Biochemical and Biophysical Research Communications 435 (2013) 69–75

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Pinocembrin inhibits angiotensin II-induced vasoconstriction via suppression of the increase of $[Ca^{2+}]_i$ and ERK1/2 activation through blocking AT_1R in the rat aorta

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ARTICLE INFO

Article history:
Received 28 March 2013
Available online 20 April 2013

Keywords:
Pinocembrin
Angiotensin II
Vasoconstriction
AT_1R
$[Ca^{2+}]_i$
ERK1/2

ABSTRACT

Pinocembrin (5,7-dihydroxyflavanone) is one of the primary flavonoids in propolis. Angiotensin II (AngII) is a biologically active peptide that induces vasoconstriction via the activation of the angiotensin type 1 receptor (AT_1R). In the present study, we investigated the vasorelaxant effect of pinocembrin on AngII-induced vasoconstriction and the molecular mechanism of action. Pinocembrin was observed to inhibit AngII-induced vasoconstriction in rat aortic rings with either intact or denuded endothelium. In endothelium-denuded tissues, pinocembrin (pD_2' 4.28 ± 0.15) counteracted the contractions evoked by cumulative concentrations of AngII. In a docking model, pinocembrin showed effective binding at the active site of AT_1R. Pinocembrin was shown to inhibit both AngII-induced Ca^{2+} release from internal stores and Ca^{2+} influx. Moreover, the increase in the phosphorylation of extracellular signal-regulated kinase (ERK1/2) and myosin light chain 2 (MLC2) induced by AngII was blocked by pinocembrin. These results demonstrate that pinocembrin inhibits AngII-induced rat aortic ring contraction, and these inhibitory effects may be related to the reduction of the AngII-induced increase in $[Ca^{2+}]_i$ and ERK1/2 activation via blocking AT_1R.

1. Introduction

Pinocembrin is one of the primary flavonoids in propolis (a natural substance collected by honeybees from buds and exudates of certain trees and plants). It has been reported to possess antimicrobial, anti-inflammatory, anti-oxidant, anti-apoptotic and vasorelaxant properties [1–5]. Preclinical experiments have shown that pinocembrin alleviates brain injury in either focal or global cerebral ischemia/reperfusion (CI/R) models in rats [2,6].

Angiotensin II (AngII), a major bioactive peptide of the renin–angiotensin system (RAS), plays an important physiological role in maintaining vascular tone by regulating immediate vasoconstriction [7]. When AngII binds to the angiotensin type 1 receptor (AT_1R) in smooth muscle cells, it induces phospholipase C activation, which increases cytoplasmic calcium concentrations and provokes muscular contraction [8]. Several studies have suggested that AngII is involved in the process of cerebral ischemia in animal models [9–11].

Others and we have previously demonstrated that pinocembrin causes relaxation of rat aortic rings that were pre-contracted with either norepinephrine (NE) or KCl via both endothelium-dependent and -independent mechanisms [1,5]. Additionally, we also observed that pinocembrin increased cerebral blood flow and improved the outcome after either focal or global CI/R injury in rats [2,12,13]. This protective effect against cerebral ischemia is similar to that observed with the AT_1R antagonist candesartan [14,15]. These observations led us to hypothesize that pinocembrin might also act as an AT_1R antagonist and interfere with AngII-induced vascular contraction. Therefore, we investigated the effect of pinocembrin on AngII-induced vasoconstriction in the rat thoracic aorta and elucidated the potential mechanisms involved.

2. Materials and methods

2.1. Materials

Pinocembrin was synthesized by the Department of Medicinal Chemistry of the Chinese Academy of Medical Sciences as

Abbreviations: AngII, angiotensin II; AT_1R, angiotensin type 1 receptor; ERK1/2, extracellular signal-regulated kinase; HBSS, HEPES buffered saline solution; MLC2, myosin light chain 2; NE, norepinephrine; VSMCs, vascular smooth muscle cells.

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http://dx.doi.org/10.1016/j.bbrc.2013.04.039

previously described (MW: 255.25; chromatographic purity > 99%) [5,16]. NE, acetylcholine, AngII and Fura2/AM were purchased from Sigma chemical co. (St. Louis, USA). Pinocembrin and Fura2/AM were dissolved in DMSO, and preliminary experiments showed that DMSO at concentrations less than 0.2% (v/v) had no effect on tension development in isolated aorta [5]. The remainder of the drugs was dissolved in distilled water. Antibodies against phospho-myosin light chain 2 (Ser19) (phospho-MLC2), MLC2, phospho-ERK1/2, and ERK1/2 were purchased from Cell Signaling Technology Inc. (Beverly, USA).

