Modulation of c-jun and jun-B Messenger RNA and Inhibition of DNA Synthesis by Prostaglandin E₂ in Syrian Hamster Embryo Cells

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ABSTRACT

Fatty acid metabolites such as prostaglandins are important regulators of DNA synthesis and cell proliferation. However, the mechanisms involved in this regulation are unclear. We have examined the effects of several fatty acid metabolites on the expression of the growth-related genes c-jun and jun-B in Syrian hamster embryo cells. Treatment of cells with prostaglandin E₂ (PGE₂) resulted in the inhibition of epidermal growth factor (EGF)-induced DNA synthesis and c-jun mRNA accumulation, whereas PGE₂ augmented EGF-stimulated jun-B mRNA and markedly stimulated jun-B accumulation in the absence of EGF. Treatment of cells with PGE₂ resulted in rapid accumulation of cyclic AMP (cAMP), whereas prostaglandin F₂α did not stimulate cAMP formation and did not alter EGF-stimulated DNA synthesis or accumulation of c-jun or jun-B mRNA. Forskolin and 8-(4-chlorophenylthio)-cAMP mimicked the effects of PGE₂ on DNA synthesis and on the expression of c-jun and jun-B, suggesting the involvement of cAMP. We have shown that EGF-induced DNA synthesis requires the formation of hydroxyeicosatetraenoic acids, formed from linoleic acid by a 15-lipoxygenase, in Syrian hamster embryo cells (Glasgow et al., J. Biol. Chem., 267: 10771-10779). Inhibition of this 15-lipoxygenase blocked EGF-dependent hydroxyeicosatetraenoic acid formation and mitogenesis but did not affect EGF-stimulated c-jun or jun-B mRNA accumulation. The data suggest that the modulation of EGF-dependent DNA synthesis by PGE₂ is associated with altered expression of c-jun and jun-B in Syrian hamster embryo cells. In contrast, hydroxyeicosatetraenoic acids appear to act downstream or divergent from c-jun and jun-B expression in the regulation of EGF-dependent DNA synthesis.

INTRODUCTION

Growth factors bind to receptors on the cell surface and initiate a series of biochemical changes within the cell which ultimately lead to DNA replication and cell division. Signaling events induced by mitogens such as EGF² include activation of the intrinsic protein tyrosine kinase activity of their receptors, with subsequent tyrosine phosphorylation of specific substrates (1), and the rapid induction of DNA-binding activity associated with various immediate-early gene products, including AP-1 transcription factor complexes (2, 3). AP-1 complexes are dimeric proteins composed of members of the jun and fos protooncogene families, which include c-jun, jun-B, and c-fos (4-6). These proteins mediate cellular responses to growth factors by binding to recognition sequences within the promoter/enhancer region of specific target genes, thereby regulating transcription (7). The cellular proto-oncogenes c-jun and c-fos share a high degree of sequence homology with the retroviral oncogenes v-jun and v-fos, respectively (8), and deregulated expression of c-jun or c-fos results in increased AP-1 activity and malignant transformation (9, 10). These observations emphasize the central importance of the AP-1 complex in the regulation of cell proliferation and the development of neoplasia.

Growth factors such as EGF and platelet-derived growth factor also induce the formation of prostaglandins in some cells. This can result from the release of arachidonic acid and from the expression of a mitogen-inducible form of the enzyme prostaglandin H synthase, which has been described as an immediate-early gene involved in the regulation of cell proliferation and differentiation (11). Prostaglandins can either inhibit (12-15) or augment (12-16-18) the mitogenic response to growth factors, depending on the type of cell and the specific prostaglandin to which the cells are exposed. Lipoxigenase-derived arachidonic acid metabolites are also involved in the mitogenic response to growth factors in some cells. Hydroxyeicosatetraenoic acids, metabolites of arachidonic acid formed by lipoxigenase enzymes, modulate cell proliferation in a variety of cell types (15, 18, 19). Fatty acid metabolites also regulate gene expression in some cells (17, 20, 21), suggesting the possibility that these compounds may be involved in mitogenic signaling by modulating the DNA-binding activity of transcriptional regulators such as AP-1.

