Changes in Components, Glycyrrhizin and Glycyrrhetinic Acid, in Raw *Glycyrrhiza uralensis* Fisch, Modify Insulin Sensitizing and Insulinotropic Actions

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We hypothesized that roasted Glycyrrhizae Radix (Glycyrrhizin Radix Praeparata, GRP) might modify anti-diabetic action due to compositional changes. Then we examined the anti-diabetic effect and mechanism of raw Glycyrrhizae Radix (GR) and GRP extracts and their major respective components, glycyrrhizin and glycyrrhetinic acid. In partial pancreatectomized (Px) diabetic mice, both GR and GRP improved glucose tolerance, but only GRP enhanced glucose-stimulated insulin secretion as much as exendin-4. Both GR and GRP extracts enhanced insulin-stimulated glucose uptake through peroxisome proliferation-activated receptor (PPAR)-γ activation in 3T3-L1 adipocytes. Consistently with the results of the mice study, only GRP and glycyrrhetinic acid enhanced glucose-stimulated insulin secretion in isolated islets. In addition, they induced mRNA levels of insulin receptor substrate-2, pancreas duodenum homeobox-1, and glucokinase in the islets, which contributed to improving β-cell viability. In conclusion, GRP extract containing glycyrrhetinic acid improved glucose tolerance better than GR extract by enhancing insulinotropic action. Thus, GRP had better anti-diabetic action than GR.

Key words: insulin; glucose; peroxisome proliferation-activated receptor (PPAR)-γ; islet; insulinotropic action

Type 2 diabetes is a heterogeneous metabolic disorder characterized by impairment of insulin secretion from pancreatic β-cells and insulin resistance in peripheral tissues such as liver, skeletal muscle, and adipose tissue. In most cases, insulin resistance usually precedes the impairment of insulin secretion in humans and experimental animals, and insulin resistance results in hyperinsulinemia. In order to prevent or delay the progression of diabetes, insulin should be sufficiently secreted to compensate for insulin resistance in peripheral tissues. Thus, anti-diabetic agents require the characteristics of insulin sensitization in peripheral tissues and/or insulinotropic action from pancreatic β-cells.

Since ancient Egyptian times, Glycyrrhizae Radix (GR) has been one of the most frequently employed botanicals in traditional medicines. The scientific name of GR is *Glycyrrhiza uralensis* Fisch. It belongs to the family Leguminosae. Glycyrrhizae Radix Praeparata (GRP) is produced by roasting GR until the outer color turns to purple. Glycyrrhizin, a glycoside of glycyrrhetic acid, is a major and active constituent of GR. However, glycyrrhizin is turned into a mixture of 18α- and 18β-glycyrrhetinic acid during roasting. The changes in its major component contribute to functional modification.

Several studies have reported that GR improved glucose tolerance in diabetic mice and that one of its pathways was to activate peroxisome proliferation-activated receptor-γ (PPAR-γ). The active compounds of PPAR-γ activation were prenyflavonoids such as glycycoumarin, glycyrin, and dehydroglyasperin C and D, in non-aqueous fractions of GR. However, glycyrrhizin and glycyrrhetinic acid worked as PPAR-γ agonists. Takii et al. found that glycyrrhizin in GR improved glucose tolerance in diabetic KK-Ay mice, but the mechanism of glycyrrhizin has not been elucidated.

Our preliminary study showed that GRP enhanced glucose-stimulated insulin secretion in Min6 insulinoma cells. Lately, exendin-4 was approved by the Food and Drug Administration of the US as an insulinotropic agent. Exendin-4, as a long-acting analog of glucagon-like peptide-1, promotes glucose-stimulated insulin gene transcription, biosynthesis, and secretion in pancreatic β-cells.
β-cells. It inhibits glucagon secretion in α-cells, decreases gastric motility and emptying, and induces satiety.10,11 Together with its insulinotrophic actions upon β-cells, glucagon-like peptide-1 and exendin-4 promotes DNA synthesis, the expression of pancreas duodenum homeobox (PDX)-1, and the differentiation pancreatic cells into insulin-producing cells in vitro.12

In this study, we identified an anti-diabetic effect of GR and GRP extracts in 55% pancreatectomized (Px) mice. In addition, the mechanism by which their extracts, glycyrrhizin and glycyrrhetinic acid, work as anti-diabetic agents, was investigated in vitro. Exendin-4 was used as a positive control.

