ELSEVIER

European Journal of Pharmacology 388 (2000) 195–208

ejp

www.elsevier.nl/locate/ejphar

Inhibition of cytosolic phospholipase A_2 attenuates activation of mitogen-activated protein kinases in human monocytic cells

Elke Burgermeister [a], Ulrich Pessara [b], Ulrich Tibes [c], Andrea Küster [d], Peter C. Heinrich [d], Werner V. Scheuer [a,*]

[a] *Department of Molecular Pharmacology, Roche Diagnostics, Nonnenwald. 2, D-82372 Penzberg, Germany*
[b] *Department of Molecular Medicine, Roche Diagnostics, Nonnenwald. 2, D-82372 Penzberg, Germany*
[c] *Department of Preclinical Research, Roche Diagnostics, Sandhoferstr. 30, D-68305 Mannheim, Germany*
[d] *Department of Biochemistry, RWTH Aachen, Pauwelstr. 30, D-52074 Aachen, Germany*

Received 22 July 1999; received in revised form 10 November 1999; accepted 16 November 1999

Abstract

Eicosanoids and platelet-activating factor generated upon activation of cytosolic phospholipase A_2 enhance activity of transcription factors and synthesis of proinflammatory cytokines. Here, we show that selective inhibitors and antisense oligonucleotides against this enzyme suppressed expression of the interleukin-1β gene at the level of transcription and promoter activation in human monocytic cell lines. This inhibitory effect was due to failure of activation of mitogen-activated protein kinases (MAPK) through phosphorylation by upstream mitogen-activated protein kinase kinases (MKK). Consequently, phosphorylation and degradation of inhibitor-κBα (I-κBα) and subsequent cytoplasmic mobilization, DNA-binding and the transactivating potential of nuclear factor-κB (NF-kB), nuclear factor-interleukin-6 (NF-IL6), activation protein-1 (AP-1) and signal-transducer-and-activator-of-transcription-1 (STAT-1) were impaired. It is concluded, that lipid mediators promote activation of MAPKs, which in turn lead to phosphorylation and liberation of active transcription factors. Since inhibition of cytosolic phospholipase A_2 ameliorates inflammation in vivo, this potency may reside in interference with the MAPK pathway.

Keywords: Phospholipase A_2; Nuclear-factor-κB; Mitogen-activated protein kinase; Interleukin-1; Inflammation

1. Introduction

In monocytic cells, multiple proinflammatory stimuli initiate the posttranslational activation by phosphorylation and subsequent translocation of cytosolic phospholipase A_2 to cellular membranes. The pivotal role of this 85-kDa enzyme in the cleavage of glycerophosphatides into arachidonic acid and lyso-phospholipids and the generation of eicosanoids and platelet-activating factor has been well established (Clark et al., 1995; Murakami et al., 1997a). In addition to proinflammatory actions mediated by binding to G-protein-coupled receptors on plasma membranes (Cunningham, 1994) these phospholipid derivatives enhance the activity of endogenous signaling molecules like MAPKs by acting as second messengers (Tournier et al., 1997; Hii et al., 1998; Yamakawa et al., 1998). Members of this serine-/threonine-kinase family including the extracellular signal-regulated kinases-1/-2 (ERK-1/-2), stress-activated p38 and c-*jun* N-terminal kinases (JNK) activate NF-κB (Baeuerle, 1998), AP-1 (Whitmarsh and Davis, 1996), NF-IL6 and STAT-1 (Akira, 1997). Thus, by affecting the phosphorylation status of essential transcription factors arachidonic acid (Danesh et al., 1994; Camandola et al., 1996), platelet-activating factor (Kravchenko et al., 1995) and leukotriene B_4 (Brach et al., 1992) positively or prostaglandins negatively (Rossi et al., 1997) influence the synthesis of the proinflammatory cytokines interleukin-1, interleukin-6 and tumor necrosis factor-α (Rola-Pleszczynski and Lemaire, 1985; Poubelle et al., 1991), adhesion molecules (Thommensen et al., 1998) and matrix metalloproteinases (Shankavaram et al., 1998). Consequently, inhibition of cytosolic phospholipase A_2

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

0014-2999/00/$ - see front matter
PII: S0014-2999(99)00816-X

should impair expression of genes coding for these proinflammatory proteins and ameliorate inflammation in vivo.

In order to verify this hypothesis three different strategies have been applied to abrogate the activity of cytosolic phospholipase A_2 in vitro and in vivo: (a) targeted disruption of the gene in knock-out mice (Uozumi et al., 1997), (b) antisense oligonucleotides against its mRNA (Roshak et al. 1994) and (c) low-molecular-weight inhibitors exemplified by the trifluoromethylketone analogues of arachidonic (C20:4-$COCF_3$), γ-linolenic (C18:3-$COCF_3$) and linoleic acid (C18:2-$COCF_3$). The latter compounds represent specific, reversible inhibitors of the catalytic S^{228} residue within the enzyme's active site (Street et al., 1993). All these approaches resulted in a reduction of stimulus-mediated release of arachidonic acid and lipid mediators from a variety of cell types (Riendeau et al., 1994; Withnall et al., 1995; Murakami et al., 1997a). However, it remains to be shown if this phospholipase A_2 is also responsible for the generation of intracellular second messengers in inflammatory cells. Referring to this, we found that beyond their primary effects on phospholipid metabolism these inhibitors suppressed the synthesis of interleukin-1β in human peripheral blood $CD14^+$monocytes and chronic inflammation in vivo (Amandi-Burgermeister et al., 1997; Burgermeister et al., 1999). In the present study, we therefore investigated the subcellular mechanisms by which the trifluoromethylketones attenuate expression of the interleukin-1β gene in human monocytic cells.

We show that inhibition of cytosolic phosholipase A_2 prevented activation of the MAPKs. This mode of action resulted in retention of inactive transcription factors in the cytosol and failure of activation of the interleukin-1β promoter. These data indicated an important function of cytosolic phospholipase A_2 in signal transduction and inflammation.

2. Materials and methods

2.1. Reagents

Trifluoromethylketones (Roche Diagnostics) (Fig. 1), pyrolidindithiocarbamate (Sigma) and acetylsalicylate (Sigma) were stored 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 dimethylsulfoxid 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, C16:0-platelet activating factor and prostaglandin E_2. Leukotriene B_4 was a 150-μM solution in ethanol. Further serial 10-fold dilutions were done in serum-free RPMI-1640 cell culture medium and instantly added to cells yielding a final ethanol concentration $< 0.1\%$.

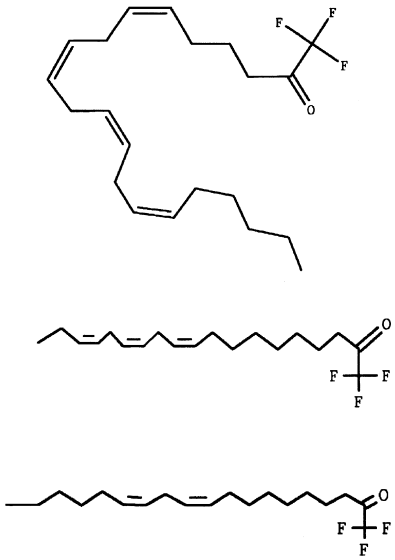

Fig. 1. Inhibitors of cytosolic phospholipase A_2. (Top) C20:4-$COCF_3$ = 1,1,1-Trifluoro-heneicosa-6,9,12,15-tetraen-2-one, $M_r = 356$; (Middle) C18:3-$COCF_3$ = 1,1,1-Trifluoro-nonadeca-10,13,16-trien-2-one, $M_r = 330$; (Bottom) C18:2-$COCF_3$ = 1,1,1-Trifluoro-nonadeca-10,13-dien-2-one, $M_r = 332$.

2.2. Cell lines

U937 and THP-1 cell lines were obtained from the American Tissue Culture Collection (Rockville, USA) and cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (Seromed Biochrom), 2 mM glutamine (Gibco), 100 IU/ml penicillin and 20 μg/ml streptomycin (Roche) in a humidified atmosphere at 37°C and 7.5% CO_2. Cells were cultivated in tissue culture flasks and polystyrole 96-well plates (Greiner). MonoMac6 cells were kindly provided by H.W.L. Ziegler-Heitbrock (Munich, Germany) and cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 9 μg/ml insulin, 1 mM oxalacetate, 1 mM pyruvate (Sigma), 1% ''minimal essential medium''-non-essential amino acids (Seromed Biochrom), 100 IU/ml penicillin and 20 μg/ml streptomycin. Cells were stimulated with 20 ng/ml phorbol-12-myristate-13-acetate (PMA) (Sigma), 0.5 μg/ml Ca^{2+}-ionophore A23187 (Roche Diagnostics), 1 μg/ml lipopolysaccharide (Sigma) or 5 U/ml of recombinant interferon-γ (Roche Diagnostics) as described in the result section and legends to figures.