Our laboratory has shown that prostaglandins are involved in the mitogenic response to EGF in BALB/c 3T3 cells (16, 17) and modulate levels of mRNA for the growth-related transcriptional regulator c-myc in these cells (17). We have also reported that EGF stimulates the formation of 9- and 13-HODEs, 15-lipoxygenase-derived metabolites of linoleic acid, in BALB/c 3T3 cells (22) and in SHE cells (23). Inhibition of 15-lipoxygenase activity blocks EGF-induced HODE formation and DNA synthesis, while the addition of HODEs greatly potentiates the mitogenic response to EGF, suggesting that the generation of HODEs is involved in mediating the proliferative response to EGF in these cells. In the present study, our objective was to determine whether prostaglandins and HODEs modulate EGF-dependent expression of the growth-related genes c-jun and jun-B. The results show differential effects of prostaglandin E₂ on c-jun and jun-B mRNA that correlate with the formation of cAMP. In contrast, EGF-stimulated HODE formation does not appear to be involved in the ability of EGF to regulate the level of mRNA for c-jun or jun-B.

MATERIALS AND METHODS

Materials. Prostaglandins and cAMP enzyme immunoassay kits were obtained from Cayman Chemical Co. (Ann Arbor, MI). Mouse natural epidermal growth factor was obtained from Collaborative Research (Bedford, MA). [32P]PdCTP was from Amersham (Arlington Heights, IL), and [3H]thymidine was from New England Nuclear (Boston, MA). Forskolin and 8-(4-chlorophenylthio)-cAMP were purchased from Sigma Chemical Co. (St. Louis, MO). cDNAs for mouse c-jun and jun-B were generously provided by Dr. Daniel Nathans of Johns Hopkins University (Baltimore, MD).

Cell Culture. The Syrian hamster embryo cell line 10WsuP8 clone 8 (24) was obtained from Dr. J. Carl Barrett (National Institute of Environmental Health Sciences). Stock cultures were grown in 75-cm² flasks.
flasks in IBR-modified Dulbecco’s medium containing 10% fetal calf serum, fungizone (1.25 μg/ml), and gentamicin (10 μg/ml) at 37°C in an atmosphere containing 5% CO₂. SHE cells were grown for 4 days to 60–80% confluence prior to serum deprivation. Cells were then made quiescent by washing and incubation for 18 h with IBR medium without serum (SFM).

**[3H]Thymidine Incorporation.** DNA synthesis was determined by measuring the incorporation of [3H]thymidine into SHE cell DNA. Serum-deprived SHE cells were washed with SFM and incubated in SFM with the agents indicated plus 1 μCi/well [3H]thymidine at 37°C for 24 h. Wells were then washed and treated with ice-cold 5% trichlor-acetic acid for 1 h. Wells were again washed and treated with 200 μl NaOH/0.1% sodium dodecyl sulfate for 30 min. Sample pH was neutralized with HCl, and [3H]thymidine incorporation into the acid-insoluble cell fraction was determined by liquid scintillation counting.

**Isolation of SHE Cell mRNA.** Following serum deprivation, quiescent SHE cells were washed with SFM and incubated with EGF in the absence or presence of prostaglandins, or other agents, in SFM at 37°C for various times. The cells were then washed twice with ice-cold phosphate-buffered saline, scraped from the dishes, and centrifuged in 10 ml of phosphate-buffered saline at 4°C. Subsequently, mRNA was isolated using oligo(dT) cellulose as described by Badley et al. (25). mRNA was quantified by measuring A₂₆₀ spectrophotometrically.

**Northern Analysis of c-jun and jun-B mRNA.** mRNA samples (750 ng) were subjected to electrophoresis in a 1% agarose gel containing 3% formaldehyde. mRNA was then transferred to a Nytran nitrocellulose membrane and cross-linked using a Stratalinker UV light source (Stratagene, Inc., La Jolla, CA). Membranes were washed with 1× standard saline citrate (20× standard saline citrate contained 3.0 M sodium chloride and 0.3 M sodium citrate) with 0.1% sodium dodecyl sulfate for 1 h at 65°C and then prehybridized in a solution containing 50% formamide, 5× standard saline-phosphate-EDTA (20× standard saline-phosphate-EDTA contained 3.0 M sodium chloride, 0.2 M sodium phosphate, and 20 mM EDTA), 5× Denhardt’s reagent (50× Denhardt’s contained 1% each of Ficoll, bovine serum albumin fraction 5, and polyvinylpyrrolidone), 100 μg/ml denatured DNA from salmon testes, and 0.1% sodium dodecyl sulfate for 2–4 h at 42°C. Hybridization with cDNA probes was then carried out in fresh prehybridization buffer overnight at 42°C. mRNA-cDNA hybrids were visualized by autoradiography and quantified by scanning densitometry.

