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LEUKOTRIENE FORMATION BY BOVINE POLYMORPHONUCLEAR LEUKOCYTES

PAULINE WALSTRA, JAN VERHAGEN, GERRIT A. VELDINK and JOHANNES F.G. VLIEGENTHART

Department of Bio-Organic Chemistry, State University of Utrecht, Croesestraat 79, NL-3522 AD Utrecht (The Netherlands)

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The leukotriene production by bovine polymorphonuclear leukocytes isolated from peripheral blood has been studied. Cells were incubated in the presence of arachidonic acid, glutathione, calcium ionophore A23187 and Ca^{2+}. The leukotrienes then formed are leukotriene C_4, leukotriene B_4, two all-*trans* isomers of leukotriene B_4 and the double dioxygenation product 12-*epi*-6-*trans*-8-*cis*-leukotriene B_4. Leukotriene C_4 is formed in such a large quantity by the bovine polymorphonuclear leukocyte that it might constitute an excellent and inexpensive source for the biosynthetic preparation of this spasmogenic leukotriene.

Introduction

Leukotrienes constitute a group of compounds formed via an initial dioxygenation of arachidonic acid by a lipoxygenase (EC 1.13.11.12). Dependent on the position of oxygen insertion, 5-, 12- and 15-series leukotrienes can arise [1–3].

So far only the biological activities of the 5-series leukotrienes have been thoroughly investigated. Leukotriene B_4 has a strong chemotactic activity for human neutrophils [4] and eosinophils [5]. Therefore it may play an important role in inflammatory processes [2,6]. The sulfidopeptide leukotrienes C_4, D_4 and E_4, being the main constituents of slow-reacting substance of anaphylaxis, have been shown to possess a strong bronchoconstrictive activity in vivo [7–9] as well as in vitro [10]. Because of these pronounced biological activities the formation of leukotrienes by various cell types has been studied. In particular the leukotriene production by polymorphonuclear leukocytes has been the subject of many investigations because these cells play an important role in immunological processes. Dependent on the species, the production of leukotriene B_4 and/or leukotriene C_4 by these cells has been reported. Rabbit polymorphonuclear leukocytes metabolize arachidonic acid to leukotriene B_4, leukotriene B_4 isomers and HETEs [11,12]. In addition to these compounds, porcine polymorhonuclear leukocytes produce 15-series leukotrienes [13]. Human polymorphonuclear leukocytes form leukotriene B_4, 20-hydroxy-leukotriene B_4, leukotriene B_4 isomers, leukotriene C_4 and small amounts of 15-series leukotrienes [14]. Recently, it has been shown that the production of leukotriene C_4 and 15-series leukotrienes by human polymorphonuclear leukocytes is almost exclusively due to the eosinophils, whereas the other leukotrienes originate from the neutrophils [14]. Horse eosinophils also produce leukotriene C_4, but in smaller quantities and beside leukotrienes D_4, B_4 and leukotriene B_4 isomers [15].

Abbreviations: leukotriene B_4, 5(*S*),12(*R*)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid; leukotriene C_4, 5(*S*)-hydroxy-6(*R*)-*S*-glutathionyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid; leukotriene D_4, 5(*S*)-hydroxy-6(*R*)-*S*-cysteinylglycyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid; leukotriene E_4, 5(*S*)-hydroxy-6(*R*)-*S*-cysteinyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid; HETE, monohydroxyeicosatetraenoic acid.

Here we report on the production of leukotrienes by bovine polymorphonuclear leukocytes. It could be shown that this leukotriene pattern is significantly different from that of other species studied so far.

Materials and Methods

Materials. Arachidonic acid (purity > 99%) was purchased from Nu Chek Prep, Inc. (MN, U.S.A.). Calcium ionophore A23187, glutathione, prostaglandin B_2 and the radical scavenger 4-hydroxy-2,2,6,6-tetramethylpiperidinooxy free radical were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Ficoll-Paque was from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Methanol and water (HPLC quality) were purchased from Merck (Darmstadt, F.R.G.). Tetrahydrofuran (HPLC quality), and octadecyl reversed-phase extraction columns (6 ml) were obtained from Baker (Deventer, The Netherlands). Synthetic leukotrienes B_4 and C_4 were a kind gift from Dr. J. Rokach (Merck-Frosst Laboratories, Pointe Claire/Dorval, Quebec, Canada). 12-*epi*-6-*trans*-8-*cis*-Leukotriene B_4, often referred to as 5(*S*)-12(*S*)-dihydroxyeicosatetraenoic acid, being the product of double dioxygenation of arachidonic acid, was biosynthesized from 12-HETE (unpublished data). All other reagents used were of p.a. quality.

