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Searching for synergistic calcium antagonists and novel therapeutic regimens for coronary heart disease therapy from a Traditional Chinese Medicine, Suxiao Jiuxin Pill

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ABSTRACT

Coronary heart disease is a vital cause of morbidity and mortality worldwide, and calcium channel blockers (CCBs) are important drugs that can be used to treat cardiovascular diseases. Suxiao Jiuxin Pill (SX), a traditional Chinese medicine, is widely used as an emergency drug for coronary heart disease therapy. However, understanding its potential mechanism in intracellular calcium concentration ($[Ca^{2+}]_i$) modulation remains a challenge. To identify the active pharmacological ingredients (APIs) and reveal a novel combination therapy for ameliorating cardiovascular diseases, the ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC/Q-TOF MS) combined with a dual-luciferase reporter $[Ca^{2+}]_i$ assay system was applied. Ligustrazine, ferulic acid, senkyunolide I, senkyunolide A and ligustilide were identified as potential calcium antagonists in SX, and the combination of ligustrazine and senkyunolide A showed synergetic calcium antagonistic activity. Additionally, the synergetic mechanism was further investigated by live-imaging analysis with the Ca^{2+} indicator fluo-4/AM by monitoring fluorescence changes. Our results indicated that ligustrazine can block voltage-operated Ca^{2+} channels (VDCCs) effectively and senkyunolide A can exert an inhibition effect mostly on ryanodine receptors (RYRs) and partly on VDCCs. Finally, an arterial ring assay showed that the combination of ligustrazine and senkyunolide A exerted a better vasodilatation function than using any components alone. In this study, we first revealed that a pair of natural APIs in combination acting on VDCCs and RYRs was more effective on vasodilatation by regulating $[Ca^{2+}]_i$.

1. Introduction

Coronary heart diseases are common cardiovascular diseases that have become a vital cause of morbidity and mortality worldwide in the past decade [1]. Regulation of the intracellular calcium concentration ($[Ca^{2+}]_i$) can modulate vascular resistance and improve pressure-induced constriction in cardiovascular diseases [2]. Hence, calcium channel blockers (CCBs) are recommended by the European Society of Hypertension (ESH) together with the European Society of Cardiology (ESC) [3] as initial therapy or one of a combination of drugs to treat cardiovascular diseases. In cardiomyocytes, $[Ca^{2+}]_i$ increases mainly *via* three calcium channels: VDCCs, RYRs and inositol 1, 4, 5-triphosphate (IP3) receptors (IP3Rs) [4]. Clinically, there are three main types of CCBs: 1,4-dihydropyridines, aralkylamines and benzothiazepines [5], which all target VDCCs, but these CCBs cause severe adverse reactions, such as cardiac arrhythmias, atrioventricular conduction disorder, nausea, headache, flushing and dizziness [6, 7]. To promote $[Ca^{2+}]_i$ modulation with fewer adverse effects, the discovery of novel CCBs or its combination in a therapeutic schedule is a meaningful and urgent issue for decreasing the treatment risk, hospitalization burden and healthcare costs of cardiovascular diseases.

Suxiao Jiuxin Pill (SX), administered by sublingual delivery, is used to cure coronary heart diseases, angina pectoris and other cardiovascular diseases [8–10] and has become one of the top five best-selling traditional Chinese medicines (TCMs) based cardiovascular drugs in China [11]. SX is composed of the essential oil extract of Ligusticum, the dried rhizome of *Ligusticum chuanxiong* Hort (Umbelliferae) and Borneol, the extract of fresh branches and leaves of *Cinnamomum camphora* (L.) Presl. Chemical studies have revealed that the primary components of SX include phenolic acids (such as ferulic acid and vanillic acid), phthalides (such as ligustilide and senkyunolide A), alkaloids (such as ligustrazine) and borneol [12, 13]. Phthalides are the

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large content constituents in SX, and they are also pharmacological active components with anti-proliferative, anti-inflammatory, and anti-oxidant effects [14–16]. Ligustrazine, a characteristic component in SX, possesses many anti-tumor effects; it protects endothelial cells, attenuates inflammation, and ameliorates oxidative organ injury and vasodilatation [17–19]. Among them, ligustrazine (tetramethylpyrazine) and some phthalides that are characterized by α, β-unsaturated γ-lactone, have been reported as potential calcium antagonists by inhibiting VDCCs or receptor-mediated Ca^{2+} influx and release [20–22]. However, suggestions on how these ingredients, with distinct structures, work together in SX to exert calcium antagonistic effects, have not been discussed. Research on calcium antagonistic compositions of SX, a classic prescription, is a much more efficient approach to discovering novel CCBs as well as effective combinations.

Due to the high efficiency separation and sensitivity detection characteristics, the UPLC/Q-TOF MS has been used widely to identify components in complex samples, especially TCMs [23, 24]. However, only chemical components study cannot reflect the comprehensive quality of TCMs. Therefore, the UPLC/Q-TOF MS combined with a dual-luciferase reporter $[Ca^{2+}]_i$ assay system has been applied for quality investigation and APIs identification of TCMs [25]. Otherwise, intracellular Ca^{2+} imaging assay which combines live-imaging analysis with the Ca^{2+} indicator dye has been widely used to monitor changes of $[Ca^{2+}]_i$ [26, 27].

In this study, we aimed to investigate the potential active constituents of SX and an optimal combination therapy with a calcium antagonist effect. Additionally, the synergetic mechanisms that ligustrazine and senkyunolide A exert on calcium inhibition by blocking VDCCs, RYRs and IP3Rs were evaluated. Then, *in vitro* arterial ring assays were carried out to validate the vasodilator effects of the combination scheme.

