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Role of Arachidonic Acid Metabolism in the Mitogenic Response of BALB/c 3T3 Fibroblasts to Epidermal Growth Factor

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SUMMARY

We have investigated the involvement of arachidonic acid release and metabolism in the mitogenic response, i.e., [^{3}H]thymidine incorporation, to epidermal growth factor (EGF) in BALB/c 3T3 cells. EGF induces release of arachidonate and prostaglandin (PG) formation after its addition to BALB/c 3T3 cells at the same concentrations that stimulate mitogenesis. Further, EGF-stimulated mitogenesis is blocked by inhibitors of arachidonate metabolism including indomethacin, eicosatetraynoic acid, and dexamethasone, whereas the addition of major arachidonate products in BALB/c 3T3 cells, PGE_2, $PGF_{2\alpha}$, and their intermediates PGG_2 and PGH_2, stimulate mitogenesis in synergism with EGF. The addition of PGs to BALB/c 3T3 cells also overcame indomethacin- and eicosatetraynoic acid-inhibited responses to EGF. Indomethacin must be added with EGF in order to block arachidonate metabolism and subsequent mitogenesis. These results suggest that the release of arachidonic acid and its subsequent metabolism is an apparent early requirement for the initiation of cell cycle traversal by EGF.

Growth factors stimulate "quiescent", nonproliferating cells that are reversibly arrested in the G_1/G_0 phase to enter the cell cycle and become committed to DNA synthesis in S phase (1, 2). Growth factors exert their effect by interacting with high affinity receptors on the plasma membrane and promote the generation of early signals in the membrane and cytosol, leading to propagation of the mitogenic signal into the nucleus and eventually to cell proliferation (3, 4).

PDGF and EGF are probably the best characterized growth factors. It has been suggested that PDGF is necessary to render density-arrested BALB/c 3T3 fibroblasts competent to initiate DNA synthesis in response to EGF and insulin-like growth factors (5, 6), whereas cells arrested at subconfluence by serum-depletion can be induced to enter the cell cycle by EGF or insulin-like growth factors alone (7, 8).

PDGF activates phospholipase C in BALB/c 3T3 cells, generating the second messengers inositol trisphosphate and diacylglyceride (3, 9). Those two compounds, which evoke release of intracellular calcium stores and stimulate protein kinase C, respectively, are important for several cell responses including mitogenesis (10, 11). However, EGF, a potent growth stimulator of fibroblasts, does not appear to activate phospholipase C in BALB/c 3T3 cells (7, 8). Transduction of the EGF signal in BALB/c 3T3 cells must therefore be via another mechanism. EGF stimulates arachidonic acid release in several mammalian cell lines (12–14) and, further, EGF is linked to the activation of phospholipase A_2 in A431 cells via its ability to phosphorylate and regulate the activity of lipocortin (15). This activity of EGF plus the ability of eicosanoids to regulate proliferation of fibroblasts (16–18), and the increased production of PG by transformed fibroblasts (19) and many malignant tumors (20, 21), led us to investigate the possible involvement of arachidonic acid metabolites in the mitogenic response to EGF in BALB/c 3T3 (A31) cells. Much of the published work on the role of eicosanoids in cultured fibroblast proliferation was done with exogenously added compounds, although there is a great deal of literature on the role of endogenous eicosanoids in the control of proliferation in other cell types. We ask the following question: is the formation of eicosanoids in BALB/c 3T3 cells necessary for EGF-induced mitogenesis, or are the two events unrelated? We report that EGF stimulates release of arachidonic acid in quiescent BALB/c 3T3 fibroblasts and that this arachidonic acid is converted by these cells into metabolites that are necessary for the mitogenic response. We postulate that these eicosanoids are important intracellular mediators of EGF-induced cellular responses in BALB/c 3T3 cells.

Materials and Methods

Materials. [^{3}H]Arachidonic acid (60–100 Ci/mmol), [^{3}H]PGE_2 (100–200 Ci/mmol), [^{3}H]$PGF_{2\alpha}$ (150–180 Ci/mmol), and [^{3}H]thymidine

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ABBREVIATIONS: PDGF, platelet-derived growth factor; DMEM, Dulbecco's modified Eagle medium; EGF, epidermal growth factor; ETYA, 5,8,11,14-eicosatetraynoic acid; FCS, fetal calf serum; NDGA, nordihydroguaiaretic acid; PG, prostaglandin; HPLC, high performance liquid chromatography.

(6.7 Ci/mmol) were from New England Nuclear (Boston, MA). EGF was from Seragen, Inc. (Boston, MA). Arachidonic acid was purchased from Nu-Chek Prep (Elysian, MN). PGE_2 and $PGF_{2\alpha}$ were from Sigma Chemical Co. (St. Louis, MO), PGG_2 and ETYA were from Biomol Research Laboratories (Philadelphia, PA), and PGH_2 was from Oxford Biomedical Research (Oxford, MI).

Indomethacin, ibuprofen, NDGA, dexamethasone, and *p*-bromophenacyl bromide were obtained from Sigma Chemical Co. Instruments for HPLC were from Waters Associates (Milford, MA) and a radioactivity flow detector from Radiomatic Instruments and Chemical Co. (Tampa, FL) was used to measure radioactivity of the HPLC eluate, with Hydrofluor (National Diagnostics, Somerville, NJ) as scintillation fluor. Solvents used included HPLC-grade methanol, HPLC-grade water, and acetic acid from Fisher Scientific (Fairlawn, NJ) and ethyl acetate from Baker Chemical Co. (Phillipsburg, NJ).

Cell culture. BALB/c 3T3 mouse fibroblasts (clone A31) obtained from the American Tissue Culture Collection (Rockville, MD) were maintained at 37° in a humidified, 5% CO_2/95% air atmosphere. The culture medium was DMEM (GIBCO, Grand Island, NY) containing 10% FCS (Hyclone Laboratories, Logan, UT, or Sigma Chemical Co.), fungizone (1.25 μg/ml; GIBCO), and gentamycin (50 μg/ml, M.A. Bioproducts, Walkersville, MD). Trypsin (GIBCO Laboratories) was used to subculture cells.

