Early Changes in Phosphatidylcholine Metabolism in Human Acute Promyelocytic Leukemia Cells Stimulated to Differentiate by Phorbol Ester

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The HL-60 leukemia cell line derived from a human acute promyelocytic leukemia, undergoes differentiation to mature neutrophils in the presence of dimethyl sulfoxide and a variety of other chemicals. In contrast, when exposed to the tumor promoter, 12-0-tetradecanoylphorbol-13-acetate (TPA), the immature HL-60 cells adhere to glass and undergo differentiation to mature neutrophils in the presence of dimethyl sulfoxide and a variety of other chemicals.

We studied early alterations (within 90 min of exposure to TPA) in phosphatidylcholine metabolism in HL-60 cells and found that phosphatidylcholine synthesis by methylation of phosphatidylethanolamine was inhibited in a dose-dependent fashion. In contrast, synthesis of phosphatidylcholine from endogenous choline was enhanced and correlated inversely with the degree of inhibition of the methylation pathway. Phorbol ester congeners of TPA caused similar alterations in phosphatidylcholine metabolism in direct relationship to their capacity to induce differentiation in HL-60 cells. Perturbation of phosphatidylcholine metabolism is an early membrane event in TPA-induced HL-60 cell differentiation.

T HE HL-60 LEUKEMIA cell line, derived from a patient with acute promyelocytic leukemia, undergoes differentiation to mature neutrophils in the presence of dimethyl sulfoxide and a variety of other chemicals. In contrast, when exposed to the tumor promoter, 12-0-tetradecanoylphorbol-13-acetate (TPA), the immature HL-60 cells adhere to glass and acquire morphological, cytochemical, and functional characteristics of macrophages. Evidence of macrophage differentiation exists within 24 hr. The mechanism by which TPA induces differentiation in HL-60 cells is unclear.

TPA is highly lipophilic and readily binds to plasma membranes. We questioned whether biochemical events within the membrane might be part of the process whereby differentiation is induced. Hirata and Axelrod have suggested that the transduction of biochemical signals across membranes may be mediated in part by the methylation of membrane phospholipids. They have identified two enzymatic steps that participate in the sequential methylation of phosphatidylethanolamine through monomethyl and dimethyl forms to form phosphatidylcholine. Although this is a quantitatively minor pathway of phosphatidylethanolamine synthesis compared to the synthesis from cytidine diphosphocholine (CDP-choline) and 1,2-diacylglycerol, a number of studies suggest that it is important. For example, increased activity of the methylation pathway is induced by agonist binding of β-adrenergic receptors to rat red blood cells and C6 astrocytoma cells, chemoattractant stimulation of rabbit neutrophils, chemotactic stimulation of guinea pig macrophages and human monocytes, concanavalin A stimulation of rat peritoneal mast cells, cyclic adenosine monophosphate (AMP) stimulation of the slime mold Dictyostelium discoideum, antigenic stimulation of rat leukemic basophils, and lectin-stimulated lymphocyte mitogenesis. Inhibition of the methylation pathway blocks cellular responses to certain stimuli.

Changes in methylation precede changes in protein, RNA, or DNA synthesis, supporting the concept that local events in the cell membrane occur in response to surface stimulation as an early response. Associated with increased methylation of phospholipids are alterations in membrane fluidity, in numbers of binding sites for agonists, in activation of adenylate cyclase, and in calcium flux. We, therefore, examined the changes in phosphatidylcholine synthesis by the transmethylation and the exogenous choline pathways in HL-60 cells stimulated to differentiate by TPA.

MATERIALS AND METHODS

Materials

[1-Methyl-1H]methionine (12-15 Ci/m mole) and [methyl-14C]choline chloride (49.5 mCi/m mole) were from New England Nuclear, Boston, Mass.; phorbol and its congeners were from Life Systems Company, Newton, Mass.; all phospholipid standards were from Supelco Co., Bellefonte, Pa.; except for egg phosphatidycholine (GIBCO, Grand Island, N.Y.), phosphatidyl-N-monomethyl ethanolamine and phosphatidyl-N,N-dimethylethanolamine (Avanti Biochemicals Inc., Birmingham, Ala.); HL-60 cells were obtained from Dr. Robert C. Gallo (National Cancer Institute, Bethesda, Md.).

