Involvement of TRPV1 in the expression and release of calcitonin gene-related peptide induced by rutaecarpine

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Abstract. The traditional Chinese herb Wu-Chu-Yu has been used to treat hypertension for hundreds of years. A previous study indicated that rutaecarpine was the effective component of Wu-Chu-Yu, which lowered blood pressure by elevating the expression level of calcitonin gene-related peptide (CGRP). The present study was performed to investigate the role of transient receptor potential cation channel subfamily V member 1 (TRPV1) in CGRP expression and release induced by rutaecarpine. Dorsal root ganglia (DRG) obtained from Sprague-Dawley rats were cultured to analyze the mRNA expression and release of CGRP. Calcium influx, as an indicator of TRPV1 activation, was measured in 293 cells with stable overexpression of TRPV1. The results demonstrated that the amount of CGRP in the cell culture supernatant and the mRNA expression of CGRPα and CGRPβ in DRG was upregulated by rutaecarpine in a concentration-dependent manner, and was inhibited by the TRPV1 receptor antagonist capsazepine. In addition, intracellular Ca2+ levels were increased by Rut in the aforementioned 293 cell line, indicating the activation of TRPV1 by Rut. Therefore, it was concluded that TRPV1 was involved in the expression and release of CGRP stimulated by rutaecarpine, which provided novel mechanistic understanding of the treatment of hypertension using the Chinese herb Wu-Chu-Yu.

Introduction

Calcitonin gene-related peptide (CGRP) is a predominant neurotransmitter in capsaicin-sensitive sensory nerves, which are widely distributed in the nervous and cardiovascular systems (1). It is known that CGRP is a potent vasodilator, which may additionally inhibit vascular remodeling and serve a protective role in the cardiovascular system (2). Studies have demonstrated that the concentrations of CGRP in plasma of spontaneous hypertensive rats and patients with essential hypertension were significantly decreased, suggesting an important role of CGRP in the development of hypertension (3,4).

It has been demonstrated that the synthesis and release of CGRP from sensory nerves is primarily regulated by transient receptor potential cation channel subfamily V member 1 (TRPV1), which is distributed in the terminals and cell bodies of sensory nerves (5-8). TRPV1 is additionally termed vanilloid receptor subtype 1; it is a ligand-gated non-selective cation channel, with a molecular weight of ~95 kDa and six transmembrane domains. TRPV1 is a multi-type signal detector, and may be activated by a variety of factors, including hydrogen ions, high temperature and aromatic acid compounds (including capsaicin) (9).

Wu-Chu-Yu, the dried unripe fruit of Evodia rutaecarpa (A. Juss.) Benth (Tetradium ruticarpum), is a well-known Chinese herbal drug that has been used to treat hypertension for hundreds of years (10). Modern pharmacological research isolated a quinazolinocarboline alkaloid from Wu-Chu-Yu, which is termed rutaecarpine (11,12). Previous studies have demonstrated the role of rutaecarpine in decreasing blood pressure in a number of animal models, including spontaneously hypertensive rats (SHR) and 2-kidney and 1-clip rats (13,14). In vitro experiments demonstrated that rutaecarpine incubation may induce marked vasodilatation in aortic rings (15). Notably, rutaecarpine produced vasodilatation concomitantly with an increase in the release of CGRP in isolated vascular tissue, and the effect of rutaecarpine was abolished by CGRP8-37, the CGRP receptor antagonist (16,17). As mentioned above, CGRP synthesis in the cell bodies of sensory nerves is regulated by the activation of TRPV1. It is likely that rutaecarpine...
induces CGRP synthesis and release via the activation of TRPV1. In the present study, therefore, the regulatory effect of rutaecarpine on the synthesis and release of CGRP was assayed in cultured dorsal root ganglia (DRG) treated with the TRPV1 antagonist capsaeline. It is known that TRPV1 regulates the synthesis and release of CGRP through the stimulation of calcium influx (18). Therefore, calcium influx is frequently used as an indicator for TRPV1 activation in 293 cells overexpressing TRPV1.

