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Effects of Flavonoids Isolated from Scutellariae Radix on the Production of Tissue-type Plasminogen Activator and Plasminogen Activator Inhibitor-1 Induced by Thrombin and Thrombin Receptor Agonist Peptide in Cultured Human Umbilical Vein Endothelial Cells

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Abstract

The effects of different flavonoids isolated from the roots of *Scutellaria baicalensis* Georgi on the production of tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1) induced by thrombin and thrombin receptor agonist peptide, Ser-Phe-Leu-Leu-Arg-Asn-Pro-Asn-Asp-Lys-Tyr-Glu-Pro-Phe, have been examined in cultured human umbilical vein endothelial cells (HUVECs).

Thrombin and thrombin receptor agonist peptide induced production of both t-PA and PAI-1 and the elevation of intracellular free calcium concentration ($[Ca^{2+}]_i$). Baicalein isolated from Scutellariae Radix dose-dependently inhibited PAI-1 production induced by thrombin and thrombin receptor agonist peptide; its concentrations for 50% inhibition (IC50) were 6·8 and 3·5 μM, respectively. Other flavonoids had no effect. In contrast, flavonoids isolated from Scutellariae Radix had no effect on production of t-PA induced by thrombin and thrombin receptor agonist peptide. Baicalein inhibited the elevation of $[Ca^{2+}]_i$ induced by thrombin and thrombin receptor agonist peptide and, at a concentration of 1000 μM, slightly increased t-PA production.

These findings suggest that the mechanism by which baicalein inhibits PAI-1 production induced by thrombin and thrombin receptor agonist peptide might be by reduction of $[Ca^{2+}]_i$ elevation. The results suggest that baicalein in Scutellariae Radix might be active as a drug in the treatment of arteriosclerosis and thrombosis.

Tissue-type plasminogen activator (t-PA) is a highly specific protease synthesized by vascular endothelial cells and secreted into the bloodstream. The enzyme plays a key role in the fibrinolytic system, the natural counterpart of the blood coagulation system, and is responsible for timely degradation of fibrin structures in blood clots and thrombus (Rijken & Collen 1981). Plasminogen activator inhibitor-1 (PAI-1) is also synthesized by vascular endothelial cells and secreted into the bloodstream. This protein is the main physiological inhibitor of both t-PA and urokinase-type plasminogen in plasma (Kruithof et al 1984). Thus t-PA and PAI-1 play an important role in the regulation of the blood coagulation system in the bloodstream. Thrombin is a serine protease, which also plays a central role in blood coagulation (Furie & Furie 1988), platelet activation (Kimura et al 1988) and pulmonary vascular injury (Tahamont & Malik 1983). It has been reported that thrombin causes production of t-PA (Levine et al 1984) and PAI-1 (Gelehrter & Sznycer-Laszuk 1986) in endothelial cells. Recently, Vu et al (1991a, b) demonstrated that thrombin receptor agonist peptide, a 14-amino-acid peptide (Ser-Phe-Leu-Leu-Arg-Asn-Pro-Asn-Asp-Lys-Tyr-Glu-Pro-Phe) with a sequence identical to that of the new amino terminus of the thrombin receptor after thrombin cleavage, is able to activate human platelets and directly cause the elevation of free calcium concentration ($[Ca^{2+}]_i$) with platelets.

In China and Japan the roots of *Scutellaria baicalensis* Georgi have been used clinically as therapeutic drugs for treatment of allergic inflammatory diseases such as asthma, atopic dermatitis, hyperlipaemia and arteriosclerosis. We reported the inhibitory action of baicalein and baicalin isolated from Scutellariae Radix in reducing levels of blood platelets and fibrinogen in endotoxin-induced disseminated intravascular coagulation (DIC)-rats (Kubo et al 1985) and the inhibitory action of baicalein on leukotriene B_4 and C_4 synthesis and leukocyte degranulation (Kimura et al 1987a, b).

In this study we have examined the inhibitory action of flavonoids isolated from Scutellariae Radix on the production of t-PA and PAI-1 induced by thrombin and thrombin receptor agonist peptide in cultured human umbilical vein endothelial cells (HUVECs). The mechanisms of the effects have also been studied.

Materials and Methods

Materials

Modified MCDB 131 (E-BM) and EGM-UV culture media of human umbilical vein endothelial cells (HUVECs) were obtained from Kurabo. Human α-thrombin, dibutyryl cyclic

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AMP, forskolin and bovine serum albumin (fraction V, fatty-acid-free) were purchased from Sigma. The protein kinase C inhibitors 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H-7) and sphingosine were purchased from Biomol Research and Wako Pure Chemical, respectively. 1-[2-(5′-amino-carboxyoxazol-2′-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)ethane-*N,N,N′,N′*-tetraacetic acid, penta-acetoxylmethyl ester (Fura 2-AM) as a calcium indicator was obtained from Dojin. Thrombin receptor agonist peptide (Ser-Phe-Leu-Leu-Arg-Asn-Pro-Asn-Asp-Lys-Tyr-Glu-Pro-Phe) was synthesized by the Peptide Institute. Cluster six-dish wells were purchased from Corning. t-PA and PAI-1 ELISA kits were purchased from Biopool. Lactate dehydrogenase kit was purchased from Wako. Other chemicals were of reagent grade. The flavonoids wogonin, wogonin-7-*O*-D-glucuronide, oroxylin-A, skullcapflavone II, 2′,5,5′,7-tetrahydroxy-6′,8-dimethoxyflavone, baicalein, baicalin, (2*S*)2′,5,6′,7-tetrahydroxyflavanone, (2*R*,3*R*)2′,3,5,6′,7-pentahydroxyflavanone were isolated from the ethyl acetate and methanol extracts of the dried roots of *Scutellaria baicalensis* Georgi according to previous reports (Kubo et al 1981; Kimura et al 1982, 1984). Test compounds were dissolved in ethanol; the final concentration of ethanol was $< 0{\cdot}25\%$.

