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Review

# The pro-apoptotic action of stilbene-induced COX-2 in cancer cells

## Convergence with the anti-apoptotic effect of thyroid hormone

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**Constitutively expressed cyclooxygenase-2 (COX-2) is a marker of tumor cell aggressiveness. Inducible COX-2 has also been described in cancer cells and localizes in the cancer cell nucleus, where formation of a complex of mitogen-activated protein kinase (MAPK) and COX-2 is antecedent to p53-dependent apoptosis. The stilbene resveratrol is a model pharmacologic activator of this pro-apoptotic mechanism. Physiological concentrations of thyroid hormone are anti-apoptotic in several types of tumor cells. A mechanism by which the hormone is anti-apoptotic is disruption of the nuclear MAPK-COX-2 complex. We review here the apoptosis-relevant effects of resveratrol and thyroid hormone and then speculate about the significance of convergence of these actions in cancer cells in the intact organism. Clinical activity of resveratrol may be modulated by normal tissue levels of endogenous thyroid hormone, and hypothyroidism in the cancer patient—whether spontaneous or induced by chemotherapeutic agents—may permit full expression of the apoptotic activity of the administered stilbene. Chronic pharmacologic inhibition of COX-2 may oppose the pro-apoptotic effect of resveratrol.**

## Cyclooxygenase and Resveratrol

The biochemical and pharmacological distinctions between cyclooxygenase (COX)-1 and COX-2 activities and the regulation of such activities that result in local prostaglandin (PG) production from arachidonic acid (AA) are well-described in inflammatory cells.[1] There has been much recent interest in the observation that constitutive *COX-2* gene expression in cancer cells appears to predict aggressiveness of tumors. A clinical corollary of this observation is the possibility that nonsteroidal anti-inflammatory drug (NSAID) therapy that inhibits COX-2 activity may improve the clinical behavior of COX-2-expressing cancer.[2-5]

Our laboratory has shown that it is possible pharmacologically to induce *COX-2* in certain tumor cells. When induced, the COX-2 protein translocates to the cell nucleus. In contrast to constitutive *COX-2* gene expression in cancer cells, this inducible form of the cyclooxygenase is pro-apoptotic and may be a clinically desirable endpoint in tumor cells. Conceivably, the pharmacologic inhibition of the inducible enzyme may be clinically undesirable.

Resveratrol is a widely-studied stilbene that has anti-cancer[6-14] and other biological properties.[15-17] The anti-cancer properties have been shown to have several mechanisms, one of which is induction of apoptosis. This stilbene is naturally occurring and its structure is in the public domain. Thus, unmodified resveratrol is not of commercial interest for pharmacologic development as a cancer chemotherapeutic agent, but reformulated analogues of resveratrol are under study.[18] We have used unmodified resveratrol as a model pharmacologic inducer of *COX-2* in cancer cells[11,13,14] and have implicated resveratrol-inducible COX-2 protein upstream in p53-dependent apoptosis.[11,13,14] We have also shown that a receptor for the stilbene exists on integrin $\alpha v\beta 3$,[10-14] a structural protein of the plasma membrane that is critical to cancer and non-cancer cell interactions with extracellular matrix proteins[19-21] and with certain growth factors.[22-25]

Thus, cancer cells may exhibit both constitutive and inducible COX-2 protein. These apparently discrete pools reflect different roles for the protein in tumor cells and may be different targets for manipulation in the setting of cancer.

## COX-2 in Tumorigenesis and Angiogenesis

As noted above, constitutive upregulation of *COX-2* gene expression has been found in a variety of cancers and may index invasiveness. These include cancers of the cervix,[26] endometrium,[27,28] prostate[29] and breast[30,31] where it has been shown to play a role in tumorigenesis.[31-33] Reports of such constitutive expression were initially surprising, given that COX-2 had been widely appreciated to be an inducible gene in non-cancer cells. It was