Purification of granulocytes. Bovine blood was obtained from a local slaughterhouse. Immediately after death of the animals, blood was collected in a vessel containing a solution of 0.15 M trisodium citrate (0.1 litre per litre blood). The anticoagulated blood was centrifuged at 485 × *g* for 20 min, after which the platelet-rich plasma was carefully removed by aspiration.

Erythrocytes were then lysed by adding 2 vol. of distilled water and shaking gently. After 30 s 1 vol. of hypertonic phosphate-buffered saline (2.7% (w/v) NaCl in 13.2 mM phoshate buffer, pH 7.4) was added [16] and the mixture was centrifuged at 485 × *g* for 10 min. The pellet was resuspended in a small volume phosphate-buffered saline (0.9% (w/v) NaCl in 8.6 mM phosphate buffer, pH 7.4) and centrifuged for 10 min at 485 × *g*. The cells were resuspended in phosphate-buffered saline (for cells from approx. 0.35 l of blood 20 ml phosphate-buffered saline were used) and brought upon Ficoll-Paque (20 ml cell suspension on 7 ml Ficoll-Paque).

After centrifugation at 675 × *g* for 45 min the lymphocytes and monocytes were removed. From the pellet the remaining erythrocytes were lysed as described above, after which the polymorphonuclear leukocytes were washed with phosphate-buffered saline. The whole procedure was carried out at room temperature.

Incubation procedure and sample preparation. Polymorphonuclear leukocytes were suspended in phosphate-buffered saline at a final concentration of $4 \cdot 10^7$ cells/ml. 4 ml of this suspension were preincubated at 37 °C for approx. 10 min. Incubation for 10 min (except for time-course experiments) at 37 °C and pH 7.4 was in the presence of 80 μM arachidonic acid, 5 mM glutathione, 20 μM calcium ionophore A23187 and 2 mM Ca^{2+} unless stated otherwise, and stopped by the addition of ice. The incubation mixture was then centrifuged at 27000 × *g* for 15 min at 4 °C. The supernatant (pH 7.4) was applied to an octadecyl (C_{18}) reversed-phase extraction column which had previously been washed with methanol and subsequently with water. After applying the supernatant to the column and washing with water (approx. 10 ml) the adsorbed leukotrienes were eluted with methanol (approx. 3 ml). Recovery for all leukotrienes was found to be better than 85% (data not shown). After addition of 4-hydroxy-2,2,6,6-tetramethylpiperidinooxy free radical (0.6 μg/ml), the eluates were stored under nitrogen at −20 °C.

Reversed-phase HPLC. This was carried out with a CP^{tm} Spher 10-C18 column (250 × 4.6 mm, Chrompack, Middelburg, The Netherlands) attached to a Perkin-Elmer LC 85 detector.

Isocratic elution was carried out with the solvent system tetrahydrofuran/methanol/water/acetic acid (25 : 30 : 45 : 0.1 by vol.) which had been brought to pH 5.5 with ammonia. This system allows an excellent separation of the leukotrienes in one run [17]. Since binding of cations to the column could lead to excessive retention of sulfidopeptide leukotrienes, 0.1% EDTA was added to the aqueous phase [18].

A flow rate of 0.9 ml/min was used and the detection was at 280 nm. A Hewlett-Packard 3390 A integrator was applied to measure peak areas.

Leukotrienes were quantified by relating the peak areas to the internal standard prostaglandin B_2, which had been added to all samples.

Molar absorption coefficients at 280 nm of 28 650 $M^{-1} \cdot cm^{-1}$ for prostaglandin B_2 and 40 000 $M^{-1} \cdot cm^{-1}$ for the leukotrienes were used.

Results and Discussion

Polymorphonuclear leukocytes were purified from 0.7 l bovine blood yielding $(22 \pm 1) \cdot 10^8$ cells (mean value ± S.D., $n = 12$) with a purity of 93 ± 3% (mean value ± S.D., $n = 12$). The remainder of cells were lymphocytes and monocytes, which do not produce leukotrienes under our conditions.

