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Mechanistic study of endothelium independent vasodilation effects of wogonin

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Scutellaria baicalensis Georgi, locally known as HuangQin, and commonly as Baikal or Chinese skullcap, is an important herb in Chinese traditional medicine. The flavonoids from this plant are main active substances responsible for its medicinal applications. Wogonin is one such active ingredient derived from this plant. Here, we investigated the mechanism of the vasodilation effect of wogonin on isolated rat thoracic aortas. For this study, endothelium intact and endothelium removed thoracic aortic rings were prepared from rats. Using a tension transducer, the tension of the rat thoracic aortic rings was recorded. Results showed that wogonin is able to relax the endothelium-intact aortic rings, but L-NAME, indomethacin (Indo), and methylene blue (MB) could not reduce the tension in these rings. Wogonin was also able to relax endothelium-removed rings. However, treatment with tetraethylammonium (TEA), $BaCl_2$, glibenclamide (Gly), 4-aminopyridine (4-AP), and verapamil (Ver) had no effect on vasodilation induced by wogonin. Using wogonin to pre-treat endothelium-removed aortic rings reduced the contraction induced by K^+. Pre-treatment of endothelium-removed aortic rings with wogonin markedly reduced the contraction induced by 10^{-6} M PE in Ca^{2+}-free solution. It could be concluded that L-type calcium channels and intracellular Ca^{2+} release is inhibited by wogonin.

Keywords: Baikal skullcap, Chinese skullcap, Herbal, Intracellular calcium release, Ca^{2+} channel

Cardiovascular Disease (CVD) is heart and circulatory system disorders, which has become a major human health issue today[1,2]. Medicines with vasodilatory effects are useful in preventing and curing this type of disease[3-5]. Several natural medicines have been reported that could potentially prevent CVDs[6,7]. *Scutellaria baicalensis* Georgi (Lamiaceae), a Chinese medicinal plant commonly called Baikal skullcap or Chinese skullcap, has heat dampness, purging fire detoxification, and tocolysis characteristics and can be used to staunch bleeding based on the theory of traditional Chinese medicine[8,9]. Pharmacological studies have shown that it protects endothelial cells[10] and exhibit anticancer[11,12], antivirus[13], anti-inflammatory effects[14,15], and has potential application in the treatment of CVDs[16,17] in addition to hepatitis, diarrhoea, vomiting and high blood pressure[8]. There are several flavone derivatives reported from the root of *S. baicalensis*, such as wogonin, wogonoside, baicalein and baicalin. Among those flavone derivatives, wogonin ($C_{16}H_{12}O_5$) has attracted the attention of lots of researchers (Scheme 1). It is reported that wogonin has therapeutic effects on ischemic brain injury[18], vascular inflammatory[19], antitumor *in vivo* and *in vitro*[20,21], in particular antithrombotic activities[22] and anti-angiogenesis[23]. Qu *et al.*[24] have reported that wogonin can exert vasodilatory effect on isolated rat aortic rings by endothelium-independent pathway, while the exact mechanisms still remain unclear.

Based on these pharmacological effects, in this study, we have investigated whether wogonin can affect vascular ion channels or factors. We used isolated rat aortic rings to test the effect of wogonin on vasodilation.

Materials and Methods

Experimental drug

Acetylcholine (ACh), L-NG-nitroarginine methyl ester (L-NAME), indomethacin (Indo), Glibenclamide (Gly) and Tetraethylammonium (TEA, >98% purity) were purchased from Sigma Aldrich (St. Louis, MO, USA). Phenylephrine hydrochloride (PE, >98% purity), 4-aminopyridine (4-AP, >99% purity) was purchased from TCI Development Co., Ltd

