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ORIGINAL ARTICLE

Phosphatase and Tension Homolog Overexpression in Insulin Resistant Diabetic Adipose Tissue[△]

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Key words: insulin resistance; phosphatase and tension homolog; adipose tissue

Objective To investigate the expression of phosphatase and tension homolog (PTEN) in adipose tissue of KKAY diabetic mice, a mouse model of type 2 diabetes.

Methods KKAY diabetic mice were fed with high fat diet for 4 weeks. After blood glucose met the criteria of diabetes (over 16.7 mmol/L), mice were randomly divided into 3 groups: a control group (without any treatment), a rosiglitazone group (treated with rosiglitazone 12.5 mg/kg·d once per day), and a metformin group (treated with metformin 3 g/kg·d twice daily). After 4 weeks, we then determined the expression of PTEN and phosphoserine 473-Akt (pS473-Akt) in the epididymal adipose tissue with Western blots. The mice in each group were further divided into the insulin (-) subgroup and insulin (+) subgroup, which were intraperitoneally injected with saline and insulin (5 mU/g body weight), respectively.

Results The expression of PTEN was elevated in the epididymal adipose tissue obtained from KKAY diabetic mice compared with that from the C57BL/6J mice ($P<0.001$). In accordance with the enhanced expression of PTEN, the level of pS473-Akt stimulated by insulin was decreased in the adipose tissue of KKAY mice compared to the C57BL/6J mice ($P<0.001$). Treatment with the insulin-sensitizing agents, rosiglitazone and metformin did not inhibit the elevated expression of PTEN in adipose tissue of KKAY diabetic mice.

Conclusion PTEN may play an important role in the development of insulin resistance in adipose tissue of type 2 diabetes mice model.

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TYPE 2 diabetes is characterized by impaired insulin action. The insulin-signaling pathway plays a key role in the development of type 2 diabetes. Thus, exploring the related molecular mechanisms and biochemical pathways involved in the insulin signaling pathway will have a high clinical value.

The activation of phosphatidylinositol 3-kinase/Akt (PI3K/Akt) in insulin signaling pathway has been interpreted from molecular to genetic aspects. The lipid products of PI3K initiate phosphorylation and activation of Akt, which is believed to act as downstream mediator of many insulin metabolic effects. Activated Akt plays a significant role in glycolysis, gluconeogenesis, protein synthesis, and adipogenesis. It has been shown that a reduced PI3K/Akt pathway may cause insulin resistance.¹⁻³

Phosphatase and tension homolog (PTEN) is a dual-specificity protein phosphatase which is involved in signal transduction and tumor suppression.⁴⁻⁶ PTEN has phosphoinositide 3-phosphatase activity, and it can suppress PI3K/Akt pathway by dephosphorylating phosphatidylinositol (3, 4, 5)-triphosphate (PIP3).^{7, 8} Adipose tissue is now recognized to exhibit multi-function that is important in the regulation of energy balance and substrate metabolism. In adipose tissue, insulin controls glucose and lipid metabolism through the intracellular mediators PI3K/Akt. PTEN, as a potent negative regulator of PI3K/Akt pathway, may play an important role in the development of insulin resistance in adipose tissue. Overexpression of PTEN in adipocytes significantly inhibits insulin-induced glucose transporter 4 (GLUT4) translocation, glucose uptake, and membrane ruffling, all of which are dependent on PI3K activity.^{9, 10} In addition, inhibition of endogenous PTEN leads to enhanced GLUT4 translocation.¹¹ One research indicated that PTEN inhibition in adipocyte is effective in the amelioration of insulin resistance induced by tumor necrosis factor- α .¹² Although there have some other reports trying to interpret the role of PTEN in insulin signaling in adipocyte, few attempts have been made to fully investigate the changes of PTEN in adipose tissue under insulin resistance conditions. In this study, through examining the PI3K/Akt activity and the PTEN expression level in adipose tissue of KKAY diabetic mice, we aimed to identify the role of PTEN in the development of insulin resistance in diabetic adipose tissue.

