Indian Journal of Experimental Biology
Vol. 58, November 2020, pp. 818-822

Mechanism of Rh-SAA mediating 3T3-L1 adipocytes insulin resistance

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Received 03 March 2020; *revised 24 June 2020*

Insulin resistance is a manifestation of both diabetes mellitus and obesity. Insulin signaling and its impairment in obesity and type 2 diabetes continue to excite researchers. Permanent increase in acute-phase serum amyloid A (A-SAA) level has been observed and correlated to both obesity and insulin resistance in humans. In this study, we explored the mechanism of recombinant serum amyloid A (Rh-SAA) mediating insulin resistance and JNK activation of 3T3-L1 adipocytes. We could observe the effect of Rh-SAA on insulin sensitivity of 3T3-LI adipocytes under the intervention of JNK inhibitors. We selected three experimental groups viz. (i) NC Group: Adipocytes without Rh-SAA intervention,;(ii) Rh-SAA Group: A dipocytes with 20μg/mL Rh-SAA intervention; and (iii) JNK Inhibitor Group: Adipocytes pretreated with 50 μmol/L JNK inhibitor SP600125 12 h before 20 μg/mL Rh-SAA intervention. All the three groups were incubated for 48 h, the glucose transport rate of the adipocytes was measured by^{3}H-2-DG, and the level of JNK activation was examined using Western blotting. Compared with the NC group, glucose uptake in the adipocytes treated with 20μg/mL Rh-SAA for 48 h decreased by 26% (P <0.01). The glucose uptake increased by 15% (P <0.05) in the JNK Inhibitor group. Western blotting showed that the expression levels of p-JNK in the NC group and Rh-SAA group and JNK inhibitor group were 100, 166 and 107%, respectively. Compared with NC group, the phosphorylation of JNK increased by 66% (P <0.01), but JNK inhibitor group showed no significant difference between Rh-SAA group (P <0.01) and NC group (P <0.05). Results suggested that intervening activity of JNK is expected to be an effective treatment for insulin resistance related diseases.

Keywords: Diabetes mellitus, Jun N-terminal kinase (JNK) pathway, Obesity, Recombinant SSA, Serum amyloid A proteins

Insulin resistance refers to the decrease in the efficiency of insulin in promoting glucose uptake and its utilization for various processes, and the compensatory secretion of excessive insulin by the body produces hyperinsulinemia to maintain the stability of blood glucose[1,2]. Insulin resistance predisposes an individual to metabolic syndrome and type 2 diabetes[3]. Plasma insulin concentrations were measured by radioimmunoassay principle and it was found that patients with lower plasma insulin levels had higher insulin sensitivity, while people with higher plasma insulin are insensitive to insulin[4]. In recent years, Jun N-terminal kinase (JNK) has emerged as an important metabolic modulator which plays a key role in obesity-related insulin resistance (IR)[5]. Reduced insulin sensitivity and the biological efficacy after binding to its specific receptors should be lower than the standard level in IR. The clinical symptoms are abnormal glucose uptake in peripheral tissues and increased hepatic glucose output[6]. IR in adipose or adipose peripheral tissue refers to its reduced ability to utilize glucose under the action of a certain concentration of insulin, resulting in low glucose tolerance[5,6]. Adipose tissue, an active endocrine organ produces adipokines, including leptin, adiponectin, endorphins, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), resistin, endofat, etc.[7] All the changes in adipokines affect the onset of biological effects of insulin, including adipose insulin sensitivity and regulation of skeletal muscle and liver insulin sensitivity through the blood[6,7].

The IR refers to a state in which normal doses of insulin produce subnormal biological effects, mainly manifested as resistance of insulin-sensitive cells to insulin-mediated glucose uptake and disposal as well as to lipid and egg white matter metabolism[3,8]. It is a common risk factor for a variety of diseases, such as diabetes and cardiovascular disease, often causing the occurrence of a variety of metabolic diseases[9]. The etiology of IR and its generation mechanism have been widely studied, mechanism of IR produced in different physiological and pathological conditions is not the same, and shows obvious heterogeneity of IR[10]. It causes defects in the structure and function of various signaling molecules in the pre-receptor, receptor and post-receptor of insulin target tissues as well as the abnormalities in the hormones regulating insulin action[11]. In the past few years, the mechanism of IR has been mainly focused mainly on insulin intracellular signaling pathway after insulin sensitive classical target organs such as liver, skeletal muscle

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and adipocyte receptors. The IR in such target organs is mainly a risk factor interfering with the IR S-1/PI3K/Akt/GLUT-4 signaling pathway, which attenuates insulin-mediated glucose uptake and subsequent disposal in these target organs and increases hepatic glucose production and output[12].