Data were normalized by scanning autoradiograms of blots probed with glyceraldehyde 3-phosphate dehydrogenase cDNA (26) radiolabeled by random oligonucleotide priming using Klenow enzyme (Boehringer Mannheim, Indianapolis, IN) and with [32P]oligo(dT)₁₀, which hybridizes with total polyadenylated RNA on the blots (27). Hybridization of [32P]oligo(dT)₁₀ was linear with mRNA amounts from 0.25 to 2.0 μg (r = 0.99). Oligo(dT)₁₀ was radiolabeled using [γ-³²P]ATP and T4 polynucleotide kinase (Sigma). Normalized results were similar using either probe. A 0.24–9.5-kilobase RNA ladder (BRL Life Sciences, Gaithersburg, MD) was used for molecular weight markers and was visualized with ethidium bromide. Mouse c-jun and jun-B cDNA (2, 5), 2.6 kilobases and 1.8 kilobases, respectively, were isolated from the EcoRI site of the pGEM-2 plasmid by restriction digestion and agarose gel electrophoresis. cDNA probes were labeled with [³²P]dCTP by random oligonucleotide priming.

**Measurement of cAMP Formation.** Serum-deprived cells were washed with SFM and incubated with the indicated agents in 0.5 ml SFM at 37°C. The assay was stopped by the addition of 0.5 ml of methanol. Cells were then scraped from the wells, transferred to microfuge tubes, and lyophilized. Samples were then resuspended in water and immediately boiled for 5 min. Insoluble debris was pelleted by centrifugation and the supernatant was assayed for cAMP by enzyme immunoassay.

**RESULTS**

**EGF Stimulates c-jun and jun-B mRNA Accumulation.** The level of c-jun and jun-B mRNA was assessed by Northern analysis in quiescent SHE cells treated with or without EGF, cDNA probes for these genes detected major bands at 2.8 kilobases and 2.0 kilobases for c-jun and jun-B, respectively. Very low levels of c-jun and jun-B mRNA were detected in control cells treated with serum-free medium in the absence of EGF. However, treatment of cells with 10 ng/ml EGF resulted in marked accumulation of mRNA for c-jun (Figs. 1 and 24) and jun-B mRNA were detected in control cells treated with SFM only (A and C) or as a percentage of the maximal response to EGF in the absence of other agents (B and D). Results from autoradiograms were quantified by scanning densitometry, normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and calculated as a percentage of mRNA in control cells treated with SFM only (A and C) or as a percentage of the maximal response to EGF in the absence of other agents (B and D). Values are mean ± SEM (n = 3). Autoradiograms from an experiment typical of those from which these data were obtained are shown in Fig. 1.

**Fig. 1.** Northern blots showing effects of PGE₂, PGF₂α, forskolin, and cpcAMP on EGF-stimulated accumulation of c-jun and jun-B mRNA. Quiescent cells were treated with either SFM (control), 100 nM PGE₂, 100 nM PGF₂α, 10 μM forskolin, or 100 μM cpcAMP or with 10 ng/ml EGF in the absence or presence of these agents for 60 min at 37°C prior to the isolation and analysis of mRNA. The autoradiogram depicts an experiment typical of those from which the data in Fig. 2 were obtained. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

**Fig. 2.** Quantification of effects of PGE₂, PGF₂α, forskolin, and cpcAMP on EGF-stimulated accumulation of c-jun and jun-B mRNA. Quiescent cells were treated as described in the legend to Fig. 1 prior to the isolation and analysis of mRNA for c-jun (A and B) and jun-B (C and D). Results from autoradiograms were quantified by scanning densitometry, normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and calculated as a percentage of mRNA in control cells treated with SFM only (A and C) or as a percentage of the maximal response to EGF in the absence of other agents (B and D). Values are mean ± SEM (n = 3). Autoradiograms from an experiment typical of those from which these data were obtained are shown in Fig. 1.
The addition of EGF, followed by a return towards basal levels (data not shown).

Prostaglandin E₂ Differentially Modulates c-jun and jun-B mRNA Levels. Simultaneous incubation of SHE cells with EGF plus PGE₂ resulted in a 75% inhibition of EGF-induced c-jun mRNA accumulation (Figs. 1 and 2B) as well as a reduction in the basal level of c-jun mRNA (Figs. 1 and 2C). These effects were specific for PGE₂, insofar as PGF₂α had comparatively little effect on c-jun mRNA levels in the absence or presence of EGF (Figs. 1 and 2A, A and B). Furthermore, PGE₂ had no effect on basal levels of c-fos mRNA or on EGF-stimulated c-fos mRNA accumulation (data not shown), suggesting that the inhibitory effects of PGE₂ on c-jun mRNA accumulation are not due to changes in EGF receptor number, affinity for EGF, or tyrosine kinase activity. In marked contrast to the results obtained for c-jun, PGE₂ induced a substantial increase in jun-B mRNA in the absence of EGF (Figs. 1 and 2C). PGE₂ also induced a small but consistent increase in EGF-stimulated jun-B mRNA accumulation (Figs. 1 and 2D). PGF₂α did not substantially alter jun-B mRNA levels in the absence or presence of EGF, as compared with the effects of PGE₂ (Fig. 1 and 2, C and D).

