Blockade of Urotensin II Receptor Prevents Vascular Dysfunction

Young-Ae Kim[1], Dong Gil Lee[1], Kyu Yang Yi[2], Byung Ho Lee[2] and Yi-Sook Jung[1,3,]*

[1]College of Pharmacy, Ajou University, Suwon 16499,
[2]Research Center for Drug Discovery Technology, Korea Research Institute of Chemical Technology, Daejeon 34114,
[3]Research Institute of Pharmaceutical Sciences and Technology, Ajou University, Suwon 16499, Republic of Korea

Abstract

Urotensin II (UII) is a potent vasoactive peptide and mitogenic agent to induce proliferation of various cells including vascular smooth muscle cells (VSMCs). In this study, we examined the effects of a novel UII receptor (UT) antagonist, KR-36676, on vasoconstriction of aorta and proliferation of aortic SMCs. In rat aorta, UII-induced vasoconstriction was significantly inhibited by KR-36676 in a concentration-dependent manner. In primary human aortic SMCs (hAoSMCs), UII-induced cell proliferation was significantly inhibited by KR-36676 in a concentration-dependent manner. In addition, KR-36676 decreased UII-induced phosphorylation of ERK, and UII-induced cell proliferation was also significantly inhibited by a known ERK inhibitor U0126. In mouse carotid ligation model, intimal thickening of carotid artery was dramatically suppressed by oral treatment with KR-36676 (30 mg/kg/day) for 4 weeks compared to vehicle-treated group. From these results, it is indicated that KR-36676 suppress UII-induced proliferation of VSMCs at least partially through inhibition of ERK activation, and that it also attenuates UII-induced vasoconstriction and vascular neointima formation. Our study suggest that KR-36676 may be an attractive candidate for the pharmacological management of vascular dysfunction.

Key Words: Urotensin II, Urotensin II receptor antagonist, KR-36676, ERK, Smooth muscle, Proliferation

INTRODUCTION

Atherosclerosis remains the leading cause of morbidity and mortality worldwide, especially in the Western world. In the normal vascular environment, vascular smooth muscle cells (VSMCs) are contractile and responsive to stimuli generated by changes in blood flow and blood pressure. However, the atherosclerotic environment, growth factors released from intimal layer of the arterial wall can proliferation of VSMCs leading to VSMCs transmigration from the media into the intima, and further formation of atherosclerotic lesions (Loirand *et al.*, 2008).

Urotensin II (UII), a cyclic peptide of 11 amino acids, was identified as the ligand for the orphan G-protein coupled receptor 14 which has been renamed UII receptor (UT) (Ames *et al.*, 1999b; Maguire and Davenport, 2002). UII and UT are expressed in a great number of cells (Coulouarn *et al.*, 1998; Ames *et al.*, 1999b; Douglas *et al.*, 2000; Maguire *et al.*, 2004), including endothelial cells and VSMCs (Ames *et al.*, 1999b; Douglas *et al.*, 2000). Up-regulation of the UII sys-

tem has been demonstrated to have association with several cardiovascular and metabolic diseases (Douglas *et al.*, 2000). In fact, increased expression of UII and UT was observed in diseased carotid arteries from patients with atherosclerosis (Ames *et al.*, 1999b; Maguire *et al.*, 2004; Hassan *et al.*, 2005), while little expression of UII is found in normal arteries. In addition, UII is known to be upregulated in atherosclerotic lesions, which results in the formation of atheroma by causing VSMCs proliferation and migration, as well as foam cell formation (Watanabe *et al.*, 2005). In this context, inhibition of the UII system has been considered as a therapeutic strategy for atherosclerotic vascular diseases.

To date, several selective UT antagonists including SB-611812, palosuran, and urantide have been developed to attenuate the detrimental events in cardiovascular pathology, such as atherosclerosis and intimal hyperplasia of restenosis lesions (Tsoukas *et al.*, 2011). Very recently, we have reported a newly synthesized UT antagonist KR-36676 (2-(6,7-dichloro-3-oxo-2H-benzo[b][1,4]oxazin-4(3H)-yl)-N-methyl-N-(2-(pyrrolidin-1-yl)-1-(4-(thiophen-3-yl)phenyl) ethyl)acetamide),

www.biomolther.org

can exert anti-hypertrophic effects against pressure overload-induced cardiac hypertrophy (Oh *et al*., 2015). The present study aimed to evaluate the effects of KR-36676 on UII-induced vascular dysfunction by investigating its effects on VSMCs proliferation, vascular contractile response and neo-intimal thickening. In addition, we further investigated the potential mechanisms responsible for the effects of KR-36676.

