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PII: S0014-2999(08)00069-1
DOI: doi: 10.1016/j.ejphar.2008.01.020
Reference: EJP 64900

To appear in: *European Journal of Pharmacology*

Received date: 12 May 2007
Revised date: 23 December 2007
Accepted date: 15 January 2008

Please cite this article as: Shi, Lei, Zhang, Wei, Zhou, Yue-Yang, Zhang, Yi-Nan, Li, Jing-Ya, Hu, Li-Hong, Li, Jia, Corosolic acid stimulates glucose uptake via enhancing insulin receptor phosphorylation, *European Journal of Pharmacology* (2008), doi: 10.1016/j.ejphar.2008.01.020

Corosolic acid stimulates glucose uptake via enhancing insulin receptor phosphorylation

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Abstract

Corosolic acid, a triterpenoid compound widely existing in many traditional Chinese medicinal herbs, has been proved to have antidiabetic effects on animal experiments and clinical trials. However, the underlying mechanisms remain unknown. Here, we investigate its cellular effects and related signaling pathway. We demonstrate that it enhances glucose uptake in L6 myotubes and facilitates glucose transporter isoform 4 translocation in CHO/*h*IR cells. These actions are mediated by insulin pathway activation and can be blocked by phosphatidylinositol 3-kinase (PI_3 Kinase) inhibitor Wortmannin. Furthermore, Corosolic acid inhibits the enzymatic activities of several diabetes-related non-receptor protein tyrosine phosphatases (PTPs) *in vitro*, such as PTP1B, T cell-PTP, src homology phosphatase-1 and src homology phosphatase-2.

Key words: corosolic acid; traditional Chinese medicinal herbs; insulin receptor; protein tyrosine phosphatase; glucose uptake.

1. Introduction

Type 2 diabetes mellitus or non-insulin-dependent diabetes mellitus is a widespread syndrome characterized by fasting and post-prandial hyperglycemia affecting increasing number of the world population (Richard, 2004). Despite considerable progress in the management of diabetes mellitus with synthetic drugs, the search for indigenous natural antidiabetic agents is ongoing. With the distinctive traditional medical opinions and natural medicines mainly originated in herbs, the traditional Chinese medicine performed a good clinical practice and is showing a bright future in the therapy of diabetes mellitus and its complications. Based on a large number of chemical and pharmacological research work, numerous bioactive compounds have been found in Chinese medicinal plants for diabetes.

Corosolic acid, a triterpenoid named 2α-hydric ursolic acid, has been discovered in many Chinese medicinal herbs, such as the *Lagerstroemia speciosa L* (Fukushima et al., 2006), *banaba leaves* (Yamaguchi and Yamada, 2006), *Tiarella polyphylla* (Park et al., 2002), etc. Recently, it has been reported to have antidiabetic activity in some animal experiments and clinical trials. Miura T reported that Corosolic acid reduced the blood glucose levels and significantly lowered plasma insulin levels in KK-Ay mice 2 weeks after a single oral dose 2 mg/kg. Furthermore, blood glucose in KK-Ay mice treated with Corosolic acid significantly decreased in an insulin tolerance test (Miura et al., 2006). The muscle GLUT4 translocation from low-density microsomal membrane to plasma membrane was significantly increased in the orally

Corosolic acid -treated mice when compared with that of the controls ($P<0.05$) (Miura and Itoh et al., 2004). Fukushima M demonstrated that Corosolic acid has an effect on lowering postchallenge plasma glucose levels in vivo in human. Corosolic acid treatment subjects showed lower glucose levels from 60 min until 120 min and reached statistical significance at 90 min (Judy et al., 2003). Although Corosolic acid was suggested to be a promising lead compound for diabetes, its underlying mechanisms remain unknown.

The aim of this study is to gain the insight into the cellular effects of Corosolic acid on glucose metabolism and related signaling pathway.

2. Materils and methods

2.1 Materials

α-Modified Eagle'Medium (αMEM)，Modified Eagle'Medium (MEM) and Ham's F12 medium (F12) were from GIBCO^{TM}. Fetal bovine serum (FBS) was purchased from Hyclone. 2-deoxyglucose (2-DOG), cytochalasin B, and Wortmannin were from Sigma. [^{3}H]-2-DOG were from Amersham. pY20, pY1162/1163 and anti-insulin receptor (IR) were from Santa Cruz Biotechnology. Anti-Akt and anti-phospho-Akt Ser^{473} polyclonal antibodies, anti-β-actin, anti-mouse IgG and anti-rabbit IgG HRP-linked antibodies were from Cell Signal Technology. Polyvinylidene Fluoride (PVDF) membranes were from Immobilon Millipore. ECL reagents were from Calbiochem.

