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Inhibitory effect of rhynchophylline on contraction of cerebral arterioles to endothelin 1: Role of rho kinase

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ABSTRACT

Ethnopharmacological relevance: Rhynchophylline (Rhy) is a major ingredient of *Uncaria rhynchophylla* (*UR*) used to reduce blood pressure and ameliorate brain ailments. This study was to examine the role of Rho kinase (ROCK) in the inhibition of Rhy on contraction of cerebral arterioles caused by endothelin 1 (ET-1).

Materials and methods: Cerebral arterioles of male Wistar rats were constricted with ET-1 for 10 min followed by perfusion of Rhy for 20 min. Changes in the diameters of the arterioles were recorded. The effects of Rhy on contraction of middle cerebral arteries (MCAs) were determined by a Multi-Myograph. Western blotting and immunofluorescent staining were used to examine the effects of Rhy on RhoA translocation and myosin phosphatase target subunit 1 (MYPT1) phosphorylation.

Results: in vivo, Rhy (30–300 μM) relaxed cerebral arterioles constricted with ET-1 dose-dependently. in vitro, Rhy at lower concentrations (1–100 μM) caused relaxation of rat MCAs constricted with KCl and Bay-K8644 (an agonist of L-type voltage-dependent calcium channels (L-VDCCs)). Rhy at higher concentrations (> 100 μM) caused relaxation of rat MCAs constricted with ET-1, which was inhibited by Y27632, a ROCK's inhibitor. Western blotting of rat aortas showed that Rhy inhibited RhoA translocation and MYPT1 phosphorylation. Immunofluorescent staining of MCAs confirmed that phosphorylation of MYPT1 caused by ET-1 was inhibited by Rhy.

Conclusions: These results demonstrate that Rhy is a potent inhibitor of contraction of cerebral arteries caused by ET-1 in vivo and in vitro. The effect of Rhy was in part mediated by inhibiting RhoA–ROCK signaling.

1. Introduction

Uncaria rhynchophylla (*UR*) which belongs to rubiaceae family is widely used in China for the treatment of hypertension (Chou et al., 2009; Zhou and Zhou, 2010). Rhynchophylline (Rhy) is one of the main bioactive ingredients of *UR* (Wang et al., 2010). It acts as a vasodilator in number of types of vessels, mainly conductive vessels, in vitro (Huang et al., 1994; Zhang et al., 2004; Zhou and Zhou, 2010; Li et al., 2013). However, little is known about the effect of Rhy on cerebral arterioles, though *UR* is often used to ameliorate cerebral ailments (Zhou and Zhou, 2010).

Endothelin 1 (ET-1) is a major vasoactive isoform in endothelin family (Yanagisawa et al., 1988). It has been implicated in hypertension, especially modest to severe hypertension, and cerebral vasospasm (Pollock, 2005; Dhaun et al., 2008; Kohan, 2010; Vatter et al., 2011). L-type voltage-dependent calcium channels (L-VDCCs) and RhoA–Rho kinase (ROCK) signaling are involved in the vasoconstriction induced by ET-1 (Yoshida et al., 1994; Gohla et al., 2000; Miao et al., 2002; Ansari et al., 2004; Fernandez-Tenorio et al., 2011). Studies show that vasodilator effect of Rhy, which is independent of the presence of the endothelium, is related to the inhibition of calcium channels, especially L-VDCCs (Zhang et al., 2004). However, the role of RhoA–ROCK pathway has not been studied, which plays an important role in calcium sensitization and is critically involved in the actions of ET-1 (Shi and Wei, 2013). When RhoA, a small G protein, is activated, it is

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translocated from cytoplasm to the membrane and activates ROCKs (Chikumi et al., 2002; Somlyo and Somlyo, 2003; Jin et al., 2004; Ying et al., 2009), followed by the inhibition of myosin light chain phosphatase (MLCP) through phosphorylation of myosin phosphatase target subunit 1 (MYPT1) at threonine 696 (T696) and threonine 853 (T853), which results in an increased Ca^{2+} sensitivity of the contractile filaments and consequently an increased vasoconstriction (Somlyo and Somlyo, 2003; Gao et al., 2007).

The present study was to determine the effect of Rhy on the responsiveness of cerebral arterioles to ET-1, particularly focusing on the role of RhoA–ROCK signaling.

2. Material and methods

2.1. Reagents

ET-1, nifedipine, Y2732, Bay K8644, sodium nitroprusside, isoprenaline, nitro-L-arginine and indomethacin were purchased from Sigma (St. Louis, MO, USA). Rhy was purchased from Kunming Feng-shan-jian Pharmaceutical Research Ltd., Kunming, Yunnan, China. According to the information provided by the company, total alkaloid was firstly extracted from *UR* with 80% alcohol and ethyl acetate. Rhy was then separated by alumina column. After recrystallized for three times, a final purity of 98.7% of Rhy was finally obtained (Batch: 2011092313).

Rhy (0.1 M) was prepared in hydrochloric acid (0.1 M) as stock solution. It was diluted using normal saline and neutralized with NaOH (0.1 M) for in vivo study (pH 7.0). For in vitro study, the Rhy stock solution was diluted with distilled water and added into the Krebs-Ringer bicarbonate buffer containing 118.3 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, and 11.1 mM glucose (from now on referred as Krebs buffer). The addition of Rhy did not affect the pH of the Krebs buffer. Indomethacin (10^{-5} M) was prepared with equimolar Na_2CO_3 in distilled water. The concentrations of Na_2CO_3 did not affect the pH of the Krebs buffer significantly in this experiment.

