Bryostatin-1 Stimulates the Transcription of Cyclooxygenase-2: Evidence for an Activator Protein-1-Dependent Mechanism

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ABSTRACT
Bryostatin-1 (bryostatin) is a macrocyclic lactone derived from Bugula neritina, a marine bryozoan. On the basis of the strength of in vitro and animal studies, bryostatin is being investigated as a possible treatment for a variety of human malignancies. Severe myalgias are a common dose-limiting side effect. Because cyclooxygenase-2 (COX-2)-derived prostaglandins can cause pain, we investigated whether bryostatin induced COX-2. Bryostatin (1–10 nM) induced COX-2 mRNA, COX-2 protein, and prostaglandin biosynthesis. These effects were observed in macrophages as well as in a series of human cancer cell lines. Transient transfections localized the stimulatory effects of bryostatin to the cyclic AMP response element of the COX-2 promoter. Electrophoretic mobility shift assays and supershift experiments revealed a marked increase in the binding of activator protein-1 (AP-1) (c-Jun/c-Fos) to the cyclic AMP response element of the COX-2 promoter. Pharmacological and transient transfection studies indicated that bryostatin stimulated COX-2 transcription via the protein kinase C—mitogen-activated protein kinase—AP-1 pathway. All-trans-retinoic acid, a prototypic AP-1 antagonist, blocked bryostatin-mediated induction of COX-2. Taken together, these results suggest that bryostatin-mediated induction of COX-2 can help to explain the myalgias that are commonly associated with treatment. Moreover, it will be worthwhile to evaluate whether the addition of a selective COX-2 inhibitor can increase the antitumor activity of bryostatin.

INTRODUCTION
COX2 catalyzes the synthesis of PGs from arachidonic acid. There are two isoforms of COX-designated COX-1 and COX-2, respectively. COX-1 is constitutively expressed in most tissues where it fulfills a homeostatic function (1). In contrast, COX-2 is not detectable in most normal tissues but is inducible by oncogenes, tumor promoters, cytokines, and growth factors (2–8).

COX-2 is an important target for treating arthritis, pain, and possibly cancer (9–12). For example, the expression of COX-2 is increased in inflamed tissues such as rheumatoid synovium (13), and selective COX-2 inhibitors are useful for the treatment of arthritis (10). COX-2-derived PGs also appear to play a significant role in inducing pain (14).

Several lines of evidence strongly suggest that COX-2 is a bona fide target for anticancer therapy (12). Increased amounts of COX-2 are commonly found in malignant tumors (12, 15–20). Overexpression of COX-2 was sufficient to induce mammary cancer in multiparous transgenic mice (21). Mice engineered to be COX-2 deficient are protected against developing both skin and intestinal tumors (22, 23). In addition to the genetic evidence implicating COX-2 in carcinogenesis, selective COX-2 inhibitors reduce the formation and growth of experimental tumors (24–28) and decrease the number of colorectal polyps in familial adenomatous polyposis patients (29). Enhanced synthesis of COX-2-derived PGs favors tumor growth by inhibiting apoptosis (30, 31), stimulating cell proliferation (32), promoting angiogenesis (33, 34), and increasing invasiveness (35, 36). With this in mind, it is reasonable to postulate that the induction of COX-2 by chemotherapy will reduce the efficacy of treatment and potentially contribute to medication-related side effects, including pain.

Bryostatin is a natural macrocyclic lactone isolated from the marine bryozoan Bugula neritina (Fig. 1; Ref. 37). It exhibits antineoplastic activity in a variety of murine tumor models (38–42). Because of these promising preclinical findings, bryostatin has been evaluated in the treatment of a variety of human malignancies. Unfortunately, the reported single agent activity has been disappointing (43). Moreover, dose-limiting myalgias have been observed (43, 44). Studies are under way to evaluate whether bryostatin will be useful when combined with cytotoxic agents (43). Ideally, a mechanism-based rationale should exist for combining bryostatin with other agents. Although the precise mechanism of action of bryostatin is uncertain, low concentrations of bryostatin activate PKC (45, 46). Prototypic activators

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2 The abbreviations used are: COX, cyclooxygenase; PG, prostaglandin; bryostatin, bryostatin-1; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; AP-1, activator protein-1; CRE, cyclic AMP response element; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal kinase; ATRA, all-trans-retinoic acid.
of PKC, including phorbol esters, induce COX-2 (12, 13). In combination, these findings raise the possibility that COX-2 is a downstream target of bryostatin.

