Appearance of an arachidonic acid 15-lipoxygenase pathway upon differentiation of the human promyelocytic cell-line HL-60

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The metabolism of arachidonic acid and 15-HPETE was studied in a human promyelocytic cell line (HL-60). Upon exposure to DMSO, HL-60 cells undergo differentiation and acquire a 15-lipoxygenase activity while undifferentiated cells challenged with either arachidonic acid or 15-HPETE did not enzymatically transform these precursors. Products of the arachidonic acid 15-lipoxygenase pathway were identified by HPLC, UV-absorption and gas chromatography-mass spectrometry. Results indicate that upon differentiation HL-60 cells express a 15-lipoxygenase activity as well as the ability to transform 15-HPETE to 8,15-DHETEs and 14,15-DHETE. Moreover, these findings suggest that products of the 15-lipoxygenase cascade may be generated by a single cell system.

Arachidonic acid *Leukotriene* *15-Lipoxygenase pathway*

1. INTRODUCTION

In recent years the metabolism of arachidonic acid in human polymorphonuclear leukocytes (PMNL) to biologically active derivatives has been extensively studied [1]. In human leukocytes, arachidonic acid is metabolized via initial lipoxygenation at either the C-5, C-12 or C-15 position giving rise to leukotrienes as well as several other mono-, di- and trihydroxy acids [1,21].

The human promyelocytic cell-line HL-60, first established by Collins et al. in 1977 [2], has been shown to possess distinct morphological and histochemical characteristics towards myeloid differentiation. Upon exposure to certain polar compounds (i.e., dimethyl sulfoxide) this cell line undergoes differentiation and becomes capable of most PMNL functions. These include chemotaxis, phagocytosis, respiratory burst activity as well as bacterial killing [4–6]. Differentiated HL-60 cells possess both cyclo-oxygenase and lipoxygenase functions, as demonstrated by the conversion of arachidonic acid to LTB_4, 5-HETE and thromboxane [3,18].

Here we present evidence indicating that differentiated HL-60 cells also metabolize arachidonic acid via initial lipoxygenation at the C-15 position to produce 15-HETE as well as 8,15-DHETEs and 14,15-DHETE.

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Abbreviations: 15-HPETE, 15(*S*)-hydroperoxy-5,8,11,13-eicosatetraenoic acid; 15-HETE, 15(*S*)-hydroxy-5,8,11,13-eicosatetraenoic acid; 5-HETE, 5(*S*)-hydroxy-6,8,11,14-eicosatetraenoic acid; 12-HETE, 12(*S*)-hydroxy-5,8,10,14-eicosatetraenoic acid; LTB_4, 5(*S*),12(*R*)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid; 8,15-DHETE, 8,15(*S*)-dihydroxy-5,9,11,13-eicosatetraenoic acid; 14,15-DHETE, 14(*R*),15(*S*)-dihydroxy-5,8,10,12-eicosatetraenoic acid; DMSO, dimethyl sulfoxide; GC-MS, gas chromatography-mass spectrometry; RP-HPLC, reverse-phase high-performance liquid chromatography; $O_2^{\cdot -}$, superoxide ion

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2. MATERIALS AND METHODS

2.1. *Materials*

Arachidonic acid was purchased from Nu-Chek Prep (Elysian, MN) and soybean lipoxygenase from Sigma (St. Louis, MO). HPLC equipment was from Waters Associated (Milford, MA: pump 6000-A, injector U6K) and from LDC, Laboratory Data Control (Riviera Beach, FL: UV-detector LDC III). HPLC solvents were purchased from Rathburn Chemicals, Walkerburn, Scotland.

2.2. *Cell preparation*

HL-60 cells, generously provided by Dr R.C. Gallo (National Cancer Institute, Bethesda, MD) were kept in continuous suspension culture using RPMI-1640 medium (GIBCO) supplemented with 15% heat-inactivated fetal calf serum, penicillin (50 units/ml) and streptomycin (50 μg/ml) as in [2]. Differentiation was induced by addition of DMSO to a final concentration of 1.3% for 6 days.

Cells cultured in the presence or absence of DMSO (6 days) were harvested by centrifugation, washed 3 times in phosphate-buffered saline (pH 7.45), counted, and resuspended at a concentration of 30×10^6 cells/ml in phosphate-buffered saline (pH 7.45).

