0026-895X/04/6502-470–478$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2004 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 65:470–478, 2004

Vol. 65, No. 2
2722/1126491
Printed in U.S.A.

Aspirin-Mediated COX-2 Transcript Stabilization via Sustained p38 Activation in Human Intestinal Myofibroblasts

Randy C. Mifflin, Jamal I. Saada, John F. Di Mari, John D. Valentich, Patrick A. Adegboyega, and Don W. Powell

Departments of Internal Medicine (R.C.M., J.I.S., J.F.D., J.D.V., D.W.P.), Pathology (P.A.A.), and Physiology and Biophysics (D.W.P.), The University of Texas Medical Branch, Galveston, Texas

Received June 9, 2003; accepted November 3, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Acetylsalicylic acid (aspirin) is a cyclooxygenase (COX) inhibitor, yet some of its therapeutic effects are thought to derive from mechanisms unrelated to prostaglandin synthesis inhibition. In human intestinal myofibroblasts, aspirin, at therapeutic doses, had the unexpected effect of inducing prolonged COX-2 expression. This induction was especially pronounced when cells were treated with interleukin-1α (IL-1) plus aspirin for 24 h. Sodium salicylate, a poor COX inhibitor, likewise enhanced IL-1–mediated COX-2 gene expression whereas 5-aminosalicylic acid (5-ASA) or indomethacin had no effect. The COX-2 transcriptional rate, measured by nuclear runoff analysis and heterogeneous nuclear RNA reverse transcription-polymerase chain reaction, was only modestly elevated by aspirin treatment. In contrast, aspirin treatment dramatically stabilized the COX-2 message. The COX-2 mRNA half-life in IL-1 treated cells was 1 h and was increased in excess of 5 h in IL-1 + aspirin-treated cells. Phosphorylation of p38 MAPK was enhanced in aspirin-treated cells (but not in cells treated with 5-ASA or indomethacin) for up to 24 h after treatment. Inhibition of p38 activity negated aspirin-mediated COX-2 mRNA stabilization and the resultant increase in COX-2 mRNA and protein levels. The modest transcriptional response seen in aspirin treated cells was also abolished by p38 inhibition. We conclude that aspirin enhances COX-2 expression via sustained activation of p38, which results in prolonged stabilization of the COX-2 message and a slightly elevated transcription rate. Aspirin also enhanced steady-state mRNA levels of other IL-1 modulated genes (IL-1β, IL-6, groα, and TNFα) that are likewise regulated at the level of message stability via p38 activation.

Acetylsalicylic acid, commonly called aspirin, is the most widely used nonsteroidal anti-inflammatory drug (NSAID) in the world. Aspirin is effective in the treatment of pain, fever, and inflammation and also for prophylaxis against coronary artery disease and cancer (Vane et al., 1998). Aspirin's therapeutic benefit in many cases derives from its ability to inhibit cyclooxygenase (COX) enzymatic activity, resulting in decreased production of prostaglandins. In vivo, however, aspirin is rapidly deacetylated to form salicylate and, although salicylate is a poor inhibitor of COX activity, both salicylic acid and aspirin are potent anti-inflammatory agents. Furthermore, the optimal therapeutic dose for aspirin in the treatment of chronic inflammation is much higher than that required to inhibit cyclooxygenase activity (Rainsford, 1984; Brooks et al., 1986; Furst et al., 1987). Several non–prostaglandin-related effects of aspirin and salicylate have been described. These effects include inhibition of NF-κB mobilization (Kopp and Ghosh, 1994; Yin et al., 1998), activation or inhibition of MAPK/SAPK signaling (Schwenger et al., 1997, 1998, 1999), and others (Pillinger et al., 1998; Cronstein et al., 1999; Marnett and DuBois, 2002).

One major effect of the proinflammatory cytokine IL-1 is activation of the local synthesis and release of eicosanoids through the cyclooxygenase (COX) pathway (Dinarello, 1996; Vane et al., 1998). At least two isoforms of COX exist in humans, each encoded by separate unlinked loci. They share similar catalytic properties (e.g., K_m, V_{max}, catalytic products), but each has a distinct biological function (Turini and DuBois, 2002). COX-1 is constitutively expressed in many cell types, whereas COX-2 expression is more restricted and

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases grant DK55783, the Gulf Coast Digestive Disease Center (DK56338), and a grant from the Crohn's and Colitis Foundation of America, Inc.

ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; NF-κB, nuclear factor κB; MAPK, mitogen-activated protein kinase; SAPK, stress-activated protein kinase; IL-1, interleukin-1α; IMF, intestinal myofibroblast; PG, prostaglandin; TNFα, tumor necrosis factor α; 5-ASA, 5-aminosalicylic acid; SB-203580, 4-(4-fluorophenyl)-2-(4-methylsulfinyl phenyl)-5-(4-pyridyl)-1*H*-imidazole; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; hnRNA, heterogeneous nuclear RNA; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; p-p38, phosphorylated p38; ASA, acetylsalicylic acid (aspirin).

frequently only observed after stimulation of cells with mitogens or proinflammatory cytokines (Vane et al., 1998; Turini and DuBois, 2002).

We have previously characterized signaling pathways responsible for IL-1–mediated induction of COX-2 gene expression in human intestinal myofibroblasts (Mifflin et al., 2002; Di Mari et al., 2003). Intestinal myofibroblasts (IMFs) modulate responses of epithelial cells and immune and nonimmune cells of the lamina propria through elaboration of growth factors, cytokines, and lipid mediators such as prostaglandins (PGs) (Powell et al., 1999). IMFs are also likely to be important in the progression of colorectal cancer because they express COX-2 early in the process (Shattuck-Brandt et al., 2000; Adegboyega et al., 2001; Sonoshita et al., 2002), and IMF activation is observed in premalignant colorectal polyps (Adegboyega et al., 2002).

Herein we report that aspirin and sodium salicylate have the unexpected effect of inducing COX-2 gene expression in human IMFs. The induction is especially pronounced in cells treated with the combination of NSAID plus IL-1. The main mechanism whereby this occurs is aspirin-mediated enhancement of p38 MAPK activity, which leads to a dramatic stabilization of the COX-2 message. Aspirin likewise enhanced expression of proinflammatory cytokines and chemokines (IL-1β, IL-6, groα, and TNFα) that are likewise regulated at the level of message stability via p38 activation. Another salicylate derivative, 5-aminosalicylate (5-ASA), did not affect this pathway, which may be one reason why 5-ASA is therapeutic for the treatment of chronic intestinal inflammation and other salicylates are not.

Materials and Methods

Materials. Recombinant human IL-1α was purchased from R&D Systems (Minneapolis, MN). The following were purchased from Sigma (St. Louis, MO): acetylsalicylic acid (aspirin), 5-aminosalicylic acid, sodium salicylate, and indomethacin. The compound SB-203580 was purchased from BIOMOL (Plymouth Meeting, PA). The COX-2 polyclonal antibody was purchased from Cayman Chemical (Ann Arbor, MI). Antibodies recognizing phosphorylated (phospho-Thr180/Tyr182) and total p38 were purchased from Cell Signaling Technologies (Beverly MA). The transcriptional inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) was purchased from Calbiochem (San Diego, CA). The monoclonal antibody to α-smooth muscle actin (clone 1A4) was purchased from Sigma.

Cell Culture. The human intestinal myofibroblast isolate 18Co was obtained from the American Type Culture Collection (Manassas, VA) and maintained as described previously (Valentich et al., 1997; Mifflin et al., 2002). Cells were cultured in Eagle's minimum essential medium supplemented with 10% NuSerum (BD Biosciences, Bedford, MA) at 37°C in a humidified incubator containing 5% CO_2 in air. For experiments, cells between passage 10 and 15 were used at confluence.

