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Cyclooxygenase 2 Rescues LNCaP Prostate Cancer Cells from Sanguinarine-Induced Apoptosis by a Mechanism Involving Inhibition of Nitric Oxide Synthase Activity

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Abstract

Expression of cyclooxygenase-2 (Cox-2), an inducible enzyme responsible for the production of prostaglandins from arachidonic acid, is elevated in human prostate tumor samples. The aim of this study was to investigate whether expression of Cox-2 is effective against prostate cancer cell apoptosis triggered by sanguinarine, the quaternary benzophenanthridine alkaloid with antineoplastic properties. Sanguinarine effectively induced apoptosis in LNCaP human prostate cancer epithelial cells as assessed by caspase-3 activation assay, Annexin V staining assay, or by visual analysis for the apoptotic morphology changes. Sanguinarine-mediated apoptosis was associated with the increase of nitric oxide (NO) formation in prostate cancer cells as assessed by measurements of nitrites with Sievers nitric oxide analyzer as well as flow cytometry analysis using NO fluorescent sensor.

Activation of NO synthase (NOS) activity was crucial for sanguinarine-induced cell death because NOS inhibitor L-NMMA efficiently protected cells from apoptosis. Adenovirus-mediated transfer of Cox-2 into LNCaP cells inhibited sanguinarine-induced apoptosis and prevented an increase in NO production. Surprisingly, NO donors failed to induce apoptosis in LNCaP cells, suggesting that constitutive NO generation is not sufficient for triggering apoptosis in these cells. Besides NO generation, NOS is also capable of producing superoxide radicals. Sanguinarine-induced production of superoxide radicals, and the addition of MnTBAP, a scavenger of superoxide radicals, efficiently inhibited sanguinarine-mediated apoptosis. These results suggest that Cox-2 expression rescues prostate cancer cells from sanguinarine-induced apoptosis by a mechanism involving inhibition of NOS activity, and that coadministration of Cox-2 inhibitors with sanguinarine may be developed as a strategy for the management of prostate cancer. (Cancer Res 2006; 66(7): 3726-36)

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Introduction

Apoptosis, or programmed cell death, is a normal physiologic process of cell deletion in embryonic development as well as in the maintenance of tissue and organ homeostasis. Inappropriate induction of apoptosis has been associated with organ injury, whereas failure to undergo apoptosis may cause cell overgrowth and malignancy (1). The ability of tumor cells to avoid apoptosis determines their resistance to cytotoxic therapies and prevents their removal from the pool of proliferating cells. Resistance to apoptotic death is a characteristic feature of prostate cancer cells and is one of the reasons for the failure of chemotherapeutic strategies of hormone refractory prostate cancer (2). The clinical improvement in men with metastatic prostate cancer following androgen ablation therapy is finite, lasting ~15 months (2, 3). The treatment of patients with relapsing cancer is usually disappointing, with chemotherapy having little or no effect due to high resistance to apoptosis by metastatic prostate cancer cells (2). The molecular mechanisms of this resistance are far from being resolved.

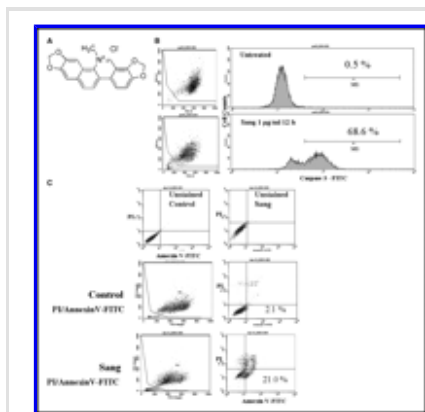
Nonsteroidal antiinflammatory drugs (NSAID) have recently attracted attention due to their putative potential in cancer chemoprevention (4, 5). The principal target of NSAIDs is cyclooxygenase (COX), the enzyme which catalyzes the first, rate-limiting step in the formation of prostaglandins from arachidonic acid (6). The inducible isoform Cox-2 and its products, especially prostaglandin E₂ (PGE₂), are involved in the inflammatory responses and in the inhibition of apoptosis (6). Several epidemiologic studies have shown that

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individuals regularly taking aspirin or other NSAIDs, pharmacologic COX inhibitors, have a reduced incidence of colon cancer than those that do not (6). Treatment of HCA-7 cells (human colon cancer cells expressing Cox-2 constitutively) with a highly selective Cox-2 inhibitor, resulted in the inhibition of growth and in the increase of the number of apoptotic cells, which could be reversed by PGE₂ stimulation (7). Thus, Cox-2 evolved as one of the pharmacologic targets for chemoprevention (8).

Similarly, the involvement of Cox-2 overexpression in prostate cancer has been suggested (9, 10). Increased levels of prostaglandins, products of COX enzymatic activity, have been found in malignant human prostate tumors and in mouse prostate cancers (9). Furthermore, increased synthesis of prostaglandins was shown to be associated with poor disease prognosis in humans (11). Inhibitors of Cox-2 reduced human prostate tumor cell invasiveness and triggered apoptosis (12, 13). Cox-2 expression was induced in a canine model of spontaneous prostatic adenocarcinoma (14) and elevated levels of Cox-2 expression were shown in cultured prostate cancer cells (15). Even more important was the finding that Cox-2 mRNA and protein are overexpressed in 83% of the human prostate tumor samples (16). Thus, the involvement of Cox-2 in prostate cancer is compelling.