Incubation of HL-60 Cells With TPA and Phorbol Congeners

HL-60 cells were grown in RPMI-1640 medium (GIBCO), 15% heat-inactivated fetal calf serum, glutamine, and vitamins plus penicillin and streptomycin. This medium contains 0.33 μM choline.
and 10 μM methionine. The cell concentrations on serial cultures ranged from 3 x 10^6 cells/ml to 2 x 10^6 cells/ml. For studies of phospholipid synthesis, aliquots of cells were centrifuged, resuspended in fresh medium plus 20 mM HEPES at a concentration of 1–2 x 10^6 cells/ml, and allowed to equilibrate with isotopic precursors (L-[methyl-3H] methionine or [methyl-^14C] choline) at 37°C prior to the addition of TPA for 20 min. Isotopic labeling was initiated by the addition of isotope bringing the final concentration to 4 μCi/ml of L-[methyl-3H] methionine or 0.5 μCi/ml of [methyl-^14C] choline, and then incubated at 37°C in a shaking water bath. TPA was diluted in medium prior to the addition of cells.

Incubations were terminated at various times by placing the test tubes on ice and adding an equal volume of 10% cold trichloroacetic acid. All experiments were run in triplicate.

Analysis of Membrane Phospholipids

One hour after the addition of trichloroacetic acid, precipitated material was sedimented at 1900 g for 10 min at 5°C and the supernatants removed. Pellets were resuspended in chloroform: methanol:H_2O (1:2:0.8) and vigorously mixed. After 1 hr at room temperature, chloroform:water (1:1) was added to establish a 2-phase system. After vigorous mixing, tubes were centrifuged at 1000 g for 10 min. The chloroform layer was washed twice with 2 ml of 0.1 M KCl/50% methanol. Aliquots of the chloroform layer were pipetted into glass vials and evaporated to dryness under nitrogen, prior to determination of radioactivity. Quenching was found minimal and constant among samples.

Cells were counted and stained with Trypan blue at the beginning and end of each experiment in control tubes and TPA-treated tubes and showed no change in number or viability. Nonspecific trapped radioactivity was less than 5% of total counts. This was negligible in a typical experiment and no corrections for these counts were made.

To determine the radioactivity in individual phospholipids, the antioxidant, 2,3-diterbutyl, 4-methyl-phenol was added to the extracts, which were then evaporated to 20 μl under nitrogen and resuspended in 100 μl of chloroform. All solvents used in thin-layer chromatography were of high performance thin-layer chromatography grade. Phospholipid separation was accomplished by ascending chromatography on preabsorbed silica gel G plates (Uniplate, Analtech, Newark, Del.) using chloroform:propionic acid:n-propanol:H_2O (2:2:3:1). This system effectively separated phosphatidic acid, phosphatidylethanolamine, phosphatidyl-N-monomethylethanolamine, phosphatidyl-N-dimethylethanolamine, phosphatidylcholine, lysophosphatidylethanolamine, and sphingomyelin. Phosphatidylserine and phosphatidylinositol migrated with phosphatidyl-N-dimethylethanolamine. An alternate solvent system of chloroform:methanol:acetic acid:H_2O (25:15:4:2) gave similar separation except that phosphatidylethanolamine overlapped phosphatidyl-N-monomethylethanolamine. Phospholipid standards and the lipid extract of the HL-60 cells were run simultaneously on the same plate, and the dried thin-layer plates were exposed to iodine to visualize individual phospholipids. The entire plate was scraped in 1 cm segments into scintillation vials and the iodine allowed to evaporate. Ten milliliters of scintillation cocktail was added and the radioactivity counted.

Statistics

Mean values are expressed as ± SEM. The difference between the means was examined by Student’s t test for paired data.

RESULTS

Phospholipid Methylation

The incorporation of L-[methyl-^1H] methionine into HL-60 phospholipids with and without TPA. As described in Materials and Methods, HL-60 cells were incubated with radiolabeled methionine at 37°C. After 20-min preincubation, TPA (10^-8 M) was added to experimental tubes. HL-60 cell phospholipids were extracted and the amount of radioactivity per 10^6 cells was determined. Data represent the means ± SEM of three experiments. O, TPA-treated cells; O, control.

Fig. 1. Incorporation of [methyl-^3H] from [methyl-^3H] methionine into HL-60 phospholipids with and without TPA. As described in Materials and Methods, HL-60 cells were incubated with radiolabeled methionine at 37°C. After 20-min preincubation, TPA (10^-8 M) was added to experimental tubes. HL-60 cell phospholipids were extracted and the amount of radioactivity per 10^6 cells was determined. Data represent the means ± SEM of three experiments. O, TPA-treated cells; O, control.

