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Cell Biology

Nonsteroidal antiinflammatory drugs cause apoptosis and induce cyclooxygenases in chicken embryo fibroblasts

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ABSTRACT **Programmed cell death (apoptosis) is an intrinsic part of organismal development and aging. Here we report that many nonsteroidal antiinflammatory drugs (NSAIDs) cause apoptosis when applied to v-*src*-transformed chicken embryo fibroblasts (CEFs). Cell death was characterized by morphological changes, the induction of tissue transglutaminase, and autodigestion of DNA. Dexamethasone, a repressor of cyclooxygenase (COX) 2, neither induced apoptosis nor altered the NSAID effect. Prostaglandin E_2, the primary eicosanoid made by CEFs, also failed to inhibit apoptosis. Expression of the protooncogene *bcl-2* is very low in CEFs and is not altered by NSAID treatment. In contrast, p20, a protein that may protect against apoptosis when fibroblasts enter G_0 phase, was strongly repressed. The NSAID concentrations used here transiently inhibit COXs. Nevertheless, COX-1 and COX-2 mRNAs and COX-2 protein were induced. In some cell types, then, chronic NSAID treatment may lead to increased, rather than decreased, COX activity and, thus, exacerbate prostaglandin-mediated inflammatory effects. The COX-2 transcript is a partially spliced and nonfunctional form previously described. Thus, these findings suggest that COXs and their products play key roles in preventing apoptosis in CEFs and perhaps other cell types.**

During apoptosis or programmed cell death, cells round and may shrink, chromatin condenses, characteristic nucleosomal-sized fragments of DNA appear, and enzymes involved in cytoskeletal crosslinking and DNA hydrolysis [e.g., transglutaminase (TGase), calpain, and endonucleases] are induced or activated by signal transduction (1, 2).

Prostaglandin (PG) E_2 has important physiological roles in inducing apoptosis during embryonic implantation into the endometrium (3), is needed for rupture of the ovarian follicle (4), and has also been shown to induce programmed cell death in cultured cells (5). Interest in eicosanoids as inducers of apoptosis arose in our laboratory through our cloning and identification of cyclooxygenase (COX) 2 from v-*src*-transformed chicken embryo fibroblasts (CEFs) (6). COX-2 and its related isoenzyme, COX-1, play crucial roles in organ, tissue, and cellular homeostasis because they catalyze the rate-limiting steps in the production of PGs and thromboxanes. COX-2 is induced in an immediate-early fashion by v-*src* transformation, phorbol ester, and serum (7) and plays an important role in inflammation and other physiological states in which cellular PG synthesis is induced by external mediators such as hormones or cytokines (8, 9).

We report herein the effect of nonsteroidal antiinflammatory drugs (NSAIDs) (competitive and noncompetitive inhibitors of COXs) on CEFs transformed with a temperature-sensitive mutant of the Rous sarcoma virus (RSV). These drugs induce programmed cell death.

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MATERIALS AND METHODS

Cells and Viruses. CEFs were isolated from virus-free 9-day White Leghorn chicken embryos as described (10). Some cells were infected with tsNY72-4 RSV, a temperature-sensitive mutant first identified and characterized by Hanafusa and coworkers (11). Temperatures permissive for transformation by this mutant are 37–35°C, whereas culture at 41.5° produces morphologically normal nontransformed cells. All assays were performed on CEFs cultured for 4–10 passages after explanting of the cells from the embryo. Cells were typically grown in Richter's medium containing insulin (Irvine Scientific) and 5% (vol/vol) calf serum (HyClone). At time of assay, cells were shifted into Dulbecco's modified Eagle's medium (DMEM) containing 0.5% calf serum. NSAIDs were added to this solution for drug testing.

