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The anti-atherosclerotic di-peptide, Trp-His, inhibits the phosphorylation of voltage-dependent L-type Ca^{2+} channels in rat vascular smooth muscle cells

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Trp-His is the only vasoactive di-peptide known to regulate intracellular Ca^{2+} ($[Ca^{2+}]_i$) and prevent the onset of atherosclerosis in mice. In this study, we showed that Trp-His reduced the $[Ca^{2+}]_i$ elevation in phospholipase C-activated vascular smooth muscle cells (VSMCs), while a mixture of the corresponding constituent amino acids did not show significant reduction. Furthermore, Trp-His suppressed calmodulin-dependent kinase II (CaMK II) activity in angiotensin II-stimulated VSMCs, resulting in the inhibition of phosphorylation of voltage-dependent L-type Ca^{2+} channels (VDCC). Therefore, Trp-His potentially regulates the VDCC phosphorylation cascade through Ca^{2+}-CaM/CaMK II.

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1. Introduction

In a series of our studies, we demonstrated that Trp-His [1], as well as His-Arg-Trp [2], exhibited potent vasorelaxant and anti-proliferative effects against vascular smooth muscle cells (VSMCs) and were involved in the reduction of elevated intracellular Ca^{2+} level ($[Ca^{2+}]_i$) in VSMCs in part by their binding to an extracellular site of voltage-dependent L-type Ca^{2+} channels (VDCC) [3]. To date, except for these peptides, no endothelium-independent vasoactive peptides have been reported, although peptides, such as Arg-Ala-Asp-His-Pro [4] and Met-Tyr [5], have been reported to evoke an endothelium-dependent relaxation. Our new findings on the vasoactive functionality of small peptides allowed us to investigate their potential role(s) in the prevention of hypertension-related vascular diseases, such as atherosclerosis, since the onset is closely associated with the proliferation and/or migration of VSMCs [6]. An oral administration study of Trp-His to apolipoprotein E-deficient mice clearly demonstrated the in vivo vasoactivity of Trp-His, since 9 weeks of administration of Trp-His (10 or 100 mg/kg/day) to mice inhibited progressive atherosclerotic lesion without any alteration of the lipid profiles [7].

As noted above, the anti-atherosclerotic di-peptide Trp-His also exhibited the ability to regulate elevation of $[Ca^{2+}]_i$ in VSMCs. The action was partly explained by its binding to VDCCs or inhibition of extracellular Ca^{2+} influx into VSMCs, since Trp-His reduced the elevation of $[Ca^{2+}]_i$ by a VDCC agonist (Bay K8644). However, it was also found that the ability of Trp-His to reduce $[Ca^{2+}]_i$ in Bay K8644-stimulated VSMCs was one-quarter of that in angiotensin II (Ang II)-stimulated VSMCs [3]. Ang II is known as one of the major vasopressor hormones, and induces diverse intracellular Ca^{2+}-signaling transductions [8]. Therefore, it can be speculated that greater effect of Trp-His to reduce $[Ca^{2+}]_i$ in Ang II-VSMCs may be caused by suppression of Ang II-related intracellular Ca^{2+}-signaling pathways in VSMCs, in addition to VDCC blocking actions. In the present study, we demonstrate that Trp-His (and not its individual constituent amino acids) inhibits phospholipase C (PLC) activated-$[Ca^{2+}]_i$ elevation in VSMCs and that the underlying mechanism involves inhibition of calmodulin-dependent kinase II (CaMK II) activity. The present study also provides the first evidence that Trp-His significantly inhibits the phosphorylation of VDCC, which pivotally regulates Ca^{2+} entry via VDCC in VSMCs, suggesting a potential role of the anti-atherosclerotic di-peptide Trp-His in the Ca^{2+}-CaM/CaMK II-VDCC phosphorylation pathways.

