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Anti-diabetic effect of ginsenoside Re in *ob/ob* mice

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Abstract

We evaluated the anti-diabetic effects of ginsenoside Re in adult male C57BL/6J *ob/ob* mice. Diabetic *ob/ob* mice with fasting blood glucose levels of approximately 230 mg/dl received daily intraperitoneal injections of 7, 20 and 60 mg/kg ginsenoside Re for 12 consecutive days. Dose-related effects of ginsenoside Re on fasting blood glucose levels were observed. After the 20 mg/kg treatment, fasting blood glucose levels were reduced to 188±9.2 and 180±10.8 mg/dl on Day 5 and Day 12, respectively (both $P<0.01$ compared to vehicle group, 229±9.5 and 235±13.4 mg/dl, respectively). The EC_{70} of ginsenoside Re was calculated to be 10.3 mg/kg and was used for subsequent studies. Consistent with the reduction in blood glucose, there were significant decreases in both fed and fasting serum insulin levels in mice treated with ginsenoside Re. With 12 days of ginsenoside treatment, glucose tolerance of *ob/ob* mice increased significantly, and the area under the curve for glucose decreased by 17.8% ($P<0.05$ compared to vehicle treatment). The hypoglycemic effect of the ginsenoside persisted even at 3 days of treatment cessation (blood glucose levels: 198±13.1 with ginsenoside treatment vs. 253±20.3 mg/dl with vehicle, $P<0.01$). There were no significant changes in body weight or body temperature. Preliminary microarray analysis revealed differential expression of skeletal muscle genes associated with lipid metabolism and muscle function. The results suggest that ginsenoside Re may prove to be useful in treating type 2 diabetes.

Keywords: Diabetes; Ginsenoside Re; Intraperitoneal glucose tolerance test; Insulin; Microarray; *ob/ob* mouse

1. Introduction

Diabetes mellitus is a serious chronic metabolic disorder that has a significant impact on the health, quality of life, and life expectancy of patients, as well as on the healthcare system. In the US, diabetes is the sixth leading cause of death [1]. Diabetes is divided into two major categories: type 1 diabetes (formerly known as insulin-dependent diabetes mellitus or IDDM) and type 2 diabetes (formerly known as non-insulin dependent diabetes mellitus or NIDDM). The overall prevalence of diabetes is approximately 6% of the US population, of which 90% have type 2 diabetes [2]. Treatment and care of diabetes represents a substantial portion of the national healthcare expenditure [3].

Data from epidemiological studies [4,5] and clinical trials [6,7] showed that hyperglycemia is the principal cause of

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the complications associated with diabetes. Thus, effective control of blood glucose level is a key step in preventing or reversing diabetic complications and improving the quality of life in both type 1 and type 2 diabetic patients [8,9]. Recently, we reported that ginseng berry extract possesses significant anti-diabetic and anti-obese activities in diabetic *ob/ob* mice [10]. We observed that after the extract administration, fasting blood glucose levels reduced markedly and the improvement in glucose levels was associated with an increase in insulin-mediated glucose disposal. A preliminary observation of the study also showed an anti-hyperglycemic effect of ginsenoside Re (Re), a major constituent of ginseng berry.

The present study was designed to evaluate the anti-diabetic effects of Re in *ob/ob* mice. In *ob/ob* mice, a mutation in the leptin gene leads to morbid obesity and metabolic abnormalities such as hyperglycemia and hyperinsulinemia that phenotypically resemble human type 2 diabetes [11,12]. These *ob/ob* mice also exhibit reduced metabolism with a lower body temperature. We tested the effects of Re on glucose tolerance and serum insulin levels, as well as body weight and body temperature. In addition, we also explored possible molecular mechanisms underlying the anti-diabetic effects of Re by genome-wide gene expression profiling in skeletal muscle.

2. Materials and Methods

2.1. Animals

The study protocol was approved by the Institutional Animal Care and Use Committee of the University of Chicago. Adult male C57BL/6J *ob/ob* mice were obtained from Jackson Laboratory (Bar Harbor, ME). Animals at 10–16 weeks of age were used. Mice were housed in environmentally controlled conditions with a 12-h light/dark cycle and had free access to standard rodent pellet food (Zeigler Brothers, Gardner, PA), except when fasted before some experiments.

