Original Article

Resveratrol reduces high glucose-induced de-novo lipogenesis through mTOR mediated induction of autophagy in HepG2 cells

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Article info:
Received: 12 Feb 2023
Revised: 17 Mar 2023
Accepted: 5 Apr 2023

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ABSTRACT

Resveratrol has been reported to decrease lipid accumulation in the liver, but the molecular mechanism underlying this effect remains unknown. In this study, the role of autophagy in high glucose (HG)-induced lipogenesis in the presence of resveratrol in HepG2 cells was investigated. Resveratrol pretreatment prevented HG-induced total lipid content, triglyceride level, apo B secretion, and key lipogenic gene expression (FAS, ACC, SREBP1c, and MTP). HG diminished p-Foxo1 and p-AMPK levels, while resveratrol reversed this effect by inducing p-Foxo1 and p-AMPK levels by 40% and 47%, respectively. HG treatment reduced autophagy markers such as LC3-II, ATG5, and ATG7 and increased p62 protein levels, whereas resveratrol significantly reversed these effects. Additionally, inhibiting autophagy with chloroquine led to enhanced total lipid and triglyceride content compared to untreated control cells. Notably, co-treatment with chloroquine inhibited the preventive effect of resveratrol on HG-induced lipogenesis in HepG2 cells. Furthermore, while HG induced p-mTOR level in HepG2 cells, resveratrol reversed this effect. Rapamycin, an inhibitor of mTOR, ameliorated HG-induced total lipid and triglyceride content and the expression of lipogenic genes. Collectively, these data demonstrate that the lipid-lowering effect of resveratrol is mediated through the induction of autophagy in an mTOR-dependent mechanism.

Keywords: High Glucose, lipogenesis, Resveratrol, autophagy, Liver, HepG2, lipid accumulation, mTOR

Abbreviation: AMPK: AMP-activated protein kinase, ACC: Acyl COA Carboxylase, CQ: chloroquine, ATG5: autophagy related 5, FAS: Fatty Acid Synthase, FBS: Fetal bovine serum, FFA: Free fatty acids, FoxO1: Forkhead box protein O1, HG: High glucose, LC3: light chain 3, LDL: Low density lipoprotein, MTP: Microsomal transfer protein, mTOR: Mammalian target of rapamycin, SREBP1c: Sterol Regulatory Element-Binding Proteins, T2D: type 2 diabetes, TG: triglyceride, VLDL: Very low density lipoprotein.

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Citation: Gorgani-Firuzjaee S, Meshkani R. Resveratrol reduces high glucose-induced de-novo lipogenesis through mTOR mediated induction of autophagy in HepG2 cells. Acta Biochimica Iranica. 2023;1(1):32-39.

https://doi.org/10.18502/abi.v1i1.14063

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Introduction

Cardiovascular diseases, such as coronary heart disease and stroke, increase the rate of death worldwide (1). Dyslipidemia is one of the key risk factors for cardiovascular diseases (2). Dyslipidemia is characterized by increased plasma concentrations of triglycerides, reduced concentrations of high-density lipoprotein cholesterol (HDL-C), and increased concentrations of small dense low-density lipoprotein (LDL) cholesterol (3). Evidence from both animal and human studies suggests that insulin resistance in adipose and liver tissues is an important underlying cause of hypertriglyceridemia in subjects with metabolic syndrome and type 2 diabetes (T2D) (3, 4). In the hyperglycemic state observed in insulin-resistant and T2D subjects, elevated glucose levels lead to increased de-novo lipogenesis and induction of very low-density lipoprotein (VLDL) secretion from the liver (5-7).

Herbal remedies have been used in many cultures to manage some metabolic diseases. In particular, previous studies have shown that dietary intake of polyphenols is positively associated with a lower risk for cardiovascular diseases (8, 9). Resveratrol is a polyphenolic compound found in many plant families. Importantly, resveratrol was found to be the main polyphenol in the root of Polygonum Cuspidatum (1). In Chinese traditional medicine, the root of this plant has been used for the treatment of atherosclerosis (10). Resveratrol is believed to have various bioactivities, such as anti-cancer, anti-carcinogenesis, and anti-inflammatory properties. Additionally, a hypolipidemic effect of resveratrol has been shown in animal models and cell culture (11), as well as some randomized clinical trials (12). However, the molecular mechanisms underlying this effect of resveratrol have not been fully investigated.

