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PKC-dependent extracellular signal-regulated kinase 1/2 pathway is involved in the inhibition of Ib on AngiotensinII-induced proliferation of vascular smooth muscle cells

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ABSTRACT

AngiotensinII (AngII) induces vascular smooth muscle cell (VSMC) proliferation, which plays an important role in the development and progression of hypertension. AngII-induced cellular events have been implicated, in part, in the activation of protein kinase C (PKC) and extracellular signal-regulated kinases 1/2 (ERK1/2). In the present study, we investigated the effect of Ib, a novel nonpeptide AngII receptor type 1 (AT_1) antagonist, on the activation of PKC and ERK1/2 in VSMC proliferation induced by AngII. MTT, and [^{3}H]thymidine incorporation assay showed that AngII-induced VSMC proliferation was inhibited significantly by Ib. The specific binding of [^{125}I]AngII to AT_1 receptors was blocked by Ib in a concentration-dependent manner with IC_{50} value of 0.96 nM. PKC activity assay and Western blot analysis demonstrated that Ib significantly inhibited the activation of PKC and phosphorylation of ERK1/2 induced by AngII, respectively. Furthermore, AngII-induced ERK1/2 activation was obviously blocked by GF109203X, a PKC inhibitor. These findings suggest that the suppression of Ib on AngII-induced VSMC proliferation may be attributed to its inhibitory effect on PKC-dependent ERK1/2 pathway.

Vascular smooth muscle cell (VSMC) proliferation induced by various growth factors contributes to a variety of pathological processes, including hypertension, atherosclerosis, and restenosis after coronary angioplasty [1]. AngiotensinII (AngII), the active component of the renin-angiotensin system (RAS), is important in physiological processes regulating blood pressure and in pathological mechanisms underlying vascular diseases. Over the past decade, *in vitro* and *in vivo* experiments have shown that AngII has numerous actions on vascular smooth muscle, causing cell proliferation, hypertrophy, differentiation, and apoptosis [2].

AngII has been shown to induce proliferation of VSMCs through the Gq protein-coupled AT_1 receptor (AT_1R). The major signaling event activated by the AT_1R appears to be phospholipase C-dependent hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2). The cleavage of PIP_2 results in the generation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol, which leads to intracellular Ca^{2+} mobilization and protein kinase C (PKC) activation, respectively. The AT_1R also activates multiple protein tyrosine kinases, including janus family kinases, focal adhesion kinase, phosphatidylinositol 3-kinase, and so on [3]. Furthermore, AngII has been shown to activate signaling cascades that activate MAPKs, including extracellular signal-regulated kinase 1/2 (ERK1/2), JNK, and p38MAPK, which are implicated in VSMC differentiation, proliferation, migration, and fibrosis [4,5]. Growth factors and other mitogenic agents usually activate ERK1/2 most strongly [6], and numerous studies have demonstrated that ERK1/2 pathway plays a critical role in VSMC proliferation induced by AngII [7]. Moreover, PKC is often found to be associated with this pathway [8].

Ib (Fig. 1), was designed and synthesized successfully by the department of Medicinal Chemistry of China Pharmaceutical University. The pharmacological study has indicated that Ib is a potentially active AT_1R antagonist [9], and has multiple pharmacological effects like anti-hypertension [10], preventing cardiac hypertrophy [11], and inhibiting vascular contraction [12]. In addition, we assessed the pharmacokinetic properties of Ib [13]. However, the molecular mechanism of its potent anti-hypertension activity remains poorly understood and warrants further investigations. In the present study, we investigated the effect of Ib on the activity of PKC and ERK1/2 in the proliferation of VSMCs induced by AngII.

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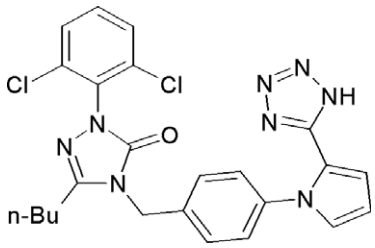

Fig. 1. Chemical structure of Ib.

