

# Effect of urotensin II on apolipoprotein B<sub>100</sub> and apolipoprotein A-I expression in HepG2 cell line

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## Abstract

**Background:** Increased apolipoprotein B100 (apo B) and decreased apolipoprotein A-I (apo A-I) production are important risk factors in atherosclerosis. Urotensin II (UII), as the most potent vasoconstrictor in human, is related with hypertension and probably atherosclerosis. Because of the relationship between the hypertension and lipoprotein metabolism in atherosclerosis, the aim of this study was to test the effect of urotensin II on apo B and apo A-I expression in hepatic (HepG2) cell line.

**Materials and Methods:** HepG2 cells were treated with 10, 50, 100, and 200 nmol/L of urotensin II ( $n = 6$ ). Relative apo B and apo A-I messenger RNA (mRNA) levels in conditioned media, normalized to glyceraldehyde-3-phosphate dehydrogenase, were measured with quantitative real-time polymerase chain reaction method. In addition, apo B and apo A-I levels were also estimated and compared with the controls using the western blotting method. Data were analyzed statistically by ANOVA and non-parametric tests.

**Results:** The apo B mRNA levels were not increased significantly following the treatment with UII. However, apo B protein levels were increased significantly after the treatment with urotensin II, especially at 100 and 200 nmol/L. The apo A-I mRNA and protein levels in conditioned media also were not significantly changed. However, there was a significant decrease in apo A-I mRNA and protein levels at 200 nM UII.

**Conclusions:** UII might increase apo B at protein level probably through participating factors in its synthesis and/or stability/degradation. In addition, UII may have decreasing effect at more than 200 nM concentrations on apo A-I.

**Key Words:** Apolipoprotein B100, apolipoprotein A-I, expression, HepG2, urotensin II

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## INTRODUCTION

Dyslipidemia, particularly increase of low density lipoproteins (LDL) and/ or decrease of high density lipoproteins (HDL) levels play a key role in pathogenesis of atherosclerotic cardiovascular diseases.<sup>[1,2]</sup> Apolipoprotein B100 (apo B) is essential for the intracellular assembly, secretion and cellular uptake of LDL.<sup>[3]</sup> Apolipoprotein A-I (apo A-I), the major protein on HDL surface, contributes in reverse cholesterol transport.<sup>[4]</sup> Therefore, increased

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apo B and /or decreased apo A-I are related to atherosclerosis.<sup>[5,6]</sup>

Urotensin II (UII), as the most potent vasoconstrictor factor in human,<sup>[7]</sup> via its receptor (UT) contributes in development of atherosclerosis and hypertension through different mechanisms, such as vascular smooth muscle cell proliferation<sup>[9]</sup> and formation of foam cells.<sup>[10]</sup>

Since the relationship between the hypertensive factors and lipoprotein metabolism in atherogenesis is not clear entirely, the aim of this study was to investigate the effect of urotensin II on apo B and apo A-I expression in hepatic (HepG2) cell line.

## MATERIALS AND METHODS

### Cell culture

Hepatic (HepG2) cells (Pasteur institute/Iran) were maintained in high glucose Dulbecco's modified Eagle medium (DMEM) (Sigma/ USA) containing 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 µg/mL), in a humidified incubator at 37°C and 5% CO<sub>2</sub>. Cell viability was determined by trypan blue exclusion and was greater than 95% in all experiments. For the subculture, the medium was removed and the cells were detached from the culture dish with 0.25% trypsin diluted in phosphate buffer saline (PBS) containing 0.2 g/l EDTA. Culture medium with FBS was added to stop trypsinization. Cells were seeded on 35 mm culture dishes for all experiments.<sup>[11]</sup>

### Experimental media preparation and treatment

After 48 h (doubling time of cells) cells were washed two times with PBS and once with serum free culture media. Then, experimental media (DMEM + 0.5% human serum albumin (HSA))<sup>[12]</sup> containing human urotensin II (hUII) (Sigma/ USA) at the following concentrations was added. Final concentrations of UII in culture media were 10, 50, 100 and 200 nM.

