Soymorphin-5, a soy-derived μ-opioid peptide, decreases glucose and triglyceride levels through activating adiponectin and PPARα systems in diabetic KKAy mice

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ADIPONECTIN IS AN ADIPOCYTOKINE derived from adipocytes with insulin resistance-improving and lipid metabolism-enhancing activities (4, 37). Adiponectin levels are decreased in obesity and type 2 diabetes. Both adiponectin receptors, AdipoR1 and AdipoR2, mediate a major part of the insulin-sensitizing action of adiponectin in the liver (10, 39, 40). The activation of AdipoR1 or AdipoR2 stimulates the adenosine monophosphate (AMP) kinase pathway or peroxisome proliferator-activated receptor-α (PPARα) pathway coupled to fatty acid β-oxidation (38); thus, we focused on orally active molecules to stimulate adiponectin signaling.

It is known that a number of bioactive peptides derived from the enzymatic digestion of food proteins exhibit various physiological functions, including opioid (3, 43, 44), antihypertensive (18, 45), cholesterol-lowering (41), memory-enhancing (24, 42), and anxiolytic-like (25, 46, 47) activities. It is reported that soy protein increases adiponectin and insulin sensitivity in rats and type 2 diabetic KKAy mice (20–22, 28); however, little is known about soy protein-derived functional molecules. In this study, we found that a low-molecular-weight peptide, soymorphin-5, derived from major soy protein, activated the adiponectin system and ameliorated hyperglycemia after long-term oral administration in KKAy mice. We previously reported that soymorphin-5 is a μ-opioid pentapeptide derived from soy β-conglycinin β-subunit, having anxiolytic-like activity after oral administration (25). In general, it is known that μ-opioid receptor agonists, including morphine and endomorphin-1 or -2, stimulate food intake after central administration in rodents (2, 16); however, soymorphin-5 did not stimulate but rather suppressed food intake after a single administration in normal ddY mice (13). Indeed, food intake was not affected by long-term oral administration of soymorphin-5 in KKAy mice. Thus, we investigated the mechanism underlying the antiadipic activity of soymorphin-5.

MATERIALS AND METHODS

Reagents. Soymorphin-5 (YPFVV) and des-Tyr-soymorphin-5 (PFVV) were synthesized by the Fmoc method and were purified by reverse-phase high-performance liquid chromatography. D-Glucose was purchased from Wako Pure Chemical Industries, (Osaka, Japan). Insulin (human) was from the Peptide Institute, (Osaka, Japan).

Animals. Five-week-old male KKAy mice obtained from CLEA Japan (Tokyo, Japan) were used for the experiments on glucose and lipid metabolism. Each mouse was housed individually under regulated conditions (23°C on a 12:12-h light-dark cycle with lights on at 7 AM) and had free access to food pellets (CE-2; CLEA Japan) and water. Four- to five-week-old male ddY mice weighing 20–26 g obtained from SLC (Shizuoka, Japan) were used for glucose and insulin tolerance tests and behavioral experiments using the elevated plus maze. All experiments were approved by the Kyoto University Ethics Committee for Animal Research Use. All mice used for the behavioral experiment were euthanized with an overdose of anesthetic after the experiments.

Experimental procedure using KKAy mice. After 1 wk of acclimatization, KKAy mice were divided into control and soymorphin-5 groups so that the averages of body weight and blood glucose levels were approximately equal. Soymorphin-5 (10 mg·kg⁻¹·day⁻¹) dissolved in drinking water was administrated to KKAy mice for 5 wk, whereas water was given to the control mice. Food and water intake...
were measured every day, and body weight was measured every third or fourth day. Plasma glucose was measured at 0, 2, 4, and 5 wk after start of the experiment from a drop of blood collected from the tail vein by using the NIPRO FreeStyle Freedom Blood Glucose Monitoring System (Nipro, Osaka, Japan). After soymorphin-5 administration for 5 wk, all KKA mice were deprived of food pellets for 3 h. Blood was then collected from the ophthalamic vein of anesthetized mice with sevoflurane (Maruishi Pharmaceutical, Japan) and placed in ice-cold heparinized tubes. The plasma was separated by centrifugation at 4,500 rpm for 10 min at 4°C. The fat tissues, including mesenteric and epididymal fat and brown adipose tissue, and the liver were removed and weighed. For the quantitative determination of mRNA expression associated with glucose and lipid metabolism, the fat tissues were quickly frozen using liquid nitrogen and stored at −80°C until RNA extraction, and the liver was stabilized using RNA Later RNA Stabilization Reagent (QIAGEN). The other samples were stored at −20°C until analysis.

