Scutellarin attenuates endothelium-dependent aasodilation impairment induced by hypoxia reoxygenation, through regulating the PKG signaling pathway in rat coronary artery

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Available online 20 Apr. 2015

[ABSTRACT] Scutellarin (SCU), a flavonoid from a traditional Chinese medicinal plant. Our previous study has demonstrated that SCU relaxes mouse aortic arteries mainly in an endothelium-depend-ent manner. In the present study, we investigated the vasoprotective effects of SCU against HR-induced endothelial dysfunction (ED) in isolated rat CA and the possible mechanisms involving cyclic guanosine monophosphate (cGMP) dependent protein kinase (PKG). The isolated endothelium-intact and endothelium-denuded rat CA rings were treated with HR injury. Evaluation of endothelium-dependent and -independent vasodilation relaxation of the CA rings were performed using wire myography and the protein expressions were assayed by Western blotting. SCU (10–1 000 μmol·L−1) could relax the endothelium-intact CA rings but not endothelium-denuded ones. In the intact CA rings, the PKG inhibitor, Rp-8-Br-cGMPS (PKGI-rp, 4 μmol·L−1), significantly blocked SCU (10–1 000 μmol·L−1)-induced relaxation. The NO synthase (NOS) inhibitor, NO-nitro-L-arginine methylester (L-NAME, 100 μmol·L−1), did not significantly change the effects of SCU (10–1 000 μmol·L−1). HR treatment significantly impaired ACh-induced relaxation, which was reversed by pre-incubation with SCU (500 μmol·L−1), while HR treatment did not altered NTG-induced vasodilation. PKGI-rp (4 μmol·L−1) blocked the protective effects of SCU in HR-treated CA rings. Additionally, HR treatment reduced phosphorylated vasodilator-stimulated phosphoprotein (p-VASP, phosphorylated product of PKG), which was reversed by SCU pre-incubation, suggesting that SCU activated PKG phosphorylation against HR injury. SCU induces CA vasodilation in an endothelium-dependent manner to and repairs HR-induced impairment via activation of PKG signaling pathway.

[KEY WORDS] Scutellarin; Endothelium-dependent vasodilation; Hypoxia reoxygenation; cGMP-dependent protein kinase; Rat; Coronary artery

[CLC Number] R965
[Document code] A

Introduction

Flavonoids are among the most ubiquitous groups of plant secondary metabolites and exhibit effects in treating cardiovascular dysfunction [1]. However, the exact mechanisms underlying flavonoids’ vascular reactivity remain unclear. Given the recent increases in the use of herbal medicines by all societies, understanding the mechanisms of action of flavonoids is an urgent demand and may have significant clinic impact.

Scutellarin (SCU), 4', 5, 6-trihydroxy flavonoid-7-glucuronide (Fig. 1), is a flavonoid glycoside found in a traditional Chinese herb medicine, Erigeron breviscapus (vant.) Hand-Mazz [2]. Compared with breviscapine (BRE, a flavonoid mixture extract from Erigeron breviscapus), SCU exhibits stronger protective effects against cardio- and cerebro-vascular ische-
mia in rats [3]. In animal studies, SCU (50 and 75 mg·kg⁻¹) has been reported to be neuroprotective in rat brain ischemia reperfusion (IR) models, and the protective effects of SCU (15–50 mg·kg⁻¹) on cardiovascular and cerebrovascular ischemia are better than that of BRE in rats [3-4].

Despite these investigations have described an effect of SCU on vascular responses, the underlying mechanism of PKG action is poorly described. In the present investigation, we studied the effects of SCU on vascular tone and HR-induced endothelium injury in isolated rat coronary artery (CA). We also tested the effects of PKG inhibitor on SCU-induced vasorelaxation and determined p-VASP protein levels in the CA to elucidate the molecular mechanisms underlying SCU’s beneficial effects.