Preparation and isolation of HUVECs

HUVECs were isolated from umbilical cords. Briefly, the umbilical vein was cannulated, washed with phosphate-buffered saline (PBS), perfused for 20 min with collagenase (1 mg mL^{-1}) in PBS at 37°C and rinsed with PBS to detach the cells. Cells were seeded on collagen-coated six-well plates and grown to confluence in modified MCDB 131 (E-BM) culture medium supplemented with 20% (v/v) foetal calf serum, 100 mg mL^{-1} bovine brain extract, 100 mg mL^{-1} porcine heparin, 100 units mL^{-1} penicillin and 100 mg mL^{-1} streptomycin. Studies were performed with cells at passage 3.

Measurement of t-PA and PAI-1 production in cultured HUVECs

Third-passage cultures of HUVECs were incubated in a 5% CO_2 atmosphere at 37°C in this study. Briefly, HUVECs were grown to confluence in collagen-coated six-dish well plates. The cells were incubated for 24 h in EGM-UV culture medium with the indicated amounts of the various flavonoids in the presence of thrombin or thrombin receptor agonist peptide. After incubation the t-PA and PAI-1 produced in the supernatant were measured by use of t-PA and PAI-1 ELISA kits, respectively.

Measurement of $[Ca^{2+}]_i$ induced by thrombin and thrombin receptor agonist peptide in cultured HUVECs

Measurement of $[Ca^{2+}]_i$ in cultured HUVECs was performed by the method of Hallam et al (1988). Briefly, HUVECs were grown on collagen-coated cover slips (13·2 mm diameter) in six-well plates. To load Fura 2 into the HUVECs, Fura 2-AM (3 μM) was added to the confluent HUVECs overlaid with Tyrode-5 mM HEPES solution containing 0·1% bovine serum albumin (BSA) (pH 7·4) and incubated at 37°C for 30 min in a 5% CO_2 atmosphere. The cells were washed twice with Tyrode-5 mM HEPES solution (pH 7·4). For the test, the cover slip was firmly positioned in a 1-cm quartz cuvette at an angle of 45°. The cuvette contained 1 mL Tyrode-5 mM HEPES solution containing 1 mM $CaCl_2$ and 0·1% BSA and was kept at 37°C. Fura-2-loaded HUVECs were preincubated for the indicated time with buffer or test compounds at 37°C with stirring at 500 rev min^{-1} and then thrombin or thrombin receptor agonist peptide was added and the mixture was incubated. Fura 2-Ca fluorescence was measured by fluorimetry (JASCO CAF-110, Ca^{2+} analyser) with a fluorescence ratio (short–long wavelengths) of dual excitation at 340 nm and 380 nm and emission at 500 nm.

Data and statistical analysis

Data are expressed as means ± standard error (s.e.). Statistical analysis was performed by Student's *t*-test.

Results

Effects of thrombin and thrombin receptor agonist peptide on t-PA and PAI-1 production in cultured HUVECs

As shown in Fig. 1, thrombin at concentrations of 5–20 units mL^{-1} dose-dependently increased t-PA and PAI-1 production. Furthermore, thrombin-induced t-PA production was enhanced by dibutyryl cyclic AMP (3 mM) (Fig. 1A). In contrast, thrombin-induced PAI-1 production was completely inhibited by dibutyryl cAMP (Fig. 1B). Fig. 2 shows that thrombin receptor agonist peptide at concentrations of 40–320 μM dose-dependently stimulated t-PA and PAI-1 production. Production of PAI-1 induced by thrombin receptor agonist peptide was completely inhibited by dibutyryl cAMP (3 mM), whereas production of t-PA induced by thrombin receptor agonist peptide was slightly enhanced by dibutyryl cAMP, although not significantly.

Tables 1 and 2 show that forskolin (50 μM) inhibited the elevation of PAI-1 production induced by thrombin (10 units mL^{-1}) and thrombin receptor agonist peptide (160 μM), but that it had no effect on the elevation of t-PA production induced by thrombin and thrombin receptor agonist peptide. Thrombin enhanced the elevation of PAI-1 induced by thrombin receptor agonist peptide (Table 1), whereas thrombin receptor agonist peptide did not cause elevation of t-PA and PAI-1 production after treatment of thrombin (Table 2). The protein kinase C inhibitors H-7 (50 μM) and sphingosine (10 μM) inhibited production of t-PA and PAI-1 induced by thrombin or thrombin receptor agonist peptide (Table 3). Furthermore, protein kinase C inhibitors inhibited spontaneous production of t-PA and PAI-1 in cultured HUVECs (Table 3).