MATERIALS AND METHODS

Chemicals and reagents

Human UII was obtained from Sigma-Aldrich (St Louis, MO, USA). KR-36676 was synthesized at the Bio-Organic Division of the Korea Research Institute of Chemical Technology (Daejon, Republic of Korea). In this study, KR-36676 was dissolved in dimethyl sulfoxide (DMSO) for in vitro, and 0.5% carboxymethylcellulose (CMC) for in vivo study, which were selected from the preliminary experiments to find out solvents for KR-36676 by using various solvents such as distilled water, saline, polyethylene glycol, DMSO and 0.5% CMC. SB202190, SP600125, and U0126 were purchased from Calbiochem (San Diego, CA, USA). Anti-phospho ERK1/2 and anti-ERK1/2 antibodies were purchased from Cell Signaling (Beverly, MA, USA).

Cell culture

hAoSMCs (ScienCell, Carlsbad, CA, USA) were incubated in growth media SmCM (ScienCell, Carlsbad, CA, USA) containing 5% fetal bovine serum at 37°C in a humidified 5% CO_2 incubator. All experimental cells were between passages 5 and 9. Cells were pretreated with UT antagonist or inhibitors for 30 min after serum starvation for 24 h. Thereafter, the cells were incubated with or without UII.

5-Bromo-2'-deoxyuridine (BrdU) incorporation

Cell proliferation was accomplished using the BrdU Cell Proliferation Assay (Calbiochem, Darmstadt, Germany), according to the manufacturer's directions. Briefly, cells were seeded at the density of 1×10^4 cells/well in 96-well plates. BrdU was increased to the stated medium for 24 h. The cells were then incubated with anti-BrdU antibody for 30 min. For quantification of the incorporated BrdU, measurement at 405 nm was carried out using a microplate reader (Molecular Devices Corp., Sunnyvale, CA, USA).

Western blot analysis

Phosphorylation of ERK1/2 was detected using western blot analysis, as previously described (Lee *et al*., 2012). Cells were dissolved using lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 1% NP-40, 150 mmol/L NaCl, 0.25% Na-deoxycholate, 2 mmol/L EDTA, 1 mmol/L NaF, 1 mmol/L Na_3VO_4, 1 mmol/L PMSF, 10 μg/mL of aprotinin, and 10 μmol/L leupeptin). Lysates were then centrifuged at 14,000 rpm for 15 min, and the supernatants were obtained. Equal volumes of protein were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and detected with the indicated antibodies (Liang *et al*., 2013). After incubation with horseradish peroxidase (HRP)-conjugated secondary antibody, proteins were visualized using an LAS 1000 (Fuji Photo Film, Tokyo, Japan). Densitometric analyses were accomplished using QuantityOne software (Biorad Laboratories, Berkeley,

CA, USA).

Measurement of contractile response in rat aorta

The descending thoracic aorta was isolated from Sprague-Dawley rats. The endothelial layer of the aorta was destroyed by gentle rubbing of the luminal surface with a cotton swab moistened with Krebs' solution (Kim *et al*., 2009). The aorta was cut into ring segments of 2-3 mm in width, and the aorta rings were suspended between wire hooks in an organ bath containing 20 ml of Krebs' bicarbonate buffer (mM: NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $NaHCO_3$, 25; $MgSO_4$, 1.2; KH_2PO_4, 1.2; and glucose, 11.0) bubbled with mixture gas (95% O_2, 5% CO_2) and maintained at 37°C. A resting tension of 2 g was applied, and the aortic preparations were allowed to equilibrate for 90 min. The aortic preparations were precontracted submaximally with 45 mM KCl. After the contraction was stabilized, acetylcholine (1.0 μM) was added to confirm the absence of the endothelium. After washing out 3 times for 45 min, and the aortic preparations were pretreated with a single concentration of KR-36676 (0.03, 0.1, 0.3 μM) for 30 min, then a cumulative concentration-contractile response curve for urotensin II (0.1-300 nM) was obtained. Stock solutions of KR-36676 were prepared in dimethyl sulfoxide and the maximum concentration of dimethyl sulfoxide in experimental Krebs'bicarbonate buffer was 0.3%. Results are expressed as percentage of the KCl-induced response. The pA2 values were determined according to the Schild equation.

