MOLECULAR TARGETS IN SIGNALING PATHWAYS MEDIATING ANTI-TUMOR EFFECTS OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDs)

Daniel Hwang, Pennington Biomedical Research Center, Louisiana State University, Baton Rouge, Louisiana, 70808, U.S.A.

Many epidemiological studies have revealed that the use of aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs) can reduce the risk of colon cancer. Since the well-documented pharmacological action of aspirin and other NSAIDs is the inhibition of cyclooxygenase [COX, the rate-limiting enzyme in prostaglandin (PG) biosynthesis], it has been inferred that the beneficial effect of NSAIDs may be mediated through the inhibition of PG biosynthesis. However, experimental evidence to support this hypothesis has not been conclusively demonstrated. Several lines of experimental observations imply that the beneficial effects of NSAIDs may be mediated through both COX-dependent and COX-independent pathways.

Results from recent studies demonstrated that NSAIDs can induce the expression of the inducible form of COX (COX-2) in epithelial cells (Meade et al., J Biol Chem 274:8328, 1999). Previously (J Biol Chem 275:28173, 2000), we demonstrated that some NSAIDs show two opposing effects on COX-2 expression; it induces the expression of COX-2 in the colon cancer cell line (HT-29) and macrophage cell line (RAW 264.7) in the absence of other inducers of COX-2 expression; conversely, it inhibits TNFα- or lipopolysaccharide (LPS)-induced COX-2 expression in HT-29 and RAW 264.7 cells, respectively. The relative magnitude of NSAID-induced COX-2 expression and inhibition of cytokine-induced COX-2 by NSAIDs varies with types of cells. NSAIDs do not activate NFκB. Thus, unlike cytokine- or LPS-induced COX-2 expression, NSAIDs- induced COX-2 expression is not mediated through activation of NFκB. NSAIDs that inhibit cytokine- or LPS-induced COX-2 expression also inhibit NFκB activation. These results suggest that the inhibition of cytokine or LPS-induced COX-2 expression is mediated at least in part through the suppression of NFκB activation which is one of major signaling pathways leading to COX-2 expression.

NSAIDs that inhibit LPS-induced COX-2 expression also inhibit expression of other inflammatory marker gene products such as iNOS and IL-1α in macrophages. These results suggest that NSAIDs inhibit not only downstream signaling pathways and target gene expression derived from the activation of pro-inflammatory cytokine receptors, but also the expression pro-inflammatory marker gene products in response to initial inflammatory stimuli.

Macrophages, important components of stromal cells in tumor tissues, can release cytokines, which in turn stimulate tumor cells and other stromal cells to induce the expression of COX-2. Our results suggest that NSAIDs can inhibit both the production of cytokines by macrophages, and the induction of COX-2 by tumor cells in response to the cytokines. These effects may represent a novel mechanism by which NSAIDs exert their anti-inflammatory and possible anti-neo-plastic effects. These results suggest that the pharmacological effects of NSAIDs are mediated through not only the inhibition of COX enzyme activity but also the suppression of the expression of COX-2 and other pro-inflammatory marker gene products.

[Supported by NIH Grants DK-41868 and CA-75613, USDA Grant 9700918, and AICR Grant 98A0978]

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 36, Issue of September 8, pp. 28173–28179, 2000
Printed in U.S.A.

Two Opposing Effects of Non-steroidal Anti-inflammatory Drugs on the Expression of the Inducible Cyclooxygenase

MEDIATION THROUGH DIFFERENT SIGNALING PATHWAYS*

Received for publication, March 20, 2000, and in revised form, June 6, 2000
Published, JBC Papers in Press, June 23, 2000, DOI 10.1074/jbc.M002329200

Ji H. Paik, Jeong H. Ju, Joo Y. Lee, Mary D. Boudreau, and Daniel H. Hwang‡

From the Pennington Biomedical Research Center, Louisiana State University, Baton Rouge, Louisiana 70808

The efficacy of non-steroidal anti-inflammatory drugs (NSAIDs) is considered to be a result of their inhibitory effect on cyclooxygenase (COX) activity. Here, we report that flufenamic acid shows two opposing effects on COX-2 expression; it induces COX-2 expression in the colon cancer cell line (HT-29) and macrophage cell line (RAW 264.7); conversely, it inhibits tumor necrosis factor α (TNFα)- or lipopolysaccharide (LPS)-induced COX-2 expression. This inhibition correlates with the suppression of TNFα- or LPS-induced NFκB activation by flufenamic acid. The inhibitor of extracellular signal-regulated protein kinase, p38, or NFκB does not affect the NSAID-induced COX-2 expression. These results suggest that the NSAID-induced COX-2 expression is not mediated through activation of NFκB and mitogen-activated protein kinases. An activator of peroxisome proliferator-activated receptor γ, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, also induces COX-2 expression and inhibits TNFα-induced NFκB activation and COX-2 expression. Flufenamic acid and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 also inhibit LPS-induced expression of inducible form of nitric-oxide synthase and interleukin-1α in RAW 264.7 cells. Together, these results indicate that the NSAIDs inhibit mitogen-induced COX-2 expression while they induce COX-2 expression. Furthermore, the results suggest that the anti-inflammatory effects of flufenamic acid and some other NSAIDs are due to their inhibitory action on the mitogen-induced expression of COX-2 and downstream markers of inflammation in addition to their inhibitory effect on COX enzyme activity.

Many epidemiological studies have revealed that the use of aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs)[1] can reduce the risk of colon cancer. Since the well documented pharmacological action of aspirin and other NSAIDs is the inhibition of cyclooxygenase (COX, the rate-limiting enzyme in prostaglandin biosynthesis), it can be inferred that the beneficial effect of NSAIDs may be mediated through the inhibition of prostaglandin biosynthesis. However, experimental evidence to support this hypothesis has not been conclusively demonstrated. Several lines of experimental observations imply that the beneficial effects of NSAIDs may be mediated through both COX-dependent and COX-independent pathways.

