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Cyclooxygenase-2 Expression by Nonsteroidal Anti-inflammatory Drugs in Human Airway Smooth Muscle Cells: Role of Peroxisome Proliferator-Activated Receptors

Linhua Pang, Mei Nie, Lisa Corbett, and Alan J. Knox

Nonsteroidal anti-inflammatory drugs (NSAIDs) have been shown to modulate cyclooxygenase (COX)-2 expression, but the mechanisms involved are controversial and may be cell specific. We show in this study that indomethacin (Indo), flurbiprofen (Flur), and the selective COX-2 inhibitor NS-398 induced COX-2 expression and markedly enhanced IL-1β-induced COX-2 expression in human airway smooth muscle (HASM) cells. These effects were not reversed by exogenous PGE2, suggesting that they are prostanooid-independent. Indeed, PGE2 also induced and enhanced IL-1β-induced COX-2 expression. Peroxisome proliferator-activated receptor (PPAR) α and PPARγ (not PPARβ) were expressed in HASM cells. PPARγ activators ciglitizone (Cig) and 15-Deoxy-Δ12,14-PGJ2 (15d-PGJ2), but not the PPARα activator WY-14643, mimicked the effect of NSAIDs on COX-2 expression. Treatment with Flur, NS-398, Cig, and 15d-PGJ2 alone, but not Indo and WY-14643, elevated COX activity; however, neither enhanced IL-1β-induced COX activity. Pretreatment with dexamethasone suppressed COX-2 expression, PGE2 release, and COX activity induced by NS-398, Cig, IL-1β, alone or in combination. Unlike IL-1β, NS-398 and Cig did not cause NF-κB (p65) nuclear translocation, nor did they further enhance IL-1β-induced NF-κB translocation, but they stimulated PPARγ translocation. Indo, NS-398, Flur, and 15d-PGJ2, but not WY-14643, induced transcriptional activity of a COX-2 reporter construct containing the peroxisome proliferator response element (PPRE) on their own and enhanced the effect of IL-1β, but had no effect on a COX-2 reporter construct lacking the PPRE. The results suggest that COX-2 expression by NSAIDs is biologically functional, prostanooid-independent, and involves PPARγ activation, and provide the first direct evidence that the PPRE in the promoter is required for NSAID-induced COX-2 expression. The Journal of Immunology, 2003, 170: 1043–1051.

Peroxisome proliferator-activated receptors (PPARs) (4–7). PPARα, predominantly localized to the liver, orchestrates β-oxidation of fatty acids. PPARγ, predominantly expressed in adipose tissue, regulates adipocyte differentiation; the physiological function of PPARβ is unclear (8). PPARγ are also expressed in other cells (5, 9), but their expression and functions in airways is unknown. PPARs are activated by a heterogeneous group of structurally dissimilar chemicals and the selectivity is activator concentration- and cell type-dependent (8). The PGD2 metabolite 15-deoxy-Δ12,14-PGJ2 (15d-PGJ2) is a direct-binding ligand for PPARγ (10). Other PPAR activators include WY-14643 (selective for PPARα), several NSAIDs, anti-diabetic drugs (thiazolidinediones), and fatty acids (e.g., AA and other eicosanoids) (8). PPARs have complex regulatory effects on inflammatory responses. PPARα-deficient mice show a prolonged inflammatory response (11), whereas PPARγ activation inhibits inducible NO synthase, TNF-α, IL-6, and IL-1β expression (9, 12) and prevents matrix metalloproteinase induction (13) in stimulated monocytes/macrophages.

The effect of NSAIDs on COX-2 expression and the role of PPARs in this process is controversial. Meade et al. (4) demonstrated that COX inhibitors (NSAIDs), substrate (AA), and products (PGD2, 15d-PGJ2, and PGF2α) induce COX-2 expression transcriptionally in epithelial cells through PPARs, whereas Staels et al. (5) found that PPARα activation by WY-14643 suppresses IL-1-induced COX-2 expression in human aortic smooth muscle cells. It has also been shown that NSAIDs induce COX-2 expression but inhibit mitogen-induced COX-2 expression in the colon cancer cell line (HT-29) and macrophage cell line (RAW 264.7) (6), and that COX-2 expression is regulated by a negative feedback loop mediated through PPARγ in the macrophage-like-differentiated U937 cells (7). Therefore, COX-2 expression is likely to be

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regulated in a cell-specific manner by different PPAR activators.

