Inducible COX-2-dependent apoptosis in human ovarian cancer cells

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Resveratrol is a naturally occurring trihydroxyl-diphenylethylene compound that has been shown experimentally to have beneficial effects in the treatment of cancer and cardiovascular disease. Resveratrol induces programmed cell death (apoptosis) in these cells and activates important signal transducing proteins including extracellular signal-regulated kinases (ERKs) 1 and 2 in cancer cells. Resveratrol also causes nuclear accumulation of the enzyme cyclooxygenase (COX)-2 and of the oncogene suppressor protein, p53. We have studied the molecular basis of the anti-cancer actions of resveratrol using human ovarian carcinoma (OVCAR-3) cells. Our findings include the following: (i) nuclear accumulation of COX-2 in resveratrol-treated cells is blocked by the ERK1/2 inhibitor, PD98059; (ii) an inhibitor of COX-2 activity, NS398, prevents accumulation of ERK1/2, COX-2, activated p53 and small ubiquitin-like modifier (SUMO-1) in the nucleus; (iii) apoptosis, quantitated by nucleosome enzyme-linked immunosorbent assay and the nuclear abundance of the pro-apoptotic protein, Bcl-xx, were inhibited by NS398. This finding implicates nuclear COX-2 in p53-mediated apoptosis induced by resveratrol. Sumoylation is important to stabilization of p53 and a COX-2–SUMO-1 interaction suggests sumoylation of COX-2 in resveratrol-treated cells and (iv) chromatin immunoprecipitation studies showed binding of induced nuclear COX-2 to the promoter region of PIG3 and Bax, pro-apoptotic gene targets of transcriptionally active p53. Nuclear accumulation of activated ERK1/2 and sumoylated COX-2 are essential to resveratrol-induced pSer-15-p53-mediated apoptosis in human ovarian cancer cells.

Introduction

Despite improvements in chemotherapy agents and schedules and new drug combinations, epithelial ovarian cancer remains a leading cause of gynecologic cancer death in Western countries (1). It is usually diagnosed at late stages of the disease, which makes complete surgical resection technically more difficult (2,3). Resveratrol is a naturally occurring stilbene found in grape skin and other plants (4). The compound has anticancer and other biologic properties, such as retardation of aging (5), neuroprotection (6) and cardio-vascular protection (7). Studies have reported that resveratrol induces p53-dependent apoptosis in several different cancer cell lines, including cells derived from cancers of the prostate (8,9), thyroid (10), breast (11) and glioblastoma (12). A cell surface receptor for the stilbene is identified on integrin αvβ3 (13) and shown to be linked to activation of the extracellular signal-regulated kinase (ERK) 1 and 2 signal transduction pathway (12,13). The mechanism of resveratrol’s action as an antimutator agent is known to involve posttranslational modification of p53 (8–12).

Cylooxygenase (COX)-2, the rate-limiting enzyme in prostaglan-din synthesis, is induced in many cells by inflammatory mediators. Two COX isoforms have been identified (14). COX-1 is a constitutively expressed form of the enzyme and is ubiquitous in its distribution. COX-2 is inducible and is present in inflammatory loci and neovascularature (15) but frequently is constitutively expressed in tumors. Expression of COX-2 in cells and animal models is associated with tumor cell growth and metastasis, enhanced cellular adhesion and inhibition of apoptosis (16,17). The mechanisms of these tumor-promoting actions of COX-2 are incompletely known. Pharmacologic inhibitors of COX-2 can decrease tumor cell growth or prevent tu-morigenesis in animal models of cancer (18–20) and may decrease growth of certain human tumors (21,22). 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 anti-apoptotic (23,24) and that pharmacologic induc- tion 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 brain cancer cells with the non-steroidal anti-inflammatory drug flurbiprofen has been shown to enhance COX-2 expression, to cause complex formation of COX-2 protein with p53 and to suppress tumor growth (18). Resveratrol has also been shown to induce expression of COX-2 and p53 and apo-poptosis in different cancer cells (12,27). Overexpression of COX-2 inhibits platelet-derived growth factor-induced proliferation via the induction of p53, as well as of p21 (24).