Two isoforms of COX have been identified: constitutively expressed COX-1 (1–5) and mitogen-inducible COX-2 (6–11). Evidence supporting the hypothesis that the beneficial effect of NSAIDs in reducing the risk of colon cancer is mediated by the inhibition of COX activity includes the fact that cross-breeding of $APC^{\Delta 716}$ knockout mice with COX-2 knockout mice, or the administration of the COX-2 specific inhibitor to $APC^{\Delta 716}$ knockouts, resulted in a dramatic reduction in the numbers and size of the intestinal polyps (12). In addition, it has been demonstrated that the overexpression of COX-2 in intestinal epithelial cells leads to enhanced tumorigenic phenotypes, metastatic potential, and angiogenesis (13–15).

It has been shown that NSAIDs have pharmacological effects other than the inhibition of COX activity. Sodium salicylate and aspirin were shown to inhibit the transcription factor NFκB (16). NSAIDs can also activate peroxisome proliferator-activated receptors (PPAR) α and γ, and induce differentiation of pre-adipocytes to adipocytes (17). Results from recent studies by Meade *et al.* (18) demonstrated that various NSAIDs, as PPAR activators, induce the expression of COX-2 in epithelial cells. However, Xu *et al.* (19) showed that aspirin and sodium salicylate suppress COX-2 expression induced by IL-1 in endothelial cells. In addition, the activation of PPARα by Wy 14643 leads to the inhibition of IL-1-induced COX-2 expression in smooth muscle cells (20).

To clarify these seemingly opposing results, we studied the effects of NSAIDs on COX-2 expression in the presence or absence of a known inducer of COX-2 expression. COX-2 expression is induced by various mitogenic stimuli in different cell types (7, 9, 11, 22). The cis-acting NFκB element is present in the 5′-flanking regions of COX-2 genes of different species (23, 24). Results from our previous studies demonstrated that the activation of NFκB is required to induce the expression of COX-2 in the lipopolysaccharide (LPS)-stimulated macrophage cell line (25). The activation of mitogen-activated protein kinases (MAPKs, ERK-1 and -2, and p38) alone is not sufficient to induce the expression of COX-2, but the inhibition of ERK-1 and -2 or p38 results in partial suppression of COX-2 expression (25). Pro-inflammatory cytokines, such as TNFα and IL-1,

* This work was supported by National Institutes of Health Grants DK-41868 and CA-75613, United States Department of Agriculture Grant 9700918, and American Institute for Cancer Research Grant 98A0978. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Pennington Biomedical Research Center, Louisiana State University, 6400 Perkins Rd., Baton Rouge, LA 70808. Tel.: 225-763-2518; Fax: 225-763-3030; E-mail: hwangdh@pbrc.edu.

[1] The abbreviations used are: NSAID, non-steroidal anti-inflammatory drug; COX, cyclooxygenase; COX-2, mitogen-inducible COX; PPAR, peroxisome proliferator-activated receptor; TNFα, tumor necrosis factor α; NFκB, nuclear factor κB; IL-1α, interleukin-1α; iNOS, inducible form of nitric-oxide synthase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; EMSA, electrophoretic mobility shift assay; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; FBS, fetal bovine serum; TPCK, *N*-tosyl-1-phenylalanine ketone.

also activate NFκB and MAPKs, and induce the expression of COX-2 in many cell types (26, 27).

Thus, we investigated signaling pathways through which NSAIDs modulate the expression of COX-2 in a colon tumor cell line (HT-29) treated with TNFα and a macrophage-like cell line (RAW 264.7) stimulated by LPS. If NSAIDs can modulate mitogen-induced expression of COX-2 in addition to inhibiting the enzyme activity of COX, this may represent a new mechanism of anti-inflammatory and anti-neoplastic actions of NSAIDs.

EXPERIMENTAL PROCEDURES

Reagents—Polyclonal antibodies for COX-1 and COX-2 were prepared and characterized as described previously (28, 29). Polyclonal antibodies for IκBα, iNOS, and IL-1α were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and polyclonal antibody for β-actin was from Sigma. Goat anti-mouse and donkey anti-rabbit immunoglobulin G (IgG) antibodies conjugated to horseradish peroxidase were purchased from Amersham Pharmacia Biotech. Enhanced chemiluminescence (ECL) Western blotting detection reagents were purchased from Amersham Pharmacia Biotech. Polyvinylidene difluoride transfer membrane was purchased from Millipore (Bedford, MA). PD98059, SB203580, and piroxicam were from Calbiochem (La Jolla, CA). NS-398 and flurbiprofen were purchased from Cayman Co. (Ann Arbor, MI). G. A. Piazza (Cell Pathways, Inc., Aurora, CO) provided sulindac sulfide. All other NSAIDs were purchased from Sigma and reconstituted in Me_2SO unless otherwise specified. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (thiazol blue) reagent was purchased from Sigma and reconstituted in PBS to give 5 μg/ml and then filter-sterilized. *N*-Tosyl-1-phenylalanine ketone (TPCK) was purchased from Sigma.

Cell Culture—HT-29 cells (a human colon adenocarcinoma cell line, ATCC HTB-38) or RAW 264.7 cells (a murine macrophage-like cell line, ATCC TIB-71) were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) heat-inactivated fetal bovine serum (FBS, Intergen) and 100 units/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Inc.) at 37 °C in a 5% CO_2/air environment. Cells (2×10^6) were plated in 60-mm dishes (Falcon) and cultured for an additional 18 h to allow the number of cells to approximately double. Cells were maintained in the serum-poor (0.25% FBS) medium for another 18 h prior to the treatment with indicated reagents.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Immunoblotting—These were performed essentially the same as described previously (25, 30, 31).