**Material and Methods**

**Chemicals and drugs**

SCU (yellow powder; purity > 99%; molecular weight, 464.4) was obtained from Kunming Longjin Pharmaceuticals Co. (Kunming, China). The nitric oxide synthase (NOS) inhibitor no-nitro-L-arginine methylester (L-NAME), the guanylyl cyclase (GC) inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one(1,0DQ), the adenylate cyclase (AC) inhibitor 9-((terahydroy-2-furanyl)-9H-purin-6-amine(SQ22536),9,11-di deoxy-11a,9α-epoxyxethano-prostaglandinF₂α (U46619), acetylciloline (ACH), and nitroglycerin (NTG) were purchased from Sigma-Aldrich (Shanghai, China). The PKG inhibitor Rp-8-Br-cGMPS (PKGl-rp) was obtained from Santa Cruz Biotechnology (Shanxi, China). Rabbit anti-p-VASP (Ser239) monoclonal antibody was purchased from Millipore (Hong Kong, China). All salts were obtained from Biyuntian Co. (Shanghai, China).

**Animals**

Sprague-Dawley (SD) rats (weighing 200–250 g) were purchased from the Experimental Animal Center at Xi'an Communication University (Xi'an, China). This study protocol was approved by the Animal Care and Use Committee of Kunming Medical University and conformed to the standards set by the Yunnan Experimental Animal Management Board. All animals were housed in microisolation under conditions of constant temperature and controlled illumination (light on 12 h light/dark cycle). Food and water were available ad libitum. All the animals used in the experiment received humane care. All surgical and experimental procedures were in accordance with institutional animal care guidelines.

**Preparation of CA rings**

The rats were sacrificed by intraperitoneal injection of 10% urethane (0.1 mL/100 g). The CA rings were removed and dissected free from connective tissue using an integrated wire myograph system (Model 620M, DMT-Asia Ltd., Shanghai, China) under a Motic SMZ168-TL stereomicroscope. 1-mm-long CA ring segments were subsequently dissected from the cleaned vessels and placed in cold (4 °C) physiological saline solution (PSS) (140 mmol·L⁻¹ NaCl, 4.7 mmol·L⁻¹ KCl, 1.6 mmol·L⁻¹ CaCl₂, 1.2 mmol·L⁻¹ MgSO₄, 1.2 mmol·L⁻¹ MOPS, 1.4 mmol·L⁻¹ Na₂HPO₄, 0.02 mmol·L⁻¹ EDTA, and 5.6 mmol·L⁻¹ D-glucose, pH7.4).

For tension recording, the CA rings were mounted with 60-μm steel wires in separate tissue baths of the wire myog-
raph system. The tissue baths were filled with PSS solution (pH 7.4) at 37 ± 1 °C and aerated with O2. The mounted CA rings were washed before each treatment by draining and replacing the bathing solution. Isometric tension signals were recorded and data were collected using the Powerlab data acquisition system (AD Instruments Asia, Shanghai, China). Tension response of each ring to U46619 (1 μmol·L⁻¹ for 10 min) was recorded, and the rings showing less than 10% variation in tension response between two measurements were used for subsequent experiments. In some experiments, when denuded rings were required, the endothelium was removed mechanically by a segment of hair into the lumen and rolling the ring for a few seconds.

**Hypoxia Reoxygenation (HR) treatment and evaluation of endothelium-dependent and -independent vasodilation relaxation in CA rings**

HR treatment of isolated CA rings was carried out as previously described with minor modifications. Blood vessel segments were incubated in glucose-free PSS pre-aerated with N2 for 60 min, and then transferred to glucose-containing PSS pre-aerated with O2 for 90 min. To assess the effect of the HR treatment on endothelium-dependent and -independent vasodilation in CA rings, ACh (0.001–100 μmol·L⁻¹) and -independent vasodilation in CA rings, ACh (0.001–100 μmol·L⁻¹) and NTG (0.01–10 μmol·L⁻¹) was accumulatively added after pre-contraction with KCl (6 × 10⁴ μmol·L⁻¹) or U46619 (1 μmol·L⁻¹) to obtain their dose-response curves. To evaluate the effect of SCU on ACh or NTG induced relaxation, CA rings were incubated with SCU for 15 min prior to pre-contraction. After HR treatment, the segments were incubated with SCU and PKG inhibitor for 15 min prior to pre-contraction to assess their effects on ACh or NTG induced relaxation.