Materials and methods

Medicine. Ib and losartan (Department of Medicinal Chemistry, China Pharmaceutical University) were dissolved in dimethyl sulfoxide (DMSO) and diluted to the indicated concentration with PBS before the experiment, and the final concentration of DMSO did not exceed 0.01% (v/v) in all experiments.

Cell culture. Primary VSMCs were obtained from the thoracic aorta of rabbit and cultured by the tissue explants method. The aorta was minced into small cube-shaped specimens and incubated with Dulbecco's modified Eagle's medium (DMEM) containing 1 mg/ml collagenase for 1 h. The individual pieces of the vessel segments were seeded in a T-25 culture flask for at least 15 min and then incubated with 3 ml of DMEM supplemented with 20% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 95% air/5% CO_2. Approximately 7–10 days later, the segments were removed and cells were placed into a 75 cm^2 flask, and the medium was changed to DMEM containing 10% FBS. Cells were characterized morphologically as VSMCs by phase contrast microscopy and immunostaining with α-actin. Cells at passages 3–6 were used for the experiments.

Reagents. AngII and MTT were obtained from Sigma Chemical Co. (St. Louis, MO, USA). [^{3}H]Thymidine was purchased from Institute of High Energy Physics, Chinese Academy of Sciences (Beijing, China). [^{125}I]AngII was obtained from Northern Biotech Institute (Beijing, China). Primary antibodies for p-ERK1/2, β-actin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and for ERK1/2 was from Chemicon. IRDye™ 800-conjugated anti-mouse, anti-rabbit, and anti-goat second antibodies were obtained from Rockland Inc. (USA). GF 109203X was obtained from Calbiochem (CA, USA). All other chemicals used were of the highest grade available commercially.

MTT and [^{3}H]thymidine incorporation assay for cell proliferation. VSMCs were counted and seeded into 96-well culture plates (6×10^3 cells/well). After 24 h, the medium was changed to DMEM containing 0.5% newborn calf serum to make them quiescent for 48 h. VSMCs were pre-incubated with or without Ib (0.1, 0.5, and 2.5 μM) for 12 h followed by stimulation with AngII (1, 10, 100, and 1000 nM) for 24 h. Then a volume of 20 μl of 5 mg/ml MTT was added to each well and incubated for 4 h at 37 °C. Formazan crystals were dissolved in 100 μl of DMSO and the absorbance was measured at a wavelength of 570 nm with an enzyme-linked immunosorbent assay (ELISA) reader (1500, Thermo Electron Corporation, USA). For [^{3}H]thymidine incorporation assay, growth-arrested VSMCs were pretreated with or without Ib (0.1, 0.5, and 2.5 μM) for 12 h before exposure to AngII (100 nM) for 24 h. The stimulated cells were added with 5 μCi/ml [^{3}H]thymidine during the last 8 h of culture. Cells were washed once with PBS and twice with ice-cold 5% trichloroacetic acid, and then solubilized in 100 μl of 0.25 N NaOH in 0.1% sodium dodecyl sulfate (SDS). Radioactivity was determined by a liquid scintillation counter (Wallac Guardian 1414, Perkin-Elmer Life and Analytical Sciences Inc., Boston, MA, USA).

Radioligand binding assay. Binding of [^{125}I]-labeled AngII was performed as previously described by Sachinidis. Quiescent and confluent cells cultured in 24-well plates were washed with 200 μl of binding buffer (50 mM Tris, 100 mM NaCl, and 0.25% BSA, pH 7.2) and incubated in the same buffer for 1 h at room temperature to allow dissociation of endogenous AngII. Then cells were washed and incubated for 1 h in 200 μl of binding buffer containing 1 nM [^{125}I]AngII (20 μl) in the presence or absence of antagonists and/or unlabeled AngII. The reaction was stopped by removing the incubation medium and washing the cells twice. The amount of AngII bound to cells was quantified by radioactivity counting in a gamma spectrometer (Wallac 1470 Wizard, Perkin-Elmer, Finland).