Preparation of Nuclear Extracts—Cells (4×10^6) were plated in 100-mm dishes and cultured in medium containing 0.25% FBS for 18 h. Fifteen minutes after the TNFα stimulation with or without various NSAIDs, cells were scraped in ice-cold PBS, pelleted, and washed with PBS one more time. Pellets were resuspended in 400 μl of buffer A (10 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 10 mM KCl, 0.3 mM EGTA, 0.3 M sucrose, 10 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 0.5% (v/v) Nonidet P-40, 1 μg/ml leupeptin) and incubated on ice for 20 min. Nuclei were pelleted by centrifugation at 7,200 × *g* for 10 min at 4 °C, followed by washing with buffer A. Nuclei were resuspended in 100 μl of high salt buffer B (20 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 320 mM KCl, 0.2 mM EGTA, 0.5 mM dithiothreitol, 1 μg/ml aprotinin, 1 μg/ml leupeptin) and incubated on ice for 15 min. After centrifugation at 13,500 × *g* for 15 min at 4 °C, the supernatants were kept at −70 °C until analyzed. The protein content of the supernatant was determined by the Bradford method.

Electrophoretic Mobility Shift Assay (EMSA)—A double-stranded oligonucleotide containing a tandem repeat of the consensus sequence for the NFκB binding site was used: 5′-GATCCAAGGGGACTTTCCATG-GATCCAAGGGGACTTTCCATG-3′, 3′-GTTCCCCTGAAAGGTACCT-AGGTTCCCCGAAAGGTACCTAG-5′. Five nanograms of double-stranded oligonucleotide were end-labeled in polynucleotide kinase buffer (60 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 15 mM β-mercaptoethanol, 0.33 μM ATP) using T4 polynucleotide kinase in the presence of 100 μCi of [γ-^{32}P]ATP. The labeled oligonucleotides were purified by G-50 Sephadex® spin columns. Five micrograms of nuclear extract were mixed with incubation buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 4% (v/v) glycerol, 0.08 mg/ml sonicated salmon sperm DNA) and incubated at 4 °C for 15 min. The labeled oligonucleotides (40,000–100,000 cpm) were added to the preincubated mixture and the incubation continued at room temperature for 20 min. Reaction mixtures were run on a 6% non-denaturing polyacrylamide gel at 150 V until the front dye reached 2–3 cm of the bottom of the gel. Following completion of running, the gel was transferred to blotter paper and dried under vacuum. The dried gel was placed in the PhosphorImager screen and exposed overnight.

Luciferase Reporter Gene Assay—Cells were plated in six-well plates (4×10^5 cells/well) and transfected with the reporter gene plasmids using SuperFect transfect reagent (Quiagen, Valencia, CA) according to the manufacturer's instruction. HT-29 cells or RAW 264.7 cells were transfected with 2.5 μg of NFκB response element-driven pGL2 luciferase reporter plasmid, and 0.5 μg of HSP70-lacZ as an internal control (kindly provided by Robert L. Modlin, UCLA, Los Angeles, CA). For COX-2 promoter assay, 2.5 μg of murine COX-2 gene promoter (nt-1,017/+93)-driven pGL2 luciferase reporter plasmid (gift from David Dewitt, Michigan State University, East Lansing, MI) was used. After 3 h, the medium was changed and further incubated for 6 h. The cells were further incubated in medium containing 0.25% FBS for an additional 15 h. The cells were treated with flufenamic acid alone or a combination of flufenamic acid with TNFα (50 ng/ml, Sigma) or LPS in the serum-poor (0.25% FBS) medium. The luciferase activity was determined using the Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's recommended protocol. Luciferase activity was normalized to the internal control plasmid HSP70-lacZ by measuring β-galactosidase activity.

Ligand Binding Assay for PPARγ—HT-29 cells or RAW 264.7 cells were transfected with 1 μg of the chimeric receptor expression construct, pcDNA3-hPPARγ/GAL4 containing the ligand binding domain of hPPARγ and the yeast GAL4 transcription factor DNA binding domain. Ligand binding activity was measured by co-transfecting 1.5 μg of the reporter gene construct, pUAS(5x)-tk-luc, which contains five copies of GAL4 response element (kindly provided by Joel Berger, Merck Research Laboratory), and 0.5 μg of HSP70-lacZ as an internal control. After 3 h, the medium was changed and further incubated for 6 h. The cells were further incubated in the serum-poor (0.25% FBS) medium for an additional 15 h. The medium was removed and fresh medium containing various NSAIDs or 1 μM of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) was added to each well and incubated for another 24 h. The luciferase activity was determined as described above.

Determination of Cell Viability—HT-29 cells or RAW 264.7 cells (2.5×10^4 cells/well) were plated in 96-well plates. After treatment with flufenamic acid for 11 h, 20 μl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide solution (5 μg/ml) were added to each well and incubated for additional 4 h. Insoluble formazan precipitates formed in the medium were solubilized with 100 μl of 10% SDS, 0.01 N HCl solution. Optical density at 595 nm was measured using Bio-Rad plate reader.

RESULTS

NSAIDs, Flufenamic Acid, and Sulindac Sulfide Inhibit TNFα-induced NFκB Activation: This Inhibition Leads to the Suppression of COX-2 Expression—Among various NSAIDs tested, flufenamic acid and sulindac sulfide were the most potent inhibitors of TNFα-induced NFκB activation determined by IκBα degradation in HT-29 cells (data not shown). Thus, we investigated these two NSAIDs in this report.

Pretreatment of HT-29 cells with flufenamic acid shows a biphasic effect on TNFα-induced COX-2 expression: enhancement at concentrations below 200 μM but inhibition at concentrations above 200 μM (Fig. 1*A*). However, flufenamic acid suppressed the TNFα-induced activation of NFκB in a dose-dependent fashion without showing the biphasic effect as demonstrated by both EMSA and NFκB reporter gene assay (Fig. 1, *B* and *C*). Flufenamic acid at concentrations up to 200 μM does not affect viability of HT-29 and RAW 264.7 cells as determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay (data not shown). However, flufenamic acid induces cell death at concentrations above 200 μM. Thus, only adherent cells were harvested and analyzed by Western blot analysis for the cells treated with flufenamic acid at concentrations above 200 μM.

