Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 65 (2003) 887–895
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Inhibition of the NF-κB signaling pathway mediates the anti-inflammatory effects of petrosaspongiolide M

Inmaculada Posadas[a], Maria Carmen Terencio[a], Antonio Randazzo[b], Luigi Gomez-Paloma[b], Miguel Payá[a], Maria José Alcaraz[a,*]

[a]*Departamento de Farmacología, Facultad de Farmacia, Universidad de Valencia, Avda. Vicent Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain*
[b]*Dipartimento di Scienze Farmaceutiche, Università di Salerno, via Ponte Don Melillo, 84084 Fisciano, Salerno, Italy*

Received 23 September 2002; accepted 5 December 2002

Abstract

Petrosaspongiolide M (PT) is a potent secretory phospholipase A_2 inhibitor and anti-inflammatory agent. This marine metabolite reduced the production of nitrite, prostaglandin E_2, and tumor necrosis factor-α in the mouse air pouch injected with zymosan. These effects were also observed in mouse peritoneal macrophages stimulated with zymosan. Inhibition of these inflammatory mediators was related to reductions in inducible nitric oxide synthase, cyclo-oxygenase-2, and tumor necrosis factor-α expression. Since nuclear factor-κB (NF-κB) appears to play a central role in the transcriptional regulation of these proteins by macrophages, we investigated the effects of PT on this transcription factor. We found that PT was a potent inhibitor of the NF-κB pathway since at 1 μM it strongly decreased NF-κB–DNA binding in response to zymosan, in mouse peritoneal macrophages. Our study also indicated that PT could interfere with a key step in NF-κB activation, the phosphorylation of IκBα, resulting in inhibition of IκBα degradation. The control of a wide range of mediators by PT suggests a potentially wide therapeutic spectrum for this marine metabolite in inflammatory conditions.

Keywords: NF-κB; Zymosan; TNFα; Petrosaspongiolide M; Mouse air pouch; Mouse peritoneal macrophages

1. Introduction

Cell activation by bacterial components, cytokines, and other stimuli releases arachidonic acid *via* phospholipase (PL) A_2 to synthesize active metabolites including platelet activating factor, PGs, thromboxanes, lipoxins, and leukotrienes [1]. $cPLA_2$ and $sPLA_2$ have been reported as the enzymes responsible for the synthesis of eicosanoids, although the relative contribution of each enzyme [2] and other phospholipases such as PLC and PLD could vary according to the stimulus and cell type [3]. Potential interactions among these enzymes in the regulation of arachidonic acid release have been explored. Therefore, a functional crosstalk has been demonstrated between $cPLA_2$ or $sPLA_2$-IIA and PLD, resulting in activation of this enzyme with increased arachidonate release and PGE_2 production [4]. In addition, a calcium-independent PLA_2 ($iPLA_2$) is also involved in arachidonic acid hydrolysis and seems to play a role in phospholipid remodeling [2].

Inflammation is a central feature of many pathological conditions. Group IIA $sPLA_2$ has been linked to inflammatory diseases such as rheumatoid arthritis [5] and asthma [6]. $sPLA_2$ present in inflammatory fluids may participate in the formation and release of mediators and thus exerts proinflammatory effects. On the other hand, this enzyme activity contributes to the antimicrobial host defence system since bacterial phospholipid degradation by this enzyme follows phagocytosis of microorganisms by neutrophils [7]. In addition, $sPLA_2$ could participate in the expression of adhesion molecules on neutrophils and thus it cooperates with PAF for exocytosis of gelatinase granules *via* the 5-lipoxygenase pathway, resulting in a synergistic increase in Mac-1 (CD11b/CD18) surface expression during inflammatory processes [8].

* Corresponding author. Tel.: +34-96-3544292; fax: +34-96-3544292.
E-mail address: maria.j.alcaraz@uv.es (M.J. Alcaraz).

Abbreviations: COX-1, cyclo-oxygenase-1; COX-2, cyclo-oxygenase-2; $cPLA_2$, cytosolic PLA_2; IL-1β, interleukin-1β; IκB, NF-κB inhibitory protein; iNOS, inducible nitric oxide synthase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor-κB; NO, nitric oxide; PGE_2, prostaglandin E_2; PL, phospholipase; PAF, platelet activating factor; PT, petrosaspongiolide M; $sPLA_2$, secretory PLA_2; TNFα, tumor necrosis factor-α.

0006-2952/03/$ – see front matter
doi:10.1016/S0006-2952(02)01659-3

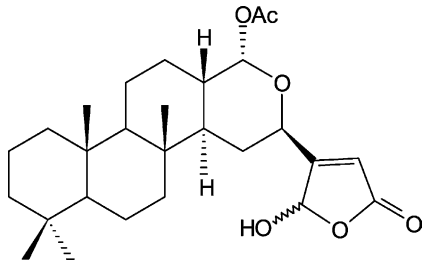

Fig. 1. Chemical structure of petrosaspongiolide M.

Marine metabolites are potent inhibitors of $sPLA_2$. The present work extends our initial report that PT (Fig. 1), isolated from the Caledonian marine sponge *Petrosaspongia nigra*, is a potent inhibitor of $sPLA_2$ with anti-inflammatory properties in experimental models [9]. We showed previously that oral administration of PT significantly inhibited the chronic inflammation induced by Freund's adjuvant in rats and acute inflammatory responses in mice, with reductions in eicosanoids and TNFα. This cytokine is a therapeutic target in rheumatoid arthritis since anti-TNFα therapy down-regulates cytokine production, including IL-1β, which plays an important role in bone and cartilage destruction, leukocyte recruitment, and angiogenesis in the rheumatoid synovium [10].

NF-κB-dependent gene expression plays an important role in immune and inflammatory responses. This family of proteins exists in most cells as homodimeric or heterodimeric complexes of p50 and p65 subunits and remains inactive in the cytoplasm of cells associated with IκB. Binding to this protein is the main cellular mechanism preventing gene transcription mediated by NF-κB. This transcription factor increases the expression of genes encoding cytokines, receptors involved in leukocyte adhesion and migration, proinflammatory enzymes such as iNOS and COX-2, etc. [11,12]. Interestingly, several lines of evidence support the notion that blocking NF-κB is an important target for the control of inflammation and cancer [13].

