Inhibition of Interleukin-1-Induced Proteoglycan Degradation and Nitric Oxide Production in Bovine Articular Cartilage/Chondrocyte Cultures by the Natural Product, Hymenialdisine

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Accepted for publication April 2, 1999 This paper is available online at http://www.jpet.org

ABSTRACT

The effects of hymenialdisine (SK&F 108752) were evaluated on interleukin-1 (IL-1)-induced proteoglycan (PG) degradation, PG synthesis, nitric oxide (NO) production, and inducible nitric oxide synthase (iNOS) gene expression in bovine articular cartilage (BAC) and/or cartilage-derived chondrocytes. Cartilage disks from 0- to 3-month-old calves were treated with IL-1α or retinoic acid. PG release was determined by measuring glycosaminoglycan release, and nitrite production was measured as a readout for NO. Inhibition of iNOS gene expression was measured by Northern blot analysis in chondrocytes grown in monolayer, and inhibition by hymenialdisine was observed with an IC_{50} of approximately 0.6 µM. Herbimycin, a protein tyrosine kinase inhibitor, also inhibited PG breakdown, whereas RO 32-0432, a protein kinase C inhibitor, had no effect. Both hymenialdisine and herbimycin also were able to inhibit retinoic acid-stimulated PG release. IL-1α-stimulated NO production in BAC was inhibited by hymenialdisine and herbimycin at similar concentrations. The effect on iNOS gene expression was determined by Northern blot analysis in chondrocytes grown in monolayer, and inhibition by hymenialdisine was observed with an IC_{50} of approximately 0.8 µM. In chondrocytes cultured in alginate beads, IL-1α inhibited PG synthesis, whereas hymenialdisine stimulated synthesis at low concentrations (0.6 and 1.25 µM), and higher doses (2.5 µM) were not stimulatory. Compounds with this profile may have utility in the treatment of osteoarthritis.

Inflammatory mediators such as interleukin-1 (IL-1) and tumor necrosis factor (TNF) α are thought to play major roles in joint diseases such as rheumatoid arthritis (RA), and there is considerable evidence that there is a role for these cytokines in osteoarthritis (OA; Loyau and Punot, 1990; Kirkham, 1991; Westacott and Sharif, 1996). IL-1 has been shown to inhibit extracellular matrix synthesis, induce matrix metalloproteinases (MMPs), and stimulate the degradation of proteoglycans (PGs; Dingle et al., 1979; Gowen et al., 1984; Krakauer et al., 1985; Tyler, 1985), resulting in damage to both cartilage and bone. One of the mechanisms by which IL-1 elicits its proinflammatory effects is by the stimulation of the production of nitric oxide (NO), which, in turn, may activate the MMPs responsible for PG degradation in articular cartilage (Murrell et al., 1995). Compounds able to inhibit the IL-1-mediated effects on matrix degradation may well have therapeutic activity in OA and RA.

Hymenialdisine (Fig. 1) was originally isolated from the marine sponges Axinella verrucosa and Acanthella aurantiaca (Cimino et al., 1982). Recently, this compound was shown to inhibit IL-8 production in the human macrophage cell line, U937, by inhibition of nuclear factor-κB (NF-κB; Breton and Chabot-Fletcher, 1997) and also to inhibit NF-κB-mediated, IL-1β-stimulated prostaglandin E_{2} in human rheumatoid synovial fibroblasts (Roshak et al., 1997). A closely related analog, debromohymenialdisine, has been shown to be effective in the adjuvant arthritic rat, and its anti-inflammatory activity was reported to be due to inhibition of protein kinase C (PKC; DiMartino et al., 1995). However, it is clear that even though these compounds are potent

Received for publication December 9, 1998.

