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Studies on Scutellariae Radix. XII. Anti-thrombic Actions of Various Flavonoids from Scutellariae Radix

MICHINORI KUBO,[a] HIDEAKI MATSUDA,*,[a] TADATO TANI,[b] SHIGERU ARICHI,[b] YOSHIYUKI KIMURA[c] and HIROMICHI OKUDA[c]

Faculty of Pharmaceutical Sciences, Kinki University,[a] Kowakae, Higashiosaka, Osaka 577, Japan, The Research Institute of Oriental Medicine, Kinki University,[b] Sayama-cho, Minamikawachi-gun, Osaka 589, Japan and 2nd Department of Medical Biochemistry, School of Medicine, Ehime University,[c] Shigenobu-cho, Onsen-gun, Ehime 791-02, Japan

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The flavonoid components, baicalein, baicalin, wogonin, wogonin-7-*O*-D-glucuronide, oroxylin A, skullcapflavone II, chrysin, (2*S*)-2′,5,6′,7-tetrahydroxyflavanone and (2*R*,3*R*)-2′,3,5,6′,7-pentahydroxyflavanone, obtained from the roots of *Scutellaria baicalensis* have been screened in comparison with two standard anti-thrombic agents, aspirin and heparin, for activity in experimental models of blood platelet aggregation or conversion of fibrinogen to fibrin.

Baicalein, wogonin, oroxylin A, skullcapflavone II and chrysin at a concentration of 1.0 mM inhibited collagen-induced blood platelet aggregation. Chrysin was also found to inhibit adenosine diphosphate (ADP)-induced blood platelet aggregation. In the case of arachidonic acid-induced blood platelet aggregation, baicalein and wogonin were found to have an inhibitory action. Baicalein and baicalin among the test substances inhibited the conversion of fibrinogen to fibrin induced by thrombin.

The effects of baicalein and baicalin on the experimental disseminated intravascular coagulation (DIC) induced by endotoxin in rats was examined; both compounds prevented the decrease of blood platelets and fibrinogen in DIC rats.

Keywords——*Scutellaria baicalensis*; baicalein; baicalin; wogonin; blood platelet aggregation; fibrin; disseminated intravascular coagulation

Scutellariae Radix ("Ogon" in Japanese), the roots of *Scutellaria baicalensis* GEORGI (Labiatae), has been used in Chinese medicine as a remedy for inflammation, suppurative dermatitis, allergic diseases, hyperlipemia and arteriosclerosis. In the previous paper,[1] we reported that the 70% methanolic extract inhibited the endotoxin-induced disseminated intravascular coagulation (DIC) in hyperlipemic rats and its fractions inhibited blood platelet aggregation and the conversion of fibrinogen to fibrin induced by thrombin.

The present paper deals with a study on the anti-thrombic actions of various flavonoids obtained from Scutellariae Radix.

Materials and Methods

Materials——The various flavonoids, baicalein, baicalin, wogonin, wogonin-7-*O*-D-glucuronide, oroxylin A, skullcapflavone II, chrysin, (2*S*)-2′,5,6′,7-tetrahydroxyflavanone (Comp. 1) and (2*R*,3*R*)-2′,3,5,6′,7-pentahydroxyflavanone (Comp. 2), of Scutellariae Radix were isolated by the method described in our previous papers.[2-4] The sources of materials were as follows: endotoxin (*Escherichia coli* 055:B5, Difco Lab., U.S.A.), thrombin (Mochida Ltd., Japan), urokinase (Midorijuji Ltd., Japan), collagen, adenosine diphosphate (ADP) disodium salt and arachidonic acid (Sigma Chemical Co., U.S.A.).

Animals——Male Wistar-King strain rats weighing between 150—200 g were used for the experiments involving

endotoxin-induced DIC and blood platelet aggregation. They were fed a standard diet (Nihon Clea, Japan) for a minimum period of 7 d and then fasted for 24 h before the start of the experiments.