2.2. Drug preparation and administration

Re (Fig. 1) was obtained from Shanghai Pharmaceutical Company, China. High performance liquid chromatography (HPLC) analysis was performed in our laboratory to confirm that Re had a purity of >99% (Shimadzu Corp., Kyoto, Japan). Re standard for HPLC assay was obtained from Indofine Chemical Company (Somerville, NJ).

Re, at the doses used in this study, was dissolved in polyvinylpyrrolidone (PVP-10) solution for intraperitoneal (IP) administration once a day for 12 consecutive days. Control animals were injected with an equivalent volume of PVP-10. No irritation, restlessness or other adverse effects (i.e., respiratory distress, abnormal locomotion, or catalepsy) were detected in mice following Re or vehicle administration.

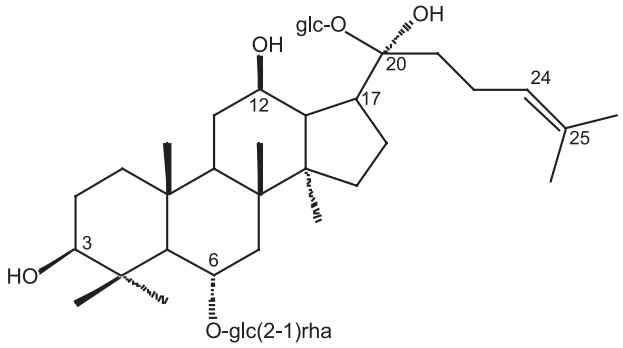

Fig. 1. Chemical structure of ginsenoside Re.

2.3. Measurement of fasting blood glucose level

Fasting blood glucose levels were measured after animals were fasted for 4 h (starting from 9:00AM) on Day 0 (before treatment), Day 5 (during treatment), and Day 12 (last day of treatment). In some experiments, fasting blood glucose levels were also measured 3 or 8 days after cessation of the treatment (i.e., on Day 15 or Day 20, respectively). Blood glucose levels were determined in blood samples from the tail vein at 1:00 PM using a Glucose Analyzer (Hemocue AB, Angelholm, Sweden).

2.4. Measurement of serum insulin level

Fed or fasting serum insulin levels were measured in tail blood samples obtained either at 9:00 AM (for fed) or at 1:00 PM following a 4 h fasting (starting from 9:00AM). Serum insulin levels were assayed with a Unitra Sensitive Insulin ELISA Kit (Crystal Chem, Chicago, IL).

2.5. Intraperitoneal glucose tolerance test (IPGTT)

On the days of the test, animals were fasted for 4 h (starting from 9:00 AM) followed by an intraperitoneal administration of glucose (2 g/kg). Blood glucose levels were determined in blood samples from the tail vein at 0 (prior to glucose administration), and 30, 60 and 120 min after glucose administration.

2.6. Measurement of body temperature

Body temperature was measured at 3:00 PM with a thermocouple probe (Physitemp, Clifton, NJ). The thermocouple probe was inserted approximately 1 cm into the rectum to obtain body temperature.

2.7. Microarray hybridization and data analysis

Total RNA was isolated from the skeletal muscles (soleus and gluteus maximus) of the *ob/ob* mice using TRIzol reagent (Invitrogen Life Technologies). Double-stranded cDNA and cRNA were synthesized according to the protocol provided by Affymetrix (Santa Clara, CA). Ten

micrograms of biotin-labeled cRNA was used to hybridize to Affymetrix MG-U74Av2 chip. The acquisition and initial quantification of array images were performed using the Affymetrix Microarray Suite Version 5.0 (MAS 5.0) with the default analytic parameters.

Subsequent data analysis included: (1) Evaluation of hybridization quality. (2) Data filtration. Genes that had signal intensity ≤100 units in all samples or genes that received an "absent" call in all samples were excluded for further analysis. (3) Identification of differentially expressed genes. D-Chip was used to identify differentially expressed genes between groups with ginsenoside Re and vehicle treatments based on the following thresholds: 1.5-fold change, and signal difference >100 units. Those genes were then classified into functional groups according to gene ontology (GO) terms.

2.8. Data and statistical analysis

Data are expressed as mean±S.E. Statistical significance between the vehicle-treated vs. Re-treated mice was determined by paired or unpaired Student's *t* test and analysis of variance (ANOVA) for repeated measures, with $P< 0.05$ considered statistically significant.