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then found that COX-2-specific inhibitors that were administered for anti-inflammatory purposes incidentally conferred the benefit of reducing the likelihood of colon cancer recurrence.[34,35] While this implied that the prostaglandin (PG) products of the COX-2 enzymatic activity might support tumor growth, other mechanisms are possible. One of these is that the arachidonic (AA) precursors of PGs suppressed tumor growth when they accumulated in cells as a result of inhibition of the enzymatic activity of COX-2.[36-38]

Another possible explanation for the anti-cancer effect of pharmacologic inhibitors of COX-2 is that previously unappreciated actions of the cellular COX-2 protein were being modulated by the COX-2 inhibitors. That this could be the case is suggested by studies of the mechanism by which the stilbene resveratrol induces apoptosis in cancer cells, as indicated above. Such studies disclose that subcellular distribution of COX-2 in resveratrol-treated cancer cells, monitored by confocal microscopy, included the intranuclear compartment,[13] as well as the perinuclear zone or nuclear envelope.[14] The nuclear accumulation of COX-2 is unaffected by nonspecific cyclooxygenase inhibitors, but is blocked by treatment of cells with a specific pharmacologic COX-2 inhibitor.[11,13,14]

That COX-2 might also be involved in angiogenesis was appreciated when aspirin, NS398 and NSAIDs were shown to reduce angiogenesis in vivo and in vitro.[39] NS398 is a specific inhibitor of COX-2, whereas aspirin and NSAIDs that are used clinically inhibit both COX-1 and COX-2.[40,41] COX-2 modulates angiogenesis by several mechanisms, including stimulating production of angiogenic factors, such as vascular endothelial growth factor (VEGF)[42] and platelet-derived growth factor.[43,44] Indeed, there may be co-localization of COX-2 and VEGF at the advancing edge of tumor cells.[43] The interface between COX-2 and molecules such as PDGF is known to be relevant to participation of pericytes and vascular mural cells (VMC) in developing vasculature.[45] Targeting of PDGF-β in combination with VEGF has antitumor efficacy in experimental models[46,47] and pharmacologic COX-2 inhibition is an additional measure that offers anti-angiogenic and anti-proliferative properties.

β-catenin is a multifunctional protein that interacts with many proteins, including the sequence-specific DNA binding transcription factor TCF and other proteins implicated in transcription and chromatin remodeling.[48] Cytoplasmic β-catenin is associated with COX-2 overexpression, supporting the role of cytoplasmic β-catenin in stabilizing COX-2 mRNA.[49] Correlation of survivin distribution with COX-2 and β-catenin expression patterns is observed in colo-rectal cancer. The co-localization of COX-2/β-catenin/survivin in the same epithelial cells in tumor samples lends credence to possible in vivo regulatory effects of COX-2 and β-catenin on the intracellular survivin levels in mouse and human colon cancer.[50]

Chemokine receptor CXCR4 is also involved in the homing of vascular progenitor cells to sites of active angiogenesis[51,52] and the receptor also has been shown to be affected by COX-2 inhibition.[45] Activation of this receptor can stimulate the PI3K/Akt pathway in a number of cell types.[53,54] In endothelium, CXCR4 mediates capillary tube formation stimulated by prostaglandin E, an effect that is disrupted by cyclooxygenase blockade.[52] Significantly lower expression of CXCR4 in SC-236-treated tumors is detected by microarray and confirmed by diminished CXCR4 immunopositivity in the abnormal, segmentally dilated tumor vessels.[45] Lee et al. have shown that a specific COX-2 inhibitor, SC-236, disrupts an early phase of VMC incorporation in tumor-related blood vessels, perhaps by blocking CXCR4-mediated incorporation of early pericytes/vascular mural progenitor cells into xenograft vessels.[45]

These various studies that link COX-2 and tumorigenesis or tumor-related angiogenesis or both are based on the concept of constitutive production of the cyclooxygenase by cancer cells. While it appears that nuclear COX-2 induced by resveratrol opposes the activity of constitutive COX-2 on tumor cell proliferation,[11,13,14] it is not yet known whether the pro-angiogenic activity of constitutive COX-2 is affected by the induction of nuclear accumulation of cyclooxygenase.

