Figure 8. Identification of galactose as a hypoglycemic compound using the hyperglycemic silkworm model. (A) Preparation protocol for the jiou extract. (B) Silkworms were fed a 10% (w/w) glucose diet for 60 min. 50 μl of Jiou extract (1 mg/ml) or human insulin (3.5 mg/ml) was injected into the hemolymph of the hyperglycemic silkworms. The silkworms were fasted for 6 h and the sugar level in the hemolymph was determined. n = 6–7 per group. Data are shown as means ± SD. (C, D) The jiou extract was treated with TFA, and analyzed by TLC. Sugars were localized with 10% sulfuric acid solution. (E) Silkworms were fed a 10% (w/w) glucose diet for 60 min. 50 μl of D-Galactose (Gal), D-glucose (Glu), D-talose (Tal), D-mannose (Man), (1 mg/ml) or human insulin (3.5 mg/ml) was injected into the hemolymph of hyperglycemic silkworms. Silkworms were fasted for 6 h and sugar level in the hemolymph was determined. n = 6 per group. (F) Structure of sugar is shown by Fischer projection in the panel. Numbers shown on the left indicate the carbon positions of the sugar. Red hydroxyl group indicates the positions that differ from D-galactose. Activity represents the hypoglycemic effect. Data are shown as means ± SD. NS; not significant. (G) Galactose (10 mg/ml, 0.5 ml i.p.) was injected to streptozotocin induced-diabetic mice, and blood glucose level was determined after 4 h of fasting. Blood glucose levels in streptozotocin-induced hyperglycemic mice were measured (blood glucose 250–400 mg/dl) and then the mice were treated with either PBS or galactose solution. Four hours after administration and the removal of diet, the blood glucose levels were measured again. The data represent the blood glucose value after treatment divided by the blood glucose value before treatment of individual animals. In all panels, the statistical significance of the difference was evaluated using Student's *t* test. (H) Blood glucose levels in streptozotocin-induced hyperglycemic mice were measured (blood glucose 250–400 mg/dl) and then the mice were treated with either PBS, galactose (200 mg/kg mouse, i.p.), or glucose (200 mg/kg mouse, i.p.) solution. Two hours after administration and removal of the diet, the mice were killed and the membrane fraction in mouse liver was isolated. GLUT2 and Na, K-ATPase were detected by Western blot analysis with anti-GLUT2 antibody or anti-Na, K-ATPase antibody. Immunoblots of GLUT2 and Na, K-ATPase (Top) and calculations of relative GLUT2 (Bottom). n = 3–4 per group. Data at the bottom of the figure are shown as means ± SD. In all panels, the statistical significance of the difference was evaluated using Student's *t* test.
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Hatched larvae were reared to the fifth instar on an artificial diet, SilkMate 2S, which contains antibiotics (Nosan Corporation), at 27°C. All experiments were performed using fifth-instar male larvae (0.9–1.0 g) fasted overnight during the fourth ecdysis, unless otherwise mentioned.

The glucose diet was prepared by mixing Silkmate 2S and D-glucose at the amounts indicated as the percentage of glucose in the total diet.

Injection experiments were performed as follows[24]. Sample solution (50 μl) was injected into the hemolymph at the second abdominal segment of the larva. Syringes (1 ml) and needles (27G×3/4) were purchased from Terumo.

Sugar quantification

Hemolymph (20 μl) was collected from the larva through a cut on the first proleg ands mixed with 9 volumes of 0.6N perchloric acid. Precipitated proteins were removed by centrifugation at 3000 rpm for 10 min at 4°C. The supernatant (hemolymph extract) was diluted with the appropriate volume of distilled water for sugar quantification.

Total sugar in the hemolymph was determined using the phenol-sulfuric acid (PSA) method[25]. Hemolymph extract (100 μl) was mixed vigorously with 100 μl of 5% phenol aqueous solution, followed by vigorous mixing with 500 μl sulfuric acid, incubation at room temperature for 20 min, and absorbance at 490 nm was measured. Serially diluted glucose solution was used as a standard.

Glucose in the hemolymph was determined using the glucose oxidase method. Hemolymph extract (20 μl) was mixed with 400 μl of reaction solution (0.12 M sodium-phosphate buffer [pH 7.4] containing 4 U/ml glucose oxidase, 3 U/ml peroxidase, and 9 mM *o*-dianisidine), followed by vigorous mixing with 100 μl of 70% sulfuric acid solution, incubation at room temperature for 40 min, and absorbance at 530 nm was measured. Serially diluted glucose solution was used as a standard.