2.2 Cell culture

The Chinese hamster ovary (CHO) cell line transfected with an expression plasmid encoding human insulin receptor (CHO/*h*IR) was a kindly gift from Dr. Michel Tremblay of McGill University. The cells were grown in F12 medium supplemented with 10% (v/v) FBS, 2 mM L-glutamine, and 50 units/ml penicillin and 50 μg/ml streptomycin. L6 myoblast cell line was a kindly gift from Dr. David James of Garvan Institute of Medical Research in Australia. The cells were grown in αMEM supplemented with 10% FBS. For differentiation, the cells were seeded in appropriate culture plates, and after sub-confluence (about 70%), the medium was changed to αMEM supplemented with 2% FBS. The medium was then changed every 2 days until the cell were fully differentiated.

2.3 Vector construction

The cDNA encoding human GLUT4 with haemaglutinin (HA) epitope tag was amplified by standard PCR techniques from pBabe-HA-GLUT4 plasmid (a kindly gift from Prof. David James of Garvan Institute of Medical Research in Australia) and subcloned into pEGFPN1 (a kindly gift from by Prof. Boliang Li, Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences in P. R. China) in the same frame to GFP. The primers were: 5'-ATCTAGAATTCGCCGCCACCATGG GACCGTCG GGCTT C-3' and 5'-ATGCAGGATCCCGGTCGTTCTCATC-3'.

2.4 Transfection

4 μg vector was transfected into 80% confluent CHO/*h*IR cells in a 3.5 cm dish with lipofectamine 2000 (Invitrogen), and transfered them into 12 well plate at the density of 20000 cells per well 24 hours later. The experiment was carried out after another 24 hours. .

2.5 [^{3}H]-2-DOG Glucose uptake assay.

The assay was initiated by the addition of [^{3}H]-2-DOG to a final concentration of 100 μM and 0.5 μCi per well in Hepes Buffered Saline (HBS) containing 20 mM HEPES pH7.4, 140 mM NaCl, 5 mM KCl, 2.5 mM $MgSO_4$,and 1.0 mM $CaCl_2$. After 10 min incubation at 37 ºC, cells were washed with ice-cold PBS, lysed using 0.1% Triton-X 100 and counted for radioactivity. Noncarrier-mediated uptake was determined in the presence of 10 μM cytochalasin B. Protein concentrations were measured by the Bradford method. Data were expressed as pmol/min/mg protein.

2.6 Immunohistochemistry

Cells were fixed with 4% polyformalin for 15 min, and blocked with 10% goat

serum in PBS for 1 hour, then incubated with mouse anti HA primary antibody (COVANCE) in 1:1000 dilution overnight at 4°C. The cells were incubated with goat anti-mouse secondary antibody conjugated with Alexa Fluor 555 (Molecular Probs) in 1:500 dilution for 30 min. Finally the nucleus was stained with 5μg/ml Hochest 33342 for 5 min. Three times 5 min washing with ice-cold PBS were conducted between every two procedures above. The images were taken by photometric camera working with *Olympas* 1X51 Fluorescence microscopy.

2.7 Western blot

Cells were rinsed twice with PBS and terminated immediately by liquid nitrogen, then lysed with 1×SDS loading buffer. Samples were electrophoresed on 10% SDS-polyacrylamide gels, and transferred to PVDF membranes. The membranes were blocked for 1 hour with 5% (w/v) BSA, incubated with the primary antibodies overnight at 4 ºC and the secondary antibodies for 1 h at room temperature. Antigen–antibody complexes were detected by the ECL kit.

2.8 Cell membrane isolation and insulin receptor activation

Cell membrane isolation and insulin receptor activation are described by Ling et al., (1999) and Morten et al., (2001).

2.9 Inhibitory effects of Corosolic acid on some PTPs

The inhibitory effects of Corosolic acid were evaluated on some PTP family members: Protein Tyrosine Phosphatase 1B (PTP1B), T-cell Protein Tyrosine Phosphatase (TcPTP), Src homology domain 2 (SH2)-containing tyrosine phosphatase-1 (SHP1), src homology phosphatase-2 (SHP2), leukocyte antigen-