The aim of this study was to further explore the mechanisms of action of PT relevant to the regulation of the inflammatory response. We have shown that this compound suppresses TNFα production as well as iNOS and COX-2 expression, *in vivo* and in primary mouse macrophages and these effects can be related to an interference with the NF-κB signaling pathway.

2. Materials and methods

2.1. Materials

PT was isolated from the sponge *P. nigra* following known procedures [14]. Stock solutions of this compound were prepared in ethanol. The maximal concentration of solvent in the final incubation media (*in vitro* experiments) was 1% (v/v). The same concentration of ethanol was included in the control group. [γ-^{32}P]ATP and [α-^{32}P]UTP were purchased from NEN Life Sciences Products, Inc. and [5,6,8,11,12,14,15(*n*)-^{3}H]PGE_2 from Amersham Biosciences. Anti-mouse TNFα and IL-1β antibodies were from Immunokontact. COX-2 and iNOS specific polyclonal antisera and 15-deoxy-$\Delta^{12,14}$-PGJ_2 were purchased from Cayman Chem. Polyclonal antibodies against p65 and IκBα, and IκBα (1–317) were purchased from Santa Cruz Biotechnology Inc., anti-phospho-(Ser^{32}) IκBα antibody from New England Biolabs and Z–Leu–Leu–Leu–CHO (MG132) was from Biomol Research Laboratories, Inc. The peroxidase-conjugated IgG was purchased from Dako and the rest of reagents were from Sigma Chemicals.

2.2. Mouse air pouch model

All studies were performed in accordance with international regulations for the handling and use of laboratory animals. The protocols were approved by the institutional Animal Care and Use Committee. Air pouch was produced in female Swiss mice (25–30 g) as previously described [15]. Six days after the initial air injection, 1 mL of sterile saline (saline group), 1 mL of 1% w/v zymosan in saline plus 10 μL ethanol (control group), or 1 mL of 1% w/v zymosan in saline plus 10 μL of drug dissolved in ethanol (treated group) was injected into the air pouch. PT was administered intrapouch at the same time as zymosan and 8 hr after, whereas dexamethasone was injected by the same route 1 hr before and 8 hr after zymosan injection. At different times after zymosan administration, animals were killed by cervical dislocation and the exudate in the pouch was collected. Leukocytes present in exudates were measured using a Coulter counter. After centrifugation of exudates (1200 *g* at 4° for 10 min) the supernatants from 2 hr were used to measure TNFα levels by time-resolved fluoroimmunoassay [16]. In the 12 hr exudates, the supernatants were used to measure nitrite by the fluorometric method of Misko *et al.* [17], PGE_2 levels by radioimmunoassay [18], and IL-1β by time-resolved fluoroimmunoassay [16]. The cell pellets from 12 hr air pouches were used to determine COX-2 and iNOS expression by Western blot analysis as described below.

2.3. Isolation and culture of mouse peritoneal macrophages

Female Swiss mice weighing 25–30 g were used to obtain highly purified peritoneal macrophages. Cells were harvested by peritoneal lavage 4 days after i.p. injection of 1 mL of 10% thioglycolate broth. Cells were resuspended in culture medium (120 mM NaCl, 4.7 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM HEPES, 1 mM arginine, and 10 mM glucose) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and incubated at

37° for 2 hr. The adherent cells were used to perform the following experiments. The mitochondrial dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan was used to asses the possible cytotoxic effects of test compounds.

2.4. Measurement of nitrite, PGE_2, and cytokine levels in supernatants

Peritoneal macrophages (4×10^5 cells/well) were incubated for different times with zymosan (0.1 mg/mL) at 37° in the presence of test compounds or vehicle. After centrifugation at 2000 *g* for 10 min at 4°, aliquots of the supernatants were used to measure different mediators. After 6 hr of stimulation, supernatants were collected to measure TNFα levels by time-resolved fluoroimmunoassay [16]. In supernatants from 18 hr zymosan-stimulated cells, nitrite was assayed fluorometrically in microtiter plates using a standard curve of sodium nitrite [17] and PGE_2 levels were determined by radioimmunoassay [18]. Cells stimulated with zymosan for 18 hr were collected to determine iNOS and COX-2 expression by Western blot analysis as described below.

2.5. Electrophoretic Mobility Shift Assay (EMSA)

Nuclear and cytosolic extracts from mouse peritoneal macrophages were prepared as described [19]. Protein was determined by the DC Bio-Rad protein reagent. The double-stranded oligonucleotide containing the consensus NF-κB sequence (Promega Corp.) was end-labeled using T4 polynucleotide kinase (Amersham Biosciences) and [γ-^{32}P]ATP, followed by purification using G-25 microcolumns (Amersham Biosciences). Incubations were performed on ice with 6 μg of nuclear extract, 100,000 c.p.m. of labeled probe, 2 μg poly(dI-dC), 5% v/v glycerol, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 100 mM NaCl, and 10 mM Tris–HCl buffer (pH 8.0) for 15 min. To assess a direct interaction of PT with nuclear proteins, in another series of experiments, PT or 15-deoxy-$\Delta^{12,14}$-PGJ_2 was incubated for 10 min with nuclear extracts from zymosan-stimulated cells prior to the addition of the oligonucleotide probe. Complexes were analyzed by nondenaturating 6% polyacrylamide gel electrophoresis in 0.5× Tris-borate buffer followed by autoradiography of the dried gel. Densitometric analysis was performed in a GS-700 densitometer (Bio-Rad).

2.6. Ribonuclease protection assay (RPA)

Total RNA was extracted using the Trizol® reagent (Life Technologies Inc.). The Riboquant™ multi-probe Rnase protection assay system was used according to manufacturer's instructions with a mouse cytokine multi-probe template set (PharMingen). Densitometric analysis was performed in a GS-700 densitometer (Bio-Rad).

