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Inhibitory effect of rhynchophylline on platelet aggregation and thrombosis

CHEN Chang-Xun, JIN Ruo-Min, Li Yi-Kui, ZHONG Jian, YUE Lei, CHEN Shun-Chao, ZHOU Ji-Yan (*Department of Pharmacology, Shanghai College of Traditional Chinese Medicine, Shanghai 200032, China*)

ABSTRACT Rhynchophylline (Rhy) inhibited rabbit platelet aggregation induced by arachidonic acid (AA), collagen, and ADP. The values of IC_{50} were 0.72, 0.74, and 0.67 mmol · L^{-1}, respectively. Rhy reduced the thromboxane B_2 (TXB_2) generation in PRP induced by collagen but failed to reduce that induced by AA. Rhy suppressed malondialdehyde (MDA) formation in platelet suspension stimulated by thrombin, inhibited the platelet factor 4 (PF4) release. It did not alter intraplatelet cAMP concentration. Rhy 10-20 mg · kg^{-1} iv showed a significant inhibition of venous thrombosis and cerebral thrombosis in rats.

KEY WORDS rhynchophylline; platelet aggregation; thromboxane B_2; malondialdehyde; adenosine cyclic monophosphate; platelet factor 4; thrombosis

Rhynchophylline (Rhy), an active component of a Chinese herbal medicine, is obtained from *Uncaria rhynchophylla* (Miq) Jacks, a Chinese herbal medicine. Our previous study[1] showed that Rhy possessed the action on platelet aggregation in rats and on pulmonary thromboembolism in mice. The present studies described the results in which Rhy was evaluated for its potential as an antiplatelet drug by determining its inhibitory effects on platelet aggregation in rabbits and on experimental thrombosis in rats using acetylsalicylic acid (ASA) as reference.

MATERIALS

Rhy, obtained from Shanghai Institute of Materia Medica, Chinese Academy of Sciences, was dissolved in HCl 0.1 mmol · L^{-1} and diluted with NS (pH 5.5). AA was purchased from Fluka Chemical Co, Switzerland. Adenosine diphosphate (ADP) and 1,1,3,3-tetramethoxy-propane were products of Sigma Chemical Co, USA. Collagen and cAMP RIA kit were provided by Research Departments of Hepatic Cirrhosis and of Nuclear Medicine, Shanghai College of Traditional Chinese Medicine, respectively. TXB_2 RIA kit was purchased from Suzhou Medical College. ASA and thrombin were dissolved in NS. The thiobarbituric acid (TBA) reagent was prepared by mixing 0.8% TBA with 7% perchloric acid at a ratio of 2:1 before use.

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METHODS AND RESULTS

Effect on platelet aggregation Platelet rich plasma (PRP) was obtained from blood of ♂ New Zealand rabbits weighing 2.2 ± *s* 0.1 kg, anticoagulated with sodium citrate (3.8%, 1:10) and centrifuged at 190 × *g* for 8 min. The remaining red cell precipitate of the blood samples was further centrifuged at 1800 × *g* for 10 min to get platelet poor plasma (PPP). The platelet counts of each PRP were adjusted to 4×10^8 / ml. The blood platelet aggregation test was performed according to the method of Born[2]. PRP 0.2 ml was placed in a cuvette and stirred with drug or control solution at 37℃ for 5 min, then aggregating agent 10 μl was added (final concentration: AA 200 μmol · L^{-1}; collagen 40 μg · ml^{-1}, and ADP 4 μmol · L^{-1}). Aggregation was measured with a platelet aggregometer (PAM-2, Danyang Electric Factory, China). The transmission at maximal aggregation after the addition of an aggregating agent was recorded. Rhy caused concentration dependent inhibition of platelet aggregation. On collagen and ADP induced aggregation it was

more potent than ASA. The values of IC_{50} were 0.74 and 0.67 mmol · L^{-1} compared to 2.89 and 2.05 mmol · L^{-1} of ASA respectively. On the AA induced aggregation it was less potent, with the IC_{50} of 0.72 mmol · L^{-1} compared to 0.15 mmol · L^{-1} of ASA (Tab 1).

