Inhibition of Cyclooxygenase-2 Decreases DNA Synthesis Induced by Platelet-Derived Growth Factor in Swiss 3T3 Fibroblasts

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ABSTRACT

NS-398 [N-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide], a selective inhibitor of cyclooxygenase-2 (COX-2), inhibited proliferation induced by platelet-derived growth factor (PDGF) in Swiss 3T3 fibroblasts. The effect of NS-398 was found to be concentration-dependent. The half-maximal effect occurred at ~0.1 μM. NS-398 decreased mitogenesis at subsaturating PDGF concentrations and the inhibitory effect of NS-398 was overcome by increasing PDGF concentration. SC-236, another COX-2 selective inhibitor, also inhibited PDGF-induced proliferation. In contrast, two selective COX-1 inhibitors, valeryl salicylate and ketorolac, had no significant inhibitory effect on PDGF-stimulated DNA synthesis. The inhibition was obtained when NS-398 was added during the first hour after PDGF addition. At 1 h, PDGF induced COX-2 protein and prostaglandin (PG)E2 synthesis, and NS-398 blocked the synthesis of PG_E2. The inhibitory effect of NS-398 on PDGF-stimulated DNA synthesis was counteracted by 280 nM PG_E2. The antimitogenic action of NS-398 and SC-236 suggests that selective inhibition of COX-2 may produce antiproliferative effects with substantial safety advantages over nonselective COX inhibitors.

Epidemiological studies, clinical observations, and animal studies demonstrate that nonsteroidal anti-inflammatory drugs (NSAIDs) can prevent colorectal cancer (Smalley and DuBois, 1997; Taketo, 1998). However, the mechanisms involved is not completely understood, although inhibition of cell proliferation, reduction of mutagenesis, inhibition of metastasis, induction of apoptosis, and inhibition of angiogenesis have been proposed (Shiff and Rigas, 1997; Tsujii et al., 1998). These mechanisms are not exclusive and the effect of NSAIDs on cancer cells may depend on a combination of them.

Inhibition of cell proliferation has been described for several NSAIDs in multiple cell types (Levy, 1997). The mechanisms underlying these effects are not clear. NSAIDs directly target cyclooxygenase (COX) (Vane, 1971), a key enzyme in the production of prostaglandins (PGs), prostacyclins, and thromboxanes (Smith, 1989). However, both COX-dependent and COX-independent mechanisms in the antiproliferative action of NSAIDs have been proposed (Shiff and Rigas, 1997; Smalley and DuBois, 1997; Elder and Paraskeva, 1998).

Two isoforms of human COX, designated COX-1 and COX-2, have been identified (Williams and DuBois, 1996; Vane et al., 1998). COX-1 is normally expressed constitutively in many different cells and tissues in contrast to the highly regulated and inducible expression of COX-2 in a subset of cell types. Both isoforms are key enzymes in the production of prostanoids. These products mediate numerous cellular responses, including modulation of cellular adhesion, differentiation, and mitogenesis. However, the contribution of the inhibition of each COX isofrom to the antiproliferative effects of NSAIDs is not clear.

The two COX isoenzymes differ markedly in their sensitivity to NSAID inhibition. Many standard NSAIDs exhibit nonselective inhibition of both COX isoforms, but new compounds, such as N-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide (NS-398) and (4-[5-(4-chlorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide) (SC-236) show potent and selective inhibition of COX-2 versus COX-1 (Futaki et al., 1993, Masferrer et al., 1994). These compounds are used to analyze the mechanisms of mitogenic stimulation by growth factors (Rozengurt, 1986).

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ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; PG, prostaglandin; NS-398, N-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide; SC-236, 4-[5-(4-chlorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide; PDGF, platelet-derived growth factor; VSA, valeryl salicylate; FCS, fetal calf serum; DMEM, Dulbecco’s modified Eagle’s medium; TBST, Tris-buffered saline/Tween 20; NF-kB, nuclear factor-kB.
togen for Swiss 3T3 cells that can stimulate DNA synthesis in the absence of any other growth factor. The effects of this factor are mediated by multiple synergistic signaling pathways, including arachidonic acid release and production of PGE₂ (Domin and Rozengurt, 1993). So, these cells provide a useful model system for the investigation of the role of cyclooxygenase and prostanoids in cell proliferation.

Recently, we reported that aspirin inhibits DNA synthesis in Swiss 3T3 fibroblasts (Castano et al., 1997). Two lines of evidence indicate that the antiproliferative action of aspirin (at concentrations < 1 mM) is mediated by inhibition of COX. First, aspirin only inhibits the mitogenic action of growth factors that increase arachidonic acid release. Second, the effects of aspirin are partially reversed by exogenous PGE₂, the major arachidonate derivative via COX in these cells. PDGF, tumor promoters, and other growth factors induce expression of COX-2 in Swiss 3T3 cells (Herschman, 1994), making them a good model to study the relationship between expression of COX-2 and cell proliferation. However, the role of COX-2 in the proliferation of these cells has not been studied.

