Resveratrol-induced cyclooxygenase-2 facilitates p53-dependent apoptosis in human breast cancer cells

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Abstract

Cyclooxygenase-2 (COX-2) is antiapoptotic and is implicated in tumorigenesis. Recent reports, however, have also ascribed a proapoptotic action to inducible COX-2. We show here for the first time that a stilbene, resveratrol, induces nuclear accumulation of COX-2 protein in human breast cancer MCF-7 and MDA-MB-231 cell cultures. The induction of COX-2 accumulation by resveratrol is mitogen-activated protein kinase (MAPK; extracellular signal–regulated kinase 1/2)- and activator protein 1-dependent. Nuclear COX-2 in resveratrol-treated cells colocalizes with Ser^{15}-phosphorylated p53 and with p300, a coactivator for p53-dependent gene expression. The interaction of COX-2, p53, and p300, as well as resveratrol-induced apoptosis, was inhibited by a MAPK activation inhibitor, PD98059. A specific inhibitor of COX-2, NS398, and small interfering RNA knockdown of COX-2 were associated with reduced p53 phosphorylation and consequent decrease in p53-dependent apoptosis in resveratrol-treated cells. We conclude that nuclear accumulation of COX-2 can be induced by resveratrol and that the COX has a novel intranuclear colocalization with Ser^{15}-phosphorylated p53 and p300, which facilitates apoptosis in resveratrol-treated breast cancer cells. [Mol Cancer Ther 2006;5(8):2034–42]

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Introduction

Resveratrol is a naturally occurring stilbene found in grape skin and other plants (1). The compound has anticancer properties and is capable of inducing apoptosis (2) and conferring cardiovascular protection (3) and neuroprotection (4). We have reported that resveratrol induces p53-dependent apoptosis in several different cancer cell lines, including cells derived from cancer of the prostate (5, 6), thyroid (7), and breast (8). We have also described a cell surface receptor for resveratrol, which is linked to activation of p53 and apoptosis in MCF-7 human breast cancer cells (9). The mechanism of resveratrol action as an antitumor agent is known to involve posttranslational modification of p53 (5–8) and, at least in selected cell lines, activation of protein kinase C (6, 10).

Cyclooxygenase-2 (COX-2), the rate-limiting enzyme in prostaglandin synthesis, is induced in many cells by inflammatory mediators. Expression of *COX-2* in cell and animal models is associated with tumor cell growth and metastasis, enhanced cellular adhesion, and inhibition of apoptosis (11, 12). The mechanisms of these tumor-promoting actions of COX-2 are not completely known but are assumed to reflect actions of prostanoids (13). Pharmacologic inhibitors of COX-2 can decrease tumor cell growth or prevent tumorigenesis in animal models of cancer (14–16) and may decrease growth of certain human tumors (17, 18). COX-2 has been shown to modulate the transcriptional function of p53, as evidenced by suppression of p53 target gene induction by COX-2 cotransfection (19). NS-398 is a selective COX-2 inhibitor that has been shown *in vitro* to induce apoptosis in several types of human cancer cells (20, 21). COX-2 has been shown to interact with p53 *in vitro* and *in vivo*, and this is inhibited by treatment of cells with NS-398 (22). Such results suggest a novel function of COX-2, which inhibits DNA damage–induced apoptosis through direct regulation of p53 function.

However, the nature of the relationship of COX-2 protein to cancer cell growth is more complex than the foregoing evidence infers and this is suggested by reports that COX-2 may be proapoptotic (23, 24) and that pharmacologic induction of *COX*-2 expression results in inhibition of colon cancer cell growth (25). Pharmacologic inhibition of COX-2 activity in colon cancer cells has been shown to increase the nuclear localization of active p53 (26). Treatment of human glioblastoma and medulloblastoma cells with the nonsteroidal anti-inflammatory drug flurbiprofen has been shown to enhance *COX-2* expression, cause complex formation of COX-2 protein with p53, and suppress tumor growth (18). Overexpression of *COX-2* inhibits platelet-derived growth factor–induced proliferation via the induction of *p53*, as well as of *p21* (23). On the

other hand, studies by Corcoran et al. (19) indicate that p53 up-regulates COX-2 and that COX-2 in turn may negatively affect p53 activity through mechanisms that could involve physical interactions between COX-2 and p53. Thus, COX-2 and p53 have a variable and not well-explained relationship that can be associated with tumor cell growth or induction of apoptosis.

In this report, we describe a novel mechanism involved in COX-2-associated apoptosis. Acting in human breast cancer cells like a nonsteroidal anti-inflammatory drug, resveratrol induced nuclear COX-2 accumulation by an extracellular signal–regulated kinase (ERK) 1/2– and activator protein 1 (AP-1)–dependent signal transduction pathway. We found that the COX-2 that accumulated in the nucleus in these cells formed complexes with Ser^{15}-phosphorylated p53 and p300, which facilitated p53-dependent apoptosis.

Materials and Methods

Cell Culture

Human breast cancer MCF-7 cells were maintained in DMEM supplemented with 5% fetal bovine serum and MDA-MB-231 cells were maintained in DMEM supplemented with 10% fetal bovine serum in a 5% CO_2/95% air incubator at 37°C. Before the study, cells were placed in 0.25% hormone-depleted serum–supplemented medium for 2 days.

Immunoblotting

The techniques are standard and have previously been described in a number of our publications (5–8). Nucleoproteins from treated or control cells were separated on discontinuous SDS-PAGE and then transferred by electroblotting to nitrocellulose membranes (Millipore, Bedford, MA). After blocking with 5% milk in TBS containing 0.1% Tween 20, the membranes were incubated with various antibodies overnight. Secondary antibodies were either goat anti-rabbit immunoglobulin G (1:1,000; Dako, Carpenteria, CA) or rabbit anti-mouse immunoglobulin G (1:1,000; Dako), depending on the origin of the primary antibody. Immunoreactive proteins were detected by chemiluminescence.

Reverse Transcription-PCR

Total RNA was isolated as previously described (5–8). First-strand complementary DNAs were synthesized from 1 μg of total RNA using oligo(dT) and avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI). First-strand cDNA templates were amplified for *GAPDH* and *COX-2* mRNAs by PCR using a hot start (Ampliwax, Perkin-Elmer, Foster City, CA). Primer sequences were *c-fos* [5′-GAATAAGATGGCTGCAGCCAAATGCCGCAA-3′ (forward) and 5′-CAGTCAGATCAAGGGAAGCCACAGACATCT-3′ (reverse)]; and *c-jun* [5′-GGAAACGACCTTCTATGACGATGCCCTCAA-3′ (forward) and 5′-GAACCCCTCCTGCTCATCTGTCACGTTCTT-3′ (reverse)]. The PCR cycle was an initial step of 95°C for 3 minutes, followed by 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute, then 25 cycles and a final cycle of 72°C for 8 minutes. PCR products were separated by electrophoresis through 2% agarose gels containing 0.2 μg ethidium bromide/mL. Gels were visualized under UV light and photographed with Polaroid film (Polaroid Co., Cambridge, MA). Photographs were scanned under direct light for quantitation and illustration. Results from PCR products were normalized to the *GAPDH* signal.

