HHS Public Access

Author manuscript

Oncogene. Author manuscript; available in PMC 2020 April 27.

Published in final edited form as:

Oncogene. 2008 December 04; 27(53): 6729–6737. doi:10.1038/onc.2008.322.

Proline oxidase, a p53-induced gene, targets COX-2/PGE_2 signaling to induce apoptosis and inhibit tumor growth in colorectal cancers

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Abstract

Proline oxidase (POX), a flavoenzyme localized at the inner mitochondrial membrane, catalyzes the first step of proline degradation by converting proline to pyrroline-5-carboxylate (P5C). POX is markedly elevated during p53-induced apoptosis and generates proline-dependent reactive oxygen species (ROS), specifically superoxide radicals, to induce apoptosis through both mitochondrial and death receptor pathways. These previous studies also showed suppression of the mitogen-activated protein kinase pathway leading us to broaden our exploration of proliferative signaling. In our current report, we used DLD-1 colorectal cancer cells stably transfected with the POX gene under the control of a tetracycline-inducible promoter and found that three pathways which cross talk with each other were downregulated by POX: the cyclooxygenase-2 (COX-2) pathway, the epidermal growth factor receptor (EGFR) pathway and the Wnt/β-catenin pathway. First, POX markedly reduced COX-2 expression, suppressed the production of prostaglandin E2 (PGE_2) and importantly, the growth inhibition by POX was partially reversed by treatment with PGE_2. Phosphorylation of EGFR was decreased with POX expression and the addition of EGF partially reversed the POX-dependent downregulation of COX-2. Wnt/β-catenin signaling was decreased by POX in that phosphorylation of glycogen synthase kinase-3β (GSK-3β) was decreased on the one hand and phosphorylation of β-catenin was increased on the other. There changes led to decreased accumulation of β-catenin and decreased β-catenin/TCF/LEF-mediated transcription. Our newly described POX-mediated suppression of proliferative signaling together with the previously reported induction of apoptosis suggested that POX could function as a tumor suppressor. Indeed, in human colorectal tissue samples, immunohistochemically-monitored POX was dramatically decreased in tumors compared with normal counterparts. Thus, POX metabolism of substrate proline affects multiple signaling pathways, modulating both apoptosis and tumor growth, and could be an attractive target to metabolically control the cancer phenotypes.

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Keywords

proline oxidase; COX-2; signaling cross talk; colorectal cancer; tumor suppressor

Introduction

Prostaglandins and other arachidonic acid metabolites participate in the regulation of normal cell growth as well as in aberrant proliferation observed in pathogenic states such as chronic inflammation and carcinogenesis (Prescott and Fitzpatrick, 2000; Gupta and Dubois, 2001; Dannenberg *et al.,* 2005). The cyclooxygenase (COX) enzyme catalyzes the key step in the conversion of free arachidonic acid to prostaglandins. Colorectal cancer as well as other solid tumors exhibits elevated COX-2 levels resulting from defects in the normal regulation of COX-2 gene expression. The relationship between COX-2 expression and carcinogenesis was first suggested by studies demonstrating the efficacy of aspirin and other nonsteroid anti-inflammatory drugs in reducing the relative risk of colon cancer and in promoting colon cancer tumor regression in both humans and experimental animal models (Williams *et al.,* 1997; Kawamori *et al.,* 1998; Gupta and Dubois, 2001). Now it is widely accepted that COX-2 and prostaglandins, especially prostaglandin E2 (PGE_2), are directly related to the development and progress of colorectal cancer as well as cancers in other tissues. Inhibition of COX-2 through pharmacological means or direct regulation of its expression/activity can limit the development or progress of human cancers. Thus, they are important targets for cancer prevention and treatment.

Colorectal cancer is the second leading cause of cancer-related deaths in both men and women in the United States (Jemal *et al.,* 2005). The development of colorectal cancer results from the sequential accumulation of mutations or deletions in the coding sequence of a number of tumor suppressor genes and oncogenes, together with aberrant activity of molecules controlling genomic stability. COX-2, p53, epidermal growth factor receptor (EGFR), β-catenin/adenomatous polyposis coli (APC), K-ras and so on, all may play important roles in colorectal carcinogenesis (Worthley *et al.,* 2007). For example, more than 80% of colorectal cancer have either β-catenin or APC mutation, leading to the activation of this pathway, although EGFR is overexpressed in more than 50% of colorectal adenocarcinomas and is associated with a more aggressive and invasive phenotype. The interaction between these signalings, such as the interaction between COX-2 and EGFR, and COX-2 and β-catenin/APC are critical (Dannenberg *et al.,* 2005). Thus, these interactions are essential for understanding colorectal cancer carcinogenesis as well as for therapeutic intervention.

Proline oxidase (POX) is a mitochondrial innermembrane flavoenzyme involved in the degradation of the amino acid proline, which constitutes more than 25% of incorporated residues of collagen, the most abundant protein in the extracellular matrix and in the human body. POX catalyzes the conversion of proline to pyrroline-5-carboxylate (P5C). Together with P5C reductase, which converts P5C back to proline, they mediate the proline cycle to shuttle redox equivalents between mitochondria and the cytosol. Moreover, P5C is the intermediate providing a direct carbon bridge connecting the tricarboxylic acid (TCA) and

urea cycles. Recent studies have revealed POX's response to energy and nutrient stress. After it was found to be one of the p53-induced genes in p53-induced apoptosis in colorectal cancer, its role in cell proliferation, apoptotic cell death, especially in cancer cells has been intensively investigated. It is induced by p53 (Polyak *et al.,* 1997), as well as by peroxisome proliferator-activated receptor-γ, another important regulator of a variety of cell processes (Pandhare *et al.,* 2006). POX may induce apoptosis through both intrinsic and extrinsic pathways. In both, the generation of reactive oxygen species (ROS), especially superoxides, plays a critical role. Downstream mediators include activation of calcineurin/nuclear factor of activated-T cell pathway and inhibition of MEK/ERK signaling (Rivera and Maxwell, 2005; Liu *et al.,* 2006b).

