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

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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

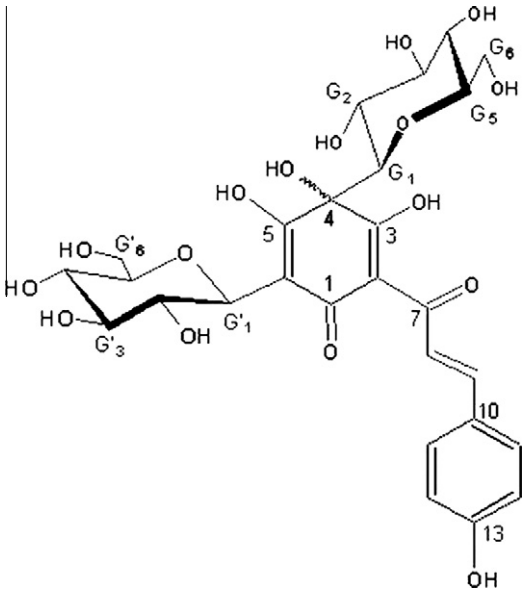

Figure 1. Structural formula of HSYA.

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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.

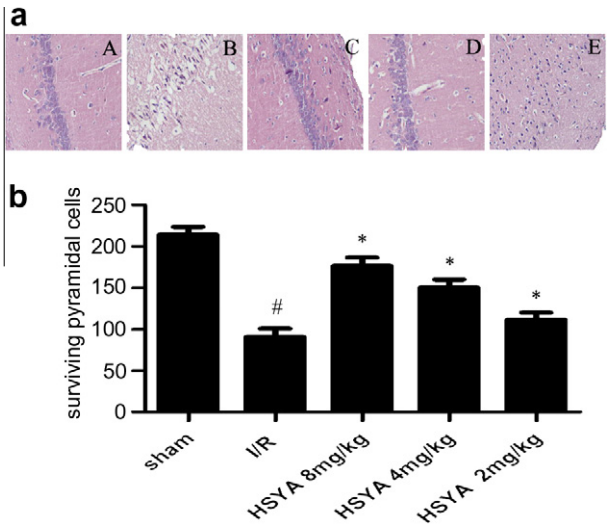

Figure 2. Effects of HSYA (8, 4, 2 mg/kg, respectively, I.V.) on the number of survival hippocampal CA1 pyramidal cells. Brains were quickly removed after 2 h of ischemia/24 h of reperfusion. Histological examination showed that normal pyramidal cells in hippocampal CA1 region which showed round and 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.).

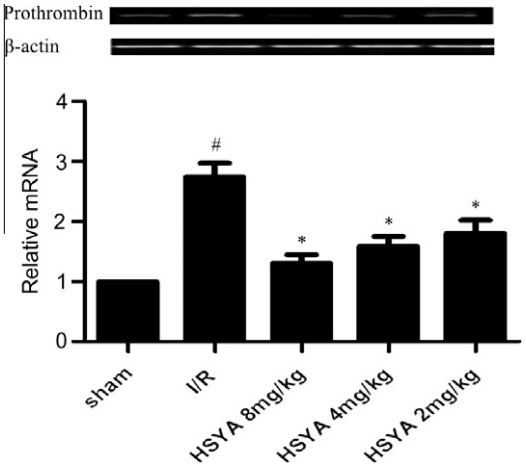

Figure 3. Effects of HSYA (8, 4, 2 mg/kg, respectively, I.V.) treatment on the expression of prothrombin mRNA. Left hippocampus of each rats were quickly removed after 2 h of ischemia/24 h of reperfusion. Semiquantitative RT-PCR analysis with β-actin was used to characterize prothrombin mRNA in the hippocampal 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.