The fat body, isolated from the dorsolateral region of the larva, was rinsed in insect saline (130 mM NaCl, 5 mM KCl, and 1 mM $CaCl_2$), and weighed. The fat body (1~10 mg) was lysed in 50 μl of 30% KOH with heating at 90°C for 10 min. Distilled water (150 μl) and ethanol (300 μl; final 60%) were added and the mixture was incubated at 90°C for 10 min. The samples were incubated at 4°C overnight and centrifuged at 15,000 rpm for 3 min. The precipitate was dissolved in distilled water to give a concentration of 50~100 mg fat body/ml by heating at 90°C for 10 min. The resulting fat body extract was used for sugar quantification by the PSA method. The amount of sugar in 1 mg of fat body was calculated.

Chemicals

Recombinant human insulin was purchased from Wako and dissolved in 0.9% NaCl containing 0.1% acetic acid. Wortmannin was purchased from Calbiochem. AICAR was purchased from Toronto Research Chemicals Inc. Jiou was purchased from Uchida Wakanyaku. D-Glucose was purchased from Nacalai Tesque. D-galactose, D-mannose, and D-talose were purchased from Wako.

In vitro fat body sugar uptake assay

The fat body (wet weight 2~10 mg) was isolated from the dorsolateral region of the larva, rinsed in insect saline, and cultured in 200 μl Grace's insect medium supplemented with 1% glucose and antibiotics (penicillin and streptomycin) at 27°C for 30 min. Test sample solution (50 μl) was added to the culture medium, and the fat body was cultured and lysed and then the amount of sugar was determined using the PSA method.

Immunoblot analysis

The fat body (wet weight 1~10 mg) was isolated from the dorsolateral region of the larva, rinsed in insect saline, and cultured in 200 μl Grace's insect medium supplemented with 1% glucose and antibiotics (penicillin and streptomycin) at 27°C for 30 min with or without wortmannin. Test sample solution (50 μl) was added to culture medium and the fat body was cultured and then transferred to NP-40 lysis buffer (10 mM Tris/HCl [pH 7.5], 150 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, 1% NP-40, 10 mM NaF, and 1 mM Na_3VO_4) and lysed by sonication. The samples were centrifuged at 15,000 rpm for 3 min and proteins in supernatants were precipitated by trichloroacetic acid followed by centrifugation at 15,000 rpm for 15 min. The precipitates were washed twice with ice-cold ethanol, dissolved in a buffer with sodium dodecyl sulfate, heat-treated, and electrophoresed in a 12.5% polyacrylamide gel according to the method of Laemmli[26]. Proteins in the gel were electroblotted onto a polyvinylidene difluoride membrane (Millipore), probed with antibody, and detected using Western Lightning (Perkin-Elmer Life Sciences). The following antibodies were used for immunoblot analysis: rabbit polyclonal antibodies to total Akt, phosphorylated Akt, total AMPK, phosphorylated AMPK, Na, K-ATPase from Cell Signaling Technology, GLUT2 from ALPHA DIAGNOSTIC; and mouse polyclonal antibody to AGEs from Cosmo Bio Co., LTD.

For immunoblot analysis of hemolymph AGEs, silkworms were fed a normal diet or a 10% (w/w) glucose diet for 4 days. Aminoguanidine was injected into the hemolymph of the silkworms at 12-h intervals. The AGEs in hemolymph were detected by immunoblot analysis using anti-AGEs antibody and proteins were stained with Coomassie brilliant blue.

For immunoblot analysis of GLUT2 in mouse liver, the mouse liver membrane fraction was prepared as follows. Approximately 10 mg of mouse liver was harvested and cut into small pieces using scissors in Tris B (10 mM Tris/HCl (pH 7.4), 10 mM NaCl, 1.5 mM $MgCl_2$) then centrifuged at 3300 rpm for 10 min. The supernatant was collected as liver extract. The extract was further centrifuged at 45,000 rpm for 1 h, and the resulting precipitate was dissolved by adding 50 µl of 1 M Tris base and used as the membrane fraction. GLUT2 and Na, K-ATPase in the membrane fraction of mouse liver were detected by immunoblot analysis using anti-GLUT2 and anti-Na, K-ATPase antibody.

Quantification of the amount of phosphorylated Akt or phosphorylated AMPK or GLUT2 was performed by densitometric scanning with Image Gauge software. The relative amount of phosphorylated Akt or phosphorylated AMPK or GLUT2 on total Akt or total AMPK or Na, K-ATPase was determined. The amount of AGEs was normalized to the lysate protein concentration.