POX caused apoptotic cell death, inhibited tumor growth *in vitro* and *in vivo* (manuscript submitted), which prompted us to ask whether these effects were connected with the aforementioned signaling that are critical for colorectal carcinogenesis. Here, we show that POX suppresses COX-2/PGE_2 activities and PGE_2 rescues cells from POX-induced apoptosis. In addition, the β-catenin/APC and EGFR signaling are also suppressed by POX. This indicates POX's effects involved multiple pathways in colorectal cells and further supports that cross talk among these pathways is critical for tumor development. The findings of reduced expression of POX in human cancer tissue samples compared with their normal tissue counterparts from the same patient suggest that POX could be an important tumor suppressor in colorectal tissues. As POX and related enzymes are such important regulators in the metabolism of extracellular matrix, these findings strongly suggest that it might modulate cancer phenotypes in situations of energy/nutrient stress.

Results

The inhibition of COX-2/PGE_2 signaling by POX

We and others (Maxwell and Rivera, 2003; Liu *et al.,* 2005, 2006b; Rivera and Maxwell, 2005) have demonstrated that POX induced apoptosis and inhibited tumor growth in a variety of types of cancers, including cancers from colorectum. As COX-2/PGE_2 signaling plays such an important role in colorectal carcinogenesis, we decided to determine whether it also is involved in POX-dependent inhibition of tumor growth. Using DLD-1 colorectal cancer cell line, in which POX could be induced by removal of doxycycline (DOX), we monitored the expression levels of COX-2 under the induction of POX. We found that after removal of DOX and induction of POX, the expression of COX-2 was markedly reduced (Figure 1a). The COX-2 expression was not affected by DOX in DLD-1 Tet-Offvector cells, where POX was not induced by the removal of DOX from culture medium (Figure 1a). On account of transcriptional activation of COX-2, for example by growth factors, transcriptional factors, proinflammatory mediators, is also an important means for regulating COX-2 activity, we employed a COX-2 promoter luciferase construct to check its potential change by POX. The data showed minimal differences between conditions with and without DOX, indicating that POX did not transactivationally suppress COX-2 activity (Figure 1b).

As the main enzymatic product of COX-2, the level of PGE_2 was also examined. As expected, PGE_2 levels were also considerably decreased by POX (Figure 1c). To further support the role of COX-2/ PGE_2 signaling in POX-induced apoptosis and growth inhibition,

we treated DLD–POX cells with different concentrations of PGE_2 and performed assays to determine cell cycle distribution and cell growth, and found PGE_2 partially reversed POX-induced apoptosis, which dropped from 19.1 to 7.7% on an average in day 3 without DOX (Figure 1d and e). It also partially reversed growth inhibition (Figure 1f). These data suggest that COX-2/ PGE_2 inhibition plays an important role in POX-induced apoptosis and growth inhibition.

The involvement of ROS/superoxides in the inhibition of COX-2/PGE_2 activity and cell growth

We have shown that POX-induced apoptosis was directly mediated by ROS/superoxides (Liu *et al.,* 2005, 2006b). Here, again we showed that POX expression caused the generation of ROS/superoxide in a time-dependent manner (Figure 2a). Manganese superoxide dismutase (MnSOD), a mitochondrial enzyme, which converts superoxides to H_2O_2, blocked this apoptosis (Figure 2b). We suspected that these effects were also, at least partially, through COX-2/PGE_2 regulation and mediated by ROS/superoxides. To prove this, we treated DLD-1-POX cells with MnSOD recombination adenovirus (Ad-MnSOD) and found that MnSOD partially reversed POX-mediated decrease of COX-2 (Figure 2c). Consistent with this, MnSOD also reversed reduced levels of PGE_2 by POX (Figure 2d), supporting the interpretation that COX-2/ PGE_2 diminution by POX was mediated by increased levels of ROS/superoxides.

The involvement of EGFR signaling and β-catenin pathway

Colorectal carcinogenesis is a complicated process that involves the deregulation of multiple signaling pathways (Kinzler and Vogelstein, 1996; Wood *et al.,* 2007). In addition to COX-2, other mediators include p53 tumor suppressor, APC/β-catenin singaling, K-Ras oncogene, EGFR tyrosine kinase and so on. We have shown that COX-2/ PGE_2 played a role in POX-induced apoptosis. Actually, there are a growing number of studies showing that interactions among COX-2, β-catenin and EGFR are critical for colorectal carcinogenesis, and all of them or specific combinations could be targets for therapeutic intervention (Castellone *et al.,* 2005; Dannenberg *et al.,* 2005; Liu *et al.,* 2006a). Thus, we further examined these pathways. We found that after induction of POX by removal of DOX, the phosphorylation (activity) of EGFR was reduced (Figure 3a). This reduction was partially restored after the introduction of MnSOD by an adenovirus vector, suggesting a role for ROS/superoxides (Figure 3b). To further determine the importance of EGFR signaling in POX-induced apoptosis and growth inhibition, we treated the POX-expressing cells with EGF. Although we did not observe the reversion of apoptosis induction or growth inhibition (data not shown), we found EGF treatment partially blocked COX-2 reduction by POX, indicating the linkage between EGF and COX-2 (Figure 3c). For the APC/β-catenin pathway, we determined the phosphorylation status of glycogen synthase kinase-3β (GSK-3β) and β-catenin and found decreased phosphorylation of GSK-3β and increased serine phosphorylation of β-catenin by POX (Figures 4a and c), both indicating reduced activity of the APC/β-catenin pathway. Both changes were partially reversed after a MnSOD adenovirus infection, indicating a critical role for ROS/superoxides (Figures 4b and d). To determine the effect of POX on this pathway at the promoter level, we performed luciferase

assay using TOPflash and FOPflash and found the TCF/LEF promoter activity was also reduced by POX (Figure 4e).