PKC activity assay. PKC activation is associated with its translocation from the cytosolic to the membrane fraction. PKC activity in the aliquots was determined by measuring ^{32}P incorporation from [γ^{32}P]ATP (ICN) into histone III-S or myelin basic protein as previously described [14]. The assay mixture contained: 25 mM Tris–Cl (pH 7.5), 10 mM $MgCl_2$, 200 μg/ml histone III-S, 80 μg/ml phosphatidylserine, 30 μg/ml diolein, [γ^{32}P]ATP (1 to 3×10^5 cpm/nmol), and 0.5–3 μg protein. After 5 min incubation at 30 °C, the reaction was stopped by spotting 25 μl of the assay mixture onto phosphocellulose discs. The discs were washed three times with 5% trichloroacetic acid, placed in 4 ml Ecolite (ICN) and radioactivity was measured in a scintillation counter. Specific PKC activity was defined as PKC activity in the presence minus that in the absence of phosphatidylserine.

Western blot analysis. Proteins were isolated by lysis buffer (100 mM Tris–Cl, pH 6.8, 4% (m/v) SDS, 20% (v/v) glycerol, 200 mM β-mercaptoethanol, 1 mM PMSF, and 1 g/ml Aprotinin) and measured using the BCA protein assay method with Varioskan spectrofluorometer and spectrophotometer (Thermo) at 562 nm. Protein samples were separated with 15% SDS–polyacrylamide gel (SDS–PAGE) and transferred onto the PVDF membranes (Millipore). Proteins were detected using specific antibodies against indicated primary antibodies at 37 °C followed by IRDyeTM800 conjugated second antibody for 1 h at 37 °C. Immunoreactive protein bands were detected by the Odyssey Infrared Imaging System (LI-COR Inc., USA). All blots were stripped and reprobed with polyclonal anti-β-actin antibody to ascertain equal loading of protein.

Statistical analysis. All experiments were performed in triplicate and values were expressed as means ± SEM. The IC_{50} values were calculated by linear regression. Statistical differences between mean values were determined using one-way analysis of variance (ANOVA). $^*P < 0.01$ was considered statistically significant. Concentration–response curves were fitted by nonlinear regression.

Results

Effects of Ib on AngII-induced VSMC proliferation

Fig. 2 showed the effects of Ib on AngII-induced VSMC proliferation measured by MTT method and [^{3}H]thymidine incorporation assay. Compared with control cells, AngII significantly stimulated VSMC proliferation in a concentration-dependent manner with the maximal effect at 100 nM (2.76-fold) (Fig. 2A). This concentration was therefore used in the following experiments. The AngII-induced VSMC proliferation was inhibited by Ib at final concentrations of 0.1, 0.5, and 2.5 μM, and the extents of inhibition were 31.0%, 45.2%, and 54.2%, respectively (Fig. 2B). Ib alone did not affect the VSMC proliferation.

