Original Article

Increased Migration of Vascular Adventitial Fibroblasts from Spontaneously Hypertensive Rats

Li LI[1)–3)], Ding-Liang ZHU[1)–3)], Wei-Li SHEN[1)–3)], and Ping-Jin GAO[1)–3)]

Experimental evidence has suggested that vascular adventitial fibroblasts (AFs) may migrate into the neointima of arteries after balloon injury in various animal models. However, the research on migration of AFs has been limited to the effects of acute vascular injury. The role of AFs in chronic vascular injury and hypertension is not yet known. In this study, the migration of spontaneously hypertensive rat (SHR)-AFs and Wistar-Kyoto rat (WKY)-AFs from the thoracic aorta was determined by a transwell technique. Our results showed that fetal calf serum, angiotensin II (Ang II), phorbol ester, basic fibroblast growth factor and platelet-derived growth factor-BB induced migration in a dose-dependent manner, and the migration of SHR-AFs was always greater than that of WKY-AFs. Ang II-induced migration of AFs was considered to have been mediated by Ang II type 1 receptor (AT1-R), because the AT1-R antagonist losartan (10^{-7}–10^{-5} mol/l) suppressed Ang II-induced migration. Ang II-induced migration was also blocked by the extracellular-regulated protein kinase 1/2 (ERK1/2) inhibitor PD98059 (10^{-5} mol/l) and p38 kinase inhibitor SB202190 (10^{-5} mol/l), indicating that ERK1/2 and p38 kinase were involved in Ang II-induced migration. Ang II (10^{-7} mol/l)-induced ERK1/2 and p38 kinase phosphorylation, both of which peaked after 5 min, were suppressed by PD98059 and SB202190, respectively. The Ang-II induced phosphorylation of both proteins was suppressed by losartan, whereas no effect was observed with PD123319, a specific inhibitor of Ang II type 2 receptor (AT2-R). Thus, in the present study, various factors stimulated the migration of SHR-AFs and, to a leber extent, WKY-AFs from the thoracic aorta, and the ERK1/2 and p38 kinase pathways are involved in Ang II-stimulated migration of fibroblasts. (*Hypertens Res* 2006; 29: 95–103)

***Key Words*: vascular adventitial fibroblast, migration, angiotensin II, extracellular-regulated protein kinase, p38 mitogen-activated protein kinase**

Introduction

There is increasing evidence suggesting that the adventitia is a mediator of vascular dysfunction and a potential therapeutic target (*1–3*). More recently, evidence has accumulated that adventitial fibroblasts (AFs) play an important role in pathogenesis after acute angioplasty. After balloon injury, the translocation of bromodeoxyuridine-labeled cells suggests that proliferating adventitial cells migrate to the neointima (*4*). This interesting observation was confirmed by implanting LacZ-positive fibroblasts into the adventitia of carotid arteries and tracking their migration from the adventitia layer, through the medial layer, and into the neointima after endoluminal injury (*5*). However, little attention has been paid to the role of the adventitia in vascular remodeling during hyperten-

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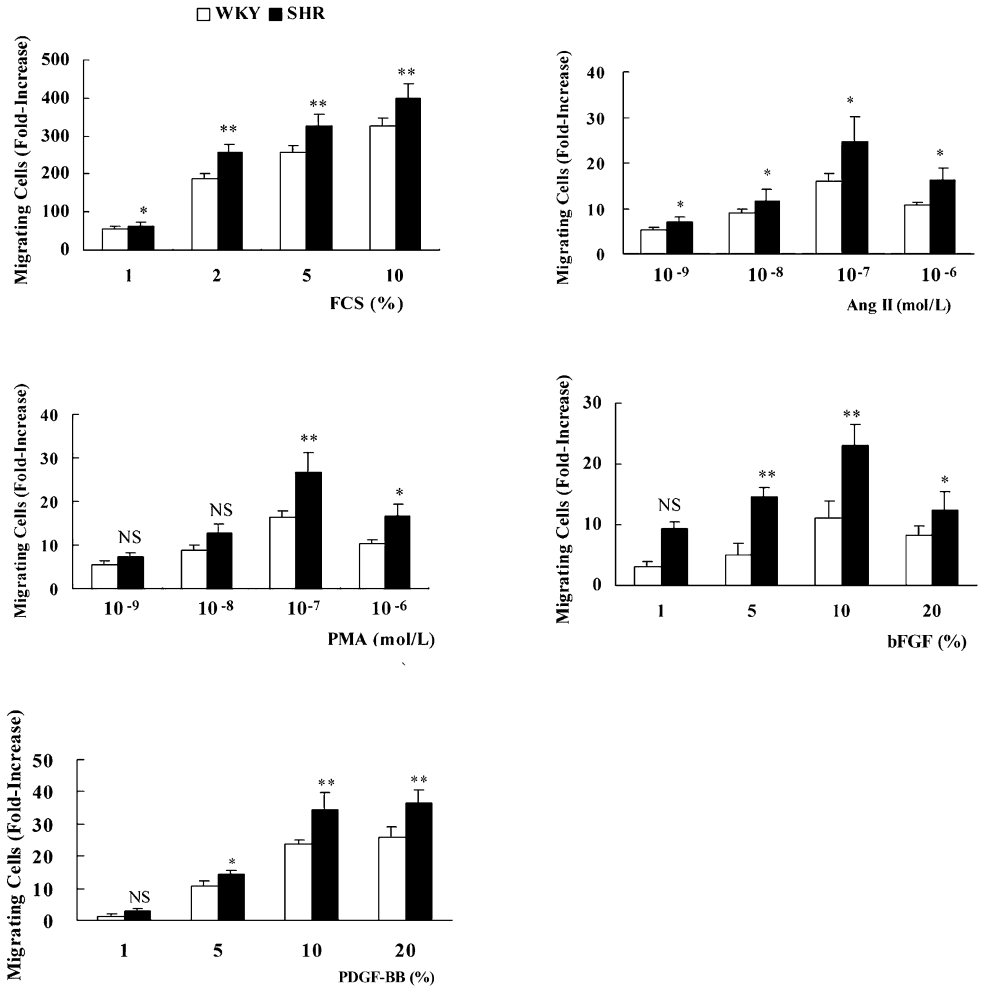