Effects of flavonoids isolated from Scutellariae Radix on production of t-PA and PAI-1 induced by thrombin- and thrombin receptor agonist peptide in cultured HUVECs

As shown in Figs 3 and 4, baicalein dose-dependently inhibited production of PAI-1 induced by thrombin and thrombin receptor agonist peptide (IC50 values were 6·8 μM and 3·5 μM, respectively) whereas it had no effect on production of t-PA induced by thrombin and thrombin receptor agonist peptide. Baicalein at high concentration (1000 μM) significantly increased t-PA production (Fig. 3A). The other flavonoids (wogonin, wogonin-7-*O*-D-glucuronide, oroxylin-A, skullcapflavone II, 2′,5,5′,7-tetrahydroxy-6′,8-dimethoxyflavone, baicalin, (2*S*)2′,5,6′,7-tetrahydroxyflavanone, (2*R*,3*R*)2′,3,5,6′,7-

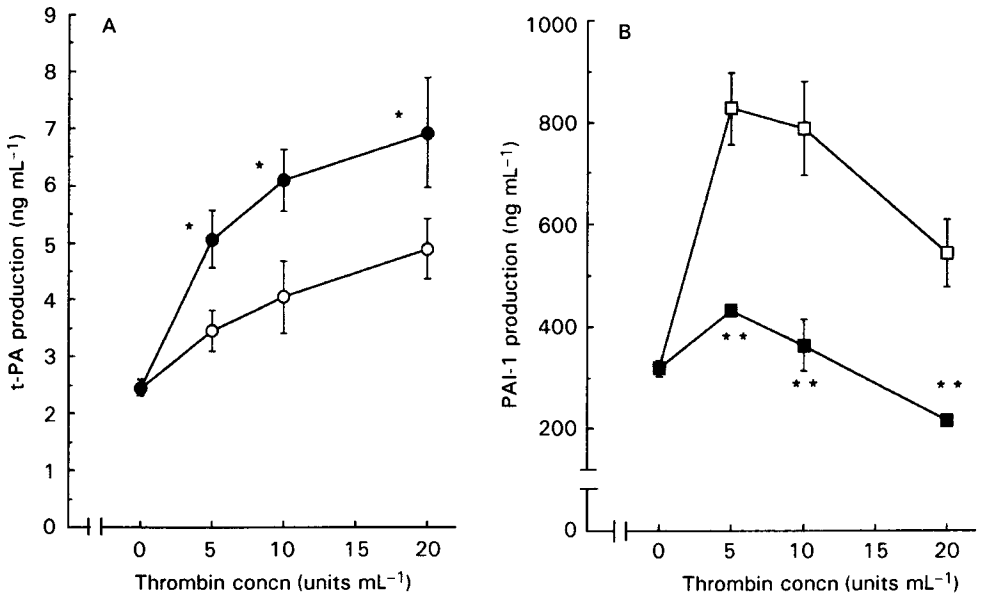

FIG. 1. Dose–response curve for effect of thrombin on production of t-PA (A) and PAI-1 (B) in cultured HUVECs. HUVECs were incubated with thrombin or thrombin plus dibutyryl cAMP (3 mM) for 24 h at 37°C in a 5% CO_2 atmosphere. ○, □ without dibutyryl cAMP; ●, ■ with 3 mM dibutyryl cAMP. Values are means ± s.e. of four to eight experiments. $*P < 0.05$, $**P < 0.01$, significantly different from thrombin alone.

pentahydroxyflavanone had no effect on t-PA and PAI-1 production induced by thrombin or thrombin receptor agonist peptide (data not shown).

Effects of thrombin and thrombin receptor agonist peptide on $[Ca^{2+}]_i$ in cultured HUVECs

As shown in Fig. 5, thrombin (10 units mL^{-1}) and thrombin receptor agonist peptide (160 μM) induced elevation of $[Ca^{2+}]_i$ in cultured HUVECs. Thrombin receptor agonist peptide did not induce $[Ca^{2+}]_i$ elevation after thrombin treatment. Forskolin (50 μM) inhibited elevation of $[Ca^{2+}]_i$ induced by thrombin and thrombin receptor agonist peptide.

Effects of baicalein isolated from Scutellariae Radix on $[Ca^{2+}]_i$ elevation induced by thrombin and thrombin receptor agonist peptide in cultured HUVECs

Of the nine flavonoids tested only baicalein strongly inhibited the production of PAI-1 induced by both thrombin and thrombin receptor agonist peptide in cultured HUVECs. The effects of baicalein on $[Ca^{2+}]_i$ elevation induced by thrombin and thrombin receptor agonist peptide were therefore investigated. As shown in Figs 6 and 7, baicalein dose-dependently

