Iranian Journal of Diabetes and Lipid Disorders; 2010; Vol 9, pp 1- 8

The Effects of PTP-1B Knockdown on Glucose Uptake and Triglyceride Levels in C2C12 Skeletal Muscle Cells

Salar Bakhtiyari[1], Reza Meshkani[2*], Mohammad Taghikhani[3], Bagher Larijani[4]

1. *Department of Clinical Biochemistry, Faculty of Medicine, Ilam University of Medical Sciences, Ilam, Iran*
2. *Department of Biochemistry, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran*
3. *Department of Clinical Biochemistry, Faculty of Medical Sciences, Tarbiat-Modares University, Tehran, Iran*
4. *Endocrinology and Metabolism Research Centre, Tehran University of Medical Sciences, Tehran, Iran*

Abstract

Background: Insulin resistance is the central defect in obesity and type 2 diabetes. In Lipid accumulation and increased Protein Tyrosine Phosphatase-1B (PTP-1B) gene expression have been reported in muscle insulin resistance. The aim of this study was to investigate the effect of PTP-1B knockdown on glucose uptake and triglyceride levels in C2C12 cells.

Methods: Reducing the expression of PTP-1B in C2C12 myoblasts was performed using the plasmids containing the shRNA against PTP-1B gene. PTP-1B protein level was assessed by western blot. The rate of glucose uptake and the intracellular triglyceride levels were evaluated in the PTP-1B knockdown and normal cells.

Results: PTP-1B protein level in PTP-1B knockdown C2C12 cells decreased by 58% compared to the normal cells. Insulin-stimulated glucose uptake was decreased by palmitate in both the control and knockdown cells, whereas PTP1B knockdown cells treated with 0.5 and 0.75mM palmitate remained sensitive to the insulin with about 2.5 and 3-fold, respectively as well as increase in glucose uptake compared to the control cells. Treatment of the cells with 0.5 and 0.75mM palmitate resulted in 1.25 and 1.42 fold increase in triglyceride levels, respectively in the knockdown cells compared to the control cells.

Conclusion: The results of this study suggest that increased insulin sensitivity in PTP-1B knockdown cells can be partly attributed to increased triglyceride levels within the muscle cells. Thus, reduced PTP-1B gene expression can be a potential therapeutic target for treating insulin resistance, type 2diabetes and metabolic syndrome.

Keywords: Protein tyrosine phosphatase-1B, Insulin resistance, Type 2 diabetes, Glucose uptake, Palmitate

[1*]**Corresponding Author:** Department of Biochemistry, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran, Email: rmeshkani@tums.ac.ir

Introduction

Type 2 diabetes (T2D) is a metabolic disorder resulting from insulin resistance and beta cells dysfunction. Insulin resistance is defined as the reduced ability of the cells or tissues in response to the physiological concentrations of insulin. Genetic and environmental factors such as increased age, obesity, physical inactivity and stress have been suggested to associate with insulin resistance (1-3). It seems that increased plasma free fatty acids (FFA) levels interferes with the insulin signaling through reducing insulin sensitivity in insulin target tissues, especially in the muscle cells (4). Palmitate is the main FFA in the circulation (5). *In vitro* and *in vivo* studies have suggested that palmitate induces insulin resistance in insulin target tissues (6-8). Muscle insulin resistance has a key role in whole body glucose homeostasis, since almost 80% of insulin dependent glucose uptake in the body occurs in this tissue (9, 10). Nevertheless, the underlying mechanism for FFA induced insulin resistance in skeletal muscle has not precisely been identified.

An ample body of human and animal studies has demonstrated a close relationship between increased skeletal muscle triglyceride (TG) content and insulin resistance (11). In contrast, it has been shown that increased TG synthesis in skeletal muscle leads to improved insulin sensitivity (12). It has been reported that palmitate increases the accumulation of intermediate lipids such as diacylglycerol (DAG) and ceramide in skeletal muscle cells (8, 13,14). More recent works in this field have revealed that these metabolites can induce insulin resistance by affecting the key elements of insulin signaling pathway (15). Therefore, identifying factors leading to a decrement in the levels of these lipid metabolites could potentially be a therapeutic target for treatment of insulin resistance condition.

