

Effect of leukotriene B₄ on arachidonate metabolism and activation of blood cells and endothelial cells¹

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ABSTRACT Leukotriene B₄ (LTB₄) induced human neutrophils (Neu) aggregation with thromboxane B₂/prostaglandin E₂ 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 umbilical vein endothelial cells. Some of its actions were inhibited by quercetin and cobra venom. These results indicate that LTB₄ 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 B₄ (LTB₄) is one of the major products of arachidonic acid metabolism via lipoxygenase pathway in animal and human Neu^(1,2). LTB₄ 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 LTB₄ takes part in various pathological processes has not well been known. In this study we have purified LTB₄ 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 [⁵¹Cr]chromate (Na₂⁵¹CrO₄) 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 B₂ (TXB₂) and 6-ketoprostaglandin F_{1α} (6-keto-PGF_{1α}) and the ELISA kit for von Willebrand factor (vWF) were prepared in our laboratory.

Preparation of LTB₄ Porcine blood was collected in 2% Na₂-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 NH₄Cl-Tris for lysis of the remaining red cells. The Neu were suspended in Hank's solution without Ca²⁺ and Mg²⁺ at 1 × 10¹¹ cells · L⁻¹, incubated with Ca²⁺ 20 mmol · L⁻¹ and Mg²⁺ 10 mmol · L⁻¹ for 5 min, then incubated with arachidonic acid 600 μmol · L⁻¹ and calcimycin 10 μmol · L⁻¹ for 5 min, and terminated by adding methanol/acetone. The supernatant was collected after centrifugation, dried, passed through silicic acid column, and then purified by reversed-phase (C-18) HPLC (LKB Co). Quantitation was done by using uv absorption (λ_{max} = 280 nm, ε = 39 500). The purity of LTB₄ 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 calcimycin) or with a combination of LTB₄ 2 μmol · 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-[³H]HT and incubated at 37°C for 30 min. Five min after adding ADP, collagen, arachidonic acid or calcimycin, the PRP was mixed

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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 $2 \mu\text{mol} \cdot \text{L}^{-1}$ 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°C 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 addition of LTB_4 .

Radiimmunoassay 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°C . 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 peroxidase 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 *lysodeicticus* solution in the spectrophotometer.

Statistics Statistical analysis for significance was calculated by t test.

RESULTS

Effect of LTB_4 on Neu activation Addition of LTB_4 induced Neu aggregation with the EC_{50} $1 \mu\text{mol} \cdot \text{L}^{-1}$ (0.85 – $1.15 \mu\text{mol} \cdot \text{L}^{-1}$). 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 $500 \mu\text{g} \cdot \text{ml}^{-1}$ (Tab 1).

Enhancing effect of LTB_4 on platelet activation by calcimycin and ADP LTB_4 20 – $50 \mu\text{mol} \cdot \text{L}^{-1}$ showed an enhancing effect of the threshold concentration of calcimycin ($10 \mu\text{g} \cdot \text{ml}^{-1}$). 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) $2 \mu\text{mol} \cdot \text{L}^{-1}$ 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, $\mu\text{g} / \text{ml}$	Neu aggregation, %	Lysozyme, $\mu\text{g} / 10^7$ Neu	TXB_2 , $\text{pg} / 10^7$ Neu	PGE_2 , $\text{pg} / 10^7$ Neu
Control	10.9 ± 1.9	17.2 ± 2.5	281 ± 65	36 ± 4
50	$6.1 \pm 2.7^{***}$	$17.9 \pm 2.4^*$	$190 \pm 43^{**}$	$19 \pm 5^{**}$
100	$3.6 \pm 1.9^{***}$	$18 \pm 3^*$	$167 \pm 59^{***}$	$17 \pm 3^{***}$
200	$1.7 \pm 0.8^{***}$	$17.7 \pm 2.7^*$	$144 \pm 31^{***}$	$15 \pm 4^{***}$
500	—	$14.1 \pm 2.1^{**}$	—	—

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

Drug	Platelet aggregation, %	5-HT release, % ($n = 10$)	TXB_2 , $\mu\text{g} / 2 \times 10^8$ cells ($n = 10$)
Adenosine diphosphate ($0.33 \mu\text{mol} \cdot \text{L}^{-1}$)			
Control	14 ± 13 (7)	22 ± 11	0.4 ± 0.3
LTB_4	11 ± 5* (7)	45 ± 18***	0.29 ± 0.21*
Collagen ($5 \mu\text{g} \cdot \text{ml}^{-1}$)			
Control	23 ± 18 (7)	26 ± 12	0.9 ± 0.7
LTB_4	14 ± 17* (7)	25 ± 14*	1.6 ± 1.7*
Arachidonic acid ($40 \mu\text{g} \cdot \text{ml}^{-1}$)			
Control	8 ± 4 (7)	39 ± 12	7 ± 5
LTB_4	7 ± 3* (5)	37 ± 11*	7 ± 6*
Calcimycin ($10 \mu\text{g} \cdot \text{ml}^{-1}$)			
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} $0.2 \mu\text{mol} \cdot \text{L}^{-1}$ ($0.16-0.24 \mu\text{mol} \cdot \text{L}^{-1}$). The LTB_4 -treated Neu stimulated 6-keto-PGF_{1 α} formation and vWF release from endothelial cells. Neu adherence stimulated by LTB_4 was significantly inhibited by preincubation with cobra venom $0.2 \text{mg} \cdot \text{ml}^{-1}$, 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 α} (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 , $\mu\text{g} \cdot \text{ml}^{-1}$	Venom, $\text{mg} \cdot \text{ml}^{-1}$	Neu-HUVEC adhesion, %	6-keto-PGF _{1α} , $\text{ng} \cdot \text{ml}^{-1}$	vWF, $\text{U} \cdot \text{ml}^{-1}$
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 LTB₄-treated Neu to endothelial cells, but such Neu remained capable of stimulating endothelial cells to generate 6-keto-PGF_{1α} 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 LTB₄ 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 LTB₄ as well as in searching new type of anti-inflammatory drugs.

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 白三烯 B₄ 对血细胞与内皮细胞花生四烯酸代谢与活化的影响
 R 373.2
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摘要 白三烯 B₄ (LTB₄)引起白细胞聚集并伴有血栓烷 B₂/前列腺素 E₂ 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 LTB₄ 的某些作用. 本文研究证明 LTB₄ 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

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