Abstract. In this study, we clarified the intracellular mechanism of angiotensin II (Ang II) in promoting migration in rat aortic smooth muscle cells (RASMCs). RASMC migration was measured with the Boyden chamber assay, and the result was confirmed with an aortic sprout assay. The activities of kinases were investigated by western blot analysis. Ang II enhanced RASMC migration, which was chemotaxis directed, and induced the phosphorylation of p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase 1/2 (ERK1/2), and heat shock protein 27 (Hsp27). Ang II-enhanced cell migration was inhibited by SB203580 (a p38 MAPK inhibitor) and piceatannol (a spleen tyrosine kinase inhibitor), but only partially by PD98059 (an ERK inhibitor) and PP2 (a Src inhibitor). The Ang II-stimulated phosphorylation of p38 MAPK and Hsp27 in RASMCs was inhibited by piceatannol and SB203580. The phosphorylation of ERK1/2 stimulated by Ang II was suppressed by PD98059, piceatannol, and PP2. Ang II increased the sprout outgrowth from aortic rings and this response was attenuated by pretreatment with SB203580, PD98059, PP2, or piceatannol. These results suggest that p38 MAPK contributes to the regulation of the Ang II-induced chemotactic migration of vascular smooth muscle cells, which is mediated by Hsp27 phosphorylation.

Keywords: angiotensin II, migration, rat aortic smooth muscle cell (RASMC), p38 mitogen-activated protein kinase (MAPK), heat shock protein 27 (Hsp27)

Introduction

Vascular smooth muscle cell migration, apoptosis, and proliferation are important events in the formation of the neointima in pathological states such as atherosclerosis and hypertension (1–3). Angiotensin II (Ang II), a physiologically important vasoconstrictor of the renin-angiotensin system, is a major mediator in the inflammation, migration, and proliferation of vascular smooth muscle cells, when they are exposed to chronic or long-term stimulation with Ang II (3–5). AT1, an Ang II receptor, is widely expressed in all organs, and this receptor mediates most of the physiological and pathophysiological actions of Ang II, including the vasoconstriction, hypertrophy, apoptosis, proliferation, and migration of vascular cells (6–8). Activation of the AT1 receptor stimulates various signaling molecules, including protein kinase C, mitogen-activated protein kinase (MAPK), and tyrosine kinases (7, 9). Moreover, tyrosine kinase activation by Ang II also leads to the activation of MAPK and consequently promotes the growth response in vascular smooth muscle cells (10, 11).

Cell migration is mediated by intracellular kinases including Src, phosphoinositide-3 kinase, and focal adhesion kinase; and MAPK is a downstream target of all these kinases (12–14). MAPKs are involved in the
regulation of the migration and proliferation of vascular smooth muscle cells (15, 16). The activation of p38 MAPK leads to the contraction, migration, and proliferation of vascular smooth muscle cells (17–19). Ang II is known to stimulate the activation of MAPKs, including extracellular signal-regulated kinase (ERK) 1/2, p38 MAPK, and stress-activated protein kinase/c-Jun N-terminal kinase in vascular smooth muscle cells (9, 20, 21). Previously, we have shown that the p38 MAPK pathway is involved in spleen tyrosine kinase (Syk)-induced migration in response to platelet-derived growth factor (PDGF) in vascular smooth muscle cells (3). Heat shock protein 27 (Hsp27) acts as a physiological substrate for p38 MAPK, and the phosphorylation of Hsp27 by p38 MAPK is associated with actin polymerization and neutrophil migration (22–24). Moreover, we recently reported that Ang II-induced migration was suppressed by the inhibition of Hsp27 in vascular smooth muscle cells (3). These results suggest that p38 MAPK is associated with the Hsp27-mediated migration of vascular smooth muscle cells in response to Ang II. However, the role of p38 MAPK in promoting Ang II-induced migration has not yet been determined in vascular smooth muscle. Therefore, in this study, we investigated the roles of p38 MAPK and its related signal molecules in the Ang II-induced migration of rat aortic smooth muscle cells (RASMCs).

