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Formation of Leukotrienes From Calcium Ionophore-A23187 Stimulated Rabbit, Rat and Mice White Blood Cells

Mahmoud A. Mansour[1]*, Fahad I. Al-Jenoobi[2], Saleh A. Al-Suwayeh[2], Abdulhakeem A. Al-Majed[1] and Othman Al-Shabanah[1]

Department of Pharmacology[1] and Department of Pharmaceutics[2], College of Pharmacy King Saud University, P. O. Box 2457, Riyadh 11451, Saudi Arabia.

Abstract

Leukotrienes (LTs) producing capacity was investigated in calcium ionophore A23187-stimulated rabbit, rat and mice peripheral white blood cells suspension. A reverse phase high performance liquid chromatography technique and computerized UV spectroscopy were employed to isolate and quantitate the released LTs namely, LTC_4 and LTB_4. Preincubation of rabbit white blood cells at 37°C for 5 min followed by calcium ionophore-A23187 (1 μM) stimulation for another 5 min produced an equal amounts of LTC_4 as compared to LTC_4 produced by human white blood cells (105 ± 11 versus 95 ± 9.5 pmol/10^7 cells respectively; mean ±SEM). In contrast rabbit white blood cells synthesized significantly lower LTB_4 in comparison with LTB_4 produced by peripheral white blood cells from healthy control (168 ± 18 versus 228 ± 19 pmol/10^7 cells respectively; mean ±SEM). When rat and mice white blood cells suspension were stimulated with calcium ionophore A23187 (1 μM) after preincubation at 37°C for 5 min, equivalent amounts of LTC_4 and LTB_4 were observed. However, LTB_4 and LTC_4 produced by rat and mice white blood cells were significantly lower in comparison with LTB_4 and LTC_4 produced by human white blood cells stimulated with calcium ionophore-A23187. These results demonstrate that rabbit, rat and mice white blood cells suspension possess the capacity to produce LTC_4 and LTB_4 from endogenous substrate after calcium ionophore-A23187 stimulation.

Introduction

Leukotrienes are a family of oxygenated metabolities of arachidonic acid, which synthesized by 5-lipoxygenase enzyme (EC 2.5.1.18)[1]. During cell activation, LTs biosynthesis is initiated by translocation of a specific high molecular weight cytosolic phospholipase A_2 ($cPLA_2$) from cytoplasm to selectively hydrolyze nuclear envelope phospholipids releasing arachidonic acid[2]. The liberated arachidonic acid binds to arachidonate transfer protein, 5-lipoxygenase-activating protein (FLAP) which makes the fatty acid available to 5-lipoxygenase enzyme[3,4]. In a calcium-and ATP-dependent reaction the fatty acid is transformed to unstable epoxide intermediate leukotriene A_4 (LTA_4)[5,6]. In various human cell types and tissues, leukotriene A_4 is metabolized via three main pathways; by cytosolic LTA_4 hydrolase (EC 3.3.2.6) to the potent leukocyte activator, LTB_4[7] or by membrane bound LTC_4 synthase (EC 2.5.1.37) to the spasmogenic cysteinyl containing leukotriene *viz* LTC_4[8] or spontaneously non-enzymaticaly hydrolysed to the biologically inactive stereoisomers namely; 6-trans LTB_4 and 12-epi-6-trans LTB_4[9]. Endogenous and exogenous stimuli including platelet activating factor, chemotactic peptide C5a and calcium ionophore A23187 have been shown to trigger the release of LTs[10].

LTC_4 once formed is exported from the cells via an active transport to the extra-cellular space[11]. The subsequent conversion of LTC_4 into LTD_4 via removal of γ -glutamyl moiety from glutathione is catalyzed by γ-glutamyl-transpepetidase, an enzyme located at the external site of plasma membrane[12]. This reaction can not be considered as biological inactivation, since LTD_4 generally possess equal or even higher potency than LTC_4[13]. The next metabolic step results in substantially loss of the biological activity through removal of glycine results in LTE_4 formation. The cysteinyl-containing leukotrienes bind to specific receptors and mediate a wide variety of inflammatory response[14]. In the airway system, LTC_4 is one of the relevant mediators involved during bronchial asthma and is responsible for many

of the cardinal symptoms of the disease[15]. It increases mucus secretion, edema formation and being a potent spasmogenic[16]. The cysteinyl-containing leukotrienes receptors antagonists are now in clinical use for pharmacological treatment of these diseases[17]

A key pro-inflammatory role has been postulated for LTB_4 in the light of its ability to recruit and activate inflammatory cells[18]. It stimulates the production of a number of pro-inflammatory mediators including cytokines[7], release of lysosomal enzymes[19], the generation of superoxide radicals in neutrophils[20], as well as it activates NADPH oxidase (EC 1.6.99.1) of human eosinophil and H_2O_2 production by human monocytes[21]. These effects reflect an ability to accentuate free radicals generation and indicating an ability to augment and prolong tissue inflammation.

