# RESISTIN STIMULATES THE EXPRESSION OF SUPPRESSOR OF CYTOKINE SIGNALING 3 GENE IN 3T3-L1 ADIPOCYTES THROUGH MULTIPLE KINASE PATHWAYS

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**Abstract -** Resistin (Rstn) and suppressor of cytokine signaling (SOCS)-3 have been reported to regulate fat cell activity and insulin resistance, respectively. Using 3T3-L1 adipocytes and the method of real-time PCR, we investigated the pathways involved in Rstn-stimulated SOCS-3 gene expression. The up-regulation of SOCS-3 mRNA expression induced by Rstn was observed. Treatment with an inhibitor of Janus kinase (JAK)-2, such as AG490, blocked Rstn-increased levels of SOCS-3 mRNA. Moreover, pretreatment with specific inhibitors of either ERK1/2 (U0126), p38 MAPK (SB203580), JNK (SP600125), or phosphatidylinositol 3-kinase/AKT (LY294002 and wortmannin) prevented Rstn-increased levels of SOCS-3 mRNA. These results imply that JAK2, PI3K, ERK1/2, p38, and JNK MAPKs are necessary for the Rstn stimulation of SOCS-3 gene expression.

**Keywords -** Resistin, Adipocyte, SOCS-3, Kinase.

## I. INTRODUCTION

Suppressor of cytokine signaling (SOCS)-3 is a polypeptide that was first cloned from the complementary DNA (cDNA) libraries of thymus and myeloid leukemia cells and was found to act negatively to inhibit cytokine signaling (1-2). The SOCS-3 protein contains 225 amino acids with a molecular mass of 24.7 kDa and its messenger RNA (mRNA) is 675 base pairs long. Since its discovery, SOCS-3 was found to possess numerous other actions (1-8). For example, SOCS-3 mediates cancer cell growth (3-4), provides negative feedback to suppress insulin signaling (5-6), and acts as a signaling molecule for adipokines (e.g., resistin and adiponectin) (7-8).

Expression of the adipocyte SOCS-3 gene is regulated by a variety of hormones and adipokines (1-12). For example, resistin (Rstn) stimulates SOCS-3 expression and increases the association between SOCS-3 and the insulin receptor (7). Insulin stimulates SOCS-3 expression through activation of signaling transducer and activator of transcription (STAT)-5b (5) and janus-activated kinases (JAKs) (9). Other adipokines and hormones, such as endothelin (ET)-1, tumor necrosis factor-α, and growth hormone were also demonstrated to upregulate SOCS-3 gene expression in adipocytes, but prolactin had no effect on SOCS-3 mRNA levels (10-13). Despite the demonstration that SOCS-3 is differentially regulated by different hormones through modulations of various kinase signaling molecules, no studies have demonstrated whether Rstn acts on SOCS-3 gene expression in adipocytes through the JAK2/STAT-3 pathways.

Resistin is a cysteine-rich peptide hormone that was first isolated from adipose tissues and used to link obesity to type II diabetes in rodents (14). In particular, the administration of exogenous Rstn into normal mice causes glucose intolerance, while immunoneutralization of Rstn hormone in obese mice improves insulin sensitivity (14). In humans, Rstn gene expression in adipose tissues of normal humans is lower than that in obese subjects (15), and Rstn genome with a single nucleotide polymorphism was associated with obesity (16) and diabetes (17). Others found the functional diversity of Rstn in different species or systems. For example, Rstn regulates hepatic insulin resistance, lipid metabolism, cancer growth, inflammation, and proliferation of endothelial cells and muscle cells (18-21).