Materials and Methods

Cell isolation and culture

All experiments and animal care were conducted in conformity with the institutional guidelines of Konkuk University, South Korea. RASMCs (used at passages 5–11) were enzymatically isolated from aorta of male Sprague Dawley rats (6-week-old, 187 g, n = 6) and were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, and 200 mM glutamine. For all experiments, RASMCs were grown to 70%–80% confluence and starved in DMEM without FBS for 24 h. After the treatment with stimulants, cells were lysed with cold extraction buffer [20 mM HEPES, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 10% glycerol, 10 mM NaF, 1 mM Na2VO4, 2.5 mM 4-nitrophenylphosphate, 0.5 mM PMSF, and 1 tablet of complete proteinase inhibitor cocktail (Roche, Indianapolis, IN, USA)].

Immunoblotting

RASMC lysates were centrifuged (13,000 × g, 15 min, 4°C), and the supernatants were collected as protein samples. Protein concentrations were determined using Bio-Rad DC protein assay reagents. The protein homogenates were diluted 1:1 (v/v) with SDS sample buffer containing 40 mM Tris-HCl (pH 6.8), 8 mM EGTA, 4% 2-mercaptoethanol, 40% glycerol, 0.01% bromophenol blue, and 4% SDS and then boiled for 5 min. Proteins (20–30 µg/lane) were separated using 12% polyacrylamide SDS gels and then transferred electrophoretically to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). The membrane was then blocked for 1 h at room temperature with PBS containing 0.05% Tween 20 and 5% fat-free dried milk. The membranes were incubated with antibodies diluted 1:2000 overnight at 4°C. Immune complexes were detected with horseradish peroxidase-conjugated antibodies (Amersham-Pharmacia, Piscataway, NJ, USA) diluted 1:1000 and incubated for 1 h at room temperature. After application of the secondary antibody, blots were incubated in enhanced chemiluminescence kits (Amersham-Pharmacia) and exposed to photographic film. Band intensity was measured by computer analysis using Quantitation software (Bio-Rad, Hercules, CA, USA).

Immunoprecipitation

Cell lysates containing 500 µg of proteins in the extraction buffer were incubated with 4 µg/ml anti-Syk antibody for 5 h at room temperature. The immunocomplex was precipitated using protein A-agarose beads (Roche) overnight at 4°C. Beads were washed with PBS containing 0.05% Tween-20, resuspended in the SDS sample buffer, and boiled for 5 min. Protein samples collected were immunoblotted with an anti-phospho tyrosine (4G10) antibody as described above.

Boyden chamber assay

Cell migration assays were carried out in 48-well microchemotaxis Boyden chambers (Neuro Probe, Cabin John, MD, USA). Polycarbonate membranes with 8-µm pores (Neuro Probe) were coated with a 0.1 mg/ml of type I collagen from rat tail tendon (BD Bioscience, San Jose, CA, USA) and then dried for 60 min. RASMCs were harvested using trypsin-EDTA (Life Technologies, Paisley, UK) and resuspended in DMEM containing 0.1% BSA and test inhibitors. The bottom chamber was loaded with 3 × 104 cells and the membrane was laid over the cells. The microchamber was then inverted and incubated at 37°C for 120 min. The chamber was then returned to an upright position, and the upper wells were loaded with DMEM containing 0.1% BSA, PDGF-BB, Ang II, and test inhibitors. The chamber was then incubated at 37°C for 90 min, and the membranes were fixed and stained using Diff-Quik (Baxter Healthcare, Miami, FL, USA). The number of
cells migrating through the membrane was determined by counting four randomly chosen regions of each well under a microscope (×400).

