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Ursolic acid and rosiglitazone combination prevent low-grade inflammation and hepatic insulin resistance by modulating cytokine expression in mice fed a high-fat diet

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Abstract

Background:
The objective of this research endeavor was to assess the effects of rosiglitazone (RSG) and ursolic acid (UA) on hepatic insulin signaling indicators and inflammatory marker concentrations in C57/BL/6J mice that were provided with a high-fat diet (HFD).

Methods:
C57BL/6J mice were fed a HFD for 16 weeks and orally administered UA (5 mg/kg BW), RSG (4 mg/kg BW), and UA (5 mg/kg BW) + RSG (4 mg/kg BW) for the last 6 weeks.

Results:
The HFD groups showed a significant increase in leptin, TNF-α, and IL-6, whereas adiponection level significantly decreased. The expression of insulin signaling markers in the liver also significantly increased in HFD mice.

Conclusions:
Combination treatment improves above said parameters than individual parameters. These data suggest that combination treatment (UA with RSG) has potential benefits for the treatment of HFD-induced insulin resistance, and its effects may be associated with improvements in the inhibition of the expression of inflammatory markers in plasma and liver.

Keywords: Rosiglitazone, Ursolic acid, High fat diet, Inflammatory markers, Insulin signaling.

Introduction

Diabetes is projected to affect 592 million individuals globally by 2035, imposing a significant burden on clinical and public health systems.[1, 2] The two most prevalent forms of diabetes are type 1 diabetes and type 2 diabetes, which collectively constitute over 85 percent of all diabetes cases. [3] Insulin resistance is the hallmark of type 2 diabetes mellitus (T2DM). Progressive inflammation in adipose tissue, liver, skeletal muscle, and pancreatic islets characterizes T2DM, and mounting evidence suggests that this is a chronic low-grade inflammatory disease.[4,5] In order to modulate insulin resistance, lipid profile, appetite, and glucose homeostasis, adipose tissue secretes an assortment of bioactive peptides called adipokines.[6] Chronic low-grade inflammation in adipose tissue is induced by obesity. This inflammation leads to an increase in the synthesis of proinflammatory cytokines, including interleukin-6 (IL-6), interleukin-10, and monocyte chemoattractant protein-1; conversely, the production of anti-inflammatory adipokines, such as adiponectin, decreases. There is a correlation between obesity, metabolic syndrome, and dysregulation of adipokine secretory patterns.[7, 8]

The levels of proinflammatory cytokines are increased in insulin target tissues and blood samples obtained from obese animals. Additionally, it has been observed that the improvement of insulin sensitivity may be achieved by the neutralization of these inflammatory cytokines. The activation of inflammatory cytokines has the potential to directly elicit insulin resistance and disrupt intracellular insulin signaling pathways. The user has provided a numerical range, specifically.[9,10] Moreover, these inflammatory factors exert a detrimental effect on the phosphorylation of insulin receptor tyrosine, impair the insulin-induced tyrosine phosphorylation of insulin receptor substrate (IRS-1), and decrease the transcription of crucial components in the insulin signaling pathway. Consequently, these actions disrupt the transmission of insulin signaling. The user did not provide any text to rewrite.

Ursolic acid (UA) is a natural pentacyclic triterpenoid carboxyl acid that is the main component of some traditional medicine herbs and is widely known to have many beneficial biological effects, such as those described above (anticancer[11], antioxidative, hepatoprotective[12], hypolipidemic, and antiatherosclerotic).[13] Pharmacological intervention is a possible new approach to preventing and/or treating insulin resistance. Type 2 diabetes mellitus is often treated with anti-diabetic medications such as rosiglitazone (RSG), a synthetic PPAR- agonist that belongs to the thiazolidinedione family of chemicals. It reduces blood sugar and cholesterol levels while increasing insulin sensitivity.[14]

This research aims to evaluate the effects of rosiglitazone and ursolic acid, both individually and in combination, on the levels of hepatic insulin resistance and certain inflammatory markers (specifically, TNF-α and IL-6), in a mouse model of HFD-induced type 2 diabetes.

Materials and methods

Ethics Statement

All animal experiments and care were carried out in accordance with the guidelines set forth by the Control and Supervision of Experiments on Animals (CPCSEA), based in New Delhi, India. All of the experiments described here were conducted with permission from the Institutional Animal Ethics Committee at Rajah Muthiah Medical College and Hospital (RMMC & H), Annamalai University, India (Reg. no.160/1999/CPCSEA).