Drug Treatment. Suitable vehicles for each drug were determined by dissolving each compound in a variety of solvents. NSAIDs that did not dissolve in water were solubilized in ethanol. In chronic administration assays for apoptosis, the concentrations indicated in Figs. 1–8 refer to the NSAID dose administered to cells (without a change of medium) every 12 h. This protocol was followed to provide continuous exposure to drugs whose pharmicokinetics in this cell system are unknown. Cycloheximide (CHX at 75 μM, final concentration), dexamethasone (DEX at 1 μM, final concentration), and PGE_2 (1–10 μM) were administered in ethanol. Control cells were treated with the appropriate solvent in each assay. In no case did organic solvents represent >1% of total medium volume. Drugs were purchased from Sigma.

RNA Isolation and Analysis. RNA for gel blot analysis and other experiments was obtained from CEFs by using the guanidinium isothiocyanate method as described (12). For Northern blot analysis, RNA was electrophoresed on denaturing formaldehyde gels, blotted, and probed. Radiolabeled cDNA probes ($2–5 \times 10^6$ cpm/ml) were hybridized to filters at 65°C in Church–Gilbert buffer (13) for 16 h. Filter washing was at 65°C in an aqueous solution containing 0.5 × SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate) and 0.4% SDS.

Reverse Transcription-Coupled PCR. A 600-base chicken tissue TGase [TGase2, EC 2.3.2.13 (14)] fragment was amplified by using a cDNA template made from the total RNA of v-*src*-transformed CEFs. The primer pair used for amplification was as follows: sense, 5′-ATGCGGATCAAGCTGTCGG-3′; antisense, 5′-CGTCAGCTTGTCGCTCTCAA-3′. The 696-bp open reading frame of *bcl-2* (15) was amplified by using the following primer pair: sense, 5′-CCACTRCGCTGCTTCCCC-TCG-3′; antisense, 5′-GGGTGACTCTACTTATGTCC-3′.

Abbreviations: COX, cyclooxygenase; CEF, chicken embryo fibroblast; NSAID, nonsteroidal antiinflammatory drug; NIA, NSAID-induced apoptosis; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RSV, Rous sarcoma virus; DEX, dexamethasone; TGase, transglutaminase; CHX, cycloheximide; PG, prostaglandin.

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Table 1. Morphological inhibition of transformation in response to NSAID treatment

NSAID	Effect 100 μM	Effect 10 μM
Oxicam		
Isoxicam	–	–
Piroxicam	\	–
Salicylate		
Aspirin	–	–
Diflunisal	X	–
Acetamidophenol	–	–
Acetaphenetidin	–	–
Salicylamide	–	–
Acetic acid		
Indomethacin	X	X
Acemetacin	X	\
Tolmetin	\	–
Sulindac	–	–
Diclofenac	X	\
Zomepirac	\	–
Fenamate		
Mefenamic acid	X	\
Flufenamic acid	X	\
Niflumic acid	X	\
Propionic acid		
Ketoprofen	\	–
Naproxen	\	–
Indoprofen	\	–
Ibuprofen	X	–
Flurbiprofin	\	–
Suprofen	\	–
Fenbufen	\	–
Carprofen	X	–
Pyrazole		
Phenylbutazone	–	–
Oxyphenbutazone	–	–

X, complete inhibition of focus formation by RSV; \, partial inhibition that was characterized by cell rounding and formation of small clumps of cells; –, no effect.

Cell Labeling and Immunoprecipitation Assays. After CEFs were grown to confluence (5×10^5 cells in a 60-mm dish), they were serum-starved, shifted to 37°C for 24 h, and washed twice in serum-free DMEM lacking methionine and then cultured for 3 h in 1.5 ml of the same solution containing [^{35}S]methionine (New England Nuclear) at 300 μCi/ml (1 Ci = 37 GBq). Cell lysates were prepared [each sample contained equal amounts (4×10^6 cpm) of radiolabeled protein measured after trichloroacetic acid precipitation) and immunoprecipitation assays were performed as described (16).