Natural alkaloid sanguinarine [13-methyl(1,3)benzodioxolo(5,6-c)-1,3-dioxolo(4,5-i)phenanthridinium] derived from *Sanguinaria canadensis*, exhibits antimicrobial, antitumor (17), antiangiogenic (18), and antiinflammatory (19) properties (Fig. 1A). Sanguinarine was reported to cause the apoptosis of immortalized human keratinocytes (20), human epidermoid carcinoma (21), and human prostate carcinoma cells (22). Like many other antitumor drugs, sanguinarine acts by interfering with the function of DNA and inducing apoptosis. Because sanguinarine is effective against multidrug resistance (23), it could be developed as an anticancer drug (21). Here, we show a novel mechanism of the antiapoptotic function of Cox-2 in cancer cells. Treatment of prostate cancer cells with sanguinarine results in apoptotic cell death accompanied with increased generation of nitric oxide (NO) and superoxide radicals. NO and superoxide are known to rapidly react to yield peroxynitrite, which exhibit a wide array of tissue-damaging effects ranging from DNA damage to protein nitration. The ability of Cox-2 overexpression to rescue LNCaP prostate cancer epithelial cells from sanguinarine-induced apoptosis may tantalizingly be explained by the prevention of peroxynitrite formation due to Cox-2-dependent decrease of NO production.



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Figure 1. Sanguinarine treatment induces apoptosis in LNCaP human prostate cancer epithelial cells. LNCaP cells were serum-starved and treated with sanguinarine (1 $\mu\text{g}/\text{mL}$) for 12 hours and assayed for caspase-3 activation and transitions in cell viability by FITC-AV/PI staining using flow cytometry. *A*, structure of sanguinarine chloride. *B*, side versus forward scatter dot plot of cells (*left*) and the gate set up for obtaining histograms of cell count versus active caspase-3 labeled to FITC (*right*). The M1 region, which represents cells with active caspase-3, is highlighted (representative of six independent experiments). *C*, untreated cells and cells treated with sanguinarine analyzed in the absence of FITC-AV/PI staining (*top*). It was necessary to modify the quadrant gate in sanguinarine-treated cells in order to compensate for sanguinarine internal fluorescence. *Left*, side versus forward scatter dot plot of untreated and sanguinarine treated cells, respectively (*middle* and *bottom*). *Right*, cells stained with FITC-AV/PI to show fractions of viable, early apoptotic, and necrotic cell populations (*middle* and *bottom*). *Bottom right quadrant*, percentile of cells in corresponding to early apoptotic cell population (representative of three independent experiments).

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Reagents. Sanguinarine chloride was purchased from Sigma Chemical Co. (St. Louis, MO) and was added to cells in a final concentration of 1 to 3 $\mu\text{g/mL}$ (2.72-8.16 $\mu\text{mol/L}$). Active caspase-3 FITC monoclonal antibody apoptosis kit containing rabbit monoclonal antibodies against cleaved caspase-3 was purchased from BD PharMingen (San Diego, CA). The Annexin-V/propidium iodide (PI) kit was purchased from Calbiochem (San Diego, CA). The NO sensor kit was purchased from BD Biosciences Clontech (Palo Alto, CA). The PGE₂ ELISA kit was purchased from Amersham Biosciences (Piscataway, NJ). L-NMMA, DETA-NONOate, and SNAP were purchased from Cayman Chemical Company (Ann Arbor, MI). MnTBAP was from Alexis Biochemicals. Dihydroethidium was from Molecular Probes, Inc. (Eugene, OR). Celecoxib was kindly provided by Pfizer, Inc. (Kalamazoo, MI). Horseradish peroxidase conjugates used in Western blotting were from Bio-Rad Laboratories (Hercules, CA). Polyclonal goat anti-Cox-2 antibodies and rabbit polyclonal antibodies against cleaved caspase-3 used in Western blotting, were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture. Androgen-dependent LNCaP and androgen nonresponsive PC-3 prostate cancer cells were obtained from the American Type Culture Collection (Manassas, VA) and were maintained in 25 mmol/L HEPES buffered RPMI 1640 supplemented with 10% fetal bovine serum and 100 units/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin and 0.29 mg/mL L-glutamine at 37°C in a humidified incubator containing 5% carbon dioxide.

Adenovirus-mediated gene transfer. The recombinant adenoviral vectors AdCox-2, AdGFP, and AdNull expressing the Cox-2, green fluorescent protein (GFP), and adenovirus vector without introduced cDNA, respectively, were either constructed as previously described (24) or were purchased from Qbiogen, Inc. Amplification of recombinant adenoviruses was done at the Medical College of Wisconsin Adenoviral Core. Prostate cancer cells were incubated with AdCox-2, AdGFP, or AdNull [at a multiplicity of infection (moi) of 100] for 1 hour at 37°C with periodic shaking, followed by the addition of serum-free medium. At 60 hours, sanguinarine was added for the indicated amounts of time and cells were taken for analysis.

Western blot analysis. Cells were washed twice with ice-cold PBS and lysed in Triton lysis buffer containing HEPES (50 mmol/L; pH 7.5), NaCl (150 mmol/L), MgCl₂ (1.5 mmol/L), EGTA (1 mmol/L), 1% Triton X-100, 10% glycerol, protease inhibitor cocktail, phenylmethylsulfonyl fluoride (1.0 mmol/L), sodium orthovanadate (0.2 mmol/L) and sodium fluoride (10.0 mmol/L) for 10 minutes on ice (60 μL lysis buffer per well of a six-well plate, 120 μL for 5-cm and 500 μL for 10-cm dishes). Cell lysates were collected in microcentrifuge tubes and cleared by centrifugation at 15,000 $\times g$ for 15 minutes at 4°C. Supernatants were then resolved by SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA). After transfer of proteins, the polyvinylidene fluoride membrane was blocked with TBS [Tris-HCl, 20 mmol/L (pH 7.8) and NaCl, 150 mmol/L] containing 3% bovine serum albumin (United States Biochemical, Cleveland, OH) for 2 hours at room temperature. Primary antibodies were added and the membranes incubated overnight at 4°C with constant rotation. The following day, the membranes were washed five times (5 minutes each) with TBS containing 0.1% Tween 20 (TBST) followed by two washes with TBS prior to the addition of secondary antibodies. Secondary antibodies were diluted in TBS containing 5% nonfat dry milk and incubated with the membrane for 1 hour at room temperature. The membranes were again washed five times with TBST and twice with TBS prior to the development of immunoreactive signal by chemiluminescence.