Protein tyrosine phosphatases (PTPs) are a group of enzymes that control the function of many metabolic pathways (16). *In vitro* and *in vivo* studies have shown that PTP-1B is the most important enzyme of this family. It serves as the main negative regulator of insulin signaling and dephosphorylates the insulin receptor (IR) and insulin receptor substrates (IRS) (17,18). Human and animal studies have demonstrated enhanced activity and expression of PTP-1B in adipose and skeletal muscle of obese and diabetic individuals (19,20). PTP1B-deficient mice are resistant to weight gain, exhibit increased insulin sensitivity and remain insulin-sensitive when subjected to a high-fat diet. In addition, decreased expression of PTP-1B using antisense oligonucleotides and nonspecific inhibitors led to increased IR tyrosine phosphorylation and insulin sensitivity (21-24). These findings provide reliable evidence that PTP-1B negatively regulates the activity of insulin signaling. Therefore, it seems that decreased the expression of PTP-1B can help to improve insulin resistance induced by factors such as FFA, inflammation, high glucose and insulin. However, the particular role of PTP-1B reduction on the lipid content in muscle cells is not well understood. In the present study the effects of PTP-1B knockdown on palmitate-induced insulin resistance as well as intracellular triglyceride levels were evaluated in C2C12 cells.

Methods

Preparation of plasmid containing shRNA against PTP-1B gene

Plasmid containing shRNA against PTP-1B gene (*ptpn1*) was purchased from OriGene Company. This plasmid was transformed into the *E.coli* strain DH5α. After selection with ampicillin, a colony was picked up and grown in LB broth and the plasmids were then extracted using the plasmid extraction kit. These plasmids were used to silence the expression of PTP-1B in the transfection experiments.

Cell culture

C2C12 myoblasts were purchased from the Pasteur Institute of Iran. The cells were cultured in DMEM containing 10% FBS (Fetal Bovine Serum) and 1% antibiotics and maintained at 37°C and 5% CO2. Differentiation of C2C12 myoblasts into myotubes was induced when the cells had achieved 70–90% confluence by replacing the media with DMEM containing 2% horse serum. Four days after the fusion, the differentiated myotubes were used for the experiments.

Calcium phosphate transfection method

Delivering the plasmids containing shRNA against *ptpn1* gene into C2C12 myoblasts were performed according to the calcium phosphate method (25,26). In brief, a day prior to transfection, 5×10^5 cells were seeded in six-well plates. The next day, transfection was performed using 15 µg of plasmids. To assess the transfection efficiency, GFP (Green Fluorescent Protein) plasmid was also delivered to the cells.

Western blot analysis

After 48 hours of transfection, proteins were extracted from the cells using RIPA buffer (50 mM Tris-HCl, 1% Triton-X100, 2% sodium deoxycholate, 1 mM Na-EDTA, 1 mM PMSF). After determining protein concentrations, equal amounts of protein were subjected to SDS–PAGE, followed by transfer onto PVDF membrane. Blocking was carried out through overnight incubation at 4 ºC with 5% non-fat dry milk in TBS with 0.5% Tween-20. The membrane was then incubated with primary antibody against PTP-1B or β-actin for 1 hour. After washing with TBST, the membrane was incubated with HRP-conjugated secondary antibody for 1 hour. Antibody binding was visualized using a chemiluminescent substrate. In the next step, the membrane was placed adjacent to the sensitive radiography film and protein bands were quantified using Scion Image software.

Palmitate Treatment

For treating the cells with FFA, palmitate should be conjugated with FFA-free BSA. Briefly, the required amount of sodium palmitate was dissolved in 50% (v/v) ethanol, diluted in DMEM containing 1% (w/v) FFA-free BSA to the final concentration. Two hours before the experiments, myotubes were placed in serum free-DMEM containing 1% BSA and then the cells were incubated in the presence or absence of 0.5 mM palmitate for 16 h.