2.3. Reporter DNA construction and stable transfection of THP-1 cell line

Upstream regulatory control sequences of the human pro-interleukin-1β gene (−3747/+12 bp) (Auron and Webb, 1994) were obtained by polymerase chain reaction from the plasmid pIL1 (interleukin-1) (kindly provided by G. Bensi, Siena, Italy) using 5′-primer (5′-CGC GCG GGA

TCC *GAA TTC* GGA GGA GAA TGG AAT GTC CCT TGG-3′) and 3′-primer (5′-CGC GCG GGA TCC *GAA TTC* CGA AGA GGT TTG GTA TCT GCC AGT TTC-3′). Resulting 3.7 kb fragments were digested with enzyme *Eco*RI and ligated 5′ to the β-galactosidase gene in the reporter plasmid pNASSβ (Clontech). Clones were analysed by restriction cut analysis and DNA sequencing. Plasmid DNA was cotransfected with pHMR272 (hygromycin resistance) into THP-1 cells using electroporation. Clones were selected with 1 mg/ml hygromycin B (Roche Diagnostics) for stable integration of plasmids and with β-galactosidase assay for functional expression of the interleukin-1β/β-galactosidase cassette. For removal of mutants, transfected THP-1 cells were cultured frequently in presence of 1 mg/ml hygromycin B.

2.4. β-Galactosidase assay

Stably transfected THP-1 cells (1×10^6 per ml) were stimulated in round-bottom 96-well plates (Nunc). Supernatants were removed after centrifugation and 150 μl of substrate solution were added to cell pellets containing 3.29 mM chlorophenol red β-D-galactopyranoside (Roche Diagnostics) in 100 mM HEPES, 150 mM NaCl, 2 mM $MgCl_2$, 1% bovine serum albumin (Roche Diagnostics), 0.1% Triton X-100 (Sigma), 0.1% sodium azide adjusted to pH 7.0. Plates were developed at 37°C for 1 h. Activity of β-galactosidase was quantified photometrically as optical density at 570 nm. Alternatively, total β-galactosidase protein was determined in crude lysates from 1×10^7 THP-1 cells by immunoassay (Roche Diagnostics) according to the manufacturer's instructions.

2.5. Transient transfection of reporter plasmids

The reporter plasmids pCMV (cytomegalovirus), 4× NF-κB-pBL2, pBL2 and pBL3 were from Roche Diagnostics (Mihm et al., 1991). Cells were seeded in serum-free RPMI-1640 cell culture medium at a density of 1×10^6 per ml in 6-well plates. Transient transfection was performed by lipofection with *N*-[(1-(2,3-Dioleoyloxy) propyl]-*N*, *N*, *N*-trimethyl-ammonium-methylsulfate (DOTAP) (Roche Diagnostics) and reporter plasmids according to the manufacturer's instructions. After 6 h cells were harvested and recovered in complete RPMI-1640 medium supplemented with 10% fetal calf serum and cultured for 24 h. Thereafter, cells were preincubated with the compounds and stimulated as described in the Results section. After 24 h cells were harvested and cell lysates were assayed for expression of the reporter protein chloramphenicol acetyltransferase using immunoassays according to the manufacturer's instructions (Roche Diagnostics).

2.6. Antisense studies with phosphorothionate oligonucleotides

U937 cells (5×10^6 per ml) were washed twice with phosphate-buffered saline (Roche Diagnostics) and seeded in round-bottom 96-well plates in serum-free RPMI-1640 cell culture medium. Phosphorothionate-modified single-stranded DNA-oligo-nucleotides against the translational initiation site within the mRNA of cytosolic phospholipase A_2 (+140/+160 bp) (Roshak et al., 1994) were synthesized and purified by Biometra.

Antisense: 5′-GTA AGG ATC TAT AAA TGA CAT-3′
Sense: 5′-ATG TCA TTT ATA GAT CCT TAC-3′

Oligonucleotides were diluted in serum-free RPMI-1640 medium and added to the cells at concentrations of 0.1 to 10 μM for 6 h. Thereafter, 5% fetal calf serum was supplemented and the cells were further incubated for 18 h. Cells were stimulated with 50 ng/ml PMA and 1 μg/ml lipopolysaccharide for another 24 h. Plates were centrifuged and interleukin-1β was quantified in the supernatants by immunoassay (Roche Diagnostics) according to the manufacturer's instructions.

2.7. Detection of p65 subunits of NF-κB by flow cytometry

Cells 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 and 0.1% Triton-X 100 for 10 min on ice. Unspecific binding sites were blocked with 50 μg/ml goat immunoglobulin G (IgG) and 50% fetal calf serum for 15 min. Cells were stained with 10 μg/ml anti-p65 monoclonal antibody (mouse IgG3, Roche Diagnostics), anti-pan histone (mouse IgG1, Roche Diagnostics) and isotype-matched control antibodies (Biozol) in phosphate-buffered saline with 10% fetal calf serum for 1 h followed by 30 μg/ml fluorescein-conjugated AffiniPure goat anti-mouse IgG $F(ab')_2$ fragment (Dianova) for 30 min. Cells were subsequently analyzed using a fluorescence-activated cell sorter (FACScan, Becton Dickinson).

2.8. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using Tripure Reagent (Roche Diagnostics). Serial 10-fold dilutions of 100 ng total RNA were reverse transcribed using the 5′-downstream primer (Biometra) specific for β-galactosidase according to the manufacturer's instructions (Geneamp-Thermostable rTh-Reverse-Transcriptase RNA PCR Kit). Resulting cDNAs were amplified by PCR (35 cycles) using the 3′-upstream primers:

5′-primer: 5′-TAA CGA CAT TGG CGT AAG TG-3′
3′-primer: 5′-AAT CCG AGC CAG TTT ACC CG-3′

Equal RNA content was confirmed by using primers specific for β-actin (Stratagene). Amplification products were separated by ethidiumbromide agarose gel electrophoresis. Band intensity in gel was estimated by video-based den-

sitometry (EasyPlus, device by Herolab). Results were calculated from arbitrary optical density units (see Results section).

2.9. Preparation of nuclear extracts

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 phenylmethyl-sulfonylfluoride and 5 mM dithiothreitol (Roche Diagnostics) for 15 min on ice. Cells were lysed by sonication ($1–2 \times 3$ s) on ice. Free nuclei were recovered by centrifugation in a microcentrifuge (7000 rpm, 10 min) and extracted in 50–100 μl (according to 4-fold 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 bovine serum albumin as a standard (Pierce) and protein assay reagent from Biorad.

2.10. Electrophoretic mobility shift assay

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

NF-κB site from the murine Ig-κ light chain enhancer (Los et al., 1995):
5′-AGC TTC AGA GGG GAT TTC CGA GAG G-3′
NF-IL6 site from the human pro-interleukin-1β enhancer (−2882/−2869 bp) (Shirakawa et al., 1993):
5′-TCT GAT ACA TAC GTT GCA CAA CCT-3′
AP-1 site (Meyer et al., 1993):
5′-TTC CGG CTG ACT CAT C-3′
NF-Y site (Dorn et al., 1987):
5′-ATT TTT CTG ATT GGT TAA-3′
STAT-1 site within the sis-inducible element from the human c-*fos* promoter (Wagner et al., 1990):
5′-GAT TGA CGG GAA CTG-3′

Single-stranded DNA-oligonucleotides (Biometra) were annealed and 50 pmol were labelled by fill-in reaction with α[^{32}P]dATP using Klenow fragment of DNA Polymerase I (Roche Diagnostics). Unincorporated nucleotides were removed by centrifugation through Sepharose G-50 using Nick-Spun-columns (Pharmacia). The gel-shift reaction for NF-κB was set up in 20 μl with 50.000 cpm of [^{32}P] labelled oligonucleotide (about 250 fmol in TEN-200 buffer), 5–10 μg of nuclear extract, 2 μg poly-dI-dC (Roche Diagnostics) as an unspecific competitor, 20 μg bovine serum albumin (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. Accordingly, for NF-IL6 0.5 μg poly-dI-dC, 10× binding buffer (100 mM Tris–HCl, pH 7.5, 500 mM NaCl, 10 mM EDTA, 50% glycerol), 15% glycerol and 20 μg bovine serum albumin, for AP-1 2 μg poly-dI-dC, 5× binding buffer (50 mM Tris–HCl, pH 7.5, 125 mM NaCl, 25 mM $MgCl_2$, 5 mM EDTA, 50% glycerol) and 20 μg bovine serum albumin, for NF-Y 2 μg poly-dI-dC, 10× binding buffer (100 mM Tris–HCl, pH 7.1, 1 mM EDTA, 1% TritonX-100, 50% glycerol, 800 mM NaCl, 30 mM $MgCl_2$) and 10 mM β-mercaptoethanol and for STAT-1 1 μg poly-dI-dC, 5× binding buffer (50 mM HEPES, pH 7.8, 5 mM EDTA, 25 mM $MgCl_2$, 50% glycerol) and 2 μg bovine serum albumin were used. For competition a 100-fold molar excess of unlabelled double-stranded DNA-oligonucleotides were added. In ''supershift'' studies 1–4 μg of polyclonal antiserum against p50, p65 and c-rel (Santa Cruz Biotechnology) and control rabbit IgG (Sigma) or monoclonal anti-p65 antibody (mouse, IgG3, Roche Diagnostics) and IgG3 isotypic control antibody (Biozol) per 5 μg of nuclear extract were applied. The reactions were incubated at room temperature for 15 min or 30 min in case of ''supershift'' assays and separated using a 4% polyacrylamide gel in 0.25× TBE-buffer (22.5 mM Tris–borate, 0.5 mM EDTA, pH 8.0). Gels were dried and exposed to X-ray film (Kodak). Relative amounts of bands in films were estimated by video-based densitometry.