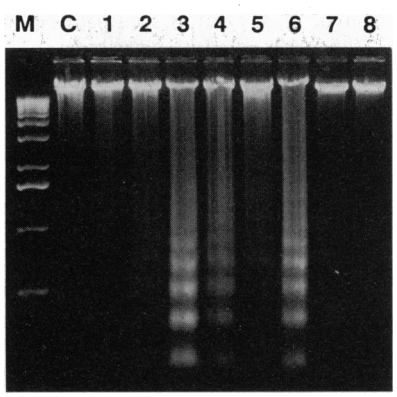

FIG. 1. Induction of DNA fragmentation in RSV-transformed CEFs by NSAIDs. Lanes: M, 1-kb molecular size marker (GIBCO/BRL); C, control (serum-starved) RSV-transformed cells without drug treatment); 1–8, serum-starved RSV-transformed cells treated, respectively, with diflunisal, indomethacin, acemetacin, diclofenac, mefenamic acid, flufenamic acid, niflumic acid, and carprofen. Genomic DNA was isolated from cultures after 30 h of exposure to 100 μM drug and analyzed on a 1% agarose gel.

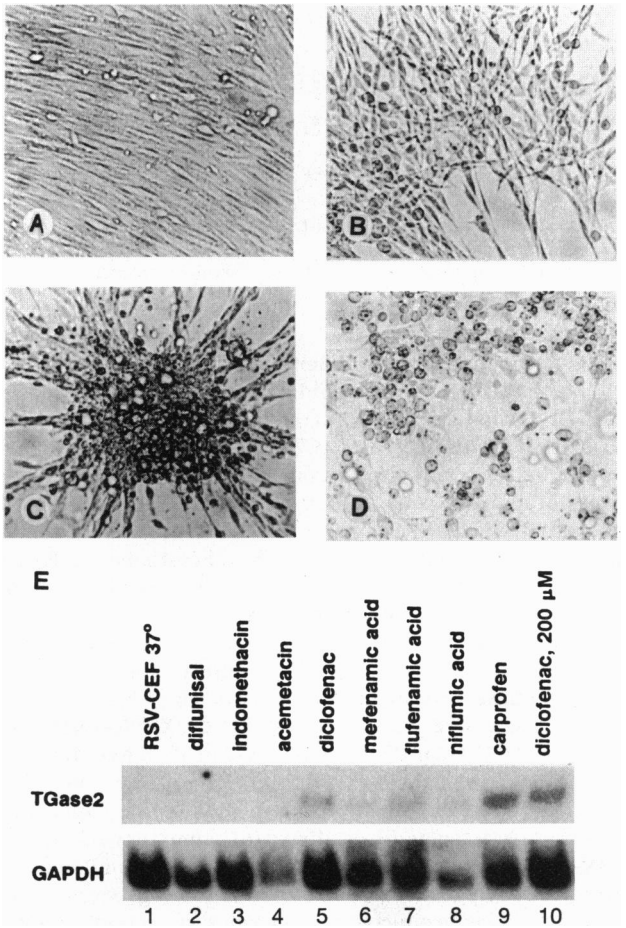

FIG. 2. Apoptotic response of transformed CEFs to NSAIDs. The sequence of morphological changes occurring during transformation by v-*src* is shown for infected cells held at the nonpermissive temperature 42°C (*A*), 30 h at the permissive temperature 37°C (*B*), or 48 h at 37°C (*C*). (*D*) Indomethacin at 100 μM for 48 h at 37°C. (*E*) Northern blot analysis of the effect of NSAIDs on the expression of chicken tissue TGase2. Total RNA was isolated 29 h after shift to 37°C in the presence of 100 μM NSAIDs. The blot was hybridized to a TGase2 cDNA probe. Lanes: 1, no drug treatment; 2–10, NSAID treatment as indicated. TGase2, 7-day exposure; GAPDH, 12-h exposure.

Morphometric Analyses and Trypan Blue Exclusion Assays. Cells were analyzed and photographed for morphological changes by using a Leitz Diavert light microscope with phase-contrast optics. Cell death was measured by trypan blue exclusion. In this assay, cells were exposed for 2 min to a solution containing 0.2% trypan blue dye, 0.06% potassium phosphate (dibasic), and 0.8% NaCl. The solution containing the cells was applied to a hemocytometer and blue cells were counted. In another assay, the trypan blue solution was added directly to cells adhering to the plate and gently aspirated, and blue cells were counted.