RNA Isolation and Northern Blot Analysis. Total RNA was isolated using the Ultraspec RNA Isolation Reagent (Biotecx Laboratories, Inc., Houston, TX). Northern blotting and hybridizations were performed as described previously (Hinterleitner et al., 1996). Gene-specific mRNA levels were detected and quantified using filmless autoradiographic analysis (PerkinElmer Life and Analytical Sciences, Boston, MA). Slight variations in signal strength resulting from differences in the amount of total RNA loaded in each well were corrected by normalization to 28S or 18S rRNA levels using digitized images of the membranes after transfer. The COX-2-specific probe was a 1.8-kilobase fragment of the human COX-2 cDNA provided by Dr. Timothy Hla (University of Connecticut, Farmington, CT). The IL-1β, IL-6, and groα-specific probes were gel-purified PCR fragments spanning nucleotides 631 to 1290 of the human IL-1β cDNA (GenBank accession number X02532), nucleotides 306 to 904 of the human IL-6 cDNA (GenBank accession number M14584), and nucleotides 105 to 690 of the human groα cDNA (GenBank accession number J03561).

Western Blot Analysis. Western blot analyses were performed as described previously (Mifflin et al., 2002). Chemiluminescent detection was performed using a chemiluminescence detection kit (Amersham Biosciences, Piscataway, NJ) according to the supplier's recommendations.

Nuclear Runoff Analysis. Nuclear runoff analyses were performed as described previously (Mifflin et al., 2002). Relative transcription levels were determined using filmless autoradiographic analysis (PerkinElmer Life and Analytical Sciences).

COX-2 Transcription Rate Using hnRNA RT-PCR. We developed an alternate method of assessing the COX-2 transcription rate based on the assay described by Elferink and Reiners (1996). Based on the fact that nascent, unspliced nuclear RNA [heterogeneous nuclear RNA (hnRNA)] is rapidly spliced to generate mature mRNA, quantitation of unspliced COX-2 transcripts is used as an indicator of the COX-2 transcriptional rate. Total RNA samples, extracted as above, were treated with DNase (Promega, Madison, WI) and reverse-transcribed using oligonucleotide primers specific for introns within the human COX-2 gene to generate hncDNA. These samples were then subjected to amplification by PCR using intron-specific primer pairs to generate products traversing COX-2 exons. For each reverse transcription reaction, a mock reaction was carried out in which the reverse transcriptase was left out to control for potential contamination by genomic DNA. The reverse transcription and PCR primers used were as follows (numbering based upon GenBank accession number D28235). Amplification across exon #6: COX2GEN L6020, 5′-AGTTTTCATTTACCACA-3′ (RT primer); COX2GENU5468, 5′-TTTCATCATTTTCTAGGTGGACTTAA-3′ (sense PCR); and COX2 GENL5597, 5′-TAAAATATGGGTATAAGCGGTAATAA-3′ (antisense PCR). Amplification across exon #9: COX2GENL7472, 5′-GCTCACGC-CTGTAATCC-3′ (RT primer); COX2GENU6942, 5′-AGCTGAATA-CAAACAGTAAATATGCCTAA-3′ (sense PCR); and COX2GENL7218, 5′-AGGGCGGCTCCATCTCGAAAA-3′ (antisense PCR).

PCR reactions were performed in the presence of [^{32}P]dCTP (1.0 μCi/20-μl reaction, to facilitate quantitation by filmless autoradiographic analysis) and fractionated in 6% acrylamide gels in Tris-borate buffer. It was empirically determined that 25 cycles of amplification were within the linear quantitative range for this assay and this number was used throughout.

RT-PCR Analysis of TNFα mRNA. TNFα mRNA was detected using commercially supplied primers for human TNFα (R&D Systems) to amplify cDNA prepared from treated cells as directed by the supplier. Included in each reaction were primers for the constitutively expressed heme oxygenase 2 (spanning nucleotides 273 to 764 of the human HO-2 cDNA, GenBank accession number S34389) as an internal control.

ELISA Assay for TNFα Secretion. TNFα secretion was monitored using a commercially available enzyme-linked immunosorbent assay (ELISA; limit of detection, ~15 pg/ml; R&D Systems). Confluent monolayers were treated for 24 h as indicated in the legend to Fig. 7. Culture medium was removed, cleared by centrifugation, and assayed for TNFα levels.

Data Analysis. All experiments were repeated a minimum of three times with similar results. Where appropriate, data were expressed as the mean ± S.E. Statistical analysis was performed using Student's t test using a P value of 0.05 as statistically significant. In all cases in which comparative data are presented, the autoradiographic images originated from the same exposure of the same gel; in some cases, for clarity of presentation, lanes containing samples not germane to this study were removed.

Results

Synergistic Induction of COX-2 Expression by IL-1 + Aspirin. Therapeutic doses of salicylates for the treatment of chronic inflammation, such as rheumatoid arthritis, are realized when serum levels are in the 1 to 5 mM range (Rainsford, 1984; Brooks et al., 1986; Furst et al., 1987). Figure 1A demonstrates COX-2 mRNA levels in cells cultured 4 and 24 h in the absence or presence of IL-1 (500 pg/ml), aspirin (5.0 mM), indomethacin (20 μM), or IL-1 plus aspirin or indomethacin. At the earlier time point, aspirin moderately enhanced (1.5-fold) COX-2 mRNA levels above those seen in control or IL-1–treated cells, but this trend was not statistically significant ($P > 0.2$; $n = 4$). However, COX-2 mRNA levels were significantly higher in aspirin-treated cells after 24 h. Aspirin treatment resulted in 6.4-fold higher ($P < 0.001$; $n = 7$) COX-2 mRNA levels over untreated cells and aspirin augmented IL-1–induced COX-2 mRNA levels by 4.8-fold ($P < 0.001$; $n = 9$). Indomethacin, at a dose that inhibits PGE_2 synthesis by >97% in these cells (20 μM), had no effect upon basal or IL-1–induced COX-2 mRNA levels (Fig. 1A). The inability of indomethacin to affect COX-2 mRNA levels argues against the possibility that PGE_2 or other COX-derived eicosanoids act in a negative feedback loop to down-regulate IL-1–mediated COX-2 induction. Figure 1B demonstrates COX-2 mRNA levels in IMFs after 24 h in the presence of IL-1 plus increasing concentrations (10 μM to 5.0 mM) of aspirin. The inclusion of IL-1 results in a more sensitive assay because slight increases over the IL-1 baseline are easier to detect. Dose response analyses, such as the one presented in Fig. 1B, indicate that aspirin enhances IL-1–mediated COX-2 induction over the 1.0 to 5.0 mM range. Higher aspirin concentrations were not included in this study because of signs of toxicity in cells treated 24 to 48 h with aspirin at levels ≥10 mM. The effect of other salicylates upon IL-1–mediated COX-2 induction is shown in Fig. 1C. Sodium salicylate (Na-Sal) likewise enhanced COX-2 mRNA levels when included at 5.0 mM, yet it had little or no effect at the 0.5 mM dose. 5-ASA (5.0 mM) did not enhance COX-2 expression. The observed aspirin-mediated COX-2 mRNA induction was also reflected at the protein level as judged by Western blotting of extracts prepared 24 h after treatment (Fig. 1D). In experiments not shown, sodium salicylate (5.0 mM) likewise increased COX-2 protein levels.

