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Effect of new-breviscapine on fibrinolysis and anticoagulation of human vascular endothelial cells[1]

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ABSTRACT Cultured confluent human umbilical vein endothelial cells were incubated with new-breviscapine (NB), a flavonoid consisting of 4-*OH*-scutellarin-7-*O*-glucuronide ($C_{33}H_{30}O_{18}$) and $FeCl_3$, $MgCl_2$, and $CaCl_2$, which is first extracted from *Erigeron breviscapus* (Vant) Hand-Mazz in China. 0, 6.25, 12.5, 25, 50, 100, and 1 000 $\mu g \cdot ml^{-1}$. The releases of tissue-type plasminogen activator (t-PA), and epoprostenol (Epo) from endothelial cells were stimulated by NB, but no significant effect of plasminogen activator inhibitor (PAI) activity was seen. NB 25-1 000 $\mu g \cdot ml^{-1}$ induced a production of thrombomodulin (TM) within the cells, an expression of TM on the surface of the cells, and a release of TM from the cells. Our data provide a new evidence that NB is a stimulant to fibrinolysis and anticoagulation of endothelial cells.

KEY WORDS new-breviscapine; fibrinolysis; vascular endothelium; tissue-type plasminogen activator; epoprostenol; cultured cells; anticoagulants

New-breviscapine (NB) with a chemical formula $C_{33}H_{30}O_{18}$ is a flavonoid consisting of 4-*OH*-scutellarin-7-*O*-glucuronide and some kinds of salts (Ferric chloride, Magnesium chloride and Calcium chloride), which is extracted from *Erigeron breviscapus* (Vant) Hand-Mazz[1]. It has been reported that NB had strong antiplatelet effects, including the inhibition of platelet activation induced by adenosine diphosphate (ADP), thrombin, arachidonic acid, and calcium ionophore calcimycin (A-23187), and the inhibition of production or release of thromboxane B_2 and 5-hydroxytryptamine by platelets *in vitro*[2]. It also had an inhibitory effect on thrombus formation *in vivo*[3]. But whether NB has a modulation effect on endothelial function has not been reported yet. In this study, we investigated the effect of NB on the fibrinolysis and anticoagulation of cultured human umbilical endothelial cells (HUVEC).

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MATERIALS AND METHODS

Culture and treatment of endothelial cells Primary cultures of HUVEC were prepared by the method of Jaffe, *et al*[4,5]. Cultured confluent HUVEC in flask were washed with RPMI-1640 medium (J R Scientific) for 3 times, and divided into 8 groups (3 flasks were used for each group). The cells were incubated at 37℃ with NB (gifted by ZHANG Ren-Wei, Yunnan Provincial Institute of Materia Medica) in serum free medium at concentrations of 0, 6.25, 12.5, 25, 50, 100, and 1000 $\mu g \cdot ml^{-1}$, respectively. In addition, another group of HUVEC was treated with NB 100 $\mu g \cdot ml^{-1}$ and dactinomycin (Sigma) 5 $\mu g \cdot ml^{-1}$. The conditioned medium was collected at 10 min, 4-h, and 24-h after incubation and centrifuged at 10 000 × *g* for 3 min to remove detached cells, and cellular debris, and was used in the determination for t-PA, PAI, Epo, and TM. After 24-h incubation, the cells were collected and treated as previously described[5].

Protein purification and iodination A monoclonal antibody specific to human TM, named SZ-53, was prepared and identified as

previously described[6]. SZ-53 IgG was purified from the ascitic fluid by affinity chromatography using protein-A Sepharose 4B (Pharmacia). Both purified SZ-53 IgG and another monoclonal antibody against human t-PA (Immunotech) were labled with (^{125}I) by the iodogen procedure[7].

Measurement of t-PA, PAI, TM, and Epo T-PA antigen in the conditioned medium and the lysate of HUVEC were measured by immunoradioassay[8]. PAI activity was determined by titration with purified t-PA (provided by Dr D Collen, University of Leuven, Belgium) and measurement of remaining t-PA activity[9]. One unit of PAI activity was defined as the amount of inhibitor that neutralises one unit of t-PA activity. The molecular number and activity of TM on the surface of HUVEC were determined by RIA[10] and chromogenic assay[5], respectively. One unit of TM activity was defined as 1 nmol · L^{-1} activated protein C formed / ml of incubation mixture per min. TM in conditioned medium was determined by Sandiwich method[11]. 6-Keto-prostagladin F_1 alpha (6-keto-PGF_1 alpha), a stable metabolite of Epo, was measured by RIA[12]. The amount of 6-keto-PGF_1 alpha was used to represent the level of Epo.

