

Olmesartan inhibits angiotensin II-induced migration of vascular smooth muscle cells through Src and MAP kinase pathways

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Abstract

Clinical studies have shown that Angiotensin receptor blockers (ARBs) reduce the risk of cardiovascular diseases in hypertensive patients. It is assumed that the reduction of the risk by ARBs may be attributed in part to the inhibition of AII-induced vascular smooth muscle cell (VSMC) migration associated with atherosclerosis. However, the effect of ARBs on AII-induced changes in intracellular signaling and resultant cell migration has not been well established. Here, we investigated the effect of olmesartan, an ARB, on AII-induced extracellular signal-regulated kinases 1/2 (ERK1/2) and c-Jun N-terminal kinase (JNK) activation and rat aortic smooth muscle cell (RASMC) migration. Olmesartan inhibited AII-induced ERK1/2 and JNK activation at lower concentrations (10 nM). On the other hand, PP2, an Src tyrosine kinase inhibitor, also inhibited AII-induced ERK1/2 and JNK activation, but its effect on ERK1/2 was less pronounced than that of olmesartan. Olmesartan, U0126 (an ERK1/2 inhibitor), SP600125 (a JNK inhibitor), and PP2 potently inhibited AII-induced RASMC migration. From these findings, it was inferred that angiotensin receptor blockade by olmesartan results in the inhibition of AII-induced activation of Src, ERK1/2, and JNK in RASMC. Olmesartan may be a potent inhibitor of AII-induced VSMC migration, which may be involved in the progression in atherosclerosis.

Key words: angiotensin II, angiotensin receptor blocker, ARB, olmesartan, cell migration

Introduction

Angiotensin II (AII) is known to induce hypertension *via* vasoconstriction and stimulation of aldosterone release; it is also associated with vascular remodeling, inflammation, and oxidative stress (1). Vascular remodeling is caused by excessive proliferation and migration of vascular smooth muscle cells (VSMCs), which are the most characteristic pathological features in experimental injury models of atherosclerosis (2, 3), and is an important step in the pathogenesis of atherosclerosis. The above-mentioned effects of AII are mainly mediated by the angiotensin type 1 (AT₁) receptor at the cell surface. Angiotensin receptor blockers (ARBs), which compete with AII for the binding site of the AT₁ receptor, are used as antihypertensive agents. Large-scale clinical investigations have shown that ARBs are efficient in reducing the risk of cardiovascular and renal complications such as stroke, ischemic heart disease, and diabetic nephropathy (4–9). Therefore, it has been suggested that ARBs function by blocking the AT₁ receptor and thereby suppressing vascular remodeling and tissue damage caused by the proliferation and migration of VSMC; this effect of ARBs, in turn, brings about a reduction in the risk of cardiovascular and renal events.

AII signaling through the AT₁ receptor involves several pathways, which are associated with G proteins and receptor- and non-receptor-tyrosine kinases; this

signaling also results in various physiological and pathological effects *via* activation of mitogen-activated protein (MAP) kinases, protein kinase C (PKC), NAD(P)H oxidases, and generation of reactive oxygen species (10). MAP kinases are reported to be critical regulatory factors for the growth and migration of various cell types including VSMCs (11–14). It has also been reported that the activation of the MAP kinase family, extracellular signal-regulated kinases 1/2 (ERK1/2) (15), and c-Jun N-terminal kinase (JNK) (16) *via* Src activation is important for AII-induced migration of VSMC (17). Excessive VSMC migration induces vascular wall remodeling or the formation of neointima and thickening of the tunica media; these lead to atherosclerosis, which in turn causes cardiovascular diseases. The role of AII in the pathogenesis of cardiovascular diseases associated with VSMC growth and migration is believed to be executed *via* the activation of these signaling pathways (18).

ARBs inhibit vasoconstriction, aldosterone release, and cell proliferation and migration by blocking the AT₁ receptor. In particular, the inhibitory effect of ARB on AII-induced cell proliferation and migration is considered to be significantly related to the reduction in the risk of cardiovascular diseases. However, the mechanism by which ARB regulates AII-mediated intracellular signaling and resultant cell migration is not well established. Activation of ERK1/2 and JNK, mediated by the phosphorylation of Src,

is an important step in AII-induced VSMC migration. Here, we examined the effects of the active form of olmesartan (RNH-6270) on AII-induced ERK1/2 and JNK activation in rat aortic smooth muscle cells (RASMCs). We also compared the effects of olmesartan on RASMC migration with those of Src and MAP kinase inhibitors.