However, no studies have shown that the peroxisome proliferator response element (PPRE) in the promoter region is required for COX-2 expression by NSAIDs.

We and others have shown that human airway smooth muscle (HASM) cells have important synthetic functions. In addition to the synthesis of cytokines/chemokines (14-16) and growth factors (17, 18), HASM cells express COX-2 and release large quantities of prostanooids (mainly PGE2) upon stimulation by proinflammatory cytokines and other mediators (19-21). PGE2 produced from COX-2 inhibits cell proliferation (22), mediates IL-1β- and bradykinin-induced attenuation of cAMP generation in response to β2-adrenoceptor agonists (23, 24), and acts as an autocrine regulator of IL-8 and vascular endothelial growth factor release (15, 18), suggesting COX-2 induction modulates airway smooth muscle function (25). In terms of the effect of NSAIDs on COX-2 expression, Bonazzi et al. (26) showed that COX inhibitor flurbiprofen (Flur) reduced IL-1β-induced COX-2 expression in HASM cells but did not perform mechanistic studies.

To investigate the regulation of COX-2 expression and function by NSAIDs in HASM cells and the role of PPAR activators in this process, we studied the effect of NSAIDs on COX-2 expression in the presence or absence of IL-1β, a COX-2 inducer, and compared the effect of NSAIDs with other PPAR activators. We show in this study that NSAIDs induce COX-2 expression and enhance IL-1β-induced COX-2 expression via a prostanoid-independent mechanism involving PPARγ, but not NF-κB, activation. We also show for the first time that deletion of the PPRE in the COX-2 promoter abolishes the effect of NSAIDs. Paradoxically, PGE2, the major COX product of these cells, also induces COX-2.

Materials and Methods

DMEM, penicillin and streptomycin, t-glutamine, amphotericin B, PGE2, SDS, PMSF, Triton X-100, glycerol, acrylamide/bis-acrylamide, Tris, EDTA, leupeptin, pepstatin, 2-ME, MT, EGTA, polyclonal rabbit antiserum, IgG coupled with HRP, polyclonal goat anti-rabbit IgG with HRP, anti-PGE2 serum, ciglitzone (Cig), indomethacin (Indo), Flur, AA, dexamethasone (Dex), and other unspecified chemicals were all purchased from Sigma-Aldrich (Poole, U.K.). FCS was purchased from Sera-Lab (Loughborough, U.K.). Recombinant human IL-1β was from R&D Systems (Minneapolis, MN). NS-398, 5004634, and 15d-PGJ2 were from Calbiochem-Novabiochem (Nottingham, U.K.). Anti-human COX-2 and COX-2 Abs were from Cayman Chemical (Ann Arbor, MI). Abs against PPARα, β(0), γ NF-κB (p65), and IκBα were from Santa Cruz Biotechnology (Santa Cruz, CA); [5, 6, 8, 11, 12, 14, 15(n)-3H]PGE2, rainbow-colored protein m.w. markers, ECL Western blotting detection reagent, and Hyperfilm-ECL were from Amersham Pharmacia Biotech (Little Chalfont, U.K.). Pure nitrocellulose membrane was from Gelman Sciences (Northampton, U.K.). The Bio-Rad protein assay reagent was from Bio-Rad (Hemel Hempstead, U.K.). FuGene 6 transfection reagent was from Roche (East Sussex, U.K.). The dual-luciferase reporter assay system was from Promega (Southampton, U.K.).

Cell culture

Primary cultures of HASM cells were prepared from explants of HASM as previously reported (19, 20). This protocol was approved by the Nottingham City Hospital Research Ethics Committee. Cells at passages 5 and 6 were used for all experiments. We have previously shown that the cells grown in this manner depict the immunohistochemical and light microscopic characteristics of typical HASM cells (19). HASM cells were also obtained from BioWhittaker (Wokingham, U.K.) and were used at passage 6.

Experiment protocols

The cells were cultured to confluence in DMEM supplemented with 10% FCS, the antibiotics penicillin (100 U/ml) and streptomycin (100 μg/ml), the antifungal amphotericin B (2.5 μg/ml), and t-glutamine (4 mM) in humidified 5% CO2/95% air at 37°C and growth-arrested in serum-free medium for 24 h before experiments. Immediately before each experiment, fresh serum-free medium was added. To test the effect of NSAIDs and PPAR activators on their own, cells were treated with the drugs for 4 h in most cases or for the times indicated in the time-course experiments. To test the effect of NSAIDs and PPAR activators on IL-1β-induced COX-2 expression, PGE2, release, and COX activity, they were added 30 min before the incubation with IL-1β. The inhibitory effect of Dex was assessed by preincubating the cells with Dex for 30 min before the incubation with NSAIDs, PPAR activators, and IL-1β, alone or in combination, for a further 4 h. All the reagents were dissolved in DMSO (final concentration ≤0.4% v/v). In all the experiments, a group of control cells were incubated with the drug vehicle for the same period of time as the experimental cells.