Fig. 1. *Comparison of migratory response of WKY-AFs and SHR-AFs in the presence of various concentrations of serum (1, 2, 5, 10%), Ang II (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} mol/l), PMA (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} mol/l), bFGF (1, 5, 10, 20%) and PDGF-BB (1, 5, 10, 20%). Migration of cells is shown as fold increases over untreated cells. NS, non-significance,* $*p < 0.05$, $**p < 0.01$ *vs. untreated cells.*

sion. Up to now, the research on migration of vascular fibroblasts has been limited to the effects of acute vascular injury. The role of vascular AFs in chronic vascular injury and hypertension is not yet known. Although Zhu *et al*. reported in 1991 that AFs from spontaneously hypertensive rats (SHR) showed greater proliferation potential than those from Wistar-kyoto rats (WKY) (*6*), there has been no report on other adventitial functions, such as the migration of vascular AFs, in hypertension.

Hsieh *et al*. (*7*) has showed that the migration of aortic smooth muscle cells (SMCs) in cultures derived from SHR is greater than that of SMCs derived from WKY. This difference also existed in young animals before the elevation of blood pressure occurred. This finding suggested that enhanced migratory response in SMCs might contribute to the development of hypertension (*7*), whereas previous studies have shown that both AFs and vascular SMCs (VSMCs) play significant roles in vascular injury (*8–10*). Therefore, we hypothesized that vascular AFs in hypertension would show an increased migratory response, and that the local renin-angiotensin system may play a pivotal role in the progrebion of vascular remodeling by stimulating the migration of AFs. In the present study, we examined whether a difference in the migration of vascular fibroblasts derived from SHR and WKY exists in the presence of serum and other stimulating conditions and whether the mitogen-activated protein kinase (MAPK) signaling pathway is involved in angiotensin II (Ang II)-induced migration of vascular fibroblasts derived from SHR.

Methods

All experimental procedures were performed according to the guidelines for the care and use of laboratory animals as estab-

lished by our university.

Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were from Gibco-BRL (Gaithersburg, USA). The cell culture materials and transwell chamber were from Costar (NY, USA). Ang II and PD 123319 were obtained from Sigma-Aldrich (St. Louis, USA). Losartan was provided by Merck & Co. (Rahway, USA). Anti-phospho-p38 MAPK and anti-extracellular-regulated protein kinase 1/2 (anti-ERK1/2) antibodies were purchased from New England Biolabs (Hitchin, UK). β-Actin and other reagents were from Sigma-Aldrich unless otherwise indicated in the text.

Animals and Cell Culture

Male, 8-week-old SHR and WKY rats were used in this study. Systolic blood pressure (SBP) was measured by the tail cuff method using a plethysmograph (Powerlab, Castle Hill, Australia), and was found to be similar in WKY and SHR rats (116±6 mmHg, 125±4 mmHg, respectively, $p>0.05$). AFs from the thoracic aortae of WKY and SHR were isolated and cultured as previously described (*6*). Cells were maintained in DMEM containing 10% fetal bovine serum and penicillin/streptomycin (100 U/ml, 100 μg/ml). Cells were passaged 3–8 times, and experiments were performed in cells passaged an equal number of times. Identification of vascular AFs was confirmed as described previously (*8*).