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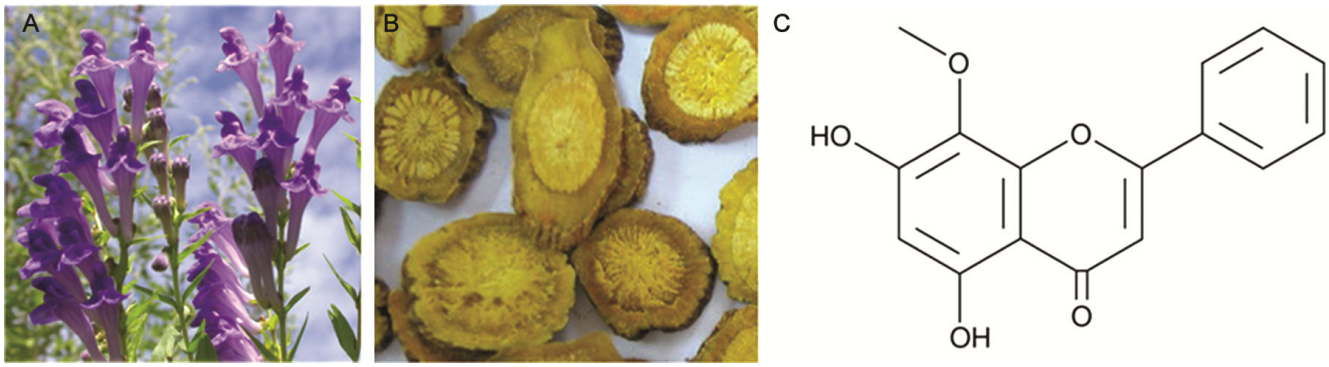

Scheme 1 — (A) *Scutellaria baicalensis* Georgi; (B) decoction pieces of *S. baicalensis*; and (C) Chemical structural formula of wogonin.

(Shanghai, China). Wogonin ($C_{16}H_{12}O_5$, MW 284.26, 99.01% purity) was bought from the Chengdu Pusi Bio-Technology Co., Ltd (Chengdu, Sichuan, China) Ethylenediaminetetraacetic acid tetrasodium salt (EDTA·4Na, 99-102% pure), verapamil (Ver, 99% pure) and methylene blue (MB, 99% pure) were obtained from Aladdin Industrial Corporation (Shanghai, China). All other reagents used were of analytical purity. ACh and PE were dissolved in deionized water, whereas wogonin was dissolved in DMSO. The Kreb-Henseleit (K-H) solution consisted of 118 mmol·L^{-1} NaCl, 25 mmol·L^{-1} $NaHCO_3$, 4.7 mmol·L^{-1} KCl, 2.5 mmol·L^{-1} $CaCl_2$, 1.2 mmol·L^{-1} $MgCl_2$, 1.2 mmol·L^{-1} KH_2PO_4, and 2.2 mmol·L^{-1} glucose (pH =7.4). The Ca^{2+}-free K-H solution and Ca^{2+}-free high concentration K^+ K-H solution (60 mmol·L^{-1} K^+) both containing 1×10^{-4} mol·L^{-1} EDTA.

Animals and aortic ring preparation

Male Sprague-Dawley rats weighing 200-230 g were provided by the Comparative Medicine Centre of Yangzhou University (Yangzhou, China). Animals were housed in a temperature and light controlled room (23±1°C; 12 h light/dark cycle) and allowed to freely access food and water. All animal experiments were strictly conducted following a protocol approved by the Animal Ethics Committee of China Pharmaceutical University.

Rats were euthanized by cervical dislocation under diethyl ether anesthesia. To isolate aortic ring, the thoracic aortas were removed immediately and immersed in K-H solution, then the attached tissue were removed. The thoracic aortas were sheared into aortic rings (3 mm long). A wooden toothpick was used to gently rub the inner portion of the aortic rings to displace the endothelial layer. The endothelium was deemed intact if 10^{-5} mol·L^{-1} of ACh induced 80% relaxation, which was pre-contracted by 10^{-6} mol·L^{-1} of PE. A relaxation expressed <5% was recorded when the endothelium was completely denuded.

The aortic rings were suspended in the K-H solution bath (5 mL), which maintained with 95% O_2 and 5% CO_2 at 37 °C. One side of the ring was connected to an L-shape hook, and the other side was connected to the tension transducer connected to a MedLab BL-420 Polygraph (Tai Meng Technology, Chengdu, China). The baseline tension loaded onto the aortic rings was 2 g. Each ring was equilibrated in the bath solution for 30 min before involving the contractile response to the addition of 60 mmol·L^{-1} of the high K^+ K-H solution. The aortic rings were washed thrice with the K-H solution, each time for 10 min. Recorded the relaxant response of the ACh concentration gradient (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} mol·L^{-1} ACh) after pre-contraction was induced by 10^{-6} mol·L^{-1} of PE. Relaxation was expressed as Relaxation %. Relaxation %= [(Maximal tension of 10^{-6} mol·L^{-1} PE – Minimal tension of X)/(Maximal tension of 10^{-6} mol·L^{-1} PE- resting tension)]*100%, Minimal tension of X is the minimum tension relaxed by reagent.