Mouse carotid ligation model

All protocols used conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and were also approved by the Committee on Animal Research at Ajou Medical Center, Ajou University, Suwon, Republic of Korea. Male 8-week-old C57BL/6 mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg), and anesthesia was confirmed by the absence of toe pinch reflex (Ferguson *et al*., 2013). The left common carotid artery was then ligated close to the carotid bifurcation with 6-0 propylene suture, as previously described (Sindermann *et al*., 2002). KR-36676 (30 mg/kg/day) was orally treated 30 min before surgery and continued for 2 or 4 weeks after surgery. The vehicle group was given oral administration of 0.5% CMC. Intimal thickening was evaluated for each ligation on days 14 and 28.

Hematoxylin and eosin staining

Carotid arteries were fixed in 4% paraformaldehyde for 24 h and paraffin embedded. Serial sections (thickness of 5 μm) were cut and samples from each 100 μm interval were collected. Slides were stained with H&E (Watanabe *et al*., 2009; Ou *et al*., 2014). The luminal surface area was measured by tracking the lumen. Thickening of the intima was analyzed using the ImageJ software (NIH). Three equally spaced cross section of the vessel were analyzed in all mice.

Statistical analysis

All data were presented as the mean ± SEM from at least 4 different experiments. Comparisons were done using Student's t-tests and ANOVA. A *p*-value of <0.05 was considered statistically significant.
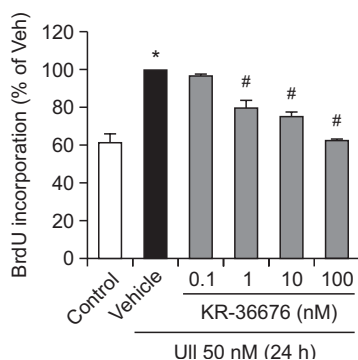
Fig. 1. Effects of KR-36676 on UII-induced proliferation of hAoSMCs. hAoSMCs were incubated with different concentrations of KR-36676 (0.1-100 nM) 30 min before treatment with UII (50 nM, 24 h).Cell proliferation was analyzed using BrdU kit as described in Methods section. Results are presented as mean ± SEM (n≥5). *$p < 0.05$ vs. Control; #$p < 0.05$ vs. Vehicle. BrdU, bromodeoxyuridine.
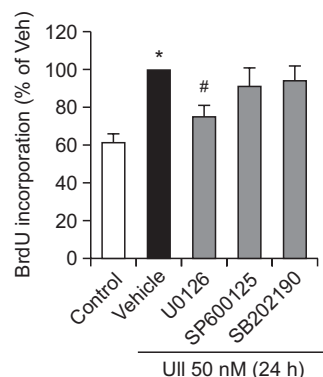
Fig. 2. Inhibitory activity of various inhibitors on UII-induced proliferation in hAoSMCs. hAoSMCs were pre-incubated with U0126 (ERK1/2 inhibitor), SP600125 (JNK inhibitor) and SB202190 (p38 MAPK inhibitor) for 30 min before treatment with UII (50 nM, 24 h). Cell proliferation was analyzed using BrdU kit as described in Methods section. Results are presented as mean ± SEM (n≥5). *$p < 0.05$ vs. Control; #$p < 0.05$ vs. Vehicle. BrdU, bromodeoxyuridine.

RESULTS

KR-36676 attenuated the proliferation of hAoSMC

The proliferation of VSMCs is the main phase for the progression of atherosclerosis and restenosis (Duran-Prado *et al*., 2013). Herein, the effects of KR-36676 on the proliferation of UII (50 nM)-stimulated hAoSMCs were assessed through analysis of DNA synthesis using the incorporation of BrdU. The pretreatment of hAoSMCs with KR-36676 (1, 10 and 100 nM) 30 min before UII exposure significantly inhibited the increase in BrdU incorporation stimulated by UII. The UII-induced proliferation was inhibited by KR-36676 in a concentration-dependent manner (Fig. 1).