The reduced expression of POX in human colorectal cancer tissues

POX inhibited colorectal tumor growth both in our *in vitro* cell culture model and in an *in vivo* xenograft mouse model (manuscript submitted). It induced apoptotic cell death through modulating multiple pathways that are critical for colorectal cancinogenesis. These observations suggest that POX may play a tumor suppressor role. To extend this idea into clinical cancer, we employed human tissue samples and performed immunostaining for POX to determine its expression and compared cancer tissues versus normal tissues from the same individual. We found that in 20 out of 24 pairs of colorectal tissues, the expression of POX in cancer tissues strikingly decreased compared with normal counterparts from the same patient whereas three cases were unchanged and only one was increased (Figure 5). Statistical analysis indicated the difference is significant ($P<0.001$; Table 1). The data strongly suggest a tumor suppressor role of POX in human colorectal tissues.

Discussion

Several lines of evidence suggest that POX plays a tumor suppressor role. It is a p53 downstream gene in p53-induced apoptosis; it induces apoptosis through activation of both intrinsic and extrinsic pathways and through the modulation of multiple signaling pathways, including MEK/ERK and calcinurin/nuclear factor of activated-T cell. Furthermore, it inhibits growth of various cultured tumor cells and suppresses tumor formation in a xenograft model (manuscript submitted). We now have further demonstrated that POX targets COX-2 for inhibition of colorectal cancer and regulates EGFR and β-catenin/Wnt signalings. Importantly, there is reduced expression of POX in cancer tissues compared with normal tissue counterparts. Although our effort in identifying genetic or epigenetic changes in a human tumor panel has yet to yield conclusive results, literature from genetic sources indicates the existence of several tumor suppressors in chromosome 22q11.2, where the POX gene is located. Chromosome 22q11 is a common breakpoint with frequent deletion or translocation in human cancers. Patients with Di George/22q11 deletion syndrome develop malignancy at much higher frequency than controls (McDonald-McGinn *et al.,* 2006). Two tumor suppressor genes have been cloned from this chromosome site, the existence of another tumor suppressor has been suggested (Sevenet *et al.,* 1999; Huang *et al.,* 2002).

Unregulated COX-2 expression is an important early step in colorectal tumorigenesis. Thus, it is a very important target for cancer chemoprevention and treatment. COX-2-specific inhibitors have been approved for use in the prevention of colorectal polyp formation in patients with familial adenomatous polyposis. Unexpectedly, the prolonged use of high doses of these inhibitors is associated with increased thrombotic events in patients, and thus was voluntarily withdrawn from the market. Thus, search for new COX-2 inhibitors is urgently needed.

Increased amounts of COX-2 are commonly found in both premalignant and malignant tissues. Overexpression of COX-2 occurs because of dysregulated transcriptional and posttranscriptional control. Growth factors, oncogenes, cytokines and tumor promoters

stimulate COX-2 transcription through protein kinase C and Ras-mediated signaling. Depending on the cell type and stimulus, different transcription factors including AP-1, NF-IL6, nuclear factor-κB, nuclear factor of activated-T cell and PEA3 can activate COX-2 transcription (Dixon *et al.,* 2000; Dixon, 2004). Although COX-2 can be upregulated by many factors, much less is known about negative effectors. Wild type, but not mutant p53, can inhibit COX-2 transcription *in vitro.* APC tumor suppressor gene status may also impact COX-2 expression (Gupta and Dubois, 2001). These findings suggest that the balance between oncogenes and tumor suppressor genes modulate the expression of COX-2 in tumors. Here, we found that POX, a candidate tumor suppressor, also suppresses COX-2 expression and activity, indicating that it acts similar to many other important tumor suppressors by targeting multiple signalings to execute its tumor suppressing role.

It's not surprising that POX also inhibits EGFR and β-catenin signalings. Certainly, POX may target and block each individually. Another consideration is the cross talk between these signalings and COX-2. The interactions are critical for colorectal carcinogenesis and are the focus for development of new combinational cancer therapies. First, COX-2/PGE2 induces transactivation of the canonical APC/β-catenin/Wnt and EGFR signaling pathways through G protein-coupled receptors and many commonly used signaling cascades (Dannenberg *et al.,* 2005). In contrast, β-catenin acts on TCF/LEF transcriptional factor to regulate COX-2 expression (Gupta and Dubois, 2001; Araki *et al.,* 2003). The signaling involved in COX-2 induction through EGFR varies depending on the type of cells and inducers, but the ras/raf/MAPK signaling pathways contribute to both increased transcriptional and posttranscriptional control (Dannenberg *et al.,* 2005). This is consistent with our previous finding that POX downregulates MEK/ERK signaling (Liu *et al.,* 2006b). Actually, a direct collaborative effect between PGE_2 and EGFR on tumor cell proliferation and invasion is also well documented (Buchanan *et al.,* 2003; Shao *et al.,* 2004, 2005).

