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Phenolics with PPAR-γ Ligand-Binding Activity Obtained from Licorice (*Glycyrrhiza uralensis* Roots) and Ameliorative Effects of Glycyrin on Genetically Diabetic KK-A^y Mice

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Abstract—The EtOAc extract of licorice (*Glycyrrhiza uralensis* roots) exhibited considerable PPAR-γ ligand-binding activity. Bioassay-guided fractionation of the extract using a GAL-4-PPAR-γ chimera assay method resulted in the isolation of two isoflavenes, one of which is a new compound named dehydroglyasperin D, an isoflavan, two 3-arylcoumarins, and an isoflavanone as the PPAR-γ ligand-binding active ingredients of licorice. The isoprenyl group at C-6 and the C-2′ hydroxyl group in the aromatic ring-C part in the isoflavan, isoflavene, or arylcoumarin skeleton were found to be the structural requirements for PPAR-γ ligand-binding activity. Glycyrin, one of the main PPAR-γ ligands of licorice, significantly decreased the blood glucose levels of genetically diabetic KK-A^y mice.

Introduction

Peroxisome proliferator-activated receptors (PPARs) are ligand-dependent transcriptional regulatory factors belonging to the nuclear receptor superfamily and regulate the expression of a group of genes that maintain glucose and lipid metabolism. There are three PPAR subtypes, commonly designated PPAR-α, PPAR-γ, and PPAR-δ.[1,2] PPAR-α is mainly expressed in the liver, skeleton muscle, and kidney, while PPAR-δ is ubiquitously expressed.[3] PPAR-γ has two isoforms, PPAR-γ1 and PPAR-γ2. PPAR-γ1 is expressed not only in adipose tissues but also in the immune system organs, adrenals, and small intestine.[3] PPAR-γ2 is specifically expressed in adipose tissues and is a master regulator of the differentiation and maturation of adipocytes.[4] PPAR-γ is the predominant molecular target for the insulin-sensitizing thiazolidinedione drugs such as troglitazone, pioglitazone, and rosiglitazone.[5,6] The thiazolidinedione derivatives activate PPAR-γ and improve insulin resistance by increasing the number of small adipocytes with normal function differentiated from preadipocytes and inducing apoptosis in large adipocytes which hyperproduce and hypersecrete adipocytokines such as leptin, TNFα, and free fatty acid.[7] Among natural PPAR-γ ligands, the polyunsaturated fatty acids such as linoleic acid, linolenic acid, arachidonic acid, and eicosapentaenoic acid are known to exhibit nonspecific PPAR ligand-binding activity.[8,9] Recently, a few flavonoids,[10,11] isoprenols,[12] and triterpene acids[13] have been reported to activate PPAR-γ. These findings suggest that the ingredients of some foods and crude drugs

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have PPAR-γ agonistic activity and are useful for the prevention and improvement of type-2 (non-insulin dependent) diabetes, a representative insulin resistance syndrome. In order to discover novel PPAR-γ ligands in natural products, we evaluated extracts of more than 70 spices and herbs for their PPAR-γ ligand-binding activity and found that the EtOAc extract of licorice (*Glycyrrhiza uralensis* roots) exhibited higher activity than the other materials tested. Bioassay-guided fractionation of the extract using a GAL-4-PPAR-γ chimera assay method[12] resulted in the isolation of several phenolic compounds, including a new isoflavene derivative, with PPAR-γ ligand-binding activity. This paper reports the isolation and identification of the licorice phenolics with PPAR-γ ligand-binding activity and the ameliorative effects of glycyrin, one of the main PPAR-γ ligands of licorice, on genetically diabetic KK-A^y mice.

