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Evidence for lipoxin formation by bovine polymorphonuclear leukocytes via triple dioxygenation of arachidonic acid

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Incubation of bovine polymorphonuclear leukocytes (PMNs) with arachidonic acid leads to the formation of four lipoxins. The same lipoxins are also formed upon incubation of bovine PMNs with 5(*S*)-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid, 5-hydroxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid, 5(*S*)-hydroperoxy, 15(*S*)-hydroxy-6,13-*trans*-8,11-*cis*-eicosatetraenoic acid or 5(*S*),15(*S*)-dihydroxy-6,13-*trans*-8,11-*cis*-eicosatetraenoic acid. A 5,6-epoxide as intermediate in lipoxin formation in the bovine PMN is highly improbable because the 5-hydroxy compounds are as good substrates as the 5-hydroperoxy compounds. Moreover, the two main lipoxins were found to coelute with the two lipoxins produced via a triple dioxygenation of arachidonic acid by soybean lipoxygenase-1. Hence the bovine PMN is the first cell for which evidence is presented that the formation of lipoxins proceeds mainly via triple dioxygenation and not via 15-hydroxy-leukotriene A_4 as is proposed for human and porcine PMNs.

Lipoxin; Triple dioxygenation; Arachidonic acid; (Bovine polymorphonuclear leukocyte)

1. INTRODUCTION

The recently discovered lipoxins [1,2] represent a novel class of oxygenated derivatives of arachidonic acid. They can be formed by combined action of 5- and 15-lipoxygenases. Because lipoxins contain a conjugated tetraene system, they have a characteristic ultraviolet spectrum with absorption maxima at 289, 302 and 316 nm. Two subclasses of lipoxins exist, 5,6,15-trihydroxy-7,9,11,13-tetraenes designated LXA and 5,14,15-trihydroxy-6,8,10,12-tetraenes designated LXB. Several biological activities of the lipoxins have been described. LXA stimulates superoxide anion generation by human PMNs without causing aggregation [2], it provokes contraction of parenchymal strips and stimulates microvascular changes [3,4]. LXA and LXB both inhibit human natural killer cell cytotoxicity [5,6].

Incubation of human PMNs with 15-HPETE leads to the formation of six LXA and at least two LXB isomers [3,4,7]. Very recently, porcine PMNs were found to produce LXB isomers after treatment with phospholipase A_2 [8]. The formation of lipoxins by these two cell types is proposed to proceed via an epoxide, namely the 15-hydroxy derivative of LTA_4 [4,9–11].

Until now, the formation of lipoxins by human PMNs has been reported exclusively from 15-HPETE and not from arachidonic acid. (After completion of the present study the formation of *S,R,S-trans,cis,trans,trans*-LXB directly from

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Abbreviations: 5-HPETE, 5(*S*)-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid; 5-HETE, racemic 5-hydroxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid; 5,15-diHETE, 5(*S*),15(*S*)-dihydroxy-6,13-*trans*-8,11-*cis*-eicosatetraenoic acid; 5HP,15-HETE, 5(*S*)-hydroperoxy,15(*S*)-hydroxy-6,13-*trans*-8,11-*cis*-eicosatetraenoic acid; 8HP,15-HETE, 8(*S*)-hydroperoxy,15(*S*)-hydroxy-5,11-*cis*-9,13-*trans*-eicosatetraenoic acid; 15-hydroxy-LTA_4, 5(*S*)-5,6-oxido-15(*S*)-hydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid; LXA, 5,6,15-trihydroxy-7,9,11,13-eicosatetraenoic acid; LXB, 5,14,15-trihydroxy-6,8,10,12-eicosatetraenoic acid; PMN, polymorphonuclear leukocyte

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arachidonic acid by rabbit reticulocytes has been reported [18].) We decided to investigate a possible lipoxin formation by bovine PMNs directly from arachidonic acid as well as from several other possible intermediates in lipoxin biosynthesis. In addition, we present evidence that the lipoxin formation by bovine PMNs proceeds mainly via triple dioxygenation.

2. MATERIALS AND METHODS

2.1. *Materials*

Arachidonic acid (>99%) was purchased from Fluka AG (Buchs, Switzerland). Calcium ionophore A23187, prostaglandin B_2 and the radical scavenger 4-hydroxy-2,2,6,6-tetramethylpiperidinooxy free radical were obtained from Sigma (St. Louis, MO, USA). Sample filters (pore size 0.45 μm) were from Nihon Millipore Kogyo KK (Yonezawa, Japan). Methanol, water (Merck, Darmstadt, FRG) and tetrahydrofuran (Baker, Deventer, The Netherlands) were of HPLC quality. Octadecyl solid-phase extraction columns (6 ml) were obtained from Baker. Racemic 5-HETE was generously provided by Mr G.A.A. Kivits (Unilever Research Laboratory, Vlaardingen, The Netherlands). 5(*S*)-HPETE was from Cayman Chemical (Ann Arbor, MI, USA). Soybean lipoxygenase-1 was purified according to [12].