Similar to flufenamic acid, sulindac sulfide inhibits TNFα-induced COX-2 expression and this inhibition correlates with the suppression of TNFα-induced NFκB activation by sulindac sulfide (Fig. 2, *A* and *B*). In addition, TNFα-induced COX-2

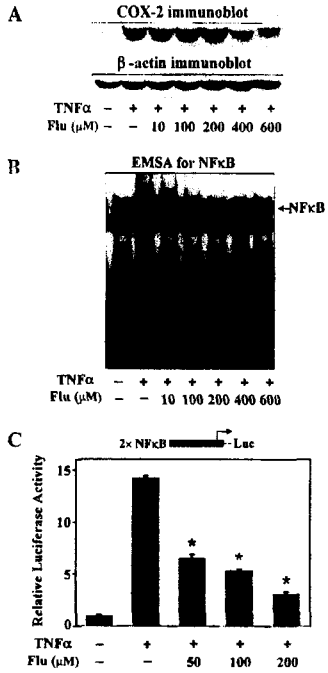

FIG. 1. **Inhibitory effects of flufenamic acid on TNFα-induced COX-2 expression and NFκB activation in HT-29 cells.** Cells maintained in the serum-poor medium were treated with flufenamic acid (*Flu*) for 3 h and then stimulated with TNFα (20 ng/ml) in the presence of flufenamic acid. *A*, after 8 h, cell lysates were analyzed by COX-2 and β-actin immunoblot. *B*, after 15 min, nuclear extracts were analyzed by EMSA for NFκB. *C*, cells were transfected with NFκB response element-driven pGL2 luciferase reporter plasmid and HSP70-lacZ as an internal control. Transfected cells were pretreated with flufenamic acid for 3 h and then treated with a combination of flufenamic acid and TNFα (50 ng/ml) for 6 h. The luciferase activity was measured using the luciferase assay system. Values are mean ± S.E. ($n = 3$). *, significantly different from the vehicle control (TNFα alone) ($p < 0.05$). The figures are representative data from more than three different analyses.

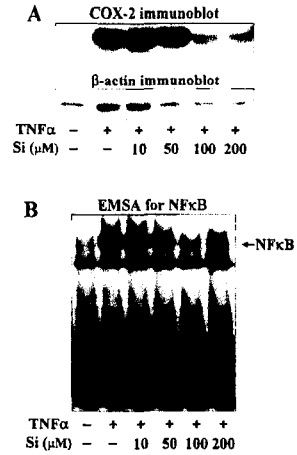

FIG. 2. **Inhibitory effects of sulindac sulfide on TNFα-induced COX-2 expression (*A*) and activation of NFκB (*B*).** Cells maintained in serum-poor medium were treated with sulindac sulfide (*Si*) for 3 h and then stimulated with TNFα (20 ng/ml) in the presence of sulindac sulfide. EMSA for NFκB and COX-2 immunoblot analyses were performed as described in Fig. 1.

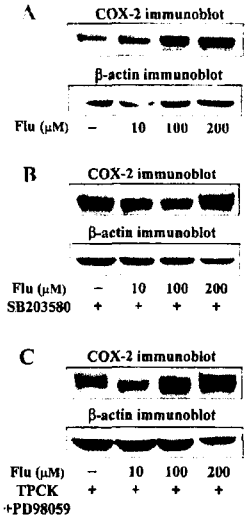

FIG. 3. **COX-2 expression induced by flufenamic acid (*Flu*) in HT-29 cells is not inhibited by the inhibitor of p38 (SB203580) or the mixture of the inhibitors of NFκB (TPCK) and MEK1 (PD98059).** *A*, cells maintained in the serum-poor medium were treated with flufenamic acid for 8 h. Cells were pretreated with SB203580 (2.5 μM) (*B*) or a combination of TPCK (15 μM) and PD98059 (10 μM) (*C*) for 3 h and then further treated with flufenamic acid in the presence of inhibitors for 8 h. Cell lysates were analyzed by COX-2 and β-actin immunoblot. The panels are representative immunoblots from more than three different analyses.

expression is suppressed by the inhibitor of NFκB (data not shown). These results suggest that the inhibition of TNFα-induced COX-2 expression by flufenamic acid or sulindac sulfide is at least in part due to its inhibitory effect on TNFα-induced NFκB activation.

Flufenamic Acid in the Absence of Other COX-2 Inducers in the Medium Induces the Expression of COX-2 in HT-29 Cells: This Induction Was Not Inhibited by the Inhibitors of MAPKs or NFκB—Next, to determine whether enhancement of TNFα-induced COX-2 expression by flufenamic acid at concentrations below 200 μM (Fig. 1*A*) is due to COX-2 expression induced by flufenamic acid itself, cells were treated with flufenamic acid alone in the absence of other COX-2 inducers. Flufenamic acid alone induces COX-2 expression in a dose-dependent fashion (Fig. 3*A*). This induction was not inhibited by the pretreatment of cells with SB203580, a specific inhibitor of p38, or a mixture of inhibitors, PD98059 and TPCK, for MEK1 and NFκB, respectively (Fig. 3, *B* and *C*). Sulindac sulfide also induces COX-2 in the serum-poor medium and this induction was not inhibited by inhibitors of MAPKs or NFκB (Fig. 4, *A–C*). Flufenamic acid and other NSAIDs alone do not induce the degradation of IκBα (data not shown). Flufenamic acid and other NSAIDs do not affect COX-1 expression in HT-29 cells (data not shown). These results suggest that the expression of COX-2 induced by NSAIDs such as flufenamic acid or sulindac sulfide is not mediated through the activation of MAPKs and NFκB signaling pathway.

Results from recent studies demonstrated that some NSAIDs including flufenamic acid can bind and activate PPARγ and PPARα (17) and induce the expression of COX-2 in epithelial cells and fibroblasts (18, 32). Our immunoblot analyses demonstrated that PPARγ, but not PPARα, was detected in HT-29 cells (data not shown). Thus, we determined the effects of another known activator of PPARγ on COX-2 expression in

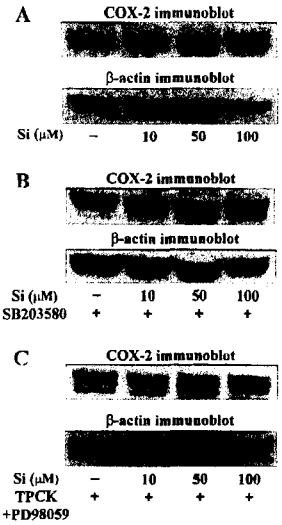

FIG. 4. **COX-2 expression induced by sulindac sulfide (*Si*) in HT-29 cells is not inhibited by the inhibitor of p38 (SB203580) or the mixture of the inhibitors of NFκB (TPCK) and MEK1 (PD98059).** Cells in serum-poor medium were treated and analyzed as described in Fig. 3. The panels are representative immunoblots from more than three different analyses.