Resistin regulates the gene expression of non-fat cells through at least a few pathways, the JAK2/STAT3-mediated pathway, the mitogen-activated protein kinase (MAPK)-regulated pathway, and the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT)-mediated pathway (22-25). The JAK-2/STAT3-mediated pathway controls Rstn-stimulated expression of the SOCS-3 in human endothelial cells (22). The ERK MAPK-regulated pathway mediates the Rstn-stimulated caspases-3 expression in porcine ovarian follicular cells (23); the p38 MAPK-regulated pathway controls the Rstn-stimulated CCL4 expression in human nucleus pulposus tissues and fractalkine (FK) expression in human endothelial cells (24-25); and the JNK MAPK/c-JUN-mediated pathway mediates the FK expression in human endothelial cells (25). The PI3K/AKT-mediated

pathway controls Rstn-stimulated caspase-3 expression (23). These studies indicate that the action of Rstn varies with cell types and kinase signaling molecules. A few recent studies have reported that toll-like receptor 4 (TLR-4) and the receptor tyrosine kinase (RTK)-like orphan receptor, but not TLR-2, could serve as receptors for actions of Rstn in human cells or murine adipocytes (26-27). Despite of these attentions, it is still not clear whether any of these signaling molecules are responsible for Rstn to act on adipocyte SOCS-3 gene expression. Thus, the hypothesis arose that Rstn may regulate SOCS-3 gene expression in adipocytes through the JAK, MAPK, and PI3K pathways.

The present study used 3T3-L1 adipocytes to understand the influence of Rstn signaling on SOCS-3 mRNA expression in fat cells. We show that Rstn stimulated SOCS-3 mRNA expression through the JAK2, ERK MAPK, p38 MAPK, JNK MAPK, and PI3K pathways. We also found that endothelin-1 (ET-1) and green tea epigallocatechin-3-gallate (EGCG), the major polyphenolic catechin, reduced the Rstn-induced up-regulation of SOCS-3 mRNA expression.

## II. DETAILS EXPERIMENTAL

### 2.1. Materials

All reagents (*e.g.*, dexamethasone, dimethyl sulfoxide, insulin, 3-isobutyl-1-methylxanthine, and so forth) were purchased from Sigma (St. Louis, MO) unless otherwise stated. DMEM, penicillin-streptomycin, calf serum (CS), fetal bovine serum (FBS), trypsin, agarose, the 1-kb plus DNA ladder marker, ribonuclease inhibitor, and the protein marker were purchased from GibcoBRL-Invitrogen (New York, NY). Trizol, T*aq* polymerase, and the 3'-RACE system were purchased from Biotools B&M Labs (Madrid, Spain) and Invitrogen Life Science Technologies (Carlsbad, CA), respectively. Inhibitors were purchased from TOCRIS Bioscience (Bristol, UK). EGCG ( > 98% pure) was isolated from green tea (*Camellia sinensis*) as previously described (28).

### 2.2. Cell culture

3T3-L1 adipocytes (American Type Culture Collection, Manassas, VA) were obtained according to a published method (10), in which 2-day post-confluent 3T3-L1 preadipocytes ($3 \times 10^6$ cells in a 10-cm plate) were treated with DMEM containing a final concentration of 10% FBS, 5 μg/ml insulin, 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine for 48 h. The medium was then changed to DMEM containing 10% FBS and 5 μg/ml insulin for an additional 8-10 days. With this protocol, greater than 90% adipocyte differentiation was achieved, as indicated by triglyceride accumulation and the expression of aP2 (10).

For all experiments as described by Chang *et al*. (10) and Tang *et al.* (29), adipocytes were serum-starved for 16 h in DMEM containing 0.1% (fatty acid-free) BSA and then, unless noted otherwise, incubated with or without Rstn at 30 ng/ml for the 2-hour periods. Resistin and ET-1 were purchased from American Peptide Co. (Sunnyvale, CA) and dissolved in sterile water containing 0.1% (w/v) bovine serum albumin (BSA). U0126 (a MEK1 inhibitor; 12.5~50 μM), SP600125 (a JNK inhibitor; 10 μM), SB203580 (a p38 MAPK inhibitor; 10 μM), LY294002 (a PI3K inhibitor; 50 μM), wortmannin (a PI3K inhibitor; 0.2 μM), and AG490 (a JAK2/STAT3 inhibitor; 10 μM) were used to inhibit the MEK1, JNK, p38 MAPK, PI3K, and JAK2 activities, respectively. In the experiments, serum-starved 3T3-L1 adipocytes were pretreated with or without the inhibitor for 30 or 60 min. Inhibitors were dissolved in 100% dimethyl sulfoxide (DMSO) and then diluted in sterile media such that the final concentrations of DMSO and BSA were 0.1% (v/v) and 0.1% (w/v), respectively. Adipocytes were then stimulated with or without Rstn (30 ng/ml) for the 2-hour period. When Rstn was added, inhibitors were also present. The SOCS-3 mRNA level was measured after treatment.