2.2. *Preparation of 5HP,15-HETE and 5,15-diHETE*

15(*S*)-HPETE was biosynthesized from arachidonic acid by incubation with soybean lipoxygenase-1 [13], and was reduced to 15(*S*)-HETE with $NaBH_4$. After purification on straight-phase HPLC (solvent system: hexane/2-propanol/acetic acid, 97:3:0.5 (v/v)), 15(*S*)-HETE was converted into 5HP,15-HETE and 8HP,15-HETE by incubation with soybean lipoxygenase-1 [13]. 5HP,15-HETE was purified on reversed-phase HPLC with methanol/water/acetic acid, 70:30:0.5 (v/v), as a solvent system. For the preparation of 5,15-diHETE, the mixture of 5HP,15-HETE and 8HP,15-HETE was reduced with $NaBH_4$ before purification on reversed-phase HPLC (same solvent system).

2.3. *Purification of PMNs*

PMNs were isolated from bovine blood, obtained from a local slaughterhouse. Purification was as described in [14].

2.4. *Incubation procedure and sample preparation*

PMNs were suspended in phosphate-buffered saline (0.9% (w/v) NaCl in 8.6 mM phosphate buffer, pH 7.4) at a final concentration of 4.0×10^7 cells/ml. The suspensions were preincubated at 37°C for approx. 10 min. Incubation was for 10 min or 30 min, as indicated, at 37°C and pH 7.4 in the presence of 20 μM calcium ionophore A23187 and 2 mM Ca^{2+}. The substrate was added, dissolved in ethanol, at indicated concentrations. The final ethanol concentration never exceeded 0.1% (v/v). The concentrations of 5-HPETE, 5-HETE, 5HP,15-HETE and 5,15-diHETE were determined spectrophotometrically using molar absorption coefficients of 29500 $M^{-1} \cdot cm^{-1}$ (monoH(P)ETEs) and 33500 $M^{-1} \cdot cm^{-1}$ (diH(P)ETEs) [13], respectively. Reactions were stopped by the addition of ice and the incubation mixture was centrifuged at $12000 \times g$ for 20 min at 4°C. Eicosanoids were extracted with octadecyl solid-phase extraction columns as described [15]. Prostaglandin B_2 was added as an internal standard. The methanol eluates were stored under nitrogen at −20°C after the addition of 0.6 μg/ml of the radical scavenger 4-hydroxy-2,2,6,6-tetramethylpiperidinooxy free radical. Before HPLC analysis, the eluates were concentrated and filtered through sample filters (0.45 μm).