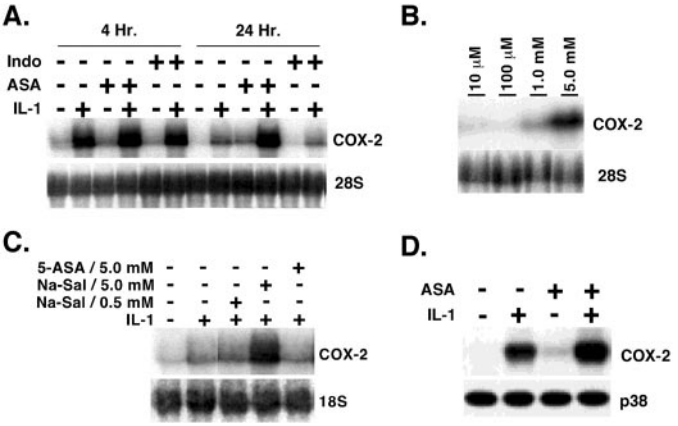

Fig. 1. Synergistic induction of COX-2 mRNA and protein levels by IL-1 plus aspirin. Confluent 18Co monolayers were incubated for the indicated time in the presence of either IL-1 (500 pg/ml), ASA (5.0 mM), or, as indicated in B, indomethacin (Indo; 20 μM), sodium salicylate (Na-Sal; 0.5 or 5.0 mM), 5-ASA (5.0 mM) or combinations thereof. Control cells received 1.0% ethanol as a solvent control. Cells were then harvested for isolation of either total RNA or protein. A, COX-2 mRNA levels. Total RNA was subjected to Northern analysis using a COX-2–specific cDNA probe. Below is an inverse image of the 28S rRNA bands on the membrane before hybridization, demonstrating equivalent loading and transfer. B, dose-response for aspirin-mediated COX-2 induction. Cells were incubated for 24 h in the presence of IL-1 (500 pg/ml) plus the indicated concentration of aspirin. Total RNA was subjected to Northern analysis using a COX-2–specific cDNA probe. Below is an inverse image of the 28S rRNA bands on the membrane before hybridization, demonstrating equivalent loading and transfer. C, effect of other salicylates. Total RNA was subjected to Northern analysis using a COX-2–specific cDNA probe. Below is an inverse image of the 18S rRNA bands on the membrane before hybridization, demonstrating equivalent loading and transfer. D, COX-2 protein levels after 24 h of IL-1 and aspirin treatment. Total protein (25 μg) was subjected to Western analysis using an antibody specific for COX-2 as described under *Materials and Methods*. The resultant blot was then stripped and reanalyzed for total p38 protein levels to ensure equivalent loading.

Effect of Aspirin upon COX-2 Transcription. Experiments were conducted to determine whether aspirin regulates COX-2 expression at the transcriptional level. A nuclear runoff analysis of the COX-2 transcriptional rate 4 h after IL-1 (500 pg/ml), aspirin (5.0 mM), or IL-1 plus aspirin treatment is shown in Fig. 2A. The results of several such experiments, summarized graphically in Fig. 2A, indicate a slight increment in the COX-2 transcriptional rate in aspirin-treated cells (2.2 ± 1.2-fold; $n = 3$). This trend was not statistically significant ($P > 0.05$), perhaps because of the inherent insensitivity of the runoff assay. The IL-1–mediated COX-2 transcriptional rate was not significantly enhanced by aspirin inclusion (18.9 ± 2.84-fold versus 18.4 ± 9.01-fold; $n = 5$) as measured by nuclear runoff analysis (Fig. 2A).

We developed a more sensitive assay to measure the transcription rate of the endogenous COX-2 gene to determine whether aspirin has any significant transcriptional effect on its own. To this end, we adopted the assay of Elferink and Reiner (1996), which measures the level of nascent, unspliced transcripts (hnRNA) from a particular gene using an RT-PCR approach that employs intron-specific reverse transcription and PCR primers. Figure 2B shows the results of one such assay in which COX-2 hnRNA levels were determined in IMFs cultured 4 h in the presence or absence of IL-1, aspirin, or both. Using this assay, IL-1 increased the COX-2 transcriptional rate 16 ± 6.3-fold ($n = 5$, $P > 0.002$) over the level observed in untreated cells. This result agrees well with the runoff data presented in Fig. 2A and published by us previously (Mifflin et al., 2002). Aspirin treatment increased the COX-2 transcription rate 2.6 ± 0.33-fold ($n = 5$, $P > 0.005$) relative to untreated cells. The augmentation of IL-1–induced COX-2 transcription by aspirin using this assay was only 1.2-fold ($n = 5$, $P = 0.04$), similar to that observed in the runoff analyses. To demonstrate that the hnRNA amplification assay is linear with respect to input hncDNA, 2-fold serial dilutions of hncDNA prepared from IL-1 treated IMFs were analyzed, and the resultant data are plotted in Fig. 2C. The data plotted in Fig. 2C had an R^2 value of 0.87.

Effect of Aspirin upon COX-2 Message Stability. Given the modest effect of aspirin upon COX-2 transcription compared with its effect upon steady-state mRNA levels, we examined whether aspirin influenced COX-2 mRNA stability. Cultures were pretreated 4 h with IL-I or IL-1 plus aspirin and then placed in serum-free medium containing the RNA polymerase II transcriptional inhibitor DRB (50 μM) with or without aspirin. Cultures were then harvested 2, 4,

and 6 h later for determination of COX-2 mRNA levels by Northern blotting. In these experiments, the COX-2 message half-life in IL-1 treated cells, as judged by decay in the presence of DRB, was approximately 1 h (Fig. 3) (Mifflin et al., 2002). This half-life was dramatically extended to a period exceeding 5 h in the presence of aspirin. The COX-2 message is stabilized by p38 MAPK activation in human IMFs (Mifflin et al., 2002) and other cell types (Ridley et al., 1998; Lasa et al., 2000, 2001). We have previously demonstrated that p38 inhibition reduces the COX-2 message half-life in IL-1–treated cells to only 20 min (Mifflin et al., 2002). To determine whether p38 plays a role in the observed aspirin-mediated COX-2 message stabilization, COX-2 mRNA decay was measured in the presence of aspirin plus the p38 inhibitor SB-203580 (20 μM). As shown in Fig. 3, the p38 inhibitor negated aspirin-mediated COX-2 message stabilization.

Effect of Aspirin upon p38 Activation. We determined whether aspirin treatment activates p38 by monitoring phosphorylated p38 (P-p38) levels in cells treated with IL-1 or IL-1 plus aspirin by Western blotting. Within the first hour of treatment, aspirin had little effect upon basal and IL-1–induced P-p38 levels (Fig. 4A). However, a gradual time-dependent increase in P-p38 levels was observed at later time points (2–24 h) in aspirin-treated cells. In cells treated with IL-1, aspirin seemed to retard the decay of IL-1–induced P-p38 levels, which remained elevated after 24 h (Fig. 4, A and D), analogous to what was observed with COX-2 mRNA levels (Fig. 1A).