2.11. Western blotting

Cells ($1–10 \times 10^7$) were washed twice with ice-cold phosphate-buffered saline supplemented with 2 mM Na_2VO_3 and 10 mM NaF (phosphatase inhibitors, Sigma). Pellets were resuspended in 50–100 μl phosphate-buffered saline containing 2 mM Na_2VO_3, 10 mM NaF, 2 mM phenylmethylsulfonylfluoride, 10 mM dithiothreitol, 50 μg/ml aprotinin and were supplemented 1:1 with Laemmli-loading buffer (5% sodiumdodecylsulfate, 5% β-mercaptoethanol, 50% glycerol, 250 mM Tris–HCl, pH 6.8, 0.05% bromphenolblue). Samples were boiled for 15 min at 100°C. Equal amounts of protein were separated using a 10% polyacrylamide gel in 1× Laemmli-buffer (25 mM Tris, 192 mM glycine, 0.1% sodiumdodecylsulfate). Proteins were transferred on polyvinylidendifluoride 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 in 1× Tris-buffered saline (50 mM Tris–HCl, 150 mM NaCl, pH 7.5) (Roche Diagnostics) for 1 h. Primary antibodies were anti-phospho-tyrosine (mouse monoclonal IgG2b), anti-ERK-1 (rabbit polyclonal), anti-ERK-2 (mouse monoclonal IgG2a, Upstate Biotechnology), anti-phospho-MKK-1/-2, -4 and -3/-6 (rabbit IgG poly-

clonal, New England Biolabs), anti-I-κBα (rabbit IgG polyclonal, Santa Cruz Biotechnology) and anti-cytosolic phospholipase A_2 (rabbit IgG polyclonal, Cayman Chemicals). Primary antibodies were added to 0.5% blocking solution at a dilution of 1:1000 for 1 h. Membranes were washed twice with Tris-buffered saline and 0.1% Tween-20 for 10 min each and twice in 0.5% blocking solution for 10 min each. The detection antibody anti-rabbit/mouse-IgG-peroxidase conjugate (1:1000, Roche Diagnostics) was applied in 0.5% blocking solution for 30 min. Membranes were washed four times with Tris-buffered saline and 0.1% Tween-20 and developped by chemoluminescence (Amersham) according to the manufacturer's instructions. Membranes were exposed to X-ray films (Cronex). Bands in films were quantified by video-based densitometry.

2.12. In vitro kinase assays

Cells (1×10^7) were lysed by a detergent buffer according to the manufacturer's instructions (New England Biolabs). The active phosphorylated forms of the kinases were precipitated from total cell lysates with antisera against the phospho-tyrosine/-threonine activation epitope of human ERK-1/-2 (p44/p42) and p38α kinase for 24 h at 4°C with constant shaking. The isoforms of JNK were pulled down by direct binding to a c-*jun*-fusion protein. Protein A-sepharose beads were added for 4 h. After centrifugation the precipitate was washed twice with lysis buffer and twice with kinase assay buffer (New England Biolabs). Kinase assays were set up in 50 μl kinase assay-buffer supplementing 1 μM ATP and the protein substrates elk-1 for detection of ERK and activating transcription factor-2 (ATF-2) fusion protein for detection of p38, respectively, and incubated for 30 min at 30°C. Samples were cooled on ice, supplemented 1:1 with Laemmli-loading buffer and boiled. Western blotting was performed with antibodies against phospho-c-*jun*, phospho-elk-1 and phospho-ATF-2.

2.13. Cytotoxicity assays

Cell viability was monitored by flow cytometry using propidiumiodide exclusion (Amandi-Burgermeister et al., 1997) and by colorimetric assay applying 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazoliumbromide (MTT) and 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzendisulfonate sodium salt (WST-1) (Roche Diagnostics) according to the manufacturer's instructions.

2.14. Statistics

Each experiment was performed at least three times using sequential log-phase passages of the cell lines. Values are expressed as means ± S.D. or S.E.M. as indicated in legends to figures.

3. Results

3.1. Arachidonic acid, leukotriene B_4 and platelet-activating factor superinduce gene expression controlled by the interleukin-1β promoter

Phorbolester (PMA) and Ca^{2+}-ionophore (A23187) increase the activity of cytosolic phospholipase A_2 and rapid release and metabolism of arachidonic acid in the human promonocytic cell line THP-1 (Schwende et al., 1996; Hichami et al., 1997). Since these cells also express receptors for eicosanoids and platelet-activating factor on the plasma membrane (Cunningham, 1994), the effect of these lipids on activation of the interleukin-1β promoter was studied. THP-1 cells were stably transfected with DNA-constructs containing β-galactosidase as a reporter gene under the control of the complete 5′-upstream regulatory sequence of the human pro-interleukin-1β gene. Synthetic lipids per se did not increase expression of β-galactosidase (not shown). In contrast, PMA + A23187 enhanced expression of the reporter enzyme from 4 ± 4 pg to 991 ± 145 pg per 10^7 cells within 24 h ($n = 6$) (not shown). In a costimulation experiment (Fig. 2A), the combination of leukotriene B_4 plus platelet-activating factor (4.5-fold) elicited a strong superinduction of β-galactosidase expression above the PMA + A23187-stimulated control. Arachidonic acid (4-fold) or leukotriene B_4 (1.8-fold) alone also enhanced, whereas prostaglandin E_2 lowered its expression ($n = 3$). The concentration of the solvent ethanol was below 0.1% and exerted no effect. These data indicated that exogenous lipid mediators require concomitant activation of endogenous cytosolic phospholipase A_2 by PMA + A23187 in order to superstimulate the interleukin-1β promoter.

3.2. Inhibitors of cytosolic phospholipase A_2 down-regulate interleukin-1β promoter-dependent gene expression

To examine the role of cytosolic phospholipase A_2 in this signaling pathway, the effect of the trifluoromethylketone inhibitors on interleukin-1β promoter-dependent gene expression was studied. Stimulation of wild-type THP-1 cells with PMA + A23187 for 24 h resulted in secretion of 0.5 ± 0.1 ng prostaglandin E_2 and 4 ± 1 ng leukotriene B_4 per 10^6 cells ($n = 3$) which was prevented by the inhibitors (not shown). Concordantly, stably transfected THP-1 cells which had been stimulated with PMA + A23187 in presence of inhibitors of cytosolic phospholipase A_2 at a final concentration of 10 μg/ml and assayed for β-galactosidase activity, failed to activate the interleukin-1β promoter. Thus, reporter gene expression was reduced by 95 ± 7% for C20:4-$COCF_3$, 98 ± 2% for C18:3-$COCF_3$ and 82 ± 17% for C18:2-$COCF_3$ compared to stimulated controls ($n = 3$) (Fig. 2B). The antioxidant and NF-κB-inhibitor pyrolidindithiocarbamate also abol-

ished reporter gene expression by 100%, whereas acetylsalicylate, an inhibitor of cyclooxygenases, was ineffective. A time-course study confirmed that β-galactosidase already failed to accumulate within 1–2 h upon stimulation and did not increase over the following 4–24 h in contrast to stimulated controls (not shown). Semi-quantitative RT-PCR further revealed that C18:2-$COCF_3$ also down-regulated levels of the β-galactosidase RNA-transcript by 73 ± 22% without affecting the mRNA levels of β-actin ($n = 3$) (Fig. 2C). Neither direct inhibition or masking of the β-galactosidase activity by the compounds nor cytotoxic effects were recorded (not shown). These results suggested that inhibitors of cytosolic phospholipase A_2 attenuate the interleukin-1β promoter at the level of transcriptional activation. Supplementation of synthetic leukotriene B_4 or arachidonic acid together with platelet-activating factor led to a partial recovery of reporter gene expression in C18:2-$COCF_3$-treated cells to 30–40% of stimulated control, whereas addition of single lipids was ineffective (Fig. 2D). These data confirmed that exogenous lipids cannot replace for a functional cytosolic phospholipase A_2.

