

Ameliorating effect of sesamin on insulin resistance of hepatic L02 cells induced by high glucose/high insulin

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Abstract: Sesamin (SES) has the ameliorating effect on L02 hepatocyte model of insulin resistance induced by high glucose and high insulin, based on insulin receptor signaling pathway IRS/PI3K/Akt. Treatment with SES (200, 100µg/ml) increased glucose consumption, glucose uptake and the intracellular glycogen synthesis of L02 hepatocyte model of insulin resistance significantly. Moreover, treatment with SES promoted the gene and protein expression levels of insulin receptor (InsR) and the post-receptor associated proteins, such as insulin receptor substrate 1 (IRS1), insulin receptor substrate 2 (IRS2), PI3K (phosphatidylinositol 3-kinase), GLUT4 (glucose transporter 4) significantly, which were determined by RT-PCR and immunoblot analysis. In conclusion, SES has the ameliorating effect on L02 hepatocyte model of insulin resistance induced by high glucose/high insulin, which might be related to its effect on promoting expression of insulin receptor and its associated proteins of IRS-PI3K-Akt passway, and thus promoting insulin sensitivity.

Keywords: Sesamin (SES), insulin resistance, L02 hepatocytes, insulin receptor, IRS/PI3K/Akt signaling pathway.

INTRODUCTION

Insulin resistance is defined as a state of reduced response of some target tissues to the circulating insulin, which plays an important role in the development of metabolic syndrome. Insulin resistance is also a feature of other health disorders, including type 2 diabetes, obesity, hyperlipidemia and hypertension (Giorgio, 2006).

Sesame seed and sesame oil have been praised and consumed by human for thousands of years for their enticing taste and nutrition. The main active part is sesamin which has multiple and beneficial functions (Kanu *et al.*, 2007) and could be extracted from sesame seed, sesame oil and sesame meal (Zhou *et al.*, 2010). Sesamin is a kind of lignans with the white needle like crystal shape, with the formula C₂₀H₁₈O₆ (fig. 1) and molecular weight 354.36. Moreover, the medicinal properties of sesamin have been demonstrated experimentally and scientifically. For instance, sesamin has been shown to have the effect of promoting immune function (Bian, 2008), anti-oxidative effect (Dong *et al.*, 2007), anti-tumor activity (Wei *et al.*, 2008), lowering serum lipids and protecting hepatocytes effects (Wang and Song, 2006; Nobuo *et al.*, 2005).

Sesamin with the high purity has been extracted and purified from sesame meal in our laboratory (Lei *et al.*, 2013a; Liu *et al.*, 2017). Our previous study has demonstrated the insulin-resistance-ameliorating effect of sesamin, exploiting a kind of insulin-resistance animal

model of type 2 diabetes (KKay mice), and hepatic membrane insulin receptors played the important role in the ameliorating-insulin-resistance effect of sesamin (Lei *et al.*, 2013b). Therefore, the present study would further investigate the mechanisms of ameliorating-insulin-resistance effect of sesamin, exploiting a kind of cellular model which is L02 hepatocyte model of insulin resistance induced by high glucose and high insulin, concentrating on insulin receptor and its associated proteins of IRS-PI3K-Akt signaling passway. The present study would provide the experimental basis for application of sesamin as the candidate for improvement of insulin resistance and treatment of the related diseases.

MATERIALS AND METHODS

Preparation of sesamin (SES) and its purity

Sesamin was purified from sesame meal by ethanol extraction combined with macroporous resin purification, followed by recrystallization technology as previously described (Lei *et al.*, 2013a; Liu *et al.*, 2017). The purity of the sesamin sample was 95.26% estimated by high performance liquid chromatography (HPLC) as shown in fig. 2. Then sesamin sample was dissolved in RPMI-1640 medium and diluted to the concentration needed.