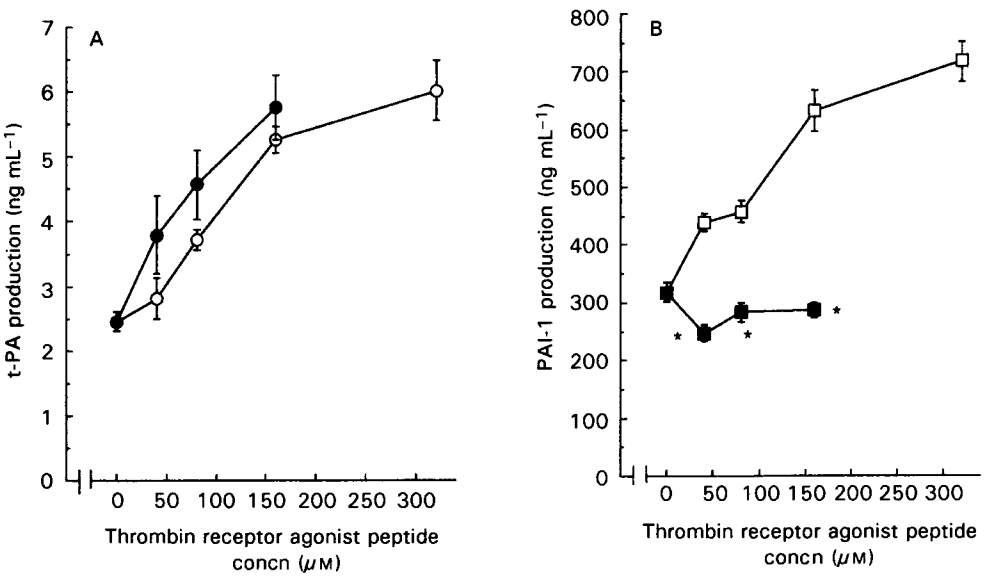

FIG. 2. Dose–response curve for effect of thrombin receptor agonist peptide on production of t-PA (A) and PAI-1 (B) in cultured HUVECs. HUVECs were incubated with thrombin receptor agonist peptide or thrombin receptor agonist peptide plus dibutyryl cAMP (3 mM) for 24 h at 37°C in a 5% CO_2 atmosphere. ○, □ without dibutyryl cAMP; ●, ■ with 3 mM dibutyryl cAMP. Values are means ± s.e. of four to eight experiments. $*P < 0.01$, significantly different from thrombin receptor agonist peptide alone.

Table 1. Effects of thrombin receptor agonist peptide and forskolin on production of tissue-type plasminogen activator and plasminogen activator inhibitor-1 induced by thrombin in cultured HUVECs.

Reaction mixture	Tissue-type plasminogen activator (ng mL^{-1})		Plasminogen activator inhibitor-1 (ng mL^{-1})	
	Mean ± s.e.	% of control	Mean ± s.e.	% of control
Control	7·55 ± 0·18*	100	406·0 ± 10·6*	100
Thrombin (10 units mL^{-1})	11·37 ± 0·18	150·6	780·7 ± 13·4	192·3
Thrombin + thrombin receptor agonist peptide[a]	12·04 ± 0·21	159·5	800·1 ± 18·3	197·1
Forskolin + thrombin[b]	9·97 ± 0·30	132·1	455·0 ± 16·5*	112·1

[a]HUVECs were preincubated with thrombin (10 units mL^{-1}) for 1 h at 37°C in a 5% CO_2 atmosphere, then thrombin receptor agonist peptide (160 μM) was added and the mixture was further incubated for 23 h at 37°C in a 5% CO_2 atmosphere. [b]HUVECs were incubated with forskolin (50 μM) in the presence of thrombin (10 units mL^{-1}) for 24 h at 37°C in a 5% CO_2 atmosphere. Values are means ± s.e. of three experiments. *$P < 0·01$, significantly different from thrombin alone.

Table 2. Effects of thrombin and forskolin on production of tissue-type plasminogen activator and plasminogen activator inhibitor-1 induced by thrombin receptor agonist peptide in cultured HUVECs.

Reaction mixture	Tissue-type plasminogen activator (ng mL^{-1})		Plasminogen activator inhibitor-1 (ng mL^{-1})	
	Mean ± s.e.	% of control	Mean ± s.e.	% of control
Control	8·20 ± 0·21*	100	459·0 ± 8·34*	100
Thrombin receptor agonist peptide (160 μM)	11·93 ± 0·44	145·5	654·3 ± 13·0	142·5
Thrombin receptor agonist peptide + thrombin[a]	12·93 ± 0·50	157·7	740·0 ± 10·2*	161·2
Forskolin + thrombin receptor agonist peptide[b]	10·02 ± 0·55	122·2	487·4 ± 9·72*	106·2

[a]HUVECs were preincubated with thrombin receptor agonist peptide (160 μM) for 1 h at 37°C in a 5% CO_2 atmosphere, then thrombin (10 units mL^{-1}) was added and the mixture was further incubated for 23 h at 37°C in a 5% CO_2 atmosphere. [b]HUVECs were incubated with forskolin (50 μM) in the presence of thrombin receptor agonist peptide (160 μM) for 24 h at 37°C in a 5% CO_2 atmosphere. Values are means ± s.e. of three experiments. *$P < 0·01$, significantly different from thrombin receptor agonist peptide alone.

Table 3. Effects of protein kinase C inhibitors H-7 and sphingosine on production of tissue-type plasminogen activator and plasminogen activator inhibitor-1 in cultured HUVECs.

Reaction mixture	Tissue-type plasminogen activator (ng mL^{-1})		Plasminogen activator inhibitor-1 (ng mL^{-1})	
	Mean ± s.e.	% of control	Mean ± s.e.	% of control
Control	4·51 ± 0·18†‡	100	271·9 ± 14·1†‡	100
H-7 (50 μM)	2·31 ± 0·08*	51·2	146·9 ± 12·1*	54·0
Sphingosine (10 μM)	2·53 ± 0·10*	56·1	134·8 ± 4·19*	49·6
Thrombin (10 units mL^{-1})	6·43 ± 0·12	142·6	568·5 ± 20·7	209·1
Thrombin + H-7	1·77 ± 0·17†	39·2	112·2 ± 1·88†	41·2
Thrombin + sphingosine	3·01 ± 0·12†	66·8	154·3 ± 5·22†	56·7
Thrombin receptor agonist peptide (160 μM)	7·70 ± 0·40	170·7	441·0 ± 9·19	162·2
Thrombin receptor agonist peptide + H-7	1·74 ± 0·12‡	38·6	152·4 ± 3·14‡	56·1
Thrombin receptor agonist peptide + sphingosine	3·91 ± 0·05‡	64·5	182·5 ± 6·09‡	67·1

HUVECs were incubated with H-7 (50 μM) and sphingosine (10 μM) in the presence or absence of thrombin (10 units mL^{-1}) and thrombin receptor agonist peptide (160 μM) for 24 h at 37°C in a 5% CO_2 atmosphere. Values are means ± s.e. of three experiments. *$P < 0·01$, significantly different from control value (spontaneous production). †$P < 0·01$, significantly different from result for thrombin alone. ‡$P < 0·01$, significantly different from result for thrombin receptor agonist peptide alone.