related phosphatase (LAR), protein tyrosine phosphatase e (PTPe). GST-fusion human PTP1B (91-1053 according to **GI190741**), TCPTP (41-1075 according to **BC008244**), SHP1 (244-570 according to **BC002523**), SHP2 (1116-2162 according to **NM002834.3**), LARD1 (1275-1613 according to **GI18860871**) and PTPeD1 (554-1630 according to **BC050062**) were cloned into pGEX-KG plasmid. PTPs were overexpressed as GST-fusion proteins in *Escherichia coli* BL21-Conden Plus (DE3) and purified through affinity chromatography. Assays were performed for PTPs using 2 mM pNPP as substrate around their Km value (PTP1B: 0.90 mM, TCPTP: 1.12 mM, SHP1: 11.76 mM, SHP2: 7.82 mM, LARD1: 0.87 mM, PTPaD1: 1.25 mM, PTPeD1: 5.38 mM) at their optimal pH, respectively, in the presence or absence of the compounds. The evaluation of inhibitory effect is described previously (Yang et al., 2005).

2.10 Statistical analysis

Data are presented as mean of triplicate assays ±S.D. Statistical analysis of the data for multiple comparisons was performed by analysis if variance (ANOVA). For single comparison, the significance of differences between means was determined by t-test. *: $P<0.05$, **: $P<0.01$, ***: <0.001 compared with control.

3. Results

3.1 Corosolic acid enhanced glucose uptake in L6 myotubes

We tested the effect of Corosolic acid on glucose uptake in L6 myotubes with or without a range of insulin concentrations. In the absence of insulin, the rate of basal glucose uptake increased in a dose-dependent manner and reached a flat roof with about 30% increment at 250 nM Corosolic acid (Fig. 2A).

To determine its effect on the insulin dose-response curve, L6 myotubes were treated with 250 nM Corosolic acid for 1 h and then stimulated with a range of insulin concentrations for 30 min. As shown in Fig. 2B, 250 nM Corosolic acid also enhanced insulin-stimulated glucose uptake at sub-maximal insulin concentrations (1 and 10 nM), whereas the same treatment had no significant effect at saturating insulin concentration (100 nM).

3.2 Wortmannin blocked Corosolic acid -stimulated glucose uptake in L6 myotubes

Wortmannin is a specific inhibitor of phosphatidylinositol 3-kinase (PI_3 Kinase) which is a key downstream kinase of insulin pathway. It was utilized to illustrate the relationship between Corosolic acid and insulin pathway in glucose uptake. L6 myotubes were pretreated with 100 nM Wortmannin for 30 min, and then incubated with 250 or 500 nM Corosolic acid for 1.5 hour. As shown in Fig. 2C, Corosolic acid -stimulated glucose uptake was abolished by 100 nM Wortmannin and had greatly significant difference comparing with that absence of Wortmannin. Meanwhile, Wortmannin abolished different concentrations of the compound-stimulated glucose uptake to similar degree as control (Fig.2C). It implied that Corosolic acid might

stimulate glucose uptake via insulin pathway.

3.3 Corosolic acid stimulates GLUT4 translocation and the effect is inhibited by Wortmannin

Glucose uptake is mainly carried out by GLUT4 translocation from cytoplasm to cell membrane for transporting glucose into cell. To investigate Corosolic acid's effect on GLUT4 translocation, we transfected the vector of human GLUT4 with HA epitope tag inserted at the extracellular loop between the first and second transmembrane sequence into pEGFP-N1 into CHO/*h*IR cells. As shown in Fig.3, comparing with DMSO treatment as a vehicle, 500 nM Corosolic acid and 100 nM insulin treatments (panel 2 and 3) induced GLUT4 translocation into cell membrane obviously, and both actions could be abolished by Wortmannin (panel 5 and 6).

3.4 Corosolic acid induces insulin receptor phosphorylation in cultured CHO/hIR cells

According to the results above, we supposed that Corosolic acid might stimulate glucose uptake via insulin pathway. CHO/*h*IR cells were incubated with different concentration of Corosolic acid for 2 hours, and then treated with or without 10 nM insulin for 10 min. As shown in Fig. 4A and 4B, Corosolic acid increased the level of tyrosine phosphorylation of insulin receptor ß, reaching the maximal effect in 500 nM, and synergistically increased the insulin-induced tyrosine phosphorylation level of insulin receptor ß.

Meanwhile, we investigated the time course of Corosolic acid-enhanced insulin receptor ß tyrosine phosphorylation level. CHO/*h*IR cells were incubated with 500

nM Corosolic acid for different time, and then stimulated with 10 nM insulin for 10 min. As shown in Fig 4C, the maximal effect was in around 2 hours.

