Amelioration of insulin resistance by Rk$_1$ + Rg$_5$ complex under endoplasmic reticulum stress conditions

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Background: Diabetes mellitus is a metabolic syndrome exaggerated by stress conditions. Endoplasmic reticulum stress (ERS) impairs the insulin signaling pathway making the diabetic conditions worsen. Pharmacological agents are supplied externally to overcome this malfunction. Ginsenosides from Panax ginseng C.A Meyer possesses many pharmacological properties and are used for the treatment of diabetes. Objective: To investigate the effects of the Rk$_1$ + Rg$_5$ complex on the amelioration of insulin resistance in 3T3-L1 cells under endoplasmic reticulum stress conditions. Materials and Methods: Heat-processed ginseng extracts are found to contain many pharmacologically active ginsenosides. Among them Rk$_1$ + Rg$_5$ is found to be present in higher concentrations than the other minor ginsenosides. The Rk$_1$ + Rg$_5$ complex was tested for its effect in the 3T3-L1 insulin-resistant model and subjected to the MTT assay, glucose oxidase assay and gene expression studies using RT-PCR and real-time PCR under endoplasmic reticulum stress conditions. Results: Rk$_1$ + Rg$_5$ treatment is found to increase the glucose uptake into the cells when compared to that of a positive control (tunicamycin treatment group, TM). Further we have analyzed the role at gene expression level. The Rk$_1$ + Rg$_5$ complex was found to show an effect on the IGF 2R receptor, CHOP-10, and C/EBP gene at a particular treated concentration (50 $\mu$M). Moreover, stress condition (about 50% decreases) was overcome by the ginsenoside treatments at 50 $\mu$M. Conclusion: The present results showed that under endoplasmic reticulum stress conditions Rk$_1$ + Rg$_5$ complex exhibits a potential protective role in insulin-resistant 3T3-L1 cells.

Keywords: 3T3-L1, dexamethasone, diabetes, insulin, insulin resistance, ginsenosides
the stress situations by regulating the insulin signaling pathway and glucose uptake in the insulin-resistant organs. Pharmacological agents, such as an external supply of IGF growth factors or the agents mimicking the function of IGF growth factors; and insulin acting on the IGF signaling receptors, are available for overcoming stress situations and affected insulin signaling pathways. IGF receptor (a kind of glucocorticoid receptor) malfunctioning is found to be ameliorated by ginsenosides. Among the well-known ginsenosides, Rg1, Rh1, and C-K are reported to show an effect on overcoming the insulin resistance. Heat processing of ginseng is a technological approach to increase the amount of pharmacologically active ginsenosides. Heat processed ginseng extracts are found to be rich in the Rk1+Rg5 complex. The Rk1+Rg5 complex is minor ginsenosides obtained from PPD type of ginsenosides by repeated heat treatment and have been reported to show multi therapeutic properties. It exhibits radical scavenging activity of about 68% by the DPPH method. Sun ginsengs containing Rk1 and Rg5 as its components are known to be tumor preventer, aging reliever and kidney protector. Here in this study, we focus on the Rk1+Rg5 complex’s effect on the insulin resistance phenomenon using the effector cells, adipocytes under endoplasmic reticulum stress. Reports of ginsenosides mediating the adipogenic pathways have been published elsewhere. In this attempt we are reporting under endoplasmic reticulum stress conditions Rk1+Rg5 complex’s efficacy on reverting the insulin-resistant conditions of the adipocytes.

MATERIALS AND METHODS

Cell culture and reagents

3T3-L1 cells were cultured in DMEM/High glucose at 10% BCS and 1% P/S (Penicillin/Streptomycin) for 2 days to reach the confluence. These cells were later immersed in differentiation media containing 10% BCS, 1% P/S, 0.5 mM IBMX, 5 μg/mL insulin and 250 nM dexamethasone. After culturing for 2 days the cells were then immersed in insulin-containing media (5 μg/mL) alone. In the following days, the cells were cultured in media containing 100 nM dexamethasone, making them insulin resistant. Later ER stress induction was carried out using tunicamycin at 2 μg/mL. The effect of ginsenosides was checked by treating them and incubating at 37°C in humidified CO2 incubators (5% CO2). The Rk1 + Rg5 complex used in the present study has been obtained by repeated heat treatment of ginseng root, kindly provided by Ginseng resource bank, South Korea.

