

Inhibition of COX-2 in Colon Cancer Cell Lines by Celecoxib Increases the Nuclear Localization of Active p53¹

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Abstract

Inactivation of the p53 tumor suppressor gene usually involves somatic mutation or binding of viral oncoproteins to the p53 protein. However, several types of malignant and premalignant tissues harbor a genetically wild-type, but transcriptionally inactive, form of p53, often localized in the cytoplasm. Electrophilic prostaglandins (PGs) are known to sequester and inactivate p53 in the cytoplasm, an effect that is likely to occur when cyclooxygenase (COX)-2 levels become elevated during colon carcinogenesis. We determined the localization and expression of p53 in the presence of PGA₁ and celecoxib, a selective COX-2 inhibitor in human colon cell lines HCT-116 (wild-type p53) and HT-29 (mutant p53). In the absence of treatment, p53 protein accumulated preferentially in the nucleus in both cell lines. We observed that the total cellular levels of p53 protein increased with exposure time and concentration of PGA₁. By contrast, p21 protein levels remained unchanged as a function of time and concentration of PGA₁. In the presence of 20 μM PGA₁, p53 accumulated preferentially in the cytosol. The nuclear:cytosol ratios of p53 were 31 and 2.1 in the controls and in the presence of PGA₁ in HCT-116 cells but were 22 and 4, respectively, in HT-29 cells. Treatment with 50 μM celecoxib for 24 h did not significantly change p53 expression and localization. However, in the presence of 100 μM celecoxib, p53 levels increased in the nucleus. The nuclear:cytosol ratios were then 31 (control) and 60 (100 μM celecoxib) in HCT-116 cells and 22 (control) and 36 (100 μM celecoxib) in HT-29 cells. These results indicate that electrophilic PGs cause wild-type p53 accumulation in the cytosol where it is inactive. Inhibition of COX-2 by celecoxib appears to alleviate this effect on p53 by reducing electrophilic PG synthesis. Thus, COX-2 inhibition of electrophilic PG formation appears to protect p53 tumor suppressor function.

Introduction

COX-2⁴ expression is induced during inflammation by pro-inflammatory cytokines and growth factors and is detectable in most tissues (1–3). Several studies have established that COX-2 overexpression is common to a variety of human malignancies, including cancer of the colon and promotes tumor cell growth, angiogenesis, tumor invasion, and metastasis (4–6). In colon carcinogenesis, COX-2 is elevated in premalignant lesions and remains at very high levels as tumors progress to malignancy (7–9). COX-2 tumorigenicity is likely to involve its production of specific PGs⁴ and their metabolites, but the role of PGs in various stages of carcinogenesis remains to be elucidated.

NSAIDs have been shown to block inflammation and also the development of colonic neoplasia by inhibiting the production of PGs

(10, 11). That COX-2 inhibition might be an important mechanism of tumor inhibition has been indicated in studies using mice with manipulation of the COX-2 gene. An inactivated COX-2 transgene produced a dramatic reduction in colon polyps, whereas wild-type COX-2 overexpression in another transgenic system enhanced tumorigenesis (6). The new generation of NSAIDs, such as celecoxib, designed to specifically inhibit COX-2 and prevent the side effects associated with COX-1 inhibition, has been shown to be particularly effective in preventing colon polyps and neoplasms in preclinical and clinical applications (12). Recent studies indicated that NSAIDs, including celecoxib, may also inhibit cell growth and cancer by COX-2-independent mechanisms (13, 14). It remains unclear how the inhibition of COX metabolites, in turn, inhibits colon tumor growth.

