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Research Paper

Effect of Urotensin II on Paraoxonase-1Gene Expression in HepG2 Cell Line

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ABSTRACT

Objective(s): Paraoxonase-1, an enzyme associated with high density lipoproteins, may have role in prevention of atherosclerosis via negative effect on lipid peroxidation in low density lipoproteins. Urotensin II, as the most potent vasoconstrictor in human, is related with hypertension and probably atherosclerosis. Since hypertension due to Urotensin II and decreased Paraoxonase-1 levels and/ or its activity are associated with atherosclerosis, the aim of this study was to test the effect of Urotensin II on Paraoxonase-1 gene expression at mRNA and protein levels in hepatic (HepG2) cell line.
Material and Methods: HepG2 cells were treated with 10, 50, 100 and 200 nanomolar of Urotensin II. Relative Paraoxonase-1 mRNA levels from cells which were extracted from conditioned media, normalized to glyceraldehyde-3-phosphate dehydrogenase, were measured with quantitative real-time PCR method. In addition, Paraoxonase-1 levels were also estimated and compared with the controls using western blotting method.
Results: The Paraoxonase-1 mRNA level increased significantly following treatment with 10 and 50 nanomolar, but it was decreased at 200 nanomolar of Urotensin II. Paraoxonase-1 concentrations in conditioned media were also significantly increased at 10 and 50 nanomolar. However, there was no significant change in Paraoxonase-1 mRNA and protein levels at 100 nanomolar concentration of Urotensin II.
Conclusions: There is controversy about the effect of Urotensin II on Paraoxonase-1 gene expression in dose dependent manner. Urotensin II may increase Paraoxonase-1 mRNA and protein at some lower concentrations in HepG2 cells. However, it may decrease Paraoxonase-1 mRNA and probably protein levels at higher amounts.

Keywords: Paraoxonase-1, Urotensin II, Gene expression, HepG2.

INTRODUCTION

High density lipoprotein (HDL) particles have antioxidant activity via enzyme activity of Paraoxonase-1 (PON1) (Durrington et al., 2001). Human serum PON1 is a calcium dependent esterase which can hydrolyze some organophosphates such as paraoxon and arylesters including phenylacetate. PON1 is synthesized in liver principally and assembled in HDL particles. PON1 probably prevents atherosclerosis by preventing LDL from oxidative modifications (Durrington et al., 2001; Mackness et al., 2004). Evidences have shown decreased level and/or enzyme activity of PON1 is related to atherosclerosis (Durrington et al., 2001).

Urotensin II (UII), the most potent vasoconstrictor factor in human (Ames et al., 1999), and its receptor (UII receptor or UT) are both present in a broad array of tissues such as brain, cardiomyocyte, vascular endothelium and smooth muscle cells, kidney and liver (Ames et al., 1999; Richards and Charles, 2004).

UII contributes in development of atherosclerosis and hypertension via different mechanisms, including induce of cell proliferation in endothelial and vascular smooth muscle cells (Bousette et al., 2004), production of inflammatory cytokines (Johns et al., 2004) and acceleration of foam cells forming (Watanabe et al., 2005).

Table 1. Quantitative RT-PCR primer sequences.

Genes	Primers	Amplicon size (bp)
GAPDH (Reference gene)	Forward: TGCACCACCAACTGCTTAGC Reverse: GGCATGGACTGTGGTCATGAG	87
PON1	Forward: ATGCTCTCCGAGAGGTACAACC Reverse: GCCAGTCCATTAGGCAGTATCTC	103

*GAPDH: Glyceraldehyde 3 phosphate dehydrogenase, PON1: Paraoxonase-1.

Although UII has been suggested to play a pathological role in the development of atherosclerosis and cardiovascular diseases, but it also may have a possible cardio-protective role, such as lower plasma UII level in patients with acute coronary syndrome than healthy controls (Joyal et al., 2006; Zoccali et al., 2006), that need to more molecular basic assessments.

Little studies to date have shown the effects of some nutrients, environmental factors and drugs on PON1 expression and or its activity *in vitro* and *in vivo* for evaluating of their effects on HDL metabolism via PON1 (Jaichander et al., 2008; Deakin et al., 2003).

