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PGE_1 NOT $PGF_{2\alpha}$ REVERSES THE ANTI-ACTIN ANTIBODY STIMULATION OF PROTEIN PHOSPHORYLATION AND DNA SYNTHESIS IN L CELLS

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The effects of prostaglandins E_1 and $F_{2\alpha}$ upon the anti-actin antibody-induced stimulation of DNA synthesis and protein phosphorylation were studied in L cells. This system was previously shown by us to exhibit a rapid turnover of arachidonic acid in phospholipids which was inhibited by non-toxic concentrations of indomethacin, suggesting participation of cyclooxygenase-derived prostaglandins (Lipids 19:239, 1984). Prostaglandin E_1 in a dose dependent manner selectively inhibited both protein phosphorylation and DNA synthesis in anti-actin antibody-stimulated cells. Prostaglandin $F_{2\alpha}$ was without effect. Indomethacin also produced a dose related inhibition of the antibody stimulation of protein phosphorylation and DNA synthesis. We conclude that prostaglandins, possibly derived from liberated arachidonic acid, play an important regulatory role in the stimulatory signal conveyed to L cells by perturbing antibody ligands.

Anti-actin antibody, when bound to surface actin molecules on a murine methylcholanthrene-transformed fibroblast (L cell), transmits a stimulatory signal to the cell interior with the resulting enhancement of several metabolic events culminating in cell growth (1,2). Important in the early metabolic events enhanced by antibody is the calcium-dependent turnover of arachidonic acid in membrane phospholipids, phosphatidylinositol > phosphatidylcholine > phosphatidylethanolamine on a molar basis (3). Involvement of prostaglandins is likely in this stimulation process since non-toxic doses of the cyclooxygenase inhibitor indomethacin (2 to 100 μM) selectively inhibited the turnover of arachidonic acid in membrane phospholipids and the burst in DNA synthesis due to antibody at 24 hr (3). Subsequent to these earlier metabolic events, a

ABBREVIATIONS: PGE_1, prostaglandin E_1; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; NM, nutrient medium; AIARS, anti-immunoselected antigen rabbit serum; CRS, control rabbit serum; C, complement; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; dThd, thymidine.

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selective stimulation of phosphorylation of cellular proteins occurs at 48 hr in antibody-stimulated cells and this preceeds increased cell proliferation which occurs at 72 hr (2). Preliminary evidence suggests that anti-actin antibody produces these effects by recruiting cells from the G_1-phase to the S-phase of the cell cycle (4). The present report describes the effects of two cyclooxygenase-produced derivatives of arachidonic acid, prostaglandin E_1 (PGE_1) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), upon discrete steps in the activation sequence, thymidine (dThd) incorporation at 24 hr and protein phosphorylation at 48 hr. A brief report of some of this work has been made (5).

MATERIALS AND METHODS

Cell line. L cells were maintained in tissue culture in nutrient medium (NM) consisting of Eagle's minimum essential medium and 10% fetal calf serum (6).

Antiserum. Rabbit antiserum to L cell actin was produced by immunization with L cell antigens immunoprecipitated by an antiserum to whole L cells (1). This anti-actin antibody preparation has been termed anti-immunoselected antigen rabbit serum (AIARS). Control rabbit serum (CRS) was obtained from adjuvant-injected animals. AIARS and CRS were heat inactivated at 56°C for 30 minutes, sterile filtered, and stored at -20°C. Normal rabbit serum was used as a source of complement (C).

Phosphorylation of L cell proteins. L cell monolayers were harvested by a 2 min exposure to a 0.05% trypsin solution and put into single cell suspension in NM by repetitive pipetting. One ml aliquots of cell suspension (5×10^4 cells/ml) were placed in tissue culture tubes, incubated at 37°C in the absence and presence of various dilutions of AIARS, CRS, 1:400 dilution of C, and various concentrations of PGE_1 or $PGF_{2\alpha}$ (Sigma Chemical Co., St. Louis, MO) for 48 hr (2). Fifty μCi of ^{32}P (orthophosphate form, 40 mCi/ml, Amersham Corp., Arlington Heights, IL) was added to the cell cultures and incubation at 37°C was continued for an additional 2 hr. Phosphorylation was stopped by the addition of 2 ml of ice-cold 0.01 M phosphate-buffered saline pH 7.4 (PBS) containing 10 mM EDTA and 10 mM NaF. Cell cultures were centrifuged at 4°C at 1000 xg for 5 min, supernatants were discarded, and the cells were washed twice more with the same buffer system. The total protein content of the cell cultures was determined by Lowry et al. (7) and were shown to be equivalent for control and antibody stimulated cells at 48 hr (2).

