosphoinositide 3-kinase; PK, pyruvate kinase; PPAR, peroxisome proliferator-activated receptor; Raptor, regulatory associated protein of mTOR; S6, S6 ribosomal protein.

The N-terminal half of IR ectodomain consists of two homologous leucine-rich repeat domains (L1 and L2), separated by a cysteine-rich region (CR). The C-terminal half of ectodomain monomer consists of three fibronectin type III domains. IR monomer forms an inverted V layout relative to the cell membrane, where one leg is formed by L1, CR, and L2 fragments and the other leg is formed by three fibronectin domains. The central β-sheets of the L1 domain and CR are contributors to insulin binding.[25] By docking IR with MCSE proteins identified in this study, we found that TI exhibited the highest docking score, compared with other MCSE proteins (data not shown). TI directly docked into the IR and fitted the L1/CR region very well (Figure 4A). Moreover, 10 amino acid residues of TI interacted with 8 residues located in the L1, CR, and L2 domains of IR via hydrophobic contacts (Table 3). These findings suggested that TI might be the IR-binding protein of MCSE.

To further elucidate whether TI was capable of binding to IR, we performed an IR kinase activity assay. IR exhibits the tyrosine kinase activity. Upon binding to the ligand, tyrosine kinase is activated and transfers phosphate from [γ-^{32}P]ATP to substrate poly(Glu,Tyr). Therefore, we evaluated the IR-binding ability of TI by counting the radioactivity incorporated into the precipitated poly(Glu,Tyr). As shown in Figure 4B, the kinase activity reached 60 units/mL in the presence of insulin. The kinase activity was increased in the presence of TI, and TI enhanced the kinase activity in a dose-dependent manner. These findings suggested that TI was a novel IR-binding protein that targeted the IR and subsequently stimulated the kinase activity of IR.

DISCUSSION

In this study, we newly identified that IR was the hypoglycemic target of MCSE and TI was the novel IR-binding protein of MCSE. MC has been used for the treatment of diabetes mellitus in Asia, South America, India, the Caribbean, and East Africa. Extracts from fruits, seeds, leaves, and whole plants of MC have shown hypoglycemic potentials in type 1 and type 2 diabetic animal models. Moreover, clinical trials have revealed that MC displays hypoglycemic effects in patients with diabetes mellitus.[1,26,27] Several tripertenoids are present in the ethanol extract of MC,[28,29] and their hypoglycemic effects have been demonstrated in vitro and in vivo.[2] However, the hypoglycemic targets of these ingredients are still unclear. Therefore, we performed transcriptomic, proteomic, and docking analyses in this study to elucidate the hypoglycemic target of MCSE and to identify the MC proteins aiming at the target.

Signal transduction pathways contributing to the hypoglycemic effect of MC have been analyzed in some studies. For example, Yibchok-anun et al. indicated that protein extracts from fruit pulps of MC display insulin secretagogue and insulinomimetic activities in STZ-induced rats.[30] Shibib et al. showed that MC depresses the hepatic gluconeogenic enzymes, such as glucose-6-phosphatase and fructose-1,6-bisphosphatase, in diabetic rats.[31] Moreover, MC activates AMPK and PI3K activities, leading to the translocation of GLUT4 and the increased glucose uptake of adipocytes or myocytes.[32,33] MC also inhibits PTP 1B activity, resulting in increased insulin signaling in *db*/*db* mice.[34] Moreover, MC up-regulates PPARγ activity, leading to the differentiation of adipocytes.[35] Because examing the level and activity of individual proteins may not fulfill the global evaluation of hypoglycemic effect of MC, we performed transcriptomic analysis to comprehensively identify the effective pathways contributing to the modulatory effect of