In this study, we show that bryostatin stimulates the expression of the COX-2 gene and thereby PG biosynthesis. Bryostatin stimulated COX-2 transcription via the PKC→MAPK→AP-1 pathway. Possibly, bryostatin-mediated induction of COX-2 will decrease the efficacy of this compound or explain, in part, the myalgias it causes.

MATERIALS AND METHODS

Materials. DMEM, Opti-MEM, and LipofectAMINE 2000 were from Life Technologies, Inc. (Grand Island, NY). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (thiazolyl blue), lactate dehydrogenase diagnostic kits, antibody to β-actin, TRI reagent, o-nitrophenyl-β-d-galactopyranoside, poly(deoxyinosinic-deoxycytidylic acid), and all-trans-retinoic acid were from Sigma Chemical Co. (St. Louis, MO). Bryostatin-1, Ro 31-8220, GF 109203X, and SP600125 were from Biomol Research Labs, Inc. (Plymouth Meeting, PA). PD 98059 (2'-amino-3'-methoxyflavone) and SB 202190 [4-(4-flurophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole] were from Calbiochem (La Jolla, CA). Enzyme immunoassay reagents for PGE2; assays were from Cayman Chemical Co. (Ann Arbor, MI). Western blotting detection reagents, [32P]ATP and [32P]CTP, were from NEN Life Sciences Products (Boston, MA). Random-priming kits were from Roche Molecular Biochemicals (Indianapolis, IN). Nitrocellulose membranes were from Schleicher & Schuell (Keene, NH). Reagents for the luciferase assay were from PharMingen (San Diego, CA). The 18S rRNA cDNA was from Ambion, Inc. (Austin, TX). Antibodies to COX-2 and c-Fos were from Santa Cruz Biotechnology, Inc. (San Diego, CA). Antibodies to phosphorylated and unphosphorylated forms of ERK1/2 (p44/p42), p38, and c-Jun were from Cell Signaling Technology, Inc. (Beverly, MA). Plasmid DNA was prepared using a kit from Promega Corp. (Madison, WI).

Cell Lines. Esophageal squamous cell carcinoma cell line 450 (SCC450) was a gift from Dr. Yutaka Shimada (Kyoto University, Kyoto, Japan; Ref. 47). SCC450 cells were routinely grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin in a 5% CO2 atmosphere at 37°C. Cells were grown to 70% confluence, trypsinized with 0.05% trypsin, 2 mM EDTA, and plated for experimental use. 1483 squamous carcinoma cells (48), A549 lung adenocarcinoma cells (49), and RAW 264.7 cells (50) were grown as in previous studies. In all experiments, cells were incubated in serum-free medium for 24 h before treatment. Treatment with vehicle (Me2SO) or bryostatin was always carried out in serum free medium. Cellular cytotoxicity was assessed by measurements of cell number, release of lactate dehydrogenase, and the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay, which was performed according to the method of Denizot and Lang (51). Lactate dehydrogenase assays were performed according to the manufacturer’s instructions. There was no evidence of toxicity under the conditions of our experiments.

PGE2 Production by Cells. A total of 5 x 10^4 cells/well was plated in 6-well dishes and grown to 70% confluence before treatment. Amounts of PGE2 released by cells were measured by enzyme immunoassay. Production of PGE2 was normalized to protein concentration.

