ELSEVIER

Preferential Formation of 13-Hydroxylinoleic Acid By Human Peripheral Blood Eosinophils

F. Engels[1], G.C.R. Kessels P.A.J. Henricks and F.P. Nijkamp*

Department of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, P.O. Box 80082, 3508 TB Utrecht, and *Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands

Lipid mediators released by inflammatory and immune cells play an important role in inflammatory and immune processes. Most attention has been focussed on arachidonic-derived mediators, including prostaglandins, thromboxanes, leukotrienes, and lipoxins. Literature data, however, suggest that also metabolites of the unsaturated fatty acid linoleic acid may be important in this respect. We have studied the formation and release of 9-hydroxy- and 13-hydroxy-linoleic acid (9-HODE and 13-HODE) by enriched populations of human peripheral blood neutrophils, eosinophils, basophils, monocytes, and lymphocytes. We demonstrate that the eosinophil preferentially produces 13-HODE, whereas the other cell types produce equal amounts of 9-HODE and 13-HODE. The biological significance of these findings is discussed.

Keywords: 13-hydrocylinoleic acid; 13 HODE; eosinophils

Introduction

Arachidonic acid-derived mediators, including prostaglandins, thromboxanes, leukotrienes and lipoxins, play an important modulatory role in inflammatory processes (1). These mediators are produced by all cell types that are involved in inflammatory conditions, and may be released under resting conditions or after cellular activation. Recently, research interest has focussed on the possible role of metabolites that are formed from another unsaturated fatty acid, i.e. linoleic acid, in inflammation. Linoleic acid has been demonstrated to be converted to hydroxy-

[1]To whom correspondence should be addressed.

Prostaglandins 52:117–124, 1996

655 Avenue of the Americas, New York, NY 10010

0090-6980/96/$15.00
PII S0090-6980(96)00057-3

derivatives, of which two have been identified as 9-hydroxy-octadecadienoic acid (9-HODE) and its 13-positional isomer (13-HODE) (2). Among the biological activities reported thusfar, HODEs appear to inhibit the adhesion of tumor cells to endothelial cells or extracellular matrix (3). We have been able to demonstrate that HODEs also modulate inflammatory cell activity, i.e. HODEs inhibit the release and/or the activity of oxygen-centered radicals from pulmonary macrophages (4), and appear to be chemotactic for polymorphonuclear leukocytes (5) and modulate their degranulatory responses (6). In addition, 13-HODE has been reported to increase smooth muscle reactivity in the airways (7). In order to characterize more clearly the cellular origin of HODEs, we investigated the differential formation of HODEs by enriched preparations of monocytes, lymphocytes, neutrophils, basophils and eosinophils obtained from human peripheral blood. Our finding that eosinophils predominantly generate 13-HODE, whilst the other cell types produce smaller amounts of both 13-HODE and 9-HODE, may be of particular interest in the light of the important role of eosinophils in airway diseases.

Materials and Methods

Purification of human blood cells. The different types of blood cells were purified as described previously (8), with minor modifications. In short, blood cells were purified from buffy coats (leukocyte-rich fractions) obtained from 500 ml of fresh blood. The buffy coat was diluted 3 times with phosphate buffered saline (PBS) containing 0.4% (w/v) trisodium citrate and was centrifuged over Percoll with a specific gravity of 1.076 g/cm^3 (1000xg, 20 min, 20°C.). Cells collected from the interface were further subjected to isolation procedures yielding enriched preparations of lymphocytes, monocytes and basophils, whilst the resulting pellet was used for isolation of neutrophils and eosinophils (*vide infra*).

Isolation of lymphocytes, monocytes and basophils. The cells from the interface were injected in an elutriator centrifugation system (Beckman J21C centrifuge with a JE-6 elutriation rotor). The elutriation medium consisted of PBS with 13 mM trisodium citrate and 0.5 % (w/v) human serum albumin. To separate the different cell populations in the elutriator, the flow rate was kept constant at 20 ml/min while the rotor speed was lowered stepwise from 4000 rpm to 1000 rpm. First, platelets were collected in a fraction of 150 ml at 3700 rpm (purity > 98%). The rotor speed was then slowly reduced to 2680 rpm, and fraction 2 was collected (300 ml). This fraction contained most of the lymphocytes (purity > 95%) and some remaining erythrocytes. Between 2680 and 1000 rpm, fraction 3 was collected (100 ml), which contained the monocytes and basophils. Fraction 3 was centrifuged at 400xg for 5 min

at room temperature. The cell pellet was suspended in 8 ml of Percoll with a specific gravity of 1.0680 g/cm^3. The cell suspension was divided into two fractions, each of which was layered on top of 2 ml of Percoll with a specific gravity of 1.0750 g/cm^3. The gradients were completed with a top layer of 0.5 ml of PBS with 13 mM trisodium citrate. After centrifugation (15 min, 1000xg, 20°C), the cells were harvested from the two interfaces in each gradient. The upper interface contained the monocytes (purity > 90%), and the lower interface contained the basophils (purity > 70%). The cells were kept on ice before use.

