RESEARCH COMMUNICATION

Leukotriene B₄ stimulates *c-fos* and *c-jun* gene transcription and AP-1 binding activity in human monocytes

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We have examined the effect of leukotriene B₄ (LTB₄), a potent lipid proinflammatory mediator, on the expression of the proto-oncogenes *c-jun* and *c-fos*. In addition, we looked at the modulation of nuclear factors binding specifically to the AP-1 element after LTB₄ stimulation. LTB₄ increased the expression of the *c-fos* gene in a time- and concentration-dependent manner. The *c-jun* mRNA, which is constitutively expressed in human peripheral-blood monocytes at relatively high levels, was also slightly augmented by LTB₄, although to a much lower extent than *c-fos*. The kinetics of expression of the two genes were also slightly different, with *c-fos* mRNA reaching a peak at 15 min after stimulation and *c-jun* at 30 min. Both messages rapidly declined thereafter. Stability of the *c-fos* and *c-jun* mRNA was not affected by LTB₄, as assessed after actinomycin D treatment. Nuclear transcription studies *in vitro* showed that LTB₄ increased the transcription of the *c-fos* gene 7-fold and the *c-jun* gene 1.4-fold. Resting monocytes contained nuclear factors binding to the AP-1 element, but stimulation of monocytes with LTB₄ induced greater AP-1-binding activity of nuclear proteins. These results indicate that LTB₄ may regulate the production of different cytokines by modulating the yield and/or the function of transcription factors such as AP-1-binding proto-oncogene products.

INTRODUCTION

Leukotriene B₄ (LTB₄) is a potent biologically active lipid mediator which exerts numerous proinflammatory and immunoregulatory activities [1–6]. Its production by polymorphonuclear and mononuclear leucocytes can be triggered by a variety of stimuli (reviewed in [2]), and the cells which produce it can also be targets of its bioactions. In monocytes, exogenous LTB₄ was shown to stimulate H₂O₂ production as well as cytotoxicity against tumour cells [7,8]. Moreover, LTB₄ could stimulate the production of cytokines such as tumour necrosis factor α [8] and interleukin 6 [9]. Endogenous 5-lipoxygenase metabolites, including LTB₄, may also be involved in mediating signals for cytokine production stimulated by other ligands, such as platelet-activating factor [10].

The activation of several cytokine genes, including those of tumour necrosis factor α and interleukin-6, can be mediated by nuclear protein *trans*-acting factors which bind to *cis*-acting DNA elements of gene promoters, such as AP-1 [11–13]. AP-1-binding factors include the heterodimeric complex of FOS and JUN proteins [14]. These proteins are products of the proto-oncogenes *c-fos* and *c-jun* respectively, which become transcriptionally active within minutes after extracellular signals and regulate the expression of genes containing AP-1-binding sites [15–18]. Thus extracellular signals and their receptors can be linked to eventual cytokine production by a cascade of events which may include transcriptional activation of *c-fos* and *c-jun*, production of FOS–JUN proteins and binding to AP-1-containing cytokine genes, which would then trigger transcriptional activation of these genes and stimulation of production of the cytokines.

In the present study we demonstrate that the lipid mediator

LTB₄ can indeed stimulate the transcriptional activation of *c-fos* and *c-jun* and we suggest that this may constitute a mechanism through which it can affect transcriptional regulation of AP-1-containing target genes.

Materials and methods

Reagents

LTB₄ was generously provided by Dr. A. Ford-Hutchinson (Merck-Frosst, Pointe-Claire, Canada). LTB₄ ω -oxidation metabolites 20-OH-LTB₄ and 20-COOH-LTB₄ were generously provided by Dr. P. Poubelle (Laval University, Ste.-Foy, Canada).

Preparation of monocytes

Human venous blood from healthy medication-free volunteers was collected on citrate/dextrose/adenine. Undiluted blood was layered over a Ficoll–Paque (Pharmacia, Baie d’Urfe, QC, Canada) cushion and centrifuged at 800 *g* for 30 min by the method of Böyum [19]. Monocytes were then purified by adherence to the surface of plastic Petri dishes previously coated with baby-hamster-kidney-cell micro-exudate. This was effective in enriching the adherent population to > 90% monocytes as assessed by non-specific esterase staining. This population was removed with EDTA (0.01 M) in RPMI 1640 medium and resuspended in RPMI 1640 medium/5% fetal-bovine serum (Flow, McLean, VA, U.S.A.). The cells were then ‘rested’ overnight in polypropylene tubes (15 ml; Becton–Dickinson Labware, Lincoln Park, NJ, U.S.A.). Since LTB₄ was dissolved in methanol before dilution in culture medium, control cultures contained methanol in equivalent maximal concentration, which never exceeded 0.01% (v/v) and had no effect in the assays used in this study.