Materials and Methods

Extraction of GR and GRP. GR and GRP were purchased from the Kyung Dong Herb Market (Seoul, Korea), finely ground, and extracted with 70% ethanol (EtOH) at 70 °C for 12 h. The extracts were filtered with a 0.4 µm filter, and concentrated by vacuum evaporation and lyophilization. The powder was dissolved in sterile dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO) to 1 mg/ml, and then further diluted with sterile phosphate buffered saline or media to the appropriate concentrations.

In vivo study to determine anti-diabetic effect of GR and GRP. Male C57BL6J mice aged 6 weeks were purchased from Dae Han Biolink (Seoul, Korea) and housed individually in a light- (12 h on/12 h off) and temperature-controlled room. All surgical and experimental procedures were performed according to the guidelines of the Animal Care and Use Review Committee of Hoseo University, Korea. After a 2-week quarantine, the entire splenic portion of the pancreas was surgically removed, resulting in an about 55% pancreatectomy, confirmed by weighing the removed and remnant portions during a pilot study.13 Partial Px mice showed type 2 diabetic symptoms. The 40 Px diabetic mice were randomly divided into control (cellulose treated), positive control (exendin-4 treated), and experimental (GR or GRP treated) groups. They were orally administered freeze-dried 70% EtOH extract of GR or GRP (1 mg/kg body weight) or cellulose (1 mg/kg body weight) on a daily basis for 8 weeks. Exendin-4 (150 pmol/kg body weight) was subcutaneously injected every 12 h.

At 3-week intervals, fasted animals were orally administrated 2g/kg of glucose to perform an oral glucose tolerance test (OGTT). Blood glucose was measured at 0, 10, 20, 30, 45, 60, 90, and 120 min after glucose loading by tail bleeding, and the area under the curve was calculated. Serum glucose and insulin levels were measured by the glucose oxidase method (Glucose Analyzer II, Beckman, Fullerton, CA) and with a radioimmunoassay kit (Linco Research, St. Charles, MO) respectively.

Quantification of glycyrrhizin and glycyrrhetinic acid. The contents of the effective components, glycyrrhizin and glycyrrhetinic acid, were identified by HPLC analysis. Glycyrrhizin and glycyrrhetinic acid were separated on a µ Bondapak TM C18 column (4.6 × 150 mm) with a gradient elution of 10% acetic acid and acetonitrile (60:40, v:v) by diluting acetic acid concentration at a 1 ml/min flow rate. Peaks were detected at a wavelength of 254 nm with a UV detector. Then the contents of glycyrrhizin and glycyrrhetinic acid in the extracts of GR and GRP were calculated using their standards, purchased from Sigma and Wako Pure Chemical Industry (Osaka, Japan). The 18α- and 18β-isomers of glycyrrhetinic acid were not separated in HPLC analysis. Since these epimers have been reported to have similar biological activities14,15 and they had comparable insulin-stimulated glucose uptake in our preliminary experiment, we used a racemic mixture of 18α- and 18β-glycyrrhetinic acid (1:1) as a reference for further experiments. The mixture was abbreviated as glycyrrhetinic acid.

3T3-L1 fibroblast differentiation into adipocytes. To determine the effect of GR, GRP, glycyrrhizin, and glycyrrhetinic acid on triglyceride accumulation, their designated concentrations were added into media with differentiation inducers for 4 d during the differentiation of 3T3-L1 fibroblasts. Then the vehicle, extracts, or compounds with the same assigned concentrations were treated without differentiation inducers for more than 6 d, as described in previous studies.16,17 Until the cells were harvested, DMSO, extracts or compounds were freshly added whenever the media was replaced. At the end of a 6 d incubation with extracts or compounds, the cells were harvested with a lysis buffer without glycerol, and the triglyceride contents in the cells were measured with a Trinder kit. (Young Dong Pharmaceutical, Seoul, Korea).

