An extract of *Perilla stem* inhibits Src homology phosphatase-1 (SHP)-1 and influences insulin signaling

Liu Peng[1,3,a], Zhang Lei[1,3,a], Xie Xiao-na[2],Wang Deli[3] ,Sun Jing[1,3], Wang Yong-sen[1,3], Wang Zhi[1], Xing Shu[1,3], Ma Jun-feng[1,3], Li Wan-nan[1,3]* and Fu Xue-qi[1,3]*

[1]Key Laboratory for Molecular Enzymology and Engineering, Ministry of Education, Jilin University, Changchun, PR China
[2]The First Hospital of Jilin University, Changchun, PR China
[3]The State Engineering Laboratory of AIDS Vaccine, Jilin University, Changchun, RP China

Abstract: Protein tyrosine phosphatases (PTPs) are enzymes that catalyze protein tyrosine dephosphorylation of which Src homology phosphatase-1 (SHP-1) is one of the best-validated, a widely distributed intracellular tyrosine phosphatase that contains two SH2 domains. Down regulation of SHP-1 tyrosine phosphatases was significantly increased sensitivity to insulin in insulin signaling pathway. Through in vitro enzymatic reaction kinetics experiment, we found that the extract of *Perilla stem* was a potential inhibitor to ΔSHP-1, the catalytic domain of SHP-1 protein tyrosine phosphatase, and its IC_{50} was 4ug/ml, and was more sensitive towards SHP-1than other PTPs, which indicated that SHP-1 might be a target of the extract of *Perilla stem*. It can strengthened the level of tyrosine phosphorylation of insulin receptor (IR) and extracellular signal-regulated protein kinase (ERK) in HepG2 cells, and then activated the insulin signaling pathway through inhibiting the protein phosphorylation of SHP-1. These results demonstrated that the extract of Perilla stem could play an important role for diabetes treatment through inhibiting the level of SHP-1 in insulin signaling pathway.

Keywords: SHP-1, *Perilla stem*, inhibitor, insulin sensitivity.

INTRODUCTION

The rapidly increasing number of patients with diabetes mellitus is becoming a serious threat to human health around the world (Li *et al.*, 2004). The control and treatment of diabetes and its complications mainly depend on chemical or biochemical agents.

In recent years, many protein tyrosine phosphatases (PTPs) have been identified as targets for therapeutic drug development. PTPs are a family of diverse enzymes that regulate various cellular processes through a common dephosphorylation catalytic mechanism (Andersen *et al.*, 2004). The dysregulation of PTP activities contributes to the pathogenesis of several human diseases, including diabetes, obesity, cancer, and immune disorders (Arena *et al*., 2005 and Zhang 2001). In a recent research, the tyrosine phosphorylation of both insulin receptor (IR) and insulin receptor substrate (IRS) 1/2 was significantly enhanced, accompanied by a specific downregulation of SYP and SHP-1 tyrosine phosphatases (Oriente *et al*., 2011). SHP-1, a widely distributed intracellular tyrosine phosphatase that contains two SH2 domains, is one of the best-validated PTPs (Szkudelski *et al*., 2001). SHP-1 has a crucial role in cell signaling (Tonks and Neel 2001). SHP-1 is a ubiquitously expressed SH2 domain-containing cytosolic PTP that positively modulates insulin signaling (Maegawa 1999). The tyrosine phosphorylation of carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), a modulator of hepatic insulin clearance, and the clearance of serum insulin were markedly increased in SHP-1-deficient mice or hepatic cells in vitro. These findings show that SHP-1 has a role in the regulation of glucose homeostasis through modulation of insulin signaling in the liver and muscles as well as hepatic insulin clearance (Dubois, 2006).

Perilla frutescens is widely cultivated in Asia (Korea, Japan, China, northeast India and the Himalayan hills) for its essential oil and anthocyanin content and is used as a spice, colorant and in Chinese medicine (Lee *et al*., 2001). The plant can be used in prevention or treatment of depression (Takeda *et al.*, 2002), vascular diseases (Makino *et al.*, 2002). Recently, many other important pharmaceutical properties of *Perilla* have been reported, including antioxidant activity (Kim *et al.*, 2007), anti-inflammatory and anti-allergic activity (Ueda *et al.*, 2002). Furthermore, the anti-diabetic effect of *Perilla frutescens* has also been found (Hisashi *et al.*, 2010). In present study, the hypoglycemic effect of aqueous-extract from the *Extract of Perilla stem* was investigated in type 2 diabetic mouse model. Through the *in vitro* enzymatic reaction kinetics experiment, its possible mechanism has also been investigated.