Fig. 1. Effect of LTB_4 on *c-fos* and *c-jun* mRNA expression in human monocytes

Northern-blot analysis was performed on 10 μg of total RNA. (a) Monocytes were stimulated for 30 min with (1) 0.003 % (v/v) methanol (vehicle for LTB_4), (2) 10^{-12} M- LTB_4 , (3) 10^{-10} M- LTB_4 , (4) 10^{-8} M- LTB_4 and (5) 10^{-6} M- LTB_4 . (b) Monocytes were stimulated with vehicle (lanes 1, 3 and 5) or LTB_4 at 10^{-8} M (lanes 2, 4 and 6) for different time periods (lanes 1 and 2 for 15 min, lanes 3 and 4 for 30 min and lanes 5 and 6 for 60 min). (c) Monocytes were used directly after overnight incubation (lane 1) or incubated for 30 min without (lane 2) or with (lane 3) LTB_4 at 10^{-6} M. In (b) and (c) the membranes were hybridized with *c-fos*, then stripped of the probe and rehybridized with *c-jun*-specific probe. The membranes were exposed for 20 h at -80°C .

mRNA studies

After appropriate treatment, cells were pelleted in 15 ml polypropylene tubes (Sarstedt, St.-Laurent, QC, Canada) and total cellular RNA was isolated by acid guanidium thiocyanate/phenol/chloroform extraction as described by Chomczynski & Sacchi [20]. RNA was separated by electrophoresis on 1 % agarose and transferred on to a Hybond-N (Amersham, Oakville, Ont., Canada) membrane for Northern analysis. The cDNA probes used were a 2.7 kb *Xho*I/*Stu* insert of *c-fos* [American Type Culture Collection (A.T.C.C.)] and a 0.9 kb *Pst* insert of *c-jun* (generously provided by Dr. C. Asselin of this University). The probes were labelled with multiprime DNA-labelling system (Amersham International), using [α - ^{32}P]dCTP (specific radioactivity > 3000 Ci/mmol; Amersham).

Membranes were prehybridized for 4 h in a mixture containing Tris (120 mM), NaCl (600 mM), EDTA (8 mM), sodium pyrophosphate (0.1 %), SDS (0.2 %) and heparin (100 $\mu\text{g}/\text{ml}$); hybridization was carried out overnight at 68°C in the same mixture, but the concentration of heparin was increased to 625 $\mu\text{g}/\text{ml}$ and dextran sulphate (10 %) was added [21]. The membranes were then washed once at room temperature for 20 min in $2 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15 M-NaCl/0.015 M-sodium citrate, pH 7), once with $0.1 \times \text{SSC}/0.1\%$ SDS at 68°C for 60 min, then rinsed at room temperature with $0.1 \times \text{SSC}$. The membranes were exposed to Kodak XAR-5 film with intensifying screens at -80°C . Signal intensity was quantified by densitometry with a Pharmacia LKB Ultrascan XL instrument (Pharmacia, Baie d'Urfe, Canada). The membranes were then stripped of the probe by washing for 60 min with $0.02 \times \text{SSC}/0.2\%$ SDS at 90°C and rehybridized.

In order to assess stability of *c-fos* and *c-jun* mRNA, cells were treated with actinomycin D (10 $\mu\text{g}/\text{ml}$) 10 min after stimulation with LTB_4 or medium to arrest transcription; the reaction was stopped after various intervals, and Northern analysis was performed as described above.

Nuclear transcription *in vitro*

The '*in vitro*' nuclear transcription assays were performed as described by Greenberg & Bender [22] with several modifications. Briefly, nuclei were prepared from human monocytes by resuspending cells in a lysis buffer containing sucrose (0.3 M), KCl

