Effect of insulin in combination with selenium on IRS/PI3K-mediated GLUT4 expression in cardiac muscle of diabetic rats

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Abstract. – Background: Oral administration of selenium is an effective treatment for diabetes in animal models. However, selenium exerts these effects at high doses and several toxic effects are produced. Low doses of selenium are relatively safe but are unable to elicit any antidiabetic effect.

Objectives: The present study explored the prospect of using low doses of insulin in combination with selenium to evaluate their antidiabetic effect, and to evaluate their effect on IRS-1, PI3K and GLUT4 levels in cardiac muscle of diabetic rats.

Materials and Methods: Diabetic rats were treated with insulin, selenium, and insulin and selenium in combination for four weeks. The effect of these antidiabetic compounds was examined on general physiological parameters and distribution of IRS-1, PI3K and GLUT4 in cardiac muscle by immunoblotting and immunohistochemistry.

Results: Insulin in combination with selenium could significantly revive normoglycemia, and restore the disturbances in IRS-1, PI3K and GLUT4 levels in cardiac muscle. Treatment with insulin was only partially effective in the restoration of diabetic alterations.

Conclusion: The treatment of diabetic rats with combined doses of insulin and selenium was most effective in controlling glycaemia, and remarkably restored GLUT4 distribution by IRS-1/PI3K-dependent pathway in cardiac muscle of diabetic rat.

Key Words:
STZ-diabetic rats, Insulin, Sodium selenite, IRS-1, PI3K, GLUT4.

Introduction

Cardiovascular disease is one of the major complication of diabetes, resulting in a high percentage of morbidity and mortality and producing significant costs for the health care system1. Hyperglycemia is the main pathogenic factors in the cardiovascular complications of diabetes2-4. Insulin receptor substrate-1 (IRS-1) appeared to be more important in the control of glucose metabolism through activation of the phosphoinositide 3-kinase (PI3K) pathway. PI3K, the pivotal kinase in insulin signal transduction pathway5, is necessary for insulin-stimulated glucose transport in cardiac muscle6. The studies demonstrate that the deletion of the p85a/p55a/p50a and p85b regulatory subunits of PI3K in the muscle severely impairs PI3K signaling and leads to decreased muscle size7, decreased insulin-stimulated muscle glucose uptake, and impaired whole-body glucose disposal8. GLUT4 is the most abundant glucose transporter isoform and primarily contributes to insulin-stimulated glucose uptake9, the reduction in the GLUT4 levels contributes significantly to the elevated glucose levels and to the myocardial dysfunction10,11. Therefore, restoration of IRS-1, PI3K and GLUT4 levels is very important to enhance the glucose metabolism in diabetic hearts and help in assuaging myocardial dysfunction.

Selenium (Se) is a trace element that could improve glucose homeostasis1] and has a protective role against lipid peroxidation and diabetes-inducing injury in several tissues13,14. Oral administration of selenium is an effective treatment for diabetes in animal models15. However, selenium exerts these effects at high doses and several toxic effects are produced16-19. Lower doses of selenium are relatively safe, but fail to effectively decrease blood glucose levels of diabetic rats20,21.
Insulin has a beneficial effect on hyperglycemia in diabetes, but administration of insulin for controlling hyperglycemia may produce hyperinsulinemia, which is considered to be the cause of diabetes-induced coronary heart disease, hypoglycemia and allergy.

The principal goal of the present study was to explore the possibility of using low doses of insulin in combination with selenium and to evaluate their effect on these parameters in diabetic rats.

