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DEXAMETHASONE AFFECTS PHOSPHATIDYLINOSITOL SYNTHESIS AND DEGRADATION IN CULTURED HUMAN EMBRYONIC CELLS

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Dexamethasone (DEX), a glucocorticoid which induces cleft palate, causes marked alterations in the synthesis and degradation of phosphatidylinositol (PI) but not phosphatidylcholine in an established fibroblastic cell line derived from a human embryonic palate. Incorporation of radiolabeled inositol into phosphatidylinositol as well as degradation of prelabeled phosphatidylinositol is stimulated by DEX. The dose-response curves for the DEX-induced effect on PI synthesis and DEX-induced inhibition of cell proliferation are nearly identical, with the maximal responses occurring at 10^{-8}M DEX. Our results suggest that DEX-induced inhibition of human embryonic palatal mesenchyme cell proliferation and alterations in synthesis and degradation of phosphatidylinositol are related.

Anti-inflammatory glucocorticoids, when administered in pharmacologic doses at midgestation, induce palatal clefting in 90-100% of the offspring of susceptible inbred strains of mice (1). Additional evidence exists which suggests that glucocorticoids may be a causative factor in the production of cleft palate in non-human primates as well (2). Although the mechanism of this teratogenic effect is not fully understood, Diewert and Pratt (3) have reported that it can be attributed primarily to a glucocorticoid-induced

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Abbreviations: DEX, dexamethasone; PI, phosphatidylinositol; HEPM, human embryonic palatal mesenchyme cells; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediamine tetraacetate; CMF-PBS, calcium-magnesium-free phosphate buffered saline; TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate.

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receptor-dependent inhibition of palatal mesenchyme cell growth and subsequent failure of palatal shelf contact. At pharmacologic levels, glucocorticoids have been shown to inhibit proliferation of primary cultures of mouse secondary palate mesenchyme cells (4) as well as proliferation of a palatal mesenchyme cell line derived from the human embryo (5). We have utilized this human embryonic palatal mesenchyme (HEPM) cell line as an in vitro model with which to investigate in greater detail the mechanism of glucocorticoid-induced inhibition of cell proliferation.

The breakdown and resynthesis of phosphatidylinositol (PI), an important plasma membrane phospholipid, is thought to play a key role in the mechanism of growth stimulation induced by a wide variety of hormones, growth factors and other mitogenic agents (6, 7, 8, for a review see 9). It is postulated that these agents first bind to and activate their plasma membrane receptor. The activated receptor stimulates a phosphatidylinositol-specific phospholipase C which degrades the phospholipid to phosphorylinositol and 1,2-diacylglycerol. Diacylglycerol is then phosphorylated to phosphatidic acid, which is used in the resynthesis of phosphatidylinositol. The activation of this "PI cycle" is thought to result in stimulated calcium entry into the cell and/or an increase in prostaglandin synthesis (10). Proliferation may be stimulated by the action of one or both of these "second messengers" within the cell. Therefore, agents which alter normal PI turnover might prevent proliferation induced by growth factors which act by stimulating the PI cycle. Since PI turnover is involved in the mechanism of action of certain growth factors and glucocorticoids are known to alter phospholipid synthesis in fetal lung tissue (11), an investigation of the effect of glucocorticoids on HEPM cell phosphatidylinositol synthesis and degradation was undertaken.

Materials and Methods

Dexamethasone (DEX) and lipid standards were obtained from Sigma Chemical Company (St. Louis, MO). Myo-[2-^{3}H (N)]-inositol (12.5 Ci/mmol) and [methyl-^{3}H] choline chloride (80 Ci/mmol) were purchased from New England Nuclear (Boston, MA). We obtained the HEPM cell line (CRL 1486) in the third passage from the American Type Culture Collection (Rockville, MD). The cells were maintained in culture in Dulbecco's modified Eagle's minimum essential medium containing

10% heat-inactivated fetal bovine serum, 50 units/ml penicillin, and 50 µg/ml streptomycin (complete DMEM) in 75 cm^2 tissue culture flasks in a humidified atmosphere containing 5% CO_2 in air at 37°C. The medium was changed every 2 days. When the cells reached confluency (4 tc 5 days after plating), they were harvested with a 0.05% trypsin - 0.02% ethylenediamine tetraacetate (EDTA) solution and subcultured. At the 8th passage the cells were removed from the flask and stored frozen in liquid nitrogen in complete DMEM containing 10% dimethyl sulfoxide. Frozen cells were thawed and used in experiments between the 9th and 12th passages.