The critical role of ROS/superoxides in POX's regulation of signaling pathways, in its induction of apoptosis and inhibition of growth has been previously reported (Liu *et al.,* 2005, 2006b). It is believed that redox status regulates many signaling pathways, including those involving COX-2, EGFR and β-catenin (Chen *et al.,* 2005; Kim *et al.,* 2005; Korswagen, 2006). MnSOD partially reverses the reduced expression/activity of these molecules/signalings by POX; further indicating superoxides are critical for the reduction of these signalings. Now, we have accumulating data suggesting the importance of ROS/superoxides in mediation of POX's effect. Whether they are the sole mediator for POX's activity is still an open question. P5C, the product of POX activity, which has many links to important cellular processes, must also be considered. On account of the close connection between the proline cycle and the TCA cycle and the potential effect of POX activity on TCA cycle and its intermediates, and because of the importance of TCA cycle for many cellular activities, POX-induced changes in TCA cycle, particularly its intermediates, is an additional consideration.

Extracellular matrix and its interaction with cells have received considerable attention in cell signaling and cancer research, and tumor microenvironment is already considered as an important target for cancer chemoprevention (Albini and Sporn, 2007). Proline, the substrate for POX, is primarily derived from extracellular matrix after the digestion of collagen and so

on, by matrix metalloproteinases and prolidase. Recently, we showed that prolidase, which catalyzes the final step in collagen degradation to produce proline, stimulates hypoxia-inducible factor signaling with a potential role in angiogenesis and tumor progression (Surazynski *et al.,* 2008). Thus, POX can also be considered as a target in cancer prevention studies. Actually, the first report to indicate that COX-2 can have a significant role in colon polyp formation presciently noted that COX-2 expression in polyp occurred in the stromal cells, rather than in the epithelium of the polyp (Oshima *et al.,* 1996).

The characterization of distinct proto-oncogenes as inputs on cell metabolism and tumor repressors, as negative regulators reveals the close connection between metabolism and cancer (Merida and Avila-Flores, 2006). On account of its close links with many important cellular metabolic processes, POX can also be considered as a metabolic target for cancer prevention. Proline release from collagen degradation takes place mainly in stress situations that is, nutrient/metabolism stress and inflammatory stress accompanying cancer progression and invasion. In contrast, proline degradation may bioenergetically support a number of necessary functions that are important for oxidative metabolism. For example, cycling of P5C and proline can transfer reducing potential derived either from glycolysis or the pentose phosphate shunt into ROS or adenosine triphosphate (Hagedorn and Phang, 1986). P5C stimulates the oxidative arm of the pentose phosphate shunt and commensurately increases purine ribonucleotide synthesis (Yeh and Phang, 1988). Thus, proline metabolism can participate in either survival or programmed cell death. Current targeted therapeutics directed against cancer mainly involve specifically blocking molecular signals that promote tumor cell proliferation, obstruct cell death, hamper cellular differentiation or facilitate angiogenesis. However, the molecular pathways that underlie cellular signaling are multifaceted and often redundant. An alternative approach may be to target tumor metabolism, because a distinct property that most tumors exhibit is abnormal energy metabolism. Cancer cells have acquired mutations that make them less sensitive to restrictive stimuli and less dependent on growth stimuli, but they may be vulnerable to metabolic stress. Thus, POX could be a useful tool to modulate metabolic targets as well as to regulate COX-2.

We will continue our current study trying to find genetic and epigenetic variations of POX gene in human cancers, which may provide new insights into the mechanism by which POX contributes to tumor development. In the mean time, we will determine the metabolic consequences of high POX activity, for example, to test its effect on the TCA cycle and its intermediates. As a major mediator in p53-induced oxidative stress (Bensaad and Vousden, 2007), and because of its special connection with extracellular matrix, metabolism, COX-2 and other signalings, POX may play a broader role than previously expected, which makes it a promising new therapeutic target for colorectal cancer as well as for other types of cancer particularly in combinational therapy.

Materials and methods

Cell culture and reagents

The DLD-1 human colon cancer cells are from America Type Culture Collection (ATCC, Manassas, VA, USA). The generation, characterization and maintainence of the DLD-1 Tet-

Off POX and DLD-1 Tet-Off vector cell lines have been previously described (Donald *et al.,* 2001; Liu *et al.,* 2005). PGE_2 was purchased from Cayman Chemical (Ann Arbor, MI, USA). EGF was purchased from Invitrogen (Gaithersburg, MD, USA). Ad-MnSOD was purchased from the Vector Core Facility of University of Iowa.

Cell growth assays

The CellTiter 96 aqueous nonradioactive cell proliferation assay (Promega, Madison, WI, USA), performed according to the manufacturer's protocol, was used to measure cell growth. Approximately 25 000 cells were seeded in wells of a 24-well plate and DOX or vehicle was added to block or induce the expression of POX by DLD-1 Tet-Off POX cells. A solution containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (20:1 v/v) was added to the cells for 2h at 37 °C and absorption at 490 nm was determined. Each data point was performed in triplicate, and the results were reported as mean absorption ± s.e.

Western blot analysis

The whole cell lysates were prepared in a buffer containing 50 mM Tris–HCl (pH 6.8), 2.0% sodium dodecyl sulfate and 10% glycerol and protease inhibitor cocktail tablets (Roche, Mannheim, Germany). Equal amounts of extract were electrophoresed on an acrylamide denaturing gel and transferred by electroblotting onto a nitrocellulose membrane. The primary antibodies used were those against COX-2 (BD, San Jose, CA, USA), EGFR, p-EGFR, GSK-3β, p-GSK-3β (Cell Signaling, Danvers, MA, USA), β-catenin, p-β-catenin and actin (Sigma, St Louis, MO, USA). The anti-POX antibody was prepared in the lab and has been described elsewhere (Liu *et al.,* 2005). Blots were developed using the chemiluminescence procedure (Amersham, Piscataway, NJ, USA).

