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$[Ca^{2+}]_i$ and PKC-α are involved in the inhibitory effects of Ib, a novel nonpeptide AngiotensinII subtype AT_1 receptor antagonist, on AngiotensinII-induced vascular contraction in vitro

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Abstract

The vasoactive peptide AngiotensinII (AngII) is an important factor in the cardiovascular system, exerting most of its effects through AngII receptor type 1 (AT_1). Ib, a new nonpeptide AT_1 receptor antagonist, has been observed to play a positive role in the treatment of hypertension in preclinical tests. In this study, the inhibitory effects of Ib on AngII-induced vascular contraction in vitro were investigated, and its molecular mechanisms were further explored. In endothelium-denuded aortic rings from rabbits, Ib produced a rightward shift in the concentration–response curve for AngII with a decrease in the maximal contractile response and the pD_2' was 7.29. In vascular smooth muscle cells (VSMCs), the specific binding of [^{125}I]AngII to AT_1 receptors was inhibited by Ib in a concentration-dependent manner with IC_{50} value of 0.96 nM. Ib could inhibit both AngII-induced Ca^{2+} mobilization from internal stores and Ca^{2+} influx. Moreover, the translocation of PKC-α stimulated by AngII was inhibited by Ib. Thus, the inhibitory effects of Ib might be related with the depression on AngII-induced increase in $[Ca^{2+}]_i$ and translocation of PKC-α through blocking AT_1 receptors.

Keywords: Ib; AT_1 receptor antagonist; AngII; Vascular contraction; $[Ca^{2+}]_i$; PKC-α

The pivotal role of the Renin–Angiotensin System (RAS) in the regulation of blood pressure has been well established. Many attempts have been made to decrease high blood pressure by inhibiting the synthesis of AngII. Several Angiotensin-Converting Enzyme (ACE) inhibitors were proved to be clinically effective in the treatment of hypertension and congestive heart failure [1]. But some evidences suggested their unwanted side effects, such as dry cough and angioedema, resulted from lack of specificity to ACE for AngI [2]. However, AT_1 receptor antagonists are selective for AT_1 receptors, and act independently on the AngII synthetic pathway [3–5]. Experimental and clinical trials have documented the efficacy of AT_1 blockers in preserving target-organ function and reversing cardiovascular remodeling [4]. Orally active AT_1 receptor antagonists have been introduced clinically as antihypertensive agents.

In order to screen a new kind of nonpeptide AT_1 receptor antagonist with high potency and low side effects, based on the principles of bioisosterism and hybridization, 5-*n*-butyl-4-{4-[2-(1H-tetrazole-5-yl)-1H-pyrrol-1-yl]phenylmethyl}-2,4-dihydro-2-(2,6-dichloridephenyl-3H-1,2,4-triazol-3-one (Ib, Fig. 1) were designed and synthesized successfully by the Department of Medicinal Chemistry of China Pharmaceutical University for the first time. The pharmacological study has indicated that Ib is a potentially active AT_1 receptor antagonist [6]. Ib has proved to be efficient in various animal models of hypertension [7], and has shown a good effect on inhibiting cardiac hypertrophy induced by L-thyroxin in rats [8]. In addition, we also do some research on the pharmacokinetics of Ib [9].

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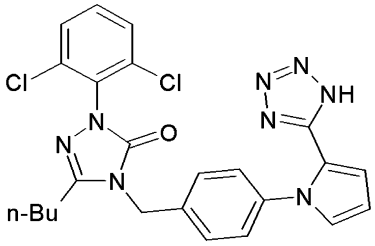

Fig. 1. Chemical structure of Ib.

Vascular contraction contributes to the regulation of vascular resistance and blood pressure, and its dysregulation may lead to hypertension. Therefore, our present study explored the possibility that Ib interfered with AngII-induced vascular contraction to control blood pressure. To test this hypothesis, the effects of Ib on AngII-induced isometric contraction of isolated endothelium-denuded aortic rings were assessed. Furthermore, effects of Ib on AngII-induced increase of intracellular free calcium concentration ($[Ca^{2+}]_i$) transients and translocation of PKC-α in VSMCs were investigated to verify the related mechanisms.

