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Leukotriene B_4 levels in the arterial blood of asthmatic patients and the effects of prednisolone

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Leukotriene B_4 levels in the arterial blood of asthmatic patients and the effects of prednisolone. K. Shindo, M. Fukumura, K. Miyakawa. ©ERS Journals Ltd 1995.

ABSTRACT: Prednisolone is very effective in controlling wheezing attacks of bronchial asthma, but its mechanism and the pathogenic role of leukotriene B_4 remain unclear.

We measured changes in plasma levels of leukotriene B_4 in an open study during the clinical course of bronchial asthma, with or without water-soluble prednisolone treatment. Two millilitres of blood was drawn from the radial artery of patients on three occasions: 1) during remission; 2) on admission to hospital with an asthma attack; and 3) 2 days after admission and treatment with intravenous prednisolone (1,000 mg·day^{-1}). Leukotriene B_4 was detected by chromatographic fractionation and radioimmunoassay.

In 11 asthmatic patients, leukotriene B_4 levels on the three occasions were 26.8 (10.7), 106.0 (39.9) and 51.6 (20.2) pg·ml^{-1} (mean (SD)), respectively. In contrast, the mean leukotriene B_4 level of 10 normal controls was 35.9 (10.5) pg·ml^{-1}. Leukotriene B_4 levels differed significantly between remission and attack treated without prednisolone, and between attacks treated with and without prednisolone. Mean arterial carbon dioxide ($Paco_2$) values were 4.8 (0.4) kPa (36.0 (3.0) mmHg), 6.1 (0.4) kPa (45.6 (2.9) mmHg), and 5.5 (0.3) kPa (41.6 (2.0) mmHg), respectively. There were significant differences between these mean $Paco_2$ values. The mean leukotriene B_4 levels on the three occasions were correlated with the mean $Paco_2$ values.

Thus, leukotriene B_4 levels in arterial blood reflect the severity of asthmatic attacks and may be affected by intravenous prednisolone.

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In 1979, the leukotrienes were discovered as a group of biologically active compounds that are formed from arachidonic acid, and one of them, leukotriene C_4 (LTC_4), was determined to be a component of slow-reacting substance of anaphylaxis (SRSA). Subsequent studies have demonstrated that leukotrienes are formed by transformation of arachidonic acid into an unstable epoxide intermediate, leukotriene A_4 (LTA_4). The leukotrienes include leukotriene B_4 (LTB_4) ((5S, 12R)-dihydroxy-6, 14-cis-8, 10-trans-eicosatetraenoic acid) which is formed from LTA_4 by the action of LTA_4 hydrolase [1, 2], and which has multiple proinflammatory actions, including chemotactic activity for neutrophils [3].

LTB_4 has only weak effects on smooth muscle, but LTB_4 induces prolonged bronchial narrowing by stimulating the cyclo-oxygenation of endogenous arachidonic acid and the generation of contractile thromboxanes, promotes local oedema formation, and evokes increased secretion of airway mucus [4, 5]. However, evidence to assess the true role of LTB_4 in bronchial asthma is not yet sufficient. Similarly, there have not been sufficient data to explain the mechanism of *in vivo* effectiveness of prednisolone in the treatment of bronchial asthma. Thus, it is important to examine the relationship between plasma levels of LTB_4 and prednisolone therapy in order to investigate the role of LTB_4 in the development of bronchial asthma and the mechanism of action of prednisolone.

The aim of this study was, therefore, to determine whether the LTB_4 level in arterial blood represents the severity of asthmatic attacks, and to investigate whether the plasma level of LTB_4 correlates with intravenous water-soluble prednisolone.

Methods

Subjects

LTB_4 was quantified in the arterial blood of 10 normal controls and 11 asthmatic patients, using a method combining high performance liquid chromatography (HPLC) and radioimmunoassay (RIA). Ten normal male volunteers aged 25–45 yrs with no history of bronchial asthma or other allergies, and with normal pulmonary function, gave their informed consent prior to the study. None of the normal controls were taking medication. Eleven asthmatic men aged 22–76 yrs who had a history

of asthma also gave their informed consent prior to the study. All of the patients met the criteria for the definition of asthma proposed by the American Thoracic Society [6]. Namely, asthma was defined by a history of episodic wheezing and a greater than 20% reversibility of resting forced expiratory volume in one second (FEV_1) after 400 μg of inhaled albuterol.