PPAR-γ agonist activity. Human embryo kidney (HEK) 293 cells were seeded into 96-well plates at 1 × 10⁴ cells per well 24 h before transfection. The cells were transiently transfected with a PPRE-luciferase construct (firefly pGL3-DR-1-luciferase; 0.12 µg DNA/well), pSV-SPORT-PPAR-γ expression vector (0.12 µg DNA/well), and pSV-SPORT-retinoid X receptor (RXR)-α vector (0.08 µg DNA/well) with a Lipofectamine PLUS reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. For assessment of transfection efficiency, renilla phRL-TK vector (10 ng DNA/well) was used (Promega, Madison, WI). After 42 h of transfection, the cells were treated in serum-free DMEM containing 0.1% BSA for 6 h and were exposed to specified concentrations of GR, GRP, glycyrrhizin or glycyrrhetinic acid for 24 h. At the end of incubation, the cells were washed three times with phosphate buffer saline and solubilized in a 1× passive lysis buffer (Promega). Cell lysates were assayed for both firefly
and 12 d after initiation of differentiation, adipocytes rendered islets from male mice were isolated by collagenase. Rosiglitazone was used as a positive control. The results were expressed as the ratio of firefly luciferase activity to renilla luciferase activity. Rosiglitazone and renilla luciferase activities using the Dual-Luciferase Reporter Assay System (Promega).

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Insulin-stimulated glucose uptake in vitro. Between 9 and 12 d after initiation of differentiation, adipocytes were used to determine insulin-stimulated glucose uptake by measuring the uptake of 2-deoxy- D-[1H] glucose in 3T3-L1 adipocytes, as described in previous studies. Insulin-stimulated glucose uptake in vitro. Between 9 and 12 d after initiation of differentiation, adipocytes were used to determine insulin-stimulated glucose uptake by measuring the uptake of 2-deoxy-D-[1H] glucose in 3T3-L1 adipocytes, as described in previous studies.

Glucose-stimulated insulin secretion in islets. Pancreatic islets from male mice were isolated by collagenase digestion as previously described, and were incubated for 24 h in high glucose DMEM media containing 10% (v/v) heat-inactivated fetal bovine serum, 50 mm 3-mercaptoethanol, penicillin, and streptomycin at 37 C in a humidified atmosphere with 5% CO2 to allow recovery and to eliminate acinar cells. Twenty islets were pre-incubated with low glucose DMEM containing 5 mg/ml bovine serum albumin for 16 h. The islets were treated with the vehicle (DMSO) or designated concentrations of GR, GRP, glycyrrhizin, or glycyrrhetinic acid in low (2 mm) or high (20 mm) glucose Krebs-Ringer buffers containing 20 mm Hepes pH 7.4 and 5 mg/ml bovine serum albumin for 30 min. Insulin concentrations in each medium were measured with a radioimmunoassay kit (Linco Research). Exendin-4 (2.5 nm) was used as a positive control. Cell viability was measured with a WST-1 assay kit (Roche, Mannheim, Germany) at 6, 12, 24, 36, and 48 h. These experiments were repeated four times.