In Fig. 1 a typical reversed-phase HPLC chromatogram of the leukotrienes formed by bovine polymorphonuclear leukocytes is shown. The yields of the leukotrienes produced, namely leukotriene C_4, leukotriene B_4, 12-*epi*-6-*trans*-8-*cis*-leukotriene B_4 and the two all-*trans*-leukotriene B_4 isomers 6-*trans*-leukotriene B_4 and 12-*epi*-6-*trans*-leukotriene B_4 are given in Table I. It should be noted that bovine polymorphonuclear leukocytes are capable of synthesizing very large amounts of leukotriene C_4. Recently it could be shown that the leukotriene C_4 formed by human polymorphonuclear leukocytes originates entirely from the eosinophils [14]. Because in our bovine polymorphonuclear leukocyte preparations relatively large amounts of eosinophils (approx. 20%) are present, it is likely that also in this case the eosinophil is responsible for the leukotriene C_4 production. Because the density distribution of bovine polymorphonuclear leukocytes differs substantially from that of human polymorphonuclear leukocytes, the purification method described for human eosinophils and neutrophils [14] has not been successful for the isolation of pure bovine eosinophils

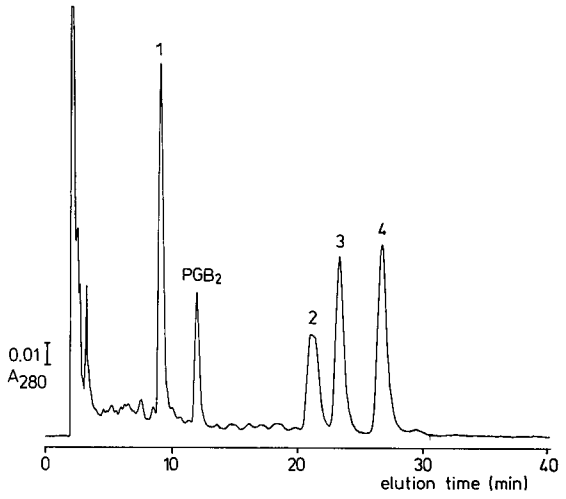

Fig. 1. Reversed-phase HPLC chromatogram of the leukotrienes produced by $64 \cdot 10^6$ bovine polymorphonuclear leukocytes. Cells were incubated at a concentration of $4.0 \cdot 10^7$ cells/ml in the presence of 80 μM arachidonic acid, 20 μM calcium ionophore A23187, 5 mM glutathione and 2 mM Ca^{2+} in phosphate-buffered saline, pH 7.4 for 10 min. Solvent system: tetrahydrofuran/methanol/water/acetic acid (25:30:45:0.1 by vol.), pH 5.5. Flow rate 0.9 ml/min/ Detection was at 280 nm, using prostaglandin B_2 (0.4 μg) as an internal standard. Peaks were identified by HPLC behaviour, coelution with the synthetic standards leukotriene C_4 (peak 1), leukotriene B_4 (peak 3) and 12-*epi*-6-*trans*-8-*cis*leukotriene B_4 (peak 4) and ultraviolet spectra. Peak 2, 12-*epi*-6-*trans*-leukotriene B_4 plus 6-*trans*-leukotriene B_4. PGB_2, prostaglandin B_2.

until now. However, preliminary experiments with purified neutrophils indicate that these cells do not produce leukotriene C_4 as a major metabolite.

The optimal incubation time for leukotriene formation by bovine polymorphonuclear leukocytes is about 10 min, as can be derived from the time courses shown in Fig. 2.

Leukotriene C_4 was found not to be metabolized into other sulfidopeptide leukotrienes by bovine polymorphonuclear leukocytes. Even after

TABLE I

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Cells were incubated according to the conditions described in the legend to Fig. 1. Results are given as molecules $\times 10^{-6}$ released per cell. For conversion of the unit 10^6 molecules/cell into pmol/10^6 cells multiply by 1.7. LT, leukotriene.

	LTC_4	LTB_4	12-*epi*,6*t*-LTB_4 + 6*t*-LTB_4	12-*epi*,6*t*,8*c*-LTB_4	Total leukotrienes
With 2 mM Ca^{2+} ($n = 10$)	17 ± 3	11 ± 4	7 ± 5	24 ± 13	59 ± 18
Without Ca^{2+} ($n = 6$)	2 ± 2	2 ± 3	1 ± 2	3 ± 3	8 ± 9

