Inhibition of NFκB-Mediated Interleukin-1β-Stimulated Prostaglandin E₂ Formation by the Marine Natural Product Hymenialdisine

AMY ROSHAK, JEFFERY R. JACKSON, MARIE CHABOT-FLETCHER and LISA A. MARSHALL

Department of Immunopharmacology, SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania

Accepted for publication July 14, 1997

ABSTRACT

Exposure of human rheumatoid synovial fibroblasts (RSF) to interleukin 1β (IL-1β) results in the coordinate up-regulation of 85-kDa phospholipase A₂ (PLA₂) and mitogen-inducible cyclooxygenase (COX II) and subsequent biosynthesis of prostaglandin E₂ (PGE₂). We have recently demonstrated, through the use of oligonucleotide decoys and antisense, the participation of the proinflammatory transcription factor, nuclear factor κB (NFκB), in the regulation of the prostanoïd-metabolizing enzymes. Hymenialdisine, a marine natural product has recently been characterized as an inhibitor of NFκB activation and exposure of IL-1-stimulated RSF inhibited PGE₂ production in a concentration-dependent manner (IC₅₀ ~ 1 μM). Alternatively, both an analog, aldisine, and the protein kinase C inhibitor, RO 32-0432, were without affect. Direct action of hymenialdisine on IL-1-induced NFκB activation was demonstrated by a significant reduction (~80%) in NFκB binding to the classical κB consensus motif (as assessed by electrophoretic mobility shift assay) and inhibition of stimulated p65 migration from the cytosol of treated cells (as assessed by Western analysis). Consistent with the role of NFκB in the transcriptional regulation of COX II and 85-kDa PLA₂, hymenialdisine-treated RSF did not transcribe the respective mRNAs as response to IL-1. This led to reductions in their respective protein levels and subsequent reductions in the ability to produce PGE₂. Specificity of action was suggested as IL-1-stimulated interleukin-8 (IL-8) production, which is known to be an NFκB-regulated event, was also inhibited by hymenialdisine, whereas IL-1-induced production of vascular endothelial growth factor, a non-NFκB-regulated gene, was not affected by exposure to hymenialdisine. Taken together, hymenialdisine inhibits IL-1-stimulated-RSF PGE₂ formation acting predominantly through modulation of NFκB activation and offers an interesting novel tool to evaluate the role of NFκB in inflammatory disease.

Rheumatoid arthritis is a complex autoimmune disease characterized by chronic inflammation, bone erosion and proliferation of the synovial lining. Inflammatory cytokines such as IL-1β are elevated in the joint fluid of patients with rheumatoid arthritis and, as such, are thought to play a critical role in the progression of the disease (Goddard et al., 1992; Sipe et al. 1994). Exposure of RSF to IL-1 induces the expression of several inflammatory genes and results in the production of a wide variety of pro-inflammatory mediators including IL-8 and PGE₂ (Dayer et al., 1986; Gilman et al., 1988; Roshak et al., 1996a, b). In the case of PGE₂, IL-1 causes the coordinate induction of RSF 85-kDa PLA₂ and mitogen-inducible COX-II mRNA and subsequent increases in protein levels. This results in nanogram quantities of PGE₂ produced by these cells (Angel et al., 1994; Hulikower et al., 1994; Roshak et al., 1996a).

IL-1β is known to act through the activation of the pro-inflammatory transcription factor, NFκB (Siebenlist et al., 1994; Thanos and Maniatis, 1995). The NFκB family of transcription factors comprises several distinct gene products including the mammalian forms, p65, p50, c-rel and Rel-B (Baldwin, 1996; Siebenlist et al., 1994). These proteins form a variety of homo- and heterodimer pairs, display different affinities for distinct DNA binding motifs and are expressed in varying levels in different tissues. Typically, NFκB dimers are confined to the cytoplasm of nonstimulated cells through sequestration of the nuclear localization sequence by its endogenous inhibitor, IκB (Miymoto and Verma, 1995). Upon cellular activation through a variety of stimuli (i.e., cytokines, viral or bacterial products, free radicals or physical