ABBREVIATIONS: IL-1, interleukin 1; TNF, tumor necrosis factor; BAC, bovine articular cartilage; DMMB, dimethylmethylen blue; GAG, glycosaminoglycans; iNOS, inducible nitric oxide; MMP, matrix metalloproteinase; NMMA, N^{G}-monomethyl-L-arginine; NO, nitric oxide; PG, proteoglycan; PTK, protein tyrosine kinase; PKC, protein kinase C; NF-κB, nuclear factor-κB; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; FCS, fetal calf serum; CPC, cetylpyridiniumchloride-precipitable; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LPS, lipopolysaccharide.
PKC inhibitors, their anti-inflammatory effects may well be through another mechanism(s). In the studies reported here, we describe the ability of hymenialdisine to protect cartilage explants from IL-1- and retinoic acid-induced matrix degradation and to stimulate PG synthesis in chondrocytes. IL-1 induced nitric oxide synthase (iNOS) gene expression and the consequent release of NO also was inhibited by hymenialdisine. In addition, we propose that the mechanism of the compound on cartilage metabolism is not due to PKC inhibition but may be due, in part, to inhibition of protein tyrosine kinase (PTK).

Materials and Methods

Reagents. Hymenialdisine (SK&F108752) was isolated from the sponge Stylotella aurantium in the Department of Biomolecular Discovery, SmithKline Beecham Pharmaceuticals (SB). RO 32-0432 was synthesized by the Department of Medicinal Chemistry at SB according to the reported synthesis (Bit et al., 1993). Recombinant human IL-1α (which was used in all the experiments) and the metalloproteinase inhibitor, BB94, were also prepared at SB. Herbimycin was obtained from Life Technologies (Grand Island, NY). Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F12 medium were obtained from GIBCO (Grand Island, NY). Fetal bovine serum (FBS) was obtained from HyClone (Logan, UT). The media were supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, and 2 mM L-glutamine (GIBCO). Dulbecco’s PBS was obtained from Gibco and contained 2× antibiotics. BSA, l-ascorbic acid, N-acetylmethyl-l-arginine (NMMA), retinoic acid, shark chondroitin sulfate, pronase E from Streptomyces griseus, and hyaluronidase type V were obtained from Sigma Chemical Co. (St. Louis, MO); collagenase D from Clostridium histolyticum was obtained from Boehringer Mannheim (Indianapolis, IN). 1,9-Dimethylmethylene blue (DMMB) was obtained from Aldrich Chemical Co. (Milwaukee, WI).

Cartilage and Chondrocyte Cultures. Carpal metacarpal joints of calves (0–3 months old) were obtained from Covance (Denver, PA). Full-thickness articular cartilage slices were aseptically collected and placed in Dulbecco’s PBS with 2× antibiotics for 30 min. Full-thickness cartilage disks (5–7 mg) were dissected from the cartilage using a sterile, leather punch (Libertyville Saddle Shop, Libertyville IL). Disks were transferred to 96-well, flat-bottom plates (Nunc, Denmark) containing DMEM supplemented with antibiotics and 10% FBS. This medium was changed 48 to 72 h later to DMEM with 0.5% FBS, and samples to be tested were added 24 h later. Glycosaminoglycan (GAG) release and nitrite levels, as a measure of NO, were determined in the cartilage explant supernatants 72 h after the addition of the samples being evaluated. The amount of IL-1 required to induce/stimulate maximum release of PGs or NO ranged from 20 to 100 ng/ml depending on the cartilage samples and the conditions being used. Therefore, we have used both of these concentrations in the experiments presented here.

Chondrocytes were isolated from bovine cartilage as described elsewhere for human chondrocytes (Kuettner et al., 1982; Aydelotte and Kuettner, 1988). Briefly, cartilage was cut into small pieces (10–20 mg) and chondrocytes were liberated by sequential treatment with hyaluronidase (0.2% in DMEM without FBS) for 30 min, pronase E (0.25% in DMEM without FCS) for 30 min, and collagenase D (0.2% in DMEM with 10% FCS) for 20 h at 37°C. Cells were washed two times in DMEM with 10% FBS.

For culture of chondrocytes in alginate beads we used a previously described method (Guo et al., 1989; Hausermann et al., 1992). Briefly, the cells were suspended in sterile-filtered, low-viscosity alginate (1% w/v) at a concentration of 4×10⁶ cells/ml and then slowly expressed through a 22-gauge needle in a dropwise fashion into a 102-mM CaCl₂ solution. The beads were allowed to polymerize further for 10 min in CaCl₂ solution, washed four times in 0.15 M NaCl, and then placed in 200 μl of complete culture medium (Ham’s F12 with antibiotics/antimycotic and 10% FBS) in 96-well round-bottom microtiter plates (3 beads/well). Cultures were fed every other day for 7 days, and then the medium was changed to Ham’s F12 with 0.01% BSA and hymenialdisine was added for an additional 5 days.

