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Original article

Scutellarin protects human cardiac microvascular endothelial cells with hypoxia-reoxygenation injury via JAK2/STAT3 signal pathway

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ABSTRACT

Objective: To investigate the antagonistic cell injury effect and molecular mechanism of scutellarin (SCU) in hypoxia reoxygenation (HR) treated human cardiac microvascular endothelial cells (HCMECs).
Methods: The method of 12 h hypoxia following by 12 h reoxygenation was used to culture HCMECs *in vitro* to built cell injury model. The groups were divided into control group, model (HR) group, and HR + SCU (0.1 μmol/L, 1 μmol/L, and 10 μmol/L) group. The cell viability was determined by MTT, and oxidative stress was detected by malondialdehyde (MDA) levels by biochemical assay kit. Protein expression of JAK2/p-JAK2 and STAT3/p-STAT3 were evaluated by Western blot.
Results: The results of MTT and MDA showed that HR decreased the cell viability ($P < 0.05$) and increased MDA level significantly ($P < 0.05$), SCU played a contrary role in these processes. Western blot analysis indicates that, the expression of JAK2 and p-JAK2, STAT3, and p-STAT3 were increased in model group when compared with control group ($P < 0.05$); Compared with model group, their expression were reduced by SCU ($P < 0.05$).
Conclusion: SCU took a protective effect on HR-treated HCMECs, and the molecular mechanism may be associated with the inhibition of JAK2/STAT3 signal transduction pathway.

1. Introduction

Ischemic heart disease (IHD) is one of the most prevalent cardiovascular diseases around the world (Benjamin et al., 2017). The main treatment for IHD is to restore the normal blood supply. However, restoration of the blood supply to ischemic heart tissue does not restore cardiac function and induces irreversible myocardial injury, which is called myocardial ischemia-reperfusion injury (MIRI) (Goldhaber & Weiss, 1992). Therapeutic approaches to treating IHD have evolved to incorporate the regulation of critical signals pathways that contribute to MIRI. For example, previous studies have identified that Janus activated kinase signal transducer and activator of transcription 3 (JAK2/STAT3) was activated in ischemia preconditioning, ischemia post-treatment, and anti-ischemia-reperfusion injury (Yang, Lei, Lu, Wang, & Xiong, 2008; Zhang, Hu, Wang, Xu, & Zeng, 2009). Therefore, regulating JAK2/STAT3 may decrease MIRI following IHD treatment.

Scutellarin (SCU) (4′,5,6-trihydroxyflavonoid-7-glucuronide) is a flavonoid glycoside compound isolated from the traditional Chinese medicinal plant *Erigeron breviscapus* (Vant.) Hand. Mazz (Zhang et al., 2000). SCU exhibits pharmacological activity as an anti-oxidative (Lin et al., 2007), anti-inflammatory (Zhang et al., 2009), and anti-apoptosis (Dai et al., 2011; Wang et al., 2016; Yang, Zhao, Wang, Liu, & Zhang, 2017) agent. SCU has also been reported to have therapeutic applications for IHD (Calvert et al., 2015). Both oral and injectable form of SCU preparations have been developed for the treatment of IHD (Zhang et al., 2000, 2006); However, the protective mechanism of SCU is still not completely understood.

Herein, we elucidate the potential therapeutic mechanism of SCU in regulating JAK2/STAT3 in human cardiac microvascular endothelial cells (HCMECs). Given that JAK/STAT3 is activated in IHD, we hypothesize that the beneficial effects of SCU are related to JAK/STAT3 inhibition.

2. Materials and methods

2.1. Reagents

SCU (purity 99%, formula weight 464.4) was obtained from Renwei Zhang of Kunming Longjin Pharmaceutical Co. (Kunming, China). HCMECs were purchased from Yanyi Biology Co., Ltd.