Vasodilation induced by wogonin and the effect of wogonin on endothelium function

To test the effect of wogonin on endothelium intact aortic rings, increasing concentrations of wogonin were applied to the rings after pre-contracting with 10^{-6} mol·L^{-1} of PE. L-NAME (10^{-4} mol·L^{-1}), Indo (10^{-5} mol·L^{-1}), and MB (10^{-5} mol·L^{-1}) were used to test the mechanism of the endothelium relaxation pathways.

Vasodilation induced by wogonin and the role of K^+ channels

To determine whether K^+ channels play a role in the vasodilation effect of wogonin, endothelium-removed

aortic rings were pre-incubated with TEA (calcium-activated K^+ channel blocker, 3×10^{-3} mol·L^{-1}), Gly (ATP-sensitive K^+ channel blocker, 10^{-5} mol·L^{-1}), 4-AP (voltage-dependent K^+ channel blocker, 10^{-4} mol·L^{-1}), or $BaCl_2$ (inward rectifier of K^+ channels, KIR, 10^{-4} mol·L^{-1}) for 20 min. Wogonin (1, 3, 10, 30, 100 and 300 μmol·L^{-1}) was added cumulatively after PE (10^{-6} mol·L^{-1}) was addition,

Vasodilation induced by wogonin and the role of Ca^{2+} channels

To illustrate the relationship between Ca^{2+} channels and the pharmacological effects of wogonin, endothelium removed aortic rings were pre-incubated with Ver (10^{-5} mol·L^{-1}) for 20 min. 10^{-6} mol·L^{-1} of PE was then added to the bath to induce vasoconstriction, and wogonin (1, 3, 10, 30, 100 and 300 μmol·L^{-1}) was added cumulatively.

The effect of wogonin on PE- or KCl-induced vasoconstriction in endothelium-removed rings

After equilibrated in K-H solution and pre-incubated with wogonin (28.58 μmol·L^{-1} or 85.74 μmol·L^{-1}) for 10 min, endothelium-removed rings were exposed to PE (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, or 10^{-5} mol·L^{-1}) or KCl (1×10^{-2}, 2×10^{-2}, 4×10^{-2} or 8×10^{-2} mol·L^{-1}). PE- or KCl induced vasoconstriction were expressed as contraction%. For PE, Contraction %= (Maximal tension of PE – resting tension)/(Maximal tension of 60 mM K^+ – resting tension)*100%. For KCl, Contraction %= (Maximal tension of KCl – resting tension)/(Maximal tension of 8×10^{-2} mol·L^{-1} K^+ – resting tension)*100%.

The effect of wogonin on intracellular calcium release

To evaluate the effect of wogonin on intracellular calcium release during vasoconstriction, endothelium-removed rings were washed thrice with Ca^{2+}-free K-H solution and incubated for 10 min, followed by addition of 10^{-6} mol·L^{-1} of PE. The rings were then washed thrice with K-H solution and equilibrated for 40 min to refill the intracellular Ca^{2+} stores. Finally, the rings were washed with Ca^{2+}-free K-H solution thrice and pre-incubated with 28.58 or 85.74 μmol·L^{-1} of wogonin for 10 min before 10^{-6} mol·L^{-1} of PE was added to induce the second contraction.

Data analysis

All data are expressed as the mean ± SD. Statistical significance was evaluated using an ANOVA followed by Dunnett's test. P <0.05 was taken as a significant difference. EC_{50} values were determined by Graphpad Prism 5.

Results

Role of the endothelium in the function of wogonin

Wogonin (1, 3, 10, 30, 100 and 300 μmol·L^{-1}) reduced the contraction of endothelium intact aortic rings caused by 10^{-6} mol·L^{-1} of phenylephrine (PE). Pre-treatment of aortic rings with L-NAME, Indo, or MB had no effect on the relaxation induced by wogonin (Fig. 1). L-NAME is a nonselective inhibitor of nitric oxide synthase, while Indo can inhibit cyclooxygenase activity by reducing the generation of prostacyclin. MB is a guanylate cyclase inhibitor. These results indicate that the vasodilatory effect of wogonin is independent of endothelium.

Role of K^+ channels in the function of wogonin

Wogonin reduced the tension in endothelium-removed aortic rings. The EC_{50} values of wogonin were calculated as the compound concentration inducing 50% of the maximum (EC_{50} = 28.58 μmol·L^{-1}). Endothelium-removed aortic rings were then treated with 4-AP, TEA, Gly and $BaCl_2$. These four blockers had no effect on the function of wogonin (Fig. 2A).

