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Resveratrol Causes COX-2- and p53-Dependent Apoptosis in Head and Neck Squamous Cell Cancer Cells

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Abstract Cyclooxygenase-2 (COX-2) content is increased in many types of tumor cells. We have investigated the mechanism by which resveratrol, a stilbene that is pro-apoptotic in many tumor cell lines, causes apoptosis in human head and neck squamous cell carcinoma UMSCC-22B cells by a mechanism involving cellular COX-2. UMSCC-22B cells treated with resveratrol for 24 h, with or without selected inhibitors, were examined: (1) for the presence of nuclear activated ERK1/2, p53 and COX-2, (2) for evidence of apoptosis, and (3) by chromatin immunoprecipitation to demonstrate p53 binding to the *p21* promoter. Stilbene-induced apoptosis was concentration-dependent, and associated with ERK1/2 activation, serine-15 p53 phosphorylation and nuclear accumulation of these proteins. These effects were blocked by inhibition of either ERK1/2 or p53 activation. Resveratrol also caused p53 binding to the *p21* promoter and increased abundance of COX-2 protein in UMSCC-22B cell nuclei. Resveratrol-induced nuclear COX-2 accumulation was dependent upon ERK1/2 activation, but not p53 activation. Activation of p53 and p53-dependent apoptosis were blocked by the COX-2 inhibitor, NS398, and by transfection of cells with *COX-2-siRNA*. In UMSCC-22B cells, resveratrol-induced apoptosis and induction of nuclear COX-2 accumulation share dependence on the ERK1/2 signal transduction pathway. *Resveratrol-inducible* nuclear accumulation of COX-2 is essential for p53 activation and p53-dependent apoptosis in these cancer cells. J. Cell. Biochem. 104: 2131–2142, 2008. © 2008 Wiley-Liss, Inc.

Key words: cyclooxygenase-2; UMSCC-22B; resveratrol; p53; apoptosis

The cyclooxygenase isoforms, cyclooxygenase-1 (COX-1) and -2 (COX-2) play a myriad of roles in normal and abnormal physiologic conditions [Dubois et al., 1998; Smith et al., 2000]. COX-1 is constitutively expressed in a wide variety of tissues, is ubiquitous in its distribution, and is thought to play a role in tissue homeostasis and maintenance levels of prostaglandins [Dubois et al., 1998] and in promotion of angiogenesis [von Rahden et al., 2005]. By comparison, COX-2 is inducible, is present in inflammatory foci [Zhang et al., 1998] and in tumors [Dannenberg and Subbaramaiah, 2003], and promotes neovascularization by enhancement of angiogenesis [Daniel et al., 1999; Masferrer et al., 2000; Dannenberg and Subbaramaiah, 2003; von Rahden et al., 2005]. Regulation of COX-2 gene expression is dependent upon both rate of gene transcription and mRNA stability at the post-transcriptional level [Dixon et al., 2000; Shao et al., 2000].

Although COX-2 overexpression is associated with tumorigenesis in animal models, and inhibition of the COX-2 pathway has been associated with reduction in tumor incidence and progression of lung cancer [Sandler and Dubinett, 2004], recent studies have suggested that overexpression of COX-2 can, under certain circumstances, exert an antiproliferative effect through the induction of *p53*, as well as of *p21* [Zahner et al., 2002]. The stilbene resveratrol causes p53-dependent apoptosis in several tumor cell lines including those from cancers of the prostate [Lin et al., 2002; Shih

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et al., 2004], thyroid [Shih et al., 2002], and breast [Zhang et al., 2004; Tang et al., 2006], as well as glial cell tumors of the central nervous system [Lin et al., 2008]. In this report, we show that resveratrol-induced apoptosis in cells derived from squamous cell carcinoma of the head and neck, specifically UMSCC-22B cells, is associated with increased cellular accumulation of COX-2 protein, and is dependent on both activation of the ERK1/2 signal transduction pathway and nuclear accumulation of COX-2. Although activation of p53 is required for resveratrol-induced cancer cell apoptosis, p53 activation is apparently not required for increased nuclear accumulation of COX-2 to occur.

MATERIALS AND METHODS

Cell Line and Reagents

The human head and neck squamous cell cancer cell line, UMSCC-22B, was provided by Dr. Thomas Carey (University of Michigan, Ann Arbor, MI). Cells were maintained in DMEM supplemented with 10% fetal bovine serum in a 5% CO_2/95% air incubator at 37°C. Resveratrol, PD98059, pifithrin-α (PFT-α) and SB203580 were obtained from Calbiochem (San Diego, CA), and NS398 and indomethacin from Sigma–Aldrich (St. Louis, MO). Antibodies for immunoblotting included polyclonal anti-phosphorylated ERK1/2 (pERK1/2), anti-phospho-p38, anti-pSer15-p53 (Cell Signaling, Beverly, MA), and anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). Anti-COX-2 was obtained from Cayman Chemical Co. (Ann Arbor, MI).

Cell Treatment and Fractionation

Subconfluent UMSCC-22B cells were cultured in DMEM with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% glutamine, and then treated as described. Nuclear fractions were prepared by our previously reported methods [Lin et al., 2002; Shih et al., 2002; Shih et al., 2004; Zhang et al., 2004]. Nuclear extracts were derived by resuspension of the crude nuclei in high salt buffer (420 mM NaCl, 20% glycerol) at 4°C with rocking for 1 h. The supernatants containing nucleoproteins were then collected after centrifugation at 13,000 rpm and 4°C for 10 min.

Immunoblotting

The techniques are standard and have been previously described [Shih et al., 2004; Zhang et al., 2004]. Nucleoproteins were separated on discontinuous SDS-PAGE (9% gels), then transferred by electroblotting to Immobilon membranes (Millipore, Bedford, MA). After blocking with 5% milk in Tris-buffered saline with 0.1% Tween, the membranes were incubated overnight with antibodies as indicated. Secondary antibodies were either goat anti-rabbit IgG (1:1,000) (Dako, Carpenteria, CA) or rabbit anti-mouse IgG (1:1,000) (Dako), depending upon the origin of the primary antibody. Immunoreactive proteins were detected by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL) and the results scanned and quantitated with the VersaDoc 5000 Imaging system (Bio-Rad Laboratories, Hercules, CA) and analyzed with Excel software (Microsoft Corp., Redmond, WA). The image intensities were linear in the concentration range of nuclear proteins examined.