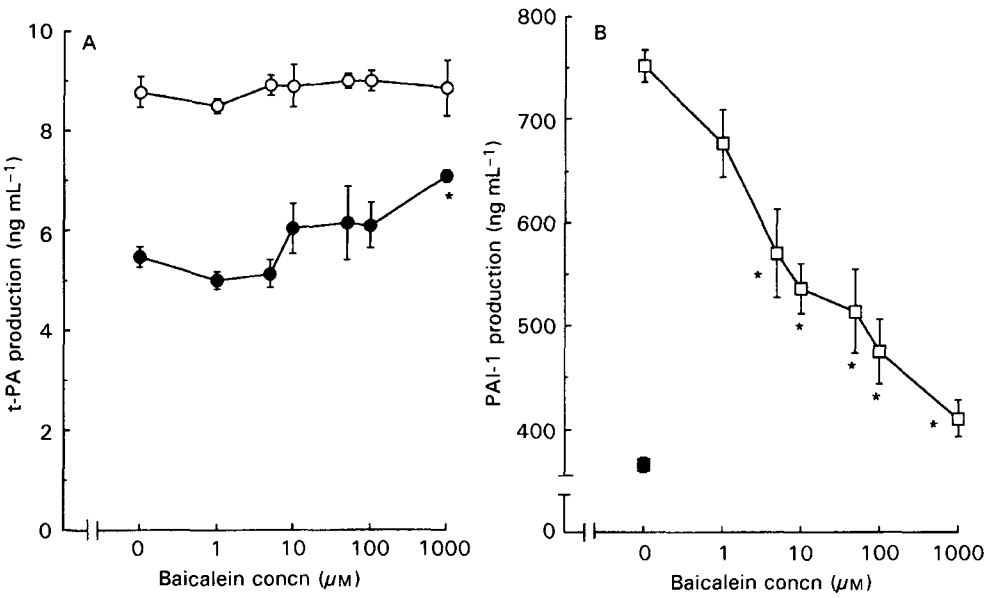

FIG. 3. Effects of baicalein isolated from Scutellariae Radix on production of t-PA (A) and PAI-1 (B) induced by thrombin in cultured HUVECs. HUVECs were incubated with baicalein in the presence or absence of thrombin (10 units mL^{-1}) for 24 h at 37°C in a 5% CO_2 atmosphere. ○, □ thrombin (10 units mL^{-1}) plus baicalein; ● baicalein alone; ■ medium alone. Values are means ± s.e. of four to eight experiments. *$P < 0.01$, significantly different from thrombin alone.

inhibited the elevation of $[Ca^{2+}]_i$ induced by thrombin and thrombin receptor agonist peptide.

Discussion

It is well known that thrombin is a serine protease which plays a central role in blood coagulation, platelet activation and thrombosis (Furie & Furie 1988; Kimura et al 1988). Vascular endothelial cells can synthesize both tissue-type plasminogen activator (t-PA) and its specific inhibitor plasminogen activator inhibitor-1 (PAI-1), which are two of several important factors involved in the regulation of fibrinolytic function in the vascular system (Rijken & Collen 1981; Kruithof et al 1984; Levine et al 1984; Gelehrter & Sznycer-Laszuk 1986). Hung et al (1992) reported that thrombin caused both phosphoinositide hydrolysis and inhibition of adenylylcyclase in a variety of responsible cells.

In this study also we found that thrombin and thrombin receptor agonist peptide dose-dependently induced production of both t-PA and PAI-1 in cultured HUVECs (Fig. 1). Thrombin-induced t-PA production was potentiated by