2.3. *Incubation conditions*

Cell suspensions were warmed for 10 min at 37°C and then exposed to either arachidonic acid (80 μM) or 15-HPETE (80 μM) dissolved in ethanol. The concentration of ethanol did not exceed 0.05% of the incubation mixture. In all experiments the duration of incubations was 30 min. Incubations were carried out in a water bath at 37°C with gentle continuous stirring. In a typical experiment approx. 50–100 ml of cells at a concentration of 30×10^6 cells/ml were used.

2.4. *Extraction and purification*

Incubations were terminated by rapid addition of 2 vols methanol. Following centrifugation and acidification to pH 3 with samples were immediately extracted with diethyl ether, evaporated and subjected to silicic acid chromatography [8]. Briefly, ethyl acetate and diethyl ether/*n*-hexane (40:60) fractions from silicic acid chromatography (CC-4, Mallinckrodt, St. Louis, MO) were concentrated and directly analyzed by RP-HPLC. The UV detector was set at 270 nm for monitoring of conjugated trienes and at 234 nm for conjugated dienes. Separation was achieved using a Polygosil C-18, 5 μm particle column (0.46 × 35 cm) using methanol/water/acetic acid (70:30:0.01) as a mobile phase, at 1 ml/min. Material eluted from HPLC chromatograms were collected, concentrated and further analyzed by UV spectrometry and GC-MS.

2.5. *UV spectrometry*

UV spectra were measured with a Hewlett-Packard 8450A spectrophotometer with methanol as a solvent. Spectra were recorded between 220 and 320 nm.

2.6. *GC-MS*

Samples co-chromatographing with known standards of 8,15-DHETE and 14,15-DHETE were treated with diazomethane and converted to trimethylsilyl ethers using pyridine, hexamethyldisilazane and trimethylchlorosilane. Samples were dried under argon, dissolved in *n*-hexane and injected into the GC-MS. The instrument (LKB 9000) was equipped with a 1% SE-30 column. The ionization beam was set at 22.5 eV.

3. RESULTS

Incubations of undifferentiated HL-60 cells with arachidonic acid did not lead to the formation of detectable amounts of products associated with either the 5- or 15-lipoxygenase catalyzed reactions. When differentiated cells (i.e., cells exposed to DMSO at a concentration of 1.3% for 6 days) were incubated with arachidonic acid under the same conditions, several monohydroxy acids were produced (fig.1). The identities of these compounds were established by UV spectrometry and co-chromatography with reference compounds (i.e., 5-HETE, 12-HETE and 15-HETE [7,20]).

RP-HPLC analysis of material eluting in the ethyl acetate fractions (obtained from incubations with differentiated cells challenged with arachidonic acid) revealed the presence of compounds which had retention times characteristic of those of 8,15-DHETE isomers as well as material with a retention time in accordance with 14,15-DHETE. UV spectrometry of these fractions showed triple peak configurations with a λ_{max} of 268 nm for the

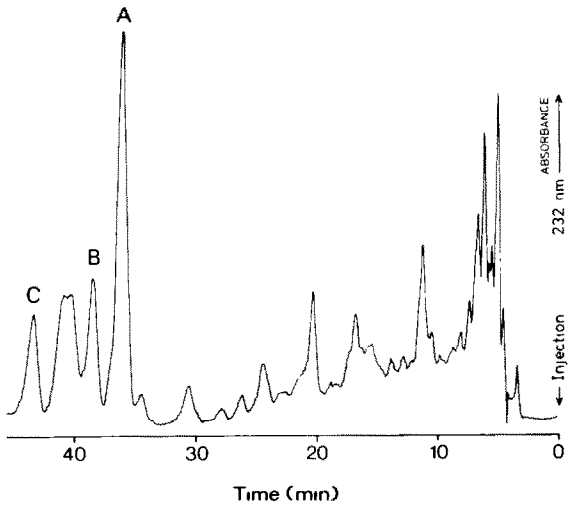

Fig.1. RP-HPLC chromatogram of monohydroxy acids obtained from incubations of differentiated HL-60 cells with arachidonic acid. 50 ml of cells (30×10^6 cells/ml) in PBS were incubated with arachidonic acid (80 μM) for 30 min. 40:60 diethyl ether/*n*-hexane fraction from silicic acid chromatography was injected on a Polygosil C-18, 5 μm column using methanol/water/acetic acid (60:40:0.01) as a mobile phase. Detector setting was 234 nm and pump flow rate 1 ml/min. Peak A represents 15-HETE, B corresponds to 12-HETE and C to 5-HETE as determined by co-chromatography with standards of proven identity.