2.7. Western blot analysis

Mouse peritoneal macrophages obtained as described above or cells present in exudates from the mouse air pouch [15] were treated with lysis buffer (1% Triton X-100, 1% deoxycholic acid, 20 mM NaCl, and 25 mM Tris, pH 7.5, sonicated and ultracentrifuged. iNOS or COX-2 protein expression was studied in the cytosolic or microsomal fractions, respectively. Protein was measured by the Bradford method using bovine serum albumin as standard. Equal amounts of protein were loaded on 12.5% sodium dodecyl sulphate–polyacrilamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes for 90 min at 125 mA. Membranes were blocked in phosphate buffer saline (0.02 M, pH 7.0)-Tween-20 (0.1%) containing 3% w/v unfatted milk. For iNOS, membranes were incubated with specific anti-iNOS polyclonal antiserum (1:1000 dilution); for COX-2, membranes were incubated with specific anti-COX-2 polyclonal antiserum (1:1000 dilution). After washing, membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG (1:20,000 dilution). The immunoreactive bands were visualized using an enhanced chemiluminescence system (ECL, Amersham Biosciences). Cytoplasmic or nuclear extracts from peritoneal cells were used for Western blotting of proteins of the NF-κB pathway. Equal amounts of protein were loaded on 15% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Membranes were blocked in phosphate buffered saline-Tween 20 containing 3% w/v unfatted milk and incubated with polyclonal antibodies against either p65 or IκBα (1/500). Anti-phospho-(Ser^{32}) IκBα antibody (1:750 dilution) was used according to manufacturer's instructions and incubation solution contained IκBα (1–317) (50 ng/mL) [20]. Finally, membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG (1:20,000 dilution). The immunoreactive bands were visualized using an enhanced chemiluminescence system (ECL, Amersham Biosciences). Densitometric analysis was performed in a GS-700 densitometer (Bio-Rad).

2.8. Statistical analysis

The results are presented as mean ± SEM; N represents the number of experiments or animals. The level of statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons.

3. Results

3.1. Effect on cell migration, nitrite, PGE_2, cytokines, and protein expression in the mouse air pouch injected with zymosan

We selected this model of inflammation to assess the effects of PT on some parameters of the inflammatory

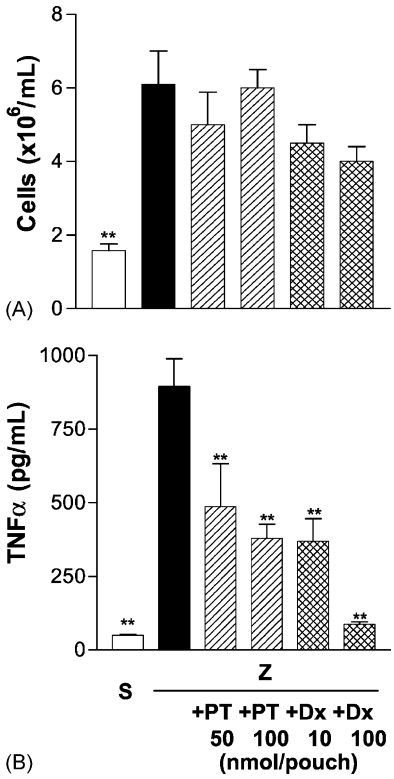

Fig. 2. Inhibitory effects of PT and dexamethasone in the 2 hr zymosan-injected mouse air pouch. (A) Cell migration and (B) TNFα levels in exudates. S, saline; Z, zymosan; Dx, dexamethasone. Results are the mean± SEM of 6–10 animals; $^{**}P < 0.01$ with respect to the zymosan control group.

response *in vivo* not addressed in our first report. According to previous studies [15] and preliminary experiments, 2 hr of stimulation with zymosan led to the highest TNFα production in the air pouch which preceded maximal cell migration. This time point was used to confirm the inhibitory effects of PT, whereas induction of COX-2 and iNOS required later times and thus, 12 hr of zymosan stimulation was selected to assess the effects of PT on protein expression and the levels of PGE_2, nitrite, and IL-1β in inflammatory exudates. At 2 hr after zymosan administration, PT did not modify cell accumulation but decreased TNFα secretion in the air pouch in a dose-dependent manner (Fig. 2). By 12 hr, cell migration was significantly inhibited by this agent, accompanied by reductions in nitrite, PGE_2, and IL-1β levels (Fig. 3). At this time point, a high expression of iNOS and COX-2 protein was detected by Western blotting in the cells accumulating in the air pouch exudate, whereas PT administration significantly decreased the expression of both proteins induced by zymosan treatment (Fig. 4A and B).

3.2. Effect on the release of nitrite, PGE_2, and TNFα induced by zymosan in mouse peritoneal macrophages. Relationship with protein or mRNA expression of iNOS, COX-2, and TNFα

We next examined whether the release of these inflammatory mediators induced by zymosan was also affected