Blood Platelet Aggregation——Whole blood samples were collected into plastic syringes by heart puncture from rats anesthetized with pentobarbital. Next, 9 ml of blood and 1 ml of heparin solution (10 U/ml) were transferred into plastic tubes. Platelet-rich plasma (PRP) was obtained by centrifugation of the mixture at 1000 rpm for 10 min. PRP was removed with a siliconized pipet, and samples were stored in capped plastic test tubes. The samples were gently stirred at 5—10 °C for 30 min prior to use. The red cell portion of the samples was centrifuged a second time at 3000 rpm for 30 min to produce platelet-poor plasma (PPP) that was used as the maximal transmittance standard.

The experiments on platelet aggregation were performed by the method of Born *et al.*[5] Aggregating agents used were collagen (500 μg/ml), ADP (0.05 μM) and arachidonic acid (50 mM). A 0.2 ml aliquot of PRP was placed in a tube and stirred at 1200 rpm, 37 °C, for 1 min, followed by addition of a 10 μl aliquot of the test solution. The control solution was dimethylsulfoxide (DMSO). After 1 min, an aggregating agent was added to the reaction mixture. Platelet aggregation was monitored by continuous recording of light transmittance in a Husm System platelet aggregometer (Rika Electric Co., Japan). The extent of aggregation was estimated from the percent increase in the transmission at maximal aggregation after addition of the aggregating agent.

Conversion of Fibrinogen to Fibrin Induced by Thrombin——Fibrinogen (500 mg) was dissolved in 100 ml of 0.05 M Tris–acetate buffer (pH 7.4) in 0.15 M NaCl. A test solution (0.1 ml) was added to 1.8 ml of a fibrinogen solution with stirring. After 1 min, 0.1 ml of thrombin solution (0.2 U/ml) was added and the whole was gently stirred until a fibrin clot appeared. The time required for clotting was recorded.

Endotoxin-Induced DIC——Experimental DIC was induced by a modification of the method of Schoendorf *et al.*[6] Baicalein, baicalin and wogonin (20 or 50 mg/kg) were administered orally to the rats 1 h before the injection of endotoxin (0.1 mg/kg) into the tail vein. Blood samples were withdrawn from the heart into plastic syringes at 4 h after the injection of endotoxin, while the rats were anesthetized with pentobarbital. As an anticoagulant, a 0.01 M solution of the sodium salt of ethylenediaminetetraacetic acid (EDTA) was used for platelet counts and a 1 : 9 volume of 3.8% sodium citrate for prothrombin time and fibrinogen determination.

Platelets were counted with an automatic blood cell counter (Coulter counter, model S-Plus, Coulter Co., U.S.A.). Fibrinogen was determined according to the method of Quick.[7] The prothrombin time was measured with a COAG-A-Mate dual-channel device (General Diagnostic, Warner–Lambert Co., U.S.A.). Fibrin degradation product (FDP) was determined by means of the latex aggregation test (FDPL test U, Teikoku Zoki, Japan).

Results

Collagen-Induced Blood Platelet Aggregation

As shown in Table I, 1.0 mM aspirin as an anticoagulant agent inhibited collagen-induced blood platelet aggregation. Oroxylin A and skullcapflavone II among the test substances showed a stronger inhibitory effect than aspirin. Baicalein, wogonin and chrysin at a concentration of 1.0 mM also inhibited the blood platelet aggregation induced by collagen.

Table I. Effects of Various Flavonoids and Aspirin on Collagen-Induced Blood Platelet Aggregation

Treatment	Inhibition (%)		
	0.1	0.5	1.0 (mM)
Baicalein		19.5±1.9	23.3±1.7
Baicalin			12.4±1.9
Wogonin		5.9±2.0	24.7±3.0
Wogonin-7-*O*-D-glucuronide			8.9±1.6
Oroxylin A	5.8±2.4	34.3±2.1	43.8±2.0
Skullcapflavone II		5.4±1.4	32.5±3.1
Chrysin		4.7±1.5	23.4±2.7
Comp. 1			1.9±0.7
Comp. 2			7.2±1.5
Aspirin		8.0±2.1	21.6±2.5

Each value represents the mean±S.E. of 5 experiments.