### 2.3. Real-time and reverse-transcriptase polymerase chain reaction (RT-PCR) for resistin mRNA

We used a real-time PCR (10) to determine the mRNA levels of SOCS-3 and GAPDH. Total RNA was isolated with a Trizol kit. cDNA was synthesized from equal amounts (5 μg) of RNA using 100 units of M-MLV reverse transcriptase (Bionovas Biotechnology, Toronto, Ontario) in the presence of 40 units of RNase inhibitor (Invitrogen), and the adapter primer was 5'-GGCCACGCGTCGACTAGTAC$(T)_{19}$-3'. The real-time PCR analysis was performed twice in duplicate using the power SYBR green PCR master mix and ABI 7300 Sequence Detection System (Applied Biosystems, Foster City, CA) under the following conditions: an initial denaturing cycle at 95 °C for 5 min, followed by 40 cycles of amplification consisting of denaturation at 95 °C for 3 s and annealing at 60 °C for 30 s. The respective forward and reverse primers were 5'-CTGGACCCATTCGGGAGTTC-3' and 5'-CACTGGGAGCTACCGACCGTTG-3' for mouse SOCS-3 (accession no. NM_007707) and 5'-CCTCTGGAAAGCTGTGGCGT-3' and 5'-TTGGCAGGTTTCTCCAGGCG-3' for mouse GAPDH (accession no. NM_008084). A relative expression ratio of the SOCS-3 transcript to the GAPDH transcript was calculated as previously described (10) and expressed as a multiple of the control. Amplification specificity and efficiency ( > 90%) of real-time PCR analysis were as previously described (10).

### 2.4. Statistical analysis

As previously described by Chang *et al.* (10), data are expressed as the mean ± SE (n=3). The unpaired

Student's *t*-test was used to examine differences between the control and Rstn-treated groups, between the control and inhibitor-treated groups, or between Rstn-treated groups and the combination group with Rstn and inhibitors. Differences were considered significant at $p < 0.05$. To convert the discontinuous normalized data to the continuous ones, they were transformed with the logarithm and then performed with statistics using SigmaStat (Jandel Scientific, Palo Alto, CA).

## III. RESULTS AND DISCUSSION

### 3.1. The JAK2/STAT3 inhibitor suppresses resistin-induced up-regulation of SOCS-3 mRNA expression

The JAK-2/STAT3-mediated pathway controls Rstn-stimulated expression of the SOCS-3 in human endothelial cells (22). Whether Rstn-induced up-regulation of SOCS-3 mRNA expression in adipocytes is related to the JAK2 pathway was assessed (Fig. 1). Adipocytes were pretreated with the specific JAK2/STAT-3 inhibitor AG490 (20 μM) for 60 min and then were treated with Rstn for 2 h. When compared with the Rstn-treated group, AG490 indeed significantly suppressed the Rstn-stimulated expression of SOCS-3 mRNA expression. Treatment with AG490 alone did not alter SOCS-3 mRNA expression. These results suggest the JAK2/STAT3-dependent effect. These findings in adipocytes are consistent with that reported in human endothelial cells (22).