## Functions of Nuclear COX-2 and Protein Complexing

Immunofluorescent studies in murine 3T3 cells and human and bovine endothelial cells by Smith et al. have indicated that COX-2 localizes in the endoplasmic reticulum (ER), Golgi complex and nuclear envelope (NE).[55,56] Catalytically active COX-1 and COX-2 are localized in the nuclear envelope and ER of $PGE_2$-releasing cells.[55,56] More recent studies have suggested that for functional coupling and $PGE_2$ biosynthesis, cytosolic $PLA_2$, COXs and PGEs appear to be localized in the perinuclear region.[57-59]

Patel et al. have shown that stimulation of murine RAW 264.7 macrophages with lipopolysaccharide (LPS) causes 90% of COX-2 to localize in the nuclear fraction and ~10% in cytoplasm.[60] In quiescent endothelial cells from human umbilical vein, porcine and human cerebral microvessels, COX-2 is also found principally in the nucleus.[61,62] When endothelial cells are treated with interleukin (IL)-1β, nuclear COX-2 relocates gradually to the nuclear envelope and cytoplasm.[62] The functioning of the longer C-terminal segment in COX-2 is distinctly more tolerant of structural change than the shorter COX-1 C-terminal segment. However, C-terminal substitutions or deletions do not change the subcellular localization of either isoform, indicating that neither of the C-terminal segments contains indispensable intracellular targeting signals.[63] Mechanisms for inducible COX-2 translocation to the nucleus are still not clear.

In the plasma membrane, COX-2 and caveolin-3 (Cav-3) are co-localized in caveolae, a microdomain in which glycosylphosphatidylinositol (GPI)-anchored proteins reside and form a caveolar protein-protein complex in human fibroblasts[64] and in primary cultures of rat chondrocytes.[65] This suggests that the caveolins might play a role in the regulation of COX-2 functions.[65] Type IIA secretory phospholipase $A_2$ ($sPLA_2$-IIA) is present in caveolae and also in the perinuclear area in proximity to COX-2. A GPI-anchored heparan sulfate proteoglycan glypican facilitates the trafficking of $sPLA_2$-IIA into particular subcellular compartments, and arachidonic acid thus released from the compartments may link efficiently to the downstream COX-2-mediated PG biosynthesis.[66]

Studies by Parfenova et al.[62] indicate that nuclear COX-2 in vascular endothelial cells is associated with the nuclear matrix that

spatially organizes chromatin; the association implies involvement of COX-2 in essential nuclear activities such as transcription, replication and regulation of gene expression.[67-69] Nuclear COX-2 has been shown to be complexed with Ser-15 phosphorylated p53 and phosphorylated ERK1/2.[11,13,14] It is well-established that resveratrol is capable of inducing apoptosis in cancer cells[10-14] and that stilbene-induced apoptosis in this setting is p53-requiring.[6,14] Surprisingly, exposure of resveratrol-treated cells to a specific COX-2 inhibitor blocked stilbene-induced apoptosis.[11,13,14] This suggested that resveratrol-inducible COX-2, rather than being anti-apoptotic—like constitutively-expressed COX-2—participated in the pro-apoptotic process.

Because COX is irreversibly inactivated following catalysis, it is assumed that COX activity is a function of the amount of the enzyme protein present and that the latter is regulated exclusively at the levels of transcription and translation. However, induction of COX-2 expression (measured as mRNA or protein) does not always correlate with prostanoid synthesis. In fact, recent studies have demonstrated that posttranslational modification of COX-2 in the form of tyrosine phosphorylation regulates COX-2 activity in cerebral endothelial cells.[70]

In contrast to such studies in non-tumor cells, experiments we have conducted in cancer cells have shown that stilbene-induced accumulation of COX-2 in the nucleus may be associated with the generation of complexes of COX-2 and ERK1/2 that are relevant to p53-dependent apoptosis.[11,14] It is not clear what the biochemical steps are between the COX-2-activated MAPK (pERK1/2) complex and activation of p53. The induction of COX-2 has been shown to be either ERK1/2- or p38-dependent.[11]

