INTRODUCTION

Neointimal hyperplasia resulting from proliferation and migration of vascular smooth muscle cells (VSMCs) contributes to the pathogenesis of various vascular diseases such as atherosclerosis, transplant vasculopathy, and restenosis after coronary angioplasty (Huang et al., 2011). Characteristic changes in the complex process of neointima formation include the migration of medial SMCs into the subendothelial layer and the accumulation of VSMCs within the intima. Such exacerbated proliferation and migration of VSMCs occur in response to various factors such as inflammatory cytokines and growth factors produced in excess after injury (Rudijanto, 2007). Although the pathogenesis of neointima formation has been extensively studied at the molecular level, clinically effective therapy for this disease is currently unavailable (Huang et al., 2011).

Human UII, a cyclic peptide of 11 amino acid (H-Glu-Thr-Pro-Asp-c[Cys-Phe-Trp-Lys-Tyr-Cys]-Val-OH), is related to a number of diseases (Loirand et al., 2008). Elevated plasma UII levels have been found in patients with cardiovascular diseases including heart failure, hypertension, and atherosclerosis (Heringlake et al., 2004; Suguro et al., 2007). UII is a potent vasoconstrictor (Ames et al., 1999), it is also a mitogenic and hypertrophic agent, partly resulting in the enlargement of cells and neointima formation (Djordjevic et al., 2005; Chen et al., 2008). Indeed, there is a strong expression of UII in a new intima of rat carotid arteries with balloon angioplasty-induced restenosis (Tsoukas et al., 2011). Therefore, UII antagonism has been considered as a significant therapy for restenosis and atherosclerosis. Up to date, several selective UT antagonists such as SB-657510A with anti-atherosclerotic efficacy have been developed (Park et al., 2013). However, a potent and safe antagonist that can be used in patient with atherosclerosis and restenosis is yet to be found. Very recently, GSK-1440115 (4′-[(1R)-1-[[6,7-dichloro-3-oxo-2,3-
dihydro-4H-1,4-benzoxazin-4-yl)acetyl][methyl]amino]-2-(4-morpholinyl)ethyl]-4-biphenylcarboxylic acid trifluoroacetate (GSK-1440115).

In the search for more effective UT antagonists, we recently synthesized N-(1-(3-bromo-4-(piperidin-4-yloxy)benzyl)piperidin-4-yl)benzo[b]thiophene-3-carboxamide (KR-36996) and found that it was a novel and potent UT antagonist (Kᵢ: 4.4 nM). The present study aimed to evaluate the effect of this novel UT antagonist KR-36996 on cell proliferation of VSMCs in comparison with GSK-1440115, a structurally different UT antagonist. In addition, we evaluated the effects of KR-36996 in comparison with GSK-1440115, a structurally different UT antagonist or inhibitors for 30 min before UII treatment.

**Cell culture**
Human aortic SMCs (HASMC) (Lonza, Walkersville, MD, USA) were cultured in growth media SmGM-2 (Lonza) in 5% fetal bovine serum at 37°C in a humidified 5% CO₂ incubator. From the preliminary test for optimum concentration of UII, we found out 50 nM UII as optimal conc. for HASMC proliferation. After serum starvation for 24 h, cells were pretreated with UT antagonist or inhibitors for 30 min before UII treatment.

**5-Bromo-2'-deoxyuridine (BrdU) incorporation**
Cell proliferation was performed using BrdU Cell Proliferation Assay (Calbiochem). Briefly, cells were seeded at 1×10⁴ cells/well in 96-well plates. BrdU was added to the conditioned medium for 24 h. Subsequently, cells were fixed and incubated with anti-BrdU antibody for 30 min. The quantification of BrdU incorporation was measured at 405 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

**Western blot analysis**
Activation of ERK1/2 was measured using western blot analysis as previously described (Lee et al., 2012). Cells were lysed 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 Na₃VO₄, 1 mmol/L PMSF, 10 μg/mL of aprolin, and 10 μmol/L leupeptin). Lysates were subsequently centrifuged at 14,000 rpm for 15 min and supernatants were collected. Then, equal amounts of protein were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and reacted with antibody (Liang et al., 2013). After probing with horseradish peroxidase (HRP)-conjugated secondary antibody, proteins were visualized using LAS 1000 (Fuji Photo Film, Tokyo, Japan). Densitometric analyses were performed using QuantityOne software (Biorad Laboratories, Inc., Hercules, CA, USA).