All patients in this study had atopic asthma. Atopy was defined by the presence of a greater than 3 mm wheal as compared with that caused by the diluent control in response to skin-prick testing with at least two of the following common aeroallergens: cat fur, mixed grass pollens, dog hair, feathers, a mixture of molds, *Dermatophagoides pteronyssinus* and *D. farinae* (Bencard, Brentford, UK).

Blood sampling and medication

In each patient, blood sampling from a radial artery was performed on three occasions: 1) during remission; 2) on admission to hospital with an asthma attack; and 3) 2 days after admission and open treatment with intravenous prednisolone (1,000 mg·day^{-1}). Firstly, two ml of blood was sampled from each patient during remission. A second blood sample was collected just after a patient was admitted to the hospital, and then 1,000 mg of water-soluble prednisolone per day was initiated immediately. On the 2nd day after administration of 1,000 mg of water-soluble prednisolone, the third blood sampling was performed. The blood was used for checking blood gas and detecting LTB_4. The dose of prednisolone was tapered to 0 mg, by 5 mg steps per every 2 or 3 days depending on the severity of clinical symptoms after changing to oral prednisolone. The cumulative dose of the water-soluble prednisolone before the 3rd blood sampling was about 2,000 mg·2 days^{-1}. Inhaled prednisolone, 400 μg·day^{-1} was administered concomitantly during the two days. Inhaled prednisolone was withdrawn together with oral treatment. Before each sampling, complete consent was obtained from each patient and volunteer.

Preparation [7, 8]

Two millilitres of blood was drawn from the radial artery and was immediately placed on ice. After centrifugation at 1,500×g for 30 min, the plasma was kept frozen at -70°C until LTB_4 analysis was performed. Plasma, 0.8 ml, was added to 4 vol of 80% cold ethanol. The solution was centrifuged at 15,000×g at 4°C for 20 min. The supernatant was evaporated to dryness under reduced pressure below 40°C. The residue was dissolved in 10 ml of distilled water, adjusted to pH 5.1 with 0.1 N HCl and passed through a pretreated disposable SEP-PAK C18 extraction column (Waters Chromatography Div., Milford, Massachusetts, USA). The column was eluted successively with 20 ml of distilled water, 10 ml of petroleum ether, and finally with 20 ml of 100% methanol. The last eluent was collected and evaporated to dryness underreduced pressure.

High pressure liquid chromatography (HPLC)

The residue was dissolved in the HPLC eluting solvent and injected into a reverse phase-high pressure liquid chromatograph (RP-HPLC) to collect the LTB_4 fraction. With this extraction procedure, the mean recovery rate of LTB_4 was 83%. The HPLC system used was a Shimazu LC6A model (Kyoto, Japan) equipped with SPD-2AS spectrophotometric detector. The column was a 2.5 mm × 25 cm Microsorbe RP18 (ODS) (Ranin Instrument Co., Inc., Woburn, Massachusetts, USA.). The injection volume was 100 μl. The composition of the solvent was as follows: acetonitrile, methanol, water, and acetic acid (45:15:39:1). The pH of the solvent was adjusted to 5.6 using HCl and NaOH. The retention time of authentic LTB_4 was about 16 min. The LTB_4 fractions that corresponded with the retention time of authentic LTB_4 were collected with a substantial band width in a chromatogram by a fraction collector. The width in a chromatogram to obtain a fraction was regularly the same in each sample. However, the LTB_4 fractions were as wide as possible to have a better recovery rate. We decided the width by performing several preliminary studies using a larger amount of authentic LTB_4. Thus, finally, the fraction was a composite of the sum of several fractions so as not to lose even a grain of LTB_4 in a sample.