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 any of the experiments.

Cell culture. Primary VSMCs were obtained from the thoracic aorta of rabbit and cultured by the tissue explants method. One section of aorta was removed and placed in Dulbecco's Modified Eagle's Medium (DMEM). Adherent fat and connective tissue were gently removed with fine sterile forceps. The aorta was minced into small cube-shaped specimens and incubated with DMEM/1 mg/mL collagenase for 1 h. The individual pieces of vessel segments were seeded in a T-25 culture flask for at least 15 min to ensure adherence to the bottom surface. They were then incubated with 3 mL of DMEM supplemented with 20% (vol/vol) fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/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 smooth muscle by phase contrast microscopy and by immunostaining with α-actin. Cells at passages 3–6 were used for the experiments.

Reagents. AngII was obtained from Sigma Chemical Co. (St. Louis, MO, USA). [^{125}I]AngII was obtained from Northern Biotech Institute (Beijing, China). Dulbecco's modified Eagle's medium (DMEM) and newborn calf serum were purchased from Invitrogen Corp. (Carlsbad, CA, USA). Fluo 3-AM was from Molecular Probes Inc. (Eugene, Oregon, USA). Antibodies for PKC-α and horseradish peroxidase-coupled goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). All other chemicals used were of the highest grade available commercially.

Functional antagonism and specificity in isolated rabbit aorta. New Zealand White male rabbits weighing 2.0–4.5 kg were killed by cervical dislocation and exsanguination. Thoracic aorta was removed and placed in Krebs–Henseleit solution (pH 7.2) containing (in mM) 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 25 $NaHCO_3$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, and 11.0 glucose. Aortas were cleaned of excess fat and connective tissue and cut into helical strips. The endothelium was removed by gently rubbing the intima of the aorta with a cotton swab. One end of each aortic strip was mounted on a stationary stainless steel rod and the other end mounted on a force transducer (JZ100; Xinhang E&M Co. Ltd., Gaobeidian, China) to measure isometric contractile force. Strips were placed in a 10 mL isolated tissue bath with warmed (37 °C), aerated (95% O_2, 5% CO_2) Krebs–Henseleit solution. Tissues were placed under optimal resting tension (2 g, determined previously) and allowed to equilibrate for an hour, with frequent washing. After equilibration, AngII from $10^{-9.5}$ M to 10^{-6} M was cumulatively added to the organ baths to obtain a concentration–response curve. Then the tissue was washed 3 times until baseline tension was recovered. After each ring was treated for 30 min with a single concentration of Ib (3 nM, 10 nM, and 30 nM), losartan (3 nM, 10 nM, and 30 nM), or vehicle (0.1% DMSO), the second cumulative concentration–response curve for AngII was established. Responses from rabbit aorta were expressed as percentage of the maximal AngII response obtained from the first cumulative concentration–response curve.

To test the specificity of Ib as an AT_1 receptor antagonist, the concentration responses to Norepinephrine and KCl were also examined in the endothelium-denuded rabbit aorta in the absence and the presence of Ib at 10 μM.

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 (Tris 50 mM, NaCl 100 mM, and BSA 0.25%, 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 attached cells were dissolved in 0.5 ml of 0.1 M NaOH. The amount of AngII bound to cells was quantified by radioactivity counting in a gamma spectrometer (Wallac 1470 Wizard, Perkin-Elmer, Finland).

Measurement of $[Ca^{2+}]_i$ by laser scanning confocal microscope. Intracellular Ca^{2+} imaging was carried out using a Ca^{2+} sensitive indicator Fluo3-AM by means of a laser scanning confocal microscope (LSCM, LSM 510, Carl Zeiss). VSMCs were grown on 24 mm glass coverslips in 6-well plates in DMEM containing 10% newborn calf serum at a density of 1×10^4/ml. After 24 h, cells were washed three times and incubated with 6 μM Fluo3-AM for 40 min in the dark at 37 °C. Extracellular Fluo3-AM was removed by washing three times. The Fluo3-AM dye was excited with a 488 nm wavelength argon laser beam and the emission fluorescence was monitored at 525 nm. $[Ca^{2+}]_i$ was expressed as a percentage of fluorescence intensity relative to basal fluorescence.