PPAR-γ Ligand-binding Assay

PPAR-γ ligand-binding activity was carried out using a GAL-4-PPAR-γ chimera assay system.[12] CV-1 monkey kidney cells from the American Type Culture Collection (ATCC) (Manassas, VA, USA) were inoculated into a 96-well culture plate at 6×10^3 cells/well, and incubated in 5% CO_2/air at 37 °C for 24 h. As medium, Dulbecco's modified Eagle medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS), 10 mL/L penicillin-streptomycin (5000 IU/mL and 5000 μg/mL, Gibco), and 37 mg/L ascorbic acid (Wako Pure Chemical, Tokyo, Japan) was used. Cells were washed with OPTI-minimum essential medium (OPTI-MEM) (Gibco) and transfected with pM-hPPAR-γ and p4×UASg-tk-luc using LipofectAMINE PLUS (Gibco). In a mock control, pM and p4 × UASg-tk-luc were transfected into CV-1 cells. After 24 h of transfection, the medium was changed to DMEM containing 10% charcoal-treated FBS and each sample,[14] and the cells were further cultured for 24 h. Then, the cells were washed with Ca^{2+}- and Mg^{2+}-containing phosphate-buffered saline (PBS+), to which LucLite (Perkin-Elmer, Wellesley, MA, USA) was added. The intensity of emitted luminescence was determined using a TopCount microplate scintillation/luminescence counter (Perkin–Elmer). The luminescence intensity ratio (test group/control group) was determined for each sample, and PPAR-γ ligand-binding activity was expressed as the relative luminescence intensity of the test sample to that of the control sample.