TLC analysis

Jiou extract (0.4 mg) was mixed with TFA solution (final 2 M) and incubated at 96°C for 2 h. The sample was dried by evaporation and dissolved in 50 µl water. The sample (5 µl) was spotted on a silica gel plate (Silica gel 60F254, Merck) and developed with a propanol solution (1-propanol:water = 85:15). The plate was sprayed with 10% sulfuric acid solution (sulfuric acid:ethanol = 10:90) and heated to detect the spots.

Streptozotocin-induced diabetic mouse

Mature male C57BL6/J mice (8 weeks of age) were purchased from SLC. Diabetes was induced by a single intraperitoneal injection of streptozotocin (150 mg/kg)[27]. Blood samples were collected from the tail vein 4–7 days after injection of streptozotocin and the blood glucose concentration was determined using a glucometer (Accu-Chek Aviva, Roche). Mice with a blood glucose level of 250 to 450 mg/dl were used to evaluate the hypoglycemic effects of test samples.

Ethics Statement

All mouse protocols followed the Regulations for Animal Care and Use of the University of Tokyo and were approved by the Animal Use Committee at the Graduate School of Pharmaceutical Science at the University of Tokyo (approval number: P21-12).

Statistical Analysis

Data are shown as means ± SD. Statistical significance between groups was evaluated using a two-tailed Student's *t* test. A p-value of less than 0.05 was considered statistically significant.

Supporting Information

Figure S1 Schematic illustration of the strategy for screening anti-diabetic agents using silkworms.
(TIF)

Figure S2 Increased hemolymph sugar levels in silkworms fed a normal diet followed by a decrease in hemolymph sugar levels induced by subsequent fasting. Silkworms were fed a normal diet for 24 h (shown in gray), then fasted. The hemolymph sugar level of silkworms before feeding, 12 or 24 h after feeding, or fasted for 12 or 24 h was determined. n = 5 per group. Data represents means ± SD.
(TIF)

Figure S3 Growth inhibition by feeding a high glucose diet in male silkworms. (A–D) Male silkworms were fed a normal diet (N.D.), a 5%, 10%, 15%, 30% (w/w) glucose diet (G.D.), or fasted for 3 days. Body size (A, B), body weight (C), and sugar level in hemolymph (D) were determined. n = 7–10 per group. Data represents mean±SD. *$p<0.0001$ versus saline injected silkworms fed a normal diet (N.D.).
(TIF)

Figure S4 Decrease in total sugar in hemolymph after injection of human insulin. (A) Silkworms were fed a 10% (w/w) glucose diet (G.D.) for 60 min (indicated by gray background) then fasted. 50 µl of human insulin (2 mg/ml) was injected into the hemolymph of the hyperglycemic silkworms, and hemolymph sugar levels were measured 0, 1, 3, and 6 h after injection. n = 5–7 per group. Data represents mean ± standard deviation. *$p<0.05$ versus saline injected silkworms fed a glucose diet (G.D.). (B) Silkworms were fed a 10% (w/w) glucose diet for 60 min. After cessation of the diet, serially diluted human insulin (0.005–0.5 mg/g larva) was injected into the hemolymph of the hyperglycemic silkworms. Hemolymph sugar levels were measured 6 h after injection. n = 8–10 per group.
(TIF)

Figure S5 Stimulation of AMPK phosphorylation in the fat body by AICAR. Isolated fat bodies from silkworm were cultured with AICAR (final conc. 0.8 mg/ml) in Grace's insect medium for 0, 60, or 120 min. Fat bodies were homogenized and extracts were prepared. Total AMPK and phosphorylated AMPK were detected by immunoblot analysis.
(TIF)

Figure S6 Effect of glucose concentration in the culture medium on total sugar in the fat body. Isolated fat body from silkworms was cultured in Grace's insect medium containing 0%, 0.5%, 1.0%, or 2.5% glucose for 3 h, and the amount of sugar in the fat body was measured.
(TIF)

Figure S7 Effect of a high fat diet in silkworms. (A–C) Silkworms were fed a normal diet (N.D.); a 7.5%, 15%, or 30% (w/w) olive oil-containing diet; or a 7.5% or 15% (w/w) oleic acid containing diet for 1 day. Sugar levels in the hemolymph (A), body weight (B), and food intake (C) were determined. n = 5 per group. Data represents mean±SD. The statistical significance of the difference was evaluated using Student's *t* test. p: P value versus silkworms fed a normal diet (N.D.).
(TIF)

Author Contributions

Conceived and designed the experiments: YM ES. Performed the experiments: YM ES TS. Analyzed the data: YM ES TS KS. Wrote the paper: YM ES KS.

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