### Total RNA extraction

Total RNA was extracted from cells 24 h after treatment using the RNeasy Plus Mini kit (Qiagen/ Germany). All steps were performed as recommended by the manufacturer. RNA was quantified using a NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies/Thermo Fisher Scientific Inc, USA), and RNA quality was assessed by electrophoresis on 1% agarose gel. The ratio of the optical density at 260/280 nm exceeded 2.0 for all RNA samples.<sup>[11]</sup>

### Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was used to quantify the

expression of specific genes [Table 1]. First, cDNA was synthesized using the QuantiTect Reverse Transcription kit (Qiagen/Germany). Reverse transcription was performed using 800 ng of total RNA and random hexamer and oligo dT primers according to the manufacturer's instructions. Then, the qRT-PCR was performed using SYBR Fast kit (Qiagen/Germany) by Rotor-Gene 6000 qPCR machine (Qiagen/Germany). The stages were 95°C for 5 min for activation of HotStart Polymerase, 40 cycles at 95°C for 10 s, and 60°C for 30 s (annealing/extension combined step). Melting curve analysis was performed by increasing the temperature (1°C/s) from 55 to 95°C, with continuous fluorescence acquisition.

Primer sequences were obtained from Gene Cards (The Human Gene Compendium) database and validated in NCBI BLAST-primer. PCR conditions for all primers were optimized, and specificities of all reactions were verified by melting curves and electrophoresis on 1% agarose gels. Primers for target genes and reference gene are shown in Table 1.

Relative changes in apo B and apo A-I mRNA levels were calculated using the  $\Delta(\Delta Ct)$  method ( $2^{-\Delta\Delta Ct}$ ) and normalized based on Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels.<sup>[13]</sup>

### SDS-PAGE

The protein samples were extracted from HepG2 cells 24 h after the treatment, by Radio-Immunoprecipitation Assay (RIPA) lysis buffer (Sigma/ USA) containing protease inhibitor cocktail (Sigma/Saint Louis, USA) and phenylmethanesulfonyl fluoride (PMSF) (Sigma/ USA). After determination of the protein concentrations by Bradford method<sup>[14]</sup> and also direct protein assay at 280 nm using a NanoDrop Spectrophotometer ND-1000, one hundred micrograms of protein from each prepared and denatured samples with modified Laemmli buffer were loaded in 7% (for apo B) and 12% (for apo A-I and GAPDH) linear tricine gels with a 5% stacking gel and the buffer system. The 7% and 12% gels were run at 35 mA for 3.5 h and 1.5 h, respectively, by BioRad Tetra cell mini protein apparatus (Bio-Rad Laboratories Inc/USA).

**Table 1: Quantitative RT-PCR primer sequences**

Genes*	Primers	Amplicon size (bp)
GAPDH (Reference gene)	Forward: TGCACCACCAACTGCTTAGC	87
	Reverse: GGCATGGACTGTGGTCATGAG	
Apo A-I	Forward: GTGGATGTGCTCAAAGACAGCG	124
	Reverse: GCTTGCTGAAGGTGGAGGTCAC	
ApoB100	Forward: AGAGGACAGAGCCTTGGTGGAT	114
	Reverse: CTGGACAAGGTCATACTCTGCC	

\*GAPDH: Glyceraldehyde 3 phosphate dehydrogenase; ApoA-I: Apolipoprotein A-I; ApoB: Apolipoprotein B100

### Western blotting

After electrophoresis, samples were transferred to a polyvinylidene fluoride (PVDF) membrane.<sup>[15]</sup> Then, the membranes were incubated by 5% skim milk (Merck/Germany) in Tris buffered saline (pH 7.4) containing 0.5 v/v Tween 20 (Panreac/Spain) TBST at room temperature overnight. The membranes were then incubated with the rabbit anti-human-apo B (1:5000), rabbit anti-human-apo A-I (1:1000) and the rabbit anti-human-GAPDH (1:2500) primary antibodies (Abcam/USA) at room temperature for 1 h. The membrane was then incubated with a horseradish peroxidase conjugated goat-anti-rabbit antibody (1:10000) (Abcam/USA) for 1 h at room temperature. All above antibodies were diluted in TBST containing 0.5% Tween 20. The membranes were then immersed in enhanced chemiluminescence (ECL plus of Perkin Elmer/Netherlands) reagent and exposed to film (HyBlot CL Autoradiography Film of Denville Scientific Incorporation/USA) at a dark room. After development, films were scanned and signals were quantified using Image J software.