Arachidonic acid metabolism: characterization of metabolites. Assays for arachidonic acid metabolism were carried out in triplicate. Cells were cultured at 7.5×10^5 cells/75-cm^2 flask (Falcon, Oxnard, CA) in 15 ml DMEM/10% FCS and grown to near confluence. [^{3}H]Arachidonic acid (5 μCi; final concentration 1 μM) was added to the cells in serum-free DMEM containing 10 ng/ml EGF and incubated for 24 hr. [^{3}H]Arachidonic acid and its metabolites were extracted from the incubation medium and the cells by acidifying to pH 3.5 with 1 M formic acid and extracting twice with 3 volumes of ethyl acetate. The organic phase was dried under vacuum and the samples were reconstituted in 30% methanol for analysis by HPLC. Recovery of internal standards during sample preparation was greater than 90%. Separation of eicosanoids by reverse phase HPLC was achieved by stepwise elution from a C_{18} Ultrasphere column (Altex Scientific Inc., Beckman Instruments, Berkeley, CA) with methanol/water, pH 5.05, at a flow rate of 1.1 ml/min as described previously (22). Eluted radioactivity was monitored using a Flow-One radioactivity detector (Radiomatic Instruments) equipped with a Qume computer (Radiomatics) for data processing. Metabolites were also separated using a normal phase HPLC system consisting of a μ-Porasil (10 μm) column (Waters Associates) eluted with hexane/ethanol/acetic acid (994:6:1) for 25 min followed by hexane/ethanol/acetic acid (90:10:1) for an additional 60 min at a flow rate of 3.0 ml/min.

Arachidonic acid metabolism: release by EGF. The cellular phospholipid fraction was labeled with [^{3}H]arachidonic acid by incubating monolayers with 5 μCi of [^{3}H]arachidonic acid (10 nM) for 16 hr. Incorporation of [^{3}H]arachidonic acid into the phospholipid fraction was confirmed by thin layer chromatographic analysis of extracted phospholipids performed according to the method of Flower and Blackwell (23). Routinely 70–75% of the [^{3}H]arachidonic acid was incorporated. The labeled monolayers were washed twice with Hank's balanced salt solution (GIBCO) to remove unincorporated [^{3}H]arachidonic acid. To study the release of endogenous arachidonic acid metabolites the cells were incubated for 1 hr in 5 ml of serum-free DMEM, in the presence or absence of EGF as described in the figure legends. Arachidonic acid and its metabolites were extracted and separated by the reverse phase HPLC system as described above.

Analysis of DNA synthesis. [^{3}H]Thymidine incorporation was used to measure the mitogenic response. Assays were carried out in quintuplicate. BALB/c 3T3 cells were cultured at 1×10^4 cells/0.32-cm^2 well in 96-well plates (Costar, Cambridge, MA) in 0.2 ml of DMEM/10% FCS. Cells were grown to near confluence (2–3 days) and then incubated for 24 hr in serum-free DMEM. The cells retained their ability to respond to EGF and therefore remained competent in serum-free medium. DNA synthesis experiments were performed by incubating the cell monolayers in 0.2 ml of serum-free DMEM containing EGF. Potential inhibitors of mitogenesis were added to the cells in serum-free DMEM 30 min before the addition of EGF. In the case of dexamethasone, a phospholipase A_2 inhibitor (24, 25), a 4-hr preincubation was used. In experiments to determine the time dependence for inhibition of mitogenesis by a cyclooxygenase inhibitor, indomethacin was added at the times indicated in Fig. 8. In other experiments, eicosanoids were added simultaneously with EGF. After incubation for 20 hr the monolayers were pulsed with 1 μCi of [^{3}H]thymidine/well and incubated for an additional 4 hr. After trypsin treatment for 15 min, cells were harvested by using a semiautomated sample harvester (Skatron; Flow Laboratories, Rockville, MD) onto glass filter paper discs. Samples were processed for liquid scintillation counting in Atomlight (New England Nuclear).

Analysis of endogenous PG. In parallel with experiments to measure DNA synthesis, experiments were conducted to estimate PG formation. BALB/c 3T3 cells were cultured at 1×10^5 cells/2-cm^2 wells in 24-well plates in 2.0 ml of DMEM/10% FCS. Cells were grown to near confluence (2–3 days) and then incubated for 24 hr in serum-free media. PG formation was examined by incubating cell monolayers with 0.5 ml of serum-free DMEM containing EGF. Inhibitors of arachidonic acid metabolism were added to the cells in serum-free DMEM 30 min before the addition of EGF. For dexamethasone, a 4-hr preincubation was used. The incubations were continued for 30 min. The medium was removed and analyzed for PGs.

PG production was measured by estimating PGE_2 with a specific radioimmunoassay using antiserum supplied by Seragen as described previously (26). Samples were assayed at several dilutions and exhibited parallelism with authentic standard PG.

Results

Characterization of arachidonic acid metabolites. Exogenous [^{3}H]arachidonic acid (1 μM) was added to the cells and incubated for 24 hr (Fig. 1A). The major arachidonic acid metabolite coeluted with PGE_2/D_2 on reverse phase HPLC. The leading peak of radioactivity represents the void volume containing uncharacterized polar phospholipids or diacylglyceride. PGE_2/D_2 and $PGF_{2\alpha}$ accounted for approximately 30 and 15%, respectively, of the total added [^{3}H]arachidonic acid. The metabolite peak that coeluted with PGE_2/D_2 was collected and rechromatographed on a normal phase HPLC system that separated PGE_2 from PGD_2. In all cases, only one peak was detected that coeluted with PGE_2.[2] It should also be noted that in many experiments small amounts of uncharacterized metabolites eluted between 60–70 min, a region in which hydroxy arachidonic acid metabolites normally elute.