2. Materials and methods

2.1. Materials

Trp-His was synthesized using an Fmoc-solid phase synthesis method, according to the manufacturer's instructions (Kokusan

Abbreviations: PLC, phospholipase C; VSMCs, vascular smooth muscle cells; CaMK II, calmodulin-dependent kinase II; VDCC, voltage-dependent L-type Ca^{2+} channels; Ang II, angiotensin II; 2-APB, 2-aminoethoxydiphenyl borate; AT_2R, angiotensin type 2 receptor; ER/SR, endoplasmic reticulum/sarcoplasmic reticulum; IP_3R, inositol 1,4,5-trisphosphate receptor

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Chemicals, Tokyo, Japan). Fura-2/AM was purchased from Dojindo (Kumamoto, Japan). Angiotensin II (Ang II), S-(+)-PD 123177, 2-aminoethoxydiphenyl borate (2-APB) and *m*-3M3FBS were obtained from Sigma–Aldrich (St. Louis, MO, USA). U-73122 was obtained from Merck (Darmstadt, Germany). RIPA buffer, Phos-tag and KN-62 were purchased from Wako Pure Chemical Industry (Osaka, Japan). Horseradish peroxidase (HRP) conjugated streptavidin and ECL prime detection reagents were obtained from GE Healthcare Biosciences (Piscataway, NJ, USA).

2.2. Cell culture

Isolation of VSMCs from an 8 week-old male Wistar rat (Charles River Japan, Kanagawa, Japan) and the cell culture were performed according to our previous study [3]. The VSMCs were maintained at 37 °C in a humidified 5% CO_2 incubator. Passage numbers 5–7 were used in this study. Animal experiments were carried out according to the Guidance for Animal Experiments in the Faculty of Agriculture and in the Graduate Course of Kyushu University and the Law (No. 105, 1973) and Notification (No. 6, 1980 of the Prime Minister's Office) of the Japanese Government.

2.3. Measurement of $[Ca^{2+}]_i$ in VSMCs

Measurement of $[Ca^{2+}]_i$ was performed using a Ca^{2+}-sensitive probe (Fura-2/AM), as described previously [3]. Briefly, quiescent VSMCs were incubated with 1 μM Fura-2/AM containing 0.1% dimethylsulfoxide and 0.04% Cremophor EL (Nacalai Tesque, Kyoto, Japan) in physiological salt solution (PSS, NaCl 145 mM, KCl 5 mM, Na_2HPO_4 1 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 0.5 mM, glucose 10 mM, and HEPES 5 mM, pH 7.4) for 60 min at 37 °C. The cells (1×10^5 cells/ml) were then added into a 3 ml-quartz cuvette, followed by pretreatment with Trp-His or inhibitors for 10 min at 37 °C. Mitogen (10 μM Ang II or 20 μM *m*-3M3FBS) was injected into the cuvette and subsequent fluorescence intensities were monitored using a fluorescence spectrophotometer (RF-5300PC, Shimadzu Co., Kyoto, Japan). $[Ca^{2+}]_i$ was calculated automatically at a dual excitation wavelength of 340/380 nm and emission wavelength of 500 nm. Unless otherwise noted, in Ang II-stimulation experiments, 1.0 μM PD123177 (an antagonist of angiotensin type 2 receptor (AT_2R)) [9] was added 10 min before the addition of Ang II to avoid activation of vasorelaxation signaling pathways via AT_2R or any unexpected $[Ca^{2+}]_i$.

2.4. Extraction of the whole protein from Ang II-stimulated VSMCs

VSMCs (2×10^5 cells/well) were grown up to 90% confluence in 6-well plates with DMEM (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Invitrogen). Quiescent VSMCs were pretreated with either vehicle or an inhibitor for 10 min, and then incubated with either vehicle or Ang II (10 μM) in each well for 60 min at 37 °C. After Ang II stimulation, the cells were washed twice with ice cold PBS. The cells were then lysed in RIPA buffer (150 mM NaCl, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium sulfate, 1.0% (w/v) NP-40 and 50 mM Tris–HCl, pH 8.0) and kept for 10 min on ice. The supernatant was collected following centrifugation at 20,000×g for 15 min at 4 °C and the protein concentration was determined with a Bio-Rad DC Protein Assay Kit (using BSA as a standard).

2.5. CaMK II activity in Ang II-stimulated VSMC extracts

The CaMK II activity in VSMCs was determined with an ELISA-based kit (CycLex CaM Kinase II Assay Kit, MBL International, Woburn, MA, USA) according to the manufacturer's protocol. Briefly, 10 μl of the VSMC extract and 90 μl of the reaction mixture containing 1 mM ATP were added to each well of a CaMK II substrate-coated plate and incubated for 40 min at 30 °C. The blank for this assay contained an equal volume of RIPA buffer. After washing five times with washing buffer, 100 μl of HRP conjugated anti-phospho-substrate antibody was incubated for 60 min at 30 °C. After additional washes, 100 μl of tetramethylbenzidine substrate solution was incubated for 20 min at 30 °C. The reaction was stopped by adding 100 μl of the stop solution and the absorbance at 450 nm was measured with a Wallac 1420-microplate reader (Perkin Elmer Life Science, Tokyo, Japan). The CaMK II activity (mU/mg protein) in VSMCs was calculated from standard curves prepared with serial dilutions of CaMK II standard.

