ELSEVIER

European Journal of Pharmacology 369 (1999) 373–386

ejp

Activation of nuclear factor-κB by lipopolysaccharide in mononuclear leukocytes is prevented by inhibitors of cytosolic phospholipase A_2

Elke Burgermeister [a], Ulrich Tibes [b], Hubertus Stockinger [c], Werner V. Scheuer [a,*]

[a] *Department of Molecular Pharmacology, Roche Diagnostics, Nonnenwald 2, D-82372 Penzberg, Germany*
[b] *Department of Preclinical Research, Roche Diagnostics, D-68305 Mannheim, Germany*
[c] *Department of Biochemicals, Roche Diagnostics, Nonnenwald 2, D-82372 Penzberg, Germany*

Received 5 October 1998; revised 31 December 1998; accepted 8 January 1999

Abstract

In monocytes, lipopolysaccharide induces synthesis and activity of the 85-kDa cytosolic phospholipase A_2. This enzyme releases arachidonic acid and lyso-phospholipids from membranes which are metabolized to eicosanoids and platelet-activating-factor. These lipid mediators increase activity of transcription factors and expression of cytokine genes indicating a function for cytosolic phospholipase A_2 in signal transduction and inflammation. We have shown previously that trifluoromethylketone inhibitors of cytosolic phospholipase A_2 suppressed interleukin-1β protein and steady-state mRNA levels in human lipopolysaccharide-stimulated peripheral blood mononuclear leukocytes. In this study, the subcellular mechanisms were analyzed by which trifluoromethylketones interfere with gene expression. We found that they reduced the initial interleukin-1β mRNA transcription rate through prevention of degradation of inhibitor-κBα. Consequently, cytosolic activation, nuclear translocation and DNA-binding of nuclear factor-κB were decreased. Trifluoromethylketones ameliorate chronic inflammation in vivo. Thus, this therapeutic potency may reside in retention of inactive nuclear factor-κB in the cytosol thereby abrogating interleukin-1β gene transcription.

Keywords: Phospholipase A_2; Phospholipase A_2 inhibitor; Nuclear factor-κB; Interleukin-1; Inflammation

1. Introduction

Phospholipases A_2 hydrolyze glycerophosphatides into lyso-phosphatides and arachidonic acid representing the rate-limiting step in the synthesis of eicosanoids and are involved in the formation of platelet-activating factor. Phospholipases A_2 are implicated in digestion, membrane remodelling, blood coagulation and signal transduction and in pathophysiological states of inflammation, cancer and autoimmune diseases. In mammals, multiple isoforms exist where among the 85-kDa cytosolic Ca^{2+}-dependent type IV phospholipase A_2 is responsible for agonist-induced receptor-mediated intracellular arachidonic acid generation (Murakami et al., 1998). Human monocytes treated with antisense oligonucleotides against cytosolic phospholipase A_2 mRNA (Roshak et al., 1994) and macrophages from knock-out mice exhibit a decreased arachidonic acid release and lipid mediator production. Furthermore, these mice exert a diminished acute allergic response (Uozumi et al., 1997). Concordantly, inhibitors of arachidonic acid metabolism like non-steroidal-(NSAID) and cytokine-suppressive-antiinflammatory drugs (CSAID®) abrogate cytokine production in vitro and prevent inflammation in vivo (Boehm et al., 1996; Osnes et al., 1996). Collectively, these data suggest that cytosolic phospholipase A_2 plays a priorized role in inflammation.

This isoform is expressed in platelets, granulocytes, monocytes, tissue macrophages, mast cells, fibroblasts, epithelial and endothelial cells and is activated by diverse proinflammatory stimuli like bacteria, cytokines, growth factors, kinins, complement and immune complexes (Clark et al., 1995; Murakami et al., 1997b). In peripheral blood mononuclear cell populations monocytes but not B- or T-lymphocytes are the major source for arachidonic acid release (Camussi et al., 1995; Surette et al., 1996). Lipopolysaccharide binding to CD14 on monocytes triggers rapid Ca^{2+}-dependent translocation of the cytosolic phospholipase A_2 from the cytosol to membranes. Mitogen-activated protein kinases and protein kinase C further activate the enzyme by phosphorylation. Within hours de

* Corresponding author. Tel.: +49-8856-60-2542; Fax: +49-8856-60-3201

0014-2999/99/$ - see front matter
PII: S0014-2999(99)00011-4

novo synthesis of cytosolic phospholipase A_2 mRNA and protein is also induced (Clark et al., 1995; Murakami et al., 1997b). The lipid derivatives generated upon cytosolic phospholipase A_2 activation either bind in an auto- or paracrine fashion to G-protein-coupled receptors on plasma membranes inducing, e.g., bronchoconstriction, vasopermeability and chemotaxis or transfer signals as intracellular 'second messengers' (Serhan et al., 1996). Thus, arachidonic acid, platelet-activating factor and leukotriene B_4 enhance whereas prostaglandin E_2 decreases activity and synthesis of the transcription factors nuclear factor-κB, nuclear factor-interleukin-6 (CCAAT/enhancer binding protein-β) and activation protein-1 (c-*Jun*/c-*fos*) (Brach et al., 1992; Camandola et al., 1996; Danesch et al., 1994; Kravchenko et al., 1995; Rizzo et al., 1995; Rossi et al., 1997). Thereby, lipid metabolites control the expression of genes for cytokines (Chaughey et al., 1997; Gormand et al., 1996; Poubelle et al., 1991; Rola-Pleszczynski and Lemaire, 1985; Rola-Pleszczynski and Stankova, 1992; Spangelo and Jarvis, 1996; Zhong et al., 1995), adhesion molecules (Milam et al., 1991) and tissue proteases (Mehindate et al., 1995) which are responsible for induction and maintenance of inflammation.

The trifluoromethylketone analogues of arachidonic acid (C20:4-$COCF_3$), γ-linolenic (C18:3-$COCF_3$) and linoleic acid (C18:2-$COCF_3$) are specific, reversible inhibitors of the cytosolic phospholipase A_2 enzyme activity by forming a stable hemiketal with the nucleophilic Ser^{228} residue of the catalytic triad (Clark et al., 1995; Street et al., 1993). These compounds do not inhibit 14-kDa secretory type IIA phospholipase A_2, CoA-independent transacylase nor lipoxygenases and cyclooxygenases (Bartoli et al., 1994; Riendeau et al., 1994; Tibes et al., 1997). They reduce arachidonic acid release and formation of eicosanoids and platelet-activating factor in platelets and leukocytes upon stimulation with lipopolysaccharide, Ca^{2+}-ionophore or thrombin (Lo et al., 1997; Murakami et al., 1997b; Shamsuddin et al., 1997; Withnall et al., 1995). We have shown previously that in addition to this primary mode of action the trifluoromethylketones display antiphlogistic effects in vitro and in vivo: they (a) exhibited therapeutic benefit in animal models of acute, allergic and chronic inflammation (Tibes et al., 1997; Tibes et al., 1995), (b) prevented chemotaxis and Ca^{2+}-ionophore-induced expression of the β_2-integrin Mac-1 (CD11b/CD18) in human neutrophils, and (c) suppressed lipopolysaccharide-stimulated protein synthesis and steady-state mRNA levels of interleukin-1β in human peripheral blood mononuclear cells ($CD14^+$ monocytes) with IC_{50} values of 10–20 μM (Amandi-Burgermeister et al., 1997). In the present study, we investigated by which subcellular mechanisms trifluoromethylketones interfere with interleukin-1β gene expression in monocytes. We show that these inhibitors of the cytosolic phospholipase A_2 enzyme activity prevent degradation of inhibitor-κBα and subsequent cytoplasmatic mobilisation, nuclear translocation and DNA-binding of nuclear factor-κB. Thereby, transcriptional activation of the interleukin-1β gene is abrogated.

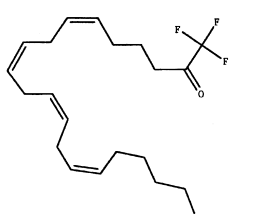
2. Materials and methods

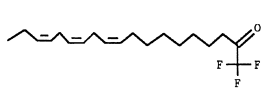
2.1. Reagents

Trifluoromethylketones (Roche Diagnostics) (Fig. 1), pyrrolidinedithiocarbamate, acetylsalicylate (Sigma) and cyclosporine A (Sandoz) were stored as 10 mg/ml stock solutions in dimethylsulfoxide at −20°C under N_2. Compounds were freshly diluted in RPMI 1640 cell culture medium and added to cells with a final dimethylsulfoxide concentration $< 0.1\%$. Synthetic lipid metabolites (Biomol) were stored at −80°C. Stock solutions of 100 mM in ethanol were freshly prepared from arachidonic acid, C16-platelet-activating factor and prostaglandin E_2, while leukotriene B_4 was a 150 μM solution in ethanol. Further serial tenfold dilutions were done in serum-free cell culture medium and instantly added to cells yielding a final ethanol concentration $< 0.1\%$.

2.2. Preparation of human peripheral blood mononuclear cells

Human peripheral venous blood was collected in Liquemin (Roche Diagnostics, 2500 IU/ml blood). Donors were anonymous, randomly selected, and confirmed not to be receiving medical treatment and did not have any





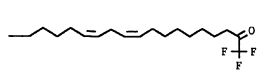


Fig. 1. Chemical structures of cytosolic phospholipase A_2 inhibitors. C20:4-$COCF_3$ = 1,1,1-Trifluoro-heneicosa-6,9,12,15-tetraen-2-one, m.w. 356; C18:3-$COCF_3$ = 1,1,1-Trifluoro-nonadeca-10,13,16-trien-2-one, m.w. 330; C18:2-$COCF_3$ = 1,1,1-Trifluoro-nonadeca-10,13-dien-2-one, m.w. 332.

allergic or chronic inflammatory diseases. Mononuclear cells were freshly isolated by density gradient centrifugation using Lymphoprep separation medium (Nycomed Pharma) according to the manufacturer's instructions. Viability after purification was greater than 98% with an average of 10–20% monocytes and 80–90% lymphocytes. Platelets were removed by several washes with phosphate-buffered saline (Ca^{2+}/Mg^{2+}-free, Roche Diagnostics). Mononuclear cells were cultivated in RPMI 1640 medium (low endotoxin, Seromed Biochrom) supplemented with 10% fetal calf serum (heat-inactivated for 45 min at 56°C, low endotoxin, Gibco Life Technologies), 2 mM glutamine (Roche Diagnostics), 1% minimal essential medium-vitamins (Gibco Life Technologies), 100 IU/ml penicillin and 20 μg/ml streptomycin (Roche Diagnostics). Cells were seeded at a density of 1×10^6 cells/ml and cultured in suspension using sterile polypropylene tubes (Nunc/Falcon) to prevent adherence of monocytes to plastic surfaces. To avoid lipopolysaccharide contamination plastic disposable equipment and cell culture grade endotoxin-free solutions were used.