Chromatin Immunoprecipitation

Aliquots of 6×10^6 cells were exposed to 1% formaldehyde for 15 min at room temperature to effect crosslinking. Monolayers were then washed twice with PBS and cell extracts were prepared by scraping cells in 1 ml of buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris [pH 8], 5 mM EDTA) containing the protease inhibitors leupeptin (10 μg/ml), pepstatin A (10 μg/ml), the phosphatase inhibitors NaF (50 mM) and 0.2 mM sodium orthovanadate, and deacetylase inhibitor, trichostatin A (5 μM; Calbiochem, San Diego, CA). Cell lysates were sonicated to yield chromatin fragments of approximately 600 bp as determined by agarose gel electrophoresis. Immunoprecipitation of DNA-associated p53 was performed with anti pSer15-p53 antibody (Cell Signaling, Beverly, MA), and resulting protein–DNA complexes then resolved by electrophoresis. The resulting DNA was amplified by PCR using primers for the *p21* promoter as follows: [5′-CCGCTCGAGCCCTGTCGCAAGGATCC-3′ (forward) and 5′-GGGAGGAAGGGGATGGTAG-3′ (reverse)] (Invitrogen, Carlsbad, CA). The 230 bp PCR product represents nucleotides −2,280 to −2,050 for the p21 promoter. The PCR product was resolved using 8% polyacrylamide gels (acrylamide:bis

acrylamide, 19:1) in 1× Tris acetate–EDTA buffer. Resulting gels were stained with ethidium bromide, and relative levels of DNA determined with QuantifyOne software (Bio-Rad).

RT-PCR

Total RNA was isolated as described previously [Lin et al., 2002; Shih et al., 2002; Lin et al., 2007]. First strand complementary DNAs were synthesized from 1 μg of total RNA using oligo dT and AMV Reverse Transcriptase (Promega, Madison, WI). First-strand cDNA templates were amplified for *GAPDH*, *c-fos*, *PIG3*, *c-Jun*, *BAD* mRNAs by polymerase chain reaction (PCR) using a hot start (Ampliwax, Perkin Elmer, Foster City, CA). Primer sequences were *GAPDH* [5′-AAGAAGATGCGGCT-GACTGTCGAGCCACA-3′ (forward) and 5′-T-CTCATGGTTCACACCCATGACGAACATG-3′ (reverse)], *c-fos* [5′-GAATAAGAT-GGCTGCA-GCCAAATGCCGCAA-3′ (forward) and 5′-CA-GTCAGATCAAGGGAAG-CCACAGACATCT-3′ (reverse)], *PIG3* [5′-TGGTCACAGCTGGCTCC-CAGAA-3′ (forward) and 5′-CCGTGGAGA-AG-TGAGGCAGAATTT-3′ (reverse)], *c-jun* [5′-GG-AAACGAC-CTTCTA-TGACGATGC-CCTCAA-3′ (forward) and 5′-GAACCCCTCCTGCTCA-TC-TGTCACGTTCTT-3′ (reverse)] and *BAD* [5′-GTTTGAGCCGAGTGAGCAGG-3′ (forward) and 5′-ATAGCGCTGTGC-TGCCCAGA-3′ (reverse)]. The PCR cycle was an initial step of 95°C for 3 min, followed by 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, then 25 cycles and a final cycle of 72°C for 8 min. PCR products were separated by electrophoresis through 2% agarose gels containing 0.2 μg of 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.

Transfection of siRNA

The small interfering RNA (siRNA) of COX-2 (Cat#: M-004557-01) was purchased from Dharmacon (Lafayette, CO) and scrambled RNA (scRNA) (Cat#: AM4615) was purchased from Ambion (Austin, TX). UMSCC-22B cells were seeded onto six-well tissue culture plates at 60–80% confluence and in the absence of antibiotic for 24 h before transfection. Immediately prior to 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 lipofectamine (5 μg/well) in Opti-MEM I medium according to the manufacturer's instructions (Invitrogen). After transfection, cultures were incubated at 37°C for 4 h and then placed in fresh culture medium. After an additional 24 h, cells were utilized for experimentation.

Confocal Microscopy

Exponentially growing UMSCC-22B cells were seeded in slide chambers. After growth overnight, cells were transfected with *COX-2-siRNA* and *scRNA* as described above. After exposure to 0.25% stripped FBS-containing medium for 2 days, cells were treated with either 10 μM resveratrol or control solvent for 24 h. Cells were fixed with 4% formaldehyde in acetone for 30 min and then permeabilized in 0.06% Triton X-100 for 30 min. The cells were incubated with monoclonal anti-BAD or anti-c-Fos, or polyclonal rabbit anti-PIG3 followed by Alexa-488-labeled donkey anti-mouse antibody or donkey anti-rabbit antibody, and the signal revealed using the Histostain SP kit, as recommended by the manufacturer (Zymed–Invitrogen, Carlsbad, CA). Nuclear staining with propidium iodide was also employed. Cells were examined under 250× magnification.

Apoptosis/Nucleosomes

An early event in apoptosis is DNA fragmentation followed by release of nucleosomes into the cytoplasm. The nucleosome is the basic unit of chromatin and results from the ordered association of histones and DNA [Salgame et al., 1997]. The double-antibody sandwich ELISA is based upon the specific recognition of nucleosomes by a pair of monoclonal antibodies (mAb) and detects cytoplasmic nucleosomes on the ELISA plate. Cells were treated with different reagents for 48 h. The medium was harvested, spun down and pellets were washed twice with phosphate-buffered saline. Pelleted cells were then lysed and the supernatants were collected and stored for at least 18 h at −20°C. From each appropriately diluted sample 100 μl were added to a 96-well plate coated with a DNA binding protein and incubated at room temperature for 3 h. After three washes with buffer, detector antibody was added for 1 h. Streptavidin conjugate was then added and incubated for 0.5 h before adding substrate.