The UII-induced proliferation of hAoSMC was inhibited by U0126

The activation of MAPK is known to have critical role in the UII-induced proliferation of VSMCs (Djordjevic *et al*., 2005; Duran-Prado *et al*., 2013). Therefore, the effects of inhibitors of p38 MAPK, JNK, and ERK1/ on VSMC proliferation were
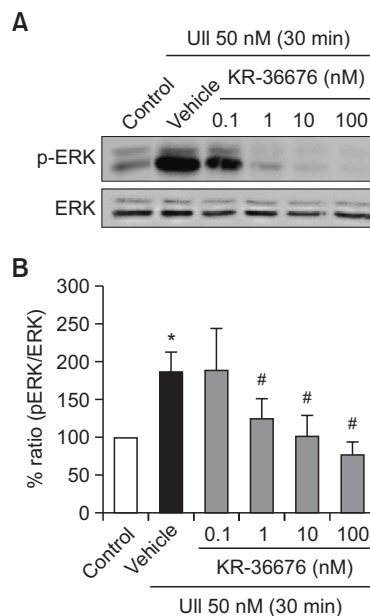
Fig. 3. Effect of KR-36676 on UII-induced ERK1/2 phosphorylation in hAoSMCs. (A) p-ERK was detected by western blotting using anti-p-ERK antibody. Equal loading was confirmed by re-probing the membranes with an anti-ERK1/2 antibody. Cells were pre-incubated with KR-36676 (0.1-100 nM) for 30 min before treatment with UII (50 nM, 24 h). (B) Summary of the effects of KR-36676 on ERK1/2 phosphorylation. Results are presented as mean ± SEM (n≥5). *$p < 0.05$ vs. Control; #$p < 0.05$ vs. Vehicle.

examined. U0126 (ERK1/2 inhibitor) largely inhibited the UII-induced proliferation of hAoSMCs, but SP600125 (JNK inhibitor) and SB202190 (p38 MAPK inhibitor) did not. These results indicated that the phosphorylation of ERK1/2 may play a role in the VSMCs proliferation induced by UII (Fig. 2).

KR-36676 inhibited the phosphorylation of ERK1/2

Next, the effect of KR-36676 on ERK1/2 phosphorylation was examined in hAoSMCs. Maximal phosphorylation of ERK1/2 occurred 30 min after induction with UII (data not shown), and the UII-induced increase in ERK1/2 phosphorylation was inhibited by KR-36676 in a concentration-dependent manner (Fig. 3). Similar to the inhibitory effect on proliferation, KR-36676 (1, 10, 100 nM) was also shown to inhibit the phosphorylation of ERK1/2 induced by UII at 30 min in a concentration-dependent manner.

Effect of KR-36676 on contractile response in rat aortia

We examined the effect of KR-36676 on constrictive responses induced by UII in rat aorta. As shown in Fig. 4, KR-36676 (100, 300 nM) inhibited the UII-induced contractions of the rat aorta in a concentration-dependent manner, with a parallel rightward shift in the concentration-response curve.

KR-36676 Inhibited VSMC Proliferation *In Vivo*

In order to investigate the influence of KR-36676 on the proliferation of VSMCs *in vivo*, the effects were examined in an induced neointimal proliferation model established by ligation of the mice carotid artery. In this model, widespread neointima formation was induced after 4 weeks (Fig. 5). The
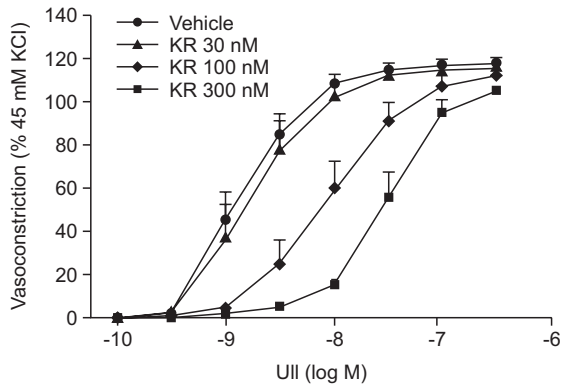
Fig. 4. Effect of KR-36676 (KR) on UII-induced constriction in rat aorta. Rat aortic ring were treated with KR-36676 (30, 100, 300 nM) 30 min before treatment with increasing concentration of UII (0.1 nM-500 nM. Then, a concentration-contractile response was measured, and results are presented as mean ± SEM (n≥6).