Annexin-V FITC and PI double staining. LNCaP (5×10^5 cells) were treated with either sanguinarine or vehicle and stained with Annexin-V (AV) labeled to FITC in combination with PI for fluorescence-activated cell sorting (FACS)

analysis of apoptosis. FITC-AV/PI staining was optimized for attached cells according to instructions outlined by the manufacturer.

Assessment of caspase-3 activation using flow cytometry. FITC conjugated to a monoclonal rabbit antibody raised against the active fragment of caspase-3 was used to determine apoptosis in cells treated with either sanguinarine or vehicle, following cell permeabilization and fixation according to the instructions outlined by the manufacturer.

Assessment of superoxide production using high-pressure liquid chromatography. The procedure of measurement of superoxide radical production was adapted as previously described (25). Briefly, 72 hours after serum starvation (80% confluent on 10 cm dish), 5 mL of fresh serum-free medium containing 1 µg/mL of sanguinarine was added for 3.5 hours. Cells were washed once with 5 mL of DPBS at 37°C. Dihydroethidium at a final concentration of 10 µmol/L was added for another 20 minutes from the 10 mmol/L stock in DMSO and all further procedures were carried out in reduced lighting. Cells were washed twice with 4 mL DPBS. DPBS was thoroughly aspirated and cells were scraped into 1 mL of DPBS, transferred into microcentrifuge tubes and centrifuged at 3,000 x g for 10 minutes. Supernatant was removed and 0.25 mL of lysis buffer (DPBS with 0.1% Triton) was added. Samples were then aspirated and released 20 times using a 1 mL syringe and 27.5-gauge needle. The protein concentration was measured, and 0.5 mL of high-pressure liquid chromatography (HPLC) grade *n*-butanol was added, and samples were subjected to vortexing (1 minute) and centrifugation (10 minutes at 8-10,000 x g). The *n*-butanol phase was transferred to another vial and evaporated with nitrogen. Dried samples were solubilized with 120 µL of molecular biology grade H₂O and centrifuged at 8 to 10,000 x g for 10 minutes. One hundred microliters of supernatant was used for HPLC analysis. HPLC peak areas in each sample were normalized to protein concentrations. Standard for dihydroethidium superoxide product (2-hydroxyethidium, 2-OH-E⁺) was prepared following a previously published method (26).

Assessment of NO production. Cells growing in six-well plates in 10% fetal bovine serum medium at ~80% confluency were treated with sanguinarine (3 µg/mL) for 6 hours. The medium was removed and cells were washed twice with HBSS at 37°C prior to incubation (30 minutes) with 1 mL of HBSS containing 25 µmol/L L-arginine at 37°C. Afterwards, 0.75 mL of HBSS from each well was taken and centrifuged at 1,000 x g for 10 minutes. The resulting supernatant (0.5 mL) was placed into microcentrifuge tubes on ice, prior to injection into the Sievers nitric oxide analyzer. Hamilton gastight syringes (100 µL) were used to inject 50 µL of sample per injection (in triplicate). Samples of dH₂O and HBSS-L-arginine solution was test-injected for levels of background nitrites. Nitrite standards of 100, 200, 400, and 600 nmol/L were prepared by serial dilution from stock solution of sodium nitrite freshly made for each experiment. Nitrite measurement protocol using KI and CuSO₄ was used according to the manufacturer's instructions (Sievers).

Analysis of PGE₂ production. Cells were kept in serum-free medium containing 30 µmol/L of arachidonic acid (Cayman Chemical Company) for 48 hours. Cells were lysed by the addition of dH₂O, scraped, and centrifuged at 14,000 x g for 10 minutes at 4°C. The media and cell lysates were combined and subjected to Amprep Octadecyl C18 minicolumns (Amersham Biosciences). PGE₂ levels were analyzed by ELISA, prostaglandin E₂ Biotrak enzyme immunoassay system, protocol 1, standard EIA procedure (Amersham Biosciences).



Results

Sanguinarine treatment induces apoptosis in human prostate cancer epithelial cells. The ability of DNA intercalator sanguinarine to induce apoptotic cell death in human prostate cancer epithelial cells (LNCaP) was established using the detection of activation of

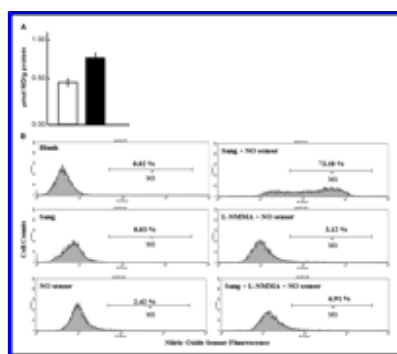
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executioner caspase-3, either by Western blot analysis with antibodies, which recognize both latent precursor and cleaved

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caspase-3 fragments, or by flow cytometry analysis using FITC-conjugated antibodies raised against the cleaved form of caspase-3 (Fig. 1B). Cleavage of executioner caspases is generally accepted as an evidence of their activation. Redistribution (externalization) of phosphatidylserine, as detected by Annexin-V binding, is another hallmark of apoptotic cell death, and presumably is dependent on activation of caspase-3. As detected by Annexin-V/PI staining, sanguinarine dramatically increases the percentile of cells staining positive for Annexin-V binding, and negative for PI staining, which corresponds to early apoptotic cell population. Sanguinarine was also effective in inducing apoptosis in PC3 human prostate cancer epithelial cells (data not shown).