ABBREVIATIONS: COX-II, cyclooxygenase II; IL-1β, interleukin-1β; PLA₂, phospholipase A₂; rh 85 kDa-PLA₂, recombinant human baculovirus expressed 85-kDa PLA₂; NFκB, nuclear factor kappa B; HEPES, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; RSF, rheumatoid synovial fibroblasts; DTT, dithiothreitol; PGE₂, prostaglandin E₂; EMSA, electrophoretic mobility shift assay; TNF, tumor necrosis factor; PDCT, pyrrolidine dithiocarbamate; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; PKC, protein kinase C; VEGF, vascular endothelial growth factor.
stress), IκB is phosphorylated and degraded via the ubiquitin proteasome pathway (Li et al., 1995; Palombella et al., 1994; Traenckner et al., 1994). Liberated NFκB dimers are then free to translocate to the nucleus, bind to specific xB motifs in target gene enhancers and induce the transcription of several pro-inflammatory genes exemplified by the cytokines IL-6 (Liberman and Baltimore, 1990) and IL-8 (Mukaida et al., 1989; Kunsch and Rosen, 1993) and adhesion molecules such as intracellular and vascular cell adhesion molecules (Ledebur and Parks, 1995; Muller et al., 1995; Shu et al., 1993). We recently demonstrated a critical role of the NFκB protein, p65, in the IL-1-regulated expression of 85-kDa PLA2 and COX-II through the use of oligonucleotide decoys and specific antisense (Roshak et al., 1996b).

NFκB activation has been described in a variety of inflammatory disease models including airway inflammation (Adcock et al., 1994; Blackwell et al., 1994) and atherosclerosis (Liao et al., 1994) and is thought to significantly contribute to the progression of the disease through the enhanced expression of target inflammatory genes. In rheumatoid synovium, immunohistochemistry identified NFκB proteins, p65 and p50, constitutively present in the nuclei of synovial lining cells (Handel et al., 1995). Further, exposure of cultured synovial cells to TNF caused increased nuclear translocation of NFκB proteins which led to expression of NFκB-dependent genes, IL-6 and intracellular adhesion molecule and proliferation (Fujisawa et al., 1996). This was inhibitable by treatment with the antioxidant, N-acetyl-l-cysteine. Further, glucocorticosteroids and salicylates have recently been shown to suppress NFκB activity through transcriptional up-regulation of IκB (Auphan et al., 1995; Scheinman et al., 1995) and prevention of its degradation (Kopp and Ghosh, 1994), respectively. Taken together, targeting NFκB activation therefore provides an attractive approach for developing novel anti-inflammatory agents.

Several agents have been shown to possess NFκB modulatory activity. Antioxidants, such as n-acetyl-cysteine and PDCT, have been reported to repress activation of NFκB through the inhibition of IκB phosphorylation (Kawai et al., 1995). Although these agents have provided researchers with tools to assess NFκB activity, they require use at high concentrations, which in some cases are toxic, and they exhibit other activities, which often makes interpretation of results difficult. Better tools would clearly be beneficial in the study of NFκB regulation. Recently, the marine natural product, hymenialdisine, and its analog, hymenialdisine and provide a novel reagent to study NFκB by hymenialdisine and provide a novel approach to therapeutically modifying inflammatory mediators.

Methods

Materials and chemicals. PDCT was purchased from Sigma Chemical Co. (St. Louis, MO). Hymenialdisine (SK&F 108752) and hisidine (BS 203063) were obtained from Suntory Ltd., Japan. RO 32–0432 was synthesized by the Department of Medicinal Chemistry, SmithKline Beecham Pharmaceuticals according to the reported synthesis (Bit et al., 1993)

Human synovial fibroblast culture. Primary cultures of human RSF were obtained by enzymatic digestion of synovium obtained from 10 adult patients with rheumatoid arthritis as described previously (Roshak et al., 1996a). Cells were cultured in Earle's Minimal Essential Medium which contained 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin (GIBCO, Grand Island, NY), at 37°C and 5% CO2. Cultures were used at passages 4 through 9 to obtain a more uniform type I fibroblast population. For some studies, fibroblasts were plated at 5 × 10⁴ cells/ml in 16-mm diameter 24-well plates (Costar, Cambridge, MA). Cells were exposed to an optimal dose of IL-1β (1 ng/ml; Roshak et al., 1996a) (Genzyme, Cambridge, MA) for the designated time. Drugs in DMSO vehicle (1%) were added to the cell cultures 15 min before the addition of IL-1. Each study represents one of two to five individual experiments with RSF from different donors unless otherwise noted.