Radioimmunoassay

The commercially available radioimmunoassay for LTB_4, the LTB_4 ^3H assay reagent system produced by Amersham International plc. (London, UK) was obtained from New England Nuclear (Tokyo, Japan) and used as described by the manufacturer with aliquots of selected HPLC fractions. The cross-reactivity of the radioimmunoassay has been specified previously [9]. Background levels from other fractions were subtracted for each patient. The assays were performed in a blinded fashion by one well-trained technician.

Chemotaxis assays

Chemotaxis was assayed in a modified multiwell chemotaxis chamber, as described previously [10]. Cells were suspended in complete Hanks' balanced salt solution (HBSS) containing 0.2% bovine serum albumin (BSA) at concentration of 1×10^6 polymorphonuclear neutrophils (PMN)·ml^{-1}. Assays were performed in which the total number of PMNs migrating through polyvinylpyrolidone-free polycarbonate membranes (5 μm pores; Nucleopore Corp., Pleasanton, CA, USA) was quantified [11]. Chambers were incubated for 30 min at 37°C in humid air. After incubation, nonmigrated PMNs were wiped off the filters, and the filters were fixed in methanol and stained. Migrated PMNs were counted. The data are presented as the mean±SD of triplicate assays from three to four samples, and represent the total number of migrating PMNs·mm^{-2} of filter surface.

Isolation of human PMN

Neutrophils were obtained from peripheral blood by modifications of a technique described previously [12]. Blood was drawn and anticoagulated with acid-citrate-dextrose, pH 4.5. Red blood cells were passively sedimented for 45 min by adding 30 ml of whole blood to 10 ml of calcium- and magnesium-free Hanks' balanced salt solution (CMF-HBSS) with 0.35% BSA and 10 ml of 3% dextran 500 in CMF-HBSS. The leucocyte-rich plasma was then removed and centrifuged at room temperature for 7 min at 250×g. The pelleted cells were washed once with 50 ml of CMF-HBSS containing 0.35% BSA, and then resuspended in 25 ml of CMF-HBSS with 0.35% BSA. The suspension was layered over 15 ml of Ficoll-paque and centrifuged for 30 min at room temperature at 400×g. Cells pelleting through the gradient were resuspended in 10 ml of cold NH_4Cl lysis solution, and placed on ice for 10 min to lyse any remaining red blood cells. The tube was then immediately centrifuged at 300×g for 7 min at 4°C. The cell pellet was resuspended in 50 ml of CMF-HBSS with 0.35% BSA. An aliquot was removed and counted using a haemocytometer. Differential cell counts revealed that these preparations were greater than 95% neutrophils.

Materials

Authentic LTB_4 purchased from Sigma Chemical Japan (Tokyo, Japan) was used as a standard.

Statistical analysis

The levels of LTB_4 in the asthmatic patients during attacks were compared with those in control subjects and in asthmatic patients during remission using one factor analysis of variance (ANOVA)-repeated measures, Scheffe F-test, and two-tailed paired statistical analysis. We also determined whether the levels of LTB_4 in the asthmatic patients during attacks were different in the presence and absence of prednisolone, using the same methods. Results are given as mean±SD.

Results

Intra-assay and interassay coefficients of variation of LTB_4 levels were between 5.1 and 13.1% and between 4.9 and 17.5%, respectively (table 1). The percentage recoveries of added known amounts of LTB_4 were 72 and 93% (table 2). The mean percentage recovery was 83%. The serially diluted plasma samples were recovered between 82 and 103% (table 3). Chemotactic assay were performed in LTB_4 fractions and other fractions. The numbers of migrated PMNs in the total of LTB_4 fraction and in the other fractions were 73.7 (13.5) and 15.0 (3.5)·mm^{-2}, respectively, fig. 1. Thus, PMN chemotaxis was more sensitive, with significant difference in the LTB_4 fractions compared to the other fractions ($p<0.05$).

Table 1. Intra- and interassay variance of plasma LTB_4 radioimmunoassay

Plasma sample	Measured LTB_4 pg·ml^{-1}		CV %
Intra-assay precision data			
1 (n=5)	20.7	(2.7)	13.1
2 (n=5)	81.8	(4.2)	5.1
3 (n=5)	148.2	(7.7)	5.2
Interassay precision data			
1 (n=3)	21.7	(3.8)	17.5
2 (n=3)	45.9	(3.4)	7.4
3 (n=3)	132.8	(13.6)	10.2
4 (n=3)	173.8	(8.5)	4.9

Data are presented as mean, and SD in parenthesis. LTB_4: leukotriene B_4; CV: coefficient of variation.