Western-blot analysis for the translocation of PKC-α. Protein-matched samples of the cytosolic and cell membrane fractions were subjected to electrophoresis on 8% sodium dodecylsulfate–polyacrylamide gels and then transferred electrophoretically to nitrocellulose membranes. The nitrocellulose membranes were incubated in 5% dried milk in phosphate buffered saline (PBS)–Tween at 22 °C for 1 h, washed with PBS–Tween 3 times for 5 min, and then incubated in the primary anti-PKC-α antibody solution at 4 °C for 24 h. The specificity of the antibodies was confirmed by the absence of specific bands when the antibody solution was supplemented with the peptide to which the antibody was raised. The nitrocellulose membranes were washed 5 times for 15 min in PBS–Tween and then incubated in horseradish peroxidase-coupled anti-rabbit IgG antibody for 1 h at room temperature. The blots were washed with PBS–Tween 5 times for 15 min and visualized with enhanced chemiluminescence (Amersham). The reactive bands corresponding to PKC-α were analyzed by optical densitometry with a GS-700 imaging densitometer (Bio-Rad).

Statistical analysis. All experiments were performed in triplicate and values are 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. The pA_2 values were determined according to the Schild equation and pD_2' was calculated as $-\log IC_{50}$ (M).

Results

Effects of Ib on AngII-induced vascular contraction in isolated rabbit aortas

Ib and losartan inhibited AngII-induced contractions of the rabbit aorta in a concentration-dependent manner (Fig. 2A and B), but with dissimilar types of antagonism. Ib (3 nM, 10 nM, and 30 nM) produced a rightward shift in the concentration–response curve to AngII with a significant reduction in the maximal contractile response by 4%, 13%, and 40% at each concentration, respectively (pD_2' value, 7.29; Fig. 2A). In contrast, losartan (3 nM, 10 nM, and 30 nM) produced a parallel rightward shift in the