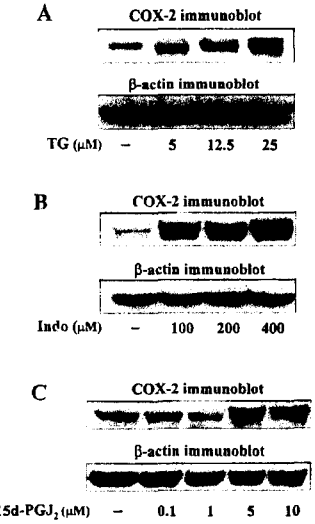

FIG. 5. **Induction of COX-2 expression by activators of PPARγ.** *A*, troglitazone (*TG*); *B*, indomethacin (*Indo*); *C*, 15d-PGJ_2. Cells were treated with troglitazone, indomethacin, or 15d-PGJ_2, and then COX-2 and β-actin immunoblot were performed as described in Fig. 3.

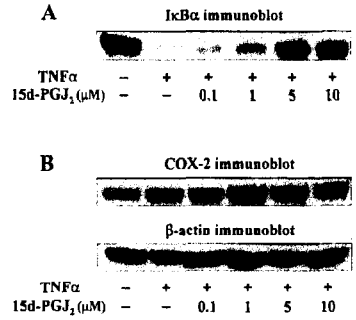

FIG. 6. **Inhibitory effects of the PPARγ activator, 15d-PGJ_2 on the TNFα-induced degradation of IκBα and COX-2 expression in HT-29 cells.** *A*, cells maintained in serum-poor medium were treated with 15d-PGJ_2 for 3 h and then stimulated with TNFα (20 ng/ml) for 15 min in the presence of 15d-PGJ_2. Cell lysates were analyzed by IκBα immunoblot. *B*, cells were treated with 15d-PGJ_2 for 3 h and then stimulated with TNFα (20 ng/ml) for 8 h in the presence of 15d-PGJ_2. Cell lysates were analyzed by COX-2 and β-actin immunoblot. The panels are representative immunoblots from more than three different analyses.

HT-29 cells. Results show that, similar to flufenamic acid, other known PPARγ activators troglitazone, indomethacin, and 15d-PGJ_2 induce COX-2 expression (Fig. 5). In addition, pretreatment with 15d-PGJ_2 results in the inhibition of TNFα-induced IκBα degradation and COX-2 expression (Fig. 6, *A* and *B*). However, indomethacin, although it induces COX-2 expression, does not inhibit TNFα-induced COX-2 expression (data not shown).

To determine whether NSAIDs bind PPARγ, HT-29 cells were transfected with the chimeric receptor expression construct, pcDNA3-hPPARγ/GAL4 and the reporter gene construct, pUAS(5x)-tk-luc as described elsewhere (33). Treatment of HT-29 cells with flufenamic acid, sulindac sulfide, or 15d-PGJ_2 resulted in a significantly increased ligand binding activity to hPPARγ (Fig. 7*A*). The same pattern of results was shown in RAW 264.7 cells (Fig. 7*B*). These results imply that flufenamic acid- and sulindac sulfide-induced COX-2 expression is mediated through the activation of PPARγ both in HT-29 cells and RAW 264.7 cells.

Flufenamic Acid Induces COX-2 Expression and Also Inhibits the LPS-induced Activation of NFκB and COX-2 Expression in the Murine Macrophage-like Cell Line (RAW 264.7)—We next determined in a cell type other than HT-29 cells whether flufenamic acid induces COX-2 expression and also inhibits activation of NFκB and COX-2 expression induced by mitogenic stimulation. Pretreatment of RAW 264.7 cells with flufenamic acid leads to a dose-dependent inhibition of LPS-stimulated transcriptional activity of COX-2 promoter and NFκB reporter genes (Fig. 8, *A* and *C*, respectively).

Flufenamic acid, in the absence of other inducers of COX-2 expression, enhances transcriptional activity of COX-2 promoter-reporter gene in RAW 264.7 cells (Fig. 8*B*). However, flufenamic acid alone does not affect the basal promoter activity of NFκB (Fig. 8*D*), indicating that flufenamic-induced COX-2 expression is not mediated through the activation of NFκB. This result corroborates with the results, obtained by Western blot analyses of endogenous COX-2 protein in HT-29 cells (Fig. 3), demonstrating that the inhibitor of NFκB does not suppress flufenamic-induced COX-2 expression.

Flufenamic Acid and 15d-PGJ_2 Inhibit the LPS-induced Expression of Other Pro-inflammatory Marker Gene Products Such as iNOS and IL-1α in RAW 264.7 Cells—Pretreatment of RAW 264.7 cells with flufenamic acid or 15d-PGJ_2 leads to a dose-dependent inhibition of LPS-induced expression of iNOS and IL-1α as determined by Western blot analyses (Fig. 9, *A* and *B*). These results suggest that NSAIDs, which inhibit mitogen-induced activation of NFκB, can suppress the expression of many genes whose induction is mediated in part through activation of NFκB.