(60 mM), NaCl (15 mM), Hepes (pH 7.5, 15 mM), EDTA (2 mM), EGTA (0.5 mM), spermine (0.15 mM), spermidine (0.5 mM), mercaptoethanol (14 mM) and 0.1 % Nonidet P40, followed by sedimentation through a 30 % (w/v) sucrose solution. Transcription *in vitro* was carried out in the following transcription mixture: $1 \times$ buffer [$5 \times$ buffer is $(\text{NH}_4)_2\text{SO}_4$ (1.5 M)/Tris/HCl (pH 7.9, 500 mM)/ MgCl_2 (20 mM)/ MnCl_2 (20 mM)/NaCl (1 M)/EDTA (2 mM)/phenylmethanesulphonyl fluoride (0.5 mM), dithiothreitol (1.2 μM), GTP (1 mM), ATP (1 mM), CTP (1 mM), creatine phosphate (10 mM), RNasin (20 units/ml) and glycerol (29 %, v/v) in the presence of 150 μCi of [α - ^{32}P]UTP (> 3000 Ci/mmol). The radiolabelled RNA was isolated by chloroform/phenol extraction and trichloroacetic acid/sodium pyrophosphate precipitation. The labelled nuclear RNA was then hybridized to linearized cDNA probes (250 ng) that were immobilized on Genescreen Plus membranes with a slot-blot apparatus. The membranes were prehybridized with the following mixture: Hepes (pH 7.5, 50 mM)/NaCl (0.75 M)/formamide (50 %, v/v) SDS (0.5 %)/EDTA (2 mM)/ $10 \times$ Denhardt's solution/salmon sperm DNA (500 $\mu\text{g}/\text{ml}$)/poly(A) (10 $\mu\text{g}/\text{ml}$), for 2 days. Hybridization was carried out for 4 days in the same mixture with the radiolabelled RNA; the membranes were then washed and exposed to X-ray film for 2 weeks.

Nuclear factors and mobility-shift assay

Nuclei were isolated from monocytes and HeLa cells (kindly given by Dr. D. Hunting) as described above. To obtain nuclear proteins, the nuclei were lysed by repeated freeze-thaw cycles (minimum three), in a buffer containing 250 mM-Tris (pH 7.8), 60 mM-KCl, 1 mM-dithiothreitol and 1 mM-phenylmethanesulphonyl fluoride. The following oligonucleotide containing the AP-1 binding site was used: 5'GTGAGTCAGTGAGTCA3' with the complementary oligonucleotide 5'TGACTCACTGACTCAC3'. A mutant $\Delta\text{AP-1}$, 5'GTGATTCAATTGATTCA3', annealed to its complementary strand, 5'TGAATCAATGATATCAC3', and an irrelevant oligonucleotide SP-1, 5'GGG-CGGGGGCGG3', annealed to its complementary strand, 5'CCGCCCCCGCCC3', were used for competition studies. For the binding reaction, the ^{32}P -labelled probe (1–2 ng) was incubated at room temperature for 30 min with 5 μg of the nuclear extract in binding buffer consisting of 10 mM-Hepes, pH 7.9,

60 mM-KCl, 4% Ficoll, 1 mM-dithiothreitol, 1 mM-EDTA and 2 μ g of poly(dI-dC). The DNA-protein complexes were separated by electrophoresis on a low-ionic-strength 4%(w/v)-polyacrylamide gel with Tris/borate electrophoresis buffer (TBE).

RESULTS AND DISCUSSION

The proto-oncogenes *c-fos* and *c-jun* have been implicated in the regulation of both cell proliferation and differentiation. These genes code for proteins which can associate in a heterodimer and function synergistically in DNA binding [23]. We examined the effect of LTB₄ on the expression of the two proto-oncogenes in human monocytes. Since adherence activates monocytes, they were incubated overnight, after their isolation, in order to allow them to return to their baseline activity. Resting monocytes expressed low amounts of *c-fos* mRNA and substantially higher amounts of *c-jun* message (Fig. 1c, lane 1) and these baseline levels varied between individuals, as has been shown by others [24]. LTB₄ augmented the expression of *c-fos* mRNA in a dose-dependent manner, showing 3–15-fold increase at 10⁻⁶ M and 2–5-fold at 10⁻⁸ M (Fig. 1a); lower concentrations of LTB₄ did not consistently modulate *c-fos* expression. The *c-jun* mRNA also showed increased accumulation after stimulation by LTB₄ at the same concentrations. Although the expression of this gene was augmented to a much lower extent (1.2–1.5-fold), the increase was consistently reproducible (Figs. 1b and 1c). The accumulation of mRNA of both genes in human monocytes can

be augmented by simple physical perturbation, such as shaking the cells in order to distribute the intended stimulus. This can be seen in Fig. 1(c) and Fig. 2 (lanes 1) where cells that have been rested overnight and directly harvested without further disturbance expressed much lower levels of mRNA than cells which had been shaken and incubated for a period of time (lanes 2).