Confocal Microscopy

Exponentially growing MCF-7 and MDA-MB-231 breast cancer cells were treated with resveratrol for 24 hours, then spun onto glass microscope slides previously precoated with Histogrip (Zymed/Invitrogen, Carlsbad, CA). The samples were immediately fixed with 5% formaldehyde in acetone for 20 minutes. Cells were permeabilized in 100% methanol for 10 minutes and rehydrated in 90% methanol for 30 minutes. The cells on the slides were incubated with anti-COX-2 antibody (green; Cayman, Ann Arbor, MI) and a propidium iodide stain for nucleus (red) and the signal was revealed using the Histostain SP kit as recommended by the manufacturer (Zymed). To detect phosphorylated p53 and COX-2 colocalization, the cells on the slides were first incubated with 5% rabbit immunoglobulin G (Zymed) in PBS to block all nonspecific sites. This was followed by coincubation with a FITC-conjugated COX-2 antibody (mouse immunoglobulin G; Cayman) and a phospho-Ser^{15}-p53 antibody (rabbit immunoglobulin G; Cell Signaling Technology, Danvers, MA) at 37°C for 2 hours. The fluorescent signals from COX-2 (red) and phospho-Ser^{15}-p53 (green) were analyzed with a laser scanning confocal microscope (Carl Zeiss, Inc., Thornwood, NY). The figures shown are representative of four fields for each experimental condition. Magnification was ×250.

Chromatin Immunoprecipitation

A total of 6×10^6 cells were exposed to 1% formaldehyde for 15 minutes at room temperature to effect cross-linking. Monolayers were washed twice with PBS. Extracts were prepared by scraping cells in 1 mL of buffer [150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mmol/L Tris (pH 8), 5 mmol/L EDTA] that contained the protease inhibitors leupeptin (10 μg/mL) and pepstatin A (10 μg/mL), phosphatase inhibitors (50 mmol/L NaF and 0.2 mmol/L sodium orthovanadate), and the deacetylase inhibitor trichostatin A (5 μmol/L; Calbiochem, San Diego, CA). Cell lysates were sonicated to yield chromatin fragments of ~600 bp as assessed by agarose gel electrophoresis. Immunoprecipitation was done with c-Jun antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Primers used in PCR were promoters of COX-2 (Cayman). PCR products were resolved using 8% polyacrylamide gels (acrylamide/bis-acrylamide, 19:1) in 1× Tris-acetate-EDTA buffer. Gels were stained with ethidium bromide. Relative levels of DNA were determined with QuantifyOne software (Bio-Rad, Hercules, CA).

Transfection of Small Interfering RNA

The small interfering RNA (siRNA) of COX-2 (PGHS2) and scrambled RNA (scRNA) were purchased from Ambion (Austin, TX). MCF-7 cells were seeded onto six-well tissue culture plates at 60% to 80% confluence and in the absence of antibiotic for 24 hours before transfection.

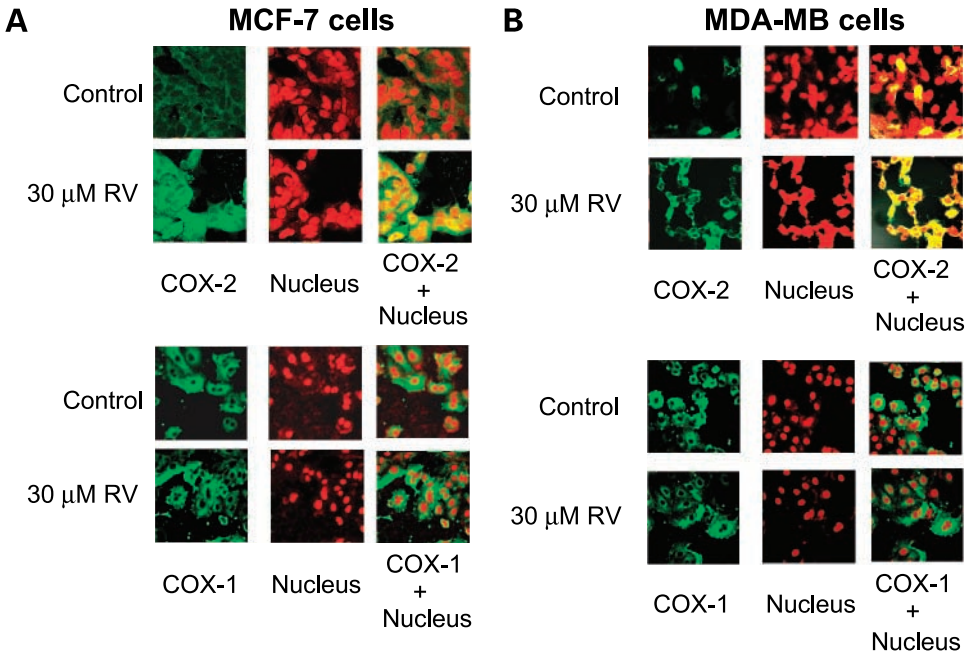

Figure 1. Resveratrol (*RV*) induces COX-2 accumulation in human breast cancer cells. MCF-7 cells (**A**) and MDA-MB-231 cells (**B**) seeded on cover slides were exposed to 0.25% hormone–depleted fetal bovine serum–containing DMEM for 48 h before starting the experiments. Cell cultures were treated with 30 μmol/L resveratrol for 24 h. COX-2 (*top*) and COX-1 (*bottom*) were immunostained with anti-COX-2 or anti-COX-1 antibodies (Cayman) and evaluated by confocal microscopy. Nuclei were stained with propidium iodide. COX-2 was evident in the cytosol of cells treated with solvent alone (*Control*) whereas resveratrol (30 μmol/L, 24 h) caused COX-2 accumulation in nuclei indicated by the yellow color. There was no effect of resveratrol on COX-1 accumulation. Magnification, ×250.

Immediately before transfection, the culture medium was removed and the cells washed once with PBS, then transfected with either scRNA or siRNA (0.2 μg/well) using Oligofectamine (2 μg/well) in Opti-MEM I medium according to the instructions of the manufacturer (Ambion). After transfection, cultures were incubated at 37°C for 4 hours and then placed in fresh culture medium. After an additional 24 hours, cells were used for experimentation.

