Effect of a novel proteoglycan PTP1B inhibitor from *Ganoderma lucidum* on the amelioration of hyperglycaemia and dyslipidaemia in *db/db* mice

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Abstract
Protein tyrosine phosphatase 1B (PTP1B) is implicated in the negative regulation of the insulin signalling pathway by dephosphorylating the insulin receptor (IR) and IR substrates. *Ganoderma lucidum* has traditionally been used for the treatment of diabetes in Chinese medicine; however, its anti-diabetic potency and mechanism in *vivo* is still unclear. Our previously published study reported a novel proteoglycan PTP1B inhibitor, named Fudan-Yueyang-*Ganoderma lucidum* (FYGL) from *G. lucidum*, with a half-maximal inhibitory concentration (IC50) value of 5.12 (SEM 0.05) μg/ml, a protein:polysaccharide ratio of 17:77 and 78% glucose in polysaccharide, and dominant amino acid residues of aspartic acid, glycine, glutamic acid, alanine, serine and threonine in protein. FYGL is capable of decreasing plasma glucose in streptozotocin-induced diabetic mice with a high safety of median lethal dose (LD50) of 6 g/kg. In the present study, C57BL/6 *db/db* diabetic mice were trialed further using FYGL as well as metformin for comparison. Oral treatment with FYGL in *db/db* diabetic mice for 4 weeks significantly (P<0.01 or 0.05) decreased the fasting plasma glucose level, serum insulin concentration and the homeostasis model assessment of insulin resistance. FYGL also controlled the biochemistry indices relative to type 2 diabetes-accompanied lipidaemic disorders. Pharmacology research suggests that FYGL decreases the plasma glucose level by the mechanism of inhibiting PTP1B expression and activity, consequently, regulating the tyrosine phosphorylation level of the IR β-subunit and the level of hepatic glycogen, thus resulting in the improvement of insulin sensitivity. Therefore, FYGL is promising as an insulin sensitisir for the therapy of type 2 diabetes and accompanied dyslipidaemia.

Key words: *Ganoderma lucidum*; Protein tyrosine phosphatase 1B; Diabetes: *db/db* Mice

Diabetes is recognised as a group of metabolic disorders with the common elements of hyperglycaemia and glucose intolerance, due to insulin deficiency or impaired insulin action(1). As such, enhancing insulin sensitivity is a primary strategy to improve metabolic control in subjects with type 2 diabetes. Insulin acts as a ligand that binds to insulin receptors (IR) of insulin-sensitive tissues such as liver, skeletal muscle and adipose tissue. The coordinated tyrosine phosphorylation of receptors is essential for signalling pathways regulated by insulin and leptin. Protein tyrosine phosphatases (PTP) are the regulators of tyrosine phosphorylation-dependent cellular events that govern numerous critical physiological processes(2,3). Among the PTP family members, PTP1B first purified from the human placenta(4) is known to be a key negative regulator of the IR signal transduction pathway(5). Vast amounts of studies on chemical, biochemical, cellular, animal

Abbreviations: BzN-EJJ-amide, N-benzoyl-L-glutamyl-(4-phosphono-(difluoromethyl))-L-phenylalanine-(4-phosphono-(difluoromethyl))L-phenylalanineamide; FYGL, Fudan-Yueyang-*Ganoderma lucidum*; JTT-551, monosodium(5-(1,1-dimethyllethy1)dihizol-2-yl)ethan-1-amino(4-[4-(4-propylbutyl)phenoxyl]methyl)phenyl-2(1H)azolin-3-one; HDL-C, HDL-cholesterol; HOMA-IR, homeostasis model assessment of IR β-cell function; HOMA-β, homeostasis model assessment of insulin resistance; IR, insulin receptor; IRK, insulin receptor kinase; IRS, insulin receptor substrate; LDL-C, LDL-cholesterol; NC, nitrocellulose; P3K, phosphatidylinositol-3-kinase; PKB, protein kinase B; PTP, protein tyrosine phosphatase; TC, total cholesterol.

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and human genetic levels have identified the importance of PTP1B for both insulin and leptin signalling. As an enzyme dephosphorylating the IR in the liver and skeletal muscle, PTP1B acts as an important mediator for the control of blood glucose levels and body weight through regulating IR and leptin signalling. PTP1B interacts with and dephosphorylates the IR as well as the IR substrate (IRS). If PTP1B did not dephosphorylate the IRS, the phosphorylated IRS would serve as an adaptor protein and recruit phosphatidylinositol 3-kinase (PI3K) via the regulatory subunit; PI3K would then catalyze the conversion of phosphatidylinositol to 3,4-bis- and 3,4,5-trisphosphate that would stimulate the activity of phosphoinositide-dependent kinase-1. Together with phosphoinositide-dependent kinase-2, phosphoinositide-dependent kinases activate serine/threonine protein kinase (Akt or protein kinase B), via the phosphorylation of a critical serine and threonine residue. If PTP1B were overexpressed, then most of the IRS would be dephosphorylated and a series of enzymes such as PI3K and PKB participating in the process of glucose uptake would be inactivated since the insulin transduction pathway is blocked. Insulin-resistant states show the increase in PTP1B activity. Mice that lack PTP1B display an enhanced sensitivity to insulin, with increased or prolonged tyrosine phosphorylation of the IR in the liver and muscle. In addition, PTP1B deficiency for PTP1B knockout suckling mice increases the content of glycogen and serum TAG in the liver, compared with that for wild-type controls. On the basis of these findings, the inhibition of PTP1B has emerged as an attractive therapeutic strategy to treat type 2 diabetes and obesity. The dependence of diabetes and obesity on PTP1B has increased research interest for searching more efficient PTP1B inhibitors during the last decade. Unfortunately, most of these small-molecule inhibitors of PTP1B have been proven difficult to be developed into effective drugs due to their low cell permeability and bioavailability; efforts on finding more efficient PTP1B inhibitors are still undergoing.