The knowledge concerning leukotrienes biosynthesis by white blood cells of different species is limited. Previously it has been demonstrated that calcium ionophore-A23187 induced LTB_4 generation from rabbit and rat white blood cells[22]. However LTC_4 formation has not been described.

The goal of the present study was to extend the previous observations to study the capacity of white blood cells from different species rabbit, rat and mice to produce LTB_4 and LTC_4. To achieve this goal, white blood cells from rabbit, rat and mice were isolated and stimulated with calcium ionophore-A23187 to produce LTB_4 and LTC_4 in comparison with LTB_4 and LTC_4 produced by peripheral white blood cells from the healthy donors were investigated.

Materials and Methods

Materials

The following chemicals were used: calcium ionophore-A23187, LTB_4 and LTC_4 (Sigma Chemical Company, St. Louis, MO, USA); dextran T500 and ficoll (Pharmacia, Freiburg, Germany); phosphate buffer saline (PBS): Dulbecco's formulation containing 0.9 mM calcium, pH 7.4 (Gibco BRL, Life Science Technologies Ltd., Paisley, Scotland); HPLC

columns (Beckman, San Roman, California, USA) and HPLC solvents (E. Merck, Darmstadt, Germany). The remaining reagents were of the highest analytical grade available.

Animals Experiments:-

White rabbit, swiss albino rats and normal mice were obtained from Experimental Animal Care Center of King Saud University, Riyadh, Saudi Arabia. They were housed in a room maintained at a temperature of 24±1°C and 55±5% relative humidity with a regular 12 h light: 12 h dark cycle and free access to standard laboratory food (Purina Chow) and water.

Preparation of white blood cells suspension

Blood samples from the rabbit, rats and mice as well as from healthy controls were taken into heparinized tubes. The white blood cells were separated following standard laboratory technique[23]. Briefly, 10 ml of whole blood was mixed with an equal volume of dextran T500 (2% in saline), then, allowed to stand for 30 min at 4 °C to sediment erythrocytes. The leukocytes-rich upper plasma layer was then aspirated and centrifuged at 280 xg for 10 min. The resultant leukocyte pellet was washed twice with PBS then, re-suspended in 20 ml of hypotonic ammonium chloride (0.74% in PBS) for another 30 min at 4°C to lyse any remaining erythrocytes. After centrifugation at 280 xg for 10 min, the cell pellets were washed twice and suspended in PBS. The cells were counted in a Neubauer counting chamber. The final white blood cell counted was adjusted at $10x10^6$ cells/ml PBS. The viability was about 95% as judged by trypan blue exclusion test.

Incubation procedure:

Human, rabbit, rat and mice white blood cells $10x10^6$ cells/ml PBS were incubated at 37°C for 5 min. After pre-incubation, the cells were immediately stimulated for the release of LTs with calcium ionophore-A23187 (Final concentration 1μM) 1μl of calcium ionophore-A23187 (1mM) was added to each aliquote (1 ml) to give final concentration of 1 μM. The release reaction was terminated after 5 min by adding 5 volumes of 99% ethanol and placing the tubes in ice. The samples were stored at –20°C until required for further processing.

Purification, Identification and Quantitation of leukotrienes: -

The samples dissolved in ethanol were centrifuged and the clear supernatants were aspirated and then evaporated to dryness. The obtained residues were dissolved in mobile phase and then centrifuged. Identification and quantitation of leukotrienes were performed by RP-HPLC and UV spectroscopy, as previously reported[24]. Chromatographic separation was performed at room temperature using Shimadizu programmable module apparatus (Japan) and a mobile phase consisting of acetonitrile/methanol/water/acetic acid (30:19:50:0.8, pH 5.6). The absorbance of the effluent was continuously monitored at 280 nm using a variable wavelength UV detector. Authentic standard of LTs was co-chromatographed with the samples for the identification and quantitation of the eluted LTs in the samples. The areas under the curve of each type of LTs in the samples were correlated with that of the standard. The results were expressed as pmol/10^7 cells.