As a tumor suppressor p53 regulates the response to various cellular associated stressors, including DNA damage and oncogenic stimulation (15, 16). As a transcription factor, its nuclear localization is essential for its activity (17). In response to stress, p53 is activated by post-translational modification and localized in the nucleus where it evokes cell cycle arrest or apoptosis via the transcriptional activation of numerous effector genes (17–19). Inactivation of p53 in cancer usually occurs by mutation, deletion, or through inhibitory binding with viral oncoproteins (20–22). The p53 functional network is extensive and includes other known tumor suppressors (*e.g.*, p21, Chk2, and BAX) as well as oncogenes (Mdm2 and Bcl-2). In many cases in which p53 is not inactivated by classical mechanisms, alternate components of its network have been targeted. Wild-type p53 may be inactivated in many malignant and premalignant tissues as a result of abnormal sequestration in the cytoplasm where it is functionally muted (23–25). Electrophilic PGs produced by COX-2 were shown to inhibit wild-type p53 activity by covalently binding and impairing p53 in cytosol (26). Taking together, these observations suggest that p53 localization and expression as well as PGs may play a critical role in colon carcinogenesis. In the present study, we examined the effect of celecoxib-directed inhibition of COX-2 on p53 localization and activity in colon cancer cell lines.

Materials and Methods

Cell Culture. The human colon cell lines, HT-29 cells (p53 mutant type) and HCT-116 cells (p53 wild type), were obtained from the American Type Culture Collection and maintained in McCoy's 5A medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum in an atmosphere of 95% air, 5% CO₂ at 37°C. All experiments were performed with cells at ~70–80% confluence. PGA₁ was purchased from Cayman Chemicals (Ann Arbor, MI); Celecoxib was a generous gift from Pharmacia (Skokie, IL).

Cell Viability Assay. Cells were cultured in 6-well plates at a density of 105 cells/well with different concentrations of celecoxib. Cultures were propagated for a period of 24 h to assess cell viability. The cell viability was determined by counting cell numbers with trypan blue exclusion and the MTT assay. The trypsinized and harvested cells were mixed with an equal volume of 0.3% trypan blue and evaluated for viability. For the MTT assay, cells were incubated with 0.5 mg/ml MTT for 4 h; the resulting formazan was dissolved in acidic isopropanol and quantitated by measuring absorbance at 540 nm (27).

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⁴ The abbreviations used are: COX, cyclooxygenase; PG, prostaglandin; MTT, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt]; PMSF, phenylmethylsulfonyl fluoride; NSAID, nonsteroidal anti-inflammatory drug.

Protein Extraction and Estimation. The HT-29 cells grown and incubated for 6 h with 0–40 μM concentrations of PGA_1 were washed with ice-cold PBS, then lysed in 400 μl of cold lysis buffer containing 20 mM Tris-HCl (pH 7.4), 2 mM EGTA, 50 mM β -glycerophosphate, 1 mM sodium orthovanadate, 5 mM NaF, 1% (volume for volume) Triton X-100, 1 mM DTT, 1 mM PMSF, 20 $\mu\text{g}/\text{ml}$ aprotinin, and 10 $\mu\text{g}/\text{ml}$ leupeptin. The lysates were homogenized and kept on ice for 30 min with vortexing at 10-min intervals. The lysates were centrifuged at $13,000 \times g$ for 15 min at 4°C . The supernatants were collected, and protein concentrations were estimated by the Bradford method (Bio-Rad, Richmond, CA).

Cytosol and Nuclear Fractions. After treatments with the various concentrations of celecoxib, HT-29 and HCT-116 cell lines were collected and washed twice with ice-cold PBS and pelleted by centrifugation at $1500 \times g$ for 5 min. The pellet was resuspended in 1 ml of PBS, transferred into an Eppendorf tube, and pelleted again by spinning for 1 min in a microcentrifuge. PBS was removed, and the cell was resuspended in 400 μl of cold buffer A [10 mM HEPES (pH 7.9); 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF] by gentle mixing with the tip of the pipette. The cells were allowed to swell on ice for 15 min, after which 25 μl of a 10% solution of Nonidet NP40 was added, and tubes were vigorously vortexed for 10 s. The homogenate was centrifuged for 30 s in a microcentrifuge. The supernatant that contained the cytosol fractions was collected. The pellet was resuspended to extract the nuclear fraction extraction in 50 μl of ice-cold buffer B (20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF), and the tubes were vigorously rocked at 4°C for 15 min on a shaking platform. Then, the contents were centrifuged at $13,000 \times g$ for 5 min at 4°C ; the resulting supernatant was collected and used as the nuclear fraction. Protein concentration in both the cytosol and nuclear fractions was estimated by the Bradford method (Bio-Rad).