2.2. Animals

Male Wistar rats weighing 240–260 g were supplied by the Laboratory Animal Center of Health Science Center of Peking University. "*N*" represented the number of rats used in each experiment. All rats were housed in a temperature-controlled room under a 12 h light/dark cycle and allowed free access to water and standard diet. This study conformed to the guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985). All the procedures and protocols in this study were approved by the Experimental Animal Ethics Committee of Health Science Center of Peking University (LA2011-38).

2.3. in vivo study

The rats were anesthetized with urethane (intramuscular, 1 ml/100 mg) and their heads were held steady in a stereotactic frame. By using a hand-held drill, a $4 \times 6\ mm^2$ cranial window was opened through an incision 1 mm behind the coronal suture and 1 mm on the right side of the sagittal suture. Two slender tubes were used for the perfusion of ET-1, Rhy, or solvent through the cranial window. The cranial window was covered by a rubber slip, and normal saline was added under the cover slip to keep moisture. The rat was put under an upright fluorescence microscope (BX51WI, Olympus, Tokyo, Japan) equipped with a color video camera (Jk-TU53H, Toshiba, Tokyo, Japan), a color monitor (20PF5120, Philips, Eindhoven, Netherlands), a video timer (VIG-33, FOR.A, Tokyo, Japan) and a DVD recorder (DVR-560H, Philips, Eindhoven, Netherlands). After 15 min of baseline observation, ET-1 (10^{-7} M) was perfused continuously for 10 min at a speed of 5 ml/h. Thereafter ET-1 was switched to solvent (normal saline) or Rhy (30, 100, 300 μM) for 20 min at a speed of 5 ml/h. The cerebral arterioles of 200 μm in length and 30–50 μm in external diameter were chosen for observation through a 20 × objective. The diameter of each arteriole was measured three times at each experimental condition using Image-Pro Plus 6.0 (Media Cybernetic, Bethesda, MD, USA). The mean of the three measurements was used as the diameter of the arteriole.

2.4. in vitro vessel tension study

Rats were sacrificed by intramuscular injection of urethane (2 ml/100 mg). The injection of urethane did not affect the vasoconstriction caused by ET-1 and vasorelaxation caused by Rhy. MCAs of rats (external diameter: 100–150 μm) were dissected free and cut into rings (length: 1.5 mm) under a stereoscope in ice-cold Krebs buffer. Isometric tension of the arteries was recorded as previously described (Liu et al., 2014). Briefly, two stainless steel wires were used to suspend the arteries in the chamber of a Multi-Myograph (620 M, Danish, Myo Technology A/S, Aarhus, Denmark) with 5 mL Krebs buffer constantly bubbled with 95% O_2–5% CO_2 and maintained at 37 °C. At the beginning of the experiment, the arteries were brought to their optimal tension (~1 mN), followed by 30 min equilibration and contracted with 60 mM KCl. Following washing three times with Krebs buffer and being equilibrated for 15 min, the arteries were contracted with KCl (60 mM), Bay K8644 (3×10^{-7} M) or ET-1 ($2-5 \times 10^{-8}$ M). Before the arteries were constricted by Bay K8644 (3×10^{-7} M), 30 mM KCl was added into the solution to enlarge the constriction caused by Bay K8644 (3×10^{-7} M) (Zhang et al., 2012). After the contraction was stabilized, the effects of Rhy were examined. Pharmacological inhibitors (nifedipine and Y27632) were administrated at least 30 min prior to testing their effects.

2.5. Western blotting

Rings of the rat aortas were incubated in Krebs buffer (37 °C, 95% O_2–5% CO_2, pH 7.4) for tranquillization for 1 h. Nitro-L-arginine (10^{-4} M) and indomethacin (10^{-5} M) were then administered to prevent the interference of endogenous nitric oxide and cyclooxygenase products. Thirty minutes later, ET-1 (2×10^{-8} M) was added. Three minutes later, Rhy (300 μM) or solvent was added. Three minutes later, the tissues were rapidly frozen. The tissues were then homogenized in a buffer containing 50 mM Tris HCl (pH 7.4), 100 μM EGTA, 1 mM sodium orthovanadate, 1% 2-mercaptoethanol, and 10% SDS. The homogenate was sonicated (5 s for 3 times, 4 °C), centrifuged (18,000 g, 15 min, 4 °C), and solubilized in 5 × loading buffer before SDS-PAGE. After separated on SDS-PAGE, proteins were electro transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Nonspecific binding of antibody was blocked by incubation with 10% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h at room temperature. The blot was then subjected to two brief washes with TBST and incubated in TBST and the primary antibody with appropriate dilution for overnight at 4 °C. After two more washes in TBST, the blot was incubated for 1 h in horseradish-peroxidase conjugated secondary antibodies at room temperature. After three washes in TBST, the blots were visualized with an enhanced chemiluminescence reagent kit (Applygen Technologies Inc, Beijing, China). Quantitation of the proteins was performed using Quantity One 4.6.2 software (Bio-Rad, Hercules, California, USA). The level of phosphorylation of MYPT1 at T853 was determined by monoclonal antibody anti-MYPT1 (1:1000; CST, Boston, USA) and rabbit anti-phosphoMYPT1 (1:1000; CST, Boston, USA).

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