Fig. 5. Effect of KR-36676 (KR) on carotid ligation-induced intimal thickening. C57BL/6J mice (n≥10) underwent ligation of the left common carotid artery, and then mice were treated with KR for 4 weeks by daily oral administration of 30 mg/kg. Quantitative analysis of intima area. Results are presented as mean ± SEM (n≥7). *$p<0.05$ vs. control; #$p<0.05$ vs. vehicle.
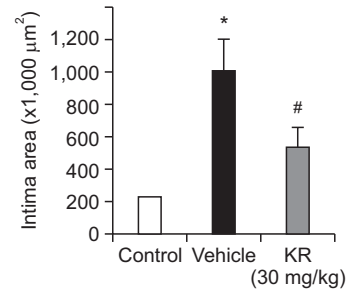
presence of decreased vessel diameter diminished the physiological relevance of this model for the investigation of human cardiovascular disease. Oral administration of mice with KR-36676 extremely reduced the neointima formation in the artery of ligated legion. These results indicate that the inhibitory effect of KR-36676 on VSMCs proliferation may be associated with its reducing effect on intima-media thickening of the carotid artery.

DISCUSSION

This study demonstrated that KR-36676, a novel selective UT antagonist, inhibits VSMCs proliferation, at least partially, through inhibiting ERK1/2 pathway. KR-36676 also revealed alleviating effect on vascular remodeling in a mouse model of carotid artery ligation.

Atherosclerosis is known to be associated with the release of growth factors and cytokines leading to VSMCs proliferation and transmigration into the intima. The subsequent accumulation of lipids, foam cells, and extracellular matrix proteins in the subendothelial layer leads to the formation of atherosclerotic plaques. Several reports have recently shown that UII plays a role in the pathogenesis of atherosclerosis by stimulating the proliferation of ECs and VSMCs (Watanabe *et al*., 2009). In addition, both *in vitro* and *in vivo* studies have reported that UII might contribute to VSMCs hyperplasia and vasoconstriction, as well as cardiomyocyte hypertrophy (Loirand *et al*., 2008). In this context, the inhibition of UII activity with UT antagonists such as SB-611812 and urantide has been suggested to attenuate VSMCs proliferation and atheroscleroric lesion. In this study, the effect of KR-36676 on UII-stimulated VSMC proliferation was assessed by analyzing DNA synthesis using BrdU incorporation. The concentration of UII (50 nM) used in this study was determined from the preliminary experiments to found out its concentration showing the best efficacy. The UII (50 nM)-induced VSMCs proliferation was significantly inhibited by pretreatment with KR-36676 (1, 10 and 100 nM) in a concentration-dependent manner.

ERK1/2 phosphorylation has been reported to play an important role in the mitogenic property of UII in several cells in-

cluding human umbilical vein endothelial cells and rat pulmonary artery smooth muscle cells (Sauzeau *et al*., 2001; Sue *et al*., 2009; Rodriguez-Moyano *et al*., 2013). The role of ERK1/2 phosphorylation in UII-induced proliferation was also revealed in our study in hAoSMCs. This study also demonstrated that UII-induced ERK1/2 phosphorylation was inhibited by KR-36676.

UII has been defined as the most potent vasoconstrictor to date as it is approximately 10-fold more potent than ET-1 (Ames *et al*., 1999a). Yet, stimulation of UT is known to be able to trigger the release of relaxant mediators such as nitric oxide (NO), prostacyclin and prostaglandin E_2 to balance the contractile effect on VSMCs (Gibson, 1987; Bottrill *et al*., 2000; Gray *et al*., 2001). Vasoconstriction is mediated by receptors on VSMCs, whereas vasodilation is endothelium-mediated (Douglas *et al*., 2004). However, in a disease state of chronic heart failure or essential hypertension, UII loses its dilatory ability (Lim *et al*., 2004; Sondermeijer *et al*., 2005). It is understood that such a loss and dysfunction of endothelial cells would favor a contractile response over a relaxant one (Lim *et al*., 2004). Hence, UII causes endothelium-independent vasoconstriction during disease state. In the present study, the contractile response of rat aorta to UII was shown to be inhibited by KR-36676 (Fig. 4), suggesting its beneficial potential to balance the vascular tone in a disease state. Further study remains to elucidate the signaling mechanisms for these beneficial effects on vascular tone.