**Materials and Methods**

**Animals and Model**

Thirty-five male Sprague-Dawley rats weighing 180-220 g provided by the Medical Experimental Animal Center of Xi’an Jiaotong University (Xi’an, PR China) were used in this research. Rats were housed individually in metal cages with constant temperature and relative humidity (22±1°C; relative humidity, 50±2%) in the Medical Experimental Animal Center of Xi’an Jiaotong University. All animals received water and chow ad libitum. After a week of acclimatization, animals were randomly grouped into control (C) diabetic (DM), insulin-treated diabetic (DM+In), selenium-treated diabetic (DM+Se) and diabetic treated with selenium and insulin (DM+In+Se). Control rats (n = 7) were injected (i.p.) with a corresponding volume of citrate buffer. Streptozotocin (STZ: 50 mg/kg body weight, dissolved in 0.02 M sodium citrate, pH 4.5; Sigma-Aldrich, St. Louis, MO, USA) was injected (i.p.) into the other group. Models were regarded as successfully established if blood glucose concentration was >16.7 mmol/L and accompanied with polyuria or other diabetic symptoms after 7 days. The insulin treatment group (n = 7) involved injection (s.c.) of insulin (1 U/kg per day); the selenium treatment group (n = 7) were treated with a dose of (180 µg/kg per day) of sodium selenite dissolved in redistilled water by gavage; and the selenium in combination with insulin treatment group (n = 7) were given both treatments. After 4 weeks of treatment, anesthesia was induced with an intravenous injection of pentobarbital sodium (25 mg/kg) and rats were killed by exsanguination of the carotid artery. After blood collection, cardiac muscle was removed, rapidly frozen in liquid nitrogen and kept at -80°C until further analysis. All animal experiments were performed with the approval of the Animal Research Committee of the University of the Xi’an Jiaotong University (PR China).

**Material**

Trihydroxymethyl aminomethane (Tris), glycine, sodium dodecyl sulfate (SDS), acrylamide and bis-acrylamide were purchased from Amresco Inc. (Amresco, Solon, OH, USA). Rabbit anti-rat-actin polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-IRS-1, Anti-PI3K and Anti-GLUT4 polyclonal antibody was purchased from Upstate Biotechnology Incorporated (Lake Placid, NY, USA). RI-PA was purchased from Biotek Corporation (Beijing, PR China). BCA™ protein assay kit, BlueRanger® pretained protein molecular weight marker mix, and Super-Signal® West Pico chemiluminescent substrate were purchased from Pierce Chemical Company (Pierce, Rockford, IL, USA). Insulin was from Novo Nordisk (Copenhagen, Denmark) and sodium selenite and STZ were purchased from Sigma (Sigma-Aldrich Chemical Co, St Louis, MO, USA).

**Detection of Blood Glucose and Hemoglobin A1c (HbA1c)**

The fasting blood glucose of each group was estimated every week using One Touch SureStep Blood Glucose meter. HbA1c of each group was measured after four weeks of treatment.

**Immunoblotting**

Total and plasma membrane proteins of cardiac muscle were extracted using RIPA agents and quantified with BCA protein assay kit. Equal amounts of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto microporous polyvinylidene fluoride membranes in running buffer with 20% methanol. After nonspecific sites were blocked with 5% milk-Tris-buffered saline Tween-20 (TBST), membranes were incubated with anti-IRS-1, anti-PI3K, anti-GLUT4 antibody and anti-β-actin antibody, respectively. Membranes were washed in TBST, A horseradish peroxidase-linked antibody was employed as a secondary antibody, and the bands of interest were detected using an enhanced chemiluminescence technique. Densities of band were analyzed by computer-assisted image quantification (GEL DOC gel 2000, BIO-RAD Company, Hercules, CA, USA).
Immunohistochemistry

Briefly, 4-µm sections were treated with 2% hydrogen peroxide to quench endogenous peroxide for 30 min and washed with phosphate-buffered saline (PBS). After several washes with PBS, sections were blocked with 10% normal goat serum in PBS, followed by overnight incubation with rabbit anti-IRS-1, anti-PI3K or anti-GLUT4 antibodies at 4°C overnight. Sections were processed using the ABC staining method. Nickel-intensified biotinylated diaminobenzidine (DAB) was used to visualize the signal.