RESULTS

Morphological and Degenerative Effects of NSAIDs. Treatment of tsNY72-4 RSV-infected CEFs with 26 NSAIDs showed that all but 9 of the drugs inhibited v-*src*-induced formation of foci when these cells were shifted to the permis-

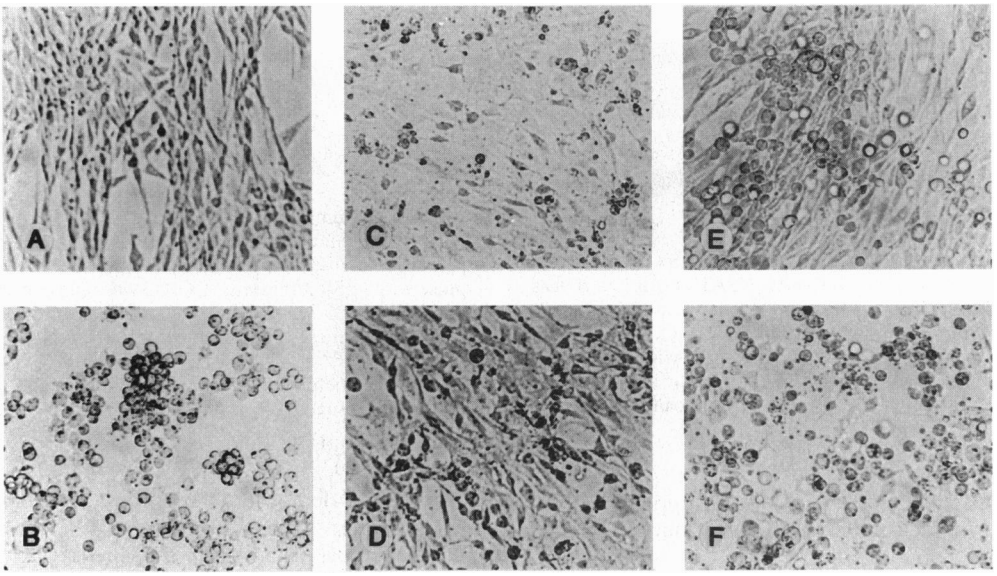

FIG. 3. Impact of DEX, CHX, or PGE_2 on NIA. (*A*) Serum-starved RSV-infected CEFs 30 h after shift to 37°C treated with 5 μM DEX. (*B*) Same as in *A* but with diclofenac at 100 μM. (*C*) RSV-infected CEFs 24 h after shift to 37°C treated with 75 μM CHX. (*D*) Same as in *C* but with 100 μM diflunisal. (*E*) RSV-infected CEFs 48 h after shift to 37°C treated with 1 μM PGE_2. (*F*) Same as in *E* but with 100 μM indomethecin.

sive temperature (Table 1). Of the effective drugs, indomethacin, diclofenac, carprofen, niflumic acid, mefenamic acid, flufenamic acid, diflunisal, and acemetacin were most potent in eliciting morphological effects. Dose–response curves were established for each of these drugs. During this study, it became clear from microscopic observation that in addition to inhibition of focus formation, higher doses of these drugs caused significant cell death within 24 h. This was found to be an apoptotic response, not necrosis. Analysis of DNA from cells treated with 100 μM doses of the 8 most potent NSAIDs showed that all but one (carprofen) caused the generation of nucleosomal-sized ladders of DNA fragments in treated cells (Fig. 1). Also, in contrast to the usual appearance and behavior of transformed cells [proliferation, aggregation, and the formation of foci (Fig. 2 *A–C*)], treated cells failed to form foci and exhibited rounding, pyknotic chromatin, and condensed cytoplasm (Fig. 2*D*)—typical of apoptotic cells. At concentrations >300 μM, nontransformed CEFs exhibited necrosis, but not apoptosis. These cells took up trypan blue but did not show DNA fragmentation (data not shown).