Glucose Uptake Assay

Glucose uptake was assayed using [3H] 2-DOG ([3H] 2-deoxyglucose). Glucose uptake measurements were performed in triplicate and in three independent experiments. After 4 days of differentiation, myotubes were treated with 0.5 and 0.75 mM palmitate for 16 h, followed by a serum starvation of 2–3 h in DMEM plus 0.1% FFA-free BSA. Myotubes were then treated with or without 100 nM insulin for 30 min and washed two times with wash buffer [20 mM HEPES (pH 7.4), 140 mM NaCl, 5 mM KCl, 2.5 mM MgSO4, and 1 mM CaCl2]. Myotubes were then incubated in the transport buffer (wash buffer containing 0.5 mCi [3H] 2-DOG/ml and 10 µM 2-DOG) for 10 min. For stopping glucose uptake reaction, the cells were washed three times with cold phosphate buffer. The cells were then lysed in 0.05 M NaOH and [3H] 2-DOG levels were counted in the cell lysate using a scintillation counter. Nonspecific uptake was determined by incubating the cells in the presence or absence of 10 µM cytochalasin B.

Triglyceride content measurement

Lipid extraction was performed based on the method of Floch *et al* (27). 10 µL of the extracted lipid was used for measuring TG content. TG measurement was performed using Randox kit according to its instructions. The TG value of each sample was then normalized to the protein concentration of the samples.

Statistical Analysis

All statistical analyses were performed using SPSS 13.0. Comparisons among all groups were performed with the one-way analysis of variance (ANOVA) test. If statistical significance was found, the Tukey post hoc test was performed. Values of $p<0.05$ were considered statistically significant.

Results

Investigating the efficiency of calcium phosphate method

For studying the efficiency of calcium phosphate method in C2C12 cells, GFP plasmid was used. The results of this experiment showed that the calcium phosphate is a highly efficient method for delivering the plasmids into C2C12 myoblasts.

Silencing the expression of PTP-1B in C2C12 cells

For confirming the reduced expression of PTP-1B in C2C12 myoblasts western blot was performed. The results showed that PTP-1B

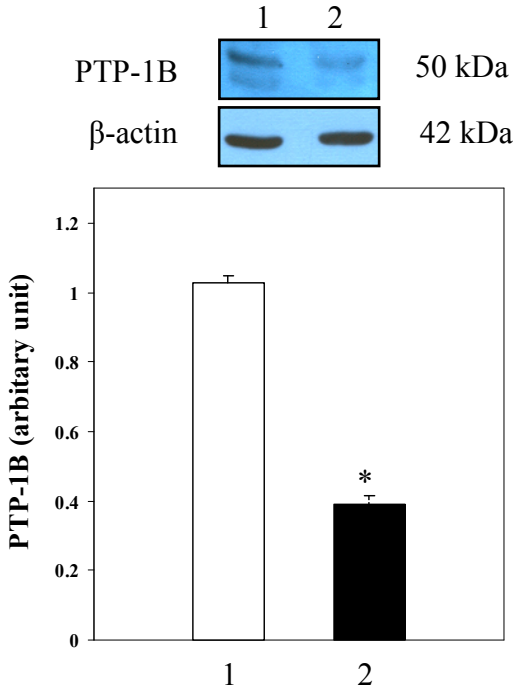
protein level in theses cells decreased to 58% of the control cells (Fig 1).

The effect of PTP-1B knockdown on glucose uptake in C2C12 cells

The results showed that the insulin-stimulated glucose uptake in the PTP-1B knockdown myotubes was increased 2.3-fold with respect to the control myotubes (P<0.001). Insulin-stimulated glucose uptake was decreased by palmitate in both the control and knockdown cells. In the PTP-1B knockdown myotubes treated with 0.5 and 0.75 mM palmitate, insulin-stimulated glucose uptake was 2.5- and 3-fold of the control cells, respectively (P<0.001). Basal glucose uptake had no significant difference between the PTP-1B knockdown and control myotubes (Fig 2).