2.3. Quantification of interleukin-1β

Interleukin-1β protein was determined in the supernatants of lipopolysaccharide-stimulated (Sigma) mononuclear cells using human interleukin-1β immunoassay (Roche Diagnostics) according to the manufacturer's instructions.

2.4. Probes and primers

Digoxigenin-11-dUTP-labelled DNA-probes for Northern hybridization were synthesized by reverse transcription-polymerase chain reaction. One microgram of total RNA extracted from lipopolysaccharide-stimulated mononuclear cells by Tripure Reagent (Roche Diagnostics) was reverse-transcribed with random hexamer primer using AMV-Reverse Transcriptase Kit (Roche Diagnostics). A 5 μl aliquot of cDNA was taken for polymerase chain reaction containing a 1:2 fraction of digoxigenin-11-dUTP and unlabelled dTTP (Roche Diagnostics) using interleukin-1β specific primers (DM155/DM156, Perkin Elmer) according to the manufacturer's instructions. The probe was purified by applying High Pure PCR-Product Purification Kit (Roche Diagnostics). Before use, 2 μl of the PCR volume per ml hybridization solution were heat-denatured for 15 min at 100°C and cooled on ice.

2.5. Northern analysis

Total RNA was extracted from 1×10^7 mononuclear cells using Tripure Reagent (Roche Diagnostics). Integrity and equal loading was checked by ethidium bromide gel electrophoresis visualizing the three ribosomal RNAs. For Northern analysis, 5–10 μg of total RNA per lane were separated electrophoretically using a 1% formaldehyde containing agarose gel in 1× gel-running buffer (20 mM 4-morpholine-propane-sulfonic acid, pH 7.0, 8 mM sodium acetate, 1 mM EDTA, pH 8.0). The RNA was blotted on nylon membrane (Roche Diagnostics) by capillary transfer overnight. The RNA was crosslinked to the membrane using UV-Stratalinker (Stratagene) and was hybridized to a digoxigenin-11-dUTP-labelled interleukin-1β DNA probe and digoxigenin-11-dUTP-labelled β-actin RNA probe (Roche Diagnostics) in digoxigenin-EasyHyb buffer (Roche Diagnostics) overnight at 50 and 68°C, respectively. Membranes were washed twice with 2× SSC buffer (300 mM sodium chloride, 30 mM trisodium-citrate-dihydrate, pH 7.0) supplemented with 0.1% sodium dodecylsulfate for 15 min at room temperature followed by two washes in 0.5× SSC buffer supplemented with 0.1% sodium dodecylsulfate for 15 min at 68°C. Detection of digoxigenin by chemoluminescence was performed according to the manufacturer's instructions (Roche Diagnostics). Membranes were exposed to X-ray film (Cronex). Relative amounts of mRNA signals on films were estimated by video-based densitometry (Easy Plus, device by Herolab) and were calculated from arbitrary O.D. units.

2.6. Semiquantitative reverse transcription-polymerase chain reaction

One hundred nanograms of total RNA together with 0, 10^3, 10^4, 10^5 or 10^6 copies of the pAW109 internal standard plasmid RNA (Perkin Elmer), respectively, were reverse-transcribed in one tube using the 5′-downstream primer (DM156, Perkin Elmer) specific for both sample interleukin-1β mRNA and pAW109 control transcript according to the manufacturer's instructions (Geneamp^R-Thermostable rTh-Reverse-Transcriptase RNA PCR Kit). Resulting cDNAs were amplified by polymerase chain reaction (35× cycles) using the 3′-upstream primer (DM155, Perkin Elmer). In parallel, serial tenfold dilutions (100, 10, 1 ng) of total RNA were reverse-transcribed with Oligo-dT_{15} primer (Roche Diagnostics) and cDNAs amplified with β-actin specific primers (Stratagene). Amplification products were separated by ethidium bromide agarose gel electrophoresis. The relative amounts of endogenous interleukin-1β mRNA were estimated in comparison to the amounts of internal standard transcript. Equal content of total RNA in the samples was confirmed by comparing β-actin levels. The band intensity in gel was estimated by video-based densitometry.

2.7. Preparation of nuclear extracts

Mononuclear cells ($1–5 \times 10^7$) were washed once with ice-cold phosphate-buffered saline and incubated in 200 μl

of hypotonic buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$) supplemented with 10 μg/ml aprotinin, 0.5 mM phenylmethylsulfonylfluoride and 5 mM dithiothreitol (Roche Diagnostics) for 15 min on ice. Cells were lysed by sonication (2 × 3 s) on ice. Free nuclei were recovered by centrifugation in a microcentrifuge (7000 rpm, 10 min) and extracted in 50–100 μl (fourfold volume of nuclei pellet) of high salt buffer B (20 mM Hepes pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 200 μM EDTA, 25% glycerol) for 30 min on ice with frequent vortexing. Debris was removed by full-speed centrifugation for 10 min and nuclear extract was diluted 1:1 with buffer D^+ (20 mM Hepes pH 7.9, 100 mM KCl, 5 μM EDTA, 20% glycerol, 1% Nonidet-P40). Aliquots were stored at −80°C. Protein content was quantified photometrically using a bovine serum albumine standard (Pierce) and protein assay reagent from Biorad.

2.8. Electrophoretic mobility shift assay

The following double-stranded DNA-oligonucleotides were used as ^{32}P-labelled binding probes:

Nuclear factor-κB site (−2752/−2743 Bp) from the human pro-interleukin-1β distal enhancer (Gray et al., 1993): 5′-TCT CGA GGG GGC ATT GCC CC-3′.

Nuclear factor-κB site (−296/−286 Bp) from the human pro-interleukin-1β proximal enhancer (Cogswell et al., 1994): 5′-TCT TCT AAC GTG GGA AAA TCC-3′.

Nuclear factor-κB site from the murine immunoglobulin κ light chain enhancer (Los et al., 1995): 5′-AGC TTC AGA GGG GAT TTC CGA GAG G-3′.

Nuclear factor-interleukin-6 (−2882/−2869 Bp) site from the human pro-interleukin-1β distal enhancer (Shirakawa et al., 1993): 5′-TCT GAT ACA TAC GTT GCA CAA CCT-3′.

Activation protein-1 site (Meyer et al., 1993): 5′-TTC CGG CTG ACT CAT C-3′.

Nuclear factor-Y site (Dorn et al., 1987): 5′-ATT TTT CTG ATT GGT TAA-3′.

Single-stranded DNA-oligonucleotides (Biometra) were annealed and 50 pmol of double-stranded oligonucleotides were labelled by fill-in reaction with α-[^{32}P]-dATP using Klenow fragment of DNA Polymerase I (Roche Diagnostics). Unincorporated nucleotides were separated by centrifugation through Sepharose G-50 using Nick-Spun-columns (Pharmacia). For nuclear factor-κB binding, gelshift reaction was set up in 20 μl with 50,000 cpm of ^{32}P-labelled oligonucleotide containing about 250 fmol in 1 × TE buffer (10 mM Tris/HCl, pH 7.5, 1 mM EDTA, pH 8.0) supplemented with 200 mM NaCl, 5–10 μg of nuclear extract, 2 μg poly-dI-dC (Roche Diagnostics) as an unspecific competitor, 20 μg bovine serum albumine (Roche Diagnostics), 2 μl of buffer D^+ and 2 μl of 10 × binding buffer (100 mM Tris/HCl, pH 7.5, 500 mM NaCl, 10 mM EDTA, 50% glycerol) supplemented with 10 mM dithiothreitol, 2 mM phenylmethylsulfonylfluoride and 50 μg/ml aprotinin. For DNA-binding of nuclear factor-interleukin-6 0.5 μg poly-dI-dC, 2 μl 10 × binding buffer (100 mM Tris/HCl, pH 7.5, 500 mM NaCl, 10 mM EDTA), 20% glycerol and 20 μg bovine serum albumine and for activation protein-1 2 μg poly-dI-dC, 2 μl 10 × binding buffer (100 mM Tris/HCl, pH 7.5, 250 mM NaCl, 50 mM $MgCl_2$, 10 mM EDTA, 50% glycerol) and 20 μg bovine serum albumine were used. For competition experiments a hundred-fold molar excess of unlabelled double-stranded oligonucleotides was added to the gel shift reaction. The reaction was incubated for 15 min at room

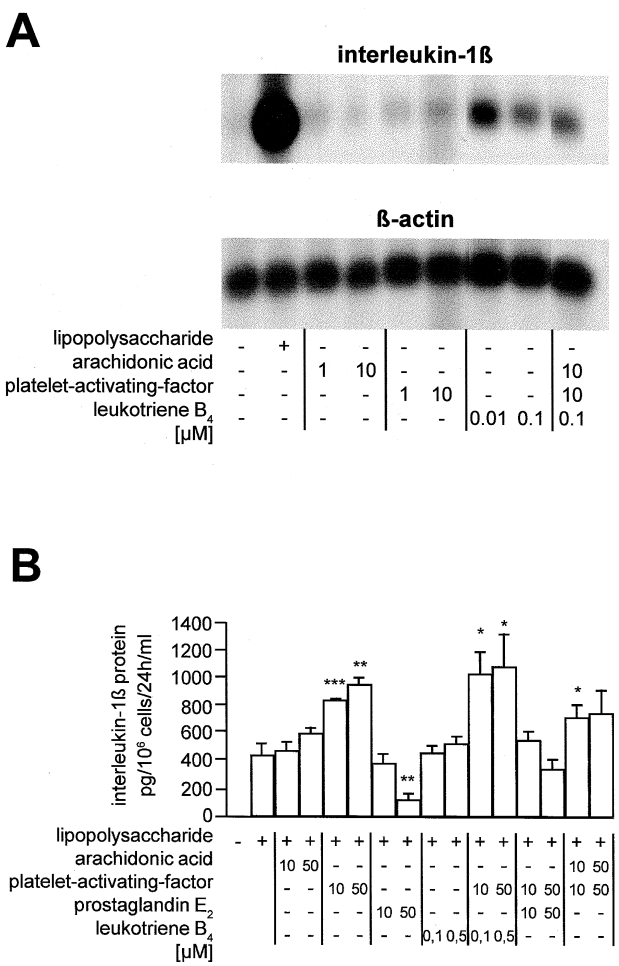


Fig. 2. Induction of interleukin-1β mRNA transcription (A) and superinduction of lipopolysaccharide-mediated interleukin-1β protein release (B) in human peripheral blood mononuclear cells by arachidonic acid, leukotriene B_4 and platelet-activating factor but not by prostaglandin E_2. (A) Cells (10^6 per ml) were incubated with concentrations indicated of the synthetic lipids or 100 ng/ml lipopolysaccharide for 1 h. Total RNA was extracted and 5 μg/lane were separated in an 1% denaturing agarose gel. The RNA was blotted on nylon membrane and hybridized against digoxigenin-labelled interleukin-1β and β-actin probes. After chemoluminescence detection, the membrane was exposed to X-ray film. One experiment out of two similar is shown. (B) Cells were treated with lipids and lipopolysaccharide for 24 h and interleukin-1β protein was quantified in the supernatants by immunoassay. Values represent mean ± S.E.M. (pg/10^6 cells/ml) of interleukin-1β protein ($n = 3$ different donors).

temperature and run in a native 4% polyacrylamide gel in 0.25× TBE (22.5 mM Tris-borate, 0.5 mM EDTA, pH 8.0) buffer for optimal separation of p65/p50 heterodimers and p50/p50 homodimers. Gel was dried and exposed to X-ray film (Kodak). For 'supershift' studies 1–4 μg of polyclonal antiserum against p50, p65 and c-rel (Santa Cruz Biotechnology) and control rabbit immunoglobulin G (Sigma) were added to the gel shift reaction. Alternatively, 1–4 μg of monoclonal anti-p65 antibody (mouse, immunoglobulin G_3, Roche Diagnostics) or immunoglobulin G_3 isotypic control antibody (Biozol) per 5 μg of nuclear extract were applied. For antibody binding, reaction time was extended to 30 min at room temperature before gel loading. Relative amounts of DNA-protein complexes were estimated by video-based densitometry.