**Evaluation of effects of inhibitors of PKG, NOS and AC/GC on SCU vasodilation**

Accumulative concentrations of SCU (10–1 000 μmol·L⁻¹) were added after pre-contraction with U46619 (1 μmol·L⁻¹) to obtain accumulative dose response curves of SCU vasodilation in CA rings. Data were normalized to that of 1 μmol·L⁻¹ U46619.

To study the involvement of PKG, NOS, and AC/GC, the equilibrated CA rings were pre-incubated for 15 min with the PKG inhibitor PKGI-rp (4 μmol·L⁻¹), the NOS inhibitor L-NAME (100 μmol·L⁻¹), the GC inhibitor ODQ (100 μmol·L⁻¹), and the AC inhibitor SQ22536 (100 μmol·L⁻¹). The control group was treated with the same volume of vehicle alone. The rings were then stimulated with U46619 (1 μmol·L⁻¹), followed by accumulative addition of SCU (10–1 000 μmol·L⁻¹).

**Evaluation of effects of SCU on PKG signaling**

We examined the influence of SCU on the PKG signaling pathway by determining p-VASP (Serine 239, phosphorylated product of PKG) levels in isolated rat CA. CA rings were collected from 14 SD rats and each CA ring was dissected into five 3-mm-long segments. The CA segments were then equally divided into five treatment groups: control, HR model, SCU (50 μmol·L⁻¹) + HR, SCU (100 μmol·L⁻¹) + HR, and SCU (200 μmol·L⁻¹) + HR. The control segments were incubated in glucose-rich PSS buffer pre-aerated with O2 at 37 °C. The HR segments were incubated in glucose-free PSS pre-aerated with N2 for 6 h, and then returned to glucose-rich PSS pre-aerated with O2 for 4 h. The SCU segments were pre-incubated with SCU for 30 min prior to HR treatment. After the treatment was completed, segments were homogenized and the p-VASP levels in the homogenates were determined by Western blot analysis. All experiments were performed in triplicate.

**Preparation of CA homogenate**

After treatment, the CA segments were wrapped in foil and stored in liquid nitrogen until analysis. Immediately after removal from liquid nitrogen, the foil-wrapped vessels were hammermed and the washed vessels were transferred into a pre-cooled glass homogenizer containing 200 μL of pre-cooled RIPA buffer (50 mmol·L⁻¹ Tris–HCl, 150 mmol·L⁻¹ NaCl, 1 mmol·L⁻¹ EDTA, 1 mmol·L⁻¹ PMSF, 1 mmol·L⁻¹ Na3VO4, 1 mmol·L⁻¹ NaF, 1% NP-40, 0.25% Na-deoxycholate, 10% glycerol, and 1 mg·mL⁻¹ each of the phosphatase inhibitors aprotinin, leupeptin, and pepstatin, pH 7.4). The tissues were then homogenized (Method ibid) for 10 min, transferred to a 1.5-mL Eppendorf tube, and centrifuged at 12 000 r·min⁻¹ for 15 min at 4 °C. The supernatant was collected and stored at −80 °C until analysis.

**Western blot analysis**

The samples with equal amount of total protein (40 μg) were subjected to Western blot analysis, using the See Blue Plus 2 Prestained Standard (7 μL) (Invitrogen, Carlsbad, CA, USA) as a molecular weight marker. The gels were electrophoresed at 80 V/100 mA for 30 min and then 120 V/160 mA for 80 min. The proteins were then semi-dry transferred to Immobilon PVDF membranes (Immobilon-P, pore size: 0.45 μm; Millipore Corporation, Billerica, MA, USA) at 15 V/10mA for 40 min. The membranes were then washed with Tris-buffered saline with Tween-20 (TBS-T; 10 mmol·L⁻¹ Tris·HCl, 150 mmol·L⁻¹ NaCl, and 0.1% Tween-20, pH 7.6), blocked with 5% skim milk at room temperature for 1.5 h, and incubated with rabbit anti-p-VASP monoclonal antibody (1 : 1 000) and anti-β-Actin antibody (1 : 5 000) at 4 °C overnight. After washing, the membranes were incubated with HRP-conjugated anti-rabbit secondary antibody at room temperature for 1 h. All the membranes were visualized with chemiluminescence detection (ECL Plus Western Blotting Detection System; GE Healthcare, Buckinghamshire, UK). The density values of bands were quantified by densitometric analysis of scanned images (Scion image 4.03). The relative intensity was calculated as percent of control.

