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THE APPEARANCE OF PHOSPHOLIPASE AND CYCLO-OXYGENASE ACTIVITIES IN THE HUMAN PROMYELOCYTIC LEUKEMIA CELL LINE HL60 DURING DIMETHYL SULFOXIDE-INDUCED DIFFERENTIATION.

Robert W. Bonser, Marvin I. Siegel, Randy T. McConnell and Pedro Cuatrecasas

Molecular Biology Department, Wellcome Research Laboratories, Research Triangle Park, North Carolina 27709

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Summary: The human promyelocytic leukemia cell line HL60 can be induced to differentiate into mature granulocytes by exposure to dimethyl sulfoxide. During differentiation a phospholipase activity, which releases arachidonic acid from membrane phospholipids, is expressed. Similarly, fatty acid cyclo-oxygenase activity increases 10-fold. In addition, there is a 40-fold increase in chemotactic formyl peptide receptor binding and a dramatic increase in glucose oxidation via the hexosemonophosphate shunt. The addition of indomethacin, a potent cyclo-oxygenase inhibitor, to the culture medium reduced the cyclo-oxygenase activity of HL60 cells exposed to dimethyl sulfoxide by 97%. However, the presence of indomethacin did not block the dimethyl sulfoxide induced increases in chemotactic formyl peptide receptor binding and hexosemonophosphate shunt activity.

In 1977 Collins et al. established a human promyelocytic leukemia cell line (HL60) from the blood of a patient with acute promyelocytic leukemia (1). This cell line has now been successfully maintained in suspension culture for more than three years and continues to show myeloid characteristics (2,3).

Several reports (2-5) have described the morphologic differentiation of this cell line into mature granulocytes on exposure to dimethyl sulfoxide (DMSO)[1] and other polar compounds. A number of functional changes have also been reported (3): these include increased superoxide generation, hexosemonophosphate shunt activity, phagocytic capability, degranulation and the ability to kill bacteria. In addition, the appearance of the formyl peptide chemotactic receptor, the ability to display a chemotactic response and specific changes in surface glycoproteins have been described (4,6-9).

[1]Abbreviations used: DMSO, dimethyl sulfoxide; AA, arachidonic acid; TXB_2, thromboxane B_2

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This report describes the appearance of two enzyme activities involved in arachidonic acid metabolism during DMSO-induced differentiation. The first of these activities is a phospholipase, which releases arachidonic acid (AA) from membrane phospholipids, and the second is fatty acid cyclo-oxygenase (8,11,14-icosatrienoate, hydrogen donor:oxygen oxidoreductase, E.C. 1.14.99.1) which converts AA to prostaglandins. The observations that DMSO-induced differentiation increases hexosemonophosphate shunt activity (3) and formyl peptide receptor binding in HL60 cells (6,8,9) have been confirmed in this study.

MATERIALS AND METHODS: Cells. The HL-60 cells were a generous gift from Dr. J. Niedel, Wellcome Research Laboratories. The HL60 cell line, which was determined to be free from mycoplasma contamination, was grown in suspension culture in RPMI 1640 medium supplemented with 10% fetal calf serum (Sterile Systems, Logan, UT), penicillin (50 units/ml) and streptomycin (50 μg/ml). Cell cultures were split every 6 days so that the cell density was maintained between 2.5×10^5 and 2.0×10^6 cells/ml. Cells were induced to differentiate by the addition of DMSO to a final concentration of 1.3%. Cells were harvested by centrifugation and washed twice in assay buffer (50 mM Tris·HCl, pH 7.4, containing 100 mM NaCl, 1.4 mM $CaCl_2$ and 0.7 mM $MgCl_2$) and finally resuspended in the same buffer, without serum, for determination of phospholipase and cyclo-oxygenase activities.

Phospholipase Assay. Cell cultures were incubated overnight with [1-^{14}C]-arachidonic acid (Amersham, 55 μCi/μmole) at a concentration of 2 μM (0.1 μCi/ml). The labeled cells were washed twice and resuspended to a final concentration of 5×10^7 cells/ml in assay buffer. The cell suspensions (1 ml) were incubated at 37°C with or without 10 μM calcium ionophore A_{23187} (Calbiochem) for 5 minutes. After the reaction was stopped by the addition of 2.4 ml of chloroform: methanol (1:1, v/v) and 0.05 ml of 2% formic acid, the cell suspension was vortexed, cooled in ice and centrifuged. The organic layer was withdrawn and evaporated to dryness under N_2. The dry extract was redissolved in a small volume of chloroform:methanol (1:1, v/v) and spotted on silica thin layer plates (Sil G25, without gypsum, Brinkman). Chromatograms were developed in an ascending fashion using the solvent systems: I, ethyl acetate:isooctane:acetic acid:water (90:50:20:100, v/v/v/v); II, chloroform:methanol:acetic acid:water (90:8:1:0.8, v/v/v/v); III, diethyl ether:methanol:acetic acid (90:1:2, v/v/v). Labeled products were located by autoradiography and the appropriate regions of the plates were scraped and counted in a liquid scintillation counter. Products were identified by co-chromatography with authentic standards. Phospholipase activity was determined by measuring the free arachidonic acid released by ionophore stimulation.