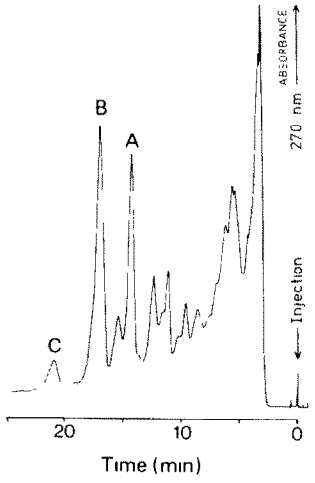

Fig.2. RP-HPLC chromatogram of products obtained from incubation of differentiated HL-60 cells with 15-HPETE. 50 ml of cells (30×10^6 cells/ml) suspended in PBS (pH 7.4) were incubated with 15-HPETE to a final concentration of 80×10^{-6} M. The ether extract was purified by silicic acid column chromatography prior to RP-HPLC. Mobile phase was methanol/water/acetic acid (70:30:0.01), flow rate 1 ml/min and detector setting 270 nm.

two former compounds and a λ_{max} of 272 nm for the latter.

Human PMNL are capable of converting 15-HPETE into mono-, di- and trihydroxy acids [9,21]. To obtain larger amounts of the dihydroxy acids formed by HL-60 cells we next examined the metabolism of 15-HPETE in this cell line. Undifferentiated HL-60 cells exposed to 15-HPETE did not convert this substrate into appreciable amounts of 14,15-DHETE. With differentiated cells, however, a pattern very similar to that of incubations with arachidonic acid and differentiated cells was observed (fig.2). Compounds A and B gave essentially identical retention times values and UV absorption spectra as the compounds obtained from incubations with arachidonic acid and differentiated HL-60 cells. These compounds were further analyzed by GC-MS. The mass spectra of compounds A and B were virtually identical with prominent ions at *m/e* 494, 479, 463, 423, 404, 394, 353, 321, 263, 237, 217, 199, 191, 173, and 129. The *C* values of the compounds were 22.3 and 24.8, respectively (SE-30). These findings suggest that compounds A and B are identical to the pair of C-8 epimers of 8,15-DHETEs [9]. GC-MS analysis of compound C revealed two isomers on GC. Their respective *C* values (SE-30) were 23.8 and 24.7. Both isomers proved to have similar mass spectra, with prominent ions at *m/e* 479, 394, 321, and 173 (fig.3). This is in accordance with previously published data for formation of 14,15-DHETE by suspensions of human PMNL [9].

The transformation was also examined of 15-HPETE (100 μM) incubated with fetal calf serum (15%, v/v) in PBS (30 min, 37°C). Here conversion of 15-HPETE to 14,15-DHETE was not observed. Under these conditions, however, 15-HPETE was converted to 8,15-DHETEs. The conversion of 15-HPETE to 8,15-DHETEs in fetal calf serum most likely proceeds via mechanisms similar to those recently observed for the interactions of hydroperoxy acids and heme-containing proteins [12].

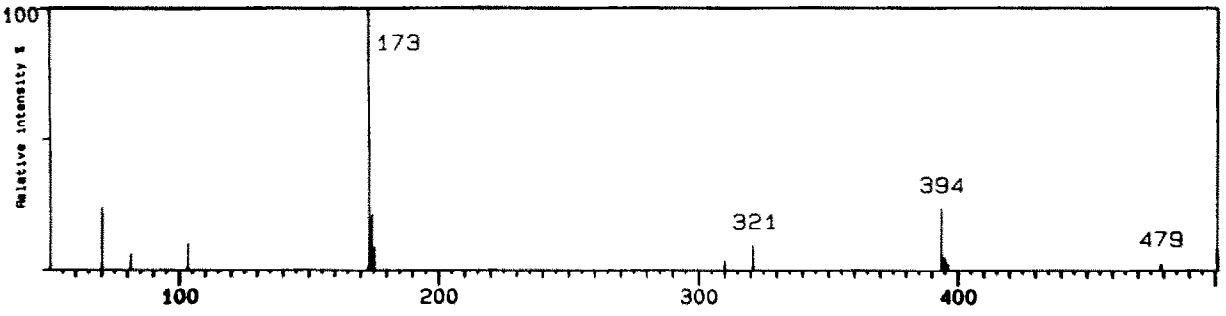

Fig.3. Mass spectrum of compound C from fig.2. Mass spectrum representative of the second isomer appearing on GC (SE-30, 1% on Supelcoport) with a *C* value of 24.7. Prior to injection on GC-MS samples were treated with diazomethane in ether and then converted to trimethylsilyl ether derivatives (see section 2).