3.5 Corosolic acid did not enhance AMPK phosphorylation

AMP-activated protein kinase (AMPK) is another pathway which increase muscle cell glucose uptake by contraction/exercise in physiological condition. We investigated whether Corosolic acid could affect AMPK phosphorylation level in L6 myotubes. As shown in Fig.4D, positive compound Berberine could stimulate AMPK Thr^{172} phosphorylation, however, Corosolic acid with the effective concentration in glucose uptake could not.

3.6 Wortmannin blocked Akt but not insulin receptor's phosphorylation induced by Corosolic acid

Akt Ser^{473} phosphorylation is an important node in signal transduction of insulin pathway for its accepting signal from upstream and passing to downstream, finally giving rise to related physiology phenomenon such as glucose uptake and glycogen accumulation. To confirm the hypothesis that Corosolic acid enhanced insulin signal pathway, we tested its effect on the phosphorylation of Akt Ser^{473} after treatment with different concentrations of insulin. As shown in Fig.5A and 5B, the increased fold of Ser^{473} phosphorylation by 500 nM Corosolic acid relative to control is 1.35, 2.13, 2.40 and 1.17 after stimulated with 0.1,1,10,100 nM insulin, respectively.

Due to increased phosphorylation on both insulin receptor and AKT induced by Corosolic acid, we used Wortmannin to investigate whether it increased AKT phosphorylation through insulin receptor activation. As shown in Fig. 5C, no matter

treated with Wortmannin or not, Corosolic acid increased phosphorylation of insulin receptor in the similar level. However, Akt Ser^{473} phosphorylation enhanced by Corosolic acid could be abolished by 100 nM Wortmannin.

3.7 Corosolic acid could not enhance insulin receptor ß phosphorylation directly

To test whether Corosolic acid enhanced insulin receptor phosphorylation directly, Sodium vanadate was utilized to inhibit PTPs in intact cells to minimize the dephosphorylation effect of PTPs on insulin receptor, and then cells were treated with 500 nM Corosolic acid for different time. As shown in figure 6A and 6B, the effect of Sodium vanadate to enhance insulin receptor phosphorylation became weak by the time, and it could stimulate insulin receptor phosphorylation on the base of Sodium vanadate's effect in long time interval (120 min), but not in short time interval (within 60 min). Meanwhile, isolated membrane of CHO/*h*IR was used to test whether Corosolic acid could directly activate insulin receptor ß. As the result shown in Fig.6C, Corosolic acid could not enhance insulin receptor ß phosphorylation at all.

3.8 Corosolic acid inhibited the activities of several insulin pathway related PTPs

The critical negative regulatory step in insulin signal transduction is the dephosphorylation of signaling molecules by PTPs, which helps to terminate insulin signaling (Bleyle et al., 1999; Cheng et al., 2002; Drake et al., 1998). Changes in the expression levels or activities of specific PTPs, including protein tyrosine phosphatase 1B (PTP1B), T-cell protein tyrosine phosphatase (TCPTP), src homology phosphatase-1 (SHP1), src homology phosphatase-2 (SHP2), leukocyte antigen-related phosphatase (LAR), protein tyrosine phosphatase ε (PTPε), have been reported to influence insulin pathway, and implicate insulin sensitivity, which is the most common inherent

pathology of type 2 diabetes mellitus and obesity (Ahmad et al., 1995, 1997,a,b; Asante-Appiah and Kennedy, 2003; Dubois and Bergeron et al., 2006; Galic et al., 2004; Nakagawa et al., 2005; Norris et al., 1997). To test the hypothesis that Corosolic acid may enhance insulin receptor tyrosine phosphorylation through inhibiting the activities of certain PTPs, we expressed and purified recombinant human PTP1B, TCPTP, SHP1, SHP2, PTPe D1 and LARD1, and determined the IC_{50} values of Corosolic acid on them. The results are shown in Table 1, Corosolic acid showed selective inhibition on PTP1B, TCPTP, SHP1 and SHP2, and no visible inhibitory activity at 100 μM towards receptor-like transmembrane phosphatases such as LAR and PTPε.

4. Discussion

Disruption of the maintenance of blood glucose concentration is a characteristic feature of non-insulin-dependent diabetes mellitus (Moller et al., 2001) and can be associated with a number of complications including cardiovascular disease (Keen,et al., 1999) and renal failure (Ritz, 1999). Therefore, controlling blood glucose levels is important for either preventing or delaying the progression of complications (The Diabetes Control and Complication Trial Research Group, 1993; UKPDS, 1998). The mechanisms responsible for producing high levels of glucose in the blood in type 2 diabetes are defects in pancreatic secretion of insulin and insulin action (American Diabetes Association, 2001; Cavaghan and Ehrmann et al., 2000) and insulin resistance in target tissues, mainly muscle and the liver.

