Arachidonic Acid Increases c-fos and Egr-1 mRNA in 3T3 Fibroblasts by Formation of Prostaglandin E₂ and Activation of Protein Kinase C*

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Arachidonic acid is one of the major polyunsaturated acids present in the mammalian cell membrane. Its rapid clearance from the cytosol by enzymatic metabolism or reesterification to phospholipids makes arachidonic acid an attractive candidate for regulation of signal transduction from the membrane to the nucleus.

As an example, arachidonic acid regulates ion channels (6, 7) and modulates the activity of enzymes and proteins such as protein kinase A (8), protein kinase C (9), NADPH oxidase (10), GTPase-activating protein (11), and the platelet-derived growth factor receptor (12). In addition, arachidonic acid has also been shown to be involved in the regulation of gene expression. The fatty acid synthase gene (13) as well as the hepatic S14 gene (14) and the gene coding for stearoyl-CoA desaturase (15) are transcriptionally repressed by polyunsaturated fatty acids including arachidonic acid. Based on data obtained with chimeric gene constructs (13, 14) an arachidonic acid response element in the promoter region of these genes has been postulated. However, neither an arachidonic acid-response element nor an arachidonic acid-regulated transacting DNA-binding protein has been identified.

Since arachidonic acid is metabolized by different pathways (16) characterized by the enzymes lipoxygenase, cyclooxygenase, and cytochrome P-450 epoxygenase, gene expression may also be modulated by arachidonic acid metabolites rather than directly by arachidonic acid.

In fact, arachidonic acid metabolites have been shown to regulate gene expression in various cell types. For instance, in rat aortic smooth muscle cells H₂O₂ increased c-fos mRNA accumulation (17) and c-jun gene transcription (18). Both effects could be prevented by nordihydroguaiaretic acid, an inhibitor of lipoxygenase and cytochrome P-450 epoxygenase, pointing to a metabolite of arachidonic acid in the regulation of c-fos and c-jun. More direct, the addition of leukotriene B₄ to human monocytes was shown to increase c-fos gene transcription (19).

In Tα1 adipocytes, 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid induced c-fos mRNA (20), and PGE₂ increased junB mRNA in Syrian hamster embryo fibroblasts (21). However, not much is known about the molecular mechanisms involved. In a few reports, well characterized signal transduction pathways and second messenger systems operating via cAMP (22), protein kinase C (17), or protein tyrosine kinases (23) have been implied to mediate the effects of arachidonic acid metabolites on gene expression.

In order to further our understanding of how arachidonic acid or its metabolites regulate gene expression and to identify the underlying mechanisms, we analyzed mRNA accumulation

Growth and development of animal cells are controlled and regulated by various growth factors. Binding of growth factors to their corresponding receptors elicits numerous physiological effects, including the release of arachidonic acid from the S,2 position of membrane phospholipids (1, 2), presumably by activation of phospholipase A₂(3).

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The abbreviations used are: PG, prostaglandin; MOPS, 4-morpholinepropane-sulfonic acid; HPLC, high performance liquid chromatography.

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of two growth-associated immediate early genes, c-fos and Egr-1, in their response to arachidonic acid in Swiss 3T3 cells. We show that arachidonic acid increases mRNA levels of both genes. This induction is due to the synthesis of the arachidonic acid metabolite PGE2 and the subsequent activation of protein kinase C.

EXPERIMENTAL PROCEDURES

Materials—Prostaglandins, calphostin C, and chelerythrine were purchased from Biomed (Hamburg, Federal Republic of Germany). Iloprost was a gift from Schering AG (Berlin, FRG). [3H]Arachidonic acid (58 mCi/mmol), [α-32P]PCTP (800 Ci/mmol), and the cAMP kit were obtained from Amersham (Braunschweig, FRG). SP6 RNA polymerase, RNasin, and RNase-free DNase I were from Promega (Heidelberg, FRG). Arachidonic acid, arachidonoyl alcohol, arachidonic acid methyl ester, eicosatetraynoic acid, 4-bromophenacyl bromide, A23187, phorbol 12-myristate 13-acetate, forskolin, dibutyril cAMP, lipoxxygenase, and cylooxygenase inhibitors as well as cell culture medium and serum were from Sigma (München, FRG).

