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# Platelet activating factor production in bovine cerebral microvascular endothelial cells and its drug inhibition

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**ABSTRACT** The production of platelet activating factor (PAF) in bovine cerebral microvascular endothelial cells (CME cells) and the effects of tetrandrine (Tet) and dauricine (Dau) on the PAF production were investigated. PAF was determined by the aggregation of washed rabbit platelets. The results showed that the CME cells produced PAF 5.93 ng / $8.5\times 10^5$ cells under the calcimycin 2.5 $\mu mol\cdot L^{-1}$ stimulation. Tet and Dau 1, 10, and 100 $\mu mol\cdot L^{-1}$ inhibited the production of PAF by 18.2%, 51.8%, 56.8%, and 26.3%, 63.3%, 65.9%, respectively. Tet concentration–dependently inhibited the PAF 9.1 $nmol\cdot L^{-1}$ induced washed rabbit platelets aggregation with the $IC_{50}$ of 3.05 $\mu mol\cdot L^{-1}$ (95% confidence limits; 0.59–15.86 $\mu mol\cdot L^{-1}$). The binding of [$^3$H]triazolodiazepine to the CME cells was partially displaced by Tet 0.02–33.00 $\mu mol\cdot L^{-1}$. It is suggested that the cerebrovascular system produces PAF at the pathological conditions and the inhibition of Tet and Dau.

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**KEY WORDS** tetrandrine; dauricine; platelet activating factor; vascular endothelium; capillaries; triazoles.

Platelet activating factor (PAF) was originally described as a potent platelet activator derived from antigen–stimulated, IgE– sensitized rabbit basophils[1]. PAF is involved in various disorders, such as asthma, shock, cardiovascular disorders, and renal ischemia[2] PAF is 1000 to 10000 times more potent on a molar basis than histamine in increasing the vascular permeability. It is produced by various cells including basophils, macrophages, monocytes, and mast cells. PAF can be released from endothelial cells of human umbilical veins, bovine aortas, and pulmonary arteries[3,4]. It constricts the pial arteries of newborn pigs[5], and decreases the cerebral blood flow of rats[6]. Our previous studies showed that the PAF specific binding

sites were existence on CME cells[7]. But the production of PAF in the cerebral microvascular endothelial cells (CME cells) has not been described. In the present study, we investigated the production of PAF and the inhibitory effects of Tet and Dau in CME cells.

## MATERIALS AND METHODS

Calcimycin (A-23187, Sigma); trypsin (Sigma); [$^3$H]triazolodiazepine (Tri) and $C_{16}$-PAF (Boehringer); Minimum essential medium (MEM, Gibco); BN 50739 (Gifted from P Braquet); Tris-Tyrode's bovine serum albumin (BSA): pH 7.4, KCl 2.62, $MgCl_2 \cdot 6H_2O$ 1.0, NaCl 137, $CaCl_2 \cdot 2H_2O$ 1.30, Tris 10, glucose 5.6 mmol·$L^{-1}$, BSA 0.25%. Tyrode's gelatin no calcium (TG-no calcium): pH 6.5, KCl 2.62, $MgCl_2 \cdot 6H_2O$ 1.0, NaCl 137, $NaHCO_3$ 12, glucose 5.6, EGTA 0.2 mmol·$L^{-1}$, gelatin 0.25%. Tris-HCl buffer: pH 7.4, KCl 140 mmol·$L^{-1}$, Tris 10 mmol·$L^{-1}$, BSA 0.1%. Acid citrate dextrose: citric acid 6.0, sodium citrate 8.5, glucose 12.3 mmol·$L^{-1}$.

**Cultivation of bovine CME cells and PAF production** Cells were cultivated by our previous method[8]. The CME cells were collected with 0.1% trypsin from culture flask, washed with Tris-Tyrode's BSA (800× *g* for 8 min), resuspended in 0.5 ml Tris-Tyrode's BSA ($1.7\times 10^6$ cells·$ml^{-1}$), and incubated 10 min at 37℃. After Tet and Dau (1-100 μmol·$L^{-1}$) were added, the cells were incubated for another 10 min further. Finally, the cells were stimulated with calcimycin (2.5 μmol·$L^{-1}$) for 20 min. Then the suspension was acidified with 20 μl acetic acid. PAF was extracted[9]. The $CHCl_3$ phase were collected by centrifugation of 1200× *g* for 15 min and dried by the stream of $N_2$. The residues were kept at −40℃ before use.

