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Formation of lipoxin A by granulocytes from eosinophilic donors

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The formation of arachidonic acid-derived lipoxygenase products was examined with human granulocytes obtained from eosinophilic donors. These eosinophil-enriched leukocyte populations, challenged in vitro with the ionophore of divalent cations A23187, transformed both exogenous and endogenous sources of arachidonic acid to several lipoxygenase-derived products, including 5(*S*),6(*R*),15(*S*)-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid (lipoxin A). Lipoxin A was detected and characterized by high-pressure liquid chromatography (HPLC), ultraviolet absorbance, and gas-liquid chromatography-mass spectroscopy. Neither lipoxin B nor 6(*S*)-LXA was consistently detected in extracts from these incubations. The amounts of lipoxin A formed were proportional to the percentage of eosinophils present in the suspension. The results indicate that granulocytes from eosinophilic donors can generate lipoxin A.

Lipoxygenase product; Eosinophil; Arachidonic acid; Leukocyte; (Human)

1. INTRODUCTION

The enzymatic oxygenation of non-esterified arachidonic acid by a wide variety of cell types results in the formation of several classes of biologically active compounds [1,2]. Mammalian leukocytes possess lipoxygenases (5-, 12-, 15-LO) which, when activated, commit non-esterified fatty acids such as arachidonate to highly specific metabolic routes of transformation [2]. Among these cell types the eosinophil, which is held to play an important role in both immune and hypersensitive reactions [3], can generate products of both the 5- and 15-lipoxygenase pathways [4-11]. Unlike human neutrophils, which can generate leukotriene B_4 as a predominant 5-lipoxygenase-derived product, human eosinophils generate leukotriene C_4 [6,8-11]. These cells can also generate 15-lipoxygenase-derived products, including 15-HETE, several 8,15-DHETE isomers, and a product of double dioxygenation (i.e. 5,15-DHETE) [5,7,9].

Recently, we have reported the isolation and complete structural characterization of several members of a novel series of tetraene-containing eicosanoids. These compounds, termed lipoxins, can be formed by interactions between the 5- and 15-lipoxygenases [12,13]. Of this series, lipoxin A

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Abbreviations: RP-HPLC, reverse-phase high-pressure liquid chromatography; GC, gas-liquid chromatography; MS, mass spectrometry; Me_3Si, trimethylsilyl; LX, lipoxin; lipoxin A (LXA), 5(*S*),6(*R*),15(*S*)-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid; 6(*S*)-LXA, 5(*S*),6(*S*),15(*S*)-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid; lipoxin B (LXB), 5(*S*),14(*R*),15(*S*)-trihydroxy-6,10,12-*trans*-8-*cis*-eicosatetraenoic acid; 15-HETE, 15(*S*)-hydroxy-5,8,11-*cis*-13-*trans*-eicosatetraenoic acid; HES, hypereosinophilic syndrome; 5,15-DHETE, 5(*S*),15(*S*)-dihydroxy-6,13-*trans*-8,11-*cis*-eicosatetraenoic acid

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and lipoxin B display stereospecific activities which include actions on human natural killer cells, neutrophils, microcirculation, lung strips, and isolated protein kinase C (Ca^{2+}/phospholipid-dependent protein kinase) [13–17]. In addition, one biosynthetic route in the formation of lipoxins and their isomers has been established [18,19]. Here we present evidence for the generation of lipoxin A by human eosinophil-rich granulocyte suspensions which were obtained from eosinophilic donors.

2. MATERIALS AND METHODS

2.1. *Materials*

Arachidonic acid was purchased from Nu-Chek, MN and [1-^{14}C]arachidonic acid from DuPont NEN (Boston, MA). Ionophore A23187 was from Sigma (St. Louis, MO). All solvents were of HPLC grade obtained from Rathburn Chemicals (Walkerburne).