ELISA measurement of PGE₂, IL-8, and VEGF. PGE₂, or IL-8 levels in cell-free medium collected at the termination of the culture period were measured directly by enzyme immunoassay kits purchased from Cayman Chemical Co. (Ann Arbor, MI) and Biosource International (Camarillo, CA) respectively, as described previously (Roshak et al., 1996b). Vascular endothelial cell growth factor levels in cell-free medium were measured with a VEGF ELISA Kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol as described previously (Jackson et al., 1997). Sample or standard dilutions were made with experimental medium and results were expressed as nanograms per milliliter of medium as mean ± S.D. of triplicate determinations unless otherwise stated and are subjected to one-way analysis of variance and Duncan's multiple range test (P < .05) for statistical evaluation where indicated.

RSF subcellular fractionation. Human RSF were removed by trypsin/EDTA, resuspended to 1.0 × 10⁶ cells/ml in cold homogenization buffer (0.34 M sucrose, 10 mM HEPES, pH 7.4, 1 mM ethyl-eneglycol-bis(β-aminathyyl ether)-N,N',N'-tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 200 µM leupeptin, 20 µg/ml soybean trypsin inhibitor and 20 µg/ml aprotinin at 4°C) and disrupted on ice by sonication with a Bransonics probe tip as described previously (Roshak et al., 1996a). The homogenate was centrifuged at 100,000 × g for 60 min at 4°C to obtain a supernatant (cytosol) and particulate fraction. The particulate fraction was resuspended in 5 volumes of homogenization buffer. Protein concentration was measured by Bradford analysis (Bio-Rad, Richmond, CA). Both fractions were flash frozen with liquid N2 and stored at –80°C for analysis.

Immunoblot analysis. Cell fractions (25–50 µg protein) and/or recombinant protein standard were analyzed by SDS-polyacrylamide gel electrophoresis (10% gels; Bio-Rad) as described previously (Roshak et al., 1996a, b) and visualized by use of the ECL Western
blotting system (Amersham, Arlington Heights, IL). Rabbit polyclonal antiserum against the rh 85-kDa PLA₂ was prepared as described previously (Roshak et al., 1996a, b). Rabbit anti-human COX-II was kindly donated by D. Dewitt (Michigan State University, East Lansing, MI) and used as described previously (Roshak et al., 1996a, b). Positive control standards included a 24-hr lipopolysaccharide-stimulated monocyte particulate fraction (25 µg) containing COX-II protein and rh 85-kDa PLA₂ (Roshak et al., 1996a, b). Rabbit polyclonal antibodies to p65 were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and used according to the manufacturer’s instructions. Gels were scanned for density with UVP Images-tore 5000 (San Gabriel, CA). Measurement of pixels in the bands was expressed as an area value.

Preparation of nuclear extracts and electrophoretic mobility shift assay. Cultured RSF in T75 flasks were stimulated for 15 min in the presence of IL-1β (1 ng/ml; 37°C), washed twice with phosphate-buffered saline, then removed by trypsinization. Nuclear extracts were prepared according to published methods (Dignam et al., 1983; Osborne et al., 1989) with some modifications. In short, cells were pelleted by centrifugation and resuspended in buffer A, 20 µg/10⁶ cells (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT, 0.1% (w/v) Nonidet P-40). The cell suspension was incubated on ice for 10 min, and the nuclei were pelleted by microcentrifugation at 3500 rpm for 10 min at 4°C. The pellet was suspended in 15 µl of buffer B (20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM MgCl₂, 25% (v/v) glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride) and gently mixed for 20 min at 4°C. The sample was microcentrifuged at 14,000 rpm for 10 min at 4°C, and the resultant supernatant (nuclear extract) was diluted to 75 µl with buffer D (20 mM HEPES, pH 7.9, 50 mM KCl, 20% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride). Samples were stored at −80°C until analysis.