DISCUSSION

Our results demonstrate that NSAIDs have two opposing effects on COX-2 expression; NSAIDs inhibit cytokine-induced COX-2 expression, while NSAIDs alone can induce COX-2 ex-

pression. Results from promoter-reporter assays demonstrate that flufenamic acid inhibits LPS-induced COX-2 expression and NFκB activation in RAW 264.7 cells (Fig. 8, *A* and *C*),

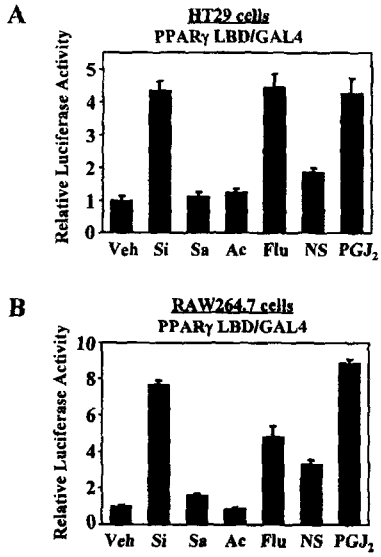

FIG. 7. **Ligand binding activity of various NSAIDs for PPARγ in HT-29 and RAW 264.7 cells.** *A*, HT-29 cells were transfected with the chimeric receptor expression construct, pcDNA3-hPPARγ/GAL4 containing the ligand binding domain of hPPARγ and the yeast GAL4 transcription factor DNA binding domain. Ligand binding activity was measured by co-transfecting the reporter gene construct, pUAS(5x)-tk-luc, which contains five copies of GAL4 response element. Transfected cells were treated with 100 μM of sulindac sulfide (*Si*), salicylic acid (*Sa*), acetaminophen (*Ac*), flufenamic acid (*Flu*), NS398 (*NS*), or 1 μM 15d-PGJ_2 (*PGJ_2*). *Veh*, vehicle. *B*, RAW 264.7 cells were transfected with the same plasmids as above. The transfected cells were treated with 50 μM sulindac sulfide, flufenamic acid, and 100 μM salicylic acid, acetaminophen, NS398, or 1 μM 15d-PGJ_2. Luciferase activity was determined as described in Fig. 1. The panels are representative data from more than three different assays. Values are mean ± S.E. ($n = 3$).

whereas it induces COX-2 expression in the absence of LPS (Fig. 8*B*). The concentrations of flufenamic acid required to inhibit LPS-induced COX-2 expression and to induce COX-2 expression are in a similar range. Thus, the magnitude of the inhibition of LPS-induced COX-2 expression by flufenamic acid might have been even greater if there was no simultaneous induction of COX-2. In HT-29 cells, enhancement of TNFα-induced COX-2 expression by flufenamic acid at 200 μM or lower is likely due to the fact that the additive induction of COX-2 expression by flufenamic acid is greater than its inhibitory effect on TNFα-induced COX-2 expression at these concentrations. However, the inhibitory effect of flufenamic acid on TNFα-induced COX-2 expression at higher concentrations may far exceed the additive induction of COX-2 expression by flufenamic acid. Flufenamic acid does not cause cell death at concentrations up to 200 μM; however, it induces cell death at concentrations above 200 μM. It has been well documented that activation of NFκB suppresses apoptotic signals in many cell types (36–39); conversely, inhibition of NFκB can induce apoptosis. Thus, it is likely that induction of apoptosis and inhibition of TNFα-induced COX-2 expression by flufenamic acid are mediated through a common signaling pathway, *i.e.* inhibition of NFκB.

Flufenamic acid does not have an opposing effect on NFκB activation. In both cell types, pretreatment with flufenamic acid leads to a dose-dependent inhibition of TNFα- or LPS-induced NFκB activation. Flufenamic acid alone does not cause NFκB activation.

Many NSAIDs bind and activate PPARs and some PPAR activators have been shown to inhibit NFκB activity (17, 20). TNFα-induced COX-2 expression in HT-29 cells was inhibited by flufenamic acid, sulindac sulfide, or 15d-PGJ_2, all of which bind PPARγ (Fig. 7). However, indomethacin, a known activator of PPARγ, does not inhibit TNFα-induced COX-2 expression and NFκB activation (data not shown). It was previously shown that, unlike sulindac sulfide, indomethacin does not inhibit IκB kinase (40, 41). Recently, it was demonstrated that not all PPAR activators inhibit NFκB activation (21) and cytokine production in macrophages (42). Together, these results suggest that the inhibition of TNFα-induced COX-2 expression or

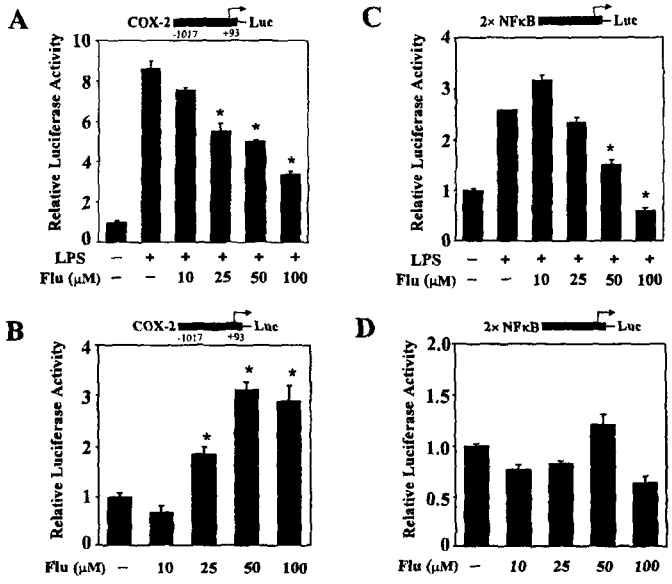

FIG. 8. **Flufenamic acid induces COX-2 expression but it inhibits LPS-induced COX-2 expression and NFκB transactivation in RAW 264.7 cells.** Cells were transfected with luciferase reporter plasmid under the transcriptional control of COX-2 promoter (*A* and *B*), or NFκB response element (*C* and *D*). Transfected cells were treated with flufenamic acid (*Flu*) and LPS (25 ng/ml for *panel A*, and 1 μg/ml for *panel C*) or flufenamic acid alone for 24 h. Panels are representative data from more than three different analyses. Values are mean ± S.E. ($n = 3$). *, significantly different from the LPS alone (*A* and *C*) and the vehicle control (*B*) ($p < 0.05$).