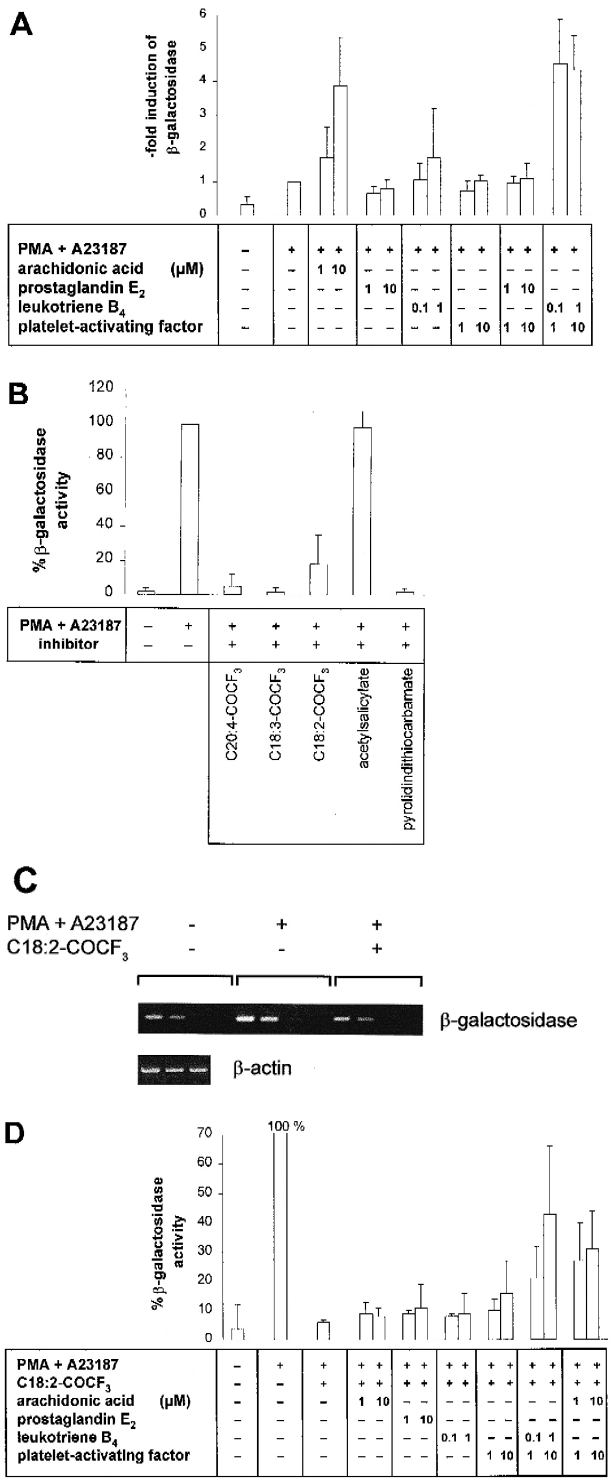

Fig. 2. Reporter gene expression under control of the upstream regulatory sequence of the human pro-interleukin-1β gene. (A) Superinduction by lipid mediators: THP-1 cells (1×10^6 per ml) were challenged with synthetic lipid metabolites, 20 ng/ml PMA and 0.5 μg/ml A23187 for 24 h. The activity of β-galactosidase was quantified photometrically in cell lysates. Values represent mean -fold induction ± S.D. compared to stimulated controls ($n = 3$). (B) Decrease of protein by inhibitors of cytosolic phospholipase A_2: Cells were preincubated with 10 μg/ml of the inhibitors and control compounds for 30 min and challenged with PMA + A23187 for 24 h. The activity of β-galactosidase is expressed as mean percent ± S.D. of stimulated controls ($n = 3$). (C) Decrease of Transcript: Semi-quantitative RT-PCR of β-galactosidase transcript. Cells were treated as in B and total RNA was extracted. 50 ng (lane 1), 25 ng (lane 2), 5 ng (lane 3) and 0.5 ng (lane 4) of RNA were reverse-transcribed and cDNA was amplified using primers specific for β-galactosidase and β-actin (only lane 1 is shown) (35 × cycles). PCR-products were separated by ethidiumbromide gel electrophoresis and quantified by video-based densitometry. One representative experiment out of three is shown. (D) Reconstitution by lipid mediators: Cells were preincubated with 10 μg/ml of C18:2-$COCF_3$ for 30 min and challenged with PMA + A23187 and lipid metabolites for 24 h. Values represent mean percent of β-galactosidase activity ± S.D. of stimulated control cells ($n = 3$).

3.3. Inhibitors of cytosolic phospholipase A_2 abolish DNA-binding of NF-κB, NF-IL6, AP-1 and STAT-1

To elucidate the molecular mechanism, how the inhibitors attenuate the interleukin-1β promoter, the DNA-binding capacity of transcription factors was investigated using electrophoretic mobility shift assays. Binding of NF-κB to the consensus motif of the murine Ig-κ light chain enhancer in nuclear extracts from PMA + A23187-challenged THP-1 cells was maximally induced within 1 h (Fig. 3). A ''supershift'' assay revealed a specific complex consisted of two bands (I + II) above a faster migrating unspecific complex (III) (Fig. 3A). The upper band (I) was supershifted by both antisera against the p50 and p65 subunits, but not by c-rel antiserum or control IgG and therefore represented the p50/p65 heterodimer. The lower band (II) corresponded to the p50/p50 homodimer since it was supershifted only by the p50 antiserum. Treatment of

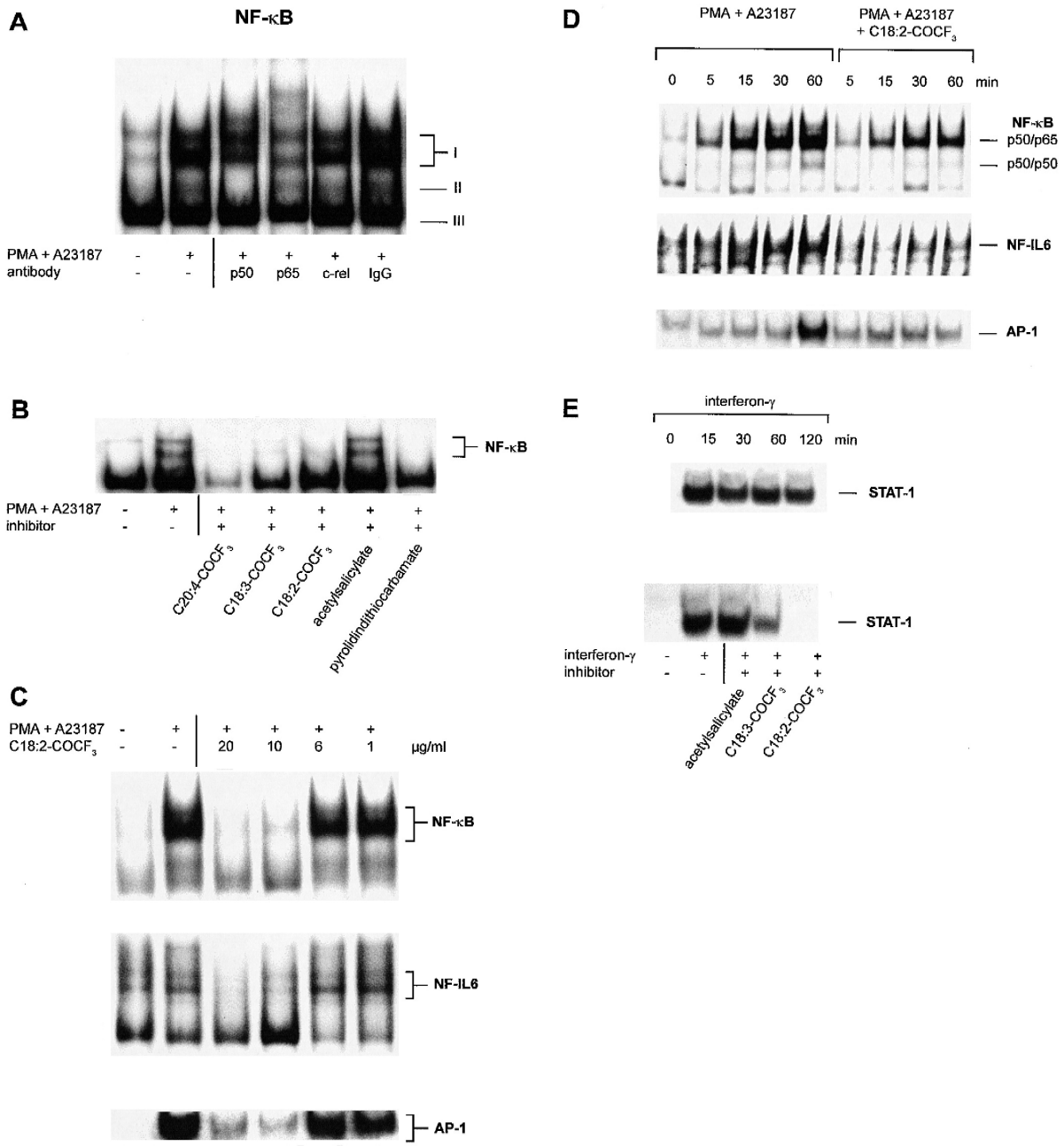

Fig. 3. DNA-binding of transcription factors using electrophoretic mobility shift assays. (A–D) THP-1 cells were preincubated with the compounds for 30 min and challenged with PMA + A23187. Nuclear extracts were incubated with the corresponding [^{32}P] labelled oligonucleotides. Samples were separated by polyacrylamide gel electrophoresis and bands were visualized on X-ray film: (A) For ''supershift''-assays 1 μg of polyclonal antisera against p65, p50 and c-rel subunits of NF-κB and IgG control antiserum were added to nuclear extracts from cells stimulated for 1 h. I = p65/p65 and p50/p65 heterodimers, II = p50/p50 homodimers, III = unspecific complex. (B) DNA-binding of NF-κB in presence of 10 μg/ml cytosolic phospholipase A_2 inhibitors and control compounds after a 1 h stimulation. (C) Dose-dependency and (D) short-time kinetics of DNA-binding of NF-κB, NF-IL6 and AP-1 in presence of 10 μg/ml C18:2-$COCF_3$. (E) DNA-binding of STAT-1 in interferon-γ-stimulated MonoMac-6 cells (upper panel). Cells were preincubated with 10 μg/ml of the cytosolic phospholipase A_2 inhibitors or the control compound and challenged with interferon-γ for 15 min (lower panel). (A–E) One representative experiment out of three is shown, respectively.

cells with inhibitors of cytosolic phospholipase A_2 at a final concentration of 10 μg/ml reduced DNA-binding of both p50/p65 and p50/p50 by 80 ± 17% compared to stimulated control ($n = 3$) (Fig. 3B). This reduction was similar to the one exerted by pyrolidindithiocarbamate, whereas acetylsalicylate was ineffective. The trifluoromethylketone inhibitors exerted a concentration-dependent decrease of DNA-bound NF-κB with IC_{50}-values of 5 μg/ml (Fig. 3C, upper panel). Short-time kinetics revealed that DNA-binding was prevented ab initio within 5 to 15 min upon challenge with PMA + A23187 in contrast to rapid induction in untreated controls (Fig. 3D, upper

panel). If increasing concentrations of the inhibitors (0.1μg/ml–1mg/ml) were added directly to nuclear extracts of stimulated control cells, DNA-binding was not impaired (not shown). This indicated that the inhibitors do not interfere with NF-κB *after* its activation. Binding of NF-IL6 to an enhancer element of the interleukin-1β promoter (−2882/−2869 bp) and of AP-1 to its cognate DNA-motif was impaired by inhibitors of cytosolic phospholipase A_2 as well yielding similar IC_{50}-values (Fig. 3 C,D, lower panels). DNA-binding of NF-Y, a constitutive control transcription factor, was not attenuated (not shown). This result together with the unaltered expression of the ''house-keeping'' genes β-actin (Fig. 2C) and glyceroladehyde dehydrogenase (not shown) indicated that the general transcription rate remains unaffected. In Mono-Mac6 cells, DNA-binding of STAT-1 to the sis-inducible element of the human c-*fos* promoter was also attenuated by the inhibitors within 15 min upon challenge with interferon-γ, whereas acetylsalicylate was without effect (Fig. 3E). These findings confirmed that inhibition of cytosolic phospholipase A_2 results in reduced DNA-binding of essential transcription factors which regulate transcription of the interleukin-1β gene in cells of the monocytic lineage.