The potential toxicity of compounds being evaluated was determined by microscopic evaluation and trypan blue exclusion of chondrocytes established in monolayer cultures and by lactic dehydrogenase release from explant cultures.

Quantitation of GAGs. GAG levels in the culture media were determined by the amount of polyanionic material reacting with DMMB (Farnadale et al., 1982). This measurement reflects both enhanced breakdown as well as enhanced synthesis. Explant supernatants were removed and 50 μl of a 1:40 dilution (made in 50 mM sodium acetate buffer) was combined with 200 μl of DMBB solution. Samples were read spectrophotometrically at 535 nm (SpectraMax; Molecular Devices, Sunnyvale, CA). Results are reported as micrograms GAG per milligram of cartilage.

[³⁵S]Sulfate Incorporation into GAGs. The synthesis of sulfated GAGs was measured in chondrocytes cultured in alginate beads by determining the incorporation of [³⁵S]sulfate into cetylpyridiniumchloride-precipitable (CPC) GAGs (van Kampen and Veldhuijzen, 1983). The cells were labeled for the last 18 h of culture with 0.5 μCi Na[³⁵S]SO₄/ml (New England Nuclear, Boston MA; specific activity 1050 Ci/mmolar). After removal of the medium, the beads were washed twice in PBS, and then 200 μl papain (Sigma; 250-μg/ml suspension) was added and incubated 60°C for 6 h. The digest then was transferred to 6-ml polypropylene tubes (Falcon, Franklin Lakes, NJ). To this digest, 1 ml of 1% CPC (Sigma) was added and incubated at 37°C for 60 min. The precipitate was washed twice with CPC (1%). The precipitate was dissolved in NCS tissue solubilizer (Amersham, Arlington Heights, IL) and placed in a 45°C water bath for 30 min. The amount of [³⁵S]sulfate was determined by liquid scintillation counting.

Nitrite Determination. NO production was measured by estimating the stable NO metabolite, nitrite, in conditioned medium using a spectrophotometric method based on the Griess reaction (Green et al., 1982). After culture of the cartilage explants for the times indicated, 50 μl of the culture supernatants or sodium nitrite standard dilutions were mixed with 50 μl Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride, and 1.25% H₃PO₄) and incubated for 10 min at room temperature. Nitrite concentrations were determined by measuring absorbance at 550 nm in an ELISA reader (Molecular Devices). The detection limit of the test was 2 μM NO₂. Values are expressed as the micromolar concentration of nitrite released per milligram of cartilage.

Isolation of Chondrocyte RNA and Northern Blot Analysis. For isolation of RNA, monolayer cultures of chondrocytes were used. Chondrocytes were seeded into six-well tissue culture-grade plates (Corning, Cambridge, MA) at a concentration of 2×10⁶ cells/ml (4 ml/well) in Ham’s F12 containing 10% FBS, ascorbic acid (25 μg/ml),
and antibiotic/antimycotic. Cells were allowed to adhere and then grown for 72 h at 37°C in 5% CO₂. The medium was then replenished and the chondrocytes were treated with varying concentrations of hymenialdisine for 30 min followed by stimulation with IL-1 in the presence of hymenialdisine. The cells were incubated at 37°C in 5% CO₂ atmosphere for 6 h. At the end of the incubation time the extracellular medium was removed and the cells were homogenized in triazol reagent at 0.5 ml/well for the isolation of RNA. Total RNA was isolated by a modified guanidine isothiocyanate extraction using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s instructions. RNA concentration and purity were determined spectrophotometrically. All RNA samples had an A260/A280 ratio of >1.8. Approximately 10 μg of the RNA samples were electrophoresed in 1% agarose gels containing 2.5% formaldehyde, 20 mM 3-(N-morpholino)propanesulfonic acid, 5 mM sodium acetate, and 1 mM EDTA, pH 7.0. After electrophoresis, the RNA was transferred to positively charged nylon membranes (Bio-Rad, Hercules, CA). Membranes were prehybridized for 1 to 2 h at 68°C with ExpressHyb (Clontech, Palo Alto, CA). Hybridization was performed under identical conditions to prehybridization with the addition of 32P-labeled (Amersham) specific probe to bovine iNOS. The 372-basepair probe was graciously provided by Dr. T. Jungi (University of Berne, Berne, Switzerland; Adler et al., 1995) and corresponds to nucleotides 682 to 1053 of the human iNOS cDNA (Geller et al., 1993). Radiolabel on the blots was analyzed using phosphorimaging technology.