**Analysis of the effects of pH on SCU vasodilation**

Since SCU (10–1 000 μmol·L⁻¹) reduces pH value in the assay mixtures, which may change vascular tone, it is necessary to determine if SCU causes CA dilation via modulating pH. In the present experiment, we simulated SCU-induced pH
changes with addition of HCl and record CA stress changes. First, pH changes in bathing solution in response to a cumulative addition of SCU (10–1 000 μmol·L⁻¹) were measured using pH and ion electrodes (Mettler-Toledo GmbH, Schwerzenbach, Switzerland). Then, we determined the volumes of cumulative addition of HCL (0.36%–0.38%), which produced the same pH profile as SCU. Finally, using the Wire Myograph system recorded the stress response when corresponsive volumes of HCl was added in isolated rat CA, and compared the response curves of HCl alone and SCU.

Statistical analysis

The results are presented as means ± SEM. Statistical analyses were performed using Statistical Software Sigma Stat 3.1. The EC₅₀ value of each CA ring was calculated from concentration–response curve by nonlinear regression analysis using a Hill algorithm in Sigma Plot 10.0. Eₘₐₓ values represented the maximal vasodilative responses (the minimal relative stresses of pre-contraction). Comparisons between two groups were performed using t-tests, while comparisons among three or more groups were conducted using one-way ANOVA or two-way ANOVA as appropriate. Differences with P < 0.05 were considered statistically significant.

Results

SCU vasodilates CA in an endothelium-dependent manner

The relaxant effects were examined in CA rings with intact or denuded endothelia. The ACh-induced response was significantly decreased in denuded CA, compared with intact CA (Fig. 2A). In denuded CA group, the Eₘₐₓ value was significantly higher than that in the intact CA group. These results suggested that CA vascular endothelium was denuded successfully in our experiments. SCU (10–1 000 μmol·L⁻¹) relaxed endothelium-intact CA rings, but not endothelium-denuded CA (Fig. 2B). The Eₘₐₓ for SCU in denuded CA was significantly increased, compared with the intact CA, suggesting that SCU-induced relaxation was largely dependent on endothelium.

Inhibitors of PKG, NOS and AC/GC modulate the SCU-induced vasodilation effects

In intact CA rings, PKGI-rp (4 μmol·L⁻¹) treatment significantly blocked SCU-induced relaxation in CA rings (Fig. 3A), leading to a significantly increased Eₘₐₓ, indicating that PKGI-rp blocked SCU-induced relaxation.

NOS inhibitor L-NAME (100 μmol·L⁻¹) resulted in slight augmentation of the relative stress level at SCU concentrations 10–1 000 μmol·L⁻¹ (Fig. 3B). The presence of L-NAME did not significantly change the calculated EC₅₀ for SCU-induced relaxation. We next tested whether the action of SCU was mediated through AC/GC. Pretreatments of CA rings with the AC inhibitor SQ 22536 (100 μmol·L⁻¹) and the GC inhibitor ODQ (100 μmol·L⁻¹) did not significantly inhibit SCU-induced relaxation (data not shown). These results indicated that the SCU-induced endothelium-dependent relaxation was in part mediated by PKG pathway.

SCU does not affect the vasodilation induced by ACh and NTG in CA rings

SCU did not alter ACh-induced endothelium-dependent dilation and NTG response in intact CA (Fig. 4).