Migration Assay

Cell migration was measured in a transwell chamber apparatus with 8 μm pore size and a polycarbonate membrane (Costar). To exclude the influence of cell proliferation, chemotaxis of AFs was measured after 6 h. Briefly, the cell suspension (2×10^6 cells/ml) was loaded in the upper compartment of the chamber, whereas DMEM containing fetal calf serum (FCS), Ang II, phorbol ester (PMA), basic fibroblast growth factor (bFGF), and platelet-derived growth factor-BB (PDGF-BB) were added only to the lower compartment of the chamber. The filters were maintained at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. After 6 h, the filters were removed, and cells remaining on the upper surface of the membrane (which had not migrated through the filter) were removed with a cotton swab. Then the membranes were washed with phosphate buffered saline (PBS) 2 times, and cells adhering beneath the membrane were fixed in methanol and stained with hematoxylin. The migration of cells was quantified by cell counts of 5 random fields at ×200 magnification in each membrane. Each experiment was performed in triplicate.

Sodium Dodecyl Sulfate (SDS)–Polyacrylamide Gel Electrophoresis (PAGE) and Western Blotting

Growth-arrested cells were pretreated with losartan (10^{-7} mol/l, 10^{-6} mol/l, 10^{-5} mol/l), 10^{-5} mol/l PD123319, 10^{-5} mol/l PD98059 or 10^{-5} mol/l SB202190 for 45 min prior to agonist exposure. After treatment cells were scraped and lysed as described previously (*8*). The whole cell lysis buffer contained 10 mmol/l Tris-HCl (pH 7.5), 150 mmol/l NaCl, 0.10% SDS, 1.00% Triton-X100, 10 ng/ml aprotinin, 2 μg/ml leupeptin and 1 mmol/l phenylmethyl sulfonyl fluoride (PMSP). The protein concentration was determined by the Bradford method (*11*). Twenty micrograms of protein was loaded in each lane and subjected to SDS-PAGE (8%), and then transferred to a nitrocellulose membrane (100 V for 60 min). The blots were probed with antibodies against phospho-ERK1/2 (1:1,000) and phospho-p38 (1:1,000). Horseradish peroxidase-conjugated secondary antibodies were used in conjunction with an enhanced chemiluminescence (ECL) detection system (Amersham, Little Chalfont, UK).

Statistical Analysis

For all experiments, data are presented as the mean±SEM of three independent experiments. For comparisons between groups, Student's *t*-test was used, with $p<0.05$ being considered statistically significant.

Results

Comparison of the Migratory Responses of AFs from SHR and WKY

The migration of AFs derived from WKY (WKY-AFs) and SHR (SHR-AFs) was examined in the presence of serum, Ang II, PMA, bFGF and PDGF-BB. The most potent stimulant was 10% serum, which had an effect approximately 10-fold greater than any other agonist. Cell migration was induced by the other agonists in a concentration-dependent manner. The migration of SHR-AFs always exceeded that of WKY-AFs (Fig. 1). The largest differences were apparent for Ang II, PMA and PDGF-BB. The fact that the migration of SHR fibroblasts was increased for a G-protein coupled receptor (Ang II) (*12*), a tyrosine kinase receptor (bFGF), and an activator of intracellular kinase (PMA) suggested that the difference was due to intracellular signaling rather than receptor number. We therefore focused our study on Ang II-stimulated migration in SHR-AFs and WKY-AFs.

Ang II-Induced Phosphorylation of ERK1/2 and P38 MAPK through AT1 Receptor

To elucidate the signaling events involved in Ang II-mediated migration, we measured the activation of ERK1/2 and p38