Plates were read at 450 nm. The nucleosome ELISA kits for these studies were purchased from Calbiochem.

RESULTS

Stimulation by Resveratrol of Nuclear Fraction COX-2 Accumulation in UMSCC-22B Cells

UMSCC-22B cells were treated with resveratrol for 24 h, and nuclear fractions then prepared. Accumulation of increased nuclear fraction COX-2 was induced by resveratrol in a concentration-dependent manner, reaching a maximal effect at a stilbene concentration of 50 μM (Fig. 1a). A decreased stilbene effect at a concentration of 100 μM may reflect more apoptosis of cells induced by resveratrol. Additional UMSCC-22B cells were treated with 10 μM resveratrol for 6–48 h, and distribution of nuclear and cytosolic COX-2 and p53 were examined. Nuclear COX-2 was low in untreated cells and increased after treatment with resveratrol, reaching a peak in 24 h; the effect was sustained for 48 h (Fig. 1b). There was an

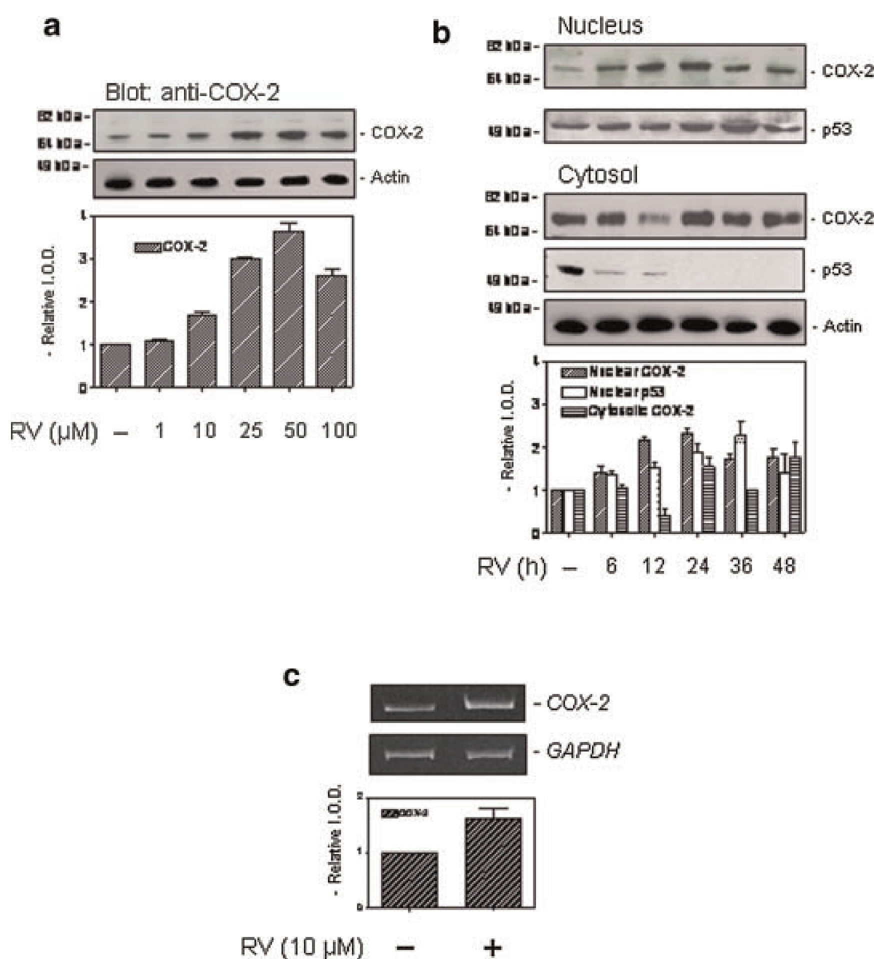

Fig. 1. Stimulation by resveratrol of COX-2 expression and nuclear accumulation in UMSCC-22B cells. **a**: Cells were treated with 1–100 μM resveratrol (RV) for 24 h, and nuclear fractions prepared. Shown in a representative immunoblot, nuclear fractions contained increased levels of COX-2 with a maximal effect seen at a resveratrol concentration of 50 μM. The accompanying graph shows increase in band integrated optical density (IOD), expressed as mean ± SE of results from three similar experiments. Actin immunoblots in this and subsequent figures serve as controls for nucleoprotein loading. **b**: RV (10 μM) caused maximal COX-2 nuclear accumulation in 24 h, and levels remained elevated for at least 48 h. Nuclear p53 levels increased in parallel. Cytosolic COX-2 levels fell in the first 12 h of resveratrol treatment, but increased above control levels in 24 and 48 h. Cytosolic p53 levels fell by 6 h, while nuclear p53 was maximal for 24–36 h of resveratrol treatment in parallel with COX-2 levels. Changes in the levels of nuclear and cytosolic p53 over time suggest trafficking of this protein between the two compartments. **c**: RV also generated increased levels of *COX-2* RNA, shown by RT-PCR of control and treated cells in one experiment. The accompanying graph shows the mean ± SE of changes in RNA levels compared to that of untreated cells in three experiments, corrected for levels of *GAPDH* in the same samples.

abundance of cytosolic COX-2 in untreated control cells that decreased in resveratrol-treated cells after 12 h and increased thereafter (Fig. 1b). Accumulation of nuclear p53 and reduction in cytosolic p53 were observed after resveratrol treatment (Fig. 1b). Resveratrol also stimulated expression of the *COX-2* gene (Fig. 1c). These results suggest that there are two pools of COX-2 in UMSCC-22B cells: one is constitutive COX-2 that resides in the cytosol and the other is resveratrol-inducible COX-2 that appears primarily in the nucleus and is found in association with nuclear p53.