Sanguinarine increases NO production in human prostate cancer epithelial cells. The excessive production of NO is often coupled to cellular injury and apoptotic cell death (27–29). We have detected the increase of NO production in LNCaP and PC3 cells treated with sanguinarine using either measurements of the level of nitrites by Sievers nitric oxide analyzer (Fig. 2A) or flow cytometry analysis of NO formation by NO fluorescent sensor (Fig. 2B). The specificity of measurement of NO generation by NO sensor was confirmed using pretreatment with NO synthase (NOS) inhibitor (L-NMMA), resulting in the disappearance of cells with increased fluorescence corresponding to enhanced NO production (Fig. 2B). Both methods show that sanguinarine-induced apoptosis of prostate cancer epithelial cells is accompanied by an increase of NO formation. Sanguinarine had similar effects in LNCaP and PC3 cells. Further analyses were carried out using androgen-dependent LNCaP prostate cancer cells.



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Figure 2. Sanguinarine increases NO production in human prostate cancer epithelial cells. *A*, NO generation was determined in control and sanguinarine-treated cells. LNCaP cells were incubated either with vehicle (*white column*) or 3 µg/mL sanguinarine (*black column*) for 6 hours before measurement of the level of nitrites using Sievers nitric oxide analyzer. The level of nitrites is shown in µmol/g of protein and is an average of triplicates. *B*, prostate cancer epithelial LNCaP cells were pretreated for 30 minutes with NOS inhibitor (L-NMMA) prior to the addition of NO fluorescent sensor (Clontech). Thirty minutes later, sanguinarine (1 µg/mL) was added for 24 hours and NO generation was determined by FACS analysis. *Blank*, untreated cells not exposed to NO fluorescent sensor. Fluorescence-activated cell sorting analysis of sanguinarine-treated cells (*Sang*) in the absence of NO fluorescent sensor shows that sanguinarine fluorescence does not interfere with the measurement of NO generation. In untreated cells (*NO sensor*) the generation of NO is low. Histograms show an increase in the amount of cells positive for the generation of NO after treatment with sanguinarine (*Sang + NO sensor*). Pretreatment with NOS inhibitor prevents sanguinarine-induced NO generation as detected by NO fluorescent sensor, confirming the specificity of FACS analysis with NO sensor (*Sang + L-NMMA + NO sensor*). Resulting histogram is similar to histogram obtained with NOS inhibitor and NO sensor in untreated cells (*NO sensor + L-NMMA*). The portion of gated cell population with increased NO level (M1 region) is indicated.

The enhancement of NO generation seems to be necessary for sanguinarine-induced apoptotic cell death of LNCaP cells. Treatment of cells with NOS inhibitor L-NMMA significantly attenuated the ability of sanguinarine to induce apoptosis as detected by Annexin-V/PI staining (Fig. 3). Because both NO-dependent transcriptional up-regulation of protective proteins (such as heat shock proteins, Cox-2, or heme oxygenase-1), and the proapoptotic effect of sustained massive NO formation has been established, NO formation is considered to play dual roles in regulating apoptotic cell death (30).

Quantification of the effect of NOS inhibitor on LNCaP apoptosis shows that NOS inhibitor has little effect on the viability of untreated LNCaP cells, but significantly decreases the percentile of early apoptotic cells after sanguinarine treatment ([Fig. 3B](#)). In accordance with Annexin V/PI staining data, NOS inhibitor also attenuated activation of caspase-3, as assessed by Western blot analysis with antibodies which recognize both latent precursor and cleaved caspase-3 fragments ([Fig. 3C](#)) or by flow cytometry analysis using FITC-conjugated antibodies raised against the cleaved form of caspase-3 (data not shown).

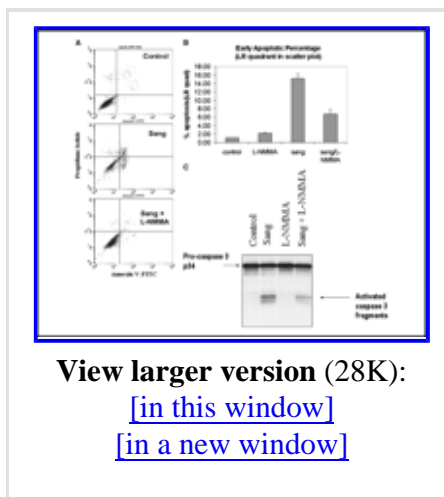


Figure 3. Inhibitor of NOS protects LNCaP cells from sanguinarine-induced apoptosis. Cells were induced to apoptosis with sanguinarine in the presence or absence of NOS inhibitor L-NMMA (5 mmol/L, 30 minutes of pretreatment) and subsequently stained with FITC-AV/PI to show fractions of cell populations corresponding to early stages of apoptosis and assayed for caspase-3 activation by Western blot analysis using antibodies raised against caspase-3. Cells that were not treated with sanguinarine are indicated as control. *A*, dot plot of control and sanguinarine-treated cells (representative of two experiments). *B*, portion of the cell population corresponding to early apoptotic cells (from lower right quadrants of scatter plots in *A*). *C*, cells were harvested and whole cell lysates were assessed for caspase-3 activation. *Arrows*, positions of procaspase-3 and activated fragments of caspase-3. Western blot results are representative of two independent experiments.