The increase in *c-fos* mRNA accumulation was transient and reached a peak at about 15 min and was often undetectable after 1 h; *c-jun* reached a peak at about 30 min and the mRNA still persisted at 2 h (Fig. 1b and results not shown). The time of appearance and length of persistence of mRNA for these two genes can vary with cell type and stimulus [25–27]. Interestingly, LTB₄ failed to elicit either *c-fos* or *c-jun* expression in adipogenic TA1 cells [28] and in peripheral-blood lymphocytes (J. Staňková, unpublished work). Distinct signals elicit different kinetics of proto-oncogene activation in monocytes; for example, macrophage colony-stimulating factor augments *c-jun* mRNA within 30 min, but the expression returns to baseline within 60 min, whereas in alveolar macrophages *c-fos* reaches a peak at 60 min after macrophage-colony-stimulating-factor stimulation [29]. Conversely, tumour necrosis factor α induced a prolonged expression of *c-jun* for up to 8 h, but *c-fos* expression is transient, returning to baseline within 60 min [27,30,31], illustrating that diverse signalling pathways may result in potentially differential effects of the oncogene products.

Human neutrophils can rapidly metabolize LTB₄ by ω -oxidation, leading to the formation of 20-OH-LTB₄ and 20-COOH-LTB₄ [32]. These metabolites had only 20–30% and 0–3% activity respectively on *c-fos* expression, compared with LTB₄ (Fig. 2), suggesting that the integrity of the LTB₄ molecule was necessary for optimal activity. A comparable level of activity for these metabolites has been found in other systems [33,34].

Both transcriptional and post-transcriptional control of *c-fos* expression in human monocytes has been described. For example, phorbol-ester-stimulated *c-fos* mRNA increase is related solely to increased transcription, whereas cycloheximide-induced increase results from both increased transcription of the *c-fos* gene and prolongation of the half-life of *c-fos* mRNA [35]. We investigated whether stabilization of the *c-fos* message contributed to the mRNA accumulation observed in LTB₄-stimulated monocytes. We measured the stability of *c-fos* mRNA by treating cells with LTB₄ for 10 min and then adding actinomycin D to block the RNA transcription *de novo*. Although the *c-fos* mRNA half-life can be extended in monocytes by fMet-Leu-Phe, a chemotactic peptide [24], the chemotactic lipid LTB₄ failed to stabilize the message for *c-fos* (Fig. 3). Similarly, the half-life of *c-jun* mRNA was not affected by LTB₄ stimulation (results not shown).

Since LTB₄ did not stabilize the proto-oncogene mRNA, we performed 'in vitro' nuclear transcription (run-on) assays in order to determine whether transcriptional mechanisms were involved in the regulation of *c-fos* and *c-jun* expression by LTB₄. Both *c-jun* and *c-fos* genes were actively transcribed in unstimulated monocytes (Fig. 4a; NS), which is in agreement with previous reports [24,30]. Stimulation with LTB₄ (10 nM) increased the rate of transcription of the *c-fos* gene by a factor of 7 and the *c-jun* gene by a factor of 1.8 (mean for six separate experiments) (Figs. 4a and 4b).

Some stimuli, such as forskolin, may enhance gene expression without producing an increase in nuclear AP-1 DNA binding, whereas the cytokine interleukin-1 induces both [36]. We therefore investigated whether the augmented expression of *c-fos* and *c-jun* in LTB₄-stimulated monocytes was associated with increased DNA-binding activity. DNA-nuclear-protein interactions were monitored with a standard electrophoretic mobility-shift assay. Unstimulated monocytes contained active factors

Fig. 2. Effect of ω -oxidation metabolites of LTB₄ on *c-fos* mRNA expression

Total RNA (10 μ g) was isolated from monocytes taken directly after overnight incubation (lane 1) or stimulated for 30 min with 3 $\times$ 10⁻⁵ % (v/v) methanol (vehicle for LTB₄) (lane 2), 10⁻⁸ M-LTB₄ (lane 3), 0.003 % (v/v) acetone (vehicle for ω -oxidation metabolites) (lane 4), 10⁻⁸ M-20-OH-LTB₄ (lane 5) or 10⁻⁸ M-20-COOH-LTB₄ (lane 6). The RNA was hybridized with *c-fos*-specific probe and exposed for 20 h at -80 °C.