Skeletal muscles account for approximately 75% of glucose absorption under insulin-stimulated conditions (DeFronzo et al., 1981) and a reduction in insulin - stimulated glucose uptake in skeletal muscles of type 2 diabetic patients has been observed both in vitro (Dohm et al., 1988) and in vivo (DeFronzo et al., 1992).The rat muscle cell line of L6 has been widely used to investigate the mechanism of insulin stimulated glucose transport (Klip and Paquet et al., 1990; Cheng et al., 1996; Oh et al., 2006) and skeletal muscle is considered an important therapeutic target tissue for non-insulin-dependent diabetes mellitus (Ritz, 1999). Thus, we employed L6 myotubes to evaluate the effect of Corosolic acid on glucose uptake. Our results validated the enhancement on glucose uptake in L6 myotubes by Corosolic acid which was consistent with its action of lowering blood glucose level in animal (Miura

et al., 2006) and clinical trials (Fukushima et al., 2006; Judy et al., 2003). Meanwhile, we found Corosolic acid was able to enhance glucose uptake stimulated by sub-maximal concentration of insulin (1 or 10 nM) and this synergic effect was covered by maximal concentration of insulin (100 nM). These results hinted us that it could improve the sensitivity of L6 myotubes to insulin. In order to validate the hypothesis, we used Wortmannin, a PI3 Kinase specific inhibitor, to block intrinsic insulin pathway. The compound induced enhancement on glucose uptake was abolished by Wortmannin completely. This hinted us that it might enhance glucose uptake via insulin pathway. Meanwhile, we tested the effect of Corosolic acid on AMPK pathway, the other distinct signaling pathway to stimulate glucose uptake which can be activated by muscle contraction/exercise in physiological condition, and the result showed AMPK could not be activated by Corosolic acid.

At the cellular level, insulin-stimulated glucose uptake results from the translocation of the GLUT4 from intracellular storage sites to the cell membrane (Cheng et al., 2002). We observed that Corosolic acid was able to promote GLUT4 translocation in CHO/*h*IR cell. It is consistent not only with our previous results on glucose uptake but also with the results in previous publication (Miura and Itoh et al., 2004). And the fact that Wortmannin abolished the effect on GLUT4 translocation confirmed the hypothesis that Corosolic acid might act on insulin pathway.

In the insulin signaling pathway, the action of insulin is mediated by a cascade of tyrosine phosphorylation events, initiated by the binding of insulin to insulin receptor (White, et al., 1997; White and Yenush et al., 1998). The insulin receptor, insulin

receptor substrate, phosphoinositide-dependent kinase 1 (PDK1), PI_3 Kinase, and the protein kinase Akt plays a central role in this pathway and the metabolic actions of insulin in many cell types (Shepherd et al., 1998). We utilized CHO/*h*IR cells to study the effect of Corosolic acid on insulin pathway. It could elevate tyrosine phosphorylation level of insulin receptor ß in absence or presence of sub-maximal concentration of insulin (10 nM). The insulin-stimulated phosphorylation level of Akt Ser^{473} was also enhanced by Corosolic acid. Wortmannin abolished its effect on Akt Ser^{473}, but not the effect at all on insulin receptor ß. These results suggested that Corosolic acid might improve insulin pathway through enhancing insulin receptor ß phosphorylation.

We consider there might be two possibilities for Corosolic acid to increase insulin receptor ß phosphorylation. It may act like an insulin mimic, to bind and activate insulin receptor directly; or act like an insulin sensitizer, to elevate insulin receptor ß phosphorylation indirectly.

To test whether Corosolic acid directly activate insulin receptor ß, we designed the experiments in cellular and molecular level. If it exerts its effect on insulin receptor ß phosphorylation as a ligand, like insulin, it would enhance insulin receptor ß phosphorylation on the base of sodium vanadate treatment compared to control. However, we did not observe any obvious increment on insulin receptor ß phosphorylation by Corosolic acid in short time treatment after pretreatment with sodium vanadate (within 60 min, as shown in Fig. 6A and 6B). Meanwhile, isolated cellular membrane from CHO/*h*IR was used to test whether Corosolic acid could

directly activate insulin receptor ß. As the result shown in Fig. 6C, Coroslic acid with different concentration could not enhance the phosphorylation of insulin receptor ß any more after the receptor was isolated. Both results hinted that Corosolic acid might not act as a ligand of insulin receptor.