PGE_2 assay

DLD-1 Tet-Off POX cells were cultured without DOX for 0, 1, 3 and 5 days. The cell lysates were prepared and the PGE_2 levels were determined using an enzyme-linked immunosorbent assay kit (Cayman Chemical) according to manufacturer's protocol.

Flow cytometry

The cells were trypsinized and washed twice in cold phosphate-buffered saline with 0.1 % bovine serum albumin. The pellets were fixed in 70% ice-cold ethanol for at least 1 h. Before running on the flow cytometer, the cells were washed twice with cold phosphate-buffered saline with 0.1 % bovine serum albumin, and incubated with 5 μl RNase (200 U/ml, DNase-free) for 15 min. The cells were stained with 10 μg/ml propidium iodide for at least 1 h in the dark. Stained cells were analyzed with an EPICS-XL-MCL flow cytometer (Beckman Coulter, Inc., Miami, FL, USA).

Each data point was performed in triplicate, and the results were reported as the mean ± s.e.

Luciferase assay

TCF (TOPflash and FOPflash) and COX-2 promoter activity in DLD-1 cells were measured using the Dual-Luciferase Reporter Assay (Promega) according to the manufacturer's

protocol. The cells were cotransfected with the TOPflash, FOPflash (kindly provided by Dr. Bert Vogelstein of the Johns Hopkins University) or Cox-2-luc (Liu *et al.,* 2004) reporter constructs and pRL-TK, a Renilla construct for normalizing of transfection efficiency. The cells were transfected using lipofectamine 2000. Transfected cells were lysed 36 h after transfection and luciferase activity was measured with equal amounts of cell extract using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA) and normalized with the Renilla activity.

Adenovirus infection

Recombinant adenoviruses, Ad-GFP, Ad-Empty, Ad-MnSOD, Ad-CuZnSOD, Ad-CAT were purchased from Gene Therapy Vector Core Facility of the University of Iowa. Ad-GFP was used to determine the infection efficiency. The multiplicity of infection of different recombinant viruses was used to reach 100% infection but did not cause dramatic cell death by virus alone.

Hydroethidine assay

The assay is designed to measure hydroethidine fluorescence, which reflects the levels of superoxide. Cells were grown in six-well plates. The cultures were washed twice with phosphate-buffered saline and then stained with 10 μM hydroethidine for the detection of superoxide for 30 min at 37 °C. The cells were washed twice with phosphate-buffered saline and fluorescence was measured using a Cytofluor II Fluorescent Multi-Plate Reader (Perseptive Biosystems, Framingham, MA, USA). The wavelengths used were 485/585 nm for hydroethidine. Each data point was performed in triplicate, and the results were reported as mean absorption± s.e.

Immunohistochemical staining of human tissue arrays

The immunostaining for POX was performed in the Pathology/Histotechnology Laboratory, NCI-Frederick. The human tissue arrays, which contained 24-paired human colorectal cancer/normal tissues were purchased from Cybridi, Co. (Frederick, MD, USA). The dilution of POX antibody was 1:1000. Pathologists in the Pathology/Histotechnology Laboratory of NCI-Frederick read and graded the expression as 5 levels: 0, +, + +, + + + and + + + +. The tumor and normal tissues from same patients were then compared, and changes, at least one level (upregulation, no change or downregulation) were decided and counted. Data were statistically analyzed using the *Z*-test.

Statistical analysis

Z-test was used for immunohistochemical staining data. Null hypothesis of proportion of decrease is equal to 50%. For all other data, Student's *t*-test was used.

Acknowledgements

This research is supported by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research. This project also has been funded in part with Federal funds from the National Cancer Institute, National Institutes of Health under Contract No. N01-CO-12400. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the US Government.

Abbreviations

APC	adenomatous polyposis coli
COX-2	cyclooxygenase-2
DOX	doxycycline
EGFR	epidermal growth factor receptor
MnSOD	manganese superoxide dismutase
P5C	pyrroline-5-carboxylate
PGE_2	prostaglandin E2
POX	proline oxidase
ROS	reactive oxygen species
TCA	tricarboxylic acid