DEX was dissolved in absolute ethanol and stored at -20°C. For each experiment, identical amounts of absolute ethanol were added to control and DEX-treated cultures. The final ethanol concentration in the medium was 0.1% which had no discernible effect on cellular morphology or proliferation. The HEPM cells were plated at a density of 5 x 10^4 per 35mm plastic tissue culture dish containing 2 ml of complete DMEM. After 4 days the monolayers were washed twice with calcium-magnesium-free phosphate buffered saline (CMF-PBS, pH 7.4), harvested with the trypsin-EDTA solution and counted in a Coulter counter (Coulter Electronics, Inc., Hialeah, FL).

To determine the effect of DEX on phospholipid metabolism, confluent radiolabeled monolayers were washed 4 times with ice-cold CMF-PBS and the cells were exposed to 2 ml 5% trichloroacetic acid (TCA) at 4°C. After removing the TCA-treated cell layer, with a rubber policeman, the TCA-insoluble material was collected by centrifugation and washed once with cold 5% TCA. The pellet was solubilized with sodium dodecyl sulfate (SDS) at a final concentration of 1% and an aliquot was saved for protein analysis while the remainder was extracted by the procedure of Bligh and Dyer (12). To ensure removal of non-lipid material, the chloroform layer was washed once with fresh upper phase prepared using identical ratios of extraction mixture components. The chloroform extract was dried at 22°C under a stream of N_2 and counted in a Beckman LS 9800 liquid scintillation counter (Irvine, CA). Lipids resolved by thin-layer chromatography were solubilized in 200 µl chloroform, spotted and separated on silica gel-60 thin-layer plates (E. Merck Reagents) and developed with the system of Pappas et al. (13). Lipid standards, including phosphatidylcholine and phosphatidylinositol, were located with iodine vapor and the appropriate regions of the plate were removed and counted in a gel containing 1 ml water and 3 ml Aquasol (New England Nuclear). Protein analysis was performed using the method of Lowry et al. (14) with bovine serum albumin (Sigma Chemical Company) solubilized in 1% SDS as standard.

Results

As shown in Figure 1, when confluent HEPM cells which had been treated with dexamethasone (DEX) for varying times were labeled with [^{3}H]-inositol, a 2.5-fold increase in label incorporated into HEPM cell lipids occurred by 30 h. Analysis of the radioactive lipids by thin-layer chromatography indicated that greater than 95% of the incorporated label chromatographed with authentic phosphatidylinositol standard in both DEX-treated and control HEPM lipid extracts. The effect of DEX on phosphatidylcholine synthesis was investigated since DEX stimulates the synthesis of that phospholipid in embryonic lung tissue (10) and since a generalized increase in the turnover of all phospholipids might explain