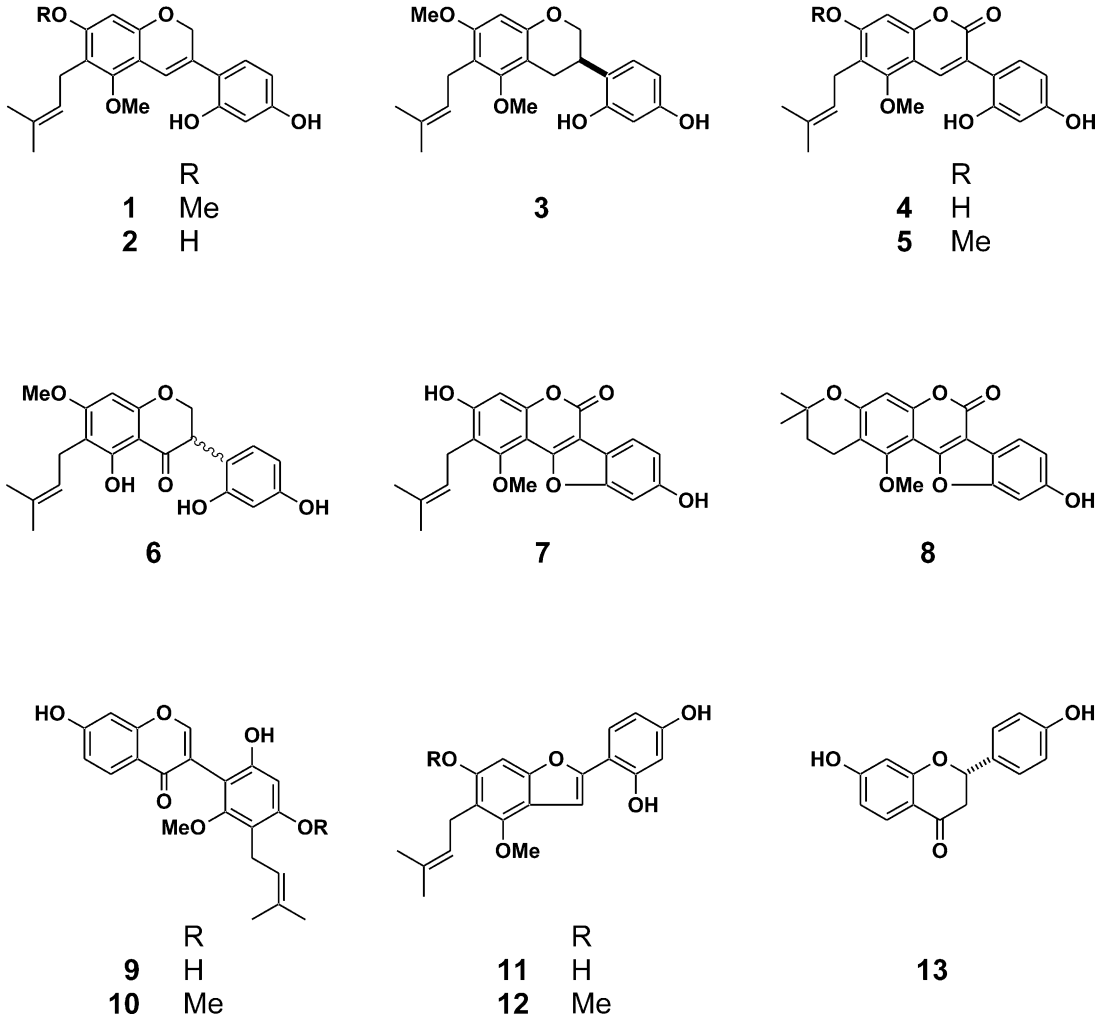

Chart 1.

In Vivo Animal Experiments

Female KK-A^{y} mice of 8 weeks old were obtained from Clea Japan (Tokyo, Japan) and housed for 7 weeks in an environmentally controlled animal laboratory. Mice of 15 weeks age, whose blood glucose levels increased to more than 470 mg/dL, were randomly divided into three groups (five mice per group) on the basis of their body weights and blood glucose levels. The mice were fed powdered CE-2 diet (Clea) in the control group, and powdered CE-2 diet containing 0.1% glycyrin (w/w), 0.1% glycyrol (w/w) or 0.02% pioglitazone (w/w) in the treated groups. Diet and water were given ad libitum for 10 days. On the 4th day, blood was taken from the tail veins of the mice and glucose concentrations were measured using a Glutest Ace blood glucose level monitor (Sanwa Kagaku, Nagoya, Japan). On the 10th day, glycyrin (100 mg/kg) or pioglitazone (20 mg/kg) suspended in 0.5% carboxymethyl cellulose-Na (w/v) was orally administrated to mice fasted overnight. In the control group, vehicle (5 mL/kg) was administrated. At 30 min after the sample administration, 40% sucrose solution was orally given at a dose of 2 g/kg. At 30, 60, and 120 min after the sucrose loading, blood glucose levels were measured. Statistical significance was determined by Dunnett's multiple comparison test using SAS/STAT computerized statistical analysis program software (SAS Institute, Cary, NC, USA).

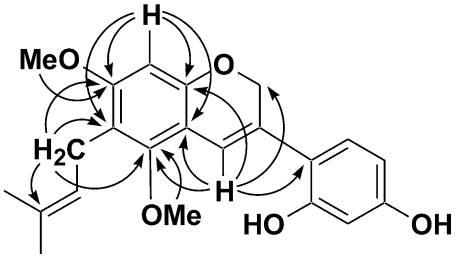

Figure 1. Important HMBC correlations of **1**.

Extraction and Isolation

Powdered *G. uralensis* (1.2 kg)[15] was extracted with EtOAc (5.5 L) at room temperature for 7 days. The extract was filtered off and concentrated (74.0 g), and then chromatographed on silica gel (Fuji-Silysia Chemical, Aichi, Japan) eluted with $CHCl_3$–MeOH gradients (19:1; 9:1; 4:1) and finally with MeOH. The $CHCl_3$–MeOH (19:1) eluate portion (55.4 g) was subjected to multiple chromatographic steps on silica gel, eluting with $CHCl_3$–MeOH and ODS silica gel (Nacalai Tesque, Kyoto, Japan) with MeOH–H_2O and MeCN–H_2O, and to reversed-phase HPLC (column: Capcell Pak C_{18}