**Measurements of reactive oxygen species (ROS) release**
Intracellular ROS were measured fluorometrically using 2',7'-dichlorofluorescein diacetate (DCF-DA) (Molecular probes, Eugene, OR, USA), which permeates cells easily and hydrolyzes to DCF after interacting with intracellular ROS (Lee et al., 2016). Cultured cells were washed twice with HEPES.
controlled salt solution (HCSS) incubated with 10 μM DCF-DA and 20% Pluronic F-127 for 30 min, and washed with HCSS, as described previously (Lee and Jung, 2012). Subsequently, cells were observed under a fluorescent microscope (Olympus, Tokyo, Japan). DCF fluorescence intensities were obtained in Fluoview FV300 software (Olympus Corporation, Shinjuku, Tokyo, Japan).

**Mouse carotid ligation model**

All animal studies 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 these were approved by the Committee on Animal Research at Ajou Medical Center, Ajou University (Suwon, Korea). Male 8-week-old C57BL/6 mice were anesthetized with intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) and the adequacy of anaesthesia was confirmed by the absence of toe pinch reflex as described previously (Ferguson et al., 2013). The left common carotid artery was ligated near the carotid bifurcation with 6-0 propylene suture as described previously (Sandermann et al., 2002). KR-36996 (10, and 30 μg/kg) and GSK-1440115 (10, and 30 μg/kg) were orally administrated 30 min before surgery and every day for 2 or 4 weeks after surgery. Vehicle group was orally treated with 0.5% CMC. Intimal thickening was assessed at 14 or 28 days after ligation.

**Hematoxylin and eosin staining**

The common carotid arteries were fixed in 4% paraformaldehyde for 24 h, and then embedded in paraffin. Serial sections (5 μm thick) were cut and collected every 100 μm interval. Slides were stained with hematoxylin and eosin (H&E) (Watanabe et al., 2009; Ou et al., 2014). Luminal area was measured by tracing along the luminal surface. Intimal thickening was analyzed using ImageJ software (NIH, public domain, USA). Three equally spaced cross-sections of the vessel in all mice were analyzed.

**Statistical analysis**

All data were expressed as the mean ± SEM from at least 4 different experiments. The comparisons were performed using Student’s t-tests. A p-value of <0.05 was considered statistically significant.

**RESULTS**

**Effects of KR-36996 and GSK-1440115 on SMC proliferation**

Proliferation of SMCs is a key step in the progression of atherosclerosis and restenosis (Duran-Prado et al., 2013). Therefore, we investigated the effects of UT antagonists on cell proliferation of primary HASMC stimulated with UII. As shown in Fig. 2A, UII stimulated the proliferation of HASMC based on BrdU incorporation. UII-induced proliferation was inhibited by KR-36996 at 1, 10, and 100 nM (inhibition of 47.4 ± 3.0%, 53.9 ± 2.3%, and 86.3 ± 4.5%, respectively) in a concentration-dependent manner, whereas GSK-1440115 only inhibited UII-induced proliferation at 10 and 100 nM (inhibition of 22.6 ± 2.2% and 56.4 ± 2.8%, respectively).