Animals and Animal Care

The C57BL/6J mice were obtained from the National Institute of Nutrition in Hyderabad. The mice were kept in a room with a regulated temperature (23 1°C) and a 12-hour light/12-hour dark cycle at Annamalai University's Central Animal House, Department of Experimental Medicine (RMMC & H). The animals were allowed unlimited access to water and food.

Experimental Design

After adaptive feeding for 1 week, animals were randomly divided into the following six groups with 10 mice in each group: normal group; normal + UA + RSG treated group; HFD group; HFD + UA group; HFD + RSG group, and HFD + UA + RSG group. For the normal and HFD groups, animals were fed with standard or HFD alone, respectively. The standard diet comprised of protein-21.1%, fat-5.1%, carbohydrates-60.0%, fiber-3.9%, minerals-7.9%, vitamins-2.0%. High-fat diet: High-fat diet (HFD) was prepared by mixing beef tallow (34.9%) with a standard pellet diet. All measures were taken to ensure uniform mixing of the additives of the diet before kneading using a little water. To develop the experimental insulin-resistant model, the mice were fed with HFD (40 % fat), which comprised of beef tallow, for 10 weeks. At the end of the experimental period, the mice were sacrificed by cervical dislocation. Blood samples, liver, and adipose tissue were collected, weighed, and then stored at -80 °C.

ELISA examination

Enzyme immunoassay kits from DIAsource, Nivelles, Belgium were utilized to quantitatively estimate insulin, adipokines (adiponectin and resistin), and cytokines (tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6)), following the instructions provided by the manufacturer.

Western blot analysis

To prepare the liver tissue for western blot analysis, it was lysed with RIPA in the presence of a phosphatase inhibitor cocktail. Following complete homogenization, samples were centrifuged at 4 °C for 30 minutes at 13000 rpm. After harvesting the supernatants, they were boiled for 5 minutes at 99 °C in loading buffer. Following SDS-PAGE separation, the protein was transferred to a PVDF membrane. Following a two-hour blocking period with 5% fat-free milk at room temperature, the membrane was incubated at 4° for two hours with primary antibodies (PI3 K, β-actin; rabbit polyclonal; dilution 1:500 in 5% BSA in TBST). Following this, membranes were subjected to a two-hour incubation at room temperature with their respective secondary antibodies, which were horseradish peroxidase-conjugated rabbit IgG. The ECL reagent was utilized to visualize the blots. The quantification of bands was performed using Image J, a public domain Java image processing software developed by Wayne Rasband, NIH, Bethesda, MD, USA. The control value for Image J was set to 1.

PCR primer design

The Entrez nucleotide database was queried for gene sequence information, and the online tool Primer 3-BLAST, accessible through GenBank, was utilized to generate primer sequences. The primers utilized in this study were procured from Sigma Chemical Company, located in St. Louis, MO, USA. The melting temperatures of sense and antisense primers for the target gene were intentionally constructed to be nearby (Table 1).

Table 1. List of primers used in RT-PCR analysis

Gene	Primer sequence (5′–3′)
TNF-α Forward	AGCCCCCAGTCTGTATCCTT
TNF-α Reverse	CTCCCTTTGCAGAACTCAGG
IL-6 Forward	TTTTCTCCACGCAGGAGACT
IL-6 Reverse	TCCACGATTTCCCAGAGAAC
GAPDH Forward	GAGAGGCCCTATCCCAACTC
GAPDH Reverse	GATTGAGCCTGCTTCACCTC

Real-time PCR analysis

Trizol was used to extract total RNA from mouse epididymal adipose tissue. RNA (2 µg) was transcribed into cDNA, and mRNA expression was measured using a realplex master cycler (Eppendrof, Germany) with the appropriate primers. Real-time PCR was carried out with an Eppendrof realplex master cycler and the SYBR Green PCR Master Mix (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's instructions. The cycle parameters were 95 degrees Celsius for 10 seconds, followed by 40 cycles of 95 degrees Celsius for 5 seconds and 60 degrees Celsius for 30 seconds. The relative measurement of gene expression using the $2^{-\Delta\Delta Ct}$ technique was used. The levels of the target genes were normalized to GAPDH, and the findings were reported as fold changes in threshold cycle (Ct) values compared to controls.