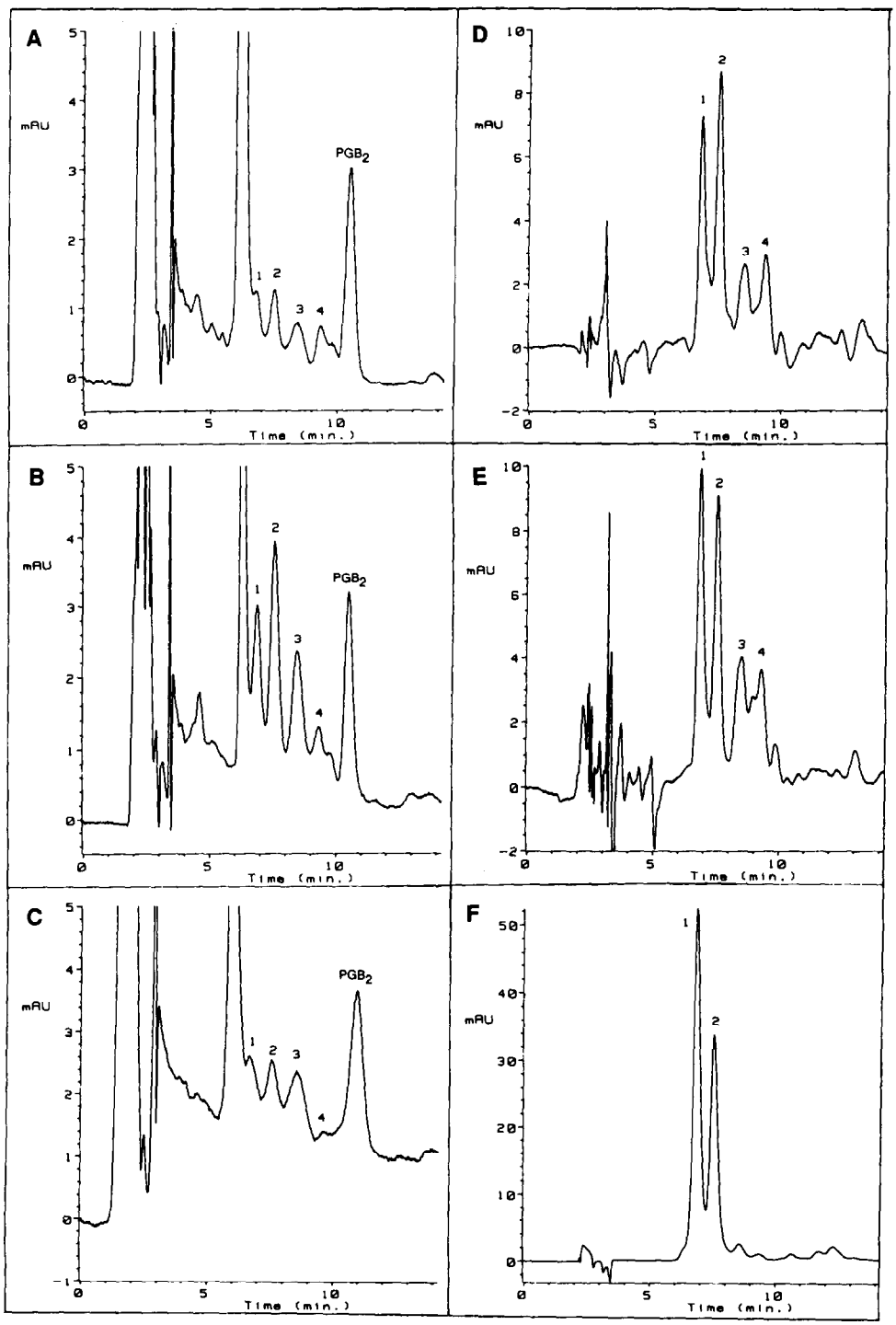
2.5. *Reversed-phase HPLC analysis*

A CP™ Spher 8-C18 column (250 × 4.6 mm, Chrompack, Middelburg, The Netherlands) was used attached to an HP 1040A diode-array detector combined with an HP 1090 solvent-delivery system. Isocratic elution was carried out using tetrahydrofuran/methanol/water/acetic acid, 25:30:45:0.1 (by vol.), which had been adjusted to pH 5.5 with ammonia [16]. The aqueous phase contained 0.1% (w/v) EDTA, which improves the recovery of the sulfidopeptide leukotrienes [17]. A flow rate of 0.9 ml/min was used and the detection was from 220 to 350 nm. Data were processed by an HP 310 SPU workstation.

3. RESULTS AND DISCUSSION

Incubation of bovine PMNs with arachidonic acid resulted in the formation of lipoxins. About 1% of the arachidonic acid was converted into lipoxins, compared to 8% conversion into leukotrienes. Four lipoxins were formed upon incubation with arachidonic acid (fig.1A) in a ratio of

Fig.1. Reversed-phase HPLC profiles of the lipoxins formed by bovine PMNs (A–E) or soybean lipoxygenase-1 (F). 4×10^7 PMNs were suspended in 1 ml phosphate-buffered saline. Incubation was in the presence of 20 μM calcium ionophore A23187, 2 mM Ca^{2+} and 5.5 μM arachidonic acid (A), 5.5 μM 5(*S*)-HPETE (B), 5.5 μM 5-HETE (C), 80 μM 5HP,15-HETE (D) or 80 μM 5,15-diHETE (E) for 10 min (A,B,C) or 30 min (D,E). Soybean lipoxygenase-1 was incubated with 500 μM 15-HETE for 24 h at 4°C (F). Solvent system was tetrahydrofuran/methanol/water/acetic acid, 25:30:45:0.1 (by vol.), pH 5.5, with a flow rate of 0.9 ml/min. Detection was at 316 nm. Correction for non-enzymatic formation of lipoxin isomers was made when 5HP,15-HETE and 5,15-diHETE were used as a substrate (D,E). Peaks 1–4 had the ultraviolet spectrum of a lipoxin.


A
B
C
D
E
F
mAU
Time (min.)
PGB2
1
2
3
4

approx. 1:2:1:1. Hence, the bovine PMN is the first cell from which lipoxin formation directly from added arachidonic acid is reported.