Main reagents

RPMI-1640 medium was obtained from Gibco. Trypsin was from Invitrogen life Technologies. Glucose or glycogen measurement kits were from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). First strand cDNA synthesis kits, PCR amplification kits, agarose,

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6×DNA loading buffer, DL Marker 2000 were from Shanghai ShengGong biological engineering Co., Ltd (Shanghai, China). RNAiso Plus kits were from Takara Bio Co., Ltd. and Reverse transcription kits were from Promega Co., Ltd. All other solvents and reagents were of analytical grade supplied by Shanghai Guoyao Chemicals Co., Ltd. Human InsR, IRS1, IRS2, PI3K, GluT4 monoclonal antibodies were obtained from Cell Signaling Co., Ltd. Actin polyclonal antibody was provided from Santa Cruz Biotechnology Co., Ltd.

Liver L02 cell culture

Human fetal liver L02 cell line was purchased from Cell Bank of the Typical Culture Preservation Committee of Chinese Academy of Sciences. Cells were cultured in RPMI-1640 medium supplemented with 25mM glucose, 15mM HEPES, 1mM sodium pyruvate, 2mM L-glutamine, 2g/L sodium bicarbonate, 100mg/L penicillin/streptomycin, 10% heat-inactivated fetal calf serum (FCS) which were adjusted to pH 7.2 and maintained in 25 cm² flasks at 37°C in a humidified atmosphere of 5% CO₂ incubators. Cells were allowed to attach the flask, the culture medium was changed every 48h and cells were passaged at 3d intervals by trypsination. Harvesting and passaging of the L02 cells were accomplished by detaching, aspirating and separating the adherent cells by mechanical agitation, followed by incubation with 0.25% trypsin and 0.02% EDTA in D-Hank's solution (pH 7.2) for 1 to 2 minutes.

Establishment of hepatocyte model of insulin resistance induced by high glucose and high insulin and Experimental design

Insulin resistance hepatocyte model was induced by high concentration of glucose and insulin. Human fetal liver L02 cells were inoculated in fresh RPMI-1640 medium and incubated at 37 °C, 5% CO₂ and saturated humidity in incubator. When 80% cells confluent, the medium was replaced by serum-free medium and incubated for another 12 hours before experiment. Then the medium was replaced with the high glucose / high insulin serum free medium (containing glucose 25mmol/L, insulin 10⁻⁶ mol/L). Establishment of insulin resistance cellular model was assessed by glucose consumption test, glucose uptake test and intracellular glycogen synthesis.

Aliquots of 1×10⁵ L02 cells were transferred into the wells of 96-well cell culture plates. After 12 h and monolayer adherence, the cells were stimulated with high glucose and high insulin solution (final concentration glucose 25mM, insulin 10⁻⁶ M) for 24h or were kept untreated as controls. Meanwhile, the cells were co-incubated in the presence or absence of sesamin (dissolved in RPMI-1640 and the final concentration 200, 100 µg/ml) for 24 h in 5% CO₂ incubator at 37 °C. Then the supernatant of culture medium was collected, the cells were washed, collected and lysed. The amount of glucose

consumption and glucose uptake were determined and the intracellular glycogen synthesis was determined by using commercial kits according to the guidelines indicated. Moreover, levels of insulin resistance related genes, such as insulin receptor (InsR), insulin receptor substrate 1 (IRS1), insulin receptor substrate 2 (IRS2), PI3K and GLUT4 mRNA and protein expression were determined by RT-PCR and immunoblot analysis.

Glucose consumption test

The glucose content of the cell culture medium was measured by glucose oxidase method, which was determined by using commercial kits according to the guidelines indicated. The amount of glucose consumed was calculated by the difference of the glucose amount of the supernatant of non-cell blank control wells and that of the inoculated cell wells.

Glucose uptake test

2-deoxy -[³H]-D glucose was added to culture medium and the final concentration was 7.5×10⁴ Bq/L to detect the specific uptake of labelled glucose. The cells were washed with cold PBS containing 10 mmol/L glucose for three times to stop the reaction, and then NaOH (0.1mol/L, 1 ml) was added to each well for 20min. Then the cell lysis was added to the scintillation liquid and the number of decay per minute (dpm) was detected by liquid scintillation counter.