Cell viability assay

The effect of the Rk1+Rg5 complex on the viability of the cells used in the study was analyzed by using MTT assay as per the previous reports. In brief, about 2 × 104 cells were plated into 96-well plates. After incubation at 37°C for 24 hours in a humidified incubator, the cells were treated with tunicamycin (2 μg/mL) and ginsenosides at varying concentrations for 24 hours. After this incubation, the MTT solution at 1 mg/mL was added to each well. Cells were then incubated at 37°C for a further 3 hours and the optical density was measured using a micro plate reader (Biotek, USA) at 570 and 630 nm. The cell viability was determined based on the absorbance values at 570 and 630 nm.

Glucose uptake assays

The glucose uptake from the media was analyzed by using the glucose oxidase reagent (Sigma G3660). Briefly, cells were differentiated using the above media (4500 mg/L of glucose) and then treated with stress agent and ginsenosides. The media was collected and incubated with glucose oxidase at 1 μM for 1.5 hours at 37°C and then with dianoisidine reagent at 37°C for 30 minutes. The absorbance was taken spectrophotometrically at 540 nm and the amount of glucose left in the medium is calculated from the standard glucose, thus giving the glucose utilization under stress and ginsenoside treatment.

Gene Expression studies using RT-PCR

Total RNA was isolated from the cells using a Qiagen RNeasy Mini Kit. The mRNA expression levels of the genes CHOP-10, C/EBP β and IGF-2R were determined by RT-PCR analysis of total RNA by using one-step RT-PCR kit with gene specific primers as given in Table 1 (included as supplementary). The intensity of the band has been calculated using Image J Software, USA. The Glut-4 gene has been quantified for 40 cycles.

| Primer | bp |
|--------|----|
| IRS-2 F-CATCGACTTGTCCCATCA | 151 |
| IRS-2 R-CCCCATCTCAAGTCAAGGG | 88 |
| PDX-1 F-AAGGAAACAGAGGACCCGTACT | 189 |
| PDX-1 R-CGGGAGAGTGTGTGTTGTTAATAGAATTC | |
| IGF-2R F-TATCAACATTCCGCCACCCG | 150 |
| IGF-2R R-CTGTCGGCTAAGCAATGAGT | |
| CHOP-10 F-AACAGAGGCTACAGCGACCACAT | 212 |
| CHOP-10 R-ACCTTCTGGCTACGCTCTCTCT | |
| C/EBP beta F-GCAACAAGCTGCTAAGCTG | 133 |
| C/EBP beta R-ATGCTCGAAGCGAAGAAGGT | |
| Glut 4F-CTAGATCCCGGAGGACCT | 196 |
| Glut 4R-AATAGGCTATGGTGCCTC | |
| Beta actin F-ATGAAAGTGACGTTGACATCC | |
| Beta actin R-CCTAAGACGTTTGGTCGACGATG |

| IRS=Insulin Receptor Substrate; PDX=Pancreatic and duodenal homeobox-1; CHOP=CHOP expression; C/EBP=AP-1 family transcription factor; Glut=Glut-4 expression |
by the real-time SYBR green dye and analyzed using the Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Science, Australia).

**Statistical analysis**

All data are expressed as mean ± S.E. The statistical significance between means of the independent groups was analyzed using the Student’s t-test and a P value less than 0.05 and 0.001 was considered to be statistically significant.

**RESULTS AND DISCUSSIONS**

**Cell viability and dose effect of the Rk₁+Rg₅ complex**

The cells were treated with Rk₁+Rg₅ complex in increasing concentration and these were found to be non-toxic until 100 micromolar concentrations were reached. When treated with tunicamycin at 2 μg/mL, this was found to cause stress to the insulin-resistant cells and at the same time by treating them with the Rk₁+Rg₅ complex the stress was found to be overcome in increasing concentrations [Figures 1 and 2]. Similar studies with other natural bioactive compounds and other ginsenosides have been reported by many other researchers with Rg₃ and Re.[20]

**Rk₁+Rg₅ effect on insulin-stimulated glucose uptake**

The media used to incubate cells with the ginsenosides and stress agents at 37°C was analyzed for the glucose uptake in to the cells. Cells treated with incubation medium (Glucose concentration 25 mM, 10% BCS, 1% P/S) are used as a control. Cells incubated with tunicamycin are used as a positive control. From Figure 3, it can been interpreted that the amount of glucose left in the medium is high for the cells under tunicamycin treatment when compared to that of cells incubated with the incubation medium alone. Parallel treatment with the Rk₁+Rg₅ complex in increasing concentrations is found to show a decrease in the glucose amount remaining in the medium where the Rk₁+Rg₅ complex aids in the glucose transport via Glut-4 translocation protein. The amount of glucose has been calculated based on the glucose standard used and the insulin-treated cells. A similar phenomenon has been shown by natural components like 3β-taraxerol, Mangifera indica ethyl acetate extract and ginsenosides such as Rg₁, Compound K, Rb₁.[21,22]