Western Blotting. Cell lysates were prepared by treating cells with lysis buffer [150 mM NaCl, 100 mM Tris (pH 8.0), 1% Tween 20, 50 mM diethyldithiocarbamate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml trypsin inhibitor, and 10 μg/ml leupeptin]. Lysates were sonicated for 20 s on ice and centrifuged at 10,000 x g for 10 min to sediment the particulate material. The protein concentration of the supernatant was measured by the method of Lowry et al. (52). SDS-PAGE was performed under reducing conditions on 10% polyacrylamide gels as described by Laemmli (53). The resolved proteins were transferred onto nitrocellulose sheets as detailed by Towbin et al. (54). The nitrocellulose membrane was then incubated with primary antisera. Secondary antibody to IgG conjugated to horseradish peroxidase was used. The blots were probed with enhanced chemiluminescence Western blot detection system according to the manufacturer’s instructions.

Northern Blotting. Total cellular RNA was isolated from cell monolayers using TRI reagent from Sigma Chemical.
Bryostatin-1 Induces COX-2 and lipoase. Immunoblots were probed with antibodies specific for COX-2. Lysate protein (100–200 μg/lane) was loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto nitrocellulose protein. The remainder of the procedure was carried out as described previously (8, 20).

Fig. 3  COX-2 protein is induced by bryostatin. (A) SCC450, (B) A549, and (D) RAW 264.7 cell lines were treated with bryostatin for 6 h. In A–D, lysate protein was from cells treated with vehicle (Lane 1) or bryostatin (1, 5, 10, and 25 nm; Lanes 2–5, respectively). Cellular lysate protein (100 μg/lane) was loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto nitrocellulose. Immunoblots were probed with antibodies specific for COX-2 and β-actin.

Fig. 4  Bryostatin induced COX-2 mRNA. Total RNA was isolated from SCC450 cells that were treated with different concentrations of bryostatin for 4 h. Lane 1 represents vehicle; Lanes 2–4 represent cells treated with 1, 5, or 10 nm bryostatin. Ten μg of RNA were added to each lane. The blot was hybridized with probes that recognized COX-2 mRNA and 18S rRNA.

Fig. 5  Localization of region of COX-2 promoter that mediated the effects of bryostatin. A, shown is a schematic of the human COX-2 promoter. B, SCC450 cells were transfected with 1.8 μg of a series of human COX-2 promoter deletion constructs ligated to luciferase (−327/+59, −220/+59, −124/+59, and −52/+59) and 0.2 μg of pSVβgal. C, SCC450 cells were transfected with 1.8 μg of either the −327/+59 COX-2 promoter-luciferase construct or the −327/+59 construct in which the CRE was mutagenized (CRM) and 0.2 μg of pSVβgal (open bars). After transfection, cells were treated with vehicle (C) or bryostatin (10 nm, □). Luciferase activity represents data that have been normalized with β-galactosidase activity. Columns, means; bars, SD; n = 6.

Oligonucleotides. The following oligonucleotides containing the CRE of the COX-2 promoter were synthesized: 5′-AACACAGTCTTTCTGCTACATGGCTTG-3′ (sense) and 5′-CAAGCCATGTGACGAAATGACTGTTT-3′ (antisense). AP-1 oligonucleotides were synthesized: 5′-CGCTTGATGAGTCAGCCGGTA-3′ (sense) and 5′-AAACAGTCATTTCGTCACATGGGCTTG-3′ (antisense). These oligonucleotides were synthesized by Genosys Biotechnologies, Inc. (The Woodlands, TX).

Plasmids. The COX-2 promoter constructs (−327/+59, −220/+59, −124/+59, −52/+59, and CRM) were a gift from Dr. Tadashi Tanabe (National Cardiovascular Center Research Institute, Osaka, Japan; Refs. 5, 55). Dr. Stephen M. Prescott (University of Utah, Salt Lake City, UT) generously provided the human COX-2 cDNA. The AP-1 reporter plasmid (2× TRE-luciferase), composed of two copies of the consensus TRE promoter, was kindly provided by Dr. Joan Heller Brown (University of California, La Jolla, CA). pSVβgal was obtained from Promega Corp.

Transient Transfection Assays. Cells were seeded at a density of 5 × 10^4 cells/well in 6-well dishes and grown to 50–60% confluence. For each well, 2 μg of plasmid DNA were introduced into cells using 2 μg of LipofectAMINE 2000 as per the manufacturer’s instructions. After 12 h of incubation, the medium was replaced with basal medium. The activities of luciferase and β-galactosidase were measured in cellular extract as described previously (56).