Fig. 3. Analysis of *c-fos* mRNA stability in monocytes treated with LTB₄

Monocytes were treated with vehicle (methanol; $3 \times 10^{-5}\%$ (v/v); control, $\blacklozenge$) or 10^{-8} M-LTB₄ for 10 min ($\square$) and the levels of *c-fos* mRNA were determined before (zero time) and at 10 min intervals after actinomycin D ($10 \mu\text{g/ml}$) was added. Densitometric analysis of the levels of *c-fos* mRNA after actinomycin D treatment are expressed as percentages of the level in monocytes at zero time. Results are means for three experiments.

Fig. 4. Analysis of *c-jun* and *c-fos* gene transcription in human monocytes stimulated with 10^{-8} M-LTB₄

Monocytes were incubated without (NS or control) or with 10^{-8} M-LTB₄ for 30 min. Nuclei were isolated and newly elongated ³²P-labelled RNA transcripts were hybridized to immobilized *c-fos* and *c-jun* cDNA. (a) A representative experiment is shown. (b) Data are presented as ratios of the densitometric units of *c-fos* and *c-jun* over glyceraldehyde-phosphate dehydrogenase (GAPDH) and represent the means for six separate experiments.

that bound to the AP-1 binding site (Fig. 5a, lane 1), but the binding activity was increased after stimulation with LTB₄ at concentrations of $1 \mu\text{M}$ and 10 nM , but not 10 fM (lanes 2–4). The specificity of the reaction was supported by the fact that the unlabelled oligonucleotide readily competed with the labelled oligonucleotide for the formation of the labelled DNA–protein complex (Fig. 5a, lanes 5–6). The second band in lanes 1–4 did not bind with specificity, as when it was present it could be competed for by unlabelled SP-1 oligonucleotide. The specificity of AP-1 binding by nuclear extracts was further addressed by comparing the degree of mobility of nuclear-protein extracts from monocytes and HeLa cells. As shown in Fig. 5(b), both

Fig. 5. LTB₄-induced AP-1 binding activity

(a) Effect of LTB₄ on the activity of nuclear factors that bind to the AP-1 element. Nuclear extracts from control (lane 1) or LTB₄-stimulated monocytes (lane 2, 10^{-6} M; lane 3, 10^{-8} M; lane 4, 10^{-14} M) were incubated with ³²P-end-labelled double-stranded AP-1 oligonucleotide. In lanes 5–7 the nuclear extracts and labelled oligonucleotide were incubated in the presence of 200-fold excess of unlabelled oligonucleotide (lane 5, control; lane 6, 10^{-8} M-LTB₄; lane 7, 10^{-14} M-LTB₄). (b) Nuclear protein–AP-1 complexes isolated from monocytes and HeLa cells migrate with a similar degree of retardation of mobility: M, monocyte extracts; H, extract from HeLa cells. (c) Nuclear-protein extracts were incubated with the labelled AP-1 oligonucleotide in the absence (lane 1) or presence (lanes 2–5) of competing unlabelled double-stranded oligonucleotides. Lane 1, no competitor; lane 2, 50-fold excess of Δ AP-1; lane 3, 50-fold excess of SP-1; lane 4, 100-fold excess of Δ AP-1; lane 5, 100-fold excess of SP-1. Protein–DNA complexes were separated from free oligonucleotide (lower part of each lane) in low-ionic-strength 4% (w/v)-polyacrylamide gels.

complexes show similar degree of retardation. In addition, mutant AP-1 oligonucleotide did not compete with AP-1 at 50–100-fold excess concentrations (Fig. 5c, lanes 2 and 4). An oligonucleotide containing the SP-1 binding site also did not decrease AP-1 binding (Fig. 5c, lanes 3 and 5). These data suggest that DNA-binding activity in monocytes stimulated with LTB₄ is specific for AP-1.

It is not surprising that unstimulated monocytes exhibited AP-1-binding activity, since the expression of *c-jun* is constitutively high and JUN/JUN homodimers bind to the consensus AP-1 sequence, although less efficiently than FOS/JUN heterodimers [34,37]. The increase in AP-1 binding of nuclear extracts from LTB₄-stimulated monocytes could be the result of greater FOS participation, due to increased gene expression.

We have shown that LTB₄ augments the expression of the proto-oncogenes *c-fos* and *c-jun*. Since the protein products of these genes are factors which may regulate the transcription of

other genes, these data give the first indication that LTB_4 may modulate monocyte function, such as cytokine gene expression, by influencing the production or composition of transcriptional factors.

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