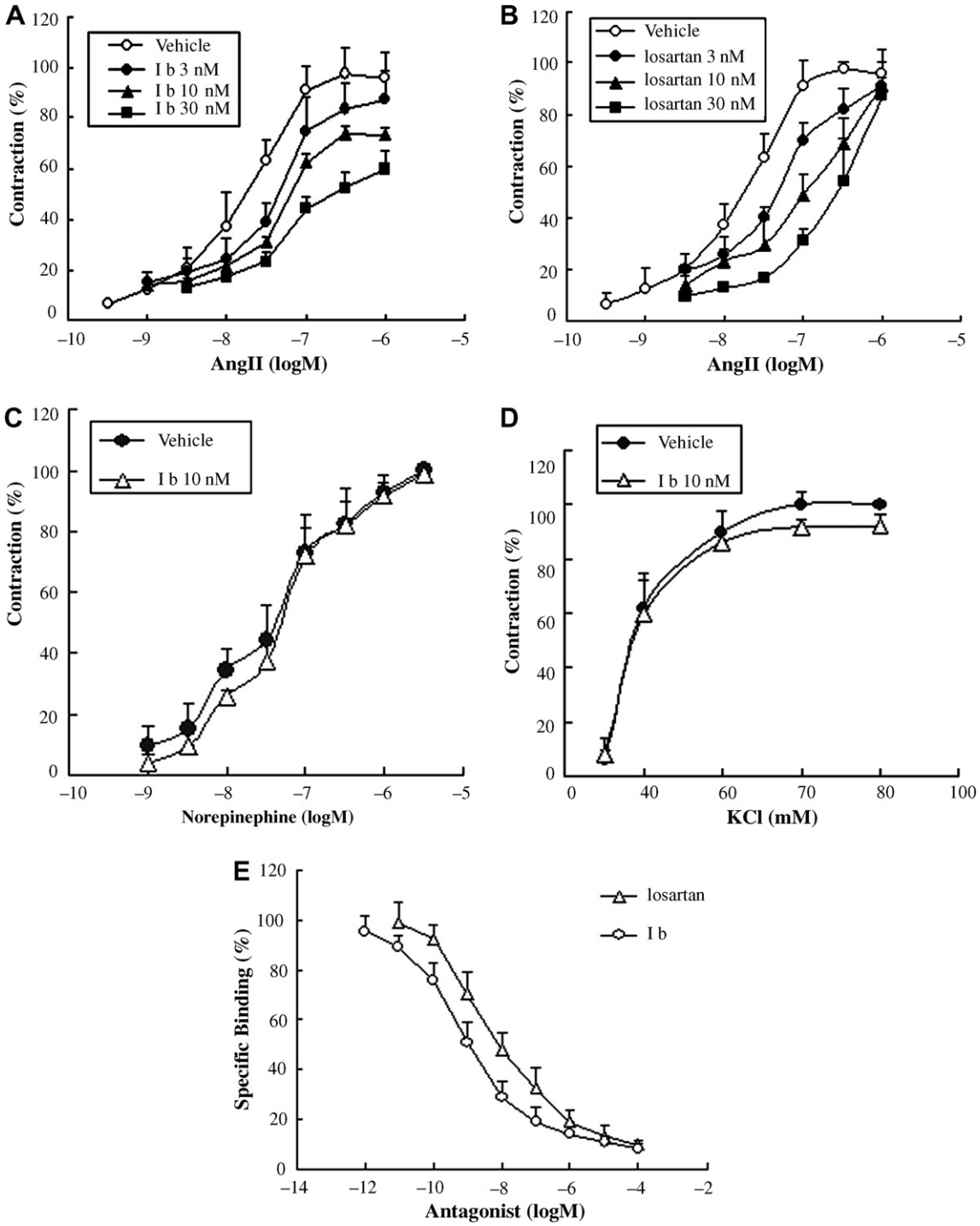

Fig. 2. Inhibitory effects of Ib on AngII in isolated rabbit aorta and in VSMCs. Effects of Ib (A) (3 nM, 10 nM, and 30 nM) and losartan (B) (3 nM, 10 nM, and 30 nM) on the concentration–response curve to AngII ($10^{-9.5}$ M–10^{-6} M) in isolated rabbit aorta. The results are expressed as a percentage of the maximal AngII response obtained with the first curve. Effects of Vehicle and Ib (10 nM) on the concentration–response curve to Norepinephrine (C) (10^{-9} M–$10^{-5.5}$ M) and KCl (D) (10 mM–80 mM) in isolated rabbit aorta. Inhibitory effects of Ib (10^{-12} M–10^{-4} M) and losartan (10^{-11} M–10^{-4} M) (E) on the specific binding of [^{125}I]AngII to AT_1 receptors in VSMCs. Each point represents means ± SEM ($n = 6$).

concentration–response curve without any changes in the maximal contractile response (pA_2 value, 8.59; Fig. 2B). Ib (10 nM) had no effect on the contraction induced by Norepinephrine or KCl (Fig. 2C and D).

Effects of Ib on specific AngII binding to AT_1 receptors in VSMCs

In VSMC cultures, the specific binding of [^{125}I]AngII was inhibited in a concentration-dependent manner by Ib and losartan (Fig. 2E). The IC_{50} values of Ib and losartan were (0.96 ± 0.17) nM and (9.6 ± 1.9) nM, respectively. It showed that Ib exhibited more affinity to AT_1 receptors than losartan because its IC_{50} value was significantly low compared with losartan's ($P < 0.01$).