Table 2. – Analytical recovery of leukotriene B_4 (LTB_4) added to plasma sample

Original conc. pg·ml^{-1}	Amounts of added LTB_4 pg·ml^{-1}	Expected rate pg·ml^{-1}	Measured rate pg·ml^{-1}	Recovery value %
160	20	90	64.8	72
	50	105	77.7	74
	100	130	109.2	84
	500	330	297	90
	1000	580	539	93
Mean recovery rate.				83

Conc: concentration

Table 3. – Analytical recovery of leukotriene B_4 (LTB_4) in diluted plasma

Plasma dilution	Expected LTB_4 pg·ml^{-1}	Measured LTB_4 pg·ml^{-1}	Recovery %
Original	120.8	120.8	–
1:2	60.4	62.4	103
1:4	30.2	26.2	87
1:8	15.1	12.3	82
1:16	7.6	9.4	111

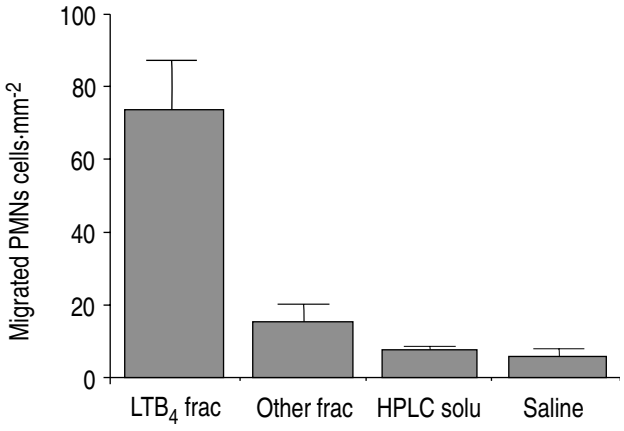

Fig. 1. – PMN chemotaxis to LTB_4 fractions (LTB_4 frac), other fractions (other frac), HPLC solution (HPLC solu), and saline. Results are given as mean±SD. PMN: polymorphonuclear neutrophils; LTB_4: leukotriene B_4; HPLC: high pressure liquid chromatography.

Table 4. – Characteristics of normal controls and asthmatic patients, and drugs administered orally

Subject No.	Age yrs	Height cm	Duration yrs	Orally administered drugs During Remission	During attack without or with prednisolone
Asthmatics (n=11, M)					
1	44	166	10	(–)	Orciprenaline 30 mg·day^{-1}
2	29	170	4	(–)	Orciprenaline 30 mg·day^{-1}
3	55	172	4	(–)	Salbutamol 24 mg·day^{-1}
4	31	164	8	(–)	Procaterol 100 μg·day^{-1}
5	38	167	4	(–)	Procaterol 100 μg·day^{-1}
6	76	177	10	(–)	Salbutamol 24 mg·day^{-1}
7	69	181	11	(–)	Salbutamol 24 mg·day^{-1}
8	42	179	4	(–)	Orciprenaline 30 mg·day^{-1}
9	22	181	5	(–)	Procaterol 100 μg·day^{-1}
10	28	177	4	(–)	Procaterol 100 μg·day^{-1}
11	30	172	9	(–)	Orciprenaline 30 mg·day^{-1}
Controls (n=10, M)					
Mean (SD)	44 (4.3)	171 (6.3)			

(a): all patients received theophylline 400 mg·day^{-1} plus orally indicated drugs: (b) all patients received water-soluble prednisolone 1,000 mg·day^{-1} plus theophylline 400 mg·day^{-1} and other drugs listed in the column, and 400 μg·day^{-1} of inhaled prednisolone; (–): no drug was used in remission. M: male.