Effect of TPA on Phospholipid Methylation

The addition of TPA (10^-8 M) decreased the incorporation of L-[methyl-^1H] methionine into HL-60 phospholipids. This was evident 20 min after addition of TPA and became more marked over the course of a 90-min incubation (Fig. 1). Differences between the means were significant (p < 0.005) at 30, 60, and 90 min. Inhibition of methyl-^1H group incorporation was 22% between 0 and 30 min, 31% between 30 and 60 min, and 45% between 60 and 90 min. The possibility of TPA-induced impairment of radioactive precursor uptake was studied. Radioactivity in the washed range of 10^6/ml to 8 x 10^6/ml at 80 min of incubation. Radioactivity was incorporated into HL-60 lipids linearly, with respect to time for 90 min (Fig. 1). After 60 min, radioactivity was found predominantly in those phospholipid fractions that cochromatographed with phosphatidyl-N-monomethylethanolamine, phosphatidyl-N-dimethylphosphatidylethanolamine, and phosphatidylcholine (Fig. 2). Another peak of radioactivity migrated with the solvent front. Little radioactivity was found in other phospholipids. These experiments indicate that the pathway that methylates phosphatidylethanolamine to phosphatidylcholine is active in HL-60 cells.
trichloroacetic acid whole cell precipitate was measured after a 60-min incubation. There was no difference between the TPA-treated and control cells.

The incorporation of radioactivity into individual phospholipids at 60 min is shown in Fig. 2. There was a reduction in counts of 49% in phosphatidylcholine, 40% in phosphatidyl-N-dimethylethanolamine, and 36% in phosphatidyl-N-monomethylethanolamine. Only a minor decrease (10%) occurred in the unidentified radioactivity at the solvent front.

The effects of various concentrations of TPA (from $10^{-5}$ to $10^{-6}$ M) on incorporation of methyl-$^3$H groups into lipids was examined after 60-min incubation. Compared to control HL-60 cells, there was a progressive reduction in incorporation of isotope from 11% at $10^{-9}$ M to 35% at $10^{-5}$ M (Fig. 3). In separate experiments, these concentrations of TPA were studied for their capacity to induce differentiation of HL-60 cells. Over 48 hr of incubation with TPA, a progressively greater percentage of cells showed differentiation at 24 hr and 48 hr with increasing concentration of TPA from $10^{-9}$ to $10^{-5}$ M. These experiments correlate the degree of decreased incorporation of methyl-$^3$H groups into lipids with the rapidity and extent of differentiation.

To assess the possibility that the phorbol ester (TPA) nonspecifically inhibited the incorporation of methyl-$^3$H groups into lipids, various congeners (all at $10^{-6}$ M) were employed (Fig. 4). After 60 min of incubation, no depression of incorporated radioactivity was found with the parent compound, phorbol (P) or its esters, 4a-phorbol-12,13-didecanoate (4a-PDD), phorbol-12,13-diacetate (PDA), and 4-0-methyl phorbol-12-myristate, 13-acetate (MPMA) compared to control HL-60 cells. In contrast, there was a 33% decline in counts with TPA, 28% with phorbol-12,13-didecanoate (PDD), and 27% with phorbol-12,13-dibutyrate (PDB). The concentration dependence of various phorbol esters in inducing HL-60 cells to differentiate has been reported. Of the phorbol esters studied, the two most active as differentiation inducers after TPA were PDD and PDB. The above experiments thus link the depression of methyl-$^3$H group
incorporation with potency of the phorbol esters as inducers of differentiation.

After preincubation of HL-60 cells with l-[methyl-\(^{3}\)H] methionine for 20 min, the cells were centrifuged at 1900 g and washed twice with cold medium (containing methionine) and resuspended in fresh medium. TPA was then added, and the stability of incorporated radioactivity in lipids was assayed at various time points over a 90-min incubation (data not shown) at 37°C. Incorporated counts rose slightly over initial values for 10 min and plateaued with no subsequent loss of radioactive counts in HL-60 lipids in control or TPA-treated HL-60 cells. This suggests that the decline in radioactivity in lipids in TPA-treated cells is not due to loss of preformed lipid.