Tab 1. Effects of rhynchophylline (Rhy) on rabbit platelet aggregation induced by AA, collagen, and ADP. $n=7$, $\bar{x} \pm s$, $^{*}P>0.05$, $^{**}P<0.05$, $^{***}P<0.01$ *vs* **control.**

Drug / mmol · L^{-1}		Platelet aggregation (%) AA	collagen	ADP
Control	0	81 ± 10	82 ± 11	52 ± 14
Rhy	0.33	74 ± 16*	75 ± 14*	45 ± 14*
	0.65	60 ± 15**	61 ± 19**	34 ± 12**
	1.30	3 ± 4***	6 ± 10***	2 ± 5***
ASA	0.07	75 ± 11*		
	0.14	51 ± 12***		
	0.28	8 ± 12***		
	0.69		72 ± 19*	45 ± 15*
	1.39		64 ± 19*	34 ± 18*
	2.78		39 ± 20**	20 ± 13***

Effect on TXB_2 generation in PRP PRP 0.2 ml and control or drug solution 10 μl were incubated at 37℃ for 5 min, and then AA (200 μmol · L^{-1}) or collagen (40 μg · ml^{-1}) was added. The mixture was stirred for 5 min and incubated for further 1 min. The reaction was terminated by addition of the same volume of 2% EDTA and placing the tube in an ice bath. After 1800 × g for 10 min, TXB_2 in the supernatant was assayed using the RIA kit according to the procedure described by the manufacturer. Rhy 0.65–1.30 mmol · L^{-1} markedly reduced the TXB_2 generation in rabbit PRP induced by collagen but was devoid of significant influence on AA induced generation, while ASA inhibited both collagen and AA induced TXB_2 generation (Tab 2).

Effect on MDA production in rat platelets The method is a modification described by Umetsu *et al*[3]. PRP was prepared from the blood of Sprague–Dawley (SD) ♂ rats weighing 355 ± s 38 g. Platelets were collected after PRP 750 × g for 10 min and washed with Ca^{2+} free Tyrode's solution containing 12.9 mmol · L^{-1} sodium citrate. The platelets were resuspended in Tyrode's solution containing 0.9 mmol · L^{-1} $CaCl_2$ and adjusted to 4 × 10^8 / ml. Control or drug solution 0.1 ml was added to 1 ml of platelet suspension. This was incubated at 37℃ for 5 min. The reaction was carried out by adding thrombin 1.7 IU · ml^{-1}, with a stirring bar driven by a magnet at 1000 rpm for 5 min, and incubated for further 10 min. The reaction was stopped by the addition of 1 ml of TBA reagent. It was then heated in boiling water for 15 min and centrifuged after cooling. Fluorescence of the supernatant was measured on a fluorescence spectrophotometer (MPF–4, Hitachi, Japan) at 533 nm for excitation and at 553 nm for emission using a standard curve of MDA. The blank tube was made by adding 1 ml of TBA reagent first and then thrombin. Rhy concentration–dependently inhibited the MDA production caused by thrombin in rat platelets with the IC_{50} of 0.71 mmol · L^{-1} (Tab 3).

Tab 2. Effect of rhynchophylline (Rhy) on AA– and collagen–induced TXB_2 in rabbit platelet rich plasma. $n=7$, $\bar{x} \pm s$, $^{*}P>0.05$, $^{***}P<0.01$ *vs* **control.**

Drug / mmol · L^{-1}		Thromboxane B_2 / ng · ml^{-1} AA	collagen
Control	0	186 ± 64	11.0 ± 7.5
Rhy	0.65	190 ± 72*	1.3 ± 1.4***
	1.30	176 ± 64*	0.4 ± 0.9***
ASA	0.28	2 ± 6***	0.1 ± 0.2***

Effect on platelet cAMP level in rats The rat platelet suspension was prepared as described above. Platelet suspension 0.2 ml was incubated with control or drug solution 20 μl at 37℃ for 10 min. Acetic acid buffer solution 0.2 ml (pH 4.75, containing 4 mmol

Tab 3. Effects of rhynchophylline (Rhy) on MDA produced by thrombin in rat platelets. $n=7$, $\bar{x} \pm s$, $^{***}P<0.01$ *vs* control.