2.9. Immune fluorescence microscopy and flow cytometry analysis of nuclear factor-κB p65 protein

Cytospin smears were prepared from mononuclear cells on cytospin slides (Shannon) by centrifugation (750 × *g*, 5 min) using cytofuge 2 (Shannon). Cells on slides were fixed with 4% para-formaldehyde for 30 min on ice followed by 70% ethanol for 10 min at −20°C. Cells were permeabilized in 0.1% sodium citrate supplemented with 0.1% Triton-X 100 for 10 min on ice. Unspecific binding sites were blocked with 50 μg/ml goat immunoglobulin G and 50% fetal calf serum for 15 min. Cells were stained with 10 μg/ml anti-p65 monoclonal antibody (mouse immunoglobulin G_3, Roche Diagnostics) and isotype matched control antibody (immunoglobulin G_3, Biozol) in phosphate-buffered saline with 10% fetal calf serum for 1 h followed by 30 μg/ml fluorescein isothiocyanate-labelled detection antibody (AffiniPure goat anti-mouse immunoglobulin G-F(ab′)$_2$ fragment, Dianova) for 30 min. Slides were photographed on Kodak film (400 DIN) using

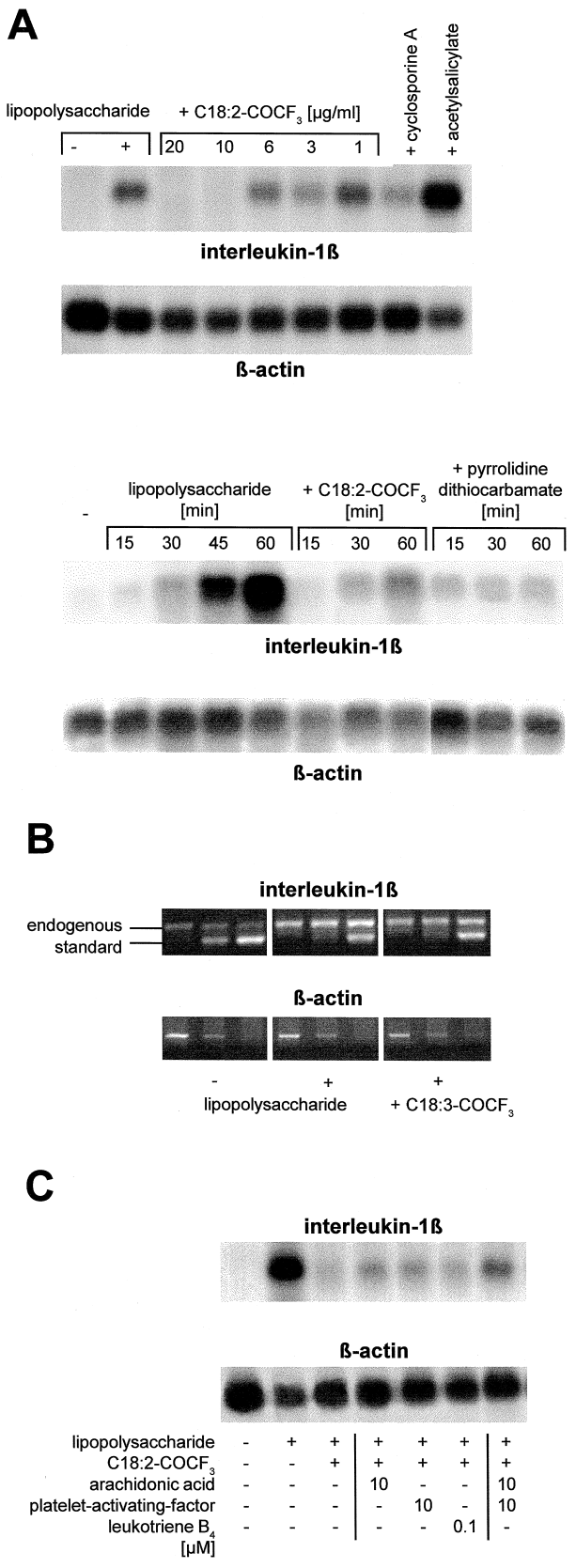


Fig. 3. Suppression of interleukin-1β mRNA expression in lipopolysaccharide-challenged mononuclear cells after treatment with C18:2-$COCF_3$ (A) and C18:3-$COCF_3$ (B), two inhibitors of cytosolic phospholipase A_2, and reconstitution by arachidonic acid, leukotriene B_4 and platelet-activating-factor (C). (A) Upper panel: concentration–response. Cells were preincubated with concentrations indicated of C18:2-$COCF_3$ or 10 μg/ml of the control compounds for 30 min and challenged with 100 ng/ml lipopolysaccharide for 1 h. Lower panel: short-time kinetics. Cells were preincubated with 10 μg/ml of C18:2-$COCF_3$ and pyrrolidinedithiocarbamate and challenged with lipopolysaccharide for the indicated times. Northern hybridization with total RNA was performed according to Fig. 2. (B) Semiquantitative reverse-transcription polymerase chain reaction. Cells were treated with 10 μg/ml of C18:3-$COCF_3$ for 30 min and stimulated with lipopolysaccharide for 1 h. 100 ng of total RNA were reverse-transcribed in presence of 10^5, 10^6 or 10^7 copies (lanes 1–3) of internal standard plasmid pAW109 RNA. The cDNA was amplified using primers for interleukin-1β (35× cycles). To ensure equal RNA content 100, 10 and 1 ng of total RNA (lanes 1–3) were reverse-transcribed and the cDNA was amplified with β-actin-specific primers. Amplified fragments were visualized by ethidium bromide gel electrophoresis. (C) Cells were preincubated with 10 μg/ml of C18:2-$COCF_3$ for 30 min and incubated with lipids and lipopolysaccharide for 1 h. Northern hybridization was performed with total RNA. (A–C) One representative experiment out of three similar is shown, respectively.

a fluorescence microscope (Zeiss). For flow cytometry analysis cells were stimulated, fixed and stained as described above. Additionally, 5 μg/ml of phycoerythrine-labelled anti-CD14 antibody (Becton Dickinson) was added for 20 min. Anti-pan histon (mouse immunoglobulin G_1, Roche Diagnostics) was used to prove permeabilization of the nuclei. Double-stained cells were analyzed gating for $CD14^+$ monocytes on a Becton Dickinson fluorescence-activated cell sorter (FACScan).

2.10. Detection of inhibitor-κBα protein by Western Blot

Mononuclear cells ($1–10 \times 10^7$) were washed twice with ice-cold phosphate-buffered saline. Cells were resuspended in 50–100 μl phosphate-buffered saline supplemented with 2 mM phenylmethylsulfonylfluoride, 10 mM dithiothreitol and 50 μg/ml aprotinin and mixed 1:1 with Laemmli-loading buffer (5% sodium dodecylsulfate, 5% β-mercaptoethanol, 50% glycerol, 250 mM Tris/HCl, pH 6.8, 0.05% bromphenol blue). Samples were boiled for 15 min at 100°C. Equal amounts of protein were separated in a 10% sodium dodecylsulfate-polyacrylamide gel in 1 × Laemmli gel running-buffer (25 mM Tris, 192 mM glycine, 0.1% sodium dodecylsulfate). Proteins were transferred on poly-vinylidene-difluoride membranes (Roche Diagnostics) by electroblotting in 1 × transfer buffer (24 mM Tris, 192 mM glycine, 20% methanol) for 2 h at 100 mA. Membranes were blocked in 1% blocking reagent (Roche Diagnostics) in 1 × Tris-buffered saline (50 mM Tris/HCl, 150 mM NaCl, pH 7.5) for 1 h. Inhibitor-κBα antiserum (rabbit polyclonal immunoglobulin G, Santa Cruz Biotechnology) was added to 0.5% blocking solution at a dilution of 1:1000 for 1 h. Membranes were washed twice with Tris-buffered saline supplemented with 0.1% Tween-20 for 10 min each and twice in 0.5% blocking solution for 10 min each. Detection antibody (anti-rabbit/mouse immunoglobulin G-horse raddish peroxidase conjugate, Roche Diagnostics) was added 1:1000 in 0.5% blocking solution for 30 min. Membranes were washed four times with Tris-buffered saline supplemented with 0.1% Tween-20 and detected by chemoluminescence using ECL reagent (Amersham) according to the manufacturer's instructions. Membranes were exposed to X-ray films (Cronex).

2.11. Statistics

Each experiment was performed at least three times using different blood donors. Values are expressed as means ± S.D. or S.E.M. as indicated in legends to figures. Significance was determined by Student's *t*-test and is indicated by asterisks (* for $P < 0.05$).