AP-1 Decoy Oligodeoxynucleotide Technique

Transcription factor decoy oligomers were synthesized *in vitro* by annealing the complementary strands in 1× annealing buffer [20 μmol/L Tris-HCl (pH 7.5), 20 μmol/L $MgCl_2$, and 50 μmol/L NaCl]. The mixture was heated to 80°C and allowed to cool to room temperature slowly over 3 hours. The AP-1 decoy sequence [(−173)-5′-TCATTTGCGTGAGTAAAGCCTGCC-3′-(−149);

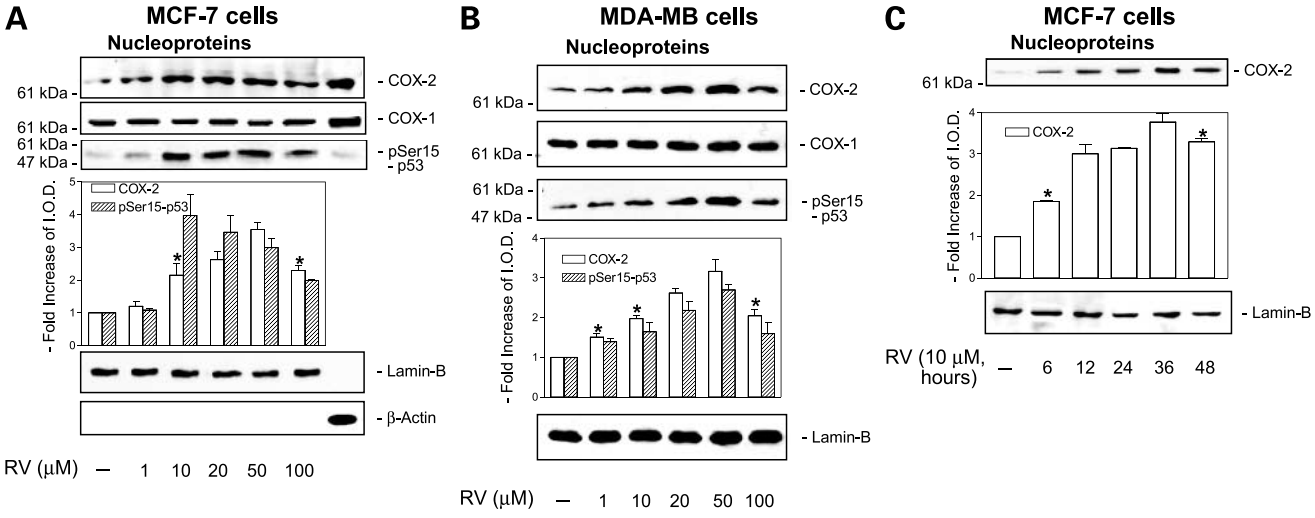

Figure 2. Resveratrol-induced COX-2 expression in breast cancer cells is concentration and time dependent. **A,** MCF-7 cells were treated with 1 to 100 μmol/L resveratrol for 24 h. Nucleoprotein fractions were separated by gel electrophoresis, electrotransferred to membranes, and immunoblotted with antibodies to COX-2, COX-1, or Ser^{15}-phosphorylated p53 (*pSer15-p53*). The right lane indicates cytosol proteins of cells treated with 100 μmol/L resveratrol. **B,** MDA-MB-321 cells were treated with 1 to 100 μmol/L resveratrol for 24 h. Nucleoprotein fractions were separated by gel electrophoresis, electrotransferred to membranes, and immunoblotted with antibodies to COX-2, COX-1, or Ser^{15}-phosphorylated p53. **C,** MCF-7 cells were treated with resveratrol (10 μmol/L) for 6 to 48 h, after which time nuclear COX-2 accumulation was measured by gel electrophoresis and immunoblotting. Nuclear protein Lamin B was used as an internal loading control. *, $P < 0.05$.

ref. 27] and a double-stranded DNA with a scrambled sequence (5′-CAGGAGAGTATCCTGCGATGCATCTGCT-3′), used as a control in the decoy experiments, were custom synthesized by Invitrogen. To be efficiently delivered to the cells, the double-stranded oligonucleotides (1 μmol/L) were mixed with LipofectAMINE reagent (Invitrogen) and incubated at room temperature for 30 minutes. The mixture was then added to the cell cultures at 200 nmol/L for oligonucleotides and at 5 μmol/L for the Lipofect-AMINE reagent for 6 hours. The cells were further incubated with resveratrol (10 μmol/L) for 16 hours and then washed. Nuclear proteins were harvested and subjected to Western blot analysis for human COX-2.

Apoptosis/Nucleosomes

The medium was harvested and washed twice with PBS. Nucleosome ELISA assays are carried out according to the protocol provided by Oncogene Research Products (Cambridge, MA). Pelleted cells were lysed. Supernatants were collected and stored for at least 18 hours at −20°C. From each appropriately diluted sample, 100 μmol/L were added to a 96-well plate coated with a DNA binding protein and incubated at room temperature for 3 hours. After three washes with wash buffer, detector antibody was added for 1 hour. Streptavidin conjugate was added and incubated for 0.5 hour before adding substrate. Plates were read at 450 nm.

Quantification of Results and Statistical Analysis

Data were quantified by phosphoimaging and with ImageQuant software on a Storm 860 Phosphoimager (Molecular Dynamics, Sunnyvale, CA). Statistical significance was determined with the Student's t test. $P < 0.05$ was considered significant.

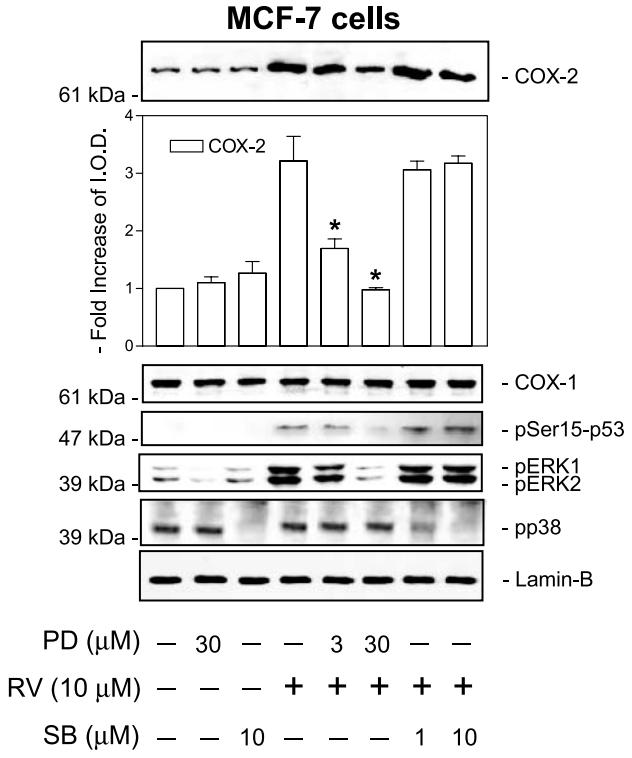

Figure 3. Activation of ERK1/2 is essential for resveratrol-induced COX-2 expression in MCF-7 cells. Resveratrol-induced COX-2 accumulation was inhibited by PD98059 [*PD*; MAPK (ERK1/2) signal transduction pathway inhibitor; *, $P < 0.05$], but not by SB203580 (*SB*; p38 pathway inhibitor). Abundance of COX-1 was not affected by resveratrol either in the presence or absence of inhibitors.

Results

Resveratrol Induces COX-2 Expression in Human Breast Cancer Cells

In confocal microscopy studies, 30 μmol/L resveratrol treatment of human breast cancer MCF-7 cells for 24 hours led to an increase in nuclear COX-2 protein in 24 hours (Fig. 1A, *top*). The treatment of resveratrol did not increase COX-1 content (Fig. 1A, *bottom*). When another breast cancer MDA-MB-231 cell line was treated with 30 μmol/L resveratrol, there was an increased accumulation of COX-2 (Fig. 1B, *top*) in nuclei and COX-1 was not affected by resveratrol treatment (Fig. 1B, *bottom*).

