Thalidomide and Its Analogues Inhibit Lipopolysaccharide-mediated Induction of Cyclooxygenase-2

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INTRODUCTION

Cox-2 catalyzes the synthesis of PGs from arachidonic acid. There are two isoforms of Cox. Cox-1 is constitutively expressed in most tissues and fulfills a homeostatic function (1). In contrast, Cox-2 is an immediate, early-response gene that is induced by a variety of stimuli including LPS, cytokines, growth factors, and tumor promoters (2–7).

Three lines of evidence suggest that Cox-2 is an important pharmacological target for the prevention and treatment of cancer. Increased amounts of Cox-2 have been detected in epithelial and stromal cells including macrophages within tumors (8–17). The forced overexpression of Cox-2 in mammary tissue is sufficient to induce cancer (18). Moreover, the formation and growth of tumors is reduced in animals engineered to be Cox-2 deficient (12) or treated with selective Cox-2 inhibitors (12, 19–22). Although the precise mechanism by which overexpression of Cox-2 predisposes to cancer is uncertain, PGs stimulate angiogenesis (23, 24) while inhibiting immune surveillance (25) and apoptosis (26, 27). It is of considerable importance, therefore, to determine whether medications with similar properties alter Cox-2 expression and PG biosynthesis.

Thalidomide (N-phthalimidoglutarimide; Fig. 1) was originally introduced as a sedative, but its use was discontinued in the 1960s because of teratogenic effects. Interest in this agent has increased with the discovery of its anti-inflammatory, immunomodulatory, and antiangiogenic activities, leading to its use in the treatment of conditions including erythema nodosum leprosum and aphthous ulcers (28–30). Thalidomide is also being evaluated as an anticancer agent (31–34). In addition to the ability of thalidomide to inhibit tumor growth in experimental animals (31), promising results have also been obtained in patients with Kaposi’s sarcoma (33) and multiple myeloma (34).

Because the mechanisms of action of thalidomide are incompletely understood, we evaluated whether it could alter the expression of Cox-2. In this report, we show that thalidomide inhibits LPS-mediated induction of Cox-2 and PG biosynthesis in murine macrophages. These results are potentially important for understanding the antineoplastic and anti-inflammatory properties of thalidomide.

MATERIALS AND METHODS

Materials. RPMI 1640 and FBS were from Life Technologies, Inc. (Grand Island, NY). Thalidomide and its analogues were provided by Celgene Corp. (Warren, NJ). Escherichia coli (strain 055:B5) LPS, arachidonic acid, Lowry protein assay kits, actinomycin D, DEAE-dextran, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (thiazolyl blue), O-nitrophenyl-β-D-galactopyranoside, and lactate dehydrogenase diagnostic kits were purchased from Sigma Chemical Co. (St. Louis, MO). Western blotting detection reagents [32P]CTP and [32P]UTP, were from NEN Life Sciences Products (Boston, MA). Random-priming kits were from Boehringer Mannheim 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). Enzyme immunoassay reagents for PGE2 assays were from Cayman Chemical Co. (Ann Arbor, MI). Antiserum to Cox-2 and secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Cellular RNA and plasmid DNA...
were prepared with kits from Qiagen (Chatsworth, CA). pSV-βgal was obtained from the Promega Corp. (Madison, WI).

Tissue Culture. The murine macrophage-like cell line RAW 264.7 was maintained in RPMI 1640 supplemented with 100 units/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 10% FBS. Treatments with vehicle (0.25% DMSO), LPS, or LPS plus thalidomide were carried out in RPMI 1640 supplemented with 3% FBS. Cellular cytotoxicity was assessed by using 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 (35). Lactate dehydrogenase assays were performed according to the manufacturer’s instructions. There was no evidence of toxicity in any of our experiments.

PGE2 Production by Cells. Cells (1 × 10^6 cells/well) were plated in 12-well dishes and grown to 60% confluence in growth medium. The levels of PGE2 released by the cells were measured by enzyme immunoassay. Production of PGE2 was normalized to protein concentrations.