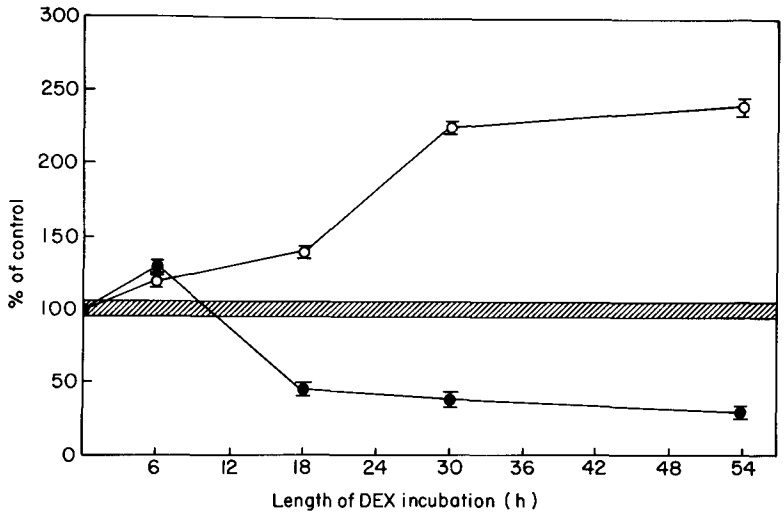

Figure 1: Effect of DEX on Transport and Incorporation of [^{3}H]-Inositol
Beginning on day 4 of culture, DEX was added at 10^{-6}M to confluent monolayers of HEPM cells at various times and were pulse-labeled with 2 μCi/ml [^{3}H]-inositol (12.5 Ci/mmol) during the final 6 h of DEX treatment on day 6. The monolayers were then harvested and the lipids extracted as described in Materials and Methods. The data points represent the mean ± S.D. of triplicate determinations. Control values were 8.6 ± 0.2 cpm/μg protein (3550 total cpm/dish) for TCA insoluble (o) and 174 ± 6 cpm/μg protein (71800 total cpm/dish) for TCA soluble (•) radiolabeled inositol.

the DEX effect on phosphatidylinositol. In contrast to the effect on phosphatidylinositol synthesis, DEX did not alter incorporation of [^{3}H]-choline into HEPM cell lipids at any time investigated (not shown). The amount of [^{3}H]-inositol in the TCA-soluble fractions of the DEX-treated monolayers increased to 130% of control values by 6 h and then decreased to about 40% of control values by 30 h (Figure 1). These results demonstrate that although DEX induced an early but transient increase in inositol uptake, the DEX-induced stimulation of inositol incorporation is not due to a stimulation in inositol transport. When similar experiments were performed using primary cultures of CD-1 mouse secondary palate mesenchyme cells in the third passage, neither a DEX-induced alteration in inositol transport nor incorporation was detected.

In order to determine whether DEX stimulated degradation of phosphatidylinositol, HEPM cells treated with DEX were incubated with [^{3}H]-inositol. After 16 h the labeled medium was removed and replaced with unlabeled conditioned medium from parallel cultures for various times. When compared to 0 h values, the decrease in radiolabeled phosphatidylinositol remaining in DEX-treated cells

TABLE 1

EFFECT OF DEX ON PHOSPHATIDYLINOSITOL DEGRADATION[a]

Time (h)	[^{3}H]-Inositol Incorporated (cpm/μg protein)	Percent of 0 h Control
Untreated		
0 h	17.3 ± 0.7[b]	—
12 h	19.3 ± 0.6	112
24 h	16.2 ± 0.5	94
DEX-treated		
0 h	26.0 ± 0.8	—
12 h	18.4 ± 1.2	71
24 h	13.5 ± 0.3	52

[a]HEPM cells grown for 3 days were incubated with DEX (10^{-6} M) for 40 h. Radiolabeled inositol (0.25 μCi/ml) was then added to the cultures. After 16 h, the [^{3}H]-inositol-containing medium was removed and unlabeled conditioned medium from parallel cultures was added for the times indicated. The cells were harvested as described in Materials and Methods except that the washed cells were first solubilized with SDS and then extracted.

[b]Mean ± S.D. for n = 3.

was much greater (29% and 48% at 12 h and 24 h, respectively) than the decrease in the untreated cells (Table 1). The increase in labeled phosphatidylinositol in the 12 h untreated cells may reflect a very slow rate of degradation coupled with continued incorporation from a relatively large [^{3}H]-inositol intracellular pool. If such is the case, the DEX-induced stimulation in phosphatidylinositol degradation may be greater than the data shown for both 12 h and 24 h. Taken together with the incorporation data (Figure 1), these results demonstrate that within 12 h DEX stimulates a large increase in the rate of phosphatidylinositol turnover in HEPM cells.