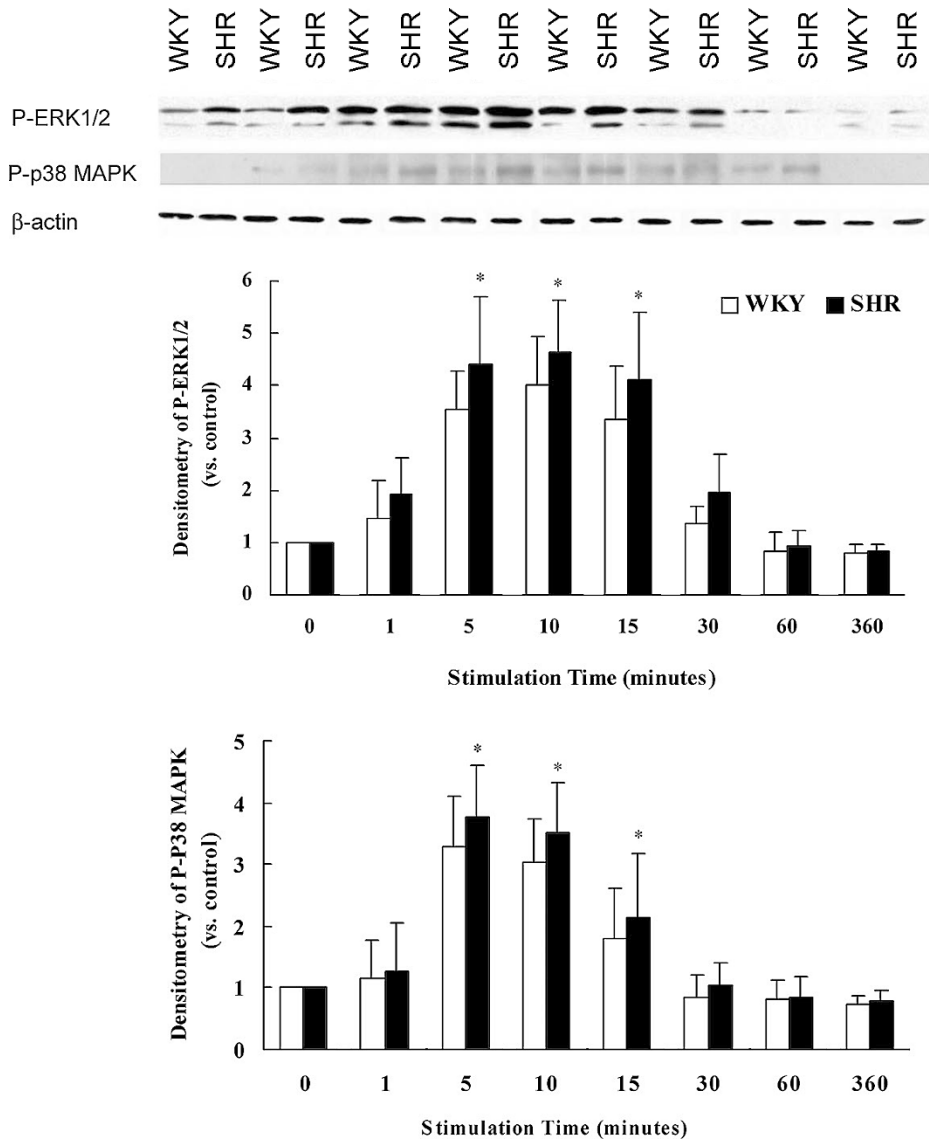

Fig. 2. *Ang II stimulated phosphorylation of ERK1/2 and p38 MAPK in WKY-AFs and SHR-AFs in a time-dependent manner. Cells were stimulated with 10^{-7} mol/l Ang II for 0, 1, 5, 10, 15, or 30 min, 1 h or 6 h. The top panel shows representative immunoblots showing that Ang II induced the phosphorylation of ERK1/2 and p38 MAPK in a time-dependent manner. β-Actin was assayed to verify the equal loading of cell lysates. The two bottom panels show the quantification of the bands by densitometry, respectively. Results are shown as fold increases from 3 independent experiments compared with unstimulated control cells.* *p < *0.05* vs. *WKY-AFs.*

MAPK in WKY-AFs and SHR-AFs by Western blotting. Ang II rapidly induced ERK1/2 and p38 MAPK phosphorylation in a time- and dose-dependent manner. The peak of phosphorylation for both kinases was reached at 5–10 min of exposure to Ang II. Furthermore, the phosphorylation of both kinases in SHR-AFs was remarkably increased at 5–15 min compared with that in WKY-AFs (Fig. 2). As shown in Fig. 3, the peak of phosphorylation of ERK1/2 and p38 MAPK was reached at 10^{-7} mol/l Ang II and Ang II-induced phosphorylation was significantly enhanced in SHR-AFs compared with WKY-AFs. Figure 4 shows that PD98059 and SB202190 inhibited the phosphorylation of ERK1/2 and p38 MAPK induced by Ang II (10^{-7} mol/l) in WKY-AFs and SHR-AFs, respectively ($p<0.05$).

To determine whether specific Ang II receptor subtypes mediate Ang II-induced ERK1/2 and p38 MAPK activation, the cells were stimulated with Ang II (10^{-7} mol/l) after pretreatment with the Ang II type 1 receptor (AT1-R) antagonist losartan (10^{-7}–10^{-5} mol/l) or Ang II type 2 receptor (AT2-R) antagonist PD123319 (10^{-5} mol/l) for 45 min. Ang II-induced