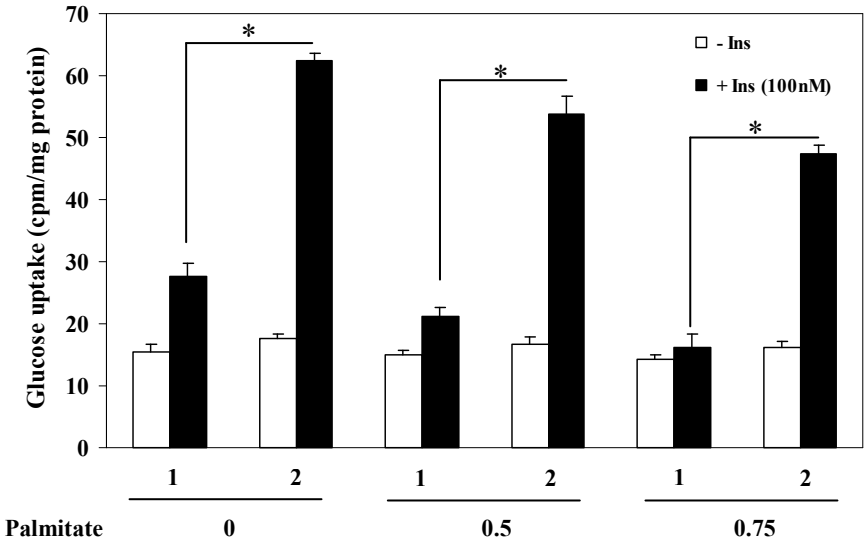
The effects of PTP-1B knockdown on triglyceride level

In order to evaluate the effects of silencing the PTP-1B gene on the intracellular lipid content, TG levels were measured. The results showed that the amount of TG had no significant difference between the PTP-1B knockdown and control myotubes in the absence of palmitate. When the cells were treated with 0.5 and 0.75 mM palmitate, TG concentration in PTP-1B knockdown myotubes was 1.25- and 1.42-fold of the control myotubes, respectively (P<0.05) (Figure 3).



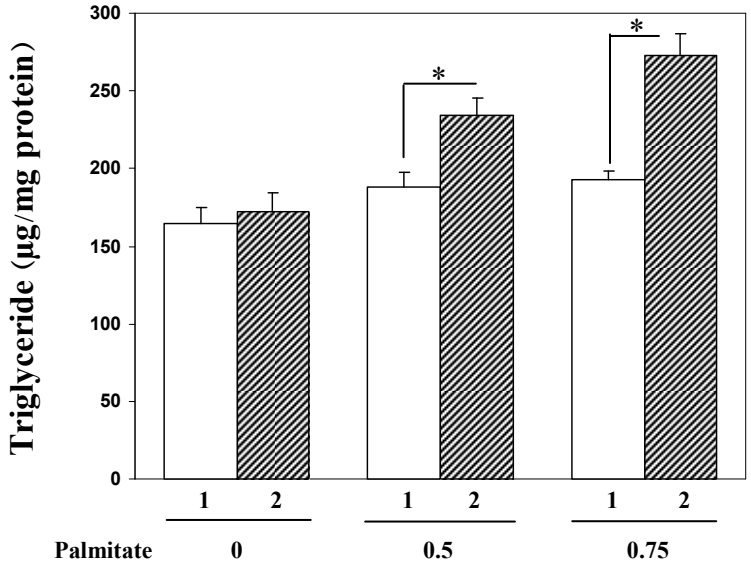
The figure shows the representative data gained from mean ± SD of three independent experiments; * P<0.01.

Figure 1- The results of western blotting of C2C12 myoblasts transfected with: (1) plasmids without shRNA (negative control) and (2) with shRNA against PTP-1B gene. C2C12 cells were transfected with 20 µg of each plasmid. Forty-eight hours post-transfection, western blot was performed using antibodies against PTP-1B and β-actin as internal control. Protein level of PTP-1B was normalized to the level of β-actin protein.