2.6. Preparation of Phos-tag

Phos-tag, a dinuclear metal complex (i.e., 1,3-bis[bis(pyridin-2-ylmethyl)amino]propan-2-olato-di-zinc(II) complex), was prepared according to the manufacturer's instructions. Briefly, 5 μl of biotin-pendant Phos-tag (10 mM) was mixed with 20 μl of $Zn(NO_3)_2$ (10 mM), 2 μl of HRP conjugated streptavidin and 473 μl of TBS-Tween20 (TBST, 20 mM Tris–HCl, 137 mM NaCl and 0.05% Tween20, pH 7.6). This solution was allowed to stand for 30 min at room temperature and then added to a centrifugal filter device cup (nominal molecular weight cutoff = 30,000 Da, Millipore) and centrifuged at 14,000×g for 10 min at room temperature. The remaining solution in the cup was diluted with 10 ml of TBST, and used as the Phos-tag solution to detect the amount of phosphorylated VDCC in Ang II-stimulated VSMCs.

2.7. Western blot analysis of phosphorylated VDCC

The determination of the amount of phosphorylation of VDCC was performed with Western blot analysis using the Phos-tag and anti-VDCC antibody on the same membrane. The VSMC extract was mixed with an equal volume of sample buffer (20% glycerol, 4% sodium dodecyl sulfate (SDS), 3% dithiothreitol, 0.002% bromophenol blue and 0.125 M Tris–HCl, pH 6.8) and incubated overnight at 4 °C. An aliquot (15 μg/lane) of the sample-treated VSMC extract was applied to 10% SDS–PAGE gels for 2 h at 20 mA and transferred onto a PVDF membrane (Hybond-P, GE Healthcare) for 1.5 h at 40 mA. The membrane was incubated with the prepared Phos-tag solution for 30 min at room temperature. After washing twice for 5 min with TBST, the phosphorylated proteins on the Phos-tag-labeled membrane were detected with ECL prime detection reagents and Image Quant LAS 4000 (GE Healthcare). For elimination of the Phos-tag, the membrane was incubated in stripping buffer (2% (w/v) SDS, 0.1 M mercaptoethanol and 62.5 mM Tris–HCl, pH 6.8) for 30 min at room temperature and complete abolishment of Phos-tag was confirmed by the lack of detection of any bands with additional ECL reagents. Then, the Phos-tag-stripped membrane was washed for 3 h and blocked for 1 h at room temperature with 5% (w/v) ECL blocking agent in TBST. The membrane was reprobed with the primary antibody for the α1 subunit of VDCC, rabbit anti-$Ca_v1.2$ antibody (1:1000, Alomone Labs, Jerusalem, Israel), and the secondary antibody, HRP conjugated donkey anti-rabbit IgG antibody (1:1000, GE Healthcare), for 1 h at room temperature, respectively, and the expression of VDCC was analyzed, as described above. Densitometry was used to quantify the amount of phosphorylation of VDCC using Image Quant TL 7.0 software (GE Healthcare). In order to determine the band of the phosphorylated VDCC (p-VDCC), the superimposed image was generated from the Phos-tag and VDCC images. Based on the image, a corresponding area was considered as the band of p-VDCC or VDCC. The amount of phosphorylation of VDCC was calculated as p-VDCC/VDCC (arbitrary unit). The respective means of the p-VDCC/VDCC in each group was described as ratio of the control VSMCs (control = 1).