EGF stimulation of arachidonic acid release and metabolism. EGF caused release of [^{3}H]arachidonic acid and its metabolites from BALB/c 3T3 cells in which [^{3}H]arachidonic acid was incorporated into cellular phospholipid (Fig. 1, B and C). The release of arachidonic acid and its metabolites was increased approximately 2-fold by the addition of EGF (10 ng/ml) (Table 1). The major metabolites of arachidonic acid released in the presence or absence of EGF coeluted with authentic PGE_2 and $PGF_{2\alpha}$ as determined by reverse phase HPLC. PGE_2 and $PGF_{2\alpha}$ accounted for approximately 8 and 0.5%, respectively, of the total radioactivity eluting on the HPLC column, whether the samples were from incubations treated with or without EGF.

PGE_2, the major metabolite of arachidonic acid in BALB/c 3T3 cells, was measured by a specific radioimmunoassay for

[2] R. D. Nolan, unpublished observations.

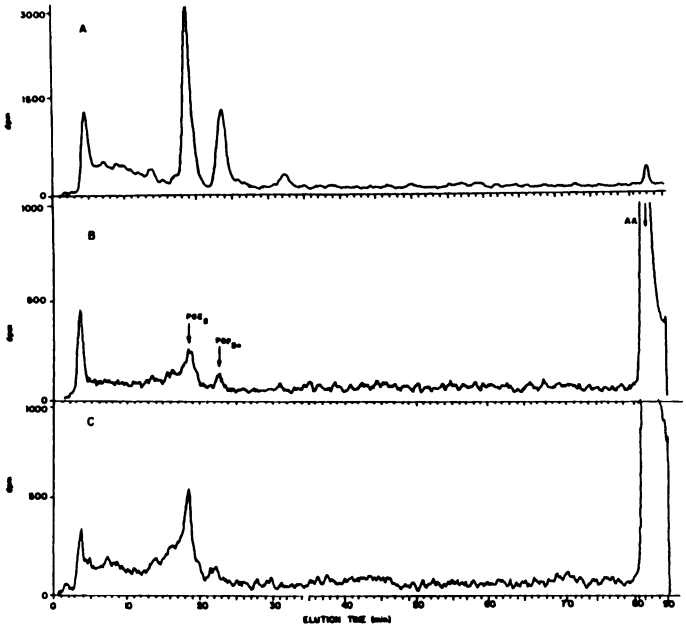

Fig. 1. HPLC radiochromatograms of [^{3}H]arachidonic acid metabolites in BALB/c 3T3 cells. A, Metabolites produced by cells after 24-hr incubation with exogenous [^{3}H]arachidonic acid. B and C, Endogenous metabolites released from cells prelabeled with [^{3}H]arachidonic acid. Cells were incubated for 1 hr in serum-free DMEM (B) or in serum-free DMEM containing EGF, 10 ng/ml (C). Media were extracted and chromatographed on the reverse phase-HPLC system as described in Materials and Methods. The results are representative of triplicate incubations in several experiments.

TABLE 1

Effect of EGF on release of arachidonic acid from resting 3T3 cells

Arachidonic acid was incorporated into the phospholipid fraction of 3T3 cells as described in Materials and Methods. Cell monolayers were then incubated for 60 min in absence and presence of EGF (10 ng/ml). The incubation medium was extracted and arachidonic acid metabolites were separated on reverse phase HPLC. Data are mean ± standard error (three determinations).

	Radioactivity released		
	Total	Arachidonic acid	PGE_2
	dpm		
Control	53750 ± 8222	45296 ± 8812	3937 ± 673
EGF	93104 ± 9365	71610 ± 5546	7475 ± 1107

PGE_2 and used as an indication of arachidonic acid metabolism and release in subsequent experiments. EGF-stimulated PGE_2 release was observed as early as 1 hr after the addition of EGF (Fig. 2). Maximal release of PGE_2 was achieved within 4–6 hr after the addition of EGF. The level of PGE_2 actually declined after 8 hr and is presumably due to conversion of PGE_2 to $PGF_{2\alpha}$. Hammarström (19) showed that BALB/c 3T3 cells slowly metabolized PGE_2 to $PGF_{2\alpha}$. This data is also consistent with the findings in the present study in which low levels of $PGF_{2\alpha}$ were detected in 1-hr incubation medium and higher levels were detected in 24-hr incubations (data not shown).

EGF stimulation of mitogenesis. EGF, at concentrations identical to those that caused release of PGE_2 (1–100 ng/ml), stimulated mitogenesis, measured by [^{3}H]thymidine incorporation into DNA in BALB/c 3T3 cells (Fig. 3). EGF-stimulated DNA synthesis was maximal at 24 hr after the addition of EGF (data not shown), compared with EGF-stimulated PGE_2 release, which was observed within 4 hr. These data suggest that eicosanoid formation may be a very early event in the initiation

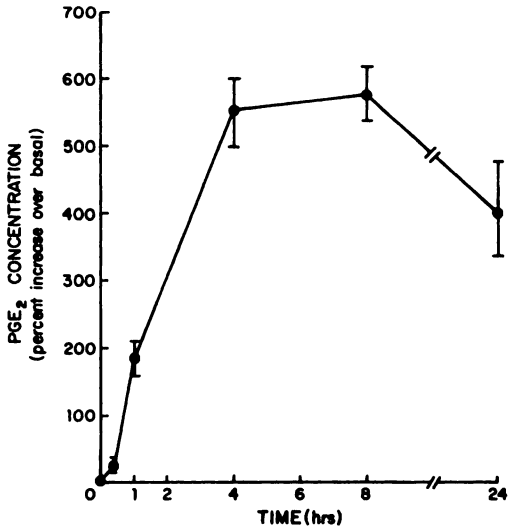

Fig. 2. Time course for PGE_2 release from BALB/c 3T3 cells. The BALB/c 3T3 cells grown to subconfluence in 24-well plates were serum-depleted for 24 hr. EGF (10 ng/ml) was added and the medium was removed at the indicated times. Release of PGE_2 into the incubation medium was measured by radioimmunoassay. The data (mean ± standard error, three experiments) are representative of several different experiments. The data are expressed as per cent increase over basal (in absence of EGF).