Cyclo-oxygenase Assay. Cells from 1, 2, 3, and 4 day old cultures were harvested, washed and resuspended in assay buffer to a concentration of 15×10^7 cells/ml. After incubation for 10 minutes at 37°C with exogenously added 40 μM [1-^{14}C]arachidonic acid in a final volume of 0.2 ml, the reaction was stopped by the addition of 2.4 ml chloroform:methanol (1:1, v/v) and 0.8 ml of 0.1% formic acid. Products were extracted as above and separated by thin layer chromatography using the procedures described for the phospholipase assay. Cyclo-oxygenase activity was determined by measuring thromboxane B_2 formation. Production of the radiolabeled metabolite co-chromatographing with authentic thromboxane B_2 was inhibited by 10 μM indomethacin, a potent cyclo-oxygenase inhibitor.

Binding Assay. The formyl peptide binding was assayed using the procedure described by Niedel et al. (6,10), with minor modifications. 1×10^6 cells

were used for each assay and the ^{125}I-labeled peptide concentration was 1 nM.

Hexosemonophosphate shunt activity. HL60 cells were washed with Krebs-Ringer-Bicarbonate, pH 7.4, containing 0.1 mM glucose. Cell suspensions (1.2×10^7 in 1 ml of Krebs-Ringer-Bicarbonate, pH 7.4) were incubated with 0.1 mM glucose containing 0.2 μCi of either [1-^{14}C]- or [6-^{14}C]glucose (61.1 μCi/μmole, Amersham) for 1 hour at 37°C. The $^{14}CO_2$ produced was trapped in hyamine and counted in a liquid scintillation spectrometer (11).

Recovery of lipids. The recovery of radiolabel from cells prelabeled overnight with [1-^{14}C]arachidonic acid, using the procedure described above, was 96.94 ± 0.25% (mean ± S.E. of 10 determinations). Experiments were also done to establish the recovery of cyclo-oxygenase products. Recoveries were monitored using [^{3}H] prostaglandin E_2 (New England Nuclear). The recovery of prostaglandin E_2 from cell suspensions, using the procedure described above, was 98.15 ± 0.47% (mean ± S.E. of 10 determinations).

RESULTS: One of the earliest characteristic biochemical changes that occurs when HL60 cells are induced to differentiate by polar compounds is the appearance of the chemotactic formyl peptide receptor (8,9). During cell differentiation, following exposure to DMSO, specific binding of the formyl peptide increases. After 4 days of exposure to DMSO there is a 40-fold increase in the chemotactic peptide receptor. In contrast, cells cultured without DMSO do not show any increase in receptor binding (Table I and ref. 8). In addition to the appearance of the chemotactic peptide receptor, HL60 cells also display an increase in hexosemonophosphate shunt activity during DMSO induced differentiation (3). We have adopted calcium ionophore (A_{23187}) stimulated glucose

Table I

The effect of indomethacin on the DMSO induced increases in cyclo-oxygenase activity, glucose oxidation and chemotactic peptide receptor binding of HL60 cells.

Cells	Indomethacin (10 μM)	[1-^{14}C]glucose oxidation	[6-^{14}C[glucose oxidation	Receptor Binding fmol/10^6 cells	TXB_2 cpm/10^8 cells
HL60	-	83	115	0.25	411
HL60	+	173	230	0.29	117
HL60 + DMSO	-	42,221	320	13.13	6,888
HL60 + DMSO	+	53,977	189	12.97	210

Cells were cultured for 4 days with or without indomethacin, washed and assayed for cyclo-oxygenase activity, glucose oxidation and chemotactic peptide receptor binding (see Materials and Methods). Glucose oxidation results are expressed as the difference between $^{14}CO_2$ release (cpm/60 min/10^7 cells) by calcium ionophore (A_{23187}, 10 μM)-stimulated cells and $^{14}CO_2$ release by resting cells. Experiments were run in duplicate and all values are within ±10% of the mean.

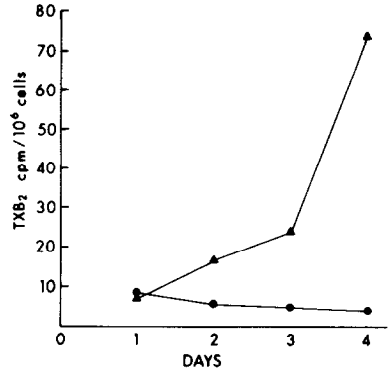

Fig. 1. Time course of cyclo-oxygenase appearance. Cell cultures were assayed for cyclo-oxygenase activity on the days indicated. 3 x 10^7 cells were used per assay. ● HL60, ▲ HL60 + DMSO. Results are the mean of duplicate experiments. All values are within ±10% of the mean.

oxidation as a measure of hexosemonophosphate shunt activity in these cells. The oxidation of [1-^{14}C]glucose was stimulated dramatically by the calcium ionophore in HL60 cells exposed to DMSO for 4 days (Table I). In contrast, cells cultured without DMSO do not show this increase in [1-^{14}C]glucose oxidation in the presence of the calcium ionophore (Table I). Ionophore stimulated [6-^{14}C]glucose oxidation was for lower in magnitude in both control and DMSO differentiated HL60 cells (Table I).