Neointima formation is known as common various forms of various vascular diseases including atherosclerosis and restenosis (Owens *et al*., 2004; Raines, 2004). It has been reported that plasma UII level is positively interrelated with carotid atherosclerosis in hypertensive patient (Suguro *et al*., 2007) and in patients with carotid artery disease (Heringlake *et al*., 2004). A high level of UII is observed in diseased carotid arteries from patients with atherosclerosis whereas little expression of UII is found in normal arteries (Ames *et al*., 1999b; Maguire *et al*., 2004; Hassan *et al*., 2005). Based on the fact that proliferation of VSMCs contributes to neointima formation, we examined the inhibitory effects of KR-36676 (30 mg/kg) on proliferation of VSMCs in *in vivo* using mouse carotid artery ligation model. This model has been characterized by rapid proliferation of VSMC (Kumar and Lindner, 1997), by showing that intimal cell proliferation and thickening continues at a high rate until 4 weeks (Choi *et al*., 2004). The concentrations of KR-36676,

30 mg/kg, was determined from the preliminary experiments to found out its concentration showing the best efficacy. This dose is the same as that used in the previous study (Oh *et al.*, 2015). Consistent with the previous studies, our results in this study showed that a significant intimal thickening in carotid artery of ligated legion due to intimal cell proliferation was observed after 4 weeks (Fig. 5). These data show the presence of decreased vessel diameter in this animal model and proves physiological relevance of this model for the investigation of human cardiovascular disease. The neointima formation was significantly attenuated by KR-36676.

In summary, this study has shown that the blockade of UII by KR-36676 has strong inhibitory effect on the proliferation of hAoSMC *in vitro*, at least partially, through inhibition of ERK1/2 signaling pathway. This study has also demonstrated that KR-36676 inhibits the UII-induced contractile response in isolated aortic ring, and attenuates vascular neointima formation in *in vivo* carotid ligation model. From these results, it is suggested that UII antagonism by KR-36676 may be an attractive candidate to prevent vascular dysfunction including atherosclerosis and restenosis.

ACKNOWLEDGMENTS

REFERENCES

Ames, N. P., Clarke, J. M., Marchylo, B. A., Dexter, J. E. and Woods, S. M. (1999a) Effect of Environment and Genotype on Durum Wheat Gluten Strength and Pasta Viscoelasticity. *Cereal Chem.* **76**, 582-586.

Ames, R. S., Sarau, H. M., Chambers, J. K., Willette, R. N., Aiyar, N. V., Romanic, A. M., Louden, C. S., Foley, J. J., Sauermelch, C. F., Coatney, R. W., Ao, Z., Disa, J., Holmes, S. D., Stadel, J. M., Martin, J. D., Liu, W. S., Glover, G. I., Wilson, S., McNulty, D. E., Ellis, C. E., Elshourbagy, N. A., Shabon, U., Trill, J. J., Hay, D. W., Ohlstein, E. H., Bergsma, D. J. and Douglas, S. A. (1999b) Human urotensin-II is a potent vasoconstrictor and agonist for the orphan receptor GPR14. *Nature* **401**, 282-286.

Bottrill, F. E., Douglas, S. A., Hiley, C. R. and White, R. (2000) Human urotensin-II is an endothelium-dependent vasodilator in rat small arteries. *Br. J. Pharmacol.* **130**, 1865-1870.

Choi, E. T., Khan, M. F., Leidenfrost, J. E., Collins, E. T., Boc, K. P., Villa, B. R., Novack, D. V., Parks, W. C. and Abendschein, D. R. (2004) Beta3-integrin mediates smooth muscle cell accumulation in neointima after carotid ligation in mice. *Circulation* **109**, 1564-1569.

Coulouarn, Y., Lihrmann, I., Jegou, S., Anouar, Y., Tostivint, H., Beauvillain, J. C., Conlon, J. M., Bern, H. A. and Vaudry, H. (1998) Cloning of the cDNA encoding the urotensin II precursor in frog and human reveals intense expression of the urotensin II gene in motoneurons of the spinal cord. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 15803-15808.