As noted above, recent studies from our laboratory also indicate that resveratrol-induced COX-2 associates with activated ERK1/2 in the nucleus of cancer cells.[13] It is not yet clear if activated ERK1/2 plays a role in nuclear COX-2 posttranscriptional modification, e.g., phosphorylation.[70,71] In addition to the p38 and MAPK signal transduction pathways, the PI-3K/Akt cascade is also involved in expression and functions of COX-2. The PI 3-K/Akt pathway is activated by COX-2 or its product, $PGE_2$.[72] $PGE_2$ increases angiogenesis by stimulating the PI-3K/Akt pathway and nitric oxide (NO) production in human umbilical vein endothelial cells (HUVEC).[73]

The finding that nuclear COX-2 can bind to the promoter region of one or more genes[11,74]—and certainly to the promoter region of its own gene—suggests that the protein may be transcriptionally active or serve as a co-factor (corepressor or coactivator) for transactivator proteins.[74] Thus, a view of this protein exclusively as an enzyme that is a critical step in the production of PGs may be too limited. Additional information is needed about the putative transcriptional role of the protein. It will also be important to determine whether constitutively expressed COX-2 protein plays a role in transcription.

An additional role for inducible COX-2 in the tumor cell nucleus was indicated by its recovery in a complex with MAPK (ERK1/2). In thyroid hormone-treated cells, we have described nuclear complexes of ERK1/2 with transcriptionally active proteins, such as nonpeptide hormone receptors,[75-78] STAT proteins[79,80] and the oncogene suppressor protein, p53.[81] Activated MAPK in this context serves to serine phosphorylate (activate) the proteins with which it is associated. We therefore considered the possibility that, following its activation by resveratrol and consequent translocation to the nucleus, ERK1/2 may play a role in the p53-dependent apoptosis that the stilbene induces (see above). Inhibition of MAPK activation pharmacologically by PD98059 or inhibition of COX-2 by NS398 blocks the nuclear complexing of COX-2, phosphorylated p53 and activated ERK1/2.[11,13]

## Thyroid Hormone-Induced Tumor and Endothelial Cell Proliferation

Acting via a plasma membrane receptor on integrin $\alpha v\beta 3$, thyroid hormone ($T_4$ and 3,5,3'-triiodo-L-thyronine, $T_3$) in physiologic concentrations causes proliferation in vitro of several tumor cell lines. These cell lines include glial cells,[13,82] human estrogen receptor (ER)-positive breast cancer (MCF-7) cells,[78] lung cancer cells,[83] thyroid cancer cells[12] and head-and-neck cancer cells (HY Lin, unpublished observations). While this thyroid hormone receptor is on the same integrin as the resveratrol receptor and both sites can lead to activation of MAPK, the binding sites are discrete.[84,85] The thyroid hormone analogue tetrac inhibits the cancer cell proliferation activity of $T_4$ and $T_3$. We have also shown that thyroid hormone can increase the growth of tumor xenografts, e.g., human breast cancer (MCF-7) cells in the nude mouse,[85] an activity that is also blocked by systemic tetrac administration. Because the hormone receptor site is at or near the Arg-Gly-Asp (RGD) recognition site on integrin $\alpha v\beta 3$, the RGD peptide can, like tetrac, inhibit the hormone effect on cancer cell proliferation. The RGD recognition site is relevant to the interaction of the integrin with important extracellular matrix (ECM) proteins and growth factors.[24,86]



Tumor cell proliferation induced by thyroid hormone is MAPK/ERK-requiring and an inhibitor of the ERK1/2 signal transduction pathway, PD98059, is effective in decreasing the proliferative action of the hormone.[78,82] The MAPK signal transduction cascade is important to a variety of biologic functions in normal cells, however, and the clinical application of a MAPK inhibitor to the thyroid hormone effect in cancer cells is likely to have an unfavorable side effect profile. It should be noted that thyroid hormone can nongenomically activate another important cellular signal transduction pathway, the phosphatidylinositol 3-kinase (PI 3-K) cascade.[86,87] However, the PI 3-K pathway effect of iodothyronines appears not be relevant to induction by the hormone of tumor cell proliferation.[86] In non-cancer cells, the activation of PI 3-K by thyroid hormone is involved downstream in transcription of certain genes that are relevant to carbohydrate handling[88] and to regulation of plasma membrane Na, K-ATPase activity.[89]