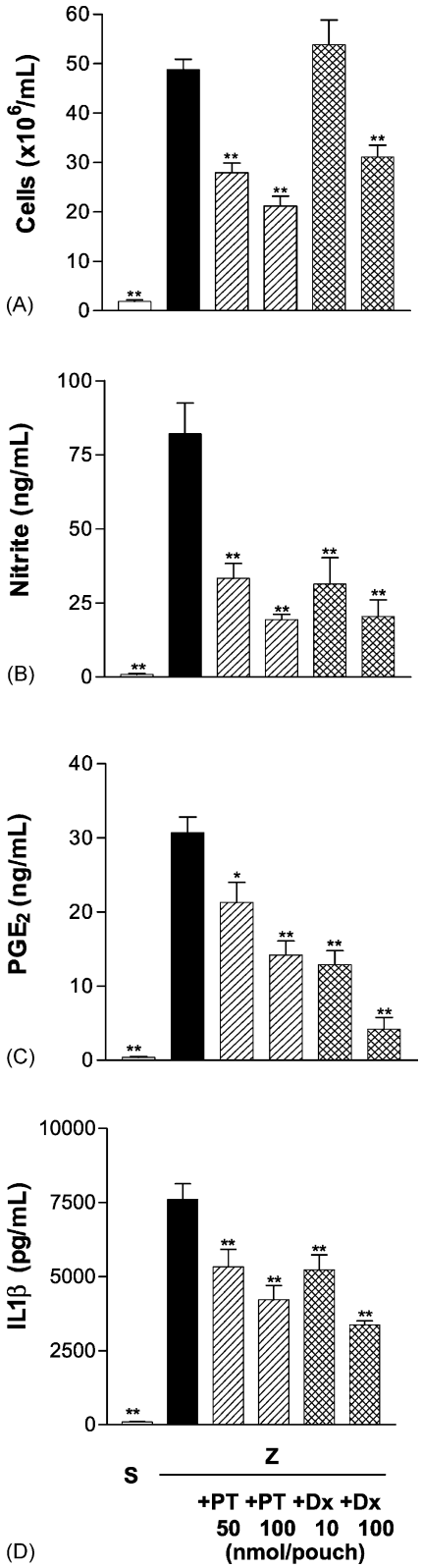

Fig. 3. Inhibitory effects of PT and dexamethasone in the 12 hr zymosan-injected mouse air pouch. (A) Cell migration, (B) nitrite, (C) PGE_2, and (D) IL-1β levels in exudates. S, saline; Z, zymosan; Dx, dexamethasone. Results are the mean ± SEM of 6–10 animals; $^{*}P < 0.05$, $^{**}P < 0.01$ with respect to the zymosan control group.

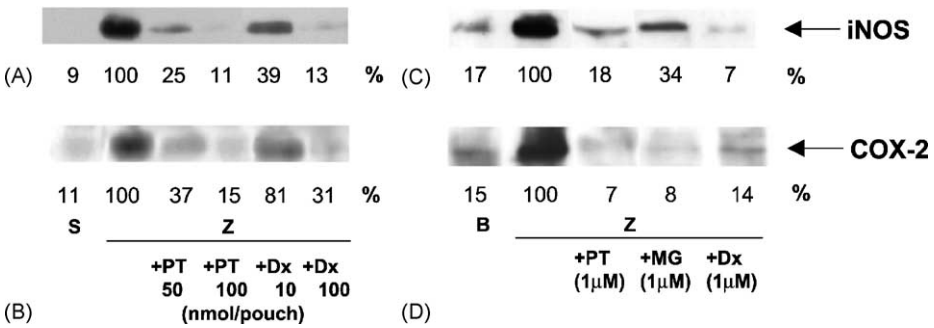

Fig. 4. Effect of PT on iNOS and COX-2 expression. Western blot and densitometric analysis of iNOS (A) and COX-2 (B) in the 12 hr zymosan-injected mouse air pouch. iNOS (C) and COX-2 (D) expression in mouse peritoneal macrophages preincubated with drugs or vehicle for 30 min and then stimulated with zymosan for 18 hr. Densitometric analysis is expressed as percentages of maximal band intensity (zymosan control group). S, saline; Z, zymosan; B, basal (nonstimulated cells); PT, petrosaspongiolide M; Dx, dexamethasone; MG, MG132. The figure is representative of three similar experiments.

by PT in mouse peritoneal macrophages. At concentrations not affecting cell viability, as assessed by the MTT test (data not shown), PT potently inhibited the release of nitrite, PGE_2, and TNFα in a concentration-dependent manner (Table 1). As shown in Fig. 4C and D, Western blot analysis confirmed that inhibition of NO and PGE_2 generation by PT was due to the reduction in iNOS and COX-2 protein expression, respectively. These effects of PT were similar to those observed for dexamethasone. By RPA analysis, we have also shown that PT inhibits TNFα mRNA expression in these cells after zymosan stimulation. Similarly, inhibition of the proteasome by MG132 reduced the mRNA levels of this cytokine in our experimental system (Fig. 5).

3.3. Effect on NF-κB–DNA binding activity

To evaluate the role of NF-κB in the mechanism of action of PT, we analyzed nuclear protein extracts from mouse peritoneal macrophages stimulated with zymosan either in the presence or absence of PT, for NF-κB–DNA binding activity using a radiolabeled NF-κB-specific oligonucleotide. Strong radioactive DNA binding to nuclear proteins was observed after 2 hr when cells were treated with zymosan. Nuclear extracts of cells incubated with PT and zymosan showed a protein–DNA complex migrating at the same mobility but the DNA-binding activity was reduced as compared to the zymosan controls and this effect was concentration dependent (Fig. 6A). To test whether this inhibitory effect in intact cells was due to a direct interaction of PT with nuclear proteins, experiments were performed incubating this compound with nuclear extracts of zymosan-stimulated mouse peritoneal macrophages. As shown in Fig. 6B, PT did not interfere with the NF-κB–DNA binding, in contrast to 15-deoxy-$\Delta^{12,14}$-PGJ_2.

Table 1
Effect of PT on TNFα, nitrite, and PGE_2 generation in zymosan-stimulated mouse peritoneal macrophages

	TNFα (IC_{50}, nM)	Nitrite (IC_{50}, nM)	PGE_2 (IC_{50}, nM)
PT	780 (735–820)	311 (295–342)	94 (89–150)
Dexamethasone	52 (49–63)	20 (17–32)	19 (12–37)

IC_{50} values and 95% confidence intervals were calculated from at least four significant concentrations (N = 6). Measurement of TNFα release was made 6 hr after zymosan stimulation (nonstimulated cells = 0.8 ± 0.1 ng/mL; zymosan-stimulated cells = 5.5 ± 0.4 ng/mL). Nitrite (nonstimulated cells = 86.2 ± 6.0 ng/mL; zymosan-stimulated cells = 291.3 ± 16.5 ng/mL) and PGE_2 (nonstimulated cells = 3.0 ± 0.5 ng/mL; zymosan-stimulated cells = 20.8 ± 1.9 ng/mL) levels were determined in 18 hr zymosan-stimulated mouse peritoneal macrophages.