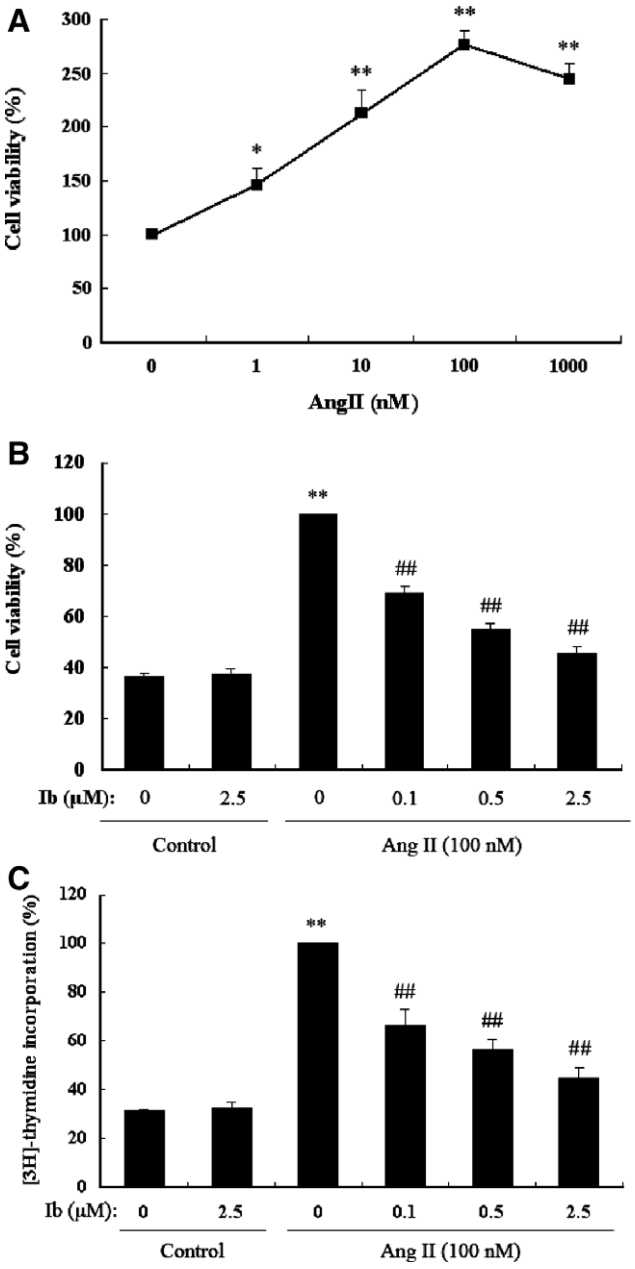

Fig. 2. Inhibitory effects of Ib on AngII-induced proliferation of VSMCs. (A) Concentration–response curve of AngII-induced proliferation of VSMCs analyzed by MTT assay. Growth-arrested VSMCs were stimulated with various concentrations of AngII (1, 10, 100, and 1000 nM) for 24 h. (B) Effects of Ib on AngII-induced VSMC proliferation analyzed by MTT assay. The cells were pretreated with Ib (0.1, 0.5, and 2.5 μM) for 12 h before stimulation with 100 nM AngII for 24 h. (C) Effects of Ib on AngII-induced DNA synthesis. Growth-arrested VSMCs were pretreated with or without Ib (0.1, 0.5, and 2.5 μM) for 12 h before exposure to AngII (100 nM) for 24 h. Radioactivity was determined by liquid scintillation counting. Data are expressed as mean ± SEM of six separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, significant difference from the control cells; $^{\#\#}P < 0.01$, significant difference from the cells treated with AngII (100 nM) only.

Fig. 2C showed the effect of Ib on AngII-induced DNA synthesis. Exposure of VSMCs to AngII (100 nM) caused 3.19-fold increase in [^{3}H]thymidine incorporation compared with the control cells. However, pretreating the cells with Ib (0.1, 0.5, and 2.5 μM) resulted in a significant decrease of the AngII-induced [^{3}H]thymidine incorporation, and the extents of inhibition were 33.3%, 43.6%, and 55.3%, respectively. As the result above, Ib alone did not inhibit DNA synthesis of quiescent VSMCs.

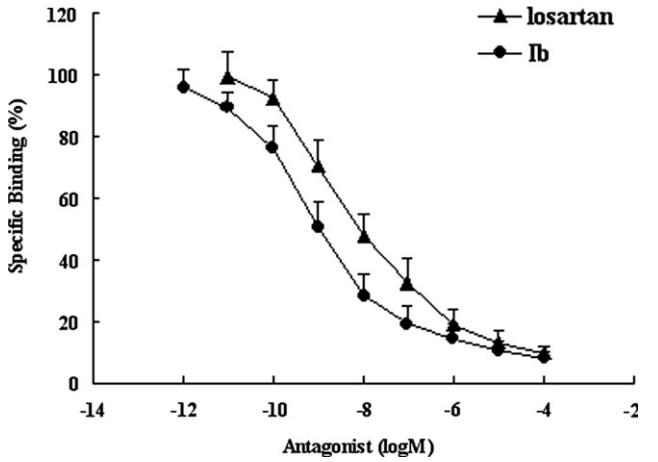

Fig. 3. Inhibitory effects of Ib (10^{-12}–10^{-4} M) and losartan (10^{-11}–10^{-4} M) on the specific binding of [^{125}I]AngII to AT_1R in VSMCs. Each point represents mean ± SEM. ($n = 6$).