2.8. Statistical analysis

The results are expressed as the mean ± S.E. (standard error). Statistical differences between the two groups were evaluated by unpaired Student's *t*-test. $P < 0.05$ was considered statistically significant. All analyses were performed with Stat View J 5.0 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. The peptide skeleton of Trp-His is crucial for the inhibition of $[Ca^{2+}]_i$ elevation

To examine the effect of Trp-His on elevated $[Ca^{2+}]_i$ control in VSMCs, the cells were incubated with Fura-2/AM, an intracellular Ca^{2+}-sensitive probe. The application of Ang II (10 μM) to Fura-2-loaded VSMCs in the presence of AT_2R antagonist yielded a marked increase in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$: Ang II, 180.0 ± 21.0 nM, $n = 8$), which was inhibited by Trp-His (300 μM) ($\Delta[Ca^{2+}]_i$: Trp-His, 18.8 ± 10.3 nM, $n = 4$, $P < 0.01$). In contrast, a mixture of the corresponding constituent amino acids (i.e., Trp + His, each 300 μM) did not show any significant inhibition compared with the di-peptide Trp-His ($\Delta[Ca^{2+}]_i$: Trp + His, 131.5 ± 15.0 nM, $n = 4$, *N.S.* vs. Ang II) (Fig. 1).

3.2. PLC-activated Ca^{2+}-signaling pathways are involved in Trp-His-induced $[Ca^{2+}]_i$ reduction

To clarify whether Trp-His is involved in the regulation of Ca^{2+} release from the endoplasmic reticulum/sarcoplasmic reticulum (ER/SR), Fura-2-loaded VSMCs were pretreated with Ca^{2+}-free PSS buffer (containing 0 mM $CaCl_2$) to avoid $[Ca^{2+}]_i$ elevation by extracellular Ca^{2+} entry and to focus on the elevation from ER/SR in Ang II-stimulated VSMCs. Trp-His did not reduce the $[Ca^{2+}]_i$ elevation under this assay condition ($\Delta[Ca^{2+}]_i$: Ang II, 87.3 ± 10.8 nM; Trp-His, 76.6 ± 12.1 nM, $n = 5$), whereas 2-APB, an inhibitor of inositol 1,4,5-trisphosphate receptor (IP_3R), abolished the $[Ca^{2+}]_i$ elevation ($\Delta[Ca^{2+}]_i$: 2-APB, 8.1 ± 1.2 nM, $n = 6$, $P < 0.01$ vs. Ang II) (Fig. 2A and B). Trp-His alone had no power for $[Ca^{2+}]_i$ elevation. Fig. 2C and D showed the changes in $[Ca^{2+}]_i$ when the angiotensin type 1 receptor (AT_1R)-coupled PLC was activated by *m*-3M3FBS, a direct PLC activator [10]. Consequently, *m*-3M3FBS (20 μM) exhibited the progressive increase in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$: *m*-3M3FBS, 221.8 ± 10.5 nM, $n = 3$), while U-73122 (a PLC inhibitor, 10 μM) abolished the $[Ca^{2+}]_i$ elevation significantly ($\Delta[Ca^{2+}]_i$: U-73122, 10.5 ± 14.6 nM, $n = 3$, $P < 0.01$). Interestingly, Trp-His significantly

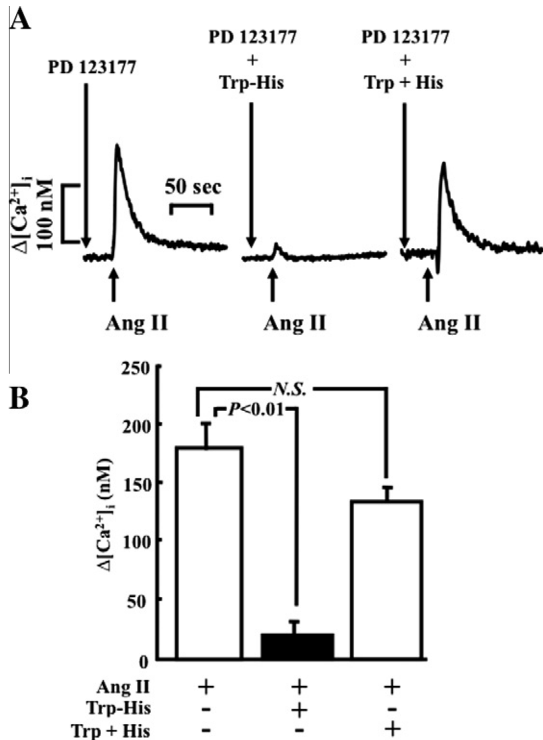

Fig. 1. The peptides skelton is required for Trp-His-induced $[Ca^{2+}]_i$ regulation in Ang II-stimulated VSMCs. (A) A real-time $[Ca^{2+}]_i$ change in Fura-2-loaded VSMCs was measured in the absence or presence of Trp-His (300 μM) or a mixture of its individual constituent amino acids (i.e., Trp + His, each 300 μM). The AT_2R antagonist (PD123177, 1 μM) and the sample were coincubated 10 min prior to the addition of Ang II. (B) Ang II-induced elevation of $[Ca^{2+}]_i$ was indicated as $\Delta[Ca^{2+}]_i$ ($P < 0.01$ compared with Ang II; $n = 4–8$).