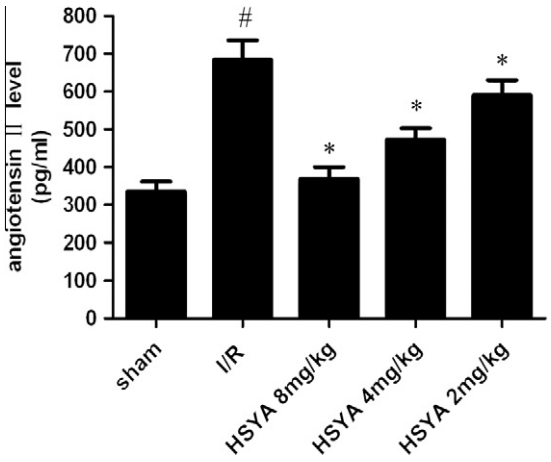

Figure 4. Effects of HSYA (8, 4, 2 mg/kg, respectively, I.V.) on plasma angiotensin II content. Plasma angiotensin II contents were measured by radioimmunoassay. Data are presented as mean ± SEM. Total *N* used in each group is 12. Groups were compared by one-way ANOVA. $^{\#}P$ <0.05, versus sham group; $^{*}P$ <0.05, versus vehicle-treated I/R group.

I.V.) significantly improved neurological function (lower score) compared with the vehicle-treated model group. The result demonstrated that HSYA protects rat brain from I/R injury in a dose-dependent manner.

And then, the number of the surviving hippocampal CA1 pyramidal cells were examined.[13] As shown in Figure 2, the number of survival neurons in hippocampus CA1 region decreased significantly in I/R group in comparison with that in sham-operated group (P <0.05), while animals treated with HSYA (8, 4, 2 mg/kg, respectively, I.V.) obviously increased in viable neurons in comparison with vehicle-treated rats. This result can also partly show HSYA has the ability to alleviate cerebral I/R injury.

Although it is demonstrated that HSYA shows promising neuroprotection against brain ischemic damage,[5] the mechanisms by which HSYA may protect brain ischemic injury have not been well elucidated. We hypothesized that the neuroprotective effect of HSYA might be due to alleviating thrombin damage in ischemic hemisphere. Therefore, we analyzed mRNA of prothrombin from hippocamp, the levels of Plasma prothrombin activation fragment 1+2 (F1+2), thrombin–antithrombin (TAT) complex, Factor VII and fibrinogen as coagulation markers after 24 h reperfusion following 2 h MCAO in rats. Thrombin generation was assessed as F1+2 and TAT complex using a commercially available enzyme immunoassay. Through the detection of plasma F1+2 and TAT complex, it can reflect the activation of thrombin. Factor VII was examined with an amidolytic method which measured Factor VIIa generated after the addition of a thromboplastin activator into the test tube.[14] Fibrinogen was determined with a polymerization time method.[15]

The results were summarized in Table 2 and Figure 3. As expected, it was found that the levels of F1+2, TAT complex and Fibrinogen after cerebral I/R were significantly increased in the vehicle-treated rats compared with sham-operated rats, which could reflect an increase in thrombin generation in rats following cerebral I/R injury. At the same time, the focal ischemia–reperfu-