PGE2 assay and COX activity

After experiments, the culture media in 24-well plates were collected and stored at −20°C until the determination of PGE2 content by RIA as described previously (19, 20). The sensitivity for PGE2 was 75 pg/ml. The anti-PGE2 antiserum had negligible cross-reactivity with other prostanooids (19) except 15d-PGJ2. COX activity was assayed functionally by washing the cells three times in PBS after the experiments and then incubating with AA (15 μM) for a further 15 min. These samples were subjected to RIA for PGE2, and the resulting PGE2 level was taken as an index of COX activity.

Preparation of whole-cell lysate

After treatment, cells in 24-well plates were washed twice with ice-cold PBS and incubated for 5 min with an extraction buffer (0.9% NaCl, 20 mM Tris-HCl, pH 7.6, 0.1% Triton X-100, 1 mM Na3PO4, 0.01% leupeptin) with gentle shaking. The samples were collected and centrifuged, and the protein concentration in the supernatant was determined using the Bio-Rad reagent.

Preparation of cytosolic and nuclear proteins

Nuclear and cytosolic extracts from the cells were prepared as described by Eickelberg et al. (27) with minor changes. After treatment, cells in 90-mm dishes were washed twice with ice-cold PBS and harvested in 1 ml of PBS with a cell scraper. The samples were centrifuged for 2 min at 1000 g, and cell pellets were resuspended in 200 µl of cold salt buffer (20 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM Na3PO4, 1 mM EDTA, 1 mM EGTA, 0.2% IGEPLA CA-630, 10% glycerol, 1 mM PMSF, and 0.01% leupeptin). After 10 min of incubation on ice, the samples were centrifuged at 7000 × g for 30 min (4°C), and the supernatants were taken as cytosolic extracts. Nuclei were then resuspended in 100 µl of high salt buffer (as low salt buffer but with 420 mM NaCl and 20% glycerol), and nuclear proteins were extracted by shaking on ice for 30 min. Samples were then centrifuged at 7000 × g for 30 min (4°C), and the supernatants were taken as nuclear extracts.

Western blot analysis

Identification of the interested proteins were performed by Western blotting analysis as described before (19, 20). Briefly, protein samples (30 μg/tube) were subjected to electrophoresis in 7.5% SDS-polyacrylamide gel. Separated proteins were electroblotted to pure nitrocellulose membranes and the blot was blocked for 2 h at room temperature with blocking buffer (wash buffer with 8% fat-free dried milk powder). The blot was then incubated with monoclonal anti-COX-2 Ab (1/2000 dilution with blocking buffer), or polyclonal Abs against PPARα, β(0), γ, NF-κB (p65), or IκBα (1/1000 dilution in blocking buffer) for 2 h, washed with wash buffer (PBS pH 7.4 with 0.3% Tween 20), and incubated with HRP-conjugated secondary Abs (1/2000 dilution with blocking buffer) for 1 h. The blot was washed again and then incubated with ECL Western blotting detection reagent for 1 min and finally exposed to Hyperfilm-ECL.

Plasmids

The 3.9-kb COX-2 firefly luciferase reporter construct (3.9-kb COX-2-Luc) in pGL3 vectors containing the putative PPRE sequence located between −3721 to −3707 (AGCGGAGACAGTGTCA) upstream of the starting point of the COX-2 gene and the 3.5-kb COX-2 firefly luciferase reporter construct (3.5-kb COX-2-Luc) lacking the PPRE were kindly provided by T. McIntyre (University of Utah, Salt Lake City, UT) and have been previously described in detail (4, 28). The internal Renilla luciferase control vector pRL-SV40 was purchased from Promega.