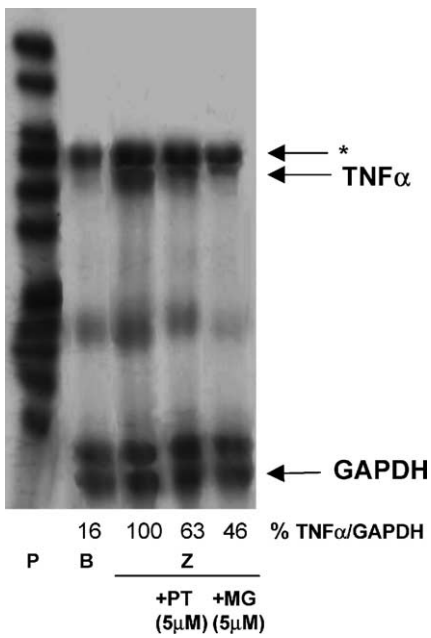

Fig. 5. Effect of PT on TNFα mRNA expression in mouse peritoneal macrophages. Cells were preincubated with drugs for 30 min and then stimulated with zymosan for 3 hr. RNA was extracted and RPA was performed as described in Section 2. Densitometric analysis is expressed as percentages of maximal TNFα/GAPDH rate (zymosan control group). P, cDNA probes; B, basal (nonstimulated cells); Z, zymosan; MG, MG132. *This band is assigned to IL-6. The figure is representative of three similar experiments.

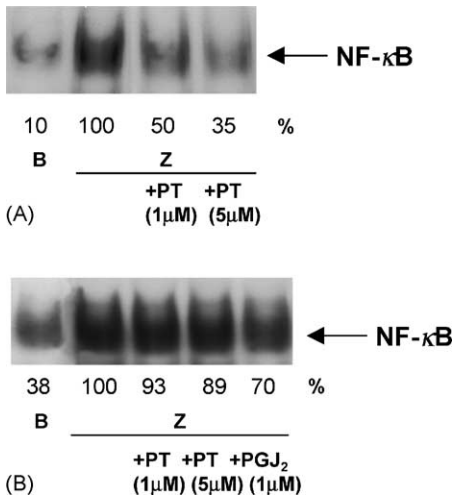

Fig. 6. Effect of PT on NF-κB–DNA binding in nuclear extracts of mouse peritoneal macrophages. (A) Intact cell treatment. Cells were preincubated with PT for 30 min followed by zymosan stimulation for 2 hr and then nuclear extracts were obtained as described in Section 2. (B) Nuclear extract treatment. Cells were stimulated with zymosan for 2 hr and then nuclear extracts were obtained as described in Section 2. Densitometric analysis is expressed as percentages of maximal band intensity (zymosan control group). B, basal (nonstimulated cells); Z, zymosan. PT or 15-deoxy-$\Delta^{12,14}$-PGJ_2 (PGJ_2) was incubated with nuclear extracts for 10 min. Results are representative of three independent experiments.

3.4. Effect on NF-κB translocation and IκBα degradation and phosphorylation

To elucidate the mechanisms involved in the inhibitory effects of PT on the NF-κB signaling pathway, we analyzed by Western blotting the influence of this compound on p65 translocation to the cell nucleus induced by zymosan. PT and the proteasome inhibitor MG132 inhibited the translocation of this protein (Fig. 7). We also examined the changes in cytoplasmic IκBα protein levels to determine whether degradation of this inhibitory protein is affected by PT. Immunoblotting analysis revealed that zymosan

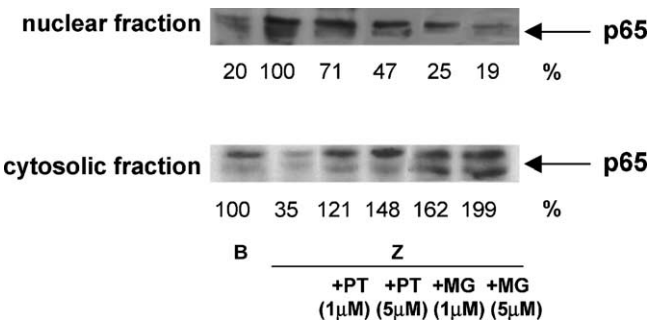

Fig. 7. Effect of PT on p65 protein expression in nuclear and cytosolic extracts of mouse peritoneal macrophages. Cells were preincubated with PT for 30 min before zymosan stimulation for 2 hr and then nuclear and cytosolic extracts were prepared. Densitometric analysis is expressed as percentages of band intensity of zymosan control group in nuclear fraction or basal group in cytosolic fraction. B, basal (nonstimulated cells); Z, zymosan. Results are representative of three independent experiments.

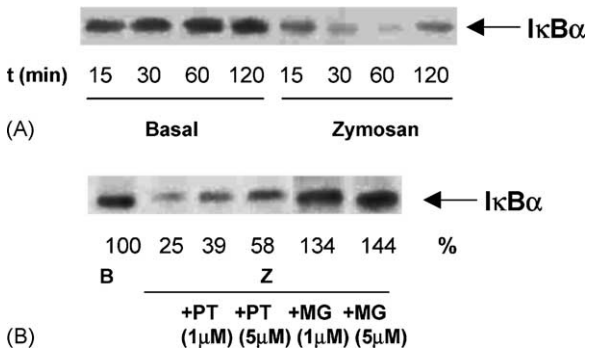

Fig. 8. Kinetic analysis and concentration effect of PT on IκBα degradation in cytosolic extracts of mouse peritoneal macrophages. (A) Cytosolic extracts from nonstimulated cells (basal) or stimulated with zymosan for 15, 30, 60, and 120 min were subjected to Western blot analysis. (B) Cells were preincubated with PT or MG132 for 30 min before zymosan stimulation for 60 min and then cytosolic extracts were prepared. Densitometric analysis is expressed as percentages of band intensity of basal group. B, basal (nonstimulated cells); Z, zymosan; MG, MG132. Results are representative of four independent experiments.

induced the proteolysis of IκBα within 15–60 min. Fig 8A shows that this was followed by newly synthesized IκBα accumulation in these cells. PT treatment increased IκBα expression, although to a lower extent than MG132 at the same concentration (Fig. 8B). Phosphorylation of IκBα is a key step for the subsequent degradation of this protein and proteasome inhibition allows accumulation of the unstable phosphorylated IκBα [21]. We used a specific anti-phospho-(Ser^{32}) IκBα antibody for Western blot analysis showing that PT treatment decreased the expression of this phosphorylated form induced by zymosan either in the presence or absence of MG132 (Fig. 9).