Materials and methods

Reagents. Capsaicin (Cap) was administered in three different dosages corresponding to the following experimental groups: Low dose group [Cap(L); 10⁻⁸ M]; medium dose group [Cap(M); 10⁻⁷ M] and high dose group [Cap(H); 10⁻⁶ M]. Capsazepine were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Rutaecarpine (Rut) was administered in three different dosages corresponding to the following experimental groups: Low dose group [(Rut(L); 10⁻⁷ M)]; medium dose group [Rut(M); 10⁻⁶ M] and high dose group [Rut(H); 10⁻⁵ M]. Rut was synthesized by the School of Pharmaceutical Sciences, Central South University (Changsha, China). The synthesis of rutaecarpine was further confirmed by high performance liquid chromatography (HPLC) and carbon-13 nuclear magnetic resonance (¹³C-NMR) (19) (data not shown). For rutaecarpine: Total yield, 60%; HPLC purity, 98.8% (Shimadzu LC-2010, Shimadzu Corporation, Tokyo, Japan; Welchom® C18S-μm column, 4.6x250 mm; CH₃CN:H₂O = 72:28; 1 ml/min); melting point, 255-256°C; mass spectrum (m/z): 287 (M⁺); ¹³C-NMR (400 MHz, CDCl₃) δ=3.23 (2H), 4.6 (2H), 7.15-8.31 (8H, Ar); 9.56 (1H, NH); ¹³C-NMR (100 MHz, CDCl₃) δ=19.7, 41.3, 112.2, 118.6, 120.1, 120.7, 121.1, 125.6, 125.7, 126.3, 126.5, 127.0, 127.3, 134.4, 138.4, 145.1, 147.3, 161.5. Reagents and conditions were as follows: i) (CF₂CO₂O), pyridine, 25°C for 15 min, 115°C for 5 min; ii) tryptamine, 115°C for 30 min; iii) HCl/HOAc, reflux; and iv) KOH/EtOH/H₂O. The reverse transcription-polymerase chain reaction (RT-PCR) kits were obtained from Beijing Biodev-tech Scientific and Technical Co., Ltd. (Beijing, China). Primers for PCR were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Radioimmunoassay kits for the measurement of CGRP were purchased from the Immunity Institute of Dongya (Beijing, China). SYBR Green PCR master mix was purchased from Takara Bio, Inc. (Otsu, Japan). The rabbit anti-TRPV1 polyclonal antibody was purchased from Abcam (Cambridge, UK; cat. no. ab74855), the goat-anti-rabbit secondary antibody was purchased from Wuhan Dr. de Bioengineering Co., Ltd. (Wuhan, China; cat. no. BA1003). The rabbit anti-neuron-specific enolase antibody was purchased from OriGene Technologies Inc. (Beijing, China; cat. no. TA309202) and the anti-rabbit immunoglobulin G secondary antibody was purchased from Sigma-Aldrich; Merck KGaA (cat. no. SAB4600099). The mouse anti-β-actin monoclonal antibody (Wuhan, China; cat. no. BM0005) and the goat-anti-mouse secondary antibody were purchased from Wuhan Dr. de Bioengineering Co., Ltd. (Wuhan, China; cat. no. BA1001). The fluorescent Ca²⁺ probe (indicator) Fluo-3/AM, Lipofectamine® 2000 transfection reagent and G-418 were purchased from Beyotime Institute of Biotechnology (Haimen, China). PCDNA3.1 and PCDNA3.1-TRPV1 plasmids were donated by Dr David Julius from the Department of Cellular and Molecular Pharmacology, University of California (San Francisco, CA, USA).