Effect of Resveratrol on Activation of ERK1/2, Serine-15 Phosphorylation of p53 and Apoptosis in UMSCC-22B Cells

After treatment of UMSCC-22B cells with 1–100 μM resveratrol in a manner similar to studies shown in Figure 1a, increased nuclear accumulation of phosphorylated ERK1/2 is demonstrated, consistent with activation of the extracellular signal-regulated protein kinase1/2 (ERK1/2) pathway by resveratrol (Fig. 2a). Nuclear accumulation of serine-15-phosphorylated p53 (pSer15-p53) is also observed. Both of these effects were resveratrol dose-dependent and similar to those which we have previously described in breast [Zhang et al., 2004], prostate [Lin et al., 2002; Shih et al., 2004] and thyroid [Shih et al., 2002] cancer cells treated with the stilbene. In order to demonstrate the binding of resveratrol-induced Ser-15 phosphorylated p53 to a p53-responsive gene such as the p21 promoter, chromatin immunoprecipitation was performed by exposure of DNA from resveratrol-treated or control UMSCC-22B cells to anti-pSer15-p53 antibody; this was followed by isolation of the immunoprecipitated DNA and identification of the *p21* promoter in that DNA by PCR. In Figure 2b, increased *p21* promoter content is seen in the resveratrol-treated immunoprecipitate compared with the content in the control sample, indicating that in UMSCC-22B cells treated with the stilbene, increased activated (serine-15-phosphorylated) p53 binds to the *p21* promoter. Studies of nucleosome ELISA indicate that apoptosis also occurred in UMSCC-22B cells treated with resveratrol (Fig. 2c).

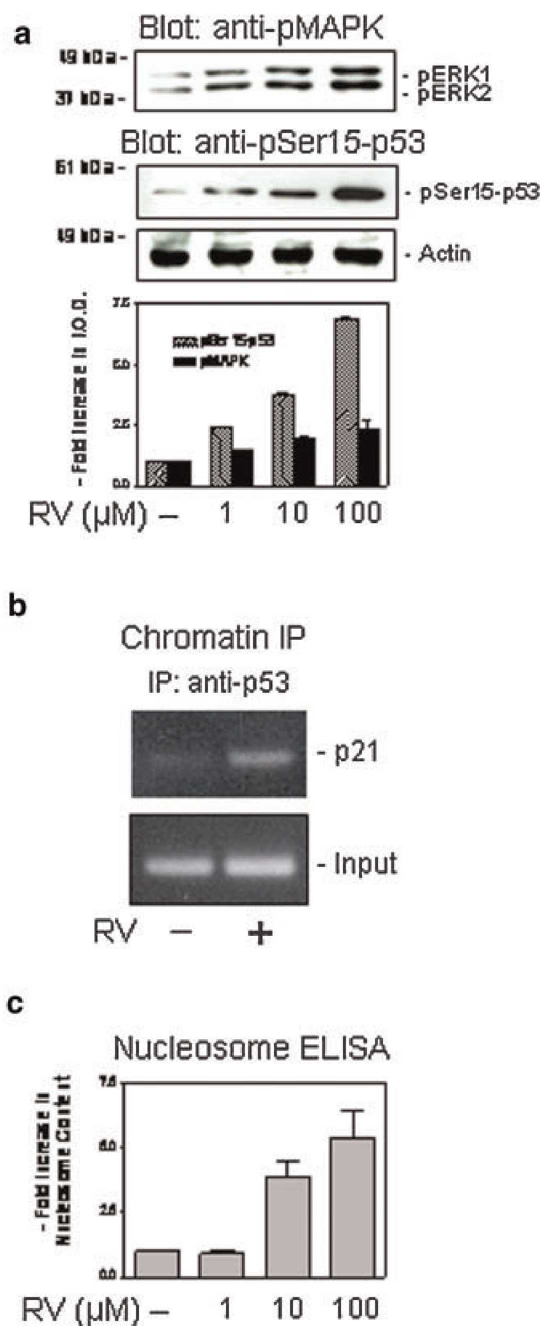

Fig. 2. Resveratrol causes activation and nuclear accumulation of ERK1/2 and p53, apoptosis, and p53 binding to the *p21* promoter in UMSCC-22B cells. **a**: Treatment of cells with 1–100 μM RV caused dose-dependent increases in nuclear accumulation of activated ERK1/2 (pERK1/2) and of serine-15-phosphorylated p53 (pSer15-p53), with maximal effects seen at a concentration of 100 μM. The immunoblots shown are representative of three experiments, and results are summarized in the graph below the blots. **b**: In results of a representative chromatin immunoprecipitation (IP) study ($n = 3$), increased Ser-phosphorylated p53 binding to the *p21* promoter is seen in cells treated with 10 μM resveratrol for 24 h. The lower panel shows controls for DNA input. **c**: Results of three studies of resveratrol-induced apoptosis, measured by nucleosome ELISA, are summarized in the graph, and show a dose-responsive effect of the stilbene on nucleosome accumulation.

Nuclear Accumulation of COX-2 in Response to Resveratrol Is Via the ERK1/2 Signal Transduction Pathway But Not the p38 Kinase Pathway

Two principal mitogen-activated protein kinase (MAPK) pathways are the extracellular signal-regulated protein kinase (ERK1/2) and the p38-MAPK cascades [Roux and Blenis, 2004; Fang and Richardson, 2005]. These pathways are also known to be involved in COX-2 expression induced by different stimuli [McGinty et al., 2000; Singer et al., 2003]. UMSCC-22B cells were treated with 10 μM resveratrol for 24 h in the presence or absence of the ERK1/2-activating kinases (MEK) inhibitor, PD 98059 (PD) or the p38 kinase pathway inhibitor, SB203580 (SB) [Singer et al., 2003]. Activation (phosphorylation) and nuclear translocation of p53 and ERK1/2, and apoptosis were inhibited by PD, as we have shown previously [Lin et al., 2002; Shih et al., 2002], but were not affected by the p38 inhibitor, even at high concentrations (10 μM [$IC_{50} = 1$ μM]) (Fig. 3). In parallel with these findings, nuclear COX-2 accumulation in response to resveratrol was inhibited by PD, but not by SB, suggesting that this process was dependent on activation of ERK1/2 and p53. The p38 pathway inhibitor, SB, only inhibited phosphorylation of p38 itself, as expected (Fig. 3).