**Statistical Analysis.** Comparisons between groups were performed using Student's t test with p < .05 considered significant. Experiments described are representative of at least three and often five or more experiments.

**Results**

**Inhibition of IL-1 and Retinoic Acid-Induced PG Breakdown.** Cartilage explants were established in 96-well plates in DMEM with 10% FBS and then transferred to medium containing 0.5% FBS. Twenty-four hours later the cultures were stimulated with 100 ng/ml IL-1α, and hymenialdisine was added at doses ranging from 0.3 to 2.5 μM. The explants were incubated for an additional 72 h, and GAG release was determined in the supernatants. In the presence of IL-1α, GAG release was increased up to 10-fold compared with that in the untreated control cultures. This increase was inhibited by hymenialdisine with an IC₅₀ of approximately 0.6 μM (results of five different experiments). A representative experiment is shown in Fig. 2A. Similar experiments were performed with the PTK inhibitor, herbimycin, and inhibition of GAG release was observed with an IC₅₀ of between 2.5 and 5 μM (Fig. 2B). The time course of the inhibitory effect of hymenialdisine is shown in Fig. 3. The compound was effective when added during the first 8 h of the 72-h culture period, but lost most of its activity if added 24 h after IL-1. To determine whether the inhibitory effect of hymenialdisine on PG breakdown was due to its ability to inhibit PKC, we evaluated the effects of the PKC inhibitor RO 32-0432 in the assay at concentrations of 0.6, 1.25, and 2.5 μM. None of these concentrations proved to be effective in protecting the cartilage from IL-1α-mediated PG breakdown (Fig. 4), and higher doses were toxic. Retinoic acid also stimulates matrix MMPs, resulting in the breakdown of cartilage matrix, and at doses of 1 and 10 μM, we found its activity to be similar to that of IL-1α. Hymenialdisine was effective in reducing the breakdown elicited by 1 μM retinoic acid to background levels, but at the 10-μM dose of retinoic acid only 78% of the GAG release was inhibited (Table 1). Therefore, we tested the ability of hymenialdisine and herbimycin to inhibit the 1-μM dose of retinoic acid. Both compounds inhibited retinoic acid-induced PG release at 2.5 and 5.0 μM and lost activity at 1.25 μM (Fig. 5). As a control for the PG-release experiments, we used the MMP inhibitor, BB84, at a concentration of 20 μM. Addition of this compound to the IL-1α-treated cultures effectively and consistently reduced PG release by about 70% (Table 2).

**Effect on PG Synthesis.** To determine the effect of hymenialdisine on PG synthesis, we used bovine chondrocytes established in alginate beads. The beads were dispersed into 96-well round-bottom plates (3 beads/well) in 200 μl of Ham’s F12 containing 10% FBS. Seven days later the medium was changed to Ham’s F12 containing 0.01% BSA. After a 24-h incubation period the beads were treated with 100 ng/ml IL-1α, 0.3 to 2.5 μM hymenialdisine, or a combination of IL-1α and hymenialdisine. The beads were cultured with compounds for 3 days and then pulsed with [35S]sulfate for 18 h. As can be seen in Fig. 6, although IL-1 inhibited PG synthesis, low doses of hymenialdisine (0.6 and 1.25 μM) stimulated the incorporation of [35S]sulfate into PGs, whereas higher doses of 2.5 μM were not stimulatory. Hymenialdisine was unable to reverse the inhibitory effect of IL-1 in this assay system (data not shown).