Transfection of HASM cells and reporter assays

All transient transfections were performed using FuGene 6 (1 μg DNA:3 μl FuGene 6) according to the manufacturer’s recommended protocol.
Results

NSAIDs enhance COX-2 expression by a prostanoid-independent mechanism

Among various NSAIDs we tested before, the nonselective COX inhibitor Indo and the selective COX-2 inhibitor NS-398 block IL-1β-induced COX-2 activity in HASM cells (19). Thus, we focused our study on these two NSAIDs. As previously demonstrated (19), IL-1β treatment (1 ng/ml, 4 h) induced a strong COX-2 protein expression in HASM cells. This was markedly enhanced by pretreatment with Indo (1–100 μM), NS-398 (1–100 μM), and another nonselective COX inhibitor Flur (10 μM) (Fig. 1A). Indo and NS-398 at 10 μM also time-dependently enhanced IL-1β-induced COX-2 expression (Fig. 1B), but COX-1 protein expression was not altered (data not shown). Because the concentrations of 1 and 10 μM of Indo and NS-398 are those required to block IL-1β-induced PGE2 synthesis in these cells (19), it is possible that the enhanced COX-2 expression is a consequence of PGE2 synthesis inhibition. If that is the case, the addition of exogenous PGE2 would reverse the enhancing effect of both Indo and NS-398, as PGE2 is the dominant prostanoid product by these cells (19). However, contrary to this hypothesis, PGE2 pretreatment enhanced IL-1β-induced COX-2 expression in a concentration-dependent manner and did not reverse the effect of NSAIDs (Fig. 1C), suggesting that the effect of NSAIDs on IL-1β-induced COX-2 expression is independent of their inhibition of the enzyme activity. We then examined if these NSAIDs could cause COX-2 expression on their own and we found that treatment with Indo and NS-398 (1 and 10 μM) for 4 h induced COX-2 expression compared with the control (Fig. 2A), and 10 μM of both Indo and NS-398 also caused COX-2 expression in a time-dependent manner, which appeared at 1 h, peaked at ~2–8 h after incubation, and declined thereafter (Fig. 2B). Flur and exogenous PGE2 (10 μM, 4 h) also induced COX-2 expression as shown in Fig. 2C.

PPARγ activators also enhance COX-2 expression

Because PPAR activators enhance COX-2 expression and a large number of NSAIDs have recently been identified as PPAR activators (4, 11), we studied the expression of PPARs in HASM cells.

Materials and Methods

Statistical analysis

Results were expressed as the mean ± SEM of n determinations from HASM cells obtained from two donors. Student two-tailed t tests were used to determine the significant differences between the means. Values of p < 0.05 were accepted as statistically significant.
We found that PPARα and PPARγ, but not PPARβ, were constitutively expressed in HASM cells and that treatment with NS-398, the selective PPARα activator WY-14643, or the selective PPARγ activator Cig for 4 and 24 h did not alter the expression (Fig. 3A). Similar results were also obtained with other NSAIDs, the selective PPARγ activator 15d-PGJ2, and IL-1β (data not shown). We then examined if PPAR activators could mimic the effect of NSAIDs on COX-2 expression and found that Cig and 15d-PGJ2 (1 and 10 μM, 4 h), but not WY-14643, caused COX-2 expression on their own (Fig. 3B) and that pretreatment with Cig and 15d-PGJ2, but not WY-14643, resulted in a similar enhancing effect on IL-1β-induced COX-2 expression (Fig. 3C) as that of NSAIDs.

**COX-2 expression induced by NSAIDs and PPAR activators is accompanied by increase in PGE2 synthesis and COX activity**

We have previously demonstrated that COX-2 expression by IL-1β is associated with increase in PGE2 synthesis and COX activity in HASM cells (19); thus, we tested if COX-2 expression induced by NSAIDs and PPAR activators was biologically functional. After 4 h of incubation, HASM cells released low levels of PGE2 in unstimulated conditions, which was significantly inhibited by Indo (10 μM, p < 0.01), not affected by NS-398 and WY-14643, but enhanced by Cig (10 μM, p < 0.01) (Fig. 4A), suggesting that the basal level PGE2 release is the result of COX-1 activity, whereas enhanced PGE2 release from Cig-treated cells is the result of COX-2 expression. Although both Indo and NS-398 caused COX-2 induction, PGE2 release was not increased due to their inhibition of the induced enzyme. The effect of another PPAR activator, 15d-PGJ2, on PGE2 release could not be interpreted, as 15d-PGJ2 had cross-reaction with the anti-PGE2 serum we used for RIA. Treatment with IL-1β (1 ng/ml, 4 h) caused a marked increase in PGE2 release, which was significantly reduced by Indo (p < 0.001) and NS-398 (p < 0.01), increased by Cig (p < 0.01), and not affected by WY-14643 (Fig. 4A), suggesting that the enhanced COX-2 expression by Cig is biologically functional. Again, as would be expected, due to the inhibition of the enzyme by the two NSAIDs, no increase on PGE2 release from the cells pretreated with Indo and NS-398 was observed despite the fact that both enhanced IL-1β-induced COX-2 expression (Fig. 1, A and B).