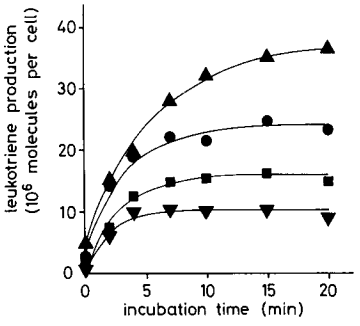

Fig. 2. The effect of the length of the incubation period on the leukotriene production by bovine polymorphonuclear leukocytes. For incubation conditions, except for the incubation time, see the legend to Fig. 1. ▲, 12-*epi*-6-*trans*-8-*cis*-leukotriene B_4; ●, leukotriene C_4; ■, leukotriene B_4; ▼, 12-*epi*-6-*trans*-leukotriene B_4 plus 6-*trans*-leukotriene B_4.

prolonged incubation (20 min) no conversion into leukotriene D_4 could be detected.

In contrast to the human polymorphonuclear leukocytes [14], the bovine leukocyte was found unable to metabolize leukotriene B_4 into 20-hydroxy-leukotriene B_4. Because the biological activities of 20-hydroxy-leukotriene B_4 are reduced as compared to leukotriene B_4 [19], it has been suggested that the ω-oxidation of leukotriene B_4 is a route of biological inactivation. Apparently the bovine polymorphonuclear leukocyte does not exhibit this pathway. Furthermore, no significant amounts of 15-series leukotrienes could be detected.

Because polymorphonuclear leukocyte preparations are always slightly contaminated with platelets, and bovine platelets possess a 12-lipoxygenase [20], it is likely that the varying amounts of 12-*epi*-6-*trans*-8-*cis*-leukotriene B_4 produced by bovine polymorphonuclear leukocytes are formed by a combined action of bovine polymorphonuclear leukocytes and platelets.

In the absence of Ca^{2+} the leukotriene production was found to be reduced by approx. 85% (Table I), in agreement with the observations that 5-lipoxygenases are Ca^{2+}-dependent [1,21].

In the absence of exogenous arachidonic acid about 35% of the amount of leukotrienes formed in the presence of 80 μM arachidonic acid was produced. From endogenous arachidonic acid relatively large amounts of leukotrienes C_4 and B_4 in addition to small amounts of 12-*epi*-6-*trans*-8-*cis*-leukotriene B_4 and the all-*trans*-leukotriene B_4 isomers were formed compared to the amounts formed in the presence of exogenous arachidonic acid. At arachidonic acid concentrations above 160 μM the leukotriene formation was found to be progressively inhibited. Optimal leukotriene production was found at approx. 80 μM arachidonic acid.

As can be seen in Fig. 3, the formation of leukotriene C_4 reaches its maximum at about 5 mM glutathione. However, the formation of leukotriene B_4, 12-*epi*-6-*trans*-8-*cis*-leukotriene B_4, 12-*epi*-6-*trans*-leukotriene B_4 and 6-*trans*-leukotriene B_4 was found to be independent of the exogenous glutathione concentration. Furthermore it can be concluded that the glutathione concentration in the cell has to be significant because in the absence of exogenous glutathione the leukotriene C_4 production is still approx. 50% of the maximal production (at 5 mM).

In summary, it can be concluded that bovine polymorphonuclear leukocytes are capable of synthesizing large amounts of the spasmogenic leukotriene C_4 beside other 5-series leukotrienes. The polymorphonuclear leukocytes isolated from 1 l of bovine blood can produce as much as 60 μg of leukotriene C_4. Because bovine blood can be easily obtained in large quantities it constitutes an inexpensive source for the biosynthetic preparation of substantial amounts of leukotriene C_4.

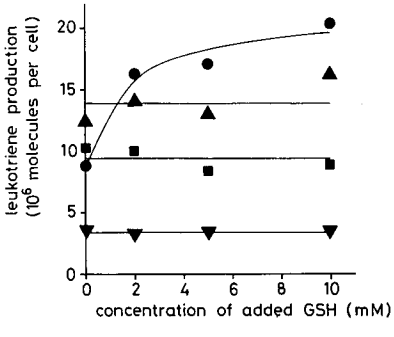

Fig. 3. The influence of glutathione (GSH) on the leukotriene production by bovine polymorphonuclear leukocytes. For incubation conditions, except for the glutathione concentration, see the legend to Fig. 1. ●, leukotriene C_4; ▲, 12-*epi*-6-*trans*-8-*cis*-leukotriene B_4; ■, leukotriene B_4; ▼, 12-*epi*-6-*trans*-leukotriene B_4 plus 6-*trans*-leukotriene B_4.

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