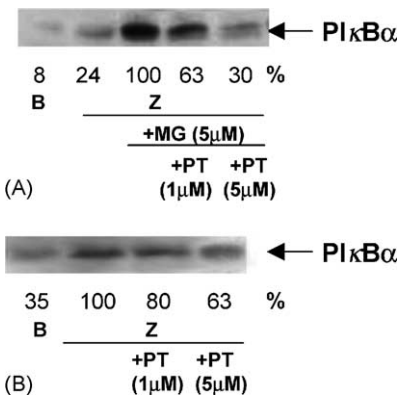

Fig. 9. Effect of PT on IκBα serine 32 phosphorylation in cytosolic extracts of mouse peritoneal macrophages. (A) Cells were preincubated with PT and MG132 for 30 min before zymosan stimulation for 60 min and then cytosolic extracts were prepared. Densitometric analysis is expressed as percentages of maximal band intensity (zymosan + MG132). (B) cells were preincubated with PT for 30 min before zymosan stimulation for 60 min and then cytosolic extracts were prepared. Densitometric analysis is expressed as percentages of maximal band intensity (zymosan control group). B, basal (nonstimulated cells); Z, zymosan; MG, MG132. Results are representative of four independent experiments.

4. Discussion

The marine metabolite PT exhibits anti-inflammatory properties in several *in vivo* models [10]. We have demonstrated in the present work that PT inhibited the response induced by zymosan in the mouse air pouch, with an anti-inflammatory behavior similar to dexamethasone, resulting in the control of cytokine, NO and PGE_2 production. This study also extends previous observations [10] and thus our results suggest that an early reduction in TNFα levels contributes to the control of cell accumulation into the air pouch exudate.

To explore the mechanisms of action of this natural product, we have used mouse peritoneal macrophages stimulated with zymosan, where we have confirmed the inhibitory effects obtained *in vivo*. The expression of inflammatory mediators is enhanced in pathological conditions by several mechanisms including gene transcription, which is the first and in many cases the most important step to control the increased expression of inflammatory proteins. Our data indicate that inhibition of NO and PGE_2 production by PT *in vivo* and *in vitro* correlated with inhibition of iNOS and COX-2 expression, respectively. We have also shown that PT effects on TNFα were related with decreased mRNA levels. Nevertheless, PT may have additional effects on TNFα production since it was more effective as inhibitor of TNFα protein levels. These effects of PT seem to be independent of $sPLA_2$ inhibition since in mouse peritoneal macrophages stimulated with zymosan, $cPLA_2$ mediates arachidonic acid release [22] and we have previously demonstrated that this enzyme is not a target for PT [10]. The interference with NF-κB activation may explain at least in part the inhibitory effects of PT observed in the present study, since this transcription factor appears to play a central role in the transcriptional regulation of iNOS, COX-2, and TNFα by macrophages [23–26].

Production of TNFα and other inflammatory cytokines is NF-κB-dependent in rheumatoid synovial tissue [27]. In chronic inflammatory conditions, activation of transcription factors such as NF-κB and activator protein-1 by cytokines, drives a regulatory loop contributing to the perpetuation of the disease [23,28]. Thus, inhibitors of cytokine production or anti-cytokine therapies exert beneficial effects in chronic inflammatory disorders [11]. In these conditions, there is also induction of COX-2 and elevated PG production in the inflamed joints. Since conventional nonsteroidal anti-inflammatory drugs inhibit COX-2 but they also affect physiological prostanoid production due to COX-1 inhibition, it is of great interest to selectively modulate COX-2 [29], which can be achieved by PT through the control of protein expression.

Nevertheless, it should be taken into account that activation of nuclear factors is cell- and stimulus-specific and little is known of the mechanisms leading to NF-κB activation after macrophage stimulation with zymosan. It has been reported recently that this stimulus induces IL-8 in human monocytes [30] and activates the TNFα promoter in RAW 264.7 macrophages through the NF-κB pathway [26]. Our results indicate that NF-κB is activated in response to zymosan in primary mouse macrophages. This transcription factor is located in the cytoplasm associated with the inhibitory protein IκB, which upon activation by inflammatory stimuli is phosphorylated and degraded. It is known that stimuli such as cytokines or lipopolysaccharide lead to the rapid proteolysis of IκB *via* a common pathway involving the phosphorylation of this molecule on critical serine residues (Ser^{32} and Ser^{36}) within the N-terminal signal response domain. Phosphorylated IκBs undergo polyubiquitination by constitutively active enzymes followed by degradation mediated by the 26S proteasome, which allows NF-κB translocation into the nucleus and binding to promoter regions of target genes [12,13].

We have observed that IκBα is phosphorylated and degraded in response to zymosan in mouse peritoneal macrophages, whereas proteasome inhibition increased IκBα expression and stabilized its phosphorylated form. However, our results do not exclude that mechanisms other than IκB degradation may participate in NF-κB activation by zymosan. In this respect, the contribution of alternative pathways is suggested in human macrophages by reports on the lack of effect of IκBα overexpression on zymosan-induced TNFα levels [31]. In addition, tyrosine phosphorylation without IκBα degradation has been reported in Jurkat cells [32]. It should be mentioned that tyrosine kinase activity has been implicated in murine peritoneal macrophage activation by zymosan [33], although its involvement in NF-κB activation has not been established.