2.2. Preparation of rat aortic rings

This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. All experimental protocols involving animals were approved by the Animal Care and Welfare Committee of the institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College. Male SD rats weighing 250–300 g were executed by decapitation, and the descending thoracic aorta was immediately excised and placed in ice-cold Krebs–Henseleit (K–H) solution as previously described [5]. After removing the adherent fat and connective tissue, the aorta was cut into rings approximately 3–4 mm lengths. For the endothelium-denuded tissues, endothelium was mechanically removed by gently rubbing the luminal surface with a cotton ball.

2.3. Measurement of isometric vascular tone

Measurement of isometric vascular tone was performed as previously described [5]. After the equilibration period, the endothelium-denuded aortic rings were constricted with a high K^+ (60 mM) solution and the contractions were allowed to stabilize. The integrity of the endothelium was confirmed by eliciting a relaxation response by administering acetylcholine (10 μM) following a recording of the contraction induced by NE (0.1 μM). Subsequently, various concentrations of AngII ranging from $10^{-9.5}$ M to 10^{-6} M were applied cumulatively to obtain concentration–response curves. To determine the effects of pinocembrin on AngII-induced vasoconstriction, either a single concentration of pinocembrin (5 μM, 25 μM or 100 μM) or vehicle (0.2% DMSO) was applied 20 min before the cumulative administration of AngII.

In separate experiments, the first temporal course of a single concentration of AngII (100 nM) was tested in both endothelium-intact and endothelium-denuded tissues. The tissue was then washed 3 times until the recovery of baseline tension. The second temporal course of a single concentration of AngII (100 nM) was tested after treating each ring with either a single concentration of pinocembrin (5 μM, 10 μM, 25 μM, 50 μM or 100 μM) or vehicle (0.2% DMSO) for 20 min.

2.4. Molecule docking analysis

The X-ray crystal structure of AT_1R (PDB ID: 1ZV0) was retrieved from the RCSB Protein Data Bank (Berman, H.; Henrick, K.; Nakamura, H. Nat. Struct. Biol., 2003, 10, 980). The protein structure was prepared by the Protein Preparation Wizard module (Schrödinger Suite 2010 Protein Preparation Wizard; Epik version 2.1; Impact version 5.6; Prime version 2.2, Schrödinger, LLC, New York, USA), and the binding site was found by the SiteMap module (SiteMap, version 2.5, Schrödinger, LLC, New York, USA) together with a consideration of the key residues in the binding pocket previously reported [17]. The ligands were prepared using the LigPrep module (LigPrep, version 2.5, Schrödinger, LLC, New York, USA). The Glide module (Glide, version 5.7, Schrödinger, LLC, New York, USA) was applied to study the molecular docking between AT_1R and pinocembrin, with losartan as a reference compound. The Glide docking was performed using the extra-precision (XP) mode, and the top-scoring pose was used for analysis.

2.5. Cell culture

Rat aortic vascular smooth muscle cells (VSMCs) were isolated from the thoracic aorta of male SD rats by enzymatic digestion and cultured in DMEM containing 10% (v/v) fetal bovine serum. Cells were used at passages 2–5. Cells were serum-starved for 24 h prior to exposure to the various treatments.

2.6. Western blotting

VSMCs were pretreated with various concentration of pinocembrin (0.1 μM, 1 μM or 10 μM) for 2 h prior to the addition of 100 nM AngII for 5 min. Rat aortic rings were pretreated with either pinocembrin (5 μM, 25 μM or 100 μM) or 0.2% DMSO (vehicle control) for 20 min prior to the exposure to 100 nM AngII for 5 min. Cellular and rat aortic ring proteins were fractionated by 10% SDS–PAGE, electrotransferred onto a PVDF membrane and probed with specific antibodies. Immunoreactive bands were visualized by an enhanced chemiluminescence assay following the manufacturer's instructions.