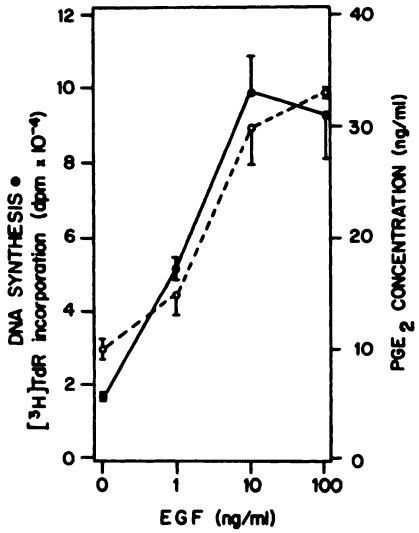

Fig. 3. Effect of EGF concentration on PGE_2 release (○) and DNA synthesis (●) in BALB/c 3T3 cells. In parallel studies BALB/c 3T3 cells were grown to near confluency in 96-well or 24-well plates. The cells were serum-depleted for 24 hr and EGF was added at various concentrations. For PG formation the media was removed from the 24-well plates 30 min after the addition of EGF and PGE_2 release was determined by radioimmunoassay. DNA synthesis was measured 24 hr after the addition of EGF in the 96-well plates as described in the Materials and Methods. The data (mean ± standard error, five determinations for [^{3}H] thymidine incorporation, three determinations for PGE_2 release) are representative of several different experiments.

of DNA synthesis. It should be noted that the amount of [^{3}H] thymidine incorporated into DNA and the amount of PGE_2 formed varied between experiments.

Effects of inhibitors of arachidonic acid metabolism on EGF-induced mitogenesis. The effect of various inhibitors of arachidonic acid metabolism on EGF-induced PGE_2 release and mitogenesis was investigated as an approach to

determine whether arachidonic acid release is essential for EGF-stimulated mitogenesis. The addition of inhibitors of arachidonic acid release and metabolism inhibited both the synthesis of PGE_2 and mitogenesis in BALB/c 3T3 cells. The possibility that these inhibitors of arachidonic acid metabolism affect cell viability was eliminated by the trypan blue exclusion test, which demonstrated greater than 85% viability in all incubations. Inhibition of phospholipase activation by dexamethasone and *p*-bromophenacyl bromide resulted in parallel inhibition of both mitogenesis and PGE_2 release (Fig. 4, A and B). Likewise, mitogenesis and PGE_2 release were both inhibited by NDGA, the cyclooxygenase and lipoxygenase inhibitor, and ETYA, the synthetic analog of arachidonic acid that blocks both the lipoxygenase and cyclooxygenase pathways (Fig. 4, C and D). Inhibition of PGE_2 release was observed at the same drug concentration as inhibition of mitogenesis for these two compounds. The cyclooxygeanse inhibitors indomethacin and ibuprofen blocked both mitogenesis and PGE_2 release at similar concentrations (Fig. 4, E and F). These data indicate that a metabolite(s) of arachidonic acid is involved in the stimulation of mitogenesis by EGF.

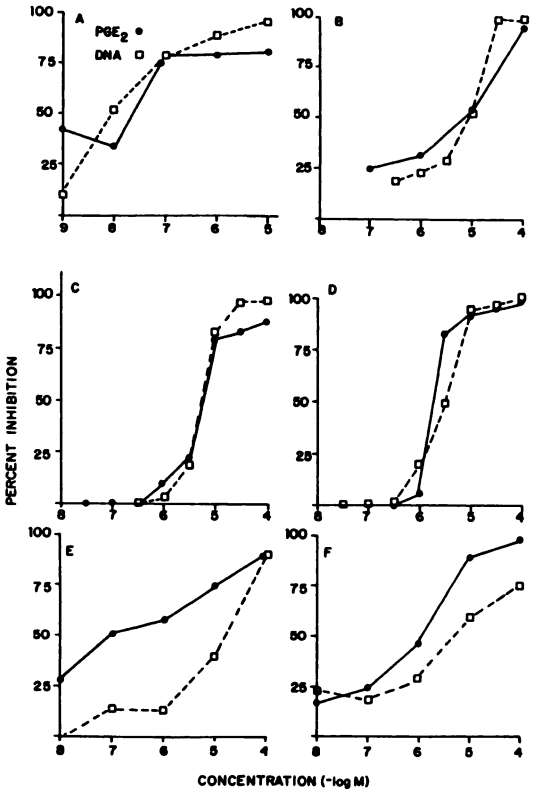

Fig. 4. Effect of inhibitors of arachidonic acid metabolism on DNA synthesis (□) and PGE_2 release (●) by BALB/c 3T3 cells. In parallel studies BALB/c 3T3 cells were grown to near confluence in 96-well or 24-well plates. The cells were serum-depleted for 24 hr. Dexamethasone was added 4 hr before the addition of EGF (10 ng/ml). All other inhibitors were added 30 min before the addition of EGF. For PG formation the media was removed from the 24-well plates 30 min after the addition of EGF and PGE_2 release was determined by radioimmunoassay. DNA synthesis was measured 24 hr after the addition of EGF in the 96-well plates as described in Materials and Methods. The data were calculated as per cent inhibition from the means of five determinations for DNA synthesis and three determinations for PGE_2 release. The standard errors for these determinations were always less than 10% of the mean and the data are representative of several experiments. A, Dexamethasone; B, *p*-bromophenacyl bromide; C, NDGA; D, ETYA; E, indomethacin; F, ibuprofen.