If aspirin-mediated p38 activation is responsible for the

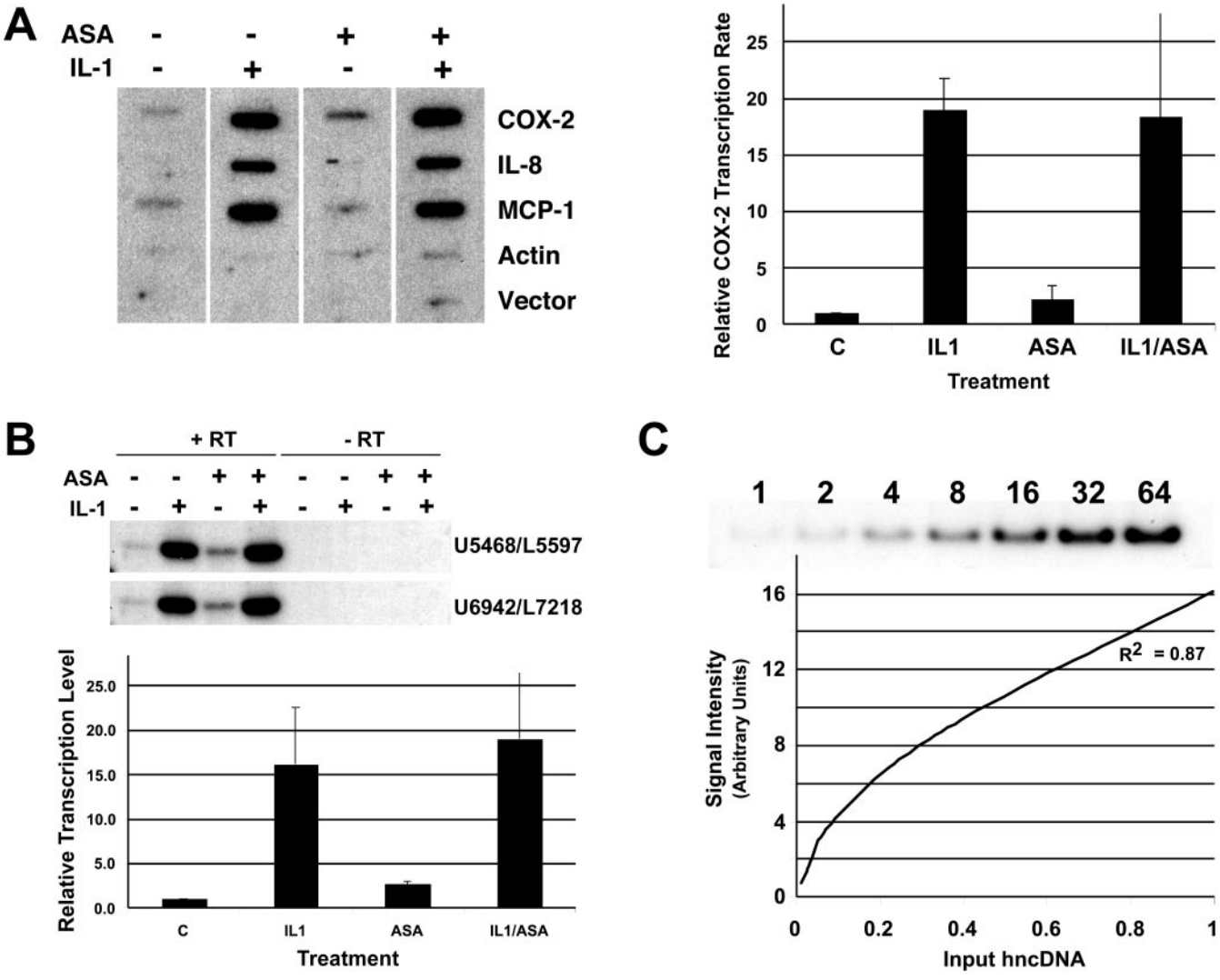

Fig. 2. COX-2 Transcription rate after IL-1, aspirin, and IL-1 + aspirin treatment. A, nuclear runoff analysis. Confluent 18Co monolayers were incubated in the presence of IL-1 (500 pg/ml), aspirin (5.0 mM), or IL-1 plus aspirin. After 4 h, nuclei were isolated and nuclear runoff analysis was performed as described under *Materials and Methods*. MCP-1, macrophage chemotactic protein 1; actin, β actin; vector-plasmid Bluescript SK$^-$ without insert, used to calculate background hybridization. The graph summarizes the results obtained from analysis of at least three independent determinations for each treatment. B, hncDNA amplification. Confluent 18Co monolayers were incubated in the presence of IL-1 (500 pg/ml), aspirin (5.0 mM), or IL-1 plus aspirin. After 4 h, total RNA was extracted, and relative COX-2 hnRNA levels were determined as described under *Materials and Methods*. A representative experiment is shown using the primers spanning exon 6 (COX2GENU5468/COX2GENL5597) and exon 9 (COX2GENU6942/COX2GENL7218) along with mock reactions that included RNA but no reverse transcriptase (RT). The graph summarizes the results obtained from analysis of five independent determinations for each treatment. C, linearity of the hncDNA assay with respect to input hcDNA. Two-fold serial dilutions of total hncDNA, synthesized from IMFs treated for 4 h with IL-1, were subjected to PCR analysis as described under *Materials and Methods* using the primers spanning exon 6. The signal strength was quantitated and results are shown in the graph below.

observed induction of COX-2 expression, then NSAIDs that did not induce COX-2 expression should not induce p38 phosphorylation. As shown in Fig. 4, indomethacin (20 μM) and 5-ASA (5.0 mM), alone (Fig. 4B) or in combination with IL-1 (Fig. 4C), had no significant effect upon p38 phosphorylation compared with aspirin-treated cells (presented graphically in Fig. 5D).

Effect of p38 Inhibition upon Aspirin-Mediated COX-2 Induction. If aspirin induces COX-2 expression via p38 activation, then p38 inhibition should retard this process. As shown in Fig. 5, aspirin-mediated induction of COX-2 expression was potently suppressed in the presence of the specific p38 inhibitor SB-203580 (20 μM) at the mRNA (Fig. 5A) and protein (Fig. 5B) levels. The degree of repression at each stage was similar (81–97% inhibition), indicating an inhibition at the level of steady-state mRNA. As shown in Fig. 5, and by others (Guan et al., 1997; Dean et al., 1999; Mifflin et al., 2002), SB-203580 also inhibits IL-1–mediated induction of COX-2 expression. In these experiments, aspirin consistently elevated IL-1–induced COX-2 message and protein levels approximately 2- to 5-fold in the presence of SB-203580. This observed increment is possibly caused by incomplete inhibition of p38 activity or to decay of the inhibitor over the 24-h course of incubation.

Effect of p38 Inhibition upon COX-2 Transcription. To determine the consequence of aspirin-mediated p38 activation upon COX-2 transcription, hnRNA amplification was performed to determine the relative transcription rate in cells treated with SB-203580. As shown in Fig. 6, SB-203580 (20 μM) inhibited the IL-1 or aspirin-induced transcription rate by 51 ± 18.3% ($n = 4$; $P < 0.05$) and 93 ± 1.2% ($n = 4$; $P < 0.01$), respectively when assayed 4 h after treatment. The COX-2 transcriptional rate in cells treated with both IL-1 and aspirin was inhibited by 82 ± 7.0% ($n = 4$; $P < 0.02$) in the presence of the p38 inhibitor. Thus, the transcriptional as well as post-transcriptional effects of aspirin upon COX-2 expression are attributable to p38 activation.

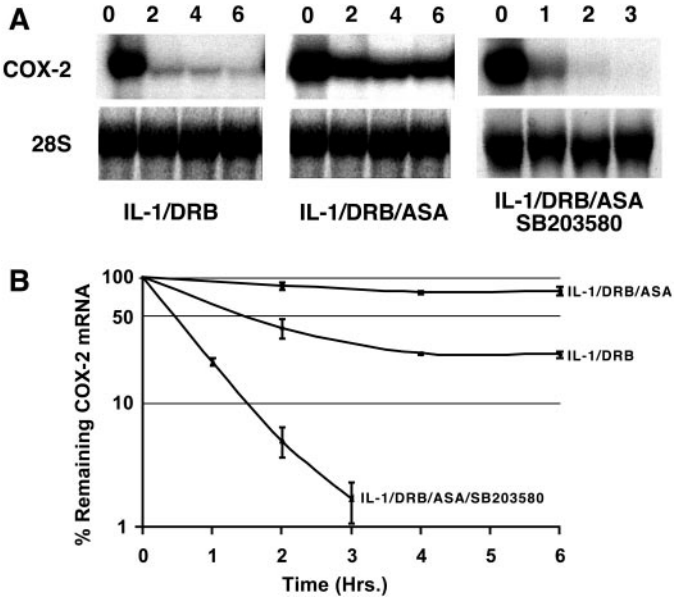

Fig. 3. Effect of aspirin upon COX-2 message stability. Confluent 18Co cultures were incubated for 4 h in the presence of IL-1 (500 pg/ml) or IL-1 plus aspirin (5.0 mM). Cells were then placed in serum-free medium containing the RNA polymerase II transcriptional inhibitor DRB (50 μM), DRB plus aspirin (5.0 mM), or DRB plus aspirin plus the p38 inhibitor SB-203580 (20 μM). Cultures were then frozen at the indicated times (in hours) for determination of COX-2 mRNA levels by Northern blotting. Note the shorter time scale for COX-2 message decay in the presence of the p38 inhibitor. Shown above are images from a representative experiment. Also shown are digitized photographs of the membranes showing equivalent levels of 28S ribosomal RNA before hybridization. Bottom, relative COX-2 mRNA levels (normalized to 28S rRNA) as a function of the time spent in the presence of each inhibitor.