DNA binding reactions and electrophoretic mobility shift assay (EMSA). A double-stranded oligonucleotide containing the sequence corresponding to the classical NF-κB consensus site (5′-agt tga ggg gac ttt ccc agg c 3′) (Santa Cruz Biotechnology Inc.) was end-labeled with γ-32P-ATP with T4 kinase (Life Technologies, Gaithersburg, MD). Unincorporated nucleotides were removed by column chromatography over two Sephadex G-50 columns (Pharmacia, Piscataway, NJ). Binding reactions were carried out in a final volume of 25 µl consisting of 10 mM HEPES, pH 7.9, 4 mM NaCl, 1.5 mM MgCl₂, 25% (v/v) glycerol, 0.2 mM EDTA, 1 mM DTT, 10% glycerol, 1.5 mg/ml bovine serum albumin and 2 µg of poly(dI-dC). Each reaction, containing 10 µg of nuclear extract, and 0.5 ng of 32P-labeled oligonucleotide probe (~50,000 cpm) was incubated for 20 min at room temperature. Binding reactions were subjected to nondenaturing polyacrylamide electrophoresis through 4% gels in a 1× Tris-borate-EDTA buffer system. Gels were dried and subjected to autoradiography. We previously demonstrated specific NF-κB binding to IL-1-treated RSF nuclear extracts which was competed by unlabeled NF-κB motif but not by an unrelated oligonucleotide motif (OCT-1) (Roshak et al., 1996b).

Northern analysis. Total RNA was isolated from RSF with Trizol reagent (Gibco/BRL, Bethesda MD) according to the manufacturer’s protocol and quantitated by spectrophotometry. RNA (20 µg) were subjected to electrophoresis in 1% agarose gel containing formaldehyde. RNA molecular weight markers (Gibco/BRL) were also included flanking the samples. After electrophoresis, gels were rinsed twice by shaking for 15 min in 300 ml of distilled water followed by a 7-min incubation in 200 ml 50 mM NaOH. Gels were next incubated in 300 ml 10× SSC (1× = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0) for 20 min. RNA was transferred to Hybond N+ (Amersham, Amersham, UK) by vacuum blotting (Bio-Rad, Hercules, CA) in 10× SSC according to the manufacturers’ protocol. After transfer, RNA was fixed to the membrane by UV cross-linking (0.12 J/cm²). RNA samples and markers were visualized on the membrane by staining with 0.02% methylene blue in 0.3 M sodium acetate, pH 5.5, for 5 min followed by destaining in distilled water for 15 min. Hybridizations were carried out in bottles in a Hybaid oven (Hybaid Ltd, Middlesex, UK). Filters were prehybridized (20 ml/blot) in 6× SSC, 5× Denhardt’s (50x = 10 mg/ml Ficoll (400), 10 mg/ml polyvinylpyrrolidone, 10 mg/ml bovine serum albumin), 0.5% SDS, 0.1 mg/ml denatured salmon sperm DNA for 3 hr or more at 68°C. Hybridizations were done in prehybridization solution (10 ml/blot) containing 30 ng of denatured specific DNA probe labeled to 1 to 2 × 10⁶ dpm/µg with 32P (see below) at 68°C for 18 hr. After hybridization, blots were washed twice with 100 ml 2× SSC, 0.1% SDS for 15 min at 68°C in bottles, and once with 100 ml 1× SSC, 0.1% SDS for 30 min at 68°C in bottles. Blots were then removed from bottles and washed once with 200 ml 0.2× SSC, 0.1% SDS for 15 min at 68°C in a tray. Filters were analyzed on a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

DNA probes. 85-kDa PLA₂ probe was a 2.5-kb HindIII into SalI cDNA fragment, COX-II probe was a 1.2-kb EcoRI fragment of the murine PGBS-2 cDNA clone kindly provided by Dr. David DeWitt, Michigan State University. Labeled probes were prepared from 20 ng of cDNA by random priming with a Rediprime kit and 50 µCi [32P]-dCTP (Amersham, Arlington Heights, IL). Unincorporated nucleotides were removed by gel filtration with Quick Spin columns (Boehringer Mannheim, Indianapolis, IN).