Autophagy is a cellular housekeeping process that eliminates destroyed organelles or subcellular particles and serves as an energy source in starved situations (13). Autophagy is essential for survival, differentiation, development, and homeostasis (14). Autophagy also plays a role in the pathogenesis of several human diseases, including obesity, diabetes, and steatosis (15). In addition to autophagy functioning as a cell survival mechanism against cell death, evidence suggests that it also regulates lipid homeostasis (15). Results from liver-specific Atg7 knockout mice revealed excessive hepatic lipid accumulation and the development of fatty liver (15). Further experimental evidence suggests that autophagy may help remove excess lipid droplets in hepatocytes, a process termed lipophagy (16). It is therefore likely that the induction of autophagy might help increase the disposal of hepatic fat. In this regard, it has been reported that polyphenols might be effective in preventing hepatic fat accumulation by inducing autophagy. The induction of autophagy by resveratrol has been suggested to be a key process in mediating many beneficial effects of resveratrol, such as reducing inflammation and inducing cancer cell death (13). However, the role of autophagy in the lipid-lowering effect of resveratrol in high glucose conditions in hepatocytes remains unknown. In this study, we aimed to investigate the effect of resveratrol on hepatic de-novo lipogenesis by focusing on the AMP-activated protein kinase (AMPK)-Mammalian target of rapamycin (mTOR) and autophagy signaling pathways. Our results showed that resveratrol reduces high glucose (HG)-induced de-novo lipogenesis via activating AMPK and autophagy pathways in HepG2 cells.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and trypsin EDTA were purchased from Life Technologies (Gaithersburg, USA). Tissue culture flasks and disposable plasticware were purchased from Greiner Bio-One (Frickenhausen, Germany). Phenylmethylsulfonyl fluoride (PMSF), protease inhibitor cocktail, and Nonidet P-40 were from Roche (Mannheim, Germany). Polyvinylidene difluoride (PVDF) membrane was from Millipore (Schwalbach, Germany). ECL reagents were from Amersham Pharmacia Corp. (Piscataway, NJ, USA). Polybrene and puromycin were from Sigma. All other reagents and chemicals were from Sigma Aldrich (Taufkirchen, Germany).

Cell culture

HepG2 cells were purchased from the Pasteur Institute of Iran. Cells were maintained at 37°C in an atmosphere of 5% CO2 in DMEM containing 10% FBS, 2mM glutamine, and 1% penicillin-streptomycin. For HG treatment, an appropriate amount of glucose and mannitol as an osmotic control was dissolved in DMEM. After filtration, HepG2 cells were treated with HG (33mM) and normal glucose (NG) (5.5mM) for 24h. HepG2 cells were pretreated with resveratrol and inhibitors for 1 hour prior to glucose treatment.

Oil red O staining of HepG2 cells

After 24h of treatment, HepG2 cells were washed in cold PBS and then fixed with a 4% paraformaldehyde solution for 1h. After washing with distilled water, the cells were incubated for 5 min in 60% isopropanol. The cells were then stained for 20 min with fresh oil red O working solution (300 mg of oil red O powder added to 100 ml of 99% isopropanol). Stained cells were thoroughly washed with distilled water prior to microscopic observation. Images were captured using an Olympus camera mounted on an Olympus upright

microscope. To quantify, 250 µl of DMSO was added to dried wells and the optical density was measured at 510 nm. The results were normalized against total protein.

Determination of triglyceride

Intra- and extracellular triglyceride content were assayed using the Biovision Triglyceride Quantification Colorimetric/Fluorimetric kit.

Determination of Apo B100

Secreted apoB100 in cell supernatant was analyzed using the apolipoprotein B human ELISA kit (Abcam, USA) according to the manufacturer's instructions. The data were normalized to intracellular protein levels. Protein concentrations were assayed by the BCA protein assay kit.

Real-Time-PCR

After treatments, cells were harvested and total RNA was extracted using the RNeasy mini kit. Total RNA was reverse-transcribed using MMulv reverse transcriptase and random hexamer or oligo dT primer (Takara, Tokyo, Japan). Gene expression levels were quantified using specific primers for acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), sterol regulatory element-binding protein 1c (SREBP1c), and microsomal triglyceride transfer protein (MTP) with SYBR Green PCR Master Mix (Takara, Tokyo, Japan). The sequences of the primers used in this study are shown in Table 1 of the supplementary file. The level of target gene transcripts was normalized relative to β-actin. The amplification protocol for 40 cycles was as follows: 10s at 95°C for initial activation, 5s at 95°C for denaturation, and 20s at 60°C for annealing/extension.