As a tumor suppressor, p53 regulates the response to various cellular stressors, including DNA damage and oncogenic stimulation (28). p53 has been termed as the ‘guardian of the genomes’ because it mediates the effects of DNA damage and, depending on cellular context, induces cell cycle arrest and DNA repair or apoptosis. p53-induced apoptosis involves the transcription of pro-apoptotic genes (such as Bax and PIG3) and the repression of anti-apoptotic genes (such as survivin and Bcl-2) (29,30). As a transcriptional factor, p53 must be localized to the nucleus for its activity (31). Inhibition of COX-2 in colon cancer cells by a selective COX-2 inhibitor increases the nuclear localization of active p53 (26). A selective COX-2 in-hibitor, NS-398, significantly enhanced genotoxic stress-induced ap-optosis in several types of p53+/− normal human cells, through a caspase-dependent pathway (32). The induction of COX-2 expres-sion results from p53-mediated activation of the Ras/Raf/mitogen-activated protein kinase (MAPK) signal transduction cascade (33).

Ubiquitin and the small ubiquitin-like modifier (SUMO) (34) are polypeptides which are attached to the lysine residues of a number of proteins, leading to the subsequent formation of poly-ubiquitinated- or poly-sumoylated-branched molecules. Ubiquitination and sumoy-lation often compete for the same lysine residues and have opposite effects in regulating the function of a variety of transcription factors by altering their intracellular targeting, their interaction with specific partners and/or their stability (34,35).

In these studies, we demonstrate that treatment of human ovarian cancer OVCAR-3 cells with resveratrol induces COX-2 expression. The inducible COX-2 translocates into the nucleus associated with activated MAPK and SUMO-1. The nuclear complex of activated MAPK and COX-2 associates with p53 and increases p53 phosphorylation. The complex also binds to the promoters of p53-responsive genes, increases the expression of anticancer proteins and induces apoptosis in human ovarian cancer cells.

Materials and methods

Cell line

The human ovarian cancer cell line, OVCAR-3, was purchased from American Type Culture Collection (Manassas, VA). Cell lines were maintained in RPMI

Abbreviations: COX, cyclooxygenase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; siRNA, small interfering RNA; SUMO-1, small ubiquitin-like modifier.

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This page contains scientific text discussing the use of resveratrol in OVCAR-3 cells, including the methods used for cell treatment, protein separation, and analysis. The text is accompanied by a figure illustrating the results of these experiments. The figure shows the induction of COX-2 expression, Ser-15-p53 phosphorylation, and apoptosis in OVCAR-3 cells treated with resveratrol at different concentrations. The text also describes the preparation of nucleoproteins, chromatin immunoprecipitation assays, and the use of small interfering RNA (siRNA) to inhibit COX-2 expression. The results are analyzed using chemiluminescence and confocal microscopy.
Resveratrol, inducible COX-2, SUMO-1 and apoptosis

Cells were harvested and washed twice with phosphate-buffered saline. Nucleosome enzyme-linked immunosorbent assay assays were carried out according to the protocol provided by Calbiochem.

Data analysis and statistics

Immunoblot and nucleotide densities were measured with a Storm 860 phosphorimager, followed by analysis with ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Student’s t-test, with $P < 0.05$ as the threshold for significance, was used to evaluate the significance of the hormone and inhibitor effects.