Quantitative RT-PCR. The recovered islets were administered DMSO, GR, GRP, glycyrrhizin, or glycyrrhetinic acid for 8 h. Total RNA was isolated from control or treated islets with Trizol (Gibco, Life Technologies, Grand Island, NY). cDNA synthesis was performed with the RETROscript kit (Ambion, Austin, TX) using oligo dT. Then quantitative realtime PCR was carried out using the QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA) on an iCycler PCR instrument (Bio-Rad Laboratories, Hercules, CA). The PCR reactions were performed following the kit manual. The primers used for the mouse insulin receptor substrate (IRS)-2, PDX-1, glucokinase, and cyclinophilin genes are the following: IRS2 forward: 5'-CATCGACTTCTCGTCCCATCA-3', IRS2 reverse: 5'-CCCATTCTCAAGGTCAAAGG-3'; PDX-1 forward: 5'-AGGAAACAAAGGGACCCGACT-3', PDX-1 reverse: 5'-CGGGAGATGTATTTGTTAATAAGAATTC-3'; glucokinase forward: 5'-CCTGAGGCTGGAGACCCATGA-3', glucokinase reverse: 5'-TGGGGTGGGACGCACTGTA-3'; cyclinophilin forward: 5'-CAGACGCCACTGTGCGCTTT-3', cyclinophilin reverse: 5'-TGTCTTTTGAAACTTTGTCGCAA-3'. Relative quantification analysis was performed by calculating the ratio of the PCR product of the gene of interest and that of internal control cyclinophilin.

Statistical analysis. All results are expressed as mean ± standard deviation (SD). Statistical analysis was performed using the SAS program. One-way analyses of variance (ANOVA) were carried out to determine the significant differences among the treatments by GR, GRP, glycyrrhizin, and glycyrrhetinic acid. Multiple comparisons of the treatments were undertaken by Tukey tests, if significant differences existed on ANOVA. Results of P < 0.05 were considered statistically significant.

Results

In vivo hypoglycemic effects of GR and GRP

The final body weights of the Px diabetic mice administered cellulose, GR, GRP, or exendin-4 were not significantly different (Table 1). Overnight fasted and post-prandial serum glucose levels of mice treated with GR or GRP decreased as much as by exendin-4 treatment, and they were significantly lower than those by cellulose treatment (the control group). However, overnight fasted serum insulin levels were elevated only by GRP treatment as much as by exendin-4 treatment.

Table 1. Body Weight and Glucose and Insulin at the End of the Experimental Period

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 10)</th>
<th>GR (n = 10)</th>
<th>GRP (n = 10)</th>
<th>Exendin-4 (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>31.6 ± 3.8</td>
<td>30.2 ± 3.5</td>
<td>33.1 ± 3.7</td>
<td>30.0 ± 3.4</td>
</tr>
<tr>
<td>Fasting serum glucose (mm)</td>
<td>8.4 ± 0.8a</td>
<td>7.5 ± 0.9b</td>
<td>6.7 ± 0.9b</td>
<td>6.2 ± 0.9b</td>
</tr>
<tr>
<td>Post-prandial serum glucose (mm)</td>
<td>20.6 ± 3.1b</td>
<td>15.2 ± 2.8b</td>
<td>14.2 ± 2.5b</td>
<td>12.6 ± 1.9b</td>
</tr>
<tr>
<td>Fasting serum insulin (mm)</td>
<td>0.26 ± 0.04</td>
<td>0.27 ± 0.04</td>
<td>0.28 ± 0.05</td>
<td>0.29 ± 0.05</td>
</tr>
<tr>
<td>Post-prandial serum insulin (mm)</td>
<td>1.01 ± 0.13b</td>
<td>1.03 ± 0.17b</td>
<td>1.29 ± 0.25b</td>
<td>1.38 ± 0.24b</td>
</tr>
</tbody>
</table>

Mean ± standard deviation

a, b Values in the same row with different superscripts (a, b) were significantly different at P < 0.05 by the Tukey test.
120 min, suggesting that peripheral insulin resistance in GR-treated diabetic mice was lower than in the control (Fig. 1A). Unlike GR, GRP or exendin-4 lowered the peak of serum glucose levels during the OGTT test as compared to the placebo. Serum glucose levels from 30 to 120 min in GRP or exendin-4 treated mice declined with the same slope as for GR treated mice (Fig. 1A). The area under the curve of serum glucose in OGTT was lower in mice treated with GR, GRP, or exendin-4 than in the control (Fig. 1B). However, the area under the curve of serum insulin was observed to be higher in GRP treated mice than the other groups. GRP extract improved glucose-stimulated insulin secretion in Px diabetic mice nearly as much as did exendin-4, a positive control. These results indicate that both GR and GRP extracts reduced serum glucose levels by enhancing insulin sensitivity, but only that GRP extract enhanced glucose-stimulated insulin secretion in diabetic Px mice with insulin deficiency.