Statistical Analysis

Values were calculated as mean ± SD. A repeated measures ANOVA was used to compare blood glucose among the control, diabetic and treated groups. A one-way ANOVA was used to analyze differences in all other parameters among the control, diabetic and treated groups. The ANOVA test followed by Dunnet’s multiple comparison test was employed for statistical comparison between Model and various groups using SPSS13.0, Chicago, IL, USA. Significance was considered at \( p < 0.05 \).

Results

Effect of Insulin in Combination with Sodium Selenite Blood Glucose and Hemoglobin A1c of Diabetic Rats

Changes in blood glucose levels and HbA1c levels are presented in Table I.

The blood glucose and HbA1c levels were significantly elevated in diabetic rats, but diabetic rats treated with selenium and insulin showed remarkably decrease in plasma glucose concentration and HbA1c levels.

Effect of Insulin in Combination with Selenium on the Level of IRS-1 Protein in Cardiac Muscle

IRS-1 protein intermediates PI3K leading to the stimulation of glucose transport, glycogen synthesis and other downstream insulin effects. In this study, expression of IRS-1 in different groups was investigated by Western blotting, and the results were analyzed by computer-assisted image quantification software. In the model group, there was a low level of IRS-1, while the IRS-1 was significantly elevated in treatment of diabetic rats with insulin and selenium in combination \( (p < 0.01) \). Treatment of diabetic rats with insulin and selenium alone could not alter the IRS-1 expression (Figure 1).

As we expected, similar results were also observed by immunohistochemistry: IRS-1 expression was decreased markedly in model group compared with the control group. Treatment of diabetic rats with insulin and selenium in combination restores the distribution of IRS-1 (Figure 2).

Effect of Insulin in Combination with Selenium on the Level of PI3K Protein in Cardiac Muscle

To examine the effect of insulin in combination with selenium on the expression of PI3K in cardiac muscle, Western blotting of the total protein was performed and the results were analyzed using an image analyzer. In the model group, a low level of PI3K expression was observed, while it was signifi-

Table I. Changes in plasma glucose (mmol/L) and HbA1c levels in control (C) and diabetic (DM) rats after treatment with insulin, selenium and insulin and selenium in combination.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose</th>
<th>FBG [mmol/L]</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>HbA1c (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Week 0</td>
<td>Week 1</td>
<td>Week 2</td>
<td>Week 3</td>
<td>Week 4</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>5.4 ± 0.8</td>
<td>5.5 ± 1.0</td>
<td>5.4 ± 0.7</td>
<td>5.5 ± 0.7</td>
<td>5.7 ± 0.7</td>
<td>4.9 ± 0.5</td>
</tr>
<tr>
<td>DM</td>
<td></td>
<td>23.5 ± 1.7**</td>
<td>23.1 ± 1.0**</td>
<td>21.7 ± 1.3**</td>
<td>22.8 ± 1.4**</td>
<td>25.0 ± 1.6**</td>
<td>8.1 ± 0.3**</td>
</tr>
<tr>
<td>DM+In</td>
<td>1 U/kg/d</td>
<td>22.3 ± 3.5**</td>
<td>15.3 ± 2.4***</td>
<td>15.6 ± 2.4***</td>
<td>14.9 ± 2.5***</td>
<td>15.0 ± 2.6***</td>
<td>6.7 ± 0.3***</td>
</tr>
<tr>
<td>DM+Se</td>
<td>180 µg/kg/d</td>
<td>20.3 ± 1.8**</td>
<td>20.0 ± 2.1***</td>
<td>19.6 ± 3.1**</td>
<td>17.1 ± 2.4***</td>
<td>15.4 ± 2.1***</td>
<td>7.0 ± 0.4***</td>
</tr>
<tr>
<td>DM+In+Se</td>
<td>1 U/kg/d + 180 µg/kg/d</td>
<td>25.5 ± 3.5**</td>
<td>18.1 ± 4.2***</td>
<td>15.3 ± 2.8***</td>
<td>10.9 ± 1.3***</td>
<td>7.6 ± 0.8***</td>
<td>5.4 ± 0.4***</td>
</tr>
</tbody>
</table>

