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Full Length Research Paper

Endothelium-independent vasodilation effect of hydroxysafflor yellow A in thoracic aorta of Wistar rats

Lin Zhang[1,2], Peihe Nie[1], Guohua Zhang[1], Weifang Rong[1], and Jianming Zhi[1]*

[1]Department of Physiology, School of Medicine, Shanghai Jiao Tong University, 280 South Chong Qing Rd, Shanghai, 200025 P.R. China.
[2]Department of molecular and cell biology, Institute for Medical Sciences, Shanghai Jiao Tong University, 280 South Chong Qing Rd, Shanghai, 200025 P.R. China.

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Hydroxysafflor yellow A, the main active component of safflor yellow, in this study is used to investigate the vascular activity of hydroxysafflor yellow A (HSYA) on rat thoracic aorta and the underlying mechanism. The tension of the isolated thoracic aorta rings of rats incubated with different concentrations of HSYA was measured using organ bath technique. The effect of HSYA (10^{-4}~10^{-6} mol/L) on the contraction induced by cumulative phenylephrine, KCl and $CaCl_2$, was respectively examined by HSYA (10^{-4}~10^{-6} mol/L) dose, which dependently inhibited the contraction induced by KCl (6×10^{-2} mol/L) or phenylephrine (PE, 10^{-6} mol/L) in both endothelium-intact and endothelium-denuded aortic rings. HSYA inhibited the $CaCl_2$-induced contraction and downward shifted concentration-response curve of aortic rings precontracted with PE. Furthermore, HSYA-induced inhibition may be partially through the blockage of IP3 receptor. These results indicated that the role of HSYA may be involved in the reduction of Ca^{2+} influx and inhibition of IP3 receptor in vascular smooth muscle cells.

Key words: Hydroxysafflor yellow A, vasodilation, Ca^{2+} channel, aorta.

INTRODUCTION

Hypertension is a major public-health challenge world-wide because of its high prevalence and concomitant risks of cardiovascular disease (Lee and Cooper 2009). An estimated 972 million adults, or 26.4% of the world's adult population had hypertension in 2000. The number of adults with hypertension in 2025 is predicted to increase by 60% to 1.56 billion (Kearney et al., 2005). In China, hypertension and prehypertension were significantly associated with increased all-cause and cardiovascular mortality. He and colleagues estimated that in 2005, 2.33 million cardiovascular deaths were attributable to increased blood pressure in China, so it is becoming the most pressing problem in the prevention of cardiovascular diseases in China (Wang et al., 2005). Recently, the interest of the general public in the use of dietary herbs has risen exponentially, due to their presumed low toxicity and good therapeutic performance. In recent years, the clinical importance of herbal drugs has received considerable attention. The dried flower of the safflower plant, *Carthamus tinctorius* L., has been used extensively in traditional Chinese medicine for its purported ability to improve blood flow and for the treatment of coronary heart disease, hypertension and cerebrovascular and gynecological diseases (Yang et al., 2009). In traditional therapy with Chinese medicine, the entire safflower is orally administrated in the way of decoction. Safflower oral solution, which is the safflower water soluble extract, was used for the treatment of stroke, coronary heart disease and angina pectoris due to blood stasis.

Safflor yellow consists of hydroxysafflor yellow A (HSYA), safflor yellow B, safflomin A, etc., while HSYA, which is the main active component of safflor yellow, has been demonstrated to have the activities of antioxidation and myocardial and cerebral protective effects (Ji et al., 2009). HSYA was chosen as an active component for controlling the quality of safflower in Chinese pharmacopoeia (The State Pharmacopoeia Commission of China, 2005).