Materials and Methods

Chemicals

Olmesartan (RNH-6270) was kindly provided by Daiichi-Sankyo Co., Ltd. (Tokyo, Japan). We purchased the following chemicals: AII (Peptide Institute, Inc., Osaka, Japan); U0126 and SP600125 (Wako Pure Chemicals, Osaka, Japan); PP2 (Calbiochem, San Diego, CA); and phospho-ERK1/2 antibody and phospho-JNK antibody (Cell Signaling Technology, Danvers, MA); Src antibody (Upstate Technology, Lake Placid, NY); Stealth select RNAi for Src, Lipofectamine RNAiMAX and Anti-Src[pY418] phosphospecific antibody, which recognizes the activated form of Src, was purchased from Invitrogen (Carlsbad, CA). All other reagents were obtained from commercial sources and used without further purification.

Cell Culture

Treatment of animals was based on the “Guide for the Care and Use of Laboratory Animals” published by the US National Institutes of Health (NIH publication No. 85-23, revised 1985) and approved by the ethical committees of Animal Laboratory of Nara Medical University. Thoracic aortae of male Sprague-Dawley rats were excised and immediately immersed in Dulbecco’s Modified Eagle Medium

(DMEM) containing penicillin (100 U/ml) and streptomycin (100 µg/ml). Connective tissue and adherent fat were removed from the specimens. Then, the isolated specimens of the aorta were cut open, and the endothelium was removed by gently rubbing off the intimal surface using sharp scissors. Denuded aortae were cut into approximately 3-mm pieces and placed in three 35-mm culture dishes (Falcon, Becton Dickinson, CA), with the intimal face down. DMEM containing 10% fetal bovine serum and penicillin/streptomycin was gently added to the dishes to cover the explants, without disturbing their orientation. VSMCs were allowed to grow from the explants for 7–10 days, and the tissues were removed using sterilized fine forceps and washed with the culture medium. Once the cells in the three 35-mm dishes reached confluence, they were harvested by brief trypsinization and grown in T-75 flasks (Iwaki, Osaka, Japan) (passage 1). Early subcultured cells (from passage 2–5) were used in the experiments, except cell migration assay, after 48 h of serum starvation. For the cell migration assay, the cells were cultured in serum-free DMEM for 24 h. Purity of the VSMCs was estimated to be >90% on the basis of cell morphology and immunoexpression of myosin, as described previously (19). Cell viability was determined to be >98% by the exclusion of 0.2% trypan blue.

RNA Interference (RNAi) Assay

Transfection was performed according to the manufacturer's protocol. RASMC cell suspension was inoculated into a 6-well plate. After 24 h, RNAi duplex and Lipofectamine RNAiMAX diluted using Opti-MEM were combined and incubated for 10–20 min at room temperature. Then, the RNAi duplex-Lipofectamine RNAiMAX complex was added to each well. The cells were incubated for 48 h and used in subsequent experiments.

Western Blot Analysis

After treatment by AII with or without examined drugs, the cells were washed once with cold phosphate-buffered saline (PBS) containing sodium orthovanadate (1 mM). The cells were lysed (for 30–60 min at 4°C) with cell-lysis buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethyleneglycotetraacetic acid (EGTA), 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM sodium orthovanadate, 1 μ g/ml leupeptin, and 1 mM phenylmethyl-sulfonyl fluoride. The lysed cells were first frozen, then thawed, and subsequently transferred into microcentrifuge tubes and centrifuged at 16,000 g for 20 min at 4°C. The protein concentration of the supernatants was measured

using a protein assay bicinchoninate kit (Nacalai Tesque, Kyoto, Japan). The cell lysates were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and the proteins were transferred on to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, CA), according to a previously described method (20). The membranes were blocked with 0.1% tris-buffered saline Tween-20 (TBS-T) containing 5% skim milk (Nacalai Tesque, Kyoto, Japan) for 1 h at room temperature. The blots were then incubated overnight with the primary antibody and then, with the secondary antibody (horseradish peroxidase conjugated) for 1 h. Immunoreactive bands were visualized using enhanced chemiluminescence (Amersham, UK) and were quantified by densitometry.