Effects of arachidonic acid metabolites on mitogenesis. Exogenous prostanoids were added to cells simultaneously with EGF (10 ng/ml) and their effects on mitogenesis were investigated. Although the PGs tested stimulate DNA synthesis to a very small extent when added alone or in the presence of indomethacin at concentrations of 10^{-4} to 10^{-5} M,[3] they have a synergistic effect when added with EGF (Fig. 5A). $PGF_{2\alpha}$ was the most potent eicosanoid ($EC_{50} \sim 5 \times 10^{-9}$ M), followed by PGG_2, PGH_2, and PGE_2 ($EC_{50} \sim 3 \times 10^{-7}$ M). PGD_2 and PGI_2, neither of which are produced by BALB/c 3T3 cells in detectable amounts, had no stimulatory effect on DNA synthesis; in fact, PGI_2 was slightly inhibitory. To determine whether the PGs could reverse the inhibition caused by indomethacin, the stimulatory effect of PGs was also investigated. Individual PGs were added in the presence of EGF to cells that had been preincubated for 30 min with indomethacin (2×10^{-5} M). $PGF_{2\alpha}$, PGE_2, PGG_2, and PGH_2 all reversed the inhibitory effect of indomethacin on EGF-stimulated mitogenesis (Fig. 5B). $PGF_{2\alpha}$ was again the most potent metabolite and provided complete restoration of the indomethacin-inhibited response at 2×10^{-8} M. The concentration of PGE_2 required for complete restoration of the indomethacin-inhibited response was 10^{-6} M (Fig. 5B). Furthermore, when added with EGF to cells in which the mitogenic response was inhibited by ETYA (5×10^{-6} M) PGE_2 and $PGF_{2\alpha}$ completely restored the mitogenic response.[4]

Temporal relationship between mitogenesis and PGE_2 biosynthesis. To test the temporal relationship between PG formation and initiation of mitogenesis, indomethacin was added to BALB/c 3T3 cells at various times after stimulation by EGF. When an EC_{50} of indomethacin (1×10^{-5} M) was added prior to or simultaneously with addition of EGF the mitogenic response was significantly reduced (Fig. 6). However, delaying addition of indomethacin after the addition of EGF had little or no effect on EGF-induced mitogenesis. This is further evidence that arachidonic acid metabolites are involved in the early initiation events that result in the mitogenic response to EGF.

Discussion

The release of arachidonic acid and its subsequent metabolism is a necessary requirement for the mitogenic effect of EGF in BALB/c 3T3 cells. This hypothesis is supported by the following observations. First, EGF stimulates the release of arachidonic acid and subsequent PGE_2 formation at concentrations very similar to those required for the stimulation of [^{3}H] thymidine incorporation. Very similar dose-response relationships were observed for PGE_2 formation and mitogenesis. Second, the addition to the cells of inhibitors of either arachidonic acid release or arachidonic acid metabolism inhibited EGF-dependent mitogenesis. Again similar dose-response relationships between EGF-dependent mitogenesis and arachidonic acid metabolism as estimated by PGE_2 formation were observed. Third, the release of arachidonic acid and its metabolites is an early response to EGF. Inhibitors of arachidonic acid metabolism added after the cessation of arachidonic acid oxidation were ineffective in inhibiting DNA synthesis. Fourth,

[3] R. D. Nolan and R. M. Danilowicz, unpublished observations.
[4] R. D. Nolan and R. M. Danilowicz, unpublished observations.