4. DISCUSSION

The studies presented above demonstrate the formation of arachidonic acid products associated with the 15-lipoxygenase pathway by differentiated HL-60 cells. This cell line has been kept in continuous suspension culture and undergoes myeloid differentiation [2]. Previous studies have shown that upon differentiation these cells develop most functional responses of human leukocytes [4–6].

When exposed to the chemotactic peptide, *N*-formyl-Nle-Leu-Phe-Nle-Tyr-Lys, HL-60 cells metabolize endogenous sources of arachidonic acid and form 5-HETE, 12-HETE and LTB_4 [18]. This study [18] clearly shows the appearance of a 5-lipoxygenase and a 12-lipoxygenase activity in the differentiated HL-60 cell. In accordance with these findings [18] we have also observed the formation of 5-HETE, 12-HETE and LTB_4. On the basis of the present study it is clear that upon differentiation HL-60 cells also acquire a 15-lipoxygenase activity and are capable of generating compounds which have previously been isolated from mixed leukocyte preparations [9].

Since the HL-60 cell line is of cancerous origin, it must be taken into consideration that the present findings may reflect fundamental abnormalities of the cell line. Nevertheless, in view of previous findings regarding restitution of leukocyte functions it is possible that the appearance of a 15-lipoxygenase activity during maturation represents part of the restitution of leukocyte function (i.e., $O_2^{\cdot-}$, degranulation).

The biological activities of compounds derived via the 15-lipoxygenase cascade have recently been examined in several systems. It has been shown that 14,15-DHETE inhibits NK cell cytotoxicity, thereby suggesting a possible immune regulating function for 15-lipoxygenase derived eicosanoids [11]. Furthermore, 14,15-DHETE inhibits LTB_4-induced superoxide anion generation [14]. However, several isomers of 8,15-DHETEs were found to be completely inactive when tested for NK cell cytotoxicity, human leukocyte aggregation, degranulation, and generation of $O_2^{\cdot-}$ [11,22].

From the present results it is clear that the formation of 15-lipoxygenase-derived arachidonate metabolites has arisen in HL-60 cells without the contribution of other cell types (i.e., RBC, platelets, etc.). Maas and Brash [19] have proposed a mechanism for generation of 14,15-DHETE from 15-HPETE involving a 12-lipoxygenase. However, since the HL-60 cell line contains both a 12-lipoxygenase and a 15-lipoxygenase activity, it is not possible to conclude on the basis of the present study whether the 14,15-DHETE observed is derived from double dioxygenation of 15-HPETE or from the formation of an allylic epoxide intermediate (cf. [9,19]).

Differentiated HL-60 cells also develop a capacity to initiate an oxidative burst [4]. This ability has been shown to parallel increments in NADPH-oxidase activity. Also, increased capacity for $O_2^{\cdot-}$ production parallels increments in the levels of cytochrome *b* in these cells [4]. Formation of the pair of C-8 epimeric isomers of 8,15-DHETE can be observed upon incubation of 15-HPETE with various heme-proteins [12]. Thus, in the present

study the contribution of heme-proteins to the formation of 8,15-DHETE cannot be ruled out since protein synthesis increases during differentiation [4] of these cells.

The biological activities of compounds derived via initial oxygenation of arachidonic acid in the C-5 position appear to be related to leukocyte activation and inflammation [1]. Human leukocytes challenged with serum-treated zymosan particles or crystals of monosodium urate generate LTB_4 and other derivatives from the 5-lipoxygenase pathway [10,16,17]. The rate-limiting step, however, for the conversion of arachidonic acid via 5-, 12- or 15-lipoxygenase pathways is at present not fully understood. Continued study of this cell line may help elucidating the mechanism whereby cells direct the lipoxygenation of arachidonic acid in response to various stimuli.

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