Wound Healing Assay

We performed a wound healing assay to evaluate cell migration, especially transverse displacement. After culturing in serum-free DMEM for 24 h, the cells were incubated in the absence or presence of MAP kinase inhibitors or Src inhibitor for 1 h; this was followed by treatment with or without olmesartan for 5 min. Then, the cells were treated with AII, wounded using a yellow pipette tip, and cultured for 24 h. Later, the wounded cultures were stained with Diff-Quick (Sysmex, Kobe, Japan) and photographed. Cells that had migrated from the rim of the dishes were counted in 10

microscope fields per dish.

Statistical Analysis

The experiments were performed in triplicate, and the values obtained are reported as the mean SD. After performing a 2-way analysis of variance (ANOVA) to determine the significance among groups, we used a modified *t* test with Fisher's post-hoc test for intergroup comparison. *P* value of <0.05 was considered to be statistically significant.

Results

Time Courses and Concentration-response Curves for the AII-induced Activation of ERK1/2, JNK, and Src in RASMC

To evaluate the relative magnitude of AII-mediated activation of MAP kinases and Src, growth-arrested RASMC were exposed to AII. ERK1/2, JNK, and Src activations in the cell lysate were determined as described under *Materials and Methods*. ERK1/2 and JNK were activated within 2 min and their activity peaked at 5 min after stimulation by AII in RASMC (6.27- and 39.1-fold increase in activity for ERK1/2 and JNK, respectively) (Fig. 1 A and B). Src activation peaked at 2 min, and the magnitude was 2.13-fold (Fig. 1 C). Thereafter, the JNK and Src activations declined. In contrast, ERK1/2 activation was sustained for 60 min. The concentration-response curves for the activation of AII-induced ERK1/2, JNK, and Src in RASMC are shown in Fig. 2 A, B, and C. AII-induced ERK1/2 and JNK activations were maximal at 100 nM of AII, whereas Src activation peaked at 10 nM of AII; however, the activation in all cases occurred in a concentration-dependent manner.

Inhibitory Effect of Olmesartan on AII-induced ERK1/2 and JNK Activation in RASMC

We examined the effect of various concentrations of olmesartan to examine its

inhibitory effect on AII-induced ERK1/2 and JNK activation (Fig. 3 A and B).

Olmesartan inhibited AII-induced ERK1/2 and JNK activations in a concentration-dependent manner, and complete inhibition of the activations was observed at an olmesartan concentration of 10 nM. These results indicated that olmesartan could inhibit AII-induced ERK1/2 and JNK activation in RASMC even at relatively lower concentration.

Comparison of the Inhibitory Effects of Olmesartan to those of PP2 or Src siRNA on AII-induced ERK1/2 and JNK Activations in RASMC

The AT₁ receptor is the origin of AII-induced intracellular signaling, and it transduces signals for Src and MAP kinases activation. Src plays an important role in AII-induced ERK1/2 and JNK activation and is a key factor regulating the migration of VSMCs. We evaluated the inhibitory effects of olmesartan and PP2, an Src tyrosine kinase inhibitor, on AII-induced ERK1/2 and JNK activations (Fig. 4). Olmesartan (10 nM) potently inhibited both AII-induced ERK1/2 and JNK activations in RASMC. On the other hand, PP2 inhibited AII-induced ERK1/2 and JNK activation, but its effect was less pronounced on ERK1/2 than on JNK activation. Similar findings were obtained in the experiments using siRNA for the inhibition of Src in RASMC (data not shown).

These findings suggest that Src is more closely associated with the AII-induced activation of JNK than that of ERK1/2 in RASMC.

Inhibitory Effect of Olmesartan, U0126, SP600125, and PP2 on AII-induced RASMC

Migration

We investigated the inhibitory effect of various concentrations of olmesartan on AII-induced RASMC migration and compared its effect with that of U0126 (an ERK1/2 inhibitor), SP600125 (a JNK inhibitor), and PP2. Olmesartan inhibited AII-induced RASMC migration in a concentration-dependent manner (Fig. 5). All the drugs, i.e., olmesartan, U0126, SP600125, and PP2, effected almost complete inhibition of AII-induced RASMC migration (Fig. 6). These findings suggest that ERK1/2, JNK, and Src play important roles in AII-induced RASMC migration and that olmesartan is a potent inhibitor for AII-induced RASMC migration.