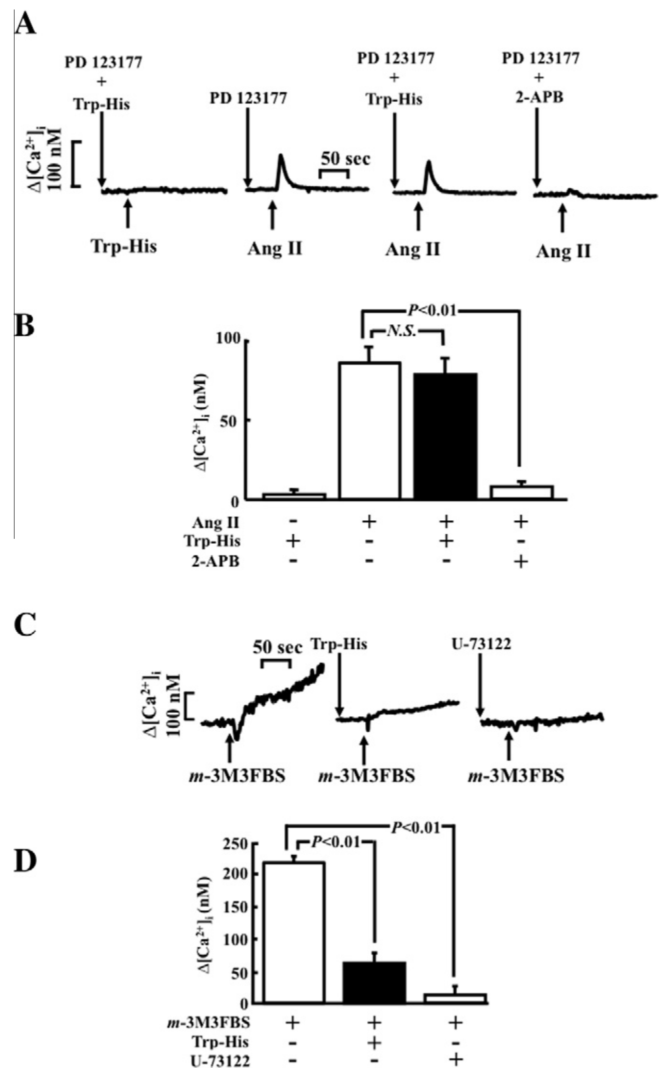

Fig. 2. Trp-His inhibited PLC-related Ca^{2+}-pathways, not Ca^{2+} release from ER/SR. (A) A real-time $[Ca^{2+}]_i$ change in Fura-2-loaded VSMCs was measured in the absence or presence of Trp-His (300 μM) or 2-APB (200 μM). PD123177 (1 μM) and the sample were coincubated in Ca^{2+}-free PSS 10 min prior to the addition of Ang II. (B) Ang II-induced elevation of $[Ca^{2+}]_i$ was indicated as $\Delta[Ca^{2+}]_i$ ($P < 0.01$ compared with Ang II; $n = 5–6$). (C) A real-time $[Ca^{2+}]_i$ change in Fura-2-loaded VSMCs was measured in the absence or presence of Trp-His (300 μM) or an antagonist of PLC, U-73122 (10 μM). The sample was coincubated in PSS 10 min prior to the addition of a PLC activator, *m*-3M3FBS. (D) The *m*-3M3FBS-induced elevation of $[Ca^{2+}]_i$ was indicated as $\Delta[Ca^{2+}]_i$ ($P < 0.01$ compared with *m*-3M3FBS; $n = 3$).