Effects of Ib on specific AngII binding to AT_1R in VSMCs

In cultured VSMCs, the specific binding of [^{125}I]AngII was inhibited in a concentration-dependent manner by Ib and losartan (Fig. 3). The IC_{50} values of Ib and losartan were (0.96 ± 0.17) nM and (9.61 ± 1.92) nM, respectively. It showed that Ib exhibited more affinity to AT_1R than losartan because its IC_{50} value was significantly low compared with losartan's ($P < 0.01$).

Effects of Ib on AngII-induced PKC activity in VSMCs

In control cells, PKC activity was greater in the cytosolic fraction than the membrane fraction. AngII activated PKC in a concentration-dependent manner, with a maximal stimulation at 100 nM (data not shown). PKC activity in the cytosolic and membrane fractions was significantly altered following the addition of 100 nM AngII in quiescent VSMCs, where maximal activation occurred at 3 min and then the cytosolic activity of PKC gradually increased and the activity of membrane fractions gradually decreased until 60 min after AngII stimulation (Fig. 4A). Ib alone did not affect the activity of PKC in quiescent VSMCs. In contrast, the pre-incubation of VSMCs with Ib (0.1, 0.5, and 2.5 μM) for 12 h before stimulation with AngII for 3 min, resulted in a significant decreasing activity in membranous fractions and increasing activity in cytosolic fractions (Fig. 4B).

Effects of Ib on AngII-induced ERK1/2 phosphorylation in VSMCs

AngII activated ERK1/2 in a concentration-dependent manner, with a maximal stimulation at 100 nM (data not shown). This concentration was therefore used in subsequent time course experiments, and ERK1/2 activity was measured with a phospho-specific antibody, which only detected the phosphorylated activated forms. AngII stimulation induced ERK1/2 activation (Fig. 4C), which peaked at 5 min (7.5-fold) and returned to basal levels within 60 min. Ib alone had no effect on ERK1/2 activity in quiescent VSMCs. However, pretreating the cells with Ib (0.1, 0.5, and 2.5 μM) for 12 h resulted in a significant reduction in ERK1/2 phosphorylation at 5 min after AngII stimulation (Fig. 4D).

The result also showed that pretreating VSMCs with the specific PKC inhibitor GF109203X (2 μM) could remarkably inhibit ERK1/2 activation induced by AngII (100 nM) (Fig. 4E).