Discussion

In the present study, we examined the effect of olmesartan on AII-induced changes in intracellular signaling and migration in RASMC. The major findings of our study are as follows: First, olmesartan inhibited AII-induced ERK1/2 and JNK activation in RASMC *via* the inhibition of Src phosphorylation. Second, the inhibition of AII-induced RASMC migration by olmesartan was similar to that of Src and MAP kinase inhibitors.

The activation of ERK1/2 and JNK has been reported to play an important role in AII-induced RASMC migration (21, 22). Cell migration requires cytoskeletal reorganization, which involves the phosphorylation of cytoskeleton-associated tyrosine kinases and formation of focal-adhesion complexes (23). In VSMC, AII-stimulated focal-adhesion complex formation and cytoskeletal reorganization are mediated by AT₁ receptor stimulation and require c-Src (24). In addition, it was reported that phosphorylation of Src is essential for AII-induced VSMC migration, through the activation of ERK1/2 and JNK (17). In the present study, we found that both olmesartan and PP2 significantly inhibited AII-induced ERK1/2 and JNK activation, but olmesartan was a more potent inhibitor than PP2 (Fig. 4). In addition, we found that Src siRNA inhibited AII-induced ERK1/2 and JNK activation (data not shown); this finding

suggests that Src exists upstream of AII-induced ERK1/2 and JNK activation. While olmesartan acts as a potent inhibitor for both AII-induced ERK1/2 and JNK activation, the inhibitory effect of PP2 on AII-induced ERK1/2 activation was lesser than that on AII-induced JNK activation. We found that AII-induced phosphorylation of Src contributes more to JNK activation than to ERK1/2 activation. These results are different from those of a previous study, which reported that the activation of ERK1/2 by AII was primarily dependent on c-Src in VSMC (25). It is difficult to explain this discrepancy at present; however, our findings suggest the possibility of the existence of AII-induced ERK1/2 activation pathways in RASMC other than those mediated by Src.

Many clinical studies have reported that angiotensin-converting enzyme (ACE) inhibitor and ARB reduce the risk for cardiovascular events in hypertensive patients (26–29). On the basis of these findings, it can be inferred that ARB and ACE inhibitors inhibit atherosclerosis formation associated with AII-induced cell migration and proliferation. This notion is supported by the findings of our present study, which showed that olmesartan significantly inhibited AII-induced RASMC migration. U0126 (30 μ M), SP600125 (3 μ M), and PP2 (1 μ M) also inhibited AII-induced RASMC migration to an extent similar to that of olmesartan (Fig. 6). These results are consistent with those of previous studies, which reported that Src and MAP kinases are crucial factors for cell

migration (13, 14). Although PP2 only had a partial inhibitory effect on AII-induced ERK1/2 activation (Fig. 4), PP2, like olmesartan, had a potent inhibitory effect on AII-induced RASMC migration; this finding points toward the existence of PP2 inhibitory mechanism(s) other than the inhibition of Src phosphorylation in AII-induced RASMC migration. In contrast, the concentrations of SP600125 and PP2 used in this study for inhibiting AII-induced phosphorylation of c-Jun and Src, respectively, were lower than those used in previous studies (17, 30, 31). This discrepancy indicates the possibility of the existence of intracellular mechanism(s) other than JNK and Src in AII-induced RASMC migration. Further, the finding that olmesartan inhibited AII-induced RASMC migration at a higher concentration of 100 μ M is inconsistent with the results of its inhibitions on AII-induced Src, ERK1/2, and JNK activations (Fig. 3 and 4). It is difficult to explain this discrepancy because Src is reported to be essential for AII-induced VSMC migration (19). However, with respect to wound healing, AII may affect mechanisms other than cell migration, such as cell proliferation or cell hypertrophy. This may explain the requirement of a higher concentration of olmesartan for inhibiting these AII-induced effects. Taken together, these findings show that ERK1/2 and JNK activation *via* phosphorylation of Src are essential for AII-induced RASMC migration.

In the present study, we showed AII-induced RASMC migration is associated with Src, ERK1/2, and JNK activation because U0126, SP600125, and PP2 potently inhibited AII-induced RASMC migration. In addition, we found that olmesartan was a more potent inhibitor of AII-induced ERK1/2 and JNK activation than PP2 and that the inhibition of RASMC migration induced by olmesartan was similar to those induced by U0126, SP600125, and PP2. These findings suggest that olmesartan, in addition to its antihypertensive properties, may prevent vascular remodeling associated with VSMC migration.