Reverse transcription polymerase chain reaction (RT-PCR)

Levels of gene expression of InsR, IRS1, IRS2, PI3K and GLUT4 of L02 cells were determined by RT-PCR. Total RNA of L02 cells was extracted with TRIzol and was reversely transcribed to synthesize cDNA with reverse transcriptase AMV. cDNA was amplified by PCR reaction with primers and Taq polymerase. PCR reaction was performed with synthesized cDNA and DNA templates. Primers were synthesized by Shanghai ShengGong Biological Engineering Co., Ltd (China). Primers, size of products and annealing temperature of the objective genes and reference gene β-actin were shown in table 1. The thermal cycle profile for InsR was as follows: denaturation at 95°C for 5 min, 30 cycles (denaturation at 95°C for 30 s, annealing at 53°C for 60 s and extension at 72°C for 60 s) and final extension at 72°C for 10 min. Thermal cycle profiles of IRS1, IRS2, PI3K, GLUT4 and β-actin were the same as those of InsR except the annealing temperature shown in table 1. PCR products of objective genes and reference gene were electrophoresed in 1% agarose gel at 120 V. Gray shades of resulting bands of PCR products were evaluated by electrophoretic image analysis system. The ratios of gray shade between objective gene and reference gene were then calculated.

Immunoblot analysis

L02 cells were treated with sesamin or vehicle for the noted times and lysed in 0.5% NP-40 lysis buffer

supplemented with protease inhibitor. Total lysates were analyzed by SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore) using the semi-dry method (Bio-Rad). Membranes were blocked with 3% bovine serum albumin in TBST buffer at room temperature for 1 h and incubated with different antibodies overnight at 4°C. The membranes were then washed and incubated with IRDye 800CW goat anti-rabbit IgG or IRDye 680RD goat anti-mouse IgG at room temperature for 1 h. Band intensity was quantified using the Licor Odyssey system.

STATISTICAL ANALYSIS

Data were expressed as means \pm S.D. Statistical analysis was evaluated using SPSS 16.0 software by one-way analysis of variance, followed by Student-Newman-Keuls test for multiple comparisons, which was used to evaluate the difference between two groups. $P < 0.05$ was considered significant.

RESULTS

Effect of sesamin (SES) on glucose consumption of L02 hepatocyte model of insulin resistance induced by high glucose/high insulin

The level of glucose consumption of L02 hepatocyte in normal, insulin resistance model induced by high glucose/high insulin, and two experimental groups (treatment with SES 200, 100 $\mu\text{g/ml}$) were shown in fig. 3. Results showed that the level of glucose consumption decreased significantly in the insulin resistance model group as compared with that of the normal control ($P < 0.01$). Administration of SES (200, 100 $\mu\text{g/ml}$) for 24 h significantly reversed the decreased level of glucose consumption ($P < 0.01$).

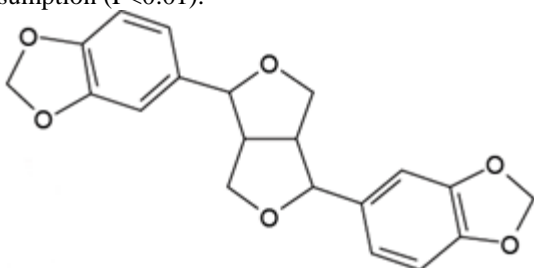


Fig. 1: Structure of sesamin.

Effect of SES on glucose uptake of L02 hepatocyte model of insulin resistance induced by high glucose/high insulin

As shown in fig. 4, the level of ^3H glucose uptake decreased significantly in the insulin resistance model group as compared with that of the normal control ($P < 0.01$). Treatment with SES (200, 100 $\mu\text{g/ml}$) for 24h exerted significant promoting effects on glucose uptake as compared with that of the insulin resistance model control ($P < 0.01$).