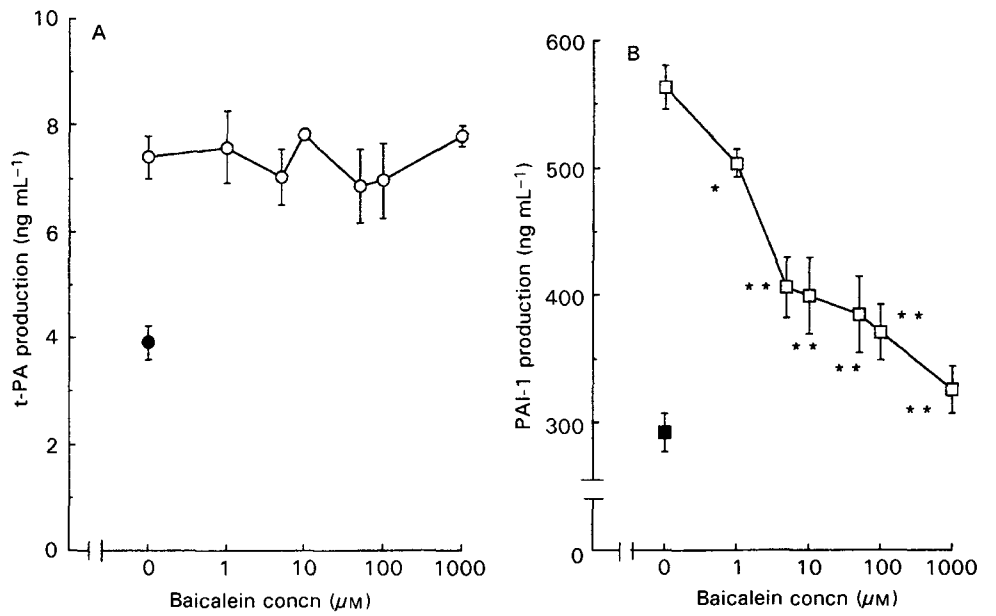

FIG. 4. Effects of baicalein isolated from Scutellariae Radix on production of t-PA (A) and PAI-1 (B) induced by thrombin receptor agonist peptide in cultured HUVECs. HUVECs were incubated with baicalein in the presence or absence of thrombin receptor agonist peptide (160 μM) for 24 h at 37°C in a 5% CO_2 atmosphere. ○, □ Thrombin receptor agonist peptide (160 μM) plus baicalein; ●, ■ medium alone. Values are means ± s.e. of four experiments. *$P < 0.05$, **$P < 0.01$, significantly different from thrombin receptor agonist peptide alone.

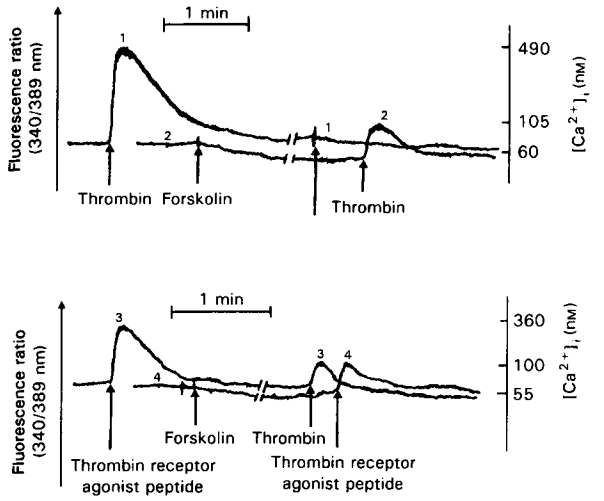

FIG. 5. Effects of thrombin and thrombin receptor agonist peptide on $[Ca^{2+}]_i$ in cultured HUVECs. HUVECs were grown on collagen-coated cover slips, and Fura 2 was incorporated into the confluent HUVECs. Fura 2-loaded HUVECs were preincubated for 10 min with thrombin (10 units mL^{-1}) or thrombin receptor agonist peptide (160 μM) at 37°C with stirring at 500 rev min^{-1}, then thrombin receptor agonist peptide or thrombin was added and the mixture was further incubated for 2 min at 37°C in the presence of 1 mM $CaCl_2$ and 0·1% BSA. Fura 2-loaded HUVECs were preincubated for 10 min with forskolin (50 μM) at 37°C with stirring at 500 rev min^{-1}, and then thrombin (10 units mL^{-1}) or thrombin receptor agonist peptide (160 μM) was added and the mixture was further incubated for 2 min at 37°C in the presence of 1 mM $CaCl_2$ and 0·1% BSA. 1, thrombin (10 units mL^{-1}) plus thrombin receptor agonist peptide (160 μM); 2, forskolin (50 μM) plus thrombin (10 units mL^{-1}); 3, thrombin receptor agonist peptide (160 μM) plus thrombin (10 units mL^{-1}); 4, forskolin (50 μM) plus thrombin receptor agonist peptide (160 μM).

dibutyryl cAMP (Fig. 1A). In contrast, thrombin-induced PAI-1 production was inhibited by dibutyryl cAMP (Fig. 1B). Dibutyryl cAMP also inhibited the PAI-1 production induced by thrombin receptor agonist peptide, but it had no effect on the production of t-PA induced by that compound (Fig. 2).

Forskolin is well known to induce elevation of intracellular cAMP levels by stimulating adenylylcyclase activity. Forskolin inhibited production of PAI-1 induced by thrombin and thrombin receptor agonist peptide but had no significant effect on production of t-PA induced by the same compounds (Table 1). Santell & Levin (1988) reported that the elevation of cAMP

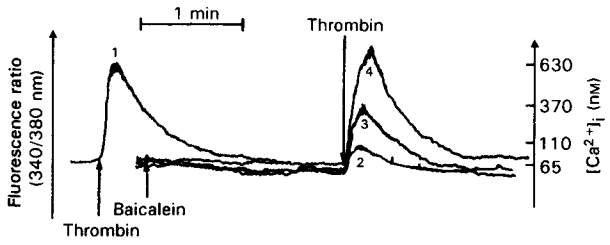

FIG. 6. Effects of baicalein on thrombin-induced $[Ca^{2+}]_i$ elevation in cultured HUVECs. Fura 2-loaded HUVECs were preincubated for 2 min with buffer or baicalein at 37°C with stirring at 500 rev min^{-1}, then thrombin (10 units mL^{-1}) was added and the mixture was further incubated for 2 min at 37°C in the presence of 1 mM $CaCl_2$ and 0·1% BSA. 1, thrombin (10 units mL^{-1}) alone; 2, baicalein (100 μM) plus thrombin; 3, baicalein (10 μM) plus thrombin; 4, baicalein (1 μM) plus thrombin.