To assess the function of induced COX-2 more accurately, after treatment with test drugs the cells were washed with PBS and incubated with 15 μM of the COX substrate AA for a further 15

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**FIGURE 3.** Effect of NS-398 and PPAR activators on PPAR expression (A), COX-2 expression (B), and IL-1β-induced COX-2 expression (C). HASM cells were treated with or without NS-398, WY-14643, or Cig for 4 and 24 h (A), or with or without WY-14643, Cig, or 15d-PGJ2 for 4 h (B), or pretreated with or without WY-14643, Cig, or 15d-PGJ2 for 30 min before the incubation with or without IL-1β for a further 4 h (C). Cell lysates were prepared and PPAR and COX-2 expression was analyzed by Western blotting as described under Materials and Methods. These blots are representative of similar results obtained three times.

**FIGURE 4.** Effect of NSAIDs and PPAR activators on PGE2 release (A) and COX activity (B and C) in the absence or presence of IL-1β. HASM cells were treated with or without Indo, NS-398, WY-14643, Cig, 15d-PGJ2, or Flur (all 10 μM) for 30 min before the incubation with or without IL-1β (1 ng/ml) for a further 4 h. PGE2 was measured by RIA and COX activity was determined by measuring PGE2 production from exogenous AA as described under Materials and Methods. Each point represents the mean ± SEM of nine determinations from three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 compared with control. + + +, p < 0.01; ++ +, p < 0.001 compared with IL-1β alone.
increased accordingly. The enzyme activity in the context of this study is not affected by NS-398, WY-14643, Cig, and 15d-PGJ2 (Fig. 4C). These results indicate that the COX-2 induced by NS-398, Flur, and the PPARγ activators alone is biologically functional and that even though NSAIDs and PPARγ activators enhance IL-1β-induced COX-2 expression, the enzyme activity in the context of this study is not increased accordingly.

Dex inhibits the induced COX-2 expression, PGE2 release, and COX activity

We have shown before that IL-1β-induced COX-2 expression, PGE2 release, and COX activity are inhibited by Dex (19), an COX-2 transcription inhibitor. In this study, we further investigated if the effects of NSAIDs and PPAR activators on COX-2 could be inhibited by Dex. As shown in Fig. 5A, COX-2 expression (4 h) by NS-398 (10 μM), Cig (10 μM), and IL-1β (1 ng/ml), individually or in combination, was strongly suppressed by Dex (1 μM). Dex also significantly inhibited the enhanced PGE2 release caused by IL-1β, Cig, and their combination (Fig. 5B, p < 0.01, p < 0.05, p < 0.001, respectively) and the enhanced COX activity caused by IL-1β, NS-398, Cig, and their combinations (Fig. 5C, p < 0.01 for all). Similar results were also observed with other Indo and Flur (data not shown).

NS-398 and Cig do not cause or enhance NF-κB translocation, but cause PPARγ translocation

As the transcriptional factor NF-κB had been shown to be critically involved in COX-2 expression (29), we went on to examine whether NF-κB activation was involved in the COX-2 expression by NS-398 and Cig. After treatment with either drug for 30 min, a slight reduction of IκBα in the cytosol was observed but NF-κB levels in both cytosol and nucleus remained unchanged (Fig. 6A). In contrast, IL-1β caused a clear reduction of both IκBα and NF-κB in the cytosol and a marked increase of NF-κB in the nucleus. However, cotreatment of either NS-398 or Cig with IL-1β did not alter the effect of IL-1β (Fig. 6A). The results suggest that NS-398 and Cig have no direct effect on NF-κB activation in HASM cells and that COX-2 expression by these two drugs is likely via a mechanism different from that of IL-1β. A possible mechanism would be the activation of PPARs, which then interact with other transcriptional factors, including NF-κB, to regulate

FIGURE 5. Effect of Dex on COX-2 expression (A), PGE2 release (B), and COX activity (C) by NS-398, Cig, and IL-1β. HASM cells were pretreated with or without Dex for 30 min before the incubation with or without NS-398, Cig, or IL-1β, alone or in combination, for a further 4 h. Cell lysates were prepared and COX-2 expression was analyzed by Western blotting. PGE2 was measured by RIA and COX activity was determined by measuring PGE2 production from exogenous AA as described under Materials and Methods. The blots in A are representative of similar results obtained three times. Each point in B and C represents the mean ± SEM of six determinations from two independent experiments: *, p < 0.05; **, p < 0.01; ***, p < 0.001 compared with corresponding treatment without Dex.