The relationship between hypertensive factors and lipoprotein metabolism in atherogenesis is not clear entirely. For *in vitro* studies on lipoprotein metabolism in hepatic cells, the HepG2 cell line was used in most researches (Jaichander et al., 2008; Hayashi et al., 2011; Xu et al., 2001; Mohammadi et al., 2014). Since decreased PON1 levels and/ or activity is an independent associated factor to atherosclerosis and there is evidences about controversy role of UII in cardiovascular system, the objective was to investigate the effect of human UII on PON1 expression, at mRNA and protein levels, in hepatic (HepG2) cell line.

MATERIALS AND METHODS

Cell culture and treatment

As we described previously (Mohammadi et al., 2014), Hepatic (HepG2) cells (Pasteur institute, Iran) were maintained in a humidified incubator at 37°C and 5% CO_2. Cell viability was greater than 95% in all experiments. For the subculture, cells were seeded on 35 mm culture dishes for all experiments (Hayashi et al., 2011; Mohammadi et al., 2014).

After 48 h (doubling time of cells) cells were washed two times with PBS and once with serum free culture media (Hayashi et al., 2011; Xu et al., 2001). For treatment experiments, we used UII (solved in PBS containing human serum albumin (HSA)) at nM concentrations which had used previously in some studies (Watanabe et al., 2005; Mohammadi et al., 2014; Watanabe et al., 2006; Tamura et al., 2003). So, experimental media containing human urotensin II at the 0, 10, 50, 100 and 200 nM concentrations was used (Mohammadi et al., 2014).

Total RNA extraction

Total RNA was extracted from cells 24 h after treatment using the RNeasy Plus mini kit (Qiagen, Germany) according to manufacturer protocol. Evaluation of total RNA was performed using a NanoDrop Spectrophotometer and by agarose gel electrophoresis, as previously described (Mohammadi et al., 2014).

Real-time PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was used to quantify the expression of specific gene using primer sequences which were obtained from Gene Cards (The Human Gene Compendium) database and validated by National Center for Biotechnology Information - Basic Local Alignment Search Tool for primers (NCBI - BLAST) (Table 1). Reverse transcription for cDNA synthesis was performed using 800 ng of total RNA and random hexamer and oligo dT primers by QuantiTect Reverse Transcription kit (Qiagen, Germany). Then real time PCR was performed using SYBR Fast kit (Qiagen, Germany) by Rotorgene 6000 qPCR machine (Qiagen, Germany). As we described previously, the PCR 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). In addition, melting curve analysis was performed by increasing the temperature (1°C/s) from 55 to 95°C, with continuous fluorescence acquisition (Mohammadi et al., 2014).

Relative changes in PON1 mRNA levels were calculated using $2^{-\Delta\Delta C_T}$ method and normalized based on glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels, as housekeeping gene.

Protein extraction and SDS-PAGE

For assessment of PON1 gene expression at protein level, the protein samples were extracted from HepG2 cells 24 h