Electrophoretic Mobility Shift Assay. Cells were harvested and nuclear extracts prepared. For binding studies, an oligonucleotide containing the CRE of the COX-2 promoter or an oligonucleotide containing an AP-1 consensus site was used. The procedure was carried out as described previously (57).
RESULTS

Bryostatin Induces COX-2. We investigated the possibility that bryostatin could stimulate PGE₂ synthesis in SCC450 cells. Treatment with 1–25 nM bryostatin caused more than a 100% increase in PGE₂ production (Fig. 2). To determine whether the change in PGE₂ production was related to differences in amounts of COX-2, Western blotting of cell lysate protein was carried out. Fig. 3A shows that treatment with bryostatin induced COX-2 protein in SCC450 cells. To confirm that this effect of bryostatin was not unique to SCC450 cells, we also investigated whether bryostatin induced COX-2 in several other cell lines. As shown in Fig. 3, B–D, bryostatin was a potent inducer of COX-2 in two other human cancer cell lines (1483 and A549) as well as in a mouse macrophage cell line (RAW264.7). Peak induction of COX-2 protein was observed when 5–10 nM bryostatin was used. To further elucidate the mechanism responsible for the changes in amounts of COX-2 protein, we determined steady-state levels of COX-2 mRNA by Northern blotting. Treatment with bryostatin caused a marked increase in amounts of COX-2 mRNA (Fig. 4).

COX-2 Transcription is Stimulated by Bryostatin. Transient transfections were performed to investigate the effects of bryostatin on COX-2 transcription. Bryostatin stimulated COX-2 promoter activity (Fig. 5). The inductive effects of bryostatin were observed with all COX-2 promoter deletion constructs, except the −52/+59 construct (Fig. 5B), suggesting that the CRE site may be responsible for mediating this effect. To test this notion, transient transfections were performed using a COX-2 promoter construct in which the CRE was mutagenized. As shown in Fig. 5C, mutagenizing the CRE site caused both a decrease in basal promoter activity and a loss of responsiveness to bryostatin.

Electrophoretic mobility shift assays were performed to identify the transcription factor that was responsible for bryostatin-mediated induction of COX-2. Bryostatin caused increased binding to the CRE site of the COX-2 promoter (Fig. 6A). Supershift analyses identified c-Jun and c-Fos, components of the AP-1 transcription factor, in the binding complex (Fig. 6B). To further evaluate the effects of bryostatin on AP-1, transient transfections were performed. Bryostatin caused dose-dependent induction of AP-1 activity (Fig. 6C) and a corresponding increase in the binding of c-Jun/c-Fos complex to a canonical AP-1 site (Fig. 6D).

Defining the Signaling Mechanism by Which Bryostatin Induces COX-2. Bryostatin is known to modulate PKC activity. Moreover, stimulation of PKC signaling results in enhanced COX-2 transcription. Hence, we determined whether two inhibitors of PKC, Ro 31-8220, and GF 109203X, blocked bryostatin-mediated induction of COX-2. Both Ro 31-8220 (Fig. 7A) and GF 109203X (Fig. 7B) completely inhibited the induction of COX-2 protein by bryostatin. To further investigate the effects of bryostatin on the PKC signal transduction pathway, we assessed its effects on the activation of MAPKs. Treatment with bryostatin activated ERK1/2, JNK, and p38 MAPKs (Fig. 7, C–E). Subsequently, experiments were done to evaluate whether bryostatin-mediated activation of MAPKs was linked to the induction of COX-2. Interestingly, SB 202190, a selective inhibitor of p38 MAPK activity, blocked bryostatin-mediated induction of COX-2 (Fig. 7F). In contrast, neither PD 98059, a compound that blocks the activation of ERK1/2, nor SP 600125, a JNK inhibitor, inhibited the induction of COX-2 by bryostatin (data not shown).
Bryostatin-1 Induces COX-2

Induction of COX-2. Retinoids elicit their biological effects, in part, by antagonizing AP-1-mediated activation of gene expression. On the basis of the evidence that bryostatin stimulated COX-2 transcription by an AP-1-dependent mechanism, we postulated that ATRA might block this phenomenon. As shown in Fig. 8, ATRA caused dose-dependent suppression of bryostatin-mediated induction of COX-2. Complete suppression was observed with 1 μM ATRA.