Table 2. Contents of Glycyrrhizin and Glycyrrhetinic Acid in Raw and Roasted Glycyrrhiza uralensis Fisch

<table>
<thead>
<tr>
<th></th>
<th>Glycyrrhiza Radix</th>
<th>Glycyrrhiza Radix Praeparata</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycyrrhizin</td>
<td>76.3 ± 5.3</td>
<td>38.9 ± 4.1</td>
</tr>
<tr>
<td>Glycyrrhetinic acid</td>
<td>10.2 ± 1.7</td>
<td>48.6 ± 3.3</td>
</tr>
</tbody>
</table>

Glycyrrhiza Radix and Glycyrrhiza Radix Praeparata represent raw and roasted Glycyrrhiza uralensis Fisch respectively. These assays were repeated four times, and the results were expressed as mean ± SD.

**Major compounds in GR and GRP**

HPLC analysis indicated that the GR extract was rich in glycyrrhizin (7.6 g/100 g extract) and low in glycyrrhetinic acid (1.0 g/100 g extract) (Table 2). During roasting, glycyrrhizin decreased by 49% and glycyrrhetinic acid increased 4.8 fold. GRP (100 g) contained 3.9 g glycyrrhizin and 4.9 g glycyrrhetinic acid.
Table 2). The chemical structures of glycyrrhizin and 18α- and 18β-glycyrrhetinic acid are given in Fig. 2.

**Insulin-stimulated glucose uptake in 3T3-L1 adipocytes**

In 3T3-L1 adipocytes, glucose uptake was stimulated at up to 10 nm of insulin in a dose-dependent manner. The glucose uptake at 0.2 and 10 nm insulin treatment was higher than baseline (no insulin treatment) by 1.6 ± 0.3 and 6.6 ± 0.8 fold, respectively. Since more than 10 nm insulin treatment did not elevate glucose uptake further than 10 nm insulin treatment, 10 nm insulin was used as an index of maximum glucose uptake.

In the absence of insulin, glucose uptake under 8 h treatment with GR, GRP, glycyrrhizin, or glycyrrhetinic acid was comparable to glucose uptake with DMSO treatment (not data shown), suggesting that its treatment did not enhance non-specific glucose uptake. When 3T3-L1 adipocytes were pretreated with GR or GRP extract for 8 h, followed by stimulation with 0.2 nm of insulin for 30 min, insulin-stimulated glucose uptake was moderately increased by both GR and GRP extracts in a dose-dependent manner (Fig. 3). GRP extract increased the uptake more than GR extract (Fig. 3). By stimulation of 0.2 nm insulin, glycyrrhizin and glycyrrhetinic acid, the major components of GR and GRP, also increased insulin-stimulated glucose uptake up to 80% of that with 10 nm insulin treatment.

