Urotensin II promotes the proliferation of endothelial progenitor cells through p38 and p44/42 MAPK activation

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Abstract. Urotensin II (UII) is a vasoactive peptide with many potent effects in the cardiorenovascular system and is also possibly involved in the pathogenesis of atherosclerosis. Endothelial progenitor cells (EPCs) are involved in angiogenesis and vascular homeostasis and may be important in the maintenance of endothelial integrity. The aim of this study was to investigate whether UII has an effect on the proliferation of bone marrow-derived EPCs and the possible signaling mechanisms involved. Bone marrow-derived EPCs were isolated from male Sprague-Dawley rats and cultured in medium containing 5% fetal bovine serum. Cells were incubated with UII for 24 h. The proliferation of EPCs was analyzed by MTT assay. Western blotting was performed to determine the phosphorylation levels of mitogen-activated protein kinases (MAPKs). The results demonstrated that UII promoted the proliferation of EPCs in a concentration-dependent manner in a certain range, and the proliferation was largely suppressed by inhibitors of GPR14 and MAPKs (p38 and p44/42). UII significantly increased the phosphorylation levels of p38MAPK and p44/42MAPK, and these effects were significantly inhibited by respective inhibitors. These findings indicate that UII promotes the proliferation of rat bone marrow-derived EPCs through a process that involves MAPK activation, and provides novel insights regarding the role of UII in the EPC-mediated repair of atherosclerotic injury.

Introduction

Urotensin II (UII) is a somatostatin-like cyclic peptide synthesized by proteolytic cleavage from a precursor molecule, prepro-UII, and has been identified as the ligand responsible for the orphan G protein-coupled receptor (GPR) 14 (1,2). mRNA for GPR14 is widely expressed in cardiovascular tissues, including myocytes, vascular smooth muscle cells (VMSCs) and endothelial cells (ECs) (1,3), as well as in endothelial progenitor cells (EPCs) (1,4). UII has been observed to be highly expressed in cardiovascular tissues and atherosclerotic plaque (3). An increased expression of the UII receptor has been reported in myocytes, ECs and fibroblasts, supporting a functional role for UII in cardiac and vascular remodeling (1,3).

Endothelial dysfunction, which is considered the functional equivalent of a disrupted balance between endothelial injury and repair, precedes overt atherosclerosis by many years. Accumulating evidence has shown that EPCs contribute substantially to the preservation of a structurally and functionally intact endothelium (5). A previous study showed that EPCs express the UII receptor and UII induces the migration of EPCs in a dose-response manner. Moreover, the chemotactic effect of UII is mediated via activation of the RhoA/Rho kinase pathway (4). In this study, we examined the potential proliferation function of UII in EPCs and the role of mitogen-activated protein kinase (MAPK) signaling in this process.

Materials and methods

Isolation and characterization of endothelial progenitor cells. EPCs were isolated from male Sprague-Dawley rats weighing 120-160 g provided by the Experimental Animal Center of Wuhan University, as previously described (4,6). The University of Wuhan's Animal Ethics Committee approved all animal procedures. Briefly, rats were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg). Bone marrow cells were harvested by flushing the femurs and tibias of rats. The mononuclear cell fraction was obtained from a Lymphoprep density gradient (EZ-Sep Mouse IX; Dakewe Biotech Company, Shenzhen, China) after centrifugation for 20 min at 2,500 rpm (centrifuge GPR; Beckman, Hettich, Germany). The mononuclear cell fraction was carded, washed and centrifuged at 1,300 rpm for 10 min.

Isolated mononuclear cells were resuspended by the EGM-2 BulletKit system (catalog no. CC-3202; Walkersville, MD, USA) consisting of endothelial basal medium-2 (EBM-2), 5% fetal bovine serum, vascular endothelial growth factor, fibroblast growth factor-2, epidermal growth factor, insulin-like growth factor-1 and ascorbic acid. Under daily observation, the first media change was performed 4 days after plating.

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The media were then changed every 3 days. After 7-10 days, confluence of the outgrowing cell population was reached and cells were divided by trypsinogen (0.125%; Gibco BRL Life-Technologies, Gaithersburg, MD, USA).

Isolation and characterization of EPCs and confirmation of endothelial cell lineage were performed as previously described (4). Fluorescent chemical detection of EPCs was performed on attached EPCs after 7 days in culture. Direct fluorescent staining was used to detect dual binding of FITC-labeled BS-1-lectin and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI)-labeled acetylated low density lipoprotein (acLDL). Cells were first incubated with 10 μg/ml DiI-ac-LDL at 37˚C for 2 h and later fixed with 2% paraformaldehyde for 10 min, followed by incubation with Ulex europeus agglutinin 1 (UEA-1) at 37˚C for 1 h. The cells were identified under a fluorescence microscope and counted at x200 magnification. Cells demonstrating double-positive fluorescence were identified as differentiating EPCs (4).