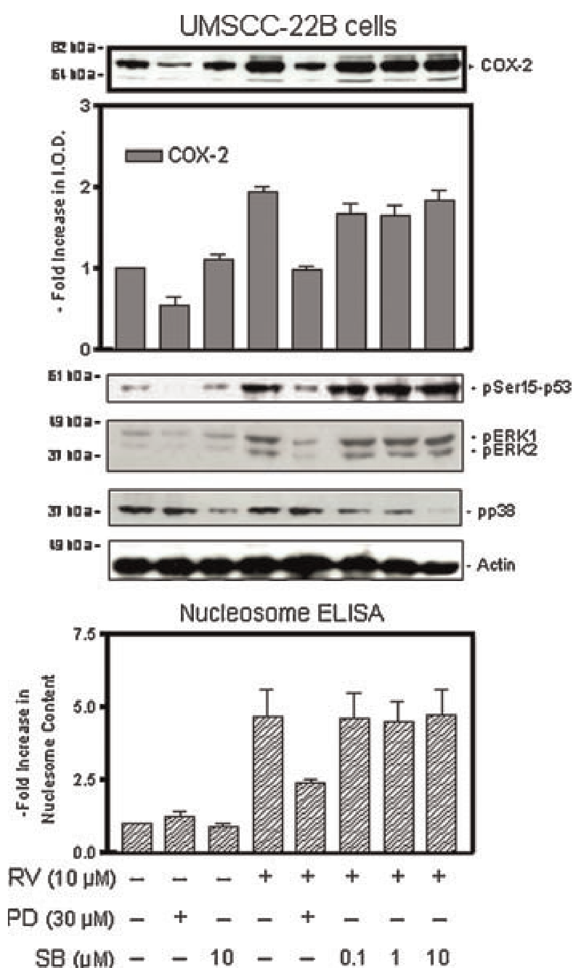

Fig. 3. Effects of resveratrol are suppressed by inhibition of the ERK1/2, but not the p38 activation pathway in UMSCC-22B cells. In this representative experiment, nuclear accumulation of COX-2, pERK1/2 and serine-15-phosphorylated p53 (pSer15-p53) in response to resveratrol (10 μM, 24 h) were suppressed by the ERK1/2 activation inhibitor, PD98059 (PD), but not by the p38 activation inhibitor, SB203580 (SB). The immunoblots are representative of three similar experiments. Studies of resveratrol-associated apoptosis by nucleosome ELISA also showed inhibition of that effect by PD, but not by SB.

The Resveratrol-Induced Increase in COX-2 Expression Is Independent of p53 Activation

When UMSCC-22B cells were treated with resveratrol in the presence of a p53 inhibitor, PFT-α (20 μM) [Komarov et al., 1999; Lin et al., 2002], resveratrol-induced nuclear COX-2 accumulation remained elevated, although PFT-α did inhibit resveratrol-induced p53 phosphorylation, total p53 accumulation and apoptosis (Fig. 4). These results suggest that resveratrol-induced nuclear accumulation of COX-2 occurs independently of p53 activation in UMSCC-22B cells. p53 has been implicated by others in the regulation of COX-2 expression [Subbaramaiah et al., 1999; Gallo et al., 2003], and overexpression of p53 has been shown in other experimental models to downregulate transcription of COX-2 in cell lines derived from head and neck squamous cell carcinoma [Lee et al., 2004]. Our results, however, indicate that in *resveratrol-treated* UMSCC-22B cells, the increase in COX-2 expression is independent of p53 activation, as shown by the use of PFT-α.

Effect of COX-2 Inhibition on p53 Activation and Apoptosis Caused by Resveratrol

We also studied the effect of COX-2 on p53 activation induced by resveratrol using NS398, a selective inhibitor of COX-2 enzyme activity [Lee et al., 2004]. Results presented in Figure 5a show that NS398 inhibited resveratrol-stimulated nuclear accumulation of COX-2 in UMSCC-22B cells as well as the activation, specifically serine-15 phosphorylation of

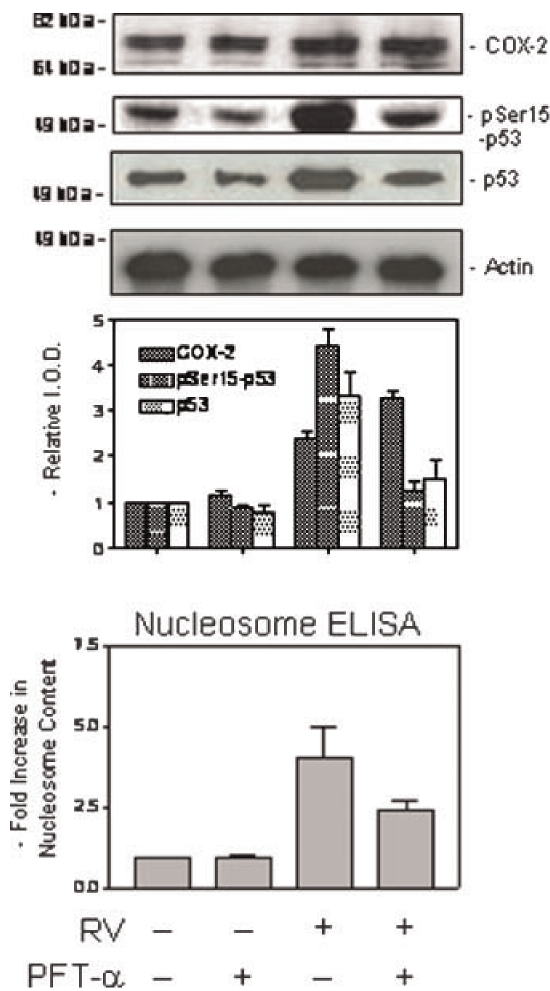

Fig. 4. Inhibition of p53 activation does not block resveratrol-induced COX-2 accumulation in UMSCC-22B cells. Resveratrol, 10 μM for 24 h, caused increased nuclear COX-2 and pSer15-p53 abundance, shown in representative immunoblots and a graph derived from results of three experiments. A parallel increase in apoptosis was also seen, as shown by results of nucleosome ELISA studies. The p53 inhibitor, pifithrin-α (PFT-α, 10 μM), suppressed nuclear accumulation of pSer15-p53 and resulting apoptosis, but did not suppress the nuclear accumulation of COX-2.