Animal preparation and cell culture. All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication no. 85-23; revised 1986). The present study was approved by the Institutional Review Board of The Third Xiangya Hospital, Central South University (Changsha, China). All surgeries were performed under general anesthesia, and all efforts were made to minimize suffering. In the present study, 50 male Sprague-Dawley rats (age, 1-3 days; weight, 9±2 g) were obtained from the Department of Animal Experiments, Central South University (Changsha, China). Animals were housed at a temperature of 25±2°C, a humidity of 50±5% and with ad libitum access to food and water. Animals were sacrificed by decapitation, and the bilateral thoracolumbar DRG were then removed under sterile conditions. Cells were cultured in neurobasal culture medium [Dulbecco's modified Eagle's medium (DMEM) F12 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA); with 1% fetal calf serum (Hangzhou Sijiqing Biological Technology Co., Ltd., Hangzhou, China), 2% B27 additive and 2 mM glutamine]. Cell culture plates were pre-coated with poly-lysine (70.000 kDa) and laminin. The 293 cell line (purchased from Cell Biology, Chinese Academy of Sciences, Shanghai, China) was cultured in DMEM high glucose medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) at 37°C in a 5% CO₂ humidified atmosphere.

RNA preparation RT-PCR analysis. The DRG on the one side of the spinal cord were frozen in liquid nitrogen and homogenized in TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA isolation and RT-semi-quantitative (sq) PCR was performed according to standard techniques. The specific primers pairs and the size of the expected products were as follows: CGRP-α forward, 5'-AAGTTTCTCC CTTTCTCTGT-3' and reverse, 5'-GGTGGGCACAAAGTT GTCTC-3' (318 bp); CGRP-β forward, 5'-GAGACCTTCAAC ACCCAAGCC-3' and reverse, 5'-GGTGGGCACAAAGTT GTCTC-3' (264 bp); and GAPDH forward, 5'-AAGCTCCCTC AAGATTGTCAACG-3' and reverse, 5'-GGAGAGTTGCTGT TGAAGTACA-3' (448 bp). The PCR amplification profiles consisted of denaturation at 94°C for 45 sec, annealing at 58°C for 45 sec and elongation at 72°C for 60 sec. The linear exponential phases for CGRP (α and β) and GAPDH were 30, 32 and 25 cycles, respectively. Equal amounts of corresponding CGRP-α, CGRP-β and GAPDH RT-sqPCR products were loaded on 1.5% agarose gels. To control for possible difference in loading of the RNA samples, the mRNA levels were normalized to those for GAPDH. Optical densities of ethidium bromide-stained DNA bands were semi-quantitatively analyzed using a ChemiDoc-It Imaging System (Vision WorksLS; UVP, Inc., Upland, CA, USA) and the results are presented as CGRP/GAPDH ratios.
The mRNA expression of TRPV1 and β-actin in 293 cells was also investigated using RT-quantitative (q)PCR analysis. The specific primer pairs were as follows: TRPV1 forward, 5'-TTACCAGAATGCGCTATGG-3' and reverse, 5'-TGA CGGTAGGGGCTCCTC-3'; and β-actin forward, 5'-TGA CGTGGACATCCGCAAAG-3' and reverse, 5'-CTGGAAAG GT GGACACGGAGG-3'. Total RNA was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and the RT reaction was performed using 1 µg samples isolated from the cells of each group. For PCR amplification, cDNA was amplified using SYBR Green Real-time PCR Master Mix and 0.4 µM of each primer pair. Amplification was performed with an initial step for 30 sec at 94°C, followed by 40 cycles of the amplification step (94°C for 30 sec, 60°C for 60 sec and 72°C for 1 min) for TRPV1 and β-actin. All amplification reactions for each sample were performed in triplicate and the means of the threshold cycles were used to interpolate curves using Applied Biosciences 7300 Real-Time PCR System Software (version 1.2.3; Applied Biosciences; Thermo Fisher Scientific, Inc.). Total RNA was quantified using the 2ΔΔCq method (20). Results are expressed as the ratio of TRPV1 to β-actin mRNA. The PCR products of TRPV1 were loaded on 2% agarose gels.

Measurement of CGRP-LI like concentration. The DRG to be tested were centrifuged at 4°C, for 15 min at 3,000 x g. The supernatant was collected and lyophilized. In order to detect immune reactive CGRP in the supernatant and DRG, a commercially available rabbit anti-rat CGRP radioimmunoassay kit (cat. no. 20140930; Immunity Institute of Dongya, Beijing, China) was used. The assay was performed according to the manufacturer's protocol. The antibody has 100% reactivity with rat CGRP-α and 79% with CGRP-β. There is no cross-reactivity with rat amylin, calcitonin, somatostatin or substance P.