3. Results

3.1. Dose-related effects of ginsenoside Re on fasting blood glucose levels

Four-hour fasting blood glucose levels were measured on Days 0, 5 and 12 after daily IP administration of 7 mg/kg Re (n=5), 20 mg/kg Re (n=7), 60 mg/kg Re (n=6), or vehicle (n=8) in male *ob/ob* mice. Diabetic *ob/ob* mice had a high baseline fasting blood glucose level of approximately 230 mg/dl. Fig. 2 shows dose-related effects of Re on fasting blood glucose levels. Compared to the vehicle group, there was a noticeable decrease in fasting glucose levels after 7 mg/kg Re administration. After 20 mg/kg Re treatment, the glucose levels were reduced to 188±9.2 and 180±10.8 mg/dl on Days 5 and 12, respectively (both P<0.01 compared to vehicle group). Increasing the Re dose to 60 mg/kg did not result in further reduction of the fasting blood glucose levels. The EC_{70} was 10.3 mg/kg, and 10 mg/kg Re was the dose used in subsequent studies.

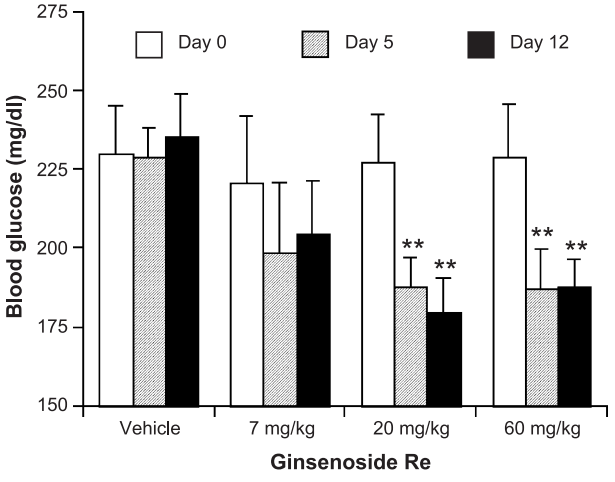

Fig. 2. Dose-related effect of ginsenoside Re on fasting blood glucose concentrations in adult *ob/ob* mice. Vehicle group, n=8; 7 mg/kg group, n=5; 20 mg/kg group, n=7; 60 mg/kg group, n=6. Compared to vehicle group, fasting glucose levels decreased significantly after 20 and 60 mg/kg ginsenoside Re treatment on Day 5 and Day 12 (**P<0.01).

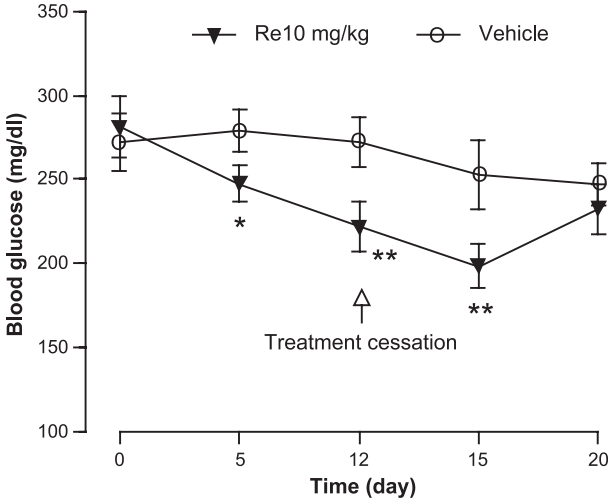

Fig. 3. Fasting blood glucose levels in adult *ob/ob* mice during and after ginsenoside Re treatment. Glucose levels decreased significantly in 10 mg/kg ginsenoside Re-treated mice (n=6) on Day 5 (*P<0.05) and Day 12 (**P<0.01) compared to vehicle-treated mice (n=6). The significant hypoglycemic effect of ginsenoside Re could still be seen 3 days after treatment cessation (i.e., on Day 15, **P<0.01), and this effect diminished 8 days after the treatment (i.e., on Day 20).