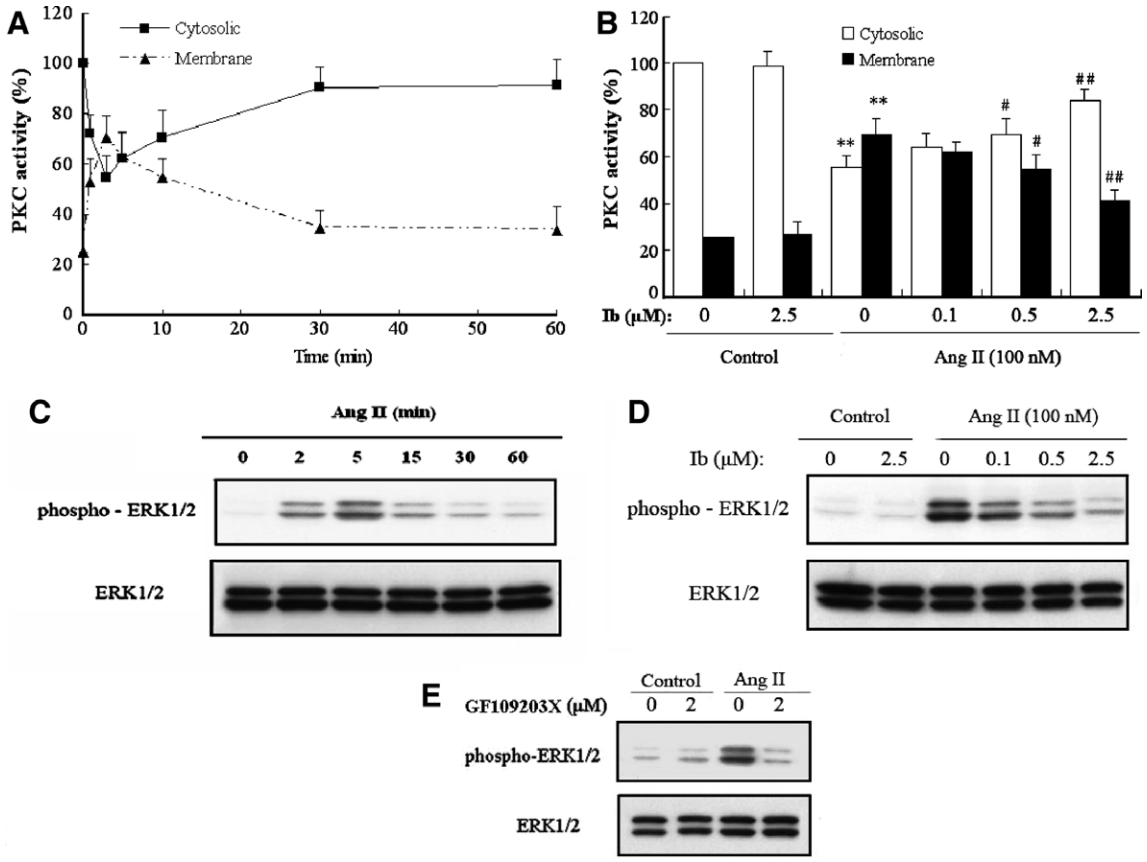

Fig. 4. Effects of Ib on AngII-induced activation of PKC and ERK1/2. (A) Time course of AngII-induced change of PKC activity. Growth-arrested VSMCs were exposed to 100 nM AngII for 0, 1, 3, 5, 10, 30, and 60 min. (B) Inhibition of Ib on the activity of PKC induced by AngII. Arrested VSMCs were pretreated with Ib (0.1, 0.5, and 2.5 μM) for 12 h, and then stimulated with 100 nM AngII for 3 min. (C) Time course of AngII-induced ERK1/2 phosphorylation. Growth-arrested VSMCs were exposed to 100 nM AngII for 0, 2, 5, 10, 30, and 60 min. (D) Inhibitory effect of Ib on AngII-induced ERK1/2 phosphorylation. Cells were pretreated with Ib (0.1, 0.5, and 2.5 μM) for 12 h before stimulation with 100 nM AngII for 5 min. (E) Specific PKC inhibitor GF109203X (2 μM, 60 min) inhibited 100 nM AngII-induced ERK1/2 activation. The results are shown as mean ± SEM. ($n = 3$). $^{**}P < 0.01$ vs Control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs AngII (100 nM) only.

Discussion

AngII is an exceptional peptide that generates signaling events to elicit pleiotropic effects in VSMCs. It plays an important role in VSMC proliferation. Abnormal VSMC proliferation in the arterial wall is importantly involved in the development of vascular diseases, such as hypertension, postangioplasty restenosis and atherosclerosis [15].

In the present study, we investigated the effects of Ib, a novel AT_1 receptor antagonist with anti-hypertension action, on AngII-induced VSMC proliferation. Our results showed that Ib was able to reduce cell viability in a concentration-dependent manner. Furthermore, by measuring AngII-induced [^{3}H]thymidine incorporation, we also demonstrated that Ib concentration-dependently inhibited DNA synthesis. These findings implied that suppression of VSMC proliferation might be one of the mechanisms for Ib to attenuate hypertension.

