Actions of Lipoxins A_4 and B_4 on Signal Transduction Events in Friend Erythroleukemia Cells (43495)

B. S. BECKMAN,[1] B. P. DESPINASSE, AND L. SPRIGGS
Department of Pharmacology, Tulane University, School of Medicine, New Orleans, Louisiana 70112

Abstract. **Earlier studies in our laboratory suggested a role for 15-lipoxygenase products of arachidonic acid, such as 15-hydroperoxyeicosatetraenoic acid and 15-hydroxyeicosatetraenoic acid, in supporting proliferative events in Friend erythroleukemia cells. Because lipoxins are also products of the same lipoxygenase enzyme, we tested their actions on signal transduction events related to DNA synthesis. Lipoxins A_4 and B_4 (10 n*M*) significantly enhanced [^{3}H]thymidine incorporation into Friend cells in the absence of fetal bovine serum without affecting cell differentiation or cell number. Lipoxin B_4 increased the duration of time that cells spent in the S phase of the cell cycle, and also significantly enhanced protein kinase C activity in nuclei, whereas c-*fos* expression was unaffected by either of the lipoxins tested. The novel, intracellular actions of lipoxins A and B on Friend erythroleukemia cells documented in this study represent a unique spectrum of effects of lipoxins on signal transduction events as compared with other eicosanoids.**
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Oxygenation products of arachidonic acid are known to exert a variety of biological effects in many different tissues. Specific roles for lipoxygenase products of arachidonate have been identified in both normal myeloid and erythroid cells (1–4) as well as in transformed hematopoietic cells. Recent evidence obtained in transformed erythroid cells (5) suggests a role for the activation of 15-lipoxygenase in supporting proliferation. In the present study, we evaluated the effects of two members of a relatively new series of oxygenated derivatives of arachidonic acid that can be formed by interactions between the 5- and 15-lipoxygenase or between the 5- and 12-lipoxygenase pathways (6, 7). Compounds of this series contain a conjugated tetraene and are called lipoxins (8). The addition of these products to human neutrophils or natural killer cells produced selective responses different from those observed with leukotrienes. Cell types known to synthesize these products include porcine leukocytes, human bone marrow cells (9) and reticulocytes, as well as macrophages from the rainbow trout (10). Most of the biological effects of lipoxins, to date, have proven to be stereospecific. In this study, lipoxins A and B were found to stimulate nuclear protein kinase C activity and entry of Friend erythroleukemia cells into the S phase of the cell cycle without affecting c-*fos* expression or proliferation, in contrast to the endogenous product of 15-lipoxygenase in these cells, 15-hydroperoxyeicosatetraenoic acid, which affects c-*fos* expression but has no apparent effect on protein kinase C activity.

Materials and Methods

Lipoxins A_4 and B_4 were purchased from Biomol Research Laboratories (Plymouth Meeting, PA). Hexamethylenebisacetamide and 3,3′-diaminobenzidine were obtained from Sigma Chemical Co. (St. Louis, MO). All culture reagents were obtained from Gibco BRL (Grand Island, NY). All enzymes, as well as the lambda *Hin*dIII/ϕ174 *Hae* III DNA marker and the 0.24–9.50-kb RNA ladder, were purchased from BRL Life Technologies, Inc. (Gaithersburg, MD).

Preparation of Cell Suspensions. Friend erythroleukemia cells (FELC), clone DS19-10, were kindly provided by Dr. Shigeru Sassa, Rockefeller University (New York, NY). Cells were cultured in Eagle's mini-

[1] To whom requests for reprints should be addressed at Department of Pharmacology, Tulane University School of Medicine, 1430 Tulane Avenue, New Orleans, LA 70112.

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mal essential medium containing Earle's salts supplemented with fetal bovine serum (5%), penicillin (100 units/ml), streptomycin (100 μg/ml), nonessential amino acids, and vitamins. Cells were maintained at 37°C with 5% CO_2 in humidified air. Cultures were diluted twice weekly to maintain the cells in a logarithmic phase of growth. The stationary or plateau phase of growth was achieved by 96 hr of culture in the absence of any media change. The fetal bovine serum consisted of a single lot of serum that had been tested for the ability to support growth and hexamethylenebisacetamide-induced differentiation of FELC (as determined by staining with 3,3′-diaminobenzidine). Cell doubling time was 11 hr.