In cell fractionation experiments, the accumulation of COX-2 in nuclei of MCF-7 cells was readily apparent at 10 μmol/L resveratrol (24 hours) and was increased further by higher concentrations of the stilbene (Fig. 2A). In contrast, nuclear COX-1 levels were unaffected by resveratrol (Fig. 2A). There was no change in the nuclear laminin protein marker (Lamin B) with resveratrol treatment (Fig. 2A). Anti-actin antibody was used to exclude contamination of the nuclear preparations with cytosolic protein (Fig. 2A). Increase in abundance of nuclear COX-2 was accompanied by accumulation of nuclear Ser^{15}-phosphorylated p53 in response to resveratrol treatment (Fig. 2A). The induction of COX-2 by resveratrol in a concentration-dependent manner was also shown in MDA-MB-231 cells (Fig. 2B). In time-course studies, the resveratrol-induced increase in nuclear COX-2 was readily apparent in 6 hours, with a maximal effect seen by 24 to 36 hours (Fig. 2C).

Activated Mitogen-Activated Protein Kinase and AP-1 Are Involved in Resveratrol-Induced COX-2 Accumulation

We have previously reported an essential role of mitogen-activated protein kinase (MAPK), specifically ERK1/2, in resveratrol-induced Ser^{15} phosphorylation of p53 and resulting apoptosis in MCF-7 cells (8). In the studies illustrated in Fig. 3, MCF-7 cells were treated with 10 μmol/L resveratrol in the presence of the MAPK kinase inhibitor PD98059 for 24 hours. The effect of this agent on p53 phosphorylation supported the role of ERK1/2 in this action. In addition, levels of nuclear COX-2 progressively decreased in cells treated with resveratrol and 3 or 30 μmol/L PD98059, indicating that COX-2 accumulation in nuclei was also MAPK-dependent.

Basal-level p38 phosphorylation is seen in Fig. 3, and this was not increased by resveratrol. This level of p38 phosphorylation in MCF-7 cells was reduced by the p38

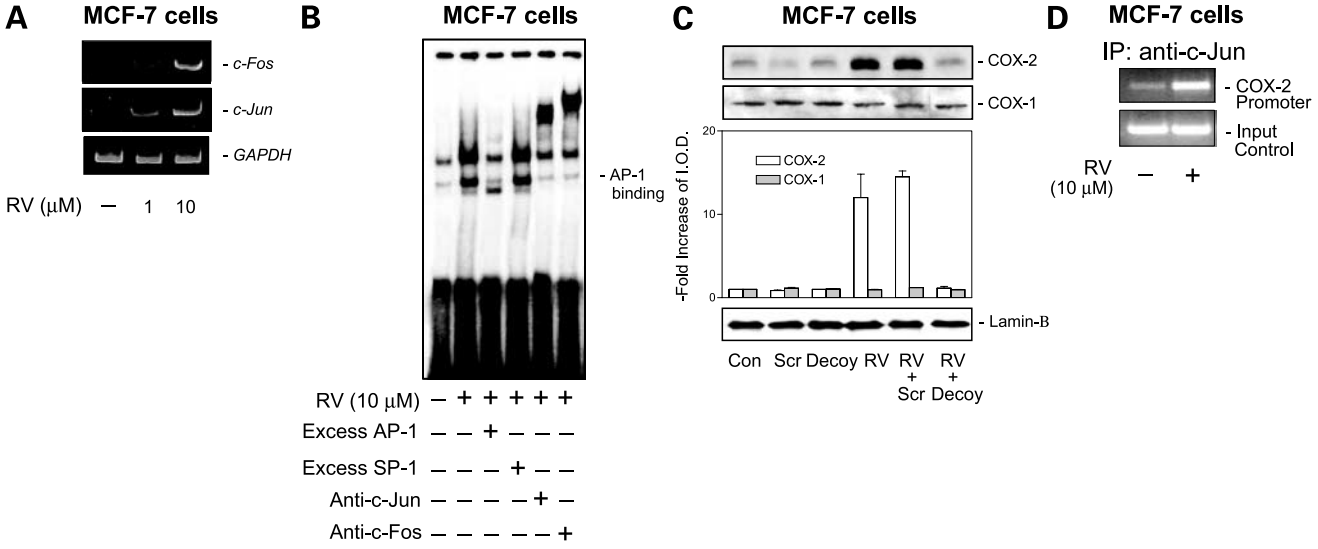

Figure 4. Role of AP-1 in resveratrol-induced COX-2 accumulation in MCF-7 cells. **A,** cells were treated with resveratrol (1 – 10 μmol/L) for 8 h and reverse transcription-PCR using *c-fos, c-jun*, and *GAPDH* was done. Induction of c-fos and c-jun was resveratrol concentration-dependent. *GAPDH* was used as an internal control. **B,** cells were treated with 10 μmol/L resveratrol for 8 h and nuclear fractions examined by electrophoretic mobility shift assay to assess binding of heterodimeric AP-1 protein to a radiolabeled AP-1 oligonucleotide. Excess unlabeled AP-1 or control SP-1 oligonucleotides were used in separate samples to determine specificity of binding. **C,** cells were treated with 10 μmol/L resveratrol for 24 h. Resveratrol-induced *COX-2* expression was blocked by the presence of an AP-1 decoy oligonucleotide. Expression of COX-1 was unchanged. *Decoy,* decoy oligonucleotide; *Scr,* scrambled oligonucleotide. **D,** in a chromatin immunoprecipitation (*IP*) study with anti-c-Jun antibody, resveratrol (10 μmol/L, 24 h) increased AP-1 binding to the COX-2 promoter in MCF-7 cell samples compared with samples from cells not exposed to resveratrol.

inhibitor SB203580 (10 μmol/L; Biosource International, Camarillo, CA), as expected. Despite this reduction in phosphorylated p38 in cells treated with SB203580, COX-2 levels were unchanged from those seen with resveratrol alone. These findings suggest that p38 activation is not directly related to the increased COX-2 expression induced by resveratrol.

We have previously shown that resveratrol promotes accumulation of c-Fos and c-Jun in thyroid cancer cells (7). In the present MCF-7 studies, 10 μmol/L resveratrol treatment for 24 hours also induced expression of *c-fos* and *c-jun* (Fig. 4A). Electrophoretic mobility shift assays, using an AP-1-specific oligonucleotide, showed that resveratrol caused AP-1 activation in MCF-7 cells (Fig. 4B). Excess unlabeled AP-1-binding, but not SP-1-binding, oligonucleotides displaced the binding of AP-1 protein by labeled AP-1-binding oligonucleotides. Supershift assays with anti-c-Fos and anti-c-Jun confirmed that AP-1 protein bound to AP-1-responsive oligonucleotides (Fig. 4B). Because AP-1 has been implicated in the induction of *COX-2* expression by cytokines and growth factors (28), we speculated that AP-1 was integral to induction by resveratrol of *COX-2* expression. Cells were treated with resveratrol in the presence or absence of AP-1 decoy or a scrambled oligonucleotide. Results presented in Fig. 4C indicate that AP-1 indeed is essential for resveratrol-induced COX-2 expression. Further, chromatin immunoprecipitation studies using antibody to c-Jun showed increased AP-1 binding to the *COX-2* promoter region in resveratrol-treated cells (Fig. 4D).