3.4. Inhibitors of cytosolic phospholipase A_2 reduce the transactivating potential of NF-κB

DNA-binding of NF-κB is followed by transactivation of promoters. To evaluate the effect of the inhibitors on this event, THP-1 cells were transiently transfected with the plasmid 4 × NF-κB-pBL2 which contained four DNA-binding sites for NF-κB in tandem array in front of the minimal promoter from the thymidine kinase gene and the reporter gene coding for chloramphenicol acetyltransferase. Stimulation of transfected cells with PMA + A23187 increased NF-κB-dependent expression of the reporter gene from 52 ± 36 pg to 2803 ± 912 pg per mg of total protein ($n = 3$) (Fig. 4). In the presence of C18:3-$COCF_3$ and C18:2-$COCF_3$ these amounts were reduced by 81 ± 25% and 76 ± 35% ($n = 3$) compared to stimulated controls. This reduction was similar to the one exerted by pyrolidindithiocarbamate. The basal expression exerted by pBL2 containing the minimal promoter and of the promoterless pBL3 was not altered by the compounds (data not shown). The strong increase of reporter protein upon stimulation of cells transfected with a plasmid, containing the immediate early enhancer and promoter region of the cytomegalovirus (pCMV), was also prevented by the inhibitors. These data indicated that inhibitors of cytosolic phospholipase A_2 abrogate the transactivating potential of NF-κB on host and viral promoters.

3.5. Cytoplasmic activation of p65 subunits from NF-κB is reduced by inhibitors of cytosolic phospholipase A_2

In this experiment, a monoclonal antibody directed against the nuclear location sequence epitope on the p65

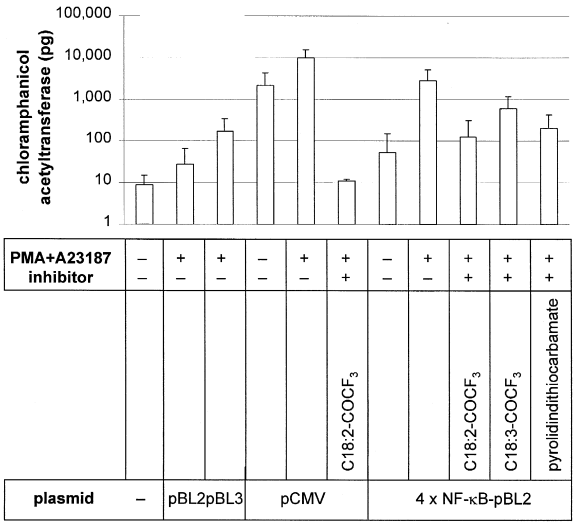

Fig. 4. Transactivation of a heterologous minimal promoter by NF-κB. THP-1 cells were transiently transfected with vehicle alone (first column) or with the reporter plasmids pBL2 (minimal promoter), pBL3 (no promoter), pCMV (promoter of the cytomegalovirus) or 4×NF-κB-pBL2 (four DNA-binding sites for NF-κB in front of a minimal promoter), respectively. Thereafter, cells were preincubated with 10 μg/ml of the compounds for 30 min and challenged with PMA + A23187 for 24 h. Total cell lysates were prepared and amounts of the reporter enzyme chloramphenicol acetyltransferase were determined. Results are expressed as means pg per mg total protein ± S.D. ($n = 3$).

subunit of NF-κB was used. In the cytosol of naive cells the antibody cannot bind to p65 because I-κB masks this epitope. After stimulation of cells I-κB is degraded and the free p65 subunit is accessible for antibody binding. Thus, the amounts of active p65 can be estimated by flow cytometry. THP-1 cells were treated for 30 min with C18:2-$COCF_3$ at a final concentration of 10 μg/ml and challenged for 1 h with PMA + A23187. Cells were fixed and stained in suspension. The mean fluorescence intensity derived from total free p65 subunits within the cytoplasma and the nucleus was enhanced 1.5-fold after 1 h of challenge compared to naive cells (Fig. 5). C18:2-$COCF_3$ lowered the mean fluorescence intensity of p65 by 88 ± 10% ($n = 3$) of stimulated control. Pyrolidindithiocarbamate also reduced the p65 signal, whereas acetylsalicylate was ineffective (not shown). An intense staining was obtained with the anti-pan histone antibody indicating that nuclear membranes were permeabilized. This experiment emphasized that cytoplasmic activation of NF-κB is diminished upon inhibition of cytosolic phospholipase A_2.

3.6. Phosphorylation and degradation of I-κBα is prevented by inhibitors of cytosolic phospholipase A_2

Phosphorylation and degradation of I-κBα is a prerequisite for liberation of active NF-κB. Therefore, we examined the amounts of I-κBα in THP-1 cells. Cells were pretreated for 30 min with C18:2-$COCF_3$ at a final concentration of 10 μg/ml and subsequently challenged

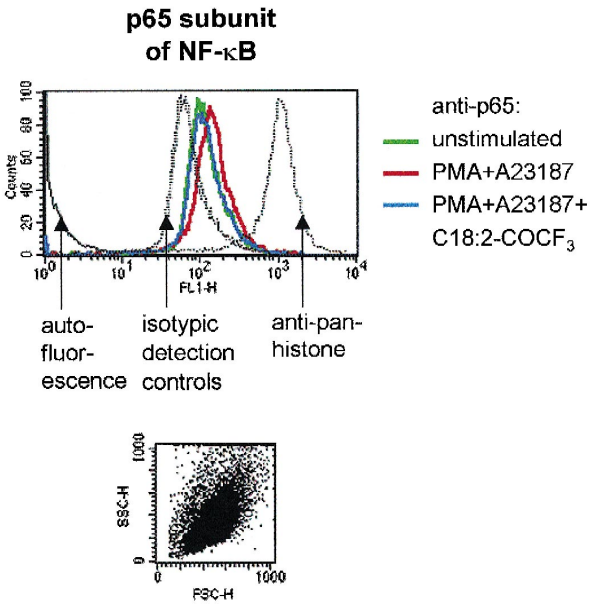

Fig. 5. Detection of active p65 subunits of NF-κB by flow cytometry. Lower Panel: Dot blot showing THP-1 cell population in a scatter profile. Upper panel: Representative histogram of the fluorescence intensity from $p65^+$ cells. Cells were preincubated with 10 μg/ml C18:2-$COCF_3$ for 30 min and challenged with PMA + A23187 for 1 h. Cells were fixed and stained with monoclonal anti-p65 or isotypic control IgG3 antibody, respectively, followed by fluorescein-labelled detection antibody. Anti-pan histone antibody was used to prove permeabilization of the nuclei. Cells were analyzed using a fluorescence-activated cell sorter. The signal curves from the unstimulated (green) and the inhibitor-treated (blue) cells superimpose, the signal from the stimulated control cells (red) is shifted to the right. One representative experiment from three similar is shown.

with A23187 + PMA for 2 min to 1 h. Total cell lysates were then subjected to Western blot analysis. In stimulated cells, the antiserum detected two adjacent bands, a faster-migrating band representing the unphosphorylated form of I-κBα and a slower migrating band corresponding to the phospho-form of I-κBα (Fig. 6). Within 2 to 5 min upon stimulation the phosphorylated form appeared and both forms were completely degraded within 1 h. In the presence of the inhibitor of cytosolic phospholipase A_2 only

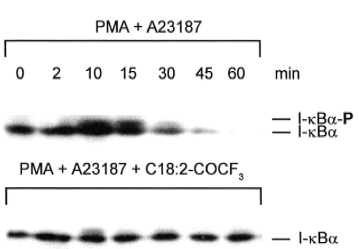

Fig. 6. Phosphorylation and degradation of I-κBα. THP-1 cells were preincubated with 10 μg/ml C18:2-$COCF_3$ for 30 min and challenged with PMA + A23187 for the times indicated. Total cell lysates were prepared and separated by sodiumdodecylsulfate polyacrylamide gel electrophoresis. I-κBα was detected by western blot using a specific antiserum and visualized on X-ray film by chemoluminescence. I-κBα-P = phosphorylated slower-migrating form, I-κBα = unphosphorylated form. One representative experiment out of two is shown.

very little of the phosphorylated form appeared and the amount of the unphosphorylated form remained unchanged for 60 min. This observation suggested that inhibition of cytosolic phospholipase A_2 abrogates phosphorylation of I-κBα by interference with protein kinases.