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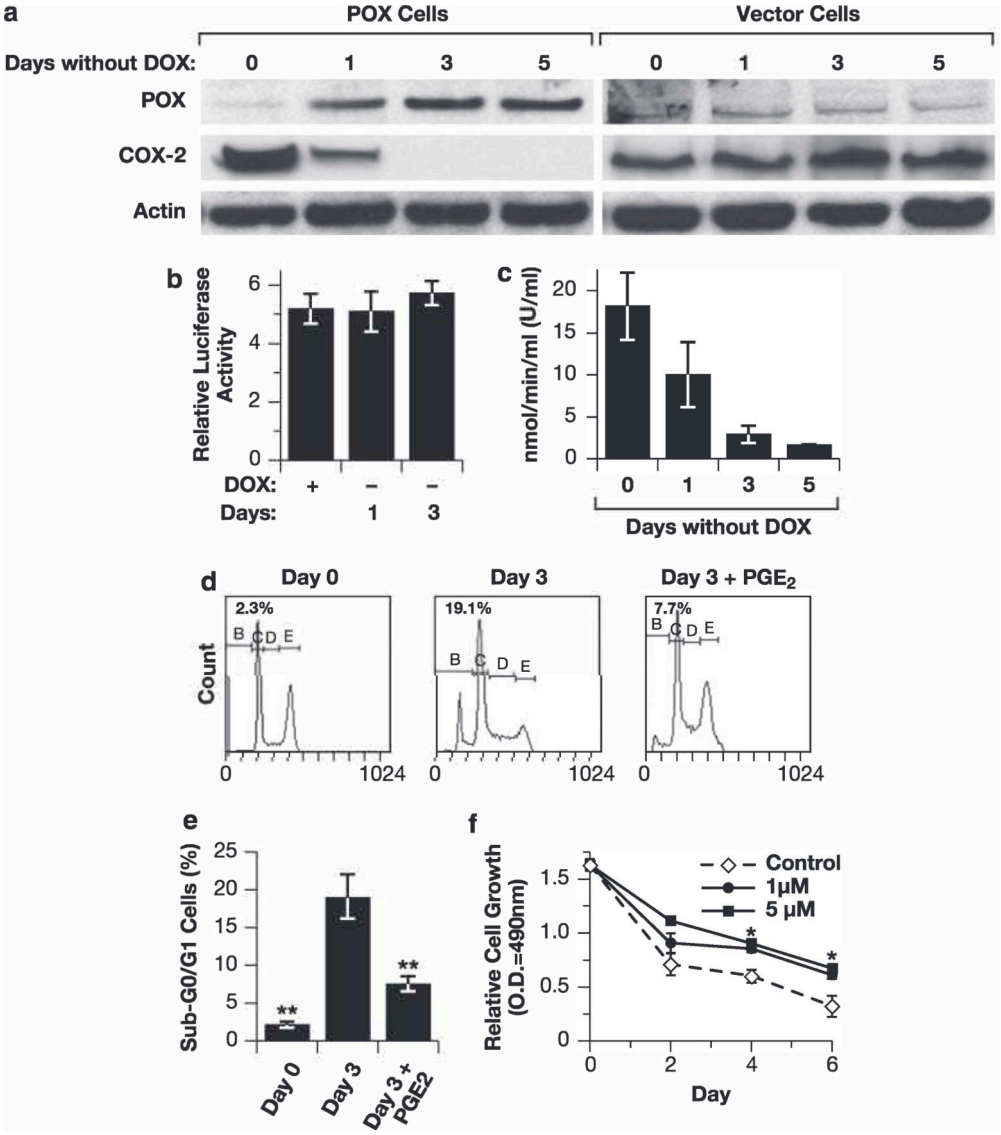

Figure 1.

The inhibition of cyclooxygenase-2 (COX-2)/prostaglandin E2 (PGE_2) by proline oxidase (POX). The DLD-1 Tet-Off POX cells and DLD-1 Tet-Off vector cells were cultured in medium without doxycycline (DOX) for 0, 1, 3 and 5 days. The cells were harvested and cell lysates were prepared. The expression levels of POX and COX-2 were determined by western blotting (**a**). The effect of POX on COX-2 transactivation activity was determined by luciferase assay (**b**). POX also suppressed PGE_2 activity, which was measured by enzyme-linked immunosorbent assay (**c**). PGE_2 treatment (5 μM) partially reversed POX-induced apoptosis, as shown here as percentages of cells in sub-G_0/G_1 phase (**d** and **e**) evaluated by flow cytometry analysis, and cell growth measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) cell proliferation assay (**f**). The asterisks indicate statistically significant difference (*$P < 0.05$; **$P < 0.01$).

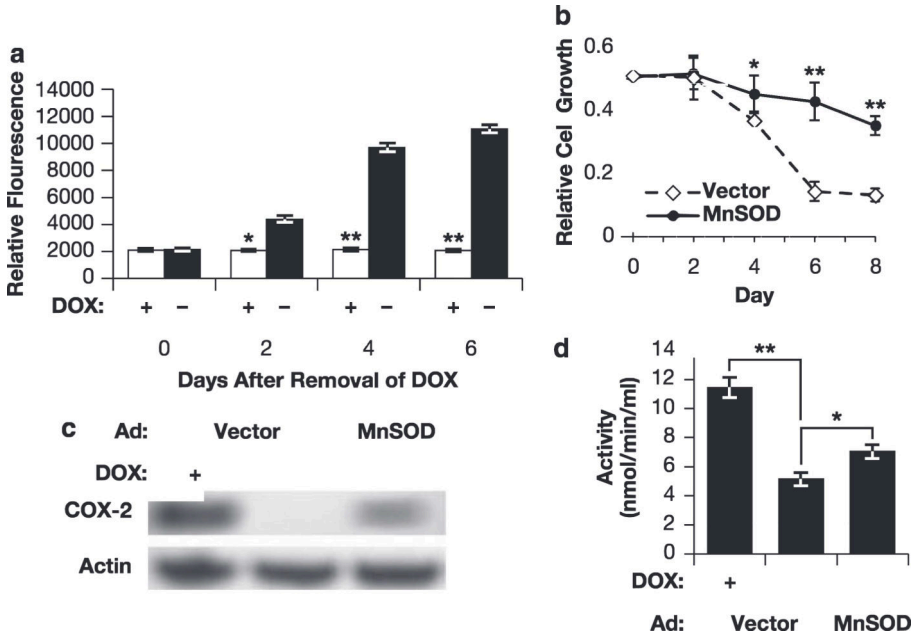

Figure 2.