2.2. *Cell preparations and incubations*

Four patients gave their consent to donate blood: one with eosinophilia from an allergic disorder, two from reactions to drugs, and one from the hypereosinophilic syndrome. Eosinophil-rich suspensions were prepared [9] with minor modifications. Heparinized blood (60 ml) was suspended in Percoll (Pharmacia, Upsala) (1.077 g/cm^3, pH 7.4) and centrifuged ($400 \times g$, 5 min). Red cells in the pellets were lysed with ice-cold NH_4Cl. In order to increase the difference in specific gravity between neutrophils and eosinophils, washed granulocytes were incubated at 37°C for 30 min in Hank's balanced salt solution (HBSS), containing 5% fetal calf serum, at pH 7.0. Next, cells were pelleted and suspended in Percoll (1.082 g/cm^3), layered over Percoll (1.100 g/cm^3) and centrifuged (20 min at $1000 \times g$). Eosinophils were recovered and smears showed that eosinophils were rarely detected in other cell fractions obtained during this process. Either arachidonic acid (final concentration 70–100 μM) or 1-^{14}C-labeled arachidonic acid was added to cell suspensions along with A23187 (2.5 μM). In the next series of experiments cells were incubated with A23187 (2.5 μM) alone (20 min, 37°C). Incubations were stopped after 20 min by addition of 2 vols ice-cold methanol. PGB_2 (50 ng) was added as an internal standard.

2.3. *Isolation and analytical methods*

Procedures for extraction and silicic acid chromatography were as described [18,19]. Briefly, fractions from silicic acid chromatography were evaporated, dissolved in methanol, treated with diazomethane and injected into a reverse-phase HPLC (RP-HPLC) column. In the HPLC system denoted as system A, the column (Nucleosil C18, 4.6 mm $\times$ 25 cm) was eluted with methanol:water (65:45, v/v) at 1.0 ml/min; in system B the column (Altex, ultrasphere - ODS, 10 mm $\times$ 25 cm) was eluted with methanol:water (65:35, v/v) at 3.0 ml/min. The presence of leukotriene C_4 in these incubations was assessed by partial purification with Sep-pak® C_{18} cartridges (Waters Associates, Milford, MA) followed by RP-HPLC analysis. Here, the column was eluted with acetonitrile:water (30:70, v/v), pH 5.7 at 1 ml/min with the UV detector set at 280 nm. The amounts of eicosanoids were assessed by measuring peaks of authentic materials obtained with varying amounts in each HPLC system. Fractions were collected and UV spectra were recorded with a Hewlett-Packard model 8450A spectrophotometer. Samples were converted to Me_3Si derivatives [20] and GC-MS was performed with a Dani 3800 gas chromatograph with a fused silica column SE-30 (20 m $\times$ 0.32 Orion) and a 7070EVG analytical mass spectrometer.

3. RESULTS AND DISCUSSION

When challenged in vitro, eosinophil-rich leukocyte suspensions obtained from peripheral blood of normal, bronchial asthmatic, and idiopathic hypereosinophilic syndrome (HES) donors generate a variety of arachidonic acid derived-lipoxygenase products [4–11]. The results of these studies prompted us to examine whether human eosinophils can generate tetraene-containing eicosanoids. Here, eosinophil-rich leukocyte suspensions were prepared from eosinophilic donors and arachidonic acid-derived lipoxygenase products formed upon activation were examined. In order to obtain quantities which would enable unambiguous identification of products, eosinophil-rich leukocyte suspensions were first incubated with exogenous

arachidonic acid (70–100 μM), 1-^{14}C-labeled arachidonic acid (in some experiments), and the ionophore A23187 (2.5 μM). Following extraction, silicic acid chromatography and treatment with diazomethane, samples were injected onto RP-HPLC columns. In addition to products previously identified [4–11,21] including 5,15-DHETE, 8,15-DHETEs and LTC_4, a component showing strong absorption (while monitoring at 301 nm) was obtained.