Prostaglandin E₂ Inhibits EGF-dependent DNA Synthesis. Quiescent SHE cells exposed to 10 ng/ml EGF for 24 h exhibited marked stimulation of DNA synthesis that was approximately 10-fold over basal (Fig. 3). EGF-stimulated DNA synthesis was inhibited by nearly 90% by coincubation with 100 nM PGE₂. This inhibitory response was specific for PGE₂ to the extent that PGF₂α did not alter the mitogenic response to EGF. There was a close correlation between the dose of PGE₂ required to block EGF-dependent DNA synthesis and the dose required to inhibit c-jun mRNA accumulation (Fig. 4). The 50% inhibitory concentration values for both effects were approximately 2 nM.

Effects of PGE₂ Correlate with cAMP Accumulation. cAMP levels were determined in SHE cells treated with either PGE₂ or PGF₂α, in the absence or presence of EGF. The results obtained demonstrated that 100 nM PGE₂ induced a nearly 4-fold increase in cAMP accumulation in SHE cells within 10 min (Fig. 5). Marked accumulation was also observed as early as 2 min following addition of PGE₂ to cells (data not shown). Thus, PGE₂ stimulates rapid formation of cAMP that precedes EGF-induced expression of c-jun and jun-B. In contrast to PGE₂, PGF₂α failed to induce cAMP formation. The presence of EGF did not alter the results obtained with either prostaglandin.

The differential effects of PGE₂ on c-jun and jun-B mRNA were mimicked by forskolin and ctp-cAMP, agents that directly activate the cAMP-generating enzyme adenyl cyclase and stimulate cAMP-dependent protein kinase, respectively. These agents decreased the basal level of c-jun and inhibited EGF-dependent c-jun mRNA accumulation (Figs. 1 and 2A, A and B), whereas they markedly stimulated the accumulation of jun-B mRNA in the absence of EGF and enhanced slightly EGF-stimulated jun-B mRNA accumulation (Figs. 1 and 2, C and D). Forskolin and ctp-cAMP also mimicked the inhibitory effect of PGE₂ on EGF-dependent DNA synthesis (Fig. 3).

Inhibition of 15-Lipoxygenase Activity Inhibits EGF-stimulated DNA Synthesis but Does Not Alter Accumulation of c-jun and jun-B mRNA. We have reported that inhibition of lipoxygenase activity blocks EGF-dependent HODE formation and DNA synthesis and that HODEs potentiate the mitogenic response to EGF, in SHE cells (23). These results suggest that...
HODEs formed rapidly (within 5 min) in response to EGF are involved in the ability of EGF to stimulate DNA synthesis in these cells. Therefore, we used ETYA, an inhibitor of 15-lipoxygenase, to determine whether EGF-dependent HODE formation is also involved in regulating the level of c-jun and jun-B mRNA. Incubation of cells with EGF in the presence of 5 μM ETYA, a concentration that blocks the formation of HODEs in response to EGF (23), resulted in an 81% inhibition of EGF-dependent DNA synthesis (Fig. 6A). However, ETYA did not alter the ability of EGF to stimulate the accumulation of mRNA for c-jun or jun-B (Fig. 6B).

**DISCUSSION**

The ability of prostaglandins either to stimulate or to inhibit the mitogenic response to growth factors has been documented in a variety of cell types (12–18). The mitogenic response to growth factors is also modulated by other arachidonic acid metabolites such as hydroxyeicosatetraenoic acids (15, 18, 19) and by linoleic acid metabolites such as HODEs (22, 23). However, less is known about the biochemical mechanisms involved in the regulation of cell proliferation by fatty acid metabolites. Our hypothesis is that fatty acid metabolites may regulate the mitogenic response to growth factors by modulating the expression of specific growth-related genes. The results of this study show that the inhibition by PGE2 of EGF-dependent DNA synthesis is associated with differential regulation of mRNA for c-jun and jun-B, both of which may serve as components of the growth-related transcription factor complex AP-1 (4, 5). In contrast, the results suggest that, although HODE formation is involved in EGF-stimulated DNA synthesis in SHE cells (23), HODE formation is not involved in the ability of EGF to stimulate the accumulation of c-jun or jun-B mRNA.