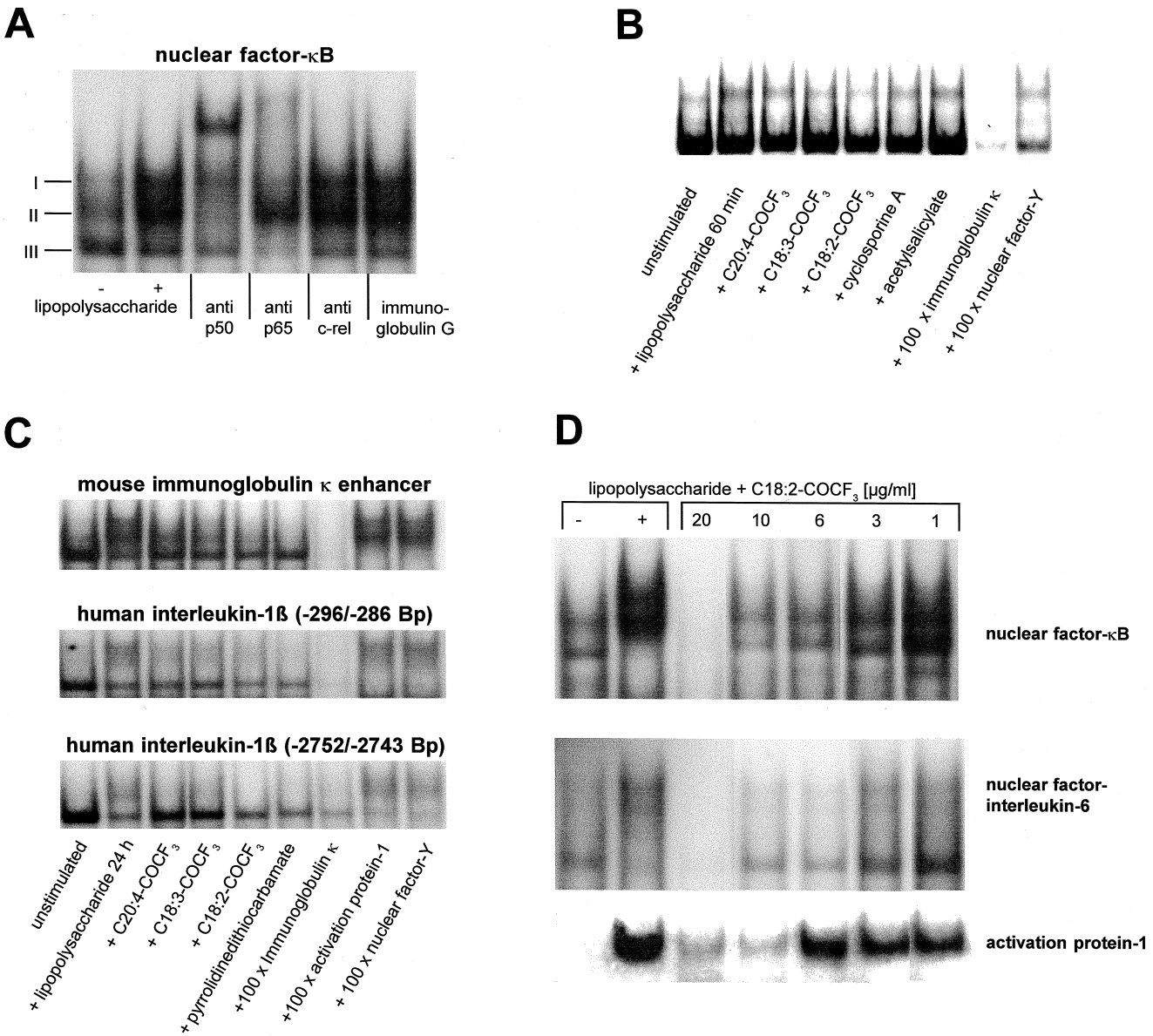
3. Results

3.1. Arachidonic acid, leukotriene B_4 and platelet-activating factor induce interleukin-1β mRNA transcription and superinduce lipopolysaccharide-mediated interleukin-1β protein synthesis in human peripheral blood mononuclear cells

We investigated the effect of exogenous lipid mediators on interleukin-1β gene expression in mononuclear cells. Synthetic lipid metabolites were added for 1 h to cells and total RNA was extracted for Northern analysis. In naive cells, either arachidonic acid (twofold) or platelet-activating factor (fourfold) weakly induced interleukin-1β mRNA expression compared to the unstimulated control (Fig. 2A). Leukotriene B_4 (15-fold) maximally enhanced mRNA levels above the unstimulated control whereas prostaglandin E_2 exerted no effect (not shown). The combination of arachidonic acid + platelet-activating factor + leukotriene B_4 (tenfold) also efficiently enhanced interleukin-1β mRNA levels. However, the lipids were less potent inducers reaching only 20% of interleukin-1β mRNA levels of lipopolysaccharide-stimulated cells. Lipopolysaccharide (100 ng/ml) also efficiently increased interleukin-1β protein in the supernatant within 4–7 h yielding 457 ± 68 pg/10^6 cells/ml ($n = 20$ different donors) after 24 h. Exogenous lipids did not induce interleukin-1β protein release in naive cells. Nevertheless, if cells were challenged simultaneously with lipopolysaccharide and either leukotriene B_4 (1.3-fold) or arachidonic acid (1.5-fold) or platelet-activating factor (2.4-fold) a superinduction of interleukin-1β protein release into the supernatant compared to lipopolysaccharide-stimulated controls was detected

Fig. 4. Electrophoretic mobility shift assays for DNA-binding of nuclear factor-κB, -interleukin-6 and activation protein-1 in mononuclear cells. (A) Cells were stimulated with lipopolysaccharide for 24 h and nuclear extracts were incubated with a [^{32}P]-labelled DNA-oligonucleotide of the nuclear factor-κB binding site from the murine immunoglobulin κ enhancer. For supershift-assays 2 μg of antisera against p50, p65, c-rel or rabbit immunoglobulin G control antiserum were added to the binding reaction. The samples were separated by polyacrylamide gel electrophoresis and visualized on X-ray film: I = specific complex p65/p50, II = specific complex p50/p50; III = nonspecific complex. (B–C) Inhibition of nuclear factor-κB binding to the immunoglobulin κ motif after a 60 min (B) and to two DNA-motifs of the human pro-interleukin-1β enhancer (−296/−286 Bp) and (−2752/−2743 Bp) upon a 24 h (C) stimulation with lipopolysaccharide in presence of 10 μg/ml of the cytosolic phospholipase A_2 inhibitors and control compounds. For competition, a 100-fold molar excess of unlabelled DNA-oligonucleotides was used. (D) Concentration-dependent inhibition of lipopolysaccharide-induced nuclear factor-κB binding to the immunoglobulin κ motif and of nuclear factor-interleukin-6 to a DNA-element of the human pro-interleukin-1β enhancer (−2882/−2869 Bp) and of activation-protein-1 in presence of C18:2-$COCF_3$. (E) Reconstitution of nuclear factor-κB binding in inhibitor-treated cells by arachidonic acid, leukotriene B_4, and platelet-activating-factor but not by prostaglandin E_2. Cells were preincubated with 10 μg/ml of C18:2-$COCF_3$ and challenged with lipopolysaccharide and lipids for 24 h. (A–E) One experiment out of three similar is shown.

($n = 3$ different donors) (Fig. 2B). In contrast, prostaglandin E_2 concentration dependently down-regulated lipopolysaccharide-mediated interleukin-1β protein levels. The maximum enhancement of interleukin-1β protein release was observed using the combination of platelet-activating factor + leukotriene B_4 (2.8-fold) or to a lesser extent using platelet-activating factor + arachidonic acid (1.8-fold). In all experiments, the concentration of the solvent ethanol was below 0.1% and had no influence on interleukin-1β synthesis.



E

lipopolysaccharide	-	+	+	+	+	+	+	+	+	+
C18:2-$COCF_3$	-	-	+	+	+	+	+	+	+	+
arachidonic acid	-	-	-	10	100	-	-	-	-	-	10
platelet-activating-factor	-	-	-	-	-	10	100	-	-	-	10
prostaglandin E_2	-	-	-	-	-	-	-	100	-	-	-
leukotriene B_4 [µM]	-	-	-	-	-	-	-	-	0.1	1	0.1

3.2. Inhibitors of cytosolic phospholipase A_2 abrogate lipopolysaccharide-induced early interleukin-1β mRNA transcription in mononuclear cells

Previously, we showed that trifluoromethylketone inhibitors of cytosolic phospholipase A_2 suppressed lipopolysaccharide-induced steady-state levels of interleukin-1β mRNA in mononuclear cells (Amandi-Burgermeister et al., 1997). Here, we investigated the effect of these compounds on the initial interleukin-1β mRNA transcription rate. Cells were preincubated with 10 μg/ml C18:2-$COCF_3$, C18:3-$COCF_3$ and control compounds for 30 min, respectively, and subsequently challenged with 100 ng/ml lipopolysaccharide for the indicated times. Total RNA was extracted and analyzed for presence of interleukin-1β and β-actin mRNA by Northern hybridization and semiquantitative reverse transcription-polymerase chain reaction. Interleukin-1β mRNA was rapidly induced 15 min upon stimulation with lipopolysaccharide (100 ng/ml) and accumulated within 30 min to 1 h. In contrast, interleukin-1β mRNA in inhibitor-treated cells remained undetectable or strongly reduced compared to lipopolysaccharide-stimulated controls (Fig. 3A, lower panel). C18:2-$COCF_3$ (Fig. 3A, upper panel) and C18:3-$COCF_3$ (Fig. 3B) at a concentration of 10 μg/ml reduced interleukin-1β mRNA levels by $90 \pm 8\%$ and $55 \pm 20\%$ compared to stimulated control cells, respectively, whereas β-actin mRNA expression remained unchanged ($n \geq 4$ different donors). From concentration–response experiments IC_{50} values of 6 μg/ml were determined for both compounds ($n = 3$ different donors). Cyclosporine A (Dwason et al., 1996) and pyrrolidinedithiocarbamate (Ziegler-Heitbrock et al., 1993) also diminished interleukin-1β mRNA expression whereas acetylsalicylate exerted a stimulatory effect. No toxic effects on human blood $CD14^+$ monocytes were observed after a 24 h treatment with 10 μg/ml of the compounds. However, C20:4-$COCF_3$ was toxic on $CD14^+$ blood monocytes and therefore applied for short-term studies.

3.3. Arachidonic acid, leukotriene B_4 and platelet-activating factor restore lipopolysaccharide-induced interleukin-1β mRNA transcription in cytosolic phospholipase A_2 inhibitor-treated mononuclear cells

Exogenous lipids were tested to rescue transcription in inhibitor-treated mononuclear cells. Cells were preincubated with 10 μg/ml of C18:2-$COCF_3$ for 30 min. Thereafter, synthetic lipid metabolites simultaneously with lipopolysaccharide were added for 1 h and total RNA was extracted. Northern analysis showed that initial lipopolysaccharide-induced interleukin-1β mRNA transcription was partially reconstituted by exogenous supplementation of arachidonic acid + platelet-activating factor. This combination of lipids maximally enhanced interleukin-1β mRNA levels to 40% of the levels of lipopolysaccharide-stimulated control cells (Fig. 3C). A weaker mRNA recovery to 20–30% was recorded supplementing either platelet-activating factor or arachidonic acid, whereas leukotriene B_4 alone was ineffective.

