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Lipoxins are major lipoxygenase products of rainbow trout macrophages

Trevor R. Pettitt, Andrew F. Rowley and Chris J. Secombes*

Biochemical and Physiological Research Group, School of Biological Sciences, University College of Swansea, Singleton Park, Swansea, SA2 8PP, Wales and **Department of Zoology, University of Aberdeen, Tillydrone Avenue, Aberdeen, AB9 2TN, Scotland*

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Rainbow trout macrophages synthesize lipoxins as major lipoxygenase products entirely from endogenous fatty acids. High-performance liquid chromatographic analysis of the supernatants from macrophages challenged with calcium ionophore A23187 revealed a range of lipoxygenase products including mono-hydroxy fatty acids, leukotrienes B_4 and B_5 and four major peaks with retention times and UV spectra characteristic of lipoxins (λ_{max} 302 nm). Cochromatography with authentic standards, UV spectroscopy and radiolabelling with [^{4}C]arachidonate and eicosapentaenoate allowed tentative identification of the two largest peaks as lipoxin A_4 and A_5.

Macrophage; Lipoxin; Leukotriene; Lipoxygenase product; (Rainbow trout, *Salmo gairdneri*)

1. INTRODUCTION

The lipoxins are a class of trihydroxytetraenes, derived from 20 carbon polyunsaturated fatty acids by the combined action of 5 and 15 lipoxygenases [1–3], which show a range of activities of possible physiological importance. Particular lipoxins have been found to inhibit natural killer cell cytotoxicity [4,5], stimulate prostacyclin generation by human endothelial cells [6], induce glomerular hyperfusion and hyperfiltration [7], cause the slow contraction of pulmonary smooth muscle [8,9], relax vascular smooth muscle [8,9] and serve as highly potent activators of protein kinase C [10].

A number of studies have examined the potential for lipoxin synthesis using a variety of mammalian cell types; however, most required the addition of a suitable substrate, usually 15-hydroperoxy-eicosatetraenoate (15-Hpete) [1,11], working on the assumption that in vivo this could be supplied by other cells which are unable to produce lipoxins themselves. Lipoxin generation would thus represent an example of synthetic collaboration between different cell types. This idea has been supported by work with mixed granulocyte-platelet suspensions where neither cell alone could produce lipoxins but together synthesized relatively large amounts entirely from endogenous fatty acid [12]. Some results have, however, suggested that certain single cell types alone may also be able to synthesize these compounds since bovine polymorphonuclear leucocytes [13] and human eosinophilic granulocytes [14] have been found capable of producing lipoxins from exogenous arachidonic acid, although at levels below those for other lipoxygenase products such as the leukotrienes. The latter cell type also synthesized lipoxins from endogenous fatty acid when challenged with calcium ionophore, although these were at best only 90% eosinophils so more than one leucocyte type may still have been involved [14]. Treatment of porcine leucocytes with phospholipase A_2 also provoked lipoxin formation from endogenous fatty acid [15]. However, none of these experiments offer convincing evidence that lipoxins can be generated in vivo in meaningful amounts by either a single or several cell types entirely from endogenous fatty acid.

Our previous work has revealed the generation of large amounts of leukotriene and related dihydroxytrienes by both whole blood and isolated leucocytes from rainbow trout [16,17] but no significant production of lipoxins was observed. Our present studies with rainbow trout macrophages have, however, discovered that this cell type can synthesize large amounts of lipoxin entirely from endogenous fatty acid and at levels in excess of those for other lipoxygenase products. This initial report is also the first finding of lipoxin synthesis by a non-mammalian cell type.

Correspondence address: A.F. Rowley, Biomedical and Physiological Research Group, School of Biological Sciences, University College of Swansea, Singleton Park, Swansea, SA2 8PP, Wales

Abbreviations: Hete, hydroxy-5,8,11,13 eicosatetraenoate; LXA_4, lipoxin A_4; LXA_5, lipoxin A_5; LXB_4, lipoxin B_4; LTB_4 leukotriene B_4; LTB_5, leukotriene B_5; LTC_4, leukotriene C_4

2. MATERIALS AND METHODS

Marcrophages were isolated from the haemopoietic head kidney of rainbow trout, *Salmo gairdneri*, and placed in short-term culture as described previously [18]. Briefly, the head kidney was removed, and pressed through a fine mesh into L-15 medium (containing 2% foetal calf serum and 1% penicillin/streptomycin): large aggregates were discarded and the cells were allowed to attach to dishes for 2 h at