**Effect on NO Production.** Cartilage explants were established in culture and stimulated with IL-1α as described for the experiments on PG breakdown. The presence of IL-1α-induced NO production in the supernatants was mea-

![Fig. 2. Dose-dependent inhibition of IL-1-induced PG release from cartilage explants by hymenialdisine (A) and herbimycin (B). IL-1 and compounds were added together at the initiation of a 72-h culture. Data are mean ± S.D. of six replicates per treatment. ns, not significant; *p < .05; **p < .01; ***p < .001.](image-url)
sured by determining the levels of nitrite in the medium. Hymenialdisine inhibited NO production with an IC\textsubscript{50} of between 1.25 and 2.5 μM (Fig. 7A). Herbigmycin was active at doses of 1.25, 2.5, and 5.0 μM with an IC\textsubscript{50} for inhibition of NO release at 2.5 μM (Fig. 7B). As a control in the NO-release experiments we used NMMA at a concentration of 1 mM. Addition of this compound to the IL-1α-treated cultures effectively inhibited NO production by 100% (Table 2).

**Effect on iNOS Gene Expression.** To determine the level at which hymenialdisine regulated NO production, the effect on iNOS mRNA was examined using Northern blot analysis. Bovine chondrocytes were isolated from articular cartilage and established in monolayer culture in six-well dishes as described in Materials and Methods. Cells were treated with various concentrations of hymenialdisine in the presence of 100 ng/ml IL-1α. Total RNA was isolated from the chondrocytes, and iNOS expression was evaluated on a Northern blot probed with a bovine cDNA probe. Strong induction of iNOS message was observed 4 h after treatment with IL-1α, which was inhibited in a dose-related manner by hymenialdisine (Fig. 8A). A bar graph of the phosphorimager counts associated with the 4.2-kb message is shown in Fig. 8B. These numbers have been normalized using integration of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) hybridization for each respective lane. These results indicate an IC\textsubscript{50} of approximately 0.8 μM for the inhibition of IL-1α-induced iNOS message by hymenialdisine.

**Discussion**

Hymenialdisine is a natural product that has been isolated from the marine sponges *A. verrucosa* and *A. aurantiaca* (Cimino et al., 1982). It has a number of effects in biological systems, in particular, the inhibition of a number of enzymes, which include PKC (Patil et al., 1997) and the PTKs p56\textsubscript{lck} (IC\textsubscript{50}, 3 μM) and epidermal growth factor receptor (IC\textsubscript{50}, 1 μM; S. Kassis, unpublished observations) but not p38 mitogen-activated protein kinase (S. Kumar, unpublished observation). Most recently, hymenialdisine has been shown to inhibit yeast YAK-1 kinase with an IC\textsubscript{50} of 1.2 μM (Kassis et al., 1998).

Hymenialdisine recently has been described as a novel inhibitor of NF-κB activation in U937 cells. The compound inhibited both receptor-mediated [TNFα and lipopolysaccharide (LPS)] and phorbol myristyl acetate-mediated luciferase expression in an NF-κB-driven luciferase reporter assay constructed in U937 cells (Breton and Chabot-Fletcher, 1997). Gel-shift analysis of nuclear extracts showed reduction in NF-κB binding but not binding of the transcription factors CCAAT/enhancer binding protein (C/EBP), activator protein-1, or Spl. In addition, hymenialdisine inhibited IL-8 production in the TNF-treated U937 cells (Breton and Chabot-Fletcher, 1997). Studies by Roshak et al. (1997) de-

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**TABLE 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GAG Release (μg/mg cartilage)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>5.4 ± 1.4</td>
</tr>
<tr>
<td>Retinoic acid (1 μM)</td>
<td>12.0 ± 3.2</td>
</tr>
<tr>
<td>Retinoic acid (1 μM) +</td>
<td>4.7 ± 0.53*</td>
</tr>
<tr>
<td>hymenialdisine (2.5 μM)</td>
<td></td>
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<tr>
<td>(100% inhibition)</td>
<td></td>
</tr>
<tr>
<td>Retinoic acid (10 μM) +</td>
<td>6.9 ± 0.7*</td>
</tr>
<tr>
<td>hymenialdisine (2.5 μM)</td>
<td>(78% inhibition)</td>
</tr>
</tbody>
</table>

*p < .001.
scribed the inhibition of NF-κB-mediated, IL-1β-stimulated prostaglandin E2 formation by the compound.

