Amelioration of insulin resistance by Rk_1 + Rg_5 complex under endoplasmic reticulum stress conditions

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ABSTRACT

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 μM). Moreover, stress condition (about 50% decreases) was overcome by the ginsenoside treatments at 50 μM. **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

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INTRODUCTION

Insulin resistance refers to the incompatible state of the cells in taking up the glucose. Insulin lowers blood glucose levels by facilitating glucose uptake into peripheral organs such as the liver, fat and skeletal muscles. Under insulin-resistant conditions, these organs are found to be functioning improperly.[1] Endoplasmic reticulum stress is the potential contributor in increasing the disease conditions, ER stress triggers transcriptional factors in the protection of the cells from the stress situations. Some of them include CHOP-10, XBP1 splicing, GRP 78. CHOP-10/GADD 153, a member in the family of CCAAT/enhancer-binding proteins. It includes other components such as α, β and γ C/EBP. One of the members C/EBP β is known to interact with CHOP-10 protein, which in turn leads to C/EBP α in increasing Glut-4 translocation. Some reports states that C/EBP β and δ are very essential for adipocyte differentiation and insulin-stimulated glucose uptake; it is also possible that they may be the therapeutic targets to treat diabetes mellitus. In addition, ER stress, by the up regulation of CHOP, impairs resistin transcription in adipocytes related to insulin resistance.[2,3] The insulin signaling pathway is initiated via insulin binding on the IGF receptor (insulin-like growth factor receptor) of the effector cells. In diabetic conditions, the insulin binding and the downstream signaling pathway are reported to be malfunctioning and in 3T3-L1 cells (effector cells) the insulin signaling pathway was found to be decreased making diabetic conditions complicated. ER stress reduces the insulin signaling pathway retaining the glucose transport.[4-6] Ginseng, a well-known medical herb famous for its pharmacological properties, overcomes

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the stress situations by regulating the insulin signaling pathway and glucose uptake in the insulin-resistant organs.[7,8] 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.[9] IGF receptor (a kind of glucocorticoid receptor) malfunctioning is found to be ameliorated by ginsenosides. Among the well-known ginsenosides, Rg_1, Rh_1 and C-K are reported to show an effect on overcoming the insulin resistance.[10,11] 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 Rk_1+Rg_5 complex. The Rk_1+Rg_5 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.[12,13] Sun ginsengs containing Rk_1 and Rg_5 as its components are known to be tumor preventer, aging reliever and kidney protector.[14] Here in this study, we focus on the Rk_1+Rg_5 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.[15,16] In this attempt we are reporting under endoplasmic reticulum stress conditions Rk_1+Rg_5 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 CO_2 incubators (5% CO_2).[17] The Rk_1 + Rg_5 complex used in the present study has been obtained by repeated heat treatment of ginseng root, kindly provided by Ginseng resource bank, South Korea.[12]

Cell viability assay

The effect of the Rk_1+Rg_5 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 2×10^5 cells were plated into 96-well plates. After incubation at 37C 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.[18]

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.[19]

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

Table 1: Primer list

Primer	bp
IRS-2 F-CATCGACTTGTCCCATCA	151
IRS-2 R-CCCATCCTCAAGGTCAAAGG	
PDX-1 F-AGGAAAACAAGAGGACCCGTACT	88
PDX-1 R-CGGGAGATGTATTTGTTAAATAAGAATTC	
IGF-2R F-TATCAACATCTGCCAGCCAC	189
IGF-2R R-CTGTCGGCTAAGCAATGAGT	
CHOP-10 F- AACAGAGGTCACACGCACAT	150
CHOP-10 R-ACTTTCCGCTCGTTCTCCTG	
C/EBP beta F-GCAACACACGTGTAACTGTC	212
C/EBP beta R-ATGCTCGAAACGGAAAAGGT	
Glut 4F-CTAGATCCCGGAGAGCCTT	133
Glut 4R-AATAGGGTATAGGGTCCGGG	
Beta actin F-ATGAAGTGTGACGTTGACATCC	196
Beta actin R-CCTAGAAGCATTTGCGGTGCACGATG	

IRS=Insulin Receptor Substrate; PDX=Pancreatic and duodenal homeobox-1; CHOP=C/EBP homologus protein-10; C/EBP-β= CCAATT/enhancer binding protein beta

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 $Rk_1 + Rg_5$ complex

The cells were treated with Rk_1+Rg_5 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 Rk_1+Rg_5 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 Rg_3 and Re.[20]

$Rk_1 + Rg_5$ 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 Rk_1+Rg_5 in increasing concentrations is found to show a decrease in the glucose amount remaining in the medium where the Rk_1+Rg_5 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 Rg_1, Compound K, Rb_1.[21,22]

Gene expression

As a next step we were interested in analyzing the protective role of the Rk_1+Rg_5 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

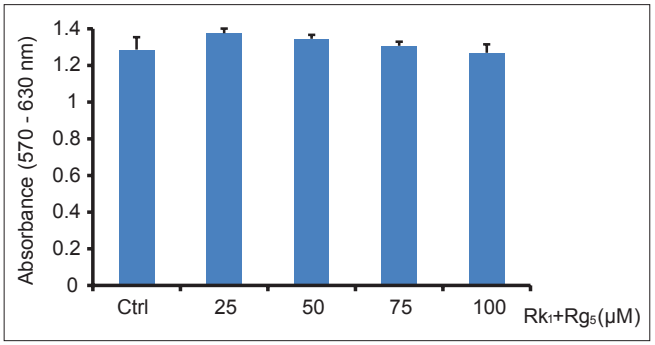

Figure 1: Effect of Rk_1+Rg_5 on insulin-resistant 3T3-L1 cells. Each bar represents the average of three independent experiments Mean ± S.E. The data have been statistically analyzed