Djordjevic, T., BelAiba, R. S., Bonello, S., Pfeilschifter, J., Hess, J. and Gorlach, A. (2005) Human urotensin II is a novel activator of NADPH oxidase in human pulmonary artery smooth muscle cells.

Arterioscler. Thromb. Vasc. Biol. **25**, 519-525.

Douglas, S. A., Dhanak, D. and Johns, D. G. (2004) From "gillstopills": urotensin-II as a regulator of mammalian cardiorenal function. *Trends Pharmacol. Sci.* **25**, 76-85.

Douglas, S. A., Sulpizio, A. C., Piercy, V., Sarau, H. M., Ames, R. S., Aiyar, N. V., Ohlstein, E. H. and Willette, R. N. (2000) Differential vasoconstrictor activity of human urotensin-II in vascular tissue isolated from the rat, mouse, dog, pig, marmoset and cynomolgus monkey. *Br. J. Pharmacol.* **131**, 1262-1274.

Duran-Prado, M., Morell, M., Delgado-Maroto, V., Castano, J. P., Aneiros-Fernandez, J., de Lecea, L., Culler, M. D., Hernandez-Cortes, P., O'Valle, F. and Delgado, M. (2013) Cortistatin inhibits migration and proliferation of human vascular smooth muscle cells and decreases neointimal formation on carotid artery ligation. *Circ. Res.* **112**, 1444-1455.

Ferguson, D., Koo, J. W., Feng, J., Heller, E., Rabkin, J., Heshmati, M., Renthal, W., Neve, R., Liu, X., Shao, N., Sartorelli, V., Shen, L. and Nestler, E. J. (2013) Essential role of SIRT1 signaling in the nucleus accumbens in cocaine and morphine action. *J. Neurosci.* **33**, 16088-16098.

Gibson, A. (1987) Complex effects of Gillichthys urotensin II on rat aortic strips. *Br. J. Pharmacol.* **91**, 205-212.

Gray, G. A., Jones, M. R. and Sharif, I. (2001) Human urotensin II increases coronary perfusion pressure in the isolated rat heart. Potentiation by nitricoxide synthase and cyclooxygenase inhibition. *Life Sci.* **69**, 175-180.

Hassan, G. S., Douglas, S. A., Ohlstein, E. H. and Giaid, A. (2005) Expression of urotensin-II in human coronary atherosclerosis. *Peptides* **26**, 2464-2472.

Heringlake, M., Kox, T., Uzun, O., Will, B., Bahlmann, L., Klaus, S., Eleftheriadis, S., Armbruster, F. P., Franz, N. and Kraatz, E. (2004) The relationship between urotensin II plasma immunoreactivity and left ventricular filling pressures in coronary artery disease. *Regul. Pept.* **121**, 129-136.

Kim, J. H., Bugaj, L. J., Oh, Y. J., Bivalacqua, T. J., Ryoo, S., Soucy, K. G., Santhanam, L., Webb, A., Camara, A., Sikka, G., Nyhan, D., Shoukas, A. A., Ilies, M., Christianson, D. W., Champion, H. C. and Berkowitz, D. E. (2009) Arginase inhibition restores NOS coupling and reverses endothelial dysfunction and vascular stiffness in old rats. *J. Appl. Physiol.* **107**, 1249-1257.

Kumar, A. and Lindner, V. (1997) Remodeling with neointima formation in the mouse carotid artery after cessation of blood flow. *Arterioscler. Thromb. Vasc. Biol.* **17**, 2238-2244.

Lee, N. Y., Rieckmann, P. and Kang, Y. S. (2012) The changes of P-glycoprotein activity by interferon-gamma and tumor necrosis factor-alpha in primary and immortalized human brain microvascular endothelial cells. *Biomol. Ther.* **20**, 293-298.

Liang, Q., Yu, F., Cui, X., Duan, J., Wu, Q., Nagarkatti, P. and Fan, D. (2013) Sparstolonin B suppresses lipopolysaccharide-induced inflammation in human umbilical vein endothelial cells. *Arch. Pharm. Res.* **36**, 890-896.

Lim, M., Honisett, S., Sparkes, C. D., Komesaroff, P., Kompa, A. and Krum, H. (2004) Differential effect of urotensin II on vascular tone in normal subjects and patients with chronic heart failure. *Circulation* **109**, 1212-1214.