Western blot analysis

Harvested cells were homogenized in modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.2% sodium deoxycholate, 0.2% SDS, 1 mM Na-EDTA, and 1 mM PMSF) supplemented with a protease inhibitor cocktail (Roche). For the detection of phosphoproteins, a buffer consisting of 50 mM HEPES, pH 7.5, 150 mM NaCl, 100 mM NaF, 10 mM EDTA, 10 mM Na4P2O7, and 2 mM NaVO4, and a protease inhibitor cocktail was used. After determining protein concentrations, equal amounts of protein were subjected to SDS-PAGE, followed by transfer onto a PVDF membrane. Blocking was carried out through a 2h incubation at room temperature with 5% non-fat dry milk or BSA for p-proteins in TBS with 0.5% Tween 20. Blots were incubated overnight with specific antibodies against SREBP1c, p-ACC, AMPK, p-AMPK, Foxo1, p-Foxo1, p-mTOR, LC3, p62, ATG5 and ATG7 (Cell Signaling Technology, Beverly, MA, USA), and β-actin (Abcam, Cambridge, MA, USA) at 4°C. After incubating with second HRP-conjugated antibodies, bands were visualized using an enhanced chemiluminescent substrate (ECL) and analyzed by ImageJ software. Each experiment was performed at least three times.

Statistical analyses

All statistical analyses were performed using SPSS18.0 (SPSS, Chicago, IL, USA). Comparisons among all groups were performed with one-way analysis of variance. If statistical significance was found, the Tukey post-hoc test was performed. Values of $p < 0.05$ were considered statistically significant. Results are expressed as the mean ± SEM of at least three independent experiments.

Results

Resveratrol reduced HG-induced lipogenesis in HepG2 cells

To investigate the importance of resveratrol in HG-induced lipogenesis, HepG2 cells were treated with 33mM glucose (HG) and resveratrol (50µM) for 24h. As shown in Fig. 1A, HG enhanced total lipid content 2.3-fold, whereas resveratrol significantly prevented the effect of HG on total lipid content. Resveratrol also significantly decreased intra- and extracellular triglyceride levels in the presence of HG (Fig. 1B-C). Additionally, resveratrol significantly reversed HG-induced apo B secretion from HepG2 cells (Fig. 1D).

Table 1: primers sequence used in the study

Primer name	Forward	Reverse
SREBP-1c	5′-TCAGCGAGGCGGCTTTGGAGCAG-3′	5′-CATGTCTTCGATGTCGGTCAG-3′
FAS	5′-CGGTACGCGACGGCTGCCTG-3′	5′-GCTGCTCCACGAACTCAAACACCG-3′
ACC	5′-TGATGTCAATCTCCCCGCAGC-3′	5′-TTGCTTCTTCTCTGTTTTCTCCCC-3′
MTP	5′-TGATATTTCAGGTGCAATGGAG-3′	5′-CGGGTTTTAGACTCACGATACC-3′
Actin	5′-ATAGCACAGCCTGGATAGCAACGTAC -3′	5′-CACCTTCTACAATGAGCTGCGTGTG -3′

Resveratrol reduced HG-induced lipogenic gene expressions

To confirm the effect of resveratrol on de-novo lipogenesis, we evaluated the expression of key genes involved in lipogenesis. While HG upregulated the expression of FAS, ACC, SREBP1c, and MTP genes in HepG2 cells, resveratrol significantly attenuated the effect of HG on the expression of these genes (Fig. 2). Importantly, the results of gene expression at the mRNA level were confirmed at the protein level, and resveratrol significantly reduced SREBP1c and p-ACC protein levels in cells treated with HG (Fig.. 3A-B).

Resveratrol enhanced p-Foxo1 and p-AMPK level in HG-treated cells

To study the mechanism of the lipid-lowering effect of resveratrol, western blot analysis for p-Foxo1 and p-AMPK was performed. Foxo1 is an important transcription factor that regulates lipogenesis, and its dephosphorylated form is active while phosphorylation changes it to an inactive form (17). In this study, HG diminished p-Foxo1 level by 0.47-fold and treatment of HepG2 cells with resveratrol reversely induced Foxo1 phosphorylation by 40% (Fig.. 3). Additionally, the results showed that resveratrol could affect p-AMPK,