SCU reduces vasodilation injury induced by HR treatment

As shown in Fig. 5A, the ACh-induced response was decreased in HR-treated CA compared with normal CA. It suggested that endothelium-dependent vasodilation in response to CAH was significantly impaired in CA exposed to HR treatment. The pre-incubation with SCU (500 μmol·L⁻¹) significantly increased ACh response in HR-treated CA (Fig. 5B), indicating that SCU restored endothelium-dependent relaxation in HR-treated CA. These results suggested that SCU attenuated HR-induced endothelium injury.
PKG and NOS are involved in SCU-induced CA vasodilation. A. CA rings were pre-incubated with the PKG inhibitor PKGI-rp (4 μmol·L⁻¹) or vehicle for 15 min, then accumulatively added with SCU (10–1 000 μmol·L⁻¹). B. CA rings were pre-incubated with the NOS inhibitor L-NAME (100 μmol·L⁻¹) or vehicle for 15 min, then accumulatively added with SCU (10–1 000 μmol·L⁻¹). The SCU-induced relaxation is expressed as a percent of pre-contraction by U46199 (1 μmol·L⁻¹). ***P < 0.001 vs vehicle, t-test of Emax. ###P < 0.001 vs vehicle, two-way ANOVA test (n = 7–8, mean ± SEM)

Fig. 3

SCU activates PKG in isolated CA

Western blot analysis of CA homogenates showed that HR treatment decreased the p-VASP protein level in isolated CA (Fig. 7), suggesting that HR impaired PKG signaling pathway. Pre-incubation with SCU (100 and 200 μmol·L⁻¹) significantly increased the levels of p-VASP, indicating that SCU restores PKG signaling in HR-treated rat CA.

SCU-associated with pH reduction is not related to SCU-vasodilation effects in CA

In CA rings, the cumulative addition of SCU (10–1 000 μmol·L⁻¹) relaxed the rings, while cumulative addition of HCL produced a slight stress increase with significant different
Fig. 5  SCU restores endothelium-dependent vasodilation injured by HR treatment in CA. Control: pre-incubated with vehicle without HR treatment. HR: pre-incubated with vehicle followed by HR treatment. HR + 500 μmol·L⁻¹ SCU groups: pre-incubated with SCU (500 μmol·L⁻¹) followed by HR treatment. A. ACh-induced response is decreased in HR-treated CA. ###P < 0.001 vs Control, two-way ANOVA test. B. SCU significantly increase ACh response in HR-treated CA. ###P < 0.001 vs HR, two-way ANOVA test. C. NTG-induced response is slightly in HR-treated CA. D. SCU slightly increase NTG response in HR-treated CA (n = 8–11, mean ± SEM).

Discussion

Vascular ED is the major contributor to the development and exacerbation of many cardiovascular diseases. In particular, ED can lead to endothelium-dependent vasodilation impairment, excessive vasoconstriction, inflammation, leukocyte adhesion, thrombosis, atherosclerosis, and proliferation of vascular smooth muscle cells. Several reports have suggested that the flavonoids can counteract any dysfunction of endothelium associated with cardiovascular diseases. SCU, one of the major bioactive constituents of flavonoid, is a well-known anti-inflammatory and cardiovascular protective agent with diverse pharmacological activities [18]. In the present study, we mainly investigated the effects of SCU on endothelium-dependent vasodilation and the related signal pathway in CA arteries isolated from rats.

First, we proved that SCU induced vasodilation in an endothelium-dependent manner, mainly via the PKG signal pathway. Our previous studies have also shown that SCU-induced vasodilation is abolished by about 80% in endothelium-denuded mouse aorta [3]. Additionally, we demonstrated that in vitro HR treatment of isolated arteries led to impaired endothelium-dependent relaxation in response to ACh by monitoring isometric tension using a wire myograph system. SCU attenuated the HR-induced ED in isolated rat CA arteries through activation of PKG signaling pathway. Our report of endothelial-dependent vasodilation by SCU was in agreement with other reports [1] about other flavonoids in aortic preparations that SCU exhibited moderate relaxation abilities, and consistent with its clinical applications.

Our result generally were consistent with these reports, but the SCU dose used was much higher than clinic dose suggesting that SCU exhibited moderate relaxation abilities,
but was consistent with its clinical applications that exclude its use in hypertension diseases. The general clinical doses of SCU are 50–200 mg per adult intravenously to treat cardiovascular disease. Based on our assuming an adult blood volume of 4.4 L, then the calculated blood concentration is 24.4–97.6 μmol L⁻¹ (intravenously); the concentrations of SCU used in these in vitro studies were not substantially different from the levels of SCU presumed in the plasma of in vivo studies humans cited. Some of the elevation of the concentration needed for effectiveness may be related to the use of a rodent model vs human tissue, but our results were in agreement with the concentration range need for SCU’s effect in other rodent model [31]. In the present study, the concentrations of SCU (1–1 000 μmol L⁻¹) used were consistent with other animal studies of SCU, reporting the effective doses of SCU in vitro in the 10–1 000 μmol L⁻¹ range.