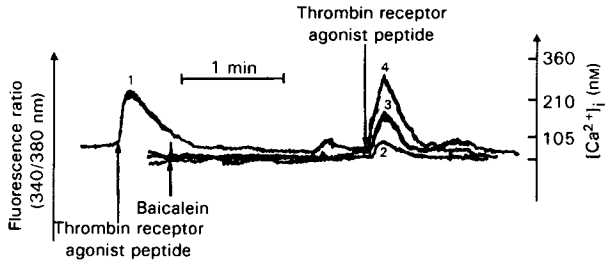

FIG. 7. Effects of baicalein on $[Ca^{2+}]_i$ elevation induced by thrombin receptor agonist peptide in cultured HUVECs. Fura 2-loaded HUVECs were preincubated for 2 min with buffer or baicalein at 37°C with stirring at 500 rev min^{-1}, then thrombin receptor agonist peptide (160 μM) was added and the mixture was further incubated for 2 min at 37°C in the presence of 1 mM $CaCl_2$ and 0·1% BSA. 1, thrombin receptor agonist peptide (160 μM) alone; 2, baicalein (100 μM) plus thrombin receptor agonist peptide; 3, baicalein (10 μM) plus thrombin receptor agonist peptide; 4, baicalein (1 μM) plus thrombin receptor agonist peptide.

potentiated t-PA production induced by phorbol myristate acetate, a protein kinase C activator, and inhibited phorbol myristate acetate-induced PAI-1 production. Thrombin is reported to activate the phosphoinositide pathway and to generate diacylglycerol, which is a physiological activator of protein kinase C (Levin & Santell 1991). These findings show that the effects of dibutyryl cAMP on t-PA and PAI-1 production induced by thrombin are similar to its effects on t-PA and PAI-1 production induced by phorbol myristate acetate. Hidaka et al (1984) reported that H-7 inhibited cyclic nucleotide-dependent protein kinase and protein kinase C. Hannun et al (1986) reported that sphingosine inhibited protein kinase C activation by thrombin in human platelets and cell growth, and that sphingosine acted as an anti-tumour promotor. Thus, H-7 and sphingosine are not specific inhibitors of protein kinase C. In this study, we examined the effects of partially selective inhibitors of protein kinase C, H-7 and sphingosine on the production of t-PA and PAI-1 induced by thrombin and thrombin receptor agonist peptide. The compounds strongly inhibited production of t-PA and PAI-1 induced by thrombin and thrombin receptor agonist peptide to below the control level and also inhibited spontaneous t-AP and PAI-1 production (Table 2). Moreover, HUVECs treated with thrombin showed transient $[Ca^{2+}]_i$ elevation, but were unresponsive to subsequent treatment with thrombin receptor agonist peptide (Fig. 4). Heller et al (1991) reported that forskolin, a direct activator of adenylylcyclase, inhibited the elevation of intracellular free calcium concentrations induced by thrombin. Thus, it is suggested that the elevation of t-PA and PAI-1 production by thrombin might be mediated via cAMP-dependent protein kinase A with $[Ca^{2+}]_i$ elevation or protein kinase C activation through thrombin receptor, or both.

We have already reported that baicalein isolated from the roots of *Scutellaria baicalensis* has anti-thrombotic action in endotoxin-induced DIC-rats (Kubo et al 1985). In this study, to clarify the mechanism of the anti-thrombotic action of various flavonoids contained in these crude drugs, we investigated their effects on t-PA and PAI-1 production induced by thrombin and thrombin receptor agonist peptide. Among the flavonoids tested baicalein dose-dependently reduced the increase of PAI-1 production induced by thrombin and thrombin receptor agonist peptide, whereas it had no effect on

the elevation of t-PA production induced by thrombin and thrombin receptor agonist peptide (Fig. 3). Furthermore, baicalein inhibited the elevation of $[Ca^{2+}]_i$ induced by thrombin and thrombin receptor agonist peptide (Fig. 4) but had no effect on intracellular cAMP level (Kimura et al 1997) or thrombin activity (Ogita et al 1995). We previously reported that baicalein and protein kinase C inhibitors such as H-7 and sphingosine reduced the reduction of t-PA and the elevation of PAI-1 production induced by interleukin 1β and tumour necrosis factor α. The flavone derivative quercetin (3,3′,4′,5,7-pentahydroxyflavone) has been reported to be a potent inhibitor of protein kinase C activity (Horiuchi et al 1986). These results suggest two possible mechanisms for the inhibitory action of baicalein on PAI-1 production induced by thrombin and thrombin receptor agonist peptide: direct inhibition of protein kinase C activity like the inhibition of protein kinase C activity by quercetin; and the reduction of $[Ca^{2+}]_i$ elevation without elevating intra-endothelial cAMP level or antithrombin activity. The results suggest that baicalein in Scutellariae Radix might be active as a drug in the treatment of arteriosclerosis and thrombosis. Further studies are needed to clarify the effects of baicalein on the fibrinolytic-coagulation system induced by thrombin in-vivo.