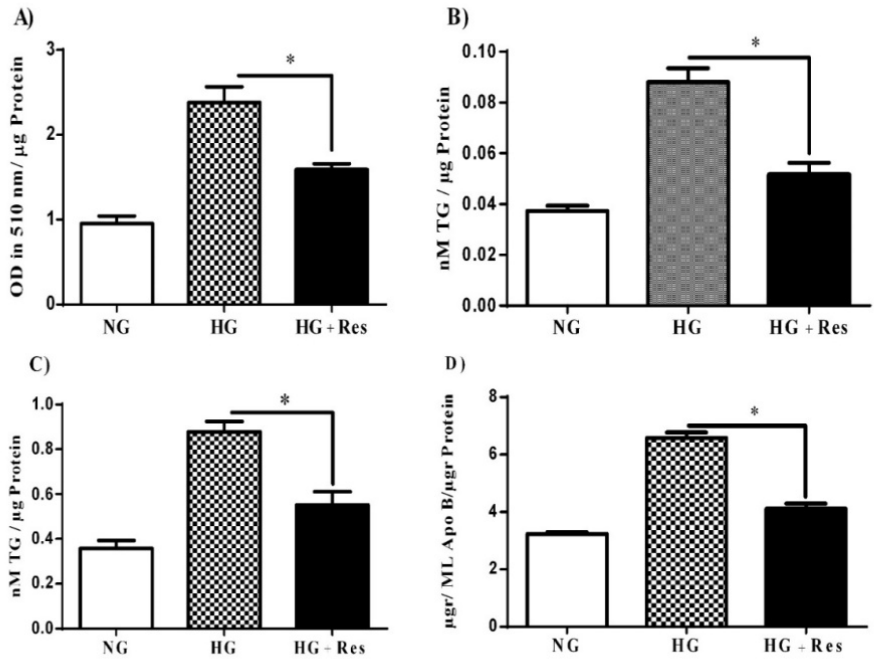

Fig.ure 1: Effect of resveratrol on HG-induced lipogenesis. (A) HepG2 cells were co-treated with 33mM glucose (HG) and 50µM resveratrol for 24h and then oil red O staining was performed. (B-C) Intracellular and extracellular triglyceride content in HepG2 cells treated with HG and resveratrol. (D) Amount of secreted apo B in the cells treated with HG and resveratrol. The results are expressed as mean ± SEM of at least three independent experiments. HG: high glucose; NG: normal glucose, Res: resveratrol. *: $p < 0.001$

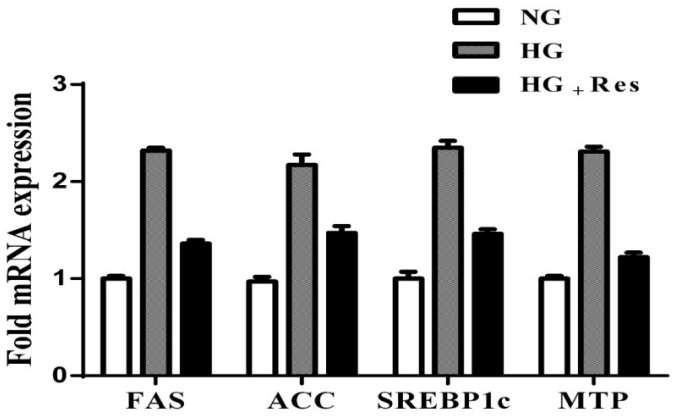

Fig.ure 2: Effect of resveratrol on expression of lipogenic genes. HepG2 cells were co-treated with 33mM glucose (HG) and 50 µM resveratrol for 24h and then real-time PCR was performed for analysis of gene expressions. The results are expressed as mean ± SEM of at least three independent experiments. HG: high glucose, NG: normal glucose, Res: resveratrol. *: $p < 0.001$

another regulator of lipogenesis in HepG2 cells. As shown in Fig. 3A and B, HG reduced p-AMPK level by about 0.33-fold, but co-treatment with resveratrol significantly increased p-AMPK level by around 47% in HG-treated cells.

Resveratrol attenuated HG-induced lipogenesis by activating autophagy pathway

To investigate the molecular mechanism by which resveratrol reduced HG-induced lipogenesis, the autophagy pathway in HepG2 cells was evaluated. HG treatment of HepG2 cells caused a reduction of LC3-II, ATG5, and ATG7 and induction of p62 protein levels (Fig. 4). Resveratrol significantly enhanced autophagy markers of LC3-II, ATG5, and ATG7 by 77%, 74%, and 47%, respectively, in HG-treated cells. Additionally, pre-treatment with chloroquine (CQ), an inhibitor of autophagy, led to enhanced total lipid and triglyceride content compared to untreated control cells (Fig. 5A and B). Importantly, co-treatment with chloroquine inhibited the preventive effect of resveratrol on HG-induced