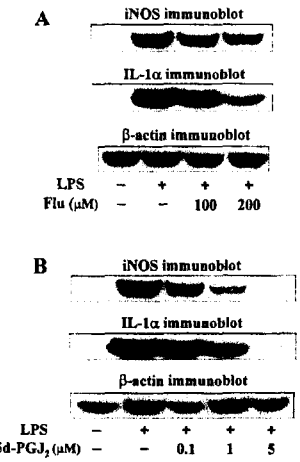

FIG. 9. **Inhibitory effects of flufenamic acid (*Flu*) and 15d-PGJ_2 on LPS-induced expressions of iNOS and IL-1α in RAW 264.7 cells.** Cells maintained in serum-poor medium were treated with flufenamic acid (*A*) or 15d-PGJ_2 (*B*) for 3 h and then stimulated with LPS (0.1 μg/ml) in the medium containing flufenamic acid or 15d-PGJ_2 for 8 h. Cell lysates were analyzed by iNOS, IL-1α or β-actin immunoblot. The panels are representative immunoblots from more than three different analyses.

NFκB activation by flufenamic acid is not mediated through the activation of PPARs. Furthermore, these results suggest that NSAIDs such as indomethacin, which do not inhibit mitogen-induced activation of NFκB, are unable to inhibit the mitogen-induced COX-2 expression.

The molecular target through which flufenamic acid inhibits TNFα- or LPS-induced NFκB activation is not known. Recent studies have demonstrated that aspirin and sodium salicylate suppress NFκB activation by inhibition of IκB kinase β (16, 40, 43). Another study has demonstrated that 15d-PGJ_2 inhibits NFκB by a covalent modification of a cysteine residue within its activation loop of IκB kinase β (21). This irreversible modification is rendered by the formation of Michael adducts between a reactive α,β-unsaturated carbonyl group in the cyclopentane ring of 15d-PGJ_2 and cellular nucleophiles such as compounds containing free SH group. Salicylate and flufenamic acid do not appear to possess such a reactive group for nucleophilic attack in their structure. Thus, the mode of action in inhibiting NFκB by flufenamic acid is likely different from that of 15d-PGJ_2.

The flufenamic acid- or sulindac sulfide-induced COX-2 expression was not affected by either the inhibitor of p38 or by inhibitors of NFκB and MEK1 (Figs. 3 and 4). These results indicate that, unlike the TNFα-induced COX-2 expression, the NSAID-induced COX-2 expression is mediated through signaling pathways that do not require the activation of MAPKs and NFκB. Some NSAIDs and 15d-PGJ_2, which are known to activate PPARγ, induce COX-2 expression in the absence of other inducers of COX-2 expression (Figs. 3–5). Aspirin and sodium salicylate which do not activate PPARs (17) were unable to induce COX-2 expression (data not shown). Together, these results suggest, but do not prove, that flufenamic acid- and sulindac sulfide-induced COX-2 expression is mediated through the activation of PPARγ. The pharmacological significance of the induction of COX-2 expression by NSAIDs is not known. Since NSAIDs inhibit the activity of COX-2 expressed in tissues in response to NSAIDs, the inhibitory effect of NSAIDs on cytokine-induced COX-2 expression would be a more important net effect.

It has been a prevailing belief that the efficacy of NSAIDs is due to their inhibitory effect on COX activity. However, the therapeutic benefit of NSAIDs is observed at plasma concentrations substantially higher than those required to inhibit COX (34). Emerging evidence now suggests that NSAIDs can also exert their anti-inflammatory and possible anti-tumor effects through COX-independent pathways (35). Our results demonstrating that NSAIDs inhibit TNFα-induced activation of NFκB signaling pathways suggest that NSAIDs can inhibit the cellular responses to pro-inflammatory cytokines by inhibiting the downstream signaling pathways derived from the activation of cytokine receptors. Furthermore, flufenamic acid inhibits not only COX-2 expression (Fig. 8) but also the expression of other inflammatory marker gene products such as iNOS and IL-1α induced by LPS in RAW 264.7 cells (Fig. 9). These results suggest that NSAIDs inhibit not only downstream signaling pathways derived from the activation of pro-inflammatory cytokine receptors, but also the expression of pro-inflammatory marker gene products in response to inflammatory stimuli.

Macrophages, important components of stromal cells in tumor tissues, can release cytokines, which in turn stimulate tumor cells and other stromal cells to induce the expression of COX-2. Our results suggest that NSAIDs can inhibit both the production of cytokines by macrophages, and the induction of COX-2 by tumor cells in response to the cytokines. These effects may represent an additional mechanism by which NSAIDs exert their anti-inflammatory and possible anti-neoplastic effects.

In summary, our results presented here suggest that the pharmacological effects of NSAIDs are mediated not only through the inhibition of COX activity but also the modulation of the expression of COX-2 and other pro-inflammatory marker gene products.

Acknowledgment—We thank Dr. Walter A. Deutsch for reading the manuscript, and Dr. Jong S. Rim, Eun Y. Kim, and Kyung H. Sohn for technical assistance.