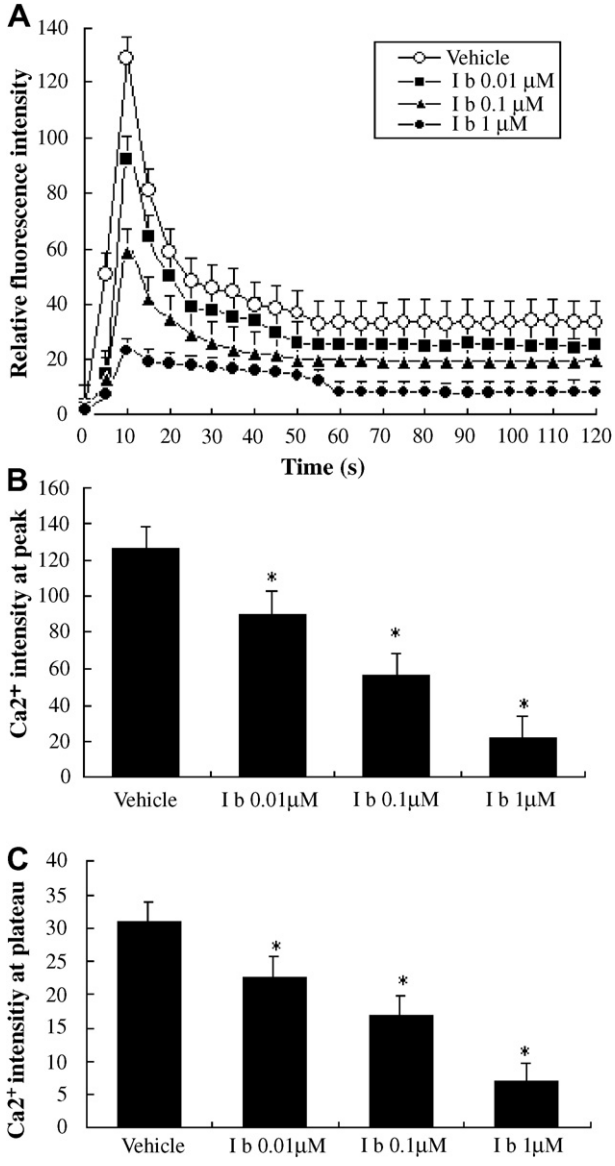

Fig. 3. Inhibitory effects of Ib on $[Ca^{2+}]_i$ in VSMCs. (A) Effects of Ib (0.01 μM, 0.1 μM, and 1 μM) on $[Ca^{2+}]_i$ induced by AngII (100 nM). $[Ca^{2+}]_i$ at peak level (B) and at plateau level (C) were calculated as the $[Ca^{2+}]_i$ value at peak and plateau, respectively, diminished by the basal $[Ca^{2+}]_i$ value. Values were expressed as means ± SEM ($n = 4$). $^*P < 0.01$, significantly different from the cells treated with AngII (100 nM) only.

Effects of Ib on AngII-induced $[Ca^{2+}]_i$ responses in VSMCs

In the presence of 1 mM $[Ca^{2+}]_{ex}$, AngII (100 nM) induced a rapid peak increase in $[Ca^{2+}]_i$ within 10 s, followed by a decrease to a sustained plateau phase. Ib alone did not affect the basal $[Ca^{2+}]_i$ level. By contrast, Ib (0.01 μM, 0.1 μM, and 1 μM) drastically attenuated both the AngII-induced transient calcium increase (expressed as the $[Ca^{2+}]_i$ value at the peak diminished by the basal $[Ca^{2+}]_i$ value) and the sustained phase (expressed as the $[Ca^{2+}]_i$ value at the plateau level diminished by the basal $[Ca^{2+}]_i$ value) in a concentration-dependent manner (Fig. 3A–C).

Effects of Ib on AngII-induced translocation of PKC-α in VSMCs

Immunoblotting assay showed that cultured VSMCs expressed PKC-α at detectable levels. In control cells, the most cellular distributions of PKC-α were in cytosolic fractions and little was detected in membrane fractions. However, PKC-α content 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 after AngII stimulation and then the cytosolic content of PKC-α gradually increased and the content of membrane fractions gradually decreased until 60 min after AngII stimulation (Fig. 4A). Ib alone did not affect the distributions of PKC-α in quiescent VSMCs. In contrast, the pre-incubation of VSMCs with Ib