On the other aspect, Corosolic acid displays a synergistic but not additive effect on glucose uptake (Fig. 2B) and AKT 473Ser phosphorylation (Fig. 5A and 5B). It increases the effect of low concentration of insulin, but not the effect of the saturated concentration of insulin, which hints that Corosolic acid might work as an insulin sensitizing reagent. We investigated the inhibitory effect of Corosolic acid on PTP1B, TCPTP, SHP1, SHP2, PTPε and LAR, which were previously reported to be able to dephosphorylate phospho-Tyr sites on insulin receptor ß. The compound displayed selective inhibition on the non-receptor PTPs, such as PTP1B, TCPTP, SHP1 and SHP2, with IC50 value 5.49, 11.31, 24.56 and 10.50 μM respectively, comparing to no visible inhibition on receptor PTPs, PTPε and LAR D1 at 100 μM. The results agreed with the recent findings that Corosolic acid is a mixed-type inhibitor of PTP1B with IC50 value 7.2 μM (Na et al., 2006 b) and that several triterpines with similar structures are inhibitors of PTP1B (Na et al., 2006a). There is some difference between Corosolic acid's effective concentration in molecular level and that in cellular level; we think that could result from the different enzyme concentrations in molecular level and cellular level assays. For example, the concentration of the recombinant PTP1B enzyme in IC50 value determining assay is above 30nM; whereas the concentration of the PTP1B might be much less in intact cells. And IC50

value is a relative parameter evaluating the inhibitory effect of a compound to enzyme. There is a fact that, for many compounds, IC50 values become higher when enzyme concentration increases. In our experiment, we also found IC50 values became higher when the enzyme concentration used in the assay increased (data not shown).

Since no obvious selectivity in molecular level assays was observed among four non-receptor-type PTPs and we could not ascertain which of these PTPs was exactly responsible for Corosolic acid's effects, we would like to suggest that Corosolic acid might enhance glucose uptake and GLUT4 translocation through enhancing insulin receptor phosphorylation via inhibiting certain PTPs, such as PTP1B, TCPTP, SHP1 and SHP2.

Results from the animal and clinical studies suggest that Corosolic acid may be a potential lead compound for the development of anti-diabetic therapeutics. Thus, it is necessary to clarify the mechanism. And that may be various. For example, it has been suggested to be an inhibitor of glycogen phosphorylase in vitro (Wen et al., 2005). Here, we reported Corosolic acid might exert its antidiabetic effects through enhancing insulin receptor ß phosphorylation by inhibiting certain PTPs. Whether other mechanism exists, still needs to be studied further.

Acknowledgements: The work was supported by National Natural Science Foundation of China Grants 30623008, 30400560 and 30472045, Shanghai Commission of Science and Technology Grants 054319910 and 06DZ22907, and Hi-Tech Research and Development Program of China Grants 2006AA02Z315 and 20060109Z4031.

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Figure Legends

Fig 1. The structure of Corosolic acid

Fig 2. Corosolic acid promotes glucose uptake of L6 myotubes. A, L6 myotubes were starved for 3 h in serum-free αMEM media, followed by incubation with different concentration of Corosolic acid in HBS buffer for 1.5 hours. B, myotubes was incubated with 250 nM Corosolic acid for 1 hours in HBS buffer, then treated with 0, 1, 10 ,100 nM insulin for anther 0.5 hour. C, myotubes were first treated with 100 nM Wortmannin for 0.5 hour in HBS buffer, then treated with 0, 250 and 500 nM Corosolic acid for 1.5 hours respectively, or treated with 100 nM insulin for 0.5 hour as control.

Fig 3. Corosolic acid increases GLUT4 translocation from cytoplasm to cell membrane and inhibited by Wortmannin. GLUT4 transfected CHO/*h*IR cells were starved for 3 hours with serum free F12 Medium, preincubated with 100 nM Wortmannin for 30min or not, then replaced by serum free F12 Medium containing 500 nM Corosolic acid for 2 hours. DMSO as negative control and 100nM insulin, as a positive control were added in last 15 min. The white arrow shows the translocated GLUT4. HA, red; GLUT4-EGFP, green; Nucleus, bule. The bar shown is 50 Micron.

Fig 4. Corosolic acid increases the phosphorylation level of insulin receptor β. Cells were starved for 3 hours with serum free F12 Medium , and treated with 0, 125, 250, 500 and 1000 nM Corosolic acid for 2 hours , followed by incubation without (A) or with (B) 10 nM insulin for 10 min. (C) Cell were treated with 500 nM Corosolic acid

for 1, 2, 3, 6 hours then treated with 10 nM insulin for 10 min. 1 mM sodium vanadate (V) and 0.2% DMSO were used as a positive and negative control, separately. (D) Corosolic acid dose not enhance phosphorylation level of AMPK in L6 myotubes. Cells were starved for 3 hours in serum-free αMEM media, followed by incubation with 0, 250 and 500 nM Corosolic acid for 1.5 hours. Berberine was positive control.

