Int J Clin Exp Med 2017;10(8):12012-12017
www.ijcem.com /ISSN:1940-5901/IJCEM0051644

Original Article

Effect of urotensin II on apoptosis of H9c2 cardiomyocytes and the underlying mechanisms

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Received January 9, 2017; Accepted July 6, 2017; Epub August 15, 2017; Published August 30, 2017

Abstract: *Objective:* To observe the effect of urotensin II (UII) on the apoptosis of cardiomyocytes and investigate the underlying mechanisms. *Methods:* H9c2 cells from rat embryonic heart cell line were cultured as an in vitro experimental model of heart cells, and were divided into four groups: the control group (C), the 10^{-8} mol/L UII-treatment group (UII), the 10^{-8} mol/L UII + 1 mmol/L SB-611812-treatment group (UII-SB), and 10^{-8} mol/L UII + 1 mmol/L KT5720-treatment group (UII-KT). The expression of caspase-3 in the four groups was detected by ELISA, cell proliferation was assessed using CKK-8 staining followed by visualization under a microscope, and the rate of apoptosis was determined by Annexin V/PI double staining and flow cytometry. *Results:* The expression of caspase-3 in group UII increased ($P<0.05$) in a dose-dependent manner as compared to that in group C. Further, UII inhibited cell proliferation and promoted apoptosis. A UII receptor antagonist, SB-611812, and a PKA-specific blocker, KT5720, protected against the effects of UII. *Conclusions:* UII may upregulate the expression of caspase-3 and the adenosine monophosphate-protein kinase A (AMP-PKA) signaling pathway, thus promoting the apoptosis of H9c2 cardiomyocytes.

Keywords: Urotensin II, H9c2 cells, caspase-3, apoptosis

Introduction

Urotensin II (UII), a neuropeptide, is a somatostatin-like vasoactive peptide [1]. It originates from the tail pituitary of goby fish [2] and has a 10-fold stronger effect as a vasoconstrictor in comparison to endothelin (ET-1) [3]. G-protein coupled receptor 14 (GPR14, UT) acts as the receptor for UII, and is widely expressed in the cardiovascular tissues [4]. In vivo and in vitro experiments have proven that UII can induce myocardial hypertrophy and promote myocardial fibrosis and the synthesis of collagens [5, 6], thus being closely related to the occurrence and development of cardiac hypertrophy, heart failure, and other cardiovascular diseases [7]. Studies have shown that UII has significant mitogenic and proliferative effects on vascular cells [8, 9]. In the cardiovascular system, it also affects apoptosis, hypertrophy, and fibrosis of various types of cells [10-12]. In a previous study, using the Langendorff isolated heart model, we have shown that UII inhibits cardiac function in rats in a dose-dependent manner. Since this inhibition could be suppressed by KT5720 (PKA antagonist), it was inferred that UII may inhibit cardiac function by acting through the PKA pathway [24]. Further, KT5720 (PKA antagonist) and SB-611812 (UT blocker) were also found to inhibit collagen synthesis in the neonatal rat cardiac fibroblasts. These data suggest that UII may promote myocardial fibrosis in pressure-overloaded rats through the cAMP-PKA signaling pathway [13]. However, the effects of UII on cardiomyocytes under pathological conditions and the underlying mechanisms remain to be elucidated.

Caspases play an important role in the regulation of apoptosis. Caspase-3 is a key effector, and the expression of caspase-3 in a cell can promote apoptosis. H9c2 cells derive from rat embryonic heart, and can highly simulate the structure and function of cardiomyocytes. Therefore, they are widely used in basic and clinical research to study cardiovascular diseases. In this study, cultured H9c2 cells were used as the experimental model to assess the

effect of different concentrations of UII on the apoptosis of cardiomyocytes and to study its regulatory effect on caspase-3 so as to investigate the mechanisms of UII-induced apoptosis of H9c2 cells.

Materials and methods

Cell culture

H9c2 cells were cultured in 10% fetal bovine serum (FBS)-containing Dulbecco's minimum essential medium (DMEM) in 50-mL cell culture flasks at a density of 2.5×10^7 cells/L. The flasks were incubated at 37°C in 5% CO_2 (95% air) for 3 days. Upon digestion with 0.25% trypsin and one subculture, the cells were seeded onto 6-well culture plates at a density of 1×10^8 cells/L. These cells were then cultured in a one-cell incubator, and the cells in the logarithmic growth phase were sampled for the experiment.