Previous work reported that HSYA could inhibit thrombosis and platelet aggregation and reduce the

*Corresponding author. E-mail: zhjm169@gmail.com. Tel/Fax: +86 2163846590-776476.

myocardial infarction size (Ji et al., 2009). Sun et al. (2010) reported that hydroxysafflor yellow A suppresses thrombin generation and inflammatory responses following focal cerebral ischemia-reperfusion in rats. Ye and Gao (2008) suggested that the protection of HSYA results from, at least in part, suppression of inflammatory responses following focal ischemia reperfusion. However, the exact mechanism of the cardiovascular effect of HSYA is still poorly understood. Accordingly, we explored the pharmacological effects of HSYA on isolated rat thoracic aortas and investigated the underlying mechanisms using the organ bath technique.

MATERIALS AND METHODS

Chemicals and drugs

HSYA was provided by Shanghai Bao Chi Tong Biological Technology Co. Ltd., China. This drug was identified by spectroscopy and its purity (>98%) was determined by HPLC. Its molecular formula is $C_{27}H_{32}O_{16}$ with a molecular weight of 611.16 Daltons. It is soluble in water, with a pH of about 5.0 (Ji et al., 2009). Phenylephrine (PE), acetylcholine (ACh), EGTA (ethylene glycol tetraacetic acid), heparin (HP) and dantrolene (Dan) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). As such, all other reagents were of analytical purity.

Preparation of rat aortas

Male Wistar rats (4 to 6 months old, weighing 250 g on average) were obtained from the Experimental Animal Center , Shanghai Jiaotong University School of Medicine (Shanghai, China). All procedures were approved by the ethics committee for the use of experimental animals in Shanghai Jiaotong University.

Rats were anesthetized with an i.p. injection of 50 mg/kg sodium pentobarbital (Sigma), sacrificed by cervical dislocation and bleeding. The thoracic aorta was rapidly dissected out and immersed in chilled Krebs' solution, following the composition (mM) of: NaCl (118.0), KCl (5), $MgSO_4$ (1.2), KH_2PO_4 (1.2), $CaCl_2$ (2), $NaHCO_3$ (25.0) and glucose (11.0); and bubbled with 95% O_2 and 5% CO_2 (pH 7.4). After excess fat and connective tissue had been removed, the aortic rings, approximately 4 mm in length, were cut. Care was taken to avoid abrading the intimal surface in order to maintain the integrity of the endothelial layer. In some arteries, the endothelium was mechanically removed by gently rubbing the intimal surface of the rings with a metal rod. Two stainless-steel triangles were inserted through each vessel ring with care to preserve the endothelial layer. Each aortic ring was suspended in 10-ml organ baths filled with Krebs' solution, at 37 ± 0.5℃, and bubbled with 95% O_2 and 5% CO_2. One triangle was attached to the bottom of the organ bath and the other was connected to an isometric force transducer (Kent Scientific, Torrington, CT, USA), which was connected to a computerized data acquisition system (PowerLab/8SP, ADInstruments, Castle Hill, NSW, Australia) and recorded on a PC using Chart 5.0 software. Each ring was stretched to a resting tension of 2 g and allowed to equilibrate for 60 to 90 min. During the equilibration period, the solution was replaced every 15 min. Before each experiment, rings were stimulated three times until a reproducible contractile resp onse wa obtained. After equilibration, rings were contracted by PE or 6 $\times 10^{-2}$ s M KCl. Thereafter, concentration-response curves to the HSYA (10^{-6}~10^{-4} M) were constructed by cumulative administration of the drugs. The presence of functional endothelium was verified by the ability of ACh (10^{-5} M) to induce more than 60% relaxation of rings precontracted with PE (10^{-6} M)

Experimental protocols

Effects of HSYA on KCl or PE – induced vascular contraction

To determine the effect of HSYA on plateau state contraction, elicited by 6×10^{-2} M KCl or PE (10^{-9}~10^{-4} M), we measured the responses in vascular tension to the cumulative increasing concentrations of HSYA (10^{-6} ~ 10^{-4} M) in endothelium-intact and -denuded tissues. The HSYA-induced dose-dependent relaxation in aortic rings was calculated as a percentage of the contraction in response to PE and KCl. In endothelium-intact aortic rings, the half-maximum effective concentration (EC_{50}) of HSYA was added to verify the time-dependent vasoactive effect on aortas precontracted with PE and KCl.