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Figure Legends

Fig. 1. Time courses for angiotensin II (AII)-induced activation of extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal kinase (JNK), and Src in rat aortic smooth muscle cell (RASMC). The cell culture medium was replaced with serum-free medium for 48 h. The cells were then stimulated by 10 nM AII for indicated periods. The activities of ERK1/2 (A) and JNK (B) and phosphorylation of Src (C) were measured as described under *Materials and Methods*. Densitometric analysis of each value was normalized by arbitrarily setting the densitometric value of control cells to 1.0. Each point represents the mean SD (n = 3)

Fig. 2. Concentration-response curves for angiotensin II (AII)-induced activation of extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal kinase (JNK), and Src in rat aortic smooth muscle cell (RASMC). The cell culture medium was replaced with serum-free medium for 48 h. Then, the cells were stimulated by indicated concentrations of AII. ERK1/2 and JNK activations were determined by a 5-min incubation period, while Src activation was determined by a 2-min incubation period. The activities of ERK1/2 (A) and JNK (B) and phosphorylation of Src (C) were measured as described under *Materials and Methods*. Densitometric analysis of each value was normalized by

arbitrarily setting the densitometric value of control cells to 1.0. Each point represents mean SD (n = 3)

Fig. 3. Inhibitory effect of olmesartan on angiotensin II (AII)-induced extracellular signal-regulated kinases (ERK1/2) and c-Jun N-terminal kinase (JNK) activation in rat aortic smooth muscle cell (RASMC). The cell culture medium was replaced with serum-free medium for 48 h. Then, the cells were pretreated with or without olmesartan of indicated concentrations for 5 min followed by stimulation with or without 10 nM AII for 5 min. The activities of ERK1/2 (A) and JNK (B) were measured as described under *Materials and Methods*. Densitometric analysis of each value was normalized by arbitrarily setting the densitometric value of control cells to 1.0. The asterisks represent values that were significantly different from the values of AII stimulation in normal RASMC (** $P < 0.01$). Each value represents mean SD (n = 3)

Fig. 4. Comparison of the inhibitory effects of olmesartan and PP2 on angiotensin II (AII)-induced extracellular signal-regulated kinases (ERK1/2) and c-Jun N-terminal kinase (JNK) activations in rat aortic smooth muscle cell (RASMC). After culturing in serum-free medium for 48 h, the cells were pretreated with or without 10 μ M PP2 for 1 h

or pretreated with or without 10 nM olmesartan for 5 min. Then, the cells were treated with or without 10 nM AII for 5 min. The activities of ERK1/2 (A) and JNK (B) were measured as described under *Materials and Methods*. Densitometric analysis of each value was normalized by arbitrarily setting the densitometric value of control cells to 1.0. The asterisks represent values that were significantly different from the values of Ang II stimulation in normal RASMC (** $P < 0.01$). Each value represents mean SD (n = 3)

Fig. 5. Inhibitory effect of olmesartan on angiotensin II (AII)-induced rat aortic smooth muscle cell (RASMC) migration. Cells were pretreated with or without olmesartan for 5 min. Then, the cells were treated with or without 100 nM AII, and wounded using a yellow pipette tip, and cultured for 24 h. After these cells were stained and photographed, the cells between the 2 rims were counted under 5 different microscope fields. Values were normalized by arbitrarily setting the counts of control cells to 1.0. The asterisks represent values that were significantly different from the values of Ang II stimulation in normal RASMC (** $P < 0.01$, * $P < 0.05$). Each value represents mean SD (n = 3)

Fig. 6. Inhibitory effect of olmesartan, U0126, SP600125, and PP2 on angiotensin II (AII)-induced rat aortic smooth muscle cell (RASMC) migration. Cells were pretreated

with or without 30 μ M U0126, 3 μ M SP600125, or 1 μ M PP2 for 1 h or 100 μ M olmesartan for 5 min. Then, the cells were treated with or without 100 nM AII, wounded using a yellow pipette tip, and cultured for 24 h. After the cells were stained and photographed, the cells between the 2 rims were counted under 5 different microscope fields. Values were normalized by arbitrarily setting the counts of control cells to 1.0. The asterisks represent values that were significantly different from the values of Ang II stimulation in normal RASMC (** $P < 0.01$). Each value represents mean SD (n = 3)