Western blot analysis. The collected cells were lysed with radio immunoprecipitation assay buffer (Beyotime Institute of Biotechnology), and total protein concentration was determined using the bicinchoninic acid assay method. Protein samples (30 µg) were separated on a 10% SDS-PAGE gel and the proteins were then transferred to polyvinylidene fluoride membranes. The membranes were then blocked using 5% non-fat milk at 25°C for 1 h. Following this, the membranes were incubated with primary antibodies against TRPV1 (1:2,000) and β-actin (1:1,000) at 4°C overnight. Membranes were then incubated with horseradish peroxidase (HRP)-anti-mouse (1:5,000) and HRP-anti-rabbit (1:5,000) antibodies at room temperature for 1 h. Proteins were then visualized using an enhanced chemiluminescent kit (Beyotime Institute of Biotechnology), and band intensities were densitometrically analyzed using a ChemiDoc XRS Protein Gel Imaging System (version 3.0; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Results are presented as the expression ratio of TRPV1 to β-actin.

Measurement of intracellular Ca\(^{2+}\). In order to monitor the effect of capsaicin and rutaecarpine on intracellular Ca\(^{2+}\) levels, 293 cells were incubated with Fluo-3/AM (5 µM; Beyotime Institute of Biotechnology) for 40 min at 37°C in Hank's buffer. Subsequently, alterations in intracellular Ca\(^{2+}\) levels in individual cells were measured by flow cytometry (version 6.1; BD FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA). Fluo-3 was excited by an argon laser light at 488 nm. Intracellular calcium alterations (F/F0) were expressed as average fluorescence (F) divided by background fluorescence (F0).

Construction of a stable cell line. 293 cells (70-80% density) were seeded in 6-well plates, and transfection was performed using the Lipofectamine\textsuperscript{\textregistered} 2000 transfection reagent (4 µl; Beyotime Institute of Biotechnology), according to the manufacturer's protocol, using either the PCDNA3.1-TRPV1 plasmid or the PCDNA3.1 empty plasmid (1.5 µg). The integrity of the plasmids was confirmed via RT-qPCR analysis and sequencing (Sangon Biotech Co., Ltd.). Cells were cultured in the presence of G418 (400 µg/ml) for 4 weeks. Positive clones were isolated and expanded to establish cell lines. Following confirmation of successful expression, the cells were continuously cultured in G418-containing medium (G418, 200 µg/ml) for subsequent experiments. RT-qPCR analysis and western blotting were used to identify successful transfections. The stably-transfected cell clones were termed 293/PCDNA3.1-TRPV1 and 293/PCDNA3.1.

Statistical analysis. All data are expressed as the mean ± standard error. All values were analyzed by t-test for two-group comparisons, or one-way analysis of variance followed by Tukey’s test for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

TRPV1 agonist capsaicin induces CGRP synthesis and release in DRG neurons. In order to investigate the role of TRPV1 in capsaicin-induced CGRP synthesis and release in DRG neurons, the mRNA expression of CGRP-α and CGRP-β in DRG neurons was assessed (Fig. 1). The results demonstrated that the mRNA expression of CGRP-α and CGRP-β were time-dependently and dose-dependently upregulated by capsaicin (10\(^{-6}\) M and 10\(^{-8}\)-10\(^{-6}\) M, respectively) compared with the control (Fig. 1C-E and F). The density of CGRP in groups Cap(M) and Cap(H) was significantly increased compared with the control ("P<0.05, **P<0.01 vs. control), which was inhibited by the TRPV1 receptor antagonist capsazepine (Fig. 1F-I) ["P<0.01 vs. Cap(H)]. The synthesis of CGRP was significantly increased by capsaicin ("P<0.01 vs. control), and this effect was inhibited by capsazepine (Fig. 1I) ["P<0.01 vs. Cap(H)].