MATERIALS AND METHODS

The recombinant *E. coli* containing ΔSHP-1 plasmid was kindly provided by Dr. Zhizhuang Joe Zhao, University of Oklahoma Health Science Center, USA. The purified

**Corresponding author:* e-mail: wyshshk@163.com;fxq@jlu.edu.cn

[a]Both authors contributed equally to this work.

ΔPTP-1B, ΔSHP-2, ΔTCPTP and ΔHePTP (the catalytic domain of PTP-1B, SHP-2, TCPTP and HePTP) were prepared as described previously (LiWan-nan 2002), *Longan* seed, *Selfheal, Perilla stem* and *Dogwood* were purchased from The Great Jilin Medicine Store. HepG2 cell was purchased from Key GEN Biotech. The antibodies of pY, p-ERK and p-IR were purchased from Santa Cruz.

Preparation of ΔSHP-1

The recombinant E. coli containing SHP-1 plasmid was constructed by Dr. Zhizhuang Joe Zhao, University of Oklahoma Health Science Center, USA. ΔSHP-1 was prepared as described previously (LiWan-nan 2002). The specific activity of the purified ΔSHP-1 was about 19,500 units/mg.

Determination of the inhibition ratios

20g herb was added to 200ml distilled water, soaked for 1 hour, heated until boiling and held for 30min. The mixture was filtered before 150ml distilled water was added to the residue and boiled for 20 more minutes. Then the mixture was filtered again. The liquors from these two steps were combined and centrifuged at 4°C and the supernate was collected as water extract of herbs. p-NPP was used as substrate for measuring the inhibitory rate of herb extract on ΔSHP-1. The reaction system contained 20mM p-NPP, Mops-NaOH buffer (25mM, pH7.0, 1mM DTT, 0.1M NaCl, 1mM EDTA 1mg/ml BSA), ΔSHP-1 (10μg/ml) and 10μl extract. The reaction condition was designed as incubation at 37 °C for 20min, and then the absorbance was measured at 405nm. Each experiment was performed in duplicate and the inhibitory rate of each extract was calculated. IC_{50} was analyzed with varying concentrations of *Perilla stem* extract. IC_{50} values are calculated by constructing dose-response curves.

Detection of the protein phosphorylation in vivo

HepG2 cells were treated with *Perilla stem* water extract at different concentrations for 1h. After 30 min of incubation, the cells were extracted in a whole cell extraction buffer containing 25 mM glycerophosphate (pH 7.3), 5 mM EDTA, 2 mM EGTA, 5 mM mercaptoethanol, 1% Triton X-100, 0.1 M NaCl, and a protease inhibitor mixture (Roche Applied Science). After centrifugation at 12,000 × g for 10 min, the cell lysates were resolved on a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel and then transferred to a polyvinylidene fluoride membrane for western blot analysis with insulin receptor β (Tyr1146) antibodies. p-ERK (E-4): sc-7383 detection was performed using the enhanced chemiluminescence method.

RESULTS

The specificity of inhibitor

The inhibitory specificity of water extract of *Perilla* stem was determined by comparing the IC_{50} value of different Protein tyrosine phosphatases (PTPs). Among the five selected PTPs. We purified ΔSHP-1, namely, the catalytic domain of SHP-1 from recombinant *E. coli* cells. The purified ΔSHP-1 as shown in fig. 1. The specific activity was about 19,500 units/mg. The purity of the ΔSHP-1 protein was over 90% as revealed by SDS-PAGE gray level analysis. The purity have been met the standard of inhibitor screening, the following experiments can be performed.

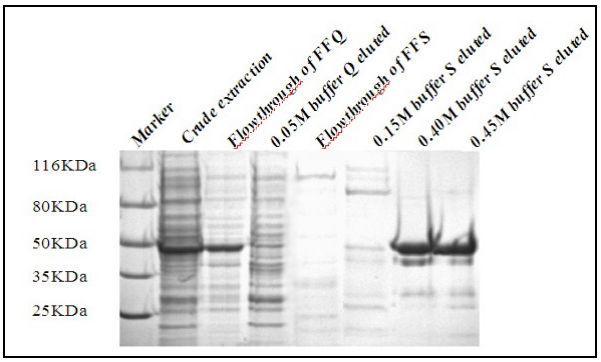

Fig. 1: The purified of ΔSHP-1 in *E. coli* through FFQ and FFS.