Ganoderma lucidum is one of the oldest mushrooms used to treat many ailments including endocrine-related diseases in traditional Chinese medicine. Scientific investigations have confirmed the beneficial effects of bioactive components isolated from G. lucidum on the prevention and treatment of human diseases. Antihyperglycaemic effects of the active extracts from G. lucidum in vitro have already been reported. As an important biologically active component in G. lucidum, polysaccharide is now arousing a great interest for its essential role in metabolism. It is reported that the polysaccharide of G. lucidum exerts its antihyperglycaemic function by influencing the metabolic pathway of hepatic glycogen. Studies have indicated that the polysaccharide of G. lucidum can promote the release of serum insulin by mediating the activity of various enzymes participating in glucose metabolism, and then decrease plasma glucose in vivo. In our previous published study, a novel proteoglycan, named Fudan-Yueyang-G. lucidum (FYGL), from the fruiting bodies of G. lucidum was also reported to be capable of decreasing plasma glucose in streptozotocin-induced mice with a high safety of median lethal dose (LD₅₀) of 6 g/kg. FYGL shows an efficient PTP1B inhibitory potency with a half-maximal inhibitory concentration (IC₅₀) value of 5·12 (SEM 0·05) µg/ml and competitive inhibition kinetics with the substrate of PTP1B in vitro. FYGL is a water-soluble proteoglycan covalently bonded with protein and polyglycan in a ratio of 17:77. Among six monosaccharides (glucose, arabinose, xylose, rhamnose, galactose and fructose) involved in FYGL, the major monosaccharide is glucose in contents of 78%, while among twenty natural amino acids, the major amino acids involved are aspartic acid, glycine, glutamic acid, alanine, serine and threonine in contents of 13, 11, 10, 9, 8 and 7, respectively. The viscosity-averaged molecular weight of FYGL is 2.6 x 10⁵.

Materials and methods

Materials and chemicals

All the dried fruiting bodies of G. lucidum were purchased from Shanghai Leiyunshang Pharmaceutical Company Limited. The kit for the analysis of insulin was purchased from Beifang Biotech Research Center. The kits for the analysis of TAG, total cholesterol (TC), LDL-cholesterol (LDL-C), HDL-cholesterol (HDL-C), NEFA and hepatic glycogen were purchased from Nanjing Jianchen Bioengineering Institute. Metformin, bovine serum albumin, porcine insulin, p-nitrophenyl phosphate, Tris, Nonidet P-40 and nitrocellulose (NC) membranes were purchased from Sigma Chemical Company. The reagents for SDS-PAGE and immunoblotting experiments were purchased from BioVision and the necessary apparatus from Bio-Rad. Protein A-Sepharose was from Pharmacia. α-PTP1B polyclonal antibody, rabbit polyclonal anti-IR β-subunit antibody and monoclonal anti-phosphotyrosine antibody (PY99) were purchased from Santa Cruz Biotechnology, Inc. Anti-rabbit IgG (IgG) conjugated with horseradish peroxidase and enhanced chemiluminescence detection reagents were from GE, Inc. Bradford protein assay reagent was purchased from Bio-Rad. All other chemicals were of the highest analytical grade.

Preparation of Fudan-Yueyang-Ganoderma lucidum from Ganoderma lucidum

FYGL was extracted from the dried fruiting bodies of G. lucidum with optimised techniques as described in Fig. 1. Briefly, 160 g dried fruiting bodies of G. lucidum were milled and then degreased with 2 litres of 95% boiling ethanol for 30 min. The residues were dried and then decocted with stirring in 3 litres of boiling water for 2 h. After filtration, the residues were treated with 2 litres of 2 M-NH₄, aqueous solution at room
Dried fruiting body of *G. lucidum*↓
2 litres boiling 95 % ethanol for 30 min
Residues↓
3 litres boiling water for 2 h
Residues
↓
2 litres of 2 m-NH$_3$ aqueous solution at room temperature for 24 h
Alkali extract↓
Neutralised, concentrated and filtered
Filtrate↓
Precipitated with ethanol in a volume ratio of 1:5
Supernatant↓
Sephadex G75 column chromatography with 2 m-NaCl solution as the eluent
FYGL

Fig. 1. Screening procedure of Fudan-Yueyang-Ganoderma *lucidum* (FYGL) from *Ganoderma lucidum*.

temperature for 24 h, then the supernatant was neutralised by 2 m-acetic acid and then dialysed, concentrated and precipitated with 80 % (v/v) ethanol. The supernatant was subjected to Sephadex G75 column chromatography with 2 m-NaCl solution as the eluent. The eluates were collected and characterised by the phenol–sulphuric acid method with UV absorption at a wavelength of 490 nm. In this analysis, three fractions were collected, and then dialysed and lyophilised. The fraction having the best PTP1B inhibition was named as FYGL. The yield of FYGL is about 0·96 % from raw material and the purity of FYGL obtained is 91 % measured by gel permeation chromatography with double detectors of UV (λ = 254 nm) and refractive index.