Herein, we analyze the effect of NS-398 and SC-236, two selective inhibitors of COX-2, on the mitogenic action of PDGF in Swiss 3T3 cells. The results show that both NS-398 and SC-236 inhibit the DNA synthesis induced by PDGF and that this effect is mediated through the inhibition of COX-2 activity and PGE₂ production.

Materials and Methods

Reagents. PDGF and PGE₂ were obtained from Sigma Chemical Co. (St. Louis, MO). NS-398 was from Biomol Research Laboratories (Plymouth, PA). SC-236 was kindly provided by Searle Research and Development (St. Louis, MO), and Ketorolac and valeryl salicylate (VSA) were obtained from Cayman Chemicals (Ann Arbor, MI). Fetal calf serum (FCS) was from Life Technologies (Rockville, MD). Dulbecco’s modified Eagle’s medium (DMEM) and Waymouth’s medium were from Biological Industries (Kibbutz Beit Haemek, Israel). [³H]Thymidine was purchased from Amersham (Buckinghamshire, UK). ¹²⁵I-PGE₂ radioimmunoassay system was obtained from New England Nuclear (Boston, MA). Anti-COX-2 antibody raised against mouse was obtained from Transduction Laboratories (Lexington, KY). All the other reagents were of analytical grade.

Cell Culture. Stock cultures of Swiss 3T3 cells were maintained in DMEM supplemented with 10% FCS, 1-glutamine (2 mM) penicillin (100 U/mL), and streptomycin (100 μg/mL) in a humidified atmosphere of 10% CO₂, 90% air at 37°C. For experimental purposes, 4 × 10⁴ cells were subcultured in 22-mm dishes with 1 ml of DMEM supplemented with 10% FCS and incubated until confluence and quiescence (6–8 days). The quiescence of the cells was confirmed by cytofluorimetric assay of DNA content with a flow cytometer (Becton Dickinson, Mountain View, CA) after staining with propidium iodide. According to this protocol, 90% of cells were in the G₀-G₁ phase of the cell cycle.

[³H]Thymidine Incorporation Assay. Determinations of DNA synthesis were performed as previously described (Gil et al., 1991). Briefly, quiescent cultures were washed twice in DMEM and incubated in DMEM/Waymouth’s medium [1:1 (v/v)] containing [³H]thymidine (1 μCi/mL; 1 nM) and various additions. Growth factors and NS-398 were added at the same time to cultures unless indicated. After 40 h, the cultures were washed twice in PBS and incubated in 5% trichloroacetic acid for 30 min at 4°C. Trichloroacetic acid was then removed and the cultures were washed twice in ethanol and extracted in 0.5 ml of 2% Na₂CO₃, 0.1 M NaOH, and 1% SDS. Incorporation was determined by scintillation counting. The results are expressed as the percentage with respect to the maximal response with 10% FCS or as the percentage of inhibition. Usually, the [³H]thymidine incorporation induced by 10% FCS was ~4000 cpm/μg protein. The [³H]thymidine incorporation in the absence of growth factors was ~40 cpm/μg protein. The number of cells assessed by crystal violet staining did not decrease significantly after 40 h of NS-398 (10 μM) treatment.

Measurement of PGE₂ Release. PGE₂ release was determined as described previously (Gil et al., 1991). Quiescent cultures were washed twice in PBS and incubated at 37°C for 1 or 2 h in the required conditions. The medium was removed and stored at −20°C. All vessels used were made of polypropylene or siliconized glassware. Measurements of PGE₂ were performed by radioimmunoassay with an ¹²⁵I-PGE₂ assay system. Aliquots of sample were diluted in assay buffer containing 0.9% NaCl, 0.1 M EDTA, 0.3% bovine γ-globulin, 0.005% Triton X-100, 0.05% sodium azide, and 25 mM phosphate buffer, pH 6.8. The samples were then bound to a rabbit anti-PGE₂ antibody with ¹²⁵I-PGE₂ as a competitive tracer for 16 h at 4°C. The immune complexes were then precipitated by the addition of 16% polyethylene glycol, 0.05% sodium azide, and 50 mM phosphate buffer, pH 6.8, for 30 min at 4°C. Samples were centrifuged for 30 min at 2000g and the supernatants were removed. The pellets were counted in a gamma counter (Wallac, Turku, Finland). Addition of growth factors to the medium had no effect on the radioimmunoassay.