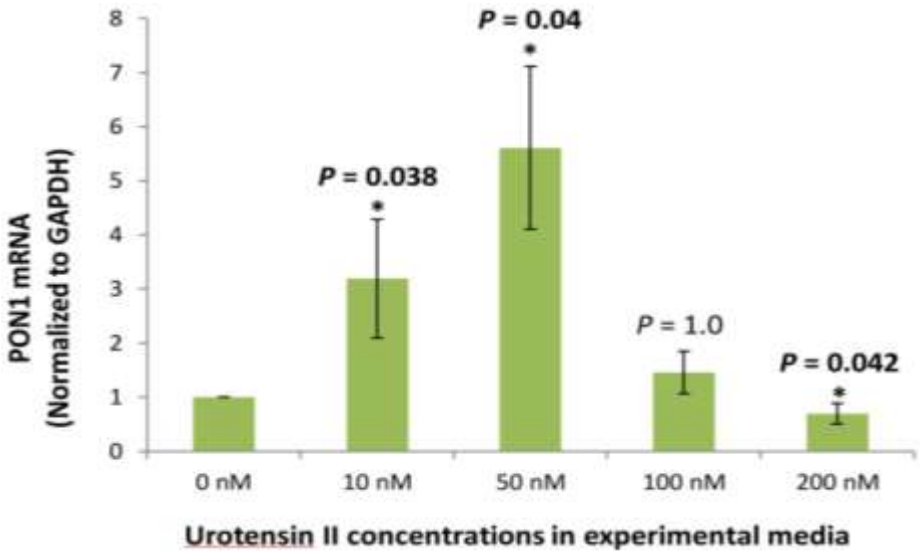

Figure 1. The effect of UII on PON1 gene expression at mRNA level. HepG2 cells were treated with the indicated concentrations of UII for 24 h. PON1 and GAPDH mRNA levels were measured by qRT-PCR. UII concentrations 10 and 50 nM were increased PON1 mRNA levels, but UII 200 nM concentration was decreased PON1 mRNA levels significantly. *P = 0.019, treated vs. control cells; n = 6.

after treatment, by complete RIPA (Radio Immuno-precipitating assay) lysis buffer (Sigma, USA) containing protease inhibitor cocktail (Santa Cruz Biotechnology, USA), as we described previously (Mohammadi et al., 2014). After determination of protein concentrations by Bradford method (Bradford, 1976) and also direct protein assay at 280 nm using a NanoDrop Spectrophotometer (ND-1000 Thermoscientific, USA), one hundred micrograms of protein from each prepared and denatured samples with modified Laemmli buffer were loaded in 12% linear tricine gels with a 5% stacking gel and the buffer system. According to GAPDH and PON1 protein molecular weight (approximately ~40 kDa for both of them), the sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) using 12% resolving gels were run at 35 mA for 1.5 h by Tetra cell mini protein apparatus (BioRad, USA) (Mohammadi et al., 2014).

Western blotting

After SDS-PAGE, samples were transferred to PVDF membrane (Towbin et al., 1979). The membranes were prepared by blocking buffer for antibody addition step at room temperature overnight. Then, the membranes were incubated with the diluted rabbit anti-human-PON1 (1:1000) and rabbit anti-human-GAPDH (1:2500) primary antibodies (Abcam, USA) and horseradish peroxidase conjugated goat-anti-rabbit antibody (1:10000) (Abcam, USA), respectively. The membranes were then immersed in enhanced chemiluminescence (ECL plus of PerkinElmer, Netherlands) reagent and exposed to film at a dark room. After development, films were scanned and signals were quantified using Image J software, as described previously (Mohammadi et al., 2014).

Data analysis

All experiments have been repeated 6 times (n=6), each culture at specific treatment being repeated 6 times. Technical repetitions were done twice for each experiment, that is each sample for quantitative real time PCR and western blot was repeated duplicate and interpretation of data was performed as mean ± SE comparing of treated culture media and basal media, with 0 nM UII, each time. Relative gene expression data comparisons at mRNA and protein levels between study and control groups were analyzed for statistical significance using Mann-Whitney and Kruskal-Wallis tests. Probability values <0.05 were considered statistically significant.

RESULTS

The effect of urotensin II on PON1 mRNA expression in HepG2 cells

After treatment of hepatic cells with different concentrations of UII which include 10, 50, 100 and 200 nM, relative expression of PON1 mRNAs were 3.19 ± 1.0, 5.61 ± 1.5, 1.46 ± 0.39 and 0.7 ± 0.19 times than control, respectively (Figure 1). These data were normalized to GAPDH mRNA levels which did not change significantly