If DEX induces a decrease in cell proliferation by first altering phosphatidylinositol turnover, the dose-response curves for the two effects should be similar. Therefore we compared concentrations at which DEX-induced increases in phosphatidylinositol synthesis with concentrations which inhibited HEPM cell proliferation. As shown in Figure 2, both the phosphatidylinositol effect and

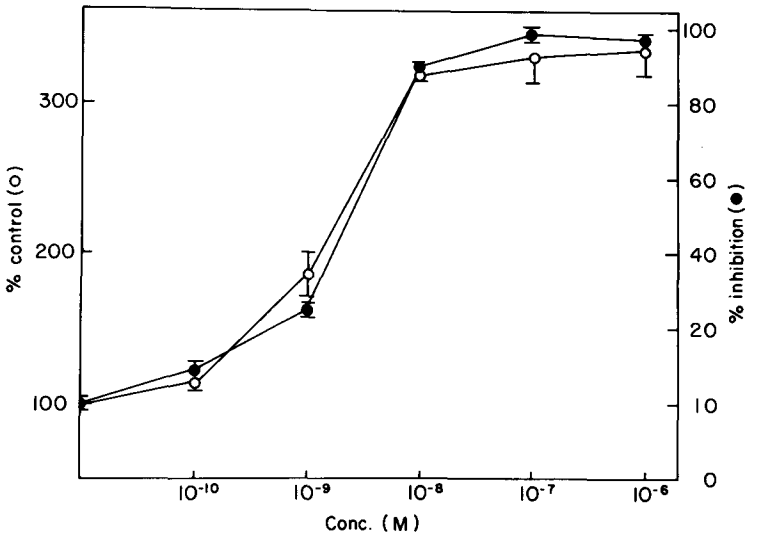

Figure 2: Effect of DEX Concentration on PI Synthesis and Growth Inhibition
HEPM cells grown for 4 days which had been treated with different concentrations of DEX from days 3-4 were pulse-labeled with 1 μCi/ml [^{3}H]-inositol for 6 h and harvested as described in Materials and Methods. Cpm/μg protein were determined and plotted as percent of control (o; 5 ± 0.1 cpm/μg protein). Data points represent the mean ± S.D. of triplicates. Alternatively, 4 day monolayers which had been treated with different concentrations of DEX for 72 h were counted as described in Materials and Methods. The data points (●) represent the means ± S.D. of quadruplicates.

growth inhibition are nearly maximal at 10^{-8}M, while significant increases in both effects were induced by 10^{-10}M DEX.

A good correlation also exists between the time courses for the two effects with nearly maximal stimulation of phosphatidylinositol synthesis occuring by 30 h (Figure 1) and nearly maximal inhibition of proliferation occurring by 24 h (not shown).

Discussion

Glucocorticoids have been reported to stimulate the synthesis of the major phospholipid component of surfactant, dipalmitoyl phosphatidylcholine, in fetal lung tissue both *in vivo* and *in vitro* (11, 15, 16). However, very little is known about the effect of DEX on the synthesis of other phospholipids. Since membrane phospholipids are important in various functions of the plasma membrane, DEX could exert its effects on the cell, in part, by altering the composition and/or turnover of plasma membrane phospholipids. The novel finding presented in the present study that levels of DEX which inhibit HEPM cell prolif-

eration also alter the synthesis and degradation of HEPM cell phosphatidylinositol is consistent with the above hypothesis.