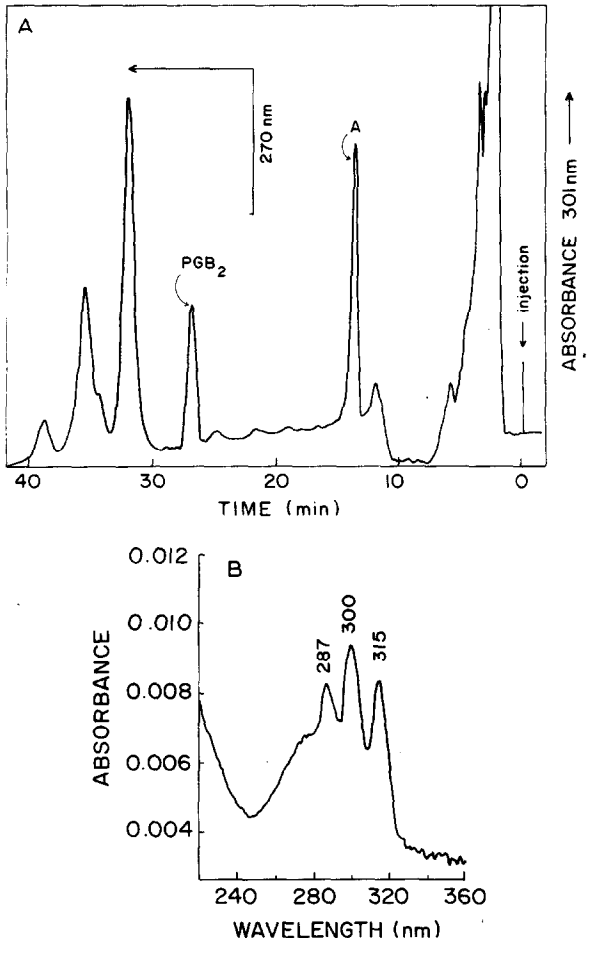

Fig.1. (A) RP-HPLC chromatogram of products obtained following incubation of a human eosinophil-rich granulocyte suspension with arachidonic acid (80 μM) and ionophore A23187 (2.5 μM) for 20 min at 37°C. The granulocyte suspension (18×10^6 cells/ml) contained 74.5% eosinophils as determined by light microscopy following purification from blood. The sample was derived from a patient with the diagnosis of hyper-eosinophilic syndrome. The incubation was stopped with methanol and the products were extracted and chromatographed in RP-HPLC system A (section 2). (B) UV spectrum of material eluting beneath the peak denoted A in the chromatogram shown in panel A. This spectrum was recorded with methanol as solvent.

Material eluting beneath the peak denoted as A was collected, transferred, and analyzed. The UV spectrum showed a triplet of absorption bands at 287, 300 and 315 nm (fig.1) consistent with that of a conjugated tetraene [12]. GC-MS analysis of the Me_3Si derivative of this material gave a *C* value of 24. The prominent ions in its mass spectrum were at *m/e* 379, 289, 203 (base peak), 173 and 171. Ions of lower intensity were at *m/e* 482, 492 and 582 M. Material from peak A also displayed the same retention time on RP-HPLC (system A) as that of the methyl ester of authentic lipoxin A. Thus, on the basis of these findings and by comaprison with published criteria [12,13,18,19] the identity of the material eluting under this peak is lipoxin A.

Neither lipoxin B nor 6(*S*)-LXA was consistently observed in extracts from these incubations; however, LXB was identified and observed in amounts less than those of LXA in two samples. Other products in these extracts which were consistently observed and identified by comparison with published criteria [21] included 8,15-DHETE isomers (which eluted under the two prominent peaks