Role of Ca^{2+} channels in the vasodilation effect of wogonin

Contraction of endothelium-removed aortic rings was induced by the addition of 10^{-6} mol·L^{-1} of PE, but addition of Ver was unable to influence vasodilation induced by 1, 3, 10, 30 or 100 μmol·L^{-1} of wogonin (Fig. 2B). This result indicates that Cav1.2 does not participate in relaxation induced by wogonin. In this study, the function of the cell surface, voltage-gated Ca^{2+} channels (VGCCs) were also studied. An

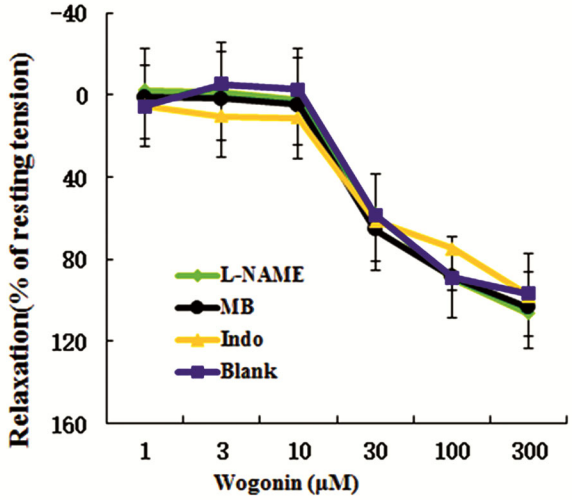

Fig. 1 — Vasorelaxation effects of wogonin on intact endothelium. [Thoracic aortic rings were pre-incubated with L-NAME, Indo, or MB before vasocontraction induced by PE. Wogonin was then added to induce vasodilation. Data are expressed as the mean ± SD, n=4-6]

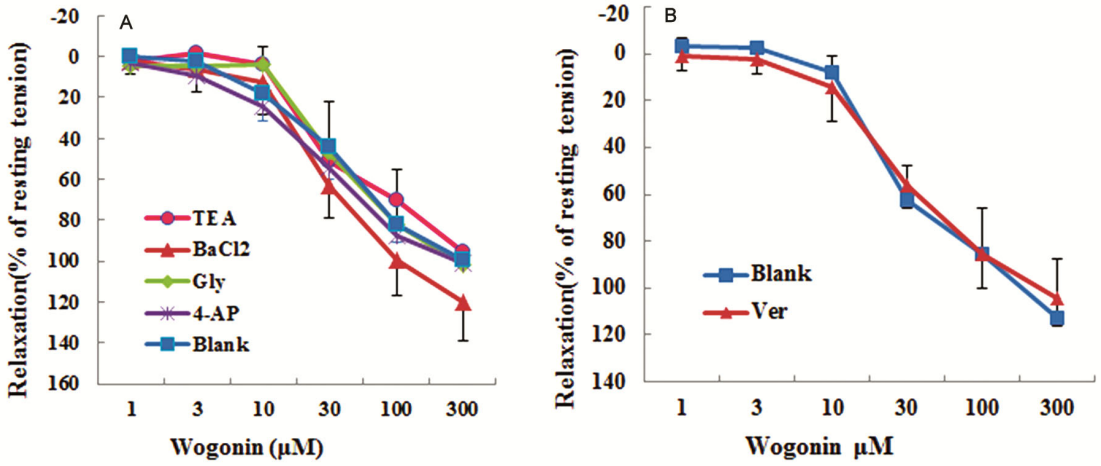

Fig. 2 — Influence of (A) potassium; and (B) calcium channel blockers on the effects of wogonin in vessels lacking endothelium. Thoracic aortic rings were pre-incubated with (A) TEA, $BaCl_2$, Gly, or 4-AP; and (B) Ver before vasocontraction induced by PE. Wogonin was then added to induce vasodilation. [Data are expressed as the mean ± SD, n=4-6]

experiment was conducted to investigate the effect of wogonin on the contractions induced by increasing concentrations of KCl. The results showed that 28.58 or 85.74 μmol·L^{-1} of wogonin reduced contractions induced by KCl (0.01, 0.02, 0.04 or 0.08 mol·L^{-1}) (Fig. 3). The contractions induced by high concentrations of K^+ rely on VGCCs and the opening of Ca^{2+} release channels (ryanodine and IP3 receptors). Thus, wogonin has an effect on the VGCCs or the opening of Ca^{2+} release channels.