Although glucocorticoids inhibit the proliferation of cultured fetal lung (15), glial cells (17), and both human and mouse embryonic palatal mesenchyme cells (4, 5), the mechanisms of DEX-induced inhibition of cell proliferation is unknown. The excellent positive correlation observed in this study between the dose-response curves for the DEX-induced phosphatidylinositol effect and growth inhibition and the similarity in time courses for the two effects suggest the possibility of a causal relationship. The inability of DEX to alter radiolabeled choline incorporation suggests the DEX effect on phosphatidylinositol is specific and further strengthens the relationship between the two effects. Thus, in stimulating the degradation of phosphatidylinositol, DEX may alter the distribution of the phospholipid in the membrane. This might result in the depletion of phosphatidylinositol from the site(s) in the plasma membrane where it can be acted upon by growth-factor stimulated degradative enzymes. Alternatively, DEX may stimulate the degradation of phosphatidylinositol while blocking subsequent metabolic steps which are necessary for stimulation of proliferation. One step might involve the phosphatidic acid-stimulated phospholipase A_2 activity which releases arachidonic acid from membrane phospholipids (10, 18, 19, 20, 21). DEX would act to block the relaease of arachidonic acid and the subsequent synthesis of arachidonic acid metabolites (prostaglandins, leukotrienes, etc.) by inducing the phospholipase A_2 inhibitor macrocortin (22, 23). In this case, DEX might stimulate phosphatidylinositol turnover directly by inducing the synthesis of PI-specific metabolic enzymes or indirectly through inhibition of arachidonic acid.

References

1. Salomon, D. S. and Pratt, R. M. (1979) Differentiation 13, 141-154.
2. Hendricks, A. G., Sawyer, R. H., Terrell, T. G., Osburn, B. I., Hendrickson, R. U., Steffek, A. J. (1975) Fed. Proc. 34, 1661.
3. Diewert V. M. and Pratt, R. M. (1981) TER. 24 (2), 149-162.
4. Salomon, D. S. and Pratt, R. M. (1978) J. Cell. Phys. 97 (3), 315-328.
5. Yoneda, T. and Pratt, R. M. (1981) J. Cran. Gen. and Dev. Biol. 1, 411-423.
6. Habenicht, Aj. J. R., Glomset, J. A., King, W. C., Nist, C., Mitchell, C. D. and Ross, R. (1981) J. Biol. Chem. 256 (23), 12329-12335.
7. Hasegawa-Sasaki, H. and Sasaki, T. (1981) Biochim Biophys Acta 666, 252-258.

8. Grove, R. I. and Schimmel, S. D. (1982) Biochim Biophys Acta 711, 272-280.
9. Michell, R. H. (1979) Trends in Biochem. Sciences 4, 128-131.
10. Lapetina, E. G. (1982) Trends in Pharm. Sciences 3(3), 115-118.
11. Farrell, P. M. (1977) J. Steroid Biochem 8, 463-470.
12. Bligh, E. G. and Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911-917.
13. Pappas, A. A., Mullins, R. E. and Gadsden R. H. (1982) Clin. Chem. 28, 209-211.
14. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
15. Funshouser, J. D. and Hughes, E. R. (1980) Biochim Biophys Acta 619, 506-514.
16. Snyder, J. M., Mendelson, C. R. and Johnston, J. M. (1981) Dev. Biol. 85, 129-140.
17. Grasso, R. J. and Johnson, C. E. (1977) Proc. Soc. Exp. Biol. Med. 154, 238-241.
18. Lapetina, E. G. and Cuatrecasas, P. (1979) Biochim Biophys Acta 573, 394-402.
19. Bell, R. L., Kennerly, D. A., Stanford, N, and Majerus P. W. (1979) Proc. Natl. Acad. Sci. USA 76 (7), 3238-3241.
20. Billah, M. M., Lapetina, E. G. and Cuatrecasas, P. (1980) J. Biol. Chem 255, 1790-1792.
21. Bell, R. L. and Majerus, P. W. (1980) J. Biol. Chem. 255, 1790-1792.
22. Hong, S. L. and Levine, L. (1976) Proc. Natl. Acad. Sci. U.S.A. 73(5), 1730-1734.
23. Blackwell, G. J., Carnuccio. R., Di Rosa, M., Flower, R. J., Parent, L. and Persico, P. (1980) Nature 287, 147-149.