Basal glucose uptake (without insulin; -Ins) and insulin-stimulated glucose uptake in the presence of 100 nM insulin (+Ins) are shown as white and black rectangles, respectively. The figure shows the representative data gained from mean ± SD of three independent experiments; * P<0.001.

Figure 2- Glucose uptake experiment in: (1) the control and (2) PTP-1B knockdown C2C12 myotubes in the absence and presence of 0.5 and 0.75 mM palmitate.



TG concentration in PTP-1B knockdown and control C2C12 myotubes are shown as white and hatched rectangles, respectively. The figure shows the representative data gained from mean ± SD of three independent experiments; * P<0.05.

Figure 3. Measuring triglyceride content in: (1) the control and (2) PTP-1B knockdown C2C12 myotubes in the absence and presence of 0.5 and 0.75 mM palmitate.

Discussion

Abundant *in vitro* and *in vivo* studies have established a role for PTP-1B in insulin resistance (28,29). PTP-1B acts as insulin antagonist through the direct dephosphorylation and inactivation of the IR and IRS (17). It has been shown that the lack of PTP-1B gene in mouse skeletal muscle leads to increased insulin sensitivity and IR and IRS tyrosine phosphorylation (21-24). This animal model also showed a phenotype of resistant to being overweight when subjected to a high-fat diet (21-24). It has been shown that the PTP-1B expression increases in skeletal muscle of mice under high-fat diet, obesity and insulin resistance condition (19, 20).

Several studies in diabetic and non-diabetic subjects have indicated that the elevated plasma FFA level leads to insulin resistance particularly in the muscle cells. Among FFAs, palmitate has the highest concentration in the blood and various studies have shown that it is one of the major factors causing insulin resistance in skeletal muscle (5-7). We have recently shown that a part of palmitate-induced insulin resistance in muscle is mediated by increasing the expression of PTP-1B in C2C12 myotubes (30, 31). Considering the important role of PTP-1B in negatively regulating the insulin signaling pathway, we aimed to investigate the role of this phosphatase in insulin resistance induced by palmitate in muscle cells. In this work we demonstrated that the reducing PTP-1B gene expression in muscle cells leads to decreased insulin resistance even when palmitate is present. Although increasing amounts of palmitate results in increased insulin resistance in muscle cells, however, decreased expression of PTP-1B caused to maintain insulin sensitivity even at high concentrations of palmitate. This phenomenon clearly demonstrates that PTP-1B is a key molecule affecting the intracellular palmitate metabolism in the muscle.

Identifying intracellular palmitate metabolism can help to better understand the role of this FFA in insulin resistance. After transporting palmitate into the myotubes, it is converted into an active form, palmitoyl-CoA. This intermediate could follow several pathways in myotubes; 1) used as fuel and enters the β-oxidation pathway, 2) the *de novo* synthesis of DAG and TG by esterification of glycerol-3-phosphate, and 3) combined with serine and eventually lead to the ceramide synthesis (32). Several studies have shown that TG itself does not impair the function of insulin in muscle cells, but rather acts as a reservoir of FFAs within the cell (12, 32, 33). If the concentration of FFA exceeds more than the reservoir capacity, the synthesis of intermediate metabolites, such as DAG and ceramide will increase. These metabolically active molecules promote the cascade of serine/threonine phosphorylation leading to the inhibition of the insulin signaling that ultimately results in decreased glucose uptake in the cells (34,35). Thus, it seems that the synthesis of TG can decrease the concentration of these intermediates and thereby maintains insulin sensitivity in the cell. In this study it was found that the reducing PTP-1B leads to significant increase in TG levels within the muscle cells treated with palmitate. These data suggest that the alterations in the intracellular palmitate metabolism prevent the activation of downstream signaling pathways inducing insulin resistance. This finding is in accordance with the other studies showing that TG accumulation reduces insulin resistance (12, 32, 33).

