Effects of Flavonoids Isolated from Scutellariae Radix on Fibrinolytic System Induced by Trypsin in Human Umbilical Vein Endothelial Cells

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Studies on the effects of flavonoids isolated from the roots of *Scutellaria baicalensis* on the fibrinolytic system induced by trypsin in cultured human umbilical vein endothelial cells (HUVECs) showed that baicalein (**1**) strongly inhibited the reduction of t-PA production and the elevation of PAI-1 production induced by trypsin. The IC_{50} for PAI-1 production was 3.7 μM. In addition, wogonin (**3**), oroxylin A (**5**), skullcapflavone II (**6**), and 2′,5,5′,7-tetrahydroxy-6′,8-dimethoxyflavone (**7**) inhibited the elevation of PAI-1 induced by trypsin, though less strongly; their IC_{50} were 105, 61, 110, and 88 μM, respectively. These findings suggest that baicalein prevents the thrombotic tendency induced by trypsin.

Tissue-type plasminogen activator (t-PA) is a highly specific protease that is synthesized by vascular endothelial cells and secreted into the bloodstream. This enzyme plays a key role in the fibrinolytic system that constitutes the natural counterpart of the blood coagulation system and is responsible for the timely degradation of fibrin structures in blood clots and thrombi.[1] Type 1 plasminogen activator inhibitor (PAI-1) is also synthesized by vascular endothelial cells and secreted into the bloodstream. PAI-1 is a physiological inhibitor of both t-PA and urokinase-type plasminogen (u-PA) in plasma.[2] Thus, it may play an important role in the regulation of a variety of fibrinolysis-dependent biological processes.[2] Trypsin, a serine protease, is abnormally secreted from the pancreas during pancreatic inflammation, resulting in pancreatic tissue injury.[3] A transient high concentration of trypsin in the bloodstream is thought to induce imbalance between blood coagulation and the fibrinolysis system by activation of Hargeman factor in acute pancreatitis.[4]

Scutellariae radix (roots of *Scutellaria baicalensis* Georgi (Labiatae)) is contained within Chinese traditional prescriptions such as "Shou-Saiko-To, Hange-Shasin-To, and Kakkon-Ougon-Ouren-To (Japanese)" and "Xiao-Chai-Hu-Tang, Ban-Xia-Xie-Xin-Tang, and Ge-Gen-Huang-Qin-Huang-Lian-Tang (Chinese)", which are used to treat acute and chronic pancreatitis. In a previous paper,[5] we have reported that nine flavonoids isolated from Scutellariae radix have anti-trypsin actions on the protease assay system using synthetic substrate (*N*-benzoyl-Phe-Val-Arg-*p*-Nitroanilide) with the IC_{50} of baicalein (**1**) being 0.5 μM. In this paper, we report on studies of the vascular physiological role of trypsin on t-PA and PAI-1 production and cell damage and a further examination of the effects of these flavonoids isolated from Scutellariae radix on t-PA and PAI-1 production in cultured human umbilical vein endothelial cells (HUVECs).

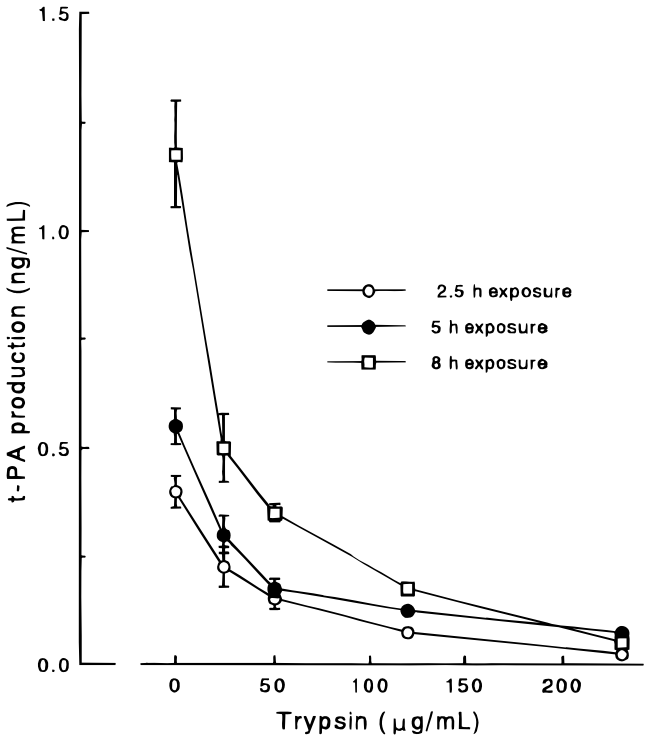

Figure 1. Effects of trypsin on t-PA production in cultured HUVECs. Values are means ± SEM of five experiments. $P < 0.01$.