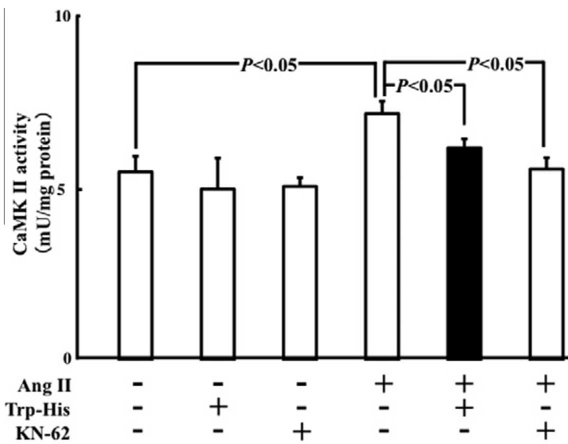

Fig. 3. Trp-His inhibited the activity of CaMK II. VSMCs were preincubated in the absence or presence of Trp-His (300 μM) or KN-62 (50 μM) for 10 min. The cells were stimulated either vehicle or Ang II (10 μM) for 60 min. CaMK II activity (mU/mg protein) in the VSMC extracts was calculated from standard curves prepared with serial dilutions of CaMK II ($P < 0.05$ compared with Ang II; $n = 3–5$).

inhibited the *m*-3M3FBS-induced $[Ca^{2+}]_i$ elevation ($\Delta[Ca^{2+}]_i$: Trp-His, 63.2 ± 11.2 nM, $n = 3$, $P < 0.01$ vs. *m*-3M3FBS).

3.3. CaMK II activity in VSMCs was inhibited by Trp-His

Effect of Trp-His on CaMK II activity in Ang II-stimulated VSMCs for 60 min was examined, since the Ang II/PLC Ca^{2+}-signal promotes the formation of Ca^{2+}-CaM complex to activate CaMK II, resulting in the direct opening of VDCC or $[Ca^{2+}]_i$ elevation [11,12]. Trp-His or KN-62 (a CaMK II inhibitor) alone did not alter the CaMK II activity in VSMCs within the experimental conditions (control, 5.51 ± 0.46 mU/mg protein; Trp-His, 5.00 ± 0.90 mU/mg protein; KN-62, 5.08 ± 0.26 mU/mg protein, $n = 3–5$). Fig. 3 also described that the addition of Ang II significantly activated CaMK II (Ang II, 7.20 ± 0.36 mU/mg protein, $n = 3$, $P < 0.05$ vs. control) and Trp-His significantly lowered CaMK II activity in Ang II-stimulated VSMCs (Trp-His, 6.21 ± 0.26 mU/mg protein, $n = 3$, $P < 0.05$ vs. Ang II), similar to KN-62 (5.59 ± 0.33 mU/mg protein, $n = 3$, $P < 0.05$ vs. Ang II).

3.4. Trp-His inhibited phosphorylation of VDCC

To confirm the involvement of Trp-His in the CaMK II/VDCC phosphorylation pathway [13,14], Phos-tag, that has already been applied for the analysis of phosphorylated potassium channels [15], was used for the detection of p-VDCC in the present study. VSMCs were pretreated with either Trp-His or KN-62, and stimulated with Ang II for 60 min. Western blot analysis (Fig. 4) revealed that both Trp-His and KN-62 had no ability to alter the p-VDCC/VDCC ratio in VSMCs (control, 1.00 ± 0.04; Trp-His, 0.97 ± 0.12; KN-62, 0.91 ± 0.08, $n = 3$). In contrast, in Ang II (10 μM)-stimulated VSMCs, Trp-His as well as KN-62 significantly reduced the p-VDCC/VDCC ratio (Ang II, 1.12 ± 0.02, $P < 0.05$ vs. control; Trp-His, 0.92 ± 0.06, $P < 0.05$ *vs.* Ang II; KN-62, 0.88 ± 0.04, $P < 0.01$ vs. Ang II, each $n = 3$).

4. Discussion

In this report, we provide the first indication of the physiological functionality of the anti-atherosclerotic small peptide, Trp-His, on vessel tone. The peptide skeleton of Trp-His plays a crucial role in the regulation of elevated $[Ca^{2+}]_i$, by which vasoconstriction (or cell proliferation) signaling pathways in VSMCs are activated. The $[Ca^{2+}]_i$ experiments using Ang II/PLC agonists or an IP_3R antagonist revealed that the reduction in elevated $[Ca^{2+}]_i$ by Trp-His was caused by suppression of the PLC-related Ca^{2+}-signaling pathways,

