Bioorganic & Medicinal Chemistry Letters 20 (2010) 4120–4124

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Hydroxysafflor Yellow A suppresses thrombin generation and inflammatory responses following focal cerebral ischemia–reperfusion in rats

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ARTICLE INFO

Article history:
Received 5 March 2010
Revised 16 May 2010
Accepted 18 May 2010
Available online 11 June 2010

Keywords:
Hydroxysafflor Yellow A
Cerebral ischemia and reperfusion
Thrombin
Inflammation

ABSTRACT

Hydroxysafflor Yellow A has been demonstrated to attenuate pressure overloaded hypertrophy in rats and inhibit platelet aggregation. Herein we found that Hydroxysafflor Yellow A prevented cerebral ischemia–reperfusion injury by inhibition of thrombin generation. In addition, treatment with Hydroxysafflor Yellow A significantly inhibited NF-κB p65 nuclear translation and p65 binding activity, both mRNA and protein levels of ICAM-1 and the infiltration of neutrophils. Mean while, Hydroxysafflor Yellow A had the capacity to improve neurological deficit scores, increase the number of the surviving hippocampal CA1 pyramidal cells and decrease the plasma angiotensin II level. These results illustrated that anti-cerebral ischemic mechanism of Hydroxysafflor Yellow A may be due to its suppression of thrombin generation and inhibition of thrombin-induced inflammatory responses by reducing angiotensin II content.

The flower of safflower plant, *Carthamus tinctorius* L. and the extracts from *C. tinctorius* have been extensively used in traditional Chinese medicine for treatment of cardiocerebrovascular diseases.[1] The extracts attract great attention owing to wide range of biological properties including antitumor, immunodepressive activities associated with chemopreventive properties and they belong to flavonoid compounds. What's more, it has strong antioxidative and anti-inflammatory activities.[2] The extracts contain several different pigments such as Hydroxysafflor Yellow A (abbreviated HSYA; Fig. 1), safflor yellow B (SYB), safflomin A, safflomin C, and prevalent pigments, etc.[3] Among them, HSYA, a pure compound, is the major active chemical component and has the highest water-solubility.

As a newly identified chemical, HSYA has been demonstrated to attenuate pressure overloaded hypertrophy in rats[4] and inhibit platelet aggregation.[5] Moreover, Our previous study reported that HSYA may provide neuroprotection against cerebral ischemia/reperfusion (I/R) injury by its anti-oxidant action.[6] However, its mechanism of action has not been well elucidated. It would be of considerable interest to explore other mechanisms through which HSYA delivers its neuroprotective effect.

Focal cerebral ischemia in stroke is a major disorder with great prevalence today. It leads to irreversible damage in infarcted brain areas. Emerging evidence illustrates that thrombin exerts physiological and pathological functions in post-ischemic cascades.[7]

Increased thrombin in brain has been shown to result in the degeneration of the hippocampal neurons.[8] Both prothrombin and its active form thrombin have been detected locally in human, mouse and rat brain.[9] Thrombin is known to be produced as a result of inflammatory reaction at injury sites[10] and serves as a

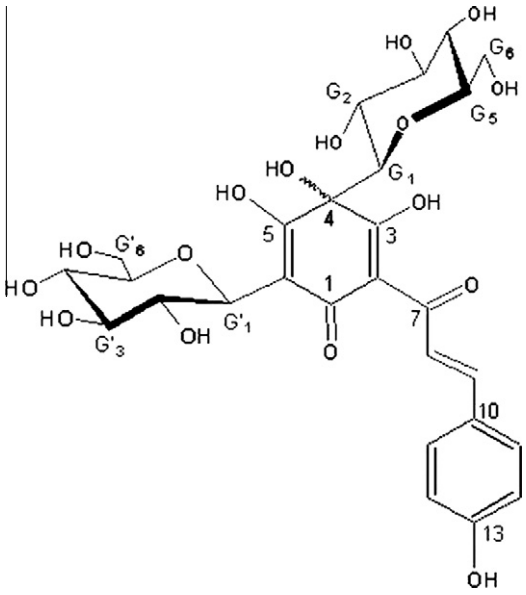

Figure 1. Structural formula of HSYA.