Grouping

The cells were divided into several groups according to the final concentrations of UII: the 10^{-9} mol/L UII-treatment group (10^{-9} M UII), the 10^{-8} mol/L UII-treatment group (10^{-8} M UII), and the 10^{-7} mol/L UII-treatment group (10^{-7} M UII), and the control group (where UII was replaced with an equal volume of the culture medium). To observe the effect of a UII receptor antagonist (SB-611812) and a PKA-specific blocker (KT5720) on the apoptosis of UII-induced H9c2 cells, several groups were set up: the control group (C), the 10^{-8} mol/L UII-treatment group (UII), the 10^{-8} mol/L UII + 10^{-3} M/L SB-611812-treatment group (UII-SB), and 10^{-8} mol/L UII + 10^{-3} M/L KT5720-treatment group (UII-KT). Six replicate wells were set in each group, and the experiment was repeated three times.

Determination of caspase-3 concentration

After digestion with 0.25% trypsin, cells from each group were collected in microfuge tubes and centrifuged for 5 min at 800 rpm. This was followed by a wash with phosphate buffered saline (PBS). Lysis solution (50 μL) was added to the tubes, which were then placed for lysis in an ice bath for 15 min; the products were then centrifuged at 3000 rpm for 10 min, and the supernatant was collected. The kit plates were equilibrated at room temperature for 20 min, and standards with different concentrations (50 μL) and the samples (10 μL) were introduced into the standard wells and the sample wells, respectively, followed by 40 μL of diluent and 100 μL of horseradish peroxidase (HRP)-labeled antibody. The plates were then closed and incubated at 37°C for 60 min. The wells were washed and 50 μL each of substrate A and B was added, and incubated at 37°C in darkness for 15 min. The stop solution was then introduced, and the OD value of each well was measured at 450 nm. The concentration of caspase-3 in each group was calculated using the standard curve obtained by following the kit instructions.

Proliferation of H9c2 by CKK-8

H9c2 proliferation was detected by CKK-8, and the cell states were determined by visualization under a microscope. H9c2 cells, under the log phase of growth, were digested with 0.25% trypsin, and 100 μL (1×10^4 cells) of each sample was loaded in 96-well plates, which were incubated for 24 hours at 37°C with 5% CO_2. The cells were then incubated in the same condition for another 48 h after being treated with different reagents. Samples were co-incubated with 10 μL of CCK solution at 37°C for 4 hours, and OD_{450} was determined.

Detection of apoptosis rate

The cell culture medium, the cells digested by 0.25% trypsin and adhering to the wall were collected in a flow tube, and the following steps were performed: 5-min centrifugation at 1500 rpm, washing with PBS twice, cell-resuspension in 200 μL of buffer, adding 15 μL of Annexin V-fluorescein isothiocyanate (FITC) for a 15-min reaction in darkness, adding 10 μL of PI for a 5-min reaction in darkness, adding 200 μL of PBS, and detection. In the analytical chart of flow cytometry, the right upper quadrant UR (FITC+/PI+) indicated the late apoptotic cells, and the right lower quadrant LR (FITC+/PI-) indicated the early apoptotic cells; the apoptotic rate was expressed as UR + LR.

Statistical analysis

The experiment was repeated three times, and SPSS18 statistical software was used for one-way analysis of variance; intergroup comparison used t-test, with $P<0.05$ considered to be a statistically significant difference.

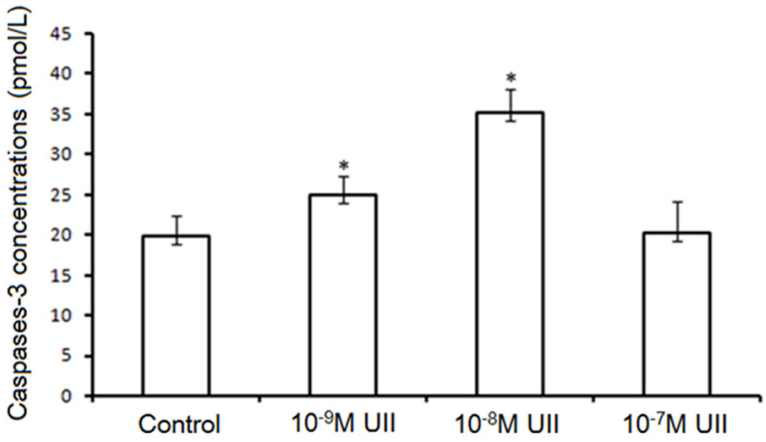

Figure 1. Expressions of caspases-3 in experimental groups. Note: Compared with group C, *P<0.05.