Drug / mmol · L^{-1}		MDA / μmol · L^{-1}	Inhibition rate / %
Control	0	2.14 ± 0.23	
Rhy	0.33	1.48 ± 0.29***	30.8
	0.65	1.19 ± 0.24***	44.4
	1.30	0.68 ± 0.12***	68.2
ASA	2.78	1.04 ± 0.22***	51.4

· L^{-1} EDTA) was added to terminate the incubation. According to the method described by Tawata *et al*[4], the indubated platelet suspension were boiled for 3 min, cooled and 1800 × *g* for 10 min. cAMP concentrations in the supernatants were determined by RIA. The results showed that the concentrations of cAMP for control, Rhy 0.65 and 1.30 mmol · L^{-1} groups were 12.13 ± 2.22, 12.71 ± 1.79, and 13.17 ± 2.46 pmol / 4 × 10^8 platelets respectively. Rhy was devoid of significant influence on intraplatelet cAMP concentration ($n=12$, $P>0.05$ *vs* control).

Effect on PF4 liberation The biological activity of PF4 was determined by evaluating the antiheparin activity in a thrombin clotting time[5]. PRP was prepared from SD ♂ rats weighing 465 ± *s* 32 g. The platelet counts were adjusted with PPP to 4 × 10^8 / ml. Drug or control solution 50 μl was added to PRP 0.5 ml. It was incubated at 37℃ and stirred with a magnetic bar at 1000 rpm. Five min after adding the collagen (40 μg · ml^{-1}) or ADP (4 μmol · L^{-1}) the reaction was stopped. After cooling it in ice bath, the mixture was centrifuged at 1700 × *g* for 10 min to produce a supernatant of PPP. To PPP 0.1 ml, heparin (0.6 IU · ml^{-1}) 0.1 ml was added, and heparin thrombin clotting time (HTCT) was recorded. Both Rhy and ASA caused prolongation of HTCT. Rhy suppressed the PF4 liberation from platelets markedly, the effect of ASA was weaker than that of Rhy (Tab 4).

Tab 4. Effect of rhynchophylline (Rhy) on platelet release reaction stimulated by ADP and collagen. $n=7$ rats. $\bar{x} \pm s$, $^{**}P<0.05$, $^{***}P<0.01$ *vs* control.

Drug / mmol · L^{-1}		HTCT / s ADP	HTCT / s collagen
Control	0	27 ± 4	30 ± 3
Rhy	0.65	38 ± 3***	48 ± 11***
	1.30	62 ± 5***	> 180***
ASA	2.78	40 ± 2***	38 ± 4**

Effect on experimental venous thrombosis The model of venous thrombosis was derived from Reyers *et al*[6]. SD rats weighing 210 ± *s* 22 g were anesthetized by ip 40 mg · kg^{-1} sodium pentobarbital. Fifteen min later, a midline incision of the abdomen was made and inferior vena cava was isolated and ligated below the left renal vein level. The abdomen was then closed. One h after ligation, drug or control solution was administered via the dorsal tail vein. One h later, the abdomen was reopened. The thrombus in the inferior vena cava was removed and put into a glass dish for measurement of wet weight. It was then placed in a drying oven at 50℃ for 20 h before measuring the dry weight. Rhy significantly inhibited the venous thrombosis. ASA showed a weaker effect on it (Tab 5).

Effect on cerebral thrombosis Experimental cerebral thrombosis was performed in a modified method according to Cahn *et al*[7]. SD ♂ rats weighing 199 ± *s* 25 g were anesthetized by ip 40 mg · kg^{-1} sodium pentobarbital. Drug or control solution was injected through the femoral vein. After fifteen min, a mixture of collagen 1 mg · ml^{-1}, ADP 2 mg · ml^{-1} and adrenaline 100μmol · L^{-1} was injected into the right carotid artery at a dose of 1 ml · kg^{-1}. Eight min later, 5 ml · kg^{-1} 1% Evans blue was injected via the same artery. The animals were killed after another 8 min and the right hemisphere of brain was excised and homogenized with a mixture (4

Tab 5. Effect of rhynchophylline (Rhy) on venous thrombosis in rats. $n=8$, $\bar{x}\pm s$, $^{**}P<0.05$, $^{***}P<0.01$ *vs* **control.**