Separation of L cell proteins with sodium dodecyl sulfate(SDS)-polyacrylamide gel electrophoresis. After the final wash, 100 μl of a cell solvent (3% SDS, 3% α-mercaptoethanol, 63 mM Tris-HCl buffer pH 6.8, 0.002% bromophenol blue, 10% glycerol) was added to cell pellets and the dissolved cell pellets were heated immediately at 100°C for 5 min. After cooling the cell extracts were quantitatively transferred to a 3% polyacrylamide stacking gel and subjected to electrophoresis on a gradient (5-15%) polyacrylamide slab gel using a running buffer system containing 0.025 M Tris-0.192 M glycine buffer pH 8.3, 0.01% SDS (8). Electrophoresis with a Bio-Rad electrophoresis apparatus was maintained at 30 mAmp for 5 hr and the leading edge was allowed to advance 11.5 cm. Gels were fixed and stained overnight in 10% acetic acid, 50% methanol, and 0.025% Coomassie blue dye and destained with 10% acetic acid and DEAE-cellulose powder. In some cases the gels were heated at 90°C for 30 min in 5% TCA to remove residual nucleic acid.

Quantitation of ^{32}P incorporation into proteins. Destained slab gels were soaked in 10% acetic acid - 1% glycerol solution for 1 hr, wiped dry with paper toweling, and vacuum dried between 2 sheets of porous cellophane on a Bio-Rad gel dryer. Dried gels were exposed to x-ray film (XAR-5, Eastman Kodak, Rochester, N.Y.) for 16 hr at -80°C. The x-ray film was developed using a Kodak RPX-OMAT processor. The black bands of the x-ray film were assessed for absorbance at 525 nm using a Quick Scan Jr. densitometer (Helena Laboratories, Beaumont, TX). The readings represent arbitrary units. In each group of gels tested, the darkest staining protein band or the darkest appearing x-ray band was used to standardize the instrument and all scans of the group of gels for the experiment were made with these settings.

Incorporation of [^{3}H]thymidine ([^{3}H]dThd) into DNA. Measurements of incorporation of [^{3}H]dThd in cells were made with the same incubation conditions used to study ^{32}P labeling of proteins. [Methyl-^{3}H] thymidine (50 Ci/mmol, Amersham Corp.) was diluted with NM. At 24 hr, each isotope was added separately to triplicate samples (0.2 μCi/ml final) and the incubation continued for an additional 24 hours prior to harvest.

Cells were harvested by filtration through polycarbonate filters (1.0 μM pore, Nucleopore Corp., Pleasanton, CA) over a suction manifold. Filters were washed with cold PBS or 6% trichloroacetic acid (same results), placed in counting vials, incubated with 0.5 ml tissue solubilizer and diluted with 5.0 ml scintillation cocktail. Retained radioactivity was measured by liquid scintillation spectrometry. We have proven that this radioactivity is contained in nuclear DNA (6).

RESULTS

Stimulation of protein phosphorylation by anti-actin antibody was measured at 48 hours of incubation in the presence of increasing concentrations of PGE_1 and $PGF_{2\alpha}$ (only two concentrations of each are shown in Fig. 1). There was a PGE_1 dose related decrease in the enhanced phosphorylation of L cell proteins due to AIARS but there was very little effect of similar doses of $PGF_{2\alpha}$. At the highest concentration of PGE_1 there was an effect on control (CRS) cultures as well but the effect upon AIARS-stimulated cultures was much greater.