### In vivo experimental design

A total of ten C57BL/6 mice and twenty-eight type 2 diabetic C57BL/6 *db/db* mice (male, 5–8 weeks old) were bought from Shanghai Institute of Materia Medica, Chinese Academy of Sciences. All animal trial procedures instituted by the Ethical Committee for the Experimental Use of Animals and for Drug Safety Evaluation in Shanghai University of Traditional Chinese Medicine were followed. The ten C57BL/6 mice were set as group I, normal mice. The twenty-eight type 2 diabetic *db/db* mice were divided into four groups: group II, diabetic control mice; group III, diabetic mice treated with 75 mg/kg low dose of FYGL; group IV, diabetic mice treated with 225 mg/kg high dose of FYGL; group V, diabetic mice treated with 200 mg/kg dose of metformin. The mice were distributed based on the mean initial blood glucose levels within the four diabetic groups. All drugs were dissolved in 0·9 % saline and administered intragastrically for 4 weeks. During the experimental period, the body weight and blood glucose of 12 h fasted mice were measured every week. The dosage was adjusted every week according to any change in body weight to maintain a similar dose per kg of mice over the entire period of study for each group.

At the end of the treatment, mice were fasted overnight, anaesthetised and killed by cervical decapitation. Before killing, all the animals were administered intraperitoneally with insulin (3 U/kg in saline with 0·1 % bovine serum albumin). After killing, the liver, soleus muscle and adipose tissue were also immediately removed from all the animals in 10 min after the insulin injection and quickly frozen in liquid N$_2$, and then the tissues were stored in a freezer at −70 °C for analysis. In the present study, all procedures used for animal trials were in accordance with the Regulations of Experimental Animal Administration issued by State Committee of Science and Technology of the People’s Republic of China on 14 November 1988. The study was approved (no. SYXK (Shanghai) 2009-0065; SCXX (Shanghai) 2007-0005) by the Ethical Committee on Animal Experimentation in the Shanghai University of Traditional Chinese Medicine.

### Measurements of plasma glucose and insulin levels

Plasma samples were obtained from the tail vein 2 h before the oral administration of the drugs every week for plasma glucose analysis. The glucose level in the plasma was measured using the glucose oxidase method (sensitivity of 0·1 mm; Sigma Diagnostics). The final fasting plasma samples were obtained by celiac puncture under halothane anaesthesia before mice were killed, and then centrifuged (3000 g, 15 min) at 4 °C for the separation of serum insulin, which was measured by the RIA method.

### Lysate preparation and protein assay in vivo

Briefly, 50 mg of the frozen liver sample were homogenised in 2 ml ice-cold lysis buffer containing 50 mm-N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (pH 7·4), 100 mm-sodium fluoride, 10 mm-sodium pyrophosphate, 4 mm-EDTA, 2 mm-sodium orthovanadate and 1 % Triton X-100, and the homogenate was centrifuged (12 000 g, 15 min) at 4 °C to remove the insoluble material. Similarly, 50 mg of the frozen muscle sample were homogenised in 50 mm-NaCl, 10 mm-sodium pyrophosphate, 2 mm-Na$_2$VO$_4$, 10 mm-NaF, 2 mm-creatine acid, 2 mm-phenylmethylsulfonyl fluoride, 10 μM-leupeptin, 1 % (v/v) Nonidet P-40 and 10 % (v/v) glycerol, and the homogenate was centrifuged (12 000 g, 15 min) at 4 °C. The supernatant homogenate from the trialed mice tissue was collected individually, and the protein concentration in the homogenate was measured with Bradford protein assay reagent, using bovine serum albumin as the standard.

### Western blotting for protein tyrosine phosphatase 1B expression analysis in vivo

Liver or muscle supernatant homogenate containing 20 μg protein was run on SDS-PAGE (10 % gel) and transferred electrophoretically onto the NC membrane for 5 h. The NC membrane was then blocked for 2 h at room temperature with the block solution provided in enhanced chemiluminescence kits. The NC membrane was incubated with anti-α-PTP1B polyclonal antibody overnight at 4 °C, and then with anti-rabbit IgG conjugated with horseradish-peroxidase in block.
solution for 1 h, and washed for 30 min with wash solution (provided in enhanced chemiluminescence kits). The immuno-reactive lanes on the NC membrane were detected by the enhanced chemiluminescence method and digitalised by the Gel Documentation System. Each membrane was blotted with 10 lanes, and two mice from the same group were analysed in two adjacent lanes in one NC membrane. In total there were four NC membranes for all trialed mice and the results were averaged by ten (for the normal group) or seven (for the other groups) mice data in one group.

The PTP1B expressions in adipose tissue were not analysed because not enough samples were obtained.

**Protein tyrosine phosphatase 1B activity in vivo**

Briefly, 100 mg of the frozen tissue sample were homogenised in 1 ml ice-cold buffer containing 50 mM-Tris and 150 mM-NaCl, and the homogenates were centrifuged (12 000g for 15 min at 4°C) to remove the insoluble material. The supernatant homogenate from the trialed mice tissue was collected individually, and the protein concentration in the homogenate was measured with Bradford protein assay reagent, using bovine serum albumin as the standard.