Receptor-mediated Ca^{2+} influx

In endothelium-removed aortic rings, wogonin (28.58 or 85.74 μmol·L^{-1}) reduced the contraction caused by increasing concentrations of PE (Fig. 4). The contraction induced by PE is mainly associated with receptor-mediated Ca^{2+} influx. It is suggested that wogonin could affect Ca^{2+} influx.

Wogonin affects intracellular calcium release

The endothelium-removed aortic rings were used to test the effects of wogonin on intracellular calcium release. First, wogonin (28.58 or 85.74 μmol·L^{-1}) was incubated with endothelium-removed aortic rings in calcium-free K-H solution for 10 min. Next, 10^{-6} mol·L^{-1} of PE was added to induce contraction. Both concentrations of wogonin were able to reduce the PE-induced contraction, which was caused by intracellular calcium release (Fig. 5). These results indicate that wogonin inhibits intracellular calcium release.

Discussion

Role of the endothelium in wogonin-mediated vasodilation of aortic rings

For endothelium intact aorta, the endothelium, and in particular the endothelial cells, are important

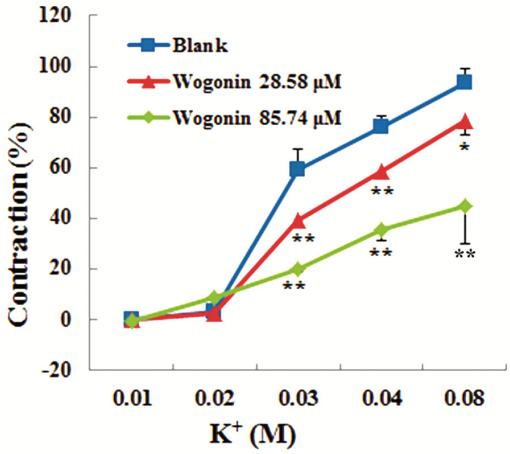

Fig. 3 — Effects of wogonin on vasodilation curves of PE in vessels lacking an endothelium. [Wogonin (28.58 or 85.74 μmol·L^{-1}) was added to pre-treat the rings, and then increasing concentrations of PE were added to induce vasocontraction. Data are expressed as the mean ± SD, n=4-6; $^{**}P$ <0.01 *vs*. Blank]

components of the vasodilation process. Endothelial cells release several factors, such as nitric oxide (NO) and prostacyclin (PGI_2) to relax the aorta[25-28]. NO released by endothelial cells activates guanylyl cyclase and elevates intracellular cyclic guanosine monophosphate (cGMP) synthesis from Guanosine triphosphate (GTP) to exert its biological effects[29,30]. In this study, wogonin relaxed the endothelium-intact aortic rings, which were contracted by prior addition of PE (10^{-6} mol·L^{-1}). This effect could not be blocked by L-NAME, an inhibitor of endothelial nitric oxide synthase (eNOS), indicating that wogonin relax the aorta via an NO independent pathway. Similarly, Indo had no effect on wogonin mediated vasodilation. Indo

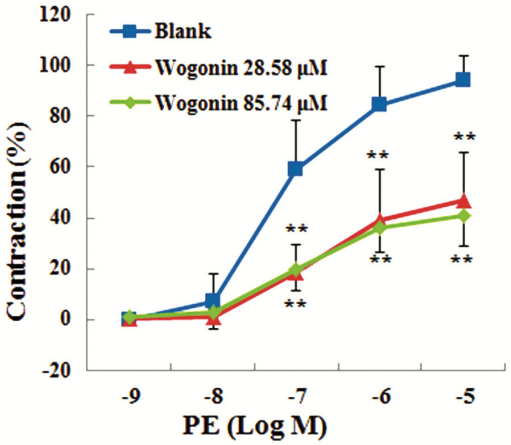

Fig. 4 — Effects of wogonin on the vasocontraction curves of KCl in vessels lacking an endothelium. [Wogonin (28.58 or 85.74 μmol·L^{-1}) was added to pre-treat the aortic rings, and then increasing concentrations of KCl were added to induce vasocontraction. Data are expressed as the mean ± SD, n=4-6; *P <0.05, ** P <0.01 *vs*. Blank]