Results

Resveratrol induces nuclear COX-2 accumulation, Ser-15 phosphorylation of p53 and apoptosis in ovarian cancer cells

Human ovarian cancer OVCAR-3 cells were treated with different concentrations of resveratrol (1–50 μM) for 24 h. Nuclear proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Resveratrol increased COX-2 levels in the nuclei. On the other hand, COX-1 expression was not affected by resveratrol (Figure 1A). Resveratrol also increased Ser-15 phosphorylation of cancer suppressor protein p53 (Figure 1A) in a concentration-dependent manner. In order to investigate if inducible COX-2 can bind to DNA sequence of p53-responsive gene promoters, chromatin immunoprecipitation was performed by exposure of DNA from resveratrol-treated or control OVCAR-3 cells to anti-COX-2 antibody, followed by isolation of the immunoprecipitated DNA and identification of the pro-apoptotic genes PIG3 and Bax promoters in that DNA by polymerase chain reaction. In Figure 1B, increased PIG3 and Bax promoter contents are seen in the resveratrol-treated immunoprecipitate compared with content in the control sample, indicating that in OVCAR-3 cells treated with resveratrol, increased COX-2 binds to pro-apoptotic protein promoter. When OVCAR-3 cells were treated with different concentrations of resveratrol (1–50 μM) for 48 h, resveratrol induced the accumulation of pro-apoptotic protein Bcl-x short form in the cytosol (Figure 1C). These results suggest that resveratrol can induce nuclear COX-2 accumulation, Ser-15 p53 phosphorylation, binding of COX-2 to the promoters of the p53-responsive genes PIG3 and Bax and apoptosis in OVCAR-3 cells.

Nuclear accumulation of COX-2 induced by resveratrol is ERK1/2 dependent in OVCAR-3 cells

Studies have previously reported an essential role of MAPK, specifically ERK1/2, in resveratrol-induced Ser-15 phosphorylation of p53 and resulting apoptosis in different cancer cells (8–12). In the studies illustrated in Figure 2A, OVCAR-3 cells were treated with 10 μM resveratrol in the presence of the MAPK kinase (MEK) inhibitor PD98059 (30 μM) or the p38 kinase inhibitor SB203580 (10 μM) for 24 h. Resveratrol-induced Ser-15 phosphorylation of p53 was inhibited by PD98059 but not by SB203580. The effect of PD 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 μM of the MEK inhibitor, PD98059, indicating that COX-2 accumulation in nuclei was also ERK1/2 dependent. Studies of confocal microscopy also indicate that inhibition of ERK1/2 activation reduced nuclear accumulation of COX-2 (Figure 2B). These results suggest that activated ERK1/2 is essential for nuclear accumulation of COX-2 and Ser-15 phosphorylation of p53 induced by resveratrol in human ovarian cancer cells.

SUMO-1 complexes with nuclear COX-2 in resveratrol-treated ovarian cancer cells

Because SUMO-1 has been shown to associate with coactivators (35) and nuclear COX-2 may play a role as a coactivator, it is of interest to study if SUMO-1 associates with nuclear-accumulated COX-2 in resveratrol-treated cancer cells. OVCAR-3 cells were treated with resveratrol (10 μM) in the presence or absence of...
Fig. 3. The COX-2 inhibitor, NS398, and the ERK1/2 inhibitor, PD98059, block nuclear colocalization of SUMO-1 and COX-2 in OVCAR-3 cells. OVCAR-3 cells were treated with 10 μM resveratrol in the presence or absence of a specific COX-2 inhibitor, NS398 (10 μM), PD98059 (PD, 30 μM) or the non-specific COX-2 inhibitor, indomethacin (Indo, 10 μM) for 24 h. Cells were fixed and permeabilized as described in Materials and Methods. Rabbit anti-SUMO-1 antibody (1:300) and mouse anti-COX-2 antibody (1:100) were added and rocked at 4°C for 24 h. Secondary antibodies (Alexa Flour 488 donkey anti-rabbit IgG, green and Alexa Flour 594 donkey anti-mouse IgG, red) were incubated on the rocker for 1 h at room temperature. Cells were viewed at ×250 magnification.