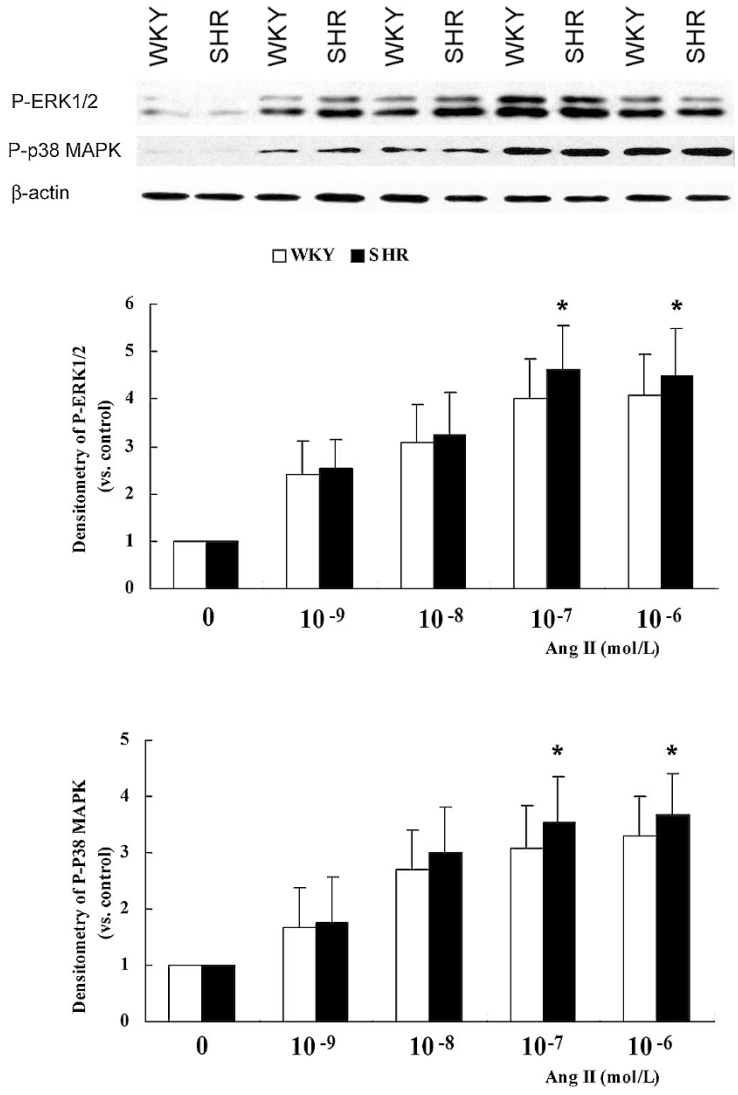

Fig. 3. *Ang II induced dose-dependent phosphorylation of ERK1/2 and p38 MAPK in WKY-AFs and SHR-AFs. Cells were stimulated with different concentrations of Ang II (0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} mol/l) for 10 min, respectively. The top panel shows representative immunoblots indicating that Ang II induced the phosphorylation of ERK1/2 and P38 MAPK in a dose-dependent manner. β-Actin was assayed to verify the equal loading of cell lysates. The two bottom panels show the quantification of the bands by densitometry, respectively. Results are shown as fold increases from 3 independent experiments compared with unstimulated control cells.* *p < *0.05* vs. *WKY-AFs.*

activation of each MAPK was completely or partly inhibited by pretreatment with losartan ($p<0.05$) both in SHR and WKY rats, whereas PD123319 had no effect ($p>0.05$), suggesting that Ang II-induced ERK1/2 and p38 MAPK activation are selectively mediated through the AT1-R (Fig. 5).

The Signaling Pathways Involved in Ang II-Induced AFs Migration

We next examined whether AT1-R and MAPK signaling were involved in Ang II-induced migration of AFs. WKY-AFs and SHR-AFs were pretreated with the AT1-R antagonist losartan (10^{-7} mol/l, 10^{-6} mol/l and 10^{-5} mol/l), PD98059 (10^{-5} mol/l) and SB202190 (10^{-5} mol/l), respectively, before the cells were stimulated with Ang II (10^{-7} mol/l). Losartan suppressed Ang II-induced migration of AFs in a dose-dependent manner (Fig. 6), indicating a key role for the AT1-R. Both PD98059 and SB202190 significantly inhibited Ang II-stimulated migration of AFs. These findings suggest that the activation of ERK1/2 and p38 MAPK were involved in Ang II-stimulated migration of AFs.