Synergistic Induction of Proinflammatory Cytokines and Chemokines by Aspirin in Combination with IL-1. Given that p38 activation leads to mRNA stabilization for a number of genes in addition to COX-2 (Frevel et al., 2003), we hypothesized that aspirin would also enhance IL-1–mediated induction of mRNAs containing AU-rich stability control elements. Preliminary microarray studies indicated that this is indeed the case and the results from a selected panel of genes are presented in Fig. 7. Northern analyses of mRNA levels for IL-1β, IL-6, and the chemokine groα after 24 h in the presence of IL-1, aspirin, or both are shown in Fig. 7A. For each gene shown, aspirin dramatically enhanced the IL-1–mediated increase in steady-state mRNA levels. Figure 7B is an RT-PCR analysis of TNFα mRNA levels after 24 h in the presence of IL-1, with and without aspirin or 5-ASA. At this time point, TNFα mRNA was only detected in cells treated with the combination of IL-1 plus aspirin. TNFα accumulation in the culture medium of cells treated as in Fig. 7B was analyzed by ELISA (Fig. 7C). Appreciable TNFα accumulation was only seen in cells treated with both IL-1 and aspirin. In experiments not shown, TNFα message was transiently detected in IL-1-treated cells 4 to 8 h after treatment, yet significant TNFα secretion into the culture supernatant was not observed (Fig. 7C). Thus, aspirin synergizes with IL-1 to induce steady-state mRNA levels for a number of genes important in inflammation.

Discussion

We have demonstrated the ability of aspirin and sodium salicylate to induce COX-2 expression in a cultured human IMF isolate, 18Co. The observed effect is especially pronounced when combined with IL-1 resulting in a synergistic induction of COX-2 mRNA and protein. Our results demonstrate that aspirin induces COX-2 expression via prolonged activation of p38, which results in a dramatic stabilization of the COX-2 message. Nuclear runoff and hnRNA RT-PCR analyses demonstrated a minor transcriptional component, but the kinetics of COX-2 mRNA and protein accumulation, combined with mRNA degradation experiments, strongly suggest that the primary mechanism underlying the observed synergistic induction is at the level of COX-2 mRNA stability. We also found that aspirin synergizes with IL-1 to enhance steady-state mRNA levels for other proinflammatory mediators that also contain AU-rich stability control elements within their mRNAs.

The mechanism by which aspirin prolongs COX-2 expression is apparently unrelated to COX inhibition because sodium salicylate, a poor COX inhibitor, also enhanced expression (Fig. 1C), and indomethacin, a potent COX inhibitor, had no effect upon steady-state COX-2 mRNA levels (Fig. 1A). Davies et al. (1997) have reported aspirin-mediated COX-2 induction *in vivo*. In that study, aspirin (250 mg/kg) administration to rats resulted in marked expression of

COX-2 mRNA and protein in the gastric lamina propria, precisely where gastric myofibroblasts are located. Indomethacin (20 mg/kg) had only a minor affect on COX-2 expression. The latter result further supports our hypothesis that salicylate-mediated COX-2 induction is not simply the consequence of COX inhibition because indomethacin is an effective COX inhibitor.

In contrast to our results, Wu and colleagues have demonstrated inhibition of COX-2 gene expression by aspirin and sodium salicylate, at doses ranging from 10 μM to 1.0 mM, in human endothelial cells, macrophages, and fibroblasts (Xu et al., 1999; Cieslik et al., 2002). In their studies, COX-2 transcription was depressed 4 h after aspirin treatment and the mechanism of salicylate-mediated inhibition was via inhibition of C/EBPβ binding to the COX-2 promoter. We saw no such effect of salicylates upon COX-2 transcription in IL-1–treated human IMFs, perhaps because in these cells, C/EBPβ binding activity is very low and does not increase upon stimulation with IL-1 (Mifflin et al., 2002).

Our results also differ from those of Faour et al. (2001), who demonstrated that in human synovial fibroblasts, IL-1β drives COX-2 expression primarily via COX-2–derived PGE_2 and that NSAIDs abrogate IL-1–mediated COX-2 induction. They demonstrate that PGE_2, acting via EP4 receptors, activates p38, resulting in COX-2 message stabilization. They further demonstrate that in their cells, IL-1β–mediated COX-2 induction occurs primarily at the post-transcriptional level, with only a minor (2-fold) transcriptional component (Faour et al., 2001). These results highlight the striking heterogeneity in mechanisms of COX-2 regulation in different cell types. In our studies, inhibition of PGE_2 synthesis by indomethacin had little effect upon COX-2 expression (Fig. 1A) and p38 phosphorylation (Fig. 5B). Furthermore, IL-1 treatment leads to dramatic COX-2 transcriptional activation in IMFs when transcription of the endogenous gene is assayed (Fig. 2) (Mifflin et al., 2002).

We should also note that salicylates can affect gene transcription by inhibiting NF-κB activation (Kopp and Ghosh, 1994; Yin et al., 1998), which would lead to decreased transcription of the COX-2 gene. We did not see such an effect because the dose of aspirin used in our studies is below the 10 to 20 mM range of salicylate necessary to inhibit IκB degradation; in experiments not shown, 5.0 mM aspirin did not inhibit IL-1–mediated induction of NF-κB binding activity.

Schwenger and colleagues have carefully studied the effect of salicylates on MAPK/SAPK activation. Their studies have demonstrated that 2.0 to 20 mM salicylate causes increased p38 phosphorylation and activity within 5 min in human foreskin fibroblasts and macrophages (Schwenger et al., 1997, 1998). While aspirin slightly enhanced p38 phosphor-