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Effect of Cox-2 overexpression on sanguinarine-induced apoptosis and NO formation in LNCaP cells. We used adenovirus-mediated gene transfer to evaluate the role of Cox-2 overexpression in protecting prostate cancer cells from sanguinarine-induced apoptosis. The adenovirus-mediated transfer of Cox-2 cDNA into cultured mammalian cells of different origin was shown to be an effective approach to study the biological significance of Cox-2 overexpression ([24](#), [31](#)). Infection of LNCaP cells with 100 moi of adenovirus encoding Cox-2 results in overexpression of Cox-2 protein as revealed by Western blot analysis ([Fig. 4A, left](#)). In cells infected with adenovirus encoding reporter gene (GFP), the Cox-2 expression was undistinguishable from untreated cells, suggesting that endogenous Cox-2 protein was not up-regulated as a result of adenovirus infection. At conditions used in this study, typically 100% of infected cells expressed the introduced gene, as confirmed by GFP fluorescence in AdGFP-treated LNCaP cells (data not shown). Adenovirus-mediated gene transfer resulted in the overexpression of functionally active protein because Cox-2 overexpression was accompanied by increased release of prostaglandins, specifically PGE₂ ([Fig. 4A, right](#)).

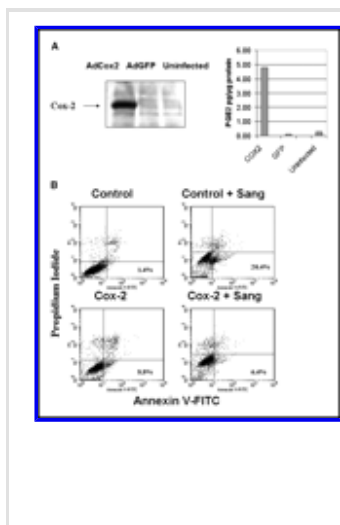
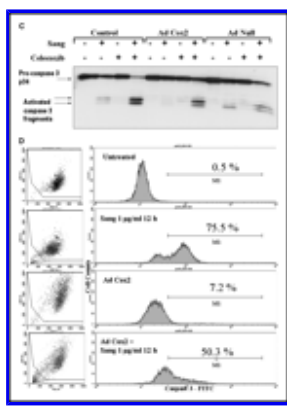


Figure 4. Adenovirus-mediated transfer of Cox-2 into LNCaP cells protects from sanguinarine-induced apoptosis. *A*, lysates from LNCaP cells, infected with 100 moi of AdCox-2 or AdGFP for 48 hours were resolved on 10% SDS-polyacrylamide gel and analyzed by Western immunoblot for Cox-2 expression (*left*). PGE₂ production was measured using prostaglandin E₂ enzyme immunoassay Biotrack system and expressed in pg/μg of protein (representative of two experiments; *right*). *B*, uninfected cells (*Control*) and cells infected with 100 moi of AdCox-2 (*Cox-2*) were induced to apoptosis with sanguinarine. Transition in cell viability was detected by FITC-AV/PI staining flow cytometry 12 hours after addition of sanguinarine. Lower right quadrant in scatter plot shows percentage of cells corresponding to early apoptotic cell population. *C*, uninfected cells (*Control*) and cells infected either with AdCox-2 or AdNull were induced to apoptosis in the presence or absence of specific Cox-2 inhibitor Celecoxib (25 μmol/L). Celecoxib was added at the time of adenovirus infection. Cells were harvested and whole cell lysates were assessed for

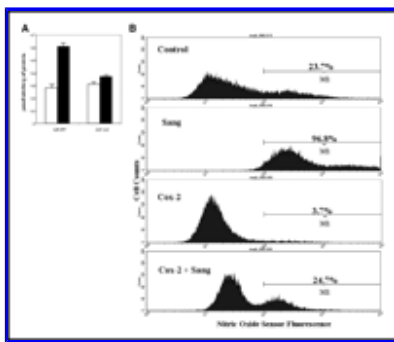


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caspase-3 activation by Western blot analysis using antibodies raised against caspase-3. *Arrows*, position of procaspase-3 and activated fragments of caspase-3. *D*, uninfected cells and cells infected with 100 moi of AdCox-2 were induced to apoptosis with sanguinarine and assayed for caspase-3 activation by flow cytometry. *Left*, side versus forward scatter dot plot of cells and specify the gate set up for caspase-3 assay. Histograms of cell count versus active caspase-3 labeled to FITC highlight M1 region, which represent cells recognized by FITC-labeled antibodies against active caspase-3. The portion of the cell population in the M1 region is indicated (representative of at least three independent experiments).

Overexpression of Cox-2 protected LNCaP cells from sanguinarine-triggered apoptosis as detected by three different methods of detection of apoptosis. First, Annexin-V/PI assay revealed a decrease in the amount of cells entering early apoptotic phase (bottom right square in quadrant gate) in cells infected with adenovirus encoding Cox-2 when compared with uninfected cells ([Fig. 4B](#)). Second, Western blot analysis with antibodies which recognize both latent precursor and cleaved caspase-3 fragments showed the appearance of cleaved active caspase-3 in sanguinarine-treated uninfected cells (*Control*) and cells infected with empty adenovirus (*AdNull*), but not in cells infected with AdCox-2 ([Fig. 4C](#)). Thus, the protective effect of AdCox-2 infection is not explained by antiapoptotic activity of core adenoviral proteins. The protective effects of adenovirus-mediated Cox-2 expression is nullified by exposure of cells to selective Cox-2 inhibitor Celecoxib (25 $\mu\text{mol/L}$). In the absence of sanguinarine, Celecoxib has no effect on caspase-3 activation in uninfected cells, cells infected with AdCox-2, or control virus. Third, flow cytometry analysis of uninfected and AdCox-2-infected cells using FITC-conjugated antibodies raised against the cleaved form of caspase-3 showed a dramatic increase of caspase-3-positive cells in response to sanguinarine in uninfected cells, which was attenuated by Cox-2 expression ([Fig. 4D](#)). Although the percentile of cells positive for caspase-3 expression still increased, it is apparent from the histograms that Cox-2 expression slows down the activation of caspase-3 in LNCaP cells. Similar experiments were carried out in which cells infected with AdCox-2 were compared with cells infected with adenovirus encoding unrelated protein. Only LNCaP cells infected with adenovirus encoding Cox-2 showed a decrease in the percentile of cells with activated caspase-3 (data not shown), providing additional evidence that resistance to apoptosis is not conferred by adenoviral backbone proteins.