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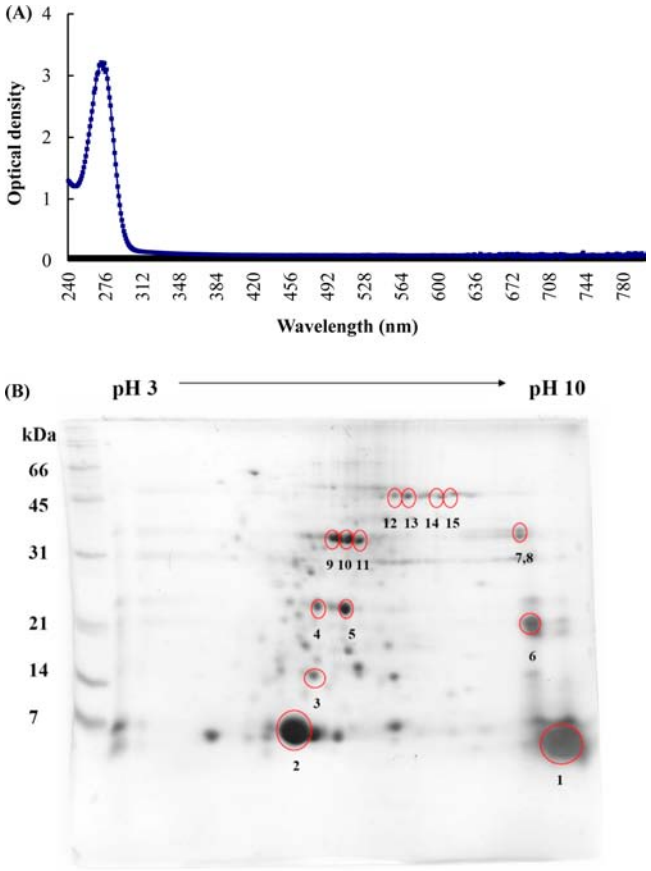

Figure 3. Analysis of constituents in MCSE: (A) sequential wavelength scanning of MCSE; (B) 2-DE image of MCSE. Protein (200 μg) was separated on a pH 3−10 strip, followed by SDS-PAGE on a 15% polyacrylamide gel. Proteins were visualized by Coomassie Brillant Blue R-250. Protein size markers (in kDa) are shown at the left. Protein spots analyzed by LC-MS/MS are indicated by red circles. Photos are representative images of three independent experiments.

MCSE on glucose metabolism in this study. Our data showed that MCSE significantly and primarily regulated the insulin signaling pathway in muscle and adipose tissues, the crucial tissues for glucose uptake. IR is a tyrosine kinase that undergoes autophosphorylation of cellular proteins, such as insulin receptor substrate and CBl. Upon tyrosine phosphorylation, these proteins interact with signaling molecules, leading to activations of the PI3K and CAP/Cbl pathways. PI3K has a pivotal role in the metabolic actions of insulin. Activation of PI3K catalyzes the phosphorylation of phosphoinositides to produce phosphatidylinositol-3-phosphates, which lead to the activation of Akt and the subsequent translocation of GLUT4. Cbl is recruited to the IR by adaptor protein CAP. Phosphorylation of Cbl recruits the CrkII-guanyl nucleotide-exchange protein (C3G) coupled to lipid rafts, where C3G specifically activates the small GTP-binding protein TC10. TC10 activation further induces the translocation of GLUT4. In addition, adiponectin plays important roles in the regulation of glucose and lipid metabolism by stimulating the phosphorylation of AMPK and the translocation of GLUT4.[18,19] Expression levels of genes encoding for proteins involved in insulin signaling pathway were up-regulated by MCSE, suggesting that MCSE stimulated glucose uptake in adipose tissues and muscles via the insulin signaling pathway.

When glucose is transported into cells by GLUT4, insulin stimulates the expression of genes encoding glycolytic and fatty acid synthetic enzymes, resulting in the utilization and storage of glucose as lipid and glycogen. In addition to glucose metabolism, insulin regulates the protein synthesis via the mTOR signaling pathway. mTOR is a Ser/Thr protein kinase that functions as an ATP and amino acid sensor to balance nutrient availability. mTOR is autophosphorylated or phosphorylated via PI3K and Akt pathway. When activated, mTOR transmits the signal to p70 S6 kinase. p70 S6 kinase further phosphorylates the S6 protein of the 40S ribosomal subunit and, in turn, initiates the translation of mRNA.[18,19] Our data showed that MCSE increased the transcription of glycolytic enzyme genes, such as PFK and PK genes, and lipogenic enzyme genes, such as the FAS gene. MCSE also increased the expression levels of mTOR, p70 S6 kinase, and S6 genes. These findings suggested that MCSE induced the translocation of GLUT4, increased the uptake of glucose, and subsequently regulated glucose metabolism and protein synthesis via the insulin signaling pathway.