Aortic ring assay
Ex vivo RASMC migrations were measured by an aortic ring assay using Matrigel with some modifications (3, 25). The endothelium and adventitia from the aorta of Sprague Dawley rats (8-week-old, n = 5) was enzymatically removed and the vessels were cut into rings (1 mm). The rings were placed and embedded in 48-well plates coated with Matrigel and simultaneously added with stimulants (PDGF-BB or Ang II) and test inhibitors in FBS-free medium. On day 5, the rings were stained with Diff-Quik and photographed and the length of sprouts was analyzed by Scion Image software.

Immunofluorescent staining
The expression of α-smooth muscle actin (α-SMA) in the aortic ring sprouts were determined by the standard immunostaining method. Briefly, the sprouts were fixed with 10% formaldehyde at room temperature for 15 min. After washing twice with PBS containing 0.05% Tween 20 and permeabilizing with 0.1% Triton X-100 for 5 min, the sprouts were treated with 1% BSA in PBS for 2 h and incubated with FITC-conjugated anti-α-SMA antibody (1:500, Sigma) overnight at 4°C. The immunostained sprouts were observed with fluorescence microscopy (Axio Observer A; Zeiss, Jena, Germany).

Materials
Piceatannol, SB203580, PP2, and PD98059 were purchased from Tocris Bioscience (Bristol, UK). PDGF-BB was obtained from R&D Systems (Minneapolis, MN, USA). Ang II, DMEM, FBS, penicillin, streptomycin, and other culture ware and chemical reagents were obtained from Hyclone (Logan, UT, USA) and Sigma. Matrigel was purchased from BD Bioscience. The following antibodies were used: monoclonal anti-Syk (Abcam, Cambridge, UK); polyclonal anti-Syk (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); anti-phospho tyrosine (Upstate, Lake Placid, NY, USA); anti-p38 MAPK, anti-phospho p38 MAPK, anti-ERK1/2, anti-phospho ERK1/2 (Cell Signaling, Beverly, MA, USA); anti-phospho Hsp27 (Affinity BioReagents, Golden, CO, USA); and anti-β-actin and monoclonal anti-FITC-conjugated α-SMA (Sigma) antibodies.

Statistical analysis
Data are presented as the mean ± S.E.M. The statistical evaluation of data was performed by Student’s t-tests for comparisons between pairs of groups and by ANOVA for multiple comparisons. P<0.05 was considered statistically significant.

Results

Ang II-stimulated migration of RASMCs and the signaling pathway involved
To evaluate the effects of Ang II on vascular responses, we examined the migration of RASMCs in response to Ang II. Ang II (0.1 – 100 nM) concentration-dependently increased RASMC migration in the Boyden chamber assay and this migration reached a maximum at 100 nM Ang II (189.9 ± 6.5% of the control, n = 8) (Fig. 1).

To determine whether Ang II-stimulated cell migration is chemotactic or chemokinetic, RASMC migration was analyzed using a checkerboard experiment. When Ang II was placed in both the upper and lower chambers or only in the upper chamber, cell migration was virtually the same. However, cell migration was increased by the presence of Ang II only in the lower chamber, indicating that Ang II induces the direct chemotaxis of RASMCs (Table 1, n = 4).

To clarify the signaling pathways of Ang II-induced cell migration, cells were pretreated with kinase inhibitors and their migration in response to Ang II was analyzed. Ang II (100 nM)-enhanced cell migration was completely inhibited in RASMCs by treatment with kinase inhibitors such as SB203580 (30 µM, a p38 MAPK inhibitor) or piceatannol (30 µM, a Syk kinase inhibitor) to a level similar to that observed in the quiescent state (n = 4), but only partly by PP2 (10 µM, a Src inhibitor) or PD98059 (30 µM, an ERK inhibitor) (Fig. 2A). Furthermore, we tested the effect of Syk inhibitor on Ang II-induced Syk activation. Cells were preincubated with piceatannol (30 µM) and then stimulated with Ang II (100 nM) for 5 min. The cell extracts were immunoprecipitated with anti-Syk antibody and