Cell proliferation assay. After pre-treatment with the p38MAPK inhibitor SB203580 (10 μM) (S1863; Beyotime Biotechnology, Haimen, China), the p44/42MAPK inhibitor PD98059 (10 μM) (S1805; Beyotime Biotechnology), the SAPK/JNK inhibitor SP600125 (20 μM) (S1876; Beyotime Biotechnology) or the GPR14 inhibitor Urantide (10 μM) (PUT-3639-PI; Phoenix Pharmaceuticals Inc., Burlingame, CA, USA) for 45 min, EPCs were stimulated with different concentrations of UII (10^{-10} to 10^{-6} M) (Phoenix Pharmaceuticals Inc.) for 24 h. The adherent cells were trypsinized and suspended in 500 μl medium. Approximately 5,000 cells/well were plated on fibronectin-coated 96-well plates and cultured for 24 h. Twenty microliters of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl -2H-tetrazolium bromide] (5 g/l) was added to each well. After incubation for 4 h at 37˚C, the supernatant was removed and replaced with 150 μl of dimethyl sulfoxide (DMSO) for 10 min, before the OD value at 450 nm was measured with a microculture plate reader (Tecan, Switzerland).

Cells were counted under a phase-contrast microscope in 10 random fields at x100 magnification, stained with 4',6'-diamidino-2-phenylindole (DAPI) and examined by fluorescence microscopy with a fluorescein isothiocyanate range barrier filter cube. The assays were performed in triplicate and repeated three times.

Western blotting. Endothelial progenitor cells were incubated with 10 μM SB203580, 10 μM PD98059 or 10 μM urantide for 45 min, followed by treatment with 10^{-7} M UII for 30 min. Whole-cell extracts were collected in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 1% deoxycholate, 5 mM ethylenediaminetetraacetic acid, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail) (P0013; Beyotime Biotechnology). The protein concentration was determined by the bicinchoninic acid protein assay. Cell lysates were boiled for 3 min and subjected to electrophoresis. Protein was transferred to nitrocellulose membranes and blocked with 5% non-fat dry milk for 2 h. Blots were probed with polyclonal antibody against GPR14 (sc-28998; Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-p38 MAPK (AM063; Beyotime Biotechnology) or phospho-p44/42MAPK (AM071; Beyotime Biotechnology), and then with the appropriate secondary antibodies (Pierce Chemical Co., Rockford, IL, USA). Proteins were detected with an ECL Plus detection kit (Pierce Chemical Co.). Protein expression levels are indicated as a ratio to GAPDH.

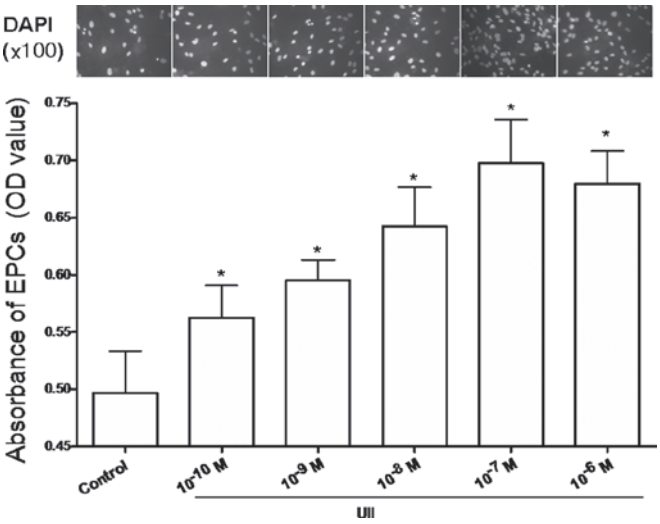

Figure 1. Urotensin II (UII) promotes the proliferation of endothelial progenitor cells (EPCs) in a concentration-dependent manner to a certain extent. A similar effect on cell proliferation was also observed as assayed by cell count. *P<0.05 vs. the control group.

Statistical analysis. Data were shown as the means ± standard error of the mean (SEM) derived from six separate experiments. Statistical significance was determined by one-way analysis of variance (ANOVA) and the Student-Newman-Keuls test. P<0.05 denoted a statistically significant difference.