2.7. Intracellular [Ca^{2+}] measurements

Measurement of $[Ca^{2+}]_i$ with Fura2/AM in VSMCs was performed as previously described [16,18] with slight modifications. Briefly, serum-starved VSMCs were loaded with 3 μM Fura2/AM in HEPES buffered saline solution (HBSS) at 37 °C for 45 min in the dark. The cells were washed 3 times with HBSS without BSA in either the presence or absence of 1.8 mM Ca^{2+} as described in the text. Cultures were pretreated with either pinocembrin (0.1 μM, 1 μM or 10 μM) or 0.2% DMSO (vehicle control) for 10 min prior to the addition of 100 nM AngII for 10 s in the presence or absence of 1.8 mM Ca^{2+}. A microplate reader (SpectraMax M5, Molecular Devices, CA, USA) was used to detect alterations in the fluorescence intensity at the excitation wavelengths of 340 and 380 nm and emission wavelength of 505 nm. Intracellular calcium ($[Ca^{2+}]_i$) concentrations were calculated using the following equation [19]: $[Ca^{2+}]_i = Kd(F380\ max/F380\ min)(R - Rmin)/(Rmax - R)$. A Kd value of 224 nM was assumed for the binding of calcium to Fura2. The *R*max and *R*min values were determined by the consecutive addition of 0.2% TritonX-100 and 8 mM EGTA, respectively.

2.8. Statistical analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by a Bonferroni post hoc test. All data are presented as the mean ± SEM. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Pinocembrin inhibits AngII-induced contraction of rat aortic rings with either intact or denuded endothelium

Pinocembrin at concentrations up to 100 μM had no effect on the basal tension of rat aortic rings. Furthermore, pretreatment with pinocembrin inhibited AngII-induced contraction of aortic rings in a concentration-dependent manner (Fig. 1A and B). In endothelium-intact aortic rings, pinocembrin at concentrations of 10 μM, 25 μM, 50 μM and 100 μM was observed to produce a significant reduction in the maximal contractile response by 42.2 ± 3.9%, 53.7 ± 5.4%, 83.3 ± 3.1% and 97.5 ± 1.5%, respectively, compared to the vehicle control (0.2% DMSO) (Fig. 1A). We also

observed that in endothelium-denuded aortic rings, pinocembrin at concentrations of 10, 25, 50 μM and 100 μM blocked the contractile response to AngII and suppressed the maximal response by 45.6 ± 8.7%, 60.6 ± 8.0%, 82.3 ± 1.2% and 90.5 ± 1.6%, respectively (Fig. 1B).

3.2. Effects of pinocembrin on the concentration–response relationships of AngII

In endothelium-denuded aortic rings, pinocembrin (5 μM, 25 μM and 100 μM) resulted in a concentration-dependent rightward shift of the concentration–response curves to AngII with a significant progressive decrease in the maximal response to AngII, yielding a pD_2' value of 4.28 ± 0.15 ($n = 6$; Fig. 1C).

3.3. Pinocembrin shows effective binding at the active site of AT_1R

A molecular docking study of pinocembrin binding to AT_1R was performed. In the docking model, losartan was observed to bind to AT_1R with a free energy (docking score) of −7.4 kcal/mol. Similarly, pinocembrin bound to AT_1R with a free energy (docking score) of −6.3 kcal/mol. The 7-OH of pinocembrin was hydrogen bonded with Thr 175 in the binding pocket. This suggests that pinocembrin can effectively bind at the active site of AT_1R (Fig. 1D).

3.4. Pinocembrin suppresses AngII-induced $[Ca^{2+}]_i$ increase in VSMCs

Pinocembrin at a concentration of 10 μM (Pin control) alone did not affect basal $[Ca^{2+}]_i$ levels. In Ca^{2+}-containing medium, 1 μM pinocembrin remarkably suppressed the AngII-induced $[Ca^{2+}]_i$ increase; this suppression was more pronounced at a concentration of 10 μM (Fig. 2A). Because intracellular release from sarcoplasmic reticulum stores and extracellular influx account for the elevation in $[Ca^{2+}]_i$ in VSMCs, these cells were stimulated with AngII in Ca^{2+}-free medium (HBSS without BSA and 1.8 mM Ca^{2+}) containing 1 mM EGTA (Fig. 2B). Approximately 2 min post-stimulation with 100 nM AngII, 1.8 mM calcium was added back to the medium and the subsequent calcium influx was measured (Fig. 2C). In Ca^{2+}-free medium containing 1 mM EGTA, there was both a lower baseline and a reduction in AngII-induced release of $[Ca^{2+}]_i$ compared to medium containing 1.8 mM calcium. However, pinocembrin markedly blocked the AngII-induced intracellular calcium release at concentration of 1 μM, with an even stronger effect at 10 μM (Fig. 2B). Additionally, we observed that despite the reintroduction of 1.8 mM Ca^{2+} into the medium, pinocembrin at concentrations of 1 μM and 10 μM significantly blocked the extracellular calcium influx activated by addition of Ca^{2+} after depletion of the intracellular Ca^{2+} stores (Fig. 2C).