We then decided to investigate lipoxin formation using possible intermediates in lipoxin biosynthesis. For this purpose, 5-HPETE, 5-HETE, 5HP,15-HETE and 5,15-diHETE were used. Incubation of 5-HPETE and of 5-HETE with bovine PMNs also resulted in the formation of four lipoxins (fig.1B and C, respectively) which were found to coelute with those formed by incubation with arachidonic acid. Moreover, also the incubation of bovine PMNs with 5HP,15-HETE or with 5,15-diHETE resulted in the formation of the same four lipoxins (fig.1D and E, respectively) although in different ratios (3:3:1:1) and amounts. In addition, up to seven other lipoxins were produced in small amounts, together representing up to 10% of the total amount of lipoxins. Incubations with 5HP,15-HETE or 5,15-diHETE in phosphate-buffered saline (pH 7.4) for 30 min at 37°C, demonstrated that these seven lipoxins can be readily formed non-enzymatically. Therefore, the lipoxin formation using 5HP,15-HETE or 5,15-diHETE as a substrate was always corrected for this non-enzymatic lipoxin formation.

Since the same four lipoxin isomers were produced when arachidonic acid, 5-HPETE, 5-HETE, 5HP,15-HETE as well as 5,15-diHETE was used as a substrate and the formation of a 5,6-epoxide is neither possible from 5-HETE, nor from 5,15-diHETE, it has to be concluded that the lipoxins produced by bovine PMNs are not formed via a 5,6-epoxide. Therefore, we investigated the possibility that the lipoxins produced by bovine PMNs are formed via triple dioxygenation, by incubating 15(*S*)-HETE with soybean lipoxygenase-1. This afforded two compounds with the ultraviolet spectrum of a lipoxin (fig.2). According to literature data [4,10], these compounds are *S,R,S-trans,cis,trans,trans*-LXB (peak 1, fig.1F) and *S,R,S,-trans,trans,cis,trans*-LXA (peak 2, fig.1F). *S,R,S-trans,cis,trans,trans*-LXB was found to coelute with lipoxin 1 and *S,R,S-trans,trans,cis,trans*-LXA was found to coelute with lipoxin 2, formed upon incubation of bovine PMNs with either of the following substrates: arachidonic acid, 5-HPETE, 5-HETE, 5HP,15-HETE or 5,15-diHETE. This is different from lipoxin formation in human and porcine PMNs,

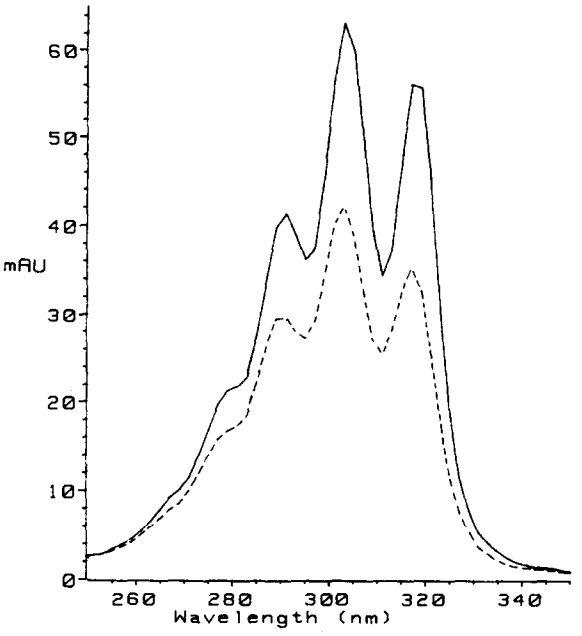


Fig.2. Ultraviolet spectra of the two lipoxin isomers formed by soybean lipoxygenase-1 upon incubation with 15(*S*)-HETE. Straight line: peak 1 (fig.1F), *S,R,S-trans,cis,trans,trans*-LXB; dotted line: peak 2 (fig.1F), *S,R,S-trans,trans,cis,trans*-LXA.

which proceeds via 15-hydroxy-LTA_4. In human PMNs, incubation of 15-HPETE led to the formation of only small amounts of *S,R,S-trans,trans,cis,trans*-LXA in addition to other lipoxins whereas no *S,R,S-trans,cis,trans,trans*-LXB was produced by these cells [4]. Treatment of porcine PMNs with phospholipase A_2 resulted in the formation of mainly *S,R,S-trans,trans,trans,trans*-LXB next to *S,S,S,-trans,trans,trans,trans*-LXB and very small amounts of *S,R,S-trans,cis,trans,trans*-LXB [8].

In summary, the bovine PMN is the first cell for which evidence is presented that the formation of lipoxins proceeds mainly via triple dioxygenation of arachidonic acid.

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