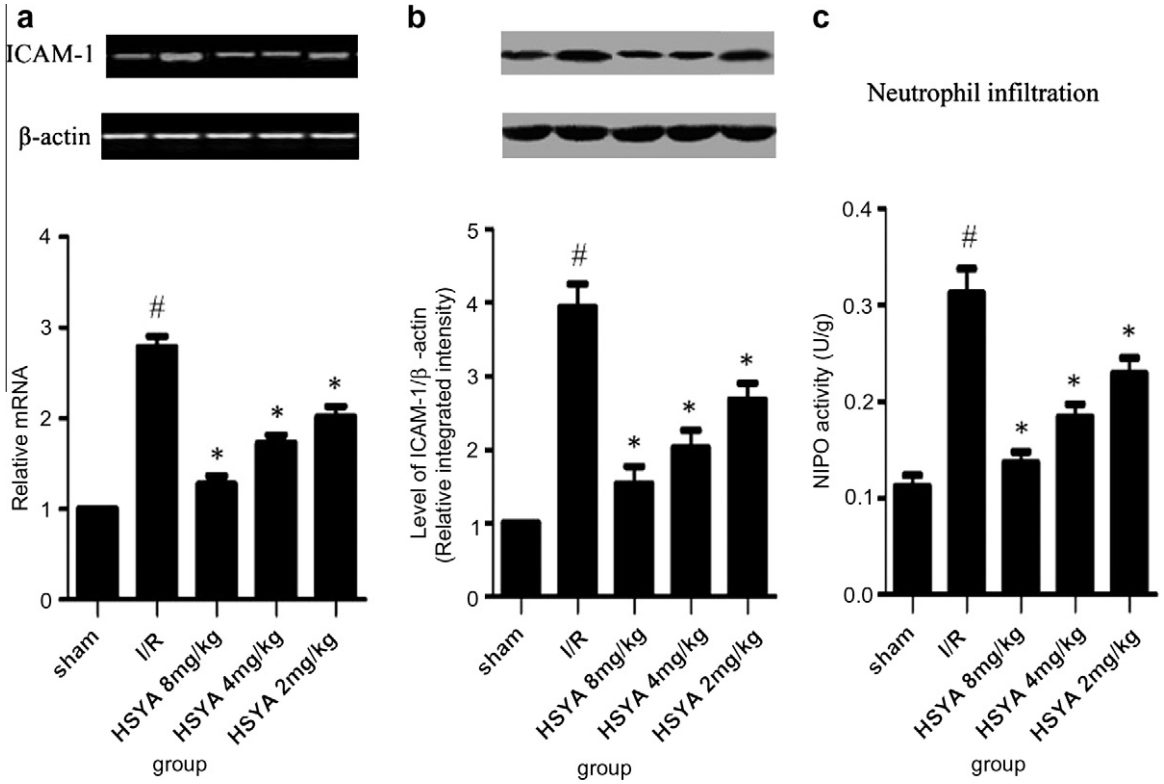

Figure 5. Decreased ICAM-1 and neutrophil infiltration in rats following focal cerebral ischemia–reperfusion by treatment with HSYA (8, 4, 2 mg/kg, respectively, I.V.). Left hippocampus of each rats were quickly removed after 2 h of ischemia/24 h of reperfusion. (a) Semiquantitative RT-PCR analysis of the expression ICAM-1 as a marker of the inflammatory response. Densitometric analysis was normalized to β-actin. Results were representative of at least three independent experiments. (b) The protein levels of ICAM-1 by Western blot were determined with specific antibody. Densitometric analysis was normalized to β-actin. Results were representative of three independent experiments. (c) Bar figures represent the marked depression of MPO activity, that is, the number of infiltrating neutrophils by HSYA (8, 4, 2 mg/kg, respectively, I.V.) treatment after MCAO. The procedures used to quantify MPO activity from rat brain samples were performed according to the description of the kit. Total *N* used in each group is 12. $^{\#}P$ <0.05, versus sham group; $^{*}P$ <0.05, versus vehicle-treated I/R group.