MATERIALS AND METHODS

Animal models

Male C57BL/6J mice and KKAY mice (Animal Research Center of Chinese Academy Medical Sciences) at an age of 10 weeks were used for the experiments and maintained under standard light (12 hours light/dark) and stable room temperature. All the mice were divided into the C57BL/6J mice group and KKAY diabetic mice group. Mice were separately housed and provided with food and water *ad libitum*. C57BL/6J mice were sacrificed until 16 weeks old. KKAY diabetic mice were fed with basic diet until week 12th, then fed with high fat diet (fat 10%, egg yellow powder 10%, sucrose 10%, and basic food 70%) for 4 weeks. Blood glucose from tail vein were measured every week from 15 weeks old. It would be diagnosed as diabetes if the blood glucose level is more than 16.7 mmol/L in the last two consecutive weeks. Food was withdrawn 12 hours before blood sampling. KKAY diabetic mice were randomly divided into the control group, metformin-treated group (intragastric administration of 3 g/kg $\cdot$ d bid for 4 weeks, Bristol-Myers Squibb, Shanghai, China), and rosiglitazone-treated group (intragastric administration of 12.5 mg/kg $\cdot$ d once per day for 4 weeks, Glaxo Smith Kline, Tianjin, China). Body weight was measured before mice were sacrificed, and the epididymal fat pads were rapidly removed and weighted. Then the adipose tissues were frozen in liquid nitrogen and stored at -80°C .

The KKAY mice in each group were further divided into the insulin (-) subgroup and insulin (+) subgroup, which were intraperitoneally injected with saline and insulin (5 mU/g body weight), respectively. The mice were sacrificed 10 minutes after injection, and the epididymal adipose tissue was rapidly excised.

Measurement of blood glucose and insulin

Fasting blood glucose (FBG) was measured with glucose meter (LifeScan, Shanghai, China). Plasma insulin was examined by enzyme-linked immunosorbent assay (ELISA). Insulin ELISA kit was purchased from LINCO Research and LINCO Diagnostic Services (MO, USA).

Western blot

Adipose tissue (50 mg) was homogenized using a homogenizer (Polytron Technologies Inc., Berlin, Germany) at 3520 $\times$ g for 1 minute on ice in 500 μ l homogenization buffer [20 mmol/L Tris, 5 mmol/L EDTA, 10 mmol/L $\text{Na}_4\text{P}_2\text{O}_7$, 2 mmol/L Na_3VO_4 , 1% NP-40, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin (pH 7.5)]. The tissue lysates were solubilized by continuous stirring for 1 hour at 4°C , and centrifuged for 10 minutes at 14 000 $\times$ g. Western blot

analysis of the supernatants was performed as described. Protein sample 20 μ l was resolved on 7.5% SDS-PAGE using a mini-gel apparatus (Bio-Rad Laboratories Inc., CA, USA) and transferred to a nylon/PVDF membrane (Millipore, Darmstadt, Germany) using a trans-blot transfer system (Bio-Rad). The residual binding sites in the membrane were blocked by incubating the membrane with PBST (PBS and 0.1% Tween 20) containing 5% non-fat dry milk powder for 1 hour. The blots were incubated with anti-PTEN, anti-phosphoserine 473-Akt (pS473-Akt), anti-Foxo3a, and anti- β -actin antibodies in PBST containing 1% non-fat milk powder at 4°C overnight. Membranes were then washed in PBST and incubated with corresponding peroxidase-conjugated anti-IgG antibody in PBS containing 1% milk powder for 1 hour. The blots were then developed using enhanced chemiluminescence Western blotting detection reagents (General Electrics, Shanghai, China)

Statistical analysis

Statistical analysis was done by using the SPSS 13.0 package. Means were calculated for each of the quantitative values, data was presented as means $\pm$ SE. The comparisons were made using analysis of variance (ANOVA) and independent sample *t*-test. *P*<0.05 was considered statistically significant.

RESULTS

Blood glucose and insulin level

The FBG and fasting insulin levels in KKAY diabetic mice were significantly higher than those in C57BL/6J mice (all *P*<0.05). Compared with the untreated KKAY diabetic mice, FBG and insulin levels of treated KKAY diabetic mice were

decreased (all *P*<0.05). But, the insulin level of metformin and rosiglitazone treated groups was still higher than that in C57BL/6J mice (all *P*<0.05, Table 1).

Change in PTEN and Foxo3a protein levels in adipose tissue of KKAY diabetic mice, rosiglitazone-treated and metformin-treated KKAY diabetic mice

PTEN expression level in KKAY diabetic mice was 1.24 times higher than that in C57BL/6J mice (*P*<0.001). Compared with the control KKAY diabetic mice, there was no significant change in PTEN expression level of rosiglitazone or metformin treated KKAY diabetic mice (both *P*>0.05, Fig. 1).