In addition, NSAID treatment of transformed CEFs induced TGase, a molecular indicator of programmed cell death. Flufenamic acid at 100 μM, carprofen at 100 μM and—in a dose-dependent fashion—diclofenac produced a large increase in the amount of 3.5-kb TGase2 (the tissue or ubiquitous type) mRNA (Fig. 2*E*). Mefenamic acid and niflumic acid were less potent inducers. Though not visible in Fig. 2, diflunisal, indomethacin, and acemetacin also induced TGase2 after prolonged autoradiographic exposure.

Effects of Known Apoptotic Agents. Among agents identified in other cell systems, we tested those known to be COX-related to see if they influenced the effects of NSAIDs in causing cell death. DEX selectively down regulates COX-2 in CEFs and other fibroblasts (16). Fig. 3 *A* and *B* shows that this corticosteroid alone had no effect on v-*src* transformation or NSAID-induced apoptosis (NIA). Conversely, CHX [known to cause a rapid decline in COX-2, which has a half-life of only 22 min in CEFs (16)], caused modest apoptosis but completely inhibited NIA (Fig. 3 *C* and *D*). Because NSAIDs are inhibitors of COX and because PGE_2 is the major PG made by CEFs (17), we tested whether PGE_2 could prevent NIA when coadministered with these drugs. We found that PGE_2 alone caused cells to round in shape (Fig. 3*E*) as described (18). However, when administered with NSAIDs, PGE_2 had no effect on NIA (Fig. 3*F*) in concentrations ranging from 1 to 10 μM.

Time and Dose Dependency. Fig. 4 shows that indomethacin, one of the most potent apoptosis-inducing NSAIDs, produced 100% cell death in a dose- and time-dependent fashion. The time required to effect 50% trypan blue permeability ranged from 12 to 48 h depending on the drug and dose. Higher concentrations of a drug caused apoptosis more quickly than lower doses. It is clear from our observations that changes affecting dye uptake are relatively late events, many hours beyond the initial commitment to cell death.

Induction of COXs. Because both NSAIDs and PGs can transcriptionally or posttranscriptionally modulate COX expression (19, 20), we tested the effects of apoptotic doses of NSAIDs on COX-1 and COX-2 mRNAs. All drugs induced COX-2 mRNA to some extent (Figs. 5 and 6). However, except for indomethacin, the predominant form induced at 29 h by these drugs was a partially spliced nonfunctional mRNA we

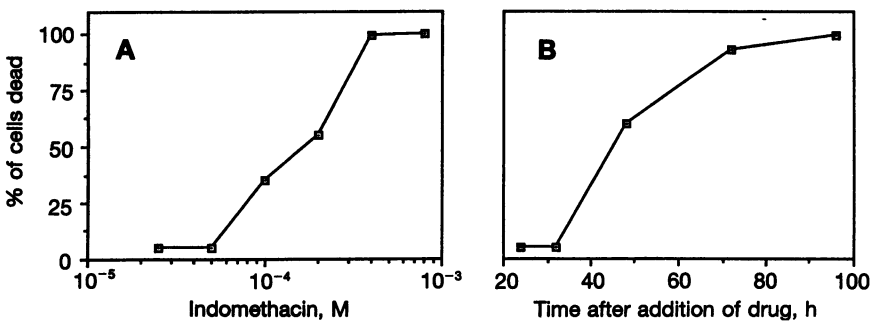

FIG. 4. Effects of concentration and exposure time on indomethacin-induced apoptosis. (*A*) Trypan blue-stained cells 37 h after shift to 37°C in cultures treated with 25, 50, 100, 200, 400, and 800 μM indomethacin every 12 h. (*B*) Numbers of dead cells were assayed as in *A*, and the percentage of dead cells was measured at 24, 32, 48, 72, and 96 h. Indomethacin (100 μM) was administered every 12 h.