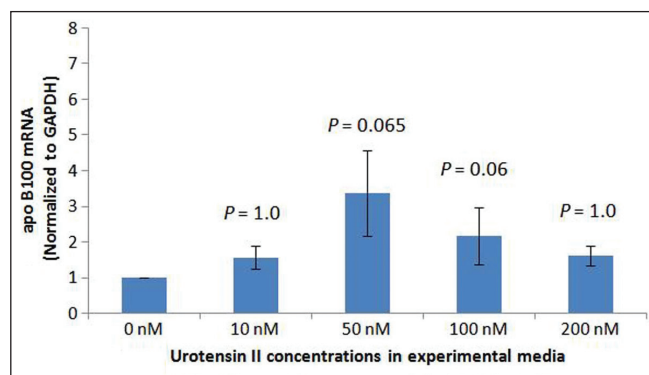
### Data analysis

All of these treatment experiments have been repeated 6 times ( $n = 6$ ) and interpretation of data was performed on mean  $\pm$  SEM. Relative gene expression data comparisons were analyzed for statistical significance using Kruskal-wallis and Mann-Whitney tests. In addition, protein synthesis levels were analyzed using the ANOVA test compared with the control group. Results are presented as least-square means and SE of the mean. Probability values  $<0.05$  were considered statistically significant.

## RESULTS

### The effect of urotensin II on apo B100 mRNA expression in HepG2 cells

Relative apo B mRNA, normalized to GAPDH mRNA, were  $1.55 \pm 0.3$ ,  $3.4 \pm 1.0$ ,  $2.2 \pm 0.8$  and  $1.6 \pm 0.28$  in cells



**Figure 1:** The effect of urotensin II on apo B100 mRNA expression. HepG2 cells were treated with the indicated concentrations of urotensin II for 24 h, and apo B and GAPDH mRNA levels were measured by qRT-PCR. Urotensin II at 50 and 100 nM concentrations increased apo B mRNA levels, but they were not significant. \* $P=0.63$ , treated vs. control cells;  $n=6$ .

treated with 10, 50, 100 and 200 nmol/L urotensin II, respectively (not significant,  $P = 1.0$ ,  $P = 0.065$ ,  $P = 0.06$ ,  $P = 1.0$ , respectively) [Figure 1]. The GAPDH mRNA levels did not change significantly with the treatment.

### The effect of urotensin II on apo B100 expression at protein level in HepG2 cells

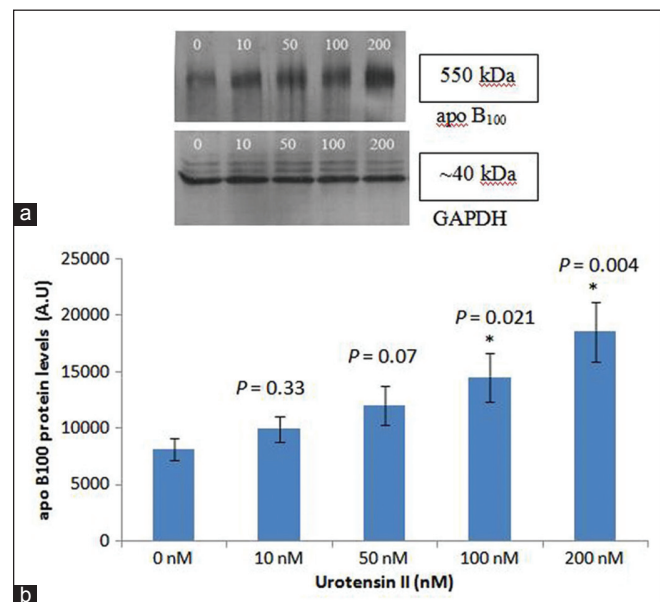
The apo B levels (in arbitrary unit; AU) increased from  $7972 \pm 971$  AU in the control cells to  $9640 \pm 1135$ ,  $11351 \pm 1742$ ,  $17646 \pm 2138$  and  $22981 \pm 2650$  AU in cells treated with 10, 50, 100 and 200 nM urotensin II, respectively (significant,  $P = 0.33$  and  $P = 0.07$ ,  $P = 0.021$  and  $P = 0.004$  respectively). GAPDH levels did not change with urotensin II treatment, suggesting that urotensin II specifically increases apo B protein [Figure 2].