Most of the known physiological effects of AngII are mediated by AT_1R. Once AngII binds to the AT_1R, it would activate a series of signaling cascades. The results of radioligand binding assay indicated that Ib could inhibit the binding of [^{125}I]AngII to AT_1R on VSMCs in a concentration-dependent manner. Moreover, Ib was 10 times as potent as losartan for displacing [^{125}I]AngII binding to AT_1R, and hence, appeared to have a better affinity for AT_1R. It is supposed that Ib may affect the various responses of AngII on VSMCs through blocking AT_1R.

In cultured VSMCs, the AT_1R couples to a wide variety of signal transduction events, leading to intracellular Ca^{2+} mobilization, PKC activation, and protein tyrosine phosphorylation of multiple substrates, including ERK1/2, the main signaling molecules of the MAPK cascades [16]. ERK1/2 activation by AngII mediates further transmission of growth signals to the nucleus, which stimulates DNA synthesis and enhances cell proliferation [16,17]. So PKC and ERK1/2 are two major targets of AngII to regulate cellular function.

PKC is not a single protein kinase but rather a family of multiple isoenzymes with different biochemical characteristics, substrate specificities, and cofactor requirements [18]. The various isoforms of PKC vary in their tissue distribution and function [19]. Increased expression/activity of PKC isoforms in VSMCs could cause excessive vasoconstriction as well as trophic vascular changes leading to increased vascular resistance and hypertension. Overexpression of α-PKC has been implicated in the pathogenesis of hypertension [20,21]. The Ca^{2+}-independent ε-PKC may increase the myofilament sensitivity to $[Ca^{2+}]_i$ in VSMCs during hypertension [22]. δ-PKC is mainly associated with the cytoskeleton and may play a role in vascular remodeling in hypertension [23]. ζ-PKC plays a central role in the regulation of cell survival and proliferation, which are associated with the pathogenesis of atherosclerosis and malignant hypertension. [21]. Our present study showed that Ib significantly inhibited the activation of PKC induced by AngII in VSMCs. These results suggest that the inhibitory effect of Ib on AngII-induced

VSMC proliferation might be attributed to its ability to suppress PKC activation. Since there are different PKC isoforms, further studies should be performed to confirm whether Ib has effects on those PKC subspecies or not.

High-affinity binding of AngII to the AT_1R activates ERK1/2 via phosphorylation within the activation loop [24]. In accordance with these reports, we observed an obviously increasing phosphorylation of ERK1/2 stimulated by AngII for 5 min. The pretreatment of the cells with Ib significantly inhibited the phosphorylation of ERK1/2 induced by AngII. Other members of MAPK superfamily, such as JNK and p38 MAPK, have been reported to be involved in AngII-induced VSMC proliferation [25]. Thus, further investigation is necessary to clarify whether the effects of Ib on VSMC proliferation is due to interference with JNK and/or p38 MAPK pathways.

Recent studies have demonstrated that AngII stimulates ERK1/2 via PKC-dependent Raf-1 pathway [26]. Furthermore, various isoforms of PKC have been shown to mediate intracellular ERK1/2 activation in response to different ligands and individual cell types [27]. To determine whether PKC activation is involved in AngII-induced ERK1/2 activation in VSMCs, we examined the effect of PKC inhibitor on ERK1/2 activation stimulated by AngII. GF 109203X at a concentration as low as 2 μM obviously blocked ERK1/2 activation. This data suggested that PKC was involved in ERK1/2 activation stimulated by AngII in VSMCs. Previous studies have shown that several members of PKC isoforms including PKC α, β, δ, ε, and ξ are expressed in VSMCs [28], and among them, PKCδ is the most abundant in rat aortic VSMCs [29]. Next, we attempt to determine which specific isotype of PKC is required for ERK1/2 activation induced by AngII.

In conclusion, our present study showed that Ib significantly inhibited AngII-induced proliferation of VSMCs. One of the possible molecular mechanisms was the inhibition of Ib on PKC-dependent ERK1/2 activation by blocking AT_1R in VSMCs. These findings may provide new insights into molecular mechanisms involved in the inhibitory effect of Ib on hypertension, and provide the pharmacological basis for the future clinical application of Ib in the treatment of hypertension.

Acknowledgments

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