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# Modulation effect of *Smilax glabra* flavonoids on ryanodine receptor mediated intracellular $Ca^{2+}$ release in cardiomyoblast cells

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ABSTRACT

*Ethnopharmacological relevance:* *Smilax glabra* rhizome, a plant material from *Liliaceae* family, is a widely used traditional Chinese medicine for anti-cardiac hypertrophy treatment. We have previously found that *Smilax glabra* flavonoids (SGF) exerted such anti-cardiac hypertrophy activity. However, the mechanism of this activity of SGF has not been clarified yet.

*Materials and methods:* This study was aimed to investigate the inhibitory role of SGF on intracellular $Ca^{2+}$ release in rat cardiomyoblast cells (H9C2). Intracellular $Ca^{2+}$ release was determined by $Ca^{2+}$ indicator fluorescence (fluo 4-AM) in H9C2 cell line.

*Results:* SGF at concentrations of 0.25, 0.5, 1.0 mg/ml significantly inhibited the phenylephrine or angiotensin II induced intracellular $Ca^{2+}$ release in a dose-dependent manner. Furthermore, SGF could also inhibit ryanodine receptor (RyR) agonist caffeine induced $Ca^{2+}$ release and phenylephrine (PE)-induced $Ca^{2+}$ release under the condition in which inositol trisphosphate (IP3) receptors were blocked with 2-Aminoethoxydiphenyl borate (2-APB). Nevertheless, SGF had no impact on PE-induced $Ca^{2+}$ release under the condition in which RyRs were blocked with tetracaine.

*Conclusions:* Our results suggest that the protective effects of SGF are mediated via targeting inhibition of RyR mediated intracellular $Ca^{2+}$ release.



## 1. Introduction

Rhizome of *Smilax glabra* Roxb (Tu-fu-ling in Chinese), a plant material from *Liliaceae* family, has been used as a traditional Chinese medicine for the treatments of cardiac hypertrophy, hypertension, and diabetic nephropathy. We have found that the flavonoids of *Smilax glabra* (SGF) are responsible for the anti-cardiac hypertrophy and anti-hypertension effects in animal models, and can significantly reduce atrial natriuretic peptide (ANP) (Wang et al., 2011). However, its mechanism of action on those diseases is still unknown to date.

Hypertrophy is a recognized complication of high blood pressure. Abnormal cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]c$) plays a central role in the development of cardiac hypertrophy and heart failure and leads to contractile and relaxation dysfunction. $[Ca^{2+}]c$ is critically regulated by a specialized organelle called sarcoplasmic reticulum (SR) from which $Ca^{2+}$ release is controlled by inositol trisphosphate receptor (IP3R) and ryanodine receptor (RyR). These two receptors have different pathways of activating $Ca^{2+}$ release. IP3Rs are activated by inositol trisphosphate (IP3) which relies on many of the G-protein-coupled receptor and receptor protein tyrosine kinase signals. RyRs are activated by small increases in $[Ca^{2+}]$ (e.g. by caffeine), which is amplified by activating more $Ca^{2+}$ release in a process of $Ca^{2+}$-induced $Ca^{2+}$ release (CICR) (Trafford et al. 1995). In this study, to clarify the $Ca^{2+}$-release associated anti-cardiac hypertrophy mechanism of SGF, we conducted a cellular experiment using H9C2 cells.

## 2. Materials and methods

### 2.1. Reagents

Caffeine, tetracaine and 2-Aminoethoxydiphenyl borate (2-APB) were purchased from Sigma Chemical Company (St. Louis, USA). Fluo 4-AM, Pluronic®F-127 and DMSO were obtained from the Dojindo laboratories (kumamoto, Japan). Phenylephrine (PE) and Verapamil was obtained from Hefong Pharmaceutical Company (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM),

*Abbreviations:* SGF, flavonoids of *Smilax glabra*; ANP, atrial natriuretic peptide; SR, sarcoplasmic reticulum; ER, endoplasmic reticulum; IP3, inositol trisphosphate; IP3R, inositol trisphosphate receptor; RyR, ryanodine receptor; RyR2, ryanodine receptor 2; CICR, $Ca^{2+}$-induced $Ca^{2+}$ release; PE, phenylephrine; 2-APB, 2-Aminoethoxydiphenyl borate; HBSS, Hanks balanced salt solution; Ang II, Angiotensin II; FCS, fetal calf serum

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fetal calf serum (FCS) and parenzyme were purchased from GIBCO (California, USA). Angiotensin II (Ang II) was obtained from GL Biochem (Shanghai, China).

### 2.2. Plant materials, Smilax glabra flavonoids extract and purification

*Smilax glabra* was purchased from Zhejiang Chinese Medical Hospital and authenticated by the authors. A voucher specimen (no. 101213) has been deposited at College of pharmacy of Zhejiang Chinese Medical University. The material was extracted with 60% ethanol twice at solid–liquid ratio of 1:20 (W/V). The extractions, suspended in 10% ethanol, were loaded on HPD-300 macroporous resin column and eluted with 50% and 70% ethanol respectively at a flow of 1 ml/min. The eluted fractions of 50% and 70% ethanol were further dried with vacuum concentration at 45 °C, which consisting mainly of total flavonoids (SGF). The deposit of SGF was dissolved by Hanks balanced salt solution (HBSS) for use, and its final content of flavonoids was determined by ultraviolet spectrophotometry using rutin as standard substance.

### 2.3. Cell culture

Rat cardiomyoblast cells (H9C2 cells from ATCC) were cultured in DMEM containing high glucose (4500 mg/L), 10% FCS, and antibiotics (penicillin and streptomycin) at a 37 °C humidified incubator with 5% $CO_2$ supply.