Statistics All measurements were expressed as $\bar{x} \pm s$. The significances were evaluated by t test.

RESULTS

Effects of NB on release and production of t-PA and PAI by HUVEC Increasing amounts of NB caused an increasingly stimulative effect on t-PA release at 10 min, 4-h, and 24-h after incubation. The amounts of t-PA within HUVEC reduced after 24-h of incubation when the amount of NB was added. PAI activity in conditioned medium did not show significant change in all groups treated with NB, although a slight decrease in PAI activity was observed in several groups treated with NB 25-1000 $\mu g \cdot ml^{-1}$. These results indicate that NB stimulates the release of t-PA from HUVEC.

Effects of NB on TM of HUVEC The effects of NB on release and production of TM by HUVEC were found to be concentration-dependent. After treatment with NB 25-1000 $\mu g \cdot ml^{-1}$, the amounts of TM were increased not only within the cells, but in conditioned medium as well. Moreover, both the molecular number and activity of TM on the surface of the cells were also increased, which were in parallel to the increasing amounts of NB. These results show that NB induces production and release of TM by HUVEC.

In addition, when HUVEC were treated with both NB and dactinomycin, the amounts of TM within the cells were reduced about 75% as compared with that treated with NB alone. Our data indicated that dactinomycin can abolish NB-induced production of TM by HUVEC. This means that NB-induced production of TM by HUVEC appears to be involved the promotion of DNA transcription within the cells.

Tab 1. Effects of new-breviscapine on molecular number and activity of thrombomodulin on surface of HUVEC. $n=3$, $\bar{x} \pm s$, $^{*}P>0.05$, $^{**}P<0.05$, $^{***}P<0.01$ *vs* **control.**

$\mu g \cdot ml^{-1}$	Molecular numbers of TM / surface per HUVEC	TM / unit · ml^{-1}
0	38 000 ± 2 200	12.1 ± 1.2
6.25	38 600 ± 2 300	13.5 ± 1.4
12.5	37 300 ± 4 600	12.9 ± 2.3
25	50 600 ± 4 000	14.2 ± 2.2
50	72 000 ± 6 600	16.2 ± 1.5
100	89 300 ± 4 700	19.5 ± 1.2
1 000	116 000 ± 6 700	24.8 ± 2.1

Effects of NB on production and release of Epo Incubation of HUVEC with NB 25-1000 $\mu g \cdot ml^{-1}$ led to an increase in