Along with preventing apoptosis, AdCox-2 infection inhibited NO formation in LNCaP cells. The effect of Cox-2 overexpression on NOS activation was shown using either measurement of the level of nitrites using Sievers nitric oxide analyzer in AdCox-2 and AdGFP-infected cells in the absence and presence of sanguinarine ([Fig. 5A](#)), or by detection of NO formation with NO sensor in uninfected and AdCox-2-infected cells using flow cytometry ([Fig. 5B](#)). These data show that inhibition of NO formation is mediated by Cox-2 expression, not by unrelated adenoviral proteins.



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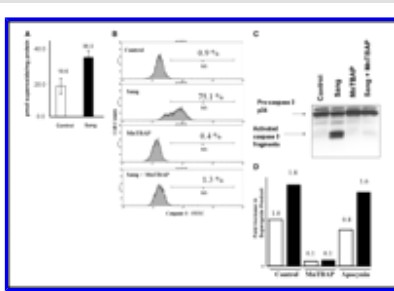
Figure 5. Effect of Cox-2 overexpression on sanguinarine-induced increase of NO production. *A*, LNCaP cells infected either with AdGFP or AdCox-2 were incubated either with vehicle (*white columns*) or 3 µg/mL sanguinarine (*black columns*) for 6 hours before measurement of the level of nitrites using Sievers nitric oxide analyzer. The levels of nitrite are shown in µmol/g of protein and is an average of quadruplicates (data shown is representative of three experiments). *B*, uninfected LNCaP cells and cells infected with 100 moi of AdCox-2 were treated with sanguinarine and NO generation was determined by FACS analysis using NO fluorescent sensor (Clontech). Histograms of cell count versus signal from NO sensor highlight M1 region, which represents cells with increased amounts of NO. The portion of gated cell population with increased NO level (M1 region) is indicated. Histograms are representative of two independent experiments.

Surprisingly, potent NO donors were not able to induce caspase-3 activation and apoptosis in LNCaP cells, suggesting that NO formation is insufficient to trigger apoptosis in these cells. Even high doses of DETA-NONOate failed to induce apoptosis in LNCaP cells (Supplementary Fig. S1). In addition to data with DETA-NONOate, we obtained similar data using another NO donor, SNAP (data not shown). In support of the conclusion that increase of NO was insufficient to induce apoptosis, the intracellular concentration of NO was monitored in cells treated with NO donors using NO sensor and flow cytometry analysis (data not shown). We have confirmed that incubation with NO donors indeed caused the increase of the concentration of intracellular NO, as detected by NO sensor, in the absence of caspase-3 activation.

Sanguinarine treatment induces formation of superoxide radicals in LNCaP prostate cancer epithelial cells.

Because activation of NOS activity is required for sanguinarine-induced apoptosis ([Fig. 3](#)), but formation of NO is not sufficient to trigger apoptosis (Supplementary Fig. S1), it is possible that NOS-dependent apoptosis is mediated via the formation of superoxide radicals, which can be generated by NOS when the concentration of substrate is low, or when NOS is uncoupled ([32](#)). To this end, we have assessed the formation of superoxide radicals in LNCaP cells treated with sanguinarine using our modification of the method described previously ([25](#)).

Sanguinarine increased the intracellular formation of superoxide as measured by dihydroethidium using HPLC with fluorescence detection ([Fig. 6A](#)). As pointed out elsewhere ([25](#)), HPLC separation prior to fluorescent measurement is required, as 2-hydroxyethidium (specific dihydroethidium-superoxide product) is not the only fluorescent product formed—the major dihydroethidium oxidation product in our system was ethidium (E^+ ; Supplementary Fig. S2). In addition, sanguinarine alone is also fluorescent, which is reflected in the appearance of an additional HPLC peak in samples containing sanguinarine (Supplementary Fig. S2). The peak corresponding to sanguinarine served as an internal control for the amount of drug penetrating LNCaP cells. The production of superoxide increased with length of incubation time with sanguinarine. Data shown are experiments with sanguinarine incubation time of 3.5 hours. Additional evidence that generation of oxidized dihydroethidium was due to the formation of superoxide radicals came from experiments in which cells were preincubated with superoxide scavenger MnTBAP prior to treatment with sanguinarine. Preincubation with MnTBAP prevented the sanguinarine-dependent formation of oxidized dihydroethidium ([Fig. 6D](#)). We also tested the ability of sanguinarine to chemically generate superoxide radicals in a cell-free system. Our data argues against this possibility because no oxidation of dihydroethidium was detected in these conditions (data not shown).