### 2.4. $Ca^{2+}$ indicator dye loading and general procedures

Fluo 4-AM was used as $Ca^{2+}$ indicator to load the cell cytoplasm. H9C2 cells were incubated with 5 μM of the membrane permeant fluo 4-AM and 0.05% Pluronic F-127 in HBSS solution without $Ca^{2+}$ for 1 h at 37 °C, followed by blank HBSS solution washing for three times to remove the residual fluo4-AM. Then the cells were covered with HBSS solution and incubated for 30 min at 37 °C in incubator in order to AM body esterify with intracellular.

### 2.5. $Ca^{2+}$ measurement and drug intervention

#### 2.5.1. PE, Ang II and caffeine induced intracellular $Ca^{2+}$ increase

H9C2 cells loaded with $Ca^{2+}$ indicator were transferred into a 96-well fluorescent plate ($5 \times 10^5$ cells per well) and cultured with SGF (0.25 mg/ml, 0.5 mg/ml, and 1.0 mg/ml) as well as 0.05 μg/ml verapamil as a positive control (5 wells for each plate). After incubation for 30 min at 37 °C, the cells were respectively added with different activators including 1 μM PE, 5 μM Ang II, and 50 mM caffeine. Meanwhile, the cells were fluorescently monitored via the VarioScan flash multifunctional fluorescence enzyme standard instrument at the excitation wavelength of 494 nm and emission wavelength of 516 nm (Thermo Fisher, Finland). Intracellular $Ca^{2+}$ release were estimated in accordance with the relative fluorescence intensity.

#### 2.5.2. PE induced intracellular $Ca^{2+}$ increase mediated by IP3 or ryanodine receptor

As described before (Ng et al., 2007; Xi et al., 2008), the cells were pretreated with 25 mM 2-Aminoethoxydiphenyl borate (2-APB) and 50 mM tetracaine for 15 min to block IP3 receptor or ryanodine receptor respectively, followed by 1 μM PE treatment. The intracellular $Ca^{2+}$ release was detected as same as above.

### 2.6. Statistical analysis

All data were expressed as means ± SD and analyzed by SPSS 16.0. One-way analysis of variance (ANOVA) test was used for the statistical comparison.

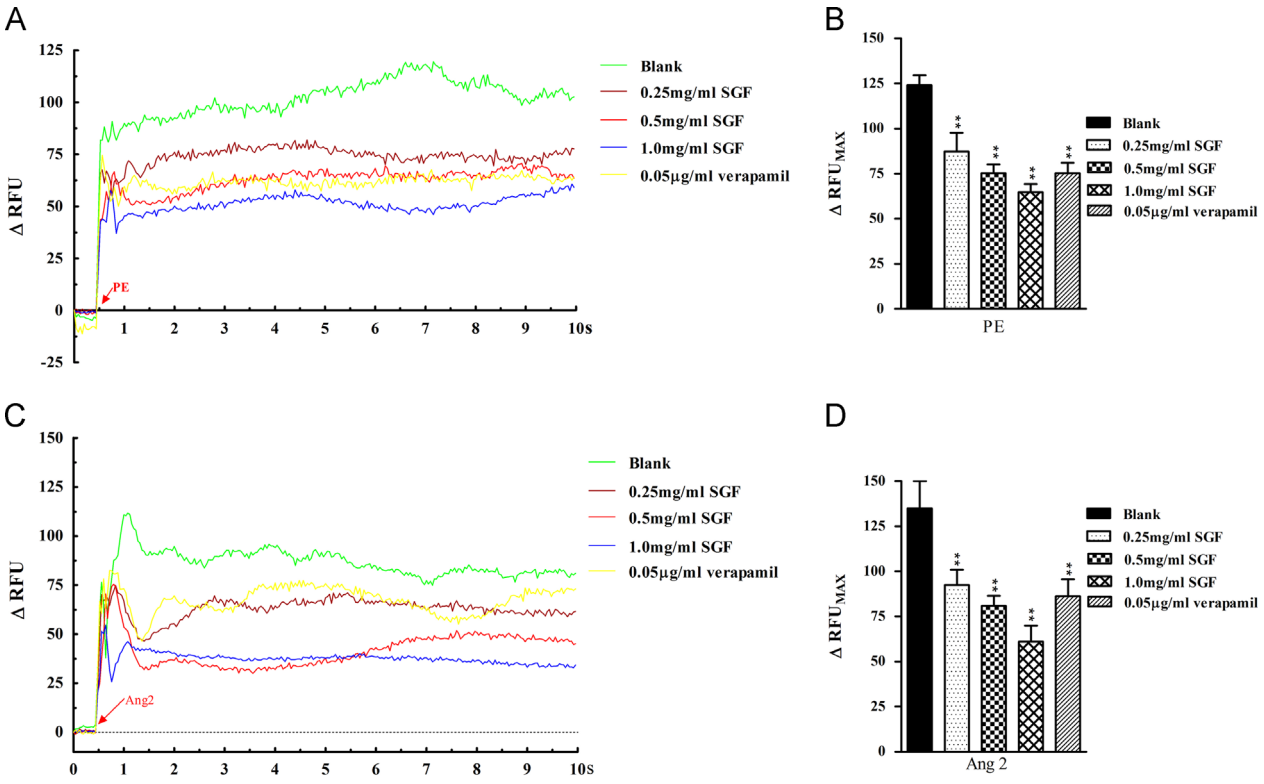


**Fig. 1.** The inhibiting effect of SGF on intracellular $Ca^{2+}$ release induced by PE or Ang II. (A) and (B) PE treated cells. (C) and (D) Ang II treated cells. Data presented as mean ± SD ($n=4$). $^{**}P < 0.01$ vs. blank control.