Results

The effect of inhibitors on IL-1-induced RSF PGE₂ formation. Antioxidants such as PDCT have been effective but nonselective inhibitors of NF-κB activation, presumably functioning through the inhibition of IκB degradation. Because we have previously demonstrated NF-κB participation in IL-1-induced RSF PGE₂ production, PDCT was evaluated for its ability to inhibit PGE₂ formation in the RSF system. Confluent RSF in Earle’s Minimal Essential Medium with 10% fetal bovine serum were incubated with DMSO vehicle or PDCT (1–300 µM) for 15 min at 25°C before addition of IL-1β (24 hr, 37°C). Cells were monitored and toxicity was observed at concentrations greater than 30 µM, as assessed by morphology and trypan blue exclusion. Figure 1 shows that pretreatment with PDCT resulted in a concentration-dependent decrease in IL-1β-stimulated PGE₂ production (IC50 ~ 15 µM). Hymenialdisine (0.03–10.0 µM), an analog, aldisine (10 µM), or DMSO vehicle alone were evaluated for their effect on IL-1-stimulated PGE₂ production. Cells were incubated with the respective reagents 15 min before exposure to IL-1β for 24 hr. Pretreatment of RSF with hymenialdisine, but not aldisine, resulted in a concentration-dependent inhibition of IL-1-stimulated PGE₂ release (fig. 2; IC₅₀ = 0.6 µM ± 0.2; confidence limits of 0.002–1.16). No toxicity was noted. Breton and Chabot-Fletcher (1997) mentioned that an analog of hymenialdisine, debromohymenialdisine, is an inhibitor of PKC (DiMartino et al., 1995). However, in their system this was not shown to be the primary mechanism of action for hymenialdisine because a selective, nonsite-type specific, PKC inhibitor, RO 32–0432 (IC₅₀ vs. human neutrophil PKC,14 nM) did not have an effect in the TNF-stimulated NFκB-luciferase reporter assays and did not affect TNF-stimulated U937 IL-8 production by the same cells. As a control, RO 32–0432 was also examined in the IL-stimulated RSF system. Figure 2 shows that RO 32–0432 (100 nM) had no effect on IL-1-stimulated prostaglandin synthesis, which demonstrates the lack of participation of PKC.

Effect of hymenialdisine on IL-1-mediated activation of NFκB in RSF. We have previously demonstrated that 15 min is the peak time for acute activation of NFκB
significant difference from IL-1 control at P < 0.05.

Fig. 1. The antioxidant, PDCCT, concentration-dependently inhibits IL-1-stimulated PGE₂ formation. RSF were preincubated with increasing concentrations of PDCCT (1–300 μM) for 15 min at 25°C before the addition of IL-1β for 24 hr (1 ng/ml, 37°C). Cell-free medium was removed and analyzed for PGE₂ levels by ELISA as described under “Methods.” Data represent the mean ± S.D., n = 3 of one experiment.

Fig. 2. Hymenialdisine inhibits IL-1β-induced RSF PGE₂ production in a concentration-dependent manner. RSF were incubated with various concentrations of hymenialdisine (0.03–10.0 μM), aldisine (10 μM) or RO 32-0432 (100 nM) for 15 min at 25°C before stimulation with IL-1β for an additional 24 hr (1 ng/ml, 37°C). Cell-free medium was removed and analyzed for PGE₂ levels by ELISA as described under “Methods.” Data are expressed as % stimulated control (nonstimulated PGE₂ control, 1980; IL-1-stimulated control, 4804; fig 3A). We have previously demonstrated that binding to the NFκB classical motif is inhibited by incubation with excess unlabeled NFκB oligonucleotide (40×) (40%). Pretreatment of RSF with hymenialdisine or aldisine resulted in an ~80% (area pixel value 2500) or 35% (area pixel value 3820) reduction in the IL-1-stimulated NFκB binding, respectively. Percent reduction is obtained by comparing the IL-1-stimulated value, corrected for constitutive levels (subtraction of 1980), to the corrected hymenialdisine value. Addition of hymenialdisine (1 μM) to binding reactions containing untreated RSF nuclear extracts did not directly interfere with NFκB binding to the classical motif (data not shown).

We have previously demonstrated that the NFκB protein, p65, is involved in the regulation of COX-II and 85-kDa PLA₂ gene expression. Therefore, the effect of hymenialdisine on the translocation of p65 from the cytoplasm to the nucleus in response to IL-1 was evaluated by Western analysis. The study was performed identically with that described above. Western analysis on the cytosolic fraction of hymenialdisine (1 μM)-treated and -untreated cells is shown in figure 3B. In the absence of IL-1, a significant amount of p65 immunoreactive protein is found in the cytosolic fraction of RSF. However, in response to stimulation with IL-1 (15 min) the majority of the immunoreactive material is lost from the cytosol.
Pretreatment of the cells (15 min) before IL-1 exposure resulted in a significant repression (60%) of the IL-1-induced p65 migration (area pixel values unstimulated control, 2399; IL-1-stimulated control, 289; 1 μM hymenialdisine + IL-1, 1556).