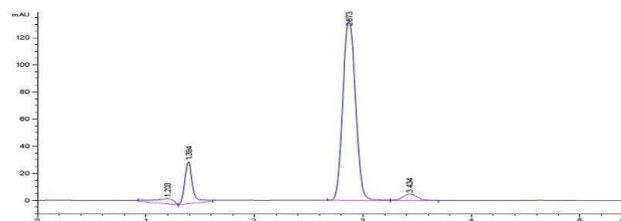
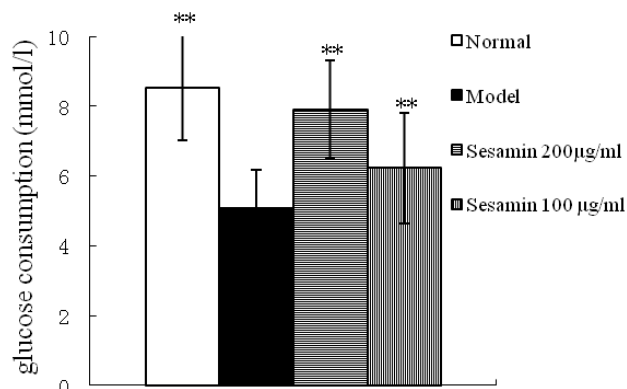
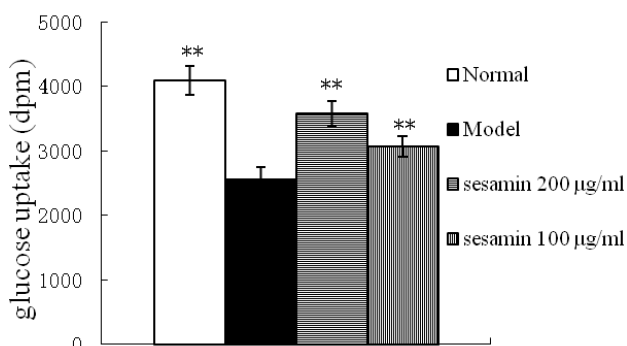


Fig. 2: HPLC diagram of sesamin recrystal sample.



Data are expressed as mean \pm S.D. (n=10). Compared with model group, $**P < 0.01$.

Effect of sesamin (SES) on ^3H glucose uptake of L-02 hepatocyte model of insulin resistance induced by high glucose/high insulin



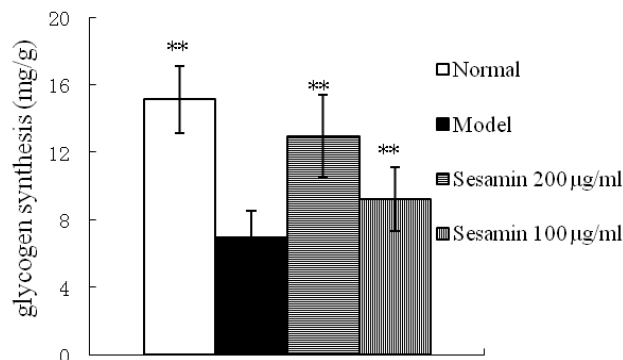
Data are expressed as mean \pm S.D. (n=10). Compared with model group, $**P < 0.01$.

Effect of SES on intracellular glycogen synthesis of L02 hepatocyte model of insulin resistance induced by high glucose/high insulin

Effect of SES on intracellular glycogen synthesis of L02 hepatocyte model of insulin resistance induced by high glucose/high insulin

The levels of intracellular glycogen synthesis of L02 hepatocyte were shown in fig. 5. As shown in fig. 5, the level of intracellular glycogen synthesis decreased significantly in the insulin resistance model group as compared with that of the normal control ($P < 0.01$). Treatment with SES significantly improved intracellular glycogen synthesis of L02 hepatocyte model of insulin resistance in a dose-dependent manner significantly. This suggested that SES is involved in promoting the synthesis

of glycogen, and thus, ameliorate insulin resistance of the L02 cells induced by high glucose/high insulin.



Data are expressed as mean \pm S.D. (n=10). Compared with model group, **P<0.01.

Fig. 5: Effect of sesamin (SES) on intracellular glycogen synthesis of L-02 hepatocyte model of insulin resistance induced by high glucose/high insulin

Effect of SES on gene expression of *InsR*, *IRS1*, *IRS2*, *PISK*, *GLUT4* of L02 hepatocyte model of insulin resistance induced by high glucose/high insulin

Gene expression of the insulin receptor associated proteins (*InsR*, *IRS1*, *IRS2*, *PISK* and *GLUT4*) of L02 hepatocyte were shown in fig. 6. Compared with the normal group, gene expression levels of *InsR*, *IRS1*, *IRS2*, *PISK* and *GLUT4* were significantly lower in the model group ($P<0.01$). Treatment with SES markedly increased the gene expression of the insulin receptor associated proteins *InsR*, *IRS1*, *IRS2*, *PISK* and *GLUT4* significantly ($P<0.01$).