ADP-Induced Blood Platelet Aggregation

As shown in Table II, the incubation of aspirin (1.0 mM) with PRP did not produce any inhibitory effect on blood platelet aggregation induced by ADP. Only chrysin among the test substances had an inhibitory effect on blood platelet aggregation induced by ADP.

Arachidonic Acid-Induced Blood Platelet Aggregation

As shown in Table III, aspirin at a concentration of 1.0 mM inhibited arachidonic acid-induced blood platelet aggregation. The inhibitions by baicalein, baicalin and wogonin at a concentration of 1.0 mM were $47.7 \pm 1.9\%$, $31.3 \pm 2.4\%$ and $45.5 \pm 2.5\%$, respectively.

Conversion of Fibrinogen to Fibrin Induced by Thrombin

As shown in Table IV, the clotting time of the control without addition of any test solution was 193 ± 4 s. The clotting time was prolonged significantly by incubation with 10 U/ml of heparin, an anti-thrombin agent, before addition of thrombin. Only baicalein and baicalin among the substances prolonged the clotting time of fibrinogen.

Endotoxin-Induced DIC

It was shown that DIC could be induced by injection of endotoxin (0.1 mg/kg) into the tail vein, resulting in a decrease of blood platelets and fibrinogen, prolongation of the prothrombin time and an increase of FDP. Before the injection of endotoxin, 20 or 50 mg/kg of baicalein or baicalin was administered orally, and the preventive effect against the

TABLE II. Effects of Various Flavonoids and Aspirin on ADP-Induced Blood Platelet Aggregation

Treatment	Inhibition (%)	
	0.5	1.0 (mM)
Baicalein	10.2±3.1	18.1±2.1
Baicalin		3.2±1.1
Wogonin		15.2±2.2
Wogonin-7-*O*-D-glucuronide		2.8±1.0
Oroxylin A		5.8±1.1
Skullcapflavone II		14.3±2.4
Chrysin	10.8±2.1	27.3±1.9
Comp. 1		15.2±1.8
Comp. 2		7.9±1.4
Aspirin		16.7±3.0

Each value represents the mean±S.E. of 5 experiments.

TABLE III. Effects of Various Flavonoids and Aspirin on Arachidonic Acid-Induced Blood Platelet Aggregation

Treatment	Inhibition (%)		
	0.1	0.5	1.0 (mM)
Baicalein	15.2±3.2	33.7±2.0	47.7±1.9
Baicalin		12.3±2.3	31.3±2.4
Wogonin	13.8±1.8	30.4±2.7	45.5±2.5
Wogonin-7-*O*-D-glucuronide		7.8±1.1	20.1±1.7
Aspirin		16.7±3.5	30.4±2.5

Each value represents the mean±S.E. of 5 experiments.

TABLE IV. Effects of Various Flavonoids and Heparin on the Conversion of Fibrinogen to Fibrin Induced by Thrombin

Treatment	Clotting time of fibrinogen(s)				
	0	0.05	0.1	0.5	1.0 (mM)
Control	193±4				
Baicalein		197±3	253±5[a)]	413±7[a)]	602±5[a)]
Baicalin		199±5	243±5[a)]	478±3[a)]	622±7[a)]
Wogonin					213±7
Wogonin-7-*O*-D-glucuronide					204±3
Oroxylin A					201±8
Skullcapflavone II					194±5
Chrysin					204±3
Comp. 1					191±4
Comp. 2					197±5
Heparin (10 U/ml)	281±6[a)]				

Each value represents the mean±S.E. of 5 experiments. Significantly different from control, *a*) $p<0.01$.

TABLE V. Effects of Flavonoid Components (Baicalein and Baicalin) and Aspirin on the Endotoxin-Induced DIC in Rats