[^{3}H]Thymidine Assay. Suspensions of cells in stationary phase were counted and diluted to a final concentration of 5×10^4 cells/ml in a total volume of 10 ml of minimal essential medium with or without serum. To each well of a 96-well microtiter plate were added 100 μl of cells to 100 μl of lipoxin solution with or without serum. Lipoxins were prepared as a stock solution (0.1 m*M*) in ethanol and additional dilutions were made in minimal essential medium. The 0.1% ethanol vehicle did not affect cell viability. The incubation period was 24 hr at 37°C. At 20 hr, cells were pulsed with [^{3}H]thymidine (1 μCi/well) and allowed to incubate for another 4 hr before they were harvested onto glass-fiber filter mats with a Skatron automatic cell harvester.

Nuclear Protein Kinase C Assay. Activity was determined using an *in vitro* assay in which the phosphorylation of endogenous substrate was measured (11). At room temperature, purified nuclei (10^6/100 μl) were preincubated for 15 min with 50 μl of ATP (50 μ*M*), 50 μl of $CaCl_2$ (17.5 m*M*), and 25 μl of either incubation buffer or 1-(5-isoquinoline sulfonyl)-2-methylpiperazine (H-7) (60 μ*M*). Following a 1-min incubation period at 30°C, 25 μl of incubation buffer containing agonist and 0.6 μCi of [γ-^{32}P]ATP were added to each tube. Final concentrations were: ATP, 10 μ*M*; $CaCl_2$, 3.5 m*M*; and H-7, 6 μ*M*. At the end of an additional incubation period of 3 min, the reaction was terminated by adding 3 ml of ice-cold trichloroacetic acid (10%) to each tube. The tubes were placed on ice for 30 min, after which time the trichloroacetic acid precipitates were collected onto a GF/C filter with a Brandel cell harvester. The filters were dried and counted in a liquid scintillation counter. Protein kinase C activity was determined to be the difference in the amount (pmol) of PO_4 incorporated into endogenous substrate in the absence and presence of H-7, an inhibitor of protein kinase C.

Proliferation Studies. Log phase cultures (10^6 cells/ml) were transferred to serum-free medium and inoculated with lipoxin B_4 (10 n*M*). An equal volume of serum-free medium alone was added to control cultures. All cultures were incubated for 24 hr at 37°C in 5% CO_2. The cell number (viable cells/ml) of each culture was determined by hemacytometer count before (0 hr) and after (24 hr) treatment. Cell viability at each time point was >90%, as assessed by trypan blue dye exclusion.

Flow Cytometry. Log phase FELC were treated with or without lipoxin B_4 (10 n*M*) for 24 hr in serum-free medium and were then subjected to flow cytometry analysis according to the manufacturer's protocol (Coulter EPICS profile analyzer).

Northern Blot Analysis and Quantitation of c-*fos* mRNA. Total RNA from 10^7–10^8 cells was isolated with RNAzol (Cinna/Biotecx Laboratories International, Inc., Friendswood, TX) before and after treatment with either lipoxin B_4 or serum-free medium. Total RNA (20 μg) was electrophoresed and blotted onto a NitroPlus (MSI) filter and probed for c-*fos* mRNA using a 2.0-kb ^{32}P-labeled c-*fos* cDNA probe. The same filter was also probed with a rat β-actin cDNA probe to ensure equal loading. The levels of c-*fos* mRNA were determined by densitometric scanning of the autoradiographs.

Statistical Analyses. Experiments were performed three to four times with duplicate determinations, and results are expressed as means ± SE. *P*-values of <0.05 derived from the unpaired Student's *t* test were considered significant.

Results

Proliferation and Differentiation of FELC in Response to Added Lipoxins. Both lipoxins A_4 and B_4 significantly enhanced [^{3}H]thymidine incorporation in the absence of fetal bovine serum, but not in its presence (Fig. 1). The maximal effect was seen at 10 n*M* for both lipoxins. At this concentration, the increase compared with control values was approximately 3-fold in three experiments and the dose-response curve was biphasic. This same concentration range of lipoxins did not affect differentiation, as measured by benzidine staining for hemoglobin (data not shown).

Cell growth was also determined after treatment of FELC with lipoxin B_4. As shown in Figure 2, no significant difference in cell number was observed in 24 hr in the absence of added serum as compared with untreated cells.