Effect of SES on protein expression of *InsR*, *IRS1*, *IRS2*, *PISK*, *GLUT4* of L02 hepatocyte model of insulin resistance induced by high glucose/high insulin

Immunoblot analysis of protein expression of the insulin receptor associated proteins (*InsR*, *IRS1*, *IRS2*, *PISK* and *GLUT4*) of L02 hepatocyte were shown in fig. 7. The level of protein expression of *InsR*, *IRS1*, *IRS2*, *PISK* and *GLUT4* decreased significantly in the insulin resistance model group as compared with that of the normal control. Treatment with SES (200, 100µg/ml) for 24h exerted significant promoting effects on protein expression as compared with that of the insulin resistance model control. These suggested that SES had the ameliorating effect on insulin resistance of the L02 cells induced by high glucose/high insulin, which were related to the effect of promoting expression of insulin receptor associated proteins.

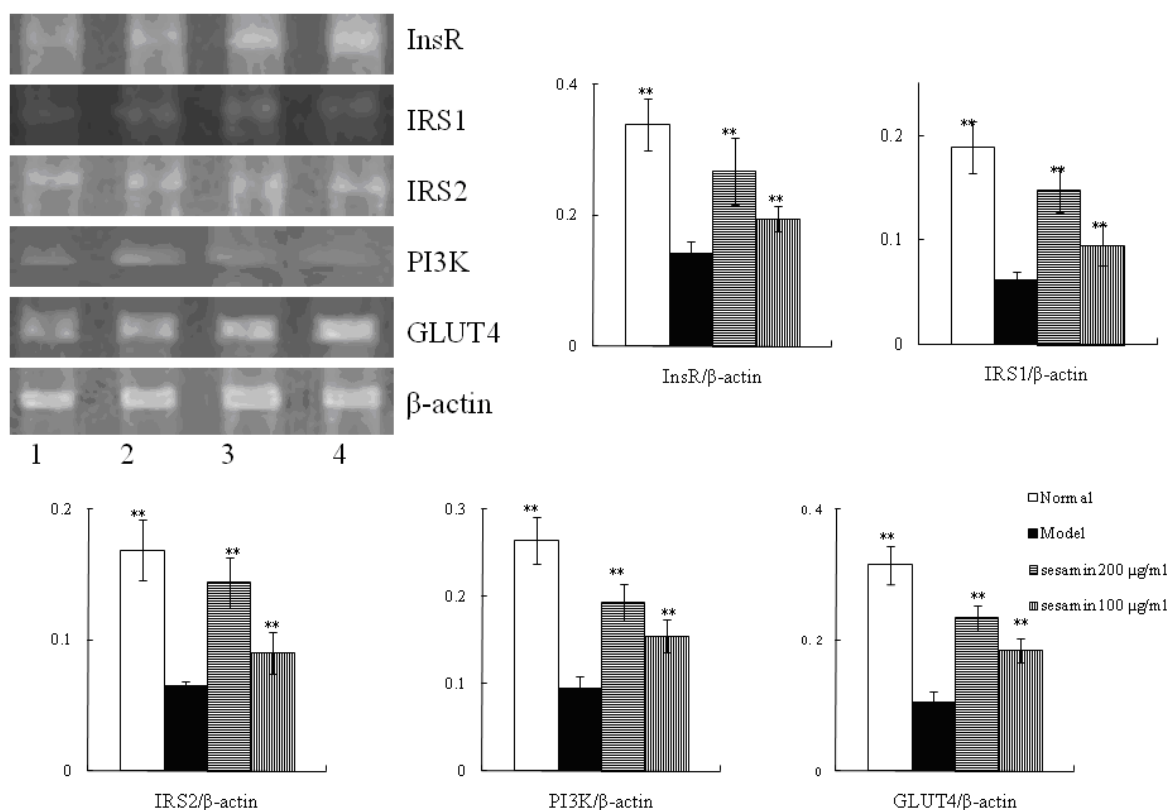
DISCUSSION

Insulin resistance is defined as the loss of normal physiological response to insulin and the body can not maintain glucose homeostasis. At present, several cellular

models of insulin resistance have been successfully established which were induced by high glucose/high insulin, high concentration of glucosamine, tumor necrosis factor alpha, free fatty acid or dexamethasone (Li *et al.*, 2013; Zhang and Tong, 2012). Normal human fetal liver L02 cells retain the basic phenotype and function of primary hepatocytes. In the present study, we successfully established the cellular model of insulin resistance induced by high glucose/high insulin, exploiting normal fetal liver L02 cells. So the present study used this kind of cellular model of insulin resistance to investigate the ameliorating effect of sesamin on insulin resistance *in vitro* and its molecular mechanisms of action.