FIGURE 6. Effect of NS-398, Cig, and IL-1β on IκBα degradation, NF-κB translocation (A), and PPAR translocation (B). HASM cells were treated with or without NS-398, Cig, or IL-1β, alone or in combination, for 30 min. Cytosolic and nuclear proteins were prepared and the expression of IκBα, NF-κB, PPARγ, and PPARα was analyzed as described under Materials and Methods. These blots are representative of similar results obtained two to three times.
NSAIDs and 15d-PGJ2 induce transcriptional activation of COX-2 promoter via PPRE

The 5′-regulatory region of human COX-2 gene contains a distal PPRE (4, 28) that interacts with PPARs. To further assess the role of PPAR activation in COX-2 expression by IL-1β, NSAIDs, and PPARγ activators, two COX-2 firefly luciferase reporter constructs were used to transfect the cells. The 3.9-kb COX-2 construct contains the PPRE (−3721 to −3707) in addition to the proximal regulatory elements required for induction by cytokines (30, 31) such as NF-κB (−223 to −214), NF-IL-6 (−132 to −124), and cAMP response element (CRE) (−59 to −53), whereas the 3.5-kb COX-2 construct lacks the PPRE but retains the proximal regulatory elements (4, 28). After cotransfection with 3.9-kb COX-2-Luc and the internal control vector pRL-SV40, relative luciferase activity was significantly induced by 10 μM of Indo (p < 0.01), NS-398 (p < 0.01), Flur (p < 0.05), and 15d-PGJ2 (p < 0.001) alone, but not by WY-14643 (Fig. 7A); IL-1β (1 ng/ml) alone also markedly increased COX-2 promoter activity and the effect was further enhanced by Indo, NS-398, Flur (p < 0.01), and 15d-PGJ2 (p < 0.001), but not by WY-14643 (Fig. 7A). In contrast, the 3.5-kb COX-2-Luc lacking the PPRE was unresponsive to these NSAIDs and 15d-PGJ2, but was still fully induced by IL-1β (Fig. 7B). These results suggest that NSAIDs and PPARγ activators induce COX-2 expression via transcriptional regulation of the COX-2 gene and that it is the presence of PPRE in the distal −3.5-kb to −3.9-kb region that confers responsiveness to these NSAIDs and 15d-PGJ2.

Cell viability

Cell viability after treatment with the chemicals used in this study was consistently >95% compared with cells treated with the vehicle.

Discussion

The major findings of this study are that NSAIDs induce the expression of biologically functional COX-2 and enhance IL-1β-induced COX-2 expression in a prostanoid-independent manner and that the effect is mimicked by PPARγ, but not PPARα, activators. Unlike IL-1β, NSAIDs and PPARγ activators do not activate NF-κB. The fact that NSAIDs stimulated the activity of a COX-2 promoter construct which was not seen when the PPRE was deleted provides the strongest evidence yet that NSAIDs induce COX-2 via PPAR activation. This study is the first to implicate PPARs in regulating any function of HASM cells.

NSAIDs can regulate COX-2 expression, but studies are conflicting. For instance, meclofenamate, mefenamic acid, ibuprofen, NS-398, and sulindac acid enhance COX-2 expression in mammary epithelial cells (4); aspirin and sodium salicylate suppress IL-1β-induced COX-2 expression in endothelial cells (32); flufenamic acid induces COX-2 expression but inhibits mitogen-induced COX-2 expression in a colon cancer cell line and macrophages (6). These data suggest that COX-2 expression is regulated differently by NSAIDs in different cell types. In this study, we report that Indo, NS-398, and Flur all induce COX-2 expression and enhance IL-1β-induced COX-2 expression independently of prostanoid synthesis inhibition. Our results differ from those of Bonazzi et al. (26) in HASM cells, who demonstrate that Flur inhibits IL-1β-induced COX-2 expression as a result of PGE2 synthesis inhibition; however, they did not study the effect of NSAIDs alone on COX-2 expression. The reasons for the discrepancy are unclear. One possible explanation could be different experimental and culture conditions. In their experiments, the cells were treated at the same time with Flur and IL-1β, whereas in ours the cells were treated with NSAIDs for 30 min before IL-1β stimulation, allowing NSAIDs to exert their effects before IL-1β. This is supported by the fact that COX-2 expression by NSAIDs occurs faster than that by IL-1β (Figs. 18 and 2B). Alternatively, Flur may have a different effect on COX-2 expression in different cells since the
same authors also report that Flur, like Indo, significantly increases COX-2 expression in corneal epithelial cells (33), and different culture conditions may have an effect on HASM cell PPAR expression and consequently their response to NSAIDS.