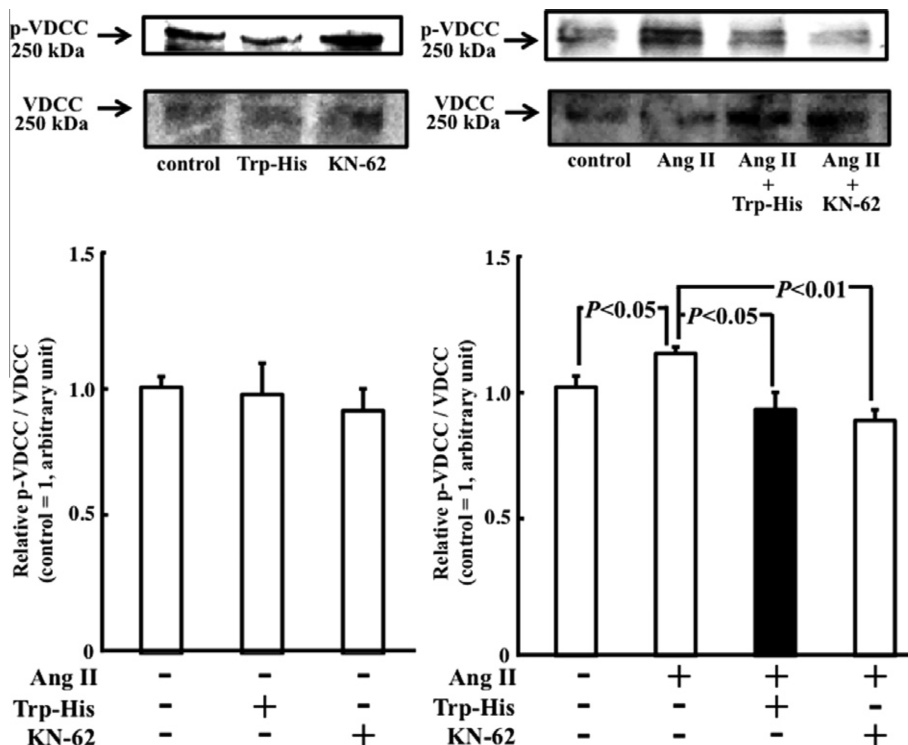

Fig. 4. Trp-His inhibited Ang II-induced phosphorylation of VDCC. VSMCs were preincubated in the absence or presence of Trp-His (300 μM) or KN-62 (50 μM) for 10 min and stimulated with Ang II for 60 min. The densitometric analysis of p-VDCC/VDCC (arbitrary unit) was performed using Image Quant TL 7.0 software. The respective means of the p-VDCC/VDCC in each group was described as ratio of the control VSMCs ($P < 0.05$, $P < 0.01$ compared with Ang II; $n = 3–5$).

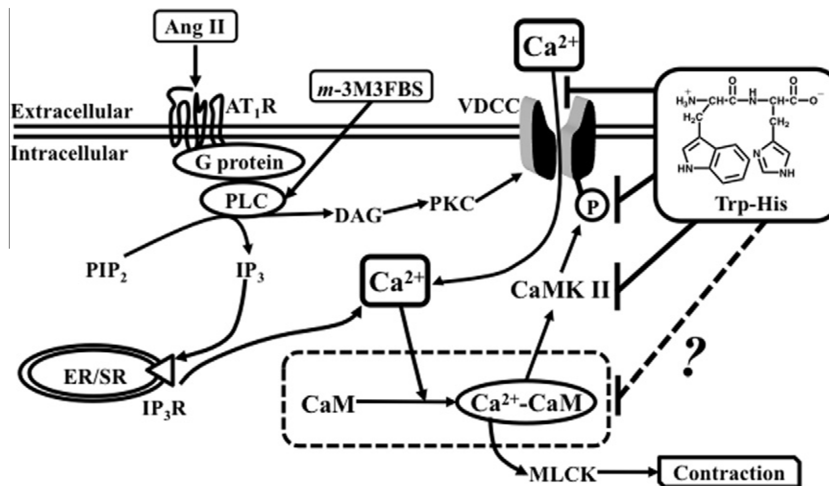

Fig. 5. Schema of the possible roles of Trp-His on CaMK II-mediated VDCC phosphorylation in VSMCs. We demonstrated that Trp-His inhibited the Ang II-induced activation of CaMK II and inhibited Ang II-induced phosphorylation of VDCCs. Considering that CaMK II is involved in the phosphorylation of VDCCs followed by the facilitation of Ca^{2+} influx via VDCCs, these data suggest that Trp-His plays a potential role in the regulation of $[Ca^{2+}]_i$ by its blockade of the CaMK II-VDCC phosphorylation pathway. The potential of Trp-His to act on the Ca^{2+}-CaM/CaMK II signaling pathways remains unclear, and further investigations are currently in progress.