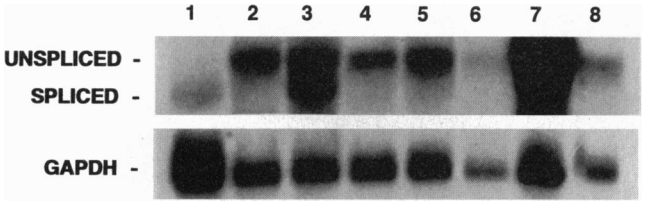

FIG. 5. Induction by NSAIDs of COX-2. For Northern blot analysis, 20 μg of total CEF RNA isolated from RSV-transformed CEFs treated with and without 100 μM NSAIDs for 29 h was hybridized to a COX-2 cDNA probe. The upper band (4.7 kb) is a partially spliced variant of the lower (4.3 kb) spliced COX-2 mRNA. Lanes: 1, no drug treatment; 2–8, cells treated with diflunisal, indomethacin, acemetacin, mefenamic acid, flufenamic acid, niflumic acid, and carprofen, respectively. COX-2, 2-day exposure; GAPDH, 12-h exposure.

have described (1). Dose–response experiments showed that the appearance of partially spliced mRNA, the upper band (4.7 kb), correlated very closely with cell rounding during apoptosis.

COX-1-like mRNA was found to be expressed at extremely low levels in chicken tissues and in CEFs (unpublished data). Relative to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control, COX-1 mRNA was induced 5- to 10-fold by 200 μM diclofenac and was increased 15- to 30-fold at 400 μM (Fig. 6). The effect of 200 μM or higher concentrations of the other apoptotic NSAIDs on COX-1 or COX-2 expression has not yet been tested. Note, however, that induction of COX-1 mRNA by diclofenac required almost an order of magnitude higher dose than did induction of COX-2 mRNA.

We have also attempted to determine what impact these mRNA changes have on levels of COX proteins. Immunoprecipitation assays using anti-COX-2 sera showed that NSAID treatment caused a marked increase in COX-2 concentrations in these cells (Fig. 7). COX-1 was not measured in these experiments due to the absence of a suitable chicken antiserum.

Effects on Other Apoptosis-Related Genes. Expression of protooncogene *bcl-2* is reported to suppress apoptosis depending on the cell type (21). Northern blot analysis and reverse transcription-coupled PCR experiments detected very low levels of a *bcl-2*-related 3.5-kb mRNA in CEFs that were not changed by NSAID treatment (unpublished data).

Culture in plasma induces CEFs to enter G_0 phase, a prolonged state of quiescence, without undergoing apoptosis. Under these conditions CEFs secrete p20, a protein related to β_2-microglobulin (22). Northern blot analysis of diclofenac-treated CEFs showed that apoptotic doses (beginning at 100 μM) sharply reduced p20 expression (Fig. 8).

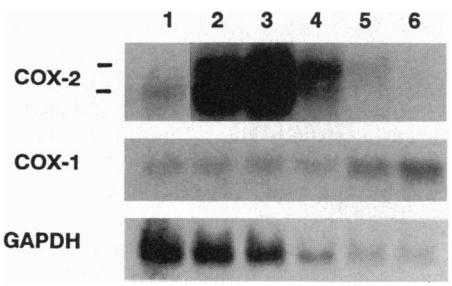

FIG. 6. Effect of concentration on diclofenac induction of COX-1 and COX-2. For Northern blot analysis, 20 μg of total RNA from RSV-transformed CEFs with and without treatment with various doses of diclofenac 29 h after shift to 37°C, was hybridized to COX-1, COX-2, or GAPDH probes. Lanes: 1, serum-starved RSV-transformed cells without drug treatment; 2–6, diclofenac treatment at 25 μM, 50 μM, 100 μM, 200 μM, and 400 μM, respectively. COX-1, 7-day exposure; COX-2, 20-h exposure; GAPDH, 6-h exposure.