References

Furie, B., Furie, B. C. (1988) The molecular basis of blood coagulation. Cell 53: 505–518

Gelehrter, T. D., Sznycer-Laszuk, R. (1986) Thrombin induction of plasminogen activator-inhibitor in cultured endothelial cells. J. Clin. Invest. 77: 165–169

Hallam, T. J., Pearson, J. D., Needham, L. A. (1988) Thrombin-stimulated elevation of human endothelial-cell cytoplasmic free calcium concentration causes prostacyclin production. Biochem. J. 251: 243–249

Hannun, Y. A., Loomis, C. R., Merrill A. H., Jr, Bell, R. M. (1986) Sphingosine inhibition of protein kinase C activity and of phorbol dibutyrate binding in vitro and in human platelets. J. Biol. Chem. 261: 12604–12609

Heller, R., Bussolino, F., Ghigo, D., Garbarino, G., Schröder, H., Pescarmona, G., Till, U., Bosia, A. (1991) Protein kinase C and cyclic AMP modulate thrombin-induced platelet-activating factor synthesis in human endothelial cells. Biochim. Biophys. Acta 1093: 55–64

Hidaka, H., Inagaki, M., Kawamoto, S., Sasaki, Y. (1984) Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide-dependent protein kinase and protein kinase C. Biochemistry 23: 5036–5041

Horiuchi, T., Fujiki, H., Hakii, H., Suganuma, M., Yamashita, K., Sugimura, T. (1986) Modulation of phorbol ester receptors in mouse skin by application of quercetin. Gann 77: 526–531

Hung, D. T., Vu, T.-K. H., Nelken, N. A., Coughlin, S. R. (1992) Thrombin-induced events in non-platelet cells are mediated by the unique proteolytic mechanism established for the cloned platelet thrombin receptor. J. Cell Biol. 116: 827–832

Kimura, Y., Okuda, H., Tani, T., Arichi, S. (1982) Studies on Scutellariae Radix VI. Effects of flavanone compounds on lipid peroxidation in rat liver. Chem. Pharm. Bull. 30: 1792–1795

Kimura, Y., Okuda, H., Taira, Z., Shoji, N., Takemoto, T., Arichi, S. (1984) Studies on Scutellariae Radix IX. New components inhibiting lipid peroxidation in rat liver. Planta Medica 50: 290–295

Kimura, Y., Okuda, H., Arichi, S. (1987a) Effects of baicalein on leukotriene C_4 biosynthesis in human lymphocytes. Phytother. Res. 1: 48–49

Kimura, Y., Okuda, H., Arichi, S. (1987b) Effects of baicalein on leukotriene biosynthesis and degranulation in human polymorphonuclear leukocytes. Biochim. Biophys. Acta 922: 278–286

Kimura, Y., Okuda, H., Arichi, S. (1988) Effects of various ginseng saponins on 5-hydroxytryptamine release and aggregation in human platelets. J. Pharm. Pharmacol. 40: 838–843

Kimura, Y., Okuda, H., Yokoi (Hayashi), K., Matsushita, N. (1997) Effects of baicalein isolated from roots of *Scutellaria baicalensis* Georgi on interleukin 1β- and tumour necrosis factor α-induced tissue-type plasminogen activator and plasminogen activator inhibitor-1 production in cultured human umbilical vein endothelial cells. Phytother. Res. In press

Kruithof, E. K. O., Tran-Thang, C., Ransijn, A., Bachman, F. (1984) Demonstration of a fast-acting inhibitor of plasminogen activators in human plasma. Blood 64: 907–913

Kubo, M., Kimura, Y., Odani, T., Tani, T., Namba, K. (1981) Studies on Scutellariae Radix part II: The antibacterial substance. Planta Medica 43: 194–201

Kubo, M., Matsuda, H., Tani, T., Arichi, S., Kimura, Y., Okuda, H. (1985) Studies on Scutellariae Radix XII. Anti-thrombic actions of various flavonoids from Scutellariae Radix. Chem. Pharm. Bull. 33: 2411–2415

Levin, E. G., Santell, L. (1991) Thrombin- and histamine-induced signal transduction in human endothelial cells: stimulation and agonist-dependent desensitization of protein phosphorylation. J. Biol. Chem. 266: 174–181

Levine, E. G., Marzec, U., Anderson, J., Harker, L. A. (1984) Thrombin stimulates tissue plasminogen activator release from cultured human endothelial cells. J. Clin. Invest. 74: 1988–1995

Ogita, Z., Takada, A., Kimura, Y. (1995) Anti-trypsin actions of various flavonoids isolated from Scutellariae Radix. J. Trad. Med. 12: 264–267

Rijken, D. C., Collen, D. (1981) Purification and characterization of the plasminogen activator secreted by human melanoma cells in culture. J. Biol. Chem. 256: 7035–7041

Santell, L., Levin, E. G. (1988) Cyclic AMP potentiates phorbol ester stimulation of plasminogen activator release and inhibits secretion of plasminogen activator inhibitor-1 from human endothelial cells. J. Biol. Chem. 263: 16802–16808

Tahamont, M. V., Malik, A. B. (1983) Granulocytes mediate the increase in pulmonary vascular permeability after thrombin embolism. J. Appl. Physiol. Respir. Environ. Exercise Physiol. 54: 1489–1495

Vu, T. H. K., Wheaton, V. I., Hung, D. T., Charo, I., Coughlin, S. R. (1991a) Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell 64: 1057–1068

Vu, T.-K. H., Wheaton, V. I., Hung, D. T., Charo, I., Coughlin, S. R. (1991b) Domains specifying thrombin-receptor interaction. Nature 353: 674–677