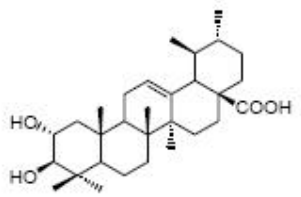
Fig5. (A) Corosolic acid increases Akt-phosphorylation in CHO/*h*IR cell. Cells were starved for 3 hours with serum free F12 Medium, followed by incubation with 0 and 500 nM Corosolic acid for 2 hours, and then stimulated with differently final concentration (0.1 to 100 nM) of insulin for 10 min. The phosphorylation level of Akt Ser^{473} was quantified by Bandscan 4.3 software and normalized by Akt. Fold: the ratio of Corosolic acid induced Akt Ser^{473} phosphorylation relative to that of DMSO in insulin treatment with different concentrations. (B)The quantified result of Corosolic acid increased Akt Ser^{473} phosphorylation was presented.(C) Wortmannin blocked elevated Akt- phosphorylation but not insulin receptor phosphorylation induced by Corosolic acid. After 3 hours starvation in serum free F12 Medium,cells were preincubated with 100 nM Wortmannin for 30 min, then changed to serum free medium containing 500 nM Corosolic acid for 2 hours ,followed by stimulation with 10 nM insulin for 10 min. 1 mM sodium vanadate (V) and 0.2% DMSO were used as a positive and negative control, separately.

Fig6. Corosolic acid did not enhance insulin receptor β phosphorylation. CHO/*h*IR cells were treated with 0.5 mM sodium vanadate for 1 hour, and stimulated with 10 nM insulin (A), followed by cleaning the sodium vanadate by 3 times wash with PBS, and then incubated with 500 nM Corosolic acid for different time(B). (C) Isolated crude cell membrane was incubated Corosolic acid with different concentration for 2 hours, followed by adding 5 μM ATP (final concentration) for 10 min, the reaction was stopped by adding 2X SDS loading buffer.

Table 1. The IC_{50} of Corosolic acid on diabetes related PTPs.

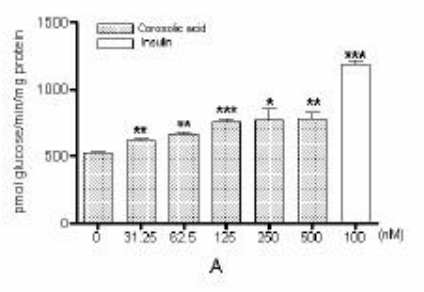
PTPs	IC_{50} (μM)
PTP1B	5.49±0.12
TCPTP	11.31±0.19
SHP1	24.56±0.56
SHP2	10.50±0.29
PTPε D1	>100
LAR D1	>100