Immunohistochemistry

BioGenex, USA, supplied a super-sensitive polymer HRP detection system kit for immunohistochemistry. The liver tissue was removed, fixed in 10% formalin for 24 hours, and paraffin was embedded. Sections were cut to a thickness of 3-4 µm, flattened, and glued to the slides. The paraffin-embedded mounted slices are deparaffinized in xylene and rehydrated with an ethanol/H_2O gradient. After a 10-minute heat-mediated antigen retrieval process, the slides were allowed to cool for another 20 minutes. This was followed by a 10-15 minute peroxidase block therapy and a 15-minute power block treatment. The sections were incubated for 2 hours in 1:200 dilution with the diluted primary antibodies (anti-TNF-α and mouse anti-IL-6) before being treated with the super-enhancer solution for 30 minutes and the super sensitive poly-HRP solution for 30 minutes. The slices were microscopically inspected and photographed at 200 magnification after color development using DABT (5 mg DAB, 10 mL of 1x TBS, and 5 µL of H_2O_2).

Statistical analysis

The significance of the differences between the samples was assessed using one-way ANOVA in SPSS version 11.5 (SPSS, Chicago, IL). Duncan's multiple range test (DMRT) was used to distinguish differences between the six groups. $P \geq 0.05$ was the recognized level of significance. The results were reported as means ± SD (standard deviation).

Result

The impact of combination therapy (UA/RSG) on the plasma concentrations of leptin and adiponectin in HFD-fed mice is illustrated in Table 2. Adiponectin levels decreased significantly in HFD-fed mice, whereas plasma leptin levels increased significantly. Plasma leptin decreased significantly in response to UA or RSG treatment alone, whereas adiponectin increased considerably; combination treatment significantly returned the aforementioned parameter to normal levels. Values are means ± S.D. for six assays from ten mice.

Table 2. Effect of UA and RSG on leptin and adiponectin levels in HFD-fed C57BL/6J mice

Groups	Leptin (ng/mL)	Adiponectin (µg/mL)
Normal	4.25 ± 0.22^{a}	7.31 ± 0.31^{a}
Normal + UA + RSG	3.87 ± 0.34^{a}	8.62 ± 0.31^{a}
HFD	36.05 ± 2.99^{b}	2.36 ± 0.16^{b}
HFD + UA	17.39 ± 1.36^{c}	5.75 ± 0.27^{c}
HFD + RSG	14.33 ± 0.54^{c}	4.47 ± 0.37^{c}
HFD + UA + RSG	7.89 ± 0.42^{d}	6.98 ± 0.29^{a}

Values not sharing a common superscript differ significantly at $p \leq 0.05$. Duncan's Multiple Range Test (DMRT)

Liver inflammatory markers.

An enormous rise in fasting plasma concentrations of IL-6 and TNF-α was observed in the HFD group (Table 3). UA and RSG reduced TNF-α and IL-6 levels significantly, both individually and in combination. The concurrent administration of both medications resulted in a more substantial reduction in TNF-α and IL-6 levels, as well as a more pronounced enhancement in insulin sensitivity, compared to the use of either drug individually.

Table 3. Effect of UA and RSG on TNF-α and IL-6 levels in HFD-fed C57BL/6J mice

Groups	TNF-α (pg/mL)	IL-6 (pg/mL)
Normal	16.34 ± 0.92[a]	71.41 ± 6.31[a]
Normal + UA + RSG	17.53 ± 1.14[a]	72.34 ± 5.62[a]
HFD	59.45 ± 3.87[b]	195.37 ± 17.73[a]
HFD + UA	37.39 ± 1.36[c]	156.62 ± 13.36[b]
HFD + RSG	34.33 ± 2.54[c]	144.82 ± 12.51[c]
HFD + UA + RSG	22.53 ± 1.42[d]	102.71 ± 9.59[a]

Values are means ± S.D. for six assays from ten mice.
Values not sharing a common superscript differ significantly at $p \leq 0.05$. Duncan's Multiple Range Test (DMRT)
The immunohistochemical examination of liver sections for the presence of TNF-α and IL-6 proteins is depicted in Figure 1. As indicated by our findings, the HFD led to an increase in the expression of the TNF-α and IL-6 genes. In comparison to treatment with UA and RSG separately, the expression levels of those genes were substantially reduced following treatment with both agents.