### The effect of urotensin II on apoA-I mRNA expression in HepG2 cells

Relative apo A-I mRNA, normalized to GAPDH, were  $1.42 \pm 0.49$ ,  $2.0 \pm 0.77$ ,  $1.13 \pm 0.21$  and  $0.63 \pm 0.09$  in cells treated with 10, 50, 100 and 200 nmol/L urotensin II, respectively (not significant,  $P = 1.0$ ,  $P = 0.3$ ,  $P = 0.3$ ,  $P = 0.04$ , respectively) [Figure 3]. The GAPDH mRNA levels did not change significantly with the treatment.

### The effect of urotensin II on apo A-I expression at protein level in HepG2 cells

The apo A-I levels (in arbitrary unit; AU) were not changed significantly from  $4986 \pm 387$  AU in the control cells to



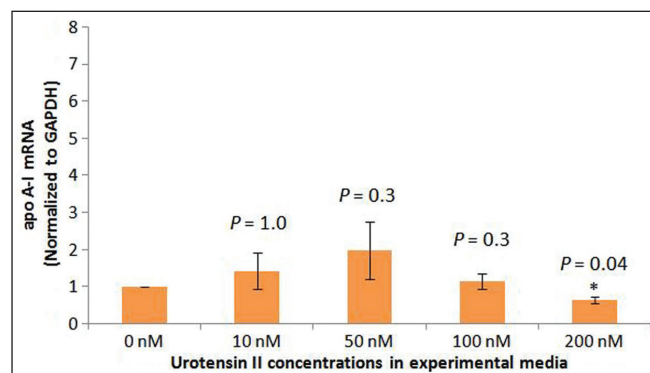
**Figure 2:** The effect of urotensin II on apo B100 expression in HepG2 cells. HepG2 cells were treated with 10, 50, 100 and 200 nmol/L urotensin II for 24 h; and apo B after total protein extraction from cells was measured by Western blot (a). Band densities were measured by densitometry (arbitrary units) and are shown in panel B. Urotensin II at different concentrations increased apo B levels significantly. \* $P < 0.005$ , treated vs. control cells;  $n = 6$

5434 ± 557, 4965 ± 476, 4621 ± 322 AU in cells treated with 10, 50 and 100 nmol/L urotensin II, respectively and decreased to 3934 ± 182 AU in the cells treated with 200 nmol/L urotensin II (not significant,  $P = 0.52$  and  $P = 0.63$ ,  $P = 0.48$  and  $P = 0.034$  respectively). GAPDH levels did not change with urotensin II treatment [Figure 4].

## DISCUSSION

Apolipoprotein B100 (apo B), the major structural component of apo B-containing lipoproteins including VLDL, IDL and LDL, has a key role in intracellular assembly and secretion of triglyceride rich lipoproteins and acts as a ligand for LDL receptors on the cell surfaces.<sup>[3,16]</sup> Hepatic regulation of apo B secretion is generally considered to occur post-translationally, via modulating the ratio of newly synthesized apo B molecules to the degraded portion within the cell. However, transcriptional regulation may also play a minor role.<sup>[17]</sup> Several factors have been suggested to play key roles in the translational and post-translational regulation of apo B secretion, including hormonal environment, and hepatic lipid availability and composition.<sup>[17,18]</sup> Plasma apo B levels are directly related to total cholesterol and LDL numbers. Thus, abnormal increases of plasma apo B can have deleterious effects on cardiovascular system.<sup>[5,6]</sup> Therefore, studies designed to understand the factors affecting hepatic apo B-containing lipoprotein production, assembly and secretion are important.<sup>[17]</sup>