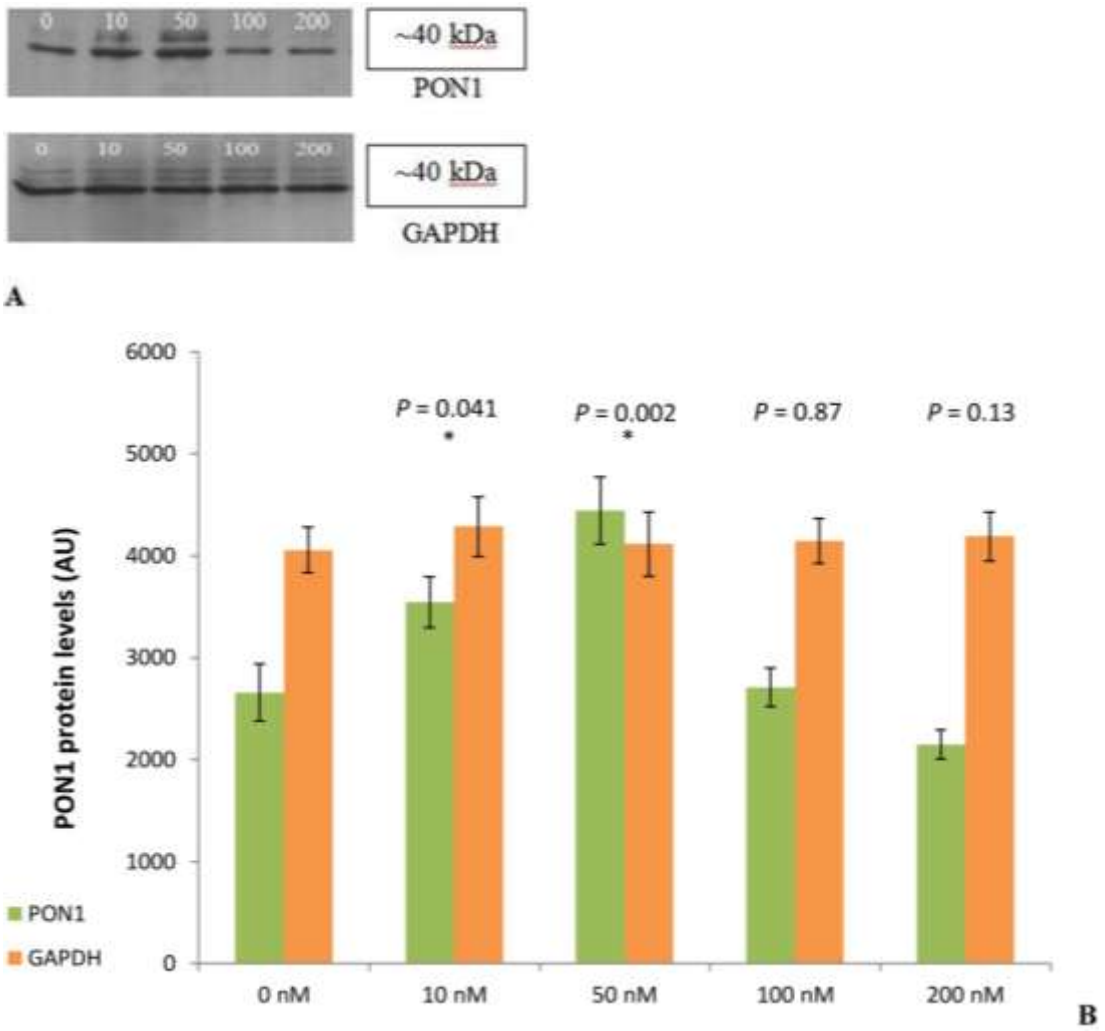

Figure 2. The effect of UII on PON1 expression at protein level. HepG2 cells were treated with different concentrations of UII at 10, 50, 100 and 200 nM for 24 hours. After total protein extraction from hepatic cells, PON1 expression at protein level was assayed by Western blot (A). Band densities were measured by densitometry (arbitrary units) and are shown in panel B. UII at different concentrations was changed PON1 levels significantly. P* < 0.01, treated vs. control cells; n = 6.

after treatment.

The effect of urotensin II on PON1 protein levels in HepG2 cells

The PON1 protein levels increased from 2657 ± 281 in control cells (without UII treatment) to 3542 ± 250 and 4444 ± 328 AU (in arbitrary unit; AU) in cells treated with 10 and 50 nM urotensin II, respectively (P = 0.041 and P = 0.004, respectively). But, PON1 protein levels were 2710 ± 190 and 2150 ± 142 AU in cells treated with 100 and 200 nM UII, respectively (P = 0.87 and P = 0.13, respectively). GAPDH protein levels did not change after treatment with UII, suggesting that UII specifically increases PON1 protein at 10 and 50 nM (Figure 2).