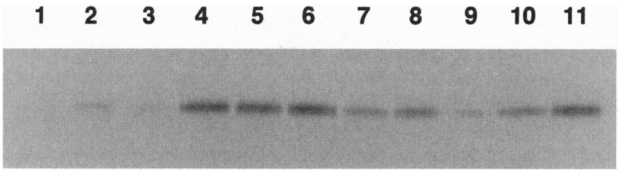

FIG. 7. Immunoprecipitation of COX-2 from v-*src*-transformed CEF cells treated with or without NSAIDs. CEFs were cultured in the presence or absence of 100 μM NSAIDs for 24 h, followed by a 3-h pulse with [^{35}S]methionine. COX-2 was then precipitated from lysates with anti-COX-2 antiserum and analyzed on a SDS/10% polyacrylamide gel. Lanes: 1, preimmune antiserum; 2, CEF + RSV at 37°C; 3, CEF + RSV at 42°C; 4–11, diflunisal, indomethacin, acemetacin, diclofenac, mefenamic acid, flufenamic acid, niflumic acid, and carprofen, respectively. Exposure, 3 days.

DISCUSSION

Our studies demonstrate that most NSAIDs cause apoptosis when applied to RSV-transformed CEFs. Although three of the eight most potent apoptosis-eliciting drugs are fenemates, they represent diverse structural subclasses (Table 1) whose only known commonality is to specifically inhibit COX-1 and COX-2. Some NSAIDs, including four salicylates (aspirin, salicylic acid, acetaphenetidin, and salicylamide), one acetic acid (sulindac), two pyrazoles (phenylbutazone and oxyphenbutazone), and 1 oxicam (isoxicam) had no detectable capacity to cause apoptosis. This is consistent with the observations that salicylic acid, acetaphenetidin, acetaphenol, isoxicam, and pyrazoles show negligible inhibition of COXs in mammalian or chicken fibroblasts (unpublished data). Similarly, sulindac is a prodrug that must be reductively metabolized to its active form, sulindac sulfide, in the liver, and fenbufen, another very weak inhibitor, is also a prodrug (23). Conversely, aspirin, which at 1×10^{-4} M is a highly effective inhibitor of COX-1 and COX-2 in fibroblasts, did not effectively cause apoptosis. It is very difficult to maintain continuous exposure to aspirin, which is extremely labile and hydrolyzes completely to salicylate and acetic acid within minutes after introduction into tissue culture medium.

There may be more than a single pathway through which these NSAIDs act, because their relative potency in producing various apoptotic effects is not the same. For example, 100 μM carprofen is a stronger inducer of TGase2 than 100 μM niflumic acid, yet the latter is a dramatically more potent inducer of COX-2 mRNA. Other drugs, however, promote DNA fragmentation more strongly than these two. Furthermore, meaningful comparisons among the drugs are difficult since their pharmacokinetics during chronic administration have not been defined.

COXs in murine and chicken fibroblasts are relatively insensitive to NSAIDs. With the exception of indomethacin, whose IC_{50} in two murine cell lines is 0.15 μM (unpublished

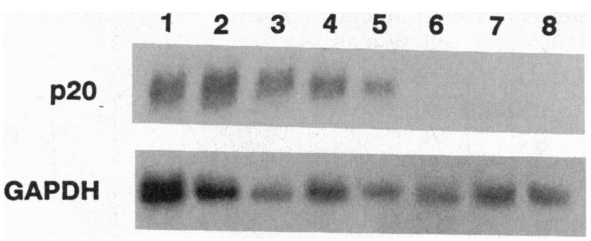

FIG. 8. Repression of p20 by diclofenac. For Northern blot analysis, 20 μg of total RNA from RSV-transformed CEFs treated with various doses of diclofenac 29 h after shift to 37°C was hybridized to a p20 probe. Lanes: 1, no drug treatment; 2–8, diclofenac treatment at 1 μM, 5 μM, 25 μM, 50 μM, 100 μM, 200 μM, and 400 μM, respectively. p20, 12-h exposure; GAPDH, 6-h exposure.

data), NSAID IC_{50} values are typically 1×10^{-4} M or greater in fibroblasts, which is in the range of concentrations used in this study. This is in contrast to macrophages, in which NSAIDs typically inhibit COX at submicromolar concentrations (24). The biochemical basis of this difference in NSAID sensitivity is unknown. All present data, therefore, suggest that the apoptosis-inducing cellular event mediated by these drugs is the inhibition of one or more COXs rather than some other mechanism of action.