At meantime, we also tested the expression level of Foxo3a in the four mice groups. No significant change was observed between control KKAY diabetic mice and rosiglitazone or metformin treated KKAY diabetic mice (both *P*>0.05), but the Foxo3a expression level in KKAY diabetic mice was 3.54 times higher than that in C57BL/6J mice (*P*<0.001, Fig. 2).

Insulin stimulation of pS473-Akt expression in KKAY diabetic mice, rosiglitazone-treated and metformin-treated KKAY diabetic mice

pS473-Akt expression in C57BL/6J mice induced by insulin was increased to 4.92 $\pm$ 0.41 times. At the same inducing condition, the pS473-Akt level was raised just 2.50 $\pm$ 0.14 times in KKAY diabetic mice without insulin induction; the pS473-Akt level decreased by 49% as compared with the C57BL/6J mice group (*P*<0.001). While, there was no significant change was observed in KKAY diabetic mice treated with rosiglitazone or metformin as compared with the control KKAY diabetic mice group (*P*>0.05, Fig.3).

Table 1. Fasting blood glucose and plasma insulin levels in C57BL/6J, untreated KKAY diabetic mice, rosiglitazone- and metformin-treated KKAY diabetic mice[§]

Groups	<i>n</i>	Glucose (mmol/L)	Insulin (ng/ml)
C57BL/6J mice	5	6.03 $\pm$ 1.89	0.64 $\pm$ 0.34
Untreated KKAY diabetic mice	6	10.52 $\pm$ 2.84*	6.12 $\pm$ 1.29**
Metformin-treated KKAY diabetic mice	6	5.70 $\pm$ 1.60 [#]	2.74 $\pm$ 1.74***
Rosiglitazone-treated KKAY diabetic mice	6	6.74 $\pm$ 1.73 ^{##}	2.40 $\pm$ 1.11***

§: Plus-minus values are means $\pm$ SD.

P*<0.05, *P*<0.001 compared with the C57BL/6J mice; [#]*P*<0.05, ^{##}*P*<0.001 compared with the untreated KKAY diabetic mice.

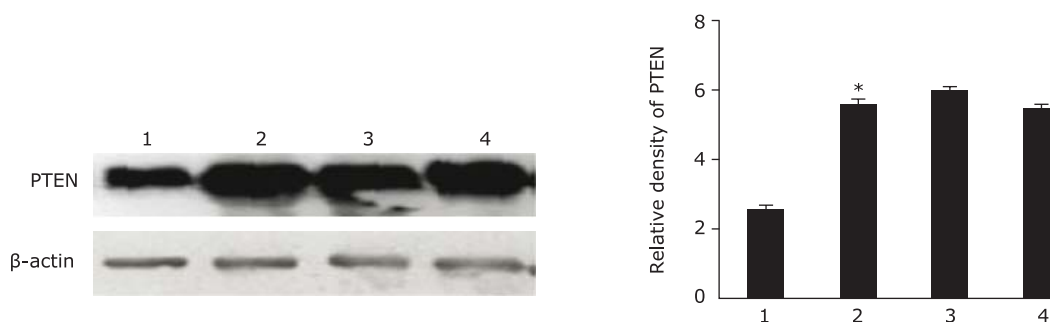


Figure 1. Phosphatase and tension homolog (PTEN) expression in the adipose tissue of C57BL/6J mice and KKAY diabetic mice analyzed by Western blot.

Whole tissue lysates, obtained from epididymal fat tissue of C57BL/6J mice and KKAY diabetic mice, were subjected to Western blot analysis as described in Materials and Methods with anti-PTEN antibody. The relative amount of PTEN protein was quantified with β -actin as an internal control. Results are expressed as means $\pm$ SE for 3 replicate determinations for each treatment group.

Lane 1. C57BL/6J mice; lane 2. KKAY diabetic mice; lane 3. rosiglitazone-treated KKAY diabetic mice; lane 4. metformin-treated KKAY diabetic mice. * P <0.001 compared with lane 1.

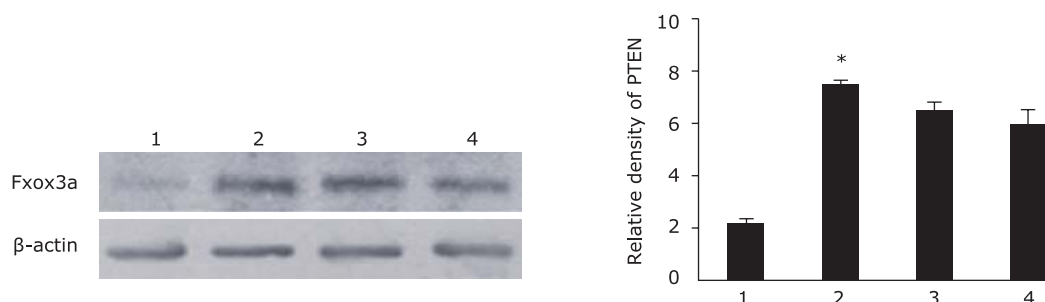


Figure 2. Expression of Foxo3a in the adipose tissue of C57BL/6J mice and KKAY diabetic mice analyzed by Western blot.