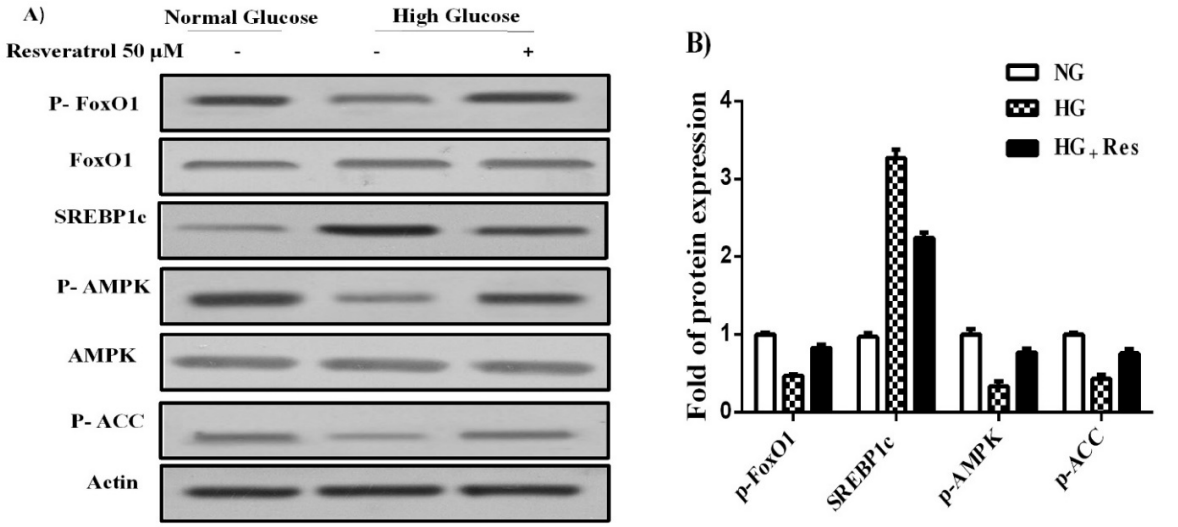

Fig.ure 3: Effect of resveratrol on the levels of important proteins involved in lipogenesis. A: HepG2 cells were co-treated with HG and resveratrol for 24h and then western blot analysis for p-ACC, p-AMPK, SREBP1c and p-Foxo1 were performed. B: Quantitative analysis of western blot results. The results are expressed as mean ± SEM of at least three independent experiments. HG: high glucose; NG: normal glucose, Res: resveratrol. *: $p < 0.001$.

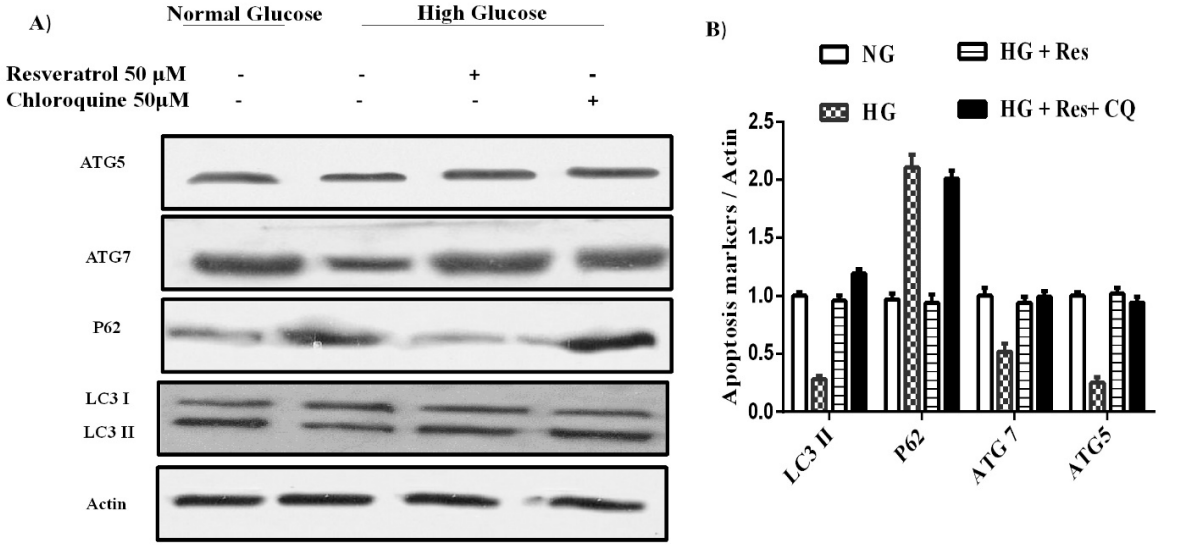

Fig.ure 4: Effect of resveratrol on autophagy markers in HG-traeted HepG2 cells. A: HepG2 cells were co-treated with HG and resveratrol for 24h and then western blot analysis was conducted using specific antibodies against autophagy markers (LC3, ATG5, ATG7 and p62). B: Quantitative analysis of western blot results. The results are expressed as mean ± SEM of at least three independent experiments. HG: high glucose; NG: normal glucose, Res: resveratrol. *: $p < 0.001$.

lipogenesis in HepG2 cells. The expression of lipogenic genes and triglyceride levels increased in resveratrol-chloroquine cells compared to cells treated with resveratrol alone (Fig. 5C). Taken together, these findings imply that the effect of resveratrol on lipogenesis in HG-treated cells might be mediated through the induction of autophagy.