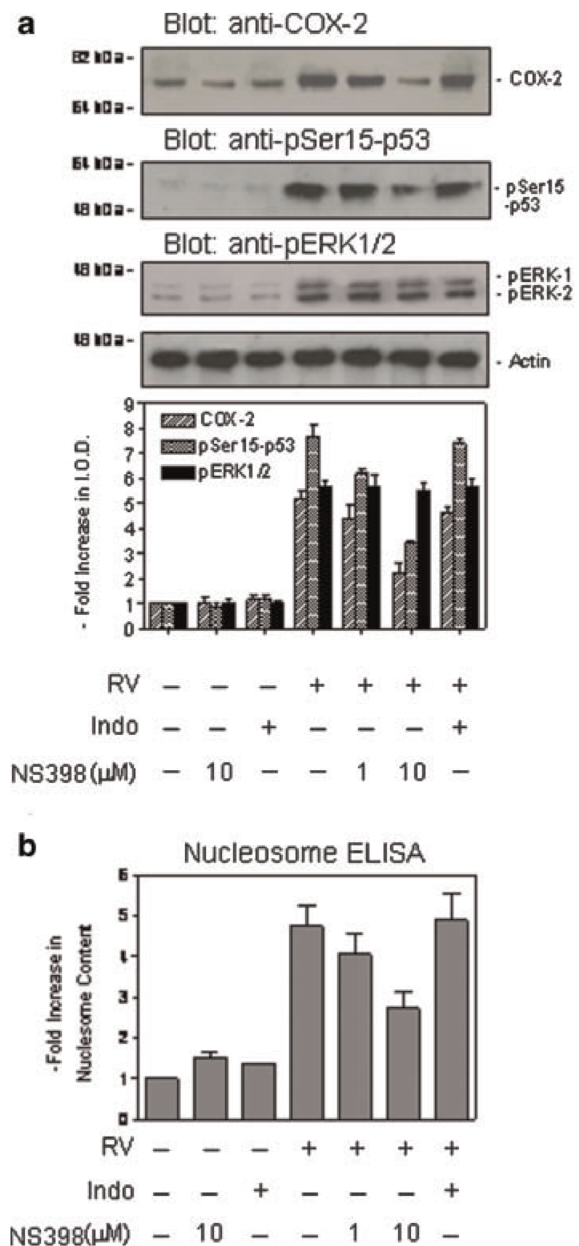

Fig. 5. Resveratrol action is suppressed by the COX-2 inhibitor, NS398. **a**: Resveratrol-induced nuclear accumulation of COX-2 and pSer15-p53 were not inhibited by the non-specific COX inhibitor, indomethacin (Indo, 10 μM), but were progressively inhibited by 1–10 μM concentrations of NS398. Nuclear accumulation of pERK1/2 was not affected by either inhibitor. Shown are representative results from one of three experiments and a graph summarizing the collective results. **b**: In comparable studies of apoptosis by nucleosome ELISA, suppression of the resveratrol effect by NS398, but not by indomethacin, is seen.

p53. This inhibitor effect was concentration-dependent. However, a non-specific inhibitor of COX activity, indomethacin, did not affect either the activation of p53 or levels of COX-2 induced by resveratrol. The resveratrol-induced ERK1/2 activation was not decreased by either NS398 or indomethacin (Fig. 5a). These results agree with our previous observation that neither NS398 nor indomethacin inhibit resveratrol-induced ERK1/2 activation in glioma C6 cells [Lin et al., 2008]. Resveratrol-induced apoptosis was monitored in the presence of NS398 or indomethacin, and was inhibited by NS398, but not by indomethacin (Fig. 5b). These results indicate that inhibition of COX-2 activity led to suppression of several of the effects of resveratrol we have observed,

including nuclear COX-2 accumulation, activation of p53 and apoptosis of UMSCC-22B cells.

Additional studies to demonstrate that inducible COX-2 is essential for resveratrol-induced p53-dependent apoptosis were conducted by transfecting UMSCC-22B cells with a COX-2-*siRNA* construct (Fig. 6a). Nuclear COX-2 accumulation and serine-15-phosphorylated p53 levels in stilbene-treated cells were diminished compared to the levels in cells transfected with *scRNA*. Expression of resveratrol-induced p53-responsive genes involved in apoptosis such as *c-fos* [Preston et al., 1996], *c-jun* [Araki et al., 1998], *PIG3* [MacLachlan et al., 2002], and *BAD* [Jiao et al., 2007] expression were blocked in *COX-2-siRNA* transfected cells, but not in untransfected or *scRNA*-transfected cells when cells were treated with resveratrol (Fig. 6b). Studies by confocal microscopy showed resveratrol-induced accumulation of pro-apoptotic proteins, BAD, c-Fos, and PIG3, in untransfected and *scRNA*-transfected cells, but not in *siRNA*-transfected cells (Fig. 7a), and the further consequence of reducing COX-2 accumulation was a reduction in resveratrol-induced apoptosis (Fig. 7b).