Isolation of neutrophils and eosinophils. After lysis of the erythrocytes in the pellet fraction with an ice-cold lysis buffer containing 155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA (pH 7.2), the granulocytes were washed twice in PBS and resuspended in incubation medium containing 132 mM NaCl, 6.0 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgSO_4$, 1.2 mM potassium phosphate, 20 mM Hepes, 5.5 mM glucose and 0.5 % (w/v) human serum albumin, pH 7.4 and kept at room temperature until use. Generally, the purity of these granulocytes was more than 97% and the percentage of eosinophils was 1-4%. Part of this mixed granulocyte preparation was used for purification of eosinophils as described by Koenderman et al. (9). In short, the mixed granulocytes were washed and suspended in Hanks medium supplemented with human serum albumin (0.5%, w/v). Subsequently, the granulocytes were incubated for 30 min at 37°C to restore initial densities of the cells. After this incubation period, the cells were washed and resuspended in PBS supplemented with human serum albumin (0.5%, w/v) and trisodium citrate (0.4%, w/v). After preincubation of the cells for 5 min at 37°C, 10 nM formyl-methionyl-leucyl-phenylalanine (fMLP, Sigma Chemical Co., St Louis, MO, USA) was added to the cell suspension and the incubation was continued for 10 min. Subsequently, the eosinophils were separated by centrifugation (15 min, 1000xg, 20°C) over an isotonic Percoll gradient with a specific gravity of 1.082 g/cm^3 (pH 7.4). The eosinophils (purity: 95%) were resuspended in incubation medium and kept at room temperature until use.

Incubations of human white blood cells and analysis of fatty acid metabolites. The purified human white blood cells were incubated at 5.10^6 cells/ml with 1 μCi/ml [^{14}C]linoleic acid (Amersham Nederland BV, Den Bosch, The Netherlands, specific activity 55.6 mCi/mmol) for 15 min at 37°C. The radiochemical purity of [^{14}C]linoleic acid was checked by adsorption TLC on Silica Gel F_{254} plates (Merck, Darmstadt, Germany) using hexane-diethyl ether-acetic acid (45:55:0.5). At the end of the incubations, the samples were immediately transferred to ice and the cells were sedimented by centrifugation at 1000xg for 15 min. The

fatty acid metabolites in the resultant supernatants were analysed by HPLC as described previously (2,10). Authentic standards of 9-HODE and 13-HODE were obtained from Oxford Biomedical Research, Inc., Oxford, MI, USA.

Results and Discussion

After addition of [^{14}C]linoleic acid ($\approx 18\mu M$) to the purified blood cells, the release of both 9-HODE and 13-HODE by all cell types could be observed. The structural identification of these metabolites was based on coelution with authentic standards on HPLC, UV absorption characteristics in keeping with those of authentic standards and the possession of a radiolabel indicating their origin from [^{14}C]linoleic acid. The HODEs were formed enzymatically by the cells, as no such metabolites were seen in control incubations without cells.

As is shown in Figure 1, small amounts of both 9-HODE and 13-HODE were released by neutrophils, basophils, monocytes and lymphocytes under non-stimulated conditions, varying from 58 pmol to 186 pmol per 5.10^6 cells. In contrast, considerably larger amounts of 9-HODE and 13-HODE were released by eosinophils, i.e. 458 pmol 9-HODE and 1212 pmol 13-HODE per 5.10^6 cells. The formation of 13-HODE far exceeds the formation of leukotriene C_4 by human eosinophils, which has been reported to amount to 290 pmol per 5.10^6 cells under stimulated conditions (11).