3.2. Time course of ginsenoside Re effect

To observe whether the hypoglycemic effect persisted after cessation of Re treatment, we measured fasting blood glucose levels 3 and 8 days after the last dose of Re injection. As shown in Fig. 3, the fasting glucose levels were significantly decreased in 10 mg/kg Re-treated mice (n=6) on Day 5 (P<0.05) and Day 12 (P<0.01) compared to vehicle-treated mice (n=6). Three days after cessation of the treatment (i.e., on Day 15), the glucose level was even lower than that on Day 12. The Re effect diminished 8 days after the treatment (i.e., on Day 20).

3.3. Effect of Re on serum insulin levels

In parallel with the reduction of blood glucose levels, there was a significant decrease in both fed (n=6) and fasting (n=6) serum insulin levels in animals treated with 10 mg/kg Re. As shown in Fig. 4A, after 12-day treatment with Re, fed serum insulin levels significantly reduced (P<0.01) compared to vehicle-treated mice (n=5). Similar to the

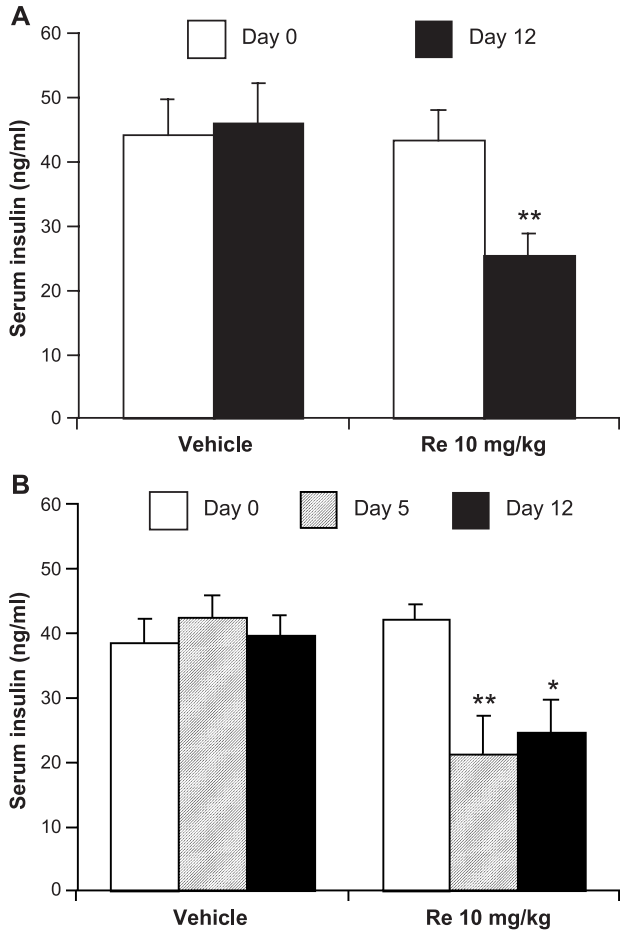

Fig. 4. Effect of ginsenoside Re on serum insulin concentrations in fed and fasting *ob/ob* mice. (A) Serum insulin levels reduced significantly under fed state after 12-day treatment with ginsenoside Re 10 mg/kg (n=6, $^{**}P<0.01$). (B) Fasting serum insulin levels also reduced significantly after 5- and 12-day ginsenoside Re treatment (n=6). $^{*}P<0.05$ and $^{**}P<0.01$ compared to vehicle-treated mice (n=5).