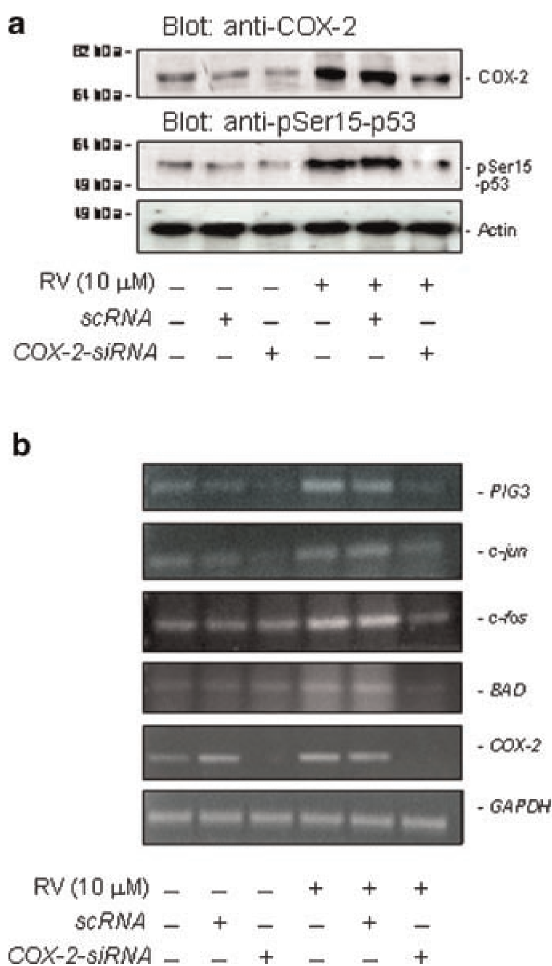

Fig. 6. Resveratrol-induced pSer-15-p53 accumulation and p53-responsive gene expression are inhibited by suppression of COX-2 expression. **a**: In UMSCC-22B cells transfected with *COX-2-siRNA*, resveratrol treatment resulted in decreased expression of COX-2 as well as suppression of pSer15-p53 accumulation in nuclei when compared to cells transfected with *scRNA*. **b**: Cells transfected with *COX-2-siRNA* and then treated with resveratrol showed diminished expression of *PIG3*, *c-jun*, *c-fos*, *BAD* and *COX-2* genes in response to the stilbene. In comparison, untransfected cells and cells transfected with *scRNA* showed increased expression of these genes after resveratrol treatment.

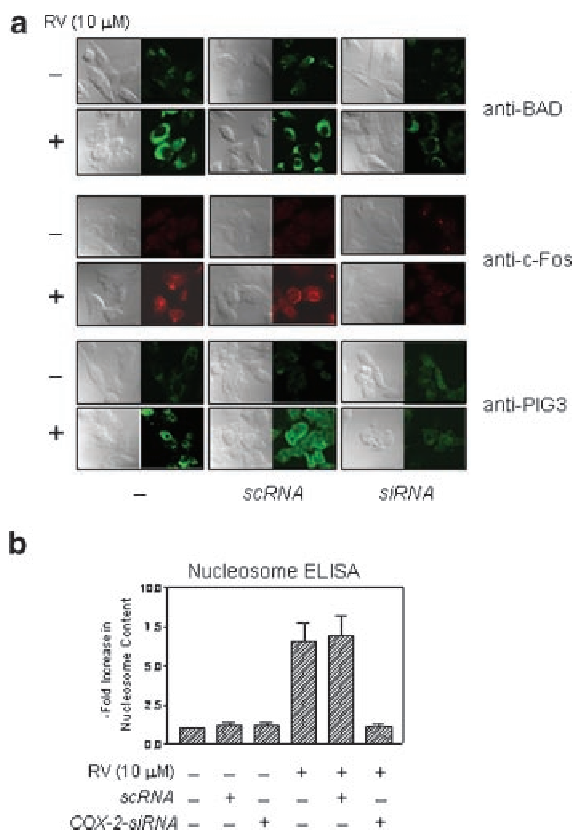

Fig. 7. Resveratrol-induced pro-apoptotic protein accumulation and apoptosis is inhibited by suppression of COX-2 expression. **a**: In UMSCC-22B cells transfected with *COX-2-siRNA*, resveratrol treatment resulted in decreased BAD (green), c-Fos (red) and PIG3 (green) accumulation, demonstrated by confocal microscopy. In contrast, transfection with *scRNA* did not result in inhibition of these resveratrol effects. The grey figures at left in each group show cell and nuclear outlines by differential interference contrast. **b**: Similar results were obtained in studies of resveratrol-induced apoptosis, in that cells transfected with *COX-2-siRNA* demonstrated no apoptosis in response to the stilbene, while cells with *scRNA* underwent apoptosis.

DISCUSSION

The results presented in this study implicate inducible COX-2 in p53 activation and p53-dependent apoptosis in UMSCC-22B cells during exposure to the stilbene, resveratrol. After 24 h of resveratrol treatment, we observed: (1) activation of the ERK1/2 pathway leading to serine-15 phosphorylation of the oncogene suppressor protein p53, (2) an increase in nuclear COX-2 content, and (3) apoptosis of cancer cells. These findings are summarized in Figure 8. The effects of resveratrol were all blocked by the ERK1/2 activation inhibitor, PD98059. Although the effects of resveratrol on serine phosphorylation of p53 and apoptosis were also blocked by the p53 inhibitor, PFT-α, the accumulation of COX-2 in nuclear fractions persisted, and therefore was not dependent on p53 activation. These findings suggest that the stilbene also exerts a primary effect on COX-2 expression in UMSCC-22B cells. Evidence for a consequent role for COX-2 in resveratrol action was shown by an inhibitory effect of NS398 not only on COX-2 accumulation in UMSCC-22B cells, but also on serine-15 phosphorylation of p53, resulting in a decrease in apoptosis.

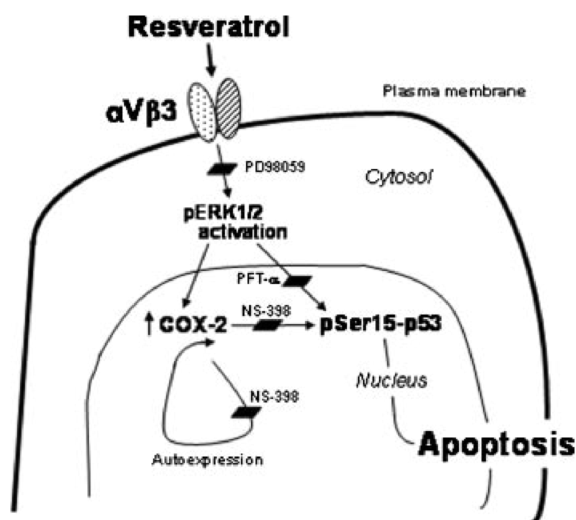

Fig. 8. Proposed signaling pathways by which resveratrol mediates increased COX-2 expression and apoptosis in UMSCC-22B cells. Resveratrol, via an integrin αvβ3 plasma membrane receptor [Lin et al., 2006], stimulates activation of pERK1/2, leading to increased expression of COX-2 in cancer cell nuclei. ERK1/2 activation and nuclear COX-2 accumulation are inhibited by PD98059. COX-2 enhances its own expression (''autoexpression''), via a mechanism which is inhibited by NS-398. COX-2 also stimulates ser15-p53 phosphorylation, an effect also inhibited by NS-398. The activated pERK1/2 complex translocates to the cell nucleus and is known to cause Ser-15 phosphorylation of p53. However, activation of p53 is blocked by PFT-α. Without activation of p53, due to reduction in COX-2 expression, inhibition of the COX-2 effect, inhibition of ERK1/2 activation or by direct inhibition of p53 activation, apoptosis in resveratrol-treated cells will not occur.