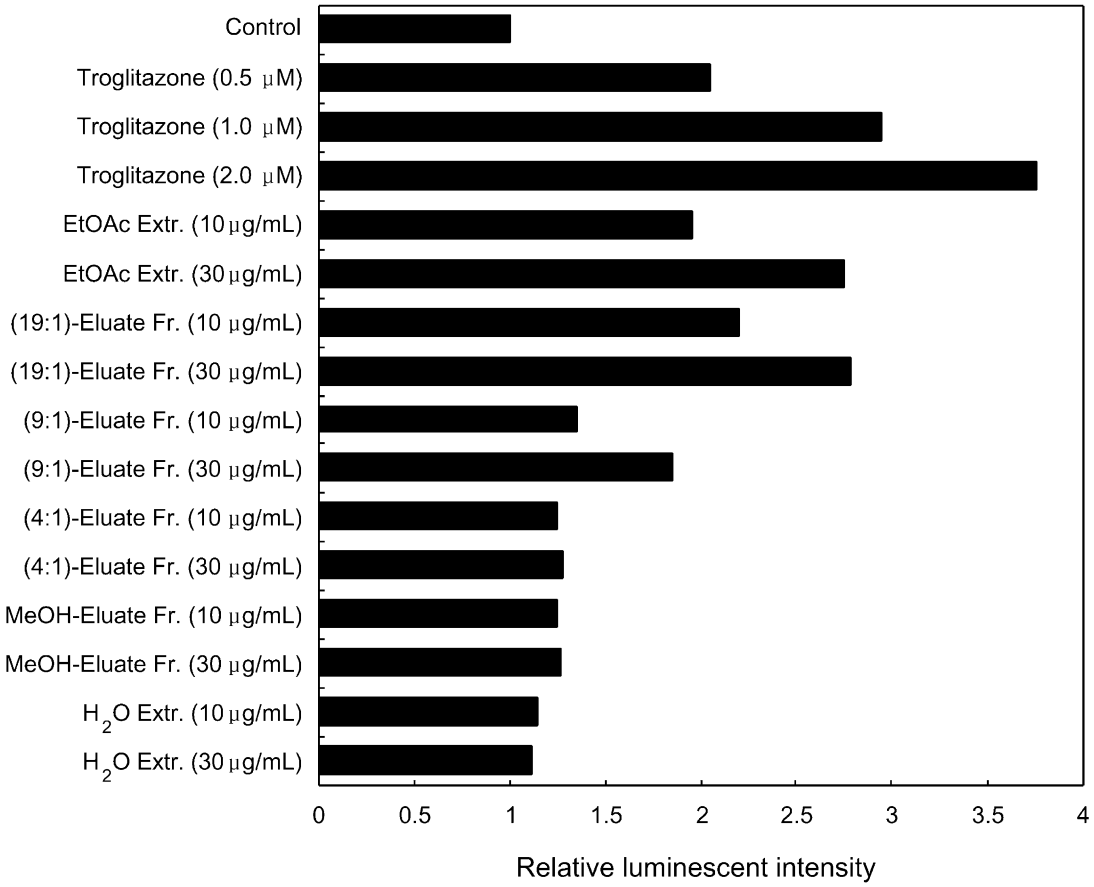

Figure 2. PPAR-γ ligand-binding activities of the licorice extracts and fractions. PPAR-γ ligand-binding activities of the licorice extracts and fractions (10 and 30 μg/mL), as well as that of troglitazone (0.5, 1.0, and 2.0 μM) used as a positive control, were measured using a GAL-4-PPAR-γ chimera assay. All samples were dissolved in DMSO, and added to medium to obtain the indicated concentrations. The luminescence intensity ratio (test group/control group) was determined for each sample, and PPAR-γ ligand-binding activity was expressed as the relative luminescence intensity of the test sample to that of the control sample. Data are means of three experiments performed in quadruplicate.