The mechanisms of COX-2 regulation by NSAIDs vary between NSAIDs and cell type. Because the main effect of NSAIDs is believed to be COX inhibition, it is reasonable to speculate that COX-2 regulation by NSAIDs is via their inhibition on COX activity, whose end products exert a feedback on COX-2 expression. For instance, Indo up-regulates endotoxin-induced COX-2 expression by removing the negative feedback of prostanoids in J774 macrophages (34). Paradoxically, we have found in this study that PGE$_2$, the main prostanoid produced by HASM cells, exerts a positive feedback effect on COX-2 expression, which is in agreement with the report of Bonazzi et al. (33) in rabbit corneal epithelial cells. Meade et al. (4) also demonstrate that NSAIDs up-regulate COX-2 expression independently of prostanoid inhibition in mammary epithelial cells. In these cases, the increased COX-2 expression by NSAIDs is clearly not a feedback mechanism because 1) it is not reversed by exogenous PGE$_2$; 2) COX-1 expression is not altered (4); 3) NSAIDs do not up-regulate COX-2 expression equally, even though they all block prostanoid generation (4). Therefore, NSAID-induced COX-2 expression in this study is a direct effect mediated by a prostanoid-independent mechanism.

Recent work has shown that NSAIDs, fatty acids, and prostanoids, compounds that are inhibitors, substrates, and products, respectively, of COX activity, regulate gene expression via PPAR activation (6, 10, 35, 36). COX-2 expression is also induced by PPAR activators (4, 33, 37), and it has been speculated that this occurs via a PPRE in the COX-2 promoter region (4), although studies deleting the PPRE and observing the effect of NSAIDs have not been previously performed. Our study is the first to show that PPAR$\alpha$ and PPAR$\gamma$, but not PPAR$\beta$, are expressed constitutively in HASM cells, providing the basis for PPAR activation. Like other nuclear receptors, PPARs are translocated from the cytosol to the nucleus upon binding with activators such as 15d-PGJ$_2$ (38). Indeed, we found that PPAR$\gamma$ activators mimicked the effect of NSAIDs on COX-2 expression and that PPAR$\gamma$, but not NF-$\kappa$B, was translocated to the nucleus by PPAR$\gamma$ activator Cig as well as NS-398. Although NSAIDs are also PPAR$\alpha$ activators, our results suggest that they mainly activate PPAR$\gamma$ in these cells, as PPAR$\alpha$ translocation results were less impressive, and PPAR$\alpha$ activation is not involved in COX-2 expression, as PPAR$\alpha$ activator WY-14643 did not have any effect. This is supported by the findings in the current study that NSAIDs and PPAR$\gamma$ activator 15d-PGJ$_2$ induced COX-2 promoter activity on their own and enhanced IL-1$\beta$-induced COX-2 promoter activity only when PPRE was included in the construct (3.9-kb COX-2-Luc). Collectively our data demonstrate that NSAID-induced COX-2 expression is likely to be mediated by PPAR$\gamma$; however, do not exclude possible interaction between PPARs and NF-$\kappa$B. The fact that the more sustained and enhanced increase of COX-2 expression was observed when NSAIDs were used together with IL-1$\beta$ (Fig. 1B) compared with NSAIDs alone (Fig. 2B) suggests that this may result from the transactivation of two transcription factors, PPAR$\gamma$ by NSAIDs and NF-$\kappa$B by IL-1$\beta$ (29), or an interaction between PPAR$\gamma$ and NF-$\kappa$B (Fig. 8). Recent studies have indeed shown that PPARs have cross-talk with transcription factors NF-$\kappa$B (6, 39) and AP-1 (39, 40) and CREB-binding protein/p300 (40). We also showed that PGE$_2$ induces COX-2 expression and enhances IL-1$\beta$-induced COX-2 expression in HASM cells. Because PGE$_2$ stimulates cAMP generation via either EP$_2$ or EP$_4$ adenylyl cyclase-coupled receptors (41) in HASM cells (26) and there is a CRE in the COX-2 gene promoter (42), it is likely that PGE$_2$ up-regulates COX-2 expression by a cAMP-dependent positive feedback loop (Fig. 8). This is supported by the findings that the $\beta_2$-adrenoceptor agonists salbutamol and salmeterol and the direct