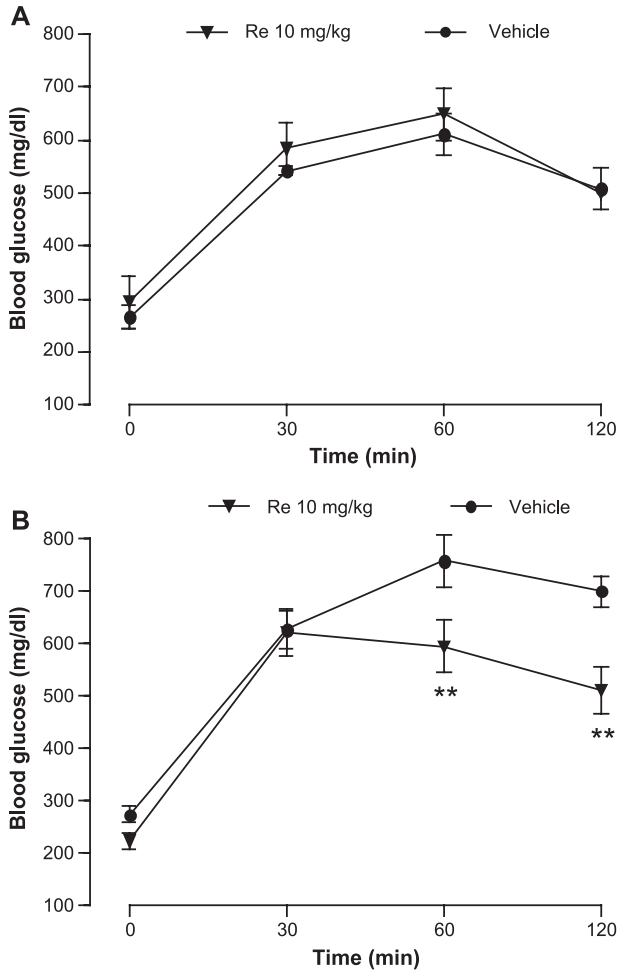

Fig. 5. Intraperitoneal glucose tolerance test in adult *ob/ob* mice before and after 10 mg/kg ginsenoside Re treatment. (A) Day 0 (before treatment). (B) Day 12 (last day of treatment), with a significantly higher rate of glucose disposal at 60 and 120 min ($^{**}P<0.01$ compared to vehicle-treated mice).

decline in fasting blood glucose levels, the fasting insulin levels (Fig. 4B) were also decreased on both Day 5 ($P<0.01$) and Day 12 ($P<0.05$), compared to vehicle-treated mice.

3.4. Effect of Re on IPGTT

Glucose tolerance was evaluated by IPGTT on Day 0 and Day 12. On Day 0, *ob/ob* mice demonstrated basal hyperglycemia. This hyperglycemia was exacerbated by the IP glucose load, and did not decrease significantly after 120 min, indicating glucose intolerance and impaired glucose disposal (Fig. 5A). After 12 days of 10 mg/kg Re treatment (Fig. 5B), the overall glucose disposal improved. There were significant differences in blood glucose levels between the Re-treated mice and the vehicle-treated mice at 60 and 120 min (both $P<0.01$). To further evaluate the overall glucose exposure, the glucose area under the curve (AUC) was calculated, which was decreased by 17.8% compared to vehicle-

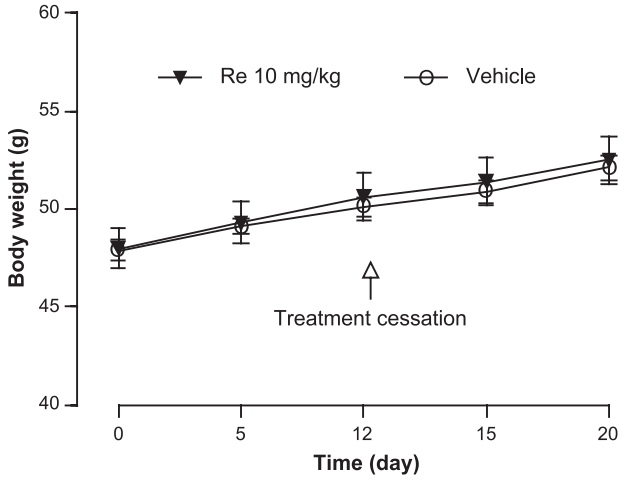

Fig. 6. Effect of ginsenoside Re on body weight in adult *ob/ob* mice. There was a continuous increase in body weight from Day 0 to Day 20 in mice that received vehicle (n=5). Compared to vehicle group, there was no significant difference in body weight of animals treated with 10 mg/kg ginsenoside Re (n=6).

treated animals. There was a significant improvement in glucose exposure from 779 mg/mL·min of Day 0 to 640 mg/mL min of Day 12 ($P<0.05$).

3.5. Effect of Re on body weight

As shown in Fig. 6, body weight of *ob/ob* mice in vehicle-treated group ($n=5$) showed a continued increase from Day 0 to Day 20. However, there was no significant difference in body weights between the vehicle group and the Re-treated group ($n=6$).