3.4. Inhibitors of cytosolic phospholipase A_2 abolish binding of nuclear factor-κB (p50/p65) to two cognate DNA motifs in the human pro-interleukin-1β enhancer region

Electrophoretic mobility shift assays were performed with nuclear extracts from mononuclear cells to determine DNA-binding activity of nuclear factor-κB to elements of the human pro-interleukin-1β gene at $-296/-286$ Bp (Cogswell et al., 1994) and $-2752/-2743$ Bp (Gray et al., 1993) and to a consensus motif from the murine immunoglobulin κ light chain enhancer (Los et al., 1995). A specific inducible complex was detected in lipopolysaccharide-stimulated cells consisting of two bands I + II above a faster migrating unspecific complex III (Fig. 4A). The upper specific band I corresponding to the p50/p65 heterodimer was supershifted by both antisera against p50 and p65 subunits but not by c-rel antiserum or control immunoglobulin G. The lower specific band II was supershifted only by the p50 antiserum representing the p50/p50 homodimers. A hundred-fold excess of the unspecific competitor oligonucleotides activation protein-1 (Meyer et al., 1993) and nuclear factor-Y (Dorn et al., 1987) resulted in disappearance of the unspecific complex III without affecting the specific complex. An equal excess of the unlabelled κB motifs resulted in disappearance of both unspecific and specific complexes. Equal protein loading was confirmed by photometric protein quantitation assay and by gel retardation experiments probing the DNA-binding of nuclear factor-Y as a constitutive control (not shown). Low levels of p50/p50 and p50/p65 preexisted in cells of several individuals. In nuclear extracts of cells derived from these donors a slight induction of nuclear factor-κB DNA-binding was visible after stimulation with lipopolysaccharide for 60 min which was undetectable in presence of the inhibitors (Fig. 4B). After a 24 h incubation with lipopolysaccharide, a marked increase in total nuclear factor-κB binding was observed in cells of all individuals tested. This induction did not occur in extracts from inhibitor-treated cells. The trifluoromethylketones at a concentration of 10 μg/ml reduced binding of nuclear factor-κB to all three elements by $70 \pm 14\%$ ($n = 4$ different donors) compared to stimulated controls (Fig. 4C). Pyrrolidine dithiocarbamate and cyclosporine A acted inhibitory as well, whereas acetylsalicylate was ineffective. DNA-binding of both p50/p65 heterodimers and p50/p50 homodimers was reduced with IC_{50} values of 0.5–1 μg/ml (Fig. 4D, upper panel). Exogenous arachidonic acid + leukotriene B_4 + platelet-activating factor completely restored nuclear factor-κB binding in inhibitor-treated cells to 100% of lipopolysaccharide-stimulated controls. Separate addition of either arachidonic acid or platelet-activating factor or leukotriene B_4 led to a recovery of nuclear

factor-κB binding to 70, 85 and 95% of control, whereas prostaglandin E_2 was ineffective (Fig. 4E). In order to exclude direct interference of the compounds with DNA-binding of preformed nuclear factor-κB, nuclear extracts from stimulated cells were set up in a gel shift reaction in presence of increasing concentrations of 0.1 μg/ml to 1 mg/ml of the compounds. No suppressive effect on DNA-binding of already activated nuclear factor-κB was recorded in this cell-free reaction (data not shown). DNA-binding of lipopolysaccharide-induced nuclear factor-interleukin-6 to an enhancer element of the human pro-interleukin-1β gene at −2882/−2869 Bp (Shirakawa et al., 1993) and of activation protein-1 to its consensus motif (Meyer et al., 1993) was also concentration dependently diminished by the trifluoromethylketones (Fig. 4D, lower panels).

3.5. Cytoplasmatic activation and nuclear translocation of activated p65 subunit of nuclear factor-κB in CD14$^+$ monocytes is suppressed by inhibitors of cytosolic phospholipase A_2

Here, a monoclonal antibody was used, which is directed against the nuclear location sequence epitope on the p65 subunit of nuclear factor-κB (Kaltschmidt et al., 1995). In naive cells, the antibody cannot bind to p65 because inhibitor-κB masks the nuclear location sequence. After stimulation, inhibitor-κB is degraded and the free p65 subunit is accessible for antibody binding. Using this antibody, one is able to monitor the amount of active p65/p50 heterodimers in their subcellular locations, whereas p50/p50 homodimers are not stained. This antibody supershifted the p65/p50 heterodimer but not the p50/p50 homodimer in an gel retardation experiment (not shown). For flow cytometry, mononuclear cells were treated with 10 μg/ml of C18:2-$COCF_3$ for 30 min and challenged for 1 h with lipopolysaccharide. Cells were fixed and stained in suspension. Flow cytometric analysis revealed that the mean fluorescence intensity derived from total free p65 subunits within the cytoplasm and the nucleus of CD14$^+$ monocytes was enhanced 1.5-fold to twofold after 1 h of lipopolysaccharide challenge compared to naive cells (Fig. 5A). Treatment with C18:2-$COCF_3$ significantly lowered the mean fluorescence inten-

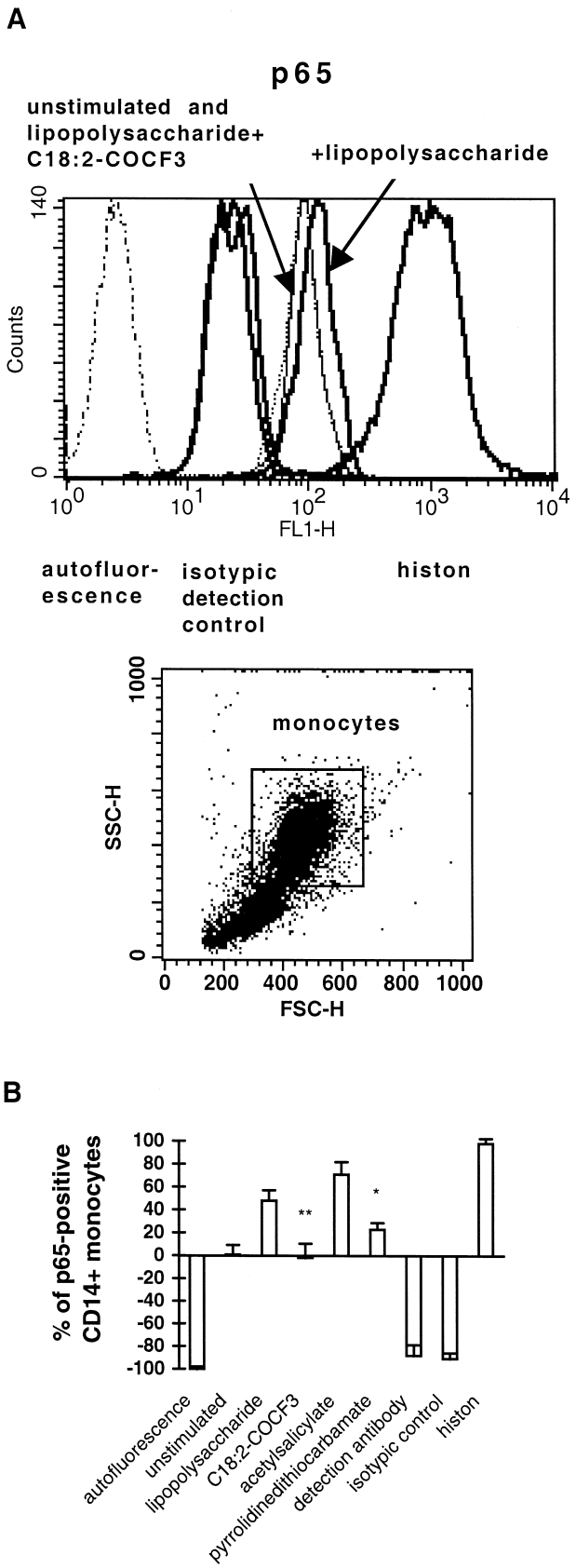


Fig. 5. Flow cytometry analysis of p65 in human CD14$^+$ blood monocytes. Mononuclear cells were preincubated with 10 μg/ml of C18:2-$COCF_3$ and control compounds for 30 min and challenged with lipopolysaccharide for 1 h. Cells were fixed and stained with monoclonal anti-p65 or isotypic control immunoglobulin G_3, respectively, followed by fluorescein-labelled detection antibody and phycoerythrine-conjugated anti-CD14. Anti-pan histon antibody confirmed permeabilization of the nuclei. Cells were analyzed using a fluorescence-activated cell sorter (FACScan). (A) Lower panel: Dot blot displaying lymphocyte and monocyte populations. Upper panel: Representative flow cytometry histogram of double-positive p65$^+$/CD14$^+$ blood monocytes. (B) The percentage of p65 positive cells corrected to the mean fluorescence intensity of unstimulated cells was determined in the monocyte gate ($n = 6$ different donors).

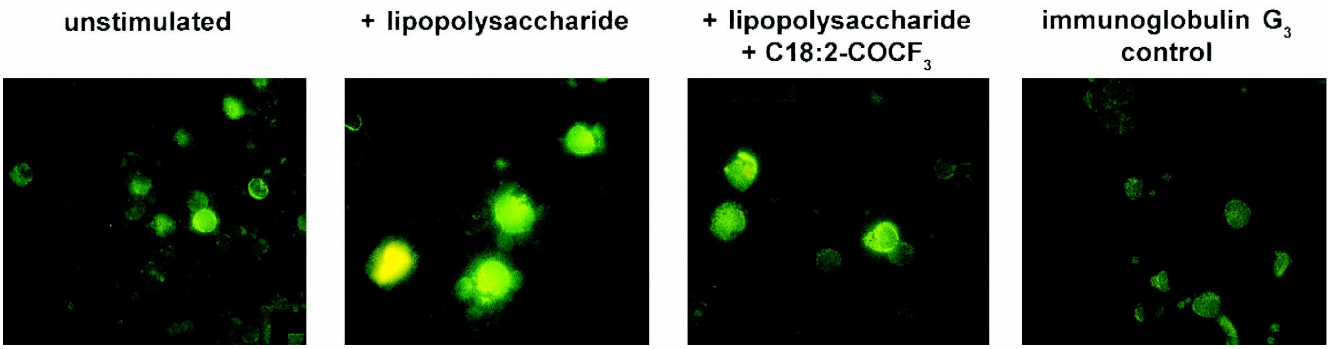


Fig. 6. Immune fluorescence microscopy of p65 in human blood monocytes. Cytospin smears were prepared from mononuclear cells treated as in Fig. 5. Cells were fixed on slides, stained with monoclonal anti-p65 or isotypic control immunoglobulin G_3 followed by fluorescein-labelled detection antibody. Cytospin slides from one representative donor are shown ($n = 8$ different donors).

sity of p65 in $CD14^+$ monocytes by $93 \pm 11\%$ (**, $P < 0.01$, $n = 6$ different donors) corrected to stimulated control cells (Fig. 5B). Pyrrolidinedithiocarbamate also reduced the p65 signal, while acetylsalicylate was ineffective. An intense staining was obtained for the anti-pan histon antibody proving the permeabilization of the nuclei. In contrast to flow cytometry, one is able to distinguish between cytosolic p65 and p65 translocated into the nucleus by performing immune fluorescence microscopy on slides (Fig. 6). Therefore, cytospin smears of mononuclear cells were prepared and stained for p65. Large monocytes (10–20%) with kidney-shaped nuclei and a broader cytoplasm were distinguished from a majority of small, round lymphocytes (80–90%) with a small cytoplasm lining the nucleus. Lymphocytes remained unstained for p65 in the nucleus with a faint staining of the cytoplasmic lining, which did not increase during lipopolysaccharide challenge. In unstimulated monocytes, the cytoplasma was either negative or slightly stained for p65 but the nuclei remained negative. After lipopolysaccharide stimulation, the fluorescence intensity of the cytoplasm was markedly increased and nuclei were stained strongly positive for activated p65 in all monocytes. In contrast, no or only a weak p65 signal was detectable in the nuclei of monocytes in presence of C18:2-$COCF_3$. A faint p65 staining of the cytoplasm was still detectable in some inhibitor-treated monocytes, while the majority remained completely negative in both cytoplasm and nucleus. The isotypic control antibody or the secondary detection antibody (not shown) alone did not stain the cells.