**Gene expression**

As a next step we were interested in analyzing the protective role of the Rk₁+Rg₅ complex in 3T3-L1 cells under endoplasmic reticulum (ER) stress at concentrations of 25 to 100 μM. Induction of CHOP-10 is involved in apoptosis of beta cells under ER stress.[23] CHOP-10, whose expression increased by tunicamycin treatment, was found to shown a decrease in expression by

![Figure 1: Effect of Rk₁+Rg₅ on insulin-resistant 3T3-L1 cells. Each bar represents the average of three independent experiments Mean ± S.E. The data have been statistically analyzed](image1)

![Figure 2: Effect of Rk₁+Rg₅ on insulin-resistant 3T3-L1 cells under tunicamycin (TM) stress treatment. Each bar represents the average of three independent experiments Mean ± S.E. ***P<0.001, *P<0.05 significant levels relative to the tunicamycin treatment group, #P<0.001 significant levels relative to the untreated group](image2)

![Figure 3: Glucose remaining in the media after stress and ginsenosides treatment. Each bar represents average of three independent experiments Mean ± S.E. *P<0.05 compared to stress treatment control (positive control). ###P<0.001 compared to that of the untreated control group](image3)

![Figure 4: Rk₁+Rg₅ treatment at 100 μM concentration](image4)
compound treatment at 100 μM mediate the apoptotic pathway to combat the stress situations. In addition, C/EBP beta gene expression was increased by compound treatment (50 μM, adjusted density of 1.00, Figure 4).\[24\] Furthermore, from Figure 5, Glut-4 gene repression has been found to be increased by Rk\(_1\) + Rg\(_5\) treatment compared to that of the control and tunicamycin stress treatment group. One of the possible mechanisms may be that of CHOP-10 (25, 50 μM) hetero-dimerizing with the C/EBPβ gene, which has been reported to have a remarkable role in Glut-4 translocation.\[25\] Thus from the above results, we can report that the ginsenoside Rk\(_1\) + Rg\(_5\) complex plays a role in making the insulin-resistant adipocytes to become sensitive to insulin. This acted by increasing C/EBPβ at 25, 50 μM concentrations and increasing the Glut-4 gene repression, thus overcoming the insulin resistance related to type 2 diabetes, obesity and endoplasmic reticulum stress.\[26\] Many reports deal with the effective role of ginsenosides in overcoming an insulin-resistant state in the peripheral organs.\[27\] The IGF-2R gene expressions have been found to be increased by TM (Tunicamycin). Importantly, treatment with Rk\(_1\) + Rg\(_5\) (μM) was found to show a greater increase in expression at 50 μM than at 25 and 100 μM (1.2 adjusted density) [Figure 4]. The IGF-2 protein is one of the proteins mimicking the function of insulin in the peripheral organs containing adipocytes.\[28\] Pharmacological agents acting in a similar way to the IGF-2 protein and ginsenosides have also been reported. Some of them include Rh\(_2\), Compound K, Rg\(_1\), Rg\(_3\) and berberine.\[25,30\] Here we are reporting on the Rk\(_1\) + Rg\(_5\) complex mimicking the function of the IGF-2 protein in binding with that of the IGF-2 receptor under ER stress. On analysis of the anti-ER stress mechanism of the complex mixtures, it was found to have no effect on the downstream signaling of the IGF signaling pathway (IRS-2) (data not shown). However, it was found to have an effect on the IGF-2R receptor gene expression correlating with the Glut-4 gene repression maximum at 50 μM. Similar results stating the role of Rg\(_5\) acting as agonist have also been reported and are related to angiogenesis competing with IGF-1R.\[31\]

In conclusion, the Rk\(_1\) + Rg\(_5\) complex treatment increases IGF-2R receptor binding sites and facilitates the Glut 4 translocation, aiding in the glucose uptake by the peripheral organs, via the CHOP-mediated signaling pathway especially in adipocytes (in this study), thus ameliorating the insulin-sensitive state of the 3T3-L1 cells and making them responsive to insulin, and thereby combating the insulin resistance closely associated with type 2 diabetes under ER stress conditions.

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