UG120, 10 mm i.d.×250 mm, Shiseido, Tokyo, Japan) using MeOH–H_2O and MeCN–H_2O to obtain compounds **1** (22.2 mg), **2** (28.3 mg), **3** (58.7 mg), **4** (225 mg), **5** (80.7 mg), **6** (12.1 mg), **7** (43.5 mg), **8** (17.5 mg), **9** (11.8 mg), **10** (74.5 mg), **11** (51.0 mg), **12** (22.1 mg), and **13** (40.1 mg) (Chart 1).

Structural Identification

Compounds **2**–**13** were identified by comparison of their physical and spectral data with those of reported compounds as dehydroglyasperin C (**2**),[16] glyasperin D (**3**),[17] glycycoumarin (**4**),[18] glycyrin (**5**),[19] glyasperin B (**6**),[17] glycyrol (**7**),[20] isoglycyrol (**8**),[20] glicoricone (**9**),[21] licoricone (**10**),[22] licocoumarone (**11**),[18] gancaonin I (**12**),[23] and liquiritigenin (**13**),[24] respectively. This is the first isolation of glyasperin B (**6**) from *G. uralensis*. The new compound **1** was obtained as a pale-yellow gum with a molecular formula of $C_{22}H_{24}O_5$ as determined by HREIMS data, with an accurate $[M]^+$ ion at *m*/*z* 368.1610, which was higher than that of **2** by 14 mass units. The UV, IR, ^1H NMR, and ^{13}C NMR spectra of **1** were quite similar to those of **2**, except for the presence of the signals for one more methoxy group at δ_H 3.75 (3H, s) and δ_C 56.3 (Me) in addition to the C-5 methoxy group at δ_H 3.67 (3H, s) and δ_C 62.3 (Me). In the HMBC spectrum, the methoxy proton signal at δ 3.75 exhibited a long-range correlation to an oxygenated quaternary carbon signal at δ 158.0, which exhibited an HMBC correlation to a singlet aromatic proton signal at δ 6.33 due to H-8 and was assigned to C-7 (Fig. 1). Thus, one more methoxy group was revealed to be located at C-7 and the structure of **1** was formulated as 3-(2,4-dihydroxyphenyl)-5,7-dimethoxy-6-(3-methyl-2-butenyl)-2*H*-chromene, which was named dehydroglyasperin D.[25]

PPAR-γ Ligand-binding Activity

The EtOAc extract of licorice (*G. uralensis* roots) exhibited stronger PPAR-γ ligand-binding activity than the other 70 spices and herbs extracts tested,[26] and its relative luminescence intensity was 2.8 at a sample concentration of 30 μg/mL, which was almost equivalent to that of 1.0 μM (=0.44 μg/mL) troglitazone, a potent synthetic PPAR-γ agonist (Fig. 2). The H_2O extract of licorice,

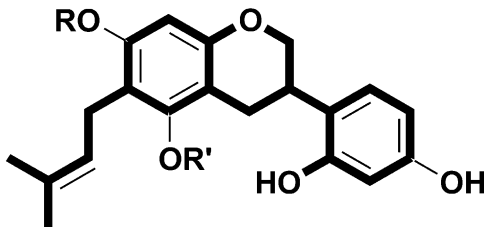

Figure 4. Structural requirements for PPAR-γ ligand-binding activity in the isoflavan skeleton.