Table 2. MCSE Proteins Identified by LC-MS/MS

spot	protein name	accession no.[a]	MW (kDa)	p*I*	score[b]
1	inhibitor against trypsin	BAB32588	7.099	10.85	57
2	napin-like protein large chain	AAB50873	7.8503	5.44	134
3	superoxide dismutase [Cu−Zn] 1	P28756	15.242	5.71	89
4	lactoylglutathione lyase	Q8H0V3	20.835	5.14	56
5	ferritin, chloroplast precursor	Q94FY2	28.035	5.73	59
6	β-momorcharin chain A	1CF5_A	28.076	9.19	133
7	ribosome-inactivating protein momordin I precursor	P16094	31.513	9.13	125
8	ribosome-inactivating protein momordin II precursor	P29339	32.012	8.93	91
9	cycloartenol-C-24-methyltransferase	Q9LM02	38.244	5.84	28
10	fructose-bisphosphate aldolase, cytoplasmic isozyme 2	P46257	38.467	6.77	91
11	fructose-bisphosphate aldolase, cytoplasmic isozyme	P08440	38.58	7.52	106
12	adenosylhomocysteinase	P35007	53.199	5.6	37
13	11S globulin β subunit precursor	P13744	54.592	6.71	197
14	aspartic proteinase precursor	O04057	55.820	5.43	66
15	glutelin type-B 1 precursor	P14323	56.515	9.26	36

[a]Protein accession number in the Swiss-Prot database. [b]MASCOT MS/MS ion score for individual peptide sequences. The significance threshold of score is 37.

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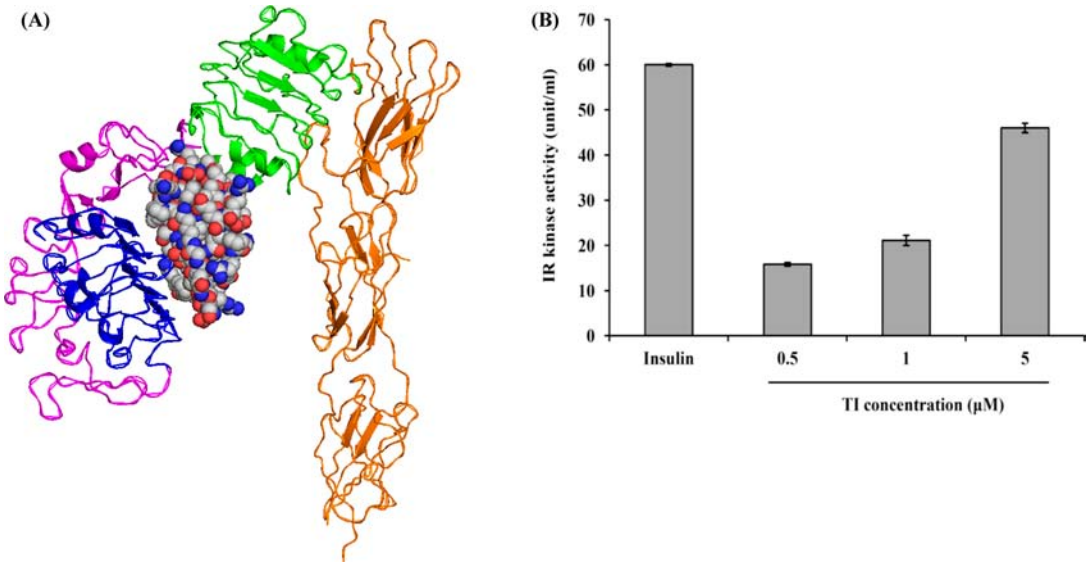

Figure 4. Physical and functional interaction between TI and IR. (A) Docking structure between IR and TI. The IR ectodomain monomer is shown in a schematic presentation. Individual domains are colored as follows: L1, blue; CR, pink; L2, green; fibronectin type III domain, orange. TI is shown in an atomic sphere representation. (B) Effect of TI on the IR kinase activity. Mixtures containing IR, insulin or various amounts of TI, [γ-^{32}P]ATP, and poly(Glu,Tyr) were incubated at 30 °C for 10 min. The radioactivity incorporated into the precipitated poly(Glu,Tyr) was counted by scintillation counter, and the kinase activity (units/mL) was calculated as described under Materials and Methods. Values are the mean ± standard error of triplicate assays.