Tissue samples, obtained from epididymal fat tissue of C57BL/6J mice and KKAY diabetic mice, were subjected to Western blot analysis as described in Materials and Methods with anti-Foxo3a antibody. The Foxo3a expression level was quantified. β -actin was used as a loading control. Results are expressed as means $\pm$ SE for 3 replicate determinations for each treatment group.

Lane 1. C57BL/6J mice, lane 2. KKAY diabetic mice, lane 3. rosiglitazone-treated KKAY diabetic mice, lane 4. metformin-treated KKAY diabetic mice. * P <0.001 compared with lane 1.

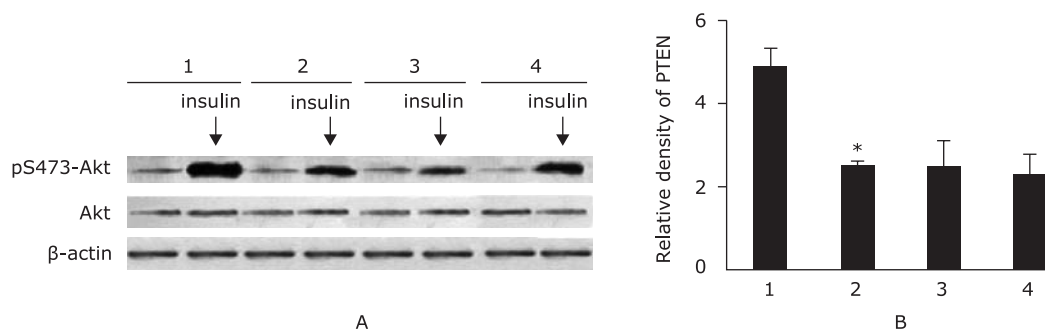


Figure 3. Insulin-induced phosphorylation of Akt at S473 in the adipose tissue of C57BL/6J mice and KKAY diabetic mice.

Tissue samples, obtained from epididymal adipose tissue of C57BL/6J mice and KKAY diabetic mice, were analyzed by Western blot as described in Materials and Methods with anti-phosphoserine 473-Akt (pS473-Akt) and anti-Akt antibody.

A. The 1st lane in each group represents C57BL/6J mice, KKAY diabetic mice, rosiglitazone-treated and metformin-treated KKAY diabetic mice without insulin stimulation. The 2nd lane in each group respectively represents insulin-induced C57BL/6J mice, KKAY diabetic mice, rosiglitazone-treated and metformin-treated KKAY diabetic mice.

B. Values are means $\pm$ SE of the increased fold of pS473-Akt after insulin stimulation in C57BL/6J mice (1), KKAY diabetic mice (2), rosiglitazone-treated KKAY diabetic mice (3) and metformin-treated KKAY diabetic mice (4), with β -actin as loading control. Results are expressed as means $\pm$ SE for 3 replicate determinations for each treatment group. * P <0.01 compared with lane 1.

DISCUSSION

Adipose tissue is increasingly being viewed as an active endocrine organ with a high metabolic activity and plays a critical role in the development of insulin resistance. In adipose tissue, insulin controls glucose and lipid metabolism through the intracellular PI3K/Akt pathway. Emerging biochemical and genetic evidences suggested that insulin resistance can be treated via modulating cell signaling transduction. PI3K/Akt pathway and its up- and downstream modulators can be as potential targets for the treatment of type 2 diabetes. PTEN shares homology with protein tyrosine phosphatases and has been shown to dephosphorylate PtdIns (3, 4, 5) P3 to PtdIns (4, 5) P2, resulting in decreased level of PtdIns (3, 4, 5) P3 and a reduction of Akt phosphorylation at threonine and serine residues. Here we found that PTEN expression level in adipose tissue was significantly increased in insulin resistant diabetic mice, indicating the augment of PTEN function may suppress the PI3K/Akt pathway, which induced the insulin resistance in diabetic mice. The PI3K/Akt pathway is crucial in various metabolic effects of insulin. Some researches suggested that there is a correlation between the blocking of PI3K/Akt pathway and the development of insulin resistance.^{2, 13-15} Our results showed the increased fasting glucose and insulin levels and decreased pS473-Akt level in adipose tissue of KKAY diabetic mice compared with C57BL/6J mice. All the evidence suggested that the PI3K/Akt pathway might be suppressed in the adipose tissue of diabetic mice.