The involvement of reactive oxygen species (ROS)/superoxides in cyclooxygenase-2 (COX-2)/prostaglandin E2 (PGE_2) inhibition by POX. (**a**) Superoxide radical generation by proline oxidase (POX) expression. The DLD-1 Tet-Off POX cells were cultured in the presence and absence of doxycycline (DOX). At different time points, hydrothidine assay was performed to measure the generation of superoxide radicals. DLD-1 Tet-Off POX cells were seeded in 24-well plates in the presence of DOX and were infected with recombinant adenoviruses containing manganese superoxide dismutase (MnSOD) with equal amount vector virus as control. DOX was removed from the medium 2 days later and cell growth was determined using, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) proliferation assay (**b**). The cells were infected by MnSOD recombination adenovirus (Ad-MnSOD) or Ad-vector and 1 day later the DOX was removed from the medium for 3 days. The cells were harvested and western blotting for COX-2 and assay to determine PGE_2 activity were performed (**c** and **d**). The asterisks indicate statistically significant difference (*P<0.05; **P<0.01).

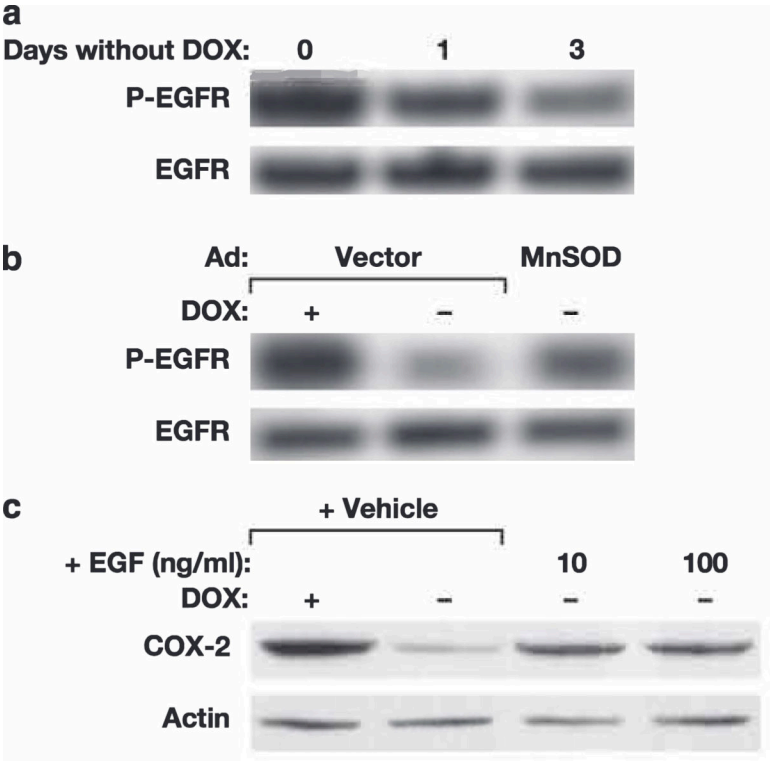

Figure 3.

The involvement of epidermal growth factor receptor (EGFR) signaling. The DLD-1 Tet-Off proline oxidase (POX) cells were fed with medium without doxycycline (DOX) for 0, 1 and 3 days. The cells were harvested and cell lysates were prepared, western blotting was performed for phosphorylated-EGFR and EGFR (**a**). Cells were infected with Ad-manganese superoxide dismutase (MnSOD) or Ad-vector and 1 day later, DOX was removed from the medium for 3 days. Then the cells were harvested and cell lysates were prepared and western blotting for EGFR and its phosphorylated form were performed (**b**). (**c**) To determine the effect of EGF on POX-reduced cyclooxygenase-2 (COX-2) expression, DOX was removed from the medium for 3 days and the cells were treated with EGF at the concentrations of 10 and l00 ng/ml. Then the cells were harvested and cell lysates were prepared and western blotting for COX-2 was performed. The western blotting for actin was used as loading control.