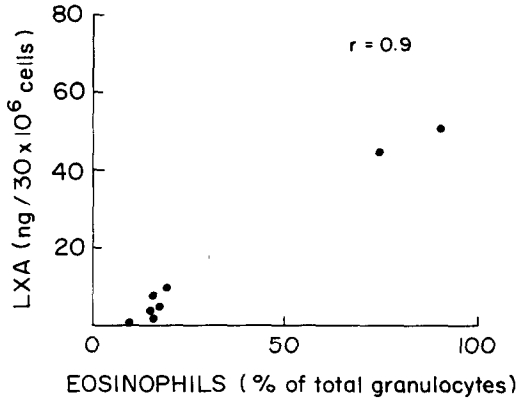

Fig.2. Relationship between amounts of LXA formed by granulocytes from HES donors following incubation with ionophore A23187 (2.5 μM) for 20 min at 37°C and the number of eosinophils (% of total cell suspension) ($r = 0.9$). Each point represents a separate cell purification. Values are expressed as LXA ng/30×10^6 cells per incubation.

following PGB_2, fig.1), 5,15-DHETE (which eluted at a retention time of 50 min), and leukotriene C_4 which was identified utilizing a separate isolation and HPLC solvent system (see section 2). It should be noted, however, that the 8,15-DHETEs were not detectable in these extracts in the absence of exogenous arachidonic acid.

We next examined the relationship between the amounts of LXA formed by eosinophil-rich leukocyte suspensions incubated with ionophore A23187 (2.5 μM) (without addition of exogenous arachidonic acid) and the percentage of eosinophils present in these suspensions (fig.2). The values obtained for LXA are expressed per 30×10^6 leukocytes per incubation, since the mean value of cells (or total cell number) obtained following isolation from whole blood was 30.9×10^6 cells. The amount of LXA formed by leukocytes correlated with the percentage of eosinophils. This suggests that the formation of LXA from endogenous sources in eosinophil-rich leukocyte suspensions, exposed to ionophore A23187, is proportional to the percentage of eosinophils present. It cannot, however, be concluded from these results whether LXA was formed exclusively from the arachidonic acid release and processed within a single cell. For example, transcellular metabolism or cell-cell interactions can contribute to the formation of various lipoxygenase-derived products [22–24]. In particular, activated leukocytes can utilize exogenous 15-HETE to generate both lipoxin A and B [18,19]. It remains possible, then, that upon addition of A23187 to eosinophil-enriched cell suspensions suitable substrate(s) can be mobilized by one cell which can be utilized and transformed to lipoxin A by another.

Previous studies have reported that leukotriene C_4 is the predominant 5-lipozygenase pathway product generated by eosinophils [6,9–11]. We have confirmed and extended these observations in the present study. When the values obtained for LTC_4 formation by HES-derived leukocytes were compared to those obtained for LXA (with cells from the same patient) LTC_4 was detected in amounts approx. 20–50-times greater than those of LXA. Also, the amounts of LTC_4 observed from incubations examined in the present study were comparable to those reported [6–11], suggesting that eosinophils from these patient categories can generate LTC_4 in amounts greater than LXA when exposed to ionophore A23187. It has been demonstrated that subpopulations of eosinophils, particularly those of low density obtained from patients with bronchial asthma during a quiescent state, can generate greater amounts of LTC_4 than those of higher densities [11]. It is not possible to conclude from our results whether eosinophils of varying densities can preferentially generate lipoxin A. Nevertheless, it is clear from the results of the present study that eosinophil-rich granulocyte suspensions can produce lipoxin A without addition of exogenous substrates. Results of our previous studies suggest that several independent biosynthesis routes may be operative in the formation of lipoxins and related compounds [12,18]. The biosynthetic route(s) involved in the formation of lipoxin A by eosinophil-rich granulocyte suspensions remain(s) to be identified. Since lipoxin A can activate neutrophils [13], constrict lung strips, cause changes in microcirculation [16,17], activate isolated protein kinase C [14] and inhibit human natural killer cell function [15], the role of eosinophils in its production may be of importance in the manifestations observed in HES.