In the studies described here, we have shown that hymenialdisine-inhibited IL-1 stimulated PG release from bovine articular cartilage with an IC₅₀ of approximately 0.6 μM. This effect clearly was not due to inhibition of PKC because RO 32-0432, a potent PKC inhibitor, was ineffective in the assay at doses as high as 2.5 μM. The IC₅₀ for RO 32-0432-mediated inhibition of PKC enzyme in human neutrophils is 14 nM, and for partially purified rat brain, PKC is 17 nM (Birchall et al., 1994). In addition, several other PKC inhibitors synthesized at SmithKline were unable to inhibit the release of PGs (data not shown). There did, however, appear to be evidence for a role for PTK in the inhibitory effect of hymenialdisine. This was provided by the finding that herbimycin, a potent PTK inhibitor (Levitzki and Gazit, 1995) was also able to inhibit IL-1-stimulated PG release from the cartilage explants. Herbimycin is an ATP analog and is a broad-spectrum tyrosine kinase inhibitor with IC₅₀ values in submicromolar concentrations in intact cells (Lockhart et al., 1998). The IC₅₀ for inhibition by herbimycin was higher than that for hymenialdisine (between 2.5 and 5 μM).

### TABLE 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GAG Release μg/mg cartilage</th>
<th>NO Production μM/mg cartilage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.2 ± 0.8</td>
<td>2.6 ± 0.8</td>
</tr>
<tr>
<td>IL-1 (100 ng/ml)</td>
<td>11.5 ± 2.7</td>
<td>16.2 ± 3.4</td>
</tr>
<tr>
<td>IL-1 (100 ng/ml) + BB94 (20 μM)</td>
<td>4.0 ± 0.9*</td>
<td>16.8 ± 3.7, N.S.</td>
</tr>
<tr>
<td>IL-1 (100 ng/ml) + NMMA (1 mM)</td>
<td>13.8 ± 0.4, N.S.</td>
<td>0.4 ± 0.1*</td>
</tr>
</tbody>
</table>

* p < .001.

Fig. 6. IL-1 (20 ng/ml) inhibits and hymenialdisine stimulates PG-synthesis ([³⁵S]sulfate incorporation), cartilage-derived chondrocytes cultured in alginate beads. Data are mean ± S.D. of 12 replicates per treatment. ***p < .001.

Fig. 7. Dose-dependent inhibition of NO production from IL-1-stimulated cartilage explants by hymenialdisine (A) and herbimycin (B). IL-1 and compounds were added at the initiation of a 72-h culture. Data are mean ± S.D. of six replicates per treatment. *p < .05; ***p < .001.

Fig. 8. Expression of IL-1-stimulated iNOS message in bovine chondrocytes is inhibited in a dose-related manner by hymenialdisine. Chondrocytes were established in monolayer culture (8 × 10⁶ in 4 ml) in six-well plates for 72 h. Cells were then stimulated with IL-1 (100 ng/ml) and treated with hymenialdisine for 6 h. A, upper, a representative Northern blot showed that the radiolabeled iNOS probe is associated with the 4.2-kilobase message and dose-related inhibition with hymenialdisine; lower, [³²P]GAPDH probe hybridized with 1.7-kilobase message on the same blot. Lane 1, control untreated; lane 2, IL-1-treated control; lanes 3 through 6, IL-1 plus hymenialdisine at 0.32, 0.8, 2.0, and 5.0 μM. B, shows the phosphorimagier counts for this Northern blot standardized with GAPDH.
compared with 0.6 μM, respectively). In addition, unlike hymenialdisine, which was able to reverse the IL-1-mediated PG release down to control levels, herbimycin could inhibit the release only by 50% at 5.0 μM, and at this dose there was some toxicity observed on isolated chondrocytes. For significant inhibition to occur, hymenialdisine had to be present during the early time course of the culture because addition 24 h after IL-1 did not result in inhibition of PG release. The inhibitory effects of hymenialdisine and herbimycin in the PG-breakdown assay were not selective for IL-1 because retinoic acid-stimulated PG breakdown was also inhibited by both compounds. In this case, the dose-response effects of the two compounds were similar, both being inhibitory at 5.0 and 2.5 μM and losing activity at 1.25 μM. We also examined the effect of hymenialdisine on PG synthesis ([35S]sulfate incorporation) in cartilage-derived chondrocytes cultured in alginate beads. Of interest was the observation that, unlike IL-1, which inhibited PG synthesis in cartilage-derived chondrocytes, hymenialdisine had positive effects and actually stimulated PG synthesis at low concentrations (0.6 and 1.25 μM), whereas higher concentrations of the compound were not stimulatory. However, the compound was unable to reverse IL-1-mediated inhibition of PG synthesis when the two were added together at the initiation of the culture (data not shown).