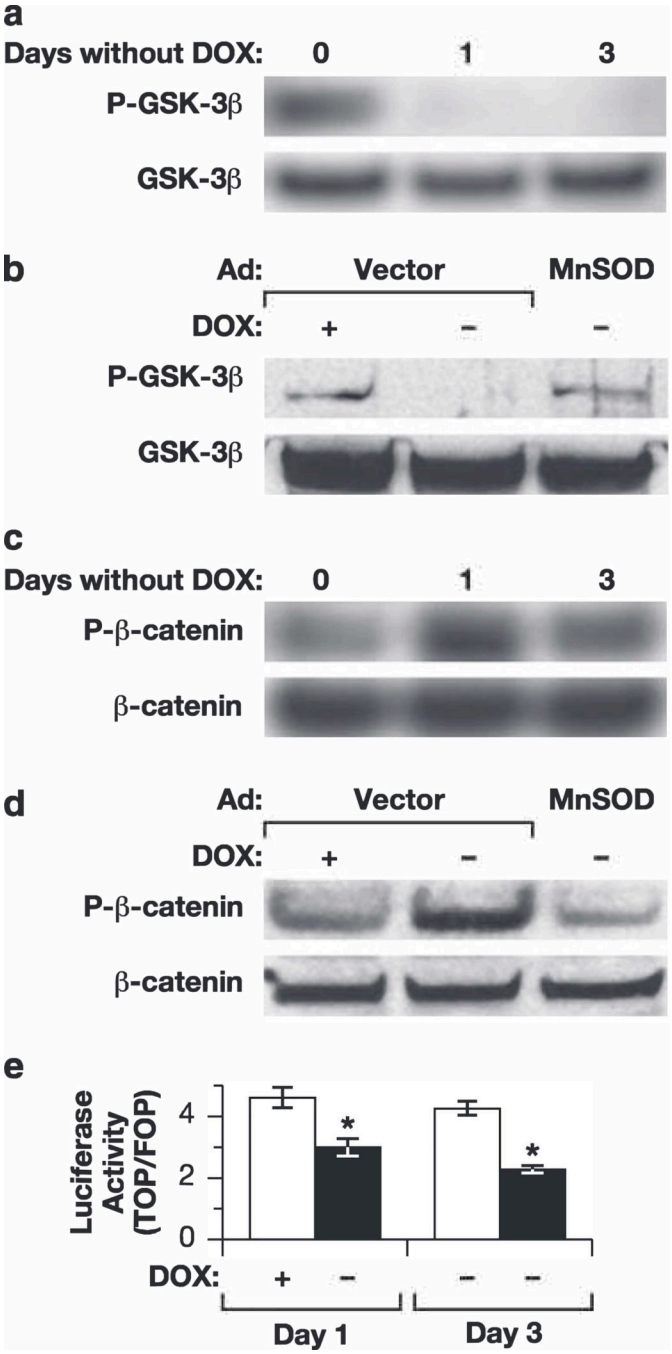

Figure 4.

The involvement of adenomatous polyposis coli (APC)/β-catenin pathway. The DLD-1 Tet-Off proline oxidase (POX) cells were fed with the medium without DOX for 0, 1 and 3 days. The cells were harvested and cell lysates were prepared and western blottings for glycogen synthase kinase-3β (GSK-3β), phosphorylated-GSK-3β and β-catenin, phosphorylated-β-catenin were performed (**a** and **c**). To determine the effect of reactive oxygen species (ROS)/superoxide on this signaling, the cells were infected with MnSOD recombination adenovirus (Ad-MnSOD) or Ad-vector as control. One day later, doxycycline (DOX) was removed from

the medium for 3 days. Then the cells were harvested and cell lysates were prepared and western blotting for GSK-3β and β-catenin and its phosphorylated form were performed (**b** and **d**). To determine the effect of POX on β-catenin/TCF/LEF transactivation activity, the TOPflash and FOPflash constructs were transfected into DLD-1 Tet-Off POX cells respectively. One day later, DOX was removed from the medium for I or 3 days. The cells were then harvested and luciferase assay was performed. The asterisk indicates statistically significant difference (**e**) (* $P<0.05$).

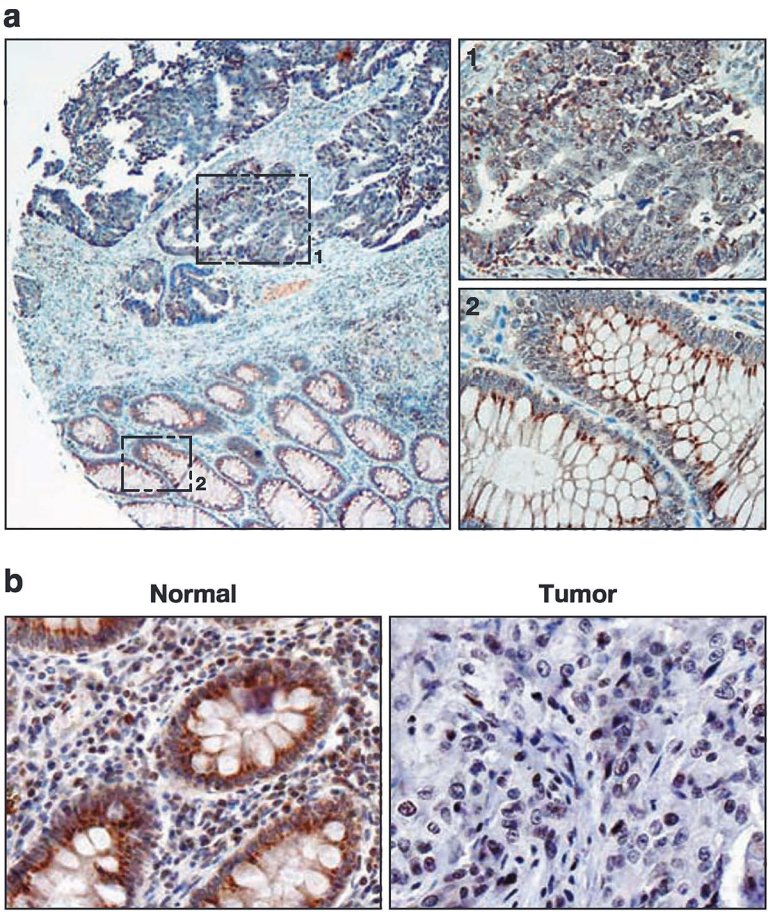

Figure 5.

The reduced expression of proline oxidase (POX) in human colorectal tumor tissues. Human tissue array slides, with 24-paired colorectal adenocarcinoma/normal tissues were immunohistochemically stained for the expression of POX. The representative images are shown. (**a**) The upper section (box 1) is poorly differentiated adenocarcinoma tissue, whereas the lower part (box 2) is normal tissue. (**b**) Immunostaining for POX of another pair of normal/cancer tissue from same patient is shown (40 × and 200 ×).

Table 1

POX expression in colorectal tissues (cancer versus normal)

Pairs	24
Down	20
No change	3
Up	1
P-value	<0.001

Abbreviation: POX, proline oxidase.

Z-test was used (null hypothesis of proportional decrease is equal to 50%). The reduced expression of POX in human colorectal tumor tissues. Twenty-four pairs of human colorectal cancer/normal tissues from same patients were immunostained for the expression of POX. The expression was graded as following five levels: 0, +, + +, + + + and + + + +. The tumor and normal tissues from same patient were then compared and changes of at least one level (upregulation, no change or downregulation) were determined. Data were statistically analyzed using the *Z*-test. Null hypothesis of proportional decrease is equal to 50%.