Results

UII promotes EPC proliferation in a dose-dependent manner. We observed that UII promotes the proliferation of EPCs with typical dose-response (all p<0.05 vs. the control group). The proliferative effect reached a peak value after treatment with 10^{-7} M UII (Fig. 1). The cell count assay revealed that a 1.4-, 1.6-, 1.9-, 2.4- and 2.2-fold increase was detected in the proliferation of EPCs with UII at a concentration of 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} M, respectively.

The MAPK signaling pathway is involved in the cell proliferation effect of UII. We examined whether the MAPK signaling pathway is involved in the cell proliferation effect of UII on EPCs. As shown in Fig. 2, inhibition of MAPK with SB203580 (10 μM) and PD98059 (10 μM) eradicated the proliferative effects of UII, and urantide (GPR14 inhibitor) had the same effect (p<0.05 vs. the 10^{-7} M UII group). The proliferative effect of UII on EPCs was not inhibited by SP600125 (20 μM) (p>0.05 vs. the 10^{-7} M UII group). A similar effect on cell proliferation was also observed as assayed by cell count.

Phosphorylation of p38MAPK and p44/42MAPK in response to treatment with UII. The phosphorylation levels of p38MAPK and p44/42MAPK were detected following

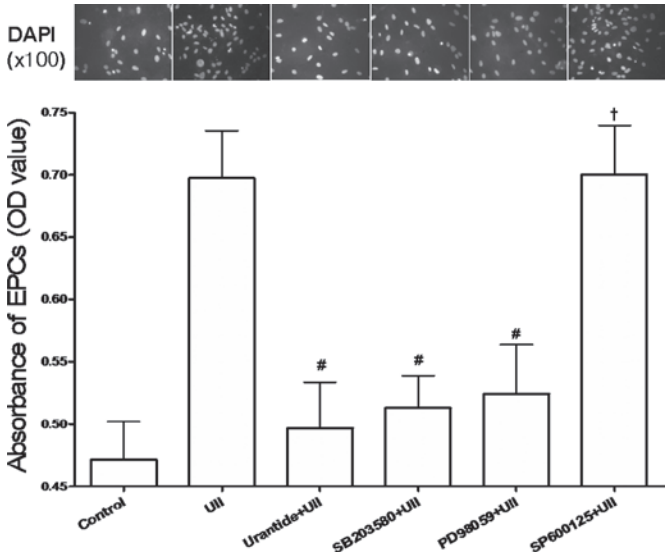

Figure 2. The MAPK pathway is involved in the proliferation of endothelial progenitor cells (EPCs). Inhibition of MAPK with SB203580 (10 μM) and PD98059 (10 μM) eradicated the proliferative effects of urotensin II (UII), and urantide (GPR14 inhibitor) had the same effect ($p<0.05$ vs. the 10^{-7} M UII group). The proliferative effect of UII on EPCs was not inhibited by SP600125 (20 μM) ($p>0.05$ vs. the 10^{-7} M UII group). A similar effect on cell proliferation was also observed as assayed by cell count. #P<0.05 vs. the 10^{-7} M UII group; †p>0.05 vs. the 10^{-7} M UII group.