Glucose and insulin tolerance tests. A glucose tolerance test (GTT) was started from 12:00 noon. Glucose solution was administered intraperitoneally at a dose of 2 g/kg in 5-wk-old male ddY mice fasted for 12 h. The tail vein blood glucose levels were measured before and 30, 60, and 120 min after glucose administration using the FreeStyle Freedom (Nipro). Soymorphin-5 was administered intraperitoneally 3 h before the glucose tolerance test. An insulin tolerance test (ITT) was started from 1500. Insulin dissolved in saline was administered at a dose of 0.75 U/kg ip to 5-wk-old male ddY mice fasted for 6 h. Soymorphin-5 was administered intraperitoneally 3 h before insulin administration. Similarly to the GTT, the tail vein blood glucose levels were measured.

Biochemical analysis. The plasma insulin level was measured using a Mouse Insulin ELISA kit (Shibayagi, Gunma, Japan). Plasma adiponectin was measured with an adiponectin ELISA kit (Otsuka Pharmaceutical, Tokushima, Japan). Plasma TG, cholesterol, and fatty free acid levels were measured by triglyceride E-test Wako, Cholesterol E-test Wako, and NEFA C-test Wako, respectively (Wako Pure Chemical Industries, Osaka, Japan). Liver lipid was extracted by the chloroform-methanol method and extracted liver TG and cholesterol were measured using the triglyceride E-test Wako and Cholesterol E-test Wako, respectively.

mRNA expression analysis. We performed quantitative RT-PCR as previously described (13, 26). Total RNA was extracted from the mouse liver with the RNeasy Lipid Tissue Kit (QIAGEN), and transcribed to cDNA with random primers and oligo(dT) by the SuperScript III Platinum Two-Step qRT-PCR kit (Invitrogen Life Technologies, Carlsbad, CA). For quantitative PCR, we amplified the cDNA using an Applied Biosystems Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) with Platinum SYBER Green qPCR SuperMix-UDG with ROX solution (Invitrogen) and each primer set specific for mouse mRNA according to the manufacturer’s instructions and previous papers (Table 1) (1, 6, 11, 14, 17, 19, 27, 33). The reactions were cycled 40 times with denaturation at 95°C for 15 s and with annealing and elongation at 60°C for 30 s. The relative expression level of each mRNA was normalized using the mRNA level of β-actin.

Elevated plus-maze test. Anxiolytic-like behavior was measured using the elevated plus-maze (EPM) test, which was performed as described previously (2, 8, 12, 25, 32, 46, 47). Four arms (25 cm long × 5 cm wide) were placed 50 cm above the ground. Two opposing arms were delimited by acrylic vertical walls (15 cm high, closed arms), and two had unprotected edges (open arms). A mouse was placed in the center of the maze facing an open arm and observed for 5 min to measure the cumulative time and frequency of entries into open and closed arms. Arm entry was defined as the entry of four paws into an arm. Open-arm entry time (time spent in open arms) was expressed as a percentage of the total entry time (% of time), and the number of open-arm entries was expressed as a percentage of the number of total entries (% of visit). Soymorphin-5 and des-Tyr-soymorphin-5 dissolved in saline were administered intraperitoneally 30 min before the test. Antagonists were co-administered with peptide intraperitoneally 30 min before the test. The total number of visits to open and closed arms and the cumulative time spent in open and closed arms were measured on a monitor using a video camera system. The EPM test was started at 1100 during the light phase of the light-dark cycle.