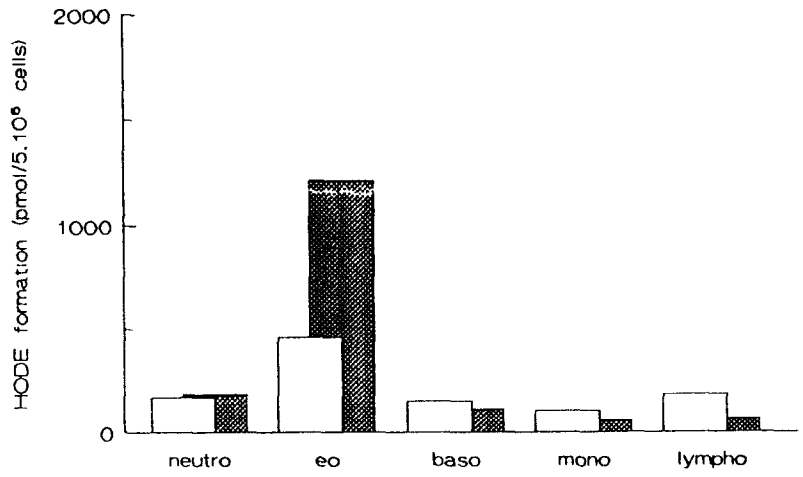

FIGURE 1. The formation of 9-HODE (open bars) and 13-HODE (filled bars) by enriched preparations of neutrophils (*neutro*), eosinophils (*eo*), basophils (*baso*), monocytes (*mono*), and lymphocytes (*lympho*) was measured by HPLC, as described in the Materials and Methods section.

The formation of linoleic acid-derived mediators can be assumed to take place via cyclooxygenase and lipoxygenase catalyzed reactions, since linoleic acid structurally resembles arachidonic acid. It is well known that arachidonic acid is metabolized to prostaglandins, thromboxanes and prostacyclin via an initial cyclooxygenase-catalyzed reaction. Leukotrienes, monohydroxy derivatives of arachidonic acid and lipoxins, on the other hand, are formed via the action of lipoxygenase enzymes. Both cyclooxygenase and lipoxygenase enzymes act upon arachidonic acid by abstracting a hydrogen atom from a C-atom that is part of a methylene-interrupted pentadiene system. Also linoleic acid possesses a methylene-interrupted pentadiene system and hence is theoretically a substrate for cyclooxygenase and lipoxygenase enzymes. Indeed, linoleic acid may be converted to HODEs by cyclooxygenase (12,13).

Alternatively, formation of HODEs has been demonstrated to be catalyzed by 12-lipoxygenase from bovine polymorphonuclear leukocytes leukocytes (14) and tracheal epithelial cells (15), and by 15-lipoxygenase from human peripheral blood leukocytes (16,17) and human and rat epidermis (18). At present biochemical data are not available on the differential formation of either 9-HODE or 13-HODE by particular enzymes in blood leukocytes. However, our present data indicate that different enzymes must be responsible for the formation of 9-HODE and 13-HODE, since in eosinophils 13-HODE formation was more substantial than 9-HODE formation, whereas in the other cell types equal amounts of the metabolites were formed. 13-HODE formation in eosinophils is most likely due to 15-lipoxygenase enzyme activity. First, eosinophils have markedly higher levels of 15-lipoxygenase activity than other cell types (19), which would be in agreement with our observation that eosinophils produce more 13-HODE than the other cell types investigated. Second, the 15-lipoxygenase enzyme is known to catalyze the formation of 15-hydroxy-arachidonic acid (15-HETE) from arachidonic acid by hydrogen abstraction at the $\omega 8$ position, which is part of a methylene-interrupted pentadiene structure (see Figure 2). The double bonds in the linoleic acid molecule have a similar structural location, allowing $\omega 8$ hydrogen abstraction to take place. This reaction would then lead to 13-HODE formation analogous to 15-HETE formation (Figure 2). 9-HODE formation must obviously also be the result of initial $\omega 8$ hydrogen abstraction from linoleic acid. The cyclooxygenase enzyme is known to also catalyze $\omega 8$ hydrogen abstraction from arachidonic acid, but resulting in the formation of an 11-HPETE intermediate that is further metabolized to prostaglandins (Figure 2). It is reasonable to suggest that 9-HODE might be formed through a cyclooxygenase-catalyzed reaction, as the rearrangements of double bonds is similar for the formation of 9-HODE and 11-HPETE (see Figure 2).