Western Blot Analysis of COX-2. Cells were lysed with Laemmli sample buffer (Laemmli, 1970) and samples were incubated for 10 min at 100°C and sonicated. Protein concentration was determined by the BCA Protein Assay (Pierce, Rockford, IL). One hundred micrograms of the protein extract was subjected to SDS-polyacrylamide gel electrophoresis (5% stacking and 8% resolving gel) and transferred to Immobilon-P (Millipore, Bedford, MA) membranes. After blocking for 1 h with 5% dried skimmed milk in Tris-buffered saline/Tween 20 (TBST) (10 mM Tris HCl, pH 7.5, 100 mM NaCl, and 0.1% Tween 20), the filters were incubated with COX-2 antibody diluted 1:2500 in 5% dried skimmed milk in TBST. Antibody binding was detected with a secondary antibody (anti-mouse Ig; Amersham, Buckinghamshire, UK) conjugated to horseradish peroxidase diluted 1:5000 in 5% dried skimmed milk in TBST and an enhanced chemiluminescence detection kit (Amersham).

Data Analysis. All data points shown are means ± S.E. of n separate experiments. Statistical significance of differences was assessed by ANOVA (Fisher’s protected least-significant difference test). Differences between absence and presence of COX inhibitors are indicated by *P < .05, **P < .01, and ***P < .001.

Results

First, we studied the effect of NS-398 on the stimulation of thymidine incorporation by PDGF in quiescent Swiss 3T3 fibroblasts. Quiescent Swiss 3T3 cells were treated with 0.3 nM PDGF and increasing concentrations of NS-398. As shown in Fig. 1, NS-398 caused a dose-dependent inhibition of thymidine incorporation, the half-maximal effect occurring at ~0.1 μM.

To analyze the effect of NS-398 on the dose-dependent curve of DNA synthesis induced by PDGF, Swiss 3T3 cells were incubated in medium containing increasing concentrations of PDGF with or without 10 μM NS-398. As shown in Fig. 2, NS-398 modified the dose-dependent stimulation curve of DNA synthesis induced by PDGF. NS-398 added to cell cultures at subsaturating concentrations of PDGF (0.4–0.5 nM) reduced thymidine incorporation. In the absence of NS-398, 0.25 nM PDGF was needed to obtain half-maximal response, whereas in the presence of the drug the concentration needed rose to 0.35 nM. The effect of NS-398 on PDGF-stimulated mitogenesis was overcome by increasing PDGF concentration. To analyze the respective roles of COX-1 or COX-2 in DNA synthesis, other
COX-selective inhibitors were used. Another COX-2-selective inhibitor, SC-236 (Gierse et al., 1996), also inhibited PDGF-stimulated thymidine incorporation (Fig. 3A). In contrast, two preferential COX-1 inhibitors, ketorolac (Warner et al., 1999) and VSA (Bhattacharyya et al., 1995), had no significant inhibitory effect on DNA synthesis induced by PDGF (Fig. 3A). In Swiss 3T3 cells stimulated by PDGF, the major arachidonic acid metabolite is PGE2, a product of the COX pathway (Domin and Rozengurt, 1993). In addition, PGE2 formation was determined in presence of these COX-1- and COX-2-selective inhibitors (Fig. 3B). Quiescent Swiss 3T3 cells were treated, for 2 h, with 0.1 nM PDGF alone or with 0.1 nM PDGF and different COX-selective inhibitors at the same time. The levels of PGE2 in the absence of PDGF were below 3 nM. Both COX-2 inhibitors, NS-398 and SC-362, blocked PDGF-induced PGE2 synthesis. However, both COX-1 inhibitors, ketorolac and valeryl salicylate, had no significant effect on PGE2 levels.

We next studied the effects of NS-398 added at different times after mitogenic stimulation (Fig. 4). The inhibitory effect of NS-398 when added 30 min after PDGF was not significantly different from that observed when added at the same time (35 and 30% reduction in [3H]thymidine incorporation, respectively). This effect was clearly lower when NS-398 was added 1 h after PDGF (15% inhibition). Addition of NS-398 two or more hours after had no effect on DNA synthesis. So, we predicted that after this time (1 h),
with 0.2 nM PDGF alone or with 0.2 nM PDGF and 10 μM NS-398 at the same time. Expression of COX-2 protein increased after activation with PDGF (Fig. 5) and was not affected by NS-398 (data not shown). PDGF increased PGE₂ levels at 1 h and NS-398 blocked PGE₂ synthesis (Fig. 5).

To determine whether inhibition of PGE₂ production is involved in the antimitogenic effect of NS-398, Swiss 3T3 cells were incubated in medium containing exogenous PGE₂. Figure 6 shows that addition of 280 nM PGE₂ counteracted the effect of NS-398 on PDGF-stimulated DNA synthesis.