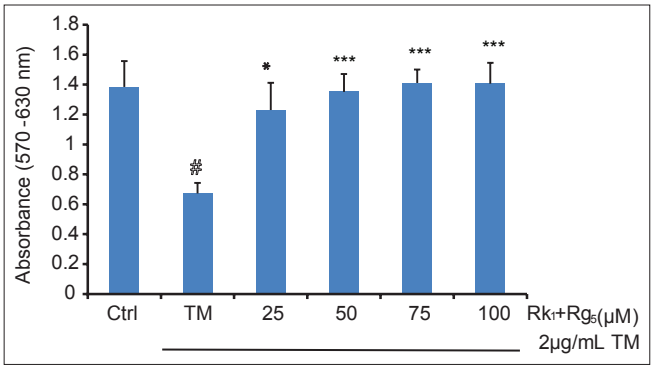

Figure 2: Effect of Rk_1+Rg_5 on insulin-resistant 3T3-L1 cells under tunicamycin (TM) stress treatment. Each bar represents the average of three independent experiments Mean ± S.E. ***P<0.001, *P<0.05 significant levels relative to the tunicamycin treatment group, #P<0.001 significant levels relative to the untreated group

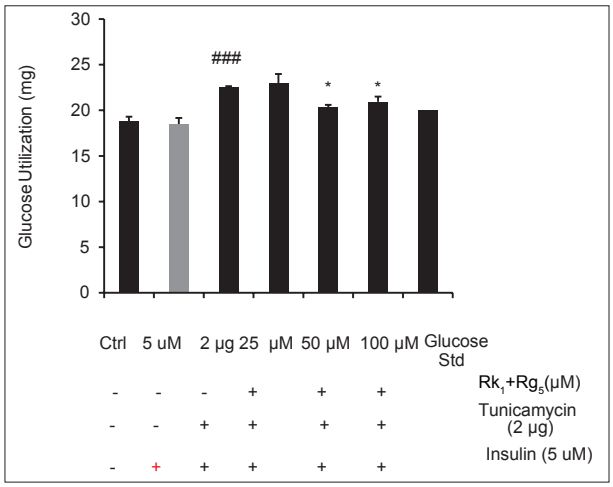

Figure 3: Glucose remaining in the media after stress and ginsenosides treatment. Each bar represents average of three independent experiments Mean ± S.E *P<0.05 compared to stress treatment control (positive control). ###P<0.001 compared to that of the untreated control group

$Rk_1 + Rg_5$ treatment at 100 μM concentration [Figure 4]. This has been found to be similar with that of another gene expression XBP1 splicing (data not shown), where

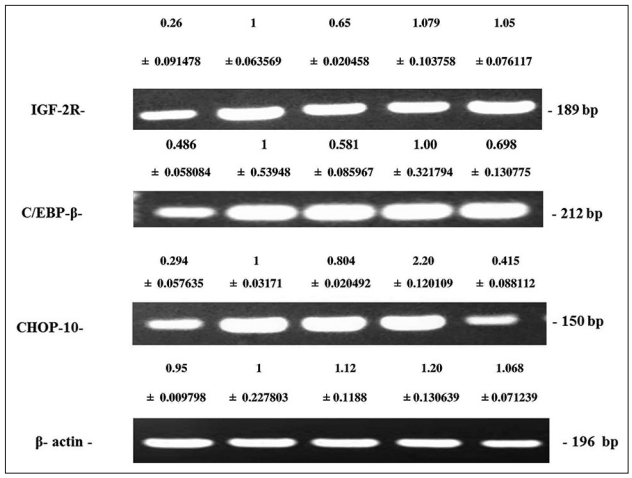

Figure 4: Effect of Rk_1+Rg_5 on the gene expression of signalling proteins: (a) IGF-2R, (b) C/EBP β, (c) CHOP-10, (d) β-actin. Number of cycle: 25, denaturation at 94°C, extension at 72°C. Intensity of the band has been calculated using Image J software and calibrated with the positive control and the adjusted density with that of the beta actin have been shown (Mean ± S.E)

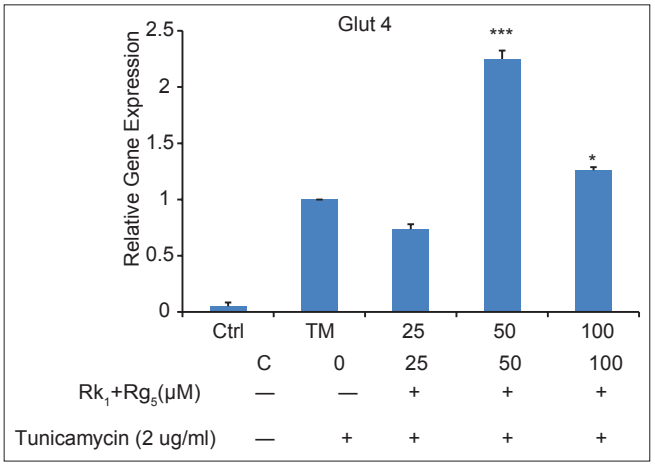

Figure 5: Effect of Rk_1+Rg_5 on Glut-4 gene expression under endoplasmic reticulum stress. Each bar represents the average of three independent experiments (Mean ± S.E). ***P<0.001, *P<0.05 significant levels relative to the tunicamycin treatment group

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/EBP β 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/EBP β 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 shown 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.[29,30] 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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