Cell Culture—Swiss 3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 2 mM l-glutamine at 37 °C and 5% CO2. Cells were maintained at high density in a humidified atmosphere of 5% CO2. Individual experiments were done with 24-h-old confluent cultures, grown for 4 days without change of medium in order to age and deplete the serum of growth factors. Cells were then washed with phosphate-buffered saline and incubated for an additional 6 h in serum-free medium. Serum-free medium was then replaced by an incubation buffer containing 20 mM Hepes, 120 mM NaCl, 2.7 mM KCl, 1 mM CaCl2, 1.4 mM KH2PO4, 25 mM NaHCO3, 0.7 mM MgSO4·7H2O, 10 mM glucose, pH 7.4, and stimuli and/or inhibitors were added. Stimulation was for 30 min, and inhibitors were added 30 min prior to stimulation. Each experiment was done separately at least three times, and the results were found to be consistent.

RNA Isolation—Swiss 3T3 cells from a confluent 100-mm tissue culture dish were washed with phosphate-buffered saline and lysed in 2 ml of 7.5 M guanidine HCl, 25 mM sodium citrate, and 0.1% Na-laurylsarcosine, pH 5.2. The lysed cell mix was passed three times through a 27-gauge needle in order to shear chromosomal DNA. The RNA was selectively precipitated with 0.5 volume of ethanol at −20 °C. Following a centrifugation at 10,000 rpm (Kontron 148.24), the RNA pellet was dissolved in 300 μl of 50 mM Tris-HCl, pH 7.5, 25 mM NaCl, 5 mM EDTA, 0.1% SDS, 0.3 M sodium acetate, pH 5.0. The RNA solution was extracted once with phenol followed by one ether extraction and was finally precipitated with 2.5 volumes of ethanol.

cAMP Assay—Cells were seeded into 24-well dishes, grown, and treated as described under “Cell Culture.” Cell extraction was done by adding 2 volume of 30% hot EDTA (400 μl) to each well (200 μl of incubation buffer). The cell extract was then transferred to a 1.5-ml micro-test tube, cleared from cell debris by centrifugation, and used for the determination of cAMP levels. The assay was performed as specified by the supplier ([125I]-cAMP assay system, nonacetylation protocol, Amersham Corp.).

RESULTS

Effect of Arachidonic Acid on c-fos and Egr-1 mRNA Accumulation—In order to assess the ability of arachidonic acid to stimulate expression of immediate early genes, we analyzed c-fos and Egr-1 mRNA levels by RNA blotting. As Fig. 1A demonstrates, arachidonic acid strongly induces c-fos as well as Egr-1 mRNA levels in a dose-dependent manner. However, the induction as well as dose dependence is more pronounced in the case of the c-fos mRNA as compared with Egr-1 mRNA.

Next, we analyzed if the stimulatory effect of arachidonic acid on c-fos and Egr-1 mRNA accumulation was arachidonic acid-specific or rather caused by nonspecific effects. Substances structurally related to arachidonic acid, arachidonic acid methyl ester, arachidonoyl alcohol, and eicosatetraynoic acid in concentrations up to 50 μM do not induce mRNA levels of c-fos and Egr-1 (Fig. 1B), indicating an arachidonic acid-specific (e.g. free unesterified arachidonic acid) stimulation of c-fos and Egr-1 mRNA accumulation.