HSYA-induced relaxation and Ca^{2+} channels

In the first set of experiments, an attempt was made to verify that the relaxation induced by HSYA involved Ca^{2+} influx. Endothelium-denuded aortic rings were washed 5 times with Ca^{2+}-free Krebs' solution (containing 1 mM EGTA) before PE (3×10^{-6} M) was applied to produce a steady contraction, and then Ca^{2+} was added cumulatively to obtain a concentration-response curve (0 to 2.5×10^{-3} M). As such, the EC_{50} of HSYA was added 30 min before the addition of PE.

In the following experiments, endothelium-denuded rings were exposed to a drug for 30 min before application of PE (3×10^{-6} M) to elicit a steady contraction. The rings were then treated with HSYA (2×10^{-5} M; n = 7 - 9) or were untreated (n = 9 - 12). Observations lasted at least 30 min. The maximal inhibition rates were calculated as a percentage of PE-induced contraction and the drugs used were heparin (HP, 50 mg/L) (Mohanty, 2002) and dantrolene (Dan, 2×10^{-5} M) (Enokizono et al., 2008).

Data analysis

All results are expressed as mean ± S.E.M. Concentration-response curves were constructed based on the responses given by the cumulative concentrations of drugs and were analyzed by the non-linear curve fitting using SigmaPlot 9.0 software (Systat software, CA, USA). The negative logarithm of the drug concentration that produced half the maximum relaxation (EC_{50}) and the maximum response (Emax) were approximated. Statistical comparisons were made using one-way ANOVA followed by Newman–Keuls test. As such, P-values less than 0.05 were considered to be statistically significant.

RESULTS

The effect of HSYA on KCl - precontracted aortas

After KCl (6×10^{-2} M) induced a steady contraction, HSYA was added accumulatively. As such, HSYA (10-7~10-4 M) induced relaxation in a dose-dependent manner in endothelium-intact aortas precontracted by KCl (Figure 1). Resultantly, the maximal inhibition rates and the EC50 values were 40.1% and 1.82×10^{-5} M,

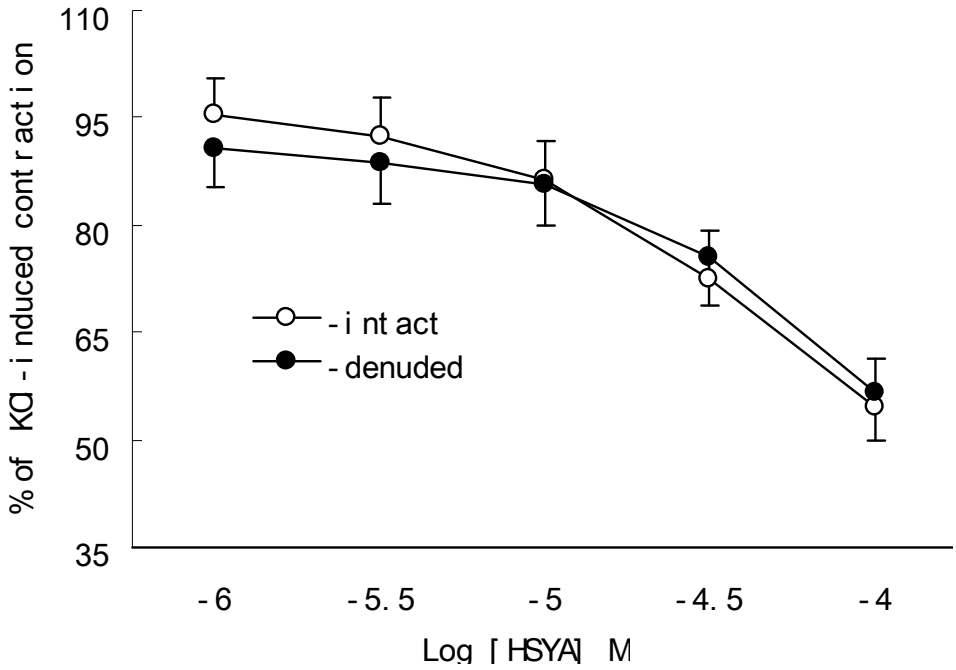

Figure 1. Contraction-relaxation by HSYA in KCl- precontracted rat aortic rings with endothelium-intact or -denuded aortas.