Serum amyloid A (SAA) is an acute-phase protein that has been recently correlated with obesity and insulin resistance[13]. Until recently, it was thought that the expression and release of SAA occurs predominantly in the liver, however, it has been found that human adipose tissue is a major SAA expression site during the non-acute-phase reactions[14]. Previous reports have shown that the insulin resistance in adipocytes could be induced by recombinant human serum amyloid A (Rh-SAA)[8,15]. However, the mechanism of Rh-SAA mediated insulin resistance in adipocytes has not been fully explained. Therefore, to examine whether Rh-SAA could induce insulin resistance in adipocytes, the JNK, an important modulator was chosen for study. Thus, the effect of Rh-SAA on JNK activity in 3T3-L1 adipocytes and effects of JNK inhibitor intervention was studied to understand the possible mechanism of Rh-SAA-induced insulin resistance in adipocytes. It might help in development of new treatment regimes for IR related disorders such as type-2 diabetes and metabolic syndrome.

Materials and Methods

Cells and main reagents

The 3T3-L1 preadipocyte cell line was procured from Shanghai Cell Bank (Chinese Academy of Sciences, Shanghai, China). The Dulbecco's Modified Eagle's medium (DMEM) medium was purchased from Gibco, USA, and the ^{3}H-2-DG was procured from Beyotime Biotechnology (Shanghai, China). The Rh-SAA (Peprotech, USA),ECL luminescence reagent (Santa Cruz, USA), Anti-JNK Rabbit mAb, p-JNK Rabbit mAb (Thr183/Tyr185), β-actin Rabbit mAb, Horseradish peroxidase-conjugated goat anti-mouse IgGandSP600125 were purchased from Cell Signaling Technology (USA).

Experimental group assignment and cell culture

In the preset study, experiment was divided into three groups (i) NC Group: Adipocytes without Rh-SAA intervention; (ii) Rh-SAA Group: Adipocyte with 20 μg/mL Rh-SAA intervention; and (iii) JNK Inhibitor Group: the adipocytes pretreated with 50 μmol/L JNK inhibitor SP600125 12 h before 20 μg/mL Rh-SAA intervention. Glucose uptake and immunoblotting was performed after 48 h of incubation[13].

Detection of glucose transport rate by ^{3}H-2-DG

Medium of adipocytes was removed and adipocytes were washed thrice with Krebs-Ringer-Phosphate (KRP) buffer. The adipocytes were incubated with KRP buffer with or without 100 nmol/L insulin at 37°C for 30 min. Next ^{3}H-2-DG was added to adjust the final concentration to 0.5 μCi/mL per well, and incubated at 37°C for 10 min. After the reaction was stopped by rapid washing thrice with pre-cooled PBS buffer, 1.0 mL of 0.1 mol/LNaOH was added and cells were incubated for 2 h[4]. Finally, NaOH was removed and 0.5 mL cell lysate was added into a scintillation vial, added with scintillation fluid and counted the number of disintegrations per minute (dpm) on a liquid scintillation counter. Meanwhile, the amount of total protein per well of the adipocytes was determined using the Kaumas brilliant blue micro protein assay. The number of disintegrations per minute per mg protein (dpm/mg protein) was used to suggest glucose transport in different experimental groups. In addition, another group was added with 10 μmol/L cytochalasin B as the non-specific uptake rate of ^{3}H-2-DG. This value was subtracted from all data as the glucose uptake value for each group of adipocytes.