Fig. 4. Inducible COX-2 forms a nuclear complex with pERK1/2, pSer-15 p53 and SUMO-1 in resveratrol-treated cells. OVCAR-3 cells were treated with 10 μM resveratrol in the presence or absence of NS398 (1–10 μM) or indomethacin (Indo, 10 μM) for 24 h. (A) Nuclear extracts were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred to nitrocellulose membrane. Membranes were incubated with rabbit anti-pSer-15-p53 or mouse anti-COX-2 antibodies and then donkey anti-rabbit or rabbit anti-mouse secondary antibodies. Lamin B was used as nuclear protein loading control. (B) Two hundred micrograms of nuclear extracts were immunoprecipitated with anti-phosphoERK1/2 or anti-SUMO-1 antibodies. After incubation overnight at 4°C with rotation, antigen–antibody mixtures were added 20 μl protein A agarose for 1 h rocking at room temperature. Immunoprecipitates were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Western blots were performed with mouse anti-COX-2 or rabbit anti-pSer-15-p53 antibodies and then rabbit anti-mouse or donkey anti-rabbit secondary antibodies.
PD98059 (PD, 30 μM), a specific inhibitor of COX-2, NS398 (10 μM) or the non-specific COX-2 inhibitor, indomethacin (Indo, 10 μM) for 24 h. SUMO-1 was immunostained with anti-SUMO-1 antibody, and COX-2 was immunostained with anti-COX-2 antibody. Resveratrol induced nuclear colocalization of SUMO-1 and COX-2. The resveratrol effect was blocked by PD and NS398 but not by Indo, the non-specific COX inhibitor (Figure 3). In order to study the effect of COX-2 inhibition on resveratrol-induced p53 phosphorylation, OVCAR-3 cells were treated with resveratrol in the presence or absence of NS398 and a non-specific inhibitor, indomethacin for 24 h. Studies indicate that resveratrol induced Ser-15 phosphorylation of p53 and nuclear COX-2 accumulation and that these effects were inhibited by NS398 but not Indo (Figure 4A). We further investigated the inhibitory effect of NS398 on resveratrol-induced complexing of COX-2 and other nucleoproteins. Resveratrol induced co-immunoprecipitation of SUMO-1 and COX-2 or COX-2 and ERK1/2 (Figure 4B). NS398 suppressed resveratrol-induced nuclear co-immunoprecipitation of pERK1/2 and pSer-15-p53 or COX-2. On the other hand, the activation of ERK1/2 per se was not suppressed by NS398. Results also indicate that there was a sumoylated COX-2 in the resveratrol-treated cells; however, the co-immunoprecipitation of nuclear SUMO-1 and COX-2 was inhibited by NS398 (Figure 4B).

**Inducible COX-2 and activated p53 collaboratively induce apoptosis in resveratrol-treated ovarian cancer cells**

We next examined if nuclear COX-2 associates with other nucleoprotein. When nuclear extracts of resveratrol-treated cells were immunoprecipitated with anti-COX-2 antibody and mouse IgG was used as a non-specific control. After incubation overnight at 4°C with rotation, antigen–antibody mixtures were added 20 μl protein A agarose for 1 h with rocking at room temperature. The immunoprecipitates were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Western blots were performed with mouse anti-COX-2, rabbit anti-pSer15-p53 or rabbit anti-p300 antibodies and rabbit anti-mouse or donkey anti-rabbit secondary antibodies. Prior to immunoprecipitation, 30 μl of each sample were set aside and separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Western blot was performed with anti-COX-2 to show amount of COX-2 and membrane was stripped and re-blotted with anti-lamin B as a loading control. (B) Two hundred micrograms of nuclear proteins were immunoprecipitated with anti-pSer-15-p53 antibody as described above and immunoprecipitates were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Membranes were incubated with anti-COX-2, anti-pERK1/2, anti-p300 or anti-pSer15-p53 antibodies and donkey anti-rabbit or rabbit anti-mouse secondary antibodies.