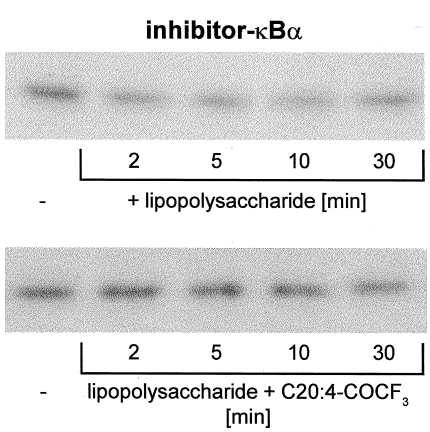


Fig. 7. Suppression of inhibitor-κBα degradation by the cytosolic phospholipase A_2 inhibitor C20:4-$COCF_3$. Mononuclear cells were preincubated with 10 μg/ml of the compound for 30 min and challenged with lipopolysaccharide for 2, 5, 10 and 30 min. Total cell lysates were prepared and separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis. Inhibitor-κBα was detected by western blot analysis using an inhibitor-κBα-specific antiserum and visualized on X-ray film by chemoluminescence. One representative experiment out of two is shown.

3.6. Lipopolysaccharide-induced degradation of inhibitor-κBα is prevented in mononuclear cells by cytosolic phospholipase A_2 inhibitors

Finally, levels of inhibitor-κBα were determined in total cell lysates by western blot analysis using a polyclonal antiserum. Mononuclear cells were treated with 10 μg/ml of C20:4-$COCF_3$ for 30 min and stimulated with lipopolysaccharide for the time periods indicated (Fig. 7). Amounts of inhibitor-κBα protein in total cell lysates decreased upon 2 to 5 min after lipopolysaccharide-challenge. About 60% of the inhibitor-κBα protein was degraded after 30 min to 1 h. Since we performed this experiments with a mixed population of monocytes and lymphocytes, inhibitor-κBα did not disappear completely. The remaining inhibitor-κBα protein may well be derived from the T-lymphocytes which were not stimulated by lipopolysaccharide. In presence of the inhibitor, the lipopolysaccharide-induced degradation of inhibitor-κBα was prevented over a time period from 5 min to 1 h.

4. Discussion

In this study, it was demonstrated for the first time that trifluoromethylketone inhibitors of cytosolic phospholipase A_2 activity interfere with lipopolysaccharide-induced nu-

clear factor-κB activation in human peripheral blood mononuclear cells by preventing inhibitor-κB degradation.

In previous work, it was stated that the inhibitors prevented arachidonic acid release and lipid mediator formation, reduced intracellular 34-kDa pre-interleukin-1β protein synthesis but not pre-protein processing or secretion of mature 17-kDa interleukin-1β and decreased steady-state levels of interleukin-1β mRNA within 24 h. In this time period, de novo synthesis of mRNA and protein of cytosolic phospholipase A_2 (Rodewald et al., 1994; Roshak et al., 1994), cyclooxygenase-2 (Lee et al., 1992) and lipoxygenases (Kaminski et al., 1996) is induced correlating with an increase in the enzyme activities. We hypothesized that if cytosolic phospholipase A_2 activity is blocked supply of precursor lipids for the down-stream enzymatic pathways is abolished thereby failing to induce expression of the interleukin-1β gene. However, these data still implicated that either the long-term mRNA stability or the transcription rate at the interleukin-1β promoter was affected. Functional activity of the cytosolic phospholipase A_2 was found to be indispensable for both the early, transient phase (10 to 30 min) and late, sustained phase (6 to 24 h) of eicosanoid (Murakami et al., 1997b) and platelet-activating factor synthesis (Camussi et al., 1995). Thus, a shorter time period was examined here representing rapid posttranslational activation of the enzyme (Clark et al., 1995). We showed that the initial interleukin-1β mRNA transcription rate was reduced by the trifluoromethylketones within 15 to 60 min upon lipopolysaccharide challenge. This transcriptional shut-down was likely to be accomplished by modulation of the transcriptional machinery. Referring to this we found that DNA-binding of both nuclear factor-κB p65/50 heterodimers and p50/p50 homodimers to enhancer elements of the pro-interleukin-1β gene at $-2752/-2743$ Bp and $-296/-286$ Bp and to the consensus motif of the murine immunoglobulin κ light chain enhancer was impaired by the trifluoromethylketones after a 1 h challenge with lipopolysaccharide. This time range corresponded to initial mobilization of preformed nuclear factor-κB protein within the cytosol. Nevertheless, the inhibition was sustained for up to 24 h indicating that de novo synthesis of p50 and p65 mRNA and protein was reduced as well. In concordance with others, we stated a constitutive DNA-binding of nuclear factor-κB in monocytes from several blood donors (Frankenberger et al., 1994). This may also point at a preactivated immune status of the individual. Nevertheless, in cells from both naive and prestimulated donors, the induction of nuclear factor-κB binding by lipopolysaccharide was diminished by the inhibitors. In contrast to the transactivating potential of p65/p50 heterodimers, p50/p50 homodimers can act as transcriptional repressors (Baldwin, 1996). We observed no induction of p50/p50 homodimers by the compounds. Instead, it was found that the amounts of activated p65 subunits were reduced in the cytoplasm of $CD14^+$ monocytes. Consequently, nuclear translocation and accumulation of p65 within the nuclei of $CD14^+$ monocytes was undetectable. Furthermore, the compounds did not directly interfere, e.g., by steric hindrance or hydrophobic adsorbance, with the DNA-binding capacity of already activated nuclear factor-κB. These results suggested that cytoplasmatic mobilization of nuclear factor-κB by upstream activation events was impaired. Interestingly, DNA-binding of nuclear factor-interleukin-6 to a site at $-2882/-2869$ Bp and of activation protein-1 was reduced as well. In contrast, DNA-binding of the signal-transducer-and-activator-of-transcription (STAT)-1-like factor to a site at $-2863/-2841$ Bp (Tsukada et al., 1996) and of the constitutive control factor nuclear factor-Y (Dorn et al., 1987) was not altered (not shown). This indicated that general transcription factor activity was not impaired.

Upon activation, cytosolic phospholipase A_2 5′-lipoxygenase (Pouliot et al., 1996) and cyclooxygenases (Song and Smith, 1996) are translocated to the nuclear membrane and the endoplasmatic reticulum. Here, the majority of liberation and metabolism of arachidonic acid occurs followed by release of lipid messengers into the nuclear matrix and the perinuclear cytoplasm (Peters-Golden et al., 1996). Moreover, platelet-activating factor synthesis was located in the nucleus (Baker and Chang, 1996). This subcellular location of the enzymes points at the mechanisms how lipid messengers transfer signals to the DNA. Lipid metabolites either directly bind to the peroxisome proliferator-activated receptors, nuclear eicosanoid receptors of the steroid hormone receptor superfamily (Jiang et al., 1998), or indirectly influence transcription factors via modulation of upstream signalling molecules. Referring to the latter case, we found that the cytosolic phospholipase A_2 inhibitors prevented lipopolysaccharide-induced degradation of the inhibitor-κBα protein within 1 h. Phosphorylation of inhibitor-κBα at serine residues is a prerequisite for its degradation (Baldwin, 1996). Therefore, we assume that lipid mediators alter protein kinase activity, an event which is then antagonized by the inhibitors. Concordantly, phospholipid metabolites induce ras-protein activity (Sermon et al., 1996), tyrosine phosphorylation (Rizzo et al., 1995) protein kinase C (Müller et al., 1995) and the mitogen-activated protein kinases (Tournier et al., 1997). Moreover, novel inhibitor-κB protein kinases have been described (Israel, 1997). Consequently, identification of the lipid-regulated kinase which phosphorylates inhibitor-κBα or the relevant transcription factors will further define the molecular mode of action of the cytosolic phospholipase A_2 inhibitors. Alternatively, one may hypothesize that the trifluoromethylketones inhibit a protease of the proteasome which degradates ubiquitinated inhibitor-κBα since they are structural similar to common serine protease inhibitors like *N*-Tosyl-L-phenylalanine chlormethyl ketone (Baldwin, 1996). In future, studies with antisense oligonucleotides or cells from knock-out mice have to prove the specifity of the effects exerted by the cytosolic phospholipase A_2 inhibitors.