In view of the discrepancy seen in [^{3}H]thymidine incorporation data as compared with actual proliferation as measured by cell number, we next utilized flow cytometry to determine whether lipoxins could affect the cell cycle. Cells were treated with lipoxin B_4 (10 n*M*) for 24 hr in serum-free medium. As shown in Figure 3 and in Table I, the percentage of cells in S phase of the cell cycle increased when the cells were treated with lipoxin B_4 as compared with untreated cells.

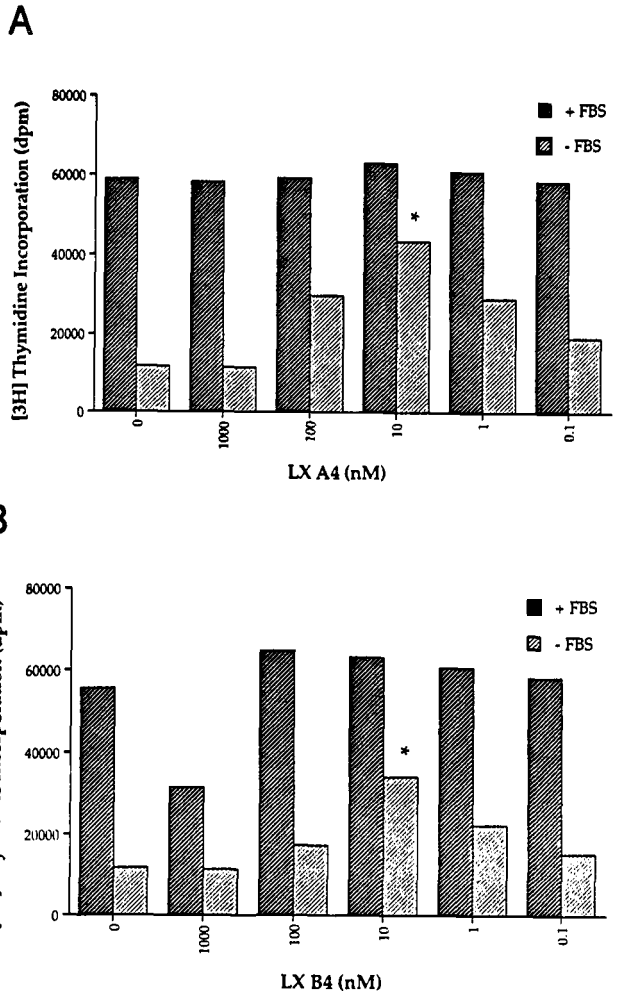

Figure 1. Effect of (A) lipoxin A and (B) lipoxin B on tritiated thymidine incorporation into Friend erythroleukemia cells. Cell suspensions in stationary phase were diluted to a final concentration of 5×10^4 cells/ml in a total volume of 10 ml of minimal essential medium plus or minus fetal bovine serum (FBS). Lipoxins A or B were added (0–1000 n*M*) for 24 hr at 37°C and cells were pulsed for 4 hr with tritiated thymidine (1 μCi/well). SE were not greater than 10% between replicates at each point, and the asterisk represents significance at $P < 0.05$. The experiment was repeated three times with six replicates per point.

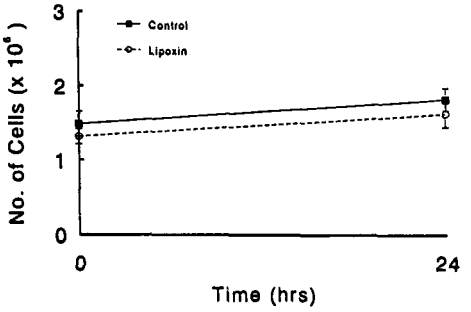

Figure 2. Effect of lipoxin B on cell number. Log phase cultures were transferred to serum-free medium and inoculated with lipoxin B_4 (10 n*M*). An equal volume of serum-free medium alone was added to control cultures. After 24 hr, cell number was determined. Viability was greater than 90%.