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18°C. Subsequently, non-adherent cells such as lymphocytes were removed by washing and the resultant monolayers were maintained for 48–72 h at 18°C in L-15 medium containing 5% heat-inactivated foetal calf serum and 1% penicillin/streptomycin. Dishes with adherent macrophages of >95% purity were thoroughly washed in Hanks' balanced salt solution (HBSS), pH 7.4, and incubated for 30 min at 18°C with 5 or 10 μM calcium ionophore A23187 in the same saline. After incubation the medium was removed, centrifuged (10000 × g for 5 min at room temperature) to pellet any cells, and eicosanoids extracted from the supernatant with C_{18} Sep-Paks (Millipore/Waters) and separated by HPLC using a reverse-phase ODS column (4.6 mm × 250 mm; Ultrasphere, Beckman RIIC) [16]. The lipoxygenase products were eluted at a flow rate of 0.6 ml/min with a gradient changing from 100% water/methanol/acetonitrile/acetic acid (45:30:25:0.05, by vol., pH 5.7) to 100% methanol in 40 min. Detection was at 235, 270 and 302 nm. Material from peaks detected at 302 nm was collected, dried under a stream of N_2, resuspended in methanol and scanned with a Beckman DU7HS spectrophotometer. Further verification of the nature of the peaks was made by cochromatography of authentic LXA_4, LXB_4, LTB_4, LTB_5, 5-Hete, 12-Hete or 15-Hete (ca. 100 ng) with the samples.

For radiolabelling experiments, 72 h macrophage cultures (ca. 5 × 10^7 cells/dish) were incubated with 0.5 μCi [1-^{14}C] arachidonate (58.3 mCi/mmol) or eicosapentaenoate (58.6 mCi/mmol) in 5 ml HBSS for 1 h at 18°C. After washing the cells twice with fresh HBSS to remove non-incorporated radiolabel, 5 μM calcium inonophore was added to the cultures and incubated for 30 min at 18°C. Supernatants were subsequently Sep-Pak extracted and separated by HPLC as previously described. Radiolabelled fractions (0.3 ml) were collected from HPLC, mixed with 4.5 ml Pico-fluor 40 (Canberra-Packard) and analysed on a Beckman LS3801 scintillation counter.

3. RESULTS AND DISCUSSION

When exposed to the calcium ionophore A23187 (under optimized conditions for leukotriene generation), rainbow trout macrophages released a range of oxygenated fatty acid metabolites into the medium, including prostaglandins, leukotrienes and lipoxins. Sep-Pak extraction followed by reverse-phase HPLC revealed a variety of lipoxygenase products including mono-hydroxy fatty acids such as 5- and 12-Hetes with λ_{max} near 235 nm, and dihydroxytrienes including LTB_4 and LTB_5 with λ_{max} near 270 nm (fig.1). Several of the components detected at 270 nm with short retention times were unexpectedly found to have UV spectra identical to those for authentic lipoxins (fig.2) even though 15-lipoxygenase activity, considered essential for lipoxin synthesis [1,2], was apparently lacking as judged by the absence of 15-Hete. To investigate this unexpected discovery, further samples were run on HPLC with detection at 302 nm (λ_{max} for the lipoxins) which revealed four major components with retention times and UV spectra corresponding to the smaller 'lipoxin-like' peaks detected at 270 nm (fig.1). Confirmation that the retention time of at least one of these peaks corresponded to that of a lipoxin was achieved by 'spiking' samples with authentic LXA_4 which was found to co-elute with peak 4 in two different solvent systems (water/methanol/acetonitrile/acetic acid; 45:30:25:0.05, by vol., pH 5.7 and water/methanol/acetic acid 70:30:0.05, by vol., pH 5.7). No peak corresponded to authentic LXB_4, however, as judged by its retention time (fig.1).

