BIBLID: ISSN 0253-9756 中国药理学报 *Acta Pharmacologica Sinica* 1993 Mar; **14** (2) : 155-158

Effect of leukotriene B_4 on arachidonate metabolism and activation of blood cells and endothelial cells[1]

WANG Zhao-Yue, CHEN De-Chun, HE Yang, LI Fu-Gang, RUAN Chang-Geng
(*Thrombosis and Hemostasis Research Unit, Suzhou Medical College, Suzhou 215007, China*)

ABSTRACT Leukotrine B_4 (LTB_4) induced human neutrophils (Neu) aggregation with thromboxane B_2 / prostaglandin E_2 formation and lysozyme release, enhanced platelet aggregation and / or serotonin release caused by threshold concentrations of calcimycin or ADP, and increased Neu adherence to human unbilical vein endothelial cells. Some of its actions were inhibited by quercetin and cobra venom. These results indicate that LTB_4 possesses pharmacological effects on blood cells as well as on endothelial cells, and would be useful for searching new type of anti-inflammatory drugs.

KEY WORDS leukotrienes B; neutrophils; platelet aggregation; vascular endothelium

Leukotriene B_4 (LTB_4) is one of the major products of arachidonic acid metabolism via lipoxygenase pathway in animal and human Neu[1,2]. LTB_4 has been found to be an important biochemical mediator in inflammatory reactions, causing chemotaxis, chemokinesis, and leukocyte adhesion[3]. It may also be involved in the process of tissue injury and thrombus formation[4]. The mechanism by which LTB_4 takes part in various pathological processes has not well been known. In this study we have purified LTB_4 from porcine Neu and observed its effects on arachidonic acid metabolism and activation of human leukocytes, platelets and human umbilical vein endothelial cells (HUVEC) in order to study its pathological and pharmacological significance.

MATERIALS AND METHODS

Calcimycin and ADP from Sigma, dextran T500 from Pharmacia, arachidonic acid from Fluka. Sodium [^{51}Cr]chromate ($Na_2{}^{51}CrO_4$ from the Atomic Energy Company of China. Cobra venom of *Naja naja atra* was a gift from the Snake Venom Institute of Guangxi Medical College. The RIA kits for thromboxane B_2 (TXB_2) and 6-ketoprostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) and the ELISA kit for von Willebrand factor (vWF) were prepared in our laboratory.

Preparation of LTB_4 Porcine blood was collected in 2% Na_2-EDTA. The platelet-rich plasma (PRP) was removed after centrifugation. The remaining blood was diluted with 2% Ficoll-hypaque and centrifuged. The Neu pellet was treated with NH_4Cl-Tris for lysis of the remaining red cells. The Neu were suspected in Hank's solution without Ca^{2+} and Mg^{2+} at 1×10^{11} cells$\cdot L^{-1}$, incubated with Ca^{2+} 20 mmol$\cdot L^{-1}$ and Mg^{2+} 10 mmol$\cdot L^{-1}$ for 5 min, then incubated with arachidonic acid 600 μmol$\cdot L^{-1}$ and calcimycin 10 μmol$\cdot L^{-1}$ for 5 min, and terminated by adding methanol / acetonitrile. The supernatant was collected after centrifugation, dried, passed through silicic acid column, and then purified by revesed-phase (C-18) HPLC (LKB Co). Quantitation was done by using uv absorption ($\lambda_{max}=280$ nm, $\delta=39\,500$). The purity of LTB_4 extracted by HPLC was 95%.

Platelet aggregation Human blood was collected in 3.8% trisodium citrate (vol : vol, 9 : 1). The PRP was taken after centrifugation. Platelet aggregation was performed either with threshold concentrations of inducers (ADP, collagen, arachidonic acid and calciumycin) or wtih a combination of LTB_4 2 μmol$\cdot L^{-1}$ and each inducer at its specific threshold concentration. The aggregation rate was measured according turbidimetry.

Platelet serotonin (5-HT) secretion PRP was mixed with 5-[^{3}H]HT and incubated at 37℃ for 30 min. Five min after adding ADP, collagen, arachidonic acid or calcimycin, the PRP was mixed

Received 1991-04-11 Accepted 1992-10-20
[1] Project supported by the National Science Foundation of China, № 3870713.

with 6% methylaldehyde and centrifuged. The radioactivity of the supernatant was measured in FJ2101G liquid scintillation counter (Efficiency 70%).