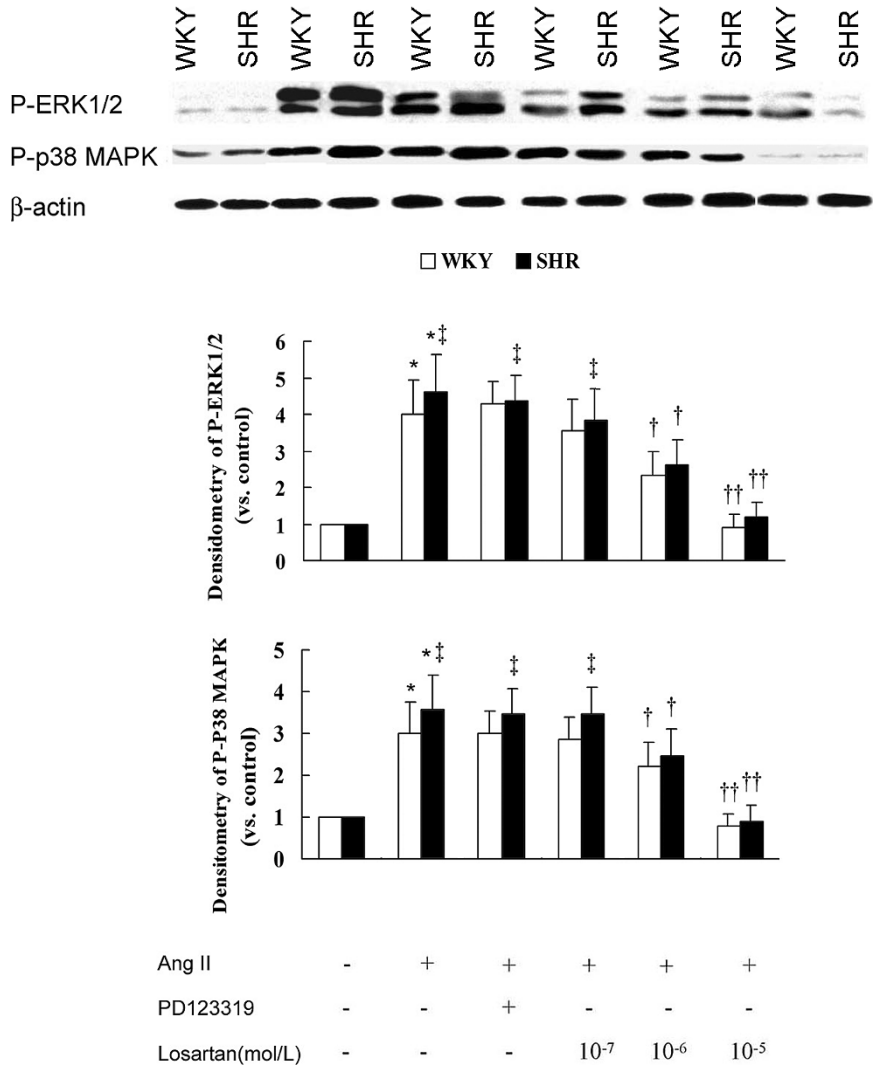

Fig. 4. *Ang II-induced phosphorylation of ERK1/2 and p38 MAPK was inhibited by losartan in a dose-dependent manner in WKY-AFs and SHR-AFs. Bands 1 and 2 were untreated cells derived from WKY and SHR. Bands 3 and 4 show the results of stimulation of WKY-AFs and SHR-AFs with 10^{-7} mol/l Ang II for 10 min. Bands 5–12 show the results of preincubation with PD123319 (10^{-7} mol/l) and different concentrations of losartan (10^{-7}, 10^{-6}, 10^{-5} mol/l) for 45 min before addition of Ang II. The representative immunoblots in the top panel show that Ang II-induced phosphorylation of ERK1/2 and p38 MAPK was inhibited by losartan. β-Actin was assayed to verify the equal loading of cell lysates. The two bottom panels show the quantification of the bands by densitometry, respectively. Results are shown as fold increases from 3 independent experiments compared with unstimulated control cells.* *$p < 0.05$ vs. *untreated cells.* †$p < 0.05$, ††$p < 0.01$ vs. *10^{-7} mol/l Ang II-treated cells.* ‡$p < 0.05$ vs. *WKY-AFs.*

Discussion

We demonstrated that SHR-AFs had a higher capacity for migration in response to Ang II or other stimulants, including serum, PMA, bFGF and PDGF-BB, compared with age-matched WKY-AFs. Moreover, the SBP did not differ significantly between the 8-week-old WKY and SHR from which the cells were derived, suggesting that the difference of migration potential was independent of blood pressure, and the enhanced migratory response of AFs could contribute to the vascular remodeling of SHR. However, the mechanisms responsible for the higher migratory response in SHR-AFs remain unclear.

The reason that we focused our interest on Ang II-induced migration of AFs is that Ang II is the main peptide of the renin-angiotensin system (RAS) during the process of vascular remodeling in hypertension (*13–16*). It has been reported that all major components of RAS are expressed in the adventitia (*17*, *18*). We found that migration of AFs was stimulated by Ang II in a dose-dependent manner, and Ang II-induced migration of AFs could be inhibited by p38 MAPK inhibitor