![Fig. 1. Ang II-stimulated migration of RASMCs. The cells were treated with Ang II (0.1 – 100 nM) or PDGF-BB (5 ng/ml) for 90 min and migration was examined using a Boyden chamber assay. Migration of RASMCs in the quiescent state is expressed as 100% (n = 8). *P<0.05 vs the quiescent state.](image-url)
then immunoblotted with anti-phospho tyrosine (4G10) antibody. Figure 2B showed that Ang II (100 nM) elevated Syk phosphorylation and this was inhibited by treatment with piceatannol (30 µM) (n = 3). Moreover, Ang II was reported to transmit its signal to cells via the PDGF receptor (9) and to activate the Syk tyrosine kinase in vascular smooth muscle (3). Therefore, we examined the contribution of the PDGF β receptor to Ang II-induced migration using a receptor neutralization method. The Ang II-stimulated migration (193.9 ± 15% of control, n = 8) was inhibited in cells neutralized with an anti-PDGF β-receptor antibody (119.1 ± 9.8% of control, n = 8).

**Table 1. Checkerboard analysis of Ang II-stimulated RASMC migration**

<table>
<thead>
<tr>
<th>Lower chamber (nM)</th>
<th>0</th>
<th>0.1</th>
<th>1</th>
<th>10</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper chamber (nM)</td>
<td>0</td>
<td>22.5 ± 1.30</td>
<td>23.5 ± 1.4</td>
<td>24.3 ± 1.4</td>
<td>18.0 ± 0.9</td>
</tr>
<tr>
<td>0.1</td>
<td>22.8 ± 0.8</td>
<td>23.5 ± 0.9</td>
<td>28.8 ± 1.0*</td>
<td>25.0 ± 2.6</td>
<td>24.5 ± 2.2</td>
</tr>
<tr>
<td>1</td>
<td>33.3 ± 1.1*</td>
<td>24.3 ± 0.6</td>
<td>23.0 ± 2.1</td>
<td>20.8 ± 1.4</td>
<td>21.8 ± 1.5</td>
</tr>
<tr>
<td>10</td>
<td>38.3 ± 1.1*</td>
<td>30.0 ± 0.9*</td>
<td>25.3 ± 1.3</td>
<td>21.8 ± 0.9</td>
<td>21.8 ± 0.8</td>
</tr>
<tr>
<td>100</td>
<td>43.8 ± 0.9*</td>
<td>34.5 ± 0.7*</td>
<td>30.3 ± 1.5*</td>
<td>26.3 ± 1.3</td>
<td>24.8 ± 1.1</td>
</tr>
</tbody>
</table>

Each data values indicates the mean ± S.E.M. of the results obtained from 4 independent experiments. * Denotes a significant increase from the responses in the quiescent states, with P<0.05.

Fig. 2. Effects of kinase inhibitors on Ang II-stimulated migration and Syk activation of RASMCs. A: Cells were pre-incubated for 120 min with one of the following kinase inhibitors: SB203580 (30 µM; a p38 MAPK inhibitor, SB), PD98059 (30 µM; an ERK inhibitor, PD), piceatannol (30 µM; a Syk inhibitor, PIC), and PP2 (10 µM; a Src inhibitor); and then they were treated with Ang II (100 nM) for 90 min. Migration was examined using a Boyden chamber assay. Migration of RASMCs in the quiescent state is expressed as 100% (n = 8). *P<0.05 vs Ang II-stimulated controls. B: Effects of Syk inhibitor on Ang II-stimulated Syk phosphorylation in cells. Cells were treated with piceatannol (30 µM) for 30 min and then stimulated with Ang II (100 nM) for 5 min. The lysates were immunoprecipitated with anti-Syk antibody and then immunoblotted with anti-phospho tyrosine (4G10) antibody (upper panel). The total expression of Syk was determined with immunoblotting using an anti-Syk antibody (lower panel) (n = 3). IB, immunoblotting; IP, immunoprecipitation; P-Syk, phosphorylated Syk; pY, phospho tyrosine; PIC, piceatannol.