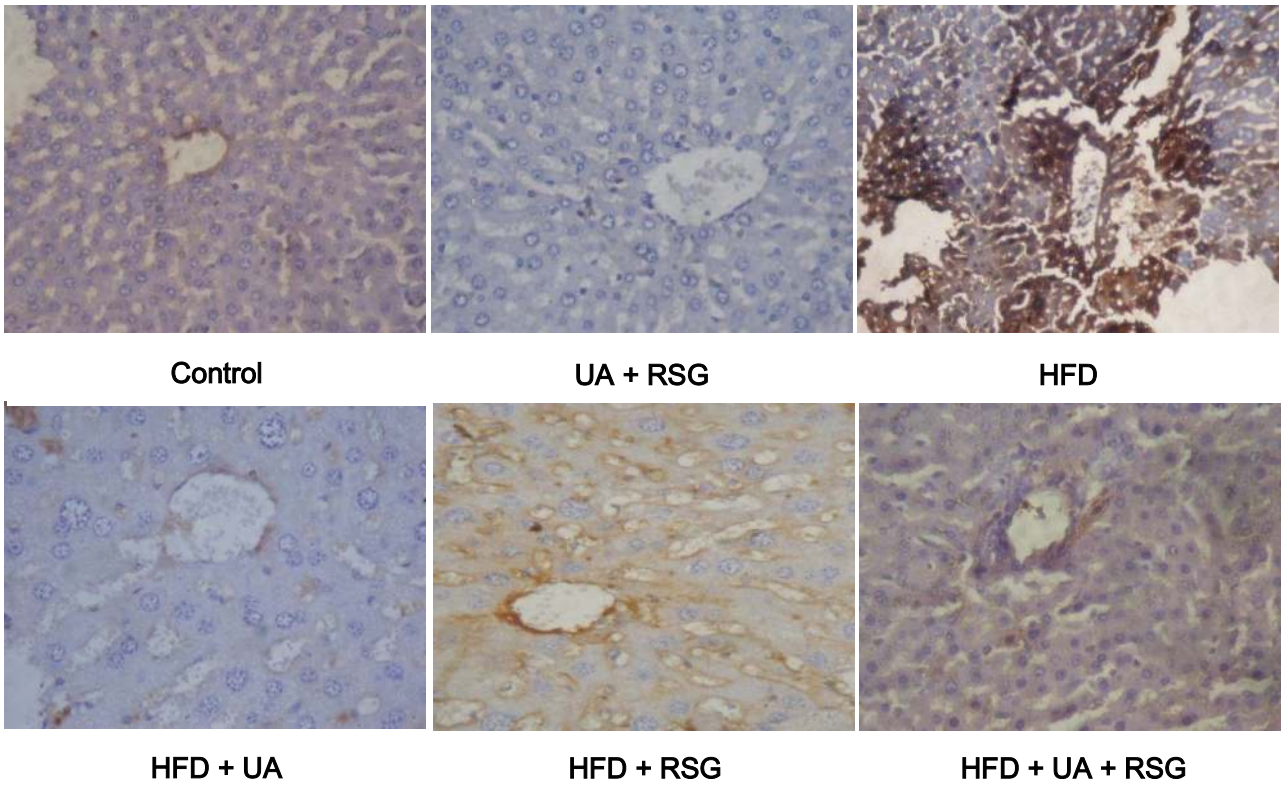

Fig.1. Effect of UA and RSG on TNF-α protein expression in the liver tissue of HFD fed C57BL/6J mice by immunohistochemistry

Liver insulin levels
The liver protein expression levels of IRS-2, PI3 kinase, and Akt in mice fed a high-fat diet are depicted in Figure 2. In rodents fed an HFD, the expressions of IRS-2, PI3 kinase, and Akt were all observed to be downregulated. The expression of IRS-2, PI3 kinase, and Akt was significantly increased in HFD-fed mice that received UA or RSG in comparison to those that were fed an HFD alone. Furthermore, the combination treatment of UA and RSG significantly increased the expression of IRS-2, PI3 kinase, and Akt.

