Resveratrol suppresses hyperglycemia-induced activation of NF-κB and AP-1 via c-Jun and RelA gene regulation

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Abstract

Background: Resveratrol (RSV) provides several important biological functions in wide variety of cells. In this study, we investigated the molecular mechanisms underlying anti-inflammatory effect of RSV on HepG2 cells by assessing the gene expression of RelA and c-Jun- subunits of NF-κB and AP-1 transcription factors.

Methods: HepG2 cells were settled in a serum- free medium with high concentrations of glucose (30 mM) and insulin (1 μM) overnight and were then incubated with RSV (5, 10, and 20 μM) for 24 and 48 hours. Real time quantitative polymerase chain reaction (qRT-PCR) was used to determine RelA and c-Jun expression.

Results: RSV diminished hyperglycemia/hyperinsulinemia stimulated expression of c-Jun dose- dependently after 24 and 48 hours (p<0.05). In addition, RelA gene expression was decreased dose-dependently in all RSV doses after 48-hour incubation (p<0.05). Our results indicated that RSV may reduce NF-κB and AP-1 activity via RelA and c-Jun gene regulation.

Conclusion: The findings of the present study demonstrated that RSV may be considered as a preventative and therapeutic agent for antagonizing inflammation in Hepatocellular carcinoma (HCC).

Keywords: AP-1, c-Jun, Hyperglycemia, Hyperinsulinemia, Inflammation, Resveratrol, NF kappa B, MAPK

Introduction

Inflammation is a normal response of the body's immune system to various agents managed by immune cells (1). It is also known as a cause of several diseases including diabetes mellitus, cancer, arthritis, atherosclerosis, and septic shock (2). Inflammation is triggered by the interactions between surface receptors and their ligands, which lead to production of cytokines, such as interleukin (IL), tumor necrosis factor (TNF), and inflammatory mediators including prostaglandin E2 (PGE2) and nitric oxide (NO) (3). Upregulation, synthesizing, phosphorylation, and activation of these inflammatory proteins are performed by inflammatory transcription factors (4).

NF-κB and AP-1 are key transcription factors in a wide range of inflammatory conditions induced by various stimuli. In NF-κB pathway, the ligand binding to cell surface receptors leads to activation of the IκB kinase (IKK), phosphorylation, and degradation of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (IκB), nuclear translocation, and DNA binding of NF-κB, respectively (5). AP-1 is the production of mitogen-activated protein kinases (MAPKs) pathway, which also initiates with ligand binding to surface receptors, leading to the phosphorylation and activation of kinases. Subsequently, AP-1 is activated, translocated to nucleus, and bind to DNA (6).

RSV (Resveratrol: 3,5,4'-trihydroxy-trans-stilbene) is a natural non flavonoid polyphenolic component found in

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↑What is "already known" in this topic:
Previous studies have presented conflicting results about RSV effects on cellular AP-1 and NF-κB activities via different biological pathways. However, there are insufficient data about RSV effects on c-Jun and RelA.

→What this article adds:
This study demonstrated that RSV decreases the expression of RelA and c-Jun and subsequently reduces NF-κB and AP-1 activity in hyperglycemia/ hyperinsulinemia condition.

diets that include grapes and other plants (7). RSV is also reported to have many multiple benefits including anti-cancer, anti-oxidative, anti-aging, anti- arthritis, and anti-diabetic properties (8). In addition, it plays anti-inflammatory roles through inhibition of gene expression in proinflammatory proteins (9). Because the expressions of numerous genes involved in inflammatory pathways are controlled by NF-κB and AP-1, many studies have investigated the effects of RSV on these transcription factors (10). RSV may interfere with NF-κB and AP-1 actions in several stages including gene expression, protein synthesizing, phosphorylation, and translocation of pathways' proteins and enzymes. Moreover, there are some controversies in RSV effects on NF-κB and MAPK pathway; in some research reports, RSV has been demonstrated to increase their activity, while in some others, it has decreased their activity (11, 12). The present study was designed to provide more evidence on the resolution of this scientific controversy.