Loirand, G., Rolli-Derkinderen, M. and Pacaud, P. (2008) Urotensin II and atherosclerosis. *Peptides* **29**, 778-782.

Maguire, J. J. and Davenport, A. P. (2002) Is urotensin-II the new endothelin? *Br. J. Pharmacol.* **137**, 579-588.

Maguire, J. J., Kuc, R. E., Wiley, K. E., Kleinz, M. J. and Davenport, A. P. (2004) Cellular distribution of immunoreactive urotensin-II in human tissues with evidence of increased expression in atherosclerosis and a greater constrictor response of small compared to large coronary arteries. *Peptides* **25**, 1767-1774.

Oh, K. S., Lee, J. H., Yi, K. Y., Lim, C. J., Lee, S., Park, C. H., Seo, H. W. and Lee, B. H. (2015) The orally active urotensin receptor antagonist, KR36676, attenuates cellular and cardiac hypertrophy. *Br. J. Pharmacol.* **172**, 2618-2633.

Ou, Y., Li, Q., Wang, J., Li, K. and Zhou, S. (2014) Antitumor and apoptosis induction effects of paeonol on mice bearing EMT6 breast carcinoma. *Biomol. Ther.* **22**, 341-346.

Owens, G. K., Kumar, M. S. and Wamhoff, B. R. (2004) Molecular

regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol. Rev.* **84**, 767-801.

Raines, E. W. (2004) PDGF and cardiovascular disease. *Cytokine Growth Factor Rev.* **15**, 237-254.

Rodriguez-Moyano, M., Diaz, I., Dionisio, N., Zhang, X., Avila-Medina, J., Calderon-Sanchez, E., Trebak, M., Rosado, J. A., Ordonez, A. and Smani, T. (2013) Urotensin-II promotes vascular smooth muscle cell proliferation through store-operated calcium entry and EGFR transactivation. *Cardiovasc. Res.* **100**, 297-306.

Sauzeau, V., Le Mellionnec, E., Bertoglio, J., Scalbert, E., Pacaud, P. and Loirand, G. (2001) Human urotensin II-induced contraction and arterial smooth muscle cell proliferation are mediated by RhoA and Rho-kinase. *Circ. Res.* **88**, 1102-1104.

Sindermann, J. R., Smith, J., Kobbert, C., Plenz, G., Skaletz-Rorowski, A., Solomon, J. L., Fan, L. and March, K. L. (2002) Direct evidence for the importance of p130 in injury response and arterial remodeling following carotid artery ligation. *Cardiovasc. Res.* **54**, 676-683.

Sondermeijer, B., Komp, A., Komesaroff, P. and Krum, H. (2005) Effects of exogenous urotensin-II on vascular tone in skin micro circulation of patients with essential hypertension. *Am. J. Hypertens.* **18**, 1195-1199.

Sue, Y. M., Chen, C. H., Hsu, Y. H., Hou, C. C., Cheng, C. Y., Chen, Y. C., Lin, S. L., Chen, T. W. and Chen, T. H. (2009) Urotensin II induces transactivation of the epidermal growth factor receptor via transient oxidation of SHP-2 in the rat renal tubular cell line NRK-52E. *Growth Factor (Chur, Switzerland)* **27**, 155-162.

Suguro, T., Watanabe, T., Ban, Y., Kodate, S., Misaki, A., Hirano, T., Miyazaki, A. and Adachi, M. (2007) Increased human urotensin II levels are correlated with carotid atherosclerosis in essential hypertension. *Am. J. Hypertens.* **20**, 211-217.

Tsoukas, P., Kane, E. and Giaid, A. (2011) Potential clinical implications of the Urotensin II receptor antagonists. *Front. Pharmacol.* **2**, 38.

Watanabe, T., Arita, S., Shiraishi, Y., Suguro, T., Sakai, T., Hongo, S. and Miyazaki, A. (2009) Human urotensin II promotes hypertension and atherosclerotic cardiovascular diseases. *Curr. Med. Chem.* **16**, 550-563.

Watanabe, T., Suguro, T., Kanome, T., Sakamoto, Y., Kodate, S., Hagiwara, T., Hongo, S., Hirano, T., Adachi, M. and Miyazaki, A. (2005) Human urotensin II accelerates foam cell formation in human monocyte-derived macrophages. *Hypertension* **46**, 738-744.