Table 1
Differential expression of genes between Re-treated and vehicle-treated mice

Gene name	Accession	Fold change (Re/vehicle)	Difference in expression
Up-regulation			
Activating transcription factor 3	U19118	4.63	498.93
Cardiac responsive adriamycin protein	AF041847	3.92	206.61
Myelin basic protein	M11533	2.47	504.18
Procollagen, type I, alpha 1	U03419	2.24	1383.44
Phospholipase A2 group VII	U34277	2.06	189.93
Aldehyde dehydrogenase family 1, subfamily A1	M74570	1.94	908.31
Receptor (calcitonin) activity modifying protein 1	AJ250489	1.94	456.79
Transforming growth factor, beta induced, 68 kDa	AV231282	1.8	149.57
RIKEN cDNA 4933436C10 gene	AI843747	1.7	577.26
Myosin, heavy polypeptide 8	M12289	1.7	209.67
T-box 14	AF013282	1.64	196.02
Aquaporin 1	L02914	1.63	323.66
Serum/glucocorticoid regulated kinase	AW046181	1.55	894.04
Down-regulation			
Troponin C, cardiac/ slow skeletal	M29793	−27.05	−2572.58
Myosin light chain, phosphorylatable	M91602	−25.63	−6135.69
Troponin T1, skeletal, slow	AV213431	−17.01	−5644.01
Troponin I, skeletal, slow 1	AJ242874	−14.84	−3122.42
Tropomyosin 3, gamma	U04541	−4.6	−4019.3
Ankyrin repeat domain 2 (stretch responsive muscle)	AJ011118	−3.96	−971.24
Stearoyl-coenzyme A desaturase 1	M21285	−3.23	−2063.46
Suppressor of K+ transport defect 3	AI837887	−3.17	−210.48
Myosin, heavy polypeptide 7, cardiac muscle, beta	AV243986	−3.03	−1068.44
Lipoprotein lipase	M63335	−2.55	−204.83
Stearoyl-coenzyme A desaturase 1	M21285	−2.5	−2863.76
Acetyl-coenzyme A synthetase 2	AW125884	−1.79	−120.5
Fatty acid synthase	X13135	−1.77	−269.27

Table 2
Significant GO terms

GO term	*P* value
Muscle development	0.000008
Troponin complex	0.000002
Striated muscle thin filament	<0.0000001
Muscle contraction	<0.0000001
Actin cytoskeleton	0.000004
Myofibril	<0.0000001
Sarcomere	<0.0000001
Muscle fiber	<0.0000001
Lipid metabolism	0.000726
Lipase	0.000072
Stearoyl-CoA desaturase	0.000899
Carboxylic acid biosynthesis	0.00029

3.6. Effect of Re on body temperature

Diabetic *ob/ob* mice exhibit significant hypothermia (35.6±0.2 °C on Day 0, $n=6$). After 12-day treatment with 10 mg/kg Re, body temperature in these animals did not change significantly (35.7±0.2 °C on Day 12).

3.7. Effect of Re on gene expression

To investigate the molecular mechanisms of action of Re, we compared the gene expression profile of Re-treated *ob/ob* mice with that of vehicle-treated *ob/ob* mice using high-density oligonucleotide arrays. As shown in Table 1, many skeletal muscle genes were differentially expressed, some of which are known to be functionally significant in the treatment of type 2 diabetes, including decreased expression of stearoyl-CoA desaturase, fatty acid synthase and lipoprotein lipase, and increased expression of lipid catabolic gene phospholipase A2 group VII and myosin heavy polypeptide 8.

Gene ontology analysis of differentially expressed genes identified 12 significant GO terms (i.e., the number of changed genes under a specific GO term is significantly enriched in comparison with a random event), which are mainly involved in muscle function and lipid metabolism (Table 2).

4. Discussion

For more than 2000 years, traditional Chinese medicine has used ginseng root to treat a variety of ailments, including symptoms similar to those of type 2 diabetes [13,14]. The active components of ginseng are considered to be ginsenosides, a group of steroidal saponins [15]. Ginsenoside Re is one of the ginsenosides and is present in a relatively high concentration in the ginseng berry [10]. The present study evaluated anti-diabetic activities of Re. We observed that Re dose dependently reduced fasting blood glucose levels in diabetic *ob/ob* mice, and this anti-diabetic effect persisted 3 days after treatment cessation. Glucose tolerance test data indicated that, after Re treatment, there was a significant

higher rate of glucose disposal. In addition, both fed and fasting serum insulin levels reduced after Re treatment.