**Differentiation and triglyceride accumulation in 3T3-L1 adipocytes and PPAR-γ agonist**

Both GR and GRP extracts increased the differentiation of 3T3-L1 fibroblasts into adipocytes and triglyceride accumulation (Fig. 4). However, the increase was less than with rosiglitazone treatment. Glycyrrhizin and glycyrrhetinic acid, the major components of GR and GRP, did not improve differentiation or triglyceride accumulation, since improvement in insulin-stimulated glucose uptake, accompanied by increased triglyceride accumulation in adipocytes, is associated with PPAR-γ activation. In 293 cells transiently transfected with a PPRE-luciferase construct, pSV-SPORT-PPAR-γ expression vector and pSV-SPORT-RXR-α vector, PPAR-γ agonists activated PPRE-luciferase construct, and
luciferase activity was measured by dual luciferase assay. Rosiglitazone, a commercial PPAR-γ agonist, was used as a positive control. Rosiglitazone (2 μM) upregulated luciferase activity 4.5 ± 0.6 folds from DMSO treatment (P < 0.001), while GR and GRP extracts enhanced the activity by 2.8 ± 0.4 and 3.3 ± 0.4 fold, respectively (Fig. 5). Consistently with the results for differentiation and triglyceride accumulation, glycyrrhizin and glycyrrhetinic acid did not increase luciferase activity. Hence, GR and GRP extracts should contain a moderate PPAR-γ agonist, other than glycyrrhizin or glycyrrhetinic acid.

**Glucose-stimulated insulin secretion in islets**
Insulin secretion increased by 4.8 ± 0.6 folds in high glucose (20 mM) DMEM media, compared to the low glucose (2 mM) media in islets isolated from mice. GR, GRP, glycyrrhizin, and glycyrrhetinic acid did not alter insulin secretion in low glucose media (Fig. 6), but in high glucose media (20 mM), 50 μg/ml GRP or 5 μM glycyrrhetinic acid significantly stimulated insulin secretion, as compared to DMSO treatment (Fig. 6). This increase did not reach the same level as did treatment with 2.5 nM exendin-4 (8.3 ± 1.0 ng/ml). In contrast to GRP and glycyrrhetinic acid, neither GR nor glycyrrhizin enhanced glucose-stimulated insulin secretion in high glucose media (Fig. 6).

The mRNA levels of IRS2, PDX-1, and glucokinase islets
GRP and glycyrrhetinic acid elevated glucose-stimulated insulin secretion similarly to exendin-4. Hence, we
determined whether 8-h incubation with GR, GRP, glycyrrhizin, or glycyrrhetinic acid elevated mRNA levels of IRS2, PDX-1 and glucokinase. In real-time PCR, GRP extract and glycyrrhetinic acid increased the expression of IRS2 and PDX-1 (Fig. 7A). In parallel with the results of glucose-stimulated insulin secretion, both GRP and glycyrrhetinic acid elevated the expression of glucokinase, but the induction was stronger in GRP than glycyrrhetinic acid. Cell viability decreased with DMSO treatment as time passed. GRP and glycyrrhetinic acid improved cell viability, as measured by WST-1 assay (Fig. 7B). However, GR and glycyrrhizin did not suppress cell death. Hence, GRP extract and glycyrrhetinic acid exhibit the possibility of working as an insulino-tropic agent like exendin-4.

Discussion
This study indicated that both GR and GRP extracts enhanced insulin-stimulated glucose uptake and activated PPAR-γ in 3T3-L1 adipocytes. Glycyrrhizin and glycyrrhetinic acid, major components of GR and GRP extracts, also improved insulin-stimulated glucose uptake. In addition, unlike GR, GRP improved anti-diabetic action by increasing glucose-stimulated insulin secretion and β-cell viability through induction of IRS2, PDX-1, and glucokinase expression in in vitro studies. These changes with GRP treatment were associated with increased glycyrrhetinic acid during roasting.

GR and GRP have traditionally been used in different remedies since they have distinct efficacies. Glycyrrhizin is the main substance of GR, which has been utilized as a Chinese herbal medicine for almost 4,000 years, a flavoring agent, and a sweetener.5) GR has been found to be effective in preventing and/or ameliorating metabolic syndromes such as diabetes, obesity, and hypertension in animal models.5,8) However, studies have not been conducted on the anti-diabetic action of GRP, roasted GR, even though GRP is different from GR due to positional modification. GRP reduced glycyrrhizin contents while glycyrrhetinic acid, a glycyrrhizin metabolite, was greatly increased in GRP.5) We compared the anti-diabetic characteristics of GR before and after roasting to clarify the anti-diabetic significance of the roasting.