In order to verify the physiological relevance of arachidonic acid and to demonstrate that endogenously generated arachidonic acid elicits the same effect on c-fos and Egr-1 mRNA levels as exogenously added arachidonic acid, we stimulated Swiss 3T3 cells for 30 min with the calcium ionophore A23187 to activate phospholipase A2 and to release arachidonic acid and extracted twice by the addition of an equal volume of chloroform. Samples were then dried under vacuum and resuspended prior to analysis in acetonitrile/water (32:68), pH 4.0. Reverse phase HPLC was performed using a Waters 600 multisolvent delivery system with a Beckman Ultrasphere ODS C18 column (4.6 mm × 25 cm). Eicosanoids were detected by UV absorption using a Kontron Uvikon 720 LC spectrophotometer and by monitoring radioactivity with a Berthold HPLC radioactivity monitor LB 506 C. Arachidonic acid metabolites were eluted with acetonitrile/water, pH 4.0, using a discontinuous gradient from 32% acetonitrile to 100% acetonitrile as described (25). Substances were identified by their retention times as well as by coelution of standards and normalized based on total recovery of radioactivity/sample.
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Fig. 2. Effect of various enzyme inhibitors of arachidonic acid metabolism on arachidonic acid-induced c-fos and Egr-1 mRNA levels. A, Swiss 3T3 cells were treated for 30 min with 0.1, 1, or 10 μM ketocozamole (Keto.), indomethacin (Indo.), caffeic acid (Caff.), and nordihydroguaiaretic acid (NDGA) and then stimulated for an additional 30 min with 10 μM arachidonic acid. As a positive control, cells were stimulated with 10 μM arachidonic acid (AA) alone. B, cells were treated as in A with various doses (μM) of the cyclooxygenase inhibitors acetylsalicylic acid (ASA), ibuprofen (Ibup.), piroxicam (Piro.), and naproxen (Napr.). Analysis of mRNA was done as described in the legend to Fig. 1. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

from membrane stores (26). It is evident from Fig. 1C that 10 μM calcium ionophore A23187 leads to a marked increase in c-fos and Egr-1 mRNA levels. 4-Bromophenacyl bromide, an inhibitor of phospholipase A2, abolishes the A23187-induced mRNA accumulation, supporting the notion that arachidonic acid released by the action of phospholipase A2 is responsible for the increase in c-fos and Egr-1 mRNA.

Inhibition of Cyclooxygenase Abolishes Arachidonic Acid-induced c-fos and Egr-1 mRNA Accumulation—Arachidonic acid is enzymatically converted to a number of eicosanoids with diverse biological properties (16, 27). It is possible that not arachidonic acid but rather one of its metabolites regulates the accumulation of immediate early gene mRNAs. To address this question, we determined the effect of various inhibitors of lipooxygenase, cyclooxygenase, and cytochrome P-450 epoxygenase on arachidonic acid-induced mRNA accumulation of c-fos and Egr-1. Pretreatment of cells with the lipooxygenase and cytochrome P-450 epoxygenase inhibitors nordihydroguaiaretic acid, caffeic acid, and ketoconazole at concentrations ranging from 0.1 to 10 μM does not significantly decrease the arachidonic acid-stimulated levels of c-fos and Egr-1 mRNA (Fig. 2A), thus ruling out the role of a lipooxygenase and cytochrome P-450 epoxygenase metabolite in the regulation of c-fos and Egr-1 mRNA accumulation. In contrast, pretreatment with 100 nM indomethacin, and inhibitor of cyclooxygenase, abolishes both c-fos and Egr-1 mRNA levels after arachidonic acid stimulation. In a dose-dependent manner, the same was found for the cyclooxygenase inhibitors ibuprofen, piroxicam, naproxen, and acetylsalicylic acid (Fig. 2B). These results strongly indicate a cyclooxygenase metabolite rather than arachidonic acid per se as the stimulatory agent in the activation of c-fos and Egr-1 mRNA levels.

PGE2 Stimulates c-fos and Egr-1 mRNA Levels—Given the inhibition of indomethacin and other cyclooxygenase inhibitors on arachidonic acid-induced c-fos and Egr-1 mRNA accumulation (Fig. 2), we tested if a prostaglandin could elicit the same stimulatory effect as arachidonic acid on c-fos and Egr-1 mRNA levels. In fact, treating Swiss 3T3 cells for 30 min with 10 μM PGE2 results in a strong induction of c-fos and EGR-1 mRNA levels (Fig. 3A).