We found that resveratrol treatment of UMSCC-22B cells prompted the binding of p53 to the p21 promoter (Fig. 2b). This suggests that the stilbene is capable of causing transcription of a p53-responsive gene such as p21. It is also possible that p21 plays a role in the pro-apoptotic action of resveratrol since, depending upon the cell line and the treatment applied to cancer cells in vitro, p21 may be pro-apoptotic or anti-apoptotic [Weiss, 2003; Gartel and Radhakrishnan, 2005]. These effects of resveratrol, including that on the COX-2-p53-apoptosis pathway, appear to be initiated at a cell membrane receptor for the stilbene that we have recently identified on integrin αvβ3 [Lin et al., 2006].

COX-2 overexpression has been associated with tumorigenesis [Dubois et al., 1998; Smith et al., 2000; Dannenberg and Subbaramaiah, 2003; von Rahden et al., 2005], and others have shown that suppression of cancer cell proliferation with agents other than resveratrol may be associated specifically with COX-2 *inhibition* [Masferrer et al., 2000; Shao et al., 2000; Dannenberg and Subbaramaiah, 2003]. It was therefore of particular interest in our studies to find that resveratrol-induced apoptosis of UMSCC-22B cells was associated with *increased* cellular accumulation of COX-2, particularly in close association with cell nuclei. Chan et al. have demonstrated almost 150-fold increases in COX-2 expression in several head and neck squamous cell carcinoma cell lines, compared to findings in cells from normal mucosa obtained from control subjects [Chan et al., 1999]. However, the location of COX-2 in the tumors studied by those authors was primarily cytoplasmic, and cell treatment was not discussed.

Our findings differ from those of Subbaramaiah et al. [1998] who studied the effect of resveratrol on COX-2 transcription and activity in human mammary epithelial cells treated with phorbol ester to induce COX-2 expression. In their studies, overexpression of protein kinase C-α led to increased levels of ERK1 and c-Jun, as well as increased *COX-2* promoter activity; all of these effects were blocked by resveratrol. Our studies, in contrast, utilized head and neck squamous cell cancer

cells treated with resveratrol in the absence of phorbol ester, and show stilbene-induced increases in cellular COX-2 concentration in close association with cell nuclei, during experimental conditions associated with UMSCC-22B cell apoptosis.

Studies with nitric oxide (NO)-releasing acetylsalicylic acid (ASA) have revealed that ASA inhibits colon cancer growth by increasing *COX-2* expression [Williams et al., 2003], providing further evidence for the role of *inducible* COX-2 in the growth inhibition of cancer cells. Apoptosis was not specifically measured in that study. Other investigators have suggested that COX-2 inhibits p53-induced apoptosis [Han et al., 2002; Choi et al., 2005]. We suggest that these divergent impressions may reflect the existence in tumor cells of two pools of COX-2, containing either: (1) ASA- or resveratrol-inducible COX-2, as demonstrated in the present study, or (2) constitutively expressed COX-2.

The signal transduction pathway involved in p53 activation and *COX-2* expression in resveratrol-treated cells is ERK1/2-dependent, as indicated by effects of the ERK1/2 pathway inhibitor, PD98059, in the studies presented above (Fig. 4). Cells treated with resveratrol and PD98059 show decreases in serine-15-p53 phosphorylation, ERK1/2 activation and nuclear accumulation of COX-2 in UMSCC-22B cancer cells. These results indicate that the changes in COX-2 levels and localization are dependent on the nuclear accumulation of ERK1/2 in response to resveratrol treatment. Inhibition of p38 function, however, had no effect on resveratrol-induced COX-2 accumulation in UMSCC-22B cells.

Increased expression of COX-2 induced by resveratrol was not affected by the p53 inhibitor, PFT-α, even though the inhibitor blocked resveratrol-induced p53 serine phosphorylation and apoptosis of UMSCC-22B cells, as we anticipated from our prior studies [Lin et al., 2002]. Those findings suggest that p53 does not play a direct causative role in the increased accumulation of COX-2 seen in resveratrol-treated cells. On the other hand, a specific inhibitor of the enzymatic activity of COX-2, NS398, and *COX-2-siRNA*, but not scrambled *RNA*, inhibited resveratrol-induced p53 activation and apoptosis, suggesting an important role for increased COX-2 activity, stimulated by resveratrol, in p53 activation and p53-dependent apoptosis of UMSCC-22B cells.

The ERK1/2 inhibitor, PD98059 blocked resveratrol-induced nuclear COX-2 accumulation (Fig. 3). On the other hand, NS398 did not inhibit resveratrol-induced ERK1/2 activation but did inhibit Ser-15 phosphorylation of p53 (Fig. 5a). These results suggest that resveratrol-induced ERK1/2 activation is upstream of nuclear COX-2 accumulation. Studies have shown that nuclear COX-2 forms a complex with phosphoERK1/2 [Lin et al., 2008] and Ser-15-phosphorylated p53 [Tang et al., 2006]. Thus, to serve as an accessory protein, nuclear COX-2 facilitates resveratrol-induced activated ERK1/2 to induce Ser-15 phosphorylation of p53.

In summary, resveratrol, via an integrin αvβ3 plasma membrane receptor [Lin et al., 2006], activates ERK1/2, leading to increased expression and accumulation of COX-2 in cancer cell nuclei. The COX-2 which accumulates in the nucleus forms complexes with pERK1/2 and p53 leading to an increase in Ser-15 phosphorylation of p53. When such activation of p53 is inhibited, either by reduction in COX-2 abundance or inhibition of COX-2 activity, apoptosis in resveratrol-treated cells will not occur.

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