**Inducible COX-2 and activated p53 collaboratively induce apoptosis in resveratrol-treated ovarian cancer cells**

We next examined if nuclear COX-2 associates with other nucleoprotein. When nuclear extracts of resveratrol-treated cells were immunoprecipitated with anti-COX-2 antibody, and the immunoprecipitated proteins then were separated by electrophoresis, a complex of COX-2 and Ser-15-phosphorylated p53 was identified in resveratrol-treated OVCAR-3 cells (Figure 5A). Nuclear complexing of the coactivator protein p300 with COX-2 was also observed in resveratrol-exposed cells (Figure 5A). The nuclear associations of COX-2 and p300 and of COX-2 and p53 were inhibited by the MEK inhibitor, PD98059 (Figure 5A). In addition, the complexing of Ser-15 phosphorylated p53 and p300, COX-2 and phosphorylated ERK1/2 induced by resveratrol were also inhibited by PD (Figure 5B), signifying the involvement of the MAPK pathway in formation of these complexes. Resveratrol-induced pro-apoptotic protein, BcL-xs (Figure 6A) and apoptosis were blocked by NS398 (Figure 6B), but there was no effect of the non-specific COX-2 inhibitor, indomethacin (10 μM). Experiments were conducted by using siRNA of COX-2 to reduce cellular COX-2 content. OVCAR-3 cells were either transfected with siRNA or with non-specific RNA, scRNA. After transfection, cells were treated with 10 μM resveratrol for 24 h. In scRNA-transfected cells,
The difference between our observations and those of others reports indicate that induction of COX-2 is ERK and p38 dependent. Phosphorylated derivatives of p38 or ERK1/2 activates the transcription of COX-2. Phosphorylated p38 or ERK1/2 can be blocked by the presence of a specific inhibitor, NS398 or NS-398. The phosphorylation of p53 is reduced, as was apoptosis (Figure 7B). These results clearly suggest that inducible COX-2 is essential for Ser-15 phosphorylation of p53 and consequently p53-dependent apoptosis.

Discussion

In addition to activation of p53-dependent apoptosis, resveratrol induces COX-2 expression in OVCAR-3 ovarian cancer cells. The inducible COX-2 associated with SUMO-1 transports into nucleus and forms complex with phosphorylated p33, phosphorylated ERK1/2 and p300. These processes are activated ERK1/2 and COX-2 dependent. The inducible nuclear-accumulated COX-2 potentiates resveratrol-induced p53-dependent apoptosis.

Although resveratrol has been shown to inhibit phorbol ester- and chemical-induced COX-2 expression and enzymatic activity (36–39), resveratrol-induced COX-2 localizes in nuclei in cancer cells (12,27,40). The induction of COX-2 by resveratrol is activated ERK1/2-dependent (12,27,40). N-acetylated phytosphingosine, a phytosphingosine derivative, elevates COX-2 expression via tyrosine kinase and Protein Kinase C with subsequent ERK activation (41). Other reports indicate that induction of COX-2 is ERK and p38 dependent (42–44). The difference between our observations and those of others in this regard is that resveratrol-induced COX-2 expression is not p38 kinase-dependent in perhaps reflecting inducer specificity of the resveratrol effects. Excessive nitric oxide production during inflammation can induce apoptosis in PC12 cells through upregulation of COX-2 expression that can be blocked by either a selective COX-2 inhibitor (SC58635) or siRNA of COX-2 (45). Our results also indicate that interference with COX-2 activity by NS398 or by siRNA knockdown of COX-2 reduces resveratrol-induced apoptosis. These results testify the importance of COX-2 in the apoptosis but do not distinguish between possible activity of the protein in transcription and enzymatic activity. As stated above, resveratrol inhibits COX-2, at both protein level and enzymatic activity, induced by phorbol 12-myristate 13-acetate and other chemicals. Thus, the nuclear COX-2 induced by resveratrol should not have enzymatic activities (46).