Resveratrol-Induced COX-2 Nuclear Accumulation Is Essential for Resveratrol-Induced p53-Dependent Apoptosis

Apoptosis of cancer cells induced by resveratrol requires posttranslational modification of p53, including phosphorylation and acetylation (8). Ser^{15} phosphorylation of p53 is dependent on the activation of MAPKs (ERKs; refs. 6, 8); subsequent to phosphorylation, the modified p53 translocates to cell nuclei, as do activated ERK1/2. These signaling steps are illustrated in confocal microscopy studies (Fig. 5A) and show that COX-2 that accumulated in the nucleus colocalized with activated (Ser^{15}-phosphorylated) p53 in resveratrol-treated cells. In addition, when nuclear extracts of resveratrol-treated cells were immunoprecipitated with anti-COX-2 antibody, and the immunoprecipitated proteins then separated by gel electrophoresis, a complex of COX-2 and Ser^{15}-phosphorylated p53 was identified in resveratrol-treated MCF-7 cells (Fig. 5B). We also observed nuclear complexing of the coactivator protein p300 with COX-2 in resveratrol-exposed cells (Fig. 5B). Because p300 is essential for p53-dependent transcription (28), these observations are consistent with the concept that inducible COX-2 is involved in or modulates the transcriptional activity of p53. We have reported elsewhere that Ser^{15} phosphorylation of p53 by ERK1/2 in resveratrol-treated tumor cells is a requirement for apoptosis (5–8). We found here that the nuclear associations of COX-2 and p300 and of COX-2 and p53 were inhibited by the MAPK kinase inhibitor PD98059 (Fig. 5C), inferring the

involvement of the MAPK pathway in the formation of these complexes. Results of apoptosis studies also indicated that PD98059 blocked resveratrol-induced apoptosis in MCF-7 cells (Fig. 5D).

COX-2 *siRNA* was then used to reduce cellular COX-2 content. In scrambled RNA–transfected cells, resveratrol induced nuclear accumulation of COX-2 as seen in untransfected cells (Fig. 6A). As expected, COX-2 *siRNA* transfection reduced COX-2 accumulation (Fig. 6A). Resveratrol-induced Ser^{15} phosphorylation was not diminished in control and scrambled *RNA*–transfected cells; however, in cells with reduced levels of COX-2, resveratrol-induced Ser^{15} phosphorylation of p53 was reduced, as was apoptosis (Fig. 6A). Additional evidence that resveratrol-induced COX-2 accumulation is essential for p53-dependent apoptosis was developed in MCF-7 cells that were treated with 10 μmol/L resveratrol in the presence of the specific COX-2 inhibitor NS398 (1–10 μmol/L; refs. 13, 14). Resveratrol-induced Ser^{15} phosphorylation of p53 was blocked by NS398 (Fig. 6B). NS398 inhibited resveratrol-induced COX-2 expression in MCF-7 cells and this is in agreement with other reports showing that NS398 reduces COX-2 abundance (29, 30). Resveratrol-induced apoptosis in MCF-7 cells was also examined in the presence of NS398; results indicate that this COX-2 inhibitor, but not a nonspecific COX inhibitor, indomethacin, blocked resveratrol-induced apoptosis (Fig. 6B). These results suggest that inducible COX-2 is essential for p53-dependent apoptosis in resveratrol-treated MCF-7 cells.

Discussion

Resveratrol has been reported to inhibit *COX-2* expression induced by carcinogens in human breast and oral epithelial