<table>
<thead>
<tr>
<th>Table 1. Primer sets used for quantitative RT-PCR</th>
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<tr>
<td><strong>Reverse</strong></td>
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<tr>
<td>Adipor1</td>
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<td>Acaca</td>
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<td>Srebp1</td>
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<td>G6pc</td>
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<td>Pck1</td>
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<td>Actb</td>
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Fig. 1. Antihyperglycemic effect of orally administered soymorphin-5 in KKA mice, a type 2 diabetes model animal. Soymorphin-5 (10 mg/kg·day−1·po) was administered to male KKA mice for 5 wk. Plasma glucose levels were measured from a drop of blood collected from the tail vein. Each value is expressed as the mean ± SE (n = 7–8). *P < 0.05, **P < 0.01 vs. the control group.
Luciferase assay. Monkey CV1 kidney cells (American Type Culture Collection) used in the luciferase assay were cultured in DMEM with 10% fetal bovine serum (FBS; JRH Bioscience, Kansas) and 1% penicillin-streptomycin mixed solution (P/S; Invitrogen) at 37°C in a humidified 5% CO2 atmosphere. The luciferase assay was performed using a GAL4/PPAR chimera system as previously described (29, 30). Briefly, we transfected p4xUASg-tk-luc (a reporter plasmid), pM-hPPAR\textsubscript{α}/H9251 (an expression plasmid for a chimera protein for the GAL4 DNA-binding domain and each human PPAR ligand-binding domain), and pRL-CMV (an internal control for normalizing transfection efficiencies) into CV1 cells cultured on 60-mm tissue culture dishes. Transfection was performed using Lipofectamine (Invitrogen) following the manufacturer’s protocol. Five hours after transfection, the transfected cells were transferred to 96-well plates and cultured in a medium containing each compound for 24 h. The luciferase assay was performed using the dual luciferase system (Promega) following the manufacturer’s protocol.

Preparation of mouse primary hepatocytes. Isolation and culture of mouse hepatocytes were performed in accordance with Watanabe’s protocol (8, 30). Briefly, C57BL/6J male mice, 5–6 wk old (SLC, Shizuoka, Japan) were anesthetized with pentobarbital sodium (Dainippon Sumitomo Pharma) intraperitoneally, and the livers were perfused with 40 ml of Liver Perfusion Medium (Invitrogen) followed by 30 ml of Liver Digestion Medium (Invitrogen), both at a flow rate of 5 ml/min. Hepatocytes were dispersed in Hepatocyte Wash Medium (Invitrogen) supplemented with 1% P/S by dissection and gentle shaking. After filtration through a 150 μm nylon mesh filter, hepatocytes were isolated by repeated centrifugation (3–5 times) at 3 × 10^6 cells/well after the viability assay (cell viability was usually >90%). After a 4- to 5-h incubation at 37°C in a 5% CO2 atmosphere, the hepatocytes were incubated with soymorphin-5 or des-Tyr-soymorphin-5 in William’s E medium containing 1% P/S for 24 h and used for mRNA quantification assay.

Statistical analysis. Values are expressed as means ± SE. The unpaired Student’s t-test and one-way ANOVA followed by Fisher’s test were used to assess differences between two and more groups, respectively. P < 0.05 was considered significant.