The incorporation of [methyl-\(^{14}\)C] choline into HL-60 cell lipids was studied with and without TPA (10 \(^{-6}\) M) stimulation. Over the time course of a 90-min incubation, progressive incorporation of isotopic label was observed in untreated cells (Fig. 5). Increased incorporation of methyl-\(^{14}\)C choline in TPA-stimulated cells compared to control cells became apparent at 30 min and increased over time. At 90 min, radioactivity was increased 130%. The differences between the means were significant at 30 min (\(p < 0.01\)), 60 min (\(p < 0.005\)), and 90 min (\(p < 0.005\)). Thin-layer chromatography, run as previously described, revealed virtually all counts to reside in the phosphatidylcholine fraction. To assess the possibility that TPA had caused a nonspecific increase in cell membrane permeability to choline rather than increased phosphatidylcholine synthesis, three experiments were performed. First, after incubation of cells for 90 min with [methyl-\(^{14}\)C] choline, HL-60 cells were centrifuged through silicone oil at 12,000 g in Eppendorf tubes. The cell pellet was excised and counted for radioactivity. There was no difference in the whole cell counts with or without TPA (data not shown). Second, after preincubation of HL-60 cells with [methyl-\(^{14}\)C] choline for 20 min, the cells were centrifuged at 1900 g and washed twice with cold medium (containing choline) and resuspended in fresh medium. TPA was then added and the radioactivity incorporated into lipids assayed at various time points over a 90-min incubation at 37°C. As shown in...
Fig. 6, radioactivity in control cell lipids increased over time with progressively smaller increments in counts. In contrast, in TPA-treated cells, after 30 min, counts rose linearly with radioactivity 128% of control at 90 min. This indicates that the TPA-induced stimulation of [methyl-\(^{14}\)C] choline incorporation into lipids is demonstrable after choline has entered the cell. Third, HL-60 cells were exposed to TPA and its congeners (all at \(10^{-6} \text{M}\)) for 90 min in the presence of [methyl-\(^{14}\)C] choline. As in the radioactive methionine experiments, the relatively inactive congeners, P, 4 \(\alpha\)-PDD, PDA, and MPMA, showed no differences from control cells; whereas there was increased incorporation of [methyl-\(^{14}\)C] choline counts into lipids compared to control cells—27% with TPA, and with PDD 29% and PDB 24%, the two congeners active in induction of differentiation.

Using a 90-min incubation time point, parallel experiments were run using [methyl-\(^{3}\)H] methionine and [methyl-\(^{14}\)C] choline in separate tubes of HL-60 cells without and with varying concentrations of TPA (Fig. 7). The incorporation of [methyl-\(^{14}\)C] choline varied directly with the concentration of TPA employed. Moreover, there was an inverse relationship between the increased counts in HL-60 lipids as [methyl-\(^{14}\)C] choline and the decreased counts as methyl-\(^{3}\)H.

Thus, the depression of phosphatidylcholine synthesis induced by TPA stimulation is specific for the N-methylation pathway and not for phosphatidylcholine synthesis in general. Moreover, the decreased phosphatidylcholine synthesis via the minor transmethylation pathway is accompanied by an increased synthesis via the major (exogenous choline) pathway.