Results and Discussion

Figures 1 and 2 show the dose and exposure time dependency of the effects of trypsin on t-PA and PAI-1 levels in cultured HUVECs. Trypsin significantly inhibited the t-PA level in a dose- and exposure time-dependent manner (Figure 1). On the other hand, the PAI-1 level was increased 1.88-, 1.88-, and 1.38-fold at 25, 50, and 120 μg of trypsin/mL, respectively, at 2.5 h

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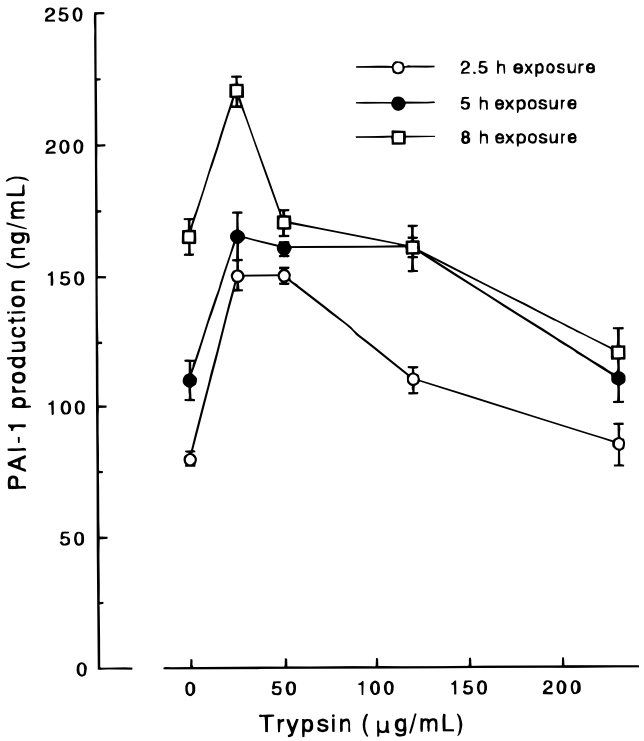
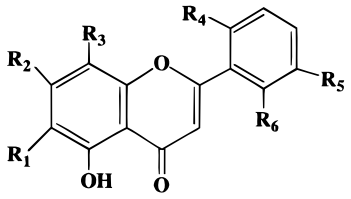


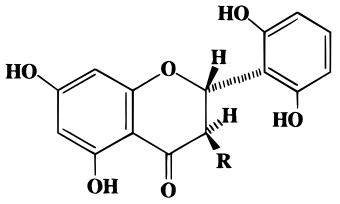
Figure 2. Effects of trypsin on PAI-1 production in cultured HUVECs. Values are means ± SEM of five experiments. $P < 0.01$.

exposure. With 5 h exposure, trypsin, at the same concentrations, significantly increased PAI-1 levels by 1.50-, 1.46-, and 1.46-fold, respectively. With 8 h exposure, trypsin at 25 μg/mL significantly increased the PAI-1 level by 1.33-fold. At 230 μg/mL, trypsin reduced the PAI-1 level to that of the control at 2.5 h and 5 h exposure, while at 8 h exposure, it significantly reduced the PAI-1 level (see Figure 2). As shown in Figure 3, trypsin increased lactate dehydrogenase (LDH) release from cultured HUVECs, in a dose-dependent and time-dependent manner. Trypsin at the concentrations of 25, 50, 120, and 230 μg/mL increased the LDH release from HUVECs at both 5 and 8 h exposure. With 2.5 h exposure time, trypsin at 120 and 230 μg/mL caused a small but significant increase in the LDH release (see Figures 1−3). Thus, it can be concluded that trypsin at the concentrations of 25 and 50 μg/mL caused the reduction of t-PA and the elevation of PAI-1 without affecting HUVEC membrane damage (LDH release) with 2.5 h exposure. On the other hand, with over 5 h of exposure, trypsin at the concentrations of 25 and 50 μg/mL caused cell damage, t-PA reduction, and PAI-1 elevation. These findings suggest that the degree of cell damage may be related to the increase of PAI-1 and the reduction of t-PA associated with the dose of trypsin and exposure time. The mechanisms whereby trypsin exerts these effects on t-PA and PAI-1 production and degree of cell damage require further clarification. To this end, the effects of nine flavonoids isolated from Scutellariae radix were measured under conditions that do not cause cell damage (LDH release): trypsin concentration, 25 μg/mL; exposure time, 2.5 h.