Western Blot Analysis of p53 Expression. Protein samples (total lysates, cytosol and nuclear) of 50 μg were separated by 8% SDS-PAGE and trans-

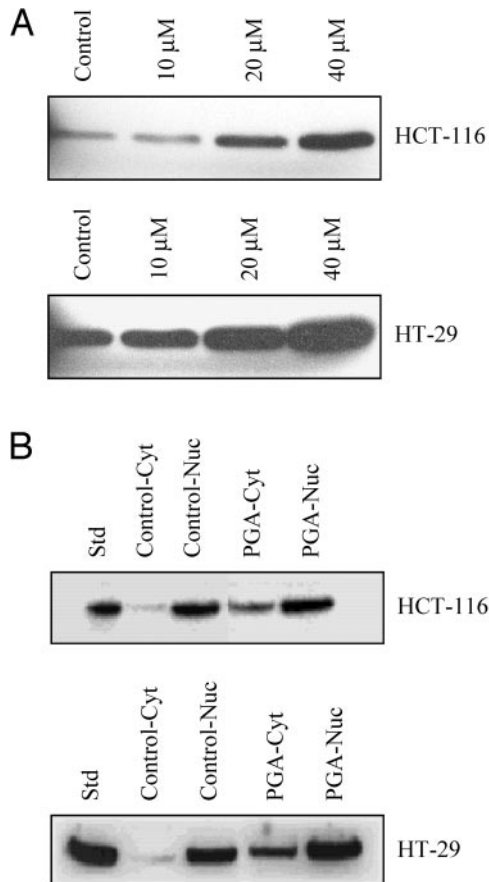


Fig. 1. A, p53 expression in cells treated with electrophilic PGA_1 at 0, 10, 20, and 40 μM . B, p53 expression in the presence of electrophilic PGA_1 (20 μM) at cytosol and nuclear levels. The nuclear:cytosol ratios of p53 in HCT-116 were 30.9 (control) and 2.14 (test) and in HT-29 were 21.92 (control) and 4.06 (test).