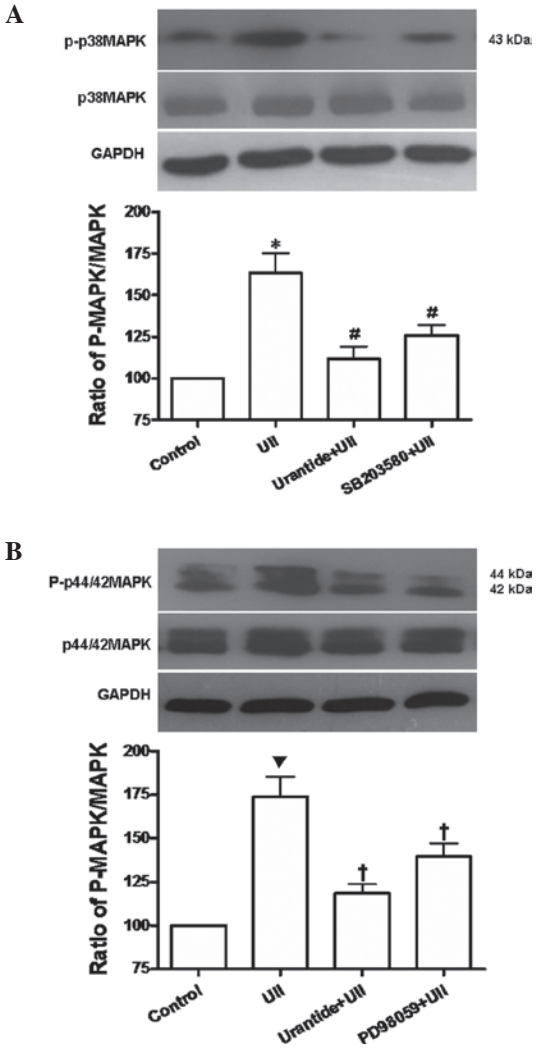

Figure 3. Urotensin II (UII) increases the phosphorylation levels of p38MAPK and p44/42MAPK. (A) UII increased the phosphorylation levels of p38MAPK, and this effect was inhibited by the inhibitors SB203580 (10 μM) and urantide, respectively. *P<0.05 vs. the control group; #p<0.05 vs. the UII group. (B) UII increased the phosphorylation levels of p44/42MAPK, and this effect was inhibited by the inhibitors PD98059 (10 μM) and urantide, respectively. ▼P<0.05 vs. the control group; †p<0.05 vs. the UII group.

the treatment of EPCs with UII. A 163.3±12.0% increase in the phosphorylation levels of p38MAPK was detected in UII-stimulated EPCs compared to control cells ($p<0.05$ vs. the control group) (Fig. 3A). The effect of UII on p38MAPK was inhibited by 41.5±7.3 and 37.5±6.3% in response to urantide (10 μM) ($p<0.05$ vs. the UII group) and SB203580 (10 μM) ($p<0.05$ vs. the UII group), respectively.

A 173.8±11.6% increase in the phosphorylation levels of p44/42MAPK was detected in UII-stimulated EPCs compared to the control cells ($p<0.05$ vs. the control group) (Fig. 3B). The effect of UII on p44/42MAPK was inhibited by 55.3±5.3 and 44.2±7.7% in response to urantide (10 μM) ($p<0.05$ vs. the UII group) and PD98059 (10 μM) ($p<0.05$ vs. the UII group), respectively.

Discussion

The present study demonstrated that UII promotes EPC proliferation in a dose-response manner. We also showed that the proliferative effect of UII is mediated via activation of the MAPK pathway.

The UII receptor (GPR14) is a seven transmembrane-spanning G protein-coupled receptor (7,8). The expression pattern of UII and GPR14 is consistent with their roles in cardiovascular homeostasis (9). Increasing evidence has indicated that, in addition to mast cells, GPR14 are expressed on and promote the proliferation of VMSCs and ECs (9,10). Other studies have reported that UII and GPR14 are expressed at high levels in atherosclerotic plaques and injured myocardium (3,11). For more than a decade, EPCs have been implicated in cardiovascular homeostasis (12-14). Expression of GPR14 in EPCs further indicated that UII exerts its function through the increase of UII concentration in circulation and local tissue (4). Results of the present study showed that UII induces EPC proliferation in a concentration-dependent manner in a certain range. This finding suggests that UII has the potential to improve the function of EPCs and may accelerate repair of the endothelial integrity in response to vascular injury or myocardial infarction.

The molecular mechanisms underlying the effects of UII and GPR14 in cardiovascular homeostasis are not entirely understood. Wang *et al* (15) reported that UII upregulates the expression of collagen-1 and downregulates the expression of matrix metalloproteinase-1 in endothelial cells. Djordjevic *et al* (16) found that UII activates NADPH oxidase and plasminogen activator inhibitor-1 in VSMCs, leading to atherosclerotic plaque formation. In addition, Tamura *et al* (17) reported that UII stimulates the phosphorylation of p44/42MAPK, but not that of focal adhesion kinase in VSMCs. Shi *et al* (9) found that UII exerts proliferative effects in human umbilical vein ECs via p44/42MAPK signaling. In the present study, we found

that UII activated p38MAPK and p44/42MAPK. The critical role of p38MAPK and p44/42MAPK in this setting was further demonstrated by the respective inhibitors SB203580 and PD98059, which significantly decreased the UII-induced phosphorylation of p38MAPK and p44/42MAPK and prevented UII-mediated proliferative effects. SB203580 and PD98059 did not completely block the phosphorylation of p38MAPK and p44/42MAPK induced by UII. This may be due to signaling crosstalk in response to UII. The present data suggest that UII may be involved in the pathophysiologic alterations in EPC function via p38MAPK and p44/42MAPK signaling.

In conclusion, UII promotes cell proliferation in EPCs via p38MAPK and p44/42MAPK signaling. These findings provide novel insight regarding the role of UII in the EPC-mediated repair of atherosclerotic injury.

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