**Role of MAPKs and ROS in UII-induced SMC proliferation**

Activation of MAPK and ROS is critically involved in UII-induced proliferation of SMCs (Djordjevic et al., 2005; Duran-Prado et al., 2013). Therefore, we examined the effects of inhibitors for MAPK family (p38 MAPK, JNK, and ERK1/2) or ROS on SMC proliferation. Results are shown in Fig. 3B. U0126 (inhibitor for ERK1/2) and trolox (ROS inhibitor) significantly inhibited UII-induced proliferation (inhibition of 55.4 ± 2.6% and 60.9 ± 4.0%, respectively), whereas neither SP600125 (inhibitor for JNK) nor SB202190 (inhibitor for p38 MAPK) significantly inhibited UII-induced proliferation, indicating that the activation of ERK1/2 and generation of ROS might play important roles in UII-induced SMC proliferation.

**Effects of KR-36996 and GSK-1440115 on ROS generation**

Next, we examined the effects of KR-36996 and GSK-1440115 on ROS generation in HASMC. Cells were treated with UII (50 nM) for 24 h in the presence or absence of KR-36996, GSK-1440115, or inhibitors. U: U0126, inhibitor of ERK1/2; SP: SP600125, inhibitor of JNK; SB: SB202190, inhibitor of p38 MAPK, Tro: trolox, scavenger for ROS. *p<0.05 vs. control (CTL), "p<0.05 vs. vehicle (Veh). N=5.
activation in HASMC. ERK1/2 activation was maximally induced by UII for 30 min (Fig. 4A). UII-induced ERK1/2 activation was significantly inhibited by KR-36996 at concentrations of 1, 10, and 100 nM (inhibition of 36.9 ± 2.8%, 70.1 ± 3.4%, and 97.6 ± 5.7%, respectively, Fig. 4B, 4E). However, UII-induced ERK1/2 activation was inhibited by GSK-1440115 only at 100 nM (inhibition of 51.7 ± 3.0%, Fig. 4C, 4E). To determine whether ROS might play a role in UII-induced activation of ERK1/2, we examined the effects of inhibitors for ROS on ERK1/2 activation. Our results revealed that UII-induced activation of ERK1/2 was significantly inhibited by trolox (Fig. 4D, 4E), indicating that ROS may act as an upstream signal of ERK1/2 phosphorylation by UII.

Effects of KR-36996 and GSK-1440115 on intimal thickening

To investigate whether KR-36996 might affect VSMCs proliferation in vivo, we examined its effect in an established neointima hyperplasia model induced by ligation of carotid artery. In this model, extensive neointima formation was induced at 2 and 4 weeks after carotid artery ligation (Fig. 5). Neointima formation was significantly reduced by oral administration of 10 mg/kg of KR-36996 for 4 weeks (reduction of 18 ± 5.1%), but not by 2 weeks of treatment. However, neointima formation was not changed by treatment with GSK-1440115 at 10 mg/kg for 2 or 4 weeks. When each drug was used at concentration of 30 mg/kg, neointima formation was significantly reduced by oral administration of KR-36996 for 2 and 4 weeks (reduction of 42 ± 4.6% and 73 ± 3.1%, respectively). Neointima formation was also significantly inhibited by 4 weeks of treatment with GSK-1440115 (reduction of 34 ± 6.3%), but not by its treatment for 2 weeks. These results indicate that KR-36996 has better beneficial effect on vascular intima-media thickening compared to GSK-1440115.

DISCUSSION

This study showed that a novel selective UT antagonist KR-36996 and a structurally different known UT antagonist GSK-1440115 could elicit inhibitory effects on VSMCs proliferation, at least partially through inhibiting ROS/ERK1/2 pathway. This study further demonstrated that KR-36996 and GSK-1440115 could attenuate vascular neointima formation in mice. Our results suggest that UT antagonist could be used as therapeutics for neointima formation during restenosis.