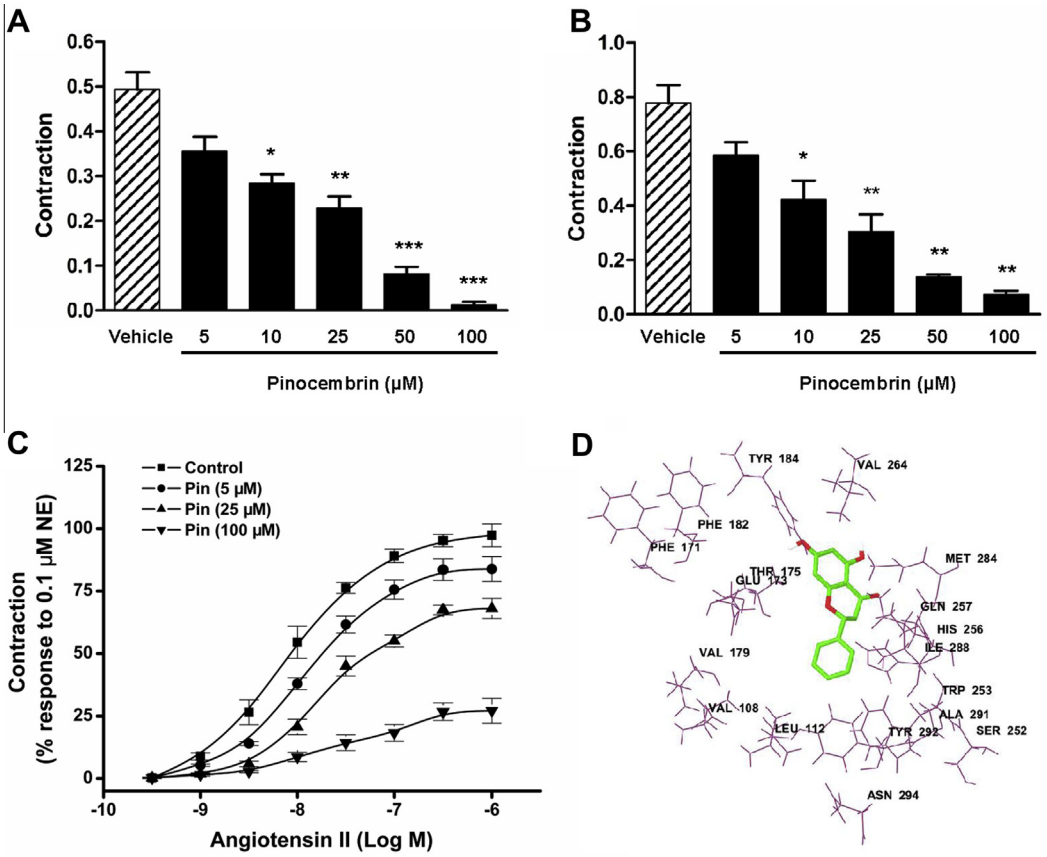

Fig. 1. Inhibitory effect of pinocembrin on AngII-induced contraction in rat aortic rings. (A and B) The rat aortic rings were bathed in K–H solution and stimulated with 100 nM AngII to obtain the initial AngII response. After each ring was treated with either a single concentration of pinocembrin (5 μM, 10 μM, 25 μM, 50 μM or 100 μM) or 0.2% DMSO (vehicle control) for 20 min, the second temporal course for AngII (100 nM) was established. The results are expressed as the ratio of the maximal AngII response obtained from the second run to that from the first run. (A) Results in rat endothelium-intact aortic rings. (B) Results in rat endothelium-denuded aortic rings. The results are presented as the mean ± SEM ($n = 6$). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to the corresponding vehicle control. (C) Concentration–response curves for AngII obtained in endothelium-denuded rat thoracic aortic rings in either the absence or presence of different concentrations of pinocembrin (Pin). Data represent the mean ± SEM ($n = 6$) and are expressed as percentage of response to 0.1 μM NE. (D) The docking model of pinocembrin binding to AT_1R. The residues in the binding pocket are colored in purple, and the ligands are shown in green (carbon atom: green, oxygen atom: red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