**Aggregation of rabbit platelet rich plasma (PRP) and washed platelets** Blood was collected from the heart of New Zealand white rabbits (3.2± *s* 0.3 kg) with plastic syringes containing acid citrate dextrose (1 to 6 vol of blood). Platelet-rich plasma (PRP) was prepared by centrifugation of 400× *g* for 6 min. Platelets were obtained by centrifugation of 1400× *g* for 15 min. After gently washed with TG-no calcium (1400× *g* for 15 min), the platelets were resuspended in TG-no calcium containing acetylsalicylic acid (ASA) 0.1 mmol·$L^{-1}$ for 15 min at 25℃, and centrifuged (1400× *g* for 15 min). The platelets were gently suspended in Tris-Tyrode's BSA at a concentration of $4\times 10^8$ platelets·$ml^{-1}$. The aggregation of platelets were induced by PAF. Creatine phosphate-creatine phosphokinase enzymatic system (CP / CPK 312.5 / 152.5 μg·$ml^{-1}$) was directly added to the platelets 30 s before PAF was added. The platelets aggregation of PRP were induced by adenosine diphosphate (ADP) 5 μmol·$L^{-1}$, or arachidonic acid (AA) 80 μmol·$L^{-1}$, or PAF 9.1 nmol·$L^{-1}$.

**Assay of PAF** PAF was assayed by the aggregation of washed rabbit platelets in an aggregometer. Extracts of the CME cells were dissolved in Tris-Tyrode's BSA, and 10 μl were added to the platelets. Platelet aggregation induced by extracts was recorded, and the amount of PAF was calculated on a calibration curve of platelet aggregation induced by synthetic PAF (0.27-9.1 nmol·$L^{-1}$).

**PAF binding assay** The cells were collected with 0.1% trypsin, resuspended in Tris-HCl buffer ($6\times 10^5$ cells·$ml^{-1}$). The cell suspension ($3\times 10^5$ cells) was added to a tube containing [$^3$H]Tri 8 nmol·$L^{-1}$, Tet, with or without 1000 fold unlabeled Tri, was incubated for 15 min at 25℃. Bound and free radioligands were separated by rapid filtration with microporous filtering film (0.8 μm). The filters were washed 5 times with ice-cold Tris-HCl buffer (no BSA). The radioactivity was measured by scintillation.

## RESULTS

**PAF activity in CME cells** In washed rabbit platelets, the action of arachidonic acid and ADP were blockaded by acetylsalicylic acid and CP / CPK. ADP 5 μmol·$L^{-1}$ or arachidonic acid 80 μmol·$L^{-1}$ induced the aggregation of PRP, but not the aggregation

of the washed platelets at the same concentration (Tab 1). The aggregations of PRP and the washed platelets were induced by PAF 0.27–9.1 nmol· $L^{-1}$, and blocked by PAF receptor antagonist Tri 10 μmol· $L^{-1}$. But Tri did not block the platelet aggregation of PRP induced by ADP or arachidonic acid (Tab 1). The aggregation of washed platelets was induced by the extracts of CME cells and blocked by Tri 10 μmol· $L^{-1}$ (Tab 1).

**Tab 1. Aggregation (%) of platelet-rich plasma (PRP) and washed rabbit platelets (WRP) induced by arachidonic acid (AA), ADP, platelet activating factor (PAF), and the antagonism of triazolodiazepine (Tri) 10 μmol· $L^{-1}$. $n$= 3 rabbits. $\bar{x}\pm s$.**

| Stimulants | PRP | WRP |
|---|---|---|
| AA 80 μmol· $L^{-1}$ | 83± 8 | 0 |
| ADP 5 μmol· $L^{-1}$ | 60± 7 | 0 |
| PAF 9.1 nmol· $L^{-1}$ | 66± 7 | 65± 6 |
| EXtracts of CME cells ($4\times 10^4$) | 45± 8 | 44± 10 |
| AA 80 μmol· $L^{-1}$ + Tri | 80± 8 | 0 |
| ADP 5 μmol· $L^{-1}$ + Tri | 58± 7 | 0 |
| PAF 9.1 nmol· $L^{-1}$ + Tri | 0 | 0 |
| Extracts of CME cells ($4\times 10^4$ + Tri) | 0 | 0 |

**PAF production induced by calcimycin** PAF was produced by CME cells under the stimulation of calcimycin, but it was not synthesized without calcimycin stimulation. The amount of PAF attained the peak at calcimycin 2.5 μmol· $L^{-1}$ after 20 min incubation, and it decreased gradually after further stimulation (Tab 2).