The clinical characteristics of the patients and normal controls are presented in table 4. Arterial plasma LTB_4 levels and corresponding $Paco_2$ for the asthmatics on the three occasions and values for controls are shown in table 5 and fig.2.

Mean plasma LTB_4 levels on admission to hospital with asthma attack were significantly raised compared to remission (Scheffe F-test, $p<0.05$). On the second day after admission and intravenous prednisolone treatment, plasma LTB_4 levels were significantly reduced (Scheffe F-test, $p<0.05$) to values not significantly different to remission. There were significant differences between the $Paco_2$ values on these three occasions (Scheffe F-test, $p<0.05$).

LTB_4 was significantly correlated with $Paco_2$ on the three occasions ($p<0.05$) (remission: $y=0.21x + 30.415$, $r^2=0.552$, attack without prednisolone; $y=0.67x + 39.025$, $r^2=0.68$, attack with prednisolone: $y=0.99x + 37.293$, $r^2=0.478$) (fig. 3).

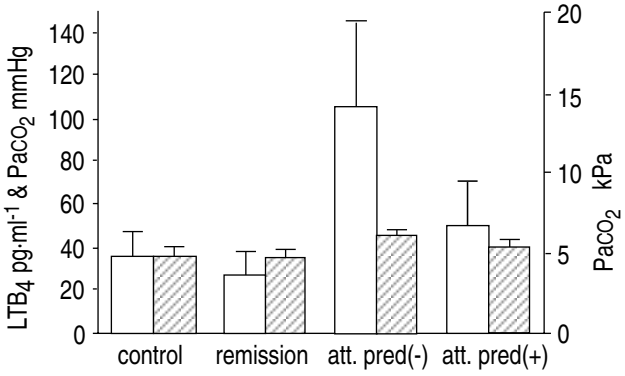

Fig. 2. – Histogram of changes in LTB_4 levels and corresponding $Paco_2$ value on the following three occasions. Results are given as mean±SD. att. pred (-): attack without prednisolone treatment; att. pred (+): attack with prednisolone treatment; LTB_4: leukotriene B_4; $Paco_2$: arterial carbon dioxide tension. □ : LTB_4; ▨: $Paco_2$.

Table 5. – Plasma levels of LTB_4 and $Paco_2$ from asthmatic patients on three occasions, and controls

	Remission		Attack without prednisolone		Attack with prednisolone					
Asthmatic No.	LTB_4 pg·ml^{-1}	$Paco_2$ kPa	LTB_4 pg·ml^{-1}	$Paco_2$ kPa	LTB_4 pg·ml^{-1}	$Paco_2$ kPa	Control No.	LTB_4 pg·ml^{-1}	$Paco_2$ kPa	$Paco_2$ mmHg
1	29.6	5.2	140.5	5.8	45.7	5.4	1	35.9	5.3	39.9
2	33.9	4.8	77.7	5.9	43.9	5.7	2	25.5	4.3	31.9
3	35.7	4.8	83.7	6.0	37.3	5.4	3	23.5	4.5	33.7
4	20.9	5.2	90.6	6.2	38.9	5.9	4	27.8	4.3	32.2
5	22.8	4.9	190.8	6.9	80.4	6.1	5	55.3	5.9	44.1
6	13.9	4.2	107.0	6.3	66.7	6.2	6	39.8	4.3	32.1
7	40.5	5.2	76.0	5.7	60.7	5.5	7	36.9	5.0	37.5
8	17.8	4.6	66.3	5.7	50.7	5.2	8	50.8	5.1	38.3
9	20.8	4.9	75.8	5.9	23.9	5.4	9	28.7	4.1	30.9
10	44.8	5.3	156.8	6.9	28.1	5.2	10	34.9	4.8	36.1
11	13.7	4.1	100.6	6.2	81.5	6.0				
Mean	26.8	4.8	106.0	6.1	51.6	5.5	Mean	35.9	4.8	35.7
(SD)	(10.7)	(0.4)	(39.9)	(0.4)	(20.2)	(0.3)	(SD)	(10.5)	(0.6)	(4.3)

LTB_4: leukotriene B_4; $Paco_2$: arterial carbon dioxide tension.