then immunoblotted with anti-phospho tyrosine (4G10) antibody. Figure 2B showed that Ang II (100 nM) elevated Syk phosphorylation and this was inhibited by treatment with piceatannol (30 µM) (n = 3). Moreover, Ang II was reported to transmit its signal to cells via the PDGF receptor (9) and to activate the Syk tyrosine kinase in vascular smooth muscle (3). Therefore, we examined the contribution of the PDGF receptor to Ang II-induced migration using a receptor neutralization method. The Ang II-stimulated migration (193.9 ± 15% of control, n = 8) was inhibited in cells neutralized with an anti-PDGF receptor antibody (119.1 ± 9.8% of control, n = 8).

**Ang II-induced kinase phosphorylation in RASMCs**

As shown in Fig. 3, the phosphorylation of p38 MAPK in RASMCs was increased in a concentration-dependent manner by treatment with Ang II (1 – 100 nM), reaching a maximal response at 100 nM Ang II (n = 4, Fig. 3: A and B). The phosphorylation of both ERK1/2 and Hsp27 also showed concentration-dependent increases (Fig. 3: A, C, and D).

To clarify how Ang II-stimulated kinase activity is affected by kinase inhibitors, RASMCs were stimulated with Ang II after treatment with kinase inhibitors. Ang II (100 nM)-induced phosphorylation of p38 MAPK and Hsp27 was inhibited by 30 µM piceatannol (n = 4, Fig. 4A). The phosphorylation of p38 MAPK and Hsp27 in response to Ang II (100 nM) was also attenuated by 30 µM PD98059, 30 µM piceatannol, or 10 µM PP2, but not by 30 µM SB203580 (Fig. 4B).

**Effect of Ang II on aortic sprout formation**

We also assessed the effects of kinase inhibitors on the Ang II-induced response in the aortic ring assay
using Matrigel to determine the role of Ang II ex vivo. As shown in Fig. 5, Ang II (100 nM) increased the formation of sprout outgrowth from aortic rings, and these sprouted cells expressed α-SMA. The response was completely inhibited by pretreatment with SB203580 (30 µM) or piceatannol (30 µM), but only partly by PD98059 (30 µM) or PP2 (10 µM).

**Discussion**

Although it is well known that Ang II induces vascular smooth muscle cell migration, it is unclear whether this response to Ang II is the result of directed migration or random movement. In this study, Ang II caused an increase in cell migration only when it was added to cells in the low Boyden chamber, but not when it was added to both the lower and upper chambers or to the upper chamber only. This result implies that Ang II has a crucial role as a chemotactic stimulant in vascular smooth muscle cell migration. Moreover, Ang II also increased both the phosphorylation of p38 MAPK and cell migration in a concentration-dependent manner in RASMCs, and these responses were significantly inhibited by treatment with a p38 MAPK inhibitor.

Although previous reports have shown that p38 MAPK is involved in the Ang II-induced migration of fibroblasts, kidney cells, and monocytes (26 – 28), this is the first report directly showing the contribution of p38 MAPK to vascular smooth muscle cell migration in response to Ang II.