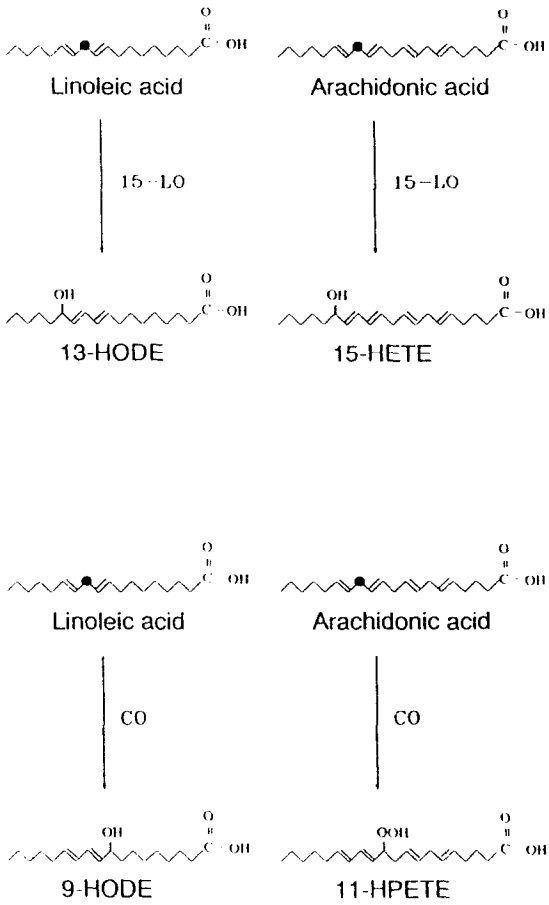

FIGURE 2. Schematic presentation of the conversion of arachidonic acid and linoleic acid by 15-lipoxygenase and cyclooxygenase enzymes. The ω8 position, where initial hydrogen abstraction takes place, is denoted by ●.

Indeed, the cyclooxygenase inhibitor indomethacin (1 μM) inhibited 9-HODE formation by eosinophils by 34%. However, 13-HODE release was also inhibited for 19%. This could indicate that cyclooxygenase activity is involved in the production of both 9-HODE and 13-HODE, and that other enzymes may play a role as well. Conclusive evidence regarding the role of cyclooxygenase and 15-lipoxygenase in the formation of 9-HODE and 13-HODE, respectively, clearly awaits the use of more selective enzyme inhibitors.

Our present finding that the eosinophil preferentially produces 13-HODE, is of special interest with respect to the airways. Thus, eosinophils are of extreme importance in airway inflammation that is thought

to play a causative role in chronic airway diseases like asthma (20,21). Lipid and protein mediators released from eosinophils may be involved in the detrimental effects on airway tissue observed in asthma (22). We have shown recently that 13-HODE is able to induce an increased responsiveness of the airways to contractile stimuli (23). Since this phenomenon is a characteristic feature of asthmatics, 13-HODE released from eosinophils may play an important role in asthma.

Acknowledgements

We thank E.F. Knol and A.T.J. Tool (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) for their assistance during cell isolations.

References

1. Cunningham, F.M., ed., *Lipid Mediators* Academic Press, London, 1994.
2. Engels, F., H. Willems, and F.P. Nijkamp. Cyclooxygenase-catalyzed formation of 9-hydroxylinoleic acid by guinea pig alveolar macrophages under non-stimulated conditions. *FEBS Lett.* 209:249. 1986.
3. Bastida, E., M.C. Bertomeu, T.A. Haas, L. Almirall, D. Lauri, F.W. Orr, and M.R. Buchanan. Regulation of tumor cell adhesion by intracellular 13-HODE: 15-HETE ratio. *J. Lipid Mediators* 2:281. 1990.
4. Engels, F., P.A.J. Henricks, H. Van der Vliet, and F.P. Nijkamp. Modulatory activity of 9-hydroxy- and 9-hydroperoxy-octadecadienoic acid towards reactive oxygen species from guinea-pig pulmonary macrophages. *Eur. J. Biochem.* 184:197. 1989.
5. Henricks, P.A.J., F. Engels, H. Van der Vliet, and F.P. Nijkamp. 9- and 13-hydroxy-linoleic acid possess chemotactic activity for bovine and human polymorphonuclear leukocytes. *Prostaglandins* 41:21. 1991.
6. Van de Velde, M.J., F. Engels, P.A.J. Henricks, P.G.M. Bloemen, and F.P. Nijkamp. The linoleic acid metabolite 13-HODE modulates degranulation of human polymorphonuclear leukocytes. *FEBS Lett.*: in press, 1995.
7. Henricks, P.A.J., F. Engels, H.J. Van der Linde, and F.P. Nijkamp. 13-Hydroxy-linoleic acid induces airway hyperreactivity to histamine in guinea-pigs. *Eur. J. Pharmacol.* 197:233. 1991.
8. De Boer, M., and D. Roos. Metabolic comparison between basophils and other leukocytes from human blood. *J. Immunol.* 136:3447. 1986.
9. Koenderman, L., P.T.M. Kok, M.L. Hamelink, A.J. Verhoeven, and P.L.B. Bruijnzeel. An improved method for the isolation of eosinophilic granulocytes from peripheral blood of normal individuals. *J. Leukocyte Biol.* 44:79. 1988.
10. Engels, F., A.A. Drijver, and F.P. Nijkamp. Modulation of the release of 9-hydroxy-octadecadienoic acid and other fatty acid derived mediators from guinea-pig pulmonary macrophages. *Int. J. Immunopharmacol.* 12:199. 1990.
11. Verhagen, J., P.L.B. Bruynzeel, J.A. Koedam, G.A. Wassink, M. de Boer, G.K. Terpstra, J. Kreukniet, G.A. Veldink, and J.F.G. Vliegenthart. Specific leukotriene formation by purified human eosiniphils and neutrophils. *FEBS Lett.* 168:23. 1984.