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(Shanghai, China). Cell culture reagents DMEM, modified RPMI-1640 medium, and fetal bovine serum (FBS) were obtained from the Hyclone (Thermo Scientific, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell culture and HR treatment

HCMECs were grown in modified RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin. Tightly (80% – 90%) confluent monolayers of HCMECs from the 4th to 15th passage were used in all experiments. In hypoxia reoxygenation (HR) treatment, HCMECs were cultured in humidified hypoxic chamber (HF100, Heal Force Biotech Co., Shanghai, China) for 12 h of hypoxia (5% CO_2 + 93% N_2 + 2% O_2) with basal medium without glucose and FBS at 37 °C followed by 12 h of reoxygenation (95% air + 5% CO_2) in complete medium containing glucose and FBS. The control cells were not exposed to hypoxic conditions but were treated with vehicle (saline). For HR + SCU groups, cells were incubated with SCU at different concentrations (0.1, 1, and 10 μmol/L) for 2 h prior to hypoxia (12 h) and reoxygenation (12 h) injury. The model group was exposed to HR treatment but treated with vehicle.

2.3. MTT assay of cell viability

HCMECs (3×10^4 cells/well) were seeded into 96-well flat-bottomed plates. After 24 h, cell were treated with SCU (0.1, 1, and 10 μmol/L) or vehicle control for 2 h. Control cells were cultured in normal culture conditions while model and SCU-treated cells were exposed to HR injury as described above. After the treatments, 20 μL of MTT solution (5 mg/mL) was added into each well and the plates were incubated for 4 h at 37°C. Then, 100 μL of lysis buffer (20% sodium dodecyl sulfate [SDS] in 50% N, N-dimethylformamide, containing 0.4% 1 mol/L HCl and 0.5% 14 mol/L acetic acid) was added into each well and incubated overnight at 37 °C. Cell viability was determined by measuring the ability of metabolically active cells to convert the yellow tetrazolium salt MTT (5 mg/mL, PH = 7.4) into purple formazan crystal with a microplate reader at 570 nm.

2.4. Detection of MDA

MDA level was determined according to manufacturer's protocol (Jiancheng Bioengineering Institute, Nanjing, China). Briefly, control or experimental HCMECs (HR-treatment with or without SCU) were analyzed and added to a chromomeric mixture. The optical density of the mixtures was measured by a microplate reader at 530 nm.

2.5. Western blot analysis

HCMEC cells were lysed with 100 μL cold lysis buffer (50 mmol/L Tris-HCl, pH 7.2, 250 mmol/L NaCl, 0.1% NP-40, 2 mmol/L EDTA, 10% glycerol, 1 mmol/L PMSF, 5 μg/mL aprotinin, and 5 μg/mL leupeptin) on ice. Cell suspensions were then centrifuged at 10 000 rpm for 5 min. The supernatant was denatured with loading buffer (Beyotime Institute of Biotechnology, China), centrifuged, and incubated at 95 °C for 5 min. A total of 20 μg proteins were loaded into each well of an 8% SDS gel that was separated electrophoretically and transferred to a PVDF membrane (Millipore, USA). The membrane was washed three times with TBST (10 mmol/L TBS with 0.1% Tween 20) for 5 min then blocked with 5% dehydrated skim milk for 2 h at room tempreture (RT). The membrane was probed over night at 4 °C with primary antibodies. The primary antibodies for JAK2 and p-JAK2 were rabbit polyclonal antibodies (1:500, Santa Cruz Biotechnology) and STAT3 and p-STAT3 were mouse monoclonal antibodies (1:1000, Cell Signaling). The membranes were incubated with HRP-conjugated goat anti-rabbit IgG or HRP-conjugated rabbit anti-mouse IgG (1:5000 or 1:10 000, Santa Cruz Biotechnology) for 2 h at RT and visualized by X-ray film with enhanced Chemiluminescence Kit (Millopore). The density of bands was quantified and normalized to GAPDH by Scion Image 4.03.

2.6. Statistical analysis

Data were expressed as means ± SEM. Statistical analysis was performed using the statistical software Sigma Stat 3.5. Three independent experiments were carried out. One-way ANOVA analysis was used for comparison.