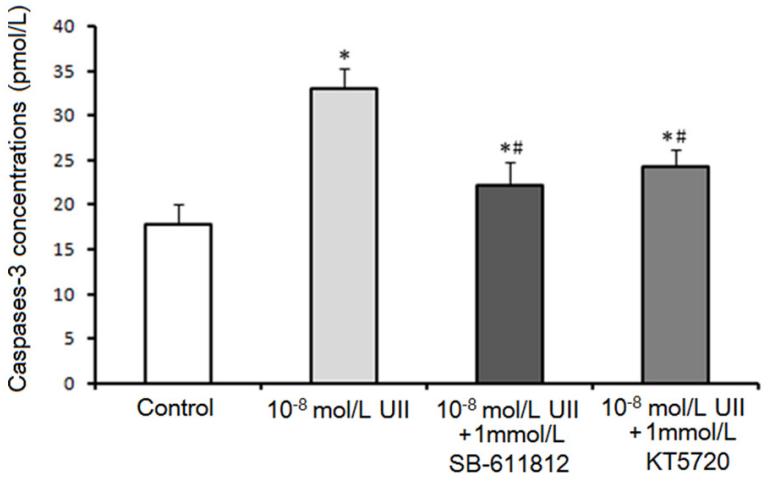

Figure 2. Impact of SB-611812 and KT5720 on expression of caspases-3. Compared with group C, *P<0.05; compared with group UII, #P<0.05.

Results

Comparison of caspase-3 concentrations

Compared with group C, the expression of caspase-3 in groups 10^{-9} M UII and 10^{-8} M UII were increased (P<0.05). Further, the caspase-3 expression increased in a dose-dependent manner with an increase in the UII concentration. Compared to group C, the expression of caspase-3 in group 10^{-7} M UII showed no statistically significant difference (**Figure 1**).

Expression of caspase-3

A comparison of the caspase-3 expression in different groups showed that it was lower in groups UII-SB and UII-KT as compared to that in group UII (P<0.05, **Figure 2**).

The proliferation of H9c2 cells

OD_{450} was lower in groups with UII at 10^{-9} mol/L, 10^{-8} mol/L and 10^{-7} mol/L in comparison to that in the control group, and the decrease in the OD correlated with the UII concentration (**Figure 3A**), while the relief from inhibition by 1 mmol/L SB-611812 and 1 mmol/L KT5720 is shown in **Figure 3B**, also confirmed by microscopy (**Figure 3C**).

Detection of apoptosis

Our results showed that 10^{-8} M UII can promote apoptosis in H9c2 cells. We also showed that the proapoptotic effects of 10^{-8} M UII can be suppressed by the introduction of 10^{-3} M SB-611812 or 10^{-3} M KT5720 (**Figure 4**).

Discussion

As a "somatostatin-like" vasoactive peptide, UII has high vasoconstrictive efficacy, and has been shown to be widely expressed in the blood vessels of the heart, kidney, pancreas, thyroid, adrenal gland, umbilical cord, and placenta [14, 15]. Studies have shown that UII expression and that of its receptor are upregulated in congestive heart failure, essential hypertension, coronary artery disease, type II diabetes, or diabetic nephropathy, indicating that the UII/UT system has great significance in the processes of cardiovascular diseases [14, 16, 17]. Studies have shown that UII can not only directly promote myocardial hypertrophy [10], but also promote myocardial fibrosis by promoting the proliferation of myocardial fibroblasts [18] and the synthesis of collagens [19]; we have found in our previous studies that UII may regulate the collagen synthesis of cardiac fibroblasts via the cAMP-PKA signaling pathway, but the effect of UII on the apoptosis of myocardial cells has not been studied.