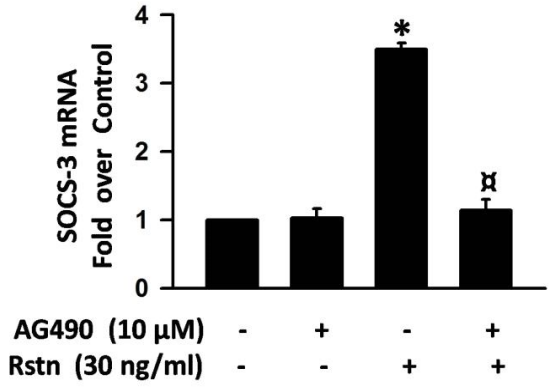


**Fig.1. The JAK2/STAT3 inhibitor reduced the resistin (Rstn)-induced increases in SOCS-3 mRNA expression. *, P < 0.05 vs the control; ¤, P < 0.05, Rstn vs Rstn + AG490.**

### 3.2. ERK, p38, and JNK MAPKs are involved in the resistin-induced up-regulation of SOCS-3 mRNA expression

Whether Rstn-induced up-regulation of SOCS-3 mRNA expression is related to the MAPK pathways was assessed (Fig. 2). Adipocytes were pretreated with either the ERK MEK1 inhibitor U0126, the p38 MAPK inhibitor SB203580, or the JNK MAPK inhibitor SP600125 for 30 min and then treated with 30 ng/ml Rstn. None of the inhibitors reduced the basal level of SOCS-3 mRNA but, in the presence of Rstn, all of them suppressed Rstn-induced increases in SOCS-3 mRNA levels. These results suggest that all the functional MAPK proteins are necessary for the effect of Rstn. These findings are consistent with those reported for the ERK-mediated pathway of Rstn on caspases-3 expression in ovarian follicular cells (23), for the p38 MAPK-regulated pathway of Rstn on CCL4 expression in nucleus pulposus tissues and FK expression in endothelial cells (24-25), and for the JNK/c-JUN-mediated pathway of Rstn on FK expression in endothelial cells (25).

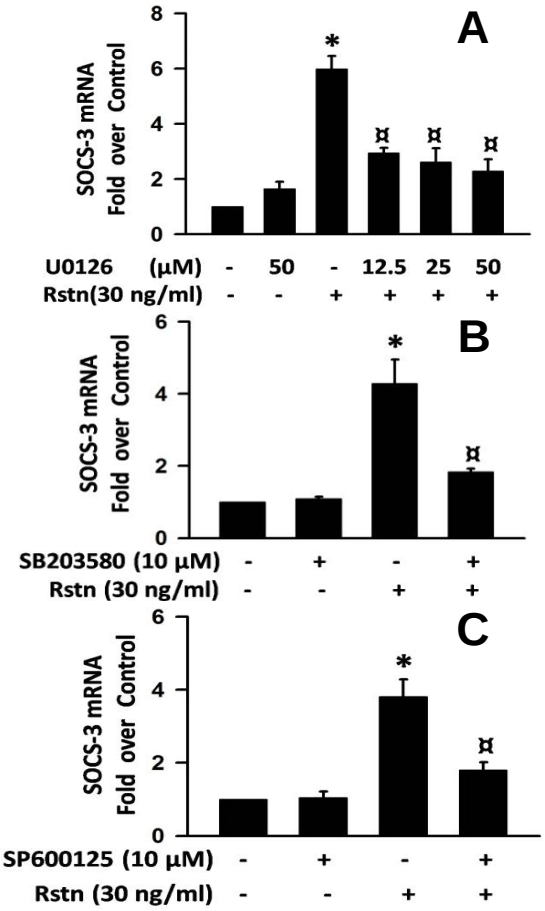


**Fig.2. MAPK inhibitors U0126, SB203580, and SP600125 reduced the resistin (Rstn)-induced increases in SOCS-3 mRNA expression. *, P < 0.05 vs the control; ¤, P < 0.05, Rstn vs Rstn + MAPK inhibitor.**

### 3.3. The PI3K/AKT inhibitor suppresses the resistin stimulation of SOCS-3 mRNA expression

We examined the possibility that the PI3K/AKT-regulated pathways can mediate the effect of Rstn on SOCS-3 mRNA expression (Fig. 3). Adipocytes were pretreated with the PI3K inhibitors, either wortmannin or LY294002, for 30 min and then treated with 30 ng/ml Rstn. Neither wortmannin nor LY294002 Wortmannin altered the basal levels of SOCS-3 mRNA but, in presence of Rstn, both of them suppressed Rstn-induced up-regulation of SOCS-3 mRNA expression. These results indicate the PI3K/AKT-dependent effect. These findings are

consistent with that reported for the PI3K/AKT-dependent effect of Rstn on caspase-3 expression in ovarian follicular cells (23).