TRPV1 antagonist capsazepine abolishes rutaecarpine-induced CGRP synthesis and release in DRG neurons. To investigate the effect of rutaecarpine on CGRP synthesis and release in DRG neurons, the mRNA expression of CGRP-α and CGRP-β in DRG neurons was assessed. The results demonstrated that the mRNA expression of CGRP-α and CGRP-β was dose-dependently upregulated by rutaecarpine (10\(^{-10}\)-10\(^{-5}\) M) compared with the control (Fig. 2A). Computational analysis confirmed that the density of CGRP in groups RUT(M) and RUT(H) was significantly increased compared with the control ("P<0.05, **P<0.01 vs. control), and
that the density of CGRP in group Capz was significantly decreased compared with the group RUT(H) [Fig. 2A-C; **P<0.01 vs. RUT(H)]. The synthesis of CGRP was significantly increased by rutaecarpine (**P<0.01 vs. control) and this effect was inhibited by the TRPV1 receptor antagonist capsazepine (Fig. 2D) [**P<0.01 vs. RUT(H)].

Overexpression of TRPV1 in 293 cells. In order to investigate the role of TRPV1 in calcium influx, the PCDNA3.1-TRPV1 overexpression plasmid was identified by qPCR amplification followed by 2% agarose gel electrophoresis, and the PCDNA3.1-TRPV1 plasmid was further proven by sequencing. The results demonstrated the success of the plasmid construction (Fig. 3A-D). The PCDNA3.1 and PCDNA3.1-TRPV1 plasmids were transfected in 293 cells to construct stable cell lines termed 293/PCDNA3.1 and 293/PCDNA3.1-TRPV1, respectively. qPCR confirmed that the mRNA levels of TRPV1 in groups 1-7 were significantly increased compared with 293/PCDNA3.1 (Fig. 3E; *P<0.05, **P<0.01 vs. 293/PCDNA3.1). The western blotting results additionally confirmed that the protein levels in the 6th group were significantly increased compared with 293/PCDNA3.1 (Fig. 3F; **P<0.01 vs. 293/PCDNA3.1).

Rutaecarpine stimulates calcium influx in TRPV1-overexpressing 293 cells. To investigate the role of TRPV1 in calcium influx and examine the involvement of rutaecarpine, flow cytometry was performed to detect the intracellular calcium content of 293 cells. The results confirmed that the calcium content in 293/PCDNA3.1-TRPV1 was significantly increased by rutaecarpine in a dose-dependent manner, compared with 293/PCDNA3.1 (Fig. 4; *P<0.05, **P<0.01 vs. control; *P<0.05 vs. 293/PCDNA3.1-TRPV1).

Discussion
The novel and potentially important findings of the present study were as follows: i) Rutaecarpine caused an increase in
Figure 2. Effect of rutaecarpine on CGRP synthesis and release in DRG neurons. (A) The effect of rutaecarpine on the mRNA expression of CGRP in DRG neurons was assessed, and the data for (B) CGRP-α and (C) CGRP-β were quantified. (D) The effect of rutaecarpine on the concentration of CGRP in the culture supernatant of DRG neurons was analyzed. Values are expressed as the mean ± standard error. n=4. *P<0.05, **P<0.01 vs. control; ++P<0.01 vs. RUT(H). CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglia; RUT(L), low-dose rutaecarpine; RUT(M), medium-dose rutaecarpine; RUT(H), high-dose rutaecarpine; Capz, capsazepine.

Figure 3. Overexpression of TRPV1 in 293 cells. (A) Identification of the PCDNA3.1-TRPV1 plasmid by reverse transcription-quantitative polymerase chain reaction. (B) Identification of the PCDNA3.1-TRPV1 plasmid by sequencing. (C) Identification of the PCDNA3.1-TRPV1 plasmid by sequencing. (D) Identification of the PCDNA3.1-TRPV1 plasmid by sequencing. (E) The mRNA level of TRPV1 in different clones of 293/PCDNA3.1-TRPV1. (F) The protein level of TRPV1 in the 6th clone of 293/PCDNA3.1-TRPV1. TRPV1, transient receptor potential cation channel subfamily V member 1; a.u., arbitrary units.
CGRP synthesis and release in DRG, which was markedly attenuated by capsazepine (a competitive TRPV1 antagonist); and ii) the activation of TRPV1 was involved in CGRP synthesis and release induced by rutaecarpine. These results provided a novel insight into the mechanism of blood pressure regulation by rutaecarpine and may facilitate the development of rutaecarpine as novel medication in the treatment of hypertension.