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0960-894X/$ - see front matter © 2010 Published by Elsevier Ltd.
doi:10.1016/j.bmcl.2010.05.076

Table 1
Effects of treatment with HSYA on neurological deficit scores in rats after focal cerebral ischemia–reperfusion injury

Group	Dose (mg/kg)	Neurological deficit scores
Sham + NS	2 ml/kg	0.00 ± 0.00
I/R + NS	2 ml/kg	2.75 ± 0.48[#]
I/R + $HSYA_H$	8	1.25 ± 0.42[*]
I/R + $HSYA_M$	4	1.58 ± 0.52[*]
I/R + $HSYA_L$	2	1.75 ± 0.42[*]

Animals received HSYA (8, 4, 2 mg/kg, respectively, I.V.) or vehicle at 20 min after the onset of ischemia. After 24 h of reperfusion, neurological deficit scores of the rats were performed according to the Longa' method. Data are presented as mean ± SEM. Total *N* used in each group is 12. Groups were compared by one-way ANOVA followed by Bonferroni tests to determine where differences among groups existed.

[#] P <0.05, versus sham group.

[*] P <0.05, versus vehicle-treated I/R group.

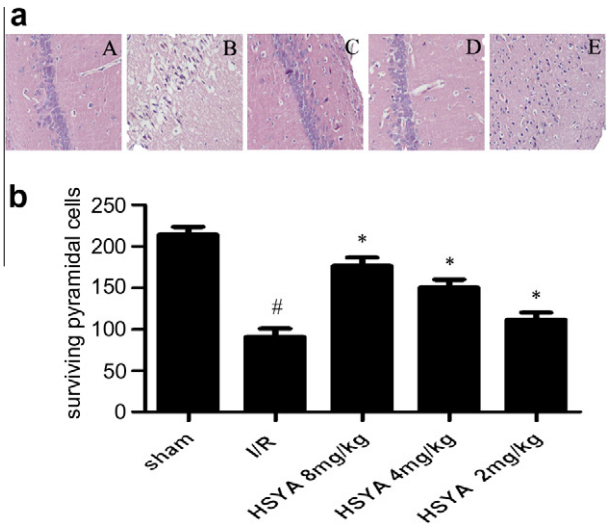

Figure 2. Effects of HSYA (8, 4, 2 mg/kg, respectively, I.V.) on the number of survival hippocampal CA1 pyramidal cells. Brains were quickly removed after 2 h of ischemia/24 h of reperfusion. Histological examination showed that normal pyramidal cells in hippocampal CA1 region which showed round and pale stained nuclei in H&E staining. (a) Morphological examination of the pyramidal cells in hippocampal CA1 region by H&E staining (×400) A: sham group; B: I/R group; C: HSYA 8 mg/kg group; D: HSYA 4 mg/kg group; E: HSYA 2 mg/kg group. (b) Bar figures represent the number of surviving hippocampal CA1 pyramidal cells in each group. Data are presented as mean ± SEM. Total *N* used in each group is 12. Groups were compared by one-way ANOVA. [#]P <0.05, versus sham group; [*]P <0.05, versus vehicle-treated I/R group.

crucial mediators of the inflammatory process through its ability to induce the expression of ICAM-1 and ICAM-dependent endothelial adhesivity toward polymorphonuclear cell (PMN).[11] Taken together, the present study was therefore undertaken to clarify a link between the neuroprotective effect of HSYA and thrombin generation-induced inflammatory responses. HSYA was obtained from *C. tinctorius* as a yellow amorphous powder, $[\alpha]_D^{25} = -54.0$ (*c* 0.1, MeOH). High-resolution ESMS exhibited an $[M-H]^-$ ion peak at *m/z* 611.1614, and the molecular formula was determined to be $C_{27}H_{32}O_{16}$. The ^1H and ^{13}C NMR spectra were consistent with other studies on HSYA. Its purity (>98%) was determined by HPLC. The powder is soluble in water, and the pH is about 5.The drug (8, 4, 2 mg/kg, respectively) was administered 20 min after middle cerebral artery occlusion (MCAO) via caudal vein injection. Sham group and model group rats received vehicle (0.9% NaCl, I.V.).