Hepatocellular carcinoma (HCC) is known as an inflammation-based carcinoma to the extent that more than 90% of HCC cases are related to chronic inflammation (13). Proinflammatory conditions increase the risk of cancer development. On the other hand, the use of nonsteroidal anti-inflammatory agents decreases the incidence of tumors (14). Recent studies have introduced NF-κB and MAPK pathways as the link between chronic inflammation and HCC (15). Hepatocellular carcinoma cell line (HepG2) is widely used to investigate inflammatory condition in HCC (16). In this study, we used HepG2 to investigate the effects of RSV on gene expression of RelA and c-Jun, the subunits of NF-κB and AP-1 transcription factors. HepG2 cells are sensitive to inflammatory conditions (13, 15) and RSV affections (17). Inflammatory situation was induced by settling the cells in high-glucose and high-insulin media with the aim of ROS overproduction, followed by activation of NF-κB and AP-1 (18). This study was designed to investigate the anti-inflammatory effects of RSV through alteration of RelA and c-Jun gene expression in liver carcinoma cells (HepG2).

Methods
Study design
This was an experimental study. All steps of this experiment have been performed in triplicate.

Chemical
RSV (200808) was provided from Santa Cruz Biotechnology-USA. Dulbecco's modified Eagle's medium F12 (DMEM-F12) was obtained from Gibco-UK. Fetal bovine serum (FBS), Dimethyl sulfoxide (DMSO), penicillin, and streptomycin were purchased from Sigma-USA. RNeasy plus mini kit (74134) was provided from QIAGEN-UK. PrimeScript™ RT reagent Kit (RR037A) and SYBR Green Real-Time PCR Master Mix (RR820L) were obtained from TaKaRa-Japan.

Cell culture
The HepG2 cell line (NCBICode: C158) was provided from national cell bank of Iran, Pasteur Institute. Cells were cultured in DMEM-F12, supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin in a 37°C humid atmosphere, with 5% CO_2. For each experiment, cells were seeded into 6 well plates at 1.2×10^6 cells per well to reach 80% confluence (19). To induce insulin resistance using GOD-POD method, HepG2 cells were treated with high d-glucose (30 mM) and high insulin (1μM) serum-free medium for 24 hours after an over-night serum-starving (20). Then, HepG2 cells were treated with different predefined optimal concentrations of RSV (5, 10, and 20 μM) for different time points (24-48 hours), according to previous studies (21, 22). DMSO, with <0.5% final concentration, was used as a solvent in all groups.

RNA extraction and qRT-PCR
Cells were scraped off the plates, and total RNA was isolated using RNeasy plus mini kit, according to the manufacture's instruction protocol. RNA was quantified measuring the ratio of 260nm and 280nm absorbance with the NanoDrop-2000c Spectrophotometer (Thermo Fisher, USA). PrimeScript™ RT reagent kit (Takara Bio, Inc.) was used to synthesize cDNA from a 500ng RNA aliquot. PCR was performed in real time PCR instrument (AB Applied Biosystem-USA) using the reaction mixture of SYBR Green Real-Time PCR Master Mix and primers, according to the kit protocol. The reaction was performed in 40 cycles, each with 95°C at 15s for denaturation, 55°C at 20s for annealing, and 72°C at 20s. The primer sequences were as follow: β-Actin, as housekeeping: forward, 5′-CGAAATCTTGCCCTTTGTCC-3′ and reverse, 5′-GTTGTGAACATCCCGAGCTAG-3′; RelA: forward, 5′-CCTGTCCTTTCTCATCCCATC-3′ and reverse, 5′-TGGTCCCGTGAAATACACCTC-3′; c-Jun: forward, 5′-GATGGAAACGACCTTCTATGACG-3′ and reverse, 5′-GTTGCTGGACTGGATTATCAGG-3′. Moreover, all melt curves were checked for the specifications of the primers in the reaction.

Statistical analysis
β-Actin was used as a reference gene, and statistical significance was determined by the value of P<0.05. The LinRegPCR 2012.1 (version; http://LinRegPCR.org.info) was applied to adjust the efficiency of the differences between PCR reactions for each sample. Pairwise fixed reallocation randomization test formula is presented below, which was used in the REST 384 (version; http://Rest.gene-quantification.org.info) and supplied expression ratio and confidence intervals.

$$R = \frac{(E_{target})^{\Delta CP\ target(MEAN\ control - MEAN\ sample)}}{(E_{ref})^{\Delta CP\ ref(MEAN\ control - MEAN\ sample)}}$$

E: Efficiency of the PCR reaction
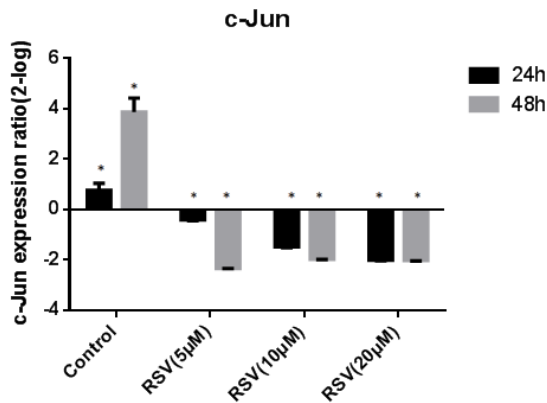CP: The crossing point above background fluorescence