Our data demonstrate that PT is a potent inhibitor of NF-κB–DNA binding. The current study suggests that PT interferes with the key step in NF-κB activation, the phosphorylation of IκB, although we have observed a lower potency on this last process. This reaction is catalyzed by a protein kinase activity specific for the N-terminal regulatory serines of IκBs, the IκB kinase (IKK) complex which is activated by phosphorylation of its IKKβ subunit [12]. Whether PT effects are mediated by interfering with this enzyme or upstream kinases such as NF-κB-inducing kinase and mitogen-activated protein kinase (MAPK)/ERK kinase kinase-1 [34] remains to be determined. The inhibitory effect of PT on IκBα phosphorylation may justify partially the NF-κB–DNA binding decrease and our results do not exclude that this compound may affect other signaling pathways resulting in modification of NF-κB activity.

Inhibition of $sPLA_2$ by PT would be dependent on the presence of the reactive pyranofuranone moiety [35]. Nevertheless, we have not observed a direct interaction of this natural product with proteins of the NF-κB complex, a mechanism participating in the inhibitory effects of cyclopentenone PGs on NF-κB activation [36].

On the other hand, inhibition of $sPLA_2$ during inflammatory states may result in anti-inflammatory effects not

dependent on the modulation of lipid mediators. $sPLA_2$ could be itself an important inflammatory mediator independently of its catalytic activity, since it may play a role in the transcription of inflammatory proteins. Thus, $sPLA_2$ could act as an important amplifier of cytokine-mediated PG production by macrophages due to an enhancement of COX-2 expression, which contributes to the severity of rheumatoid synovial inflammation [37].

We have previously demonstrated that PT is an interesting anti-inflammatory agent [10]. In the present work, we have shown that PT reduces the release of a number of proinflammatory mediators such as eicosanoids, NO and cytokines, suggesting a potentially wide therapeutic spectrum. This compound also displays the advantage of lacking ulcerogenic effects, as compared with nonselective COX inhibitors.

Acknowledgments

This work was supported by Grant SAF-2001 2639 (Ministerio de Ciencia y Tecnología, Spain).

References

[1] Serhan CN, Haeggström JZ, Leslie CC. Lipid mediator networks in cell signaling: update and impact of cytokines. FASEB J 1996;10:1147–58.
[2] Balsinde J, Balboa MA, Insel PA, Dennis EA. Regulation and inhibition of phospholipase A_2. Annu Rev Pharmacol Toxicol 1999;39:175–89.
[3] Ribardo DA, Crowe SE, Kuhl KR, Peterson JW, Chopra AK. Prostaglandin levels in stimulated macrophages are controlled by phospholipase A_2-activating protein and by activation of phospholipase C and D. J Biol Chem 2001;276:5467–75.
[4] Ueno N, Murakami M, Kudo I. Functional crosstalk between phospholipase D(2) and signaling phospholipase A(2)/cyclooxygenase-2-mediated prostaglandin biosynthetic pathways. FEBS Lett 2000;475:242–6.
[5] Bomalaski JS, Clark MA. Phospholipase A_2 and arthritis. Arthritis Rheum 1993;36:190–8.
[6] Bowton DL, Seeds MC, Fasano MB, Goldsmith B, Bass DA. Phospholipase A_2 and arachidonate increase in bronchoalveolar lavage fluid after inhaled antigen challenge in asthmatics. Am J Respir Crit Care Med 1997;155:421–5.
[7] Weiss J, Inada M, Elsbach P, Crowl RM. Structural determinants of the action against *Escherichia coli* of a human inflammatory fluid phospholipase A_2 in concert with polymorphonuclear leukocytes. J Biol Chem 1994;269:26331–7.
[8] Takasaki J, Kawauchi Y, Masuho Y. Synergistic effect of type II phospholipase A_2 and platelet-activating factor on Mac-1 surface expression and exocytosis of gelatinase granules in human neutrophils: evidence for the 5-lipoxygenase-dependent mechanism. J Immunol 1998;160:5066–72.
[9] García Pastor P, Randazzo A, Gomez-Paloma L, Alcaraz MJ, Payá M. Effects of petrosaspongiolide M, a novel phospholipase A_2 inhibitor, on acute and chronic inflammation. J Pharmacol Exp Ther 1999;289:166–72.
[10] Feldmann M, Maini RN. The role of cytokines in the pathogenesis of rheumatoid arthritis. Rheumatology (Oxford) 1999;38(Suppl 2):3–7.
[11] Karin M. The beginning of the end: IκB kinase (IKK) and NF-κB activation. J Biol Chem 1999;274:27339–42.
[12] Abraham E. NF-κB activation. Crit Care Med 2000;28:N100–4.
[13] Yamamoto Y, Gaynor RB. Therapeutic potential of inhibition of the NF-κB pathway in the treatment of inflammation and cancer. J Clin Invest 2001;107:135–42.
[14] Randazzo A, Debitus C, Minale L, García-Pastor P, Alcaraz MJ, Payá M, Gomez-Paloma L. Petrosaspongiolides M-R: new potent and selective phospholipase A_2 inhibitors from the new Caledonian marine sponge *Petrosaspongia nigra*. J Nat Prod 1998;61:571–5.
[15] Posadas I, Terencio MC, Guillén I, Ferrándiz ML, Coloma J, Payá M, Alcaraz MJ. Co-regulation between cyclo-oxygenase-2 and inducible nitric oxide synthase expression in the time-course of murine inflammation. Naunyn Schmiedebergs Arch Pharmacol 2000;361:98–106.
[16] Pennanen N, Lapinjoki S, Palander A, Urtti A, Monkkonen J. Macrophage-like RAW 264 cell line and time-resolved fluoroimmunoassay (TRFIA) as tools in screening drug effects on cytokine secretion. Int J Immunopharmacol 1995;17:475–80.
[17] Misko TP, Schilling RJ, Salvemini D, Moore WM, Currie MG. A fluorometric assay for the measurement of nitrite in biological samples. Anal Biochem 1993;214:11–6.
[18] Moroney MA, Alcaraz MJ, Forder RA, Carey F, Hoult JRS. Selectivity of neutrophil 5-lipoxygenase and cyclo-oxygenase inhibition by an anti-inflammatory flavonoid glycoside and related aglycone flavonoids. J Pharm Pharmacol 1988;40:787–92.
[19] López-Collazo E, Hortelano S, Rojas A, Boscá L. Triggering of peritoneal macrophages with IFN-α/β attenuates the expression of inducible nitric oxide synthase through a decrease in NF-κB activation. J Immunol 1998;160:2889–95.
[20] Castrillo A, Diaz-Guerra MJ, Hortelano S, Martin-Sanz P, Bosca L. Inhibition of IκB kinase and IκB phosphorylation by 15-deoxy-delta(12,14)-prostaglandin J(2) in activated murine macrophages. Mol Cell Biol 2000;20:1692–8.
[21] Chen Z, Hagler J, Palombella VJ, Melandri F, Scherer D, Ballard D, Maniatis T. Signal-induced site-specific phosphorylation targets IκBα to the ubiquitin-proteasome pathway. Genes Dev 1995;9:1586–97.
[22] Gijon MA, Spencer DM, Siddiqi AR, Bonventre JV, Leslie CC. Cytosolic phospholipase A_2 is required for macrophage arachidonic acid release by agonists that Do and Do not mobilize calcium. Novel role of mitogen-activated protein kinase pathways in cytosolic phospholipase A_2 regulation. J Biol Chem 2000;275:20146–56.
[23] Drouet C, Shakhov AN, Jongeneel CV. Enhancers and transcription factors controlling the inducibility of the tumor necrosis factor-alpha promoter in primary macrophages. J Immunol 1991;147:1694–700.
[24] Xie QW, Kashiwabara Y, Nathan C. Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. J Biol Chem 1994;269:4705–8.
[25] Caivano M, Gorgoni B, Cohen P, Poli V. The induction of cyclooxygenase-2 mRNA in macrophages is biphasic and requires both CCAAT enhancer-binding protein beta (C/EBPbeta) and C/EBPdelta transcription factors. J Biol Chem 2001;276:48693–701.
[26] Young SH, Ye J, Frazer DG, Shi X, Castranova V. Molecular mechanism of tumor necrosis factor-alpha production in 1 → 3-beta-glucan (zymosan)-activated macrophages. J Biol Chem 2001;276:20781–7.
[27] Bondeson J, Foxwell B, Brennan F, Feldmann M. Defining therapeutic targets by using adenovirus: blocking NF-κB inhibits both inflammatory and destructive mechanisms in rheumatoid synovium but spares anti-inflammatory mediators. Proc Natl Acad Sci USA 1999;96:5668–73.
[28] Monks BG, Martell BA, Buras JA, Fenton MJ. An upstream protein interacts with a distinct protein that binds to the cap site of the human interleukin 1 beta gene. Mol Immunol 1994;31:139–51.
[29] Chan CC, Boyce S, Brideau C, Charleson S, Cromlish W, Ethier D, Evans J, Ford-Hutchinson AW, Forrest MJ, Gauthier JY, Gordon R, Gresser M, Guay J, Kargman S, Kennedy B, Leblanc Y, Leger S,