Resveratrol alleviated HG-induced lipogenesis by induction of autophagy via mTOR suppression

Mammalian target of rapamycin (mTOR) has been shown to negatively regulate autophagy, and inhibition of mTOR has been shown to induce autophagic activity (18). To test whether the effect of resveratrol on autophagy is mediated through the mTOR signaling pathway, the phosphorylation of mTOR was assessed. The results showed that HG treatment induced p-mTOR level, while resveratrol significantly reversed this effect. To confirm these findings, cells were pretreated with rapamycin (20nM), an inhibitor of mTOR. As shown in Fig. 6, rapamycin induced autophagy pathway activity as measured by protein expression of LC3-II, ATG5, and ATG7. Additionally, rapamycin could ameliorate HG-induced lipogenesis in HepG2 cells. Total lipid and triglyceride levels and the expression of lipogenic genes were significantly lower in rapamycin-treated cells compared to cells treated with HG only (Fig. 7A-C). Overall, these results demonstrate that resveratrol could attenuate HG-induced lipogenesis through the induction of autophagy in an mTOR-dependent manner.

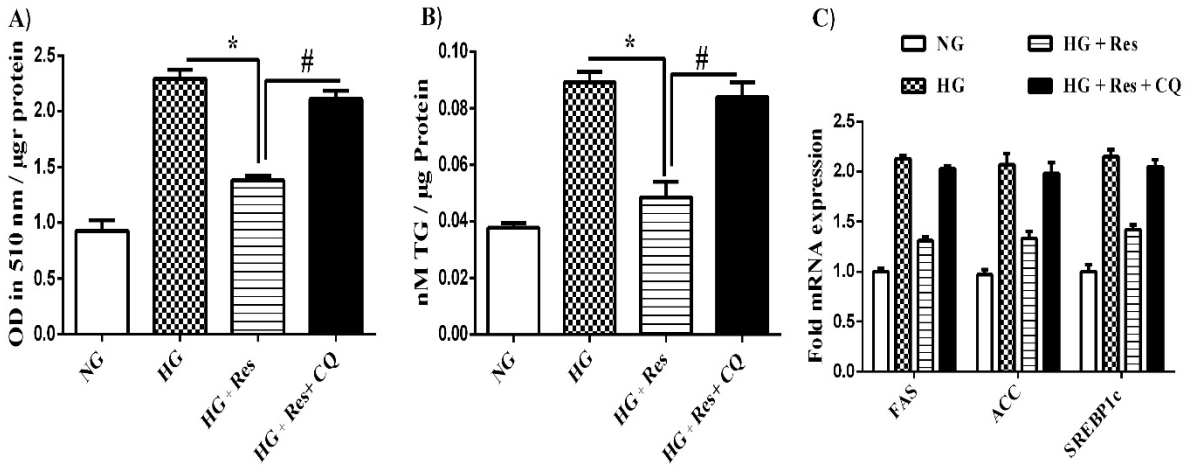

Fig.ure 5: Effect of autophagy inhibitor (chloroquine) on HG-induced lipogenesis in HepG2 cells. A: HepG2 cells were co-treated with 33mM glucose (HG), 50 µM resveratrol and 50µM chloroquine (CQ) for 24h and then oil red O staining was performed. B: Intracellular triglyceride content in HepG2 cells treated with HG, resveratrol and CQ. C: Lipogenic gene expression level in the cells treated with HG, resveratrol and CQ. The results are expressed as mean ± SEM of at least three independent experiments. HG: high glucose; NG: normal glucose, Res: resveratrol, CQ: chloroquine, *: $p < 0.001$