Neu aggregation Huan Neu aggregation was performed as described elsewhere[6]. The Neu were first preincubated in the presence or absence of quercetin for 5 min prior to the addition of LTB_4 2 $\mu mol \cdot L^{-1}$ and Neu aggregation rate was measured with turbidity method.

Neu-endothelial cell adherence HUVEC were isolated from intimal lining of umbilical vein and grown to confluence on 24-well culture plates (Costar) at 37℃ under 5% CO_2. Neu were labeled with sodium [^{51}Cr]chromate. The Neu-endothelial cell adhesion was assayed as previously described[7]. LTB_4 was incubated either with Neu for 15 min, or with HUVEC for 1 h, before Neu-endothelial cell adherence assay. In the adherence inhibiting-experiments, Neu or HUVEC were pretreated with venom of *Naja naja atra* before the additin of LTB_4.

Radioimmunoassay for TXB_2, PGE_2 and 6-keto-$PGF_{1\alpha}$ The samples (or standard), ^{125}I-labeled TXB_2, PGE_2 or 6-keto-$PGF_{1\alpha}$ and corresponding antibody were mixed and incubated overnight at 4℃. Separation of bound from free ligand was performed by adding polyethylene glycol 4000. After centrifugation, the supernatant was removed, and the pellet of ^{125}I-tracer combined with antibody was counted in FJ-2008 gamma counter[8].

vWF assay (ELISA) The plate wells were coated with an anti-vWF monoclonal antibody overnight and washed thoroughly. The samples and another anti-vWF monoclonal antibody which had been linked with horseradish peroxydase was added and incubated. The o-phenyl diamide was added. After the substrate was colored, its A was determined at 492 nm[9].

Neu lysozyme assay The supernatant of Neu was incubated with *Micrococcus lysodeicticus* solution. The amount of lysozyme was determined according to turbidity alternation of *lysodicticus* solution in the spectrophotometer.

Statistics Statistical analysis for significane was calculated by t test.

RESULTS

Effect of LTB_4 on Neu activation Addition of LTB_4 induced Neu aggregation with the EC_{50} 1 $\mu mol \cdot L^{-1}$ (0.85-1.15 $\mu mol \cdot L^{-1}$). The Neu activation was accompanied by both release of lysozyme and formation of TXB_2 and PGE_2 in a concentration dependent manner. It affected lysozyme release from Neu only at a concentration of 500 $\mu g \cdot ml^{-1}$ (Tab 1).

Enhancing effect of LTB_4 on platelet activation by calcimycin and ADP LTB_4 20-50 $\mu mol \cdot L^{-1}$ showed an enhancing effect of the threshold concentration of calcimycin (10 $\mu g \cdot ml^{-1}$). TXB_2 formation by platelet was not altered under these conditions. On the other hand, LTB_4 did not augment the action of either collagen or arachidonic acid (Tab 2).

Tab 1. Neutrophil aggregation, lysozyme release, and thromboxane B_2/ prostaglandin E_2 (TXB_2/ PGE_2) formation induced by leukotriene B_4 (LTB_4) 2 $\mu mol \cdot L^{-1}$ and their alternation in the presence of quercetin. n= **10 samples.** $\bar{x} \pm s$. $^{*}P > 0.05$, $^{**}P < 0.05$, $^{***}P < 0.01$ *vs* **control.**

Quercetin, μg / ml	Neu aggregation, %	Lysozyme, μg / 10^7 Neu	TXB_2, pg / 10^7 Neu	PGE_2, pg / 10^7 Neu
Control	10.9± 1.9	17.2± 2.5	281± 65	36± 4
50	6.1± 2.7***	17.9± 2.4*	190± 43**	19± 5**
100	3.6± 1.9***,	18± 3*	167± 59***	17± 3***
200	1.7± 0.8***	17.7± 2.7*	144± 31***	15± 4***
500	–	14.1± 2.1**	–	–

Tab 2. Enhancing effect of LTB_4 2 μmol·L^{-1} on platelet activation induced by some inducers. *n*= samples in parentheses. $\bar{x}\pm s$. $^{*}P>0.05$, $^{**}P<0.05$, $^{***}P<0.01$ *vs* control.