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 twice for 20 s on ice and centrifuged at 10,000 × g for 10 min to sediment the particulate material. The protein concentration of the supernatant was measured by using the method of Lowry et al. (36). SDS-PAGE was performed under reducing conditions on 10% polyacrylamide gels as described by Laemmli (37). The resolved proteins were transferred onto nitrocellulose sheets as detailed by Towbin et al. (38). The nitrocellulose membrane was then incubated with a goat polyclonal antibody to Cox-2. The membrane was subsequently incubated with a secondary antibody conjugated to horseradish peroxidase and developed as described previously (14).

Northern Blotting. Total cellular RNA was isolated from cell monolayers using an RNA isolation kit from Qiagen. Inc. Ten μg/lane of total cellular RNA were electrophoresed in a formaldehyde-containing 1.2% agarose gel and transferred to nylon-supported membranes. After baking, the membranes were prehybridized overnight in a solution containing 50% formamide, 5× SSC, 5× Denhardt’s solution, 0.1% SDS, and 100 μg/ml single-stranded salmon sperm DNA. Hybridization was carried out for 24 h at 65°C with radiolabeled cDNA probes for Cox-2 and 18S rRNA. The Cox-2 cDNA was a generous gift of Dr. Harvey Herschman (University of California Los Angeles, Los Angeles, CA). Cox-2 and 18S rRNA probes were labeled with [32P]CTP by using random priming. After hybridization, membranes were washed twice for 1 min at room temperature in 1× SSC and 1% SDS; twice for 1 h in the same solution at 65°C; and once for 1 h in 0.1× SSC and 1% SDS at 65°C. The washed membranes were then subjected to autoradiography. The density of the bands was quantified with densitometry.

Nuclear Run-Off Assay. Cells (2.5 × 10^5) were plated in four T150 dishes for each condition. Cells were grown in growth medium until they were ~60% confluent. Nuclei were isolated and stored in liquid nitrogen. For the transcription assay, nuclei (3.6 × 10^7) were thawed and incubated in reaction buffer [10 mM Tris (pH 8), 5 mM MgCl2, and 0.3 mM KCl] containing 100 μCi of [32P]UTP and 1 mM unlabeled nucleotides. After 30 min, labeled nascent RNA transcripts were isolated. The Cox-2 and 18S rRNA cDNAs were immobilized onto nitrocellulose and prehybridized overnight in hybridization buffer. Hybridization was carried out at 42°C for 24 h using equal cpm/ml of labeled nascent RNA transcripts for each treatment group. The membranes were washed twice with 2×...
examine PGE2 synthesis in more detail, we measured the effects omide caused dose-dependent suppression of this effect. To lipase A2 activity to the rate of production of PGE2. As shown of LPS and thalidomide on PGE2 production when an excess of exogenous arachidonic acid was added to the culture medium. This was done because PGE2 production can be affected by changes in the activity of phospholipase A2, the enzyme that provides arachidonate for Cox-catalyzed reactions. Adding excess arachidonic acid minimizes any contribution of phospholipase A2 activity to the rate of production of PGE2. As shown changes in the activity of phospholipase A2, the enzyme that provides arachidonate for Cox-catalyzed reactions. Adding excess arachidonic acid minimizes any contribution of phospholipase A2 activity to the rate of production of PGE2. As shown

SSC buffer for 1 h at 55°C and then treated with 10 mg/ml RNase A in 2× SSC at 37°C for 30 min, dried, and autoradiographed.

**Transient Transfection Assays.** Cells were seeded at a density of 5 × 10^4 cells/well in six-well dishes and grown to 60% confluency. The cells were washed in RPMI 1640 and then suspended in 0.5 ml of transfection solution containing 50 mm Tris (pH 8.0) and 500 mg/ml DEAE-dextran. Subsequently, 4.5 μg of Cox-2 promoter construct (−327/+59) and 0.5 μg of pSV-βgal were added, and the mixture was incubated at 37°C for 45 min (14). DMSO (100 μl/ml of transfection mixture) was added for 1 min at room temperature, and the reaction was stopped by the addition of 10 volumes of RPMI 1640. Transfected cells were plated in 100-mm dishes and incubated in RPMI 1640 containing 10% FBS for 24 h. The activities of luciferase and βgal were measured 24 h later in cellular extract as described previously (39).

**Statistics.** Comparisons between the groups were made with the Student’s t test. A difference between the groups of P < 0.05 was considered significant.