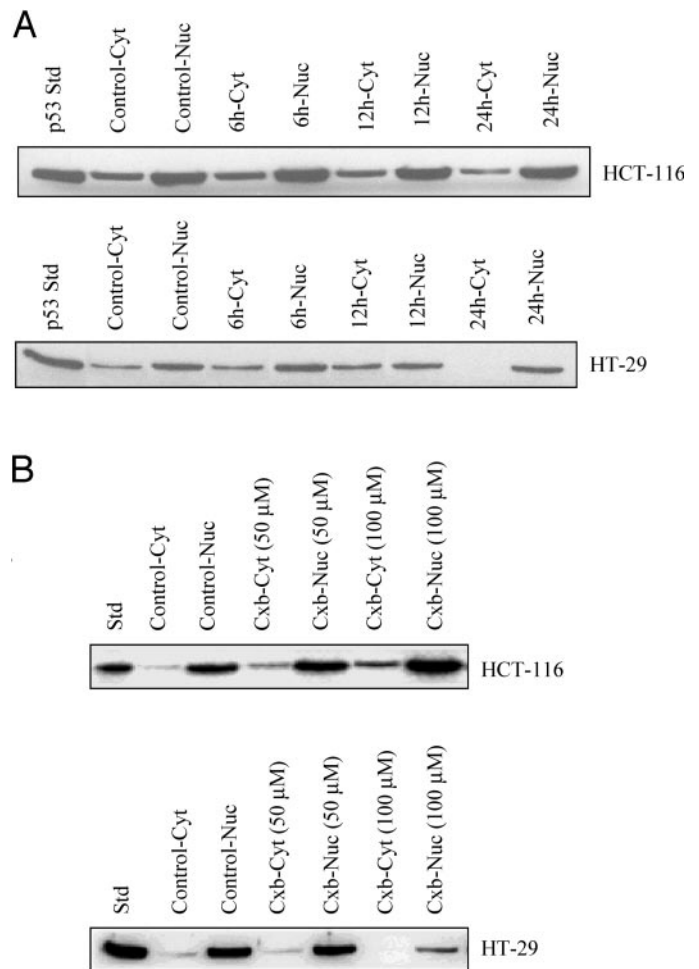


Fig. 2. A, p53 expression in the presence of celecoxib (100 μM) at cytosol and nuclear levels with different time intervals. B, p53 expression in the presence of various concentrations of celecoxib at cytosol and nuclear levels for 24 h. The nuclear:cytosol ratios of p53 in HCT-116 were 30.9 (control) and 60.6 (100 μM) and those in HT-29 were 21.92 (control) and 35.6 (100 μM).

ferred onto a nitrocellulose membrane (Millipore Corp., Bedford, MA). The membranes were blocked with TBST blocking solution containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% (volume for volume) Tween 20, and 5% (w/v) dry milk for 1 h. The membranes were probed with rabbit polyclonal antibodies of p53 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1000 dilution for 1 h. After being washed three times at 10-min intervals, the membranes were treated with horse radish peroxidase at 1:5000 dilution for 1 h. The proteins were illuminated with an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech). α -Tubulin (Ab-1) mouse monoclonal antibody (Oncogene, San Diego, CA) was used at a 1:1000 dilution as the internal standard for all Western blot analysis.

Results

Cytotoxicity. We determined the effect of celecoxib on the growth of HT-29 and HCT-116 cell lines. In MTT and cell viability assays, celecoxib had a concentration- and time-dependent inhibitory effect on both cell lines. This inhibitory effect of celecoxib in both cell lines was maximal at concentrations $> 200 \mu\text{M}$ after 24 h of treatment (data not shown). Subsequent experiments used noncytotoxic doses of celecoxib.

p53 Expression and Subcellular Localization in the Presence of PGA_1 . HCT-116 and HT-29 cells were treated with 10, 20, or 40 μM PGA_1 , after which p53 protein levels were determined. Levels of p53 expression from total lysate increased in a dose-dependent manner

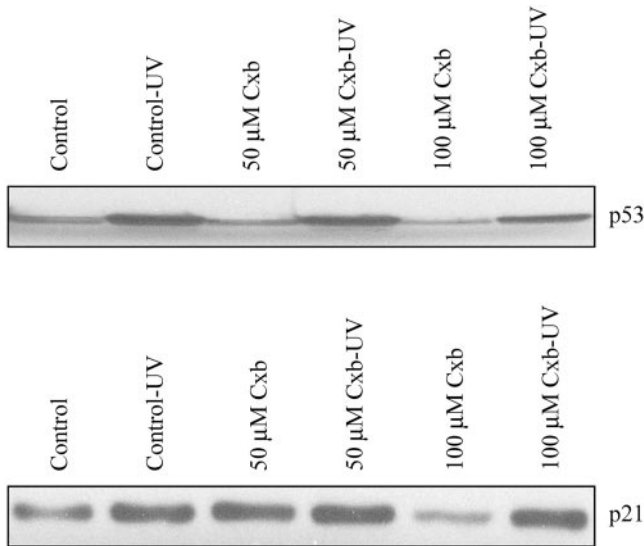


Fig. 3. p53 and p21 expression in HCT-116 cells treated with celecoxib in the presence of UV. p53 and p21 expression levels were measured 12 h after exposure to 75 J/m² of UVB.

with PGA₁ (Fig. 1A). However, p21 expression remained unchanged as a function of time and concentration of PGA₁ (data not shown). We separated the lysates into nuclear and cytosolic fractions and determined the relative distribution of p53 protein. p53 accumulated preferentially in the nucleus in both cell lines in the absence of treatment; however, in the presence of 20 μM PGA₁, p53 was significantly redistributed into the cytosol. The nuclear:cytosol ratios of p53 in the presence of PGA₁ fell from 30.9 to 2.14 in HCT-116 cells and 21.92 to 4.06 in HT-29 cells (Fig. 1B).