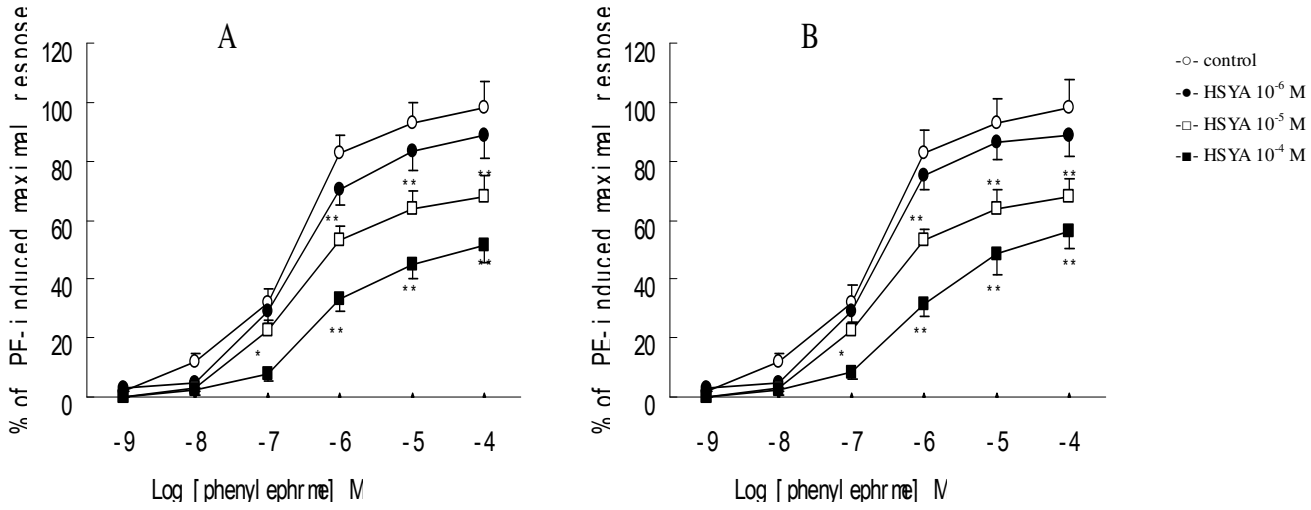

Figure 2. Effects of HSYA on the PE-induced contractile response in rat aortic rings. (A) Endothelium-intact and (B) Endothelium-denuded. *P<0.05, **P<0.01 vs. control group.

respectively. Also, HSYA (10^{-7} ~ 10^{-4} M) induced relaxation in a dose-dependent manner in endothelium-denuded aortas precontracted by KCl (6×10^{-2} M) (Figure 1). Resultantly, the maximal inhibition rates and the EC50 values were 42.3% and 1.74×10^{-5} M, respectively. However, the effects of HSYA did not differ in endothelium-intact and -denuded aortas.