Apoptosis is a type of programmed cell death, which maintains the dynamic balance between

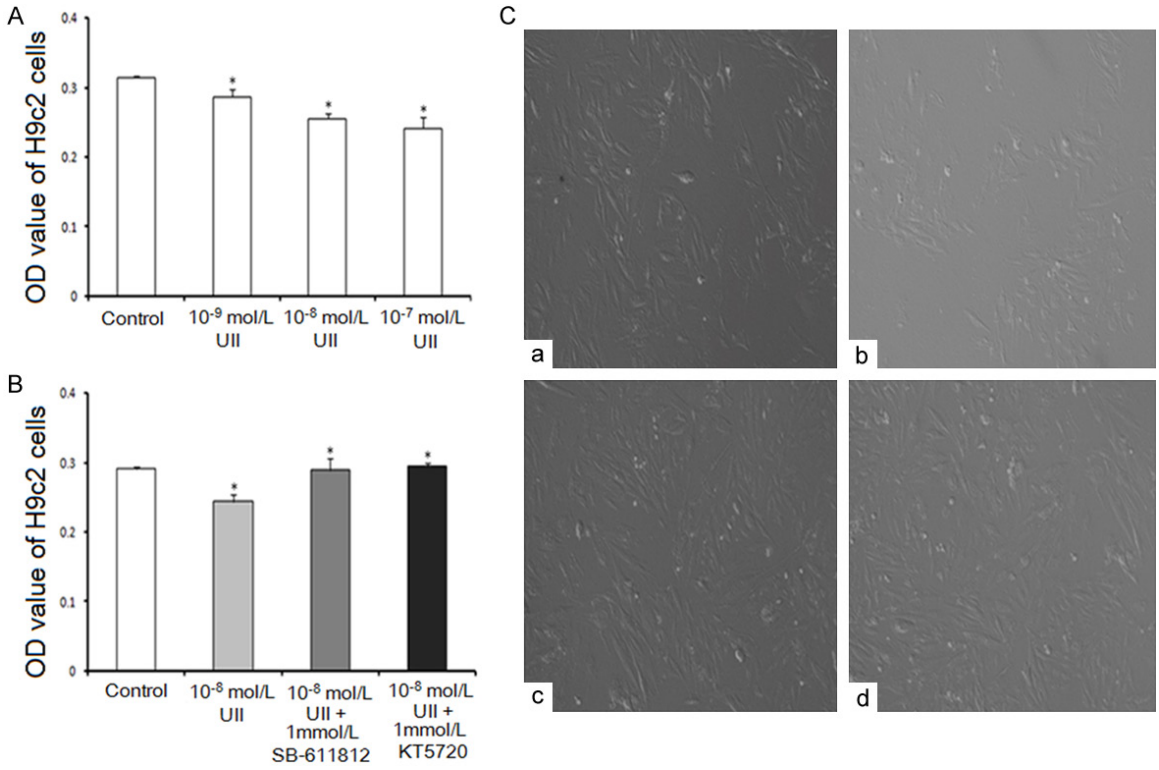

Figure 3. The proliferation of H9c2. A: OD value of different concentration of UII, B: OD value of SB-611812 and KT5720, C: Microscope figures.