The assay protocol of PTP1B activity in the tissue homogenate followed the kit’s instruction. Briefly, PTP1B activity was measured by adding 50 μl of 1 mg/l PY99 overnight at 4°C to remove the insoluble material. The supernatant from the homogenate was collected individually, and the protein concentration in the homogenate was measured with Bradford protein assay reagent, using bovine serum albumin as the standard.

The PTP1B enzyme reaction was terminated by the addition of 200 μl of 3 N-NaOH. The amount of p-nitrophenol produced was measured by UV absorption at a wavelength of 405 nm with a microplate reader.

**Tyrosine phosphorylation level of insulin receptor β-subunit in vivo**

Liver or muscle supernatant homogenate containing 1 mg protein was immunoprecipitated overnight with 2 μg anti-IR β-subunit antibody coupled to protein A-Sepharose at 4°C. The immune complex was washed twice with PBS (pH 7–4) containing 1% Nonidet P-40 and 2 mM-Na3VO4, and resuspended in Laemmli buffer, and boiled for 5 min. The protein was quantified and run on SDS-PAGE (10% gel), and then electrotransferred from the gel to the NC membrane. The NC membrane was incubated with 1 mg/l PY99 overnight at 4°C. The following steps were performed as described in the section ‘Western blotting for PTP1B expression analysis in vivo’.

The tyrosine phosphorylation levels of the IR β-subunit in adipose tissue were also not analysed because not enough samples were obtained.

**Measurement of the lipid profile in the serum**

Plasma samples for lipid profile analysis were obtained from mice killed at the end of the treatment. TAG, TC, LDL-C and HDL-C levels in the serum were measured following the commercial kit’s instructions.

**Assay of the lipid profile and glycogen in the liver**

Briefly, 100 mg of the frozen liver sample were homogenised in 1 ml of 0.9% saline and shaken for 30 min at 4°C. The homogenate was centrifuged (12 000g for 15 min at 4°C) and total lipids of the liver homogenates were extracted with a mixture of chloroform and methanol (2:1, v/v) on the basis of the method of Folch et al., and the supernatant homogenate was collected. NEFA, TAG and TC levels in the liver were measured according to the commercial kit’s instructions.

Then, 50 mg of the frozen liver sample were digested in 500 μl of 30% KOH in boiling water for 15 min, and then glycogen in the liver was measured following the commercial kit’s instructions.

The lipid profile and glycogen in the skeletal muscle were not analysed because not enough samples were obtained.

**Statistical analysis**

Data are expressed as means with their standard errors. The statistical differences between the mean values in the two groups were analysed by Student’s t test and one-way ANOVA using Origin 7 Software (MicroCal Software). A P value less than 0.05 or 0.01 is considered as a significant or very significant difference between the means.

**Results**

**Plasma glucose levels, homeostasis model assessment of insulin resistance and insulin concentration in vivo**

The antihyperglycaemic potency of FYGL showed dose-dependency against diabetic control mice, as shown in Fig. 2. The results of the drug treatments for 4 weeks are summarised in Table 1. As can be observed from Table 1, the fasting plasma glucose level was significantly higher (P<0.001) in diabetic control mice (27.5 (SEM 2.8) mmol/l) than in normal mice (6.9 (SEM 0.4) mmol/l). The level of fasting plasma glucose in diabetic mice treated with a high dose of FYGL daily for 4 weeks was significantly decreased (19.5 (SEM 2.1) mmol/l, P<0.01), compared with diabetic control mice (27.5 (SEM 2.8) mmol/l) and metformin-treated mice (13.9 (SEM 1.4) mmol/l) without any statistically significant difference.

It was also noted that the homeostasis model assessment of insulin resistance (HOMA-IR) was significantly higher (P<0.001) in diabetic control mice (15.8 (SEM 0.9)) than in normal mice (3.8 (SEM 0.3)), while homeostasis model assessment of β-cell function (HOMA-β) was significantly lower (P<0.001) in diabetic control mice (10.8 (SEM 0.5)) than in normal mice (72.4 (SEM 1.6)). After the mice were treated with FYGL and metformin for 4 weeks, serum insulin concentration and HOMA-IR were significantly lower in diabetic mice treated with a high dose of FYGL (9.7 (SEM 1.0) μIU/ml, P<0.05 and 8.4 (SEM 0.9), P<0.01, respectively) than in diabetic control mice (13.0 (SEM 0.7) μIU/ml and 15.0 (SEM 1.5), respectively) while
FYGL had no effect on HOMA-β. HOMA-IR (9.3 (SEM 0.9), \( P<0.001 \)) in metformin-treated diabetic mice was significantly reduced and HOMA-β (28.8 (SEM 1.2)) was significantly higher \((P<0.001)\) than that in diabetic control mice \((10.8 (SEM 0.5))\). In addition, there was no significant difference in the final body weight and body-weight growth trend among the diabetic control mice and the FYGL- and metformin-treated diabetic mice (see Table 1 and Fig. 3).