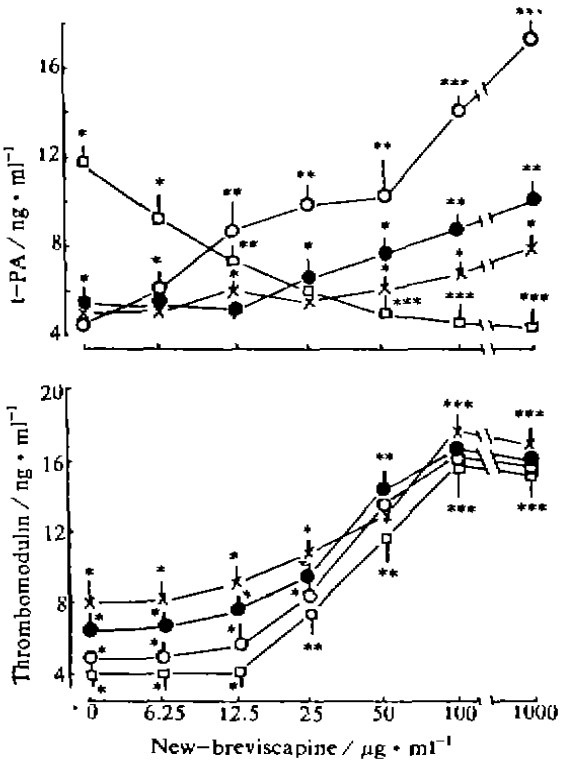

Fig 1. Effects of new-breviscapine (NB) on production and release of tissue-type plasminogen activator (t-PA) and thrombomodulin (TM) by HUVEC. t-PA and TM in cell culture medium after 10-min (○), 4-h (●), and 24-h (·) incubation of HUVEC with NB, and t-PA or TM within the cells after 24-h incubation (□), $n=3$, $\bar{x} \pm s$, $^{*}P>0.05$, $^{**}P<0.05$, $^{***}P<0.01$ *vs* control.

release of Epo at 10 min and 24-h, but a decrease in the release of Epo was seen at 4-h of incubation. The amount of Epo within HUVEC was also decreased after 24-h of incubation.

DISCUSSION

Both TM and Epo derived from endothelial cells are potent anticoagulants[13]. From Chinese traditional herbal drugs, we found that NB is a new stimulator to express TM by HUVEC, so it may be useful in the study of the mechanism of TM expression. In this study, a delayed period of Epo release for several hours between the rapid release phase and slow release phase was observed when HUVEC were treated with NB 100 and 1 000 $\mu g \cdot ml^{-1}$. The mechanism is not clear. It should be noted that Epo synthesis in endothelial cells is variable and is regulated by many factors including "self-regulation" via the inhibitory effect of Epo on its own synthesis in endothelial cells by inducing an increase of cAMP concentration[14]. We supposed that NB induces rapid release of Epo from HUVEC at 10 min, and then Epo in conditioned medium inhibits the production and release of Epo by the cells at 4-h via "self-regulation", once depression phase is over, a stimulation phase appears by turns. Our data provide a new evidence that NB enhances

Tab 2. Effects of new-breviscapine on production and release of 6-keto-$PGF_{1\alpha}$ by HUVEC. $n=3$, $\bar{x} \pm s$, $^{*}P>0.05$, $^{**}P<0.05$, $^{***}P<0.01$ *vs* control.

New-breviscapine / $\mu g \cdot ml^{-1}$	6-Keto-prostaglandin F_1 alpha, $pg \cdot ml^{-1}$ In conditioned medium 10 min	4 h	24 h	Within HUVEC 24 h
0	2.0 ± 0.2	21.0 ± 2.9	25.5 ± 3.2	64.9 ± 7.4
6.25	2.1 ± 0.2	19.5 ± 2.1	27.1 ± 3.1	64.8 ± 6.8
12.5	2.1 ± 0.2	22.0 ± 2.6	33.9 ± 4.3	64.4 ± 6.8
25	3.4 ± 0.4	16.5 ± 1.7	39.1 ± 3.8	53.9 ± 3.7
50	3.6 ± 0.4	15.0 ± 1.4	46.2 ± 5.1	49.1 ± 3.1
100	4.2 ± 0.4	13.5 ± 1.1	44.6 ± 6.8	32.4 ± 4.3
1 000	7.8 ± 0.5	11.8 ± 0.9	47.2 ± 4.6	31.3 ± 2.9

fibrinolysis and anticoagulation of HUVEC via increases in t-PA and Epo release, and TM expression by the cells. In light of the effects of NB on the modulation of endothelial cell function and on the inhibition of platelet activation and thrombus formation, we consider that NB may be served as a potent antithrombotic agent.

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新灯盏花素对人血管内皮细胞纤溶和抗凝作用的影响

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提要 培养的人脐静脉内皮细胞分别与剂量为 0、6.25、12.5、25、50、100 和 1000 $\mu g \cdot ml^{-1}$ 的新灯盏花素孵育后，t-PA 和 Epo 释放增加，而 PAI 活性无明显变化。当新灯盏花素浓度为 25-1 000 $\mu g \cdot ml^{-1}$ 时能诱导内皮细胞合成、表达和释放血栓调节蛋白。本研究提供了新灯盏花素作为内皮细胞纤溶和抗凝血促进剂的新证据。

关键词 新灯盏花素；纤维蛋白溶解；血管内皮；组织型血纤维蛋白溶酶原激活剂；依前列醇；培养的细胞；抗凝剂