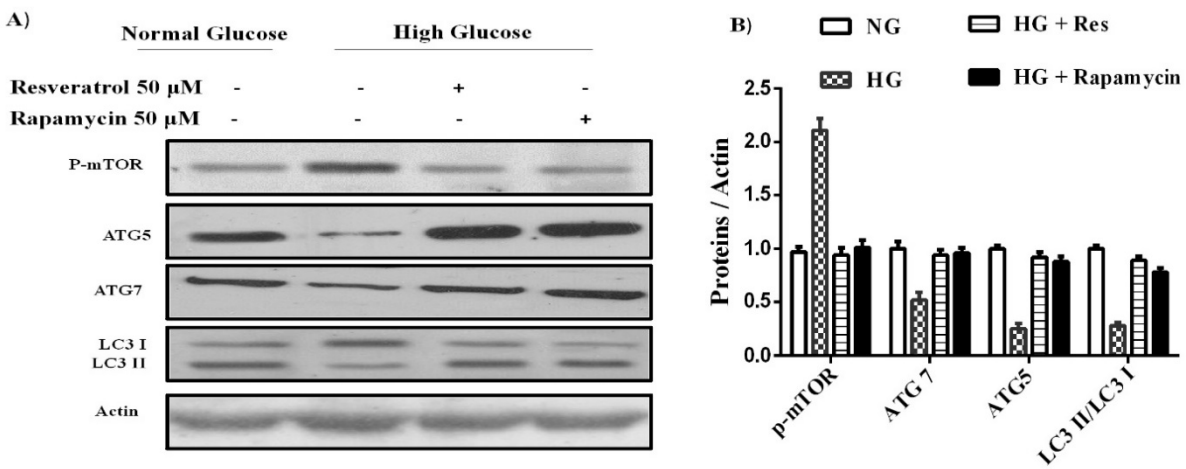

Fig.ure 6: Effect of rapamycin on autophagy markers in HepG2 cells. A: HepG2 cells were co-treated with HG, resveratrol and 50µM rapamycin for 24h and then western blot analysis was conducted using specific antibodies against autophagy markers (LC3, ATG5, ATG7, p62 and p-mTOR). B: Quantitative analysis of western blot results. The results are expressed as mean ± SEM of at least three independent experiments. HG: high glucose; NG: normal glucose, Res: resveratrol. *: $p < 0.001$.

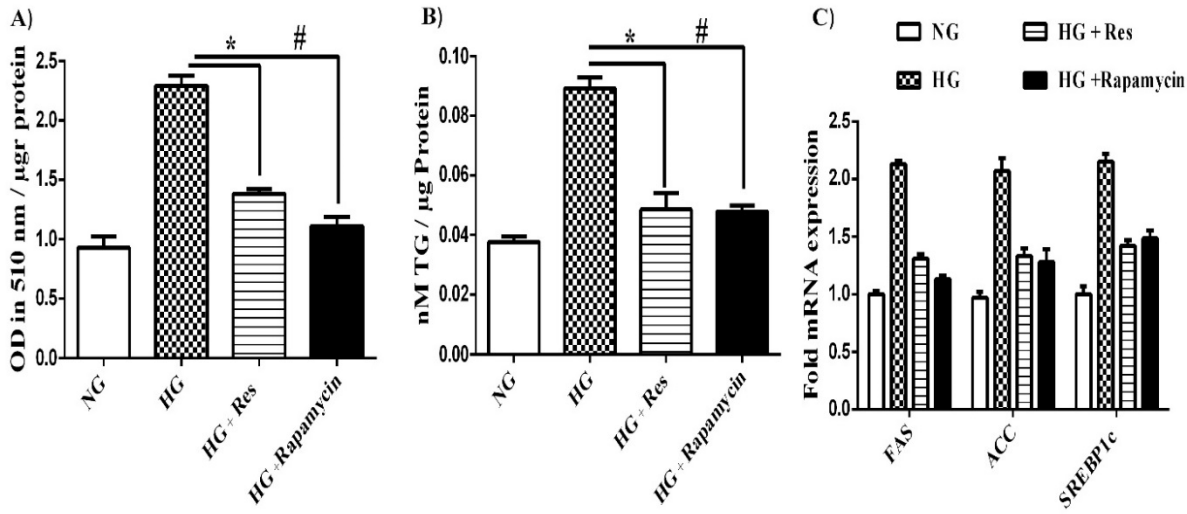

Fig.ure 7: Effect of rapamycin on HG-induced lipogenesis in HepG2 cells. A: HepG2 cells were co-treated with HG, resveratrol and 50µM rapamycin for 24h and then then oil red O staining was performed. B: Intracellular triglyceride content in HepG2 cells treated with HG, resveratrol and rapamycin. C: Lipogenic gene expression level in the cells treated with HG, resveratrol and rapamycin. The results are expressed as mean ± SEM of at least three independent experiments. HG: high glucose; NG: normal glucose, Res: resveratrol. *: $p < 0.001$.