Western blotting and analysis

The whole proteins from adipocytes were extracted by radioimmunoprecipitation assay (RIPA) buffer, and the concentrations were quantified using BCA protein assay kit. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (10% resolving gel) was conducted to separate the whole proteins (50 μg/lane)[7]. After the SDS-PAGE, the protein lanes were transferred from the gel onto polyvinylidene difluoride (PVDF) membranes at 90V. After 80 min of transferring process on PVDF membranes, 5% skimmed milk was used to block the membrane over an hour. Then JNK, p-JNK and β-actin primary antibodies (diluted by 1:1000) were used to incubate the membrane overnight at 4°C. Furthermore, 1X Tris-Buffered Saline, 0.1% Tween 20 detergent (TBST) buffer was used for washing the membranes. The membranes were incubated in horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody for 1 h at room temperature (25°C). Finally, ECL reagents and film was used to exposure and detect the protein bands. The protein

bands were analyzed by Gel Pro Analyzer software. The gray ratio of the p-JNK protein band to the JNK protein band was used as the relative expression of p-JNK, and β-actin was used as a reference. The relative expression of the target protein in NC group was set at 100%, and used as the standard.

The study was approved and carried out as per guidelines of Ethics Committee, Drum Tower Hospital, affiliated to Nanjing University Medical School, Jiangsu province, China.

Statistical analysis

All the data were analyzed using SPSS21.0 software (SPSS Inc., Chicago, IL, USA), and presented as the mean ± SEM. One-way analysis of variance (ANOVA) was used among multiple groups comparison followed by Student-Newman-Keuls post hoc test. The value of P <0.05 was considered as statistically significant.

Results and Discussion

Effects of JNK inhibitor on Rh-SAAmediated insulin resistance in 3T3-L1 adipocytes

The glucose uptake ability of adipocytes treated with 20 μg/mL Rh-SAA for 48 h decreased by about 26% compared with the NC group, and glucose uptake was increased by 15% in the JNK inhibitor group compared to Rh-SAA group (*P<0.05*). These results suggested that JNK inhibitor could restore the decrease of insulin sensitivity of adipocytes induced by Rh-SAA, and Rh-SAA could induce insulin resistance partly mediated by JNK as a consequence (Table 1).

The data suggested that Rh-SAA induced insulin resistance was partly related to JNK signaling pathway. JNK inhibitor could partially restore Rh-SAA induced insulin resistance, treated 3T3-L1 adiposities with 20 μg/mL Rh-SAA for 48 h, and then detected the phosphorylation of JNK by Western blotting. Adochio *et al.*[16] also found that reduced expression of p85α or p70 S6 kinase rescued adipocytes from IR induced by serine phosphorylation of insulin receptor substrate-1 and restored phosphatidylinositol-3-kinase activity and glucose uptake. In another study, inflammatory kinase c-jun N-terminal kinase (JNK) was found to respond to various cellular stress signals activated by cytokines, free fatty acids and hyperglycemia. Besides, JNK mediates both insulin resistance and ß-cell dysfunction, thus a potential target for type 2 diabetes therapy[17].

Many studies have shown that cell signaling pathway activated by insulin receptor cross-interacts with the signal transduction by inflammatory factors[18]. Inflammatory factors generated by non-specific inflammation induce insulin resistance by inducing the phosphorylation of serine and threonine of insulin receptor substrate-1 (IRS-1) in insulin-sensitive cells, impeding the tyrosine phosphorylation of IRS and blocking insulin signaling[19].

Effect of Rh-SAA on phosphorylation of JNK in 3T3-L1 adipocytes

The protein level of p-JNK was 100, 166 and 107% in NC group, Rh-SAA group and JNK inhibitor group, respectively. Results revealed that the phosphorylation of JNK increased by 66% in Rh-SAA group compared with NC group (P <0.01). Furthermore, there was significant difference between JNK inhibitor group and Rh-SAA group (P <0.01), however, the difference was not significant compared JNK Inhibitor group with NC group (P >0.05). The Rh-SAA increased the activity of JNK in adipocytes, and JNK inhibitor partially restored the cellular insulin resistance induced by Rh-SAA. Hence, Rh-SAA mediated insulin resistance is related to the activation of JNK pathway (Table 2). In another study, recombinant serum amyloid A (rSAA) enhanced the proliferation and inhibited differentiation in 3T3-L1 preadipocytes and also altered the insulin sensitivity in differentiated cells[20].