Table 3. Intramolecular Interaction between IR and TI

TI	IR	IR domain	distance[a] (Å)
SER8	ARG14	L1	3.06
GLN11	ARG271	CR	1.91; 2.63
VAL13	GLN272	CR	2.04
GLY14	ARG270	CR	2.61
SER15	ARG270	CR	2.7
THR16	GLU329	L2	2.63
ALA18	GLU329	L2	2.53
LYS44	TYR91	L1	2.22
PHE46	ARG118	L1	3.14
GLU57	ARG331	L2	1.95; 2.46

[a]Distance between IR and TI was calculated using PatchDock algorithm.

Several low molecular weight proteins with hypoglycemic effects have been identified from MC. For examples, polypeptide-p (11 kDa) displays hypoglycemic activity when administered subcutaneously to laboratory animals.[11,36] MC6 (3.5 kDa) decreases blood sugar levels when administered intraveneously to mice.[12,37] Charantin, a peptide resembling insulin, lowers fasting blood sugar levels when administered orally to rabbits.[27] M.Cy protein, a 17 kDa protein with a p*I* of 5, is identified as the antihyperglycemic agent when administered intravenously to normal or diabetic rats.[13] Although these MC proteins exhibit hypoglycemic effects, their mechanisms remain to be clarified, and no protein targeting IR has been identified so far. Here we found that TI was an IR-binding protein of MCSE by proteomic, docking, and IR kinase activity assays. TI is a protein consisting of 68 amino acid residues with a molecular mass of 7 kDa and a p*I* of 10.85. TI is a storage and protective protein in MC.[38] In this study, we newly found that TI was a novel IR-binding protein that might trigger the insulin signaling transduction pathway and subsequently lead to the hypoglycemic activity of MCSE. We proposed that TI might exhibit the hypoglycemic effect in mice by oral administration for three reasons. First, TI is resistant to trypsin digestion because TI is found as an inhibitor against trypsin. Its main physiological function is believed to protect plants via inhibiting trypsin digestion in the gastrointestinal tract.[38] Moreover, because TI is a small protein and there are no disulfide bonds or other post-translational modifications in TI, TI might rapidly renature to normal conformation after passing through the acidic condition in the stomach. Second, the length of predicted motif responsible for TI and IR interaction is 10−20 residues by docking analysis (data not shown). Therefore, even if TI is digested by digestive enzymes in the gastrointestinal tract, the resulting small motif might interact with IR. Finally, Forgue-Lafitte et al. found that the intestine might be a target tissue for insulin because insulin-binding sites are present in rat intestinal epithelial cells.[39] Therefore, TI might directly interact with the insulin-binding sites located in the intestinal tract and subsequently exhibit the hypoglycemic effect. In addition to the direct effect in the intestine, because some proteins have been shown to be transported across the epithelial lining of the intestine via passive diffusion or endocytosis,[40,41] intact TI or digested TI fragments might be absorbed by the small intestine by these mechanisms and enhance the glucose uptake via binding to IR in muscle and adipose tissue.

In conclusion, although several constituents of MC have displayed hypoglycemic effects in vivo, the therapeutic targets of these constituents are still unclear. In this study, we analyzed the gene expression profiling of MCSE by DNA microarray and found that MCSE exhibited hypoglycemic activity mainly via the insulin signaling transduction pathway. Furthermore, we newly identified that TI was the novel IR-binding protein of MCSE that might activate the insulin signaling pathway by binding to IR.

■ ASSOCIATED CONTENT

Supporting Information

Expression levels of MCSE-regulated genes in adipose tissue. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

Patent applications for hypoglycemic protein TI have been approved by the Taiwan Patent Office (Patent I 342781) and the Japan Patent Office (Patent 4772884). The U.S. and European patents were filed in 2009 and are now under review. The authors declare no competing financial interest.

■ ABBREVIATIONS USED

AMPK, AMP-activated protein kinase; C3G, guanyl nucleotide-exchange protein; CAP, Cbl adaptor protein; FABP4, fatty acid binding protein 4; FAS, fatty acid synthase; GLUT4, glucose transporter 4; KEGG, Kyoto Encyclopedia of Genes and Genomes; MC, *Momordica charantia*; MCSE, aqueous extract of MC seeds; mTOR, mammalian target of rapamycin; PBS, phosphate-buffered saline; PFK, phosphofructokinase; PI3K, phosphoinositide 3-kinase; PK, pyruvate kinase; PPARγ, peroxisome proliferator-activated receptor γ; PTP 1B, protein tyrosine phosphatase 1B; Raptor, regulatory associated protein of mTOR; STZ, streptozotocin

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