### Table 2. Comparison of control and soymorphin-5 groups in total food intake and body weight gain after 5 wk’ administration of soymorphin-5

<table>
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<tr>
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<th>Total Food Intake</th>
<th>Body Weight Gain</th>
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<tr>
<td>Control</td>
<td>220.3 ± 6.9</td>
<td>11.3 ± 1.3</td>
</tr>
<tr>
<td>Soymorphin-5</td>
<td>213.1 ± 4.4</td>
<td>11.8 ± 0.8</td>
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Values are means ± SE in g; n = 7–8.
RESULTS

Soymorphin-5 improves glucose and lipid metabolism in diabetic KKAy mice. The blood glucose levels in control KKAy mice gradually increased, and the hyperglycemic conditions persisted after the fourth week (Fig. 1). In contrast, soymorphin-5 decreased the elevated blood glucose levels in the fourth and fifth weeks (Fig. 1). Total food intake and body weight showed no differences between groups (Table 2). Water intake increased in response to hyperglycemia in the control group, whereas no increase was observed in the soymorphin-5 group (Fig. 2). The difference in water intake between control and soymorphin-5 groups seemed to be first observed on day 20 and was observed almost every day after that. The sum of the water consumption in the soymorphin-5 group during the latter half of the experimental period was significantly lower than in the control group. In addition, soymorphin-5 decreased blood glucose levels in the GTT and ITT after acute intraperitoneal administration (Fig. 3), implying that soymorphin-5 might increase insulin sensitivity.

Plasma insulin levels as well as blood glucose decreased after oral administration of soymorphin-5 in KKAy mice, a model animal of type 2 diabetes (Fig. 4A), suggesting that insulin exhibits glucose-lowering activity more effectively by soymorphin-5. Plasma TG levels were also decreased after soymorphin-5 administration (Fig. 4B). Plasma levels of adiponectin, a fat tissue-derived hormone increasing insulin sensitivity, were also elevated by soymorphin-5 (Fig. 4C). There were no differences in free fatty acid and cholesterol levels between groups (2.1 ± 0.1 vs. 2.0 ± 0.1 meq/l and 144 ± 10 vs. 133 ± 9 mg/dl, respectively). We then focused on the organs on which adiponectin is known to act, including liver and fat tissues.

The weight of the liver in the soymorphin-5 group was lower than in the control group (Fig. 4D); however, there were no differences in the weights of fat tissues, including the mesenteric and epididymal fat and brown adipose tissue (Table 3). The liver TG contents seemed to decrease in the soymorphin-5 group (Fig. 4E). Next, we investigated mRNA expression associated with glucose and lipid metabolism in the liver and fat tissues.

Soymorphin-5 activates adiponectin and PPARα systems. We found that soymorphin-5 increased the mRNA expression of Adipor2 but not Adipor1 among two receptor subtypes for adiponectin in the liver (Fig. 5, A and B). It has been reported that stimulation of adiponectin signaling via AdipoR2 activates the PPARα system (35, 36). Thus, we measured the mRNA expression of nuclear receptor PPARα (Ppara) and its target genes in the liver. Ppara mRNA expression was increased in the soymorphin-5 group (Fig. 5C). Furthermore, mRNA expressions of acyl-CoA oxidase (Acox1) and carnitine palmitoyltransferase 1A (Cpt1a), which are well-known PPARα target genes, were increased after soymorphin-5 administration (Fig. 5, D and E). The mRNA expression of uncoupling protein-2 (Ucp2) tended to be increased (Fig. 5F). We also

Table 3. Comparison of control and soymorphin-5 groups in ratio of fat weight (%g/body wt) in male KKAy mice after 5-wk administration of soymorphin-5

<table>
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<tr>
<th></th>
<th>Control</th>
<th>Soymorphin-5</th>
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<tr>
<td>Mesenteric fat</td>
<td>2.4 ± 0.2</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td>Epididymal fat</td>
<td>4.2 ± 0.2</td>
<td>3.8 ± 0.1</td>
</tr>
<tr>
<td>Brown adipose tissue</td>
<td>0.7 ± 0.1</td>
<td>0.7 ± 0.0</td>
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Values are means ±SE; n = 7–8.
which stimulates gluconeogenesis, was not decreased. These results suggest that soymorphin-5 activates the PPARα system downstream of AdipoR2 rather than AdipoR1.