The effect of HSYA on PE - precontracted aortas

The effect of HSYA on PE - precontracted aortas was similar to that on KCl - precontracted aortas (Figure 2). As such, the maximal inhibition rates were 35.4 and 36.8% and the EC50 values were 1.87×10^{-5} and 1.79×10^{5} M for endothelium-intact and endothelium-denuded

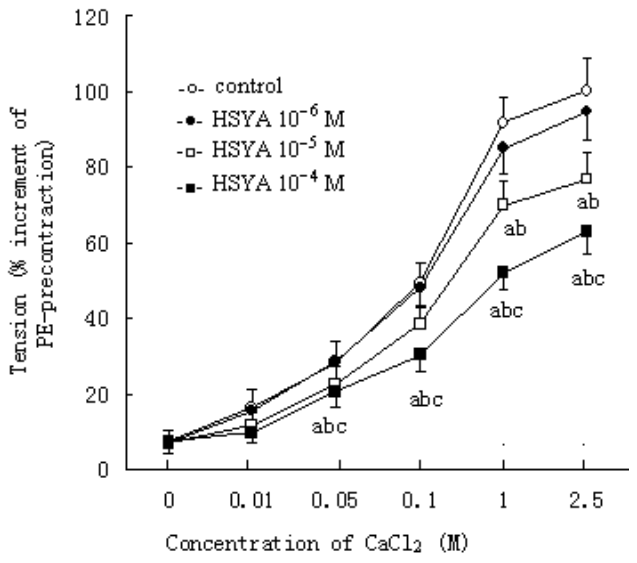

Figure 3. Effects of HSYA on CaCl2 induced contractile response in endothelium-intact aortic rings. (a) P<0.05 vs control group, (b) P<0.05 vs HSYA10-6 group and (c) P<0.05 vs HSYA10-5 group.

aortas, respectively. So the following experiments were carried out in endothelium-denuded aortas except when indicated otherwise and the concentration of HSYA was 2×10^{-5} M.

Relationship between the vasoactive effects of HSYA and the influx of Ca^{2+}

In Ca^{2+}-free Krebs solution, after PE induced a stable aortic contraction and Ca^{2+} (10^{-5} to 3×10^{-3} M) was cumulatively added to the chamber, the tension generated by the aortic rings increased with the concentration of Ca^{2+}. Incubation of the rings with HSYA for 30 min, before PE was applied, inhibited the Ca^{2+}-dependent contraction when compared with the control group and dose-response curve downward (Figure 3).

Relationship between the vasoactive effects of HSYA and the intracellular Ca^{2+} release

In order to determine the effects of IP3 and ryanodine receptors on HSYA-induced vasorelaxation, we administered the IP3 receptor inhibitor heparin and the ryanodine receptors blocker dantrolen, together with HSYA, before applying PE. Both HSYA (2×10-5 M) and HP (50 mg/L) inhibited PE (3×10^{-6} M) induced contraction of arterial rings, with the average inhibition rate of 10.95 and 27.07%, respectively. As such, there was a significant difference between HSYA and HP treatment (P <0.05), [Emax (4.31 ± 0.37) (g) vs (3.53 ± 0.39) (g)]. The average inhibition rate was 25.21% by pretreatment of HP together with HSYA [Emax 3.62 ± 0.41 (g)], but when compared to HP treatment, there was no significant difference (P>0.05). Dan (50 mg/L) alone also inhibited the PE (3×10^{-6} M) induced contraction with the inhibition rate of 19.39%, but when compared to pretreatment with Dan plus HSYA, the Dan treatment was smaller (P <0.05) [Emax (3.95 ± 0.41) (g) vs (3.42 ± 0.34) (g)].

DISCUSSION

Hydroxysafflor yellow A (HSYA), which is the main chemical component of the safflower yellow pigments, plays a pivotal role in the treatment of cardiovascular disease. However, the direct effect of HSYA on the blood vessels and the mechanisms are rarely reported. Previous studies have demonstrated that PAF may trigger platelet release and aggregation when intracellular calcium concentration was increased (Jin et al., 2004). Meanwhile, HSYA is the PAF receptor antagonist that inhibited the increase of PAF-induced platelet calcium concentration and alleviated the blood circulation disorder. Moreover, it is closely related to PE contraction mechanism of vascular smooth muscle cells and the calcium concentration. Nonetheless, it is not clear whether HSYA reduces the calcium concentration of smooth muscle cells and then inhibits the vasoconstriction by PE.