A great number of studies have suggested that hypoxia and vascular dysfunction involve PKG signaling cascades that alter vascular tone [32]. In the present study, the ACh-induced response was decreased in HR-treated CA, compared with control normal CA. It suggested that endothelium-dependent vasodilation in response to ACh was significantly impaired in

Fig. 6  PKG inhibitor PKGI-rp blocks SCU’s vasorelaxation in HR-treated CA. Control: pre-incubation with vehicle without HR treatment; Model: pre-incubation with vehicle followed by HR treatment; SCU: pre-incubation with SCU (500 μmol L⁻¹) after HR treatment; PKGI-rp: pre-incubation with PKGI-rp (4 μmol L⁻¹) after HR treatment; PKGI-rp + SCU: pre-incubation with PKGI-rp (4 μmol L⁻¹) and then SCU (500 μmol L⁻¹) after HR treatment. *$P < 0.05$ vs Model, Kruskal-Wallis One Way Analysis of Variance on Ranks of Emax; **$P < 0.05$ vs SCU, Kruskal-Wallis One Way Analysis of Variance on Ranks of Emax; ###$P < 0.001$ vs Model, two-way ANOVA test; $$$P < 0.001$ vs SCU, two-way ANOVA test ($n = 9–12$, mean ± SEM)

Fig. 7  SCU activates PKG signaling in HR-treated CA. Control: pre-incubation with vehicle without HR treatment; HR Model: pre-incubation with vehicle followed by HR treatment; SCU (50, 100, or 200 μmol L⁻¹) groups: pre-incubation with SCU followed by HR treatment. *$P < 0.05$, **$P < 0.01$ vs HR Model, ANOVA Fisher Least-Significant Difference test ($n = 3$, mean ± SEM)
vascular tone, ultimately leading to tissue damage [22]. In addition, there are two splice variants of the endothelium-dependent vasodilator (ACh) and endothelium-independent NO donor vasodilators (NTG). The results showed that SCU had no significant effects on ACh or NTG induced dilations in normal CA rings; experimental HR treatment showed that HR-induced ED was accompanied by impaired PKG signaling. The treatment with SCU restored the endothelium-dependent relaxation in HR-treated rat CA via PKG activation. Our results from Western blot analysis also showed that SCU treatment increased the levels of p-VASP in HR-treated rat CA, indicating that SCU activated the PKG signaling pathway.

Intracellular and extracellular pH changes have considerable impact on the tone and reactivity of mammalian vascular smooth muscle. In general, a decrease in either intracellular pH or extracellular pH relaxes blood vessels independently of the endothelium, whereas an increase in pH contracts them [40-43]. In the present study, with the increase in the SCU pH reduction by SCU, the key mechanism of SCU vasodilation might not be due to pH reduction by SCU.

In summary, our results support the notion that SCU could modulate CA vascular tone in an endothelium-dependent manner and repair ED induced by HR injury via activating PKG signal pathway. To our best knowledge, this is the first report that SCU protects CA from HR-induced ED through activation of the PKG signaling pathway.

Acknowledgments

We thank Mr. ZHANG Ren-Wei and FANG Xian-Er for providing the SCU compound as a gift. We also thank Dr. CAO Yong-Xiao and his group for technical assistance with operation of the wire myograph system.

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Cite this article as: CHEN Ya-Juan, WANG Lei, ZHOU Guang-Yu, YU Xian-Lun, ZHANG Yong-Hui, HU Na, LI Qing-Qing, CHEN Chen, QING Chen, LIU Ying-Ting, YANG Wei-Min. Scutellarin attenuates endothelium-dependent vasodilation impairment induced by hypoxia reoxygenation, through regulating the PKG signaling pathway in rat coronary artery [J]. Chinese Journal of Natural Medicines, 2015, 13(4): 264-273