DISCUSSION

As the results shown, UII may have increasing effects on PON1 gene expression at some concentrations (10 and 50 nM), but it also can have lowering effects at higher doses (200 nM). So, UII may protect cardiovascular system through PON1 at some concentrations, because the PON1, an enzyme associated with HDL, can inhibit lipid peroxides accumulation on LDL (Durrington et al., 2001; Mackness et al., 2004). Experimental evidences suggest that a decrease in serum PON1 activity may have role in atherosclerosis (Gupta et al., 2011; Watson et al., 1995).

So, decrease of PON1 gene expression at higher concentrations of UII may suggest that UII, as the most potent vasoconstrictor (Ames et al., 1999), probably is involved in atherosclerosis.

Many researches have shown UII may participate in development of atherosclerosis via its different effects (Ames et al., 1999; Richards and Charles, 2004), such as inducing the proliferation of vascular smooth muscle cells particularly with oxidized LDL (Watanabe et al., 2006; Zhang et al., 2007), acceleration of foam cell formation in atheroma plaques via acyl-CoA-acyl transferase-1 (ACAT1) (Watanabe et al., 2005) and increase of apolipoprotein B100 gene expression in hepatic cells (Mohammadi et al., 2014).

However, the association between hypertensive factors such as UII and different aspects of HDL metabolism, as an independent anti-risk factor for atherosclerosis (Durrington et al., 2001), is less studied.

Some evidences have suggested that UII may have a possible cardio-protective role. Joyal et al. (2006) have shown that plasma UII level is lower in patients with acute coronary syndrome than that in patients with stable coronary artery disease and healthy controls.

Plasma UII level is also found to be an independent inverse predictor of cardiovascular adverse events in patients with end-stage renal disease (Zoccali et al., 2006). In addition, Khan et al. (2007) have shown that although plasma UII levels in patients with acute myocardial infarction were increased, a lower UII level was associated with a higher risk of adverse clinical outcome.

Many studies have been indicated to date on the role and effect of different contributing factors, particularly lipoproteins metabolism, in atherosclerosis.

Disturbance of HDL metabolism, particularly proteins associated with HDL such as apo A-I and PON1, is one of the most important mechanisms in atherosclerosis development. Therefore, controlling the participating factors in this process seems as a therapeutic approach in suffering patients.

Deakin et al. (2003) have shown that simvastatin, a drug that can reduce cholesterol in patients with hypercholesterolemia, can upregulate PON1 gene expression in HepG2 cells at dose-dependent manner.

They have also reported that administration of simvastatin in hypercholestrolemic patients can increase the enzyme activity of PON1. So, simvastatin can have increasing effect on PON1, in addition to its cholesterol lowering effect. Jaichander et al. (2008) have shown that administration of aspirin can induce apo A-I and PON1 gene expression in hepatic cells of both HepG2 cells and mice model. They also have reported that PON1 enzyme activity was increased significantly (Jaichander et al., 2008).

Like Jaichander's study, in previous study (Mohammadi et al., 2014) we shown that UII at 200 nM concentration can reduce apo A-I gene expression in HepG2 cells. In addition, in present study UII was shown lowering effect on PON1 gene expression at 200 nM concentration.

These data are suggested that UII can have negative effect on HDL metabolism via decrease of apo A-I and PON1 gene expression.

Conclusion

The results of the experiments described here indicate that urotensin II at concentrations of 10 and 50 nM increases PON1 protein significantly in HepG2 cells to 3.6 and 5.8 times, respectively. This is probably the result of increased production as the PON1 mRNA concentrations increased. In addition, PON1 mRNA levels were decreased significantly at highest concentration of UII, 200 nM. In addition, PON1 protein level was decreased at 200 nM UII, but it is not significant. The main concern with the present study is the discrepancy of the PON1 mRNA and proteins levels after treatment with different concentrations of UII.

These data show UII may have cardio-protective role at 10 and 50 nmol/L concentration through increase production of PON1 perhaps by increase PON1 mRNA levels. However, we saw a significant decreasing effect of 200 nM UII on PON1 mRNA level. This suggests that UII in higher concentrations may play independently negative role in HDL metabolism via decrease of PON1.

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