Recently, we have synthesized N-(1-(3-bromo-4-(piperidin-4-yloxy)benzyl)piperidin-4-yl)benzo-[b]thiophene-3-carboxamide (KR-36996), a novel selective UT antagonist. Results from radioligand binding assay indicated that KR36996 had greater binding affinity for hUT (Ki value: 4 nM) than GSK-1440115 (Ki: 31 nM) (data not shown). Consistently, this study showed that KR-36996 could elicit remarkable inhibitory effect on VSMC proliferation with greater potency than GSK-1440115 (IC50: 3.5 nM and 82.3 nM, respectively). The accumulation of SMCs in the intimal space of arteries as a result of their migratory and proliferative activities is a critical event not only in atherosclerosis, but also in restenosis after angioplasty. For more than several decades, balloon angioplasty
and stent technology are widely used in the treatment of atherosclerosis. Many patients still have trouble with restenosis, such as having recurrent neointimal lesions consisting of hypoperiplastic VSMCs and abundant extracellular matrix (Huang et al., 2011). Such exacerbated proliferation and migration of VSMCs can occur in response to various factors including inflammatory cytokines and UII. Our results in this study raise the possibility that KR-36996 might be able to prevent neo-intima formation through inhibiting VSMCs proliferation.

To investigate whether KR36996 could prevent UII-induced neointima formation in vivo, we used a carotid artery ligation model. This model is characterized by medial SMC replication followed by SMC migration from the media to the intima and SMC proliferation within intima, leading to intimal thickening (Louis and Zahradka, 2010). It has been reported that both UII and UT are found within the regions of human atherosclerotic plaque in carotid arteries (Rakowski et al., 2005). UII expression has been found to be upregulated in rat carotid arteries with balloon angioplasty-induced restenosis (Rakowski et al., 2005). Taken together, it is suggested that the increase of UII or UT level has relation with development of neointima forma-

Consistent with previous results, our study showed that SMC migration from media to intima occurred within 1 week after ligation. Intimal cell proliferation continued at a high rate at 2 weeks after ligation. It was present for 4 weeks after ligation. Similarly, intimal thickening due to intimal SMC proliferation was observed extensively after 4 weeks. This intimal thickening was significantly attenuated by KR-36996 30 mg/kg at 2 weeks and 4 weeks after ligation. The reason for the usage of high dose KR-36996 in this in vivo study is based on the preliminary study to determine the optimum dose for in vivo study. Even though 10 mg/kg of KR-36996 showed slightly inhibitory effect on neointima formation, the effect of 30 mg/kg KR-36996 was much remarkable. Therefore, we compared the effect of KR-36996 and GSK-1440115 in in vivo study at the dose of 30 mg/kg, and found greater efficacy of KR-36996 than GSK-1440115 (Fig. 5). These results implicate that KR-36996 may act as a more effective UT antagonist in preventing neointima formation.

It is known that UII can induce the proliferation of VSMCs via multiple mechanisms such as RhoA/Rho kinase and ERK1/2 signaling (Sauzeau et al., 2001). UII can upregulate the ex-
pression levels of NADPH oxidase subunits p22phox and NOX4, indicating that ROS plays a role in the mitogenic effect of UII in vascular SMCs (Pendyala et al., 2009). UII-induced generation of ROS is dependent on NADPH oxidase activity, which also contributes to UII-induced activation of ERK1/2, p38 MAPK, c-Jun N-terminal kinase, and Akt (Djordjevic et al., 2005). Consistent with these reports, our study showed that phosphorylation of ERK1/2 was inhibited by trolox, whereas generation of ROS was not affected by U0126 (Fig. 3, 4), supporting that ROS generation acts as an upstream signal of activation of ERK1/2 during UII-induced proliferation in VSMCs. The ROS/ERK1/2 signaling pathway involved in UII-induced proliferation was significantly inhibited by KR-36996 and GSK-1440115.

In summary, this study demonstrates that blockade of UII binding to UT by KR-36996 elicits potent inhibitory effect on VSMC proliferation in vitro and neointima formation in vivo with greater potency than GSK-1440115. These results suggest that KR-36996 may be an attractive candidate to prevent vascular remodeling in the pathogenesis of atherosclerosis and restenosis.

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