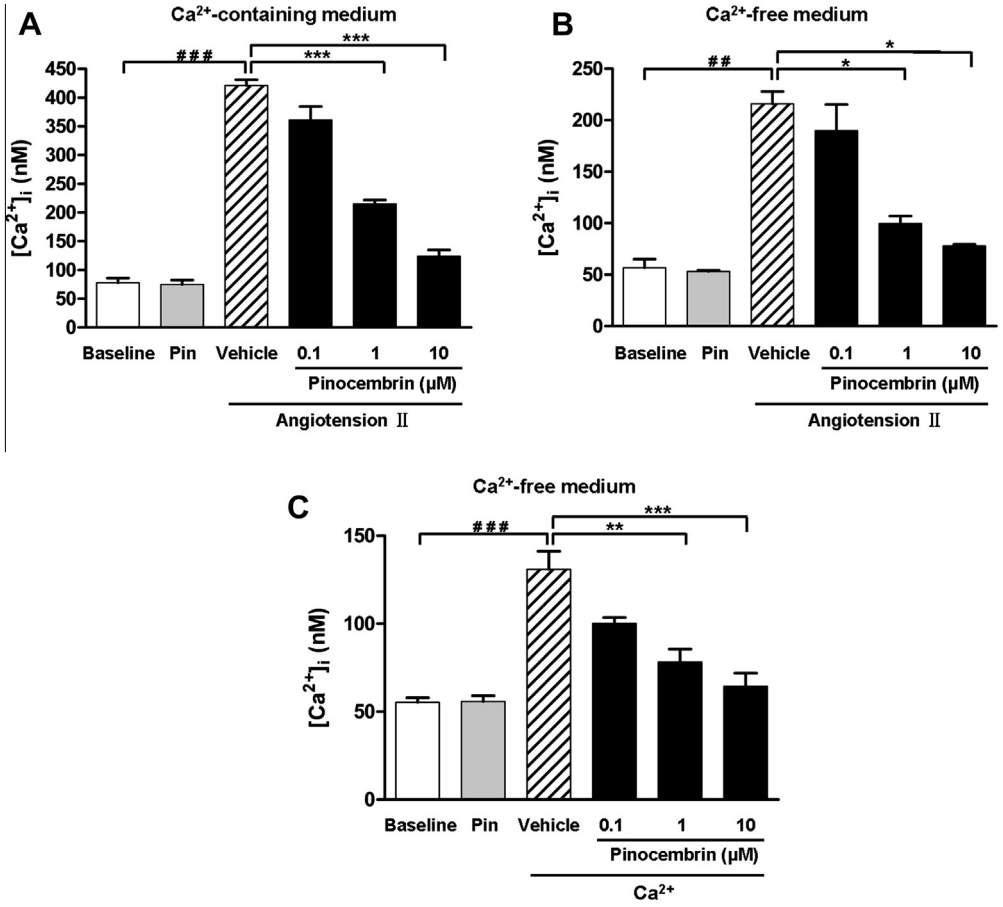

Fig. 2. Effect of pinocembrin on the Ca^{2+} influx stimulated by AngII in VSMCs. VSMCs were serum-starved for 24 h and loaded with Fura-2/AM. Cells were then washed with HBSS in either the presence (A, Ca^{2+}-containing medium) or absence (B, Ca^{2+}-free medium) of 1.8 mM Ca^{2+}. Cultures were pretreated with either pinocembrin (0.1, 1 or 10 μM) for 10 min or 0.2% DMSO (vehicle control) prior to treatment with 100 nM AngII for 10 s in the presence (A) or absence (B) of 1.8 mM Ca^{2+}. Approximately 2 min post-stimulation with 100 nM AngII, 1.8 mM Ca^{2+} was reintroduced into the medium without 1.8 mM Ca^{2+}. The values of $[Ca^{2+}]_i$ at 20 s after the addition of 1.8 mM Ca^{2+} are shown (C, Ca^{2+}-free medium). Bar graphs of data from Ca^{2+} increase (A), intracellular Ca^{2+} release (B) and extracellular Ca^{2+} influx (C) are shown. VSMCs treated with 0.2% DMSO (baseline) or pinocembrin (Pin; 10 μM) alone serve as controls. The results are presented as the mean ± SEM ($n = 3$). $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared to the baseline control; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to the corresponding vehicle control.

3.5. Pinocembrin reduces the increase in ERK1/2 phosphorylation in response to AngII in both rat aortic rings and VSMCs

Western blot analysis showed that AngII-induced ERK1/2 activation in rat aortic rings was significantly blunted by pinocembrin treatment (25 μM and 100 μM) in a dose-dependent manner (Fig. 3A and B). The inhibitory effect of pinocembrin on AngII-induced ERK1/2 phosphorylation was also observed in VSMCs pretreated with 0.1 μM, 1 μM and 10 μM pinocembrin for 2 h (Fig. 3C).