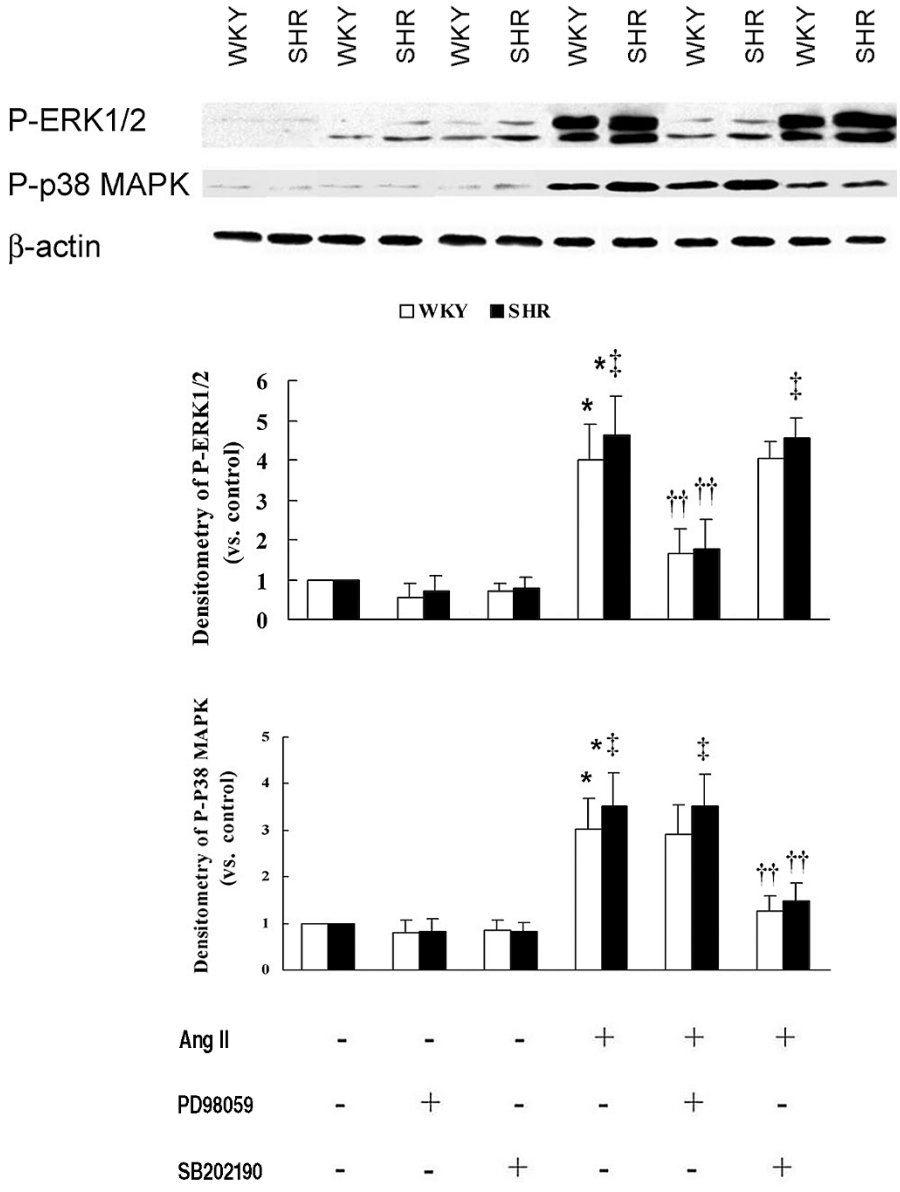

Fig. 5. *Phosphorylation of ERK1/2 and p38 MAPK induced by Ang II is inhibited by PD98059 or SB202190 in WKY-AFs and SHR-AFs. Cells were preincubated with PD98059 (10^{-5} mol/l) or SB202190 (10^{-5} mol/l) for 45 min before addition of 10^{-7} mol/l Ang II, respectively. Bands 1 and 2 were untreated cells derived from WKY and SHR. Bands 3–8 show the results of stimulation of WKY-AFs and SHR-AFs with PD98059 (10^{-5} mol/l) for 45 min, SB202190 (10^{-5} mol/l) for 45 min and Ang II (10^{-7} mol/l) for 10 min, respectively. Bands 9–12 show the results of preincubation with PD98059 (10^{-5} mol/l) and SB202190 (10^{-5} mol/l) for 45 min before addition of Ang II. The representative immunoblots in the top panel show that Ang II-induced phosphorylation of ERK1/2 and p38 MAPK was inhibited by PD98059 or SB202190. β-Actin was assayed to verify equal loading of cell lysates. Two bottom panels show the quantification of the bands by densitometry, respectively. Results are shown as fold increases of control from 3 independent experiments compared with unstimulated control cells.* $^{*}p < 0.05$ *vs. untreated cells.* $^{\dagger\dagger}p < 0.01$ *vs.* 10^{-7} *mol/l Ang II-treated cells.* $^{\ddagger}p < 0.05$ *vs. WKY-AFs.*

SB202190 and ERK1/2 inhibitor PD98059, respectively, except when the cells were pretreated with the AT1-R antagonist losartan, suggested that phosphorylations of ERK1/2 and p38 MAPK were involved in the Ang II-induced migration of AFs.