Hymenialdisine inhibits IL-1-induced up-regulation of the prostanoid metabolizing enzymes. In the following studies, hymenialdisine was used at a concentration greater than its PGE2 inhibitory IC50 value (3 μM) to maximally effect the expression levels of the COX-II and 85-kDa PLA2 genes. The effect of hymenialdisine on the mRNA levels for COX-II and 85-kDa PLA2 in IL-1β-stimulated RSF was analyzed by Northern blotting. Figure 4A shows one representative of two studies where COX-II mRNA was undetectable in unstimulated control cells and highly induced after 8 hr stimulation with IL-1β as reported previously (Roshak et al., 1996a, b). Hymenialdisine reduced this induction to levels evident in unstimulated control RSF. Pretreatment with aldisine had no effect (data not shown). A minor amount of 85-kDa PLA2 mRNA was detectable in the absence of IL-1β stimulation and a marked induction followed the 8-hr treatment with IL-1β (fig. 4B). This induction was completely blocked by hymenialdisine. PGE2 levels measured in cell-free media reflect the Northern blot data (unstimulated control PGE2, 0.6 ng/ml; IL-1-stimulated control PGE2, 10.7 ng/ml; 3 μM hymenialdisine + IL-1 PGE2, 0.6 ng/ml).

Inhibition of mRNA induction for the two prostanoid-metabolizing enzymes correlated with a reduction in protein levels. Figure 5A shows one of two representative experiments demonstrating that exposure to IL-1β caused the accumulation of COX-II immunoreactive protein whereas very little COX-II was evident in nonstimulated RSF as reported previously (Roshak et al., 1996a, b). Pretreatment with hymenialdisine (3 μM) reduced the IL-1β-induced increase in COX II immunoreactive protein levels to near basal levels. The reduction in protein levels corresponded to reduced PGE2 levels measured in the study (unstimulated control PGE2, 0.2 ng/ml ± 0.1; IL-1β-stimulated PGE2, 6.4 ng/ml ± 2.0; 3 μM hymenialdisine, 1.7 ng/ml ± 0.5). Western analysis of the 85-kDa PLA2 revealed no change in protein levels with hymenialdisine pretreatment after 8 hr (data not shown). This is consistent with our previous findings which suggest a long protein half-life for the 85-kDa PLA2 enzyme. However, evaluation of samples treated identically but exposed to IL-1β for 24 hr rather than 8 hr showed that stimulated 85-kDa PLA2 protein was reduced to below basal levels as assessed by scanning gel densitometry (pixel values: nontreated control, 2013; IL-1β-stimulated control, 3224; 3 μM hymenialdisine, 1706; fig. 5B, one representative of two experiments).

Hymenialdisine inhibits IL-1-induced RSF IL-8 but not VEGF production. To verify that hymenialdisine was acting specifically on NFkB-regulated genes and not as a general inhibitor of transcription in this system, the effect of the compound on other IL-1-inducible genes was evaluated. RSF produce IL-8 and VEGF in response to IL-1 exposure (Jackson et al., 1997). Transcription of IL-8 has been shown to be highly regulated by NFkB (Kunsch and Rosen, 1993) whereas VEGF expression is not reported to be NFkB dependent (Tischer et al., 1991). Incubation of RSF from one representative donor with IL-1 caused a marked production of IL-8 which was ~60% inhibited by pretreatment with hymenialdisine (10 μM) but not the inactive analog, aldisine (10 μM; fig. 6A). In contrast, although stimulation with IL-1 resulted in a 1.7-fold increase in VEGF levels in the cell-free medium, this was not affected by pre-

![Fig. 4. Treatment of RSF with hymenialdisine inhibits IL-1β-induction of COX-II and 85-kDa PLA2 transcripts. RSF were exposed to DMSO vehicle (lanes 1 and 2) or 3 μM hymenialdisine for 15 min at 25°C before an 8-hr exposure to IL-1β (1 ng/ml) for all but the untreated control cultures (lane 1). Total RNA was isolated and subjected to Northern analysis [COX-II (A); 85-kDa PLA2 (B)] as described under “Methods.” One representative of two individual studies.](image)
treatment with 10 μM hymenialdisine, a concentration which resulted in total inhibition of PGE$_2$ production (fig. 6B).