To correlate these effects of prostaglandins upon protein phosphorylation with other aspects of cell metabolism, we performed [^{3}H]dThd incorporation studies (DNA synthesis) in anti-actin antibody stimulated cells in the presence of increasing concentrations of PGE_1 and $PGF_{2\alpha}$ (Fig. 2). PGE_1 exhibited a dose-dependent inhibition of AIARS-stimulated DNA synthesis which was significantly different from the control at concentrations greater than 1 μM (maximal $p < 0.05$). At concentrations greater than 10 μM, PGE caused a slight decrease in DNA synthesis of control cells. $PGF_{2\alpha}$ had very little effect upon DNA synthesis in either AIARS or CRS cell cultures.

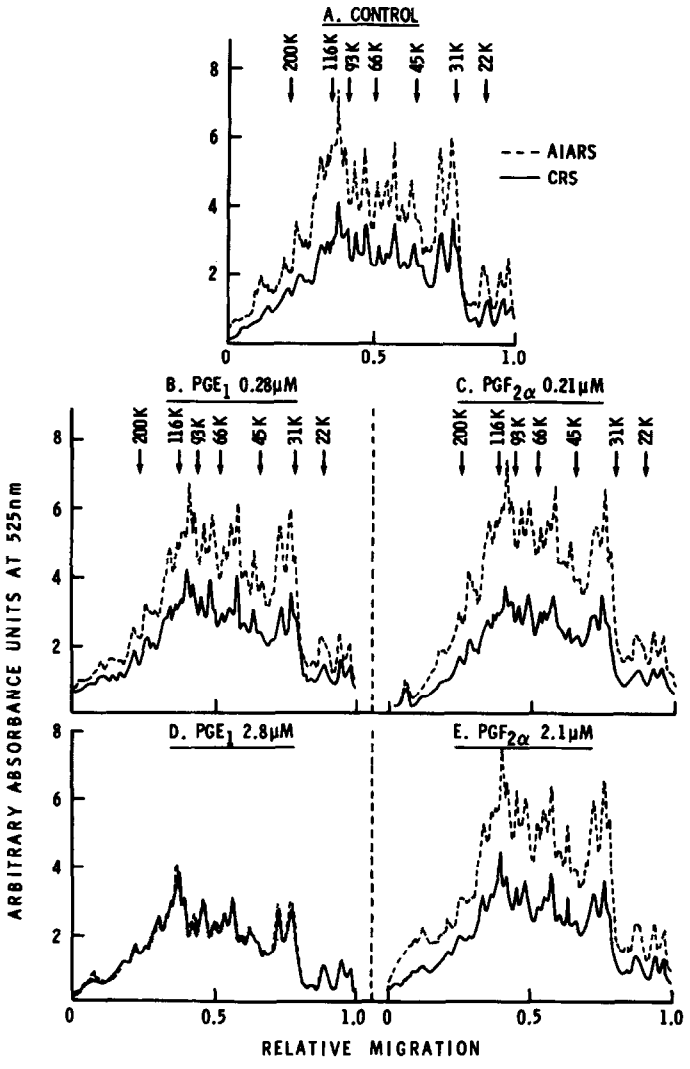

Fig. 1: Effects of prostaglandins upon AIARS stimulated protein phosphorylation in L cells: A, control AIARS and CRS cultures. B, PGE_1 0.28 μM (0.1 μg/ml). C, $PGF_{2\alpha}$ 0.21 μM (0.1 μg/ml). D, PGE_1 2.8 μM (1.0 μg/ml). E, $PGF_{2\alpha}$ 2.1 μM (1.0 μg/ml). CRS and AIARS were diluted 1:100 and incubated at 37°C for 48 hr with 5 x 10^5 L cells in 1 ml of NM containing 1:400 C. ^{32}P (50 μCi) was added at 48 hr and incubation was continued for an additional 2 hr.

We also performed phosphorylation experiments in the presence of increasing concentrations of indomethacin (1 to 100 μM) and observed a dose-related elimination of the antibody augmented phosphorylation of cellular proteins (data not shown).