Drug	Platelet aggregation, %	5-HT release, % ($n=10$)	TXB_2, μg/2×10^8 cells ($n=10$)
Adenosine diphosphate (0.33 μmol·L^{-1})			
Control	14± 13 (7)	22± 11	0.4± 0.3
LTB_4	11± 5 (7)	45± 18***	0.29± 0.21*
Collagen (5 μg·ml^{-1})			
Control	23± 18 (7)	26± 12	0.9± 0.7
LTB_4	14± 17* (7)	25± 14*	1.6± 1.7*
Arachidonic acid (40 μg·ml^{-1})			
Control	8± 4 (7)	39± 12	7± 5
LTB_4	7± 3* (5)	37± 11*	7± 6*
Calcimycin (10 μg·ml^{-1})			
Control	7± 6 (6)	46± 17	4.3± 2.9
LTB_4	55± 20***(9)	65± 16**	4.4± 4.7*

Effect of LTB_4 on Neu-endothelial cell adherence LTB_4 promoted Neu adherence to HUVEC in dose dependent manner with the EC_{50} 0.2 μmol·L^{-1} (0.16-0.24 μmol·L^{-1}). The LTB_4-treated Neu stimulated 6-keto-$PGF_{1\alpha}$ formation and vWF release from endothelial cells. Neu adherence stimulated by LTB_4 was significantly inhibited by preincubation with cobra venom 0.2 mg·ml^{-1}, but such Neu retained their ability to promote arachidonic acid metabolism in, and vWF release from, HUVEC. In marked contrast, preincubation of HUVEC with LTB_4 alone or with a combination of LTB_4 and cobra venom did not modify their subsequent adhesion for Neu, nor the release of vWF and 6-keto-$PGF_{1\alpha}$ (Tab 3).

DISCUSSION

In the present study, we prepared LTB_4 from the porcine Neu and purified it by HPLC. It is generally believed that LTB_4 is not capable of activating platelets. However, we found that LTB_4 could enhance platelet aggregation and / or 5-HT release induced by threshold concentrations of calcimycin and ADP, while it alone did not cause platelet activation. It is well known that platelets play a crucial role in hemostasis. Our results suggested that LTB_4 might take part in the thrombus formation in a way of enhancing the effect of some platelet activating agents when leukocytes were involved in this process.

LTB_4 and certain agents have been reported to promote Neu adherence to the endothelial cells. However, it has been controversial whether LTB_4 gives its efficiency by activating Neu or by stimulating endothelial cells[10,11]. In this study, it is the Neu, not the endothelial cells, that were stimulated by LTB_4 to increase their adherence. The reason of this phenomenon might be that there is no receptor for LTB_4 on the surface of endothelial cells[12]. Venom

Tab 3. Effect of LTB_4 on neutrophil (Neu)-endothelial cell interaction and its alternation in the presence of cobra venom. $\bar{x}\pm s$. $^{*}P>0.05$, $^{**}P<0.05$, $^{***}P<0.01$ *vs* control. HUVEC= human umbilical vein endothelial cells. vWF= von Willebrand factor.

LTB_4, μg·ml^{-1}	Venom, mg·ml^{-1}	Neu-HUVEC adhesion, %	6-keto-$PGF_{1\alpha}$, ng·ml^{-1}	vWF, U·ml^{-1}
Neu were treated with LTB_4 and / or venom ($n=8$ samples)				
0	0	33± 24	0.36± 0.19	0.51± 0.16
2	0	66± 20**	1.6± 0.4***	1.1± 0.5**
2	0.2	25± 9*	2.2± 0.6***	1.0± 0.3**
HUVEC were treated with LTB_4 and / or venom ($n=12$ samples)				
0	0	26± 19	0.29± 0.15	0.9± 0.5
2	0	30± 17*	0.29± 0.14*	1.0± 0.4*
2	0.2	25± 18*	0.26± 0.18*	1.05± 0.19*

of *Naja naja atra* can activate Neu as has been demonstrated by its ability to promote Neu aggregation. It blocked the adherence of LTB_4-treated Neu to endothelial cells, but such Neu remained capable of stimulating endothelial cells to generate 6-keto-$PGF_{1\alpha}$ and to induce vWF release, suggesting that, in addition to adherence, Neu can act on endothelial cells probable by means of producing certain mediators, even if no adherence occurs. In this study, we found that LTB_4 possessed direct and / or indirect effects on various blood cells and that these activities can be inhibited by some biochemically active substances. These finding would be of some importance in further studying the mechanism of action of LTB_4 as well as in searching new type of anti-inflammatory drugs.