**Discussion**

We showed previously that IL-1 induces the migration of NFκB proteins to the nucleus in RSF (Roshak et al., 1996b). Further, we demonstrated NFκB involvement in the IL-1-mediated up-regulation of 85-kDa PLA$_2$ and COX-II resulting in subsequent prostanoid formation. Specific antisense against the NFκB protein, p65, but not p50 or c-rel caused a marked reduction in the expression of these genes, reducing PGE$_2$ production and demonstrating a key role of this transcription factor (Roshak et al., 1996b).

With the recent description of hymenialdisine as a novel inhibitor of NFκB activation, this natural product was evaluated for its effect in a physiologically relevant *in vitro* model of disease. Hymenialdisine produced a potent concentration-dependent inhibition of IL-1-induced RSF PGE$_2$ production (IC$_{50}$, 0.6 μM) whereas the less active analog aldisine was without significant effect. This was considerably more potent than the nonspecific antioxidant, PDCT (IC$_{50}$, 15 μM), which acts partly through modulation of NFκB. Electrophoretic mobility shift assays and Western analysis of p65 confirmed a direct effect of hymenialdisine on IL-1-induced RSF NFκB activation and translocation to the nucleus. Pretreatment with hymenialdisine before IL-1 exposure clearly resulted in a decrease in nuclear protein binding to the radiolabeled κB motif. Complementary Western analysis of cytosolic p65 levels demonstrated that hymenialdisine inhibited IL-1-stimulated RSF p65 nuclear translocation.

Hymenialdisine inhibition of NFκB activation correlated with RSF reduced ability to transcribe 85-kDa PLA$_2$ or COX-II in response to IL-1. This resulted in a marked depletion of protein levels for both enzymes, which severely compromised prostanoid formation by these cells. These data are consistent with our previous findings with specific p65 antisense that inhibition of NFκB activation results in a reduction in IL-1-driven transcriptional up-regulation of the prostanoid-metabolizing enzymes.

Inhibition was not restricted to PGE$_2$ production because IL-1-stimulated IL-8 formation was also reduced by exposure to hymenialdisine. This was not unexpected because IL-8 is known to be an NFκB-regulated gene (Kunsch and Rosen, 1993) and this suggests a possible common mechanism of action of hymenialdisine on NFκB activation. Higher concentrations of hymenialdisine were required for IL-8 inhibition than for PGE$_2$ reduction. This could be because these studies were optimized to observe PGE$_2$ production and not IL-8. Further, PGE$_2$ formation requires the up-regulation of two distinct enzymes before the conversion of AA to prostanoid, whereas IL-8 is directly synthesized. In addition, several other transcription factors are known to participate in the regulation of IL-8 expression, e.g., NF-IL6 (Matsusaka et al., 1993), and one cannot rule out that these may function in a compensatory fashion in the absence of NFκB.

Hymenialdisine appears to act specifically at the level of NFκB inhibition and not as a general inhibitor of transcription. Breton and Chabot-Fletcher (1997) recently reported that hymenialdisine did not inhibit the transcription of the housekeeping gene, G3PDH or of PAI-1, a TNF-stimulated, NFκB-independent gene. Similarly in RSF, exposure to hymenialdisine did not affect the IL-1-induced up-regulation of VEGF whose promoter region contains AP-1 binding sites (Tischer et al., 1991) and appears to be activated thru src-associated kinase pathways (Mukhopadhyay et al., 1995). Finally, the lack of effect of hymenialdisine on IL-1-stimulated VEGF production also demonstrates that hymenialdisine is functioning downstream of the initial IL-1 receptor signaling.

In conclusion, hymenialdisine effectively attenuated the formation of RSF PGE$_2$ in response to IL-1 acting predominately through the inhibition of NFκB activation and 85-kDa PLA$_2$ and COX-II gene transcription. These data provide...
strong support that modulation of NF-kB activation would be therapeutically beneficial in attenuating pro-inflammatory mediator formation.

Acknowledgments
We acknowledge Brian Bolognese for technical assistance, Dr. Eugene Mochan for kindly providing rheumatoid synovial fibroblasts and Dr. David Dewitt for providing COX-II cDNA and polyclonal antisera.

References