High density lipoproteins (HDL), as antiatherogenic lipoproteins, act in prevention of atherosclerosis, in particular through reverse cholesterol transport from peripheral tissues to the liver.<sup>[4]</sup> Apolipoprotein A-I, as the major protein on HDL surface, is a ligand for adenosine triphosphate-binding cassette (ABC) transporters which mediate efflux of cholesterol from cells to HDL particles.<sup>[19]</sup>



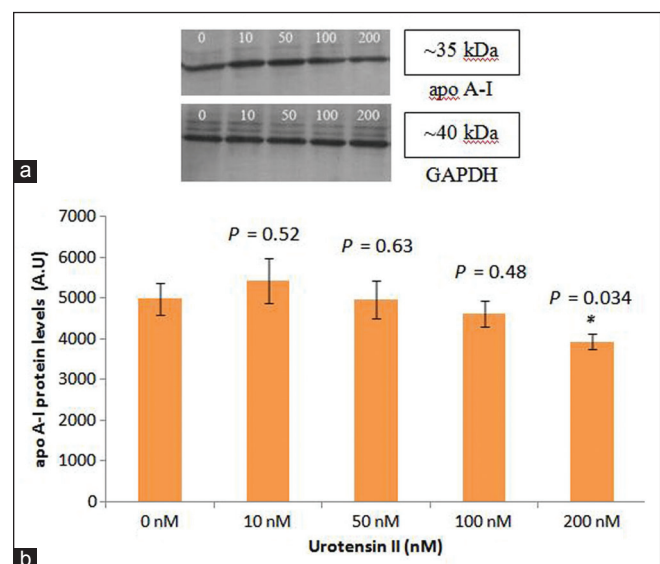
**Figure 3:** The effect of urotensin II on apo A-I mRNA expression. HepG2 cells were treated with the indicated concentrations of urotensin II for 24 h, and apo A-I and GAPDH mRNA levels were measured by qRT-PCR. Urotensin II at 200 nM concentration decreased apo A-I mRNA levels significantly. \* $P = 0.15$ , treated vs. control cells;  $n = 6$

Many factors may participate in development of hypertension. Vasoconstrictors, including angiotensin II, serotonin, thromboxane A2, endothelin-1 and urotensin II, are major factors in hypertension development and accelerate atherosclerosis process via inflammatory mechanisms.<sup>[20]</sup> Among these, urotensin II is the most potent vasoconstrictor known to date;<sup>[7]</sup> it was first isolated from the urophysis of teleost fish.<sup>[7,21]</sup> The human urotensin II (h-UII) isoform, is the endogenous ligand of the orphan G-protein coupled receptor, known as UT receptor.<sup>[22]</sup>

UII /UT system are both present in wide array of tissues, including brain, cardiomyocyte, endothelium and vascular smooth muscle cells, kidney and the liver.<sup>[21,23]</sup> It has prompted investigations on UII as a potential regulator of cardiovascular function and pathophysiology.<sup>[23]</sup>

UII acts through autocrine/paracrine mechanism on the cells, the plasma concentration of UII in physiologic condition is very low (at picomolar range).<sup>[9]</sup> In addition, different *in vitro* studies<sup>[10,24-26]</sup> have used UII at nanomolar concentrations. Hence, we used UII at nmol/L concentrations which had used previously.

UII may play a role in atherosclerosis via several mechanisms and many researchers have studied on the effect of UII on participating mechanisms in atherosclerosis.