Fig. 1. c-Jun expression ratio in HepG2 cells after treatment with RSV. HepG2 cells were settled in high-glucose (30 mM) and high-insulin (1 μM) concentration media an over-night and then were treated with RSV (5, 10, 20 μM) for 24 and 48h. c-Jun mRNA levels were analyzed by real-time PCR. *denote statistically significant difference (p<0.05) compared to control cells, treated with high dose of glucose and insulin. DMSO was used as a solvent in all the groups. 2-Log of all data was used for better visual of the graph.

Fig. 2. RelA expression ratio in HepG2 cells after treatment with RSV. HepG2 cells were settled in high-glucose (30 mM) and high-insulin (1 μM) concentration media an over-night and then were treated with RSV (5, 10, 20 μM) for 24 and 48h. RelA mRNA levels were analyzed by real-time PCR. *denote statistically significant difference (p<0.05) compared to control cells, only treated with high dose of glucose and insulin. DMSO was used as a solvent in all the groups. 2-Log of all data was used for better visual of the graph.
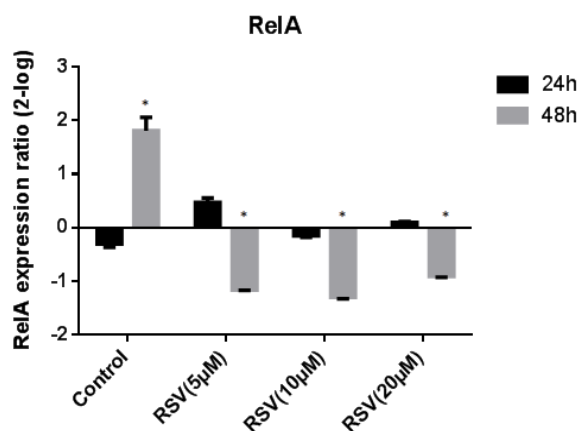
Results

First, inflammatory induction was performed according to the protocol we published recently (23). Briefly, HepG2 cells were treated with a high dose of glucose (30mM) and insulin (1μM) for 48 and 72 hours to simulate a long-term exposure as in Type 2 diabetes mellitus (T2DM) (20). This situation is related to redox status and is interpreted as an inflammatory agent, which leads to induction of MAPK pathway via gene regulation.

After an overnight incubation of HepG2 cells with high-glucose and high-insulin concentrations, cells were treated with 5, 10, and 20μM of RSV for 24 and 48 hours. RSV decreased c-Jun gene expression in a dose-dependent manner after 24 and 48 hours (Fig. 1). Also, we found that after 48 hours of treatment, all doses of RSV decreased RelA gene expression, but changes were not significant after 24 hours (Fig. 2).

Discussion

This study was designed to investigate the AP-1 and NF-κB transcription factors activity through c-Jun and RelA gene regulation in response to RSV treatments on HepG2 cells in an in vitro model of hyperglycemia and hyperinsulinemia. There are some published conflicting results regarding RSV effects on cellular AP-1 and NF-κB activities (12, 24). On the other hand, numerous studies have investigated these pathways concerning regulation, synthesizing, phosphorylation, and activation of IKK and JNK (25). However, there are insufficient data about RSV effects on c-Jun and RelA in different steps of biological functions. Thus, in the present study, we used q-RT-PCR to detect the expression of c-Jun and RelA genes.

First, we used high-insulin and high-glucose media to induce inflammation. Recent reports show that hyperglycemia, as a result of impaired insulin secretion and insulin resistance of T2DM, could result in a vicious circle named glucose toxicity (23). According to the previous studies, the most hyperglycemia is induced in high concentration of glucose and insulin (21, 23). In cells, hyperglycemia leads to ROS overproduction and imposes stress on the endoplasmic reticulum (ER), followed by unfolded or misfolded protein accumulation

in the ER lumen (26,27). To inhibit the pathological stress effects and return to normal functions, ER employs a protective mechanism named unfolded-protein response (UPR) (27,28). Some studies depicted that MAPK pathways play a critical role in crosstalk between ER stress and the UPR, which leads to activation of AP-1 and NF-κB (29). Our results indicated that high-insulin and high-glucose media induced MAPK pathway via c-Jun up-regulation in 24 and 48 hours. The previously mentioned condition upregulated RelA after 48 hours, which is in line with the findings of some studies that have shown that most changes in gene expression happen 48 hours after treatment (30). We interpreted the situation as an inflammatory condition.