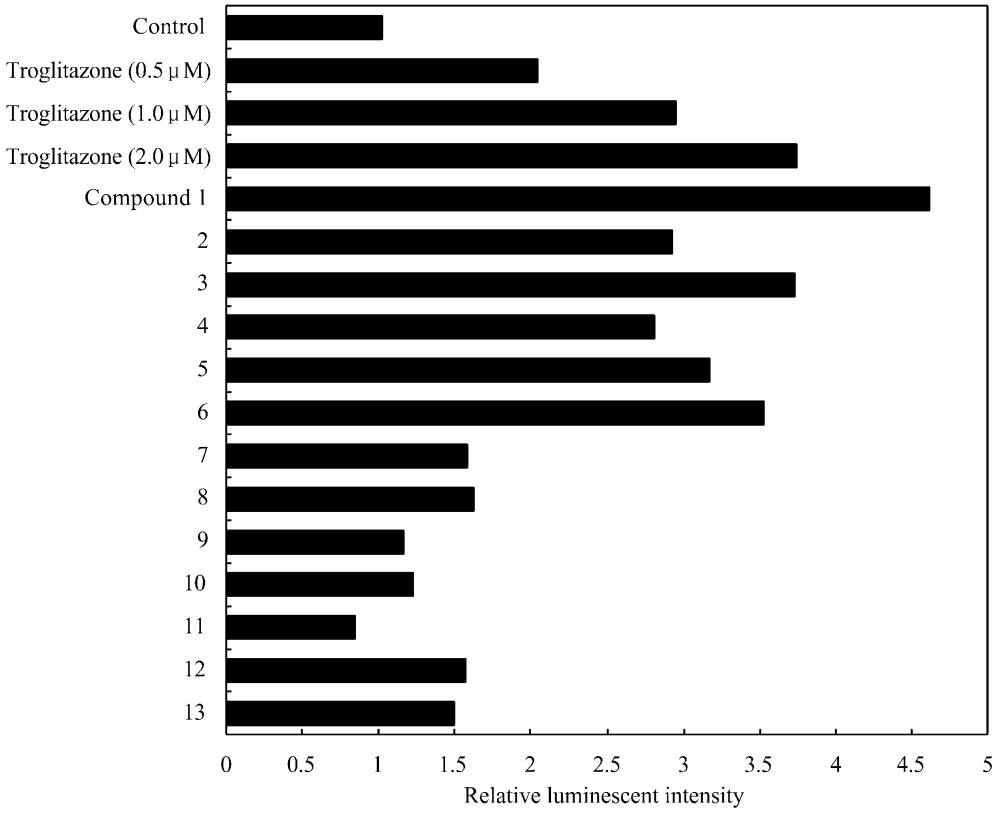

Figure 3. PPAR-γ ligand-binding activity of compounds **1**–**13**. PPAR-γ ligand-binding activities of compounds **1**–**13** (5.0 μg/mL), as well as that of troglitazone (0.5, 1.0, and 2.0 μM) used as a positive control, were measured using a GAL-4-PPAR-γ chimera assay. All samples were dissolved in DMSO, and added to medium to obtain the indicated concentrations. The luminescence intensity ratio (test group/control group) was determined for each sample, and PPAR-γ ligand-binding activity was expressed as the relative luminescence intensity of the test sample to that of the control sample. Data are means of three experiments performed in quadruplicate.

Table 1. Ameliorative effect of glycyrin on hyperglycemia in diabetic KK-A^y mice[a]

	Control	Pioglitazone 0.02%	Glycyrin 0.10%	Glycyrol 0.10%
Body weight (g)				
Day 0	52.6±0.53	55.1±0.69	54.1±1.78	52.6±1.07
Day 4	48.9±0.48	53.6±1.07	50.4±1.58	49.1±1.02
Day 7	50.4±0.56	55.8±1.50	51.9±1.62	50.3±0.91
Day 10	46.9±0.42	52.5±1.41	48.1±1.57	46.8±0.08
Blood glucose level (mg/dL)				
Day 0	476±22	486±26	474±27	427±24
Day 4	420±14	191±6**	278±14**	421±19
Average food intake (g/mouse/day)	5.43	6.34	5.34	5.47
Average sample intake (mg/kg/day)	0	23	102	108

[a]Body weights and blood glucose levels are expressed as means±SE of five mice. Average food intake was calculated from total intake amount/days/number of mice, and average sample intake was calculated from average food intake/average body weight of mice. Data for Days 0, 4, and 7 are body weights of non-fasted mice, and data for Day 10 are body weights of fasted mice. Statistical significance is indicated as ** ($p<0.01$) as determined by Dunnett's multiple comparison test.