Previous studies10–21) reported that absorption of glycyrrhizin and glycyrrhetinic acid in humans, rabbits, rodents was confirmed by metabolic pharmacokinetics in serum using liquid chromatography-electrospray ionization-mass spectrometry or HPLC, after GR or GRP was consumed. Glycyrrhizin was absorbed per se from the small intestine, and its active metabolite glycyrrhetinic acid was then absorbed from the large intestine. Even though glycyrrhizin was metabolized into glycyrrhetinic acid in the large intestine, most glycyrrhizin was absorbed in the small intestine.21) After consumption of GR containing more glycyrrhizin than glycyrrhetinic acid, serum glycyrrhizin levels were higher than serum glycyrrhetinic acid levels by 6.9 fold.21) Thus the amounts of glycyrrhizin and glycyrrhetinic acid in GR and GRP are important for detection in the blood.

Some evidence has been reported that EtOH extracts of GR have anti-diabetic action. A study by Takii et al.9) demonstrated that glycyrrhizin had a significant inhibitory effect on the post-prandial blood glucose rise in normal and diabetic mice. Other studies showed that EtOH extract of GR improved glucose homeostasis by activating PPAR-γ in animal models.5,7,8) However, the active compounds were not glycyrrhizin, but prenylflavonoids such as glycycomaririm, glycin, dehydroglyasperin C, and dehydroglyasperin D. Consistently with Mae et al.,8) GR activated PPAR-γ but neither glycyrrhizin nor glycyrrhetinic acid had PPAR-γ agonistic activity in 3T3-L1 adipocytes in our study. GRP had PPAR-γ agonistic activity at the same degree of GR. The PPAR-γ agonistic activity was not as high as that of rosiglitazone, a potent PPAR-γ agonist, but it was sufficient to alleviate glucose intolerance. Thiazolididine-
Dione drugs such as pioglitazone and rosiglitazone, which have potent PPAR-γ agonistic activity, have been approved for treating type 2 diabetes. Many studies of these drugs indicate that PPAR-γ agonists have various effects on metabolic syndromes and other diseases, such as inflammation and cancer. Unlike GR extract, GRP extract increased glucose-stimulated insulin secretion in islets. This difference was associated with increased glycyrrhetinic acid. Both 18α- and 18β-glycyrrhetinic acid work as specific gap junction inhibitors, which block cell-to-cell communication between β-cells. Disruption of cell-to-cell communication through connexin-36 modifies Ca^{2+} oscillation and insulin secretion in pancreatic β-cells. Delivery of Ca^{2+} oscillation between cells is important in amplifying insulin secretion, after glucose raises the ATP/ADP ratio that closes ATP-sensitive K^+ channels (K_{ATP} channels) in the plasma membrane in a triggering step. Conversely, some evidence suggests that gap-junctions are not required for insulin secretion in response to glucose. Thus the function of the gap junction in insulin secretion is still controversial. Squires et al. reported that up to 1 mm of heptanol, an uncoupler of the gap junction, increased glucose-stimulated insulin secretion in a dose-dependent manner by increasing the frequency of Ca^{2+} oscillations in Min6 cells without having any marked effects in the synchrony of the oscillations. Although 18α-glycyrrhetinic acid is a more potent uncoupler of the gap junction than heptanol, it did not block Ca^{2+} oscillations, nor did it re-synchronize the oscillatory activity between adjacent cells. One h treatment with 10 μm 18α-glycyrrhetinic acid did not change glucose-stimulated insulin secretion in a study of Squires et al. However, in our study, 8 h of treatment

Fig. 7. Expression of IRS2, PDX-1, and Glucokinase and Cell Viability in Islets.