To further prove that PGE2 mediates the effect of arachidonic acid on c-fos and Egr-1 mRNA accumulation, we tested if PGE2 could overcome the inhibition of indomethacin on arachidonic acid-induced mRNA levels. Swiss 3T3 cells were preincubated with 100 nM indomethacin followed by the addition of 10 μM arachidonic acid and 10 μM PGE2. As Fig. 3B demonstrates, PGE2 reverses the inhibitory effect of indomethacin. Since the stimulatory effect of PGE2 on c-fos and Egr-1 mRNA levels was not inhibited by indomethacin, the possibility is ruled out that indomethacin affects the cell unspecifically by mechanisms unrelated to the inhibition of the enzyme cyclooxygenase.

To determine that Swiss 3T3 cells are able to synthesize PGE2 from arachidonic acid, cells were stimulate with 10 μM AA (1 μCi of [14C]arachidonic acid supplemented with 6.5 μM unlabeled arachidonic acid). After 20 min, eicosanoids were extracted and analyzed by reverse phase HPLC. Apart from arachidonic acid only one prominent peak can be detected (Fig. 4A). This peak coelutes with a nonradioactive PGE2 standard, demonstrating PGE2 synthesis in Swiss 3T3 cells. Preincubation of cells with 100 nM indomethacin totally blocked synthesis of PGE2 (Fig. 4B). These results support our notion that endogenously synthesized PGE2 mediates arachidonic acid-induced c-fos and Egr-1 mRNA accumulation.

PGE2 Does Not Stimulate c-fos and Egr-1 mRNA Accumulation via cAMP and Activation of Protein Kinase A—PGE2 is reported to increase intracellular cAMP levels in various cells by stimulation of adenyl cyclase (28, 29). We tested if an increase in cAMP is responsible for the stimulation of c-fos and Egr-1 mRNA accumulation by treating Swiss 3T3 cells with forskolin, iloprost, and dibutylryl cAMP (Fig. 5), agents known...
to increase cellular cAMP levels. None of these substances at concentrations ranging from 10 to 100 μM is able to stimulate c-fos or Egr-1 mRNA levels in a manner comparable with arachidonic acid or PGE₂, suggesting that c-fos and Egr-1 cannot significantly be induced by cAMP in Swiss 3T3 cells. Furthermore, generation of cAMP is only observed after stimulation with forskolin but not with PGE₂ or arachidonic acid (Table I). This observation strongly suggests that PGE₂ stimulated c-fos and Egr-1 mRNA accumulation either directly or by a second messenger system unrelated to adenyl cyclase activation.

PGE₂ Activates c-fos and Egr-1 mRNA Accumulation through the Activation of Protein Kinase C—Since it is known that PGE₂ can activate protein kinase C by a PGE₂ receptor subtype coupled to phospholipase C (29, 30), the role of protein kinase C in PGE₂-induced mRNA accumulation of c-fos and Egr-1 was determined. Down-regulation of protein kinase C with 800 nM phorbol 12-myristate 13-acetate for 16 h greatly diminishes the stimulatory effect of 10 μM arachidonic acid and 5 μM PGE₂ on c-fos and Egr-1 mRNA accumulation (Fig. 6A). The same is observed when Swiss 3T3 cells were preincubated for 30 min with the specific protein kinase C inhibitors chelerythrine (20 μM) and calphostin C (0.5 μM) prior to arachidonic acid or PGE₂ stimulation (Fig. 6B). These results suggest that PGE₂ stimulates c-fos and Egr-1 mRNA levels by activating protein kinase C.

**DISCUSSION**

Arachidonic acid has recently been identified as a novel second messenger regulating ion channels (6, 7), enzyme activity (8–11), and cell growth (4, 31). Arachidonic acid as well as its metabolites has also been implicated in the regulation of gene expression either directly (19, 21, 23) or as mediators of various stimuli including growth factors, tumor necrosis factor, phorbol 12-myristate 13-acetate, and H₂O₂ (17, 20, 25). However, the underlying mechanism of action remains elusive.