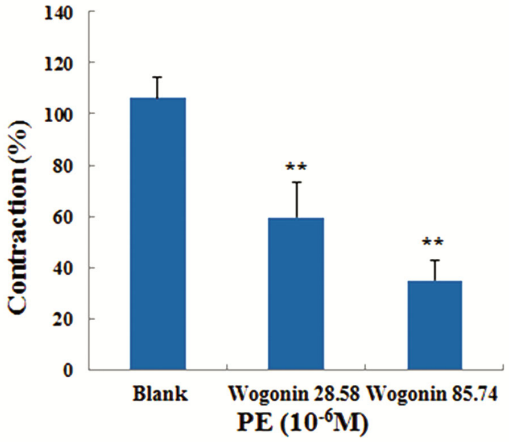

Fig. 5 — Effects of wogonin on intracellular Ca^{2+} in aortic rings lacking an endothelium. [Aortic rings were pre-treated with wogonin (28.58 or 85.74 μmol·L^{-1}) in Ca^{2+}-free K-H solution. PE was then added to induce vasocontraction. Data are expressed as the mean ± SD, n=4-6; **P <0.01 *vs*. Blank]

inhibits cyclooxygenase to reduce the production of PGI_2. Thus, the vasodilatory effects of wogonin is independent of prostacyclin (PGI_2). To further verify the results described above, we used methylene blue (MB), a guanylate cyclase inhibitor, to test the role of guanylate cyclase in the vasodilation effect of wogonin. Results showed that MB can not affect the vasodilatory effect of wogonin.

Function of smooth muscle pathways in the mechanism of wogonin

K^+ channels, Ca^{2+} channels and intracellular calcium release are all vital for vasoconstriction and vasodilation of endothelium-removed aortic rings[31,32]. In the vascular smooth muscle, there are 4 types of K^+ channels: voltage dependent K^+ channels[33], ATP sensitive K^+ channels[34], calcium activated K^+ channels[35], and inward rectifier potassium channels[36]. In this study, we used 4-AP, TEA, Gly and $BaCl_2$ to block the all four types of K^+ channels, respectively. The results showed that these four blockers had no effect on the function of wogonin. It can be concluded that the vasodilation function of wogonin is independent of these four types of K^+ channels.

In this study, verapamil, a Cav1.2 blocker[37,38], particularly the voltage dependent, L-type, alpha1C subunit (Cav1.2), was used to test the effect of wogonin on endothelium removed aortic rings. The results showed that Ver had no effect on the vasodilation induced by wogonin when rings were pre-contracted with PE (10^{-6} mol·L^{-1}). Clearly, wogonin mediated vasodilation is independent with Cav1.2. When pre-incubated endothelium-removed aortic rings with wogonin (28.58 or 85.74 μmol·L^{-1} for 20 min), the vasoconstriction caused by increasing concentrations of KCl and PE was reduced. Vasoconstriction caused by high concentrations K^+ is mediated by VGCCs and the opening of Ca^{2+} release channels (ryanodine and IP3 receptors)[39,40]. The vasoconstriction caused by PE is mainly a result of receptor-mediated Ca^{2+} influx. Together, these results indicate that wogonin exerts its vasodilation effects through the opening of Ca^{2+} release channels, as well as the receptor operated Ca^{2+} influxes. The blocking effect of low dose of wogonin (28.58 μM) on the opening of Ca^{2+} release channels is slightly weaker than the receptor-mediated Ca^{2+} influx. When the dose of wogonin is increased to 85.74 μM, the blocking effect increased and the effect on the two kinds of channels was similar. The effects of wogonin on intracellular calcium release was tested by adding PE (10^{-6} mol·L^{-1})[41], which causes contraction in calcium free K-H solutions. Results showed that wogonin reduced PE-mediated contraction, suggesting that wogonin could block intracellular calcium release. Wogonin 28.58 μM and 85.74 μM both could block the release of intracellular calcium, and the effect of 85.74 μM is stronger. The vasodilatory effect of wogonin on isolated rat aortic rings and the rat uterine smooth muscle also confirmed by other researcher's study[24,42], but more information of wogonin was studied in this research work, summarized the following points: Confirmed

the effect of wogonin to prostacyclin, guanylate cyclase, the 4 types of K^+ channels, Cav1.2 and the receptor-mediated Ca^{2+} influx.

Conclusion

Based on the results, it could be concluded that wogonin exerts vasodilation effect via blocking the opening of Ca^{2+} release channels, receptor operated Ca^{2+} influxes and intracellular calcium release. The present findings will be meaningful for revealing the pharmacological effects of wogonin on the cardiovascular system.

Acknowledgement

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Conflict of Interest

Authors declare no competing interests.

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