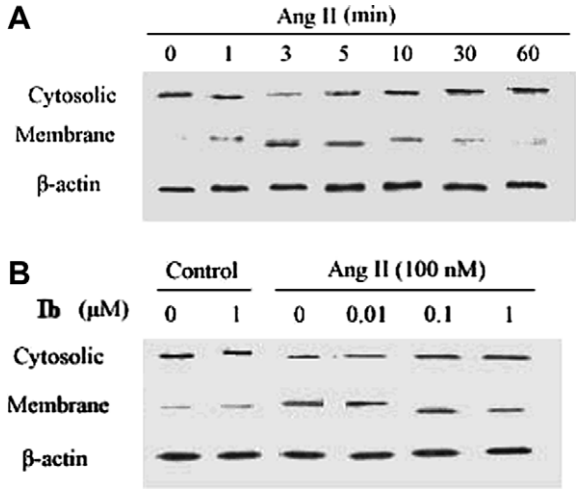

Fig. 4. Effects of Ib on AngII induced translocation of PKC-α. (A) Time course of AngII-induced translocation of PKC-α. Growth-arrested VSMCs were exposed to 100 nM AngII for 0, 1, 3, 5, 10, 30, and 60 min. (B) Effects of Ib on the translocation of PKC-α. Cells were pretreated with Ib (0.01 μM, 0.1 μM, and 1 μM) for 24 h before stimulation with 100 nM AngII for 3 min. The cell lysates were subjected to SDS–PAGE and analyzed by Western-blot.

(0.01 μM, 0.1 μM, and 1 μM) for 24 h before stimulation with AngII for 3 min, resulted in a significant decrease of immunoreactivity in membranous fractions and increase of PKC-α content in cytosolic fractions in a concentration-dependent manner (Fig. 4B).

Discussion

In our previous studies, using rat liver membrane (expressing only AT_1 receptors), we found that Ib could decrease the maximal binding of [^{125}I]AngII to AT_1 receptors from 71.59 fmol/mg protein to 44.55 fmol/mg protein. Its IC_{50} was 2.67 nM and K_i was 2.49 nM. However, losartan did not reduce the maximal binding ($B_{max} = 71.87$ fmol/mg protein), its IC_{50} was 14.7 nM and K_i was 13.8 nM (data not shown). Similarly, using bovine cerebellar membrane (expressing only AT_2 receptors), we found that both Ib and losartan had low affinity with AT_2 receptors. Therefore, they could not inhibit the binding of [^{125}I]AngII to AT_2 receptors (data not shown). These results suggested that Ib specifically and competitively antagonized AngII to AT_1 receptors.

In our present studies, the AngII antagonistic property of Ib was demonstrated in a functional test on isolated rabbit aortas by the concentration-dependent inhibition of AngII-induced contraction. Ib (3 nM, 10 nM, and 30 nM) produced a rightward shift in the concentration–response curve to AngII with a significant reduction in the maximal contractile response at each concentration, suggesting an insurmountable antagonism of AngII-induced contraction. Moreover, Ib was a specific antagonist for AngII, as it did not inhibit the contraction induced by Norepinephrine or KCl. Its distinct pharmacologic behavior was also reported previously for other nonpeptide AT_1 receptor antagonists, such as SK-1080, BIBR 277 or GR 138095 [10,11]. By contrast, losartan produced a rightward parallel shift in concentration–response curve to AngII without reduction in the maximal response. Several hypothetical mechanisms proposed to explain insurmountable antagonism exhibited by AT_1 receptor antagonists included the action on multiple receptors, a slow dissociation of the receptor–antagonist complex, and allosteric modification of receptors [12]. The insurmountable antagonism might not be limited to a single mechanism and might be influenced by factors such as the agonists/antagonists used, tissues, species and experimental conditions, as suggested by Bond [13].

VMSCs were selected to estimate the characteristic of Ib on the level of cells. The results of competitive radioligand binding assay indicated that Ib and losartan could inhibit the binding of [^{125}I]AngII to AT_1 receptors on VMSCs in a concentration-dependent manner. However, Ib was significantly more potent as 10 times as losartan for displacing [^{125}I]AngII binding to AT_1 receptors, and hence, appeared to have a better affinity for AT_1 receptors. It is supposed that Ib may affect the various responses of AngII on VMSCs through blocking AT_1 receptors.