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Figure 6. Sanguinarine increases superoxide radical production in human prostate cancer epithelial cells and effect of superoxide radical scavenger MnTBAP on sanguinarine-induced apoptosis. Untreated LNCaP cells and LNCaP cells incubated with sanguinarine (1 $\mu\text{g}/\text{mL}$, 3.5 hours) were assayed for superoxide radical anion production by staining with dihydroethidium and measurement of 2-hydroxyethidium by reversed phase HPLC with fluorescence detection. *A*, calculation of increase of sanguinarine-induced superoxide production using 2-hydroxyethidium peak area. The amount of superoxide trapped by dihydroethidium was calculated as pmol superoxide per mg protein assuming the reaction stoichiometry 1:2 [dihydroethidium-superoxide; representative of eight experiments; ref. (26)]. *B*, LNCaP cells were pretreated with MnTBAP (100 $\mu\text{mol}/\text{L}$) or vehicle prior to addition of sanguinarine (1 $\mu\text{g}/\text{mL}$, 12 hours) and assayed for caspase-3 activation by flow cytometry using FITC-labeled antibodies against active caspase-3. Histograms of cell count versus active caspase-3 labeled to FITC highlight M1 region, which correspond to apoptotic cells. The portion of cell population in M1 region is indicated. Histograms are representative of three independent experiments. *C*, untreated cells (*Control*) and cells incubated with sanguinarine in the presence or absence of MnTBAP were harvested and whole cell lysates were assessed for caspase-3 activation by Western blot analysis using antibodies raised against caspase-3. *Arrows*, position of procaspase-3 and activated fragments of caspase-3. Western blot results are representative of two independent experiments. *D*, LNCaP cells were pretreated with MnTBAP (100 $\mu\text{mol}/\text{L}$) or apocynin (300 $\mu\text{mol}/\text{L}$) for 30 minutes prior to the addition of sanguinarine (1 $\mu\text{g}/\text{mL}$, 3.5 hours; *black columns*) and superoxide radical production was assayed by HPLC with fluorescence detection of 2-hydroxyethidium. The level of superoxide in the absence of sanguinarine (*white columns*) is also shown. Results are expressed as fold increase with regard to the level of superoxide production in control untreated cells (representative of two experiments).

Because sanguinarine induces superoxide generation in LNCaP cells, it was important to test whether production of superoxide is necessary for sanguinarine-induced apoptosis. Pretreatment of LNCaP cells with superoxide scavenger MnTBAP protected them from caspase-3 activation triggered by sanguinarine (Fig. 6B). The protection was quite efficient as shown both by assessment of caspase-3 activation using flow cytometry with FITC-labeled antibodies against active caspase-3 (Fig. 6B) and by Western blot analysis using antibodies raised against caspase-3 (Fig. 6C).

The source of superoxide generation in cells treated with sanguinarine was addressed using specific inhibitors of NADPH oxidase activity, apocynin. Apocynin (300 $\mu\text{mol}/\text{L}$) failed to inhibit superoxide radical production induced by sanguinarine in LNCaP cells (Fig. 6D). We have also tested whether superoxide radicals are produced by dysfunctional or uncoupled NOS in sanguinarine-treated cells. Treatment with NOS inhibitor L-NMMA did not significantly decrease the level of superoxide radicals in sanguinarine-treated cells, arguing that a different source of superoxide is employed (data not shown).

To evaluate the role of superoxide production in triggering apoptosis in LNCaP cells, we added xanthine and recombinant xanthine oxidase to LNCaP cells, and quantified cellular superoxide content and caspase-3 activation in the same experiment. Xanthine oxidase catalyzes the oxidation of xanthine to urate and requires the reduction of molecular oxygen, thereby generating superoxide. Thus, xanthine oxidase serves as one of the potential sources of superoxide in cells (33). Due to the short intracellular lifetime of superoxide, the superoxide radical detected in cells does not originate directly from extracellular recombinant xanthine oxidase. Nevertheless, regardless of the source of the detected intracellular superoxide, our data suggest that intracellular concentration of superoxide, similar to those induced by sanguinarine

(Supplementary Fig. S3A), is not sufficient to increase the caspase-3 activation and apoptosis in LNCaP cells (Supplementary Fig. S3B).

Discussion

To date, several mechanisms have been proposed to explain the antiapoptotic effect of Cox-2 (34). Particularly, (a) depletion of arachidonic acid, which prevents the activation of neutral sphingomyelinase and production of ceramide (35), (b) modulation of expression of the antiapoptotic protein Bcl-2 (15, 36), and (c) regulation of Akt activation (13). However, none of these proposed mechanisms could completely explain the postulated Cox-2-mediated resistance to apoptosis of LNCaP prostate cancer cells for the following reasons: (a) treatment of LNCaP cells with neutral or acidic sphingomyelinase or addition of C8- or C2-ceramide, two cell-permeable analogues of endogenous ceramide, failed to induce apoptosis, although they caused inhibition of cell proliferation (37); (b) a mitogen-dependent survival-regulating, signal transduction pathway independent of phosphatidylinositol 3'-kinase and Akt kinase was found in LNCaP cells (38); and (c) apoptosis induced by Cox-2 inhibitor celecoxib was independent of Bcl-2 in LNCaP and PC3 cells (13).

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Our data suggest that Cox-2 expression rescues LNCaP cells from sanguinarine-induced apoptosis at least partially via attenuation of NO production. Previously, the ability of Cox-2 to counteract NO-mediated apoptotic cell death was shown in two other cell systems: in rat pheochromocytoma PC12 cells in which Cox-2 acted via modulation of expression of prosurvival gene capable of inhibiting production of NO (24), and in RAW 264.7 macrophages via regulation of cellular susceptibility toward NO (39).