MAPK isoforms participate in a variety of cell functions (29 – 31). MAPKs are believed to be associated with the migration and proliferation of vascular smooth muscle cells (15, 16). Migration, proliferation, and MAPK activation can occur in vascular smooth muscle cells stimulated by Ang II (5, 20, 21). Growth factors and cytokines activate the p38 MAPK pathway, which mediates cell migration in tracheal smooth muscle cells (22). Recently, we also found that PDGF increases the phosphorylation of p38 MAPK and that PDGF-mediated migration is inhibited by a p38 MAPK inhibitor (3). These results support the hypothesis that p38 MAPK and its related signals play crucial roles in vascular cell migration in response to stimulants, including growth factors and Ang II. It has also been reported that p38 MAPK acts as an upstream of the protein kinases that lead to the phosphorylation of Hsp27 in various types of cells (32). The activation of Hsp27 plays a key role in...
modulating actin polymerization and the cytoskeletal remodeling associated with cell migration (23, 33). It has been reported that p38 MAPK-mediated smooth muscle cell migration is also mediated by the phosphorylation of Hsp27 (22). Vascular cell migration in response to PDGF and Ang II was also inhibited in cells transfected with siRNA-Hsp27 (3). Moreover, in the present study, the p38 MAPK inhibitor appeared to inhibit the phosphorylation of Hsp27 that had been elevated by Ang II. These results strongly suggest that p38 MAPK contributes to Ang II-induced migration in vascular smooth muscle cells via the phosphorylation of Hsp27.

We also showed here that the phosphorylation of p38 MAPK/Hsp27 and the migration induced by Ang II were significantly inhibited by treatment with a Syk inhibitor, which was confirmed by the sprout formation assay using rat aortic strips. These results suggest that Syk mediates vascular smooth muscle cell migration in response to Ang II. In a previous report, we first demonstrated that Syk inhibition suppresses the activity of p38 MAPK and Hsp27 in the response of RASMCs to PDGF (3). Syk kinase also plays a crucial role in the increase in vascular contraction in response to endothelin-1 (34).

These results imply that Syk kinase acts as an upstream molecule in regulating the p38 MAPK/Hsp27 pathway, which mediates Ang II-induced vascular cell migration. Ang II transmits its signal to cells via the PDGF receptor (9) and elevates the tyrosine phosphorylation of the receptor. Our previous results show that both Ang II and PDGF activate the Syk tyrosine kinase and MAPK pathways in vascular smooth muscle (3, 34). In the present study, Ang II-stimulated migration was attenuated by the treatment cells with PDGF receptor antibody. Therefore, it can be inferred that Ang II-induced signal pathways, for example, Syk or MAPK, are mediated by the direct activation of the PDGF receptor by Ang II. Conversely, in this study, the inhibition of ERK1/2 partly diminished cell migration and did not affect the phosphorylation of Hsp27 and p38 MAPK in response to Ang II, implying that ERK1/2 participates in vascular cell migration via an Hsp27-independent pathway. Moreover, the Syk inhibitor completely abolished the phosphorylation of ERK1/2 elevated by Ang II. These results indicate that Syk kinase participates in both the p38 MAPK/Hsp27 pathway, which plays a critical role, and the ERK1/2 pathway, which is a partial player in Ang II-induced migration.
vascular cell migration. This study demonstrates that the treatment of cells with a Src inhibitor partially attenuates cell migration and completely inhibits ERK1/2 phosphorylation, but does not affect the phosphorylation of p38 MAPK or Hsp27 in response to Ang II. This is similar to the effects of the ERK1/2 inhibitor on cell migration, the phosphorylation of p38 MAPK/Hsp27, and aortic sprouting growth. Src and Syk are protein kinases associated with the functions of vascular smooth muscle cells (35). Therefore, our present findings suggest that Src signaling is involved in Ang II-induced migration through the activation of ERK1/2, but not that of p38 MAPK/Hsp27.

In summary, we have demonstrated that Ang II induces vascular cell migration in a chemotactic manner via the phosphorylation of p38 MAPK and Hsp27 and that this migration is suppressed by inhibitors of p38 MAPK and Syk. Moreover, we have confirmed that an EKR1/2 inhibitor partly attenuates cell migration in response to Ang II. Therefore, we conclude that p38 MAPK acts as a critical regulator of the Ang II-induced chemotactic migration of vascular smooth muscle cells, which is mediated by Hsp27 phosphorylation.

Acknowledgment

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References