3.6. Effect of pinocembrin on the phosphorylation of MLC2 induced by AngII in both endothelium-intact and -denuded aortic rings

The AngII signaling cascade culminates in activation of myosin light chain kinase, which leads to smooth muscle cell contraction via phosphorylation of Ser19 on MLC2 [20]. As shown in Fig. 4A and B, pinocembrin at concentrations of 25 μM and 100 μM strongly inhibited this increase of MLC2 phosphorylation after a 5 min exposure to 100 nM AngII in both endothelium-intact and -denuded rat aortic rings.

4. Discussion

In the present study, we have demonstrated that pretreatment with various concentrations of pinocembrin inhibited 100 nM AngII-induced vascular contractions in both endothelium-intact and -denuded rat aortic rings, with a maximal effect at 100 μM. The molecular mechanism of this effect might be attributed to a reduction in both the AngII-induced increase in $[Ca^{2+}]_i$ and ERK1/2 activation by blocking AT_1R.

The vascular AT_1R is a central component of the renin-angiotensin system. AngII activates AT_1R to induce vasoconstriction [7]. Pinocembrin (5 μM, 25 μM and 100 μM) produced a rightward shift in the concentration-response curve of AngII with a significant reduction in the maximal contractile response at each concentration, which suggests noncompetitive antagonism of AngII-induced contractions. Noncompetitive antagonism similar to that exerted by pinocembrin has also been observed for some nonpeptide AT_1R antagonists such as candesartan, EXP 3174, etc. [21]. The pD'_2 value (concentration of antagonist that causes 50% reduction of the maximal response) of these nonpeptide AT_1R antagonists varied between 8 and 10 [22], but pinocembrin showed a decreased pD'_2 value of 4.28 ± 0.15.

Molecular modeling studies have provided a platform to study the interaction of AngII and its antagonists with AT_1R [23]. The results of the present study revealed that pinocembrin was capable of interacting with the active site of AT_1R and might serve as a natural source for blocking the angiotensin receptor. However, the inhibitory effect of pinocembrin on the response to AngII is a non-specific vasodilating effect because in the absence of AngII,