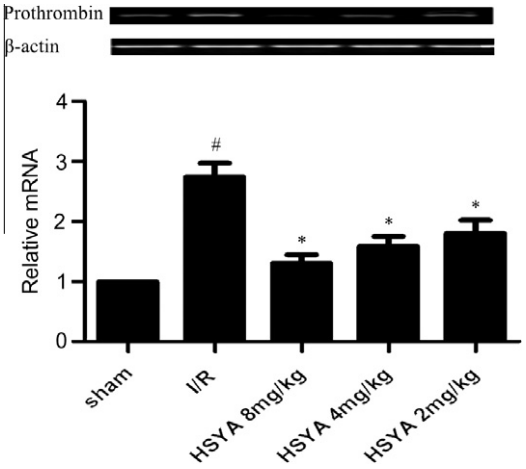

Figure 3. Effects of HSYA (8, 4, 2 mg/kg, respectively, I.V.) treatment on the expression of prothrombin mRNA. Left hippocampus of each rats were quickly removed after 2 h of ischemia/24 h of reperfusion. Semiquantitative RT-PCR analysis with β-actin was used to characterize prothrombin mRNA in the hippocampal formation. Results were representative of at least three independent experiments. [#]P <0.05, versus sham group; [*]P <0.05, versus vehicle-treated I/R group.

Adult male Wistar rats (SPF grade, certificate No.: 2007006, purchased from the Laboratory Animal Center, Shandong University of Traditional Chinese Medicine) weighing 270–320 g were used for experiment. All the animals used in this work received humane care complying with The Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication No.: 85-23, revised 1985.) Transient focal cerebral ischemia was conducted by the MCAO procedure as described by Longa et al.[12] and Wei et al.[6] Occlusion was done for a period of 2 h. For reperfusion the nylon suture was withdrawn. Sham-operated animals received the same surgical procedures without the suture inserted.

In order to test its neuroprotective effects, we examined the neurological deficit score of each rat by using a standard scale for a five-point neurological assessment[12] at 24 h after reperfusion. The result was summarized in Table 1. Neurological deficit scores were significant higher than that of the sham group 24 h after reperfusion. Treatment with HSYA (8, 4, 2 mg/kg, respectively,

Table 2
Effects of treatment with HSYA on F1+2, TAT complexes, Factor VII, and fibrinogen

	Sham group	I/R group	HSYA 8 mg/kg	HSYA 4 mg/kg	HSYA 2 mg/kg
F1+2 (μg/L)	0.28 ± 0.08	1.97 ± 0.44[#]	0.54 ± 0.15[*]	0.80 ± 0.24[*]	1.38 ± 0.31[*]
TAT complexes (μg/L)	1.15 ± 0.31	3.45 ± 0.57[#]	1.48 ± 0.24[*]	1.89 ± 0.34[*]	2.39 ± 0.59[*]
Factor VII (mg/L)	0.76 ± 0.21	1.24 ± 0.34[#]	1.01 ± 0.28	1.19 ± 0.30	1.21 ± 0.31
Fibrinogen (g/L)	1.45 ± 0.35	2.51 ± 0.48[#]	1.55 ± 0.36[*]	1.90 ± 0.45[*]	2.06 ± 0.56[*]

Thrombin generation was assessed as thrombin–antithrombin (TAT) complex and prothrombin activation fragment 1+2 (F1+2) using a commercially available enzyme immunoassay. Factor VII was analyzed with an amidolytic method. Fibrinogen was determined with a polymerization time method. Data are presented as mean ± SEM. Total *N* used in each group is 12. Groups were compared by one-way ANOVA.

[#] P <0.05, versus sham group.

[*] P <0.05, versus vehicle-treated I/R group.