### Effects of Fudan-Yueyang-Ganoderma lucidum on protein tyrosine phosphatase 1B activity in the liver, muscle and adipose tissue

PTP1B activity in the tissues of normal mice is referred to as 100%. As can be seen from Fig. 4, PTP1B levels in the liver and skeletal muscle of the diabetic control were increased by 21 and 62% \((P<0.01)\), respectively, compared with those of normal mice. In contrast, in diabetic mice treated with a high dose of FYGL, the PTP1B level was significantly reduced by 34% \((P<0.05)\) in the liver (Fig. 4(a) and (a)) and 38% \((P<0.001)\) in the skeletal muscle (Fig. 4(b) and (b)), compared with that of diabetic control mice, but metformin had no significant effect on the PTP1B content in the tissues.

### Effects of Fudan-Yueyang-Ganoderma lucidum on the relative active ratio of protein tyrosine phosphatase 1B in the liver and skeletal muscle

When considering the active ratio of PTP1B which is calculated by PTP1B activity over the PTP1B expression, we found that 225 mg/kg FYGL significantly decreased the relative active ratio of PTP1B by 22% \((P<0.001)\) in the liver (Fig. 6(a)) and 41% \((P<0.01)\) in the skeletal muscle (Fig. 6(b)), compared with that in the diabetic control.

### Table 1. Characteristics of the trial animals after 4 weeks of drug treatments†

| Groups          | Plasma glucose (mmol/l) | Insulin (µIU/ml) | Initial body weight (g) | Final body weight (g) | HOMA-IR§ | HOMA-β|| |
|-----------------|-------------------------|------------------|-------------------------|-----------------------|---------|-------|
| Normal          | 6.9                     | 0.4              | 12.3                    | 1.1                   | 27.0    | 1.4   |
| Diabetic control| 27.5***                 | 2.8              | 13.0                    | 0.7                   | 26.9    | 1.0   |
| 75 mg/kg FYGL   | 22.9***                 | 1.2              | 11.7                    | 1.2                   | 26.9    | 1.0   |
| 225 mg/kg FYGL  | 19.5***†††              | 2.1              | 9.7†                    | 1.0                   | 26.7    | 1.7   |
| 200 mg/kg Metformin | 13.9***†††            | 1.4              | 15.0                    | 1.5                   | 26.1    | 1.5   |

HOMA-IR, homeostasis model assessment of insulin resistance; HOMA-β, homeostasis model assessment of β-cell function; FYGL, Fudan-Yueyang-Ganoderma lucidum.

***Mean values were significantly different from those of the normal group \((P<0.001)\).

†††Mean values were significantly different from those of the normal group \((P<0.05)\).

§The statistical differences between the mean values in the two groups were analysed by Student’s t-test and one-way ANOVA.

|| HOMA-β = (20 × plasma insulin level (µIU/ml) × plasma glucose level (mmol/l)) / (plasma glucose level (mmol/l) – 3.5).
The effects of the drug treatments on the lipid profile and glycogen in the liver are summarised in Table 3. As can be observed from Table 3, the TAG, TC, NEFA and glycogen levels in the liver were significantly increased \((P<0.05)\) in diabetic control mice, compared with that in normal mice. After 4 weeks, the TAG, TC and NEFA levels were significantly decreased by 65.6\% \((P<0.001)\), 60.7\% \((P<0.001)\) and 31.5\% \((P<0.05)\), respectively, while the hepatic glycogen level was significantly decreased by 52.8\% \((P<0.01)\), 75.2\% \((P<0.001)\) and 41.7\% \((P<0.05)\), respectively, in db/db diabetic mice treated with a high dose of FYGL, compared with those for the diabetic control, and the potency of FYGL was dose-dependent. However, metformin had no obvious effect on the serum lipid profiles of db/db diabetic mice.

**Effects of Fudan-Yueyang-Ganoderma lucidum on the serum lipid profile in vivo**

The effects of the drugs including FYGL and metformin on the serum lipid profile *in vivo* after 4 weeks of treatment are summarised in Table 2. The TAG, TC, LDL-C and HDL-C levels were significantly decreased by 52.8\% \((P<0.01)\), 65.9\% \((P<0.001)\), 75.2\% \((P<0.001)\) and 41.7\% \((P<0.05)\), respectively, in db/db diabetic mice treated with a high dose of FYGL, compared with those for the diabetic control, and the potency of FYGL was dose-dependent. However, metformin had no effect on the serum lipid profiles of db/db diabetic mice.

**Effects of Fudan-Yueyang-Ganoderma lucidum on the tyrosine phosphorylation level of the insulin receptor β-subunit in the liver and skeletal muscle**

The tyrosine phosphorylation level of the IR β-subunit in normal mice is referred to as 100\%. It was observed that the tyrosine phosphorylation levels of the IR β-subunit in the diabetic control were 89\% in the liver (Fig. 7(a) and (a′)) after stimulation with insulin. The tyrosine phosphorylation level (129\%) in the liver of diabetic mice treated with a high dose of FYGL was significantly higher \((P<0.05)\) than that (89\%) of control mice, as shown in Fig. 7(a) and (a′). Similarly, the tyrosine phosphorylation level (130\%) in the skeletal muscle of mice treated with a high dose of FYGL was significantly higher \((P<0.01)\) than that (82\%) of control mice, as shown in Fig. 7(b) and (b′). The effect of FYGL on the tyrosine phosphorylation level of the IR β-subunit *in vivo* was dose-dependent; in contrast, metformin had no effect on the tyrosine phosphorylation level of the IR β-subunit *in vivo*.