Central to the direct vasoconstrictor action of AngII on smooth muscles is its capacity to increase $[Ca^{2+}]_i$. After interaction with AT_1 receptors, AngII-stimulated Ca^{2+} signaling is complex and occurs via multiple pathways to elicit an integrated Ca^{2+} signal. AngII typically mediates a biphasic $[Ca^{2+}]_i$ response comprising a rapid initial transient phase and a sustained plateau phase [14,15]. The first $[Ca^{2+}]_i$ transient is generated primarily by IP_3-induced mobilization of intracellular Ca^{2+} and to a lesser extent by Ca^{2+}-induced Ca^{2+} release [16]. The second $[Ca^{2+}]_i$ phase, which appears to contribute to the sustained AngII-induced vasoconstriction, is dependent on external Ca^{2+} and is the result of transmembrane Ca^{2+} influx [17–21]. AngII-induced $[Ca^{2+}]_i$ variations presented here (Fig. 3) were consistent with what has been previously reported [22,23]. Ib did not affect the basal level of $[Ca^{2+}]_i$, but they inhibited AngII-induced Ca^{2+} movements. Ib tremendously reduced AngII-induced transient Ca^{2+} elevation (i.e., AngII-induced Ca^{2+} mobilization from internal stores) and also weakened the sustained phase (i.e., AngII-induced Ca^{2+} influx) to inhibit the vascular contraction. Intracellular Ca^{2+} mobilization by AngII has been reported to be mediated by IP_3 receptors [24], therefore, IP_3 receptors are needed to verify the effect of Ib on intracellular Ca^{2+} mobilization. In addition, exact mechanisms whereby AngII stimulates Ca^{2+} influx are unclear but may involve voltage-dependent calcium channels, which are directly or indirectly activated by AngII, nonspecific dihydropyridine-insensitive cation channels, receptor-gated Ca^{2+} channels, Ca^{2+}-activated Ca^{2+} release channels, and activation of the Na^+/Ca^{2+} exchanger [25]. In our future work, further studies are needed to done to clarify the detailed mechanisms of Ib to attenuate AngII-induced elevation of $[Ca^{2+}]_i$.

PKC is a particularly intriguing protein kinase as it comprises a family of Ca^{2+}-dependent and Ca^{2+}-independent isoforms. Several studies suggest a role for PKC in VSM contraction [26,27]. PKC activation by phorbol esters has been shown to cause significant contraction in isolated vascular preparations [28]. Also, PKC inhibitors cause significant inhibition of agonist-induced vascular contraction [26,27].

PKC isoforms have different tissue and subcellular distribution, and undergo differential translocation during cell activation. PKC translocation to the cell surface may trigger a cascade of protein kinases such as mitogen-activated protein kinase (MAPK) and MAPK kinase (MEK) that ultimately interact with the contractile myofilaments and cause VSM contraction. Among the eleven PKC isoforms expressed in VSMCs, we paid attention to the 'conventional' Ca^{2+}-dependent PKC-α. PKC-α enhances Ca^{2+}-dependent VSM contraction, and its overexpression has been implicated in the pathogenesis of hypertension [29]. Addition of 100 nM AngII caused the translocation of PKC-α, significantly from the cytosol to the membrane. However, Ib inhibited the translocation of PKC-α induced by AngII. Thus, Ib might affect the translocation of PKC-α

to inhibit the contraction induced by AngII. Since there are some other PKC isoforms, further studies should be performed to confirm whether Ib has effects on those PKC subspecies or not. Besides, we are also interested in the relationship between Ca^{2+} and PKC-α in the inhibitory effects of Ib on AngII-induced vascular contraction.

In conclusion, our present study showed that Ib significantly inhibited AngII-induced vascular contraction in vitro. One possible molecular mechanism was the inhibition on elevation of $[Ca^{2+}]_i$ and translocation of PKC-α through blocking AT_1 receptors in VSMCs. These findings might offer a new insight into the anti-hypertension mechanisms of Ib, and provide the pharmacological basis for the future clinical application of Ib in the treatment of hypertension.

Acknowledgments

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