There was good correlation between DNA synthesis and the expression of c-jun under several conditions. First, c-jun mRNA levels were low in untreated quiescent cells and increased rapidly in cells treated with a mitogenic dose of EGF. Second, PGE2 inhibited both EGF-dependent DNA synthesis and EGF-stimulated c-jun mRNA accumulation in a dose-dependent manner with nearly identical 50% inhibitory concentrations. Third, both EGF-dependent DNA synthesis and c-jun mRNA accumulation were inhibited by forskolin and cpt-cAMP. Finally, exposure of cells to PGF2α did not alter the mitogenic response to EGF or the ability of EGF to stimulate c-jun mRNA accumulation. In contrast, the level of DNA synthesis could not be strictly correlated with jun-B mRNA levels, which were elevated in response to mitogenic doses of EGF but which increased further in response to doses of PGE2 that blocked EGF-dependent DNA synthesis. The differential regulation of c-jun and jun-B mRNA and inhibition of EGF-dependent DNA synthesis by PGE2 correlate with increased levels of cAMP in SHE cells. Exposure of SHE cells to PGE2 resulted in the rapid accumulation of cAMP, which preceded EGF-induced expression of c-jun and jun-B, whereas PGF2α did not increase intracellular cAMP concentration, did not alter the accumulation of c-jun or jun-B mRNA, and did not inhibit EGF-stimulated DNA synthesis. Both forskolin and the cAMP analog cpt-cAMP mimicked the regulatory effects of PGE2 on c-jun and jun-B expression and the inhibitory effect of PGE2 on DNA synthesis. Thus, there is good correlation between the elevation of intracellular cAMP concentration, the differential regulation of mRNA for c-jun and jun-B, and the inhibition of DNA synthesis.

Our findings are similar to results from other cell lines in which elevation of the intracellular cAMP concentration results in enhanced jun-B expression and inhibition of phorbol ester-stimulated c-jun expression (28, 29). In contrast, PGE2 increased c-jun expression in HL-60 cells (30), and 8-bromo-cAMP had a similar stimulatory effect on c-jun expression in rat parotid acinar cells and in the rat submandibular cell line RSMT-A5 (31). The mechanisms responsible for these cell-specific differences in the effects of cAMP on c-jun expression are unclear but may relate to the effect of cAMP on jun-B expression. Chiu et al. (28) have demonstrated that, although phorbol esters induce the expression of both c-jun and jun-B in NIH 3T3 cells, forskolin augments jun-B expression and jun-B in turn negatively regulates phorbol ester-induced expression of c-jun in these cells. While it is unclear whether enhanced expression of jun-B in response to PGE2 is responsible for the inhibitory effect of PGE2 on c-jun expression in SHE cells, our data suggest this possibility.

We have shown that EGF stimulates the formation of HODEs in SHE cells, that inhibition of 15-lipoxygenase activity blocks HODE formation and DNA synthesis in response to EGF, and that HODEs potentiate the mitogenic response to EGF in these cells (23). These results indicate that EGF-induced formation of HODEs is involved in the mitogenic

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3 W. C. Glasgow, National Institute of Environmental Health Sciences, Research Triangle Park, NC, personal communication.
response to EGF in SHE cells. Since marked HODE formation occurs rapidly and before detectable expression of c-jun and jun-B, we hypothesized that HODEs may act by modulating these genes. However, incubation of SHE cells with a concentration of ETYA that abolishes EGF-induced HODE formation does not modulate the expression of c-jun or jun-B in SHE cells. Thus, HODEs appear to act at a point in the EGF signaling pathway that either is downstream from the stimulation of c-jun and jun-B expression or diverges upstream from the expression of these genes.

Our laboratory has shown that prostaglandins and HODEs are important regulators of cell growth in both BALB/c 3T3 cells (16, 17, 22) and SHE cells (23). In SHE cells, PGE2 differentially regulates the expression of c-jun and jun-B as described here. These studies provide important insights into the biochemical mechanisms by which prostaglandins may regulate DNA synthesis and cell proliferation, particularly since the genes for c-jun and jun-B encode nuclear-binding proteins that regulate the transcription of a variety of growth-related and regulatory genes. One intriguing possibility is the regulation of the gene encoding prostaglandin H synthase, the rate-limiting enzyme in prostaglandin biosynthesis, which contains several AP-1 binding sites in the 5'-flanking regulatory domain (32).

Thus, in some cells prostaglandins may modulate prostaglandin H synthase gene expression by altering the DNA-binding activity of AP-1 transcription factor complexes, thereby regulating arachidonic acid metabolism and cell proliferation.

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