Discussion

Hepatic overproduction of VLDL and consequent hypertriglyceridemia are commonly observed in T2D and metabolic syndrome. It is well established that dyslipidemia is recognized as a prominent risk factor for cardiovascular diseases (19). Therefore, therapeutic strategies targeting hepatic lipogenesis could play an effective role in the management of these pathologies. Recently, natural plant resources have received attention as sources of biologically active substances, including antioxidants (20). In this regard, resveratrol, a natural compound present in grapes and other fruits and food products, has been shown to have antioxidant, anti-aging, anti-inflammatory, and anti-cancer properties (20). In this study, we aimed to evaluate the molecular mechanism of the hypolipidemic effect of resveratrol in hepatocytes exposed to HG concentrations.

The results of this study demonstrated that resveratrol significantly prevented HG-induced de-novo lipogenesis, as demonstrated by reduced expression of lipogenic genes such as FAS and ACC and decreased triglyceride production in HepG2 cells. FAS and ACC are key enzymes in de-novo fatty acid and triglyceride synthesis in mammals. SREBP-1c is a transcription factor responsible for gene expression of these lipogenic enzymes in the liver (21). AMPK is a major regulator of energy metabolism. AMPK suppresses SREBP-1c cleavage and nuclear translocation via SREBP-1c Ser372 phosphorylation, repressing SREBP-1c target gene expression in hepatocytes exposed to high glucose, leading to reduced lipogenesis and lipid accumulation (22). Our results showed that the effect of resveratrol on HG-induced hepatic lipogenesis in HepG2 cells is mediated through the enhancement of p-AMPK level followed by decreased expression of SREBP-1c and its downstream lipogenic genes such as FAS and ACC.

Another finding of this study was that resveratrol could ameliorate HG-induced VLDL secretion in HepG2 cells. Foxo1 regulates the transcription of MTP, which is involved in the hepatic assembly of VLDL. Insulin activation of Akt leads to phosphorylation of Foxo1, resulting in nuclear exclusion and loss of transcriptional activity. Reduced insulin action increases Foxo1 activity and induces MTP, favoring VLDL assembly (23). In this study, resveratrol was able to increase phosphorylation of Foxo1 and decrease apo B content and MTP expression in HepG2 cells. Supporting these findings, it has been reported that resveratrol administration was able to increase Foxo1 protein levels and decrease the generation of reactive oxygen species (24) and reverse changes associated with fatty acid overloading in 3T3-L1 adipocytes (25). Taken together, these findings suggest that resveratrol leads to decreased VLDL production in high glucose conditions by reducing lipid content (triglyceride) and protein content of VLDL (apoB) and suppressing the responsible protein in VLDL assembly (MTP).

Autophagy is a degradative process that targets cellular components to the lysosome, and recent studies have indicated a role for autophagy in regulating hepatic lipid metabolism (26). Studies in cultured hepatocytes that lacked autophagy by pharmacological inhibition with 3-methyladenine or by using RNA interference against ATG5 and ATG7 have revealed that inhibition of autophagy leads to increased hepatocellular triglyceride accumulation (15). To address the molecular mechanism by which resveratrol prevents lipid accumulation in HepG2 cells, we targeted the autophagy pathway. In this study, accumulation of LC3-II followed by reduced

p62 protein levels indicates that resveratrol enhances autophagic flux in hepatocytes. Further experiments using an inhibitor of autophagy, chloroquine, also suggested that resveratrol-induced autophagy could reduce lipid accumulation in HepG2 cells.

Autophagy is regulated by complex signaling networks, and mTOR is a well-established negative regulator of autophagy. To reveal the role of mTOR signaling in resveratrol-induced autophagy, the phosphorylation of mTOR was evaluated. The results showed that resveratrol suppressed the level of p-mTOR in hepatocytes, indicating that the mTOR-dependent pathway may participate in resveratrol-induced autophagy. Furthermore, rapamycin, an inhibitor of mTOR, could reduce HG-induced lipogenesis in HepG2 cells. Taken together, these findings suggest that resveratrol could attenuate HG-induced lipogenesis by activating autophagy in an mTOR-dependent pathway.

In conclusion, this study demonstrated that resveratrol significantly enhances p-Foxo1 and p-AMPK levels and reduces the expression of lipogenic genes, leading to a reduction of lipid accumulation in HepG2 cells. The lipid-lowering effect of resveratrol can be attributed to autophagy induction by an mTOR-dependent mechanism. These findings suggest that resveratrol administration may be beneficial for the prevention of hyperglycemia-induced dyslipidemia in patients with metabolic syndrome and T2D.

Acknowledgement

This work was financially supported by a grant (grant 95-01-30-31664) from the Deputy of Research, Tehran University of Medical Sciences.

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