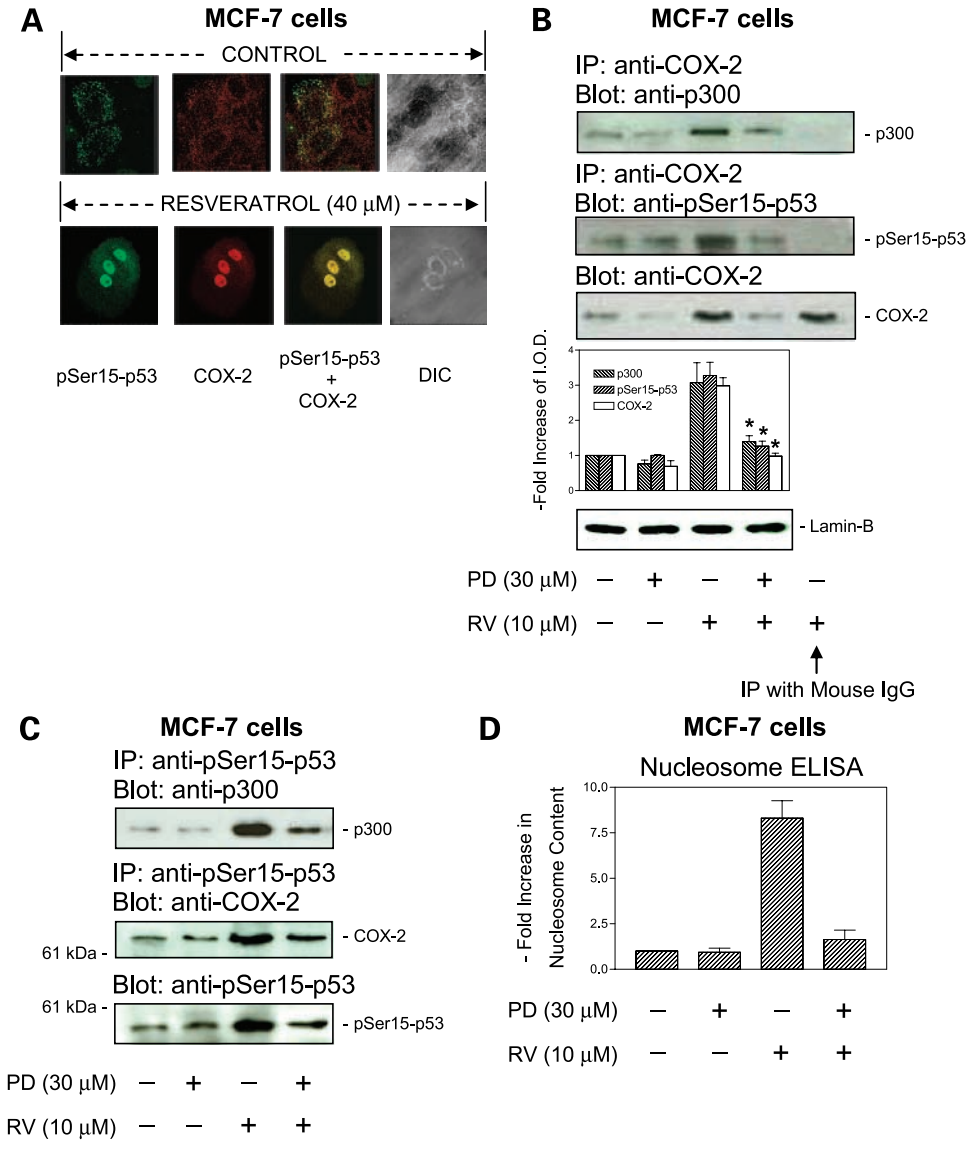

Figure 5. Inducible COX-2 forms a nuclear complex with p53 and p300 in resveratrol-treated cells. **A,** MCF-7 cells were treated with 40 μmol/L resveratrol for 24 h. Confocal microscopy showed colocalization of Ser^{15}-phosphorylated p53 and COX-2 in the nuclei of resveratrol-treated cells (yellow color). Magnification, ×250. **B,** MCF-7 cells were treated with 10 μmol/L resveratrol in the presence or absence of PD98059 (30 μmol/L) for 24 h. Nuclear extracts were immunoprecipitated with anti-COX-2 antibody and immunoprecipitates were separated by SDS-PAGE. Western blots were done with anti-p300 or anti–phospho-Ser^{15}-p53 antibodies. Nuclear extracts were also immunoprecipitated with mouse immunoglobulin G as a negative control. Before immunoprecipitation, 30 μL of nuclear extract from each sample were set aside as nucleoprotein controls for the immunoprecipitation step. Resveratrol increased nuclear complexing of COX-2, Ser^{15}-phosphorylated p53, and p300; this effect was inhibited by PD98059 (*, $P < 0.05$). **C,** nuclear extracts were immunoprecipitated with anti–phospho-Ser^{15}-p53 antibody and immunoprecipitates were separated by SDS-PAGE. Western blots were done with anti-p300 or anti-COX-2 antibodies. Resveratrol increased nuclear complexing of COX-2, Ser^{15}-phosphorylated p53, and p300; this effect was inhibited by PD98059. **D,** apoptosis was measured by nucleosome ELISA. Resveratrol caused 7-fold increases in nucleosome content, indicating apoptosis, and this effect was inhibited by PD98059. PD98059 alone had no significant effect on apoptosis.

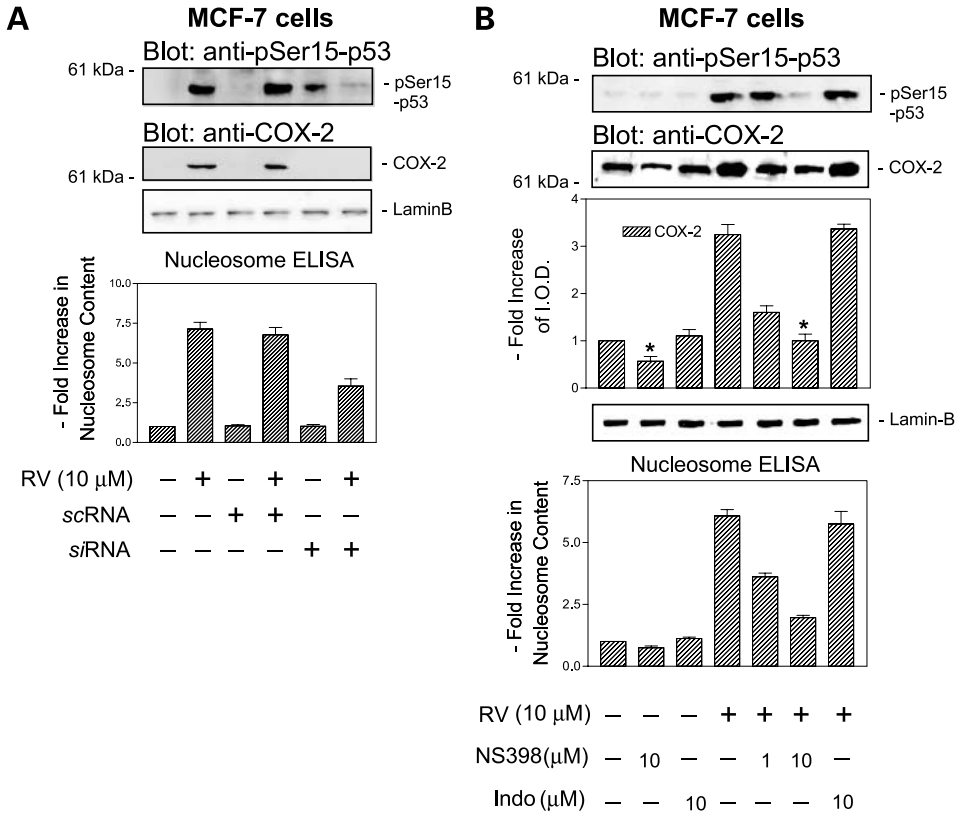

Figure 6. COX-2 is required for resveratrol-induced p53 phosphorylation and apoptosis to occur in MCF-7 cells. **A,** MCF-7 cells transfected with a COX-2-specific siRNA were then treated with 10 μmol/L resveratrol for 24 h, resulting in reduced COX-2 levels in nuclei of cells with the siRNA, and inhibition of the resveratrol effect on Ser^{15}-p53 phosphorylation and apoptosis in COX-2-deficient cells. **B,** MCF-7 cells treated with the COX-2-specific inhibitor NS398 (1 – 10 μmol/L) and 10 μmol/L resveratrol for 24 h showed inhibition of resveratrol-induced Ser^{15}-phosphorylated p53 and COX-2 accumulation in nuclei (*, $P < 0.05$) and inhibition of apoptosis. NS398 itself inhibited COX-2 accumulation in untreated MCF-7 cells (*, $P < 0.05$). In contrast, the nonspecific COX inhibitor indomethacin (10 μmol/L) did not affect resveratrol-induced Ser^{15}-phosphorylated p53 accumulation or apoptosis.

cells (31, 32). Resveratrol is a specific inhibitor of COX-1 (33) and has been suggested to be a COX-2 inhibitor.[5] That resveratrol can induce COX-2 expression in cancer cells has not previously been appreciated. We show here for the first time that resveratrol is capable of inducing *COX-2* expression in an established cancer cell line. The intracellular distribution of COX-2 protein has previously been thought to be largely perinuclear and in endoplasmic reticulum based on studies in fibroblasts and endothelial cells (34). Our results indicate that resveratrol-induced COX-2 also localizes in nuclei of cancer cells (Figs. 1 and 2). The presence of COX-2 in nuclei of resveratrol-treated human tumor cells was shown using a propidium iodide stain (Fig. 1A and B). The COX-2 in resveratrol-treated cells seems to be evenly distributed throughout the nuclei, except for its absence from nucleoli, rather than distributed along the nuclear membrane, as described by Morita et al. (34) in noncancer cells.

Resveratrol-induced *COX-2* expression was inhibited by a MAPK kinase inhibitor, PD98059 (Figs. 3 and 5B, C, and D). The latter agent also blocked resveratrol-induced p53 activation (Fig. 3) in MCF-7 cells, as it does in thyroid and prostate cancer cell lines (5–7). Kim et al. (35) showed that phytosphingosine derivatives, *N*-acetyl phytosphingosine and tetra-acetyl phytosphingosine, elevate *COX-2* expression via tyrosine kinase and protein kinase C, with subsequent ERK activation. p38 is not involved in *N*-acetyl phytosphingosine–mediated *COX-2* expression but does play a role in tetra-acetyl phytosphingosine–mediated expression. Other reports indicate that induction of COX-2 is ERK- and p38-dependent (36, 37). We did not find p38 dependence of COX-2 accumulation in resveratrol-treated MCF-7 cells (Fig. 3). Resveratrol increased DNA binding of AP-1, which is essential for resveratrol-induced COX-2 expression in MCF-7 cells (Fig. 4). Similarly, Li et al. (38) reported that treatment of cultured rat pheochromocytoma (PC12) cells with sodium nitroprusside, a nitric oxide–releasing compound, up-regulates COX-2, and this up-regulation is blocked by pretreatment with *c-fos* antisense oligonucleotide.