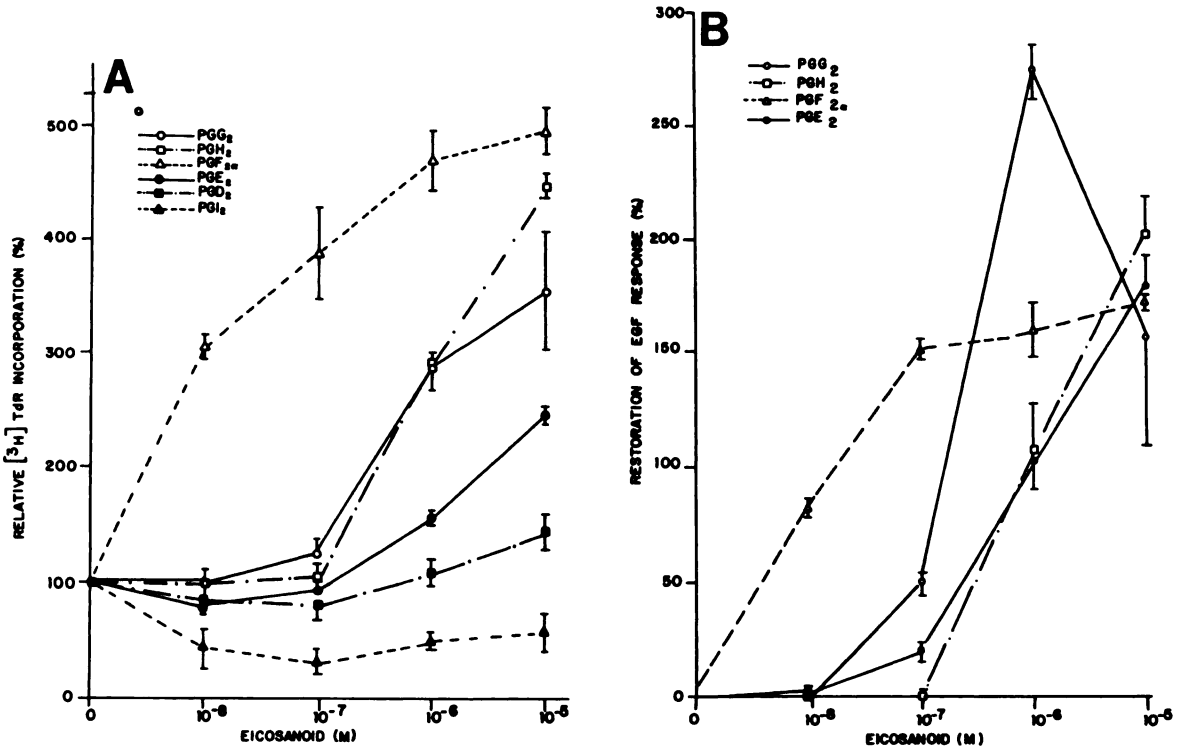

Fig. 5. Effects of eicosanoids on EGF-stimulated DNA synthesis in BALB/c3T3 cells. Cells were grown to near confluence in 96-well plates and were serum-depleted for 24 hr. Cells were then treated in two different ways. A, Eicosanoids and EGF (10 ng/ml) were simultaneously added to the cells in serum-free DMEM. DNA synthesis was measured by [^{3}H]thymidine incorporation after 24 hr. Data (mean ± standard error, five determinations) are expressed relative to stimulation by EGF alone (which is designated 100%). B, Cells were treated with indomethacin (2×10^{-5} M) for 15 min in serum-free DMEM before simultaneous addition of EGF (10 ng/ml) and eicosanoids. DNA synthesis was measured by [^{3}H]thymidine incorporation after 24 hr. Data (mean ± standard error, five determinations) are expressed as per cent restoration of the response to EGF alone. The response to EGF alone is 100% whereas the response to EGF with indomethacin is 0%. The data are representative of several different experiments.

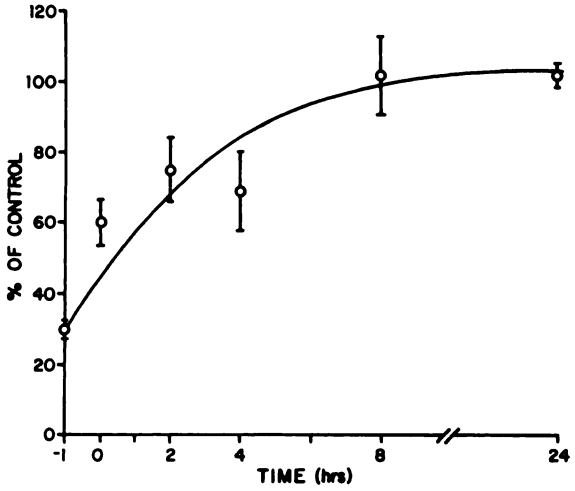

Fig. 6. The addition of indomethacin at various times during EGF stimulation of DNA synthesis. Cells were grown to near confluence in 96-well plates and were serum-depleted for 24 hr. EGF (10 ng/ml) was added at time 0. Indomethacin (1×10^{-5} M) was added to the cells at the indicated times before or after the addition of EGF. DNA synthesis was measured by [^{3}H]thymidine incorporation after 24 hr. Data (mean ± standard error, five determinations) are expressed as percentage of the control, which is EGF-stimulated DNA synthesis in the absence of indomethacin. Results are representative of several different experiments.

the addition of PGs, although at higher concentrations than released into the incubation medium by the cells, not only restored but potentiated indomethacin-inhibited EGF-dependent DNA synthesis. We think that these data strongly support the involvement of arachidonic acid metabolism in EGF-dependent mitogenesis in these cells.

Arachidonic acid, either exogenous or liberated from cellular phospholipid, is oxidized primarily via the cyclooxygenase pathway, although very minor uncharacterized metabolites do elute in the hydroxy-fatty acid region of the HPLC profile. The major stable metabolite is PGE_2, although some $PGF_{2\alpha}$ was observed, particularly in 24-hr incubations. No additional metabolites of arachidonic acid were observed in the 24-hr incubations. Hence, the formation of PGE_2 was used to estimate arachidonic acid metabolism and its detection implies the formation of the unstable endoperoxides PGG_2 and PGH_2. Metabolism of arachidonic acid is an early event, with maximum PGE_2 concentrations achieved within 4–6 hr, whereas DNA synthesis occurs and is measured 24 hr after the addition of EGF. The addition of arachidonic acid inhibitors during the initial phase subsequently leads to an inhibition of EGF-stimulated DNA synthesis.

The addition of a phospholipase A_2 inhibitor or pretreatment with dexamethasone, which induces lipocortin activity (24, 25), inhibits both mitogenesis and PG formation induced by EGF. The dose-response relationship between PGE_2 formation and DNA synthesis was similar for all inhibitors except indomethacin and ibuprofen. This suggests a possible involvement of

lipoxygenase products although these metabolites are not characterized and are produced in nearly undetectable levels. NDGA and ETYA, inhibitors of both lipoxygenase and cyclooxygenase, give very similar dose response relationships for both PGE_2 production and DNA synthesis. These data support the hypothesis that arachidonic acid metabolism is a requirement for EGF-stimulated mitogenesis in the cell.

In the absence of EGF, the PGs were very weakly mitogenic. However, exogenous PGs potentiated EGF-induced mitogenesis and not only restored but potentiated indomethacin-inhibited EGF-induced mitogenesis. $PGF_{2\alpha}$ was the most potent PG and exhibited a markedly different dose-response relationship than observed for other PGs. In this regard $PGF_{2\alpha}$ but not other PGs can stimulate phosphoinositide metabolism in Swiss 3T3 cells (27). Thus the difference in dose-response curves observed in this study may be related to $PGF_{2\alpha}$-specific stimulation of phosphoinositide metabolism.