**Figure 4:** The effect of urotensin II on apo A-I expression in HepG2 cells. HepG2 cells were treated with 10, 50, 100 and 200 nmol/L urotensin II for 24 h; and apo A-I after total protein extraction from cells was measured by Western blot (a). Band densities were measured by densitometry (arbitrary units) and are shown in panel B. Urotensin II at 200 nM concentration decreased apo A-I levels significantly. \* $P = 0.144$ , treated vs. control cells;  $n = 6$

Urotensin II can induce endothelial cells and VSMC proliferation,<sup>[9]</sup> inhibits endothelial cells apoptosis,<sup>[26]</sup> increases cardiomyocyte hypertrophy through increase in the inflammatory cytokines,<sup>[27]</sup> induces inflammation-related atherosclerosis via increase of the inflammatory cytokines (such as interleukin-6),<sup>[26]</sup> and accelerates the formation of macrophage-derived foam cells through up-regulation of acyl coenzyme A cholesterol acyl transferase-1 (ACAT1).<sup>[10]</sup> Evidence has been shown that UII with oxidized LDL has a synergistic effect on the VSMC proliferation.<sup>[24]</sup> Further, the levels of UII and UT receptor are up-regulated within the atherosclerotic plaque and injured vascular wall.<sup>[26,28]</sup> These data support the idea that UII may be involved in the pathogenesis of atherosclerosis.

Since apo B overproduction and low production of apo A-I is two important risk factors for atherogenesis,<sup>[6,29]</sup> and urotensin II also has been suggested to have a role in atherosclerosis,<sup>[21,23]</sup> we investigated the effect of UII on apo B and apo A-I expression in hepatic (HepG2) cells.

To date, many studies have been performed in related to effects of different factors, including endogenous and exogenous factors (such as drugs), on apo B and apo A-I metabolism, their gene expression, synthesis in, and their secretion from hepatic cells.

Evidence shows that UII levels are elevated in hyperlipidemic patients.<sup>[30]</sup> Kiss *et al.* in their study on UII knockout (UII KO) mice have shown that UII KO macrophages had enhanced ACAT activity and LDL uptake in the short term. In addition, cholesterol amounts and apo B secretion from UII KO hepatocytes were lower than wild type mice.<sup>[31]</sup> This data suggest that UII perhaps contributes in cholesterol metabolism and also apo B-containing lipoproteins synthesis and secretion from hepatocytes.

Wilcox and her coworkers have shown that the ACAT inhibitor, CI1011, inhibits cholesteryl ester (CE) mass in HepG2 cells via inhibiting ACAT activity and decreases apo B secretion through increase of intracellular degradation of apo B. However, another ACAT inhibitor, DuP128, inhibited ACAT and CE mass more than CI1011, without any effect on apo B secretion.<sup>[17]</sup> According to them, one of the important mechanisms which affect apo B secretion is degradation of this protein. Therefore, in our study, also UII might have increased the apo B levels via decrease of its degradation. Wilcox *et al.* also have shown that both CI1011 and DuP128 did not affect apo B expression at the mRNA level.<sup>[17]</sup> We also did not see any significant effects of UII on apo B mRNA concentrations.

Wilcox *et al.* also showed the effects of HMG-CoA reductase inhibitors, atorvastatin and simvastatin, on apo B secretion from hepatocytes.<sup>[32]</sup> In their study, atorvastatin, like CI1011,<sup>[17]</sup> decreased apo B secretion via increasing its degradation; however, simvastatin did not show any effect. In their study, they showed atorvastatin had reduced the microsomal triglyceride transfer protein (MTP) mRNA, but had no effect on apo B mRNA levels. Since MTP is one of the key factors for apo B-containing lipoprotein assembly and has an important role in secretion and stability of apo B, atorvastatin might have decreased apo B secretion via decreasing MTP gene expression.<sup>[32]</sup> According to our results, UII could increase the apo B protein levels. Since, one of the important mechanisms in apo B stability in hepatocytes is dependent to MTP activity; UII may affect apo B intracellular stability via MTP expression.