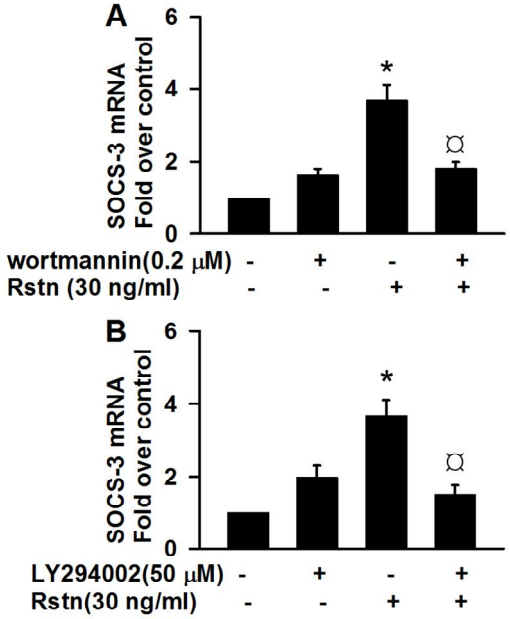


**Fig.3. The PI3K/AKT inhibitors wortmannin and LY294002 reduced the resistin (Rstn)-induced increases in SOCS-3 mRNA expression. *, P < 0.05 vs the control; ¤, P < 0.05, Rstn vs Rstn + inhibitor.**

### 3.4. EGCG suppresses the resistin stimulation of SOCS-3 mRNA expression

Green tea EGCG was previously found in 3T3-L1 adipocytes to suppress Rstn gene expression through the ERK pathway (28). Whether the EGCG is like the ERK inhibitor able to mediate the effect of Rstn on SOCS-3 mRNA expression (Fig. 2A) was assessed (Fig. 4). Adipocytes were pretreated with 20 μM EGCG for 2 h and then treated with 30 ng/ml Rstn. In the presence of Rstn, EGCG indeed inhibited Rstn-induced up-regulation of SOCS-3 mRNA expression. Because EGCG was found to reduce the activity of MEK1 (28) and because the inactivation of MEK1/ERK proteins by U0126 blocked Rstn-induced increase of SOCS-3 mRNA levels (Fig. 2A), the anti-Rstn effect of EGCG on SOCS-3 gene expression may be attributable to its lowering the activity of MEK1/ERK proteins.

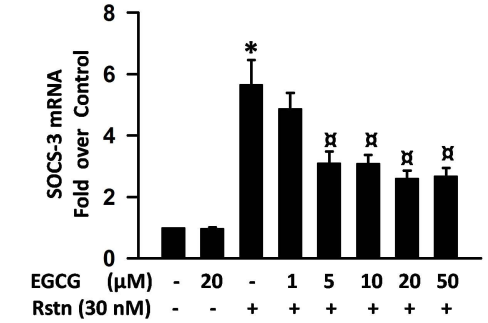


**Fig.4. Green tea epigallocatechin-3-gallate (EGCG) reduced the resistin (Rstn)-induced increases in SOCS-3 mRNA expression. *, P < 0.05 vs the control; ¤, P < 0.05, Rstn vs Rstn + EGCG.**