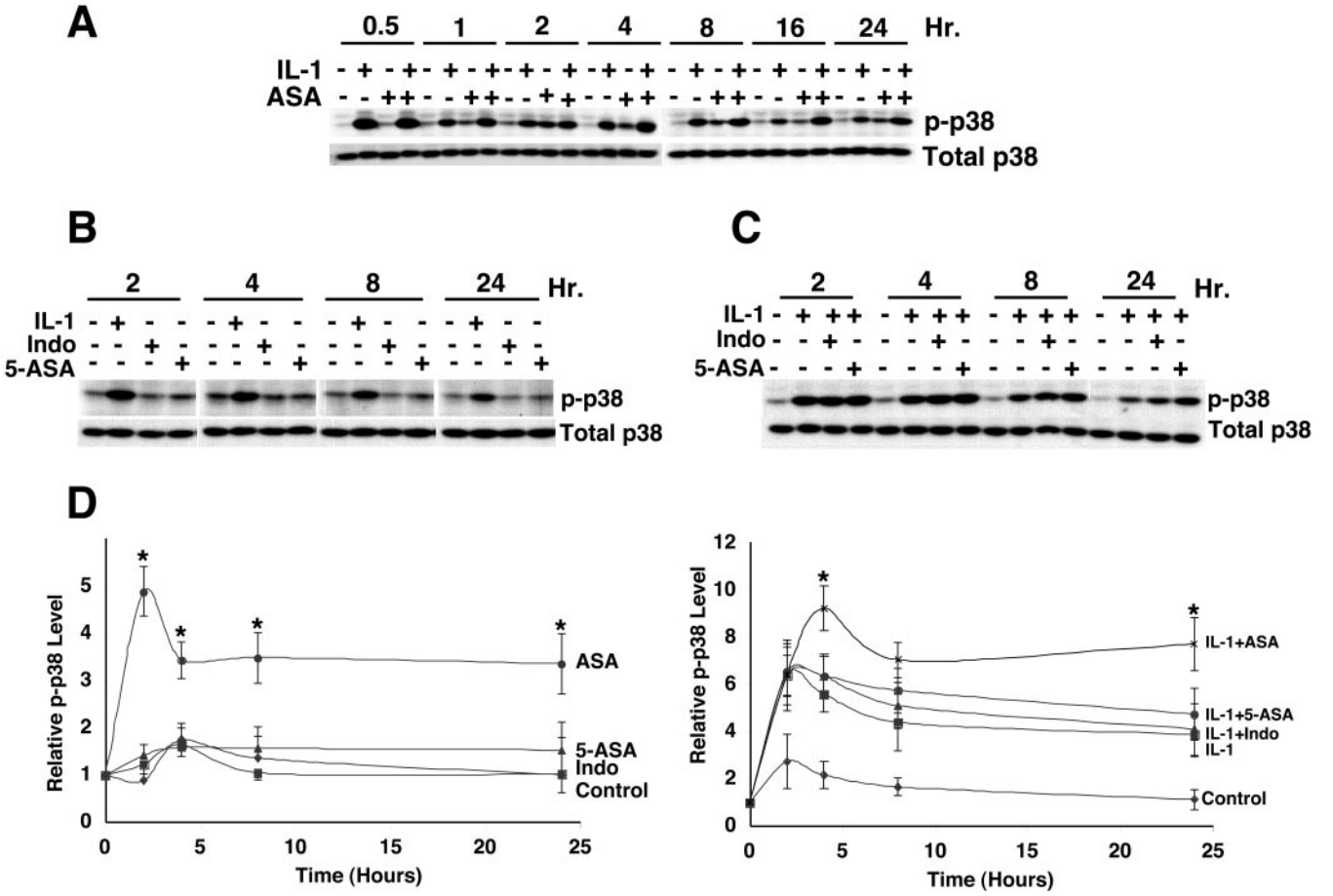

Fig. 4. Effect of IL-1 and aspirin upon p38 phosphorylation. Confluent 18Co monolayers were incubated for the indicated time periods in the presence of IL-1 (500 pg/ml), aspirin (5.0 mM), or both (A), IL-1 (500 pg/ml), indomethacin (Indo; 20 μM), or 5-ASA (5.0 mM) (B), or IL-1 in combination with either indomethacin or 5-ASA (C). Agents were delivered in serum-free medium. Cells were harvested and Western analysis was performed to determine levels of phosphorylated p38 (phospho-p38) and total p38 as described under *Materials and Methods*. D, densitometric data graphically depicting the p-p38/p38 ratio after each treatment as a function of time. *, $p < 0.05$ relative to samples not containing ASA.

ylation after 30 min in our studies, aspirin-induced p38 phosphorylation was more evident at later time points and continued for at least 24 h after treatment. Perhaps this is attributable to the lower dose of aspirin used in our studies, a higher level of basal p38 phosphorylation in our cells, or to slight differences in signal transduction pathways among different cell types. Schwenger et al. (1997, 1998) did point out, however, that increased p38 phosphorylation was observed as long as 8 h after salicylate treatment.

The time course of phospho-p38 accumulation in our studies suggests that aspirin might inhibit the phosphatase activity or activities that restore p38 activity to resting levels. For example, aspirin did not significantly enhance IL-1–mediated p38 phosphorylation (Fig. 4), yet the decay of the phosphorylated state was retarded in the presence of aspirin. Phosphatase inhibition has been shown to induce COX-2 expression in other cell types with kinetics similar to those seen with aspirin (Mahboubi et al., 1997; Miller et al., 1998). Alternatively, the rapid p38 phosphorylation observed by Schwenger and colleagues suggests that salicylates affect kinase activities responsible for p38 phosphorylation. Although the possibilities are not mutually exclusive, future studies will be needed to determine how salicylates modulate p38 activity.

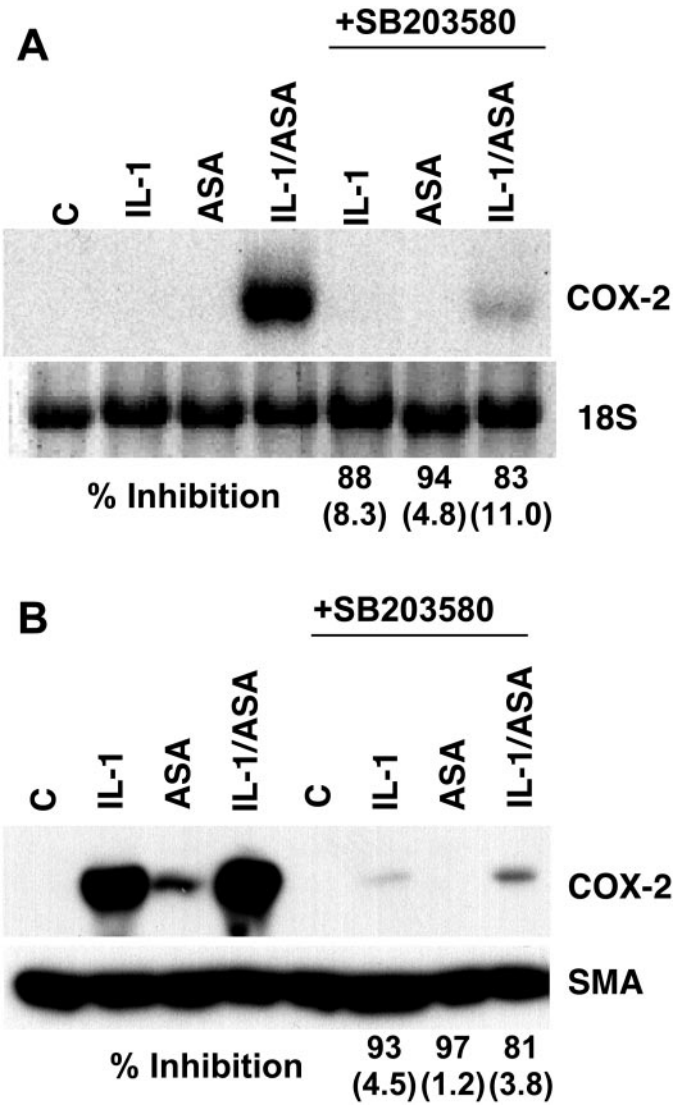

Fig. 5. Effect of p38 inhibition upon IL-1 and aspirin-mediated COX-2 induction. Confluent 18Co monolayers were incubated 24 h in the presence of either IL-1 (500 pg/ml), aspirin (5.0 mM), or both, with or without the p38 inhibitor SB-203580 (20 μM). Cells were then harvested and COX-2 mRNA (A) and protein (B) levels were determined by Northern and Western blotting, respectively, as described under *Materials and Methods*. Also shown in A is an inverse image of the 18S rRNA on the membrane before hybridization, demonstrating equivalent loading and transfer. B, the blot was stripped and reprobed with an antibody specific for α-smooth muscle actin (SMA) demonstrating equivalent loading and transfer. Below each panel is shown the percentage inhibition of COX-2 message or protein levels, with S.D. in parentheses, as quantitated by phosphorimager or densitometric analysis, respectively.

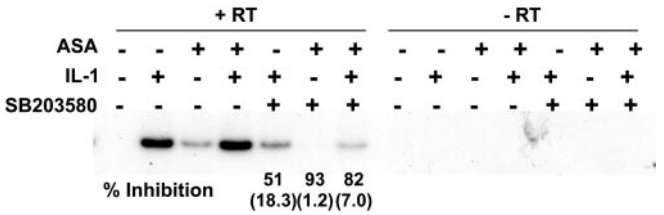

Fig. 6. Effect of p38 inhibition upon COX-2 transcription. Confluent 18Co monolayers were incubated in the presence of IL-1 (500 pg/ml), aspirin (5.0 mM), or IL-1 plus aspirin with or without the p38 inhibitor SB-203580 (20 μM). After 4 h, total RNA was extracted and relative COX-2 hnRNA levels were determined as described under *Materials and Methods* along with mock reactions, which included RNA but no reverse transcriptase (RT) using the primers spanning exon 9. The graph summarizes the results obtained from analysis of four independent determinations for each treatment. Below each panel is shown the percentage inhibition of COX-2 transcription rate, with S.D. in parentheses.