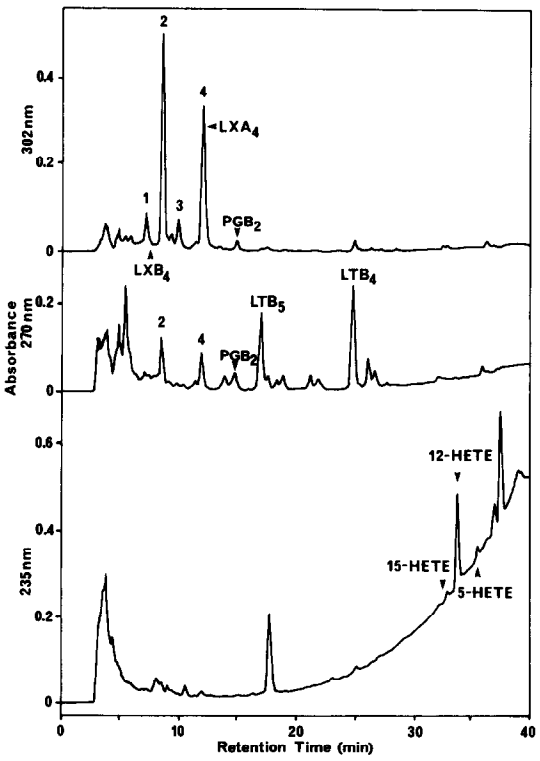

Fig.1. Lipoxygenase products released from rainbow trout macrophages after stimulation with 10 μM calcium A23187, Sep-Pak extraction and separation by reverse-phase HPLC using a gradient of 100% water/methanol/acetonitrile/acetic acid (45:30:25:0.05, by vol., pH 5.7) changing to 100% methanol in 40 min with a flow rate of 0.6 ml/min. Peaks 1–4 show spectra characteristic of the lipoxins. Prostaglandin B_2 (PGB_2), 25 ng, was added as an internal standard. Authentic LXA_4 co-eluted with peak 4. The elution positions for authentic LXB_4 and 15-Hete are arrowed.

Further confirmation that peak 4 is a 4-series lipoxin was obtained from incubation of the macrophage cultures with [1-^{14}C] arachidonate prior to ionophore challenge and HPLC separation. A number of radiolabelled components were detected, one of which corresponded to HPLC peak 4 and another which corresponded to peak 3. Other radiolabelled components corresponded to LTB_4 and its isomers, 12-Hete and free arachidonate.

Since eicosapentaenoate is present at relatively high levels in fish tissues and oils [19,20] and our own studies have shown that it is present at ca. 5% of total fatty acid in rainbow trout blood leucocytes compared to ca. 6% for arachidonate [16], the synthesis of 5-series lipoxins derived from eicosapentaenoate [21,22] would be expected. Incubations with [1-^{14}C] eicosapentaenoate confirmed this prediction since radiolabelled components were found to correspond to HPLC peaks 1 and 2 (but not 3 and 4). The provisional identification of the major arachidonate-derived lipoxin, peak 4, as LXA_4 suggests that the major

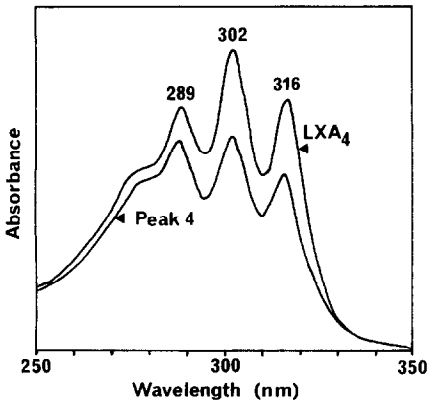

Fig.2. Ultraviolet spectra of authentic LXA_4 in methanol compared with that for peak 4 isolated from ionophore-challenged trout macrophages. Both have absorbance maxima at 289, 302 and 316 nm. Peaks 1–3 show similar spectra.

eicosapentaenoate-derived lipoxin, peak 2, is probably LXA_5 assuming the same synthetic pathway.

Quantification of the lipoxins, assuming ϵ_{302} = 50000 M^{-1} cm^{-1} [23], gave values of 19 ± 3, 103 ± 46, 19 ± 5 and 87 ±41 ng/10^7 macrophages (mean values ±SD, $n=4$) for the components of peaks 1–4, respectively. These lipoxin levels are higher than those for human eosinophilic granulucytes (up to ca. 18 ng LXA/10^7 cells) [14] and bovine polymorphonuclear leucocytes (ca. 4 ng lipoxin/10^7 cells from 5.5. μM exogenous eicosatetraenoate) [13]. However, perhaps most importantly, more lipoxin than leukotriene is produced by rainbow trout macrophages (51 ± 7 and 65 ± 13 ng/10^7 cells for LTB_5 and LTB_4, respectively; mean values ±SD, $n=4$) whereas 20–50 times more LTC_4 than LXA_4 is produced by human eosinophilic granulocytes [14] and about 8 times as much leukotriene as lipoxin by bovine leucocytes [13].

Although more than 95% of the cultured cells used in the present study for lipoxin generation were macrophages as judged by morphological appearance and non-specific esterase staining, the possibility still exists that the contaminating cells, mainly granulocytes, might play some role in the production of these molecules. This seems unlikely, however, because initial experiments with mixtures of macrophages and granulocytes produced less lipoxin than purified macrophages alone. This contrasts with results published for rat alveolar macrophages where exogenous 15-Hpete had to be supplied before any lipoxin synthesis was observed [11]. This may thus indicate a fundamental difference between fish and mammalian macrophages, leading to the possibility that the lipoxins are of greater importance in fish than in mammals and may be evolutionarily more primitive than other lipoxygenase products.

Finally, as rainbow trout macrophages can apparently synthesize lipoxins without the assistance of other cell types, these cells should provide a potentially important model system for determining how such molecules are formed in vivo.

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