All the data were shown as the mean ± SD, n = 7. FBG = Fasting Blood Glucose. *Significant different from Control group, \( p < 0.05 \). **Significant different from Control group, \( p < 0.01 \). #Significant different from DM group, \( p < 0.05 \). ##Significant different from DM group, \( p < 0.01 \).
Insulin in combination with selenium on IRS/PI3K-mediated GLUT4 expression in cardiac muscle

Significantly elevated following 4 weeks of treatment with insulin and selenium in combination (p < 0.01). Insulin and selenium alone could not revive the PI3K content in cytoplasm (Figure 3).

Similar results were observed by immunohistochemistry: there was a remarkable decrease in the PI3K level of the diabetic rats. Treatment of diabetic rats with insulin and selenium in combination restores the distribution of PI3K. Insulin and selenium alone could not alter the PI3K expression (Figure 4).

**Effect of Insulin in Combination with Selenium on the Content of GLUT4 Protein in the Membrane Fraction of Cardiac Muscle**

The effect of diabetes on GLUT4 distribution was monitored by measuring the GLUT4 protein

![Figure 1](image1.png)

*Figure 1.* Effect of insulin in combination with selenium on IRS-1 level in cardiac muscle of diabetic rats. Diabetic animal models were established by STZ to Sprague-Dawley rats, and insulin and selenium were administrated to this model. IRS-1 level in different groups was detected by Westernblotting, and the films were analyzed by computer-assisted image quantification. Results are presented as ± SD. *p < 0.05 compared with control group, **p < 0.01 compared with control group; *p < 0.05 compared with DM group, **p < 0.01 compared with DM group.

![Figure 2](image2.png)

*Figure 2.* Immunohistochemical analysis of IRS-1 in the cardiac muscle of (A) control (B) diabetic rats and diabetic rats treated with (C) insulin (D) selenium and (E) insulin + selenium. (× 400).
in the membrane fraction of the cardiac muscle of STZ-diabetic rats by immunoblotting. As has been reported earlier, there was a 47% decrease in GLUT4 levels in the membrane fraction of cardiac muscle of diabetic rats. 4 weeks of treatment of diabetic rats with insulin and selenium in combination resulted in normalization of GLUT4 levels in the cardiac muscle membrane fractions. Insulin only partially revived the GLUT4 content in the membrane. Selenium could not revise the GLUT4 levels in the membrane of cardiac muscle (Figure 5).

GLUT4 level in the cardiac muscle of control, diabetic, and treated rats was also measured by immunohistochemistry. The results were in agreement with those obtained with immunoblotting. In the diabetic state, there was a marked decrease in the GLUT4 content in the membrane.

Figure 3. Effect of insulin in combination with selenium on PI3K expression in cardiac muscle of diabetic rats. Diabetic animal model was established by injection of STZ to rats, and insulin and selenium were administered to these animals. The expression of PI3K in the different animal groups was detected by Western blotting, and the films were analyzed by computer-assisted image quantification. Results are presented as ± SD. *p < 0.05 compared with control group, **p < 0.01 compared with control group; *p < 0.05 compared with DM group, **p < 0.01 compared with DM group.

Figure 4. Immunohistochemical analysis of PI3K in the cardiac muscle of (A) control (B) diabetic rats and diabetic rats treated with (C) insulin (D) selenium and (E) insulin + selenium. (x 400).
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Combined treatment remarkably corrected the content of GLUT4, but insulin alone only partially altered the GLUT4 expression in plasma membrane of cardiac muscle (Figure 6). These results suggest that the enhancive effect of the two in combination on cardiac glucose uptake may be associated with an increase in PI3K-mediated GLUT4 in cardiac muscle. The data suggest that a combination of insulin and selenium may prove useful as a new therapy for diabetic patients but much more work needs to be done.