Presently, we can only speculate about the in vivo biological significance of the data presented. In the context of inflammation, it is interesting that although high-dose aspirin inhibits PG synthesis, it also leads to elevated COX-2 protein levels. Cessation of therapy could potentially lead to a burst of COX activity, resulting in a transient increase in PGs, thromboxanes, and prostacyclin. In the case of aspirin, increased levels of acetylated COX-2 throughout the treatment regimen would result in synthesis of bioactive 15-epi-lipoxins (Serhan, 1997; Vane et al., 1998). It is also very likely that salicylates would affect the expression of other genes subject to p38 regulation (Fig. 7) (Frevel et al., 2003). Of the salicylates surveyed, the only drug that did not affect COX expression is 5-ASA, a mainstay in the treatment of inflammatory bowel disease. 5-ASA inhibits COX activity, and serum and mucosal PG levels decline after 5-ASA treatment (Stenson and MacDermott, 1991). Patients with inflammatory bowel disease treated with other COX inhibitors likewise experience reduction in serum and mucosal PG levels, yet their clinical condition does not improve (Stenson and MacDermott, 1991) and can even be exacerbated (Evans et al., 1997). One explanation for this is that in addition to inhibition of PG synthesis, lipoxygenase inhibition by 5-ASA is the major benefit of this drug, distinguishing it from other NSAIDs (Sharon and Stenson, 1985). An alternate explanation suggested by this study is that nontherapeutic salicylates, via their ability to activate MAPK/SAPK pathways, may affect expression of other genes that exacerbate inflammation.

Acknowledgments

We are grateful to Drs. Timothy Hla and Stephen Prescott for providing plasmids used in this study. We thank Terri Kirschner for assistance in the preparation of this manuscript.

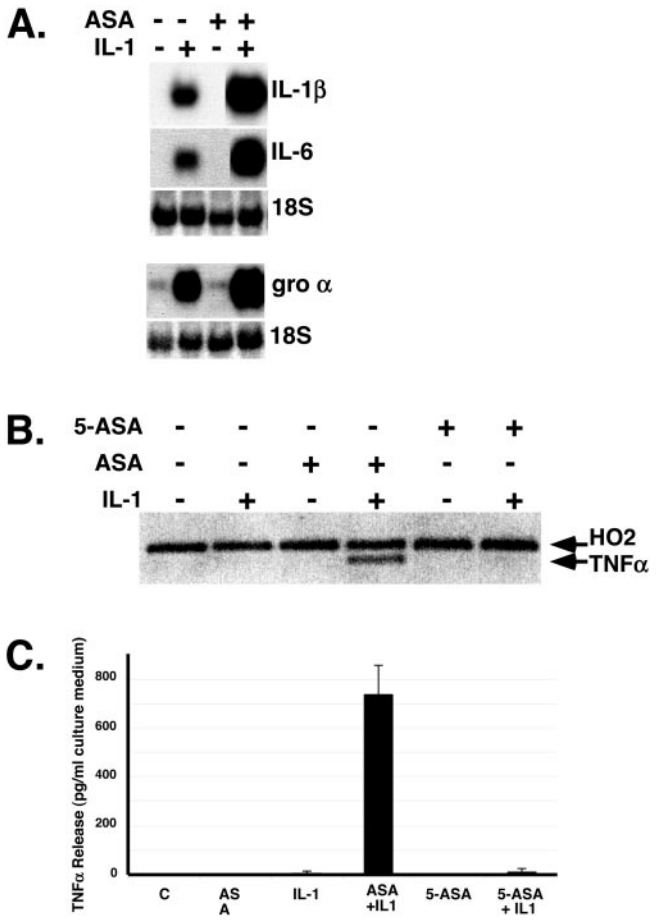

Fig. 7. Synergistic induction of other proinflammatory cytokine and chemokine mRNA and protein levels by IL-1 plus aspirin. A, IL-1β, IL-6, and groα mRNA levels. Confluent 18Co monolayers were incubated 24 h in the presence of either IL-1 (500 pg/ml), aspirin (5.0 mM), or both, and total RNA was analyzed by Northern blotting using specific probes for either IL-1β, IL-6, or groα. In the experiments shown, the same blot was used for both the IL-1 and -6 determinations. Below each is an inverse image of the 18S rRNA on the membranes before hybridization, demonstrating equivalent loading and transfer. B, TNFα mRNA detection. RNA samples isolated from cells treated as in A, with additional 5-ASA (5.0 mM) treatments were analyzed for the presence of TNFα mRNA using RT-PCR analysis. Also included in each reaction were primers for the constitutively expressed heme oxygenase 2 (HO2). Shown is an inverse image of a representative ethidium bromide-stained agarose gel demonstrating the presence of the constitutive 512 nucleotides of HO2 product in all lanes and the 414 nucleotide TNFα product only in the IL-1 plus ASA lane. C, TNFα secretion. Medium from confluent 18Co monolayers cultured 24 h in the presence of either IL-1 (500 pg/ml), ASA (5.0 mM), 5-ASA (5.0 mM), or combinations thereof were analyzed for TNFα levels by ELISA (limit of detection, ~15 pg/ml). The results from three separate experiments, each done in triplicate, are shown. Significant TNFα release was observed only in cells treated with the combination of IL-1 + ASA (735 $\pm$ 86 pg/ml).

References

Adegboyega PA, Mifflin RC, DiMari JF, Saada JI, and Powell DW (2002) Immunohistochemical study of myofibroblasts in normal colonic mucosa, hyperplastic polyps and adenomatous colorectal polyps. *Arch Pathol Lab Med* **126:**829–836.

Adegboyega PA, Mifflin RC, Saada JI, DiMari JF, and Powell DW (2001) COX-2 is localized to stromal cells in human colorectal adenomas (Abstract). *Gastroenterology* **120:**A162.

Brooks PM, Kean WF, and Buchanan WW (1986) *The Clinical Pharmacology of Anti-Inflammatory Agents*. Taylor and Francis, Philadelphia.

Cieslik K, Zhu Y, and Wu KK (2002) Salicylate suppresses macrophage nitric-oxide synthase-2 and cyclooxygenase-2 expression by inhibiting CCAAT/enhancer-binding protein-β binding via a common signaling pathway. *J Biol Chem* **277:** 49304–49310.

Cronstein BN, Montesinos MC, and Weissmann G (1999) Salicylates and sulfasalazine, but not glucocorticoids, inhibit leukocyte accumulation by an adenosine-dependent mechanism that is independent of inhibition of prostaglandin synthesis and p105 of NFkappab. *Proc Natl Acad Sci USA* **96:**6377–6381.

Davies NM, Sharkey KA, Asfaha S, Macnaughton WK, and Wallace JL (1997) Aspirin causes rapid up-regulation of cyclooxygenase-2 expression in the stomach of rats. *Aliment Pharmacol Ther* **11:**1101–1108.

Dean JLE, Brook M, Clark AR, and Saklatvala J (1999) P38 mitogen-activated protein kinase regulates cyclooxygenase-2 mRNA stability and transcription in lipopolysaccharide-treated human monocytes. *J Biol Chem* **274:**264–269.

Di Mari JD, Mifflin RC, Saada JI, Adegboyega PA, and Powell DW (2003) IL-1alpha-induced COX-2 expression in human intestinal myofibroblasts is dependent on a PKCzeta-ROS pathway. *Gastroenterology* **124:**1855–1865.