DISCUSSION

Our observations indicate that products of arachidonic acid metabolism, prostaglandins, profoundly influence discrete steps in the activation sequence

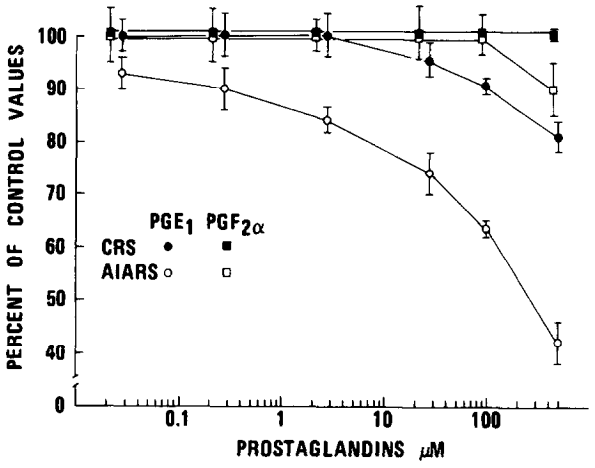

Fig. 2: Effects of prostaglandins upon AIARS stimulated [^{3}H]dThd incorporation into DNA in L cells. Conditions are similar to those described in Fig. 1 except a wider range of prostaglandin concentrations is presented. [^{3}H]dThd (0.2 μCi) was added at 24 hr and incubation was continued for an additional 24 hr. The results are expressed as the percent of control (no prostaglandin) values (means ± standard errors) of 4 experiments each performed in triplicate (n = 12). Control CRS value was 6,119 ± 182 counts per min (cpm) and control AIARS value was 44,060 ± 2,455 cpm.

produced in L cells when bound by anti-actin antibody. PGE_1 produces inhibition in the burst of DNA synthesis at 24 hr and the normally resulting increase in cellular protein phosphorylation at 48 hr. $PGF_{2\alpha}$ produced no discernable effects. When cellular cyclooxygenase was inhibited with indomethacin in non-toxic concentrations, protein phosphorylation was inhibited in antibody stimulated cells and DNA was selectively inhibited in the same cells (3). These observations imply that a balance of prostaglandins is essential for phosphorylation to take place. Disruption of that balance by the addition of PGE_1 or by shutting off arachidonic acid degradation by cyclooxygenase prevents phosphorylation events in the antibody induced activation of cells.

In other cell systems involving peptide hormone stimulation, transmembrane signaling is thought to be mediated by a calcium-dependent turnover of phospholipids resulting in activation of a protein kinase which regulates protein phosphorylation (9). Protein growth factors also produce sudden changes in phospholipid and arachidonic acid metabolism in quiescent normal fibroblasts which are stimulated to divide (10). The binding of surface actin in the

transformed L cell by high affinity IgG antibody likewise initiates a cascade of cellular events involving early release of arachidonic acid and its subsequent enzymatic conversion to prostaglandins. Delineation of the metabolic steps involved in activation of transformed cells by anti-actin antibody in vitro may prove useful in understanding the interactions of malignant cells and the immune system in vivo.

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REFERENCES

1. Shearer, W.T., Moore, E.G., Ulrich, R.A., Green, C.G., and McClure, J.E. (1984) Cell. Immunol. 86, 230-241.
2. Shearer, W.T., Gilliam, E.B., and McClure, J.E. (in press) Cell. Immunol.
3. Shearer, W.T. and Ulrich, R.A. (1984) Lipids 19, 239-249.
4. Ulrich, R., Shearer, W.T., Gilliam, E.B., and Green, C.G. (1984) Fed. Proc. 43, 1931.
5. Shearer, W.T., Gilliam, E.B., and Green, C.G. (1984) Fed. Proc. 43, 1931.
6. Shearer, W.T., Philpott, G.W., and Parker, C.W. (1975) Cell. Immunol. 17, 447-462.
7. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265.
8. Laemmli, V.K. (1970) Nature 227, 680.
9. Nishizuka, Y. (1983) Phil. Trans. R. Soc. Lond. B 302, 101.
10. Habenicht, A.J.R., Glomset, J.A., King, W.C., Nist, G., Mitchell, C.D., and Ross, R. (1981) J. Biol. Chem. 256, 12329.