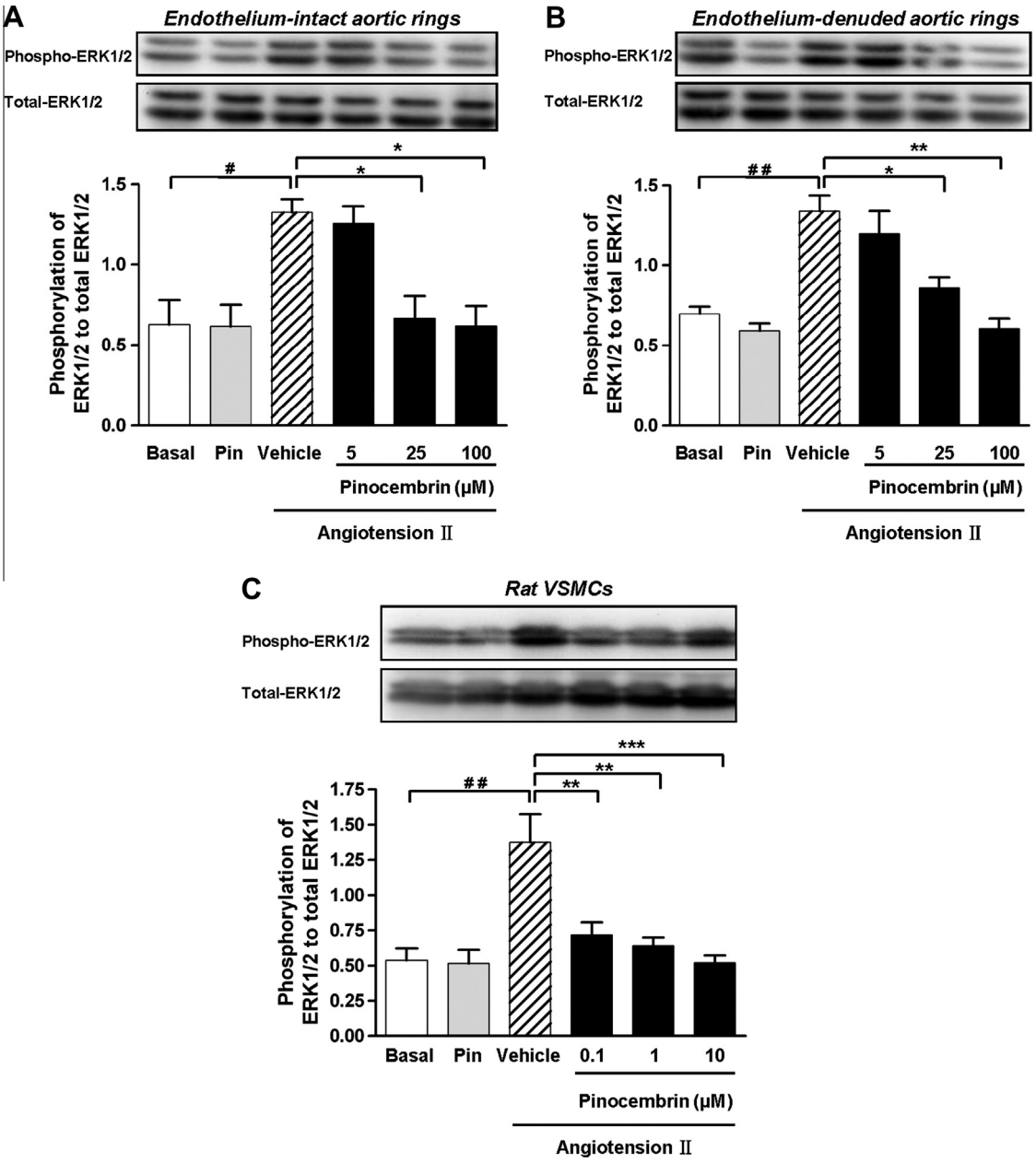

Fig. 3. Effect of pinocembrin on ERK1/2 phosphorylation induced by AngII. Rat endothelium-intact (A) or -denuded (B) aortic rings and VSMCs (C) were pretreated with pinocembrin for either 20 min (aortic rings) or 2 h (VSMCs) prior to the addition of 100 nM AngII for 5 min to induce ERK1/2 activation. The lysates from pooled samples of aortic rings or VSMCs were subjected to SDS–PAGE and analyzed by western blotting. Rat aortic rings and VSMCs treated with 0.2% DMSO (basal control) or pinocembrin (Pin; 100 μM in rat aortic rings and 10 μM in VSMCs) alone serve as controls. Representative western blots for phospho-ERK1/2 (44/42 kDa) and ERK1/2 (44/42 kDa) (upper panel) are shown. Densitometric analysis of the western blot is shown in the lower panel. The results are presented as the mean ± SEM ($n = 3$ with 4 rats in each pool (A and B) or $n = 4$ (C)). $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to the corresponding basal control; $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to the corresponding vehicle control.

pinocembrin alters the contractile responses to both NE and KCl [5].

Blockade of AT_1R in the acute phase of experimental ischemic stroke reduces ischemic injury, and this protective effect is not directly related to the decrease in blood pressure produced by these compounds [14,24]. Pinocembrin was observed to improve the outcome in global CI/R-injured rats without decreasing the mean blood pressure [13]. Hence, the inhibitory effect of pinocembrin on AT_1R and AngII-induced vascular contraction could be beneficial in the treatment of cerebral ischemia, though its effects on cerebral vessels need to be further investigated.

The increase in $[Ca^{2+}]_i$ is thought to play a direct role in the AngII-induced vasoconstriction in smooth muscle. Increased $[Ca^{2+}]_i$ facilitates Ca^{2+}-calmodulin binding, which activates the myosin light chain kinase, promoting the interaction of myosin II with actin and enhancing cross-bridge cycling with consequent contraction [25]. After interacting with AT_1R, AngII typically mediates a biphasic $[Ca^{2+}]_i$ response consisting of a rapid initial transient phase followed by a sustained plateau. The initial transient phase is primarily characterized by IP_3-induced intracellular Ca^{2+} mobilization from the sarcoplasmic reticulum whereas the sustained phase is dependent on an influx of Ca^{2+} from the extracellular medium [26]. In our experiment, pinocembrin did not affect the basal level of $[Ca^{2+}]_i$ but suppressed the AngII-induced increase in $[Ca^{2+}]_i$ in VSMCs. Pinocembrin prevented both the AngII-induced intracellular calcium release and extracellular calcium influx.