The effect of sesamin on the cellular model of insulin resistance could be assessed by glucose consumption test, glucose uptake test and intracellular glycogen synthesis. Results of our studies showed that the level of glucose consumption and glucose uptake decreased significantly in the insulin resistance cellular model group as compared with those of the normal cellular control. Administration of sesamin (200, 100µg/ml) for 24 h significantly reversed the decreased level of glucose consumption and glucose uptake. Moreover, decreased glycogen synthesis is an important index of insulin resistance, and our results showed that sesamin also involved in promoting the synthesis of glycogen of L02 cells. These suggested that treatment with sesamin significantly ameliorated insulin resistance of the L02 cells induced by high glucose/high insulin.

Insulin mediated cellular glucose uptake and utilization mainly through *IRS/PI3K/Akt* insulin receptor signaling pathway. Dysfunction of any molecules of *IRS/PI3K/Akt* signaling pathway may result in signal transduction defects of insulin receptor, leading to the occurrence of insulin resistance (Meshkani and Khosrow, 2009; Awad *et al.*, 2009). Liver is one of the important insulin-target organs with abundant insulin receptors on the membrane. Insulin bound with the alpha subunit of insulin receptors on the surface of liver cell membrane, leading to phosphorylation of tyrosine residues of beta subunit of insulin receptors. Activation of the tyrosine kinase activity of insulin receptor would recognize and recruit its substrate *IRS* family members (Kovacs, 2005). *IRS* is an important pivotal molecule of the insulin signaling pathway *IRS/PI3K/Akt* which amplifies insulin signaling and has two subtypes *IRS1* and *IRS2*. *IRS1* mainly promotes the uptake and utilization of glucose and *IRS2* mainly promotes the synthesis of liver glycogen and inhibition of gluconeogenesis (Mlinar *et al.*, 2007). *PI3K* is also an important signaling molecule in the insulin signaling pathway and is a downstream target protein of *IRS1* (Yoshiaki *et al.*, 2001). *GLUT4*, a specific carrier protein on the cell membrane, mediates the translocation of extracellular glucose from the cell membrane to the cytoplasm. Abnormal transposition of *GLUT4* is an



Data are expressed as mean \pm S.D. (n=10). Compared with model group, **P<0.01.

(Line 1: model group; Line 2: SES 100 µg/ml; Line 3: SES 200 µg/ml; Line 4: Normal L02 cells group.)

Fig. 6: Effect of sesamin (SES) on gene expression of key cascade proteins of IRS-PI3K-Akt signal pass way (InsR, IRS1, IRS2, PISK, GLUT4) of L02 hepatocyte model of insulin resistance induced by high glucose/high insulin determined by RT-PCR.

Table 1: Sequences of primers and the information of objective genes and reference gene

Genes	Sequence of primers	size of product	annealing temperature
β-actin	forward 5' -TCAGAAGGACTCCTATGTGG-3'	500bp	53°C
	reverse 3' -TCTCTTTGATGTCACGCACG-5'		
InsR	forward 5' -CCCCTCATCTTTGTCTTCCTC-3'	318bp	55°C
	reverse 3' -TCGAAGACTGGCTGACTCATT-5'		
IRS1	forward 5' -CCAGAGTCAAGCCTCACACA-3'	413bp	55°C
	reverse 3' -CCCAACTCAACTCCACCACT-5'		
IRS2	forward 5' -TGTGTTTTTCCCCACTCAAT-3'	181bp	51°C
	reverse 3' -TGGAAGTCGCCAGAATGTAT-5'		
PI3K	forward 5' -TGGGACCTTTTTGGTACGAG-3'	466bp	53°C
	reverse 3' -TTCTTTGCTGTACCGCTCCT-5'		
GLUT4	forward 5' -GCTGAAGGATGAGAAACGGA-3'	304bp	53°C
	reverse 3' -AGGCCACAAAGCCAAATATG-5'		

important feature of insulin resistance (Giorgino *et al.* 2005). So InsR, IRS1, IRS2, PISK and GLUT4 are the key cascade proteins of IRS-PI3K-Akt signal pass way.