**Inhibitory effects of Tet and Dau on production of PAF in CME cells** CME cells produced PAF 5.93 ng/$8.5\times 10^5$ cells under the stimulation of calcimycin 2.5 μmol· $L^{-1}$ for 20 min. Tet and Dau 1, 10, and 100 μmol· $L^{-1}$ incubated for 10 min with CME cells inhibited the production of PAF by 18.2%, 51.8%, 56.8%, and 26.3%, 63.3%, 65.9%, respectively (Fig 1).

**Tab 2. Stimulation of PAF production by calcimycin 2.5 μmol· $L^{-1}$ for 20 min. $n$= 3, $\bar{x}\pm s$. $^{**}P<0.05$ *vs* 10 min group, $^{***}P<0.01$ *vs* 20 min group, a: $P<0.01$ *vs* calcimycin 0.1 μmol· $L^{-1}$ group, b: $P<0.01$ *vs* calcimycin 1.0 μmol· $L^{-1}$ group, c: $P<0.01$ *vs* calcimycin 2.5 μmol· $L^{-1}$ group with Newman–Keuls test.**

| Time, min | PAF, ng/ $8.5\times 10^5$ cells | Calcimycin, μmol· $L^{-1}$ | PAF, ng/ $8.5\times 10^5$ cells |
|---|---|---|---|
| 10 | 0.97± 0.21 | 0.1 | 0.39± 0.10 |
| 20 | 5.08± 0.78$^{**}$ | 1.0 | 1.02± 0.29 |
| 45 | 2.21± 0.38$^{**,***}$ | 2.5 | 6.04± 1.20$^{a,b}$ |
| 60 | 1.60± 0.52$^{***}$ | 10.0 | 2.85± 0.36$^{a,b,c}$ |

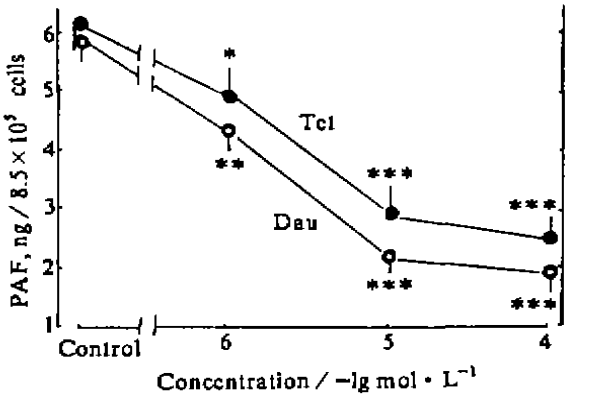


**Fig 1. Inhibition of tetrandrine (Tet) and dauricine (Dau) on PAF production in cerebral microvascular endothelial cells (CME cells). $n$= 5, $\bar{x}\pm s$. $^{*}P>0.05$, $^{**}P<0.05$, $^{***}P<0.01$ *vs* control with Dunnett test.**

**Inhibition of Tet on aggregation of washed platelets induced by PAF** Tet 0.4135, 4.135, 41.35, and 82.7 μmol· $L^{-1}$ or PAF receptor antagonist BN 50739 1, 10, 100, and 1000 nmol· $L^{-1}$ preincubated for 1 min with platelets concentration-dependently inhibited PAF induced platelet aggregation by 24.6%,

45.9%, 86.9%, 100%, and 1.6%, 32.3%, 88.7%, 100%, respectively, their $IC_{50}$ were 3.05 $\mu$mol· $L^{-1}$ (95% confidence limits: 0.59– 15.86 $\mu$mol· $L^{-1}$) and 18.73 nmol· $L^{-1}$ (95% confidence limits: 4.23–82.17 nmol· $L^{-1}$), respectively (Fig 2).