Treatment	Dose	Route	No. of rats	Blood platelets ($\times 10^4$/mm^3)	Fibrinogen (mg/dl)	Prothrombin time (s)	FDP (μg/ml)
Normal		*p.o.*	8	88±4	179±22	19.4±2.4	3±1
Control		*p.o.*	8	34±4	50±9	32.4±3.9	45±8
Baicalein	20 mg/kg	*p.o.*	8	39±8	65±18	25.7±2.9	41±11
Baicalein	50 mg/kg	*p.o.*	8	41±8	90±18[a)]	29.7±3.4	63±19
Baicalin	20 mg/kg	*p.o.*	8	36±8	58±12	30.1±6.1	46±15
Baicalin	50 mg/kg	*p.o.*	8	42±5	82±13[a)]	28.6±4.5	35±10
Normal		*p.o.*	8	82±12	280±17	12.6±0.3	2±1
Control		*p.o.*	8	32±5	77±12	26.7±3.2	60±17
Aspirin	50 mg/kg	*p.o.*	8	44±4[a)]	141±17[b)]	24.4±1.7	43±10

Each value represents the mean±S.E. Significantly different from control, *a*) $p<0.05$, *b*) $p<0.01$.

endotoxin-induced DIC was examined (Table V).

The blood platelets count was $88\pm4\times10^4$/mm^3 in normal rats injected with saline only. It was reduced to $34\pm4\times10^4$/mm^3 in rats injected with 0.1 mg/kg of endotoxin. When rats were orally given 50 mg/kg of baicalein or baicalin, the reduction of the blood platelet count was smaller. The level of fibrinogen was 179±22 mg/dl in normal rats given saline only. The level decreased to 50±9 mg/dl in DIC rats. The decrease of fibrinogen level was significantly less in rats orally given 50 mg/kg of baicalein or baicalin. Prothrombin time was 19.4±2.4 s in the normal rats, and was prolonged to 32.4±3.9 s in the DIC rats. No shortening of prothrombin time was observed in rats orally given 20 or 50 mg/kg of baicalein or baicalin as compared with the control. The FDP level was 3±1 μg/ml in normal rats injected with saline only. The level increased to 45±8 μg/ml in the DIC rats. When 20 or 50 mg/kg of baicalein or baicalin was administered to rats 1 h before the injection of endotoxin, the FDP level was not reduced.

A clear preventive effect of aspirin (used as a standard drug) was recognized on the changes in blood platelets and fibrinogen, but not on those in prothrombin time and FDP.

Discussion

Scutellariae Radix is believed to be effective in the treatment of diseases such as inflammation, hyperlipemia and arteriosclerosis. It is well known that the syndrome of DIC (closely related to arteriosclerosis and hyperlipemia) is an acquired hemorrhagic disorder characterized by the apparent activation and pathologic consequences of fibrin deposition in the microcirculation. In the previous paper,[1] studies were conducted on the effect of the 70% methanolic extract and its fractions obtained from Scutellariae Radix against experimental DIC, which is closely related to thrombosis. Accordingly, in this work, the effects of flavonoids, the major components of Scutellariae Radix on the blood platelet aggregation and conversion of fibrinogen were examined *in vitro*. Baicalein, wogonin, oroxylin A, skullcapflavone II, and chrysin were shown to have an inhibitory effect on collagen-induced blood platelet aggregation. Further, inhibitory effects of chrysin on ADP-induced blood platelet aggregation and of baicalein, baicalin and wogonin on arachidonic acid-induced blood platelet aggregation were recognized. Sekiya and Okuda[8] reported that baicalein and baicalin had an inhibitory effect on platelet lipoxygenase. These results suggest that the mechanism of action of baicalein and baicalin as regards arachidonic acid-induced blood platelet aggregation may be an inhibitory effect on the metabolism of arachidonic acid.

Baicalein and baicalin also inhibited the conversion of fibrinogen to fibrin induced by thrombin. Based on the *in vitro* experimental results, it is suggested that the *in vivo* effects of Scutellariae Radix on DIC induced by endotoxin[1] might be partly due to the inhibitory actions of these flavonoids on blood platelet aggregation and the conversion of fibrinogen to fibrin. Accordingly, the effects of baicalein and baicalin on the experimental DIC induced by endotoxin were examined in rats. As compared with the control, a preventive effect against experimental DIC was noted in two parameters (but not prothrombin time or FDP) in rats orally given 50 mg/kg of baicalein or baicalin.

These results suggest that Scutellariae Radix may be an effective crude drug for the treatment of DIC. The effective components may be the flavonoids, which are major components of Scutellariae Radix.

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