### 3.5. Endothelin-1 suppresses the resistin stimulation of SOCS-3 mRNA expression

Endothelin-1 was previously found in 3T3-L1 adipocytes to stimulate SOCS-3 and Rstn gene expressions (10, 29). Whether the ET-1 can mediate the effect of Rstn on SOCS-3 mRNA expression was assessed (Fig. 5A). Adipocytes were pretreated with 100 nM ET-1 for 4 h and then treated with 30 ng/ml Rstn. Both ET-1 and Rstn were found to increase the level of SOCS-3 mRNA. Interestingly, in presence of Rstn, ET-1 suppressed Rstn-induced up-regulation of SOCS-3 mRNA expression. When adipocytes were pretreated with 30 ng/ml Rstn for 2 h and then treated with 100 nM ET-1 for 4 h, we found that Rstn suppressed ET-1-induced up-regulation of SOCS-3 gene (Fig. 5B). To our knowledge, SOCS-3 gene expression can be rapidly (< 2 h) upregulated by either ET-1 (10) or Rstn (14), and it can inhibit cytokine or hormone signaling through negative feedback (1-8). The negative feedback mechanism of the SOCS-3 may explain the down-regulatory effect of the combination of ET-1 and Rstn on adipocyte SOCS-3 expression when compared to the effect of the individual ET-1 or Rstn hormone on SOCS levels.

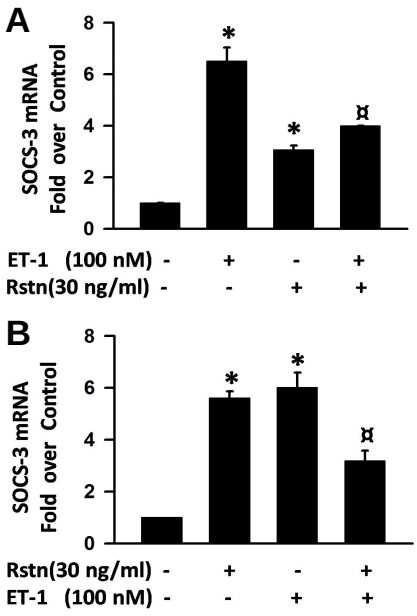


**Fig.5. The combination of endothelin (ET)-1 and resistin (Rstn) caused the level of SOCS-3 mRNA that was significantly less than that or the sum of the individual ET-1 or Rstn effect. (A) Serum-starved 3T3-L1 adipocytes were pretreated with 100 nM ET-1 for 4 h and then treated with 30 ng/ml Rstn for 2 h. (B) Adipocytes were pretreated with 30 ng/ml Rstn for 2 h and then treated with 100 nM ET-1 for 4 h. *, P < 0.05 vs the control; ¤, P < 0.05, Rstn vs Rstn + ET-1 (A), or ET-1 vs ET-1 + Rstn (B).**

Resistin regulates the expression of cellular genes through modulations of a variety of kinase signaling molecules (22-25). Our study supports the hypothesis that Rstn stimulated SOCS3 gene expression in 3T3-L1 adipocytes through the JAK2, MAPK, and PI3K pathways. These findings also indicate that Rstn acts

on adipocyte SOCS-3 gene expression through modulations of multiple kinase signaling molecules. Recently, TLR-4 and the RTK-like orphan receptor, but not TLR-2, have been reported to act as Rstn receptors in human cells or murine adipocytes (26-27). Whether the Rstn signal to regulate adipocyte SOCS-3 expression is mediated through the TLR-4 and the RTK-like orphan receptor pathways was not demonstrated in this study.

## CONCLUSIONS

We conclude that the Rstn activation of SOCS-3 gene expression in 3T3-L1 adipocytes is dependent on the JAK2, ERK MAPK, p38 MAPK, JNK MAPK, and PI3K pathways. Because SOCS-3 functions to cause insulin resistance (5-6), results of our study showing the signaling pathway of Rstn to stimulate SOCS-3 expression from murine secondary adipocytes appear to support the findings that Rstn regulates insulin sensitivity and diabetes (7,14-17). To our knowledge, Rstn was found to regulate fat cell functions, such as lipid metabolism and insulin sensitivity (7,14-19), results of our study showing the anti-Rstn effects of ET-1 and EGCG on adipocyte SOCS-3 expression seem to support the findings that ET-1 and EGCG regulate insulin sensitivity, diabetes, or obesity (30-32). It would be of interest to demonstrate whether either ET-1 and EGCG interacts with Rstn to control insulin sensitivity, diabetes, and obesity in a further *in vivo* study.

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