![FIGURE 8. Schematic of proposed mechanism by which NSAIDs, COX products, and IL-1$\beta$ regulate COX-2 expression in HASM cells. COX-2 expression is up-regulated by 1) NSAIDs, binding PPAR$\gamma$, which forms a heterodimer with retinoid X receptor (RXR) and binds to PPRE of COX-2 promoter; 2) IL-1$\beta$, activating NF-$\kappa$B; 3) PGE$_2$, stimulating cAMP and binding to CRE. COX-2 expression leads to the generation of prostanoids, of which PGE$_2$ and 15d-PGJ$_2$, in an autocrine manner, enhance COX-2 expression by a cAMP- and a PPAR$\gamma$-dependent mechanism, respectively.](http://www.jimmunol.org/)
adenyl cyclase activator forskolin also induce COX-2 (our unpublished observation) and that PGE₂ up-regulates COX-2 gene expression (43, 44) and potentiates mitogen-induced COX-2 expression (9, 45, 46). Because PGE₂ up-regulates COX-2 expression, NSAID inhibition of IL-1β-induced PGE₂ production would, in theory, reduce COX-2 expression; however, because the effect is compensated by NSAID activation of PPARγ, COX-2 expression is, in fact, enhanced (Fig. 8). Moreover, 15d-PGJ₂, the natural ligand of PPARγ, is a metabolite of PGE₂ and we have shown that IL-1β stimulates PGD₂ synthesis in a COX-2-dependent manner in HASM cells (19), it is possible that COX-2 expression is self-regulated in an autocrine manner by PGE₂ and 15d-PGJ₂ via a cAMP- and PPAR-γ-dependent mechanism, respectively (Fig. 8).

We also demonstrated that the PPARγ activator Cig significantly increases PGE₂ accumulation either alone or in combination with IL-1β, in line with the increase in COX-2 expression. No PGE₂ increase could be detected with NSAIDs because its synthesis was blocked. Because one possibility that could account for the low PGE₂ levels seen in other studies (33, 37) is the lack of substrate AA and because NSAIDs abolish PGE₂ generation from endogenous AA, the PGE₂ levels could not reflect the increase in COX-2 enzymatic activity under these conditions. To measure COX activity more accurately, we examined COX activity by measuring the PGE₂ generation from exogenous AA in the absence of NSAIDs. Under these conditions, COX activity was significantly increased in 15d-PGJ₂-, Cig-, NS-398-, and Flur-treated cells compared with control cells. As far as we are aware, this is the first evidence that COX-2 induced by NSAIDs is biologically functional when the drugs are no longer present. The reason that Indomethacin did not increase COX activity even though they did induce COX-2 expression can be explained by the fact that its inhibition on COX-2 is time-dependent and irreversible (47, 48). However, despite the fact that both NS-398 and Flur are also time-dependent, irreversible inhibitors of COX-2 (47, 49), our results clearly show that their effect can be washed away, but the reason remains unknown. Increased COX activity was also observed with IL-1β; however, addition of NSAIDs and PPAR activators did not further increase COX activity even though they did enhance COX-2 expression. This is probably because IL-1β is a stronger inducer of COX-2 than NSAIDs and PPAR activators and the COX activity has reached its maximum. Alternatively, NSAIDs and PPAR activators might indirectly inhibit cytokine-induced COX activity by modulating other enzyme and signal transduction pathways that regulate prostanoïd metabolism (37).

In summary, we have demonstrated that NSAIDs induce COX-2 expression in HASM cells through PPARγ activation. The fact that NSAIDs are not effective in treating airway inflammation in asthma suggests that the inflammatory process is not mainly mediated by prostanoïds and/or that NSAIDs may intensify the inflammatory response by activating PPARs and subsequently amplifying the response via COX-2 expression and proinflammatory prostanoïd production. Our observations may explain in part the lack of efficacy of NSAIDs in the treatment of airway inflammation.

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References