REFERENCES

1 Borgeat P, Samuelsson B. Transformation of arachidonic acid by rabbit polymorphonuclear leukocytes: formation of a novel dihydroxyeicosatetraenoic acid. *J Biol Chem* 1979; **254** : 2643-6.

2 Borgeat P, Samuelsson B. Arachidonic acid metabolism in polymorphonuclear leukocytes: Effects of ionophore A23187. *Proc Natl Acad Sci USA* 1979; **76** : 2148-52

3 Maclouf J, Mencia-Huerta JM. Les leucotrienes et autres derives des lipoxygenases: une famile de mediateurs lipidiques pharmacologiquement tres actifs. *Pathol Biol* 1985; **33** : 5-8.

4 Wang ZY, Lehuu B, He Y, Ruan CG. The variation of arachidonic acid metabolism of PMN leukocytes in cerebral thrombosis. *Thromb Haemost* 1987; **58** : 102.

5 Maclouf J, Fruteau de Laclos B, Borgeat P. Stimulation of leukotriene biosynthesis in human blood leukocytes by platelet-derived 12-hydroperoxy-icosatetraenoic acid. *Proc Natl Acad Sci USA* 1982; **79** : 6042-6.

6 Berliner S, Weinberger M, Ben-Bassat M, Lavie G, Weinberger A, Giller S, *et al*. Amphotericin B causes aggregation of neutrophils and enhances pulmonary leukostasis. *Am Rev Respir Dis* 1985; **132** : 602-5

7 Wallis WJ, Hickstein DD, Schwartz BR, Junc CH, Ochs HD, Beatty PG, *et al*. Monoclonal antibody-defined functional epitopes on the adhesion-promoting glycoprotein complex (CDDw 18) of human neutrophils. *Blood* 1986; **67** : 1007-13.

8 Wang ZY, Cheng DC, He Y, Ruan CG. Radioimmunoassay of ^{125}I-labeled thromboxane B_2. *Acta Acad Med Suzhou* 1986; **6**(3) : 13-6.

9 Xi XD, Ruan CG, Li PX, Gu JM, Cheng DW. An enzyme-linked immunosorbent assay using monoclonal antibodies to von Willebrand factor and its clinical application. *Clin J Med Lab Technol* 1988; **11** : 272-5.

10 Zimmerman GA, McIntyre TM. Neutrophil adherence to human endothelium *in vitro* occurs by CDw18 (Mol. MAC-1· L^{-1} FA-1 / GP150,95) glycoprotein-dependent and -independent mechanisms. *J Clin Invest* 1988; **81** : 531-7.

11 Hoover RL, Karnovsky MJ, Austen KF, Corey EJ, Lewis RA. Leukotriene B_4 action on endothelium mediates augmented neutrophil / endothelial adhesion. *Proc Natl Acad Sci USA* 1884; **81** : 2191-3.

12 Modat G, Muller A, Mary A, Gregroie C, Bonne C. Differential effects of leukotriene B_4 and C_4 on bovine aortic endothelial cell proliferation *in vitro*. *Prostaglandins* 1987; **33** : 531-8.

155-158 (16)

白三烯 B_4 对血细胞与内皮细胞花生四烯酸代谢与活化的影响

R 375.2

王兆钺, 陈德春, 何 杨, 李福刚, 阮长耿
(苏州医学院血栓与止血研究室, 苏州 215007, 中国)

摘要 白三烯 B_4 (LTB_4)引起白细胞聚集并伴有血栓烷 B_2 / 前列腺素 E_2 生成与溶菌酶释放, 增强阈值浓度的钙离子载体与 ADP 诱导的血小板聚集和 / 或 5-羟色胺释放, 增加白细胞对人脐静脉内皮细胞的粘附率. 槲皮素与中华眼镜蛇毒可抑制 LTB_4 的某些作用. 本文研究证明 LTB_4 对血细胞与内皮细胞的药理作用并有助于探索新的抗炎药物.

关键词 白细胞三烯 B 类; 嗜中性白细胞; 血小板聚集; 血管内皮