Mancini J, O'Neill GP, Ouellet M, Patrick D, Percival MD, Perrier H, Prasit P, Rodger I. Rofecoxib [Vioxx, MK-0966; 4-(4′-methylsulfonylphenyl)-3-phenyl-2-(5*H*)-furanone]: a potent and orally active cyclooxygenase-2 inhibitor. Pharmacological and biochemical profiles. J Pharmacol Exp Ther 1999;290:551–60.

[30] Friedland JS, Constantin D, Shaw TC, Stylianou E. Regulation of interleukin-8 gene expression after phagocytosis of zymosan by human monocytic cells. J Leukoc Biol 2001;70:447–54.

[31] Bondeson J, Browne KA, Brennan FM, Foxwell BM, Feldmann M. Selective regulation of cytokine induction by adenoviral gene transfer of IκBα into human macrophages: lipopolysaccharide-induced, but not zymosan-induced, proinflammatory cytokines are inhibited, but IL-10 is nuclear factor-κB independent. J Immunol 1999;162: 2939–45.

[32] Imbert V, Rupec RA, Livolsi A, Pahl HL, Traenckner EB, Mueller-Dieckmann C, Farahifar D, Rossi B, Auberger P, Baeuerle PA, Peyron JF. Tyrosine phosphorylation of IκB-α activates NF-κB without proteolytic degradation of IκB-α. Cell 1996;86:787–98.

[33] Glaser KB, Sung A, Bauer J, Weichman BM. Regulation of eicosanoid biosynthesis in the macrophage. Involvement of protein tyrosine phosphorylation and modulation by selective protein tyrosine kinase inhibitors. Biochem Pharmacol 1993;45:711–21.

[34] Nakano H, Shindo M, Sakon S, Nishinaka S, Mihara M, Yagita H, Okumura K. Differential regulation of IκB kinase alpha and beta by two upstream kinases, NF-κB-inducing kinase and mitogen-activated protein kinase/ERK kinase kinase-1. Proc Natl Acad Sci USA 1998;95:3537–42.

[35] Soriente A, De Rosa MMC, Scettri A, Sodano G, Terencio MC, Paya M, Alcaraz MJ. Manoalide. Curr Med Chem 1999;6:415–31.

[36] Rossi A, Kapahi P, Natoli G, Takahashi T, Chen Y, Karin M, Santoro MG. Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of IκB kinase. Nature 2000;403:103–8.

[37] Bidgood MJ, Jamal OS, Cunningham AM, Brooks PM, Scott KF. Type IIA secretory phospholipase A_2 up-regulates cyclooxygenase-2 and amplifies cytokine-mediated prostaglandin production in human rheumatoid synoviocytes. J Immunol 2000;165:2790–7.