2. Materials and methods

2.1. Chemicals and reagents

UPLC grade acetonitrile, methanol and analytical grade acetic acid were purchased from Merck (Darmstadt, Germany). Deionized water was purified with the Milli-Q system (Bedford, MA, USA). Reporter plasmid PGL4.30 and Renilla luciferase reporter vector plasmid pRL-TK were purchased from Promega (WI, USA). Ligustrazine (TMP), ferulic acid (FA), senkyunolide I (Sen I), senkyunolide A (Sen A) and ligustilide (Lig) were acquired from Tianjin Solomon Bio-technology Co., Ltd. (Tianjin, China). Fluo-4/AM and anhydrous dimethyl sulfoxide (DMSO) were bought from Dojindo Molecular Technologies Inc. (Tokyo, Japan). Pluronic F-127 was obtained from Genecopoeia (Germantown, MA, USA). (R)-(-)-Phenylephrine (hydrochloride) (PE) and norepinephrine (NE) were purchased from MCE (Milano, Italy). 2-aminoethoxydiphenyl borate (2-APB) was purchased from Gene Operation (Ann Arbor, MI, USA). Caffeine (Caf) was purchased from Abcam (Cambridge, MA, USA). Tetracaine (TEA) was bought from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). Nimodipine (Nimo) was acquired from Bayer Pharma AG (Leverkusen, Germany). All reagents for cell cultures were bought from Gibco BRL Life Technologies (Rockville, MD, USA). The condensed extract of SX (lot no. 20160309) was kindly provided by the Zhongxin Pharmaceutical Group sixth TCM factory (Tianjin, China).

2.2. Sample preparation

The extract of SX (10 mg) was dissolved in 1 mL methanol, vortexed for 1 min and then was subjected to ultrasonic treatment for 30 min. The solution was centrifuged at 12,000 rpm, at 4 °C for 10 min and the supernatant was used for UPLC analysis. Samples used for cell experiments were prepared by dissolving standards in complete cell culture medium.

2.3. UPLC/Q-TOF-MS analysis

2.3.1. UPLC separation

The samples were analyzed with a Waters ACQUITY UPLC system (Waters Co., USA) equipped with a photo diode array detector (DAD) (190–400 nm), and the system was operated by MassLynx V4.1 software (Waters Co., USA). A Waters ACQUITY BEH C_{18} column (2.1 mm × 100 mm, 1.7 μm, Waters Corporation, Milford, USA) was used for sample separations. The column was maintained at 30 °C, and the flow rate was 0.4 mL/min as 10 μL aliquots of the sample were injected. The optimal mobile phase consisted of a linear gradient system of (A) 0.1% formic acid in water and (B) acetonitrile: 0–7.0 min, B 5–20%; 7.0–25.0 min, B 20–60%; 25.0–28.0 min, B 60–100%; and 28.0–30.0 min, B 100%. After column separation, the effluent was unequally split into 1:9. The 10% fraction was directly transported to the Q-TOF-MS for the component identification, and the other fraction was collected in a 96-deep-well plate (2.2 mL). Almost 60 collections were obtained and then dried in a vacuum drying oven at 45 °C. The residue in every well was dissolved in 50 μL of the cell culture medium for the cell test.

2.3.2. Mass spectrum analysis

Accurate mass and MS/MS measurements were obtained by the detector with a dual electrospray ionization (ESI) probe and a Micromass Q-TOF micro Synapt High Definition Mass Spectrometer from Waters (Waters Corporation, Milford, USA). The mass spectra were acquired in both positive (ESI^+) and negative (ESI^-) ionization modes and the optimal condition of analysis was set as follows: the source temperature was set at 120 °C; desolvation gas temperature and desolvation gas flow were 400 °C and 800 L/h, respectively; capillary voltage was 3.0 kV for the negative mode and 2.5 kV for the positive mode; the sampling cone voltage was 30 V; the extraction cone voltage was 4.0 V; the cone gas flow was set at 50 L/h; and the collision energy was set at 6.0 eV. All the analyses were performed using a LockSpray interface to ensure accuracy and reproducibility. The Q/TOF Premier acquisition rate was 0.1 s with a 0.02 s inter-scan delay, and the first resolving quadrupole was performed in the wide-pass mode (50–1200 Da). Leucine encephalin amide acetate (200 pg/μL) was used as the lock mass ($[M - H]^- = 553.2775$, $[M + H]^+ = 555.2931$), with a 20 μL/min flow rate.

2.4. Cell culture

Human embryonic kidney 293 (HEK 293) cells that were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (V/V) fetal bovine serum (FBS), 100 U/mL penicillin and 0.1 mg/mL streptomycin in a 37 °C humidified incubator with 5% CO_2. Cells were fed every 2 days and were sub-cultured once they reached 80–90% confluency.

Rat cardiomyoblast cells (H9C2 cells from ATCC) were cultured in DMEM containing high glucose (4500 mg/L), 10% FBS, 100 U/mL of penicillin, 0.1 mg/mL of streptomycin and 2 mmol/L L-glutamine in a 37 °C humidified incubator with 5% CO_2. The H9C2 cells were fed every 2 days and were sub-cultured when they reached 80–90% confluency.

2.5. Luciferase reporter assay for calcium antagonism

The HEK 293 cells were co-transfected with 100 ng/well luciferase reporter plasmid PGL 4.30 (Promega, USA) and 10 ng/well Renilla luciferase reporter vector pRL-TK plasmid (Promega, USA) in fresh serum free medium. Lipofectamine 2000 was used in the co-transfection according to the manufacturer's instructions (Invitrogen, USA). After 24 h co-transfection, the cells were pretreated with different effluents and were simultaneously stimulated with ionomycin (1 mmol/L) and 12-myristate 13-acetate (PMA, 1 mg/mL) for 6 h. The cells were then