One of the most important effects of insulin is its ability to stimulate glucose transport into muscle and fat. Prospective studies of populations at high risk for type 2 diabetes have suggested that in most patients, the initial inherited lesion is insulin resistance. Insulin-stimulated glucose disposal is markedly reduced in patients with type 2 diabetes [16]. In *ob/ob* mice, by 6 weeks of age, insulin resistance and hyperinsulinemia are well developed [11]. Our experimental animals were profoundly hyperinsulinemic, since the average value for lean control is under 5 ng/ml. After Re treatment, serum insulin levels were significantly reduced under both fed and fasting conditions, suggesting that Re improved insulin resistance in the *ob/ob* mouse model.

The insulin resistance exhibited by type 2 diabetic patients is complicated by obesity. Past studies have shown that insulin sensitivity in type 2 diabetes patients improves with weight loss [17], possibly due to an improvement in insulin-stimulated glucose transport into muscle [18]. While the whole extract of ginseng berry possesses both anti-diabetic and anti-obese activity in *ob/ob* mice [10], our results showed that Re administration did not affect body weight. Thus, different molecules in the extract may mediate the anti-diabetic and anti-obese effects in ginseng berry. The mechanisms of actions of Re for the improvement of insulin resistance, which appears to be different from those of ginseng berry extract, remain to be elucidated.

When studying a chronic disease like diabetes, it is also important to evaluate the maintenance of lower blood glucose levels with long-term treatment as well as acute hypoglycemic effect [19,20]. In this study, we measured fasting blood glucose 5 and 12 days after Re treatment. Unlike the short-term treatment study, we found that these compounds progressively reduced blood glucose levels in *ob/ob* mice. The treatment effects could be seen on Day 5, and became more evident on Day 12. Interestingly, the Re-induced hypoglycemic effect continued for 3 days after cessation of the treatment, and the fasting blood glucose level was even lower than on Day 12, the last day of the treatment. The pharmacokinetics of Re have been investigated in rabbits, with elimination half-life of approximately 50 min [21]. Further studies are needed to address precisely the discrepancy between relatively short half-life and rather prolonged hypoglycemic effect of Re. However, this difference could be linked to intermediary mechanisms that Re initiated pharmacodynamic changes, which are supported by the microarray results.

Our microarray data suggest that Re treatment induced differential expression of genes mainly involved in muscle and lipid-related metabolic pathways. Some changes seem coordinated. Among the lipid metabolic genes, the expression of lipid catabolic gene, phospholipase A2 group VII, was increased, while lipid biosynthesis gene, fatty acid synthase, was decreased. Treatment with Re also drastically suppressed the expression of lipoprotein lipase, which is known to be up-regulated in diabetic patients. Another gene worthy of comment is stearoyl-CoA desaturase, expression level of which was reduced 2.9-fold after Re treatment. Stearoyl-CoA desaturase is a central lipogenic enzyme catalyzing the synthesis of monounsaturated fatty acids. It has been shown that stearoyl-CoA desaturase −/− mice have reduced body adiposity, increased insulin sensitivity, and are resistant to diet-induced obesity [22]. Thus, inhibition of its expression by Re might be of benefit for the treatment of obesity and diabetes. Although many Re-induced changes in gene expression are related to diabetic pathways, the functional significance of these changes remains to be investigated. In addition, muscle-related genes were down-regulated after Re treatment, the significance of which is under further evaluation.

Ginseng is one of the most commonly used herbal medicines in the United States. Many currently used pharmaceutical agents were derived from natural products. Currently available drugs for type 2 diabetes have a number of limitations such as adverse effects and high rates of secondary failure [3]. This has led us to the search for new compound(s) from natural products that may have a similar degree of efficacy as the anti-diabetic drugs without the troublesome side effects. It has been shown that compound(s) identified from natural products are the most consistently successful source of drug leads. While further animal studies and clinical trials are required, the identification of compound(s) from medicinal plants with anti-hyperglycemic activity, like Re, may provide an opportunity to develop a new class of anti-diabetic agents.

In summary, we have demonstrated that Re administration in *ob/ob* mice significantly reduced fasting blood glucose levels, improved glucose tolerance and systemic insulin sensitivity without affecting body weight. These events are mediated, at least in part, by the changes in skeletal muscle gene expression. The mechanism by which Re affects gene expression remains to be determined. Our results suggest that Re may be useful in improving glucose tolerance and insulin resistance in patients with type 2 diabetes.

Acknowledgements

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