Resveratrol-induced COX-2 accumulation in our studies was associated with an increase in nuclear accumulation of Ser^{15}-phosphorylated p53 (Fig. 5A). Direct interaction of p53 and COX-2 was confirmed by coimmunoprecipitation of these two proteins in nuclear fractions of cells, and p300 was also found to be present in the complex (Fig. 5B). These are shown to be ERK1/2-dependent actions of resveratrol. Continuous (constitutive) overexpression of exogenous COX-2 in cells containing $p53^{wt}$ does not seem to affect cytoplasmic or nuclear levels of p53 (19). However, inhibition of COX-2 activity by nonsteroidal anti-inflammatory drugs has been shown to increase accumulation in the nucleus of both COX-2 and p53 (18). These results

[5] H.J. Cao et al., unpublished observations.

suggest that there are different mechanisms of p53 accumulation when the latter is the result of constitutively overexpressed COX-2, such as that obtained with plasmid transfection, and when COX-2 is induced by either a nonsteroidal anti-inflammatory drug or resveratrol.

Inhibition of COX-2 activity by NS389 suppressed not only p53 phosphorylation but also p53-dependent apoptosis in MCF-7 breast cancer cells (Fig. 6). These results agree with the studies by Munkarah et al. (39) showing that NS398 inhibits paclitaxel-induced apoptosis in epithelial ovarian cancer cell lines. Thus, from the standpoint of cancer cell growth, COX-2 seems to have dual actions: *constitutive* expression of COX-2 is growth-promoting, but *inducible* nuclear COX-2 is a factor that supports resveratrol-induced, p53-dependent apoptosis. We have begun to address the functions of nuclear COX-2 in the present studies. We find that complexes of COX-2 with other nucleoproteins can be recovered in stilbene-treated cancer cells and that a specific inhibitor of COX-2 disrupts p53-mediated apoptosis that is initiated by resveratrol. The interactions in the nucleus of COX-2 with phosphorylated p53 and with the nuclear coactivator protein p300 suggest that roles exist for COX-2 in the nucleus that may involve transcription.

In addition to COX-2 expression, sodium nitroprusside also induces apoptosis (38). Pretreatment with a selective COX-2 inhibitor (SC58635) and COX-2 siRNA rescues PC12 cells from apoptotic cell death induced by nitric oxide and suggests that excessive nitric oxide production during inflammation can induce apoptosis in PC12 cells through up-regulation of COX-2 expression. Our results also indicate that interference with COX-2 activity by NS398, or by siRNA knockdown of COX-2, reduces resveratrol-induced apoptosis (Fig. 6). These results testify to the importance of COX-2 in the nucleus but do not distinguish between possible activity of the protein in transcription and enzymatic (prostaglandin-producing) activity.

Treatment of cells with ceramide causes a significant arrest in G_0-G_1 phase of the cell cycle (40) and induces apoptosis (41). Ramer et al. (37) have shown that *R*(+)-methanandamide induces COX-2 expression in human neuroglioma cells via production of ceramide. Treatment of human mammary epithelial cells with C_2-ceramide increases levels of COX-2 protein and mRNA and enhances prostaglandin E_2 synthesis (42). Recently, other studies have shown that resveratrol induces apoptosis through a ceramide signal transduction pathway (43, 44). It is therefore reasonable to speculate that the induction by resveratrol of COX-2–dependent apoptosis requires ceramide signaling.

In summary, in addition to activation of p53-dependent apoptosis, resveratrol induces COX-2 expression in breast cancer cells. Resveratrol-induced COX-2 studied here is associated with apoptosis. Stilbene-induced nuclear COX-2 forms complexes with the transactivator p53 and coactivator p300, suggesting that COX-2 protein in the nucleus may play a role in p53-responsive gene transcription that is proapoptotic. Recently, we have shown that COX-2 can bind to its own promoter and induce COX-2 expression in cancer cells.[6] In contrast, constitutive overexpression of *COX-2* in cancer cells may be the basis for cancer growth promotion by the COX (11–18).

[6] Unpublished observations.

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References

1. Jang M, Cai L, Udeani GO, et al. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science 1997;275:218–20.

2. Dong Z. Molecular mechanism of the chemopreventive effect of resveratrol. Mutat Res 2003;523–4:145–50.

3. Bradamante S, Barenghi L, Villa A. Cardiovascular protective effects of resveratrol. Cardiovasc Drug Rev 2004;22:169–88.

4. Virgili M, Contestabile A. Partial neuroprotection of *in vivo* excitotoxic brain damage by chronic administration of the red wine antioxidant agent, *trans*-resveratrol in rats. Neurosci Lett 2000;281:123–6.

5. Lin HY, Shih A, Davis FB, et al. Resveratrol-induced serine phosphorylation of p53 causes apoptosis in a mutant p53 prostate cancer cell line. J Urol 2002;168:748–55.

6. Shih A, Zhang S, Cao HJ, et al. Inhibitory effect of epidermal growth factor on resveratrol-induced apoptosis in prostate cancer cells is mediated by protein kinase C-α. Mol Cancer Ther 2004;3:1355–63.

7. Shih A, Davis FB, Lin HY, Davis PJ. Resveratrol induces apoptosis in thyroid cancer cell lines via a mitogen-activated protein kinase- and p53-dependent mechanism. J Clin Endocrinol Metab 2002;87:1223–32.

8. Zhang S, Cao HJ, Davis FB, Tang HY, Davis PJ, Lin HY. Oestrogen inhibits resveratrol-induced posttranslational modification of p53 and apoptosis in breast cancer cells. Br J Cancer 2004;91:178–85.

9. Lin HY, Lansing L, Merillon J-M, et al. Integrin $\alpha_V\beta_3$ contains a receptor site for resveratrol. FASEB J. Express, in press, August 2006.

10. Aggarwal BB, Bhardwaj A, Aggarwal RS, Seeram NP, Shishodia S, Takada Y. Role of resveratrol in prevention and therapy of cancer: preclinical and clinical studies. Anticancer Res 2004;24:2783–840.

11. van der Donk WA, Tsai AL, Kulmacz RJ. The cyclooxygenase reaction mechanism. Biochemistry 2002;41:15451–8.

12. Dixon DA. Dysregulated post-transcriptional control of COX-2 gene expression in cancer. Curr Pharm Des 2004;10:635–46.

13. Hla T, Bishop-Bailey D, Liu CH, Schaefers HJ, Trifan OC. Cyclooxygenase-1 and -2 isoenzymes. Int J Biochem Cell Biol 1999;31:551–7.

14. Evans JF. Rofecoxib (Vioxx), a specific cyclooxygenase-2 inhibitor, is chemopreventive in a mouse model of colon cancer. Am J Clin Oncol 2003;26:S62–5.

15. Ruegg C, Zaric J, Stupp R. Non-steroidal anti-inflammatory drugs and COX-2 inhibitors as anti-cancer therapeutics: hypes, hopes and reality. Ann Med 2003;35:476–87.