Effect of Celecoxib on p53 Expression and Subcellular Localization. We next studied whether celecoxib could modulate the expression and subcellular localization of p53. HCT-116 and HT-29 cells were treated with 50 or 100 μM concentrations of celecoxib for 6-, 12-, or 24-h intervals, after which p53 expression was determined for the cytosolic and nuclear fractions. p53 expression levels and

localization were not affected by celecoxib after 6 and 12 h of exposure. However, nuclear levels of p53 were substantially increased after 24 h of treatment with 100 μM in both cell lines (Fig. 2A). The nuclear and cytosol ratios of p53 increased from 30.9 (control) to 60.6 (100 μM) in HCT-116 cells and 21.9 (control) to 35.6 (100 μM) in HT-29 cells. There was no noticeable effect on p53 expression and localization in either cell line after treatment with 50 μM celecoxib. Fig. 2B shows the effect of celecoxib at different concentrations on p53 expression and localization levels with 24-h intervals in both cells.

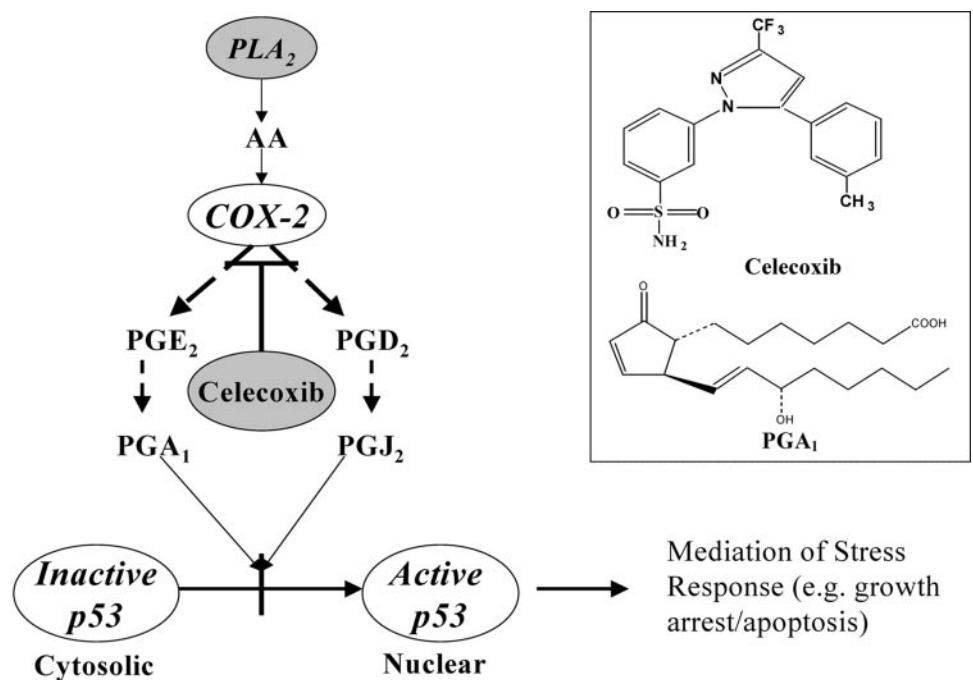
Expression of p53 and p21 Levels in the Presence of UV. We determined whether p53 activity was affected by celecoxib. To do this, the expression of p53 and p21 was measured in UV-treated cells in the presence or absence of 100 μM celecoxib in HCT-116 cells (p53 wild type). UV is a well-characterized stressor that signals for p53 activation, increased cellular levels, and consequential induction of p21 expression. p53 and p21 levels were measured 12 h after exposure to 75 J/m² UVB. Both p53 and p21 expression levels were significantly increased by UV treatment in the presence and absence of celecoxib, but celecoxib did not significantly affect the extent to which UV activated p53-induced p21 expression. (Fig. 3).