**RESULTS**

**Thalidomide Inhibits LPS-mediated Induction of Cox-2.** The possibility that thalidomide could inhibit LPS-mediated induction of PGE2 was investigated. As shown in Fig. 2A, treatment of RAW 264.7 cells with LPS (2 ng/ml) for 16 h caused a >20-fold increase in the spontaneous production of PGE2 from endogenous arachidonic acid. Thalidomide caused dose-dependent suppression of this effect. To examine PGE2 synthesis in more detail, we measured the effects of LPS and thalidomide on PGE2 production when an excess of exogenous arachidonic acid was added to the culture medium. This was done because PGE2 production can be affected by changes in the activity of phospholipase A2, the enzyme that provides arachidonate for Cox-catalyzed reactions. Adding excess arachidonic acid minimizes any contribution of phospholipase A2 activity to the rate of production of PGE2. As shown in Fig. 2B, treatment with LPS caused a 2-fold increase in the synthesis of PGE2 in the presence of excess arachidonic acid. This effect was inhibited by thalidomide in a dose-dependent manner. To determine whether the above effects on the production of PGE2 could be related to differences in the amounts of Cox-2, Western blotting of cell lysate protein was performed. Fig. 3A shows that LPS induced Cox-2. Treatment with thalidomide caused a dose-dependent decrease in LPS-mediated induction of Cox-2. The suppressive effects of thalidomide were also evaluated as a function of time. Interestingly, the time course for the effects of LPS and thalidomide differed. Maximal induction of Cox-2 by LPS occurred at 6 h, but the inhibitory effect of thalidomide required longer treatment. As shown in Fig. 3B, treatment with thalidomide for 12–18 h was required for maximal suppression of LPS-mediated induction of Cox-2.

To further elucidate the mechanism responsible for changes in amounts of Cox-2 protein, we determined the steady-state levels of Cox-2 mRNA by using Northern blotting. Treatment with LPS enhanced levels of Cox-2 mRNA, an effect that was suppressed by thalidomide in a concentration-dependent manner (Fig. 4).

**Thalidomide Inhibits Cox-2 mRNA Stability.** Nuclear run-off assays were performed to determine whether differences in the amounts of Cox-2 mRNA reflected altered rates of transcription. As shown in Fig. 5A, we detected higher rates of synthesis of nascent Cox-2 mRNA after treatment with LPS, consistent with the differences observed with Northern blotting. However, thalidomide did not inhibit the de novo synthesis of Cox-2 mRNA induced by LPS. Transient transfections were performed to confirm that thalidomide had no effect on LPS-mediated induction of Cox-2 transcription. Treatment with LPS stimulated Cox-2 promoter activity; this effect was not suppressed by thalidomide (Fig. 5B). Taken together, these results suggested that thalidomide inhibited LPS-mediated induction of Cox-2 by a posttranscriptional mechanism. To further evaluate this possibility, the effects of thalidomide on Cox-2 mRNA stability were investigated. We measured the rates of degradation of Cox-2 mRNA after treatment with LPS or LPS plus thalidomide (Fig. 6). Cells were treated with vehicle, LPS, or LPS plus thalidomide for 3 h, then transcription was stopped with the addition of actinomycin D. RNA was isolated for 1, 2, and 3 h after treatment with actinomycin D and subjected to Northern blot analysis (Fig. 6). In LPS-treated cells, treatment

![Fig. 3](image-url)

**Fig. 3** Thalidomide inhibits LPS-mediated induction of Cox-2 protein. A, cells were treated with vehicle (Lane 1), LPS (2 ng/ml; Lane 2), or LPS plus thalidomide (10, 15, 25, and 50 μM; Lanes 3–6) for 16 h. B, cells were treated with vehicle (Lane 1), LPS (2 ng/ml; Lanes 2, 4, and 6), or LPS plus thalidomide (50 μM; Lanes 3, 5, and 7) for 6 h (Lanes 1–3), 12 h (Lanes 4 and 5), and 18 h (Lanes 6 and 7). Cellular lysate protein (25 μg/lane) was loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto nitrocellulose. Immunoblots were probed with antibody specific for Cox-2.