Statistics

Data are expressed as (means±SEM). Statistical comparisons of leukotrienes production in suspension of rabbit, rat and mice white blood cells and human white blood cells were performed by using student's *t*-test. Significance was accepted at $P< 0.05$.

Results

Leukotrienes formation in suspension of human and rabbit white blood cells.

When human and rabbit white blood cells were stimulated with 1 μM calcium ionophore-A23187 for 5 min, a release of 5-lipoxygenase derived products *viz*, LTC_4 and LTB_4 can be identified. LTB_4 was the major compound formed. Rabbit white blood cells produced an equivalent amount of LTC_4 as compared to that produced by peripheral white blood cell separated from the control healthy donor. Thus, 105±11 (n=8; mean ± SEM) and 95±9.7

(n=4) pmol $LTC_4/10^7$ cells were produced by rabbit white blood cells and white blood cells separated from healthy controls respectively (Fig 1). However, the production of LTB_4 was significantly lower in rabbit white blood cells suspension as compared to the human white blood cells. Thus, 168±18 versus 228±19 pmol $LTB_4/10^7$ cells were produced by rabbit and human white blood cell suspensions respectively (Fig 1).

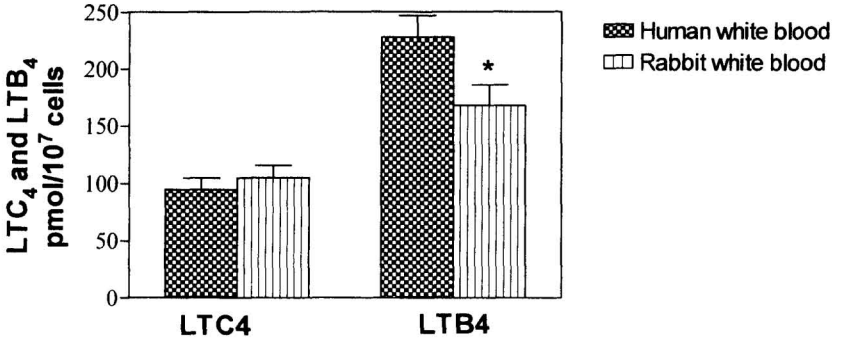

Fig (1): Leukotrienes formation by rabbit and human peripheral white blood cells suspension stimulated with calcium ionophore-A23187.

Peripheral white blood cells separated from rabbit and control healthy donors (10 $x10^6$/ml PBS) were preincubated at 37°C for 5 min, then stimulated with A23187 (1 μM) for leukotriene release for another 5 min. The reaction was terminated by addition of 5 ml of ethanol 99% and placing the tube in ice. Leukotrienes were identified and quantified by RP-HPLC.

Leukotrienes formation in suspension of human, rat and mice white blood cells.

Rat and mice white blood cells preincubated at 37°C for 5 min and stimulated with calcium ionophore-A23187 for another 5 min, produced a similar amounts of LTC_4 and LTB_4. However, stimulation of white blood cells separated from healthy donor produced LTC_4 5 times more than LTC_4 produced from rat and mice (Fig 2). In addition, human white blood cells produced 5 and 7 times LTB_4 as compared to LTB_4 produced from rat and mice, respectively (Fig 2).

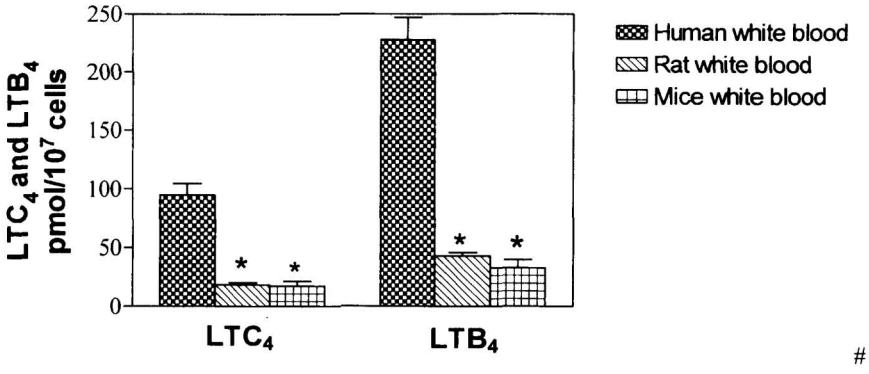

Fig (2): Leukotrienes formation by rat, mice and human peripheral white blood cells suspension stimulated with calcium ionophore-A23187.