It could be found that the extract of *Perilla* stem's IC_{50} value on ΔSHP-1 was 4ug/ml, which was significantly lower than those of other tyrosine phosphatases (fig. 2), which indicated that ΔSHP-1 is more sensitive towards the *Perilla* stem extract than other PTPs.

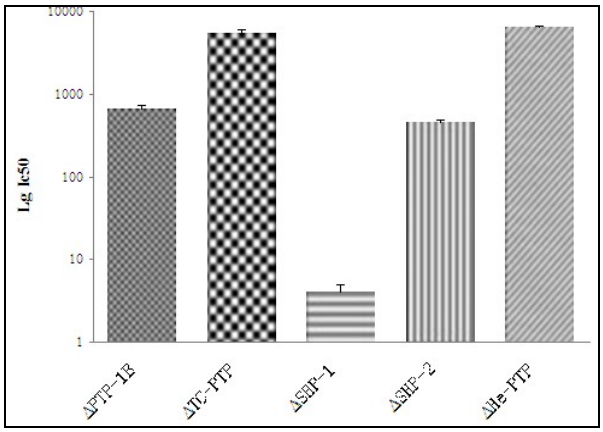

Fig.2: IC_{50} value of *Perilla stem* on different PTPs.

Concentration-dependence of the inhibitor

Different concentration of the *Perilla stem* extract is used to determine its inhibitory effect on the ΔSHP-1 (fig. 3a). The results indicated that the IC_{50} value of *Perilla stem* extract was 4μg/ml.

Evaluation of the inhibition mode of Perilla stem extract on ΔSHP-1

The Michaelis-Menten constant and maximum velocity of ΔSHP-1 were determined by Lineweaver-Burkreciprocal plots to evaluate the inhibition mode of *Perilla* stem

extract on ΔSHP-1. The Lineweaver-Burk plot showed a family of lines intercepting on the 1/v axis, suggesting a typical noncompetitive inhibition (fig. 3b).

Protein phosphorylation induced by Perilla stem extract treatment

The liver is a important organ related to glucose metabolism, HepG2 cells (Human hepatoma cells), were treated with different concentrations of *Perilla stem* extract for 30 min and it could be observed that the treatment can induce a dose-dependent significant increase in tyrosine phosphorylation of total cellular proteins by Western blotting. An extremely large variation in the level of tyrosine phosphorylation of total cellular proteins could be observed in (fig. 4a). Since the extract of *Perilla stem* is a potent inhibitor of PTPases, and an increase in the level of tyrosyl phosphorylation of several key proteins such as IR and IRS, as early step in triggering the insulin signaling cascade, we determined the effect of Perilla stem extract on the tyrosine phosphorylation of IR. As shown in fig. 4(b), the phosphorylation for IR was extremely increased by *Perilla stem* extract treatment. The experiment of SHP-1 is more sensitive towards the Perilla stem extract could be found that *Perilla stem* extract's IC_{50} value on SHP-1 was significantly lower than those of other tyrosine phosphatases. We also determined the effect of *Perilla stem* extract on the tyrosine phosphorylation of ERK. As shown in fig. 4(c), the phosphorylation for ERK was extremely increased by *Perilla stem* extract treatment.

DISCUSSION

In both insulin and leptin signaling pathways PTP1B is a negative regulator. PTP1B dephosphorylates IR and IRS-1 in the insulin signaling pathway; TCPTP is structurally the most similar to PTP1B in the PTP super family. SHP-1 and SHP-2 are highly homologous cytosolic protein tyrosine phosphatase (sharing 60% overall sequence identity). SHP-1 is mostly restricted to hematopoietic and epithelial cells, whereas SHP-2 is expressed in almost all cell types. SHP-1 is expressed in insulin target tissues and that it modulates whole-body and tissue insulin sensitivity for glucose metabolism as well as hepatic insulin clearance and SHP-2 also positively modulates insulin signaling (Uehara *et al.*, 2002); HePTP the only pTyr-specific PTP known to dephosphorylate ERK2 in lymphocytes, is critical for modulating T cell receptor (TCR) activation through mitogen-activated protein (MAP) kinase signaling (Francis *et al.*, 2011). Purified SHP-1 exhibits low PTP activity owing to inhibition by its SH2 domains and the C-terminal tail. We found that the extract of *Perilla stem* was a potential inhibitor to ΔSHP-1, and was more sensitive towards SHP-1than other PTPs,