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The effects of the drugs including FYGL and metformin on the serum lipid profile *in vivo* after 4 weeks of treatment are summarised in Table 2. The TAG, TC, LDL-C and HDL-C levels were significantly decreased by 52.8\% \((P<0.01)\), 65.9\% \((P<0.001)\), 75.2\% \((P<0.001)\) and 41.7\% \((P<0.05)\), respectively, in db/db diabetic mice treated with a high dose of FYGL, compared with those for the diabetic control, and the potency of FYGL was dose-dependent. However, metformin had no obvious effect on the serum lipid profiles of db/db diabetic mice.
increased by 109.6% (P<0.001) in diabetic mice treated with a high dose of FYGL, compared with that of the diabetic control, and the potency of FYGL was dose-dependent. It was also observed that the TAG and hepatic glycogen levels were decreased by 60.2% (P<0.001) and 19.1%, respectively, while the TC and NEFA levels were increased by 21.4 and 8.2%, respectively, in metformin-treated diabetic mice, compared with those of the diabetic control.

Discussion

Type 2 diabetes is a metabolic disorder characterised by insulin resistance, hyperglycaemia and dyslipidaemia. PTP1B is a 50 kDa cytosolic tyrosine dephosphorylase consisting of 435 amino acids that is widely expressed in human organs (28). It is well known that PTP1B dephosphorylates both the phosphorylated IR β-subunit and the phosphorylated IRS to regulate negatively the insulin signal transmission (9). PTP1B inhibitors can be
Proteoglycan inhibitor treats diabetes

To date, finding a selective small-molecule competitive inhibitors and non-competitive inhibitors. Most of the small-molecule competitive inhibitors mimic the phosphorylated tyrosine IR, while most of the non-competitive inhibitors act via oxidation of the catalytic cysteine Cys215 or by preventing the closure of the WPD loop (29). Unfortunately, most of the small-molecule inhibitors fail to succeed in vivo. To date, finding a selective small-molecule phosphatase inhibitor with good cell permeability and oral bioavailability in vivo is difficult mainly due to the highly polar phosphatase active site and the shallowness of the surrounding protein surface, which do not allow for the productive bindings of the typical lipophilic membrane with the permeable small-molecule phosphatase inhibitor (15, 16). Significantly, FYGL, a water-soluble macromolecular proteoglycan (24) different from those small-molecule PTP1B inhibitors, does successfully inhibit the PTP1B activity and decrease the plasma glucose in vivo.

Possible interaction sites of protein tyrosine phosphatase 1B with Fudan-Yueyang-Ganoderma lucidum

There exist various interactions between PTP1B and its inhibitors. Seiner et al. (30) found an acrolein PTP1B inhibitor binding to PTP1B via the conjugation of the carbon–carbon double bond of acrolein to the catalytic cysteine residue. Liu et al. (31) reported a PTP1B analogue of 6-(phosphonodifluoromethyl)-2-naphthoic acid binding to PTP1B with its 2-carboxyl group through a bridge of a water molecule. Liu et al. (32) designed a bidentate PTP1B inhibitor called SNA. The authors proposed that electrostatic interactions are more likely to be present between PTP1B and SNA than van der Waals interactions, and

Table 2. Effects of the drugs on the serum lipid profile in vivo after 4 weeks of treatment†

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<thead>
<tr>
<th>Groups</th>
<th>TAG (mmol/l)</th>
<th>TC (mmol/l)</th>
<th>LDL-C (mmol/l)</th>
<th>HDL-C (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean SEM</td>
<td>Mean SEM</td>
<td>Mean SEM</td>
<td>Mean SEM</td>
</tr>
<tr>
<td>Normal</td>
<td>0.45 0.02</td>
<td>0.76 0.12</td>
<td>0.43 0.02</td>
<td>0.35 0.05</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>1.44*** 0.17</td>
<td>1.73*** 0.07</td>
<td>1.01*** 0.11</td>
<td>0.48 0.07</td>
</tr>
<tr>
<td>75 mg/kg FYGL</td>
<td>1.35*** 0.18</td>
<td>1.37*** 0.13</td>
<td>1.00*** 0.07</td>
<td>0.49 0.06</td>
</tr>
<tr>
<td>225 mg/kg FYGL</td>
<td>0.68†† 0.13</td>
<td>0.69†† 0.07</td>
<td>0.25††† 0.05</td>
<td>0.28† 0.02</td>
</tr>
<tr>
<td>200 mg/kg Metformin</td>
<td>1.68*** 0.10</td>
<td>1.63*** 0.08</td>
<td>1.05*** 0.09</td>
<td>0.54 0.08</td>
</tr>
</tbody>
</table>

TAG, total triglycerides; TC, total cholesterol; LDL-C, LDL-cholesterol; HDL-C, HDL-cholesterol; FYGL, Fudan-Yueyang-Ganoderma lucidum.

Mean values were significantly different from those of the normal group: *** P< 0.001.††† P< 0.001.