16. Yao M, Lam EC, Kelly CR, Zhou W, Wolfe MM. Cyclooxygenase-2 selective inhibition with NS-398 suppresses proliferation and invasiveness and delays liver metastasis in colorectal cancer. Br J Cancer 2004;90:712–9.

17. Bol DK, Rowley RB, Ho CP, et al. Cyclooxygenase-2 overexpression in the skin of transgenic mice results in suppression of tumor development. Cancer Res 2002;62:2516–21.

18. King JG, Jr., Khalili K. Inhibition of human brain tumor cell growth by the anti-inflammatory drug, flurbiprofen. Oncogene 2001;20:6864–70.

19. Corcoran CA, He Q, Huang Y, Sheikh MS. Cyclooxygenase-2 interacts with p53 and interferes with p53-dependent transcription and apoptosis. Oncogene 2005;24:1634–40.

20. Gao J, Niwa K, Sun W, et al. Non-steroidal anti-inflammatory drugs inhibit cellular proliferation and up-regulate cyclooxygenase-2 protein expression in endometrial cancer cells. Cancer Sci 2004;95:901–7.

21. Liu XH, Yao S, Kirschenbaum A, Levine AC. NS398, a selective cyclooxygenase-2 inhibitor, induces apoptosis and down-regulates bcl-2 expression in LNCaP cells. Cancer Res 1998;58:4245 – 9.

22. Choi EM, Heo JI, Oh JY, et al. COX-2 regulates p53 activity and inhibits DNA damage-induced apoptosis. Biochem Biophys Res Commun 2005;328:1107 – 12.

23. Zahner G, Wolf G, Ayoub M, et al. Cyclooxygenase-2 overexpression inhibits platelet-derived growth factor-induced mesangial cell proliferation through induction of the tumor suppressor gene p53 and the cyclin-dependent kinase inhibitors p21waf-1/cip-1 and p27kip-1. J Biol Chem 2002;277:9763 – 71.

24. Trifan OC, Smith RM, Thompson BD, Hla T. Overexpression of cyclooxygenase-2 induces cell cycle arrest. Evidence for a prostaglandin-independent mechanism. J Biol Chem 1990;274:34141 – 7.

25. Williams JL, Nath N, Chen J, et al. Growth inhibition of human colon cancer cells by nitric oxide (NO)-donating aspirin is associated with cyclooxygenase-2 induction and β-catenin/T-cell factor signaling, nuclear factor-κB, and NO synthase inhibition: implications for chemoprevention. Cancer Res 2003;63:7613 – 8.

26. Swamy MV, Herzog CR, Rao CV. Inhibition of COX-2 in colon cancer cell lines by celecoxib increases the nuclear localization of active p53. Cancer Res 2003;63:5239 – 42.

27. Adderley SR, Fitzgerald DJ. Oxidative damage of cardiomyocytes is limited by extracellular regulated kinases 1/2-mediated induction of cyclooxygenase-2. J Biol Chem 1999;274:5038 – 46.

28. Kobet E, Zeng X, Zhu Y, Keller D, Lu H. MDM2 inhibits p300-mediated p53 acetylation and activation by forming a ternary complex with the two proteins. Proc Natl Acad Sci U S A 2000;97:12547 – 52.

29. Yao R, Rioux N, Castonguay A, You M. Inhibition of COX-2 and induction of apoptosis: two determinants of nonsteroidal anti-inflammatory drugs' chemopreventive efficacies in mouse lung tumorigenesis. Exp Lung Res 2000;26:731 – 42.

30. Rafiee P, Heidemann J, Ogawa H, et al. Cyclosporin A differentially inhibits multiple steps in VEGF induced angiogenesis in human microvascular endothelial cells through altered intracellular signaling. Cell Commun Signal 2004;2:3.

31. Subbaramaiah K, Chung WJ, Michaluart P, Tanabe T, Telang N, Dannenberg AJ. Resveratrol inhibits cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells. J Biol Chem 1998;273:21875 – 82.

32. Banerjee S, Bueso-Ramos C, Aggarwal BB. Suppression of 7,12-dimethyl-benz(*a*)anthracene-induced mammary carcinogenesis in rats by resveratrol: role of nuclear factor-κB, cyclooxygenase 2, and matrix metalloprotease 9. Cancer Res 2002;62:4945 – 54.

33. Szewczuk LM, Forti L, Stivala LA, Penning TM. Resveratrol is a peroxidase-mediated inactivator of COX-1 but not COX-2: a mechanistic approach to the design of COX-1 selective agents. J Biol Chem 2004;279: 22727 – 37.

34. Morita I, Schindler M, Regier MK, et al. Different intracellular locations for prostaglandin endoperoxide H synthase-1 and -2. J Biol Chem 1995; 270:10902 – 8.

35. Kim HJ, Shin W, Park CS, Kim HO, Kim TY. Differential regulation of cyclooxygenase-2 expression by phytosphingosine derivatives, NAPS and TAPS, and its role in the NAPS or TAPS-mediated apoptosis. J Invest Dermatol 2003;21:1126 – 34.

36. Subbaramaiah K, Hart JC, Norton L, Dannenberg AJ. 2000. Microtubule-interfering agents stimulate the transcription of cyclo-oxygenase-2. Evidence for involvement of ERK1/2 and p38 mitogen-activated protein kinase pathways. J Biol Chem 2000; 275:14838 – 45.

37. Ramer R, Weinzierl U, Schwind B, Brune K, Hinz B. Ceramide is involved in *r*(+)-methanandamide-induced cyclooxygenase-2 expression in human neuroglioma cells. Mol Pharmacol 2003;64: 1189 – 98.

38. Li MH, Jang JH, Surh YJ. Nitric oxide induces apoptosis via AP-1-driven up-regulation of COX-2 in rat pheochromocytoma cells. Free Radic Biol Med 2005;39:890 – 9.

39. Munkarah AR, Genhai Z, Morris R, et al. Inhibition of paclitaxel-induced apoptosis by the specific COX-2 inhibitor, NS398, in epithelial ovarian cancer cells. Gynecol Oncol 2003;88:429 – 33.

40. Jayadev S, Liu B, Bielawska AE, et al. Role for ceramide in cell cycle arrest. J Biol Chem 1995;270:2047 – 52.

41. Verheij M, Bose R, Lin XH, et al. Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. Nature 1996; 380:75 – 9.

42. Subbaramaiah K, Chung WJ, Dannenberg AJ. Ceramide regulates the transcription of cyclooxygenase-2. Evidence for involvement of extracellular signal-regulated kinase/c-Jun N-terminal kinase and p38 mitogen-activated protein kinase pathways. J Biol Chem 1998;273: 32943 – 9.

43. Scarlatti F, Sala G, Somenzi G, Signorelli P, Sacchi N, Ghidoni R. Resveratrol induces growth inhibition and apoptosis in metastatic breast cancer cells via *de novo* ceramide signaling. FASEB J 2003; 17:2339 – 41.

44. Dimanche-Boitrel MT, Meurette O, Rebillard A, Lacour S. Role of early plasma membrane events in chemotherapy-induced cell death. Drug Resist Update 2005;8:5 – 14.

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