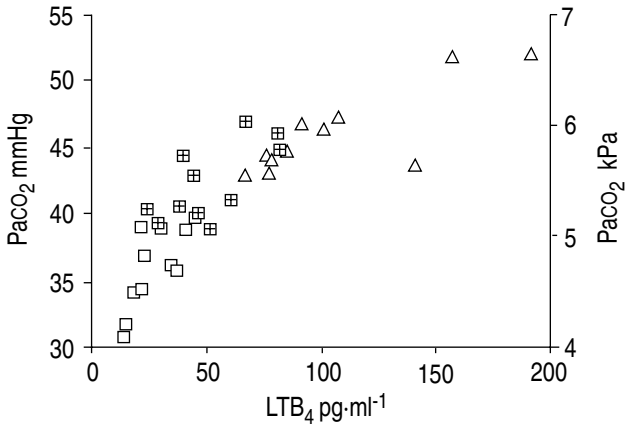

Fig. 3. – The correlation between (LTB_4) level and $Paco_2$ (mmHg and kPa). ❑: during remission; Δ: an attack without prednisolone. ⊞ : an attack with prednisolone. Note that ordinate does not start at zero.

Discussion

In this study, we measured LTB_4 levels in the arterial blood of asthmatic patients, and found that the LTB_4 concentration during an attack without prednisolone was significantly higher than that during an attack with prednisolone, during remission, and in normal controls. In addition, we showed the significant reduction in LTB_4 levels between the attacks with or without prednisolone treatment. The amount of LTB_4 during an attack with prednisolone was still higher than that during remission.

Unfortunately, the cellular origin of LTB_4 detected in this study cannot be confirmed at this time. Similarly, FERRERI *et al.* [13] could not determine the source of increased LTB_4 in nasal fluids of aspirin-sensitive asthmatic patients during reaction to aspirin. However, it is known that bronchial epithelial cells, neutrophils and alveolar macrophages can produce LTB_4 predominantly [14–17]. Thus, it is conceivable that most of the LTB_4 in arterial blood is derived from alveolar macrophages and neutrophils accumulated in asthmatic lung tissue, as well as from bronchial epithelial cells. Consequently, these results suggest the possibility that LTB_4 levels in arterial blood may, at least in part, reflect the extent to which macrophages and neutrophils are accumulated and stimulated in asthmatic lung tissue.

Although glucocorticoids induce phospholipase inhibitory proteins that reduce arachidonic acid metabolism in some tissues [18], recent reports have shown that glucocorticoids do not inhibit prostaglandin synthesis by renal glomeruli [19] and cultured fibroblasts [20]; moreover, urinary excretion of prostaglandin E_2 and prostaglandin F_2 actually increases in rats and humans receiving glucocorticoids [21, 22]. Thus, the true *in vivo* effect of glucocorticoids on cyclo-oxygenase metabolites remains unclear. Moreover, there is insufficient evidence to explain the *in vivo* effect of glucocorticoids on 5-lipoxygenase metabolites, even though the clinical effects are well-documented. The data shown in this study suggest the following: 1) that LTB_4 levels naturally reduce with attenuation of symptoms, being independent of effectiveness of the water-soluble prednisolone; 2) that the prednisolone acts on cells accumulated in asthmatic lung and inhibits them from producing and releasing LTB_4. Further investigated is needed to explain the mechanism of the reduction.

Additionally, we found a good correlation between LTB_4 and $Paco_2$, which reflects the severity of bronchial asthma. We presume that mucus secretion, oedema, and other inflammatory changes induced in the bronchi by LTB_4 affect $Paco_2$. However, we have observed that reoxygenation increases the contraction in bronchial smooth muscle induced by LTC_4 and LTD_4 under hypoxic conditions [23]. Therefore, we suggest that changes in blood gases (from hypoxic to hyperoxic, or from hyperoxic to hypoxic) may play an important role in stimulating cells to release LTB_4, particularly cells accumulated in the asthmatic lung.

In conclusion, we demonstrated that LTB_4 levels in arterial blood reflect the severity of asthmatic attacks and may be affected by intravenous prednisolone.

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