Another consequence of IL-1 treatment of cartilage and chondrocytes is the induction of iNOS and the release of NO, both of which were inhibited by hymenialdisine. As observed with the experiments measuring IL-1-induced PG breakdown, hymenialdisine was able to reverse IL-1-stimulated NO production down to the background control levels in cartilage explants, whereas herbimycin inhibited by only about 50% at the 2.5-μM dose. Hymenialdisine also inhibited IL-1-stimulated release of NO in chondrocytes established in monolayer and in agarose (data not shown). At the higher dose of 5.0 μM, there was some toxicity with hymenialdisine because about 10% of chondrocytes established in monolayer exclude trypan blue. The dose-response effects for both compounds in the PG-release and NO-production assays were quite similar. The inhibitory activity of hymenialdisine on NO production was at the transcriptional level as IL-1-induced iNOS gene expression in chondrocytes cultured in monolayer and measured by Northern blot was inhibited by the compound. The IC50 for the inhibition of iNOS gene expression by hymenialdisine was approximately 0.8 μM after a 6-h incubation of chondrocyte monolayers with IL-1. In these experiments we used NMMA as a control compound for inhibition of NO production and the MMP inhibitor BB94 for inhibition of PG release (Table 2). Under the conditions described in this paper, NMMA does not have any effect on IL-1-stimulated PG breakdown, and this is reflective of the data described previously in bovine and rabbit articular cartilage (Stefanovic-Racic et al., 1996, 1997).

Because both hymenialdisine and herbimycin had similar effects under the experimental conditions described here, it appears that the activity of hymenialdisine may well be mediated, at least in part, by its ability to inhibit PTK activity. Additional evidence for the role of PTK inhibition as a potential mechanism is that the PTK inhibitors have been shown to inhibit NO production in a number of cell types. Cloned murine microglial cells (N9) stimulated with combined LPS/interferon-γ produce a significant amount of NO, which can be inhibited by the tyrosine kinase inhibitors herbimycin, genistein, and tyrphostin (Lockhart et al., 1998). Herbimycin has been shown to inhibit LPS/interferon-γ-induced iNOS production activation in retinal epithelial cells (Faure et al., 1998) and in murine macrophages (Dong et al., 1993). The effects observed with hymenialdisine on NF-κB activation (Breton and Chabot-Fletcher, 1997) have also been observed with herbimycin and other PTK inhibitors in different assay systems. Herbimycin was shown to block IL-1-induced NF-κB DNA-binding activity in lymphoid cell lines (Mahon and O’Neill, 1995) as well as IL-1/PMA activation of NF-κB in Jurkat lymphoma cells (Natarajan et al., 1998). Genistein and erbstatin, two inhibitors of PTK, inhibited TNF-mediated NF-κB activation in the human myeloid U-937 cells (Natarajan et al., 1998). However, it should be noted that the PTK inhibitor herbimycin was not as effective as hymenialdisine in its ability to inhibit IL-1-induced GAG release or NO production at nontoxic concentrations. This would indicate that, although PTK may play a role in the IL-1-mediated events in cartilage metabolism, it is not the sole participant, and the possible role of a yet unidentified kinase or inhibition of NF-κB as the mechanism cannot be overlooked.

In summary, hymenialdisine inhibits IL-1- and retinoic acid-induced stimulation of PG release and IL-1-stimulated NO production. The latter effect is at the level of iNOS gene transcription. These anti-inflammatory effects in cartilage may be due, in part, to the ability of the compound to inhibit PTK and indicate that compounds with this profile of activity may well have beneficial effects in the treatment of RA and/or OA.

Acknowledgment

We thank Nancy Cosgrove for secretarial assistance.

References


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