Metabolic insulin signal transduction occurs through activation of the insulin receptor, including autophosphorylation of tyrosine (Tyr) residues in the insulin-receptor activation loop (Saltiel and Pessin 2002). PTPs negatively regulated metabolic insulin signal transduction, and is a general mechanism for down regulation of receptor tyrosine kinase (RTK) activity (Ostman and Bohmer 2001). We found that there was a dose-dependent strengthen the phosphorylation of IR in HepG2 cells, that maybe contributed to the *Perilla stem* extract inhibited the activity of SHP-1. Insulin stimulates the phosphorylation of SHP-1, presumably because of SHP-1 acting on the insulin receptor. Activating insulin signaling pathway by deficiency of SHP-1 was associated with increased insulin receptor (Bousquet *et al.*, 1998). Recent research suggests that the effect of insulin on positive lens compensation is likely to be mediated by activation of the MEK/ERK pathway (Alexandra Marcha Penha *et al.*, 2012). Since extracellular signal-regulated protein kinase mitogen-activated protein kinase (ERK MAPK) is key mediators of insulin signaling (Denner *et al.*, 2012).

CONCLUSIONS

Through *in vitro* enzymatic reaction kinetics experiment, it could be found that SHP-1is more sensitive towards the

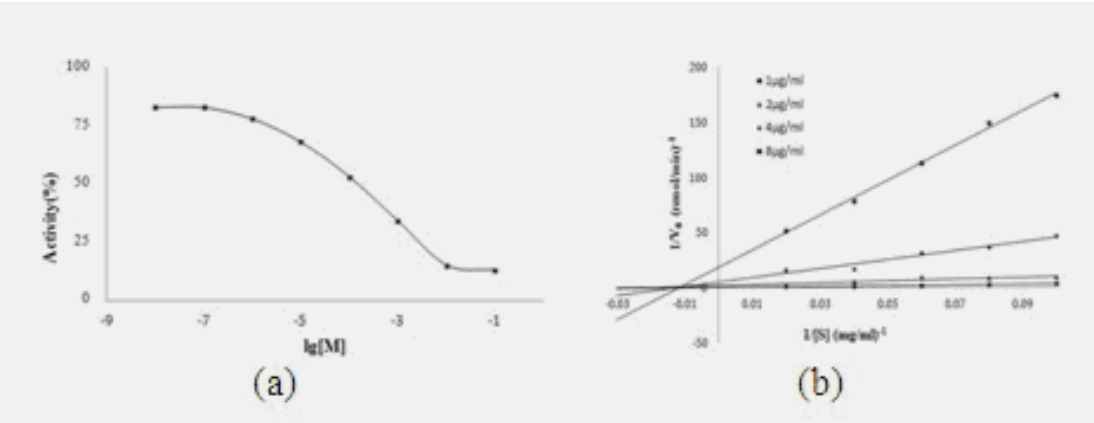

Fig. 3: *Perilla stem* extract on ΔSHP-1.a: The IC50 value of *Perilla stem* to SHP-1.b: The inhibition type of *Perilla stem* to SHP-1.

traditional Chinese medicine *Perilla stem* extract than other PTPs and its IC50 was 4μg/mL, which indicated that SHP-1 might be a potential target of *Perilla* extract and aqueous-extract from the *Perilla stem* increased the phosphorylation level of related proteins (IR, and ERK) and the total protein in insulin signaling pathway by stimulating HepG2 cells, thereby improving insulin sensitivity. Therefore, we presumed that *Perilla stem* affects the phosphorylation level of related proteins in the insulin signaling pathway by inhibiting SHP-1 activity. Screening effective drugs based on their direct targets is one of the methods for new drug discovery, which has multiple advantages including precise efficacy, clear mechanism as well as reliable quality. Besides its specificity and accuracy, this method can be utilized to find out the novel potency of existing drugs, which may lead to the secondary development of Chinese traditional herbs.

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