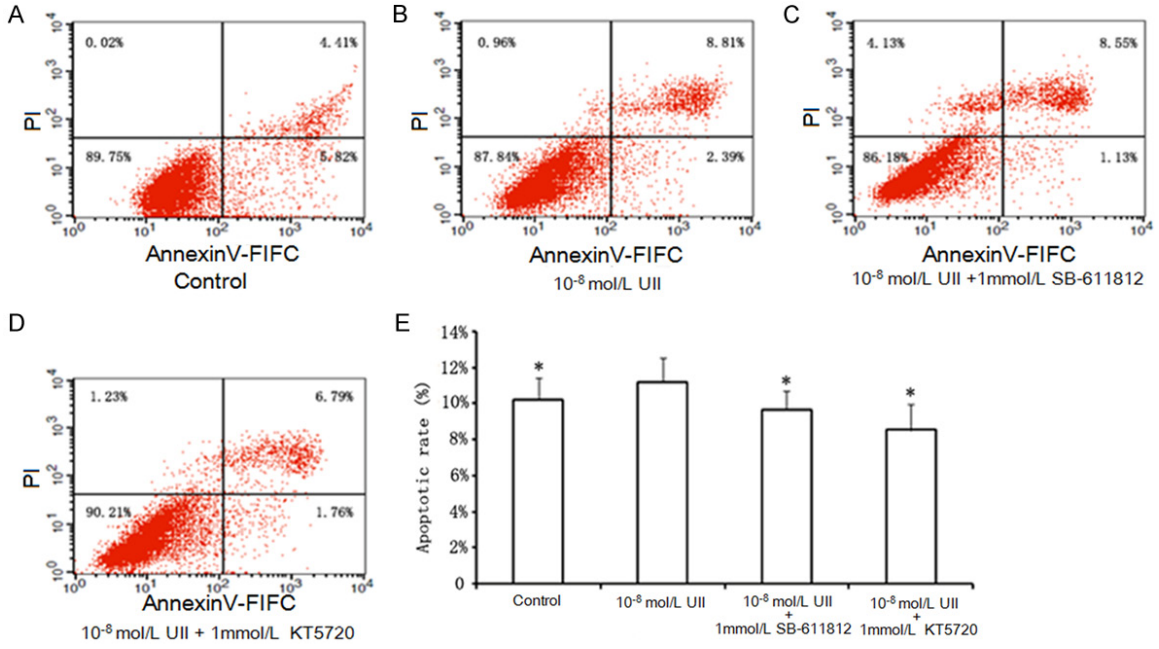

Figure 4. Comparison of apoptotic rate among different groups. Note: Compared with group UII, *P<0.05.

cell proliferation and differentiation. The mechanisms of apoptosis are mainly classified as caspase-dependent and caspase-independent. Caspase-3 is an important factor in the regulation of apoptosis and is a key protease in the process. Caspase-3 performs the function of

splicing intracellular structural proteins, thus playing an important role in apoptosis. Therefore, it is often used as a marker of apoptosis [20]. In cells programmed to die by apoptosis, caspase is activated which induces apoptosis by cascade amplification. In the process of apoptosis, caspase-3 is upregulated, and it interacts with other apoptotic regulators, causing the cleavage of many structural proteins which is followed by apoptosis [21]. The cAMP-PKA pathway is one of the most important cell signal transduction pathways, participating in the regulation of the expression of various genes and important cellular functions. Its regulatory roles are realized by activating the PKA system [22]. It has been reported that UII may inhibit the heart functions in normal and heart failure rats though the PKA pathway [23, 24].

In this study, we found that caspase-3 expression in H9c2 cells significantly increased upon induction with UII, and the magnitude of the upregulation correlated with the final concentration of UII (10^{-9} M, 10^{-8} M, and 10^{-7} M UII), exhibiting a dose-dependent increase. However, H9c2 cultured with 10^{-7} M UII showed no significant difference with respect to group C in terms of caspase-3 expression. Our results also show that with 10^{-8} M UII, 10^{-3} M SB-611812 and 10^{-3} M KT5720 can inhibit the UII-induced upregulation of caspase-3 and the proapoptotic effects. These results indicate that the UII antagonist SB-611812 and the PKA-specific blocker KT5720 can significantly suppress the biological effects of UII. Therefore, it is likely that the mechanisms underlying UII-promoted apoptosis of H9c2 involve the cAMP-PKA signaling pathway. Through our CKK-8 assay, we have found that H9c2 proliferation was inhibited by UII at different concentrations, which could be reversed by SB-611812 and KT5720.

At the same time, this study also assessed the rate of apoptosis in treated cardiomyocytes. The results of AnnexinV/PI double staining and flow cytometry reveal that the rate of apoptosis in UII-treated cells was significantly increased, but 10^{-3} M KT5720 and 10^{-3} M SB-611812 can act on CFs and inhibit the proapoptotic effects of UII. In summary, UII can promote the apoptosis of myocardial cells, and this effect may be realized by upregulating caspase-3 expression and modulating the cAMP-PKA signaling pathway. These data can provide interesting theories for the effect of UII in cardiovascular disease research, but the specific mechanisms still need further investigation.

Acknowledgements

This study was supported by the Natural Science Foundation of Shanxi Province (No.2012011036-1, No.201601D0111), the Shanxi Provincial University Scientific Research Projects Foundation of Abroad-Studying and Returning Personnel (No.2011-63), Research Project Supported by Shanxi Scholarship Council of China (No.2016-059).

Disclosure of conflict of interest

None.

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