3. Results

3.1. SCU treatment protects HCMECs against HR injury

To evaluate the effects of HR treatment on HCMECs viability, we exposed HCMEC cells to HR injury. Compared to control, HR treatment (model) caused a significant decrease ($P < 0.01$) in HCMEC viability (Fig. 1). Next, we determined the effect of SCU on cell viability in HR-treated cells. Pretreatment with SCU inhibited the HR-treatment-induced decrease in cell viability in a concentration-dependent manner. However, only 10 μmol/L of SCU significantly ($P < 0.01$) prevented a decrease in cell viability (Fig. 1).

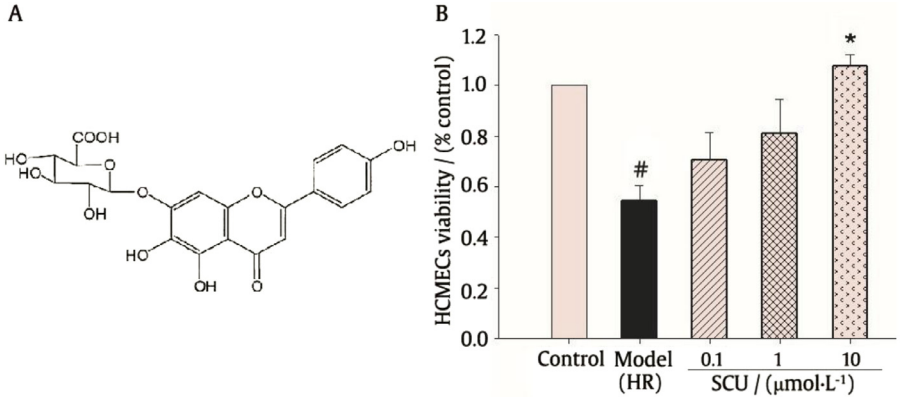

Fig. 1. Chemical structures of SCU (A) and effect of SCU on viability of HCMECs exposed to HR injury (B). Data were shown as means ± SEM ($n = 3$), independent experiments. One-way ANOVA followed by Fisher LSD test was used. $^{\#}P < 0.05$ *vs* control group. $^{*}P < 0.05$ *vs* model group.

3.2. SCU prevents MDA production in HCMECs treated with HR

To determine the severity of HR-treatment, we performed the MDA assay given that MDA is associated with hypoxia/oxidative damage (Roesner, Hankeln, & Burmester, 2006). HR cells showed a significant increase ($P < 0.001$) in MDA levels (Fig. 2) when compared with control group. Pretreatment with SCU (0.1, 1, and 10 μmol/L) resulted in a significant decrease ($P < 0.05$) in MDA levels when compared to model group.

3.3. JAK2, STAT3, and its phosphorylation protein down-regulated by SCU after HR

The data indicated that pretreatment with SCU preserved cell viability and protected against MDA production. Thus, we evaluated the JAK2/STAT3 pathway as a potential mechanism for SCU. As shown in Fig. 3A, the expression of JAK2 and p-JAK2 protein in the model was significantly higher than the control group ($P < 0.05$). Pretreatment with 1 or 10 μmol/L SCU significantly decreased JAK2 protein expression ($P < 0.01$ and $P < 0.001$, respectively). Pretreatment with SCU also inhibited JAK2 phosphorylation in a concentration-dependent manner. HR + SCU (10 μmol/L) significantly ($P < 0.05$) decreased p-JAK2 protein levels when compared to the model group. Similarly, HR treatment increased the expression and activation of STAT3, when compared to control group ($P < 0.05$). Pretreatment with SCU (0.1, 1, and 10 μmol/L) significantly ($P < 0.01$) inhibited STAT3 phosphorylation and 10 μmol/L SCU significantly ($P < 0.05$) decreased STAT3 protein levels, as shown in Fig. 3B.