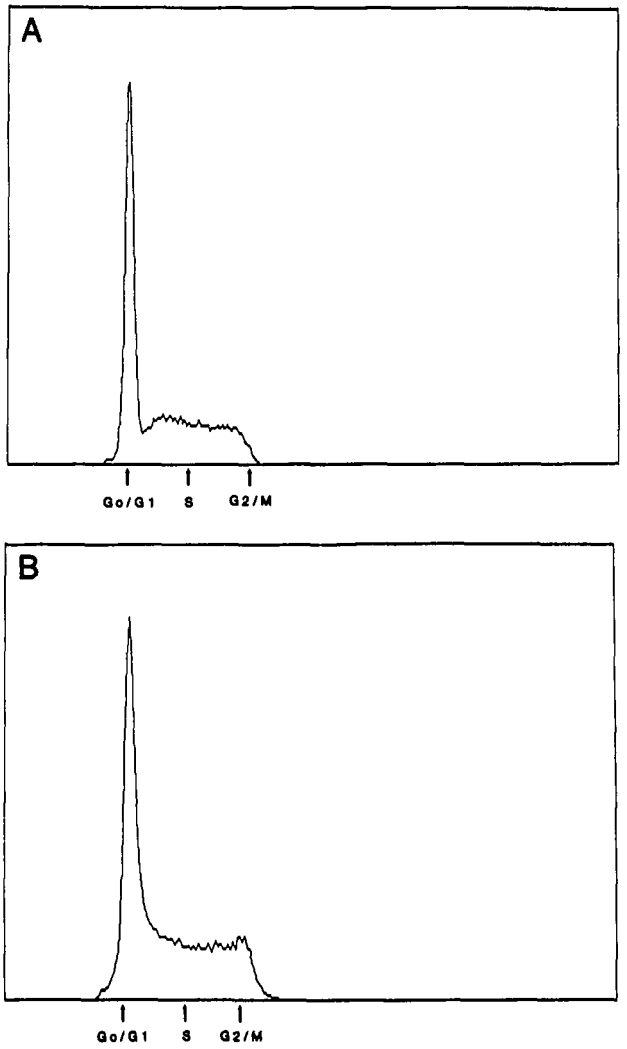

Figure 3. Effect of lipoxin B on cell cycle progression in serum-starved Friend erythroleukemia cells. Log phase FELC were treated (A) without or (B) with lipoxin B_4 for 24 hr in serum-free medium. The cells were then subjected to flow cytometric analysis for cell cycle according to the manufacturer's protocol (Coulter EPICS profile analyzer).

Table I. Flow Cytometric Analysis of Friend Erythroleukemia Cells

	%Go/G1		S		G2/M	
	%	Cell *n*	%	Cell *n*	%	Cell *n*
Control	50	7,844	47	7,311	3	398
Lipoxin B_4	44	8,965	52	10,575	4	904

Effects of Lipoxins A_4 and B_4 on Protein Kinase C in Isolated Nuclei. Earlier studies in our laboratory and others (11–13) have indicated an important role for protein kinase C activation in the signal transduction events associated with proliferation of erythroid cells in response to growth factors such as erythropoietin. Since lipoxins have been shown to activate protein kinase C in an *in vitro* assay (14), it was of interest to

Table II. Effects of Lipoxins A and B on Protein Kinase C Activation in Isolated Nuclei of Friend Erythroleukemia Cells

Treatment	pmol $PO_4/10^6$ nuclei/3 min[a]
Control	0.324 ± 0.106
Lipoxin A	
10^{-8} *M*	0.502 ± 0.225
10^{-9} *M*	1.146 ± 0.120[b]
10^{-10} *M*	0.716 ± 0.108[b]
Lipoxin B	
10^{-8} *M*	0.398 ± 0.211
10^{-9} *M*	2.442 ± 0.534[b]
10^{-10} *M*	1.366 ± 0.108[b]

[a] Values shown are mean ± SE from three replicate experiments.
[b] $P < 0.05$.

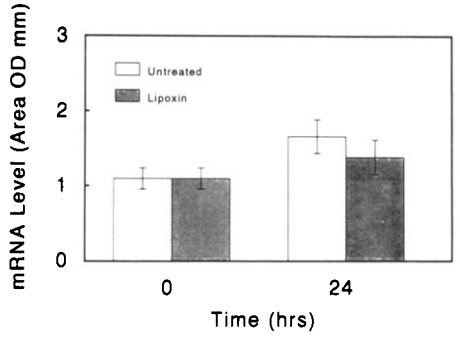

Figure 4. Effect of lipoxin B_4 on c-*fos* gene expression. Log phase FELC were incubated for 24 hr with and without lipoxin B_4 (10 n*M*) in serum-free medium. Total RNA was extracted and 20 μg of RNA was electrophoresed, blotted, and hybridized with a ^{32}P-labeled c-*fos* cDNA probe. Messenger RNA levels were determined by densitometric scanning of the autoradiographs and are expressed as the area under the curve ($n = 5$).

determine whether or not lipoxins could activate protein kinase C in the nuclei of FELC. Activity was determined in the presence or absence of a selective inhibition of protein kinase C, H-7. Table II illustrates the effects of lipoxins A_4 and B_4 on protein kinase C activity in the concentration range of 10^{-10}–10^{-8} *M*. A biphasic response was seen with significant increases in activity measured at 10^{-9} and 10^{-10} *M*. Added 12-hydroxyeicosatetraenoic acid, 15-hydroxyeicosatetraenoic acid, and 15-hydroperoxyeicosatetraenoic acid had no significant effect on protein kinase C activity in these cells (data not shown).