† The statistical differences between the mean values in the two groups were analysed by Student’s t test and one-way ANOVA.
amino acid residues of Lys41, Arg47 and Asp48 play important roles in the determination of the SNA conformation, potency and selectivity. Asante-Appiah et al. designed a peptide PTP1B inhibitor, N-benzoyl-L-glutamyl-(4-phosphono-(difluoromethyl))-l-phenylalanine-(4-phosphono-(difluoro-methyl))-l-phenylalanineamide (BzN-EJJ-amide). The crystal structure of PTP1B in the complex of PTP1B with BzN-EJJ-amide showed that the hydrogen bond between BzN-EJJ-amide and Asp48 in PTP1B is of particular significance.

**FYGL** is constructed covalently by protein and polysaccharide. Generally, proteoglycan is formed by the protein backbone and polysaccharide side chains. The protein is linked with polysaccharide by either O-linkages or N-linkages. The biological activities of proteoglycan are controlled by the interactions of polysaccharide side chains with the surrounding proteins through secondary bonding. Although proteoglycans are difficult to pass through the cell membranes, they could be dissociated by the glycosidase to enter through the cells. Actually, we found that **FYGL** can interact with glycosidase in vitro (data not shown), suggesting that **FYGL** could be hydrolysed by the glycosidase into polysaccharide and protein in the stomach and small intestine in vivo; consequently, the dissociated protein motifs in **FYGL** might enter into the plasma through pinocytosis in the terminal position of the small intestine and then interact with PTP1B to inhibit the PTP1B activity in the insulin target tissues in vivo.

The viscosity-averaged molecular weight of **FYGL** is larger than the molecular weight of PTP1B (50 kDa). However, the molecular weight of the protein motif in **FYGL** might be about 44 kDa evaluated on the protein:polysaccharide ratio of 17:77. On the other hand, the molecular weight of the protein motif in **FYGL** is larger than the molecular weight of PTP1B (50 kDa). How-
glucose level without any gain in body weight in both ob/ob and db/db mice. Also, there are some reports about natural PTP1B inhibitors. Wang et al.\(^\text{43}\) found an ethanol extract of Artemisia dracunculus L. (PMI 5011), which can decrease the blood glucose and insulin levels in KK-A\(^+\) mice through the inhibition of PTP1B activity in the skeletal muscle. Also, Astragalus polysaccharide exerts insulin-sensitised and antihyperglycaemic activities in type 2 diabetic rats through the inhibition of PTP1B in the liver\(^\text{44}\) and skeletal muscle\(^\text{45}\).

Therefore, we suggest that FYGL decreases the expression and activities of PTP1B in the insulin-sensitive tissues of the liver and skeletal muscle due to the reduction in both the expression and the relative active ratio, leading to the increase in the tyrosine phosphorylation level of the IR \(\beta\)-subunit and the improvement of insulin sensitivity. So far, no PTP1B inhibitor such as FYGL has been found to be effective in multi-tissues in vivo.

**Beneficial effects of Fudan-Yueyang-Ganoderma lucidum on the serum glucose level and insulin sensitivity through the inhibition of protein tyrosine phosphatase 1B**

As can be observed from Table 1, a high dose of FYGL decreases considerably the serum glucose levels of diabetic mice, while there are no significant differences in serum insulin concentration between the diabetic control mice and normal mice. In general, the serum glucose level is maintained within a very narrow range through the tightly coordinated secretion of insulin and glucagon in normal physiology\(^\text{40}\). Hyperglycaemia can be induced by the overexpression of glucagon because the development of hyperglycaemia is positively dependent on the glucagon:insulin ratio\(^\text{26}\). In addition, the serum insulin concentration and HOMA-IR are significantly lower in diabetic mice treated with a high dose of FYGL than those in control mice, while FYGL has no effect on HOMA-\(\beta\). There are also some reports about synthetic and natural insulin sensitisers that can decrease both plasma insulin concentrations and HOMA-IR. For example, Kim et al.\(^\text{47}\) reported that selective agonists of PARα/γ, PAR-55α, reduced plasma insulin and HOMA-IR in high-fat diet-induced obese mice. Bhavsar et al.\(^\text{48}\) isolated saponins from Helicteres isora. Saponins can decrease serum insulin and enhance insulin sensitivity in C57BL/KsJ-db/db mice through increasing the expressions of adipisin, PPAR\(\gamma\) and GLUT4. These reports indicate that insulin resistance is associated with insulin signal transduction factors including PPAR, GLUT and PTP1B. Insulin sensitivity can be improved through either the inhibition of negative factors such as PTP1B or the activation of positive factors such as PPAR and GLUT.

In the present study, the extent of the reduction in HOMA-IR (47%) and the increase in the tyrosine phosphorylation level of the IR \(\beta\)-subunit (45% in the liver and 58% in the skeletal muscle) are identical with those of the decrease in PTP1B activities (49% in the liver and 55% in the skeletal muscle) in the insulin-sensitive tissues of FYGL-treated diabetic mice and diabetic control mice, therefore leading to a 29% reduction in plasma glucose. Accordingly, it has been supposed that FYGL improves insulin sensitivity in vivo through the inhibition of PTP1B in insulin-sensitive tissues. On the other hand, it has been found that HOMA-IR in metformin-treated db/db mice is increased, and the serum insulin concentration and HOMA-\(\beta\) are also higher than those in diabetic control mice, indicating that metformin can improve insulin sensitivity as well as insulin secretion. The results are identical with those reported by Matveyenko et al.\(^\text{49}\) who found that metformin was capable of inhibiting pancreatic \(\beta\)-cell apoptosis and preserving \(\beta\)-cell function. It is generally known that metformin is an insulin sensitiser which is better than an insulin sensitisier. Nevertheless, it has been supposed that FYGL acts as an insulin sensitiser through the inhibition of PTP1B instead of an insulin secretagogue which is better than an insulin sensitisier through the inhibition of PTP1B instead of an insulin secretagogue through the recovery of pancreatic \(\beta\)-cells in db/db diabetic mice.