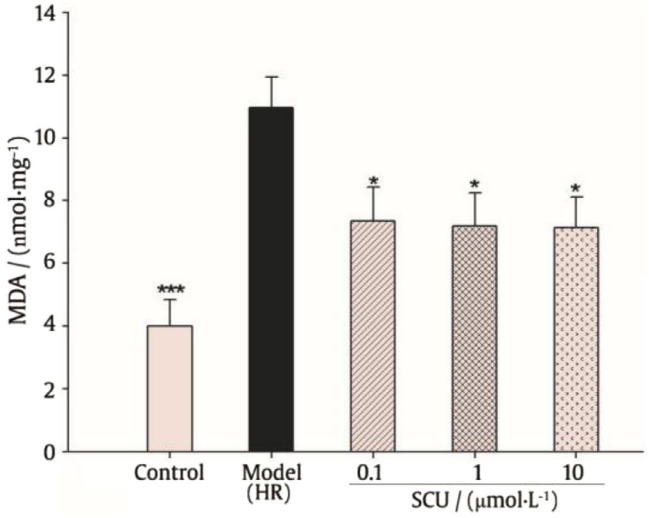

Fig. 2. Effect of SCU on MDA level in HR-treated HCMECs. Data were shown as means ± SEM ($n = 3$), independent experiments. One-way ANOVA followed by Fisher LSD test was used. $^{*}P < 0.05$ and $^{***}P < 0.001$ *vs* model group.

4. Discussion

SCU is a flavonoid glycoside that successfully used in clinic in China for treatment of ischemic diseases. For example, it was previously reported that SCU elicits a protective effect on myocardia induced by HR injury (Calvert et al., 2015).The authors observed that the viability of HCMECs was decreased significantly after HR treatment, and the content of MDA was increased significantly. We therefore determined if SCU could reverse or decrease the oxidative damage to HCMECs exposed to HR treatment. Indeed, we found that SCU pretreatment preserved cell viability and decreased MDA levels.

The JAK-STAT signal pathway, which is comprised of Janus-activated kinases (JAKs: include JAKl, JAK2, JAK3, and TYK2) and signal transducer and activators of transcription (STATs: include STAT1 through STAT6), transduce cell signal from membrane to nuclear, and play an important role in regulating gene expression involved in intracellular pathways (Boykin et al., 2011; Kunisada et al., 1996; Stephanou et al., 2000). A research reported that abnormal activation of STAT3, induced by myocardial ischemia, can protect the cardiomyocytes against ischemic injury (Chakraborty & Tweardy, 1998). The JAK-STAT pathway has well-established roles in many pathological processes of the heart, such as