12. Hamberg, M., and B. Samuelsson. Oxygenation of unsaturated fatty acids by the vesicular gland of sheep. *J. Biol. Chem.* 242:5344. 1967.
13. Hamberg, M., and B. Samuelsson. Stereochemistry in the formation of 9-hydroxy-10,12-octadecadienoic acid and 13-hydroxy-9,11-octadecadienoic acid from linoleic acid by fatty acid cyclooxygenase. *Biochim. Biophys. Acta* 617:545. 1980.
14. Walstra, P., J. Verhagen, M.A. Vermeer, G.A. Veldink, and J.F.G. Vliegenthart. Demonstration of a 12-lipoxygenase activity in bovine polymorphonuclear leukocytes. *Biochim. Biophys. Acta* 921:312. 1987.
15. Hansbrough, J.R., Y. Takahashi, N. Ueda, S. Yamamoto, and M.J. Holtzman. Identification of a novel arachidonic 12-lipoxygenase in bovine tracheal epithelial cells distinct from leukocyte and platelet forms of the enzyme. *J. Biol. Chem.* 265:1771. 1990.
16. Soberman, R.J., T.W. Harper, D. Betteridge, R.A. Lewis, and K.F. Austen. Characterization and separation of the arachidonic acid 5-lipoxygenase and linoleic acid w-6 lipoxygenase (arachidonic acid 15-lipoxygenase) of human polymorphonuclear leukocytes. *J. Biol. Chem.* 260:4508. 1985.
17. Reinaud, O., M. Delaforge, J.L. Boucher, F. Rocchiccioli, and D. Mansuy. Oxidative metabolism of linoleic acid by human leukocytes. *Biochem. Biophys. Res. Comm.* 161:883. 1989.
18. Nugteren, D.H., and G.A.A. Kivits. Conversion of linoleic acid and arachidonic acid by skin epidermal lipoxygenases. *Biochim. Biophys. Acta* 921:135. 1987.
19. Holtzman, M.J., A. Pentland, N.L. Baenziger, and J.R. Hausbrough. Heterogenity of cellular expression of arachidonate 15-lipoxygenase: implications for biological activity. *Biochim. Biophys. Acta* 1003:204. 1989.
20. Butterfield, J.H., and K.M. Leiferman. In: *Immunopharmacology of Eosinophils*. (H. Smith, and R.M. Cook eds.) Academic Press, London, 1993, p.151.
21. Kroegel, C., J.A. Warner, J.-C. Virchow Jr, and H. Matthys. Pulmonary immune cells in health and disease: The eosinophil leucocyte (Part II). *Eur. Respir. J.* 7:743. 1994.
22. Kroegel, C., J.-C. Virchow Jr., W. Luttmann, C. Walker, and J.A. Warner. Pulmonary immune cells in health and disease: The eosinophil leucocyte (Part I). *Eur. Respir. J.* 7:519. 1994.
23. Henricks, P.A.J., F. Engels, H.J. Van der Linde, J. Garssen, and F.P. Nijkamp. 13-Hydroxy-linoleic acid induces airway hyperresponsiveness to histamine and methacholine in guinea-pigs in vivo. *J. Allergy Clin. Immunol.* 96:36. 1995.

Editor: Dr. W. Smith Received: 08-04-95 Accepted: 03-28-96