REFERENCES

1. DeWitt, D. L., and Smith, W. L. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 1412–1416
2. Merlie, J. P., Fagan, D., Mudd, J., and Needleman, P. (1988) *J. Biol. Chem.* **263,** 3550–3553
3. Yokoyama, C., Takai, T., and Tanabe, T. (1988) *FEBS Lett.* **231,** 347–351
4. DeWitt, D. L., el-Harith, E. A., Kraemer, S. A., Andrews, M. J., Yao, E. F., Armstrong, R. L., and Smith, W. L. (1990) *J. Biol. Chem.* **265,** 5192–5198
5. Yokoyama, C., and Tanabe, T. (1989) *Biochem. Biophys. Res. Commun.* **165,** 888–894
6. Xie, W. L., Chipman, J. G., Robertson, D. L., Erikson, R. L., and Simmons, D. L. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 2692–2696
7. Kujubu, D. A., Fletcher, B. S., Varnum, B. C., Lim, R. W., and Herschman, H. R. (1991) *J. Biol. Chem.* **266,** 12866–12872
8. O'Banion, M. K., Sadowski, H. B., Winn, V., and Young, D. A. (1991) *J. Biol. Chem.* **266,** 23261–23267
9. Hla, T., and Neilson, K. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 7384–7388
10. Jones, D. A., Carlton, D. P., McIntyre, T. M., Zimmerman, G. A., and Prescott, S. M. (1993) *J. Biol. Chem.* **268,** 9049–9054
11. Feng, L., Sun, W., Xia, Y., Tang, W. W., Chanmugam, P., Soyoola, E., Wilson, C. B., and Hwang, D. (1993) *Arch. Biochem. Biophys.* **307,** 361–368
12. Oshima, M., Dinchuk, J. E., Kargman, S. L., Oshima, H., Hancock, B., Kwong, E., Trzaskos, J. M., Evans, J. F., and Taketo, M. M. (1996) *Cell* **87,** 803–809
13. Tsujii, M., and DuBois, R. N. (1995) *Cell* **83,** 493–501
14. Tsujii, M., Kawano, S., and DuBois, R. N. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 3336–3340
15. Tsujii, M., Kawano, S., Tsuji, S., Sawaoka, H., Hori, M., and DuBois, R. N. (1998) *Cell* **93,** 705–716
16. Kopp, E., and Ghosh, S. (1994) *Science* **265,** 956–959
17. Lehmann, J. M., Lenhard, J. M., Oliver, B. B., Ringold, G. M., and Kliewer, S. A. (1997) *J. Biol. Chem.* **272,** 3406–3410
18. Meade, E. A., McIntyre, T. M., Zimmerman, G. A., and Prescott, S. M. (1999) *J. Biol. Chem.* **274,** 8328–8334
19. Xu, X. M., Sansores-Garcia, L., Chen, X. M., Matijevic-Aleksic, N., Du, M., and Wu, K. K. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 5292–5297
20. Staels, B., Koenig, W., Habib, A., Merval, R., Lebret, M., Torra, I. P., Delerive, P., Fadel, A., Chinetti, G., Fruchart, J. C., Najib, J., Maclouf, J., and Tedgui, A. (1998) *Nature* **393,** 790–793
21. Rossi, A., Kapahi, P., Natoli, G., Takahashi, T., Chen, Y., Karin, M., and Santoro, M. G. (2000) *Nature* **403,** 103–108
22. Feng, L., Xia, Y., Garcia, G. E., Hwang, D., and Wilson, C. B. (1995) *J. Clin.*

Invest. **95,** 1669–1675
23. Fletcher, B. S., Kujubu, D. A., Perrin, D. M., and Herschman, H. R. (1992) *J. Biol. Chem.* **267,** 4338–4344
24. Yamamoto, K., Arakawa, T., Ueda, N., and Yamamoto, S. (1995) *J. Biol. Chem.* **270,** 31315–31320
25. Hwang, D., Jang, B. C., Yu, G., and Boudreau, M. (1997) *Biochem. Pharmacol.* **54,** 87–96
26. Schwenger, P., Alpert, D., Skolnik, E. Y., and Vilcek, J. (1998) *Mol. Cell. Biol.* **18,** 78–84
27. Schwenger, P., Bellosta, P., Vietor, I., Basilico, C., Skolnik, E. Y., and Vilcek, J. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 2869–2873
28. Lee, S. H., Soyoola, E., Chanmugam, P., Hart, S., Sun, W., Zhong, H., Liou, S., Simmons, D., and Hwang, D. (1992) *J. Biol. Chem.* **267,** 25934–25938
29. Hwang, D., Scollard, D., Byrne, J., and Levine, E. (1998) *J. Natl. Cancer Ins.* **90,** 455–460
30. Chanmugam, P., Feng, L., Liou, S., Jang, B. C., Boudreau, M., Yu, G., Lee, J. H., Kwon, H. J., Beppu, T., Yoshida, M., Xia, Y., Wilson, C. B., and Hwang, D. (1995) *J. Biol. Chem.* **270,** 5418–5426
31. Laemmli, U. K. (1970) *Nature* **227,** 680–685
32. Lu, X., Xie, W., Reed, D., Bradshaw, W. S., and Simmons, D. L. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 7961–7965
33. Berger, J., Leibowitz, M. D., Doebber, T. W., Elbrecht, A., Zhang, B., Zhou, G., Biswas, C., Cullinan, C., Hayes, N. S., Li, Y., Tanen, M., Ventre, J., Wu, M. S., Berger, G. D., Mosely, R., Marquis, R., Santini, C., Sahoo, S. P., Tolman, R. L., Smith, R. G., and Moller, D. E. (1999) *J. Biol. Chem.* **274,** 6718–6725
34. McEvoy, G. K. (ed) (1997) *AHFS 97 Drug Information*, American Society of Health-Systems Pharmacists, Bethesda, MD
35. Jiang, C., Ting, A. T., and Seed, B. (1998) *Nature* **391,** 82–86
36. Ferreira, V., Sidénius, N., Tarantino, N., Hubert, P., Chatenoud, L., Basi, F., and Körner, M. (1999) *J. Immunol.* **162,** 6442–6450
37. Schneider, A., Martin-Villalba, A, Weih, F., Vogel, J., Wirth, T., and Schwaninger, M. (1999) *Nat. Med.* **5,** 554–559
38. Mayo, M. W., Wang, C., Cogswell, P. C., Rogers-Graham, K. S., Lowe, S. W., Der, C. J., and Baldwin, A. S., Jr. (1997) *Science* **278,** 1812–1815
39. Liu, Z., Hsu, H., Goeddel, D. V., and Karin, M. (1996) *Cell* **87,** 565–576
40. Yin, M. J., Yamamoto, Y., and Gaynor, R. B. (1998) *Nature* **396,** 77–80
41. Yamamoto, Y., Yin, M. J., Lin, K. M., and Gaynor, R. B. (1999) *J. Biol. Chem.* **274,** 27307–27314
42. Thieringer, R. Fenyk-Melody, J., Le Grand, C., Shelton, B., Detmers, P., Somers, E., Carbin, L., Moller, D., Wright, S., and Berger, J (2000) *J. Immunol.* **164,** 1046–1054
43. Grilli, M., Pizzi, M., Memo, M., and Spano, P. (1996) *Science* **274,** 1383–1385