**Effect of Fudan-Yueyang-Ganoderma lucidum on obesity, dyslipidaemia and glycogen synthesis**

As has also been observed from Table 1 and Fig. 3, there is no significant difference in the final body weight and body-weight growth trend between the diabetic control mice and the FYGL-treated diabetic mice, indicating that FYGL would not result in overweight gain perhaps due to its decreasing effect on lipogenic enzyme activity such as fatty acid and glucose 6-phosphate dehydrogenase. The anti-obesity result is similar to that of those mono- and disalicylic acid derivatives which are potential PTP1B inhibitors as the anti-obesity drugs that suppress the weight gain by the inhibition of I\(\kappa\)B kinase-\(\beta\) and PTP1B activity\(^\text{50}\).

For diabetic patients, hyperglycaemia is often accompanied with dyslipidaemia\(^\text{51}\), which increase TAG, TC, LDL-C and decrease HDL-C. These serum lipid profiles are important plasma biochemistry indices for metabolic disorders. Abnormal values of these indices result in a series of complications. From Table 2, we found that there are higher values for TAG, TC, LDL-C and HDL-C in diabetic control mice, and Kobayashi et al.\(^\text{52}\) also observed that those indices increased in db/db mice, but FYGL significantly reduced serum lipid profiles, and the reduction potency of FYGL on LDL-C, which is highly responsible for dyslipidaemia, is about 2-fold stronger than that on HDL-C, which is an anti-arteriosclerosis factor, leading to an efficient treatment for dyslipidaemia. Unlike FYGL, metformin has no effect on those plasma biochemistry indices in vivo, indicating that the signal transduction pathway of metformin is different from that of FYGL.

There are some extracts from natural plants that have also been reported to have an antihyperlipidaemic effect on serum in vivo. For instance, Tao et al.\(^\text{52}\) isolated a polyphenol from Balanophora polyandra Griff. The extract from Balanophora polyandra Griff ameliorated hyperlipidaemia through reversing the defect expressions of anti-\(\beta\)-subunit, IRS-1, PTP1B and acetyl-CoA carboxylase \(\beta\) and increasing the phosphorylated AMP kinase. Therefore, dyslipidaemia can be ameliorated by the inhibition of PTP1B.

In addition, it has been observed from Table 3 that hepatic lipid profiles in the liver of diabetic control mice are several fold higher than those in normal mice, and decreased considerably by FYGL, implying the presence of dyslipidaemia in the liver of diabetic control mice and the effect of FYGL on hyperlipidaemia. Similarly, some natural plant extracts have...
also been reported that are able to reduce hepatic lipid profiles in vivo. Yamabe et al.\(^{53}\) evaluated loganin, an iridoid glycoside from Corni fructus. Loganin (100 mg/kg) led to a marked decrease in glucose, TAG and TC levels in the liver of db/db mice through the alleviation of oxidative stress and PPAR activation. Actually, we found that FYGL also possessed antioxidant potency (data not shown), implying that FYGL reduces hepatic lipid profiles via a multi-target mechanism including alleviation of oxidative stress and inhibition of PTP1B in db/db mice.

Besides lipid profiles, hepatic glycogen level is also an important biochemistry index in the liver. The acceleration of hepatic glycogen synthesis is one of the factors for decreasing blood glucose. The elevation of hepatic glucokinase activity and insulin sensitivity could increase the utilisation of blood glucose for glycogen storage in the liver. Table 3 shows the effect of the drug treatments on hepatic glycogen. FYGL improves the hepatic glycogen content, maybe resulting from increasing the activity of glycogen synthase via PKB and glycogen synthase kinase-3 as well as decreasing the transcription of gluconeogenic enzymes via PI3K and PKB like PTP1B antisense oligonucleotides\(^{34}\), therefore increasing glycogen synthesis.

**Conclusion**

In the present study, we demonstrated in vivo pharmacological profiles of FYGL, which is a novel proteoglycan extract from the fruiting bodies of G. lucidum, and efficient in PTP1B inhibition. After 4 weeks of oral treatment in type 2 diabetic db/db mice, FYGL could decrease considerably the plasma glucose values (P<0.01), serum insulin concentration and HOMA-IR (P<0.001), which in turn could decrease the TAG, TC, LDL-C and HDL-C levels in the serum as well as the TAG, TC and NEFA levels in the liver, indicating the improvement of hyperlipidaemia. The mechanism of plasma glucose lowered by FYGL is on the basis of inhibiting the PTP1B expression and activity, consequently regulating the tyrosine phosphorylation level of the IR β-subunit and the hepatic glycogen level. Thus, FYGL is a potential candidate for the treatment of diabetes mellitus and the accompanying metabolic disorders.

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**References**