In summary, the data suggested a positive feedback mechanism which may shift acute to chronic inflammation (Tibes et al., 1997; Wijkander et al., 1995). In agonist-activated monocytes and macrophages, endogenous lipid mediators induce the synthesis of cytokines within the cytosol where generated and in an auto- and paracrine fashion upon release into the interstitium (Chaughey et al., 1997; Poubelle et al., 1991; Rola-Pleszczynski and Lemaire, 1985). Then, interleukin-1β and tumor necrosis factor-α are secreted which further stimulate synthesis and activity of cytosolic phospholipase A_2 in neighbour cells (Clark et al., 1995). Referring to this amplification loop, we showed that exogenous lipid metabolites induced interleukin-1β mRNA transcription in naive mononuclear cells, in concert with lipopolysaccharide superinduced interleukin-1β protein release into the medium and partially reconstituted interleukin-1β gene transcription in inhibitor-treated cells. Leukotriene B_4 and platelet-activating factor were most effective in paracrine induction of interleukin-1β mRNA and protein presumably by signalling through their high-affinity G-protein-coupled receptors on plasma membranes of monocytes. However, in reconstitution experiments single lipids like leukotriene B_4 alone were ineffective. Instead, both downstream products of the eicosanoid- and lyso-phospholipid-pathways were necessary to yield the maximal recovery, i.e., arachidonic acid plus platelet-activating factor. Still, interleukin-1β synthesis could not be fully reconstituted to the level of lipopolysaccharide-challenged cells. This may be explained by the fact that not the full range from the spectrum of bioactive lipids (e.g., lyso-phosphatids, lipoxins, thromboxanes, prostacyclin, hydroxy/peroxy-fatty acids) was offered which are generated upon phospholipase A_2 activation and are strong activators of cytokine synthesis as well (Chaughey et al., 1997; Gormand et al., 1996; Spangelo and Jarvis, 1996). Moreover, exogenous lipid mediators themselves promote restimulation of synthesis and activity of cytosolic phospholipase A_2 and consequently restimulate their own endogenous synthesis (Murakami et al., 1997a; Wong et al., 1998). This positive feedback loop is interrupted in inhibitor-treated cells. Therefore, paracrine addition of lipids will be less effective to reconstitute interleukin-1β transcription in inhibitor-treated cells than to induce transcription in untreated cells with intact amplification mechanisms. In contrast, nuclear-factor-κB DNA-binding was completely reconstituted by the lipids to the stimulated level. Concordantly, IC_{50} values for cytokine mRNA suppression were higher than for nuclear factor-κB inhibition. This emphasizes that nuclear factor-κB inhibition is not the sole target for the inhibitors. Instead, the profound actions of the inhibitors may be achieved by addressing multiple targets from the rel-, jun/fos- and CCAAT/enhancer binding protein-families of lipid-regulated transcription factors. This may be in line with the pleiotropic actions of acetylsalicylate. Acetlysalicylate in very high millimolar ($\leq$ 200 mM) concentrations interacts with peroxisome proliferator-activated receptors and prevents inhibitor-κBα degradation and nuclear factor-κB mobilization by inhibition of inhibitor-κBβ kinase and mitogen-activated kinases (Osnes et al., 1996; Pillinger et al., 1998; Yin et al., 1998). Here, it was found that acetylsalicylate at lower micromolar concentrations was either ineffective or even enhanced interleukin-1β synthesis. We propose that at low concentrations acetylsalicylate prevents prostaglandin E_2 production, an endogenous suppressor of interleukin-1β synthesis, only by classical inhibition of cyclooxygenases failing to exert additional actions. Concomitantly, formation of leukotrienes and other bioactive lipids which induce interleukin-1β synthesis is increased by redirection/shunting of precursor lipids into alternative pathways. Thus, only the interference with the key enzyme cytosolic phospholipase A_2 may short-cut this divergent inflammatory cascade, a benefit which is not accomplished by classical non-steroidal anti-inflammatory drugs (Pettipher and Wimbery, 1994). Finally, due to the lack of phospholipid mediator formation, the transcription factors nuclear factor-κB, -interleukin-6 and activation protein-1 remain inactive, which are otherwise indispensable for expression of genes involved in induction and maintenance of inflammation like interleukin-1, -6, -8, tumor-necrosis factor-α (Poubelle et al., 1991; Spangelo and Jarvis, 1996), Mac-1 (CD11b/CD18) (Amandi-Burgermeister et al., 1997) and collagenases (Mehindate et al., 1995). Consequently, a novel therapy of chronic inflammation may be constituted by modulation of transcription factor activity using cytosolic phospholipase A_2 inhibitors.

Acknowledgements

We are grateful to W. Kinle and A. Schmid for excellent technical assistance. We want to thank K. Bauer and M. Frankenberger for helpful technical advice and W.-G. Friebe for synthesis and continual supply of compounds. We highly appreciate the encouraging discussion and advice from P.C. Heinrich.

References

Amandi-Burgermeister, E., Tibes, U., Kaiser, B.M., Friebe, W.G., Scheuer, W.V., 1997. Suppression of cytokine synthesis, integrin expression and chronic inflammation by inhibitors of cytosolic phospholipase A_2. Eur. J. Pharmacol. 326, 237–250.

Baker, R.R., Chang, H.Y., 1996. Alkylglycerolphosphate acetyltransferase and lyso-platelet-activating factor acetyltransferase, two key enzymes in the synthesis of platelet-activating factor are found in neuronal nuclei isolated from cerebral cortex. Biochim. Biophys. Acta 1302, 257–263.

Baldwin, A.S., 1996. The nuclear factor-κB and inhibitor-κB proteins: new discoveries and insights. Annu. Rev. Immunol. 14, 649–681.

Bartoli, F., Lin, H.-K., Ghomashchi, F., Gelb, M.H., Jain, M.K., Apitz-Castro, R., 1994. Tight binding inhibitors of 85-kDa phospholipase

A_2 but not 14-kDa phospholipase A_2 inhibit relese of free arachidonic acid in thrombin-stimulated human platelets. J. Biol. Chem. 269, 15625–15630.

Boehm, J.C., Smietana, J.M., Sorenson, M.E., Garigipati, R.S., Gallagher, T.F., Sheldrake, P.L., Bradbeer, J., Badger, A.M., Laydon, J.T., Lee, J.C., Hillegass, L.M., Griswold, D.E., Breton, J.J., Chabot-Fletcher, M.C., Adams, J.L., 1996. 1-substituted 4-aryl-5-pyridinylimidazoles: a new class of cytokine suppressive drugs with low 5-lipoxygenase and cyclooxygenase inhibitory potency. J. Med. Chem. 39, 3929–3937.

Brach, M.A., de Vos, S., Arnold, C., Gruß, H.J., Mertelsmann, R., Herrmann, F., 1992. Leukotriene B_4 transcriptionally activates interleukin-6 expression involving nuclear factor-κB and nuclear factor-interleukin-6. Eur. J. Immunol. 22, 2705–2711.

Camandola, S., Leonarduzzi, G., Musso, T., Varesio, L., Carini, R., Scavazza, A., Chiarpotto, E., Baeuerle, P.A., Poli, G., 1996. Nuclear factor-κB is activated by arachidonic acid but not by eicosapentaenoic acid. Biochem. Biophys. Res. Commun. 229, 643–647.

Camussi, G., Mariano, F., DeMartino, A., Bussolati, B., Montrucchio, G., Tobias, P.S., 1995. Lipopolysaccharide binding protein and CD14 modulate the synthesis of platelet-activating factor by human monocytes and mesangial and endothelial cells stimulated with lipopolysaccharide. J. Immunol. 155, 316–320.

Chaughey, G.E., Pouliot, M., Cleland, L.G., James, M.J., 1997. Regulation of tumor necrosis factor-α and interleukin-1β synthesis by thromboxane A_2 in non-adherent human monocytes. J. Immunol. 158, 351–358.

Clark, J.D., Schievella, A.R., Nalefski, E.A., Lin, L.-L., 1995. Cytosolic phospholipase A_2. J. Lipid Mediators Cell Signalling 12, 83–117.

Cogswell, J.P., Godlevski, M.M., Wisely, G.B., Clay, W.C., Leesnitzer, L.M., Ways, J.P., Gray, J.G., 1994. Nuclear factor-κB regulates interleukin-1β transcription through a consensus nuclear factor-κB binding site and a nonconsensus CRE-like site. J. Immunol. 153, 712–723.

Danesch, U., Weber, P.C., Sellmayer, A., 1994. Arachidonic acid increases c-*fos* and Egr-1 mRNA in 3T3 fibroblasts by formation of prostaglandin E_2 and activation of protein kinase C. J. Biol. Chem. 269, 27258–27263.

Dorn, A., Bollekens, J., Staub, A., Benoist, C., Mathis, D., 1987. A multiplicity of CCAAT box-binding proteins. Cell 50, 863–872.

Dwason, J., Hurthenbach, U., MacKenzie, A., 1996. Cyclosporine A inhibits the in vivo production of interleukin-1β and tumor necrosis factor-α but not of interleukin-6 by a T-cell independent mechanism. Cytokine 8, 882–888.

Frankenberger, M., Pforte, A., Sternsdorf, T., Passlick, B., Baeuerle, P.A., Ziegler-Heitbrock, H.W.L., 1994. Constitutive nuclear factor-κB in cells of the monocyte lineage. Biochem. J. 304, 87–94.

Gormand, F., Chabannes, B., Moliere, P., Perrinfayolle, M., Lagarde, M., Pacheco, Y., 1996. Uptake of 12-hydroxyeicosatetraenoic acid (12-HETE) by human bronchial epithelial cells (HBEC): effects on HBEC cytokine production. Prostaglandins 51, 263–273.

Gray, J.G., Chandra, G., Clay, W.C., Stinnett, S.W., Haneline, S.A., Lorenz, J.J., Patel, I.R., Wiseley, G.B., Furdon, P.J., Taylor, J.D., Kost, T.A., 1993. A CRE/ATF like site in the upstream regulatory sequence of the human interleukin-1β gene is necessary for induction in U937 and THP-1 monocytic cell lines. Mol. Cell. Biol. 13, 6678–6689.

Israel, A., 1997. Inhibitor-κB kinase all zipped up. Nature 388, 519–521.

Jiang, C., Ting, A.T., Seed, B., 1998. Peroxisome proliferation-activated receptor-γ agonists inhibit production of monocyte inflammatory cytokines. Nature 391, 82–86.

Kaltschmidt, C., Kaltschmidt, B., Henkel, T., Stockinger, H., Baeuerle, P.A., 1995. Selective recognition of the activated form of transcription factor nuclear factor-κB by a monoclonal antibody. Biol. Chem. Hoppe-Seyler 376, 9–16.

Kaminski, W.E., Jendrasch, E., Baumann, K., Kiefl, R., Fischer, S., Marcus, A.J., Broekman, M.J., von Schacky, C., 1996. Human mononuclear cells express 12-lipoxygenase: coordinated mRNA regulation with 5′-lipoxygenase and 5′-lipoxygenase-activating protein genes. Blood 87, 331–340.

Kravchenko, V.V., Pan, Z.X., Han, J.H., Herbert, J.M., Ulevitch, R.J., Ye, R.D., 1995. Platelet-activating factor induces nuclear factor-κB activation through a G-protein coupled pathway. J. Biol. Chem. 270, 14928–14934.

Lee, S.H., Soyoola, E., Chanmugam, P., Hart, S., Sun, W., Zhong, H., Liou, S., Simmons, D., Hwang, D., 1992. Selective expression of mitogen-inducible cyclooxygenase in macrophages stimulated with lipopolysaccharide. J. Biol. Chem. 267, 25934–25938.

Lo, C.J., Cryer, H.G., Fu, M.J., Kim, B., 1997. Endotoxin-induced macrophage gene expression depends on platelet-activating factor. Arch. Surgery 132, 1342–1347.

Los, M., Schenk, H., Hexel, K., Baeuerle, P.A., Dröge, W., Schulze-Osthoff, K., 1995. Interleukin-2 gene expression and nuclear factor-κB activation through CD28 requires reactive oxygene production by 5-lipoxygenase. EMBO J. 14, 3731–3740.

Mehindate, K., Al-Daccak, R., Dayer, J.-M., Kennedy, B.R., Kris, C., Borgeat, P., Poubelle, P.E., Mourad, W., 1995. Superantigen-induced collagenase gene expression in human interferon-γ treated fibroblast-like synoviocytes involves prostaglandin E_2: evidence for a role of cyclooxygenase-2 and cytosolic phospholipase A_2. J. Immunol. 155, 3570–3577.

Meyer, M., Schreck, R., Baeuerle, P.A., 1993. H_2O_2 and antioxidants have opposite effects on activation of nuclear factor-κB and activation protein-1 in intact cells: activation protein-1 as secondary antioxidant-responsive factor. EMBO J. 12, 2005–2015.