To understand the role of arachidonic acid in the regulation of gene expression, we studied two growth-related immediate early genes, c-fos and Egr-1. Both genes are responsive to arachidonic acid stimulation as evidenced by the marked increase of c-fos and Egr-1 mRNA levels following incubation of Swiss 3T3 cells with exogenous arachidonic acid or with the calcium ionophore A23187, which predominantly releases arachidonic acid from endogenous phospholipid pools. In support of this observation the phospholipase A₂ inhibitor 4-bromophenacyl bromide reduces A23187-stimulated increases in mRNA accumulation. Lack of compounds structurally similar to arachidonic acid, such as arachidonic acid methyl ester, arachidonyl alcohol, and eicosatetraynoic acid, to induce c-fos and Egr-1 mRNA levels, even at concentrations up to 50 μM, supports the notion of arachidonic acid as a specific stimulus.

Further analysis of the molecular mechanism of arachidonic acid-induced increases in mRNA levels of c-fos and Egr-1 revealed that in our strain of Swiss 3T3 fibroblasts endogenous metabolism of arachidonic acid to PGE₂ and subsequent activation of protein kinase C is required. This observation parallels recent reports indicating that unesterified arachidonic acid itself but its enzymatically produced metabolites modulate...
Egr-1
Acrtate.

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menes of PGE2 and PGF2, in 3T3 fibrohlasts. In contrast to 
Swiss 3T3 fibroblasts. In conclusion, our data demonstrate that in 
Swiss 3T3 cells as inhibitors of the respective 
receptors are coupled to adeny1 cyclasr, while thr  suhtypr 
activate the expression of c-fos. This further confirms the importance of endogenous 
eicosanoid formation in the regulation of gene expression and 
eicosanoids may act either as autocrine 
and paracrine me-
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Fig. 6. Effect of protein kinase C down-regulation on c-fos and 
Egr-1 mRNA levels following stimulation with various agents. A. Swiss 3T3 cells were stimulated with 10 µM arachidonic acid (AA), 5 µM PGE2, or 8 µM phorbol 12-myristate 13-acetate (PMA) either alone or following a 16-h preincubation with 800 nM phorbol 12-myristate 13-acetate. B. cells were stimulated with 10 µM arachidonic acid, 10 µM PGE2, or 8 µM phorbol 12-myristate 13-acetate either alone or following a 30-min preincubation with either 0.5 µM calphostin C (Caphl) or 20 µM 
cherythrine (Chel). Analysis of mRNA was as described in the legend to Fig. 1. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

gene expression. However, in contrast to findings in rat aortic smooth muscle cells (17), mesangial cells (25), or adipogenic TA1 cells (20), our results clearly demonstrate that cyclooxygenase activity is required to mediate the effect of arachidonic acid on mRNA accumulation. 1) Indomethacin and other cyclo-
clooxygenase inhibitors abolish the effect of arachidonic acid; 2) inhibition by indomethacin is completely reversed by addition of 
PGE2; and 3) exogenously added arachidonic acid is sponta-
neously metabolized to a prominent compound that coeluted on reverse phase HPLC with authentic PGE2. Lipoxigenase or cytochrome P-450-derived eicosanoids are unlikely to regulate mRNA levels in Swiss 3T3 cells as inhibitors of the respective pathways do not affect mRNA accumulation, and cells treated with [14C]arachidonic acid synthesize only minute amounts of eicosanoids coeluting with non-cyclooxygenase metabolites. Even though PGE2 is by far the predominant eicosanoid pro-
duced in 3T3 fibroblasts stimulated with arachidonic acid alone, it is possible that other cyclooxygenase or even non-
cyclooxygenase metabolites, synthesized at very low levels, might also contribute to the mRNA accumulation of c-fos and 
Egr-1. In fact, we observed that 3T3 fibroblasts, prelabeled for 16 h with [14C]arachidonic acid and stimulated with the 
calcium ionophore A23187, synthesize metabolites coeluting with 
PGE2, PGD2, 12-hydroxyheptadecatrienoic acid, and hydroxye-
cosatetraenoic acid (data not shown). This may explain why inhibition of cyclooxygenase does not reduce c-fos mRNA lev-
als to the same degree as c-fos mRNA indicating that Egr-1 mRNA levels might also be responding to non-cyclooxygenase metabolites.