Thyroid hormone has recently been appreciated to be pro-angiogenic.[24,90,91] This action may be desirable in the contexts of processes such as wound-healing[92,93] or improvement of blood flow in ischemic tissues, but is undesirable in the environment of cancers. Tetrac is a potent anti-angiogenic agent[25,85] and inhibits the action of thyroid hormone on new blood vessel growth. Interestingly, tetrac is anti-angiogenic even in the absence of

thyroid hormone, serving to block the actions of VEGF and basic fibroblast growth factor (bFGF).[24] We have proposed that this action of tetrac relates to crosstalk between the integrin receptor for thyroid hormone and specific vascular growth factor receptors that may be clustered with integrin αvβ3.

That thyroid hormone can induce and that tetrac can block angiogenesis around tumor cell masses in vitro has been shown in studies conducted in the chick chorio-allantoic membrane (CAM) model of angiogenesis.[25] Such supportive neovascularization is attributable to the release of pro-angiogenic growth factors by tumor cells.

## Convergence of Integrin-Dependent, COX-2-Requiring Actions of Resveratrol and Thyroid Hormone at Apoptosis in Cancer Cells

It is apparent that resveratrol is an effective pro-apoptotic factor in certain cancer cells. Others and we have shown that this activity of the stilbene is p53-dependent.[6,7,94] As described above, a resveratrol-inducible pool of COX-2 is a component of this process, forming nuclear complexes with activated MAPK (pERK1/2) (Fig. 1). In contrast, thyroid hormone is a proliferative factor for cancer cells and functionally anti-apoptotic by a mechanism that is also pERK1/2-dependent (Fig. 1). These pro-apoptotic and anti-apoptotic actions, respectively, of the stilbene and iodothyronine begin nongenomically at discrete receptors on cell surface integrin αvβ3.

We have begun to address the particular anti-apoptotic actions of $T_4$ and $T_3$ in resveratrol-treated cancer cells. It is now clear that thyroid hormone decreases or prevents the formation of nuclear complexes of MAPK and inducible COX-2 in stilbene-exposed cells.[13,14] Such complexes are upstream of p53-dependent induction of expression of such genes as *BcL-X* short form whose effects are pro-apoptotic. The mechanism by which thyroid hormone antagonizes COX-2-MAPK complex formation in the tumor cell nucleus is not yet clear, but it is possible that the hormone re-directs to other cellular functions the pool of MAPK committed in resveratrol-treated cells to nuclear COX-2 complex formation. Such an anti-apoptotic re-direction might be to cell proliferation, since induction of tumor cell proliferation by thyroid hormone is MAPK-dependent.[12,78,82,86,87]