3.7. Inhibition of cytosolic phospholipase A_2 alters phosphorylation of cellular proteins at tyrosine residues

The kinases affected by the inhibitors should be encircled through comparison of the band pattern deriving from phosphorylated cellular proteins. THP-1 cells were pretreated with C20:4-$COCF_3$ at a final concentration of 10 μg/ml and stimulated with PMA + A23187 for 15 min. Total cell lysates were then subjected to Western blotting using an anti-phospho-tyrosine antibody. The inhibitor did not alter the general phosphorylation pattern of all proteins but rather affected distinct bands (Fig. 7A). Determination of the molecular weight revealed that phosphorylation at tyrosine residues appeared in several bands between 38 and 55 kDa upon challenge with PMA + A23187, whereas this induction was not observed in inhibitor-treated cells. This effect was most evident in a band of 46 kDa (see marker line). A Western blot using the same cell lysate as in Fig. 7A revealed that the 46-kDa band superimposed with the bands derived from the ERK-1/-2 (Fig. 7B). The electrophoretic mobility of these kinases was slightly delayed upon treatment with PMA + A23187 due to their enhanced phosphorylation (see arrow). In contrast, the bands in the inhibitor-treated sample were identical to those of the unstimulated sample. These data strongly indicated that inhibition of cytosolic phospholipase A_2 interferes with the MAPK pathway.

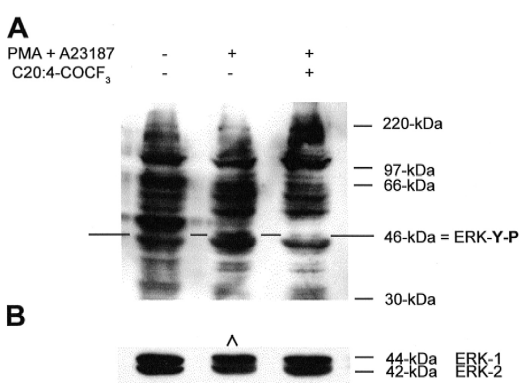

Fig. 7. Tyrosine phosphorylation of proteins in presence of an inhibitor of cytosolic phospholipase A_2. THP-1 cells were preincubated with 10 μg/ml C20:4-$COCF_3$ for 30 min and challenged with PMA + A23187 for 15 min. Total cell lysates were prepared and proteins were detected by western blot. (A) Detection of phosphorylated cellular proteins using a antibody specific for phosphotyrosine residues. A line marks the predominant 46-kDa band which is altered in presence of the inhibitor. (B) Detection of ERK-1/-2 (p44/p42) within the same cell lysate as in A. (A,B) One representative experiment out of three is shown.

3.8. Activation but not the activity of MAPKs is reduced by inhibitors of cytosolic phospholipase A_2

To define the critical event in this signalling pathway, which is affected by the inhibitors, the activity of the three MAPK-subfamilies was recorded. The active phospho-forms of the human ERK-1/-2, JNKs and p38α were trapped in lysates from THP-1 cells by immunoprecipitation. The respective antibodies were specific for the phospho-tyrosine/threonine residues within the activation ''lip'' of these kinases. The precipitates were then used for in vitro kinase reactions with the corresponding substrate proteins. Subsequently, Western blotting with antisera against the phospho-forms of the transcription factors elk-1, c-*jun* and ATF-2 was performed (Fig. 8A,B). Two to five minutes upon challenge with PMA + A23187 the activity of all three kinases increased, was maximal within 15 min and decreased beyond 30 min. This enhancement relied on increased amounts of the kinase's phospho-forms which in turn cause increased phosphorylation of the substrate proteins. In inhibitor-treated cells this induction response of the ERKs, JNKs and p38α was markedly reduced by $74 \pm 23\%$, $72 \pm 17\%$ and $66 \pm 19\%$ compared to stimulated controls, respectively ($n = 3$). If the inhibitors were added directly to the in vitro kinase reaction using immunoprecipitates of PMA + A23187-stimulated control cells, the catalytic activity of the three kinases was not impaired (not shown). Antibodies against the active phospho-forms of MKK-1/-2, MKK-4 (Fig. 8C) and MKK-3/-6 (not shown), which in turn phosphorylate the ERKs, JNKs and p38α, respectively, detected equal amounts of these upstream kinases in lysates from stimulated controls and inhibitor-treated cells. These data underlined that the inhibitors do not impair the intrinsic activity but rather prevent the phosphorylation of MAPKs through functional MKKs.

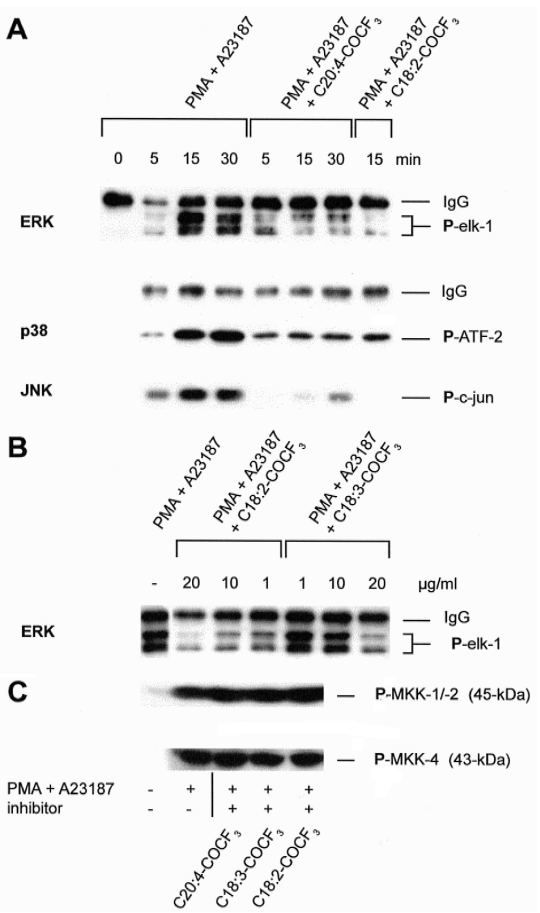

Fig. 8. Phosphorylation of transcription factors by MAPKs. THP-1 cells were preincubated for 30 min with the cytosolic phospholipase A_2 inhibitors and stimulated with PMA + A23187. The active phospho-forms of ERK-1/-2 and p38α kinase were immunoprecipitated from total cell lysates with antisera specific for the phospho-tyrosine/threonine activation motif. JNK was pulled down by binding to c-*jun*. The precipitates were used for in vitro kinase reactions with elk-1, ATF-2 and c-*jun*, respectively. The amounts of phosphorylated transcription factors were detected by western blot: (A) Short-time kinetics with 10 μg/ml of the inhibitors. (B) Concentration–response after 15 min stimulation. (C) Western blot of total cell lysates with antisera against the phosphorylated forms of MKK-1/-2 and MKK-4. (A–C) One representative experiment out of three is shown.

3.9. Synthesis of interleukin-1β is also reduced by antisense-oligonucleotides against the mRNA of cytosolic phospholipase A_2

The specificity of the effects exerted by the inhibitors should be confirmed by applying antisense technique. In this experiment, the promonocytic cell line U937 was used, which can be stimulated by PMA and lipopolysaccharide to enhance expression of cytosolic phospholipase A_2, turnover of phospholipids (Wiederhold et al., 1988; Nicholson et al., 1992) and synthesis of interleukin-1β (Lozanski et al., 1992). Antisense and sense oligonucleotides against the translational initiation site within the mRNA of cytosolic phospholipase A_2 were added to the cells for 6 h with final concentrations of 0.1 to 10 μM and under serum-free conditions. After addition of serum, cells were further incubated for 18 h and stimulated with PMA and lipopolysaccharide for another 24 h. Total cell lysates and supernatants were collected. Immunoassays detected a release of 0.3 ± 0.1 ng prostaglandin E_2, 1.3 ± 0.3 ng leukotriene B_4 and 0.6 ± 0.2 ng interleukin-1β per 10^6 control cells ($n \geq 4$). In contrast, we observed a concentration-dependent decrease of interleukin-1β release from antisense-treated cells by maximal $90 \pm 16\%$ ($n = 4$) (Fig. 9A). At high concentrations the sense oligonucleotides exerted some inhibition as well, whereas low concentrations superinduced production of interleukin-1β. The latter effect was not observed with low concentrations of antisense oligonucleotides. Levels of prostaglandin E_2 were maximally reduced in presence of antisense oligonucleotides by $40 \pm 19\%$ ($n = 4$) (Fig. 9B). In contrast, the amounts of leukotriene B_4 remained unchanged (not shown). Western blot analysis of total cell lysates con-