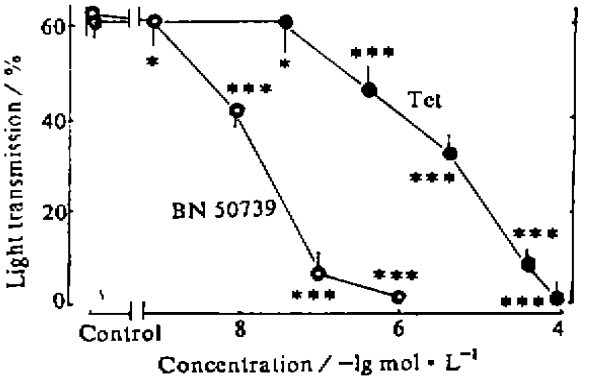


**Fig 2. Effects of Tet and BN 50739 on aggregation of washed rabbit platelets induced by PAF.** $n$= 3, $\bar{x}\pm s$. $^{*}P>0.05$, $^{**}P<0.05$, $^{***}P<0.01$ *vs* **control with Dunnett test.**

**Inhibition of Tet on the [$^3$H]Tri binding to CME cells** The specificity of [$^3$H]Tri binding to CME cells was estabolished by displacing [$^3$H]Tri 8 nmol· $L^{-1}$ by Tet in competition experiment. The binding of [$^3$H]Tri was partially displaced by Tet (0.017–33.00 $\mu$mol· $L^{-1}$) (Tab 3).

## DISCUSSION

PAF could be produced by bovine and rat brain. The existence of PAF in the brain was characterized by GC–MS, HPLC, and TLC, quantified the amount of PAF by bioassay[10,11]. Our results firstly showed that the CME cells synthesized PAF 5.93 ng / (8.5 × $10^5$ cells) under calcimycin 2.5 $\mu$mol· $L^{-1}$ stimulation. But human umbilical cord veins endothelial cells produced PAF 21.5 ng / (5 × $10^5$ cells) at calcimycin 2.5 $\mu$mol· $L^{-1}$ stimulation[12]. This might be the difference between the large blood vessels and the cerebral microvessels. The amount of PAF reached maximum after 20 min of stimulation and decreased thereafter gradually. This might be due to a rapid increase in the acetyltransferase activity and an unchangement of acetylhydrolase activity[13]

**Tab 3. Inhibition of tetrandrine (Tet) on [$^3$H]Tri binding to cerebral microvascular endothelial cells (CME cells).** $n$= 3, $\bar{x}\pm s$. $^{*}P>0.05$, $^{**}P<0.05$ *vs* **control with Dunnett test.**

| Tet, $\mu$mol· $L^{-1}$ | Bound, dpm / 3× $10^5$ cells | Inhibition, % |
|---|---|---|
| Control | 22 318± 2 032 | |
| 0.02 | 18 716± 1 892* | 16.1 |
| 0.17 | 14 494± 2 838* | 35.1 |
| 1.65 | 11 870± 1 378** | 46.8 |
| 33.00 | 10 562± 943** | 52.7 |

In the bioassay of PAF, the extracts of CME cells was added directly to the washed rabbit platelets. The aggregation of platelets induced by the extracts could be blocked by PAF receptor antagonist Tri. It is similar to the reaction of synthetic PAF. These suggested that the activity of the extracts was that of PAF.

Tet and Dau inhibited the production of PAF. Tet inhibited the specific binding of [$^3$H]Tri to CME cells and the aggregation of washed rabbit platelets induced by PAF. Our previous studies showed that Tet and Dau inhibited the proliferation and the DNA synthesis of bovine cerebral microvascular smooth muscle cells induced by PAF. Dau inhibited the binding of [$^3$H]Tri to the cerebral microvascular smooth muscle cells. It suggested that Tet and Dau might inhibit the PAF action in cerebral vascular system.

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(3)

## 血小板激活因子在脑微血管内皮细胞上的产生及药物的抑制作用

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**摘要** 在体外培养的脑微血管内皮细胞上研究血小板激活因子(PAF)的产生. 结果表明，该细胞在卡西霉素的刺激下可产生大量 PAF，粉防己碱和蝙蝠葛碱对 PAF 的产生具有抑制作用，而且粉防己碱能够抑制 PAF 诱导的挽兔洗涤血小板的聚集和[$^3$H]triazolodiazepine 与内皮细胞的结合. 结果提示，粉防己碱能够抑制 PAF 的作用.

**关键词** 粉防己碱；蝙蝠葛碱；血小板激活因子；血管内皮；微血管；三唑类

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