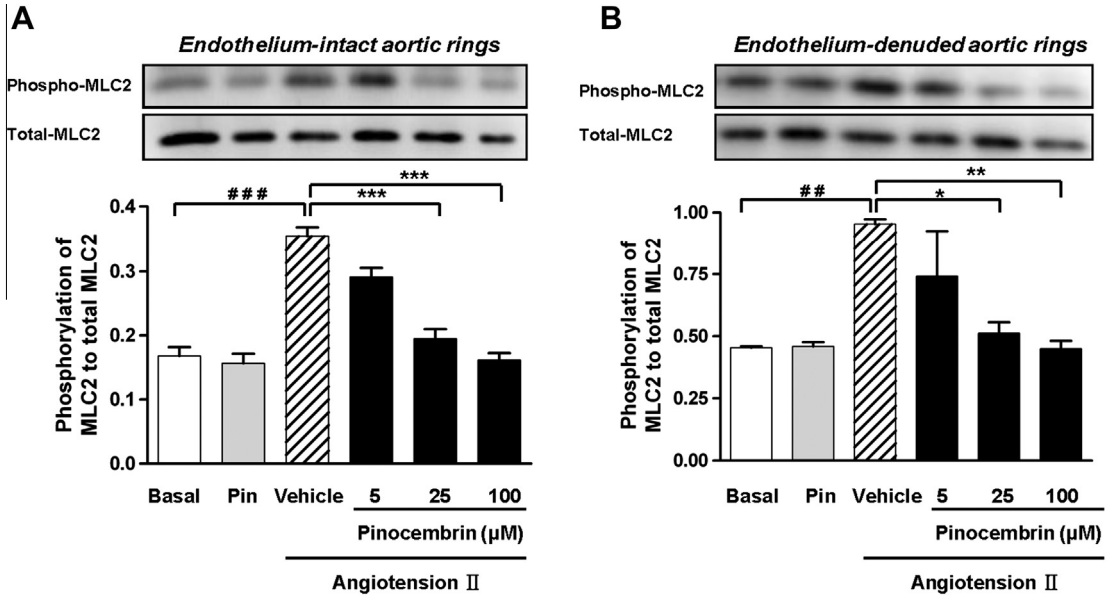

Fig. 4. Effect of pinocembrin on the phosphorylation levels of MLC2 in AngII-treated aortic rings. Rat aortic rings were pretreated with either pinocembrin (5 μM, 25 μM or 100 μM) or 0.2% DMSO (vehicle control) for 20 min prior to addition of 100 nM AngII for 5 min. The lysates from pooled samples of aortic rings were subjected to SDS–PAGE and analyzed by western blotting. Rat aortic rings treated with 0.2% DMSO (basal control) or pinocembrin (Pin; 100 μM) alone serve as controls. Representative western blots for phospho-MLC2 (18 kDa) and total-MLC2 (18 kDa) (upper panel) in endothelium-intact (A) and -denuded (B) aortic rings are shown. Densitometric analysis of the western blot is shown in the lower panel. The results are presented as the mean ± SEM (n = 3 with 4 rats in each pool). $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared to the corresponding basal control; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to the corresponding vehicle control.

These findings are in agreement with our previous study, in which we demonstrated that pinocembrin reduced NE-induced transient contraction in a Ca^{2+}-free solution and inhibited contractions induced by increasing external calcium concentrations in a Ca^{2+}-free medium containing 60 mM KCl [5].

The AngII-induced vascular contraction involves the activation of ERK1/2 and tyrosine kinases through increases in $[Ca^{2+}]_i$ [27]. Furthermore, Zhou et al. observed that ERK1/2 activation in VSMCs was dependent on the release of Ca^{2+} from intracellular stores and its influx from extracellular compartments [28]. In this study, we have shown that pinocembrin blocked both the AT_1R-mediated increase in $[Ca^{2+}]_i$ and ERK1/2 activation induced by AngII in VSMCs, suggesting that it exerts its effect through disrupting the AngII-AT_1R-Ca^{2+}-ERK1/2 signal pathway.

Taken together, our results suggest that pinocembrin prevents AngII-induced vascular contraction in vitro by inhibiting both the AngII-induced elevation in $[Ca^{2+}]_i$ and ERK1/2 activation by blocking AT_1R activation. We believe that our study contributes to a better understanding of the molecular mechanism of action of pinocembrin.

Acknowledgments

We are grateful to Dr. Xin Wang (University of Manchester, UK) and Prof. Humphrey Rang (University College London, UK) for their advice and careful reading of the manuscript.

This work was supported by the Major Scientific and Technological Special Project for "Significant New Drugs Creation" (No. 2009ZX09302-003, 2009ZX09102-034), the Special Fund of the National Laboratory of China (No. 2060204), the National Natural Science Foundation of China (No. 81102444) and the Research Fund for the Doctoral Program of Higher Education of China (No. 20091106120033).

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