It is known that CGRP is composed of 37 amino acid residues, and is widely distributed in the nervous and cardiovascular systems (21). There are two isoforms, termed CGRP-α and CGRP-β, which share similar physiological functions (22). In the peripheral nervous system, DRG neurons are the primary site of the synthesis of CGRP. In the cardiovascular system, CGRP has a broad and complex role, including reducing blood pressure (13), inhibiting the proliferation of vascular smooth muscle cells (23) and endothelial cellular apoptosis (24), and protecting against myocardial ischemia and reperfusion injury. Previous studies have demonstrated that in SHR, rutaecarpine significantly decreased blood pressure concomitantly with a decrease in the expression of CGRP, which was attenuated by capsaicin (13). To evaluate the effect of rutaecarpine on CGRP mRNA expression and release, cultured DRG were used. The results of the present study demonstrated that the amount of CGRP in the cell culture supernatant and the mRNA expression of CGRP-α and CGRP-β in DRG may be upregulated by rutaecarpine in a concentration-dependent manner, and the effects of rutaecarpine were abolished by the TRPV1 antagonist capsaicin, indicating that the stimulatory effect of rutaecarpine on the synthesis and release of CGRP may be mediated by TRPV1. Additionally, it was observed that CGRP expression in DRG may be upregulated by nerve growth factor, bradykinin/prostaglandin, protein kinase A agonists (dibutylryl cAMP) and protein kinase C agonists (phorbol ester) (25-28). The precise mechanism of CGRP expression regulation requires further detailed investigation.

TRPV1 is a ligand-gated ion channel that integrates multiple stimuli, including capsaicin, protons and heat (29-31). An increase in calcium influx has been reported to be involved in the activation of TRPV1 and has been considered to be an indicator of TRPV1 activation (9). To further confirm the role of TRPV1 in the synthesis and release of CGRP by rutaecarpine, 293 cell lines with TRPV1 overexpression were utilized. In the present study, intracellular Ca$^{2+}$ levels were increased by rutaecarpine in 293 cell lines with TRPV1 overexpression, suggesting an activation of TRPV1 by rutaecarpine. The upregulation of CGRP by rutaecarpine in DRG may be inhibited by the TRPV1 receptor antagonist capsazepine. The results of the present study suggested a role for TRPV1 in the release of CGRP stimulated by rutaecarpine and other factors. TRPV1 activation maybe a potential strategy for blood pressure regulation.

CGRP is considered to be the most potent vasodilator, and identifying novel drugs targeting CGRP for the treatment of hypertension is of interest. Previous studies have indicated that rutaecarpine exerts a hypotensive effect in rats and dilates isolated aortic rings and superior mesenteric artery rings (32,33). It was previously identified that the vasodilatory effects of
rutacarpine in normotensive rats were abolished or markedly attenuated by the competitive TRPV1 antagonist capsazepine and the selective CGRP receptor antagonist CGRPR37, and that cross-tachyphylaxis occurred between capsaicin and rutacarpine, indicating that the effects of rutacarpine are mediated by endogenous CGRP, which may be associated with activation of TRPV1 in rats (34). However, the precise mechanisms underlying the hypotensive effect of rutacarpine remain to be completely elucidated. The present study further provided experimental evidence of the effect of rutacarpine on CGRP and the role of TRPV1. There remain a number of problems regarding to the drug development of rutacarpine; for example, its low solubility and low oral bioavailability limit the progress. Structural transformation is the subject of current research. With a deeper mechanistic understanding of blood pressure regulation by rutacarpine, novel medications targeting TRPV1/CGRP may be successfully developed in the future.

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