Discussion

We investigated the mechanistic relationship between COX-2 and p53 function in colon cancer cells. We have shown that electrophilic PGA₁ treatment of HCT-116 and HT-29 colon cancer cells causes p53 to localize in the cytosol in an inactive state. COX-2 overproduction of electrophilic PGs is considered to be a contributing factor in colon carcinogenesis. These results are in agreement with a previous study showing that cells exposed to electrophilic PGs accumulate p53 protein in their cytosol under basal conditions, with no evidence of oxidative/genotoxic stress (26). In a recent study, we have shown that celecoxib inhibits PGE₂ and its electrophilic metabolites in a dose-dependent manner in colon cancer cell lines (28). This information suggests that one mechanism of COX-2-induced colon carcinogenesis may involve the impairment of p53 activity by electrophilic PGs.

The inhibition of COX-2 by NSAIDs is a potentially important

Fig. 4. Celecoxib protects p53 function by inhibiting COX-2 activity and production of PGs. In this model, the aberrant cytosolic sequestration of p53 by electrophilic PGs is prevented by celecoxib. This mechanism may contribute to the colon cancer inhibitory activity of celecoxib.



mechanism in colon cancer prevention and therapy. Here, we show that celecoxib treatment of colon cancer cell lines facilitates the nuclear localization of p53 enabling its activity as a transcription factor. This effect enhances the responsiveness of p53 to DNA damage, suggesting that celecoxib sensitizes cells to p53-responsive stresses by promoting p53 localization in the nucleus. This supports the notion that celecoxib protects p53 activity by inhibiting COX-2 production of electrophilic PGs (Fig. 4).

The mechanism of p53 inhibition by PGs remains to be clarified but appears to depend on its structural derangement. Recently, one potential mechanism was reported to involve the covalent modification of TrxR by the electrophilic PGs produced by COX-2 (29). TrxR is a selenoprotein that maintains the redox state of redox-sensitive proteins, including p53, NF- κ B, and SPK kinases. The inactivation of TrxR by PGs was shown to account for the accumulation of nonfunctional p53. This potential epigenetic mechanism of p53 inactivation could occur in the early stages of colon cancer when COX-2 levels become elevated. p53 mutations do occur as a late stage event in colon cancer which may result from different selective stresses.

It has been reported that germ-line inactivation of p53 (knockout) does not increase the incidence of colon tumors in *Min*-mice (30). Our results are consistent with this finding suggesting that p53 wild-type littermates may incur p53 inactivation via a COX-2-mediated mechanism. It remains to be demonstrated, however, that PGs reach levels that significantly alter p53 activity. Such circumstances are most likely to arise focally during inflammation (31–33). Recent studies also suggest that impairment of p53 is not limited to electrophilic PGs and a number of lipid molecules possess α , β -unsaturated carbonyls derived from lipoxynase and monoxynase enzymes (29). Other mechanisms of inactivating the sequestration of p53 in to the cytosol have been reported (34), e.g., the inducible nitric oxide synthase metabolite, nitric oxide, and its reactive metabolites impair p53 sequestration into cytosol (35). It has also been shown that a p53-associated parkin-like cytoplasmic protein is expressed at abnormally high levels in neuroblastomas, where it binds the COOH terminus of p53 and prevents its nuclear import (17).

In summary, these results suggest that COX-2 metabolites at least in part contribute to the dysfunction of p53 and that a COX-2 selective inhibitor (celecoxib) protects the p53 functional activity. Additional studies are warranted to examine *in vivo* models in support of the above mechanisms.

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