In this study, it was found that the phosphorylation level of JNK in Rh-SAA group was 66% higher than that in NC group. However, pretreated with JNK

Table 1 — Effects of JNK inhibitor on Rh-SAAmediated insulin resistance in 3T3-L1 adipocytes (Glucose transport)

Group	Sample	Glucose Transport Rate (%)	
		Normal	insulin stimulation
NC	3	46.34±6.49	265.13±13.58
Rh-SAA	3	41.97±5.82	196.63±13.99*
JNK Inhibitor	3	39.28±7.94	237.16±18.87△
F value		0.822	14.50
P value		0.484	0.005

[Compared with NC group, *P <0.01; Compared with Rh-SAA group, △P <0.05]

Table 2 — Effects of JNK inhibitor on Rh-SAA mediated insulin resistance in 3T3-L1 adipocytes (2-DG transport)

Group	Sample	2-DG Transport Rate (%)	
		Normal	insulin stimulation
NC	3	17.47±2.45	100.00±5.13
Rh-SAA	3	15.84±2.19	74.20±5.30*
JNK Inhibitor	3	14.82±3.00	89.49±7.12△
F value		0.822	14.50
P value		0.484	0.005

Table 3 — Effect of Rh-SAA on the phosphorylation of JNK in 3T3-L1 adipocytes

Group	Gray value	Relative percent
NC	16021±993	100±6
Rh-SAA	26555±2375	166±15*
JNK Inhibitor	17222±1506	107±9△
F value		33.655
P value		0.001

[Compared with NC group, **P* <0.01; Compared with Rh-SAA group, △*P* <0.05]

inhibitor followed by Rh-SAA, the phosphorylation of JNK decreased by 59% compared with Rh-SAA group. That is, JNK inhibitor essentially restored the increase of JNK phosphorylation induced by Rh-SAA (Table 3). It could be seen that the activation of JNK signaling pathway might be a key signaling molecule for Rh-SAA mediated insulin resistance in adipocytes. Therefore, the intervention of the activity of JNK is expected to be an effective method of treating insulin resistance related diseases. Yet in another study, JNK is an essential component of the pathway responsible for SAA-induced insulin resistance in 3T3-L1 adipocytes. Such studies suggest that a selective interference with JNK activity may help in development of treatment for Type 2 diabetes and other insulin-resistant states[21].

Multiple studies have shown that increased JNK activity in adipose tissue of obese mice and obese people. In hereditary and diet-induced obese mice, the activity of JNK is significantly elevated in the liver, skeletal muscle and adipose tissue[22]. Compared with wild type obese mice, HFD JNK-KO obese mice showed significant reductions in both plasma glucose and insulin levels as well as serine phosphorylation level of IRS-1, but their tyrosine phosphorylation of IRS-1 and the sensitivity to insulin increased[23]. Genetically obese mice (ob/ob) with targeted mutations in JNK have also acquired similar results and the JNK deficient dietary and hereditary diabetic animal models have some improvement in insulin resistance in the environment[6]. In addition, negative regulation of insulin signaling by JNK was further confirmed by blocking the JNK pathway using adenovirus-mediated dominant-negative JNK or JNK inhibitory polypeptide and the obese diabetic animal models C57BL/KsJ-db/db mice treated with Ad-DN-JNK exhibited low non-fasting blood glucose levels[24,25]. Besides, in Ad-DN-JNK treated mice, IRS-1 serine phosphorylation was significantly reduced and tyrosine phosphorylation was significantly increased in the liver compared with the control group, and Akt-serine 473 phosphorylation was decreased. In contrast, excessive expression of JNK in the liver of normal mice reduces insulin sensitivity. Taken together, blocking JNK pathway could attenuate insulin resistance and improve glucose tolerance in obese diabetic mice[26,27].

Conclusion

This study showed that the JNK inhibitor could restore the decreased insulin sensitivity of adipocytes induced by Rh-SAA, and Rh-SAA could induce insulin resistance (IR) partly mediated by JNK. Furthermore, the Rh-SAA increased the activity of JNK in adipocytes, and JNK inhibitor partially restored the cellular IR induced by Rh-SAA. Thus, the activation of the JNK signaling pathway may be a key signaling molecule for Rh-SAA-mediated insulin resistance in adipocytes.

Conflict of interest

The authors declare no conflict of interests.

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