A, Isolated islets were administered with vehicle (DMSO), raw Glycyrrhiza uralensis Fisch (GR) extract (50 μg/ml), roasted GR (GRP; 50 μg/ml), glycyrrhizin (5 μm), glycyrrhetinic acid (5 μm), or 2.5 nm exendin-4 in high glucose DMEM media for 8 h. Total RNA extracted from islets was reverse transcribed to make cDNA, and mRNA levels of IRS2, PDX-1, glucokinase, and cyclophilin were measured using primer sets for the full length of IRS2, PDX-1, glucokinase, and cyclophilin genes. The results represented the ratio of mRNA levels of the gene of interest (IRS2, PDX-1, or glucokinase) and those of the housekeeping gene (cyclophilin). B, Cell viability was measured with a WST-1 assay kit at 4, 8, 12, 16, and 24 h. These experiments were repeated four times, and the results were expressed as mean ± SD. *Significantly different among groups on ANOVA at P < 0.05. a,b Values of bars with different letters (a, b) were significantly different at P < 0.05 by the Tukey test.
with glycyrrhetinic acid ranged from 0.1 μm to 5 μm increased glucose-stimulated insulin secretion. Thus long-term treatment with glycyrrhetic acid can modulate other pathways to stimulate glucose-induced insulin release in islets.

Another possible approach is to induce mRNA levels of IRS2, PDX-1, and glucokinase in isolated islets, which are involved in β-cell survival and function. Induction of IRS2 expression has been known to occur by the activation of CREB in Min6 cells and islets. For example, exendin-4 extracted from lizards promoted IRS2 induction through CREB phosphorylation. It was also demonstrated that IRS2 induction activated an IGF-1/insulin signaling cascade, which increased the expression of PDX-1 and glucokinase. Many studies have shown that the expression levels of PDX-1 in islets were consistent with the proliferation of β-cells, leading to improved growth. In islets, glucokinase acts as a glucose sensor utilizing glucose to generate ATP and close the KATP channel, resulting in efficient insulin secretion when serum glucose was elevated. Thus induction of IRS2, PDX-1, and glucokinase expression promotes β-cell growth and functions to regulate glucose-stimulated insulin secretion. GRP and glycyrrhetinic acid induced expression of IRS2, PDX-1, and glucokinase, resulting in enhanced glucose-stimulated insulin secretion in islets and Px mice in this study. Thus GRP and glycyrrhetinic acid acted as insulinotropic agents in islets, but not GR or glycyrrhizin.

In conclusion, in in vivo and in vitro studies, GR and GRP extracts improved impaired glucose tolerance, possibly by enhancing insulin sensitivity. Surprisingly, GRP extract, not GR extract, greatly improved glucose-stimulated insulin secretion when serum glucose was elevated. Thus induc-tion of IRS2, PDX-1, and glucokinase expression promotes IRS2 induction through CREB phosphorylation. It was also demonstrated that IRS2 induction activated an IGF-1/insulin signaling cascade, which increased the expression of PDX-1 and glucokinase. Many studies have shown that the expression levels of PDX-1 in islets were consistent with the proliferation of β-cells, leading to improved growth. In islets, glucokinase acts as a glucose sensor utilizing glucose to generate ATP and close the KATP channel, resulting in efficient insulin secretion when serum glucose was elevated. Thus induction of IRS2, PDX-1, and glucokinase expression promotes β-cell growth and functions to regulate glucose-stimulated insulin secretion. GRP and glycyrrhetinic acid induced expression of IRS2, PDX-1, and glucokinase, resulting in enhanced glucose-stimulated insulin secretion in islets and Px mice in this study. Thus GRP and glycyrrhetinic acid acted as insulinotropic agents in islets, but not GR or glycyrrhizin.

In conclusion, in in vivo and in vitro studies, GR and GRP extracts improved impaired glucose tolerance, possibly by enhancing insulin sensitivity. Surprisingly, GRP extract, not GR extract, greatly improved glucose-stimulated insulin secretion in islets of Px mice nearly as much as exendin-4 did. Thus GRP extracts are better than GR extracts in anti-diabetic action in that they increase the amounts of glycyrrhetinic acid in in vitro and in vivo studies.

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