DISCUSSION

In the present experiments, we showed for the first time that bryostatin induced COX-2 and PG biosynthesis. Induction of COX-2 was observed in several cancer cell lines, including both squamous cell carcinoma and adenocarcinoma as well as in macrophages. Macrophages were evaluated because of evidence that stromal COX-2 contributes to tumorigenesis (22) in addition to inflammation. In regard to the underlying mechanism, bryostatin stimulated COX-2 transcription via the PKC→MAPK→AP-1 pathway (Fig. 9). Previous studies from this laboratory (56–58) and others (2, 5) have provided evidence for the importance of PKC signaling in regulating COX-2 transcription. For example, prototypic inducers of PKC activity, including phorbol esters and bile acids, induce COX-2 (56–58). The fact that PKC is known to be a principal target of bryostatin is consistent with this idea. There is also considerable evidence that the expression of COX-2 can be affected by changes in MAPK activity (12, 20, 55, 57). Treatment with bryostatin stimulated the activities of ERK1/2, JNK, and p38 MAPKs. A selective inhibitor of p38 MAPK blocked bryostatin-mediated induction of COX-2.

The AP-1 transcription factor complex consists of a collection of dimers of members of the Jun and Fos families. Electrophoretic mobility shift analyses showed that treatment with bryostatin augmented the binding of c-Jun and c-Fos to the CRE of the COX-2 promoter as well as to a canonical AP-1 site. This result is consistent with previous findings implicating AP-1 in the activation of COX-2 transcription. Tumor promoting phorbol esters, microtubule interfering agents, and oncogenes also activate COX-2 transcription by stimulating the binding of AP-1 to the CRE site in the human COX-2 promoter (12, 57, 59). The functional significance of AP-1 was established because ATRA, a prototypic AP-1 antagonist, inhibited bryostatin-mediated induction of COX-2. This result is also consistent with prior evidence that RA blocked the induction of COX-2 by phorbol esters and EGF (8, 56, 57). Clearly, the finding that bryostatin stimulated AP-1 activity is important for understanding how bryostatin induced COX-2. AP-1 has been implicated in the activation of COX-2 transcription by stimulating the binding of dimers of members of the Jun and Fos families.
Fig. 9 Schematic of proposed mechanism by which bryostatin stimulates AP-1-mediated induction of COX-2 transcription. Treatment with bryostatin activates PKC and thereby MAPK activity. This leads, in turn, to increased binding of AP-1 to the CRE site of the COX-2 promoter resulting in enhanced transcription. ATRA antagonizes AP-1 and thereby inhibits bryostatin-mediated induction of COX-2.

carcinogenesis (60, 61). It is reasonable to postulate, therefore, that bryostatin-mediated activation of AP-1 could reduce its antineoplastic activity.

The products of COX-2 activity, i.e., PGs, inhibit apoptosis (30, 31), stimulate angiogenesis (33, 34), and increase the invasiveness of malignant cells (35, 36). Possibly, bryostatin-mediated induction of COX-2 will decrease the efficacy of bryostatin as an anticancer agent. It will be worthwhile, therefore, to evaluate whether the addition of a selective COX-2 inhibitor can increase the antitumor activity of bryostatin. COX-2-derived PGs also contribute to inflammation and pain (9–11, 14). The results of this study suggest that the toxicity of bryostatin (e.g., myalgias) could be mediated, in part, by COX-2-derived PGs. Additional studies are warranted to determine whether COX-2 inhibitors can decrease the side effects of bryostatin. Our results also suggest that coadministration of an AP-1 antagonist might reduce the side effects of bryostatin or increase its efficacy as an antitumor agent.

REFERENCES


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