DEX had no effect on NIA. This may mean that inhibition of COX-2 does not cause, or is necessary but not sufficient for, apoptosis. Similarly, it is clear that inhibition of the synthesis of PGE_2, the primary eicosanoid made by CEFs after RSV transformation, is not the apoptotic signal, because addition of PGE_2 up to 10 μM did not prevent NIA. Thus both the NSAID inhibitory target (i.e., COX) and its associated cytoprotective metabolite are unknown.

The finding that chronic treatment with the eight strong NSAID inducers of apoptosis caused induction of COX mRNAs and protein was unexpected. NSAIDs elevated COX-2 protein levels in a manner that qualitatively mirrored levels of COX-2 mRNA. Although it is not clear how the drugs may affect the kinetics of COX-2 transcript processing or protein stability, we presume that the induction of COX-2 protein was always less than COX-2 mRNA because most of COX-2 mRNA induced by NSAIDs was nonfunctional (Fig. 5). A COX-1-like mRNA was also significantly elevated by diclofenac, one of the most potent apoptosis inducers (Fig. 6).

The mechanism by which induction of COXs occurs is not known. However, one or more COX-produced products may repress COX expression in a negative feedback loop. Removal of negative feedback by NSAID treatment would result in COX induction. By this model, negative mediators must be isoenzyme-specific, since COX-1 was induced at NSAID concentrations that were nearly 10 times that needed to induce COX-2.

The fact that CHX generally inhibits NIA is consistent with the notion that protein induction is required.

There are two clinically important cytotoxic or antiproliferative effects of NSAIDs in which apoptosis may be relevant. The first effect is ulceration and hemorrhage of the gastrointestinal tract (25). PGs, particularly PGE_2 and PGI_2, protect the gastric mucosa. It has been proposed that inhibition of COX-1 by ulcerogenic NSAIDs may be responsible for this effect (26), a hypothesis supported by the recent finding that NS-398, a COX-2-selective NSAID lacks ulcerogenic activity (27). However, because ulcerogenic NSAIDs inhibit both COX-1 and COX-2, it is presently unknown whether inhibition of COX-1 alone is sufficient to produce this effect.

Second, multiple large epidemiological studies have shown the efficacy of chronic NSAID treatment in reducing the incidence of colon adenomas, adenocarcinomas, and other tumors of the gastrointestinal tract in humans (28). Although the mechanism of this effect is unknown, our data raise the possibility that NSAIDs may directly act on nonimmortalized morphologically neoplastic cells to cause apoptosis. Our finding that neoplastic cells transformed by v-*src* were highly susceptible to NIA is unusual since in most reported studies oncogene expression (including expression of v-*src*) antagonized apoptosis (3, 21). In this regard it is of interest that colorectal tumors at virtually all stages of progression exhibit elevated levels of c-*src* activity (29). Reduction of gastrointestinal tumors was epidemiologically associated with less-intense NSAID treatment than was used in this study. However, fibroblasts are relatively insensitive to these drugs, and other cells *in vivo* may be more sensitive.

Finally, these studies have identified markers, in addition to TGase2, which in all cases correlated perfectly with NIA. Inhibition of p20 proved to be a useful marker because its expression is linked to the cessation of cell division and entrance into G_0 phase. Treatment of cells in G_0 phase by NSAIDs caused apoptosis and also caused a sharp decline in p20 expression (Fig. 8). The dose threshold for this effect was the same as that needed to induce NIA. Thus p20 and other quiescence-specific proteins that protect cells in G_0 phase from cell death may be down-regulated by apoptotic stimuli.

The second marker was the retention of intron 1 in COX-2 mRNA. This intron is unique among known eukaryotic introns in that its splicing occurs efficiently in dividing cells even though it completely lacks a 3′ splice acceptor site. It is a naturally occurring example of AG-independent splicing, a phenomenon previously described only in reduction-of-function mutants of lower organisms (30). Our studies indicate that splicing of this intron is regulated by signal transduction and that the default program in dividing fibroblasts is to splice this intron.

The fact that a variety of NSAIDs cause apoptosis suggests that COXs play critical roles in maintaining cellular integrity in eukaryotes.

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