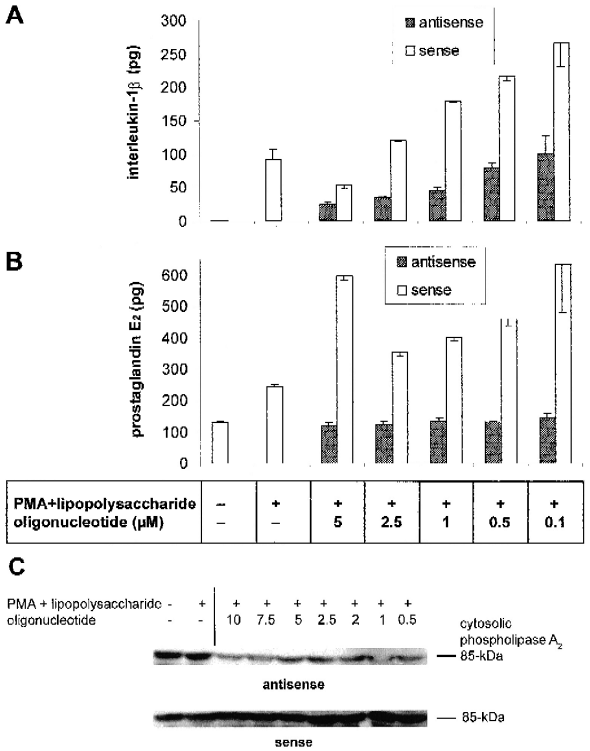

Fig. 9. Effects of antisense oligonucleotides against the mRNA of cytosolic phospholipase A_2. U937 cells were incubated with antisense and sense oligonucleotides at concentrations indicated for 24 h and were stimulated with lipopolysaccharide and PMA for another 24 h. In the supernatants interleukin-1β (A) and prostaglandin E_2 (B) were quantified by immunoassay. Results are expressed as means pg/10^6 cells/ml ± S.E.M. ($n = 4$). (C) The amount of cytosolic phospholipase A_2 protein was detected in total cell lysates by western blot using a polyclonal antiserum. One representative experiment out of four is shown.

firmed that the amounts of cytosolic phospholipase A_2 were concentration-dependently diminished upon antisense-treatment by maximal 49 ± 5% ($n = 4$) compared to stimulated controls (Fig. 9C). In summary, these findings underlined that suppression of interleukin-1β synthesis is achieved by either inhibiting the catalytic activity of cytosolic phospholipase A_2 or by lowering expression of its gene.

4. Discussion

In the present study, it was shown for the first time that inhibition of cytosolic phospholipase A_2 in human monocytic cells results in impaired activation of MAPKs. All stimuli applied here, namely phorbolester, Ca^{2+}-ionophore, interferon-γ and lipopolysaccharide converge on phosphorylation and translocation of activated cytosolic phospholipase A_2 to the nuclear membrane (Clark et al., 1995). From there the majority of arachidonic acid is released, indicating that endogenous lipid metabolites transfer signals to the DNA by acting as second messengers within the nucleus. Supporting this model, we could show that exogenous arachidonic acid, leukotriene B_4 and platelet-activating factor per se did not induce expression of a reporter gene under control of the 5′-upstream regulatory region of the human pro-interleukin-1β gene in stably transfected THP-1 cells. However, if these cells were costimulated with lipids and PMA + A23187 a strong superinduction took place. The importance of a functional cytosolic phospholipase A_2 and endogenous lipid mediators was further supported by the finding that expression of the reporter gene was down-regulated by the trifluoromethylketone inhibitors of cytosolic phospholipase A_2 within 1 to 2 h upon stimulation with PMA + A23187. In contrast to interleukin-1β, the reporter protein is not secreted. Concordantly, this attenuation was due to a decrease of cytoplasmic protein synthesis and RNA transcription. The interleukin-1β mRNA contains $A(U)_nA$-sequences at the 3′-terminus which regulate the effectivity of translation and degradation of mRNA (Auron and Webb, 1994). The reporter gene transcript not only lacks these sequences but is instead stabilized by a polyadenylation signal. Therefore, one could argue that the cytosolic phospholipase A_2 inhibitors block transcriptional activation at the interleukin-1β promoter rather than cause rapid degradation of the newly synthesized RNA transcript. Despite simultaneous supplementation of leukotriene B_4 plus platelet-activating factor, both metabolites from the two major metabolic pathways of arachidonic acid and lyso-phospholipids, only a partial recovery of reporter gene expression was achieved in inhibitor-treated cells. This may be explained by the establishment of positive feedback mechanisms by lipid mediators. Therein, exogenous lipids restimulate the synthesis and activity of cytosolic phospholipase A_2 by signalling through G-protein-coupled receptors on plasma membranes and consequently further amplify their own endogenous synthesis (Wijkander et al., 1995; Murakami et al., 1997a; Wong et al., 1998). Therefore, paracrine addition of lipids will be less effective to restore activity of the interleukin-1β promoter in inhibitor-treated cells where this positive feedback loop is interrupted than to superstimulate it in untreated cells with intact amplification mechanisms.

This transcriptional down-regulation could be attributed to the failure of rapid DNA-binding of essential transcription factors which regulate expression of the interleukin-1β gene. In THP-1 cells DNA-binding of NF-κB, NF-IL6 and AP-1 was simultaneously abolished by inhibitors of cytosolic phospholipase A_2 within 15 min to 1 h upon challenge with PMA + A23187. This impact on more than one single target implicated that these inhibitors block a common upstream regulatory molecule within the activation pathway of these transcription factors. The three transcription factors investigated here are consistently activated by phosphorylation through serine/threonine-protein kinases (Whitmarsh and Davis, 1996; Akira, 1997; Baeuerle, 1998). Supporting this, we found that the inhibitors prevented serine-phosphorylation and subsequent degradation of I-κBα within 2 min to 1 h. Consequently,

the amounts of activated, I-κB-deprived p65 subunits of NF-κB in the cytoplasm were diminished as was its transactivating potential on a heterologous minimal promoter.

Encircling the relevant kinases, it was stated here that inhibitors of cytosolic phospholipase A_2 reduced phosphorylation of cellular proteins within the range of 38–55 kDa at tyrosine residues. Due to this molecular weight and due to the fact that MAPKs are regulated by dual phosphorylation of a single tyrosine and threonine residue within an exposed activation lip (Whitmarsh and Davis, 1996) it became evident that the affected proteins represent members of this kinase family. The MAPK signalling cascade is organized in three levels of hierarchy (Whitmarsh and Davis, 1996). Thus, it had to be elucidated which event is affected upon inhibition of cytosolic phospholipase A_2. It turned out that the amounts of the active phosphorylated forms of all three subfamilies of MAPKs, namely ERK-1/-2 (p44/p42), the isoforms of JNK (p46–p55) and p38α kinase were reduced in lysates of inhibitor-treated THP-1 cells. However, the inhibitors did not directly block the intrinsic catalytic activity of these kinases on the transcription factors elk-1, c-*jun* and ATF-2. Furthermore, the compounds did not interfere with the generation of active phosphorylated forms of the upstream MKKs. This indicated that signalling pathways further upstream including the mitogen-activated protein kinase kinase kinases (MKKKs) like raf-1 are not blocked by inhibitors of cytosolic phospholipase A_2. Stimulation by PMA and A23187 converges on the level of MKKKs (Whitmarsh and Davis, 1996). Hence, these membrane-proximal events mediated by phorbolester-sensitive protein kinase C isoforms and Ca^{2+}-regulated protein kinases will remain functional as well.

Therefore, we conclude that inhibition of cytosolic phospholipase A_2 results in failure of post-translational activation of the MAPKs by abrogating the critical phosphorylation event through functional MKKs.

On the other hand, this mode of action indicates a positive function for lipid metabolites in promoting activation and/or activity of MAPKs (Tournier et al., 1997; Hii et al., 1998; Yamakawa et al., 1998). Some detailed mechanisms how arachidonic acid affects signalling molecules have been characterized. This fatty acid inhibits the GTPase-activating protein ($p120^{GAP}$) by a direct competitive association with the nucleotide binding site thereby increasing $p21^{ras}$ activity (Sermon et al., 1996). Arachidonic acid competes with GTP for the nucleotide binding site in the α-subunit of $G_{i/z}$-proteins (Glick et al., 1996). It also enhances diacylglycerol-induced activation of protein kinase C by promoting its stable Ca^{2+}-dependent association with phospholipid vesicles (Schachter et al., 1996). This effect is mediated by regulatory lipid-binding domains within this kinase (Bottomley et al., 1998). However, the exact molecular mechanism if and how endogenous eicosanoids and platelet-activating factor physically interact with MAPKs remains to be elucidated in future experiments. Little evidence existed, if the cytosolic phospholipase A_2 is also involved in activation of STAT-1. Referring to this, we show here for the first time that inhibition of this enzyme abolished DNA-binding of STAT-1 within 15 min upon challenge of MonoMac6 cells with interferon-γ. In contrast to the other transcription factors examined here, STAT-1 is activated by interferon-γ through dual phosphorylation at tyrosine residues triggered by cytokine receptor-activated janus-kinases (JAK) and at serine residues through MAPKs (Kovarik et al., 1998).

Therefore, the suppressive effect of the inhibitors may reside in failure of serine phosphorylation of STAT-1 due to impaired MAPKs. In contrast, the same kinases have been recently shown to prevent mobilisation of STAT-3 by interleukin-6 through serine phosphorylation (Sengupta et al., 1998). Antagonistic regulatory mechanisms for STAT-1 and STAT-3 induction may account for this discrepancy. In any case, this failure of phosphorylation leads to retention of inactive transcription factors in the cytosol. Since eicosanoids promote expression of the immediate early promoter of the cytomegalovirus (Kline et al., 1998) by host transcription factors, the inhibitors were shown here to abolish transactivation of this promoter as well. These multiple attenuating effects on gene expression of the host and eukaryotic viruses may explain the in vivo potency of inhibitors of cytosolic phospholipase A_2 to ameliorate acute and chronic inflammation (Amandi-Burgermeister et al., 1997).