In the present study, we demonstrated that HSYA inhibited PE and KCl induced contraction of rat thoracic aorta in a dose dependent manner, which was not associated with vascular endothelium. These results suggested that HSYA may directly be involved in the mechanisms of vascular smooth muscle construction.

Excitation-contraction coupling (ECC) is a feature of myocyte, which relies on the triplet. The key coupling factor of ECC is calcium. Calcium is necessary in the contraction of the vascular smooth muscle cells, which includes extracellular calcium influx and intracellular calcium release (Said et al., 2008). Extracellular calcium influx may be accomplished through calcium channels and calcium influx in the resting period, in that the latter amount is too small to be ignored. The increase in intracellular calcium then triggers further release of calcium from sarcoplasmic reticulum (SR) stores via ryanodine receptors. The release of calcium can be further enhanced by activation of IP_3 receptor. Calcium released by the SR increases the intracellular calcium concentration from about 10^{-7} to 10^{-5} M (Dohare et al., 2008).

The voltage-dependent calcium channels can be activated by high extracellular KCl, which induces extracellular calcium influx and then causes contraction of smooth muscle cells (Xue et al., 2007). In this study, we observed that HSYA inhibited the KCl-induced contraction of rat thoracic aorta, indicating that HYSA antagonized vasoconstriction through inhibition of the voltage-dependent calcium channels and prevention of the extracellular Ca^{2+} influx.

The PE-induced vasoconstriction was very weak in calcium free buffer. With the increase of calcium concentration, the tension of vasoconstriction also increased. These results indicated that PE-induced contraction is closely related to extracellular calcium influx. Pretreatment with HSYA significantly decreased the CaCl2 -induced contractile response in rat aortic rings, which confirmed that HSYA reduced the PE-induced contraction by inhibition of both voltage gated channels and receptor gated channels.

Phenylephrine (PE) activates both the alpha adrenergic receptors in the vascular smooth muscle cells and the receptor gated Ca2+ channels. As such, it leads the extracellular calcium influx to induce the contraction of rat thoracic aorta. In addition, PE activates protein kinase C (PKC), a ubiquitous family of serine/threonine protein kinases (Gusev and Niggli, 2008). Activated Gq with GTP bound activates its downstream target phospholipase C (PLC) to hydrolyze the membrane lipid (PIP2), producing IP3 and diacylglycerol (DAG). IP3 is water-soluble and diffuses through the cytoplasm to the ER, where it binds to and opens a calcium channel that releases the calcium stored inside the ER into the cytoplasm.

Jia et al. (2009) observed that HSYA had a similar anticoagulant effect with HP, but effect of HSYA was lower than that of HP. We observed that HP group and HSYA+ HP group could inhibit PE-induced contraction, but the inhibition ratios of both groups were higher than HSYA group, indicating that HSYA can partly inhibit the IP3 receptors to reduce the calcium release of sarcoplasmic reticulum, and subsequently decrease the concentration of intracellular calcium and antagonize the contraction of vascular rings. However, the pretreatment of Dan, blocker of ryanodine, together with HSYA had no similar effect with that of HP plus HSYA, indicating that HSYA did not block the ryanodine receptors.

The following mechanisms may be involved in the HSYA inhibited PE-induced endothelium-independent vasoconstriction: (1) HSYA inhibited the extracellular calcium influx and (2) HSYA inhibited the IP3 receptor, but not the ryanodine receptors, on the endoplasmic reticulum of vascular smooth muscle cells to reduce the intracellular calcium release.

In summary, HSYA inhibited KCl and PE-induced contraction of vascular rings, and this had nothing to do with the endothelium. HSYA inhibited the contraction of vascular rings through the inhibition of voltage gated and receptor gated channels of vascular smooth muscle cells and IP3 receptor on sarcoplasmic reticulum, which led to the decrease of extracellular Ca^{2+} influx and intracellular calcium release.

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