**DISCUSSION**

The above experiments establish that in the HL-60 cell line, as in other cells studied, the N-methylation pathway functions to add methionine-derived methyl groups to phosphatidylethanolamine (with the intermediate of S-adenosyl-L-methionine) to form phosphatidylcholine. Stimulation of HL-60 cells by TPA leads within minutes to an early and marked reduction in the incorporation of [methyl-\(^{3}\)H] methionine groups into phosphatidylcholine. Wash experiments prior to TPA addition indicate that the reduction in incorporated counts is not due to increased degradation of newly formed phosphatidylcholine by this pathway. That depression of this synthetic pathway is not a nonspecific event is shown by the fact that phorbol congeners inactive or minimally active as differentiation inducers did not impair the methylation pathway, whereas active congeners revealed depressed synthesis in correlation with their known potency as differentiation inducers of the HL-60 cell line. Generalized depression of phosphatidylcholine synthesis did not occur. In fact, accelerated incorporation of labeled choline into phosphatidylcholine was demonstrated. Wash experiments and whole cell counts showed that the TPA effect was not due to enhanced membrane permeability to the radioactive precursor. The accelerated synthesis of phosphatidylcholine via exogenous choline was inversely correlated with the depressed synthesis via methylation of phosphatidylethanolamine in a dose-dependent fashion. Wash experiments prior to TPA addition indicate that the reduction in incorporated counts is not due to increased degradation of newly formed phosphatidylcholine by this pathway. That depression of this synthetic pathway is not a nonspecific event is shown by the fact that phorbol congeners inactive or minimally active as differentiation inducers did not impair the methylation pathway, whereas active congeners revealed depressed synthesis in correlation with their known potency as differentiation inducers of the HL-60 cell line. Generalized depression of phosphatidylcholine synthesis did not occur. In fact, accelerated incorporation of labeled choline into phosphatidylcholine was demonstrated. Wash experiments and whole cell counts showed that the TPA effect was not due to enhanced membrane permeability to the radioactive precursor. The accelerated synthesis of phosphatidylcholine via exogenous choline was inversely correlated with the depressed synthesis via methylation of phosphatidylethanolamine in a dose-dependent fashion over a range of TPA concentrations. Whether these opposing directions in phosphatidylcholine synthesis are independently triggered events or are the cell's response to maintain the integrity of the phosphatidylcholine compartment of the membrane is uncertain. In the latter event, the data also do not reveal which of the alterations are primary. Recent work with the methylation inhibitor, 3-deazaadenosine, suggests that control mechanisms do exist to balance the major and minor pathways of phosphatidylcholine biosynthesis. Blockade of the methylation pathway in hamster or rat liver leads to increased choline incorporation into phosphatidylcho-
Changes in membrane phospholipid metabolism are among the earliest events to occur after cell surface stimulation. TPA application to mouse skin or exposure to bovine lymphocytes produces a rapid stimulation of incorporation of choline into phosphatidylcholine and increased synthesis of phosphatidylethanolamine and phosphatidylcholine. The altered phospholipid metabolism occurs despite inhibition of RNA or protein synthesis and in the face of membrane immobilization by cytochalasin B. Similarly, exposure of HeLa cells to TPA rapidly produces increased phosphatidylcholine synthesis from choline. The stimulation by TPA of choline incorporation in bovine lymphocytes is blocked by 5,8,11,14-eicosatetraynoic acid, an agent that blocks arachidonic acid metabolism. This inhibition is reversed by arachidonic acid, suggesting that one of its metabolites plays a role in the stimulation of phospholipid synthesis. The rapid changes induced by TPA in phosphatidylcholine synthesis by the CDP-choline pathway and the changes in the transmethylation pathway found in other stimulated cell systems led us to examine both pathways in TPA-stimulated HL-60 cells.

TPA causes the HL-60 cell line to differentiate to more mature cells with morphological, functional, and cytochemical characteristics of macrophages over a 24–48 hr period. How this differentiation sequence is initiated or how this chemical, which is a tumor promoter and inhibitor of differentiation in a number of cell lines, can cause differentiation in some cell lines is unknown. TPA is highly lipophilic and it partitions strongly into membranes making it difficult to demonstrate specific binding. However, using the less lipophilic but highly active congener phorbol 12,13-dibutyrate, specific binding to chicken embryo fibroblasts and to mink lung and murine BALB/3T3-A31 cells has been demonstrated. Regulation of cell differentiation after TPA binding to the cell membrane could occur through alterations in membrane characteristics such as fluidity or ion transport. Increases in membrane fluidity have been associated with activation of membrane enzymes. Although TPA application to rat embryo cells resulted in a decrease in fluorescence polarization of a DPH probe, TPA did not alter fluidity in artificial phospholipid vesicles but an early and rapid alteration in calcium ion transport and intracellular concentration in chick embryo myoblasts. Calcium ion shifts have been implicated as important regulators of cell and cell membrane function.

Exactly how TPA mediates the changes in phospholipid synthesis is unclear. Nevertheless, the data establish that depressed methylation of phosphatidylethanolamine to form phosphatidylcholine and enhanced synthesis of phosphatidylcholine from choline serve as useful markers of early membrane events that are part of the TPA-induced differentiation process of HL-60 cells seen morphologically 24–48 hr later. These changes can therefore be used to study other early membrane alterations in the search for the primary biochemical event that triggers HL-60 cell differentiation and for the investigation of signal transduction across the membrane.

REFERENCES


Early changes in phosphatidylcholine metabolism in human acute promyelocytic leukemia cells stimulated to differentiate by phorbol ester

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