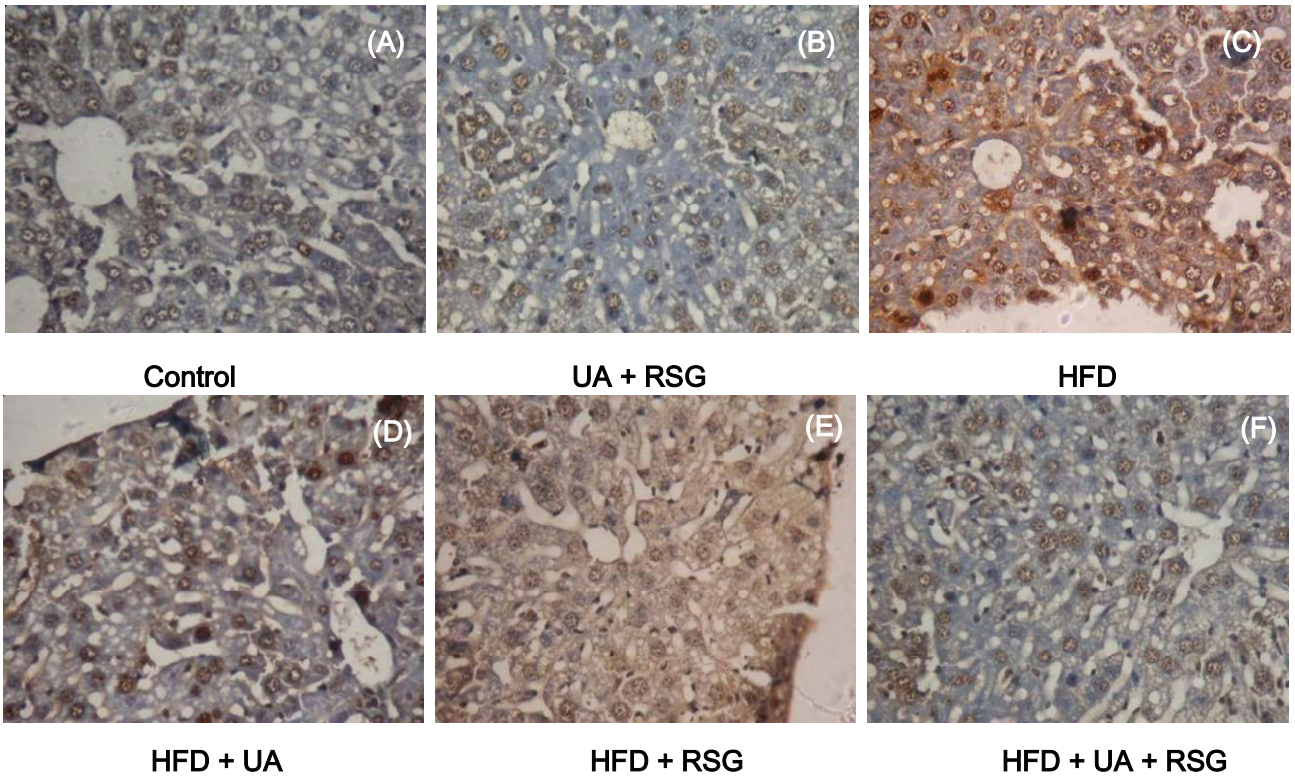

Fig.2. Effect of UA and RSG on IL-6 protein expression in the liver tissue of HFD fed C57BL/6J mice by immunohistochemistry