This hypothesis is substantiated by the findings that $PGF_{2\alpha}$ is the most potent PG in stimulating the initiation of DNA synthesis and cell division in Swiss 3T3 cells (28) and that PGE_1 and PGE_2 act in synergism with $PGF_{2\alpha}$ in producing these responses (18). EGF-stimulated mitogenesis is not associated with the inositol cycle in BALB/c 3T3 cells (7, 8), as has been shown with PDGF (3, 9). However, recent data suggest EGF stimulates the formation of inositol phosphates in density-arrested BALB/c 3T3 pretreated with activators of adenylate cyclase (29). Also EGF and PDGF produce additive or synergistic mitogenic effects in many cells (5, 6). That the endoperoxides PGG_2 and PGH_2 were significantly more potent than PGE_2 in stimulating DNA synthesis may suggest that the endogeneously formed endoperoxides may be involved in EGF-stimulated mitogenesis. Higher concentrations of exogenous PGs, PGE_2, $PGF_{2\alpha}$, PGG_2, and PGH_2, were required to potentiate or restore EGF mitogenesis than were released into the incubation medium by these cells in response to EGF. This is somewhat perplexing, but PG formation usually occurs as a burst, suggesting a higher but short-lived intracellular concentration. Measurement of intracellular PG concentration is not possible due to the rapid movement of PG to the extracellular medium. There is little evidence to indicate active transport of PGs from the extracellular to the intracellular space, except notably in lung cells (30), indicating that high extracellular PG concentrations are required to reproduce the levels seen inside the cell after endogenous synthesis.

The data presented in this paper support a role for endogenous arachidonic acid metabolism in EGF-induced mitogenesis in BALB/c 3T3 cells. One can envision a model for EGF-induced mitogenesis as follows: EGF binds to its receptor, which by means of its protein tyrosine kinase activity phosphorylates the phospholipase A_2 inhibitor lipocortin (15). Phosphorylation inactivates lipocortin, phospholipase A_2 activity releases arachidonic acid, and arachidonic acid is oxidized to the endoperoxides and PG formation ensues. The biochemical events that subsequently lead to mitogenesis are unclear but the importance of arachidonic acid release and metabolism in these processes are supported by our findings as well as others (1).

One can speculate on eicosanoid formation and involvement in the actions of other mitogens. PDGF and fibroblast growth factors stimulate the release of arachidonic acid and subsequent metabolism in BALB/c 3T3 cells as well as other cells (9, 31, 32). In other studies, indomethacin and phenadone did not block PDGF-induced DNA synthesis (33). This suggests stimulation of DNA synthesis and the involvement of eicosanoids could depend, not only on the mitogen, but on the cells under examination. Extrapolation of dexamethasone effects on DNA synthesis to other fibroblasts must be carefully considered because dexamethasone and other glucocorticoids affect fibroblasts in many ways. Recently, α-adrenergic stimulation of cell replication in a rat thyroid cell line has been associated with PG formation (34). The present study, as far as we know, is the first to demonstrate the involvement of endogenously derived eicosanoids in induction of mitogenesis in BALB/c 3T3 fibroblasts by EGF. These results may be important in understanding the control of fibroblastic proliferation in wound healing and may provide an insight into the mechanism for oncogenic transformation. Certainly, some carcinomas have been shown to possess an elevated number of EGF receptors (35) and BALB/c 3T3 cells transformed by oncogenic DNA viruses exhibit increased rates of synthesis of PGE_1, PGE_2, and $PGF_{2\alpha}$ compared with their normal counterparts (19). These findings stress the importance of finding the connection between EGF, eicosanoids, and cell proliferation. Although other studies are clearly required to elucidate the mechanism involved in EGF-induced mitogenesis, we conclude that arachidonic acid metabolism is necessary but not sufficient for initiation of DNA replication in BALB/c 3T3 fibroblasts.

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References

1. Rozengurt, E. Early signals in the mitogenic response. *Science (Wash. DC)* **234**:161–166 (1986).
2. Pardee, A. B., R. Dubrow, J. L. Hamlin, and R. F. Kletzien. Animal cell cycle. *Annu. Rev. Biochem.* **47**:715–750 (1978).
3. Hokin, L. E. Receptors and phosphoinositide-generated second messengers. *Annu. Rev. Biochem.* **54**:205–235 (1985).
4. Carpenter, G., and S. Cohen. Epidermal growth factor. *Annu. Rev. Biochem.* **48**:193–216 (1979).
5. Stiles, C. D., G. T. Capone, C. D. Scher, H. N. Antoniades, J. J. Van Wyk, and W. J. Pledger. Dual control of cell growth by somatomedins and platelet-derived growth factor. *Proc. Natl. Acad. Sci. USA* **76**:1279–1283 (1979).
6. Leof, E. B., W. Wharton, J. J. Van Wyk, and W. J. Pledger. Epidermal growth factor (EGF) and somatomedin C regulate G1 progression in competent BALB/c-3T3 cells. *Exp. Cell Res.* **141**:107–115 (1982).
7. Besterman, J. M., S. P. Watson, and P. Cuatrecasas. Lack of association of epidermal growth factor-, insulin-, and serum-induced mitogenesis with stimulation of phosphoinositide degradation in BALB/c 3T3 fibroblasts. *J. Biol. Chem.* **261**:723–727 (1986).
8. Ran, W., M. Dean, R. A. Levine, C. Henkle, and J. Campisi. Induction of c-fos and c-myc mRNA by epidermal growth factor or calcium ionophore is cAMP dependent. *Proc. Natl. Acad. Sci. USA.* **83**:8216–8220 (1986).
9. Habenicht, A. J. R., J. A. Glomset, W. C. King, C. Nist, C. D. Mitchell, and R. Ross. Early changes in phosphatidylinositol and arachidonic acid metabolism in quiescent Swiss 3T3 cells stimulated to divide by platelet-derived growth factor. *J. Biol. Chem.* **356**:12329–12335 (1981).
10. Berridge, M. J. Inositol trisphosphate and diacylglycerol as second messengers. *Biochem. J.* **220**:345–360 (1984).
11. Nishizuka, Y. The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature (Lond.)* **308**:693–698 (1984).
12. Levine, L., and A. Hassid. Epidermal growth factor stimulates prostaglandin biosynthesis by canine kidney (MDCK) cells. *Biochem. Biophys. Res. Commun.* **76**:1181–1187 (1977).
13. Hirata, Y., M. Uchihashi, H. Nakashima, T. Fujita, and S. Matsukura. Stimulatory effect of epidermal growth factor on prostaglandin E_2 production in mouse fibrosarcoma cell line ($HSDM_1$ C_1). *Horm. Metab. Res.* **17**:448–450 (1985).
14. Lorenzo, J. A., J. Quinton, S. Sousa, and L. G. Raisz. Effects of DNA and prostaglandin synthesis inhibitors on the stimulation of bone resorption by epidermal growth factor in fetal rat long-bone cultures. *J. Clin. Invest.* **77**:1897–1902 (1986).
15. Pepinsky, R. B., and L. K. Sinclair. Epidermal growth factor-dependent phosphorylation of lipocortin. *Nature (Lond.)* **321**:81–84 (1986).
16. Jimenez de Asua, L., D. Clingan, and P. S. Rudland. Initiation of cell