Figure 4 shows the dose-response curve of the effect of baicalein (**1**) on t-PA and PAI-1 production induced by trypsin. Compound **1** significantly inhibited, in a dose-dependent manner, trypsin-induced t-PA reduction at the concentrations of 10−100 μM (Figure 4a). On the other hand, the flavonoids baicalin (**2**), wogonin (**3**), wogonin 7-*O*-D-glucuronide (**4**), oroxylin-A (**5**), skullcapflavone II (**6**), 2′,5,5′,7-tetrahydroxy-6′,8-dimethoxyflavone (**7**), *(2S)*-2′,5,6′,7-tetrahydroxyflavanone (**8**), and *(2R,3R)*-2′,3,5,6′,7-pentahydroxyflavanone (**9**) did not inhibit the reduction of t-PA (Table 1). As shown in Figure 4b, baicalein (**1**) inhibited in a dose-dependent manner the elevation of PAI-1 induced by trypsin with an IC_{50} of 3.7 μM. Furthermore, compounds **3**, **5**, **6**, and **7** inhibited the elevation of PAI-1 induced by trypsin, though less strongly with IC_{50} values of 105, 61, 110, and 88 μM, respectively (see Table 1).



	R_1	R_2	R_3	R_4	R_5	R_6
Baicalein (**1**)	OH	OH	H	H	H	H
Baicalin (**2**)	OH	O-Glu-acid	H	H	H	H
Wogonin (**3**)	H	OH	OCH_3	H	H	H
Wogonin 7-*O*-D-glucuronide (**4**)	H	O-Glu-acid	OCH_3	H	H	H
Oroxylin-A (**5**)	OCH_3	OH	H	H	H	H
Skullcapflavone II (**6**)	OCH_3	OCH_3	OCH_3	OH	H	OCH_3
2',5,5',7-Tetrahydroxy-6',8-dimethoxyflavone (**7**)	H	OH	OCH_3	OH	OH	OCH_3



	R
2',5,6',7-Tetrahydroxyflavanone (**8**)	H
2',3,5,6',7-Pentahydroxyflavanone (**9**)	OH

In summary, of the nine flavonoids tested, baicalein (**1**) was found to inhibit t-PA reduction and PAI-1 elevation induced by trypsin most strongly. Further, the inhibitory effects of **1** on trypsin-induced t-PA reduction and PAI-1 elevation could be explained by its anti-trypsin actions. It seems likely that this compound prevents the thrombotic tendency caused by trypsin. Further work is required to clarify whether baicalein (**1**) can exert such an effect *in vivo*.

Experimental Section

Materials. Trypsin was purchased from Nacalai Tesque, Inc. (Japan). Collagenase and lactate dehydrogenase (LDH) kits were purchased from Wako Pure Chemical Co. (Japan). Modified MCDB 131 (E-BM) culture medium and fetal calf serum were purchased from Kurabou Co. (Japan). and Cosmobio Co. (Japan), respectively. Serum-free culture medium ASF 301 was obtained from Ajinomoto Co. (Japan). Bovine brain extract, porcine heparin, penicillin and streptomycin were purchased from Sigma Co. (USA). Cluster 6-well dishes were purchased from Corning Glass Works (Japan). Tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1) enzyme-linked immunosorbent assay (ELISA) kits were purchased