Together with other reports it becomes obvious that arachi-
donic acid and its metabolites seem to be involved in gene 
regulatory events caused by many different stimuli in a wide 
range of cell types and that gene expression can be regulated by 
different classes of endogenously synthesized eicosanoids (17– 
23, 32, 33). It is therefore possible that the eicosanoids involved are characteristic for a given cell type. The observation that in various fibroblast cell lines prostaglandins seem to modulate early gene expression of c-fos, c-jun, and c-myc (21, 22, 34) points in this direction, even though the mode of action is unclear and incongruous.

Apart from demonstrating a role of arachidonic acid and 
arachidonic acid metabolites in the regulation of various genes, 
not much is known about the underlying mechanism. In 
general, eicosanoids may act either as autocrine or paracrine me-
diators by activation of specific membrane receptors or by di-
rect intracellular interaction with various signaling systems. 
Considering an autocrine or paracrine effect of PGE2, two 
different second messenger systems are activated following bind-
ing of PGE2 to different receptor subtypes; the EP2 and EP3 receptors are coupled to adenyl cyclase, while the subtype EP1 stimulates phospholipase C, inositol phosphate formation, and subsequently activation of protein kinase C (30). Given our results, activation of adenyl cyclase by PGE2 and accumulation of c-fos and Egr-1 mRNA by CAMP are very unlikely since PGE2 does not increase CAMP levels significantly. In parallel, mRNA levels are almost unaffected by agents known to stimu-
late CAMP formation although there is a slight increase in 
Egr-1 mRNA levels following forskolin, iloprost, and dibutyryl 
CAMP stimulation of Swiss 3T3 fibroblasts.

In contrast, mRNA accumulation proved to be dependent on protein kinase C activation. Both down-regulation of protein 
kinese C by prolonged incubation with phorbol 12-myristate 13-acetate as well as inhibition by calphostin C and cherythrine abolish the stimulatory effect of arachidonic acid and PGE2 on c-fos and Egr-1 mRNAs. Although we did not characterize 
PGE2 receptors on Swiss 3T3 fibroblasts, our findings are compatible with PGE2 binding to the EP1 or even the PGE2a recep-
tor FF, since all these receptor types are recognized by PGE2 and coupled to phospholipase C (30). Similarly Kacich et al. (22) 
reported that arachidonic acid induced c-fos gene expression in 
Swiss 3T3 fibroblasts. In contrast to our results, c-fos was also 
induced by 8-bromo-cAMP, forskolin, and PGE2 in their system 
of Swiss 3T3 cells depleted of protein kinase C. This led the authors to the conclusion that arachidonic acid and PGE2, acti-
ated c-fos through elevation of the second messenger cAMP; 
however, no data were presented that actually showed an in-
crease in cAMP levels following arachidonic acid or PGE2, treat-
ment of Swiss 3T3 cells. As neither arachidonic acid nor PGF2 
was tested as a stimulus in protein kinase C-depleted cells, one 
cannot exclude the possibility that even in their cells protein 
kinese C is involved in arachidonic acid and PGE2 stimulation 
of c-fos. In our system an effect of CAMP is clearly excluded, and 
 furthermore, the addition of PGE2 does not increase mRNA 
levels of c-fos and Egr-1 (data not shown).

A further example for the involvement of prostaglandin syn-
thesis in the stimulation of immediate early gene expression in 
prostaglandins was reported by Handler et al. (34). The addition 
of PGG2 and PGF2a to BALB/c 3T3 fibroblasts led to a stimulation 
of c-myc mRNA expression (34). Although the authors did not 
address the activation of the downstream signaling pathways, 
a role of protein kinase C in the expression of c-myc in BALB/c 
3T3 fibroblasts can be assumed since the PGF2a receptor is 
directly coupled to phospholipase C and inositol lipid metabo-
(30, 35).

In conclusion, our data demonstrate that in Swiss 3T3 fibro-
blasts arachidonic acid regulates c-fos and Egr-1 mRNA levels 
via conversion to its cyclooxygenase metabolite PGE2, and sub-
sequent activation of protein kinase C, possibly involving activi-
tion of a prostaglandin receptor subtype coupled to phosphi-
lipase C. This further confirms the importance of endogenous 
eicosanoid formation in the regulation of gene expression and 
indicates that known second messenger systems may ultimate-
ly transduce the eicosanoid signal.

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