Drug / mg · kg^{-1}		Thrombus weight / mg Wet	Inhibition(%)	Dry	Inhibition(%)
Control	0	9.8±2.2		3.2±0.6	
Rhy	10.0	5.8±1.2**	42.1	1.8±0.4***	43.8
	20.0	3.2±1.2**	67.3	1.1±0.3***	65.6
ASA	30.0	7.8±0.9**	20.4	2.4±0.4***	25.0

ml / g brain) of acetone and water (7:3). After standing for 2 h, the homogenate was centrifuged. The absorbance of the supernatants at 620 nm on a 721 spectrophotometer were measured. Rats of normal group were managed by the same operative procedures except injection of platelet aggregating agents into the right carotid artery. The concentration of the dye in the right hemisphere of model control rats was obviously higher than that of normal rats. Rhy 20 mg · kg^{-1} iv reduced the concentration of the dye in the infarct hemisphere. ASA 30 mg · kg^{-1} iv only slightly reduced the concentration. These results revealed that Rhy inhibited the experimental cerebral thrombosis in rats but ASA did not (Tab 6).

Tab 6. Effect of iv rhynchophyl line (Rhy) on cerebral thrombosis in rats induced by injecting the mixture of collagen, ADP and adrenaline via carotid artery. $n=8$, $\bar{x}\pm s$, $^{*}P>0.05$, $^{***}P<0.01$ *vs* **control.**

Drug / mg · kg^{-1}		Absorbance / (g brain)
Control	0	0.110±0.041
Rhy	10.0	0.079±0.013*
	20.0	0.059±0.010***
ASA	30.0	0.077±0.025*
Normal	0	0.043±0.012***

DISCUSSION

The present study confirmed that Rhy inhibited the platelet aggregation. It was more potent than ASA in inhibiting collagen- and ADP-induced rabbit platelet aggregation, but less potent than ASA in AA-induced aggregation. Rhy reduced the TXB_2 generation in rabbit PRP induced by collagen but had no obvious influence on AA-induced TXB_2 generation. These results indicate that, unlike ASA, Rhy do not block the activity of cyclo-oxygenase, it may effect some processes which occur from the stimulation of platelet membrane to release of AA. Thrombin also stimulates platelet membrane to release AA, which is the precursor of MDA. MDA production in platelets may be the index for production of TXA_2. It showed that Rhy markedly impeded the MDA formation stimulated by thrombin. Rhy did not increase platelet cAMP level, thus, it does not seem to exert its antiplatelet action by activation of platelet adenylate cyclase. PF4 is a protein stored in the alpha granules of platelets and released during their activation. Rhy suppressed the release of PF4. Platelet activation plays an important role in thrombosis. In order to prove its antithrombotic action, we studied Rhy with experimental models of thrombosis. Rhy was reported to possess an effect on hypertension. It decreased the blood pressure of cats at the dose of 20 mg · kg^{-1} iv and of hypertensive rats at 20 mg · kg^{-1} ip[8]. The present data showed that Rhy at about the effective dose of antihypertension limited the extension of venous thrombi and inhibited the cerebral thrombosis. The reason that ASA showed a lesser effect on venous thrombosis and failed to modify cerebral thrombotic infarct may be due to its inhibiting

PGI_2 activity from the vessel wall. Our previous studies[1] proved that Rhy did not reduced the plasma PGI_2 level.

In conclusion, Rhy inhibited the platelet aggregation and thrombosis. The mechanism may be due to the suppression of AA liberation from platelet membrane and the reduction of other release products. Rhy may be a promising antithrombotic drug.

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钩藤碱对血小板聚集和血栓形成的抑制作用

陈长勋、金若敏、李仪奎、钟 健、岳 锷、陈顺超、周吉燕 (上海中医学院药理教研室，上海 200032，中国)

提要 钩藤碱抑制 AA，胶原和 ADP 诱导的兔血小板聚集，IC_{50} 分别为 0.72，0.74 及 0.67 mmol · L^{-1}. 钩藤碱能抑制胶原诱导兔血小板生成血栓素 B_2，但对 AA 诱导血栓素 B_2 的生成无影响. 钩藤碱抑制凝血酶诱导血小板生成丙二醛，抑制血小板因子 4 释放，对血小板内 cAMP 浓度无影响. 静脉注射 10–20 mg · kg^{-1} 钩藤碱明显抑制大鼠静脉血栓及脑血栓形成.

关键词 钩藤碱；血小板聚集；血栓素 B_2；丙二醛；腺苷环一磷酸；血小板因子 4；血栓形成