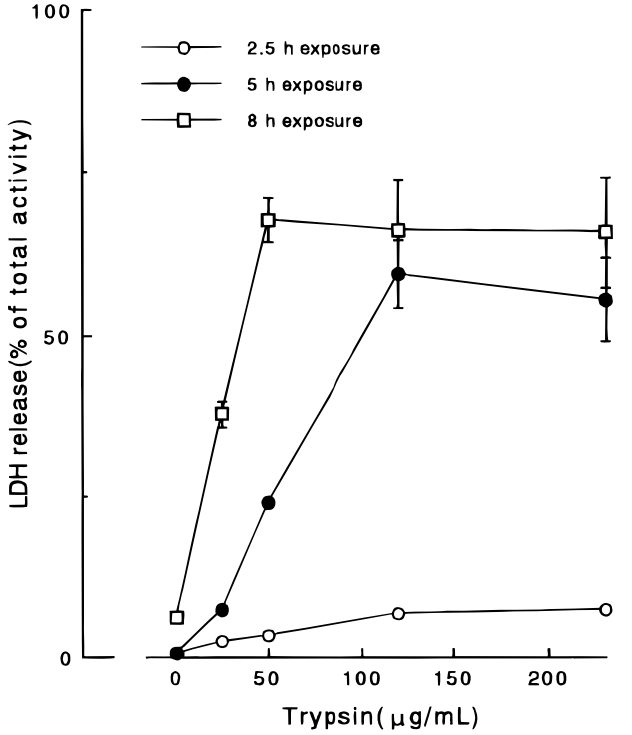


Figure 3. Effects of trypsin on LDH release in cultured HUVECs. Values are means ± SEM of five experiments. $P < 0.01$.

Table 1. Effects of Flavonoids Isolated from *Scutellariae baiclensis* Root on Trypsin-Induced PAI-1 Production in Cultured HUVECs[a]

compds	IC_{50} (μM) trypsin-induced PAI-1 production
baicalein (**1**)	3.7
baicalin (**2**)	>1000
wogonin (**3**)	105
wogonin 7-*o*-D-glucuronide (**4**)	>1000
oroxylin-A (**5**)	61
skullcapflavone II (**6**)	110
2′,5,5′,7-tetrahydroxy-6′,8-dimethoxyflavone (**7**)	88
2′,5,6′,7-tetrahydroxyflavanone (**8**)	>1000
2′,3,5,6′,7-pentahydroxyflavanone (**9**)	>1000

[a] Results are expressed as means of four experiments. Compounds were not tested at concentrations higher than 10^{-2}M.

from Biopool Co.(Sweden). Other chemicals were of reagent grade. Baicalein (**1**), baicalin (**2**), wogonin (**3**), wogonin 7-*O*-D-glucuronide (**4**), oroxylin-A (**5**), skullcapflavone II (**6**) , 2′,5,5′,7-tetrahydroxy-6′,8-dimethoxyflavone (**7**), 2′,5,6′,7-tetrahydroxyflavanone (**8**), and 2′,3,5,6′,7-pentahydroxyflavanone (**9**) were isolated from the ethyl acetate and methanol extracts of the dried roots of *S. baicalensis* Georgi (Labiatae) according to previous reports.[6−8] Test compounds were dissolved in dimethyl sulfoxide (final concentration of dimethylsulfoxide was less than 0.25%).

Preparation and Isolation of Human Umbilical Vein Endothelial Cells (HUVECs). Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords according to the method of Gimbrone.[9] Briefly, the umbilical vein was cannulated, washed with phosphate buffered saline (PBS), perfused for 20 min with collagenase (1 mg/mL) in PBS at 37 °C, and rinsed with PBS to detach the cells. Cells were seeded on collagen-coated 6-well plates and grown to confluence in modified MCDB 131 (E-BM) culture medium supplemented with 20% (v/v) fetal calf serum, 100 μg/mL of bovine brain extract, 100 μg/mL of porcine heparin, 100 units/mL of penicillin, and 100 μg/mL of streptomycin. Studies were carried out with cells at the third passage.