proliferation in cultured mouse fibroblasts by prostaglandin $F_{2\alpha}$. *Proc. Natl. Acad. Sci. USA* **72**:2724–2728 (1975).
17. Johnson, G. S., and I. Pastan. Change in growth and morphology of fibroblasts by prostaglandins. *J. Natl. Cancer Inst.* **47**:1357–1364 (1971).
18. Otto, A. M., M. Nilsen-Hamilton, B. D. Boss, M.-O. Ulrich, and L. Jimenez de Asua. Prostaglandins E_1 and E_2 interact with prostaglandin $F_{2\alpha}$ to regulate initiation of DNA replication and cell division in Swiss 3T3 cells. *Proc. Natl. Acad. Sci. USA* **79**:4992–4996 (1982).
19. Hammarström, S. Prostaglandin production by normal and transformed 3T3 fibroblasts in cell culture. *Eur. J. Biochem.* **74**:7–12 (1977).
20. Tan, W. C., O. S. Privett, and M. E. Goldyne. Studies of prostaglandins in rat mammary tumors induced by 7,12-dimethylbenz(*a*)anthracene. *Cancer Res.* **34**:3229–3231 (1974).
21. Levine, L., P. M. Hinkle, E. F. Voelkel, and A. H. Tashjian, Jr. Prostaglandin production by mouse fibrosarcoma cells in culture: inhibition by indomethacin and aspirin. *Biochem. Biophys. Res. Commun.* **47**:888–896 (1972).
22. Henke, D. C., S. Kouzan, and T. E. Eling. Analysis of leukotrienes, prostaglandins, and other oxygenated metabolites of arachidonic acid by high-performance liquid chromatography. *Anal. Biochem.* **140**:87–94 (1984).
23. Flower, R. J., and G. J. Blackwell. The importance of phospholipase-A_2 in prostaglandin biosynthesis. *Biochem. Pharmacol.* **25**:285–291 (1976).
24. Hirata, F., E. Schiffman, K. Venkatasubramanian, D. Salomon, and J. Axelrod. A phospholipase A_2 inhibitory protein in rabbit neutrophils induced by glucocorticoids. *Proc. Natl. Acad. Sci. USA* **77**:2533–2536 (1980).
25. Blackwell, G. J., R. Carnuccio, M. DiRosa, R. J. Flower, L. Parente, and P. Persico. Macrocortin: a polypeptide causing the anti-phospholipase effect of glucocorticoids. *Nature (Lond.)* **287**:147–149 (1980).
26. Nolan, R. D., and T. E. Eling. Inhibition of prostacyclin synthesis in cultured bovine aortic endothelial cells by vitamin K_1. *Biochem. Pharmacol.* **35**:4273–4281 (1986).
27. MacPhee, C. H., A. H. Drummond, A. M. Otto, and L. Jimenez de Asua. Prostaglandin $F_{2\alpha}$ stimulates phosphatidylinositol turnover and increases the cellular content of 1,2-diacylglycerol in confluent resting Swiss 3T3 cells. *J. Cell. Physiol.* **119**:35–40 (1984).
28. Jimenez de Asua, L., A. M. Otto, J. A. Lindgren, and S. Hammarström. The stimulation of the initiation of DNA synthesis and cell division in Swiss mouse 3T3 cells by prostaglandin $F_{2\alpha}$ requires specific functional groups in the molecule. *J. Biol. Chem.* **258**:8774–8780 (1983).
29. Olashaw, N. E., and W. J. Pledger. Epidermal growth factor stimulates formation of inositol phosphates in BALB/c/3T3 cells pretreated with cholera toxin and isobutylmethylxanthine. *J. Biol. Chem.* **263**:1111–1114 (1988).
30. Anderson, M. W., and T. E. Eling. Prostaglandin removal and metabolism by the isolated perfused rat lung. *Prostaglandins* **11**:645–677 (1976).
31. Rozengurt, E., P. Stroobant, M. D. Waterfield, T. F. Deuel, and M. Keehan. Platelet-derived growth factor elicits cyclic AMP accumulation in Swiss 3T3 cells: role of prostaglandin production. *Cell* **34**:265–272 (1983).
32. Shier, W. T. Serum stimulation of phospholipase A_2 and prostaglandin release in 3T3 cells is associated with platelet-derived growth-promoting activity. *Proc. Natl. Acad. Sci. USA* **77**:137–141 (1980).
33. Shier, W. T., and J. P. Durkin. Role of stimulation of arachidonic acid release in the proliferative response of 3T3 mouse fibroblasts to platelet-derived growth factor. *J. Cell Physiol.* **112**:171–181 (1982).
34. Burch, R. M., A. Luini, D. E. Mais, D. Corda, J. Y. Vanderhoek, L. D. Kohn, and J. Axelrod. α_1-Adrenergic stimulation of arachidonic acid release and metabolism in a rat thyroid cell line. *J. Biol. Chem.* **261**:11236–11241 (1986).
35. Hendler, F. J., and B. W. Ozanne. Human squamous cell lung cancers express increased epidermal growth factor receptors. *J. Clin. Invest.* **74**:647–651 (1984).

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