In conclusion, the findings of this study show that reduced expression of PTP-1B in muscle cells decreases insulin resistance that results in preserved insulin sensitivity even in the presence of increasing concentrations palmitate. It seems that PTP-1B knockdown reduces palmitate-induced insulin resistance through increasing the intracellular accumulation of TG in the muscle cells. These set of findings would favor PTP-1B as a suitable therapeutic target for the treatment of patients with insulin resistance and T2DM.

Acknowledgements

This work has been financially supported by a research grant from the Endocrinology and Metabolism Research Center of Tehran University of Medical Sciences.

References

1. Mlinar B, Marc J, Janez A, et al. Molecular mechanisms of insulin resistance and associated diseases. *Clin Chim Acta* 2007; 375(1-2): 20-35.
2. Meshkani R, Adeli K. Hepatic insulin resistance, metabolic syndrome and cardiovascular disease. *Clin Biochem* 2009; 42:1331–1346.
3. Meshkani R, Adeli k. Mechanisms linking the metabolic syndrome and cardiovascular disease: Role of hepatic insulin resistance. *J Univ Heart Ctr* 2009; 2:77-84.
4. Czech MP. Fat targets for insulin signaling. *Mol Cell* 2002; 9(4):695-6.
5. Paolisso G, Tataranni PA, Foley JE, et al. A high concentration of fasting plasma non-esterified fatty acids is a risk factor for the development of NIDDM. *Diabetologia* 1995; 38(10): 1213-7.
6. Chavez JA, Summers SA. Characterizing the effects of saturated fatty acids on insulin signaling and ceramide and diacylglycerol accumulation in 3T3-L1 adipocytes and C2C12 myotubes. *Arch Biochem Biophys* 2003; 419(2):101-9.
7. Reynoso R, Salgado LM, Calderon V. High levels of palmitic acid lead to insulin resistance due to changes in the level of phosphorylation of the insulin receptor and insulin receptor substrate-1. *Mol Cell Biochem* 2003; 246(1-2): 155-62.
8. Schmitz-Peiffer C, Craig DL, Biden TJ. Ceramide generation is sufficient to account for the inhibition of the insulin-stimulated PKB pathway in C2C12 skeletal muscle cells pretreated with palmitate. *J Biol Chem* 1999; 274(34):24202-10.
9. Randle PJ, Garland PB, Hales CN, et al. The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet* 1963; 1(7285):785-9.
10. Randle PJ, Newsholme EA, Garland PB. Regulation of glucose uptake by muscle. 8. Effects of fatty acids, ketone bodies and pyruvate, and of alloxan-diabetes and starvation, on the uptake and metabolic fate of glucose in rat heart and diaphragm muscles. *Biochem J* 1964; 93(3):652-65.
11. Alessi DR. Discovery of PDK1, one of the missing links in insulin signal transduction. Colworth Medal Lecture. *Biochem Soc Trans* 2001; 29(2):1-14.
12. Liu L, Zhang Y, Chen N, Shi X, Tsang B, et al. Upregulation of myocellular DGAT1 augments triglyceride synthesis in skeletal muscle and protects against fat-induced insulin resistance. *J Clin Invest* 2007; 117(6):1679-89.
13. Kitamura T, Ogawa W, Sakaue H, et al. Requirement for activation of the serine-threonine kinase Akt (protein kinase B) in insulin stimulation of protein synthesis but not of glucose transport. *Mol Cell Biol* 1998; 18(7):3708-17.
14. Roden M, Price TB, Perseghin G, et al. Mechanism of free fatty acid-induced insulin resistance in humans. *J Clin Invest* 1996; 97(12):2859-65.
15. Storz P, Doppler H, Wernig A, et al. Cross-talk mechanisms in the development of insulin resistance of skeletal muscle cells palmitate rather than tumor necrosis factor inhibits insulin-dependent protein kinase B (PKB)/Akt stimulation and glucose uptake. *Eur J Biochem* 1999; 266(1):17–25.
16. Tonks NK. PTP1B: from the sidelines to the front lines! FEBS Lett 2003; 46(1):140-8.
17. Seely BL, Staubs PA, Reichart DR, et al. Protein tyrosine phosphatase 1B interacts with the activated insulin receptor. *Diabetes* 1996; 45(10):1379-85.
18. Goldstein BJ. Protein-tyrosine phosphatase 1B (PTP1B): a novel therapeutic target for type 2 diabetes mellitus, obesity and related states of insulin resistance. Curr Drug Targets Immune Endocr *Metabol Disord* 2001; 1(3):265-75.
19. Ahmad F, Azevedo JL, Cortright R, et al. Alterations in skeletal muscle protein-tyrosine phosphatase activity and expression in insulin-resistant human obesity and diabetes. *J Clin Invest* 1997; 100(2):449-58.
20. McGuire MC, Fields RM, Nyomba BL, et al. Abnormal regulation of protein tyrosine phosphatase activities in skeletal muscle of insulin-resistant humans. *Diabetes* 1991; 40(7): 939-42.
21. Delibegovic M, Bence KK, Mody N, et al. Improved Glucose Homeostasis in Mice with Muscle-Specific Deletion of Protein-Tyrosine Phosphatase 1B. *Mol Cell Biol* 2007; 27(21): 7727–7734.
22. Nieto-Vazquez I, Fernandez-Veledo S, Alvaro C, et al. Protein–Tyrosine Phosphatase 1B–Deficient Myocytes Show Increased Insulin Sensitivity and Protection Against Tumor Necrosis Factor-α–Induced Insulin Resistance. *Diabetes* 2007; 56(2):404-413.
23. Xue B, Kim YB, Lee A, et al. Protein tyrosine phosphatase 1B (PTP1B) deficiency reduces insulin resistance and the diabetic phenotype in