**Discussion**

COX-2 is thought to have a role in cell proliferation. The COX-2 gene is induced after growth factor or tumor promoter stimulation (Herschman, 1994). Furthermore, COX-2 expression is induced by the oncogenes src (Xie and Herschman, 1995) and ras (Sheng et al., 1998), and inhibited by the tumor suppressor gene p53 (Subbaramaiah et al., 1999). COX-2 expression is induced by the oncogenes src (Xie and Herschman, 1995) and ras (Sheng et al., 1998), and inhibited by the tumor suppressor gene p53 (Subbaramaiah et al., 1999). COX-2 expression is increased dramatically in a significant number of colorectal cancers (Eberhart et al., 1994). The demonstration that a null mutation for COX-2 reduces the number and size of intestinal tumors in the APCΔ176 knockout mice, a murine model of familial adenomatous polyposis, provides genetic evidence for the role or COX-2 in tumorogenesis (Oshima et al., 1996). In agreement with these results, selective inhibition of COX-2 reduces proliferation of different cell lines (Tsuji et al., 1996; Martinez et al., 1997; Sheng et al., 1997; Vadlamudi et al., 1999).

However, whether inhibition of COX-2 underlies the effects of selective inhibitors of COX-2 on cell proliferation is a matter of controversy and several reports have proposed that these events are not related (Hanke et al., 1996; Coffey et al., 1997; Erickson et al., 1999). Two of these reports indicate that COX-2-derived PGs are not involved in mitogenesis in different cell types because addition of exogenous PGs had no effect on the decrease in DNA synthesis induced by COX-2 inhibitors (Hanke et al., 1996; Coffey et al., 1997).

Our results show that the selective COX-2 inhibitors NS-398 and SC-236 reduce the mitogenic action of PDGF in Swiss 3T3 fibroblasts. Three lines of evidence indicate that the antiproliferative action of NS-398 in Swiss 3T3 fibroblasts is mediated via inhibition of COX-2. First, the dose response for the effect of NS-398 on DNA synthesis is coincident with the reported IC₅₀ values for inhibition of COX-2. So, the IC₅₀ for DNA inhibition (this article) and COX-2 inhibition (Patrignani et al., 1997) are both 0.1 μM. Second, NS-398 did not inhibit PDGF-induced DNA synthesis when it was added after 2 h, time sufficient to the synthesis of a significant amount of PGE₂. Third, the antiproliferative effect of NS-398 is reversed by exogenous PGE₂, the major arachidonate derivative from the COX pathway in these cells.

Previously, we demonstrated that in Swiss 3T3 cells aspirin inhibits PDGF-stimulated DNA synthesis (Castaño et al., 1997). Because the COX-1 inhibitors did not inhibit PDGF-induced DNA synthesis (this article), we can conclude that
the COX-dependent effects of aspirin on DNA synthesis are mostly mediated via inhibition of COX-2. In agreement with these results, mitogen-induced synthesis of \( PGE_2 \) in Swiss 3T3 cells requires expression of COX-2 (Reddy and Herschman, 1994). However, COX-1 may have a role in the proliferation of other cells. It has been reported that resveratrol, an inhibitor showing preference for COX-1, inhibits proliferation of HL-60 (Jang et al., 1997), and that \( PGE_2 \) produced through COX-1 promotes crypt stem cell survival and proliferation (Cohn et al., 1997).

The effect of aspirin was consistently higher than that of \( \text{NS-398} \); moreover, the addition of aspirin \( \beta \) 8 after PDGF can inhibit PDGF-stimulated DNA synthesis (Castano et al., 1997). So, we cannot rule out COX-2-independent mechanisms. Cyclooxygenase-independent mechanisms have been implicated in the inhibition of the transcription factor nuclear factor-\( \kappa \)B (NF-\( \kappa \)B) by aspirin and salicylate (Kopp and Ghosh, 1994). Recently, it has been reported that aspirin binds and inhibits IkB kinase-\( \beta \) (Yin et al., 1998). Remarkably, NF-\( \kappa \)B is induced by PDGF during G0-to-G1 transition in fibroblasts (Olashaw et al., 1992). The involvement of inhibition of NF-\( \kappa \)B in the antiproliferative action of aspirin in Swiss 3T3 cells needs further investigation.

Inhibition of COX activity by currently marketed NSAIDs is equipotent to COX-1-specific. This may explain their propensity to cause unwanted side effects such as gastric and renal damage. Because selective inhibitors of COX-2 provide an effective DNA synthesis inhibition in Swiss 3T3 cells and other cell types, selective inhibition of COX-2 may produce antiproliferative effects of cancer cells with substantial safety advantages over nonselective COX inhibitors.

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**References**