HL60 cells synthesize barely detectable levels of cyclo-oxygenase products from exogenous AA, whereas the same cells exposed to DMSO show a dramatic, time dependent increase in cyclo-oxygenase activity (Fig. 1). Exposure to DMSO for 4 days increases cyclo-oxygenase activity 10-fold. The major cyclo-oxygenase product synthesized by these cells from exogenous AA is thromboxane B_2. The most dramatic increase in cyclo-oxygenase activity occurs after 3 to 4 days of exposure to DMSO (Fig. 1).

The calcium ionophore A_{23187} stimulates the release of AA from membrane phospholipids in a number of cell types, including polymorphonuclear leukocytes and platelets (12,13). AA release is believed to be due to the activation of a phospholipase A_2 enzyme in these cells. Following exposure to DMSO, HL60 cells slowly begin to express the ability to release AA from endogenous phospholipids. Undifferentiated and differentiated cells incorporate over 95% of

Table II

Appearance of Phospholipase Activity in HL60 Cells Treated with DMSO.

Cells	A_{23187} (10 μM)	c.p.m. Free AA	c.p.m. TXB_2
HL60	-	294	77
HL60	+	514	108
HL60 + DMSO	-	916	1138
HL60 + DMSO	+	25,946	3626

Cells from 4 day old cultures were assayed for phospholipase activity as described in Materials and Methods. Experiments were run in duplicate. All values are within ±10% of the mean.

the [1-^{14}C]AA into their phospholipids and triglycerides after overnight labeling and both cell types contain very low levels of free AA. However, only those cells which had been exposed to DMSO for 4 days release any significant amounts of AA from their lipids upon stimulation with the calcium ionophore (Table II). Consequently, only those cells which had been induced to differentiate metabolize endogenous AA to cyclo-oxygenase products. It is interesting to note that the release of AA is not tightly coupled to cyclo-oxygenase in DMSO differentiated HL60 cells.

To determine whether the appearance of cyclo-oxygenase was directly involved in DMSO induced differentiation, HL60 cells were cultured in the presence of DMSO and 10 μM indomethacin, a potent cyclo-oxygenase inhibitor. The cyclo-oxygenase activity of HL60 cells cultured for 4 days under these conditions was inhibited by 97% (Table I). However, the addition of indomethacin to the culture medium did not block the appearance of the chemotactic peptide receptor or the increase in hexosemonophosphate shunt activity (Table I).

DISCUSSION: Arachidonic acid metabolism in granulocytes has been the subject of extensive research recently. An activatable phospholipase, which releases arachidonic acid from membrane phospholipids, has been described in neutrophils

(12,14) and these cells are known to metabolize free arachidonic acid via the cyclo-oxygenase and lipoxygenase pathways (15-18). The lipoxygenase products have been shown to be chemotactic for neutrophils (17,19-21) and to induce degranulation of specific granules (22). Furthermore, arachidonic acid and its metabolites have been implicated in a number of other physiological functions of the neutrophil. These include aggregation and degranulation (23-25), the chemotactic process (26), changes in the permeability of the plasma membrane to calcium (27), stimulation of the hexosemonophosphate shunt oxidation of glucose and regulation of superoxide generation (28).

This study describes the appearance of two arachidonic acid metabolizing enzymes, phospholipase and cyclo-oxygenase, in HL60 cells which have been induced to differentiate into mature granulocytes by exposure to dimethyl sulphoxide. The observation that indomethacin, a potent cyclo-oxygenase inhibitor, does not block the DMSO induced increases in chemotactic peptide receptor binding and hexosemonophosphate shunt activity, indicates that the appearance of cyclo-oxygenase activity is not directly involved in the process of DMSO induced differentiation of HL60 cells into mature granulocytes.

The absence of a phospholipase activity in undifferentiated HL60 cells would prevent further metabolism of endogenous esterified arachidonic acid via both the cyclo-oxygenase and lipoxygenase pathways. Since undifferentiated HL60 cells do not display any of the functions characteristic of mature granulocytes, such as degranulation, superoxide generation or a stimulated hexosemonophosphate shunt activity (3), it is tempting to speculate that the absence of these functions may be due to their inability to metabolize endogenous arachidonic acid. Therefore, the HL60 cell line may serve as a useful model for studying the role of arachidonic acid metabolism in granulocyte function.

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