Discussion

Diabetes mellitus is a clinical disorder of sugar and fat metabolism. It is well known that in-

Figure 5. GLUT4 levels in the cardiac muscle membrane fraction of control (C), diabetes (DM) and diabetic rats treated with insulin (DM+In), selenium (DM+Se), Combined dose of insulin and selenium (DM+In+Se). Results are presented as ± SD. *p < 0.05 compared with control group, **p < 0.01 compared with control group, *p < 0.05 compared with DM group, **p < 0.01 compared with DM group.

Figure 6. Immunohistochemical analysis of GLUT4 in the cardiac muscle of (A) control (B) diabetic rats and diabetic rats treated with (C) insulin (D) selenium and (E) insulin + selenium. (× 400).
sulin deficiency changes cardiac energy metabolism and this is reversed by insulin, and insulin-mimetic substances. Basically, two promising insulin-mimetics, selenium and vanadate, have been studied. Due to wide variations in the circulating glucose concentrations, the blood glucose measurement does not give clear data for overall glycemic control. The amount of HbA1c reflects the average level of blood glucose over previous 1-2 months and is, therefore, a commonly used laboratory test for assessing long-term diabetic control. Therefore, we firstly studied the effect of insulin and selenium combined dose on blood glucose levels and HbA1c levels in STZ-diabetic rats. The results showed that STZ-diabetic rats showed an approximate fivefold increase in plasma glucose concentration compared with that in control rats. The two in combination produced normoglycemia in diabetic rats. According to some reports, HbA1c was significantly elevated in diabetic rats ($p < 0.01$), but its levels remarkably decreased after treatment with insulin and selenium in combination. The combined dose of insulin and selenium was most effective in controlling glycaemia.

In cardiomyocytes, glucose metabolism contributes up to 30% of the total ATP, which are provided for the contractile function of the beating heart. After insulin binding, the insulin receptor (IR) undergoes autophosphorylation of tyrosine residues, stimulating its kinase activity and association of the adaptor proteins insulin receptor substrate (IRS)-1, IRS-2 and the Grb2-associated protein, which themselves are tyrosine-phosphorylated by the receptor. Downstream signaling occurs via the PI3-kinase pathway, which is pivotal in the stimulation of glucose transport. PI3K have been implicated in the translocation of GLUT4 glucose transporters to the plasma membrane, which underlies insulin-stimulated glucose uptake. GLUT4 is the most abundant glucose transporter isoform and primarily contributes to insulin-stimulated glucose uptake in cardiomyocyte. Our study showed that the level of IRS-1, PI3K and GLUT4 protein significantly decreased in cardiac muscle of diabetic rats suggesting the obstacle of signal transduction on the level of post-receptor in cardiac muscle of diabetic rats. Treatment of diabetic rats with insulin and selenium in combination restored IRS-1, PI3K and GLUT4 levels close to normal values, suggesting that a combined dose of insulin and selenium alleviated the obstacle of signal transduction on the level of post-receptor in cardiac muscle of diabetic rats.

Similar results were obtained by immunohistochemical analyses of IRS-1, PI3K and GLUT4 in cardiac muscle. In the diabetic state, IRS-1, PI3K and GLUT4 contents decreased drastically when compared to that in control. Treatment with the two in combination restored IRS-1, PI3K and GLUT4 content in cardiac muscle. These results demonstrate that combined treatment was more effective than insulin and selenium alone in restoring IRS-1, PI3K and GLUT4 levels.

The use of insulin and selenium in combination was based on the idea that the therapeutic efficiency of selenium in STZ-diabetic rats and Selenium is an integral part of glutathione peroxidase and protects various cells against oxidative damage. Its insulin mimicry has been shown in the areas of glucose uptake, glucose metabolism and signal transduction. Insulin for effectively controlling hyperglycemia might produce some side effects. Therefore, combined doses of insulin and selenium were superior to administering them alone.

We hoped that this would provide new insights for the clinical management of diabetes and its complications.

References

Insulin in combination with selenium on IRS/PI3K-mediated GLUT4 expression in cardiac muscle