which contained glycyrrhizinic acid abundantly, exhibited no activity compared with vehicle control. The EtOAc extract was divided by silica gel column chromatography into four fractions, and activity was concentrated in the $CHCl_3$–MeOH (19:1) eluate portion (Fig. 2). Further fractionation of the active portion resulted in the isolation of two isoflavenes (**1** and **2**), an isoflavan (**3**), two 3-arylcoumarins (**4** and **5**), an isoflavanone (**6**), two coumestans (**7** and **8**), two isoflavones (**9** and **10**), two 2-arylbenzofurans (**11** and **12**), and a flavanone (**13**). Among the isolated compounds, including one new compound (**1**), **1**–**6** exhibited significant PPAR-γ ligand-binding activity, and activities were in the following decreasing order: **1**, **3**, **6**, **5**, **2**, **4** (Fig. 3). The activity of **1** with a sample concentration of 5.0 μg/mL (=13.6 μM) was stronger than that of 2.0 μM (=0.88 μg/mL) troglitazone. The coumestan derivative **7**, which was less active, was structurally related to the active constituent **4**, and the only difference recognized between **4** and **7** lay in the formation of a five-membered ether ring between C-4 and C-2′ in **7**, implying that the presence of a hydroxyl group at C-2′ in the isoflavan, isoflavene, or arylcoumarin skeleton is essential for activity. On the other hand, since the isoflavones **9** and **10** with a hydroxyl group at C-2′ and no isoprenyl group at C-6 did not exhibit activity, the isoprenyl group at C-6 also appears to be involved in the activity. In conclusion, the isoprenyl group at C-6 and the C-2′ hydroxyl group in the aromatic ring-C part in the isoflavan, isoflavene, or arylcoumarin skeleton are the structural requirements for PPAR-γ ligand-binding activity (Fig. 4).

Ameliorative Effects on Diabetic KK-A^y Mice

Ameliorative effects of glycyrin (**5**) on genetically diabetic KK-A^y mice were investigated using pioglitazone as a positive control. No difference was observed in food intake or body weights of mice between the treated groups and the control group. Sample intake calculated from food intake and body weights of the mice was approximately 100 mg/kg/day for the glycyrin and glycyrol groups, and 23 mg/kg/day for the pioglitazone group. After 4 days feeding, blood glucose levels were significantly decreased in both the glycyrin- and pioglitazone-treated groups in comparison with the control group, but not in the glycyrol-treated group (Table 1). In the oral sucrose tolerance test, glycyrin and pioglitazone strongly suppressed increase in blood glucose levels in mice after sucrose loading (Fig. 5). Pioglitazone, a potent PPAR-γ agonist, activates PPAR-γ, resulting in the amelioration of insulin resistance and type-2 diabetes mellitus. Glycyrin has significant PPAR-γ ligand-binding activity and appears to reduce blood glucose levels of genetically diabetic KK-A^y mice by the same biological mechanism as pioglitazone. This was supported by the finding that glycyrol, a licorice phenoilic structually related to glycyrin but without PPAR-γ ligand-binding activity, failed to improve the hyperglycemia of KK-A^y mice.