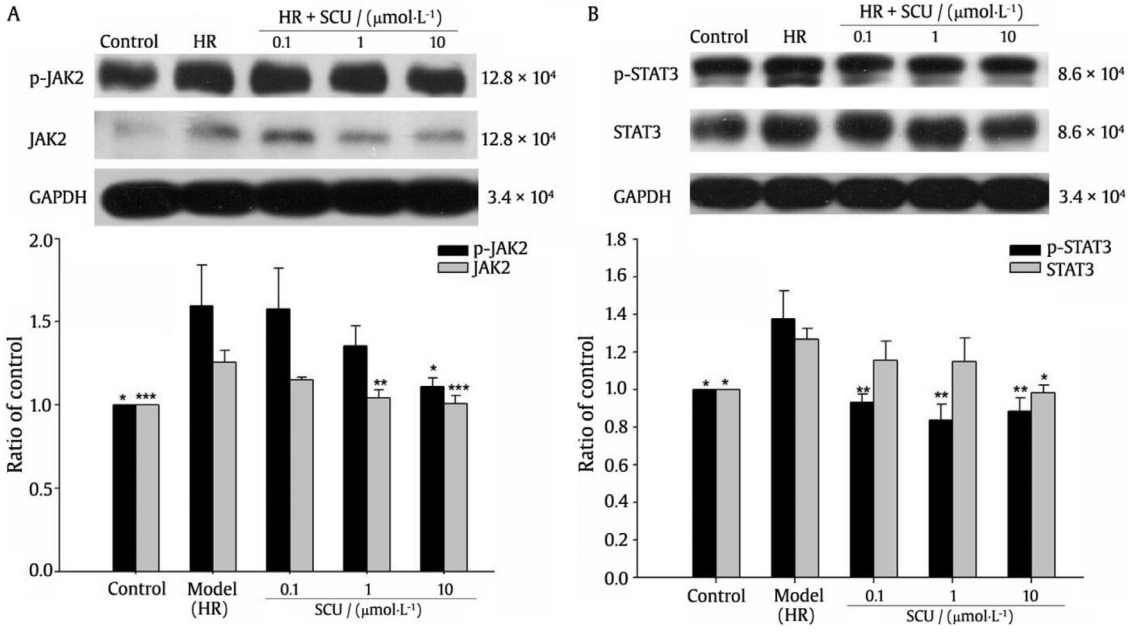

Fig. 3. Effect of SCU administration on protein expression of JAK2, p-JAK2, STAT3, and p-STAT3 in HR-treated HCMECs. (A) Representative immunoblot and quantification of JAK2 and p-JAK2 protein expression in HCMECs. (B) Representative immunoblot and quantification of STAT3 and p-STAT3 protein expression in HCMECs. Data were shown as means ± SEM ($n = 3$), independent experiments with independent culture. One-way ANOVA followed by Fisher LSD test was used. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ *vs* model group.

myocardial hypertrophy (Kunisada et al., 1998; Pan et al., 1997), apoptosis (Chin, Kitagawa, Kuida, Flavell, & Fu, 1997; Kumar, Commane, Flickinger, Horvath, & Stark, 1997), ischemia-reperfusion (Bolli, Dawn, & Xuan, 2001; Mascareno et al., 2001), pretreatment (Bolli, Dawn, & Xuan, 2003) and post-treatment (Boengler et al., 2008). The JAK-STAT pathway plays an important role in vascular injured by HR (Kim et al., 2017), and the studies have shown that cardiac microvascular endothelial cell injury played an important role in myocardial ischemia reperfusion injury (MIRI) (Wang et al. 2016). When the body occured ischemic diseases, inflammatory factors (IL-1β, IL-6 and TNF-α) and oxygen-free radicals (MDA and SDH) release increasing, and these factors stimulates JAK occurs tyrosine phosphorylation and dimerization of cytosolic STAT monomers. The STAT dimers readily translocate to the nucleus, bind to the promoter regions of the DNA, and regulate transcription of genes (Imada, 2000). The present study showed that SCU at a concentration of 10 μmol/L inhibited Jak2 expression and phosphorylation, resulting in the inhibition of phosphorylation of its downstream substrate STAT3. STAT3 phosphorylation at Y705 provokes its homo- or hetero-dimerization, and then nuclear translocation in order to bind gene-specific response elements in target gene promoters (Darvin et al., 2015). We have identified JAK2 and STAT3 as a potential mechanism for the protective effects of SCU. These results are consistent with the findings of Wang et al. (2016). The findings of this research are consistent with the results of ZHAO GL *et al.* (Satriotomo, Bowen, & Vemuganti, 2006; Yuan et al., 2015; Zhao et al., 2016; Zhao, Zhang, Li, Su, & Hang, 2011). On the other hand, it have been reported an opposite results were observed in other cell types. JAK2 was activated in cerebral ischemia-reperfusion, but STAT3 was inhibited (Li, Li, & Li, 2015; Liu et al., 2014). The reason for this difference may be related to the experimental object or drugs. For example, the animal models used in previously described reports were exposed to 0.5 h or 24 h of reperfusion. This indicates that the JAK2-STAT3 pathway may be affected in a time-dependent manner as well as fluctuate based on cell type. Therefore, it is critical to characterize these differences and study the effects of SCU in various models and cell types.

5. Conclusion

The results have shown that pretreatment with SCU may potentially attenuate the effects of oxidative damage as the result of HR treatment in HCMECs. Furthermore, we have identified JAK2-STAT3 as the potential mechanism for SCU. To deepen the understanding of SCU, future studies should include more models (*i.e. in vivo* animal models and/or various cell types) before more advanced clinical applications.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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