mice with polygenic insulin resistance. *J Biol Chem* 2007; 282(33):23829-23840.
24. Na MK, Kim KA, Oh H, et al. Protein Tyrosine Phosphatase 1B Inhibitory Activity of Amentoflavone and Its Cellular Effect on Tyrosine Phosphorylation of Insulin Receptors. *Biol Pharm Bull* 2007; 30(2):379-381.
25. Graham FL, van der Eb AJ. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 1973; 52(2):456-67.
26. Chen C, Okayama H. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol Cell Biol* 1987; 7(8):2745-52.
27. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 1957; 226(1):497-509.
28. Gum RJ, Gaede LL, Koterski SL, et al. Reduction of protein tyrosine phosphatase 1B increases insulin-dependent signaling in ob/ob mice. *Diabetes* 2003; 52(1):21-8.
29. Sun C, Zhang F, Ge X, et al. SIRT1 improves insulin sensitivity under insulin-resistant conditions by repressing PTP1B. *Cell Metab* 2007; 6(4):307-19.
30. Mohammadtaghvaei N, Meshkani R, Taghikhani M, et al. Palmitate Enhances Protein Tyrosine Phosphatase 1B (PTP1B) Gene Expression at Transcriptional Level in C2C12 Skeletal Muscle Cells. *Inflammation* 2010; 24(8):128-34.
31. Parvaneh L, Meshkani R, Bakhtiyari S, et al. Palmitate and inflammatory state additively induce the expression of PTP1B in muscle cells. Biochem Biophys Res Commun 2010; 30(5):254-9.
32. Timmers S, Schrauwen P, de Vogel J. Muscular diacylglycerol metabolism and insulin resistance. *Physiol Behav* 2008; 94(2): 242-51.
33. Ellis BA, Poynten A, Lowy AJ, et al. Long-chain acyl-CoA esters as indicators of lipid metabolism and insulin sensitivity in rat and human muscle. *Am J Physiol Endocrinol Metab* 2000; 279(3):554-60.
34. Dresner A, Laurent D, Marcucci M, et al. Effects of free fatty acids on glucose transport and IRS-1-associated phosphatidylinositol 3-kinase activity. *J Clin Invest* 1999; 103(2):253-9.
35. Itani SI, Ruderman NB, Schmieder F, et al. Lipid-induced insulin resistance in human muscle is associated with changes in diacylglycerol, protein kinase C, and IkappaB-alpha. *Diabetes* 2002; 51(7):2005-11.