Transcriptional activation of COX-2 gene occurs quickly and transiently in response to a wide range of stimuli (14). The signal transduction pathways and activation of different kinases involved in COX-2 expression are well documented (16). Resveratrol promotes accumulation of c-Fos and c-Jun in cancer cells (8–10) that have been demonstrated to be involved in resveratrol-induced COX-2 expression (27). Chromatin immunoprecipitation studies using antibody to c-Jun showed increased activator protein-1 (AP-1) binding to the COX-2 promoter region in resveratrol-treated cells (27). Resveratrol has been shown to inhibit nuclear factor-kappaB activity (47). There are nuclear factor-kappaB, AP-1 and cyclic adenosine 3′,5′-monophosphate response elements (CREs) binding sites on the 5′-UTR (untranslated region) of the COX-2 gene (48). Transcription factors bind to these sites in a variety of combinations depending on cell type and also on which regulatory pathway has been activated. It is not surprising that resveratrol-induced COX-2 expression depends on AP-1 but not NF-kB, which differs from other inducers.

The intracellular distribution of COX-2 protein has been thought previously to be largely perinuclear and in endoplasmic reticum, based on studies in fibroblasts and endothelial cells (25). The mechanisms involved in nuclear COX-2 accumulation by resveratrol are not understood. Sumoylation imparts repressive properties on transcriptional regulatory proteins. However, recent studies indicate that sumoylation has also been associated with transcriptional activation such as with p53 (49), glucocorticoid receptor (50) and NF-E2 (51). Sumoylation maintains transactivators and other proteins in their nuclear localization. Nuclear colocalization of SUMO-1 and COX-2 was observed in resveratrol-treated OVCAR-3 cells. Immunocomplex of
Inhibition of ERK1/2 activation reduces colocalization of nuclear SUMO-1 and COX-2 and sumoylated COX-2 formation. Activated ERK1/2 is also involved in phosphorylation, acetylation of p53 and p53-dependent apoptosis (8–10). MAPK directly activates transcription factor Tec1 in the yeast, resulting in a decrease of Tec1 sumolation and a concurrent increase of Tec1 transcriptional activity (52).

Resveratrol-induced COX-2 accumulation is associated with an increase in nuclear accumulation of Ser-15 phosphorylated p53. Direct interaction of p53 and COX-2 was confirmed by co-immunoprecipitation of these two proteins in nuclear fractions of cells, and p300 is also found to be present in the complex. These are shown to be COX-2- and ERK1/2-dependent actions of resveratrol. Constitutive overexpression of exogenous COX-2 in cells containing wild-type p53 does not appear to affect cytoplasmic or nuclear levels of p53 (54). However, inhibition of COX-2 activity by non-steroidal anti-inflammatory drugs has been shown to increase accumulation of both COX-2 and p53 in the nucleus (22). These results suggest that there are different mechanisms of p53 accumulation when the latter is the result of constitutively overexpressed COX-2, such as that obtained by plasmid transfection, and when COX-2 is induced by either a non-steroidal anti-inflammatory drug or resveratrol.

The COX-2-specific inhibitor, NS398, which not only inhibits COX-2 enzymatic activity but also reduces abundance of COX-2, inhibits Ser-15 phosphorylation of p53 and p53-dependent apoptosis in OVCAR-3 ovarian cancer cells. It does not affect ERK1/2 activation in resveratrol-treated cells (12,40). Studies by Munkarah et al. (55,56) also show that NS398 inhibits paclitaxel- and docetaxel-induced apoptosis in epithelial ovarian cancer cell lines when NS398 is used before or concurrently with anticancer drugs. The difference from their conclusion in which NS398 might reduce the expression of the pro-apoptotic gene bax, in our observation is that inducible COX-2 by resveratrol blocked by NS398 inhibits resveratrol-induced p53-dependent apoptosis.

Studies find that complexes of COX-2 with other nucleoproteins can be recovered in resveratrol-treated cancer cells and reduced abundance of nuclear COX-2 disrupts p53-mediated apoptosis. 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. COX-2 has been shown to be associated with nuclear matrix suggesting that nuclear COX-2 is involved in essential nuclear activities such as transcription, replication and regulation of gene expression in vascular endothelial cells (57). Put together, from the standpoint of cancer cell growth, COX-2 appears 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.

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