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REFERENCES

[1] Samuelsson, B. (1983) Science 220, 568–575.
[2] Needleman, P., Turk, J., Jakschik, B.A., Morrison, A.R. and Lefkowith, J.B. (1986) Annu. Rev. Biochem. 55, 69–102.
[3] Olsson, I. and Venge, P. (1979) Allergy 34, 353–367.
[4] Jorg, A., Henderson, W.R., Murphy, R.C. and Klebanoff, S.J. (1982) J. Exp. Med. 155, 390–402.
[5] Turk, J., Maas, R.L., Brash, A.R., Roberts, L.J. and Oates, J.A. (1982) J. Biol. Chem. 257, 7068–7076.
[6] Weller, P.F., Lee, C.W., Foster, D.W., Corey, E.J., Austen, K.F. and Lewis, R.A. (1983) Proc. Natl. Acad. Sci. USA 80, 7626–7630.

[7] Henderson, W.R., Harley, J.B. and Fauci, A.S. (1984) Immunology 51, 679-686.
[8] Borgeat, P., Fruteau de Laclos, B., Rabinovitch, H., Picard, S., Braquet, P., Hebert, J. and Laviolette, M. (1984) J. Allergy Clin. Immunol. 74, 310-315.
[9] Verhagen, J., Bruynzeel, P.L.B., Koedam, J.A., Wassink, G.A., De Boer, M., Terpstra, G.K., Kreukniet, J., Veldink, G.A. and Vliegenthart, J.F.G. (1984) FEBS Lett. 168, 23-28.
[10] Shaw, R.J., Cromwell, O. and Kay, A.B. (1984) Clin. Exp. Immunol. 56, 716-722.
[11] Kajita, T., Yui, Y., Mita, H., Taniguchi, N., Saito, H., Mishima, T. and Shida, T. (1985) Int. Arch. Allergy Appl. Immunol. 78, 406-410.
[12] Serhan, C.N., Hamberg, M. and Samuelsson, B. (1984) Biochem. Biophys. Res. Commun. 118, 943-949.
[13] Serhan, C.N., Hamberg, M. and Samuelsson, B. (1984) Proc. Natl. Acad. Sci. USA 81, 5335-5339.
[14] Hansson, A., Serhan, C.N., Ingelman-Sandberg, M. and Samuelsson, B. (1985) Biochem. Biophys. Res. Commun. 134, 1215-1222.
[15] Ramstedt, U., Serhan, C.N., Nicolaou, K.C., Webber, S.E., Wigzell, H. and Samuelsson, B. (1987) J. Immunol. 1, 266-270.
[16] Dahlen, S.E., Raud, J., Serhan, C.N., Bjork, J. and Samuelsson, B. (1986) Acta Physiol. Scand., submitted.
[17] Badr, K.F., Serhan, C.N., Nicolaou, K.C. and Samuelsson, B. (1987) Biochem. Biophys. Res. Comman., in press.
[18] Serhan, C.N., Nicolaou, K.C., Webber, S.E., Veale, C.A., Dahlen, S.E., Puustinen, T.J. and Samuelsson, B. (1986) J. Biol. Chem. 261, 16340-16345.
[19] Serhan, C.N., Hamberg, M., Samuelsson, B., Morris, J. and Wishka, D.G. (1986) Proc. Natl. Acad. Sci. USA 83, 1983-1987.
[20] Hamberg, M. (1971) Anal. Biochem. 43, 515-526.
[21] Maas, R.L., Turk, J., Oates, J.A. and Brash, A.R. (1982) J. Biol. Chem. 257, 7056-7067.
[22] Marcus, A.J., Broekman, M.J., Safier, L.B., Ullman, H.L., Islam, N., Serhan, C.N., Rutherford, L.E., Korchak, H.M. and Weissmann, G. (1982) Biochem. Biophys. Res. Commun. 109, 130-137.
[23] McGee, J.E. and Fitzpatrick, F.A. (1986) Proc. Natl. Acad. Sci. USA 83, 1349-1353.
[24] Feinmark, S.J. and Cannon, P.J. (1986) J. Biol. Chem. 261, 16466-16472.