Yanagita *et al.* have reported that addition of taurine to HepG2 cells decreases triglyceride and cholesterol levels, inhibits ACAT activity and reduces secretion of apo B.<sup>[33]</sup> In fact, the decrease of TG due to inhibition of (<sup>14</sup>C) oleate incorporation into lipogenesis by taurine, may have caused reduction of apo B secretion from cells.<sup>[33]</sup> We did not examine the effect of UII on TG synthesis; since there was a significant increasing effect of urotensin II on apo B protein levels, it is possible that UII has increased apo B incorporation into VLDL particles through an increase in lipogenesis in hepatic cells.

We investigated the effect of UII on apo B expression, at mRNA and protein levels, in HepG2 cells. According to the results of this study, UII at 50 and 100 nmol/L concentrations can increase apo B mRNA levels 3.4 and 2.2 times, respectively. But, these results were not significant. However, UII increased apo B protein levels to 1.2, 1.4, 1.7 and 3.1 times of the control at 10, 50, 100 and 200 nmol/L of UII, respectively. We observed a discrepancy between the mRNA and protein of apo B after the treatment with UII. This is not surprising, since cellular levels of an expressed protein depend on several factors (other than mRNA abundance), such as translational efficiency of the coding mRNA and protein stability/degradation.<sup>[11]</sup>

According to the results of this study, we did not see any effect of UII on apo A-I expression at mRNA and protein levels. However, there was a significant decrease in apo A-I mRNA and protein levels at 200 nmol/L urotensin II compared with the control; therefore, UII at higher concentrations may have more decreasing effects on apo A-I levels. Since the UII has decreasing effect on both mRNA and protein levels of apo A-I; it

may decrease apo A-I production through affecting on transcription.

Haas *et al.* have shown that apo A-I mRNA and protein levels were increased after treatment of HepG2 cells by nicotinic acid. They have also measured the kinetics of apo A-I mRNA induction following nicotinic acid treatment in time-dependent manner. Another potent part of their study was the determination of molecular mechanism of the effects of nicotinic acid via its responsive element on apo A-I promoter.<sup>[19]</sup> We measured the apo A-I mRNA levels after treatment with UII in time-dependent experiments, but there were not significant changes between treated cells compared with control cells (Data not shown).

Matsuura, Oku and their coworkers have shown that adiponectin, one of the important molecules for inhibiting of atherosclerosis development which released by adipose tissue, accelerates the synthesis of apo A-I in HepG2 cells. Therefore, it is suggesting that adiponectin might increase HDL assembly in the liver.<sup>[34,35]</sup> In present study, we observed no significant effect of UII on apo A-I mRNA and protein levels. However, UII could decrease apo A-I mRNA and protein levels at the highest experimental concentration, significantly.

In other studies about the effects of other molecules on apo A-I expression and secretion, Lamou-Fava has reported that phytoestrogens, in particular genistein, can increase apo A-I secretion from HepG2 cells in dose-dependent manner.<sup>[36]</sup> Qin *et al.* also have shown that pioglitazone, an anti-diabetic drug, can increase the production and secretion of apo A-I in HepG2 cells. Therefore, this drug may increase the HDL in diabetic patients via stimulating the de novo hepatic synthesis of apo A-I.<sup>[37]</sup>

As it was seen above, *in vivo* and *in vitro* studies have done about the effects of administrative drugs (such as statins) on lipoproteins and lipids metabolisms. It seems combined effects of anti-hyperlipidemic and anti-hypertensive drugs, such as combined treatment by statins and UT antagonists may be useful for controlling of simultaneous hypertension and atherosclerosis.

In summary, we did not see any significant effect of UII on apo B mRNA; however apo B protein levels were increased significantly following the treatment with UII, particularly in higher concentrations. In addition, we did not see any significant effect of UII on apo A-I expression at both mRNA and protein levels; however, there was a significant decrease of apo A-I mRNA and protein level in the highest concentration,

200 nmol/L UII. Therefore, UII may be involved in atherogenesis via affecting the intracellular apo B stability/degradation. Urotensin II may also affect the HDL in high concentrations, via decreasing apo A-I.

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