RSV is a polyphenolic phytoalexin, which is synthesized by some plants including mulberries, blueberries, peanuts, Japanese knotweed, Eastern white pine, and grapes in response to environmental stress such as fungal infection (31). Recent studies have introduced RSV as a preventive component for some common pathological conditions including cardiovascular diseases (32), pancreatitis (33), carcinogenesis (34), and inflammation (35). Recently, anti-inflammatory properties of RSV have been investigated and RSV has been recognized as a protection against ROS and nitrogen species (RNS) production, leading to a decrease in proinflammatory transcription factors and cytokines (36).

NF-κB is a family of transcription factors that regulates various cellular functions. Active form of NF-κB is classically composed of a RelA-p50 heterodimer. In the absence of stimuli, NF-κB complex is disabled by attaching to an inhibitor subunit termed IκB. In response to inflammatory agents such as ROS, a chain kinase including IKK is phosphorylated and activated. IKK phosphorylates the IκB and leads to separation and degradation of IκB in ubiquitin pathway. Subsequently, activated NF-κB is translocated into nucleus and regulates target genes through binding to promoter and enhancer sequences of DNA (37).

The anti-inflammatory effects of RSV are not cell type specific and are performed by different mechanisms: RSV suppresses IKK activity and phosphorylation, IκB degradation, NF-κB subunits nuclear translocation and DNA binding activity, RelA phosphorylation, and transcriptional coactivation and repression (38). However, some studies have not reported any effects of RSV on NF-κB cascade (24, 39). Zhang and Pellegatta have reported that RSV did not affect nuclear translocation and the nuclear appearance of RelA (24, 39). According to a surprising result, RSV enhanced NF-κB activity in cytokine-stimulated mesangial cells (40). RSV has been widely recognized as a potential therapeutic agent for inflammatory diseases (11). In the present study, our results revealed that long-term exposure of RSV (for 48 hours) inhibited RelA gene expression. This decline occurred when incubating cells with high-glucose and high-insulin media had increased RelA gene expression. On the other words, the anti-inflammatory effect of RSV on RelA gene expression was only observed when

inflammation had increased gene expression. To the best of our knowledge, this was the first study to demonstrate diminution of RelA gene regulation by RSV.

Most inflammatory agents also activate MAPK inflammatory pathway, which is known as a major signaling pathway in mammalians (41). MAPK, according to the MAPK enzyme, is divided into c-Jun N-terminal kinases (JNK), stress-activated protein kinase (SAPK), extracellular-signal-regulated kinase (ERK), and P38MAPK family. In all of them, inflammatory agents result in phosphorylation and activation of MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK), and MAPK, leading to dimerization and activation of transcription factors. AP-1 is a major transcription factor of JNK pathway, which is mainly composed of c-Jun homodimers and heterodimers of c-Jun/c-Fos (12). The effects of RSV on AP-1 activity are widely demonstrated in different cell types (5, 42). The important mechanism is the inhibition of JNK phosphorylation and activation (5, 42), but some studies reported no effects (24). There are conflicting results in RSV effects on c-Jun expression; some studies have reported that RSV enhances c-Jun expression (43), while others indicated that RSV strongly prevents c-Jun expression (12). Hence, in the present study, we investigated the effect of RSV on c-Jun gene expression induced by hyperglycemia condition. Our data demonstrated that RSV blocks JNK pathway through diminishing AP-1 activity by c-Jun downregulation. At 20μM, RSV showed maximum anti-inflammatory effect on HepG2 cells in the first 24 hours; all doses of RSV exerted such effect by decreasing AP-1 activity at 48 hours.

The limitation of this study was that it only determined gene regulation using q-RT-PCR, while response to stimuli may occur at other cellular function levels such as protein transcription and phosphorylation as well. Therefore, it is recommended to use immunoblot (Western blotting) and enzyme-linked immunosorbent assay (ELISA) to investigate relevant protein expression in the future studies.

Conclusion

In summary, we investigated the hypothesis that anti-inflammatory effects of RSV may mediate through RelA and c-Jun gene regulation. The findings of the present study revealed that RSV could decrease the expression of RelA and c-Jun on human HepG2 cells in an in vitro model of hyperglycemia and hyperinsulinemia and subsequently may reduce NF-κB and AP-1 activity. So, RSV may be a preventative and therapeutic option for inflammatory diseases.

Acknowledgments

Conflict of Interests

The authors declare that they have no competing interests.

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