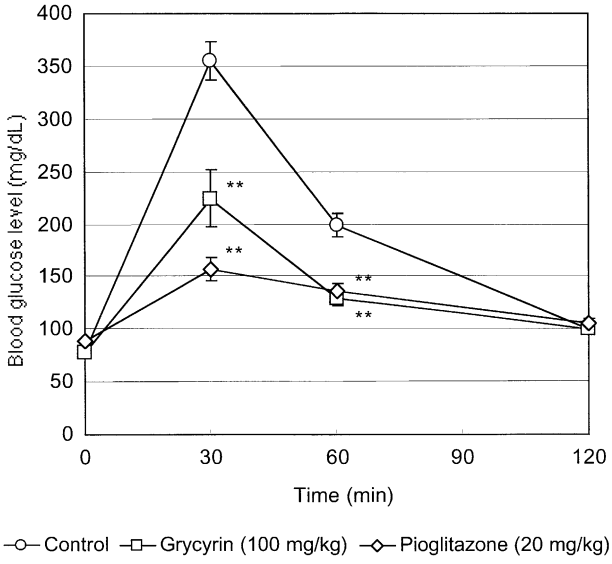

Figure 5. Ameliorative effect of glycyrin on impaired sucrose tolerance in diabetic KK-A^y mice. Data are means±SE of five mice. Statistical significance is indicated as ** ($p<0.01$) as determined by Dunnett's multiple comparison test.

Conclusion

Through screening and fractionation using a GAL-4-PPAR-γ chimera assay, the EtOAc extract of licorice

(*G. uralensis* roots) was found to exhibit significant PPAR-γ ligand-binding activity, and six phenolic compounds (**1**–**6**) were isolated from the extract as PPAR-γ ligands. When the six compounds were mixed at the same concentrations as in the licorice EtOAc extract, the PPAR-γ ligand-binding activity of the mixture accounted for more than 90% of that of the EtOAc extract (data not shown). Glycyrin, one of the active phenolics, reduced blood glucose levels of genetically diabetic KK-A^y mice in association with its PPAR-γ ligand-binding activity. Licorice has been used as not only a herbal medicine but also a sweetening agent for over 4000 years since ancient Egyptian times.[27] Here, we discovered a possible new application of this 4000-year traditional plant and its ingredients, to the amelioration of type-2 (non-insulin dependent) diabetes, a representative insulin resistance syndrome, which is becoming a serious problem in public health-care in modern society.

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25. Dehydroglyasperin D (**1**): HREIMS *m*/*z* 368.1610 $[M]^+$ (calcd for $C_{22}H_{24}O_5$: 368.1624); UV (MeOH) λ_{max} 330 (log ε 4.25) nm; IR (film) ν_{max} 3375 (OH), 2929 (CH), 1613, 1516, 1458, 1308, 1198, 1164, 1114, 1092, 1024, 978, 837 cm^{-1}; ^1H NMR (DMSO-d_6) δ 7.06 (1H, d, J = 8.4 Hz, H-6′), 6.68 (1H, s, H-4), 6.34 (1H, d, J = 2.3 Hz, H-3′), 6.33 (1H, s, H-8), 6.26 (1H, dd, J = 8.4, 2.3 Hz, H-5′), 5.09 (1H, br t, J = 6.9 Hz, H-10), 4.90 (2H, s, H_2-2), 3.75 (3H, s, C-7-OMe), 3.67 (3H, s, C-5-OMe), 3.18 (2H, br d, J = 6.7 Hz, H-9), 1.71 (3H, s, Me-13), 1.63 (3H, s, Me-12); ^{13}C NMR (DMSO-d_6) δ 67.9 (C-2), 128.8 (C-3), 114.7 (C-4), 110.4 (C-4a), 154.9 (C-5), 115.6 (C-6), 158.0 (C-7), 95.8 (C-8), 153.2 (C-8a), 22.6 (C-9), 124.1 (C-10), 130.4 (C-11), 26.0 (C-12), 18.1 (C-13), 116.8 (C-1′), 156.7 (C-2′), 103.3 (C-3′), 158.7 (C-4′), 107.4 (C-5′), 129.2 (C-6′), 62.3 (C-5-OMe), 56.3 (C-7-OMe).
26. According to *The Pharmacopoeia of Japan* (14th ed.), Glycyrrhizae Radix is prepared from the roots of *G. uralensis* or *G. glabra* and is described as containing more than 2.5% (w/w) of glycyrrhizinic acid. The HPLC chromatogram of the EtOAc extract of *G. uralensis* roots was quite different from that of *G. glabra* roots.
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