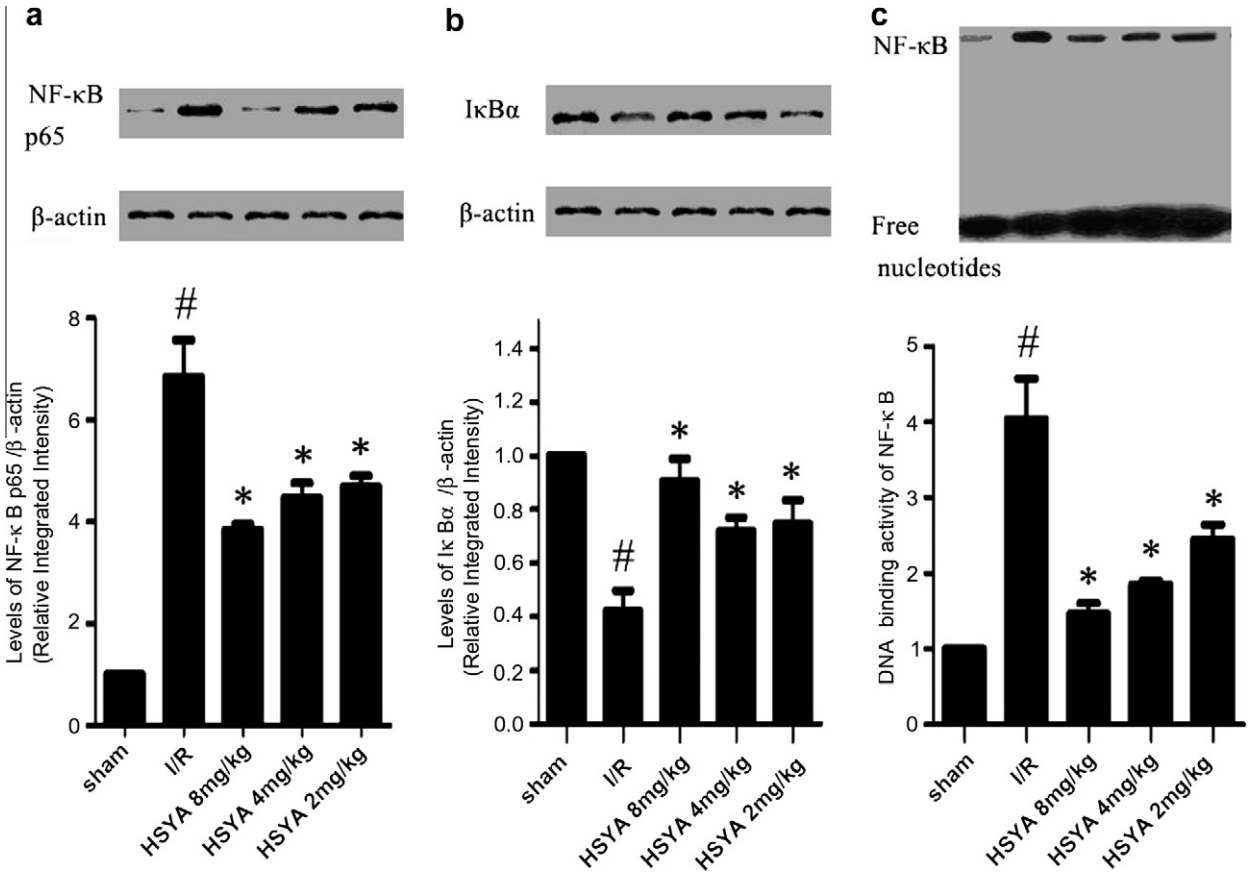

Figure 6. Effects of HSYA (8, 4, 2 mg/kg, respectively, I.V.) on NF-κB p65 subunit nuclear translocation, IκBα protein expression and NF-κB DNA biding activity after 2 h ischemia and 24 h reperfusion. (a) Western blot analysis of NF-κB p65 subunit nuclear translocation in hippocampus after I/R in rats with anti-p65 antibody. Intensity of the NF-κB bands was quantified by densitometry. (b) Western blot analysis of IκBα in hippocampus after I/R in rats with anti-IκBα antibody and the effects of HSYA on it. Loading conditions were confirmed by actin content. Bar figures represent protein expression of IκBα in all groups. (c) Electrophoretic mobility shift assay (EMSA) analysis of NF-κB DNA biding activity in nuclear extracts of rat hippocampus induced by I/R and the effect of HSYA on it. $^{\#}P$ <0.05, versus sham group; $^{*}P$ <0.05, versus vehicle-treated I/R group. Results were representative of three independent experiments.

sion injury induced an up-regulation of prothrombin mRNA in the hippocampal formation. HSYA (8, 4, 2 mg/kg, respectively, I.V.) dramatically decreased the levels of F1+2, TAT complex, and fibrinogen (Table 2), and inhibited the expression of prothrombin mRNA (Fig. 3) compared with model group, but did not influence Factor VII. Furthermore, inhibition of thrombin generation by HSYA cancelled the neurodegeneration on neurological deficits score. The results might give us a better understanding of its inhibition on the formation of thrombosis in vivo.

The molecular mechanism regulating thrombin generation in focal cerebral ischemia is not clear. In this study, plasma angiotensin II was upregulated 24 h of reperfusion in model group, which is in accordance with the alteration of thrombin generation. Plasma angiotensin II contents were analyzed by radioimmunoassay as previously described.[16] Experimental studies suggested that activation of the renin–angiotensin system activated the coagulation system, and Larsson et al observed platelet activation and signs of increased thrombin generation following a short-term intravenous infusion of angiotensin II in healthy volunteers.[17] We speculate angiotensin II caused mild activation of the coagulation cascade with increases in plasma levels of F1+2 and TAT complex. In this regard, thrombin may play important roles in the interplay between angiotensin II and cerebral ischemia–reperfusion. HSYA treatment significantly suppressed the increase of angiotensin II content induced by I/R (Fig. 4). However, further studies are clearly needed to elucidate the possible mechanism(s) of the effect of HSYA on thrombin.