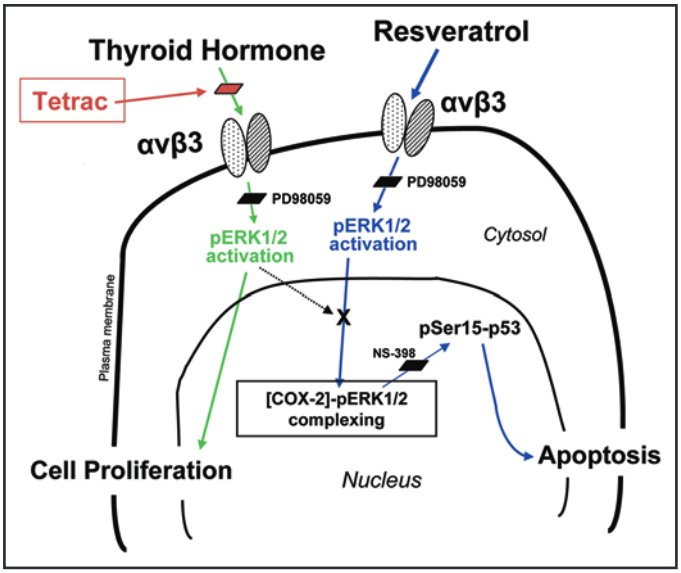


Figure 1. Signaling pathways by which resveratrol induces apoptosis and thyroid hormone mediates proliferation in cancer cells. Thyroid hormone stimulates cancer cell proliferation via a hormone receptor on an integrin (αvβ3)[23] that is expressed in plasma membranes of tumor cells and endothelial and vascular smooth muscle cells. ERK1/2 activation (pERK1/2) is required for thyroid hormone-dependent cell proliferation, as shown by the action of ERK1/2 cascade inhibition by PD98059. A discrete stilbene receptor also is present on integrin αvβ3,[10] by which resveratrol activates ERK1/2 and induces nuclear accumulation of COX-2. In resveratrol-treated cancer cells, pERK1/2 also translocates to the cell nucleus and complexes with inducible COX-2. Formation of this complex is an essential upstream feature of induction by resveratrol of p53-dependent apoptosis. The latter requires phosphorylation of p53 at Ser-15. NS-398 is a specific COX-2 inhibitor that blocks resveratrol-induced activation of p53 and apoptosis. Thyroid hormone inhibits formation of the intranuclear complex of ERK1/2 and COX-2 and, thus, resveratrol-induced, p53-requiring, apoptosis. The mechanism of the inhibition by the hormone of ERK1/2-COX-2 nuclear complexes in resveratrol-exposed cells is not yet known, but may involve competition for ERK1/2 by thyroid hormone and resveratrol and diversion of the kinases to the cell proliferation pathway.

## Conclusions

The pro-apoptotic function of stilbenes has previously been recognized and may involve several mechanisms.[95] However, the anti-apoptotic capacity of thyroid hormone has only recently been appreciated in the laboratory.[12,13] The tumor-promoting activity of thyroid hormone has also been suggested by clinical observations.[96-98] An inducible pool of COX-2 that translocates to the tumor cell nucleus is involved in the pro-apoptotic, p53-dependent action of resveratrol we have recently described and is the focus of at least one of the anti-apoptotic effects of thyroid hormone.

The possible clinical consequences of these observations are several. First, any clinical applications of stilbenes as cancer chemotherapeutic agents may be opposed by circulating endogenous levels of thyroid hormone in the euthyroid patient. It should be noted that development of spontaneous hypothyroidism or chemical induction of mild hypothyroidism in the absence of stilbene may result in slowing of tumor growth.[96,98,99] The clinical utility of resveratrol-like agents may be enhanced by reduction in thyroid hormone levels by low-dose antithyroid therapy or, if it is introduced clinically, by tetrac. The latter acts at the *cell surface* integrin αvβ3 thyroid hormone receptor to reduce the proliferative effect of the hormone, but will not reduce the desirable *intracellular* actions, both genomic and nongenomic, of thyroid hormone, itself.

Second, in patients who may participate in trials of stilbenes, the finding of coincidental biochemical hypothyroidism, i.e., mild elevation of serum thyrotropin (TSH) concentration without symptoms of hypothyroidism, may not be an immediate indication to introduce thyroid hormone replacement.[98,99] The incidental induction of *biochemical* hypothyroidism by chemotherapeutic tyrosine kinase inhibitors such as sunitinib does not in our view mandate full or partial thyroid hormone replacement.[100-102] This point of view may also be relevant to combinations of tyrosine

kinase inhibitor therapy and stilbenes, should such combination therapy come to clinical trial.

Finally, the existence of an inducible intracellular/intranuclear pool of COX-2 that is relevant to apoptosis raises the issue of how to utilize specific COX-2 inhibitors in the setting of colon or other cancers that may in part be dependent upon constitutive COX-2 production. That is, one can conceive of intermittent COX-2 inhibitor administration in such patients to permit briefly the chemical induction, such as has been modeled with resveratrol, of the nuclear pool of COX-2 and cycles of apoptosis.

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