![Fig. 4](image-url)

**Fig. 4** LPS-mediated induction of Cox-2 mRNA is inhibited by thalidomide. Cells were treated with vehicle (Lane 1), LPS (2 ng/ml; Lane 2), or LPS plus thalidomide (25 and 50 μM; Lanes 3 and 4) for 6 h. Total cellular RNA was isolated; 10 μg of RNA were added to each lane. The Northern blot was hybridized sequentially with probes that recognized Cox-2 mRNA and 18S rRNA.
with thalidomide enhanced the rate of degradation of Cox-2 mRNA.

**Structural Analogues of Thalidomide Also Inhibit LPS-mediated Induction of Cox-2.** A variety of structural analogues of thalidomide (also known as IMiDs) have been developed. Experiments were performed to determine whether these compounds could inhibit LPS-mediated induction of Cox-2 and PG biosynthesis. As shown in Fig. 7, these related compounds partially suppressed the induction of Cox-2 and PGE2 synthesis by LPS. On the basis of the results of several experiments, the potency of these compounds appeared to be similar to thalidomide.

**DISCUSSION**

Selective inhibitors of Cox-2 possess both anti-inflammatory and antineoplastic properties (19–22, 40, 41). Compounds that block the expression of Cox-2, such as thalidomide, should also inhibit carcinogenesis and decrease inflammation. In fact, some of the known properties of thalidomide can potentially be explained by its ability to inhibit Cox-2 expression and PG production. For example, Cox-2 promotes angiogenesis (24, 42) and inflammation (41, 43), whereas thalidomide inhibits both of these effects (44–46). In this context, it is noteworthy that both thalidomide and selective Cox-2 inhibitors suppress basic fibroblast growth factor-induced angiogenesis (42, 44).

Macrophages are abundant in the stroma of many tumors. Tumor-associated macrophages can express Cox-2 and are a potentially significant source of PGs (10, 11, 47). The importance of Cox-2 as a therapeutic target in macrophages was highlighted by studies of intestinal tumorigenesis in Apc-deficient mice (12, 48). In this model, Cox-2 appears to be expressed primarily in tumor-associated macrophages (47); knocking out or pharmacologically inhibiting Cox-2 protected against intestinal tumor formation (12, 48). In addition to potentially promoting angiogenesis (23) or inhibiting apoptosis (27), PGs derived from tumor-associated macrophages could be important mediators of impaired immune surveillance. PGs have been reported, for example, to inhibit several T-cell and natural killer cell functions (49–51). Therefore, compounds like thalidomide that inhibit the production of PGs by macrophages would be expected to enhance immune surveillance.

This study has potentially important implications from a
mechanistic standpoint. We found that thalidomide decreased the stability of Cox-2 mRNA. This result is consistent with a previous observation that thalidomide decreased the stability of TNF-α mRNA (52). As is the case for TNF-α, the 3'-untranslated region of Cox-2 mRNA contains a series of AUUUA sequences that can alter message stability (53). Future studies will be needed to identify the 3'-sequence(s) and binding proteins that are affected by thalidomide. The fact that several analogues of thalidomide also inhibited the expression of Cox-2 suggests that it may be possible to define a relationship between structure and function. Developing drugs that target the stability of Cox-2 or TNF-α mRNA could represent a new strategy for disease management.

Cox-2 is a bifunctional enzyme possessing both cyclooxygenase and peroxidase activities. Although NSAIDs inhibit PG biosynthesis, most do not affect the peroxidase activity of Cox, which can generate proximate carcinogens (54). One way to overcome this potential limitation of NSAIDs is to identify compounds similar to thalidomide that reduce the expression of Cox-2 and thereby inhibit both functions of the enzyme. Because thalidomide did not completely inhibit Cox-2 expression or PG biosynthesis, a therapeutic regimen combining a selective Cox-2 inhibitor or NSAID with thalidomide might be more effective than using either agent alone.

Recently, thalidomide was observed to decrease the dose-limiting gastrointestinal side effects, e.g., diarrhea, of irinotecan in patients with metastatic colorectal cancer (55). There is a considerable amount of preclinical evidence that inhibiting Cox-2 reduces the growth rate of colorectal cancer (42, 56). Hence, the results of this study strengthen the rationale for determining whether thalidomide will enhance the efficacy, in addition to decreasing the side effects, of irinotecan in patients with colorectal cancer.

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