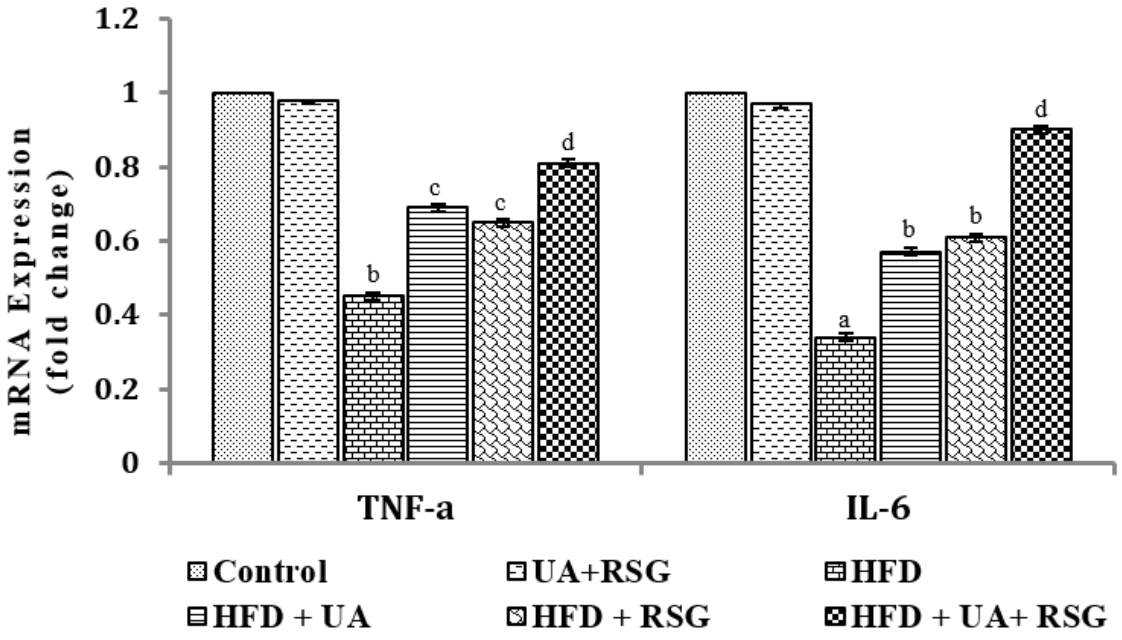

Fig.3. Histogram showing relative TNF-α and IL-6 gene expression in liver

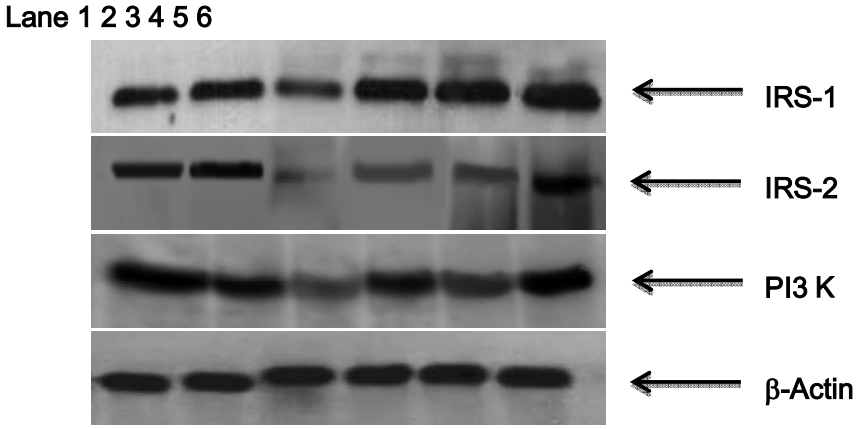

Fig.4a. Effect of UA and RSG on IRS-1 and IRS-2 protein expressions in the adipose tissue of HFD fed C57BL/6J mice.
Lane 1: Control; lane 2: Control + UA + RSG; lane 3: HFD; lane 4; HFD + UA; lane 5: HFD + RSG; lane 5: HFD + UA + RSG.