Previously, we identified some of the downstream mediator(s) of Cox-2-mediated resistance to apoptosis using human and rat cDNA expression arrays (Clontech; refs. 24, 31). The screening showed an enhanced expression of the cytoplasmic dynein light chain (DLC) [or LC8, also known as protein inhibitor of neuronal NOS (PIN)] gene. DLC/PIN protein expression was not only elevated in PC12 cell lines stably overexpressing Cox-2, but also in the PC12 cells and human mesangial cells infected with adenovirus encoding Cox-2 (24). Furthermore, PC12 cells overexpressing DLC/PIN (PC-DLC) were resistant to apoptosis induced by nerve growth factor withdrawal as compared with parental cells (PC-Off). Coimmunoprecipitation assays showed increased association of DLC/PIN protein with nNOS in PC12 cells expressing PCXII or PC-DLC cells, which decreased nNOS activity. Taken together, these results provided a novel molecular mechanism underlying the antiapoptotic role of Cox-2 in differentiated PC12 cells in response to nerve growth factor withdrawal. Even though Cox-2 overexpression resulted in the induction of DLC/PIN synthesis and a decrease in NO production with concomitant antiapoptotic effect in PC12 cells, whether this mechanism is engaged in LNCaP prostate cancer cells is not known. nNOS is constitutively expressed in both neuronal and nonneuronal tissues, but in contrast to PC12 cells, which express only nNOS, LNCaP cells also express endothelial NOS (eNOS). Although iNOS expression in LNCaP cells was not shown (40), six primary prostate neoplastic cultures showed moderately to markedly higher iNOS mRNA levels than did their paired nonneoplastic cultures (41). Thus, up-regulation of iNOS in LNCaP subjected to apoptotic stimulus can't be excluded. Notably, because DLC/PIN is supposed to be specific for nNOS, Cox-2 must employ different mechanisms to attenuate NO production by either eNOS or iNOS. Although all three NOS isoforms are subject to transcriptional control, regulation at the posttranscriptional and posttranslational levels (especially with respect to eNOS) is viewed as an important locus of control. The source of NO in sanguinarine-treated LNCaP cells is currently unknown.

Evidence keeps accumulating for the involvement of some multidrug resistance mechanisms in the chemoresistance of prostate cancer *in vitro* and *in vivo* (42). It is well established that the multidrug resistance phenotype is associated with the expression of members of the family of ABC transporter proteins, such as P-glycoprotein (also termed multidrug-resistant protein 1, MDR1), the multidrug resistance-associated protein (MRP1), the lung resistance protein (LRP), and the breast cancer resistance protein (BCRP). We have previously shown that adenovirus-mediated transfer of Cox-2 gene results in an increase of expression of MDR1 in glomerular mesangial cells (31), suggesting the role of Cox-2 in regulating the multidrug resistance phenotype in cancer cells (43). It is implausible, however, that Cox-2 mediates the efflux of sanguinarine from LNCaP cells by up-regulation of MDR1 because sanguinarine does not seem to be a substrate of MDR1 (23). Thus, sanguinarine is an effective inducer of cell death in cells expressing MDR1, but not those overexpressing Cox-2.

The most relevant radicals in physiologic control of cell function are superoxide radical and NO. Reactive oxygen species contribute to apoptosis in various cell systems and we have shown that induction of superoxide radicals by sanguinarine is indispensable for its ability to trigger apoptosis in LNCaP cells. Among the potential sources of superoxide generation are mitochondrial respiratory chain, xanthine oxidoreductase, cytochrome P450, dysfunctional NOS, and NADPH oxidases (33). Our studies with specific NADPH oxidase inhibitor apocynin and NOS inhibitor L-NMMA suggest that neither NADPH oxidases nor uncoupled NOS serve as a source of superoxide radicals in LNCaP cells treated with sanguinarine. The most probable source of superoxide radicals in sanguinarine-treated cells is the mitochondria because positively charged sanguinarine is accumulated near the external side of inner mitochondrial membranes during membrane energization and by neutralization of negative membrane charges, arising just as the inner mitochondrial membranes become energized, inhibits ATP synthesis, and uncouples oxidative phosphorylation (17).

Superoxide radical and NO rapidly react to yield peroxynitrite, which reacts with proteins, lipids, and DNA. Peroxynitrite, a strong oxidant, plays an important role in the induction of apoptosis in a number of cell types (28). The effect of peroxynitrite is mediated, at least partially, via the activation of stress-activated kinases, such as *c-Jun*-NH₂-kinase (44). We have previously shown that Cox-2 overexpression inhibits *c-Jun*-NH₂-kinase/stress-activated protein kinase activation in PC12 pheochromocytoma cells, presumably due to inhibition of NO production by NOS (45). However, peroxynitrite is not the only reactive intermediate produced by the reaction of superoxide radical and NO, and it was suggested that this reaction does not produce the powerful effects of peroxynitrite. It is generally accepted that the role of NO in tumor biology is far from being completely understood (46).

It was reported that serine-threonine kinase Akt, which phosphorylates various signaling molecules, including the Bcl-2 family member Bad, protected cancer cells from apoptosis induced by anticancer therapies (47). Molecular mechanisms for the regulation of Akt involve activating phosphorylation of Thr³⁰⁸ and Ser⁴⁷³ (48). Because chemotherapeutic agent-induced Akt inactivation was shown to contribute to the induction of apoptosis in renal cancer cells (49), sanguinarine could also be acting via Akt inactivation. In endothelial cells, sanguinarine strongly suppressed basal and vascular endothelial growth factor-induced Akt phosphorylation (18). NO contributes to apoptosis via suppression of Akt Ser⁴⁷⁴ phosphorylation (50) and the effect of sanguinarine on Akt could be mediated by increased NO production. In view of the fact that the contributions of Cox-2 in tumor angiogenesis include the inhibition of apoptosis by stimulation of Bcl-2 or Akt activation (51), the protective effect of Cox-2 could be partially explained by restoring Akt signaling either by inhibiting NO production, or by stimulating PGE₂-dependent Akt Thr³⁰⁸ phosphorylation (52).

In summary, we have shown that sanguinarine induces apoptosis in prostate cancer epithelial cells and that the ability of this drug to induce apoptosis depends on the production of NO and superoxide radicals. Cox-2 expression rescues prostate cancer cells from sanguinarine-induced apoptosis by a mechanism involving inhibition of NOS activity. The findings

support the suggestion that coadministration of Cox-2 inhibitors with sanguinarine may be developed as a strategy for the management of prostate cancer.

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Footnotes

† Deceased. 

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