Effect of Lipoxin B_4 on c-*fos* Gene Expression. Since the expression of the proto-oncogene c-*fos* is closely linked to proliferation in Friend erythroleukemia cells (15), it was of interest to determine whether or not treatment of FELC with lipoxins affected c-*fos* messenger RNA expression. As shown in Figure 4, c-*fos* expression did not change in response to treatment with lipoxin B_4 (10 n*M*). Messenger RNA is constitutively expressed in these cells. As shown in Figure 3 and Table I, the percentage of cells in S phase of the cell cycle increased when the cells were treated with lipoxin B_4 as compared with untreated cells.

Conclusions

The findings from this study support the hypothesis that lipoxins activate selective signal transduction events within target cells. One of the first biochemical effects reported to occur in response to lipoxins was an activation of protein kinase C (14). One of the most notable effects seen in this study was the significant activation of protein kinase C in nuclei. Normal erythroid progenitor cell proliferation and differentiation in response to the growth factor, erythropoietin, also results in an increase in nuclear protein kinase C activation (11). Changes in protein kinase C activity have also been reported in Friend erythroleukemia cells (16, 17). The β-II isoform was shown to be the predominant isoform, and its expression increased during differentiation induced by hexamethylenebisacetamide accompanied by a shift from a predominantly nuclear localization to a cytoplasmic localization. One of the earliest nuclear responses of quiescent cells exposed to growth factors is a rapid and transient increase in c-*fos* gene expression (18). Since c-*fos* expression has been linked to protein kinase C in a variety of studies (19, 20), it was anticipated that c-*fos* expression might also change in response to treatment of FELC with lipoxins. Since there was no change in proliferation or in c-*fos* expression, these findings suggest that although protein kinase C activity is affected, there is a dissociation between this event and proliferation in these cells. The transformation process itself may affect the coupling of signal transduction events in Friend erythroleukemia cells, and thereby obscure the relationship between biochemical events and their role in proliferation. For example, in many normal cell types, there is no c-*fos* expression until a growth factor stimulates quiescent cells to proliferate, whereas c-*fos* expression is constitutive in Friend erythroleukemia cells (15). Several growth factors, including platelet-derived growth factor and epidermal growth factor, are known to stimulate c-*fos* expression within 5 min with a peak of expression at 30 min. The transcriptional regulation of the c-*fos* gene depends on several upstream enhancer elements that bind sequence-specific protein factors that consequently control gene transcription. The serum response element is required for induction of the c-*fos* gene by serum, epidermal growth factor, platelet-derived growth factor, or insulin. The 12-*O*-tetradecanoylphorbol-13-acetate-responsive element, also known as the AP-1 consensus sequence, is required for c-*fos* induction by phorbol esters, presumably acting through a protein kinase C-dependent pathway, whereas the cAMP-responsive element controls the transcription of c-*fos* induced by cAMP. It has been demonstrated in 3T3 fibroblasts that c-*fos* transcription in response to bom-

besin or epidermal growth factor can be achieved through protein kinase C-independent pathways. In addition, high levels of c-*fos* mRNA expression are not obligatory for the mitogenic response. *fos*-Related proteins such as *fos* B or *fra* may substitute for c-*fos*. The regulation of *fos* gene expression is a complex process that relies on subtle interaction between distinct second messenger systems.

The recent finding (9) that human bone marrow cells produce lipoxins A and B, and that these compounds stimulate granulocyte-macrophage colony-stimulating factor-induced proliferation of myeloid progenitor cells at the same concentrations effective in the Friend erythroleukemia cells, supports a generalized role for lipoxins in hematopoietic cell physiology. In addition, a human megakaryocyte cell line can generate lipoxins via the 12-lipoxygenase enzyme and the intermediate, leukotriene A_4 (6).

A generalized biological role for lipoxins in mediating signal transduction events in hematopoietic cells is beginning to emerge.

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