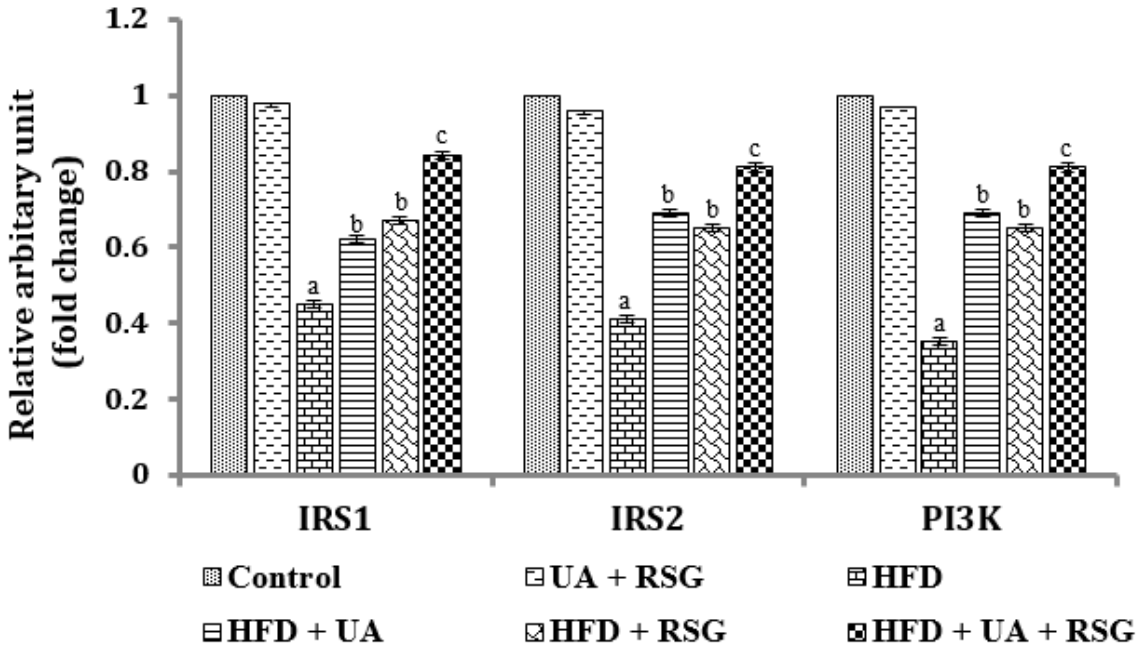

Fig.4b. Band intensities scanned by densitometer
Histogram depicts quantization of three independent experiments (means ± S.D.), with IRS-1, IRS-2 and PI3 K by defining the control group with IRS-1, IRS-2 and PI3 K protein, as 1 unit. Values not sharing a common superscript differ significantly at $P \leq 0.05$ (DMRT).

Discussion

Prolonged nutrient excess leads to metabolic inflammation, which is characterized by the dysfunction and expansion of adipocytes. It is important to note that metabolic inflammation in obesity is not limited to adipose tissue, as the liver also significantly contributes to its development.[15, 16] The combined effect of UA and RSG on HFD-induced inflammation and hepatic insulin resistance in C57BL/6J mice fed an HFD is investigated in this study. Leptin, which is secreted by adipose tissues, plays a significant role in the regulation of body weight.[17] In both humans and rodents, leptin levels are directly correlated with alterations in body weight and adiposity.[18] There are multiple reports indicating that elevated serum leptin concentrations in rodents may be the result of obesity or an HFD.[19-21] In this study, an HFD group exhibited elevated leptin levels, whereas combination therapy with UA and RSG reduced leptin levels significantly. In our prior research, we found that the concurrent administration of UA and

RSG resulted in a substantial reduction in body weight and visceral adiposity; this finding is consistent with the decreased leptin levels observed in the current study. Adiponectin is a protein hormone secreted by adipocytes that regulates glucose and fatty acid oxidation, among other metabolic processes.[22-24] In mouse models, there is substantial evidence linking low levels of adiponectin to the development of insulin resistance.[25] Furthermore, there have been reports of decreased plasma concentrations of adiponectin in individuals with insulin resistance, such as those with type 2 diabetes in humans.[26] Our data demonstrated conclusively that the combination of UA and RSG significantly increased plasma adiponectin levels.

Obesity is strongly associated with a state of systemic, low-grade inflammation characterized by the activation of inflammatory signaling pathways and the production of aberrant cytokines in adipose tissue, according to accumulating evidence.[27] Cytokine concentrations that are generated by adipocytes are typically increased in individuals who are obese.[28] In this study, we discovered that plasma and liver expressions of pro-inflammatory cytokines are significantly increased in HFD rodents; furthermore, we discovered that the combination treatment of UA and RSG eliminated these increases. The observed outcomes align with the established anti-inflammatory properties of UA[29] and RSG[30] in mice. In addition, our findings suggested that the combination of UA and RSG treatment may have had a greater anti-inflammatory effect than either treatment alone.

Plasma concentrations of inflammatory cytokines, including TNF- and IL-6, induce serine kinases in the insulin signaling pathway to phosphorylate threonine and serine residues on proteins of the insulin receptor substrate (IRS) and PI3K pathway. This inhibits the tyrosine phosphorylation of IRS and PI3K in response to insulin stimulation, thereby impeding insulin signal transduction and preventing the development of IR.[31,32] In the current investigation, an HFD impairs insulin signaling mediated by the IRS in liver tissues, whereas treatment with UA and RSG restores insulin signaling. A negative correlation was observed between the tyrosine phosphorylation of IRS-1 and the expression of TNF-α and IL-6 in the liver. The findings of our study indicate that the concurrent administration of UA and RSG can enhance hepatic insulin sensitivity by inhibiting TNF-α. In summary, the administration of UA/RSG to HFD mice resulted in substantial enhancements in both low-grade inflammation and hepatic insulin signaling.

Conflict of Interest

All the authors stated that there is no conflict of interest.

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