but not by both AT_1R antagonistic effect or suppression of the PLC/ IP_3R pathway. Further experiments in Ang II-stimulated VSMCs supported that Trp-His attenuated CaMK II activity. The finding that Trp-His significantly inhibits Ang II-induced phosphorylation of α1 subunit of VDCC by Phos-tag analysis supports that Trp-His plays a potential role in the regulation of $[Ca^{2+}]_i$ in VSMCs by its blockade of the PLC/CaMK II-VDCC phosphorylation pathway, as illustrated in Fig. 5.

A new Ca^{2+}-related signaling pathway, by which CaMK II preferably phosphorylates the intracellular side of VDCC [13,14], allowed us to consider the overall mechanism underlying the ability of Trp-His to reduce $[Ca^{2+}]_i$, since phosphorylation of VDCC promotes the opening of extracellular Ca^{2+} influx route across the VSMC membrane [16]. As shown in Fig. 3, Trp-His-treatment of VSMCs significantly lowered CaMK II activity. There are two possible mechanisms for the lowering of CaMK II activity by the bioactive peptide, Trp-His, i.e., direct inhibition of CaMK II and inhibition of Ca^{2+}-CaM formation leading to CaMK II activation. As for the former mechanism, it has been reported that longer peptides such as Lys-Lys-Ala-Leu-Arg-Arg-Gln-Glu-Ala-Val-Asp-Ala-Leu [17] inhibited CaMK II activity. In the latter mechanism, pea protein hydrolysate has been reported to inhibit the formation of Ca^{2+}-CaM complexes [18], although potential candidates (i.e., peptides) responsible for the action have not been elucidated. Thus, further investigations are necessary to clarify whether Trp-His can inhibit CaMK II or Ca^{2+}-CaM formation. In addition, considering that some subtypes of total protein kinase C (PKCs) may facilitate the phosphorylation of VDCC [19], specific inhibition of the subtypes by Trp-His must be also included for further investigations.

CaM is a Ca^{2+}-binding protein that possesses four binding sites. Once Ca^{2+} binds to CaM, it causes a dramatic change in the structural conformation, known as EF hands [20]. The binding sites of Ca^{2+} to CaM are mainly composed of Asp and Glu, which means that the binding domains of CaM are highly negative [20]. As pK_a of imidazole in His is 6.0 [21], Trp-His itself may occur as an equilibrium mixture of the peptide bearing the uncharged and positive-charged moieties at physiological pH. Although further detailed investigations on the mechanism of the Trp-His-induced $[Ca^{2+}]_i$ reduction are needed, the CaM-binding ability of Trp-His to prevent Ca^{2+}-CaM formation cannot be ruled out as once such mechanism in VSMCs. The configuration and/or sequence of peptides can drastically affect their inhibitory potential on $[Ca^{2+}]_i$ regulation in VSMCs. Our previous finding that His-Trp, the reversed sequence of Trp-His, evoked a reduction of $[Ca^{2+}]_i$ similar to Trp-His [3] suggested that the amino acid residues would be essential for the action. From these findings, the imino group and/or indole moiety of Trp-His may be responsible for the suppression of the PLC/CaMK II/ VDCC phosphorylation pathway. Studies on structure-activity relationship using Trp-His analogues are currently in progress using Ang II-stimulated VSMCs.

In conclusion, we demonstrated for the first time that evena di-peptide, Trp-His, that has been shown to have an anti-atherosclerotic effect in vivo and to reduce $[Ca^{2+}]_i$ in AngII-stimulated VSMCs, has the potential to suppress the PLC/Ca^{2+}-CaM/CaMK II/VDCC phosphorylation pathway, likely through inhibition of the formation of Ca^{2+}-CaM complexes and/ or CaMK II activity in VSMCs. The involvement of Trp-His in such Ca^{2+}-signaling pathways should be, thus, addressed to allow new insights of the vasophysiological functionalities of small peptides.

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