We also found that Ang II rapidly activated ERK1/2 and p38 MAPK phosphorylation in a dose-dependent manner in vascular AFs both from SHR and WKY. This activation of MAPK could be inhibited by pretreatment with the specific AT1-R antagonist losartan, whereas the AT2-R antagonist

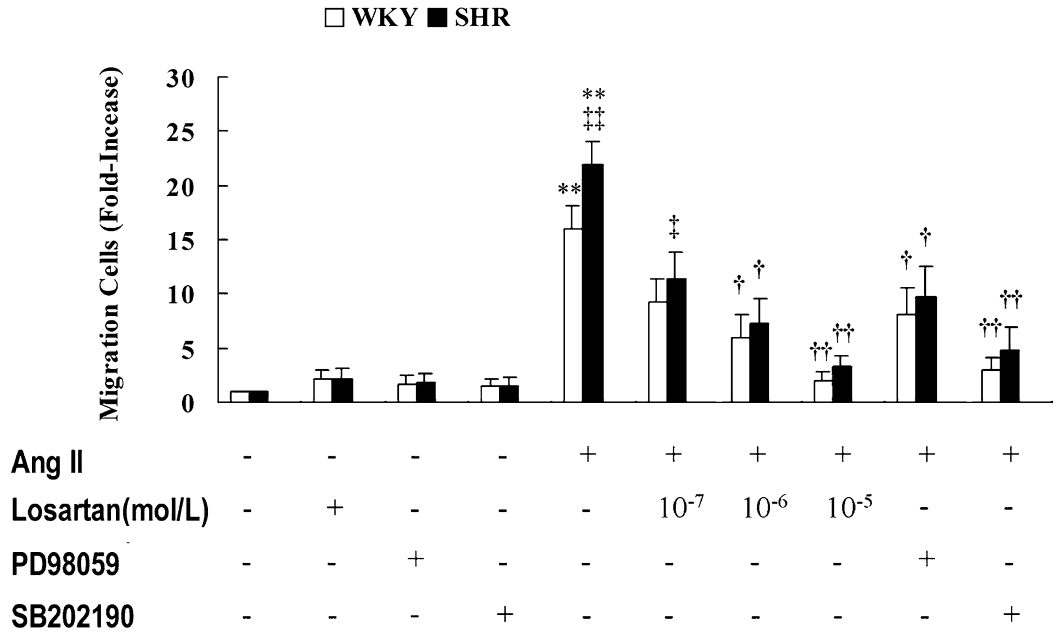

Fig. 6. *Ang II-induced cell migration was inhibited by losartan, PD98059 and SB202190 in WKY-AFs and SHR-AFs. Cells were placed in the upper chambers of a transwell and then incubated for 45 min with losartan (10^{-7}, 10^{-6}, 10^{-5} mol/l), 10^{-5} mol/l PD98059 and 10^{-5} mol/l SB202190, before exposure to Ang II (10^{-7} mol/l), respectively. Migrated cells were quantified by counting nuclei in five random high power fields. Data are presented as the mean±SEM from six independent experiments.* **$p < 0.01$ vs. *untreated cells.* †$p < 0.05$, ††$p < 0.01$ vs. *10^{-7} mol/l Ang II-treated cells.* ‡$p < 0.05$, ‡‡$p < 0.01$ vs. *WKY-AFs.*

PD123319 had no effect on MAPK activation, suggesting that Ang II-induced ERK1/2 and p38 MAPK activation are selectively mediated through the AT1-R. Although it is recognized that Ang II-induced activation of MAPK signaling is involved in VSMC proliferation (*19*, *20*), its role in VSMC migration has been little studied. It has been well documented that cell migration plays a more important role than proliferation during neointimal formation (*4*, *5*, *21*). The vascular wall is composed of phenotypically heterogeneous subpopulations of endothelial cells, VSMCs and fibroblasts. Recent studies have demonstrated that neointimal formation may be the result of fibroblast differentiation and migration (*22*). According to our recent observation, Ang II can induce phenotypic transition of vascular AFs to myofibroblasts and p38 MAPK signaling is involved in this differentiation as well (*23*). To our knowledge, the present study is the first to report that SHR-AFs showed a higher level of migration than WKY-AFs in response to Ang II, and that MAPK signaling is involved in the process of AF migration. However, the intracellular signaling mechanisms between phenotype and migration need to be further clarified.

In summary, the major finding of this study is that the migratory potential of aortic SHR-AFs was greater than that of WKY-AFs. Furthermore, the ERK1/2 and p38 pathways were shown to involved in Ang II-induced migration *via* AT1-R.

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