The cytosolic phospholipase A_2 knock-out mice exert reduced inflammatory responses in vivo and fail to release arachidonic acid, eicosanoids and platelet-activating factor from ex vivo macrophages (Uozumi et al., 1997). Accordingly, our data showed that treatment of U937 cells with antisense oligonucleotides against the translational intiation site within the mRNA of cytosolic phospholipase A_2 (Roshak et al., 1994) down-regulated the enzyme's protein levels and subsequent synthesis of prostaglandin E_2 and interleukin-1β. In contrast, sense oligonucleotides exerted a superinducing effect. This may be explained by formation of triple helices with unwound DNA at promoter regions, which may stabilize an open initiation complex for RNA-polymerase II (Probst and Skutella, 1996). In concordance to other studies (Marshall et al., 1997), leukotriene B_4 synthesis was not reduced by antisense treatment.

This lack of effect is discussed by the authors as a preferential coupling of cytosolic phospholipase A_2 to cyclooxygenases rather than to 5′-lipoxygenase (Marshall et al., 1997; Murakami et al., 1997b). However, a discrepancy remains to the potency of cytosolic phospholipase A_2 inhibitors to effectively block production of both leukotrienes and prostaglandins (Amandi-Burgermeister et al., 1997; Murakami et al., 1997b). Nevertheless, both strategies to abrogate cytosolic phospholipase A_2 activity by antisense or inhibitors converged in effective suppression of interleukin-1β production.

We conclude, that an active cytosolic phospholipase A_2 is a prerequisite for the generation of lipid messengers which support the activation status of MAPKs and thereby the recruitment of transcription factors to genes involved in inflammation.

Acknowledgements

We are grateful to W. Kinle and A. Schmid for their excellent technical assistance. Special thanks refer to G. Bensi for the plasmid pIL1 and to G. Lang for establishment of the transfected THP-1 cell line. We also thank K. Bauer for helpful technical advice and W.-G. Friebe for synthesis of compounds.

References

Akira, S., 1997. Interleukin-6 regulated transcription factors. Int. J. Biochem. Cell. Biol. 29, 1401–1418.

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.

Auron, P.E., Webb, A.C., 1994. Interleukin-1: a gene expression system regulated at multiple levels. Eur. Cytokine Network 5, 573–592.

Baeuerle, P.A., 1998. Proinflammatory signalling: last pieces in the NF-κB puzzle. Curr. Biol. 8, R19–R22.

Bottomley, M.J., Salim, K., Panayotou, G., 1998. Phospholipid-binding protein domains. Biochim. Biophys. Acta 1436, 165–183.

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 NF-κB and NF-IL6. Eur. J. Immunol. 22, 2705–2711.

Burgermeister, E., Tibes, U., Stockinger, H., Scheuer, W.V., 1999. Activation of NF-κB by lipopolysaccharide in mononuclear leukocytes is prevented by inhibitors of cytosolic phospholipase A_2. Eur. J. Pharmacol. 369, 373–386.

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

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

Cunningham, F.M., 1994. Lipid Mediators, The Handbook of Immunopharmacology. 2nd edn. Academic Press, London.

Danesch, U., Weber, P.C., Sellmayer, A., 1994. Arachidonic acid increases c-*fos* and Egr-1 mRNA in 3T3 fibroblasts by formation of PGE_2 and activation of PKC. 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.

Glick, J., Santoyo, G., Casey, P.J., 1996. Arachidonate and related unsaturated fatty acids selectively inactivate the guanine nucleotide-binding regulatory protein G(z). J. Biol. Chem. 271, 2949–2954.

Hichami, A., Duroudier, V., Leblais, V., Vernhet, L., Legoffic, F., Ninio, E., Legrand, A., 1997. Modulation of platelet-activating factor production by incorporation of naturally occurring 1-*O*-alkylglycerols in phospholipids of human leukemic monocyte-like THP-1 cells. Eur. J. Biochem. 250, 242–248.

Hii, C.S.T., Huang, Z.H., Bilney, A., Costabile, M., Murray, A.W., Rathjen, D.A., Channing, J.D., Ferrante, A., 1998. Stimulation of p38 phosphorylation and activity by arachidonic acid in HeLa cells, HL60 promyelocytic leukemic cells and human neutrophils. J. Biol. Chem. 273, 19277–19282.

Kline, J.N., Hunninghake, G.M., He, B., Monick, M.M., Hunninghake, G.W., 1998. Synergistic activation of the human cytomegalovirus major immediate early promoter by prostaglandin E_2 and cytokines. Exp. Lung. Res. 24, 3–14.

Kovarik, P., Stoiber, D., Novy, M., Decker, T., 1998. STAT-1 combines signals derived from interferon-γ and lipopolysaccharide receptors during macrophage activation. EMBO J. 17, 3660–3668.

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

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

Lozanski, G., Ballou, S.P., Kushner, I., 1992. Effect of flurbiprofen on cytokine production by human monocytes and U937 and THP-1 cell lines. J. Rheumatol. 19, 921–926.

Marshall, L.A., Bolognese, B., Winkler, J.D., Roshak, A., 1997. Depletion of human monocyte 85-kDa cytosolic phospholipase A_2 does not alter leukotriene formation. J. Biol. Chem. 272, 759–765.

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

Mihm, S., Ennen, J., Pessara, U., Kurth, R., Dröge, W., 1991. Inhibition of human immunodeficiency virus-1 replication and NF-κB activation by cysteine and cysteine derivatives. AIDS 5, 497–503.

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. Critic. Rev. Immunol. 17, 225–283.

Nicholson, D.W., Ali, A., Klemba, M.W., Munday, N.A., Zamboni, R.J., Ford-Hutchinson, A.W., 1992. Human leukotriene C_4 synthase expression in dimethylsulfoxide-differentiated U937 cells. J. Biol. Chem. 267, 17849–17857.

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. Immunol. 72, 181–187.

Probst, J.C., Skutella, T., 1996. Elevated messenger RNA levels after antisense oligodeoxynucleotide treatment in vitro and in vivo. Biochem. Biophys. Res. Commun. 225, 861–868.

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 cytosolic phospholipase A_2 blocks production of arachidonic acid and 12-hydroxyeicosatetraenoic acid by calcium ionophore challenged platelets. J. Biol. Chem. 269, 15619–15624.

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

Roshak, A., Sathe, G., Marshall, L., 1994. Suppression of monocyte 85-kDa PLA_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 NF-κB by prostaglandin A_1: An effect associated with heat shock transcription factor activation. Proc. Natl. Acad. Sci. USA 94, 746–750.

Schachter, J.B., Lester, D.S., Alkon, D.L., 1996. Synergistic activation of protein kinase C by arachidonic acid and diacylglycerols in vitro: Generation of a stable membrane-bound cofactor-independent state of protein kinase C activity. Biochim. Biophys. Acta 1291, 167–176.

Schwende, H., Fitzke, E., Ambs, P., Dieter, P., 1996. Differences in the

state of differentiation of THP-1 cells induced by phorbol ester and 1,12-dihydroxyvitamin D_3. J. Leukocyte Biol. 59, 555–561.

Sengupta, T.K., Talbot, E.S., Scherle, P.A., Ivashkiv, L.B., 1998. Rapid inhibition of interleukin-6 signalling and STAT-3 activation mediated by MAPK. Proc. Natl. Acad. Sci. USA 95, 11107–11112.

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.

Shankavaram, U.T., DeWitt, D.L., Wahl, L.M., 1998. Lipopolysaccharide induction of monocyte matrix metalloproteinases is regulated by the tyrosine phosphorylation of cytosolic phospholipase A_2. J. Leukocyte Biol. 64, 221–227.

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

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 cytosolic phospholipase A_2. Biochemistry 32, 5935–5940.

Thommensen, L., Sjursen, W., Gasvik, K., Hanssen, W., Brekke, O.-L., Skattebol, L., Holmeide, A.K., Espevik, T., Johansen, B., Laegreid, A., 1998. Selective inhibitors of cytosolic and secretory phospholipase A_2 block tumor necrosis factor-induced activation of transcription factor NF-κB and expression of intercellular adhesion molecule-1. J. Immunol. 161, 3421–3430.

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 MAPK (ERK and JNK). Eur. J. Biochem. 244, 587–595.

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.

Wagner, B.J., Hayes, T.E., Hoban, C.J., Cochran, B.H., 1990. The SIF binding-element confers sis/platelet-derived growth factor inducibility onto the c-*fos* promoter. EMBO J. 9, 4477–4484.

Whitmarsh, A.J., Davis, R.J., 1996. Transcription factor AP-1 regulation by MAPK signal transduction pathways. J. Mol. Med. 74, 589–607.

Wiederhold, M.D., Anderson, K.M., Harris, J.E., 1988. Labelling of lipids and phospholipids with [^{3}H] arachidonic acid and the biosynthesis of eicosanoids in U937 cells differentiated by phorbol ester. Biochim. Biophys. Acta 959, 296–301.

Wijkander, J., O'Flaherty, J.T., Nixon, A.B., Wykle, R.L., 1995. 5′-lipoxygenase products modulate the activity of the 85-kDa cytosolic 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., Choy, P., 1998. Lysophosphatidylcholine stimulates the release of arachidonic acid in human endothelial cells. J. Biol. Chem. 273, 6830–6836.

Yamakawa, T., Eguchi, S., Yamakawa, Y., Motley, E.D., Numaguchi, L., Utsunomiya, H., Inagami, T., 1998. Lysophosphatidylcholine stimulates MAPK activity in rat vascular smooth muscle cells. Hypertension 31, 248–253.