In adipose tissue of insulin resistant KKAY diabetic mice, Western blot results suggested that the PTEN expression level increased significantly, while the PI3K/Akt signaling pathway was impaired. Some reports from cultured adipocyte and non-diabetic mice supported our results. In 3T3-L1 adipocytes, overexpression of wild-type PTEN inhibited insulin-induced activation of Akt and glucose uptake. Knocking down of PTEN expression level by siRNA can enhance insulin-induced phosphorylation of Akt and glucose uptake.⁹ In adipose tissue-specific PTEN knockout mice, loss of PTEN results in improved systemic glucose tolerance and insulin sensitivity, decreased fasting insulin levels, increased recruitment of the glucose transporter isoform 4 to the cell surface in adipose tissue, and decreased serum resistin levels.¹² These results indicated that PTEN may negatively regulate insulin signaling in adipose tissue. Suppression of the PI3K/Akt pathway by elevated PTEN expression level in adipose tissue is an important mechanism of insulin resistance. The data from our recent researches

and other reports suggested that PTEN expression level was elevated in the muscle and live tissue when insulin resistance occurred.^{16, 17}

Our results also showed that the expression of Foxo3a in KKAY diabetic mice was increased significantly. As a PI3K/Akt pathway downstream factor, Foxo3a had been reported to play an important role in insulin receptor pathway.¹⁸ Another report revealed that the expression of Foxo3a was upregulated by hyperglycemia and in the myocardium of fatty rat fed with high fat diet. Foxo3a also involved in the other organ impairments induced by diabetes.¹⁹ Therefore, we presumed that upregulated Foxo3a in KKAY diabetic mice might be related to insulin resistance, and might be a downstream target of PTEN.

After 4-week treatment with rosiglitazone or metformin, the FBG and fasting insulin levels were decreased in each KKAY diabetic mice group, but the insulin resistance was improved comparing with C57BL/6J mice group. However, the pS473-Akt level and PTEN expression level in KKAY diabetic mice adipose tissue had no significant changes after treatment with rosiglitazone or metformin. Two reports observed the activity of PI3K and Akt in adipose tissue and vastus lateralis muscle of Goto-Kakizaki (GK) diabetic rat treated with rosiglitazone. The results suggested rosiglitazone treatment has no effects on insulin-induced PI3K and pS473-Akt activation in GK rats, which can further confirm the hypothesis of our study.^{20, 21} Our results suggested that rosiglitazone, a PPAR γ agonist, had no effects on the expression of PTEN in KKAY diabetic mice adipose tissue. One report showed that the expression of PTEN mRNA and protein was upregulated by rosiglitazone through PPAR γ in hepatocellular carcinoma cells accompanying with the downregulation of PI3K-dependent signaling.²² There may be an unusual cell signaling response in KKAY diabetic mice, which lead to insulin resistance directly or indirectly. Another study reported that PPAR γ agonists can suppress PTEN expression level in adipose and skeletal muscle tissue of C57BL/6J-ob/ob mice, meanwhile, the plasma glucose levels were decreased after treatment.²³ Overexpressed PTEN in KKAY diabetic mice may result in the unresponsive effects of PPAR γ agonist, even though PPAR γ agonist can regulate PTEN expression level in C57BL/6J-ob/ob mice.

Metformin is used alone or with other medications, including insulin, to treat type 2 diabetes. In this work, fasting glucose and insulin levels of KKAY diabetic mice were decreased significantly after 4 weeks of metformin treatment, while PTEN expression level was not affected and insulin-induced pS473-Akt expression was not reversed. These results suggested that the effect of

metformin, at lower blood sugar level, may not be associated with PTEN and PI3K/Akt pathway. Not much information is available about the relationship between PTEN and resiglitazone or metformin, so further investigation is needed to identify the causes.

In conclusion, this study demonstrated that the expression of PTEN was upregulated in adipose tissue of high fat diet induced diabetic mice. PTEN may be a potent negative modulator of PI3K/Akt pathway and play an important role in the development of insulin resistance in type 2 diabetes. PTEN may also as a therapeutic target for insulin resistance.

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