Peripheral white blood cells separated from rat, mice and control healthy donors (10 x10^6/ml PBS) were preincubated at 37°C for 5 min, then stimulated with A23187 (1 μM) for leukotriene release for another 5 min. The reaction was terminated by addition of 5 ml of ethanol 99% and placing the tube in ice. Leukotrienes were identified and quantified by RP-HPLC

Discussion

Stimulation of human white blood cells with calcium ionophore-A23187 (1 μM) led to the generation of leukotrienes, predominantly LTB_4 which is known as the major mediator released from human leukocytes[25]. By virture of its calcium ionopheric property, A23187 produces an elevation of intracellular calcium concentration that is required for the binding of 5-lipoxygenase to an activating protein (FLAP)[3]. This binding activates enzyme results in its association with the nuclear membrane and consequently increases the synthesis of LTs from arachidonic acid[26,27].

The present study showed that, rabbit, rat and mice white blood cells suspension possess the capacity to produce LTC_4 and LTB_4 after calcium ionophore-A23187 stimulation. This finding indicates that calcium ionophore A23187 activates 5-lipoxygenase enzyme in white blood cells isolated from rabbit, rat and mice and the stimulus is sufficient to make endogenous arachidonic acid available for leukotrienes synthesis in these cells.

The results of the present study demonstrate that rabbit white blood cells produced an equivalent amount of LTC_4 as compared to human peripheral white blood cells. In contrast, the synthesis of LTB_4 was significantly lowered in rabbit white blood cells suspension as compared with LTB_4 produced by human white blood cells (Fig. 1). Stimulation of rat and mice white blood cells suspensions produced similar amounts of LTC_4 and LTB_4. However, LTC_4 produced from calcium ionophore-A23187 stimulated human white blood cells was 5 times more than LTC_4 produced by rat and mice white blood cell suspension. In addition human white blood cells produced 5 and 7 times LTB_4 more than LTB_4 produced from white blood cells separated from rat and mice respectively.

The present results are consistent with the previous study demonstrated that rabbit and rat white blood cells produced LTB_4 from endogenous arachidonic acid after calcium ionophore-A23187 (1μM)[22]

White blood cells suspension contains heterogeneous cells populations mainly neutrophils which produce LTB_4[28], while esinophils and basophils produce LTC_4[29]. In addition, it has been shown that B-lymphocytes produce leukotrienes only when stimulated with calcium ionophore-A23187 in the presence of exogenous arachidonic acid and reducing agents[30]. Thus, in the present study, LTB_4 and LTC_4 were produced by neutrophils and eosinophils respectively. However, when studying in vitro leukotrienes producing capacity, samples containing identical number of the cells and a similar percentage of different cell types should be compared. Therefore, it has been assumed that the decreased level of LTC_4 and LTB_4 produced by rat and mice white blood cells suspension in the present study may be due to the difference in the percentage of various cell types in comparing with human white blood cell suspensions. In the present study, differential cell identification count revealed that there was no significant difference between number of neutrophils, eosinophils and mononuclear cells in the different white blood cells suspensions (data not shown).

The reason why the rat and mice cells produced significantly lowered LTC_4 and LTB_4 as compared to human white blood cells may be due to decreased arachidonic acid content in phospholipid of nuclear envelope of white blood cells of rat and mice. Thus less of the substrate (arachidonic acid) will be available for 5-lipxygenase enzyme. In addition, the identity of the specific phospholipaseA_2 involved in the release of arachidonic acid utilized for leukotrienes synthesis is still a matter of controversial between different species[31]. Moreover it has been demonstrated that leukotrienes synthesis in intact cells from various species depend on the activity of 5-lipoxygenase-activating protein (FLAP), which binds arachidonic acid, thereby facilitating the 5-lipoxygenase-catalyzed conversion of fatty acid into LTA_4[4,32]. These may indicate a difference in the level of arachidonic acid content or difference in 5-lipoxygenase activating protein level in rat and mice as compared to human white blood cells.

In conclusion: This investigation demonstrates that white blood cells of rabbit, rat and mice possess the capacity to produce different amounts of leukotrienes from endogenous substrate after short term incubations with calcium ionophore-A23187.

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