In our experiments, Factor VII levels were not altered by HSYA treatment (Table 2), indicating that the attenuated generation of thrombin was not owing to a decreased availability of Factor VII. Other mechanisms are more likely to be operating. HSYA treatment improves endothelial function in hypoxia and normoxia condition.[18] We speculated that HSYA treatment could improve the anticoagulating properties of the endothelium. For example, through inhibition of platelet activated factor-induced platelet adhesion and aggregation, it can inhibit platelet activation resulting in reduced platelet microparticle formation and attenuated thrombin generation.[19] Further studies are needed to clarify the possible mechanism(s).

Fibrinogen is the major coagulation protein in blood, the precursor of fibrin, and an important determinant of blood viscosity and platelet aggregation.[20] Our observations suggested that reductions in fibrinogen as a result of HSYA inhibition would thus act in an antithrombotic direction, in consistent with the antithrombin effects observed in the present study (Table 2). The reduced thrombin generation and the decrease in fibrinogen concentration that occured during treatment with HSYA could be of importance in reducing the risk of thromboembolic events. It appeared likely that HSYA exerted therapeutic activity in I/R injury benefited from its action of anticoagulation. Indeed, these findings might contribute to the protection on experimental myocardial infarction in rats.[21]

Leucocyte–endothelial interactions play a central role in inflammation.[22,23] To evaluate whether the previously described beneficial effect of HSYA treatment on cerebral I/R can be at least

partially attributed to its anti-inflammatory action, we examined the expression of ICAM-1 mRNA and protein, MPO activity as described.[24,25] MPO activity was used as an indicator of tissue neutrophils accumulation following cerebral I/R. The results were provided in Figure 5. The mRNA expression and protein of ICAM-1, MPO activity were elevated significantly in I/R rats. Otherwise, HSYA treatment could turn over these outcomes. This finding illustrates that HSYA can reduce cerebral I/R injury by inhibiting the post-ischemic inflammatory process.

Our work provides further support of the role of HSYA on inflammatory responses in I/R rats. In order to determine whether HSYA treatment can interfere with the I/R-induced activation of the transcription factor, we evaluated the binding of the NF-κB p65 subunit to DNA in the injured ipsilateral hippocampus of animals as described.[26,27] Results were shown in Figure 6. Accordance with its anti-inflammatory action, and in line with previous observations in rats with cerebral cortex ischemia,[28] we found that the administration of HSYA after MCAO inhibited the p65 translocation activity and the phosphorylation of IκBα. At the same time, HSYA suppressed p65 binding activity. In this study, therefore, these data, taken together with suppression of HSYA on thrombosis formation followed by its inhibition on thrombin generation and inflammatory responses, at least in part, could be used to explain the mechanisms underlying the therapeutic effects afforded by HSYA in cerebral I/R rats.

In brief, intravenous administration of HSYA protected focal cerebral I/R injury in rats and the underlying mechanisms might partly be associated with its inhibitory effects on thrombin generation and thrombin-induced inflammatory responses by reducing angiotensin II content. This finding may provide the basis for future initiates aimed at finding the new treatment target and raising awareness of beneficial outcome of HSYA in patients with ischemia stroke.

Acknowledgments

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13. The rats were anesthetized with 10% chloral hydrate (350 mg/kg, ip), then the rats were transcardially perfused with 100 ml 0.9% NaCl solution and subsequently with 4% paraformaldehyde in 0.1 mol/L phosphate buffer at pH 7.4. Brains were removed and post-fixed for 24 h in 10% formalin. Paraffin sections were prepared and stained with hematoxylin and eosin. The sections were examined with a light microscopy and the number of the surviving hippocampal CA1 pyramidal cells were examined.
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