Involvement of μ-opioid receptor in the antihyperglycemic effect of soymorphin-5. NH₂-terminal Tyr is important for the μ-opioid activity of soymorphin-5 (YPFVV). Soymorphin-5 dose-dependently suppressed contraction in the guinea pig ileum by electrical stimulation, indicating that soymorphin-5 has μ-opioid activity. In contrast, des-Tyr-soymorphin-5, which is an NH₂-terminal Tyr-truncated tetrapeptide (PFVV), did not suppress ileum contraction, suggesting that des-Tyr-soymorphin-5 lost μ-opioid activity (data not shown). Then, we investigated whether the soymorphin-5-induced antihyperglycemic effect is associated with μ-opioid receptor, using the Tyr-truncated soymorphin-5. Soymorphin-5, but not des-Tyr-soymorphin-5 (10 mg/kg), did not lower blood glucose levels (Fig. 6). Thus, we assumed that soymorphin-5 induces antihyperglycemic activity in KKAγ mice through μ-opioid activation.

We previously reported that orally administered soymorphin-5 exhibits anxiolytic-like activity (25). To investigate whether the soymorphin-5-induced anxiolytic-like activity was mediated by μ-opioid receptor, we used an antagonist of μ-opioid receptor, naloxone. Soymorphin-5 (10 mg/kg) increased the percentages of time and visits to open arms after intraperitoneal administration; however, the total number of visits to both open and closed arms was not affected (Fig. 7), indicating that soymorphin-5 exhibits anxiolytic-like activity. The anxiolytic-like activity of soymorphin-5 was blocked by naloxone (Fig. 7); however, des-Tyr-soymorphin-5 without μ-opioid activity did not have anxiolytic-like activity (data not shown). Thus, soymorphin-5, but not des-Tyr-soymorphin-5, had anxiolytic and anti-diabetic activities.

We also investigated whether soymorphin-5 directly activates the PPARα system. A bolus dose of soymorphin-5 (10⁻³ mg·kg⁻¹·day⁻¹) increased the percentages of time and visits to open arms, whereas des-Tyr-soymorphin-5 (10 mg/kg) showed similar effects to soymorphin-5 (10 mg/kg) (data not shown). These results suggest that soymorphin-5 activates the PPARα system downstream of AdipoR2 rather than AdipoR1. We also investigated whether soymorphin-5 directly activates the PPARα system. A bolus dose of soymorphin-5 (10⁻³ mg·kg⁻¹·day⁻¹) increased the percentages of time and visits to open arms, whereas des-Tyr-soymorphin-5 (10 mg/kg) showed similar effects to soymorphin-5 (10 mg/kg) (data not shown). These results suggest that soymorphin-5 activates the PPARα system downstream of AdipoR2 rather than AdipoR1.

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M) in the luciferase assay using a GAL4/PPAR chimera system dose-dependently activated PPARα (Fig. 8); however, in primary culture derived from liver cells, mRNA expression of Acox1, one of the target genes of PPARα, was increased after des-Tyr-soymorphin-5 without antihyperglycemic and μ- opioid activities rather than soymorphin-5 with these activities. Thus, we found that somorphin-5 marginally activated PPARα; however, the direct activation of PPARα induced by somorphin-5 might not contribute to its antihyperglycemic effect.

DISCUSSION

Soymorphin-5, a μ-opioid agonist peptide derived from soybean, ameliorated hyperglycemia after long-term oral administration for 5 wk to KKAy mice, a type 2 diabetes model. Plasma insulin levels were also decreased in the soymorphin-5 group, suggesting that insulin seems to become more effective at lowering blood glucose after oral administration of soymorphin-5. In diabetes, water intake is generally known to increase, and we confirmed that increased water intake as well as blood glucose levels were observed in control KKAy mice. In contrast, no increase in water intake was observed in the soymorphin-5 group during our experimental period, suggesting that soymorphin-5 improved the diabetic state. Observing the animals for periods longer than 5 wk might provide valuable insight.