Our previous study has demonstrated that the insulin receptors on the liver membrane played an important role in the ameliorating-insulin-resistance effect of sesamin, exploiting an insulin-resistance animal model of type 2

diabetes (KKay mice). So in the present study, the key proteins of insulin receptor signaling cascade pathway IRS/PI3K/Akt would be investigated. RT-PCR and Immunoblot analysis were employed to analyze the gene mRNA and protein expression of key insulin receptor cascade proteins of signal passway IRS-PI3K-Akt, which were InsR and its downstream signaling molecules IRS1, IRS2, PISK and GLUT4. Results of the present study

showed that compared with the normal cellular group, gene and protein expression levels of InsR, IRS1, IRS2, PISK and GLUT4 decreased significantly in the insulin resistance cellular model group. Treatment with sesamin (200, 100µg/ml) exerted significant promoting effects on gene and protein expression of InsR, IRS1, IRS2, PISK and GLUT4 significantly as compared with those of the insulin resistance model control. These suggested that sesamin had the ameliorating effect on insulin resistance of the L02 cells induced by high glucose/high insulin, and the mechanism of action might be related to the effect of promoting expression of insulin receptor and its associated proteins of IRS-PI3K-Akt passway.

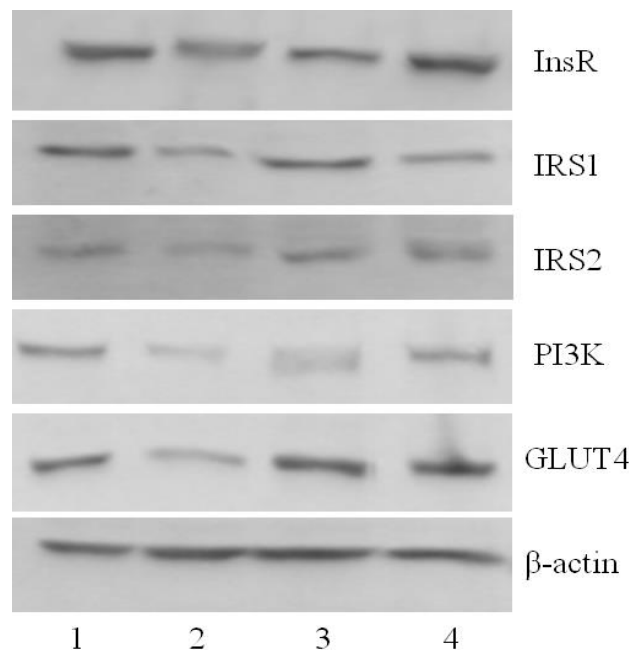


Fig. 7: Effect of sesamin (SES) on protein expression of InsR, IRS1, IRS2, PISK, GLUT4 of L02 hepatocyte model of insulin resistance induced by high glucose/high insulin determined by immunoblot analysis. (Line 1: normal L02 cells group; Line 2: model group; Line 3: SES 200 µg/ml; Line 4: SES 100µg/ml.)

CONCLUSION

Treatment with sesamin increased the amount of glucose consumption, glucose uptake and the intracellular glycogen synthesis of L02 hepatocyte model of insulin resistance induced by high glucose/high insulin, as well as promoted the gene and protein expression levels of insulin receptor (InsR) and the post-receptor associated proteins, such as insulin receptor substrate 1 (IRS1), insulin receptor substrate 2 (IRS2), phosphatidylinositol 3-kinase (PI3K), glucose transporter 4 (GLUT4) significantly. Sesamin has the ameliorating effect on L02 hepatocyte model of insulin resistance induced by high glucose/high insulin, which might be related to its effect on promoting expression of insulin receptor and its associated proteins of IRS-PI3K-Akt passway.

ACKNOWLEDGEMENTS

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