Milam, S.B., Magnuson, V.L., Steffensen, B., Chen, D., Klebe, R.J., 1991. Interleukin-1β and prostaglandins regulate integrin mRNA expression. J. Cell. Physiol. 149, 173–183.

Müller, G., Ayoub, M., Storz, P., Rennecke, J., Fabbro, D., Pfizenmaier, K., 1995. Proteinkinase C-ζ is a molecular switch in signal transduction of tumor necrosis factor-α, bifunctionally regulated by ceramide and arachidonic acid. EMBO J. 14, 1961–1969.

Murakami, M., Kuwata, H., Amakasu, Y., Shimbara, S., Nakatani, Y., Atsumi, G., Kudo, I., 1997a. Prostaglandin E_2 amplifies cytosolic phospholipase A_2 and cyclooxygenase-2 dependent delayed prostaglandin E_2 generation in mouse osteoblastic cells. J. Biol. Chem. 272, 19891–19897.

Murakami, M., Nakatani, Y., Atsumi, G., Inoue, K., Kudo, I., 1997b. Regulatory functions of phospholipase A_2. Crit. Rev. Immunol. 17, 225–283.

Murakami, M., Shimbara, S., Kambe, T., Kuwata, H., Winstead, M.V., Tischfield, J.A., Kudo, I., 1998. The functions of five distinct mammalian phospholipase A_2s in regulating arachidonic acid release. J. Biol. Chem. 273, 14411–14423.

Osnes, L.T.N., Foss, K.B., Joo, G.B., Okkenhaug, C., Westvik, A.B., Ostebo, R., Kierulf, P., 1996. Acetylsalicylic acid and sodium salicylate inhibit lipopolysaccharide-induced nuclear factor-κB/c-rel nuclear translocation and synthesis of tissue factor and tumor necrosis factor-α in human monocytes. Thrombosis and Haemostasis 76, 970–976.

Peters-Golden, M., Song, K., Marshall, T., Brock, T., 1996. Translocation of cytosolic phospholipase A_2 to the nuclear envelope elicits topographically localized phospholipid hydrolysis. Biochem. J. 318, 797–803.

Pettipher, E.R., Wimbery, D.J., 1994. Cyclooxygenase inhibitors enhance tumor necrosis factor production and mortality in murine endotoxic shock. Cytokine 6, 500–503.

Pillinger, M.H., Capodici, C., Rosenthal, P., Kheterpal, N., Hanft, S., Philips, M.R., Weissmann, G., 1998. Modes of action of aspirin-like drugs: salicylate inhibit erk activation and integrin-dependent neutrophil adhesion. Proc. Natl. Acad. Sci U.S.A. 95, 14540–14545.

Poubelle, P.E., Gingras, D., Demers, C., Dubois, C., Harbour, D., Grassi, J., Rola-Pleszczynski, M., 1991. Platelet-activating factor-acether enhances the concomitant production of tumor necrosis factor-α and

interleukin-1 production by subsets of human monocytes. Immunology 72, 181–187.

Pouliot, M., McDonald, P.P., Krump, E., Mancini, J.A., McColl, S.R., Weech, P.K., Borgeat, P., 1996. Colocalization of cytosolic phospholipase A_2, 5-lipoxygenase and 5-lipoxygenase-activating protein at the nuclear membrane of A23187-stimulated human neutrophils. Eur. J. Biochem. 238, 250–258.

Riendeau, D., Guay, J., Weech, P.K., Laliberte, F., Yergey, J., Li, C., Desmarais, S., Perrier, H., Liu, S., Nicoll-Griffith, D., Street, I.P., 1994. Arachidonyl trifluoromethylketone a potent inhibitor of 85-kDa phospholipase A_2 blocks production of arachidonic acid and 12-hydroxyeicosatetraenoic acid by calcium ionophore challenged platelets. J. Biol. Chem. 269, 15619–15624.

Rizzo, M.T., Boswell, H.S., Mangoni, L., Carlo-Stella, C., Rizzoli, V., 1995. Arachidonic acid induces c-*Jun* gene expression in stromal cells stimulated by interleukin-1 and tumor necrosis factor-α: evidence for a tyrosine-kinase-dependent process. Blood 86, 2967–2975.

Rodewald, E., Tibes, U., Maas, G., Scheuer, W., 1994. Induction of cytosolic phospholipase A_2 in human leukocytes by lipopolysaccharide. Eur. J. Biochem. 223, 743–749.

Rola-Pleszczynski, M., Lemaire, I., 1985. Leukotrienes augment interleukin-1 production by human monocytes. J. Immunol. 135, 3958–3961.

Rola-Pleszczynski, M., Stankova, J., 1992. Leukotriene B_4 enhances interleukin-6 production and interleukin-6 messenger RNA accumulation in human monocytes in vitro: transcriptional and posttranscriptional mechanisms. Blood 80, 1004–1011.

Roshak, A., Sathe, G., Marshall, L., 1994. Suppression of monocyte 85-kDa phospholipase A_2 by antisense and effects on endotoxin-induced prostaglandin biosynthesis. J. Biol. Chem. 269, 25999–26006.

Rossi, A., Elia, G., Santoro, M.G., 1997. Inhibition of nuclear factor-κB by prostaglandin A_1: an effect associated with heat shock transcription factor activation. Proc. Natl. Acad. Sci. U.S.A. 94, 746–750.

Serhan, C.N., Haeggstrom, J.Z., Leslie, C.C., 1996. Lipid mediator networks in cell signalling: update and impact of cytokines. FASEB J. 10, 1147–1158.

Sermon, B.A., Eccleston, J.F., Skinner, R.H., Lowe, P.N., 1996. Mechanism of inhibition by arachidonic acid of the catalytic activity of ras GTP-ase activating proteins. J. Biol. Chem. 271, 1566–1572.

Shamsuddin, M., Chen, E., Anderson, J., Smith, L.J., 1997. Regulation of leukotriene and platelet-activating factor synthesis in human alveolar macrophages. J. Lab. Clin. Med. 130, 615–626.

Shirakawa, F., Saito, K., Bonagura, C.A., Galson, D.L., Fenton, M.J., Webb, A.C., Auron, P.E., 1993. The human prointerleukin-1β gene requires DNA Sequences both proximal and distal to the transcription start site for tissue-specific induction. Mol. Cell. Biol. 13, 1332–1344.

Song, I., Smith, W.L., 1996. C-terminal Ser/Pro–Thr–Glu–Leu tetrapeptides of prostaglandin endoperoxide H synthases-1 and -2 targets the enzymes to the endoplasmatic reticulum. Arch. Biochem. Biophys. 334, 67–72.

Spangelo, B.L., Jarvis, W.D., 1996. Lysophosphatidylcholine stimulates interleukin-6 release from rat anterior pituitary cells in vitro. Endocrinology 137, 4419–4426.

Street, I.P., Lin, H.-K., Laliberte, F., Ghomashchi, F., Wang, Z., Perrier, H., Tremblay, N.M., Huang, Z., Weech, P.K., Gelb, M.H., 1993. Slow- and tight-binding inhibitors of the 85-kDa human phospholipase A_2. Biochemistry 32, 5935–5940.

Surette, M.E., Nadeau, M., Borgeat, P., Gosselin, J., 1996. Priming of human peripheral blood mononuclear cells with lipopolysaccharides for enhanced arachidonic acid release and leukotriene synthesis. J. Leukocyte Biol. 59, 709–715.

Tibes, U., Vondran, A., Rodewald, E., Friebe, W.-G., Schäfer, W., Scheuer, W., 1995. Inhibition of allergic and non-allergic inflammation by phospholipase A_2 inhibitors. Int. Arch. Allergy Immunol. 107, 432–434.

Tibes, U., Scheuer, W.V., Thierse, H.-J., Burgermeister, E., Schramm, S., Friebe, W.-G., Diez, E., 1997. Role of cytosolic phospholipase A_2, secretory phospholipase A_2 and nitric oxide synthase in inflammation. In: Uhl, W., Nevalainen, T.J., Büchler, M.W. (Eds.), Progress in Surgery: Phospholipase A_2—Basic and Clinical Aspects in Inflammatory Diseases, Vol. 24. Karger, Basel, pp. 153–167.

Tournier, C., Thomas, G., Pierre, J., Jaquemin, C., Pierre, M., Saunier, B., 1997. Mediation by arachidonic acid metabolites of the H_2O_2-induced stimulation of mitogen-activated protein kinases (extracellular-signal-regulated kinase and c-*Jun* NH_2-terminal kinase). Eur. J. Biochem. 244, 587–595.

Tsukada, J., Waterman, W.R., Koyama, Y., Webb, A.C., Auron, P.E., 1996. A novel STAT-like factor mediates lipopolysaccharide, interleukin-1 and interleukin-6 signaling and recognizes a interferon-γ activation site-element in the interleukin-1β gene. Mol. Cell. Biol. 16, 2183–2194.

Uozumi, N., Kume, K., Nagase, T., Nakatani, N., Ishii, S., Tashiro, F., Komagata, Y., Maki, K., Ikuta, K., Ouchi, Y., Miyazaki, J.-I., Shimizu, T., 1997. Role of cytosolic phospholipase A_2 in allergic response and parturition. Nature 390, 618–622.

Wijkander, J., O'Flaherty, J.T., Nixon, A.B., Wykle, R.L., 1995. 5′-Lipoxygenase products modulate the activity of the 85-kDa phospholipase A_2 in human neutrophils. J. Biol. Chem. 270, 26543–26549.

Withnall, M.T., Pennington, A., Wiseman, D., 1995. Characterization of cytosolic phospholipase A_2 as mediator of enhanced arachidonic acid release from dimethylsulfoxide-differentiated U937 cells. Biochem. Pharmacol. 50, 1893–1902.

Wong, T.J., Tran, K., Pierce, G.N., Chan, A.C., O, K., Choy, P., 1998. Lysophosphatidylcholine stimulates the release of arachidonic acid in human endothelial cells. J. Biol. Chem. 273, 6830–6836.

Yin, M.J., Yamamoto, Y., Gaynor, R.B., 1998. The anti-inflammatory agents aspirin and salicylate inhibit the activity of inhibitor-κB kinase-β. Nature 396, 77–80.

Zhong, W.W., Burke, P.A., Drotar, M.E., Chavali, S.R., Forse, R.A., 1995. Effects of prostaglandin E_2, cholera toxin and 8-bromo-cyclic AMP on lipopolysaccharide-induced gene expression of cytokines in human macrophages. Immunology 84, 446–452.

Ziegler-Heitbrock, H.W.L., Sternsdorf, T., Liese, J., Belohradsky, B., Weber, C., Wedel, A., Schreck, R., Bäuerle, P., Ströbel, M., 1993. Pyrrolidine dithiocarbamate inhibits nuclear factor-κB mobilization and tumor necrosis factor production in human monocytes. J. Immunol. 151, 6986–6993.