Dinarello CA (1996) Biologic basis for interleukin-1 in disease. *Blood* **87:**2095–2147.

Elferink CJ and Reiners JJ Jr. (1996) Quantitative RT-PCR on CYP1A1 heterogeneous nuclear RNA: a surrogate for the in vitro transcription run-on assay. *Biotechniques* **20:**470–477.

Evans JM, McMahon AD, Murray FE, McDevitt DG, and MacDonald TM (1997) Non-steroidal anti-inflammatory drugs are associated with emergency admission to hospital for colitis due to inflammatory bowel disease. *Gut* **40:**619–622.

Faour WH, He Y, He QW, de Ladurantaye M, Quintero M, Mancini A, and Di Battista JA (2001) Prostaglandin E_2 regulates the level and stability of cyclooxygenase-2 mRNA through activation of p38 mitogen-activated protein kinase in interleukin-1β-treated human synovial fibroblasts. *J Biol Chem* **276:**31720–31731.

Frevel MAE, Bakheet T, Silva AM, Hissong JG, Khabar KSA, and Williams BRG (2003) P38 mitogen-activated protein kinase-dependent and -independent signaling of mRNA stability of AU-rich element-containing transcripts. *Mol Cell Biol* **23:**425–436.

Furst DE, Blocka K, Cassell S, Dromgoole S, Harris ER, Hirschberg JM, Josephson N, Rupp PA, Paulus HE, and Trimble RB (1987) A strategy for reaching therapeutic salicylate levels in patients with rheumatoid arthritis using standardized dosing regimens. *J Rheumatol* **14:**342–347.

Guan Z, Baier LD, and Morrison AR (1997) P38 mitogen-activated protein kinase down-regulates nitric oxide and up-regulates prostaglandin E2 biosynthesis stimulated by interleukin-1-β. *J Biol Chem* **272:**8083–8089.

Hinterleitner TA, Saada JI, Berschneider HM, Powell DW, and Valentich JD (1996) IL-1 stimulates intestinal myofibroblast cox gene expression and augments activation of Cl- secretion in T84 cells. *Am J Physiol* **271:**C1262–C1268.

Kopp E and Ghosh S (1994) Inhibition of NF-kappa-β by sodium salicylate and aspirin. *Science (Wash DC)* **265:**956–959.

Lasa M, Brook M, Saklatvala J, and Clark AR (2001) Dexamethasone destabilizes cyclooxygenase 2 mRNA by inhibiting mitogen-activated protein kinase p38. *Mol Cell Biol* **21:**771–780.

Lasa M, Mahtani KR, Finch A, Brewer G, Saklatvala J, and Clark AR (2000) Regulation of cyclooxygenase 2 mRNA stability by the mitogen-activated protein kinase p38 signaling cascade. *Mol Cell Biol* **20:**4265–4274.

Mahboubi K, Young W, and Ferreri NR (1997) Induction of prostaglandin endoperoxide synthase-2 by serine-threonine phosphatase inhibition. *J Pharmacol Exp Ther* **282:**452–458.

Marnett LJ and DuBois RN (2002) COX-2: a target for colon cancer prevention. *Annu Rev Pharmacol Toxicol* **42:**55–80.

Mifflin RC, Saada JI, Di Mari JF, Adegboyega PA, Valentich JD, and Powell DW (2002) Regulation of COX-2 expression in human intestinal myofibroblasts: mechanisms of IL-1-mediated induction. *Am J Physiol* **282:**C824–C834.

Miller C, Zhang M, He Y, Zhao J, Pelletier JP, Martel-Pelletier J, and Di Battista JA (1998) Transcriptional induction of cyclooxygenase-2 gene by okadaic acid inhibition of phosphatase activity in human chondrocytes: co-stimulation of AP-1 and CRE nuclear binding proteins. *J Cell Biochem* **69:**392–413.

Pillinger MH, Capodici C, Rosenthal P, Kheterpal N, Hanft S, Philips MR, and Weissmann G (1998) Modes of action of aspirin-like drugs: salicylates inhibit ERK activation and integrin-dependent neutrophil adhesion. *Proc Natl Acad Sci USA* **95:**14540–14545.

Powell DW, Mifflin RC, Valentich JD, Crowe SE, Saada JI, and West AB. (1999) Myofibroblasts. II. Intestinal subepithelial myofibroblasts. *Am J Physiol* **277:** C183–C201

Rainsford KD (1984) *Aspirin and the Salicylates*. Butterworth and Co., London.

Ridley SH, Dean JL, Sarsfield SJ, Brook M, Clark AR, and Saklatvala J (1998) A p38 MAP kinase inhibitor regulates stability of interleukin-1-induced cyclooxygenase-2 mRNA. *FEBS Lett* **439:**75–80.

Schwenger P, Alpert D, Skolnik EY, and Vilcek J (1998) Activation of p38 mitogen-activated protein kinase by sodium salicylate leads to inhibition of tumor necrosis factor-induced I-kappa-b alpha phosphorylation and degradation. *Mol Cell Biol* **18:**78–84.

Schwenger P, Alpert D, Skolnik EY, and Vilcek J (1999) Cell-type-specific activation of c-Jun N-terminal kinase by salicylates. *J Cell Physiol* **179:**109–114.

Schwenger P, Bellosta P, Vietor I, Basilico C, Skolnik EY, and Vilcek J (1997) Sodium salicylate induces apoptosis via p38 mitogen-activated protein kinase but inhibits tumor necrosis factor-induced c-Jun N-terminal kinase/stress-activated protein kinase activation. *Proc Natl Acad Sci USA* **94:**2869–2873.

Serhan CN (1997) Lipoxins and novel aspirin-triggered 15-epi-lipoxins: a jungle of cell-cell interactions or a therapeutic opportunity? *Prostaglandins* **53:**107–137.

Sharon P and Stenson WF (1985) Metabolism of arachidonic acid in acetic acid colitis in rats. Similarity to human inflammatory bowel disease. *Gastroenterology* **88:**55–63.

Shattuck-Brandt RL, Varilek GW, Radhika A, Yang F, Washington MK, and DuBois RN (2000) Cyclooxygenase 2 expression is increased in the stroma of colon carcinomas from IL-10(−/−) mice. *Gastroenterology* **118:**337–345.

Sonoshita M, Takaku K, Oshima M, Sugihara K, and Taketo MM (2002) Cyclooxygenase-2 expression in fibroblasts and endothelial cells of intestinal polyps. *Cancer Res* **62:**6846–6849.

Stenson WF and MacDermott RP (1991) Inflammatory bowel disease, in *Textbook of*

Gastroenterology (Yamada T, Alpers DH, Owyang D, Powell DW, Silverstein FE eds) pp 1588–1644, Lippincott, Philadelphia.
Turini ME and DuBois RN (2002) Cyclooxygenase-2: a therapeutic target. *Annu Rev Med* **53:**35–57.
Valentich JD, Popov V, Saada JI, and Powell DW (1997) Phenotypic characterization of an intestinal subepithelial myofibroblast cell line. *Am J Physiol* **272:**C1513–C1524.
Vane JR, Bakhle YS, and Botting RM (1998) Cyclooxygenases 1 and 2. *Annu Rev Pharmacol Toxicol* **38:**97–120.
Xu XM, Sansores-Garcia L, Chen XM, Matijevic-Aleksic N, Du M, and Wu KK (1999) Suppression of inducible cyclooxygenase 2 gene transcription by aspirin and sodium salicylate. *Proc Natl Acad Sci USA* **96:**5292–5297.
Yin MJ, Yamamoto Y, and Gaynor RB (1998) The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)b kinase-beta. *Nature (Lond)* **396:**77–80.

Address correspondence to: Randy C Mifflin, Department of Internal Medicine, Division of Gastroenterology, University of Texas Medical Branch, Galveston, TX 77555-1058. E-mail rmifflin@utmb.edu