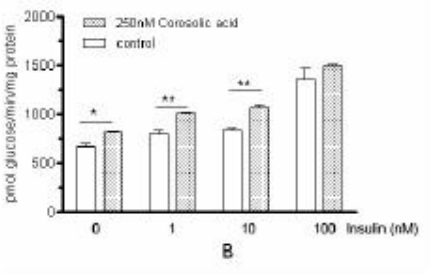
Figure 1



Corosolic acid

Fig.2 TIFF





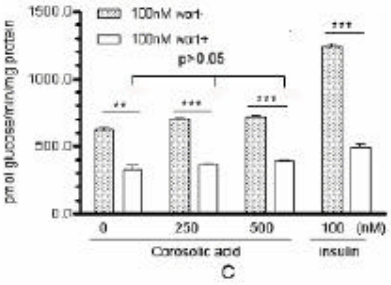


Fig.3 TIFF

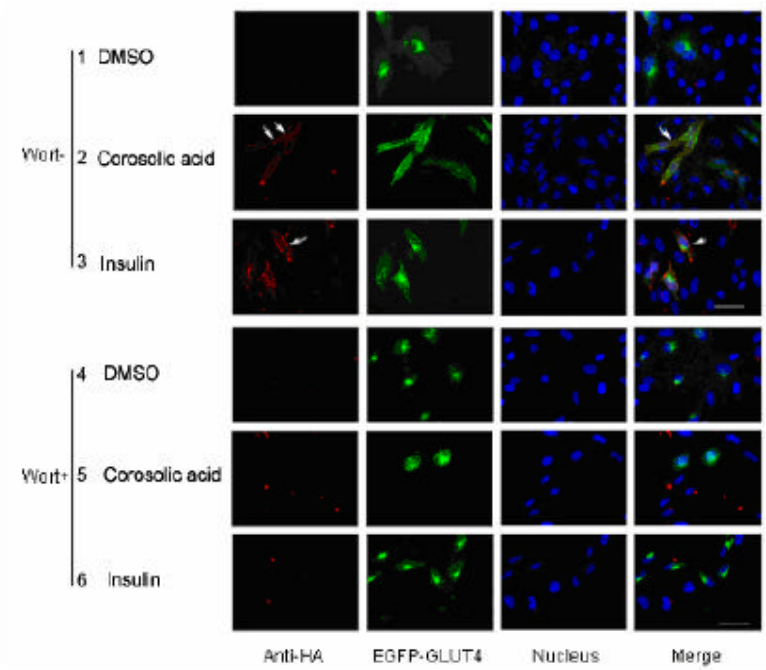


Fig.4 TIFF

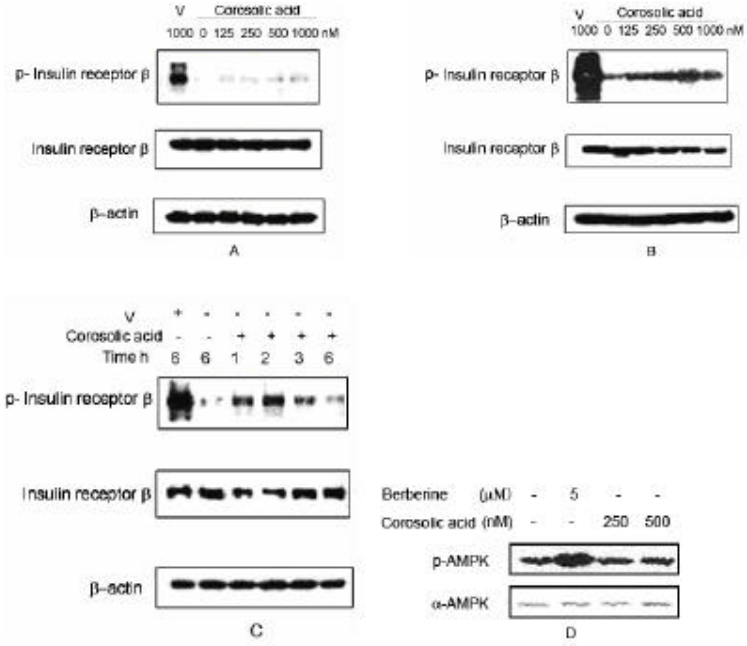
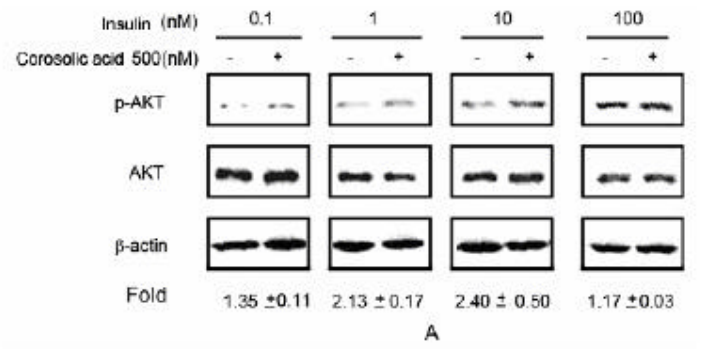
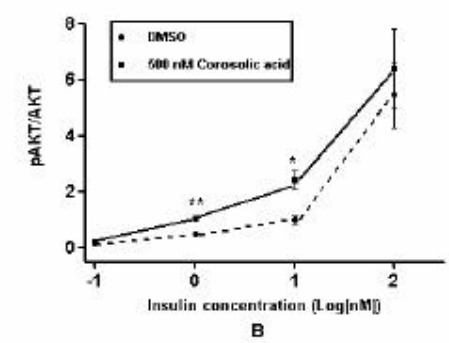


Fig.5 TIFF





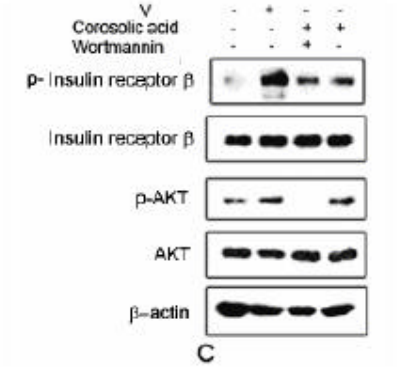


Fig.6 TIFF