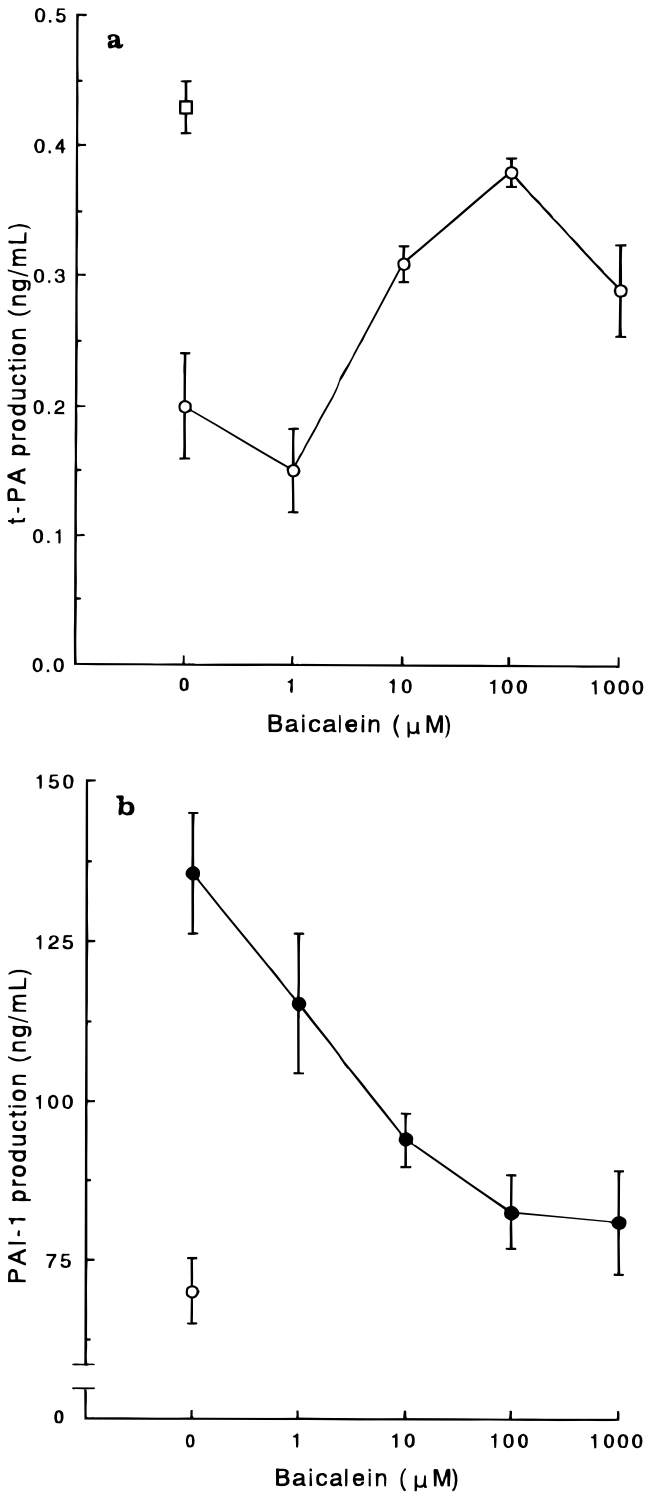


Figure 4. Effects of baicalein on t-PA and PAI-1 production induced by trypsin in cultured HUVEC. Values are means ± SEM of four experiments. (a): □, spontaneous t-PA production at 2.5 h exposure with medium alone; ○, t-PA production at 2.5 h exposure with trypsin (25 μg/mL) plus various concentrations of baicalein. Baicalein at 10 μM, $P < 0.05$; at 100 μM, $P < 0.01$. (b): ○, spontaneous PAI-1 production at 2.5 h exposure with medium alone; ●, PAI-1 production at 2.5 h exposure with trypsin (25 μg/mL) plus various concentrations of baicalein. Baicalein at 10, 100, and 1000 μM, $P < 0.01$.

Determination of Tissue-type Plasminogen Activator, Plasminogen Activator Inhibitor-1 and Lactate Dehydrogenase in Cultured HUVECs. Determination of tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1) in cultured HUVECs was performed using enzyme-linked immunosorbent assay (ELISA) kits, respectively. HUVEC damage was determined in terms of the release of lactate dehydrogenase (LDH) using LDH kits. In this study, serum-free culture medium ASF 301 was used for the determination of the physiological action of trypsin without the effects of trypsin inhibitors in serum containing culture medium. Confluent HUVECs were incubated for the indicated time in ASF 301 culture medium with trypsin alone or trypsin plus test compounds. After incubation, t-PA and PAI-1 production and LDH activity in the supernatant were measured.

Data and Statistical Analyses. Values are expressed as means ± standard errors of means ($n = 4$ or 5 experiments) of duplicate. Statistical analysis was performed with Student's *t*-test.

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