We found that plasma adiponectin concentrations were significantly increased by soymorphin-5. The downregulation of both AdipoR1 and AdipoR2 receptors for adiponectin, which have been identified recently, is reported to decrease in insulin sensitivity (39, 40). In the current study, liver AdipoR2 but not AdipoR1 mRNA expression was significantly increased by soymorphin-5 administration. AdipoR2 activation is reported to stimulate the PPARα pathway and fatty acid β-oxidation (40). Indeed, soymorphin-5 increased the hepatic mRNA expression of PPARα and its target genes, which increase fatty acid β-oxidation and energy expenditure, resulting in TG and glucose level decrease. Since soymorphin-5 marginally activated the PPARα system in the luciferase assay and primary hepatocytes (Fig. 8), we could not rule out the possibility that its direct activation by soymorphin-5 potentially induces antidiabetic activity; however, the NH2-terminally truncated tetrapeptide des-Tyr-soymorphin-5, which more potently activates the PPARα system, does not exhibit antihyperglycemic activity. We also observed that acute administration of soymorphin-5 lowered blood glucose levels in GTT and ITT, implying that soymorphin-5 might increase insulin sensitivity. These are consistent with the fact that soymorphin-5 decreased
glucose and insulin levels after long-term oral administration in diabetic KKAv mice. The changes in mRNA expression associated with gluconeogenesis, including G6pc, might be explained as compensation after long-term administration of soymorphin-5. Another group reported that fenofibrate, a PPARα agonist, improved plasma glucose and lipid metabolism after oral administration at a dose of 300 mg·kg⁻¹·day⁻¹ (4 days) but not at a dose of 100 mg·kg⁻¹·day⁻¹ (14 days) in KKAv mice (15). Bezafibrate, a hydrophobic drug to activate PPARα, in the blood and liver could be measured using the LC-MS-MS system (unpublished data); however, soymorphin-5 was under the detection limit after a single oral administration. In this study, we found that soymorphin-5, which more weakly activates PPARα than fenofibrate (29), decreases blood glucose and TG levels at a dose of 10 mg·kg⁻¹·day⁻¹ in KKAv mice. Taken together, the contribution of the direct activation of PPARα by soymorphin-5 might be small.

The μ-opioid agonist peptide soymorphin-5 has anti-hyperglycemic and anxiolytic-like activities, whereas des-Tyr-soymorphin-5, without μ-opioid activity, does not. Thus, we hypothesized that soymorphin-5 has antidiabetic activity through activation of the μ-opioid receptor associated with anxiolytic-like activity. The mechanism of how the adiponectin and PPARα pathways are activated by soymorphin-5 downstream of the μ-opioid receptor is still unknown. A few reports have suggested a relationship between μ-opioid receptor activation and the onset of diabetes (31, 35). Excess mental stress increases the risk of diabetes (31, 35). Excess mental stress increases the risk of diabetes (31, 35). Excess mental stress increases the risk of diabetes (31, 35).

It was reported that μ-opioid receptor is present in not only the central nervous system but also the peripheral tissues, including the gastrointestinal tract and liver (5, 13). We also detected mRNA expression of μ-opioid receptor in the mesenteric fat by quantitative RT-PCR (unpublished observation). Thus, we cannot exclude the possibility that soymorphin-5 directly activates adiponectin release from the fat tissues and/or increases the expression of the AdipoR2 in the liver downstream of peripheral μ-receptor. Further investigations will reveal how central or peripheral μ-opioid activates the adiponectin and PPAR systems. Furthermore, there are a number of splicing variants in μ-opioid receptor (36). The potency of μ-opioid activity of bioactive peptides is not necessarily consistent with their physiological activities. It remains to be clarified whether opioid peptides previously identified from enzymatic digestion of food proteins exhibit antidiabetic activities similarly to soymorphin-5.

In conclusion, orally administered soymorphin-5, a μ-opioid agonist peptide derived from soy β-conglycinin β-subunit, improved glucose and lipid metabolism in KKAv mice, probably via activation of the adiponectin and PPARα pathways, followed by increases in β-oxidation and energy expenditure.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


